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Examination of *in vivo* protein-DNA interactions and CpG-methylation patterns in the EBER promoter region of latent Epstein-Barr virus genomes

Ph.D. dissertation

of

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FREQUENTLY USED ABBREVIATIONS

$5^{m}C$	5-methyl-cytosine
AID	Activation-induced cytidine deaminase
ATF	Activating transcription factor
BL	Burkitt's lymphoma
ChIP	Chromatin immunoprecipitation
Cp	C promoter
CpG	5'-cytosine-guanine-3'
CTCF	CCCTC Binding Factor
DNMT	DNA-(5-cytosine)-methyltransferase
EBER	EBV-encoded small RNA
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
H3Kn	lysine 'n' of the histone H3
HAT	Histone acetyltransferase
HD	Hodgkin's disease
HDAC	Histone deacetylase
LCL	Lymphoblastoid cell line
LMP	Latent membrane protein
LRS	LMP regulatory sequence
LSD1	Lysine-specific histone demethylase
MAR	Matrix attachment region
MeCP	MeCpG-binding protein
MI	Mononucleosis infectiosa
NPC	Nasopharyngeal carcinoma
*ori*P	Latent replication origin
Pol II, Pol III	DNA-dependent RNA Polymerase II and III
Qp	Q promoter
VAI	(Adeno)Virus-associated RNA-I
Wp	W promoter

1. INTRODUCTION

Multicellular organisms consist of different cell types. In contrast to the diversity of cellular phenotypes, all the somatic cells carry the same or almost the same genome. Cell type specific expression of the genetic information accounts for the observed phenotypic diversity. The activation and silencing of genes in the right time and place is indispensable for the proper development and function of cells. This complicated process requires the fine-tuned regulation of all genes within a single cell. Disregulation of gene expression may lead to pathologic conditions, including the development of malignant tumours.

Gene expression is regulated by genetic and epigenetic mechanisms. Genetic regulation is based on the primary nucleotid sequence of genes and their regulatory sequences. Transcription factor binding sites occupied by different proteins have an impact on the activity of the promoters. Epigenetic regulatory mechanisms, however, involve processes which are either dependent on the post-synthetic modifications of the genome, influencing promoter activity through the alteration of protein-DNA interactions and the chromatin structure as well, or on the binding of protein complexes to certain DNA regions in an inherited manner.

EBV is the causative agent for infectious mononucleosis and is closely associated with the develpoment of B cell lymphomas and neoplasms of non-B cell origin as well. EBV is able to exist in lytic and latent forms within the host cells. Viral latency can further be categorized according to different gene expression patterns. In case of Epstein-Barr virus (EBV), a human herpesvirus the alternative promoter usage regulated by genetic and epigenetic mechanisms results in host cell dependent viral gene expression patterns. Among the latently transcribed genes of the virus the EBER transcripts (EBER1 and 2) are the most abundant viral products. It was demonstrated, that EBERs are tightly involved in oncogenic alterations initiated by the virus.

Because EBV and EBERs are associated with various human neoplasms, it is important to understand the regulatory mechanisms influencing the expression of viral oncogenes like EBERs. My work aimed to promote the understanding of the hardly clarified regulation of RNA polymerase III governed transcripts, and the possible ways to influence the activity of these tumorigenic genes.

2. THEORETICAL BACKGROUND

2.1. Epstein-Barr virus

Epstein-Barr virus (EBV; also referred to as Human Herpesvirus 4) is a double-stranded DNA virus that belongs to the *Lymphocriptovirus* genus of the *Gammaherpesvirinae* subfamily of the *Herpesviridae* family. The entire sequence of its cca. 172 Kbps long genome is known (Baer *et al*., 1984).

EBV was discovered in 1964 by Tony Epstein and Yvonne Barr in cell lines established form certain unusual childhood lymphoma samples collected by Denis Burkitt in Central Africa. Under electron microscope herpesvirus-like particles were noticed showing new serological characteristics (Epstein *et al*., 1964; Burkitt, 1958). The above mentioned, EBV-related lymphoma was later named Burkitt's lymphoma (BL). Based on recent estimations, the virus is detectable in the 96% of endemic BLs. BL also occurs sporadically in other parts of the world, albeit EBV-positivity is more infrequent than in endemic cases (Rickinson and Kieff, 1996).

According to evaluations more than 90% of the adult human population is infected by EBV. Childhood infections are generally symptom-free, while adolescence or adulthood infections often ends up in mononucleosis infectiosa (MI), also called "kissing disease". EBV infection can be transmitted from person to person by contact with infectious body fluids, by sexual contact or exposure to breast milk, however, oral contact with infectious saliva is the most common route of transmission. EBV mainly infects B cells and certain types of epithelial cells. The **infection** occurs by binding of the viral glycoprotein gp350/220 to the CR2/CD21 cell surface molecule. After infection, the virus persists in a latent form for the entire life in the human organism, however, the activation of the productive lytic cycle of the virus occasionally occurs. During the productive phase lytic genes are activated in a hierarchial cascade. Depending on the order of activation immediate early, early and late lytic genes can be distinguished. In non-productive latency, viral DNA is replicated by the enyzmatic machinery of the host. In this form the EBV genome is silenced and only a few viral products are expressed out of its approximately 100 genes.

Although the virus does not cause any disease in most cases, it may contribute to the development of certain clinical manifestations. Thus, it probably plays a central role in the pathogenesis of Burkitt's lymphoma, nasopharyngeal carcinoma (NPC), Hodgkin's disease (HD) and lymphoproliferative diseases of immunodeficient patients. EBV infection is thought to be correlated to several autoimmun illnesses, like sclerosis multiplex, reumatoid arthritis, too (Rickinson and Kieff, 2001; Middeldorp *et al*., 2002; Niller *et al*., 2008).

An important characteristic of **BL** cells is the translocation of the c-myc gene (located on chromosome 8) to one of the immunoglobulin loci (2p11-12, 14q32, 22q11). This phenomenon has both non-immunological and immunological consequences. The first means, that B cells carrying the c-myc translocation are constantly proliferating, in spite of the fact that their cell surface molecule profile is similar to that of resting memory B cells. The immunological component is manifested in blocking the expression of the HLA-I (Human Leukocyte Antigene-I) antigens and certain adhesion molecules. The latent EBV genomes also show a restricted gene expression pattern in BLs that may hinder the development of an effective immune response. It is important to note that EBNA1, the only EBV protein product detectable in BLs blocks antigen processing and presentation due to its long Gly-Ala repeats. Restricted EBV gene expression may permit the virus to persist in resting memory B cells, too (Klein, 1995; Kieff, 1996).

The product of the constitutively activated c-myc gene does not only have a proliferation supporting effect, but it also sensitizes the cells to apoptosis. This effect can be compensated in EBV-infected cells by elevated Bcl-2 and IL-10 expression, assuring an antiapoptotic impact. Furthermore, EBV-positive BL cells express the EBV-encoded small RNAs (EBERs), which also possess an **antiapoptotic effect** (Kitagawa *et al*., 2000; Nanbo and Takada, 2002; Yajima *et al*., 2005; Wong *et al*., 2005).

In addition to the translocation of the c-myc gene, other genetic alterations, like the mutation of the p53 gene may also contribute to the development of BLs. Normal B cells involved in the germinal center reaction undergo clonal expansion, isotype switching and receptor affinity maturation. Then the unnecessary or harmful, potentially malignant B cell clones are eliminated by apoptosis, thus more than 90% of the newly formed cells are deleted. This selection is essential, because the somatic recombination steps controlled by the RAG-1, -2 (Recombination Activating Gene 1 and 2) genes are not accurate, and several harmful mutations, providing basis for malignant

transformation, can occur. On the other hand, as a consequence of the EBV infection, the genes coding for the activation-induced cytidine deaminase (AID) and for the polymerase η – both participating in DNA-modifying processes like Ig class switch or somatic hypermutation – are activated as well, rising the possibility to accumulate **oncogenic mutations** (Epeldegui *et al*., 2007). One may speculate, that B cells dying during the selection steps under normal circumstances, could escape from apoptosis through the activation signals provided by the proteins or non-translated RNAs produced by EBV. In this way, the chance to develop an inappropriate lymphoproliferation may be multiplied (Cherney *et al*., 1994; Martinez-Valdez *et al*., 1996; Goossens *et al*., 1998).

2.1.1. EBV latency types

During EBV infection, the linear virus genome entering the cell circularizes by joining the so-called 'terminal repeats' located at its two ends, thus creating a covalently closed circular DNA molecule (episome). During latent infection, the EBV episomes use their latent replication origin (*ori*P) and replicate only once in a cell cycle – similarly to chromosomal DNA – with the help of the replication enzyme system of the host cell.

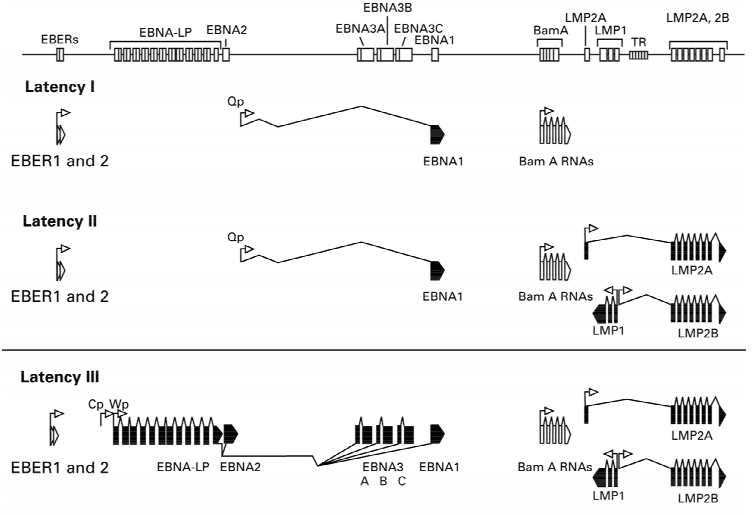
EBV can infect a variety of host cells. It was found, that there are three major EBV latency types, based on the viral gene expression pattern and the related alternative promoter usage. In EBV latency out of the nearly 100 EBV gene products about 10 latent transcripts can be generated in different patterns as categorized below (**Figure 1**.).

In **latency type I**, only the EBNA1 gene is transcribed out of the genes coding for EBV nuclear antigens, using the Q promoter (Qp). The alternative promoters Cp and Wp do not function. The promoters of genes coding for latent membrane proteins (LMPs) are also inactive, namely the LMP1 and LMP2B promoters that share a common regulatory region (LRS), and the LMP2A promoter (LMP2Ap). As a consequence, LMP-encoding mRNAs are not transcribed. This latency type is characteristic to BL biopsies and stable latency type I BL cell lines derived from the biopsies.

In **latency type II**, the EBNA1 gene is transcribed using Qp, similarly to latency type I. In this latency type, however, one could observe the generation of one or more LMP (LMP1 and/or LMP2A and/or LMP2B) transcripts as well, from the appropriate promoter. Latency type II is characteristic for NPC, HD and EBV-positive T/NK cell lymphomas.

In **latency type III,** six EBV-related nuclear antigens (EBNA1, 2, 3A, 3B, 3C, LP), three latent membrane proteins (LMP1, 2A, 2B) are expressed. The EBNA1-6 transcripts are initiated at the W (generally in the early stage of the infection) or the C promoter. This expression pattern can be observed not only in LCLs, but also in type III BL cell lines and mononuclear cells of mononucleosis infectiosa patients. The immature tonsillar B cells of symptom-free virus carriers and the EBV-associated lymphoproliferations also belong to latency type III.

Figure 1.: Latency types of the Epstein-Barr virus



On the upper line the position of latent transcripts are indicated on the linear EBV genome. Underneath, the active initiation sites are indicated with arrows showing the direction of transcription and the splicing structure between exons in the case of the main 3 latency types. Protein-coding exons are black, while non-coding exons are white (Young *et al*., 2000).

It is important to note, that EBER1 and EBER2 RNAs together with the RNA transcripts of *Bam*HI A region (except for the miRNAs encoded in the region, Cai *et al*., 2006) are always expressed without any reference to the latency type. The overviewed categories are not obligate, transient forms exist, which often differ only in the extent of gene expression (Klein, 1995; Kieff, 1996; Rickinson and Kieff, 1996; Young *et al*., 2000).

2.1.2. Structure and function of the EBER RNAs

As mentioned above, the two EBV-encoded small RNAs are always actively transcribed independently of the latency type. Although the activity of the EBER promoters significantly decreases during lytic replication, the halflife of the transcripts extends significantly. Thus, 72 hrs after the initiation of the lytic cycle the abundancy of EBERs is virtually unchanged (Greifenegger *et al*., 1998). Because approximately 10^6–10^7 EBER molecules can be present within a single cell, EBER1 and 2 represents the most abundant gene product of EBV (Lerner *et al.*, 1981). The EBER transcription units are transcribed by RNA polymerase III (Pol III; Jat and Arrand, 1982). Both RNA polymerase II (Pol II) and Pol III elements can be found in their regulatory region and all these elements are needed for full promoter activity (see 2.1.3.). EBER gene products are 166 and 172 bp long non-polyadenylated RNAs without a guanine cap. EBER1 and EBER2 are not translated into protein products, thus, they function as non-coding RNAs. They are localized dominantly within the nucleus and the perinuclear space (Howe and Steitz, 1986; Schwemmle *et al*., 1992; Nanbo and Takada, 2002). Within the cell EBER RNAs exist in ribonucleoprotein complexes with different binding partners (Glickman *et al*., 1988). These partners are the systemic lupus erythematosus antigen (La-antigen), known to bind and stabilize Pol III-transcribed RNAs (Lerner *et al*., 1981), the L22 protein, a component of the ribosomes (Toczyski *et al*., 1994), and the dsRNA-activated protein kinase (PKR, Clarke *et al*., 1991). In addition to their nuclear localization the EBER1 transcripts were shown to be secreted from the cells together with the La-antigen (Iwakiri *et al*., 2009).

Although our knowledge about the EBER RNAs continuously broadens, their exact role still needs to be elucidated. The nuclear localization of the EBER products

suggests, that they may participate in nuclear processes like DNA replication, transcription or RNA processing. Recent data on the EBER secretion suggests, however, a new possible role outside of the cell. Binding partners and putative functions are summarized in **Table 1**.

U4 and U6 are Pol III-transcribed RNAs and part of the spliceosome complex responsible for RNA processing. EBER2 contains the the same sequence as the U6 RNA for binding to U4 and it was also demonstrated that EBER2 is able to bind U4. Thus, one could conclude that EBER2 may take part in the maturation of different RNA molecules. In addition, similarly to EBERs, U6 is also stabilized by the La-antigen. This function could not be veryfied, however, in case of EBNA transcripts, because in B cell lines infected with EBER-deleted EBV the maturation of EBNA2 and EBNA-LP mRNAs were unaltered compared to cells infected with EBER-carrying EBV (Swaminathan *et al*., 1991).

In 1981 Lerner *et al*. could detect the *in vivo* association of the EBER RNAs and the **La-antigen** in immunoprecipitation experiments using anti-La antibody. The highly abundant EBER gene products may severely affect the amount of free La-antigens, resulting thereby in a diminished lifetime of other Pol III-transcribed RNAs, as speculated by Glickman *et al*., (1988). Albeit, it was found, that the La-antigen actively shuttles between the cytoplasm and the nucleus, the EBER RNAs stay permanently inside the nucleus (Bachmann *et al*., 1989; Fok *et al*., 2006). Interestingly, Iwakiri and coworkers (2009) demonstrated, that EBER1 RNA is exported and secreted in a complex formed with the La-antigen. EBER2 was only hardly detectable in the culture supernatant of EBV-carrying cells. As a consequence, the secreted EBER1 outside the cells could potentially affect the immune system through TLR3 (Toll-like receptor 3) which is the surface sensor molecule of dsRNAs. It was also confirmed, that EBER1-positive sera and culture medium activated the TLR3 pathway and induced IFN-β, IFN-γ and TNFα production (Iwakiri *et al*., 2009).

On the other hand, in experiments with dendritic cells (DC) EBER1 induced the maturation of DCs, their IFN-β and IL-12 production and enhanced their capacity for antigen presentation in a TLR3-dependent manner via the NFκB and the IRF3 signal transduction pathway (Iwakiri *et al*., 2009). This raised the possibility that EBER1 can complement the tumorigenic effect of latent membrane protein 1 (LMP1), the oncoprotein of Epstein-Barr virus.

L22, a human ribosomal protein, has also been shown to be associated with the EBER1 transcripts (Glickman *et al*., 1988). In EBV-negative cells the L22 protein localizes to the nucleoli and the cytoplasm. In EBV-infected cells a significant portion of the L22 protein is relocalized to the nuclear space by binding to the stem loops of the EBER1 RNA. Due to its spatial structure 3 molecules of L22 is able to bind to a single EBER1 RNA (Toczyski *et al*., 1994; Fok *et al*., 2006). *In vitro* mutagenesis of the stem-loop sequences of the EBER1 molecule led to impaired relocalization of the L22 protein to the nucleus and caused a significant growth-reduction of transfected EBV-negative BL cells in soft agar (Houmani *et al*., 2009).

EBER1 can substitute for certain functions of the adenovirus VAI RNA (Virus-associated RNA-I), as proven by experiments using VAI-deleted adenovirus and EBER-transfected cells (Wang *et al*., 2005). The structural similarities between the VA RNAs and EBERs suggested that they might have the same biological function. The VA RNAs are known to play important role in the inhibition of the interferon-induced antiviral reaction of the cell. In this reaction the eIF-2α – the initiation factor of the eukariote protein translation system – is phosphorylated by the **PKR** (dsRNA-activated protein kinase) molecule. In spite of the structural similarities, the intracellular localization of the VA and EBER RNAs is different. The VA RNAs show an even distribution within the nucleus and the cytoplasm, while the EBERs are mainly confined to the nucleus (Glickman *et al*., 1988). In addition, the level of VA RNA is elevated during the lytic adenovirus replication cycle (when there is a need to ensure the expression of viral proteins), whereas the EBERs are highly expressed shortly after EBV infection and their amount remains constant throughout the latent replication phase (Greifenegger *et al.*, 1998). In interferon-treated cells VAI RNA could prevent the activation of the eIF-2α, but the EBER RNAs were not able to do so, suggesting different functions of the examined RNAs (Howe *et al*., 1986). EBER1 could complement, however, VAI in other experiments, where tranfected EBER1 enabled the replication of VAI-deleted adenovirus. This experiment raised the idea to treat EBV-caused tumors with VA deleted adenoviruses to insure its selective lytic replication exclusively in EBV-infected cells (Wang *et al*., 2005). Bhat *et al*. also observed that EBERs can substitute for VA RNAs during lytic adenovirus growth (Bhat *et al*., 1983, 1985).

Exportin-5, which is the main nucleocytoplasmic transport molecule for miRNAs was shown to supply the transport of the VA RNAs to the cytoplasm. This mechanism suggests a possible role for VA RNAs in the regulation of RNA-

interference. A similar role of the EBER RNAs was disclaimed by Fok *et al*. (2006), as the binding of the EBERs to exportin-5 could not be proven. The role of the EBERs in the inhibition of the antiviral reaction by preventing the activation of PKR seems to be controversial. Nanbo *et al*. could verify the *in vivo* physical interaction between EBERs and the PKR protein leading to the phosphorylation and inactivation of the PKR in Daudi and Akata BL cell lines (Nanbo *et al*., 2002, McKenna *et al*., 2006). Contrary, Swaminathan and coworkers (1992) observed that the EBER RNAs did not block the antiviral reaction as in EBV-positive and -negative LCLs the replication of vesicular somatitis virus (VSV) was equally inhibited after IFN treatment. In addition, the results of Ruf *et al*. (2005) suggest that the putative antiviral effect is not carried out via the PKR pathway, as EBERs do not inhibit the phosphorylation of the PKR.

In transfection experiments the EBERs were shown to induce the expression of IGF-1 (Insulin-like growth factor-1, a growth factor for epithelial cells) in EBV-negative NPC cells. On this way, the small EBV-encoded RNAs can enhance the growth potential of epithelial cells and contribute, thereby, to tumorigenesis. Treatment with a PKR inhibitor did not induce IGF-1 expression, suggesting that a **PKR-independent** mechanism is involved in this tumorigenic effect of EBERs (Iwakiri *et al*., 2005).

Samanta *et al*. (2006) found that both EBER transcripts are recognised by the product of the retinoic acid inducible gene I (**RIG-1**), that is a cytosolic protein responsible for the detection of dsRNAs and for the initiation of the antiviral IFN response through the NFκB and the IRF-3 pathway. This mechanism was further studied and was evaluated that the recognition of EBERs by RIG-1 resulted in the activation of the IL-10 promoter through the IRF-3 but not the NFκB pathway. This EBER-dependent production of IL-10, the autocrin growth factor for B lymphocytes, can also provide an explanation for the tumorigenic effect of the EBER RNAs (Kitagawa *et al*., 2000; Samanta *et al*., 2008).

Beside B cells, EBV is associated with various T cell proliferations. In these cases EBV expresses the latency program II. *In vitro* studies showed an increased production of the T cell growth factor IL-9 in EBV-carrying T cells. Infection of T cells with EBER-deleted EBV caused significantly decreased IL-9 production, supplying evidence for the EBER-dependent activation of the IL-9 promoter (Yang L. *et al*., 2004).

