Regulation of the Epstein-Barr virus C promoter by the *OriP*-EBNA1 complex

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till Mattias och Matilda

Always remember: you're braver than you believe, and stronger than you seem, and smarter than you think.
A. A. Milne

ABSTRACT

Epstein-Barr Virus (EBV) is an exclusively human, lymphotropic herpes virus that infects more than 90% of the population worldwide. Primary infection usually occurs during the early years of life and does not result in any recognized disease. EBV is the causative agent of infectious mononucleosis and is associated with various malignancies including Burkitt's lymphoma (BL), Hodgkin's disease (HD), nasopharyngeal carcinoma (NPC), and immunoblastic lymphomas in immunocompromised individuals. In most immunocompetent individuals the virus is, however, harbored for life within latently infected resting memory B cells, causing no symptoms. *In vitro*, EBV efficiently transforms resting B cells to activated lymphoblasts. These perpetually dividing cells express a repertoire of viral antigens (EBNA1-6 and LMP1), all of which have been directly implicated in the immortalization process. Immediately post-infection, EBNA2 and -5 are the expressed from the W promoter (Wp), within 36 hours there is a switch in promoter usage from Wp to the upstream C promoter (Cp). Transcription from Cp leads to a concomitant expression of all EBNAs from a polycistronic transcription unit that is spliced to yield the different EBNA proteins. EBNA1 forms multiple homodimers that bind to a portion of the latency origin of replication (*oriPI*) that functions as an EBNA1-dependent enhancer of the Cp. The mechanism for the interaction between the *oriPI*-EBNA1 complex and the Cp is not completely understood at the molecular level. EBNA1 has no apparent enzymatic activities and is thought to fulfill its functions by mediating interactions with specific host cellular proteins, only few of which have been characterized. The aim of this thesis was to identify and characterize the interaction partners of this macromolecular complex.

The interactions of the transcription factors NF-Y and Sp1 with the promoter-proximal region of the Cp were previously established in our lab. In paper I we studied these interactions further using transient transfections, establishing that NF-Y and Sp1 co-stimulate Cp and that the *oriPI*-EBNA1-induced transactivation of Cp requires concomitant expression of both proteins. Furthermore, using the lymphoblastoid cell line EREB2-5, in which EBNA2 function is regulated by estrogen, we demonstrated that inactivation of EBNA2 resulted in decreased expression of NF-Y and down-regulation of Cp. Knowing that resting B cells do not express NF-Y and observing that this factor is essential for Cp activation, we suggest that its up-regulation post-infection may contribute to the Wp-to-Cp switch in primary EBV infection. The *oriPI* contains 20 repeats of the EBNA1 binding domain. In paper II we used a series of *oriPI*-deletions in *oriPI*-CpCAT reporter plasmids in transient transfections to determine the number of EBNA1 binding repeats necessary for efficient transactivation of the Cp. We showed that eight or more repeats were necessary for this effect, which underscores the complexity of the transactivation process.

In papers III and IV we set out to identify novel interaction partners of the *oriPI*- and -170Cp regions using EMSA and DNA affinity purification coupled with mass spectrometry. Three novel protein interactions with the *oriPI* and the Cp were identified. The transcription factors Bright, E2F1 and Oct-2 were found to bind both sequences *in vitro* and *in vivo*, opening up a possibility of mediating a link between the *oriPI* and the Cp. The binding sites of all three proteins were mapped to a short segment of Cp in close proximity of each other. This region was previously shown to be essential for both *oriPI*-dependent and -independent transcriptional activation, indicating that the interactions are important for Cp activity. In transient transfections, we demonstrated that exogenous Oct-2 or Bright expression up-regulated *oriPI*-dependent Cp activation in the absence of EBNA1. Finally, endogenous Bright expression was shown to correlate with latency III but not latency I and II expression patterns in EBV positive cell lines, further supporting the notion that Bright expression is important for Cp transcriptional activity *in vivo*.