It was an open question for a long time, whether the EBER genes contribute to the tumorigenic potential of the Epstein-Barr virus. Transfection of B cells with EBER-deleted EBV could transform B cells to LCLs and caused cell proliferation indistinguishable from the complete, EBER-carrying virus. However, in a later experiment the cells infected by EBER-deleted virus grew remarkably slower than cells carrying the wild-type virus, particularly at lower cell density (Swaminathan *et al*., 1991; Yajima *et al*., 2005). The transfection of the EBER locus into EBV-negative Akata (BL) cells restored the growth capacity and tumorigenicity of the cell line with a parallel elevation of the Bcl-2 antiapoptotic protein (Komano *et al*., 1999). In a further study Komano and Takada (2001) demonstrated that the elevation of the Bcl-2 protein level in BL cell lines is an important but not the only pathway involved in tumorigenesis. Using the same system, an other group found that stable expression of the EBER RNAs had a significant effect on the tumorigenic potential, but did not completely compensate for intact EBV. Regarding that PKR is suggested to have a tumour suppressor activity, it is conceivable that EBERs contribute to tumorigenesis through the inactivation of the antiviral PKR protein. This tumorigenic effect seemed to be independent of the modulation of apoptotic sensitivity (Ruf *et al*., 2000; 2005). The production of different cell cycle regulatory proteins can be modulated by activating Fas expression and the activation of the JAK/STAT (Janus-kinase/„signal transducers and activators of transcription") pathway in an IFNα-dependent manner. As a consequence, the multistage process of malignant cell transformation can be promoted by the inhibition of the PKR protein (Nanbo *et al*., 2002). Wu *et al*. (2007) transformed B cells using recombinant EBV constructs carrying either EBER1 or EBER2 and concluded that EBER2, but not EBER1 is responsible for the high transforming ability. They also observed an enhanced IL-6 expression probably mediated by the activation of the **RIG-I** protein.

Table 1.: Binding partners and putative functions of EBERs

Binding partner	Pathway, process affected	Consequence
U4	RNA splicing	Failure in RNA maturation ?
L22	Ribosome assembly	Impaired protein translation ?
La-antigen	Pol III transcript stability TLR-3, IFN	Contribution to tumorigenicity Immunactivation, IL-12 ↑
PKR	eIF-2α	blocking the IFN antiviral resp ?
PKR ?	IGF-I	Epithelial growth promotion

RIG-I	IRF-3 ?	IL-10 ↑, B cell growth IL-6 ↑
?	?	IL-9 ↑, T cell growth Bcl-2 ↑, avoid apoptosis

Although there are several known binding partners for EBERs, their exact role in tumorigenesis is not completely clarified. Probably our knowledge in this field will further expand and we are going to get closer to the understanding of the pathogenetic function of the EBER genes.

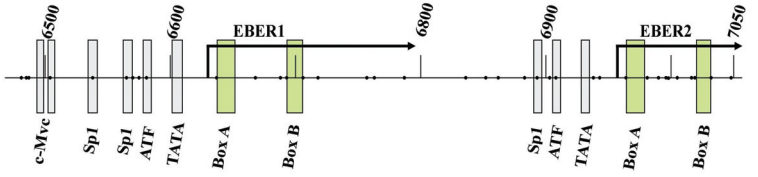
2.1.3. The EBER promoter

The regulatory regions of the EBER genes are considerably complex and this complexity may contribute to the high level of EBER expression in various cell lines. 5' from the transcription initiation site typical RNA polymerase II (Pol II) motives (TATA-box, activating transcription factor (ATF) binding site, Sp1 binding site, etc.) are located. The coding regions contain so called Box-A and Box-B elements, which are characteristic for a certain class of RNA polymerase III (Pol III)-transcribed genes (see **Figure 2**.; Howe and Shu, 1989). RNA polymerase III-transcribed genes can be divided into 4 types according to their structural characteristics. "Type 1" promoters consist of three intragenic elements, the Box A, Box C and the intermediate "I" element, like the 5S rRNA genes. "Type 2" promoters have the Box A and Box B sequences also intragenic. Most tRNA genes, the VA RNA-coding genes of adenoviruses belong to this group. The U6 and 7SK genes, however, have upstream regulatory sequences. These genes are termed "type 3" Pol III genes. In the case of "type 4" promoters the regulatory sequences consist of intragenic and external promoter sequences as well. EBER genes of EBV among others, are members of this pol III transcribed group of genes (see **Figure 2**., Paule and White, 2000; Vilalta *et al*., 2004).

In experiments with the Pol II blocking agent α-amanitine no inhibition of **EBER transcription** could be observed at a concentration that inhibited the activity of RNA polymerase II (Lindell *et al*., 1970). This suggested that the EBER genes are transcribed by Pol III (Jat and Arrand, 1982). Contrary, the upstream (Pol II) regulatory elements are also indispensable for the highly active promoter usage. According to

transfection experiments, deletion of the upstream sequences resulted in >95% inhibition of the EBER1 and EBER2 activity. Deletion of sequences upstream from the -80th position did not have significant effect on gene activity showing that the most important activating factors bind within the close proximity of the initiation site (Howe and Shu, 1989). In addition, our research group described the enhancing effect of the oncoprotein c-Myc on the EBER1 promoter activity, when bound to its binding site cca. 130 bp upstream from the initiation site (Niller *et al*., 2003). The latent replication origin (*ori*P) in the presence of the EBV nuclear antigen 1 (EBNA1) could increase EBER gene activity 2-4 fold, too (Wensing *et al*., 2001). In addition one can suspect, that the CTCF (CCCTC Binding Factor) binding sequence cca. 350 bp upstream of the EBER1 start site may enable the realization of the enhancing effect of *ori*P on EBER transcription (Day *et al*., 2007; also see 2.2.). Interestingly, the substitution of the non-consensus TATA sequence with a typical TATA-box permitted the transcription of the EBER genes by RNA polymerase II (Howe and Shu, 1993). Presumably, the TATA binding protein (TBP) is responsible for this phenomenon, because it binds to the unconventional TATA sequence in the EBER promoter region in an opposite orientation (Wang and Stumph, 1995). It was also demonstrated that transfected EBER1 in itself, i.e. not in the context of the EBV genome, showed a decreased transcriptional activity compared to the level achieved in cells carrying latent EBV genomes. This could be explained by the elevated expression of TFIIIC (Transcription Factor IIIC) and the BDP1 – a subunit of TFIIIB – proteins in EBV-carrying cells. TFIIIC and BDP1 are indispensable for Pol III-driven transcription (Felton-Edkins *et al*., 2006).

Figure 2.: EBER regulatory region



Grey rectangles indicate Pol II elements (binding sites for c-Myc, Sp1, ATF; TATA sequence), green rectangles indicate Pol III elements (Box A and Box B). Arrows indicate transcription initiation sites and the direction of transcription. Numbers above the line indicate positions within the prototype B95-8 EBV sequence (Baer *et al*., 1984).

The **epigenetic** regulatory **mechanisms (**see 2.2.) effecting the activity of the EBER promoters were not extensively studied yet. Minarovits *et al*. (1992) determined that all CpGs within CCGG, GCGC sequences of the EBER locus were unmethylated in all of the examined cell lines with the exception of a single *Hpa* II site. Because there are both methylation-sensitive and -insensitive Pol III transcribed genes, and the EBER genes were not found to be silenced and methylated in any *in vivo* samples, it is not obvious whether the EBER promoters are sensitive to methylation. Histone modifications within the EBER region were examined by Chau and Lieberman (2004) who observed a general enrichment of the activating modification, methylated lysine 4 of histone H3. The acetylated H3 and H4 was also enriched within the EBER region in a latency type I BL and a lymphoblastiod cell line, but later it could not be veryfied (Day *et al*., 2007). The acetylation of lysine 9 of histone H3 – generally associated with open chromatin – was also abundant in the EBER region of latency type I and type III BL cells lines. The inhibitory lysine 9 methylation of histone H3 was excluded from the EBER region in a latency type I and a type III cell line (Chau and Lieberman, 2004; Day *et al*., 2007).

The active expression and the activating epigenetic characteristics of the EBER locus are further supported by micrococcal DNase digestion, showing a nucleosome-poor chromatin state within the Matrix Attachment Region (**MAR**) of the EBV that includes the latent replication origin (*ori*P) and the EBER region (Jankelevich *et al*., 1992; Wensing *et al*., 2001).

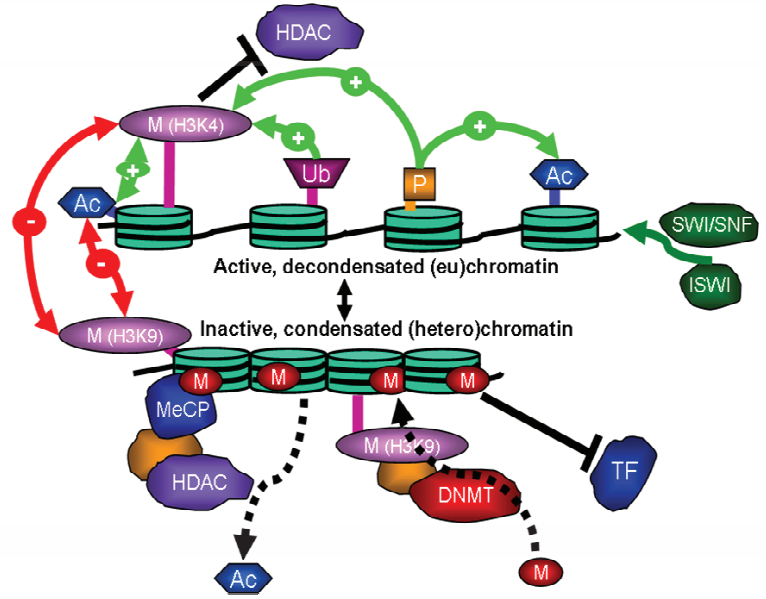
Taken together, the regulatory mechanisms affecting EBER expression are fairly versatile and seem to be complex.

2.2. Epigenetic gene regulation

The genome, besides genetic information determined directly by the sequence of bases, contains further epigenetic information due to post-synthetic modifications. The epigenetic modifications affect gene expression through the modulation of the chromatin structure and the binding of different transcription factors. Their two fundamental types are DNA-methylation and histone modifications, which are however,

shown to be tightly connected with the nowadays extensively studied RNA-interference (RNAi) and the Polycomb repressor protein complexes (Guitton and Berger, 2005). In plants the mechanisms of RNAi-directed DNA-methylation is extensively studied and one can speculate that a similar mechanism may be present in mammalians as well. In addition, the existence of an RNAi-based chromatin modification machinery was also described to act and promote chromatin remodelling together with Polycomb group proteins (Fire *et al*., 1998; Bartel, 2004; Kim *et al*., 2008; Verdel *et al*., 2009). In this respect, the RNA-interference and the Polycomb proteins can be regarded as complementing mechanisms of the fundamental epigenetical regulatory ways, i.e. DNA-methylation and histone modifications. In eukaryotes the methylation of the DNA occurs at CpG dinucleotides and in most cases causes the inactivation of the chromatin region. The modulation of the chromatin structure is influenced directly by the modification of the histone amino acid side chains with acetyl, methyl, phosphoryl, ubiquitine and sumoyl groups (Strahl and Allis, 2000). The main **role of epigenetic regulations** is to assure and coordinate the expression of genetic information at the right time and in the right place and amount. These mechanisms manifest at the gene expression level and influence all the important biological processes within a cell. Epigenetic dysregulation can be involved however, in tumorigenic processes (Laird and Jaenish, 1996; Pfeifer *et al*., 2000; Robertson and Jones, 2000). The main interactions and the mechanism of action of the DNA-methylation and the histone modifications are summarized in **Figure 3.**

Figure 3.: Mechanisms of the epigenetic regulation at the chromatin level



In case of activated chromatin structure, DNA-demethylation and -acethylation (Ac), phosphorylation (P), ubiquination (Ub) of certain histone amino acid side chains, furthermore methylation (M) of some postions (e.g. histone 3 lysine 4: H3K4) can be observed. DNA-methylation can block transcription through direct inhibiton of transcription factor (TF) binding and forming a condensed chromatin structure. This occurs through recruiting histone deacethylases (HDAC) to a specific location mediated by methylated cytosine binding proteins (MeCP). Due to H3K9 methylation, the surrounding DNA becomes methylated. Characteristic modifications can be observed among certain histone side chain modifications (red arrow: inhibition, green arrow: activation). SWI/SNF (SWItch/Sucrose NonFermentable) and ISWI (Imitation SWItch): ATP-dependent chromatin remodelling complexes.

2.2.1. CpG-methylation

Beside the 4 basic building blocks of the DNA, so called rare nucleotides also can be found within the genomes of various organisms. These are generated via a post-replicative modifications after the synthesis of the polynucleotide chain.

In eukaryotes the most widely studied modification is the methylation of cytosines at the 5th position, converted into 5-methyl-cytosines (5^{m}C). The majority of methylated cytosines is located within the 5'-cytosine-guanine-3' (CpG) palindrome dinucleotide. 5^{m}C is about 2-10% out of the total amount of cytosines found in vertebrate DNA (Gruenbaum *et al.*, 1981). Multiprotein complexes containing DNA-(5-cytosine)-methyltransferases (DNMT) participate in the generation of the global methylation pattern of the genome (Razin and Riggs, 1980; Ehrlich and Wang, 1981). For the maintenance of the original methylation pattern of the entire genome during cell division the DNMT1 enzyme is responsible. This protein ensures, that the newly synthesized DNA strand has not only the same genetic but also the same epigenetic information content as the template DNA. There are cases when new methylation patterns need to be formed, like in the steps of cell differentiation and tissue development. This process requires other enzymes – the DNMT3A and DNMT3B – that configure the new methylation pattern according to the actual needs, using their *de novo* methyltransferase activity. The mutation of the DNMT3B gene causes ICF syndrome, which is a rare recessively inheritable genetic disease (Immundeficiency, Centromeric heterochromatin instability and Facial disorder, Robertson, 2001). Because the methylation pattern of the genome undergoes dynamic changes, it is obvious, that CpG-methylation is reversible. The demethylation can happen passively during replication, if the maintenance methyltransferase (DNMT1) does not methylate the doughter DNA strand according to the template. Other mechanism can be the active demethylation of the 5^{m}cytosine by the MBD2b (methyl-CpG-binding domain 2b) protein (Ramchandani *et al*., 1999) or with base excision mechanism mediated by the 5-methyl-cytosine-DNA-glycosylase (5-MCDG, Jost *et al*., 1995). These active processes could not be veryfied by others, so the mechanism of demethylation is still unexplored (Ng *et al*., 1999; Wade *et al*., 1999).

In 1972 an other modification was described in animals, but until a while it could not be detected by any other groups (Penn *et al*., 1972; Gommers-Ampt *et al*.,

1995). The hydroxymethylation of cytosines (**hmC**) is discovered again and was found at 40% frequency compared to the 5^{m}C in Purkinje neuronal DNA (Kriaucionis and Heintz, 2009). Yet there is no information about the role of this modification and is only ahead of deep experimental studies.

There are different models how the methyl group of cytosines at the 5^{th} position can affect the transcriptional activity of a certain gene. One and possibly the most staightforward mechanism is the **direct way**, when the transcriptional factor is simply not able to bind to its recognition sequence, because of the methyl group (e.g. c-Myc/Myn, E2F, NFκB). This means a sterical interference between the methyl moiety and the DNA binding protein. The surface of the recognition site is altered due to the methyl group and the binding pocket is not able to bind it anymore. This theory can only supply explanation for the inhibition of protein binding, in case when there is at least one CpG motif within the recognition sequence (Prendergast *et al.*, 1991). This way of inactivation can be suitable for a single gene or smaller genomic regions but the inactivation of larger gene clusters or even complete chromosomes can not be explained. On the other hand, the binding of the CTCF protein to the mouse Igf-2 regulatory region was shown to be impaired by promoter methylation allowing the transcription of the mRNA, suggesting a methylation-sensitive binding of the protein. The role of CTCF in genomic imprinting at the mouse H19/Igf-2 locus was also proven and as followes, in a methylation-intolerant manner. The CTCF protein was shown to act by blocking the binding of enhancers to the promoter region; the enhancers loop to the sequence from Kbases away (Bell and Fenseld, 2000). Our research group could not verify, however, that CTCF binding is inhibited by CpG-methylation as in EBV-carrying cells the CTCF could bind to the strongly methylated Rep*-Cp sequence (Salamon *et al.*, 2009). These results were important to understand the connection between local methylation and the long-range regulation of gene activity.

Other results showed that in the majority of cases DNA-methylation can influence gene activity from a larger distance with some delay, causing the time consuming formation of a compact chromatin structure. This mechanism (**indirect way**) was supposed to be induced by proteins that are able to selectively recognise and bind methylated cytosines. Methyl-CpG binding protein 2 (MeCP2) was the first described protein having the above mentioned function, with a 5^{m}CpG-binding-domain (MBD) and a transcriptional repression domain (TRD, Lewis *et al.*, 1992). Having these two functional subunits, the binding of activating transcription factors – with a CpG-

containing recognition sequence – can simply be impaired by occupying the methyl-CpG site by MeCP2. In addition, the recruitment of chromatin remodelling enzymes (SWI/SNF) or histone deactylases (HDAC) to the promoter region by the TRD of MeCP2 triggers the condensation of the chromatin, inhibits the assembly of the transcriptional initiation complex and as a consequence, results in the subsequent inactivation of the region (Robertson, 2001; Razin, 1998; Ehrlich and Wang, 1981).

The ancient **function** of the DNA-methylation possibly differed from its recent role. This can be speculated, because in bacteria it serves to mark the own genomic DNA and to discriminate foreign nucleic acids. As a consequence, bacteria are using their methylation-sensitive restriction enzymes to digest foreign DNA, but their own "modified" genome is preserved. According to different theories, the role of DNA-methylation in higher eukariots was to act as a defensive system against parasite DNAs, transposones and viruses. With the expanding number of genes, the function of DNA-methylation has modified; it became involved in the inactivation of genes intended to be silent at a time. Recent data show that DNA-methylation plays a key role in regulating several biological processes, like embriogenesis and tissue differentiation (Li *et al*., 1992; Okano *et al*., 1999), the appropriate structural organisation of chromosomes (Jones and Wolffe, 1999), X-chromosome inactivation (Panning and Jaenisch, 1996), genomic imprinting (Li *et al*., 1993), inactivation of transposones, retroviruses, integrated viral genomes (Walsh *et al*., 1998; Yoder *et al*., 1997; Doerfler *et al*., 1995), and it has an important role in tumorigenesis, too. The effects of DNA-methylation on the transcription are responsible for the majority of the listed functions. This is fulfilled through the methylation of CpGs located in the promoter region of genes. Hence CpG-methylation has a proven fundamental physiological role, the examination of this process could be essential.

In the formation of the appropriate chromatin structure at promoters, **DNA-methylation and the histone modifications** act together. The MeCP2 and the later described MBD1-3 proteins have the ability to form complexes with histone deacetylases (Jones *et al*., 1998; Ng *et al*., 1999; Wade *et al*., 1999), leading to the deacetylation of the amino acid side chains in their proximity and the inhibition of activating factor binding by the formation of a compact chromatin structure (Fischle *et al*., 2003). In this way, huge protein complexes are included in the methylation-dependent gene silencing processes (Li, 1999; Jones and Wolffe, 1999; Robertson, 2001). It was also observed, that the activating di- and trimethylation of H3K4 is

excluded from the 200bp proximity of the DNA methylation to make a free way for inactivating mechanisms (Okitsu and Hsieh, 2007).

The inhibitory effect of **CpG-methylation** for the RNA polymerase II transcribed promoters is supported by the vast majority of experimental data. In the case of **Pol III**-transcribed RNAs the situation is not that obvious. There are only limited and controversial observations and no general conclusion can be made in this respect. The *in vitro* methylation of the chicken $tRNA^{lys2}$ gene at the *Hpa*II and *Hha*I sites had no inhibitory impact on the transcription, while the methylation of all the CpG dinucleotides resulted in a complete transcriptional block. The methylation of the *Xenopus laevis* somatic and oocyte type 5S rRNA-coding sequence did not prevent the transcription of the transfected gene, however (Besser *et al.*, 1990). Similar results could be experienced in the case of 5S rRNA-coding gene of *Arabidopsis thaliana in vitro* (Mathieu *et al.*, 2002). *In vitro* and *in vivo* approaches showed that the CpG-methylation can silence the transcription of Alu elements depending on the gene it is associated to. Alu elements of the human angiogenin, α_1-globin and tissue plazminogen activator (tPA) genes are sensitive for promoter methylation (Kochanek *et al.*, 1993; 1995) that leads to changes in protein binding within the terrirory of the Box B *in vitro* (Kochanek *et al.*, 1995). In contrast, interference of DNA-methylation with the transcriptional activity and protein binding to the Box B of the Alu sequence located within the first intron of the ACTH (Adrenocorticotropic hormone) gene could not be observed (Kochanek *et al*., 1995). Several other Alu elements were found to be methylation-sensitive (Li *et al.*, 2000; Liu and Schmid, 1993; Englander *et al.*, 1993). The VAI RNA-coding gene of the type 2 Adenovirus is quite similar to the EBER RNAs and is also transcribed by Pol III. The methylation of the *Hha*I and *Hpa*II sites inhibited the RNA transcription under *in vitro* circumstances but did not affect the activity when transfected. The methylation of all the CpGs in the complete region resulted the inactivation of the promoter in transfection and *in vitro* experiments as well (Jüttermann *et al.*, 1991).

Because of the controversial data between DNA-methylation and the gene activity of Pol III-transcribed genes and the importance of the understanding of regulatory mechanisms behind disease-connected genes, it is indispensable to examine such genes as the EBERs in epigenetical respect.