PUBLICATIONS

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

I. Functional Interaction of Nuclear Factor Y and Sp1 is Required for Activation of the Epstein-Barr virus C promoter.
    Boreström, C., Zetterberg, H., Liff, K., and Rymo, L.
    Journal of Virology (2003) 77(2): p. 821-9

II. Multiple EBNA1-binding sites within *oriPI* are required for EBNA1-dependent transactivation of the Epstein-Barr virus C promoter.
    Zetterberg, H., **Boreström, C.**, Nilsson, T., and Rymo, L.
    International Journal of Oncology (2004) 25 (3): p 693-6

III. Functional Interaction of Oct transcription factors with the Family of Repeats in Epstein-Barr virus *oriP*.
    Almqvist, J., Zou, J., Linderson, Y., **Boreström, C.**, Altiok, E., Zetterberg, H., Rymo, L., Pettersson, S., and Ernberg, I.
    Journal of General Virology (2005) 86(5): p. 1261-7

IV. Bright, E2F1 and Oct-2 bind the Epstein-Barr virus C promoter and the *oriPI*, linking the promoter to the enhancer
    Boreström, C., Rüetschi, U., and Rymo, L.
    In manuscript (2008)

ABBREVIATIONS

ATP	adenosine triphosphate
AUF1	AU-rich element RNA binding protein 1
ARID3A	AT-rich interaction domain DNAbinding protein 3A, a.k.a. Bright
bp	base pair
BART	*Bam*HI A rightward transcripts
BCR	B cell receptor
BL	Burkitt's lymphoma
Btk	Bruton's tyrosine kinase
CAT	chloramphenicol acetyl transferase
CBF	C promoter binding factor
CBP	CREB binding protein
CDK	cyclin dependent kinase
cDNA	complementary DNA
ChIP	chromatin immuniprecipitation
Cp	the EBV C promoter
CpG	cytosine and guanine separated by a phosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DS	dyad symmetry, i.e. *oriPII*
E2F	a eukaryotic transcription factor family
E2RE	EBNA2 respons element
EBER	Epstein-Barr virus encoded RNA
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
Egr-1	early growth response protein 1
EMSA	electromobility shift assay
FACS	fluorescence-activated cell sorting
FR	family of repeats, i.e. *oriPI*
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	germinal center
GRE	glucocortikoid response element
HAT	histone acetyltransferase
HD	Hodgkin's disease
HDAC	histone deacetylase
HMT	histone methyltransferase
Ig	immunoglobulin
IM	infectious mononucleosis
JNK	c-Jun N terminal kinase
kb	kilobase pair

LCL	lymphoblastoid cell line
LCR	locus control region
LMP	latent membrane protein
LSD1	lysine-specific demethylase 1
LUC	luciferase
MAPK	mitogen-activated protein kinase
MAR	matrix association/attachment regions
mRNA	messenger RNA
m/z	mass-to-charge ratio
NE	nuclear extract
NFκB	nuclear factor κB
NK cell	natural killer cell
NMP	nuclear matrix proteins
NPC	nasopharyngeal carcinoma
nt	nucleotide
NF-Y	nuclear factor Y
Oct-1	octamer binding factor 1
Oct-2	octamer binding factor 2
OriP	origin of replication
OriPI	part of *OriP* with family of repeats
PAGE	polyacrylamide gel electrophoresis
p300	transcriptional co-activator closely related to CBP
P/CAF	p300/CBP associated factor
PCR	polymerase chain reaction
PIC	preinitiation complex
PTLD	post transplant lymphoproliferative disease
PVDF	polyvinylidene fluoride
Qp	the EBV Q promoter
Q PCR	quantitative PCR
Rb	retinoblastoma protein
RBPJκ	recombination signal binding protein for immunoglobulin kappa J region
RNA	ribonucleic acid
RNA pol II	RNA polymerase II
RT PCR	reverse transcription PCR or real time PCR
TFII-I	transcription factor II-I
TR	terminal repeats
TRAFs	tumor necrosis factor receptor–associated factors
Wp	the EBV W promoter