2.2.2. The role of DNA-methylation in the regulation of latent EBV promoters

In the DNA sequence of the Epstein-Barr virus the abundance of CpG dinucleotides is relatively low compared to the average TpG and CpA frequency. This observation suggests that similarly to other gammaherpesviruses the CpGs of the EBV genome are strongly methylated in latently infected host cells and play an important role in EBV gene regulation (Honess *et al.,* 1989; Takacs *et al.,* 2001). In the case of several latent EBV promoter regions the role of CpG-methylation in the regulation of gene activity was proven using methylation-sensitive and -insensitive restriction enzyme paires and subsequent Southern-blotting technique. The fine mapping of the CpG-methylation pattern in the latent regulatory sequences was later also performed using the bisulphite sequencing method (see 4.4.). In these experiments a wide range of EBV-carrying cell types and tissues were used as follows:
Burkitt's lymphoma biopsies and BL-derived cell lines, lymphoblastoid cell lines established *in vitro*, biopsies of nasopharyngeal carcinomas and cell lines derived from the biopsies, tumours grown in nude mice, gastric carcinoma biopsies, Hodgkin's lymphoma samples, lethal midline granulomas, lymphoproliferative manifestations of immuncompromised patients and B cells isolated from the blood samples of healthy individuals.

According to the experimental results the majority of the latent regulatory sequences of the EBV: the LMP promoter regulator sequence (**LRS**) (Minarovits *et al.,* 1991; 1994a; 1994b; Hu *et al.,* 1991), the **C promoter** (Minarovits *et al.,* 1994a; 1994b; Robertson *et al.,* 1995; 1996; Robertson and Ambinder, 1997a; 1997b; Takacs *et al.,* 1998; Bakos *et al*., 2007) and the **W promoter** (Tierney *et al.,* 2000) are extensively hypomethylated or completely free of CpG-methylation when transcriptionally active. In addition, the same promoters are strongly methylated when inactive, showing methylation-sensitive gene expression. On the contrary, the **Q promoter** was found to be completely unmethylated independently of the latency type and promoter activity (Tao *et al.,* 1998a; 1998b; Salamon *et al*., 2001; Bakos *et al*., 2007), although *in vitro* methylation of the promoter inhibited its transcriptional activity (Tao *et al*., 1998b). In the case of the **LMP2A promoter** there was a good correlation between the promoter activity and the CpG-methylation pattern. Active promoters were unmethylated, while

silent promoters were found to be methylated with variable hypomethylated patches (Salamon *et al*., 2003; Gerle *et al*., 2007). The locus coding for the **EBER1 and EBER2** small RNAs of EBV was also unmethylated, with the exception of a single *Hpa*II site in all the examined samples (Minarovits *et al.,* 1992). The DS element of *ori*P the latent DNA replication origin of EBV – and the approximately 100bp long flanking regions on both sides were also unmethylated, independently of the latency program. On the other hand, CpGs between the DS sequence and the Family of Repeats (FR) located approximately 1kb upstream from the DS element were unmethylated in cell lines with latency type III and are variantly methylated in latency type I cells (Salamon *et al*., 2000).

A role for DNA-methylation in the regulation of viral gene expression pattern was demonstrated by the treatment of the BL cell line Rael (latency type I) using the DNA-methyltrasferase inhibitor 5-azacytidine (AzaC), resulting in the activation of the lytic replication program in a subpopulation of cells, while a bigger portion switched to a latency type III-like expression of genes with the induction of EBNA2-6 and the LMP proteins with a parallel demethylation and activation of the W and C promoters (Masucci *et al*., 1989; Robertson *et al*., 1995b; Robertson and Ambinder, 1997b).

In vitro methylation of the W, C and Q promoters, as well as the LRS had an inhibitory effect on promoter activity (Jansson *et al*., 1992; Minarovits *et al.,* 1994b; Robertson *et al.,* 1995; Robertson and Ambinder, 1997b; Tao *et al*., 1998b). In contrast, methylation of the *ori*P region of the EBV enhanced the activity of Cp, located approximately 2kb distance downstream of the latent replication origin (Robertson and Ambinder, 1997b).

2.2.3. Histone modifications

Chromatin is composed of DNA, histones and non-histone proteins, respectively. The genomic DNA is wrapped around an octamer of histone proteins (dimers of histone H2A, H2B, H3, H4) forming the core of the nucleosomes. These ~150 bp long repetitive units are linked to each other with the so-called linker DNA, coupled with the 5th histone protein H1. The distinct levels of chromatin organization

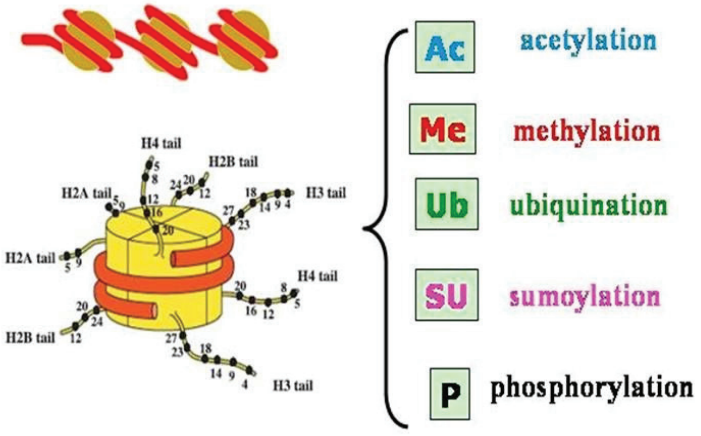
are dependent on the dynamic higher order structuring of nucleosomes. Due to their essential function, histone sequences are highly conserved (Luger *et al*., 1997).

Histone modifications affect lysine amino acids predominantly at the N-terminal tail of histones. The ε-amino group of lysine may become acetylated, methylated, ubiquitinated and sumoylated, while the serin residues may undergo phosphorylation (**Figure 4.**). Furthermore, the methylation of the arginine side chains is also possible (Lachner *et al.*, 2003; Ehrenhoffer-Murray, 2004). While histone acetylation and arginine-methylation can be correlated to the gene expression activity, phosphorylation is connected to mitotic chromosome condensation (Goto *et al.*, 1999; Wei *et al.*, 1999), the effect of histone methylation can not be generalized. The methylation of certain lysines (K) (e.g. H3K4me1-3) can be related to the formation of euchromatin with active promoter usage, while others (e.g. H3K9me2-3 or H3K27me2-3) associate to the heterochromatin state of a chromosome region, which harbours inactive DNA sequences (Barski *et al.*, 2007). Ubiquitination of side chains leads to increased activity through the degradation of histones and the dissassembly of the chromatin, whereas sumoylation causes transcriptional inhibition by reversible binding to the histone and non-histone proteins of the chromatin (Lee and O'Connell, 2006; Gill, 2005; Iniguez-Lluhi, 2006). Furthermore, it was observed, that the ADP-ribosylation level of histones increased following DNA-damage, suggesting a role of this modification in DNA-repair mechanisms (Althaus *et al*., 1994). Because histone modifications are reversible processes, the histone modification patterns are coordinated by antagonistic enzyme pairs.

Out of the numerous modifications the acetylation of the histones is the most intensively studied. Previously, it was only thought to participate in the control of gene activity (Struhl, 1998), but recently, its participation in gene silencing (Deckert and Struhl, 2001; Mulholland *et al.*, 2003; VanLint *et al*., 2006; Lallemand *et al*., 2006), in cell cycle regulation and DNA-repair mechanisms has been confirmed as well (Carozza *et al*., 2003).

Histone acetylation is mediated by histone acetyltransferase (**HAT**) enzymes by catalyzing the translocation of an acetyl group of acetyl-CoA to the ε-amino group of a lysine predominantly in the N-terminal region of histones. Most of the HAT enzymes are found in huge protein complexes containing several subunits *in vivo*. The non-catalytic subunits coordinate the function of the catalytic subunits (Roth *et al*., 2001; Carozza *et al*., 2003).

Figure 4.: Types of histone modifications (Verdone *et al*., 2005)



Like histone acetyltransferases, histone deacetylases (**HDAC**) operate as parts of enormous complexes involving different repressors and corepressors like the Polycomb complexes. HDAC enzymes have different classes in mammals. Enzymes belonging to the class I. group are located almost exclusively in the nucleus, while class II HDACs are shuttling between the nucleus and the cytoplasm. Furthermore, different HDAC levels can be observed during the different stages of the embryonic development, what could refer to the participation of various HDACs in the sequential steps of embryogenesis (McGraw *et al*., 2003).

In most cases, **transcription**al activity increases due to the influence of histone acetylation, which can be discussed in different ways. On one hand, the positively charged lysine side chain is neutralized by the acetyl group, thereby the interactions between the lysine side chain and the negatively charged DNA-phosphate backbone and also between the negatively charged amino acids of different histone tails dissociate. As a consequence, the compact chromatin structure loosens, thus the DNA becomes accessible for the transcriptional protein complex. Besides, it facilitates the sliding of the nucleosomes setting the transcriptional initiation site free (Kingston and Narlikar, 1999). On the other hand, the acetylated histone provides a new surface for histone-

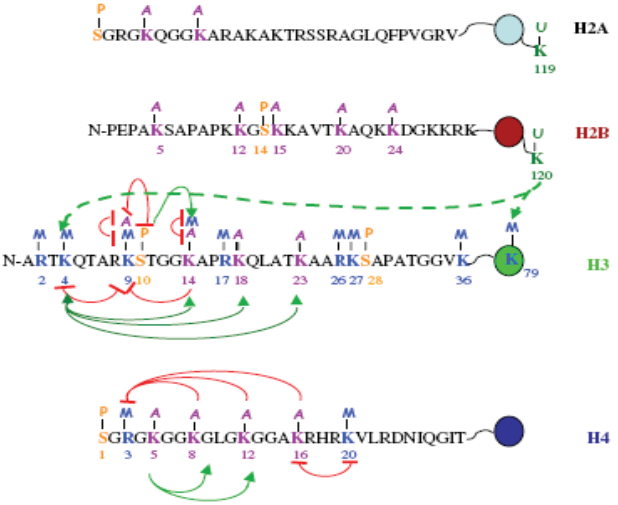
binding proteins with the appropriate binding site for recognition. One of the known protein domains can specifically recognize acetylated lysine residues is the so-called bromodomain, being an elemental part of several proteins involved in the transcription and chromatin regulating processes (Yang X-J., 2004).

Beside acetylation, **methylation** is an other frequently occurring modification in the genome. The first enzymatic histone methyltransferase activity was described by using histone H3 as a substrate (Rea *et al.*, 2000). As opposed to other covalent histone modifications, this modification was considered to be irreversible, because the N-CH_3 binding is thermodynamically stable. This idea was discarded, however, when Shi and coworkers discovered a lysine-specific histone demethylase (LSD1) – a homologue of amine oxidases – inducing the **demethylation** of the methylated histone 3 lysine 4 site (H3K4) according to the first experiments (Shi *et al*., 2004). One, two or three methyl groups can be attached to the lysine side chains. The substrate for recombinant LSD1 is the mono- or dimethyl H3K4, whose demethylation is performed by the catalyzation of an oxidative reaction. The specifity of LSD1 may be influenced by different cofactors found in the same protein complex. The distinct histone H3 modifications of other lysine side chains may have an impact on the activity of LSD1. Although H3K4-demethylation is not affected by the methylation of H3K9 – having a repressor effect on transcriptional activity –, other modifications like the acetylation of H3K9 or the phosphorylation of Ser10 decrease the affinity of LSD1 for methylated H3K4 synergistically (Forneris *et al*., 2005). Based on recent studies, it became clear that the methylation of certain lysines (e.g. H3K4me1-3, H3K27me1, H3K36me3, H3K79me3) can be connected to gene activation, while others (e.g. H3K9me2-3, H3K27me2-3) to gene silencing. In addition, using the method of ChIP-on-chip it was also mapped that there are differences in the localization of variant modified histones in the regulatory and coding regions of genes. For instance, it was found that the H3K4me2 shows a significant discrepancy up- and downstream of the transcription initiation site, as opposed with the H3K4me3 modification, which was enriched mainly around the transcription initiation site (Barski *et al*., 2007).

The mechanisms of histone acetylation, and -methylation are interconnected in the regulation of transcription, as supported by the fact, that there are histone deacetylases in the CoREST/LSD1 complex as well. Since LSD1 binds to deacetylated substrate with a greater affinity, it is thought, that H3K4-demethylation catalyzed by LSD1 is preceded by a histone deacetylation step (Kuppuswamy *et al*., 2008).

If we consider the many possible positions for modification and the possible kinds of modifications, it is easy to imagine that the regulation of gene activity by histone modifications is extremely complex. How these modifications can be integrated and interpreted is still a matter of debate. Two competing **hypotheses** suggest possible explanations. According to one of them, the effect of histone modifications is based only on a *structural principle*, while the other one suggests the existence of a so-called "*histone code*". The latter one means, that various combinations of different histone modifications have distinct effects on the chromatin structure and as a consequence on the transcriptional activity. (Santos-Rosa and Caldas, 2005; also see **Figure 5**). Due to the great number of combinations the information content of the chromatin is extremely high according to this hypothesis, and can serve a good regulatory way for the fine-tuning of gene expression (Strahl and Allis, 2000; Jenuwein and Allis, 2001).

Figure 5.: Mutual interactions of histone modifications.



The amino acid sequences (S=Serine, K=Lysine, R=Arginine) of the corresponding histone proteins (H2A, H2B, H3, H4) and their modifications (acetylation: **A**; methylation: **M**; ubiquitination: **U**; phosphorylation: **P**) are illustrated above. Green arrows mark positive, red arrows indicate negative effects between the different modifications (Santos-Rosa and Caldas, 2005).

2.2.4. The role of histone modifications in the regulation of latent EBV promoters

Within the latently infected host cells the double-stranded Epstein-Barr virus genome persists as an episome. Using the proper protein products of the host it forms a chromosome-like structure. The expression of several latent viral gene products is regulated by various histone-modifying cellular enzymes that affect the local chromatin structure and the binding of proteins inhibiting or activating transcription (see below).

The exact role of histone modifications in the regulation of EBV latency and the corresponding gene expression pattern was not deeply investigated yet, although, the importance of this epigenetic mechanism was evaluated in different EBV-carrying cell lines. Histone modifications and their importance can be studied among others with immunoblotting, Western-blotting, chromatin immunoprecipitation (see: 4.10.; using highly specific antibodies against different modifications), or with the application of the histone deacetylase inhibitor Trichostatin A (TSA).

Examinations of the LMP promoter regulatory sequence (**LRS**) showed that the level of acetylated histone 3 and histone 4 (acH3, acH4) correlates well with the LMP1 gene activity in BL-derived cell lines and lymphoblastoid cells (Sjöblom-Hallén *et al*., 1999; Wang *et al.*, 2000; Park and Faller, 2002; Chau and Lieberman, 2004), and in a nasopharyngeal carcinoma cell line as well (Nishikawa *et al.*, 2004). In NPCs the activating effect of the histone deacetylase inhibitor, TSA was also demonstrated on the expression of the LMP1 protein. Besides, it was evaluated that the EBNA2 protein has a positive impact on the LMP1 promoter activity in concert with histone acetyltransferases (Wang *et al*., 2000; Alazard *et al*., 2003). A positive correlation could be observed between histone-acetylation levels and transcriptional activity in the case of the **C promoter**, too (Alazard *et al*., 2003; Chau and Lieberman, 2004; Fejer *et al*., 2008). This was further supported by the results of Knight *et al*. (2003), as EBNA3C – possessing an inhibitory effect on the Cp – was proven to form complexes with histone deacetylases. According to our present knowledge the **Wp,** the **Qp** and the **EBER** regulatory sequences are enriched in acetylated histones when actively used, while the inactive Wp and Qp are poor in these activating modifications (Chau and Lieberman, 2004; Day *et al*., 2007; Fejer *et al*., 2008; for more details on EBERs also see 2.1.3.). In lymphoid cell lines the **LMP2Ap** activity could be correlated to histone acetylation, but

in the C666-1 NPC cell line, that does not use this promoter, a highly acetylated regulatory sequence was mapped. This suggests a different way of setting the promoter activity in B and NPC cells, and a complex mechanism behind, playing together with the DNA-methylation regulatory pathway (Gerle *et al*., 2007). The level of acetylated histones in the EBV genome was experienced to increase with the onset of the lytic viral cycle. As follows, the deacetylated state of histones presumably play a very important role in the retention of the latent form of the virus and in keeping the lytic viral promoters silent until the activation of the productive cycle, with a parallel increase of acetylated histones on the whole EBV genome (Chang and Liu, 2000; Jenkins *et al*., 2000).

Regarding the different methylations of histones, mainly H3K4 and the H3K9 modifications were examined on the EBV genome. While methylated H3K9 was highly enriched within inactive promoters, the active latent promoters were associated with a high level of methylated H3K4. This tendency was confirmed in case of **LRS**, **C promoter**, **W promoter**, **Q promoter** and the always actively trascribed **EBER promoters**, respectively. Unexpectedly, in C666-1 NPC cells inactive **LMP2A promoter** showed an enrichment of H3K4me2 modification, that suggests a role for the highly methylated LMP2A sequence in the silencing of the promoter. Gerle *et al*. noticed that the latency type I BL cell line Mutu I, that shows a higher abundancy of H3K4me2 in the LMP2Ap region compared to other latency type I cells, can be more easily activated by TSA treatment, suggesting that this modification may predispose genes to turn on promoter activity (Gerle *et al*., 2007). A similar observation was made by Fejer and coworkers in the case of Cp in Mutu I cells, supporting the importance of H3K4me2 modification in making the promoters susceptible for activating influences (Fejer *et al*., 2008). Probably due to the perpetual binding of the origo recognition complex (ORC) and EBNA1 to the latent replication origin of EBV, the *ori*P region was enriched in acetylated histones and methylated H3K4 (Chau and Lieberman, 2004; Day *et al*., 2007).

The acetylated H3K9 was found to be abundant only in the EBER region bearing a correlation with active promoter usage (Chau and Lieberman, 2004; Day *et al*., 2007).

3. OBJECTIVES

Because several lines of evidence suggest that the constitutively transcribed EBER genes contribute to oncogenesis, I aimed to study the possible mechanisms involved in the regulation of these genes in B cell lines of different origin (BL, LCL) and a nasopharyngeal carcinoma cell line.

1) Using the bisulphite sequencing method, I wished to **generate the detailed CpG-methylation maps of the EBER region** in different cell lines.

2) Applying quantitative real-time PCR, I planned to **determine the activity of the EBER1 and the EBER2 genes** in different cell lines.

3) I wished to transfect *in vitro* CpG-methylated EBER-carrying vector into EBV-negative cells in order to **analyse the effect of global CpG-methylation on the activity of the EBER1 and 2 promoters.**

4) **I wished to analyse protein-DNA interactions at the regulatory sequences of EBER1 in** the nasopharyngeal carcinoma cell line **C666-1**, using the *in vivo* genomic footprinting.

5) Using the method of *in vitro* DnaseI footprinting and electrophoretic mobility shift assay, I planned to **examine the binding of proteins to unmethylated and *in vitro* methylated EBER1 promoter** .

6) Using chromatin immunoprecipitation assay, I wished to **study the binding of selected transcription factors to the EBER1 promoter sequence *in vivo.***

7) I proposed **to quantify the abundancy of modified histones at the regulatory and the coding regions of the EBER locus.**

4. MATERIALS AND METHODS

4.1. Cell lines and cell culturing

The cell lines used in my experiments were well characterized, Epstein-Barr virus-carrying Burkitt's lymphoma cell lines (BL) and lymphoblastoid cell lines (LCL). I also studied C666-1, a nasopharyngeal carcinoma-derived cell line (**Table 2.**) The EBV-negative cell lines Hela (derived from a cervix carcinoma) and DG75 (BL) were used in transfection experiments. The EBV-positive cells kept their original latency type during *in vitro* culturing as judged by repeated analyses of their EBV latency promoter usage.

Cells were cultured in RPMI 1640 medium containing 10% FCS (Fetal Calf Serum), 2mM glutamine, 50 unit/ml streptomycin at 5% CO_2 content and 37 ^{o}C.

Rael and Mutu-BL-I-Cl-216 (Mutu I) cells expressed only the EBNA1 nuclear antigen (latency type I; Gregory *et al*., 1990; Altiok *et al*., 1992). Raji and Mutu-BL-III-Cl-99 (Mutu III) expressed EBNA1-6 and (latency type III). Mutu III was generated during a spontaneous latency switch of *in vivo* cultivated latency type I Mutu clones, thus, they carry the same EBV strain (Gregory *et al*., 1990; Altiok *et al*., 1992). The Raji cell line has a deletion in its EBV genome and as a consequence it can not enter into the lytic replication cycle (Hatfull *et al.*, 1988). The lymphoblastoid cell line CB-M1-Ral-Sto (CBM1) harbors the same EBV strain as the Rael cell line (Ernberg *et al*., 1989), whereas LCL-721 cells carry the B95-8 prototype strain (Baer *et al*., 1984; Metzenberg, 1990; Altiok *et al.*, 1992). The C666-1 nasopharyngeal carcinoma cell line (abbreviated as C666) was described initially as a latency type II cell line (Cheung *et al*., 1999), but later it was characterized as a latency type I cell line (Gerle *et al*., 2007). Hela is an EBV-negative cell line with an epithelial origin, while DG75 is an EBV-negative Burkitt's lymphoma cell line. All the EBV-positive cell lines included in my study show a highly active EBER1 and EBER2 transcription regardless of the latency type (Minarovits *et al*., 1992).

Table 2.: Cell lines and their main characteristics

Cell line	Cell type	Latency type	EBV genome
Mutu I	Endemic Burkitt's lymphoma	I	+
Rael			
Mutu III	Endemic Burkitt's lymphoma	III	+
Raji			
CBM1	Lymphoblastoid cell line	III	+
LCL-721			
C666	Epithelial cell line	I	+
Hela	Epithelial cell line	-	-
DG75	Sporadic Burkitt's lymphoma		

I, II, III means latency type I, II and III; '+' or '-' signs indicate the presence or absence of EBV genomes.

I determined the nucleotide sequences of the EBER region (nucleotides 6271-7174 of the B95-8 prototype strain, Baer *et al*., 1984) in the analysed cell lines (GenBank accession numbers: Rael, AJ315773; Mutu I, AJ315775; Mutu III, AJ315776; CBM1, AJ315774; Raji, AJ315772; C666, AM397661). LCL-721 was found to be identical with the B95-8 reference sequence.

4.2. DNA and RNA purification

DNA

The cell suspension or the scratched adherent cells (10^7) were centrifuged for 10 min at 1500g at 4°C. Pellet was washed 2x in ice cold Phosphate Buffered Saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, pH 7.4 in 1 liter of dH_2O) and after the last centrifugation step it was resuspended in 1 ml TE buffer (10mM Tris-HCl, pH 8.0; 1mM EDTA). Cells were lysed by adding 1.1 ml of lysis buffer (50mM Tris-HCl, pH 8.0; 20mM EDTA, pH 8.0; 2% SDS) and homogenized by repeated pipetting. Subsequently, the lysate was incubated at 55°C for 1-2 hours with Proteinase K (Roche) at a final concentration of 100 µg/ml. Samples were chilled and 400 µl saturated NaCl was added. After inverting the tubes several times they were let on the lab bench for 5

min. After centrifuging the samples at 2500g for 15 min at 4°C, the supernatant was transferred to a new tube and centrifuged again. Thereafter the supernatant was RNase-digested at 37°C for at least 1 hour. The samples were phenol-extracted by adding 1 volume of 0.5M Tris-HCl (pH 8.0) saturated phenol, followed by 10 min rotating at room temperature and centrifuged for 15 min at 5000g. Then the upper aqueous phase was transferred to a new eppendorf tube. The phenol extraction step was repeated two times. To get rid of any remaining traces of phenol the last transferred aqueous phase was thoroughly mixed with 1 volume of chloroform and centrifuged as in the phenol extraction step. The genomic DNA in the supernatant was then ethanol precipitated with 2 volumes of 100% ethanol and centrifuged at 4000g for 15 min. The formed pellet was rinsed with 70% ethanol and after drying the DNA was dissolved in DNase-free water. The concentration and purity of the DNA sample was meassured using spectrophotometer at the wavelengths of 260 and 280 nm.

RNA

The cell suspension or the scratched adherent cells (10^7) were centrifuged for 10 min at 1500g at 4°C. The pellet was resuspended in 1 ml of TRI Reagent (Sigma) by repeated pipetting. The samples were incubated at room temperature for 10 min and after the addition of 0.2 ml chloroform they were vortexed thoroughly. After 10 min incubation the samples were centrifuged for 15 min at 12000g and 4ºC. Three different phases could be observed: a lower, red one containing the proteins; a middle DNA phase; and an upper colorless phase with the RNA. The latter one was transferred into a new eppendorf tube and mixed with 0.5 ml isopropanol. After 10 min incubation at room temperature it was centrifuged with 12000g at 4ºC for 10 min. The pelleted RNA was washed with 70% ethanol and dried. The dry pellet was dissolved in RNase-free water (Gibco).

The purity and concentration of the samples were determined by meassuring the absorbances at 260 and 280 nm using a spectrophotometer.

In order to avoid contamination with genomic DNA, 5µl RNA was treated with 1U of RQ1 DNase (Promega) according to the manufacturer's instruction in 20 µl final volume for 30 min at 37°C. The reaction was stopped with the DNase Stop solution (2 µl of 20mM EGTA, pH 8.0) and the enzyme was inactivated at 65°C for 10 min. As a

consequence, the amplimers generated during the subsequent PCR reactions originated only from the RNA transcripts.

4.3. *In vitro* DNA-methylation and transfection

Using the enzyme M*Sss*I it is possible to methylate the cytosines of CpG motives within a sequence, *in vitro*. Because there is not a single known example for extensive methylation of the EBER region *in vivo*, I used an *in vitro* approach to compare the activity of the unmethylated and methylated EBER gene. After the *in vitro* methylation of an EBER region-harbouring plasmid, I could transfect the DNA transiently into EBV-negative cells and check for gene activity.

The plasmid pBS-EBER, containing the EBER sequence (nucleotides 6456-7162 of the B95-8 EBV genome, Baer *et al*., 1984), was a gift from Professor Hans Helmut Niller (Regensburg, Germany). 20 µg of pBS-EBER DNA was methylated (or mock-methylated) using the M*Sss*I CpG-methyltransferase (New England Biolabs) as advised by the manufacturer. The efficiency of methylation was confirmed using 1/10 of the reaction volume using the isoschisomers *Hpa*II and *Msp*I as described previously (Minarovits *et al*., 1991; 1992; 1994a; 1994b). Both enzymes recognize the same sequence (CCGG) but *Hpa*II is sensitive to CpG-methylation, while *Msp*I is methylation-insensitive. Thus, when I cut the methylated and mock-methylated plasmid DNA with these enzymes I could assess the effectivity of the methylation reaction by comparing the agarose gel run results of the restriction enzyme reactions. Methylated and mock-methylated pBS-EBER (9 µg, each) was cotransfected together with 1 µg of the plasmid pEGFP (encoding GFP, green fluorescent protein; BD Biosciences) into $5x10^6$ DG75 cells using DEAE dextrane as described earlier (Niller *et al*., 1991) or $3x10^5$ HeLa cells using Fugene reagent (Roche) according to the manufacturer's recommendation. The EGFP plasmid was used as an internal control of the transfection efficiency. DNA and RNA was isolated from the cells using TRI-reagent (Sigma) 48 hrs after transfection. The isolated DNA was analysed for CpG-methylation using bisulphite sequencing (see 4.4.). EBER1 and EBER2 RNA levels and expression of the cotransfected GFP gene and the cellular β-actin gene were analysed on a LightCycler 2.0 instrument (Roche) by real-time quantitative PCR (as detailed in 4.5., 4.6.). The

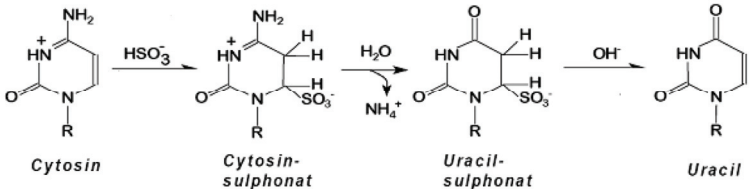
relative level of EBERs was calculated after correcting to GFP (transfection efficiency) and β-actin expression levels (RNA input).

$$\text{EBER promoter activity} = \frac{\text{EBER expression}}{\text{ACTB expression x GFP expression}}$$

4.4. Bisulphite sequencing

Using the bisulphite sequencing method it is possible to distinguish between methylated and unmethylated cytosines within the analysed DNA region. In single-stranded DNA methyl-cytosines ($5^{m}C$) resist to modification by bisulphite and hidroquinone and will appear as cytosines during PCR amplification, while unmethylated cytosines will be converted to uraciles (**Figure 6.**; Wang *et al*., 1980) and PCR-amplified as thymines. As a consequence, when one compares the bisulphite sequence with the unmodified sequence, it is possible to assess which of the CpG motives were methylated or unmethylated and the abundance of the methylation at a given position can be estimated.

Figure 6.: Sodium-bisulphite conversion of unmethylated cytosine



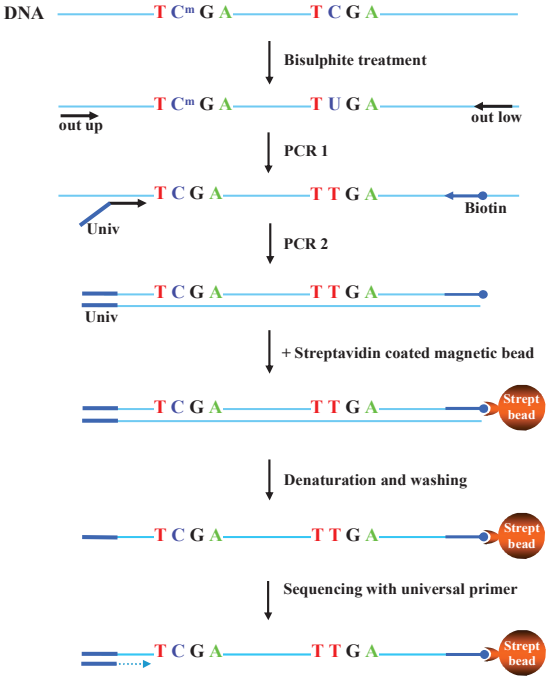
In single-stranded DNA under alkalic conditions unmethylated cytosines convert to uraciles in the presence of Na-bisulphite and hydroquinone. 5-methyl cytosines are resistant to bisulphite treatment (Based on: http://www.methylogix.com/genetics/protocols.shtmlDateien/schumachersguide1_files/image002.gif).

The modification reaction was performed according to Frommer *et al*. (1992) and Clark *et al*. (1994) with slight adaptations. 5 µg of sample DNA was diluted to 50 µl using steril destilled water and was supplemented with 5.5 µl freshly prepared 3M

NaOH. After incubation at 37°C for 15 min, 30.5 µl freshly made 10 mM hydroquinone (Sigma) and 530 µl 3.6M Na-bisulphite (pH 5.0, Sigma) was pipetted to the denatured DNA. The mixture was cautiously homogenized and split into 500 µl PCR tubes and covered with a mineral oil layer. Tubes were put into a PCR machine set to the following program: 3 min 95°C, 57 min 55°C, repeated four times. Next to the cyclic modification, 6 µl GeneClean glassmilk (BIO 101) was added to the Na-bisulphite containing DNA solution. Samples were let on the lab bench for 5 min and spin down at 5000g. The supernatant was discarded and the pellet was washed 3 times with 500 µl GeneClean wash solution (BIO 101). DNA bound on the surface of the glass beads was eluted in 50µl DNase-free water for 3 min at 50°C. 11 µl 3M NaOH was added subsequently and incubated for 15 min at 37°C. Finally, the DNA was precipitated overnight at -20°C after adding 66 µl 3M ammonium acetate and 720 µl absolute ethanol. After centrifugation for 20 min at 4°C, 12000g the precipitate was washed with 1 ml 70% ethanol and when dryed, 50 µl DNase-free water was added to dissolve the modifyed DNA. The outline of the method is illustrated in the **Figure 7.**

Following the modification step the sequences of interest were amplified using nested-PCR. Primers and circumstances used for the amplification of the EBER region are listed in **Table 3.**, while the primers and the PCR programs for the Z and R promoter to control the efficiency of the bisulphite treatment are shown in **Table 4**. The first amplification step of the nested-PCR was performed in 50 µl final volume containing 1 µl modified DNA sample, 0.2mM dNTP of each nucleotides, 40pmol of the sequence-specific outer primers, 2U of *Taq* polymerase (Promega) and 5 µl of the 10X PCR Buffer supplied by the manufacturer (1X: 10mM Tris-HCl, pH 9.0; 50mM KCl; 2.5mM $MgCl_2$; 0.1% Triton X-100). During this step the unmethylated cytosines converted to uraciles in the modification reaction are amplified as thymines, while the methyl-cytosines that remain unaffected are amplified as cytosines. The first step PCR products were diluted to 1:25 and 1.5 µl was used in the 2nd amplification step. One of the oligos used in this step was conjugated with biotin at their 5' end for the subsequent purification. The other oligos contained a 16 nucleotide long fragment of the M13 Universal primer sequence at its 5' end for the sequencing reaction (also see in **Figure 7.**). 10pmol of the oligos were used in this amplification, otherwise the composition of the reaction mixture was identical to the outer PCR. 5 µl of the final reaction was loaded on a 2% agarose gel to control the effectivness of the nested-PCR.

Figure 7.: Schematic overview of bisulphite sequencing



out up; **out low**: outer primers

Univ: nested primer flanked by M13 universal sequence : 5'-gta aaa cga cgg cca g-3'

Biotin: nested primer labelled with biotin

The purification of the biotinylated PCR product was performed according to Myöhänen *et al*. (1994). 40 µl biotinylated PCR product (the second reaction of the nested PCR) was purified using streptavidin coated magnetic beads (Dynal AB): 40 µl magnetic bead / sample was washed 2x in „binding & washing" (B&W) buffer (10mM Tris-HCl, pH 7.5; 1mM EDTA; 2M NaCl) using a magnetic rack. The vial was resuspended in 40 µl 2X B&W buffer. 40 µl PCR product was mixed with the beads and incubated for 15 min at room temperature with occassional shaking. During this incubation the biotinylated PCR products bind to the magnetic beads through strong biotin-streptavidin interaction. Samples were washed 2 times with 40 µl 1X B&W

buffer and resuspended in 8 µl freshly prepared 0.1M NaOH. After 10 min denaturation the solid phase was washed in 50 µl 0.1M NaOH and 3x in 50 µl TE buffer (10mM Tris-HCl, pH 7.5; 1mM EDTA). The biotinylated strand was dissolved in 13 µl dH_2O.

Table 3.: List of the primers (**A)** and the PCR conditions (**B**) used for the methylation mapping of the EBER region.

A

Primers, (Positions)*	**Sequence of primers 5'- 3'**
1. (6304-6326)	agg tta ggt tgt aag gtg gat gg
2. (7162-7133)	ccc tta cat att ata aat aca aaa cta acc
3. (6365-6387)	Bio-ggt gag aga gat ttt gga atg tg
4. (6363-6387)	Bio-tgg gtg ata gag att tta gaa tgt g
5. (6588-6613)	Uni-tgt ttt gag gag atg tag att tgt ag
6. (7051-7033)	Uni-cct ctc ttc tcc tcc ccc
7. (7051-7033)	Bio-acc tct ctt ctc ctc ccc c
8. (6720-6743)	Uni-ggt gtt tgt ggt tgt ttt ttt aga
9. (6789-6764)	Uni-tac raa cca cca act aat act taa cc
10. (6507-6530)	Uni-ggg gaa atg agg gtt agt ata gg
11. (6356-6380)	Uni-gta gag ttg ggt gat aga gat ttt ag
12. (6354-6377)	Uni-gtg tag tgt tgg gtg aga gag att
13. (6641-6664)	Uni-gtt tta gag gtt ttg tta ggg agg

B

Primer paires	**PCR conditions**			**Cycle number**
1-2	95°C 40"	63°C 40"	72°C 100"	35
4-5	95°C 40"	63°C 40"	72°C 50"	32
7-8	95°C 40"	65°C 40"	72°C 60"	32
3-6; 7-10	95°C 40"	63°C 40"	72°C 80"	32
7-11; 7-12	95°C 40"	65°C 40"	72°C 80"	32
3-9; 4-9; 7-13	95°C 40"	65°C 40"	72°C 65"	32

In all cases PCR was started with a 2.5 min denaturation at 95 °C and finalized with 8 min 72 °C.
* Numbers in brackets refer to nucleotide positions within the prototype B95-8 sequence (Baer *et al*., 1984). The sign „Uni" at the beginning of the oligo sequences stands for the 16 nucleotids of the M13 Universal primer sequence (5'-gta aaa cga cgg cca g-3'), the sign „Bio" means the biotinylation of the 5' end of the oligos. 1-2. primers: outer primers; 3-13. primers: inner/nested primers.

Table 4.: List of the primers (**A)** and the PCR conditions (**B**) used for the methylation mapping of the control regions, Z promoter (Zp) and R promoter (Rp) .

A

Promoter	PCR primer set	Primers, (Positions)*	Sequence of primers 5'- 3'
Zp	Outer	1. (103092-103115)	agg tat ttg gta tgg gtt agg tg
		2. (103800-103778)	ccc cta cct acc tct tta act cc
	Inner	3. (103415-103390)	Uni-cca tac ata ttt caa cta aac tat ct
		4. (103182-103205)	Bio-ggt aag gtg taa tgt tta gtg agt
Rp	Outer	5. (105920-105945)	tgt agt tgg ata ata ttt tag gat gg
		6. (106736-106713)	aat tac ctt caa tcc ctt aaa aca
	Inner	7. (106613-106591)	Uni-ctc acc taa aat aac acc caa ac
		8. (106133-106155)	Bio-tgt ttg tgt agt gag gtg ttg tg

B

Primer paires	PCR conditions			Cycle number
1-2	95°C 40"	63°C 40"	72°C 80"	35
3-4	95°C 40"	63°C 40"	72°C 50"	32
5-6	95°C 40"	63°C 40"	72°C 100"	35
7-8	95°C 40"	63°C 40"	72°C 65"	32

* Numbers in brackets indicate nucleotide positions within the prototype B95-8 sequence (Baer *et al*., 1984). The „Uni" stands for the 16 nucleotids of the M13 Universal primer sequence (5'-gta aaa cga cgg cca g-3'), „Bio" indicates the biotinylation of the 5' end of the oligos.

The sequencing reaction was performed using the „AutoRead Sequencing Kit" (Pharmacia Biotech). To the single-stranded sample DNA – bound by magnetic bead – 2 µl fluorescein-labelled „M13 Universal primer" (5'-fluorescein-cga cgt tgt aaa acg acg gcc agt-3'; 1.5µM) and 2 µl Annealing buffer (1M Tris-HCl, pH 7.6; 100mM $MgCl_2$) was added. To anneal the sequencing primer to the „Uni"-complementer sequence the mixture was incubated for 5 min at 74°C and 10 min 37°C. Thereafter, 1 µl Extension buffer (304mM citric acid; 324mM DTT; 40mM $MnCl_2$, pH 7.5) and 1 µl T7 polymerase (11.5 U/µl) was pipetted to the samples. After mixing thoroughly 4,1-4,1 µls were split into the 2.5-2.5 µl A, C, G, T Termination mix (ddNTP-containing termination mixture) and put into 37°C for 5 min. The sequencing reaction was

terminated with 5 µl „Stop" solution (deionized formamide; 5mg/ml Dextrane Blue 2000) and placed on ice until loading.

The polyacrylamide gel for the DNA fragment separation was prepared in the following way: 9 ml 40% acrylamide solution (acrylamide:bisacrylamide / 19:1; Sigma); 25.2 g urea and 3.6 ml 10x TBE buffer was supplemented with destilled water upto 60 ml. The solution was filtered through a 0.22 µm pore-sized filter and 60 µl TEMED, 210 µl 10% ammonium persulphate (APS) was added to start the polymerization and poured in between the appropriate-sized glass plates, which were previously cleaned and treated with bind-silane solution (4 ml absolute ethanol; 1ml 10% acetic-acid; 15 µl Bind-Silane (Pharmacia LKB)) in a segment contacting the comb.

The sequencing products stored at 4°C were put into 85-90°C for 3 min to denature the DNA and make all synthetized Cy5-labelled products single-stranded and ready to load. Gel running was performed in 1X TBE buffer (89mM Tris; 89mM boric acid; 2mM EDTA), using A.L.F. automatic sequencer (Pharmacia Biotech). The obtained data were analysed with the A.L.F. 2.0 software.

The methylation level of a cytosine at a given position was estimated by comparing the observed cytosine/thymine ratio at the position of interest and indicated as the % of the total amount (C+T). On this way we could assess the methylation level at a 25% punctuality.

4.5. Reverse transcription

The reaction was performed using 1 µg DNase-treated RNA. Gene-specific primers used for the reverse transcription are listed in **Table 5**. 1-1 µl primers (2pmol/µl) and 1 µl of 10mM dNTP (Promega) was added to the RNA, and the reaction mixture was supplemented with DNase- and RNase-free water (Gibco) to a final volume of 13 µl. To denature the RNA, samples were incubated for 5 min at 65°C and put on ice for at least 1 min afterwards. The reactions were supplemented with 4 µl 5X „First-Strand" Buffer (Invitrogen; 250mM; Tris-HCl, pH 8.3; 375mM KCl; 15mM $MgCl_2$), 1 µl 0.1M dithiothreitol (DTT, Invitrogen), 1 µl RNaseOUT recombinant ribonuclease inhibitor (Invitrogen, 40 U/µl), and 1 µl SuperScript III reverse transcriptase

(Invitrogen, 200 U/µl). Samples were then placed into a PCR machine for 50 min 42°C and 72°C 15 min program. The RT samples used for subsequent amplification reactions were diluted 10-fold and stored at -20°C.

Table 5.: Primers used for the RT reactions

Gene	Name of the primer (position in sequence)*	Sequence of primers 5'-3'
EBER1	EBER1-RT (6785-6770)	gac cac cag ctc gta c
EBER2	EBER2-RT (7119-7104)	gga caa gcc gaa tac c
GAPDH	GAPDH-RT (360-343)	ctt gat ttt gga ggg atc
β-actin	ACTBe5-RT (1226-1207)	tgt aac gca act aag tca tag
GFP	GFP-RT (1004-987)	ttg tac agc tcg tcc atg

* Numbers in brackets refer to nucleotide positions within the EBV prototype B95-8 sequence (Baer *et al.*, 1984) for EBER1 and 2; within the GAPDH gene (GenBank: NM_002046); within the beta-actin gene (GenBank: NM_001101); and within the GFP sequence (GenBank: U76561).