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EPSTEIN-BARR VIRUS

1.1 The early days of EBV

In the late 1950s a young British "bush surgeon" named Denis Burkitt practiced medicine in Mulago Hospital in Kampala, the capital of Uganda. During his line of work, he came into contact with a couple of young patients displaying swellings in the four angles of the jaws, one of which also had abdominal tumors. Intrigued by this observation, Burkitt conducted a study of the records department of the hospital, discovering documentation regarding a large number of children with jaw tumors, often associated with other tumors at unusual sites. Back in England he enlisted the help of a pathologist who diagnosed the tumors as unusual lymphomas (1). Around this time Anthony Epstein came into contact with Denis Burkitt and a collaboration was initiated. The strange tumors discovered by Burkitt caught Epstein's eye, and the idea that a novel virus could be involved in the development of these tumors was born. During a couple of years in the early 1960s the Epstein lab regularly received biopsy samples from Burkitt in Uganda, and from one of these deliveries the first Burkitt's lymphoma (BL) derived continuous cell line was derived; EB1 (2). By examining this first cell line by electron microscopy, Epstein and his co-workers Yvonne Barr and Bert Achong found virus particles in the cancer cells and observed that the virus had the typical morphology of a herpes virus. In order to study this virus further, Epstein contacted one of the foremost US virus diagnostic and research laboratories, the Henle lab in Philadelphia. EBV carrying cells were shipped from England to Philadelphia and through a joint effort it was determined that this new virus indeed was the newest member of the herpes virus family (3). In 1968 the virus received its proper name Epstein-Barr virus after Michael Anthony Epstein and Yvonne Barr, who together with Bert Achong, discovered the virus in the EB1 cell line (4).

1.2 The virus life cycle

Epstein-Barr Virus (EBV) is an exclusively human, lymphotropic herpes virus that infects more than 90% of the population worldwide (5). Primary infection usually occurs during the early years of life and does not result in any recognized disease. However, a dramatic immune response can occur if the primary infection is delayed through adolescence or adulthood, which frequently results in infectious mononucleosis, a self-limiting lymphoproliferative disorder (6). EBV enters the host through the oropharynx and is believed to initially infect either squamous epithelial cells or resting B cells sitting at or close to the surface of tonsillar epithelia or other lymphoid organs in Waldeyer's ring (see Fig. 1). The viral glycoprotein gp350 binds to the B cell receptor CD21. Upon binding the virion partly enters the cell, and partly remains on the B cell surface, enabling efficient transfer to CD21-negative epithelial cells (7). Thus, EBV has developed a way to access both B cells and squamous epithelial cells of the oropharynx, simultaneously. However, B cells are currently considered to be necessary and sufficient for EBV infection, while epithelial cells may be seen as helpful enhancers for transferring the virus to others, and for establishing latency in B cells (8, 9). If an EBV-infected memory B cell differentiates to a plasma cell, the virus is released for further infectious spread (10).

The default viral pathway for EBV is latency and in most immunocompetent individuals the virus is harbored for life within latently infected resting memory B cells, without causing any symptoms (11, 12). In this state of latency, the virus resides as chromatin-associated, multicopy episomes in the infected cells that occasionally produce viral particles that are released in the saliva (13). The EBV episomes replicate once per cell cycle and segregate stably during cell division (14). *In vitro*, EBV efficiently transforms resting B cells to activated lymphoblasts. These, perpetually dividing cells, express a repertoire of viral antigens (EBNA1-6 and LMP1), all of which have been directly implicated in the immortalization process (14). Transformed lymphoblasts are susceptible to immune control by cytotoxic T cells and are abundant only during primary infection and in individuals with impaired cell mediated immunity (11).

The viral genome contains six latency promoters (Wp, Cp, Qp, LMP1- and LMP2A/B promoters) that regulate the expression of all viral genes essential for viral genome persistence and cell survival in the infected host (fig. 2). The promoters are on their own, or in combination with each other, active during defined periods of the infection and are tightly regulated by viral and cellular transcription factors as well as by epigenetic events. The products of all latent EBV genes are summarized in table 1.
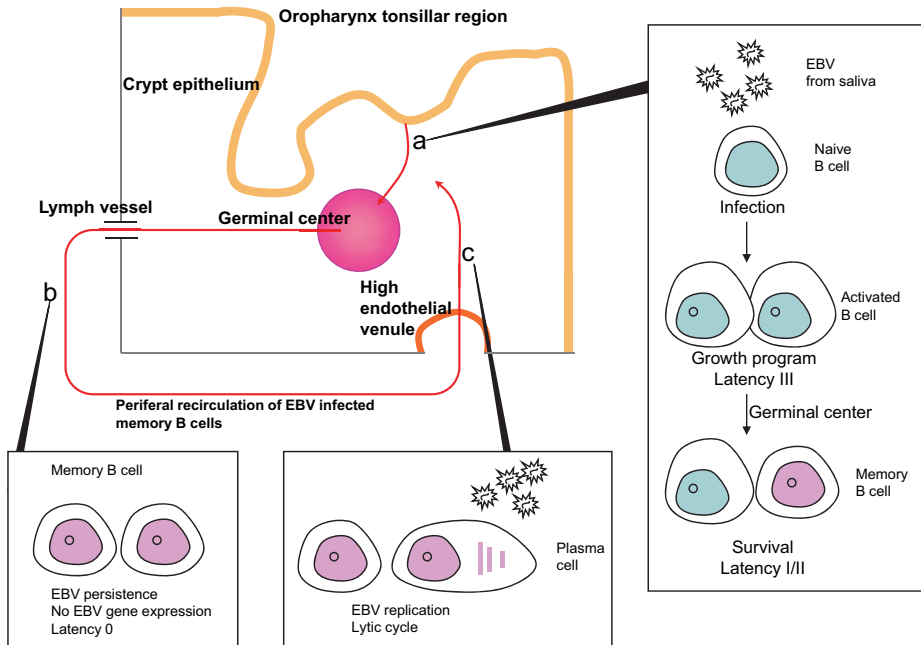
Figure 1. A model for Epstein–Barr virus (EBV) infection and persistence.
(a) In the oropharynx, EBV infects naive B cells and expresses a full spectrum of latent proteins (latency III, EBNAs1-6, LMPs 1 and 2A). The virus can thereby drive the activation and proliferation of the infected B cells. At this stage, many of these lymphoblasts are killed by a cytotoxic T cell response. However, some of the cells escape the immune response and undergo a germinal center (GC) reaction where a more limited set of viral genes are expressed (latency II/I) (b) The infected GC cells are subsequently rescued and develop into memory B cells, where no EBV gene expression is detected (latency 0), allowing the virus to persist within the B cells but to evade a host immune response. Intermittent expression of EBNA1 within dividing B cells allows the virus genome to be distributed to each of the daughter B cells (latency I). (c) As B cells recirculate to the oropharynx, a switch into the EBV lytic cycle may occur, possibly triggered by maturation of B cells into plasma cells, allowing for virus replication, shedding into saliva and transmission both to new hosts and to previously uninfected B cells within the same host. Adapted from (15).
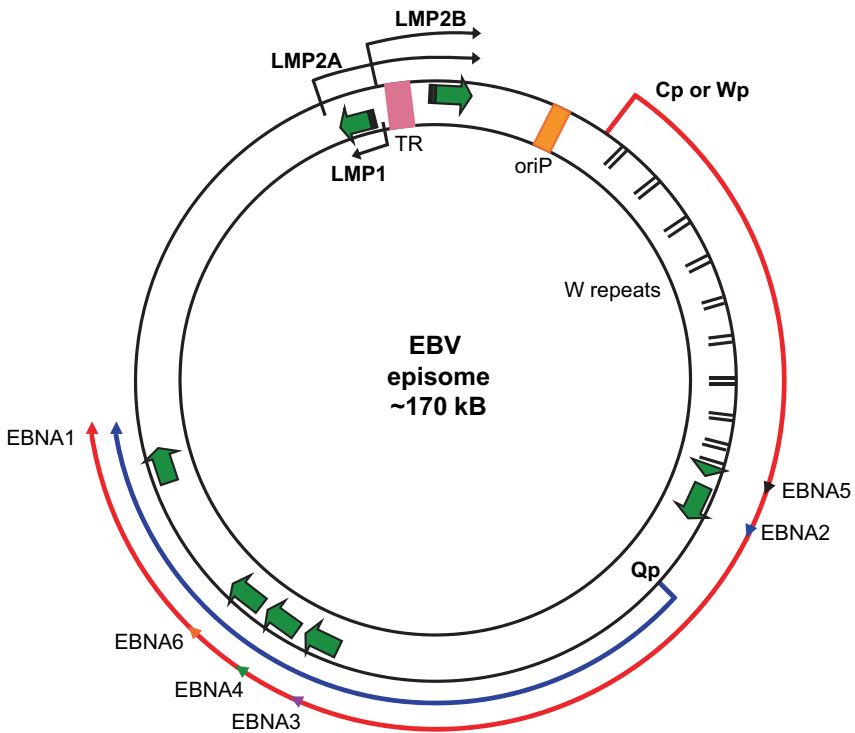
Figure 2. The Epstein-Barr virus genome
A schematic representation of the EBV double-stranded DNA episome. The linear viral DNA circularizes at the terminal repeats (TR-pink box) upon cell entry. The origin of plasmid replication (*oriP*) is shown in orange. The six latent antigens (EBNAs1-6) are produced by alternative splicing of one long primary transcript initiated from either the W promoter (Wp) or the C promoter (Cp) in cells with a latency III expression pattern. The Q promoter (Qp) drives the expression of EBNA1 in cells with a latency I and II expression pattern. The exons of the latent membrane proteins (LMP2s) A and B are located on either side of the terminal repeat (TR) regions, therefore their expression require circularization of the viral DNA. The LMP1 protein is transcribed in a leftward direction downstream of the TR. Adapted from (16).