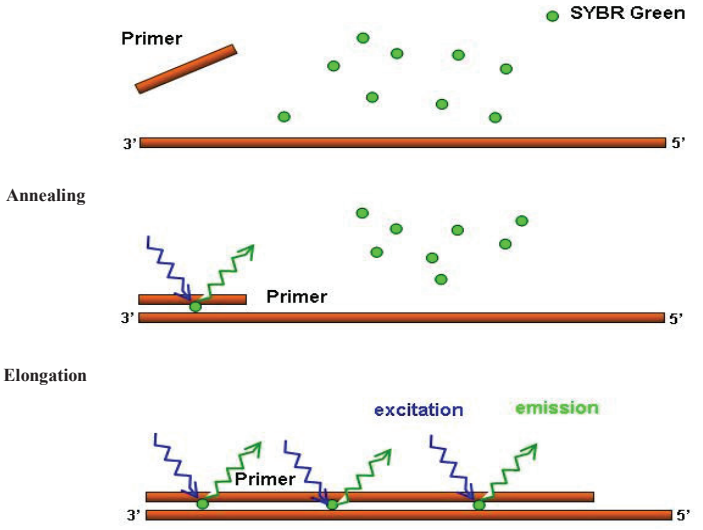
4.6. Real-time PCR

Real-time PCR is appropriate for quantitative analysis of DNA or cDNA. I used this method for defining the level of the DNA, RNA transcripts and the abundance of DNA fragments co-immunprecipitated with proteins in chromatin immunoprecipitation experiments.

The reaction was performed in 20 µl final volume. To 10.8 µl PCR-grade water (Roche), 3.2 µl $MgCl_2$ (25mM, Roche), 1-1 µl 5pmol/µl forward and reverse primers and 2 µl of LightCycler FastStart DNA Master SYBR Green I 'Hot Start' reaction compound (Roche), which contains FastStart *Taq* DNA polymerase, reaction buffer, dNTP, 10mM $MgCl_2$ and SYBR Green I dye were added. This mix was transferred into 20 µl LightCycler capillary, then 2 µl of the cDNA or the ChIP DNA template was suspended into the mix by multiple pipetting. Capillaries were centrifuged at 4500g for several seconds in Eppendorf centrifuge, so the reaction compounds could get into the

bottom of the capillaries, which were then placed in the carousel and the PCR reaction was carried out in a LightCycler 2.0 (Roche) instrument. The molecular background of SYBR Green-based real-time PCR is shown in **Figure 8**.

Figure 8.: The molecular background of the SYBR Green-based real-time PCR method



SYBR Green binds double-stranded DNA, and upon excitation emits light. When the DNA is denaturated, the SYBR Green dye floats free and does not emit light. The elongation phase begins as primers anneal, then SYBR Green dye binds to the double-stranded product and emits light.

I started the reaction with a 10-min-95°C-denaturation. FastStart *Taq* DNA polymerase is a modified form of recombinant *Taq* DNA polymerase, to which an antibody is bound to keep polymerase activity inhibited below the appropriate temperature. During the denaturation step the blocking group is removed from the enzyme, meanwhile, the double-stranded DNA is denatured to provide single-stranded templates. The following step was 45 cycles of amplification. Each cycle included 3 phases, one 2 sec 95°C denaturation step, one annealing step for 3 sec at a temperature

adequate for the used specific primers, and one 72°C elongation step for some seconds depending on the length of the product. Afterwards, melting curve analysis took place to supervise product specifity. This involved 95°C, 15 sec denaturation following quick cooling to 40°C, then 0.2°C/s reheating to 95°C.

SYBR Green I converts excess energy won by exposure to laser into rotation energy in solution, thus it does not emit, until rotation is arrested due to binding to double-stranded DNA. The excess energy transmitted as photon in this way can be detected on 530 nm. The amount of the bound SYBR Green I and thus the intensity of the detectable signal are proportional to the amount and length of the double-stranded DNA. Determination of the fluorescent signal intensity took place at the end of the elongation steps in each cycle. Throughout melting curve plotting fluorescence was measured continuously during heating from 40°C to 95°C.

The concentration of the samples was determined with the help of a plotted standard curve obtained from a 10X dilution series of a chosen DNA, total input chromatin or cDNA. Primers and the PCR conditions used to amplify templates from different experiments are listed in **Table 6.** For ChIP experiments EBER-CTCF, EBER1 and EBER2 primer were used. For the quantitation of DNA amount of samples β-actin, EBER1, EBER2 and Cp oligus were used. For expression analysis I applied GAPDH, β-actin, EBER1, EBER1 and GFP oligos. Relative concentration of the samples was calculated using the LightCycler Software 4.05 software.

Table 6.: Primers **(A)** and conditions **(B)** used for quantitation with real-time PCR

A

Gene	Name of the primer (position in sequence)*	Sequence of primers 5'- 3'
EBER-CTCF	EBER-CTCF up (6136-6156)	tca cag cta aat gcc cac cag
	EBER-CTCF low (6339-6319)	ctc aga aaa cac gcc atc cac
EBER1	EBER1 up (6656-6676)	cta ggg agg aga cgt gtg tgg
	EBER1 low (6777-6757)	gct ggt act tga ccg agg acg
EBER2	EBER2 up (6988-7004)	acc gcc aac gct cag tg
	EBER2 low (7111-7091)	cga ata ccc ttc tcc cag agg
GAPDH	GAPDH up (107-127)	gga agg tga agg tcg gag tca
	GAPDH low (261-239)	atg ggt gga atc ata ttg gaa ca
β-aktin	ACTB e4 (974-994)	ggc ggc acc acc atg tac cct
	ACTB e5 (1175-1155)	agg ggc cgg act cgt cat act
GFP	GFP up (760-780)	cag aag aac ggc atc aag gtg
	GFP low (942-924)	gtg atc gcg ctt ctc gtt g

*Numbers in brackets indicate nucleotide positions within the EBV prototype B95-8 sequence (Baer *et al.*, 1984) for EBER1 and 2; within the GAPDH gene (GenBank: NM_002046); within the beta-actin gene (GenBank: NM_001101); and within the GFP sequence (GenBank: U76561).

B

Primer paires	PCR conditions			Cycle number
EBER-CTCF up/low	95°C 4"	63°C 5"	72°C 6"	45
EBER1 up/low	95°C 2"	63°C 5"	72°C 6"	45
EBER2 up/low	95°C 2"	60°C 5"	72°C 6"	45
GAPDH up/low	95°C 2"	64°C 3"	72°C 7"	45
ACTB e4/e5	95°C 2"	64°C 3"	72°C 8"	45
GFP up/low	95°C 5"	62°C 5"	72°C 8"	45

4.7. DMS *in vivo* footprinting

Genomic footprinting was performed essentially as described previously (Niller *et al*., 1995). For each footprinting reaction 10^7 exponentially growing cells were harvested, washed 2x with phosphate buffered saline, resuspended in 1 ml of PBS and incubated at room temperature for 1 minute with 5 µl of dimethyl sulfate (DMS). The reaction was stopped by the addition of 5 ml DMS stop solution containing 1% bovine serum albumin and 100µM β-mercaptoethanol in PBS. Cells were washed once more in DMS stop solution and twice more with PBS. Finally, cells were resuspended in 1 ml of PBS and genomic DNA was prepared. Footprinted DNAs were subjected to piperidine treatment to break the strands at the DMS-modified guanines (Maxam *et al*., 1980). For visualization of footprints by Ligation Mediated-PCR (LM-PCR), 2 µg of sequenced (DMS-treated naked DNA) or footprinted (*in vivo* treated) DNA were analyzed as described previously with slight modifications (Mueller and Wold, 1989.; Niller *et al*., 1995).

The coordinates of the LM-PCR primers are listed in **Table 7**. The first strand primer extension reaction was done in Vent buffer (10mM KCl; 10mM $(NH4)_2SO_4$; 20mM Tris-HCl; 2mM $MgSO_4$; 0.1% Triton X-100, pH 8.8, New England Biolabs), containing 0.3pmol of Primer 1. (Metabion), 240µM each dNTP, and 1 unit of (exo-) Vent DNA polymerase (New England Biolabs) for 5 minutes at 94°C, 30 minutes at 60°C and 10 minutes at 72°C. For ligation of the common linker (Mueller and Wold, 1989), the sample was transferred to ice and 5 µl of PCR linker-mix, 2 µl of ligation buffer (660mM Tris-HCl; 50mM $MgCl_2$; 10mM dithioerythritol; 10mM ATP, pH 7.5, New England Biolabs), 1 µl of T4 DNA ligase (5 U/µl, New England Biolabs), and 12 µl of water were added. After overnight incubation at 4°C the DNA was ethanol precipitated, washed once with 75% ethanol, dried, and then resuspended in water. PCR amplification was done in 100 µl in 1X Vent buffer containing 10 pmol of Primer 2. and the longer linker primer, 240µM of each dNTP and 1 unit of Vent (exo-) DNA polymerase for 20 cycles for 1 minute at 94°C for 1.5 minutes at 60°C, and 3 minutes at 72°C. For labelling, the sample was transferred to ice and 5 pmol of γ-^{32}P-ATP labelled Primer 3. (labelled by using T4 kinase, New England Biolabs), 2.5 nmol of each dNTP, and 0.5 units Vent (exo-) DNA polymerase were added in a volume not exceeding 15 µl. Then the sample was heated to 94°C for 1.5 minutes, subjected to 8 cycles of 2

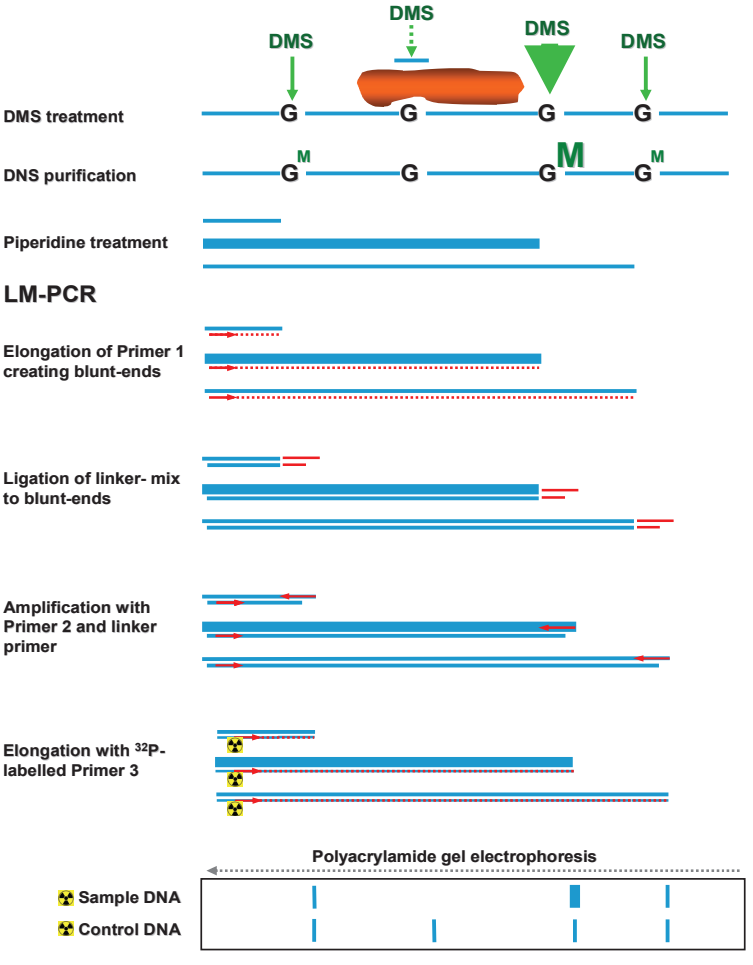
minutes at 94°C, 2 minutes at 62°C, 5 minutes at 72°C and kept at 72°C for 5 more minutes. Samples were phenol/chloroform-extracted, ethanol-precipitated, washed with 70% ethanol and resuspended in 30 µl loading dye. One tenth of each sample was separated on a 5% sequencing gel, and the gels were transferred to a 3MM paper, covered with plastic wrap and dried under vacuum at 70°C for 2 hrs. To visualize the gel running result, the gel was scanned using Personal Molecular Imager (Bio-Rad). The overview of the whole procedure is shown in **Figure 9.**

Table 7.: List of primers used in the *in vivo* footprinting reactions

Primer set (DNA strand)	**Name of primers (position in sequence)***	**Sequence of primers 5'- 3'**
EBER1 upper Primer set (+ strand)	1. (6425-6449)	acg ggt tcc cag aga ggg taa aag
	2. (6446-6470)	aaa gag ggg gcc ata aag ccc agg g
	3. (6475-6499)	aac acc gac cgc gcc acc aga tgg
EBER1 lower Primer set (- strand)	1. (6749-6725)	gca gag tct ggg aag aca acc aca g
	2. (6732-6708)	aac cac aga cac cgt cct cac cac c
	3. (6690-6666)	acg ggt ggc tac agc cac aca cgt c

*Numbers in brackets indicate positions within the B95-8 prototype EBV genome (Baer *et al.*, 1984).

Figure 9.: Schematic overview of the DMS *in vivo* footprinting



: γ-^{32}P label

: Primer 1., 2., 3.)

: linker-mix

4.8. *In vitro* DNase I footprinting

The control mock-methylated and the CpG-methylated pBS-EBER plasmids were cut with the restriction endonuclease EcoRI. The 0.7 kb *Eco*RI–*Eco*RI fragments carrying both EBER genes were gel-purified and 5'-end labelled using γ-^{32}P-ATP, cut with *Pvu*II, and the gel-purified *Eco*RI–*Pvu*II fragments (324 bp) were digested with RNase-free DNase I.

The pBS-EBER plasmid was *in vitro* methylated and mock-methylated as described in 4.3. The EBER region was cut out with the restriction endonuclease *Eco*RI, using 5 µg plasmid in 25 µl at 37°C for 2.5 hrs. The reaction product was treated with Antarctic phosphatase as recommended by the manufacturer (New England Biolabs) to avoid recircularization of the plasmid and to get 5' ends without phosphate groups for the subsequent labelling reaction. Samples were checked for cutting effectivity by electrophoretic separation on an agarose gel. DNA was then labelled with γ-^{32}P at both ends of the fragment using T4 Kinase (New England Biolabs) and γ-^{32}P-ATP. To get the needed fragments labelled at only one end, the total amount of DNA was digested with PvuII (New England Biolabs) in 50 µl at 37°C for 1.5 hrs and desalted using the Micro bio-spin chromatography column P6* (Bio-Rad) thereafter. The total amount of the samples were run on a 5% polyacrylamide gel and after developing the X-ray film the appropriate EBER1 fragment (324 bp) labelled at its 5' end was cut and the radioactive DNA was 2x eluted with an overnight incubation in 500 µl dH_2O. DNA was purified using Poly-prep chromatography columns (Bio-Rad) and precipitated with isopropanol and washed with 70% ethanol. The pellet was dissolved in 100 µl dH_2O. To have a "DNA ladder" – the so-called A+G lane – a portion (20 µl) of the probe was treated with formic acid for 5 min at room temperature; the reaction was stopped with hydrazine stop solution (0.3M Na-acetate; 0.1M EDTA; 100µg/ml tRNA) and put on ice. After the reaction the sample was ethanol precipitated and washed 3 times with 70% ethanol. 100 µl 10X diluted pyperidine was pipetted onto the pellet and put to 90°C for 30 min. The reaction was stopped by putting the sample on ice. DNA was lyophilised and resolved in 50 µl dH_2O and lyophilised 3 more times resolving the DNA in 50 µl dH_2O in between. At the latest step the samples were dissolved in 20 µl formamide-containing loading dye. During this treatment the labelled DNA fragment

was partially broken at adenines and guanines and could be used as an A+G lane or DNA ladder (Maxam *et al*., 1980).

The *in vitro* footprinting reaction was made based on the Core Footprinting System (Promega), Niller and Henninghausen, (1991), Henninghausen and Lubon, (1987). 10.000 cpm DNA probe (methylated or unmethyaletd) was incubated for 10 min on ice with or without 4µg nuclear extract (prepared as described by Dignam *et al*., 1983) in 1X Footprinting buffer (12mM Hepes-KOH, pH 7.9; 10% Glycerol; 0,12mM EDTA; 60mM KCl; 6mM $MgCl_2$; 1,6mM DTT) and 2 µg of dIdC competitor in 50 µl final volume. 50 µl Ca/Mg solution (5mM $CaCl_2$; 10mM $MgCl_2$) was added, mixed thoroughly and incubated at room temperature for 1 min. Samples without protein were treated with 0,25 U RQ1 DNase for 1 min, while the samples in the presence of proteins were incubated with 0,5 U DNase for 1 min 20 sec. Digestion was stopped by adding 150 µl Stop solution (100mM Tris-Cl, pH 7.5; 100mM NaCl; 10mM EDTA; 1 % SDS; 10 µg/µl Proteinase K; 100 µg/ml E. Coli DNA) and incubated for 1 h at 37°C. After a 3 min incubation at 90°C, samples were phenol-extracted and ethanol-precipitated, dissolved in 20 µl formamide-containing loading dye and loaded on 6% sequencing gel together with the A+G lane. After electrophoretic separation the radioactive fragments were visualized using a Personal Molecular Imager (Bio-Rad).

4.9. EMSA and Antibody Supershift

To find out if a DNA sequence can be bound by a given protein, one can use the antibody supershift assay. In this method a short double-stranded DNA is incubated together with nuclear proteins in the presence or absence of an antibody recognizing the protein of interest. Protein complexes are separated on an acrylamide gel. If the antibody makes a "bandshift" compared to the antibody-negative sample, one can conclude that the protein tested binds to the sequence of interest *in vitro*.

The preparation of nuclear extracts was essentially based on the method of Dignam *et al*. (1983). The positive and the negative strand of the oligos (CpG-methylated or unmethylated) harbouring the **ATF binding site** located upstream from the EBER1 gene (ATF-box-oligo, **Table 8**.) were hybridized to each other after the T4 Kinase labelling reaction using γ-^{32}P-ATP. On this way we gained a radioactive

labelled, unmethylated, double-stranded ATF binding DNA fragment (ATF M-) and the corresponding CpG-methylated form (ATF M+). Unlabelled ATF oligo was used as a specific competitor and a 218 bp long unrelated sequence (a PCR product corresponding to nucleotides 169372–169590 of the B95-8 EBV genome, Baer *et al*., 1984) was used as a non-specific competitor.

The putative **CTCF binding site** on the EBER region was prepared using γ-^{32}P-ATP-labelled oligos (see **Table 8**.) with T4 Kinase as in 4.7. After the amplification step the PCR product was run on an agarose gel, the band with the appropriate size (104 bp) was cut out and eluted. Unlabelled PCR product was used as specific competitor and the unspecific competitor was identical as in the ATF experiment.

Gel retardation assay was performed as described by Henninghausen and Lubon (1987) with slight modifications: 1 µg of crude nuclear proteins were incubated with 1-5 µg poly(dI-dC), 1 ng of labelled fragment and 50X excess of unlabelled double-stranded competitor in 25 µl bandshift buffer (10mM Tris-HCl, pH 7.5; 5mM $MgCl_2$, 80mM KCl; 1mM DTT; 1mM EDTA; 12.5% glycerol) for 20 min. For antibody supershift, 3.2 µg of antibody (anti-ATF-2, **Table 9.**) was pre-incubated with 1 µl of nuclear extract for 1 hour at 30°C. Immediately before loading the gel, the binding reaction was centrifuged for 2 minutes at 14.000 rpm in a centrifuge at room temperature. Protein complexes were resolved by electrophoresis on native 5% polyacrylamide gels (29:1) in 0.25x TBE buffer at 20mA for 1.5 hours. The visualization steps were the same as earlier (see 4.7.).

Table 8.: List of the oligos used in the EMSA and Antibody supershift experiments

Name of primers	Position in sequence*	Sequence of primers 5'- 3'
ATF oligo	(6568–6599)	ccc gtc acg gtg acg tag tct gtc ttg agg ag
EBER-CTCF up	(6236–6256)	tca cag cta aat gcc cac cag
EBER-CTCF low	(6339–6319)	ctc aga aaa cac gcc atc cac

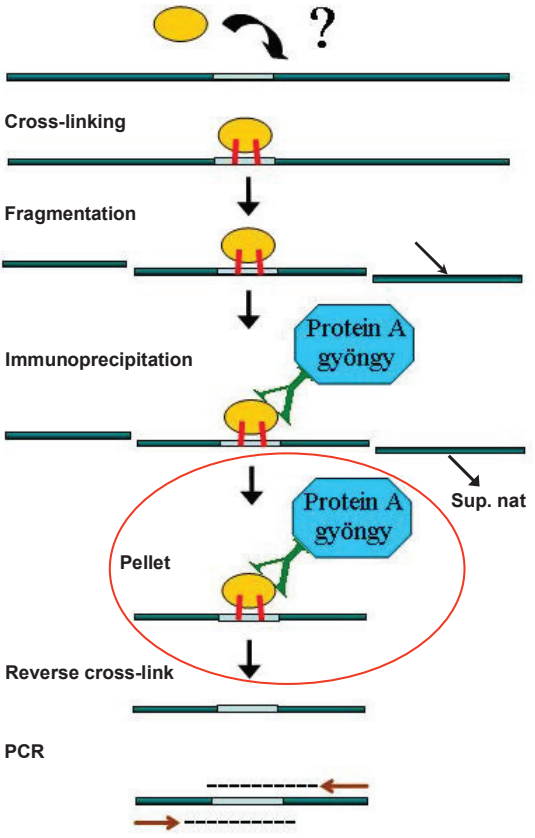
* The numbers in brackets refer to the nucleotide positions in the B95-8 EBV sequence (Baer *et al*., 1984).