Upon *in vitro* infection of resting B cells the latent W promoter (Wp) is the first to be activated and produces all EBNAs. The Wp is present in multiple copies that are thought to give the EBNA- transcription a jump-start in the early infection process. EBNA5 and EBNA2 are the first two proteins detected, and within 36 hours there is a switch in promoter usage to the Cp (17). The mechanism behind this switch is not fully understood at the molecular level, but EBNA2 appears to play an important role (18).

Table 1. Latent EBV gene products and their possible functions (14)

Viral gene product	Function	Required for immortalisation	Latency
EBNA1	▪ viral genome replication and maintenance ▪ transcriptional activation of Cp and LMP1 promoter via interaction with *oriPI* ▪ downregulates transcription from the Qp	+	I-III
EBNA2	▪ Viral oncogene ▪ Transactivates latent viral as well as cellular genes	+	III
EBNA3 (-3A)	▪ Transactivates cellular genes	+	III
EBNA4 (-3B)	▪ Transactivates cellular genes	-	III
EBNA6 (-3C)	▪ Viral oncogene ▪ Increases LMP1 expression	+	III
EBNA5 (-LP)	▪ Co-activates EBNA2-responsive genes ▪ Implicated in inhibition of pre-mRNA cleavage and polyadenylation	+/-[b]	III
LMP1	▪ Viral oncogene ▪ Induces B cell activation and adhesion ▪ Protects from apoptosis	+	II-III
LMP2A and -2B	▪ Repression of lytic cycle ▪ Enhances B cell survival	-	0, II-III
EBERs	▪ Non-translated RNAs ▪ May regulate splicing and/or translation	-	0-III
*Bam*HI A RNAs	▪ Functions unknown	-	I-III

[b] not essential but enhances efficiency of B cell transformation

The variability in gene expression during the EBV latent cycle provides a mechanism for viral persistence and immune evasion (19, 20). The different patterns of gene expression in latency are referred to as latency types (0-III), and correlate with cellular context and tumor classification. Promoter selection plays a key role in determining latency type, and this selection depends on the interplay of host-cell-specific transcription factors and poorly defined epigenetic events that reinforce gene expression patterns over multiple generations (17, 21).

In endemic and sporadic BL, EBV typically expresses a restricted pattern, type I latency, characterized by expression of the EBNA1, EBERS, and BART genes (22). In NPC, the EBV expression pattern is typically a type II latency which in addition to type I genes expresses LMP1 and LMP2A. In PTLD, EBV typically expresses a type III latency program with expression of a full panel of latent viral genes including EBNAs1-6 genes (23). Type 0 latency is reserved for quiescent, memory B cells where no viral genes are expressed (24). The fact that EBV can access and persist in memory B cells without causing disease is the key to its success in infecting most of the human population and is evidence of its co-evolution with man.