4.10. Chromatin immunoprecipitation

Chromatin Immunoprecipitation (ChIP) is a method used to detect DNA-protein interactions *in vivo*. During this procedure, proteins binding to the DNA are covalently cross-linked via a chemical reaction, and the isolated DNA is broken into pieces between 300-1,500 bps in length, thereafter. The protein of interest and the covalently cross-linked DNA are precipitated by an antibody specific to the protein and by Protein A beads binding the Fc fragment of the antibody. After these, we can identify the DNA fragments and determine the degree of binding of the examined protein to a given sequence *in vivo*. The schematic overview of the ChIP assay is illustrated in **Figure 10**.

I performed ChIP according to the method published by Farnham *et al*. (2002) with slight modifications. 44 ml of a suspension cell culture (10^6 cells per ml) was used for the ChIP experiment. 1.22 ml 37% formaldehyde (Sigma) was added to the cells in Falcon tubes and incubated at 37°C for 10 min with continuous stirring. During this time stable covalent bonds were formed between the DNA and the proteins in direct physical interaction *in vivo*. To arrest the formation of aspecific bonds the reaction was stopped with 2.38 ml 2.5M glycine (Sigma). After centrifuging the samples (600g for 5 min, at 4 °C), they were washed with ice-cold PBS, then centrifuged, 1 ml PBS was added to the pellet and the samples were transferred into 1.5 ml Eppendorf tubes. Following centrifugation for 5 min at 4°C and 1500g, the supernatant was resuspended in 1 ml cell lysis buffer (5mM PIPES, pH 8.0; 85mM KCl; 0.5% NP40) supplemented with protease inhibitor. The cell lysate was incubated on ice for 5 min and centrifuged at 1500g and 4°C for 5 min. The pellet was resuspended in cell lysis buffer incubated for 5 min and centrifuged again. Then it was taken up in 2 ml nucleus lysis buffer (50mM Tris-HCl, pH 8.1; 10mM EDTA; 1% SDS) containing protease inhibitor. Samples were incubated on ice for 5 min, next they were sonicated (10x30 sec, with 30 sec breaks). After centrifuging at 15000g for 5 min at 4°C, the supernatant was transferred into new Eppendorf tubes.

Figure 10.: Schematic overview of the chromatin immunoprecipitation (ChIP) assay



During the ChIP method, first the DNA-binding proteins are cross-linked with the DNA by adding formaldehyde. Next, the fragmentation of the isolated DNA takes place, and then the covalently cross-linked DNA is precipitated by an antibody specific to the protein of interest using Protein A beads. After these, cross-linking is reverted, thus the DNA fragments can be examined by PCR assay.

6 µl 5M NaCl and 2 µl RNase were added to 100 µl sonicated lysate. Next it was incubated at 65°C for 4 hours to disrupt DNA-protein connections. After adding 4 µl Proteinase K (20mg/ml) and incubating at 50°C for an hour, DNA was purified using the classical phenol/chloroform isolation method. The quality of the sonicated DNA was examined by gel electrophoresis using a 1% agarose gel. For the subsequent assays, I used the lysate which was broken into 500-1,500 bps in length.

100 µl of the supernatant was supplemented with 900 µl IP dilution buffer (0.01% SDS; 1.1% Triton X-100; 1.2mM EDTA; 16.7mM Tris-HCl, pH 8.0; 167mM NaCl), 20 µl (10µg/µl) sonicated salmon sperm DNA, 18 µl (11mg/ml) tRNA and 80 µl activated 50% protein A-agarose beads.

To prepare the 50% protein A-agarose beads 0.2 g lyophilized protein A-agarose beads (P9269, Sigma) were swollen in a mixture containing 900 µl dilution buffer (1% Triton X-100; 150mM NaCl; 2mM EDTA; 20mM Tris-HCl, pH 8.0), 100 µl lysis buffer (1% SDS; 10mM EDTA; 50mM Tris-HCl, pH 8.0), 40 µl 25X protease inhibitor (Roche, Complete), 1mg/ml BSA and 400µg/ml sonicated salmon sperm. It was rotated at 4°C overnight, and washed in 2X dilution buffer on the next day.

During the pre-clearing step the chromatin was incubated for 30 min at 4°C in a rotating wheel. The samples were centrifuged at 1500g for 10 sec and the supernatant was transferred into new Eppendorf tubes afterwards. The supernatant was split into 2 equal portions; to one of its halves 2 µl (1µg/µl) specific antibody was given directed against c-Myc or CTCF or the different histone modifications (acH3, acH4, H3K4-me2, also see **Table 9.**), to the other halves 2 µl (1µg/µl) control non-immun rabbit serum was added, used as a negative control. Then both samples were vortexed and incubated overnight at 4°C on a rotating wheel.

After the incubation 30 µl activated 50% protein A-agarose beads were added to the samples, then they were rotated for 30 min at room temperature. During this time the antibody specific against the examined protein and the protein-DNA fragment complexes stabilized by covalent bonds, bound to the protein A molecules with their Fc parts. On this way, it is possible to purify all the DNA fragments bound by the protein of interest *in vivo* out of the total DNA. This was performed by centrifugation for 1 min, at 1500g. I stored the supernatant of the negative control sample as the total input chromatin (TIC, "positive control").

Table 9.: List of antibodies used in ChIP and Antibody supershift experiments

Antibody specificity	Source and type of the antibody
anti-c-Myc	rabbit polyclonal IgG (N-262X, *Santa Cruz Biotechnology,* sc-764X)
anti-ATF-2	rabbit polyclonal IgG (C-19, *Santa Cruz Biotechnology,* sc-187)
anti-CTCF	rabbit polyclonal IgG (*Upstate Biotechnology*, 07-729)
anti-acetyl-Histone H3	rabbit polyclonal IgG *(Upstate,* 06-599*)*
anti-acetyl-Histone H4	rabbit polyclonal IgG *(Upstate,* 06-598*)*
anti-dimethyl-Histone H3 K4 (Lys4)	rabbit polyclonal IgG *(Upstate,* 07-030*)*
non-immun rabbit serum	rabbit polyclonal IgG, *(Santa Cruz Biotechnology,* sc-2027*)*

The obtained pellet was washed 1x with 1 ml low salt buffer (0.1% SDS; 1% Triton-X 100; 2mM EDTA; 20mM Tris-HCl, pH 8.0; 150mM NaCl), 1x with high salt buffer (0.1% SDS; 1% Triton-X 100; 2mM EDTA; 20mM Tris-HCl, pH 8.0; 500mM NaCl), 2x with LiCl detergent buffer (10mM Tris-HCl, pH 9.0; 1mM EDTA; 250mM LiCl; 1% IGEPAL; 1% deoxicholic acid). Between the washing steps, the samples were incubated for 10 min at room temperature and centrifuged at 1500g for 1 min. Thereafter, they were washed 2x with 1 ml TE buffer. Samples were rotated in 250 µl elution buffer (0.1M $NaHCO_3$; 1% SDS) for 15 min at room temperature. Throughout this, the antibody-protein-DNA complexes separated from the beads. The samples were centrifuged at 1500g for 1 min, and the supernatants were transferred into new Eppendorf tubes. As the elution step was repeated twice, the final volume turned up to be 500 µl.

To revert covalent bonds established between the protein and DNA molecules 20 µl NaCl (5M) was added to the samples - including the TIC (positive control) - and was incubated overnight at 65^{o}C. Next, DNA purification was performed using the method described in the quality assay of the lysate. The obtained DNA was dissolved in 30 µl sterile distilled water. TIC samples were diluted 100-fold, the antibody-positive samples were diluted 10-fold and the mock samples were were stored in the original 1-fold dilution. All of the samples were then subjected to real-time PCR as described above (4.6.). The antibody-positive result were compared to TIC and illustrated in a chart as the percentage of the appropriate TIC sample.

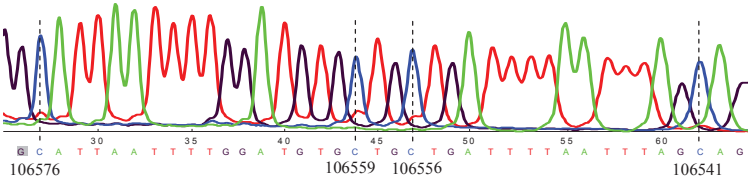
5. RESULTS

5.1. Fine-mapping of the CpG-methylation pattern of the EBER locus in latent EBV-carrying cell lines

During my work I used well charaterized, EBV-carrying cell lines (also see 4.1.) showing the latent gene expression patterns of latency type I or latency type III (for detailes see: 2.1.1.) To exclude any switch between latency types of cell lines during culturing, our research group monitored EBNA2 expression by immunoblot analysis. The ZEBRA immunoblotting experiments verified that the vast majority of the cells did not activate their lytic cycle. All the cell lines of B cell origin were supposed to have active EBER promoters and were earlier mapped for methylation using *Hha*I, *Hpa*II and *Msp*I restriction endonucleases (Minarovits *et al*., 1992).

The fine-mapping of the CpG-methylation pattern was performed as desribed in 4.4. To check if the conversion of unmethylated cytosines was completed during the bisulphite treatment, and the methylated cytosines remined unaltered in all cell lines, I control bisulphite-sequenced the R and Z promoters, that are inactivated and are known to be hypermethylated in viral latency. The control bisulphite sequencing of the Z promoter in Mutu III is shown in **Figure 11**. Methylated cytosines were amplified as cytosines, showing that they resisted to the chemical treatment as expected; no other cytosines appear, however, in the modified sequence, indicating that the treatment was successfully performed.

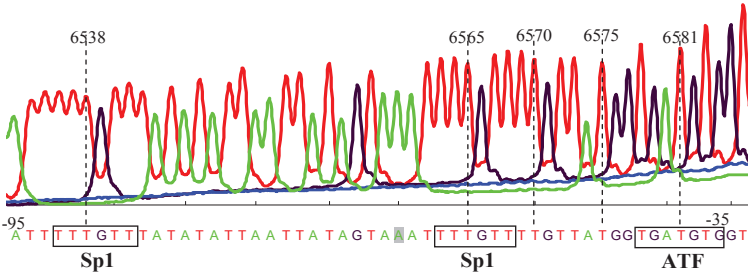
Figure 11.: Bisulphite sequencing of the Zp in Mutu-BL-III-Cl-99 cell line.



Vertical, dashed lines and numbers below the sequence indicate the positions of cytosines in CpG dinucleotides in the B95-8 prototype EBV genome (Baer *et al*., 1984).

During my work I performed the direct sequencing of the EBER region (positive strand) between the nucleotides of 6378 and 7032 of bisulphite-treated DNA isolated from Rael, CBM1-Ral-STO, Mutu-BL-I-Cl-216, Mutu-BL-III-Cl-99, Raji, LCL-721 and C666-1 cells. This region includes the upstream regulatory sequences and the coding sequence of the EBER1 gene and the upstream and inner regulatory sequence of the EBER2 gene (also see **Figure 2., 13., 18., 22.**). **Figure 12.** shows the bisulphite sequenced 5' regulatory region of the EBER1 gene in the CBM1 cell line.

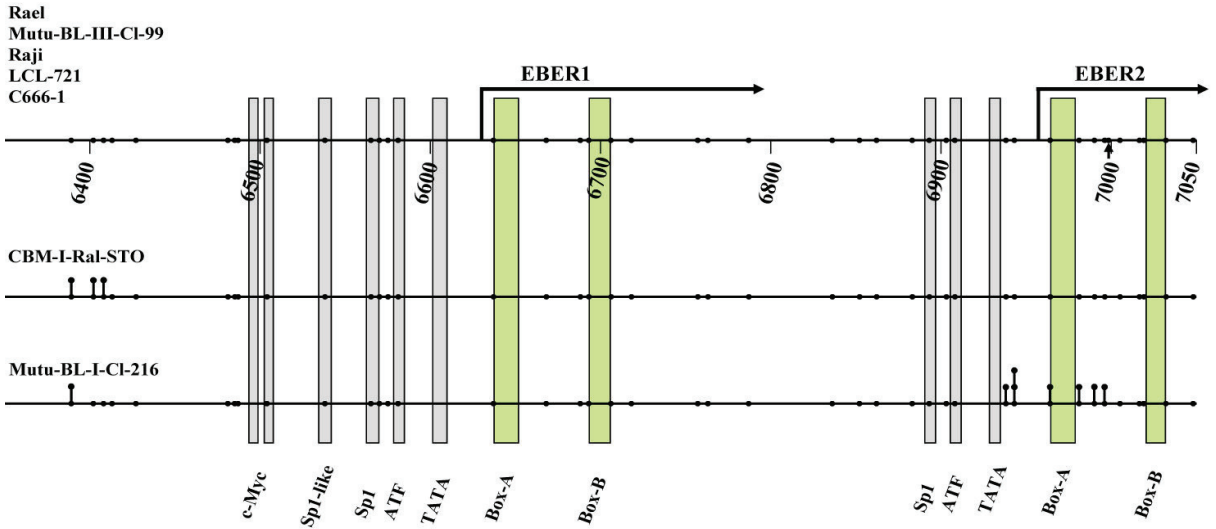
Figure 12.: Bisulphite sequencing of the 5' regulatory region of the EBER1 gene in the CBM1-RAL-STO cell line.



Vertical, dashed lines and numbers above the sequence indicate the positions of cytosines in CpG dinucleotides in the B95-8 prototype EBV genome (Baer *et al.*, 1984). The most important transcription factor binding sites (Sp1 and ATF) are indicated with rectangles in the sequence text.

The results of the fine-mapping of the positive strand of the EBER1 and 2 genes (nucleotides of 6378 and 7032) in the Rael, CBM1-Ral-STO, Mutu-BL-I-Cl-216, Mutu-BL-III-Cl-99, Raji, LCL-721 and the C666-1 cell lines are summarized in **Figure 13**.

Figure 13.: CpG-methylation map of the EBER region



Cell lines with the corresponding methylation pattern are indicated on the left side, above the "sequence". • indicate the positions of unmethylated CpGs, ┃ indicate the position of CpGs with 0-25% methylation, ┃ indicate the position of CpGs with 25-50% methylation, arrows heading to right show the initiation and direction of transcription. Numbers indicate positions in the B95-8 prototype EBV genome (Baer *et al*., 1984). Grey rectangles show the position of Pol II transcription factor binding sites (c-Myc, Sp1, Sp1-like, ATF) and the TATA-box, while green boxes indicate Pol III elements (Box-A, Box-B).

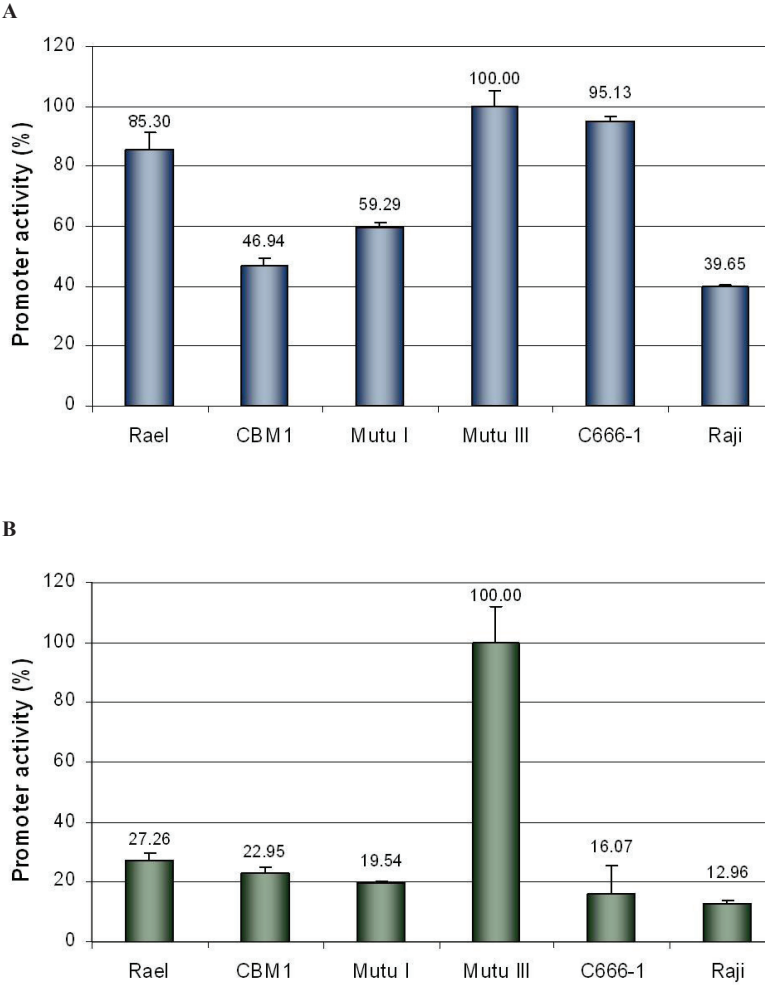
All of the cytosines within CpG dinucleotides were found to be completely unmethylated within the examined region in the Rael, Mutu III, Raji, LCL-721 and C666 cell lines. An extra CpG site was sequenced during control sequencig in the C666 cell line at the position 7048, which was also unmethylated. In the CBM1 lymphoblastoid cell line the CpGs at the positions 6389, 6402 and 6408 were partially methylated at about 0-25% of total cytosine amount, but all the other CpGs in the mapped region were totally unmethylated. The cytosines within CpGs of the EBER region were also unmethylated in the Mutu III cell line with the exception of the positions 6389, 6938, 6964, 6981, 6990 and 6996 that showed a slightly elevated level of methylation (0-25%) and the CpG at the position 6943 that was moderately methylated (25-50%).

5.2. Activity of EBER promoters in different cell lines

The amount of EBER transcripts in different cell lines was estimated by Minarovits *et al*. (1992) based on radioactive densitometry corrected to GAPDH RNA levels. Taking into consideration that different cell lines carry variable number of EBV episomes in a single cell, to assess EBER promoter activity I adjusted the meassured amounts of viral transcripts to the EBV copy number.

First of all, using the RT-real-time PCR (see 4.5. and 4.6.), I determined the EBER1, EBER2 and GAPDH RNA levels. The EBER results were corrected to the product of the houskeeping gene GAPDH to get the relative EBER RNA amounts in proportion to cell number. To assess the relative episome number per cell in each cell line, I used real-time PCR. The relative amount of the β-actin coding gene was meassured as a cell number control, and the Cp and the EBER1 gene, that are present in a single copy per viral DNA, served as a control for EBV copy number per cell. Comparing the ratio of Cp/β-actin and EBER/β-actin I could estimate the relative EBV copy number per cell in each cell line. According to the real-time PCR results I estimated, that I have to correct EBER RNA amounts with the following relative EBV copy numbers: Mutu I: 13, Mutu III: 2, Rael: 11, CBM1: 3, C666: 7. The EBER1 and EBER2 promoter activities, determined as described above, are illustrated in **Figure 14**.

Figure 14.: Activity of the EBER1 **(A)** and the EBER2 promoters **(B)** in different cell lines



EBER1 and EBER2 promoter activities were expressed in the percentage of promoter activity in the Mutu III cell line.

According to these results, there were only moderate differences among the EBER1 activity of the examined cell lines. In contrast, the EBER2 activity was found to be prominently high in the Mutu III cell line, while the C666 nasopharyngeal carcinoma cell line showed a considerably decreased EBER2 promoter usage.

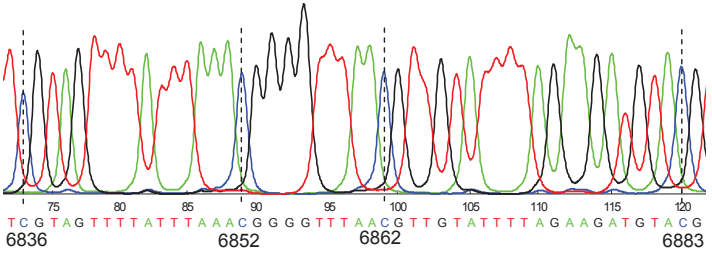
5.3. Activity of the EBER promoters in an *in vitro* methylated construct transfected into EBV-negative cells

Because the EBER locus was found to be extensively hypomethylated in all the cell lines analysed, we assessed the effect of CpG-methylation on EBER1 and EBER2 expression by introducing either an *in vitro* methylated or a mock-methylated plasmid (pBS-EBER), carrying both EBER genes, into EBV-negative cells according to the protocol desribed in 4.3. The M*Sss*I-methylated EBER locus was examined 48 hrs after transfection to check if the methylation state has not been changed by any cellular processes. Results of the bisulphite sequencing of these constructs are presented in **Figure 15**.

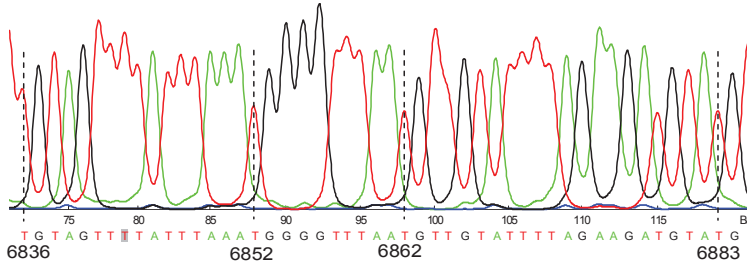
In the M*Sss*I-methylated pBS-EBER plasmid all cytosines located within CpG dinucleotides resisted to bisulfite modification and appeared as cytosines in the final genomic sequence. On the other hand, all the cytosines outside the CpG dinucleotides in the M*Sss*I-methylated construct (**Figure 15. A**), and all of the cytosines in the mock-methylated construct were converted to uracils and sequenced as T instead of C after PCR amplification (**Figure 15. B**). These results indicated, that the transfected M*Sss*I-methylated plasmid remained fully methylated, while the mock-methylated construct has also not changed and remained unmethylated 48 hrs after transfection.

Figure 15.: Methylation analysis of the M*Sss*I-methylated **(A)** and mock-methylated **(B)** pBS-EBER constructs

A



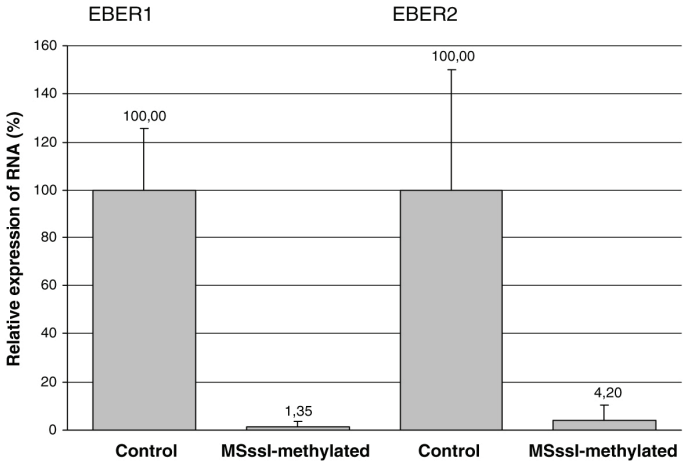
B



A region of the EBER locus situated 50 from the EBER2 transcription unit is shown (positions 6808–6885). Vertical, dashed lines and numbers below the sequence show the positions of cytosines in CpG dinucleotides within the B95-8 EBV genome (Baer *et al*., 1984).

RNAs prepared from transfected cells were reverse transcribed to meassure the amount of green fluorescent protein (GFP) transcripts as a control for transfection efficiency, β-actin RNA as a cell number control and the EBER1 and EBER2 products. The results were calculated for EBER activity as described in 4.5. **Figure 16**. shows the EBER1 and EBER2 promoter activities of methylated and unmethylated DNAs in transfected Hela cells 48 hrs after transfection.

Figure 16.: Relative EBER promoter activity in M*Sss*I-methylated and mock-methylated pBS-EBER plasmid in Hela cells



The promoter activity of EBER1 and EBER2 was calculated after correcting for cotransfected GFP gene expression and endogenous β-actin RNA expression levels. Results were expressed in the percentage of unmethylated promoter activity.

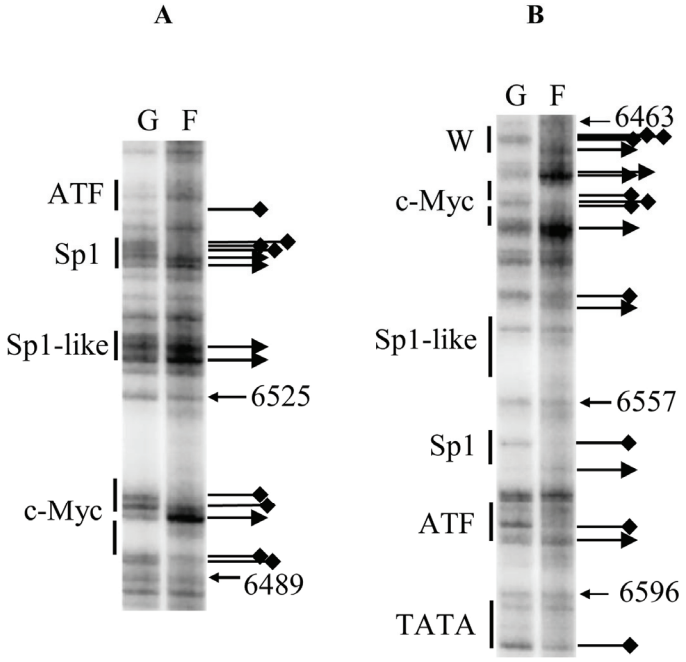
Our data shows that the methylation of the CpG dinucleotides within the EBER regulatory region strongly suppresses both EBER1 and EBER2 expression in DG75 (data not shown) and HeLa cells.

5.4. *In vivo* protein binding at the 5' regulatory region of the EBER1 promoter in C666-1 cells

In BL-derived and LCL cell lines carrying latency type I and latency type III EBV genomes, the *in vivo* protein binding pattern at the EBER locus was determined by Niller *et al*. (2003). The pattern of protected and hypersensitive guanines were generally identical between cell lines in the EBER1 region. To examine the protein footprints of the nasopharyngeal carcinoma cell line C666 and to compare the result with the earlier described footprints of EBER1 in cell lines of B cell origin, I performed *in vivo*

genomic footprinting following the protocol described in the chapter 4.7. The developed radioactive gel-photo in **Figure 17**. shows the footprinting pattern of the 5' region of the EBER1 promoter in the C666-1 cell line.

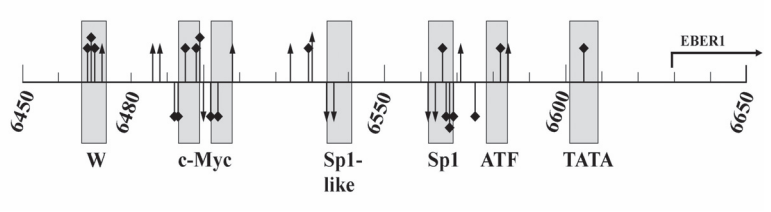
Figure 17.: *In vivo* genomic footprinting result of the EBER1 upstream regulatory region in the C666-1 cell line



Panel A shows the lower (negative) strand, panel B shows the upper (positive) strand. G on the left of both panels indicates the G-lane or naked DNA broken at guanines in the absence of proteins, F (Footprint lane) on the right indicates genomic DNA broken at guanines under *in vivo* conditions in the presence of cellular proteins. On the left side of the G-lanes the main transcription factor binding sites are marked, on the right, the numbers denote the positions of certain guanines within the B95-8 EBV genome (Baer *et al*., 1984). ——◆ Indicates protected guanines, ——▶ indicates hypersensitive positions.

The result of the *in vivo* genomic footprinting analysis shown in **Figure 17.** is summarized in **Figure 18.** for the sake of better perspicuity.

Figure 18.: Summary of the genomic footprinting analysis of the 5' region of the EBER1 gene in C666-1 cells.



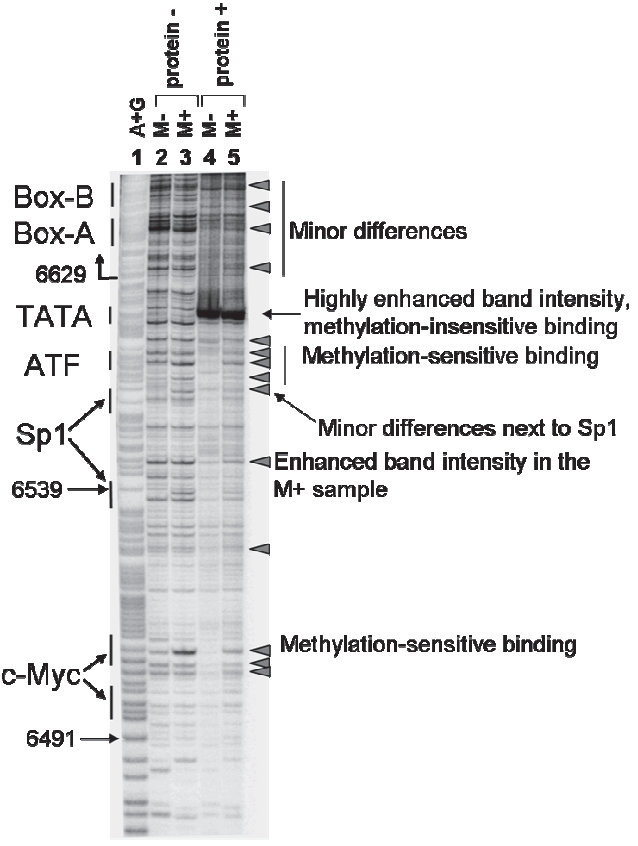
The line in the middle represents the double-stranded EBV genome between the positions on the two ends. Numbers below the line indicate nucleotid positions according to the B95-8 prototype EBV genome (Baer *et al*., 1984). The horizontal arrow on the right marks the initiation site and direction of EBER1. Grey rectangles indicate transcription binding sites, named underneath. ↑ and ↓ mark protected guanines, ↑ and ↓ mark hypersensitive positions on the upper and the lower strand.

In the 5' regulatory region of the EBER1 gene carryed by C666-1 nasopharyngeal carcinoma cells, I found footprints on both DNA strands at the TATA-box and the earlier described protein binding sites: ATF, Sp1, Sp1-like, c-Myc and W. The footprinting result was strongly similar to the protein binding pattern at the same region of EBV-carrying cell lines of B cell origin, published by our research group (Niller *et al*., 2003).

5.5. *In vitro* footprinting of M*Sss*I-methylated and mock-methylated EBER1 regulatory sequence

The preparation of the radioactive ^{32}P-labelled EBER1 sequence and the footprinting reaction was performed as detailed in 4.8. M*Sss*I-methylated and mock-methylated EBER1 DNA fragments were incubated in the presence or absence of nuclear proteins and incompletely digested with DNaseI. The products were run on an acrylamide gel near a Maxam-Gilbert A+G lane from the EBER1 fragment for orientation within the sequence. The resulting gel was visualized and shown in **Figure 19**.

Figure 19.: *In vitro* DNase I footprinting of the CpG-methylated and unmethylated EBER1 regulatory sequence



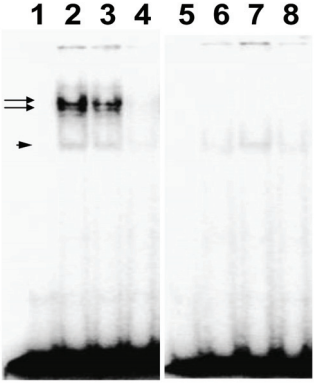
The major transcription factor binding sites are indicated on the left side of the gel with numbers standing for the nucleotide positions in the B95-8 EBV sequence (Baer *et al*., 1984); M- : unmethylated DNA; M+ : CpG-methylated DNA. "Protein –" samples were treated in the absence, "Protein +" samples in the presence of nuclear proteins. An arrow on the left side shows the transcriptional start site. Arrows and arrowheads on the right of the figure indicate differences in the DNase I footprints of protein-bound unmethylated and CpG-methylated samples.

I found several differences between the protein binding patterns of unmethylated and methylated EBER1 promoter sequence. Clear changes were found at the proximal c-Myc binding site near the distal and at the proximal Sp1 recognition site and the ATF binding site. Minor differences were observed at the Pol III sequences called Box-A and Box-B, but the bigger distance from the radioactive label resulted in a lower resolution in this region. The TATA-binding site in both cases (methylated and unmethylated) showed strong and uniform hypersensitivity in the samples incubated in the presence of nuclear extract.

5.6. Analysis of specific *in vitro* protein binding at the EBER1 promoter with electrophoretic mobility shift assay

Electromobility shift assay (EMSA) experiments were performed according to the protocol detailed in 4.9. The binding of the activating transcription factor (ATF) protein was first analysed, that showed a methylation-sensitive binding to its recognition sequence within the EBER1 promoter in *in vitro* footprinting experiments (see **Figure 19.**). The gel-photo of ATF bandshift experiment is shown in **Figure 20**.

Figure 20.: Binding of the ATF protein to unmethylated and CpG-methylated EBER1 promoter sequence.

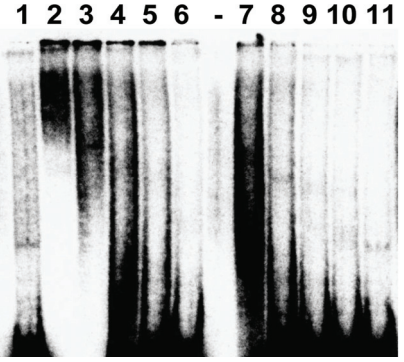


Binding of proteins of Mutu-BL-I-Cl-216 cell nuclear extract to unmethylated and CpG-methylated ATF-box-oligo is shown. Arrows indicate the sequence-specific complexes. An arrowhead indicates an unspecific complex. Lanes 1–4: unmethylated ATF-box-oligo; lanes 5–8: CpG-methylated oligo. Lanes 1 and 5: probe only, no protein added; lanes 2–4 and 6–8: nuclear extract added. Lanes 3, 7: unlabelled non-specific competitor added; Lanes 4, 8: unllabeled specific competitor added.

As seen in **Figure 20**., the unmethylated oligo harbouring the ATF binding sequence formed specific complexes with the Mutu I nuclear extract which could not be eliminated by the unspecific competitor, but the excess of the same unlabelled sequence (specific competitor) disrupted the labelled oligo-protein interaction. On the other hand, no specific binding could be observed using the CpG-methylated double-stranded oligo, even without any competitors. Using an antibody against the ATF-2 protein of the ATF family we could not observe any bandshift or alteration in the intensity of the specific DNA-protein complex (data not shown).

Because Day *et al*. (2007) observed the binding of the CTCF protein to the 5' regulatory region of the EBER1 in latency type I and III BL cell lines and in an LCL, I wished to clarify if CTCF binding to this site was strong or weak. EMSA results using Mutu I and Mutu III nuclear extracts with a CTCF binding fragment of EBER1 promoter is shown in **Figure 21**.

Figure 21.: EMSA of the putative CTCF binding site located in the 5' promoter sequence of EBER1 using nuclear proteins of the Mutu I and Mutu III cell lines.



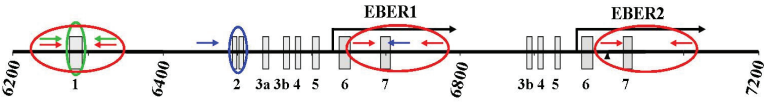
In each lane the same unmethylated CTCF binding sequence was loaded. Lane 1: probe only; no protein added. Lanes 2–6: labelled probe in the presence of Mutu I nuclear extract; 7–11: labelled probe in the presence of Mutu III nuclear extract. In these samples increasing amounts of unspecific competitor were added in the order of 2 ⟶ 6, and 7 ⟶ 11.

In this experiment I could not observe any specific protein binding to the CTCF binding sequence of the EBER1 promoter in the absence or in the presence of increasing amounts of the unspecific competitors.

5.7. Analysis of protein binding at the EBER locus using ChIP assay

ChIP assay was performed according to the protocol detailed in 4.10. Using the listed antibodies (**Table 9**.) directed against c-Myc and CTCF proteins and various histone modifications, three regions of the EBER locus was examined, i.e. the 5' regulatory region, the EBER1 and the EBER2 coding sequences (also see **Figure 22.)**.

Figure 22.: Examined regions within the EBER locus using ChIP assay

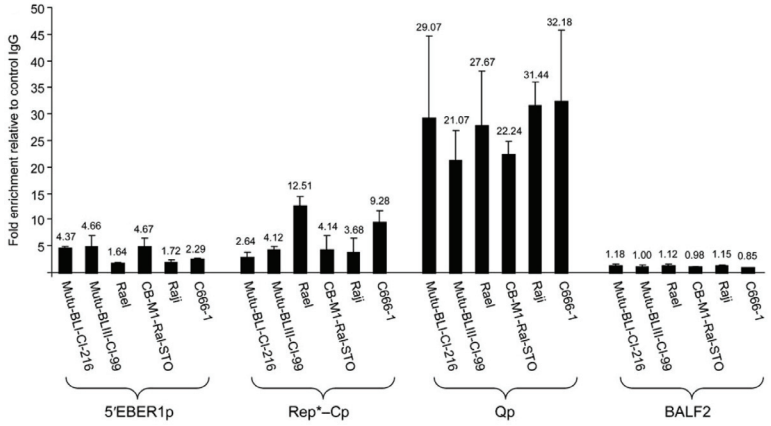


The horizontal line in the middle represents a section of the double-stranded genome of EBV between the nucleotides 6200 and 7200 according to the B95-8 prototype EBV genome (Baer *et al*., 1984). Grey rectangles symbolize transcription factor binding sites. 1: CTCF; 2: c-Myc; 3a and 3b: Sp1-like and Sp1; 4: ATF, 5: TATA-box; 6: Box-A; 7: Box-B. Thick arrows indicate the initiaton and direction of the EBER transcripts. Coloured ovals mark the regions examined with ChIP assay. Blue oval: c-Myc binding site, Green oval: CTCF binding site, Red ovals: regions examined for histone modifications. The coloured arrows represent primers (for details see **Table 6**. and Niller *et al*., 2003) used for the quantitation of the ChIP samples.

The result of chromatin immunoprecipitation with the antibody directed against the **c-Myc** protein in a latency type III Burkitt's lymphoma cell line is published (Niller *et al*., 2003). In this paper we demonstarted, that the putative c-Myc binding site is occupied by the c-Myc protein in Raji cells *in vivo*.

The binding of **CTCF** to the 5' region of EBER1 was compared to the BALF2 lytic gene, that is inactive in latency types serving as a negative control of the experiment, and the Q promoter region which was seemed to be strongly occupied by the CTCF protein (positive control). We found a weak interaction between the 5' EBER1 promoter and the CTCF protein in 6 different cell lines under *in vivo* conditions, because only a moderate level of enrichment of the EBER1 promoter could be meassured. The result of the ChIP experiment using the anti-CTCF antibody is shown in **Figure 23**.

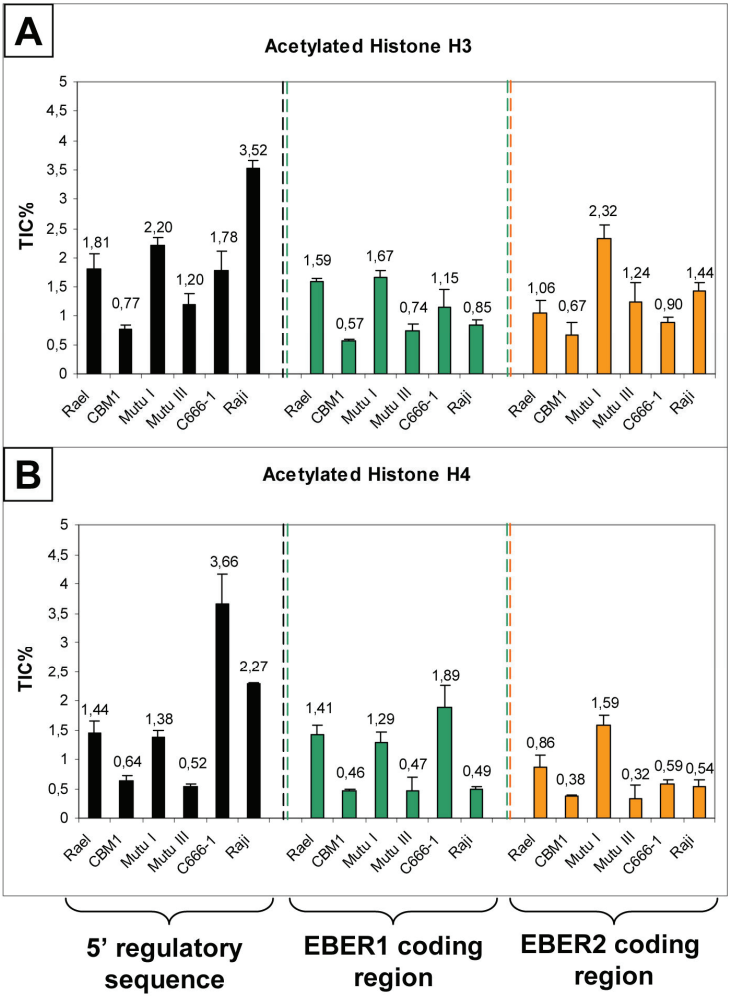
Figure 23.: ChIP analysis of CTCF binding to selected regions of the EBV genome



Binding of the CTCF protein to the 5' region of the EBER1 promoter, the Rep*-Cp region, the Q promoter and the lytic BALF2 region relative to the non-immun rabbit serum (Salamon *et al.*, 2009).

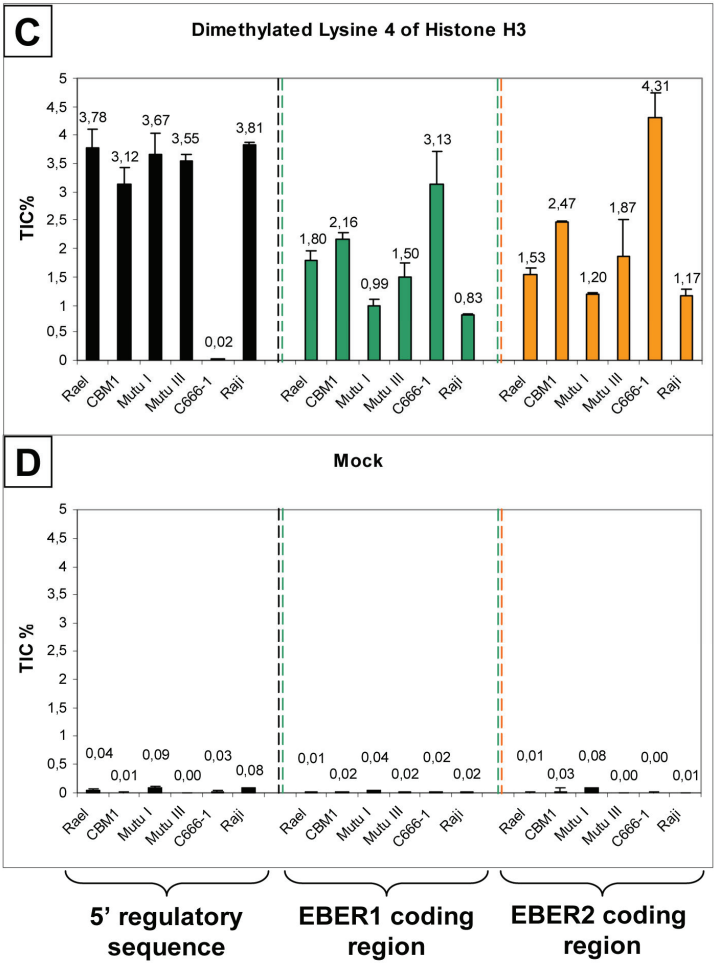
Specific antibodies directed against different **histone modifications** can be used to fish out histones enriched in the given modification together with the DNA sequence to which the histone protein binds. Chromatin immunoprecipitates of acetylated histone H3, acetylated histone H4 and dimethylated lysine 4 of histone H3 were quantified using real-time PCR with primers listed in **Table 6**. (also see **Figure 22.)**. The abundance of different histone modifications in the 5' regulatory region and the coding sequence of the EBER1 gene and the coding region of the EBER2 gene was expressed as the percentage of the total input chromatin (see **Figure 24**.).

Figure 24.: The relative abundance of activating histone modifications in the regulatory and coding sequences of the EBER locus in cell lines carrying latent EBV genomes



The abundance of different histone modifications is expressed as the percentage of the total input chromatin. **Panel A**: acetylated histone H3, **panel B**: acetylated histone H4.

Figure 24.: The relative abundance of activating histone modifications in the regulatory and coding sequences of the EBER locus in cell lines carrying latent EBV genomes



Panel C: dimethylated lysine 4 of histone H3, **panel D**: Mock-precipitated samples. On the left side, the amount of modifications in the 5' regulatory sequence (black columns), in the middle the EBER1 coding sequence (green columns), on the right the EBER2 coding sequence (orange columns) is shown in the examined cell lines.

All of the examined histone modifications were found to be present in great amounts within the entire EBER locus in all of the cell lines investigated. There were minor differences between the individual cell lines in all cases. The latency type I Rael and Mutu I was more enriched in AcH3 throughout the EBER locus, compared to the CBM1 and Mutu III latency type III cells that carry the same EBV strains. The same tendency was observed in case of acetylated H4. The abundance of these histone modifications was variable in the C666-1 and the Raji cell lines. The H3K4me2 modification was approximately equally abundant in the 5' promoter sequence of the lymphoid cell lines, but the C666-1 nasopharyngeal carcinoma cells lacked this modification in that region. In the coding sequences the latency type I cell lines (Rael, Mutu I) had lower amounts of the H3K4me2 modification compared to their latency type III counterparts (CBM1, Mutu III). The C666-1 cells showed the highest amount, and the Raji cells showed the smallest amount of this modification in the EBER coding sequences.

6. DISCUSSION

DNA-methylation contributes to the transcriptional regulation of latent EBV genes in a promoter-dependent manner. The *in vitro* methylation of the C, W, Q and LMP1 promoters result in the inhibition of the corresponding gene (Minarovits *et al*., 1994b; Robertson *et al*., 1997b; Jansson *et al*., 1992; Tao *et al*., 1998b; Tierney *et al*., 2000). The same inverse relationship could be verified between the promoter methylation and the gene activity in the case of C, W and LMP promoters *in vivo* (Takacs *et al*., 1998; 2001; Salamon *et al*., 2003; Bakos *et al*., 2007; Gerle *et al*., 2007). The Q promoter is, however, unmethylated independently of its gene activity in every cell line (Tao *et al.,* 1998a; 1998b; Salamon *et al.,* 2001; Bakos *et al*., 2007). Based on the above mentioned data, it was found to be interesting to investigate the relationship between the DNA-methylation status and the promoter activity of the EBER1 and EBER2 genes.

Examinations with methylation-sensitive restriction endonucleases showed, that within the EBER locus with only a single exception all the CpG dinucleotides (within CCGG sequences) were unmethylated in the examined lymphoid cell lines (Mutu-BL-I-Cl-216, Mutu-BL-III-Cl-99, Rael and CBM1-Ral-STO; Minarovits *et al*., 1992). With bisulphite sequencing I could assess the methylation status of all the CpGs in the region (nucleotides 6378-7032). In concert with the earlier publication of Minarovits *et al*. I found, that with some exceptions of certain CpG positions, where a moderate level of methylation could be observed, the entire EBER locus was unmethylated in all of the examined cell lines. This extensive hypomethylation in the region suggests, that the methylation of the EBER promoters could inhibit their activity, similarly to other “type 2” and “type 4” Pol III promoters with regulatory sequences within the coding region as well.

According to earlier studies the EBER genes are always actively transcribed *in vivo*. The amount of the EBER transcripts in BL and LCL cell lines were meassured and compared to the GAPDH housekeeping gene in the study of Minarovits *et al*. (1992) using radioactive densitometry. With the more sophisticated and quantitative reverse transcription followed by real-time PCR I meassured the amount of the EBER1 and EBER2 RNAs, corrected the results to EBV copy number per cell and the GAPDH gene product to get the activity of the promoters. According to this, in all of the examined

cell lines both promoters were proven to be actively transcribed with only slight differences in activity. The Mutu III cell line, however, showed an exceedingly high EBER2 promoter activity. To find out the reason for this high activity, a more detailed examination of the EBER2 is needed with special regard to the protein binding pattern at the promoter.

As there is no *in vivo* methylated EBER sequence described in the literature, I used *in vitro* methylated constructs to determine, if the EBERs are sensitive or insensitive to CpG-methylation. Using the methylated construct I could assess, that the methylation of the EBER region causes the complete inhibition of EBER1 and EBER2 transcription, suggesting that the transcription of these genes is methylation-sensitive. Within the 5' regulatory region of the EBER1 promoter the binding of ATF and c-Myc proteins was described to be sensitive to the methylation of the respective recognition sequence (Tierney *et al*., 2000; Prendergast and Ziff, 1991b). C-Myc has two binding sequences in the EBER promoter, one without a CpG motif and one with a CpG motif in the sequence. The binding of Sp1 protein, however, is insensitive to CpG-methylation (Holler *et al*., 1988). Although, these sequences have a remarkable effect on the activity of the EBER genes, as EBERs are transcribed by Pol III, it is difficult to evaluate the significance of the methylation-sensitive Pol II transcription factor binding sites that are present within the EBER regulatory region. (Howe and Shu, 1989; Niller *et al*., 2003).

CpG-methylation can act via different mechanism leading to the inactivation of certain genes. On the one hand, the methyl-group can directly interfere with the binding of transcription factors to their recognition sequence. On the other hand, CpG-methylation can attract methyl-CpG binding proteins resulting in chromatin remodelling into a compact form. To explore the consequences of the methylation on the EBER1 promoter, and to extend our knowledge about the proteins binding to that promoter, I examined the protein-DNA interactions in the EBER1 regulatory region in the C666-1 nasopharyngeal carcinoma cell line. This seemed to be interesting, because epithelial cells can also be infected by EBV and they have different intracellular conditions (proteome, transcriptome), than B cells. Our research group has already published the *in vivo* footprints of the EBER locus in differernt cell lines of B cell origin (Niller *et al*., 2003). The observed pattern of footprints in the C666-1 cell line was found to be identical with the earlier described patterns observed in B cell lines, suggesting that proteins binding to the promoter of EBER1 in different cell lines are not B cell-specific, and the binding of these transcription factors do not depend on the cellular origin.

To see if CpG-methylation acts through interfering with the binding of transcription factors to their cognate recognition sequences, I performed *in vitro* footprinting, as there is no *in vivo* methylated EBER sequence described in the literature. According to the *in vitro* footprinting results the binding of the c-Myc and the ATF proteins to the EBER1 promoter is hindered by methylated cytosines. These *in vitro* observations are further supported by electrophoretic mobility shift assays as well (Niller *et al*., 2003, see: 5.6.). As the binding of c-Myc to the EBER1 promoter enhances the activity of the gene (Niller *et al*., 2003) and the proximal upstream sequence including the ATF site is necessary for full EBER1 promoter activity (Howe and Shu, 1989), the methylation-sensitive binding of these factors to their recognition sequences flanking the EBER1 transcription unit implies that hypomethylation of these sequences *in vivo* may be a precondition for EBER1 transcription. The minor differences in the *in vitro* footprints of the Pol III elements (Box A and B) can also be meaningful, because they may also contribute to the inactivation of CpG-methylated EBER sequences in the *in vitro* experimens.

The binding of the c-Myc protein to the EBER1 upstream promoter region was confirmed *in vivo* with chromatin immunoprecipitation (Niller *et al*., 2003). This binding may have several important consequences.

Translocation of the c-myc gene in the germinal center can be regarded as a byproduct of the germinal center reaction (Goossens *et al*., 1998), because of the elevated activity of the RAG (Recombination Activating Gene) and other genes e.g. AID (Activation-Induced (Cytidine) Deaminase) or Pol η involved in processes that alters the germ line genetic information. In germinal center B cells the level of pro-apoptotic proteins is remarkedly high, perhaps because the arising useless and harmful clones have to be deleted. Translocation of the c-myc gene to an active chromosome region – to one of the immunglobulin loci in BLs – results in the highly elevated level of the pro-apoptotic and also tumorigenic c-Myc protein. We propose that in EBV-positive GC B cells (Germinal Center B cells) the high amount of c-Myc produced due to the translocated c-myc gene, can transactivate EBER1 and EBER2 that promote antiapoptotic and tumorigenic alterations. As a consequence, the fastidious balance between the apoptotic and antiapoptotic processes may come out of controll, leading to an increased statistical probability of the survival of oncogenic clones and the onset of malignant manifestations (Niller *et al*., 2004a; 2004b).

In addition, c-Myc contributes to the enchorage of certain DNA regions to the nuclear matrix, making it easier for the transcription factors to access their binding sites and activate gene expression (Van Straaten and Rabbitts, 1987). In the EBV genome the matrix attachment region (MAR) is located around the EBER genes and the closely located latent replication origin *ori*P. This region was found to be more accessible for the micrococcal nucleases than the other parts of the viral genome, indicating an open chromatin state of the region (Wensing *et al*., 2001). Because the c-Myc is involved in chromatin remodelling processes as well (Amati *et al*., 2001), one may speculate that c-Myc may contribute to the activity of the EBER1 promoter, via the alteration of chromatin structure, keeping the region in an open conformation.

As mentioned above the EBER region is in an open chromatin state *in vivo*. In addition, the unmethylated CpGs are not restricted to the transcription factor binding sites, but the entire EBER region was found to be extensive hypomethylated similarly to Qp. This may imply, that the unmethylated state of the region is important not only in facilitating the binding of activator proteins to the promoter, but also in keeping the complete region in an open conformation.

As demonstrated in EMSA and chromatin immunoprecipitation experiments, the binding of CTCF to its putative binding site on the 5' regulatory sequence of the EBER1 could not be unequivocally verified, at best a week binding could be observed.

The examination of activating histone modifications of the region showed, that the complete EBER region is enriched in acetylated histone H3 (acH3), acetylated histone H4 (acH4) and dimethylated lysine 4 of histone H3 (H3K4me2) in all the cell lines studied. This further supports the idea, that the region is in an open chromatin conformation. In the C666-1 cell line, however, the H3K4me2 modification was found to be excluded from the 5' region of the EBER1 promoter. Interestingly, in all cell lines the level of acH3 and acH4 in the EBER coding regions had a good correlation with the amount of the EBER transcripts within the cell, and not with the activity of the promoter. Different EBV-carrying cell lines have different copy numbers of the viral episomes within a single cell. As a consequence, the same promoter activity may result in different amounts of certain EBV-encoded RNAs. Because of this, it is important to distinguish between relative RNA amounts and promoter activity.

The H3K4me2 is quite abundant in the 5' regulatory sequence in all cell lines (except for C666) and its amount in the coding regions was inverse with acH3 and acH4 in the Rael, CBM1 and the Mutu I, Mutu III cell line paires. These observations raise

the possibility that the activity control of the EBER genes through the acetylation of histone H3 and H4 within the region is sensitive to the amount of the gene product. This means, that the acetylation state of the EBER genes may set the global amount of transcripts derived from the EBER1 and 2 promoters in a cell and not the tuning of the activity of the single promoters, or that the amount of gene products may set the acetylation state of the regulatory region. In addition, the amount of the acetylated histones in the EBER region may be regulated via a latency type-dependent manner, as can be suspected because of the elevated acetylated histone amounts of the latency type I (Rael, Mutu I) cell lines compared to their latency type III (CBM1, Mutu III) cell line paires. The same but the inverse relation can be found in the H3K4me2 modification, as it is more enriched in the latency type III cell lines then in their latency type I cell line paires.

7. SUMMARY

During my work, I used EBV-negative and EBV-carrying cell lines, showing different gene expression program. I studied the activity of the EBER1 and EBER2 genes, the epigenetic characteristics, including the CpG-methylation pattern and activating histone modifications of the 5' EBER1 regulatory, the EBER1 and EBER2 coding regions, and the protein binding pattern at the EBER1 promoter in the C666-1 nasopharyngeal carcinoma cell line.

The conclusions of my results are the followings:

1. High resolution CpG-methylation mapping of CpGs located within the EBER locus showed an extensive hypomethylation independently of the cellular phenotype.

2. In all of the examined cell lines the EBER1 and EBER2 genes were found to be highly active and abundantly transcribed, suggesting a methylation-sensitive transcription of these Pol III driven genes.

3. *In vitro* CpG-methylation of the EBER region resulted in a dramatical decrease in the transcription of EBERs in transfected cells, suggesting that the EBER promoters are methylation-sensitive.

4. *In vivo* genomic footprinting of the EBER locus showed, that in the nasopharyngeal carcinoma cell line C666-1 the protein binding pattern was similar to that of B cells of different origin. This may indicate that the binding of transcription factors to the EBER1 promoter does not depend on the cell type.

5. The altered protein binding to many regulatory protein binding sites (c-Myc, Sp1, ATF, Box-A and -B) in the *in vitro* methylated EBER1 promoter sequence raised the possibility, that DNA-methylation blocks the binding of activating transcription factors to the promoter.

6. Using the electrophoretic mobility shift assay I demonstrated, that the binding of ATF to the EBER1 promoter is impaired by CpG-methylation *in vitro* and under *in vitro* conditions the transcription factor bound to unmethylated EBER1p is different from ATF-2.

7. In similar EMSA experiments specific CTCF binding to the EBER promoter could not be observed, although a very week binding could be detected with ChIP experiments.

8. The binding of the transcription factor and oncoprotein c-Myc to the 5' promoter region of the EBER1 gene was demonstrated *in vivo* using chromatin immunoprecipitation. This binding may contribute to the survival of certain clones formed during the GC reaction and may lead to the development of malignant disorders. In addition, this binding can be important in keeping the region in an open conformation, and in the maintenance of latent EBV episomes by facilitating their attachment to the nuclear matrix.

9. The activating histone modifications were found to be highly enriched in the entire EBER locus in all the examined cell lines. A comparison of these results with the EBER RNA amounts and EBER gene activity suggest, that the acetylated H3 and H4 can regulate the gene activity on a product-dependent way, which may be latency type-specific.

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My Wife, my new family. Without the instinction originating from their liking and loving I could hardly get to the point of writing these lines.

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SUMMARY IN HUNGARIAN AND ENGLISH

In vivo fehérje-DNS kölcsönhatások és CpG-metilációs mintázatok vizsgálata látens Epstein-Barr vírus genomok EBER promóter régiójában

A soksejtű szervezetek különféle sejttípusokból épülnek fel, amelyek pontosan ugyanazt vagy szinte ugyanazt a genetikai információt hordozzák. A sejtek fenotípusos sokszínűsége a gének eltérően kontrollált szabályozásán keresztül valósul meg. Bár az RNS polimeráz II által átírt gének genetikai és epigenetikai szabályozási mechanizmusai és a génaktivitás között fennálló kapcsolatokat széles körben tanulmányozták, az RNS polimeráz III által átírt gének szabályozása kevésbé ismert.

A daganatos és egyéb megbetegedésekkel kapcsolatba hozható Epstein-Barr vírus (EBV) által kódolt, az RNS polimeráz III segítségével átírt, nem transzlálódó EBER1 és 2 RNS-ek hozzájárulnak a vírus patogenitásához. Munkám során az EBER gének aktivitását befolyásoló szabályozó mechanizmusok feltérképezését végeztem.

Megállapítottam, hogy az általam vizsgált sejvonalakban az EBER gének magas aktivitásuk mellett metilálatlanok voltak, vagy csak kis mértékű metilációt mutattak. Transzfekciós kísérletekkel igazoltam, hogy a CpG-metiláció gátolja az EBER gének transzkripcióját. Vizsgálataim alapján valószínűsíthető, hogy az EBER gének inaktiválódását a régió *in vitro* metilációját követően, a metiláció-érzékeny szabályozó szekvenciákhoz kapcsolódó transzkripciós faktorok kötődésének gátlása okozta. A C666-1 nazofaringeális karcinóma sejtvonal vizsgálata során azt találtam, hogy az EBER1 szabályozó régiójának fehérje-kölcsönhatásai megegyeznek a B sejtek esetén leírtakkal, vagyis nagy valószínűséggel nem sejttípus-specifikusak. Igazoltam a c-Myc onkoprotein kapcsolódását az EBER1 promóteréhez, ami közrejátszhat az endémiás Burkitt limfóma kialakulásában, és fontos szerepet játszhat az EBV látens életciklusában is. Az inzulátor szerepet játszó CTCF fehérje ugyanakkor csak gyengén vagy közvetett módon kapcsolódik az EBER lókuszhoz. Az aktiváló hiszton módosulások szintje jó korrelációt mutatott az EBER régióról átíródó RNS-ek mennyiségével. Az aktiváló hiszton módosítások jelenléte az EBER régió teljes területén – a hipometilált állapot és az állandó EBER RNS transzkripció mellett – arra utal, hogy az EBER lókusz nyitott szerkezetű kromatin doménként jellemezhető.

Examination of *in vivo* protein-DNA interactions and CpG-methylation patterns in the EBER promoter region of latent Epstein-Barr virus genomes.

Multicellular organisms consist of different cell types, carrying exactly the same or nearly the same genetic information. This phenotypic diversity can be attributed to the differentially controlled regulation of gene activity within a single cell. Although the relation between the genetic and epigenetic regulatory mechanisms and the activity of RNA polymerase II-transcribed genes is widely studied, the regulation of RNA polymerase III-transcribed genes is less understood.

Epstein-Barr virus (EBV) is connected to malignant and other disorders. EBV codes for the non-translated EBER1 and 2 RNAs, transcribed by RNA polymerase III. These RNAs contribute to the pathogenic potential of the virus. During my work I focused on the regulatory mechanisms influencing the activity of the EBER genes.

I determined that in all of the examined cell lines the EBER genes were either unmethylated or showed a moderate level of methylation, and they were actively transcribed in each case. My transfection experiments supplied evidence that the transcription of the EBER genes is inhibited by CpG-methylation. Based on my *in vitro* studies I suggest, that the *in vitro* methylation-mediated inactivation of the EBER genes was a consequence of the impaired binding of methylation-sensitive transcription factors to their regulatory sequences. The analysis of the C666-1 nasopharyngeal carcinoma cell line showed that the protein interactions of the EBER1 regulatory region are presumably not cell type-specific, because they were identical to those described B cells. I could verify the binding of the oncoprotein c-Myc to the EBER1 promoter. This interaction may participate in the development of endemic Burkitt's lymphoma and may play an important role in the latent life cycle of EBV as well. I found, however, that the binding of CTCF, an insulator protein, to the EBER locus was week or indirect. The levels of activating histone modifications showed a good correlation with the RNA amounts transcribed from the EBER region. The presence of activating histone modifications in the entire EBER region, together with the hypomethylated status and the constitutive expression of EBERs, supports the existance of an open chromatin conformation.

THIS DISSERTATION IS BASED ON THE FOLLOWING PUBLICATIONS

1. Niller, H.H., Salamon,D., Ilg, K., Koroknai, A., **Banati, F**., Bäuml, G., Rücker O. L., Schwarzmann, F., Wolf, F., Minarovits, J. (2003). The *in vivo* binding site for oncoprotein c-Myc in the promoter for Epstein-Barr virus (EBV) encoded RNA (EBER) 1 suggest a specific role for EBV in lymphomagenesis. Med. Sci. Monit. 9(1):HY1-9.

2. **Banati F**, Koroknai A, Salamon D, Takacs M, Minarovits-Kormuta S, Wolf H, Niller HH, Minarovits J (2008). CpG-methylation silences the activity of the RNA polymerase III transcribed EBER-1 promoter of Epstein-Barr virus. FEBS Lett. 582(5):705-9.

3. Salamon, D., **Banati, F***., Koroknai, A., Ravasz, M., Szenthe, K., Bathori, Z., Bakos, A., Niller, H.H., Wolf, H., Minarovits, J. (2009). Binding of CTCF *in vivo* to the region located between Rep* and C-promoter of Epstein-Barr virus is unaffected by CpG methylation and does not correlate with Cp activity. J. Gen. Virol. 90(Pt 5):1183-9.

APPENDIX