1.3 EBV and the cell cycle

The series of events that takes place as one cell becomes two is referred to as the cell cycle. This cycling phenomenon is divided into separate steps called G1, S, G2 and M. Most differentiated cells withdraw from the cell cycle during G1, entering the resting state called G0. For multicellular organisms, the initiation and progression of the cell cycle is tightly regulated by external stimuli, ensuring that the cell division happens only in the desired systems and compartments. The antigen-response in a naive B cell is an example of such a stimulus. Upon contact with an antigen, the naive B cell enters the cell cycle to produce a colony of cells, most of which differentiate into antibody-producing plasma cells and clear away the infection. The remaining cells persist as memory cells that can survive for years, or even a lifetime. In a normal adult, cells divide only when and where they are needed; however loss of normal controls on cell replication is the fundamental defect in cancer. The progression of the cell cycle is tightly controlled by checkpoint surveillance mechanisms that prevent initiation of each step until earlier steps on which it depends have been completed. The master controllers of the progression of the cell cycle are a small number of heterodimeric protein kinases that contain a regulatory subunit (cyclin) and a catalytic subunit (CDK-cyclin dependent kinase). These kinases regulate the activity of multiple proteins involved in DNA replication and mitosis by phosphorylation. CDK levels are relatively constant during the cell cycle, whereas cyclin levels fluctuate. Furthermore, large multisubunit ubiquitin protein ligases poly-ubiquitinylate key cell cycle regulators, marking them for degradation by the proteasome. This irreversible process forces the cells through the cell cycle in one direction only. In G1/S and G2/M the cell cycle can be halted by check point proteins such as p53. These types of check point proteins recognize imperfections in the replication such as DNA damage and induce cell cycle arrest (25).

The current model of EBV cell cycle regulation suggests that the virus utilizes its latency proteins to drive B cell proliferation, inducing a phenotype that closely mimics antigen-driven B cell activation and expansion. This process occurs in the absence of B cell receptor (BCR) engagement and T cell signaling (26). EBNA5 is the first latent protein expressed upon EBV infection of naive B cells *in vitro*, tightly followed by EBNA2. This expression pattern suggests that both of these latent proteins are involved in early B cell activation events. Together they induce the G0-G1 transition in primary B cells, by activating the expression of cyclin D2 (27). This activation is executed indirectly through the ability of EBNA2 to activate the proto-oncogen c-Myc, a known regulator of cyclin D2 (28). To add to the importance of EBNA2 in cell cycle progression, Kempkes et al. showed in 1995, that disruption of EBNA2 function in established LCLs causes a block in cell cycle progression in both G1 and G2, and that restoration of EBNA2 promotes S phase entry (29). Furthermore, EBNA5 binds p53 and Rb *in vitro* (30, 31), however the functional importance of these interactions is yet to be discovered. A third gene product of the Cp, EBNA6, has also been implicated in cell cycle regulation and B cell proliferation. Similar to EBNA5, EBNA6 binds Rb *in vitro*, and is also functionally linked to Rb-pathways inducing E2F dependent promoters and stimulating primary rodent fibroblast transformation with H-ras (32). Latent membrane protein 1 (LMP1) is another latent protein that is expressed early after primary infection. LMP1 is considered the major oncoprotein of EBV, as it transforms rodent fibroblasts to tumorigenicity in nude mice and is expressed in HD, NPC, and immunosuppression-associated tumors (5). In B cells, LMP1 mimics CD40 signaling, and both LMP1 and CD40 are essential for EBV-mediated B cell transformation (33-35). While CD40 interacts with CD40 ligands expressed on activated T cells to induce B cell activation and differentiation, LMP1 acts as a constitutive signal through ligand-independent oligomerization. LMP1 and CD40 interact with the same tumor necrosis factor receptor–associated factors (TRAFs) leading to activation of NFκB, c-Jun N terminal kinase (JNK), and p38 MAPK signaling pathways. All of these pathways are in one way or another involved in proliferation (36-40). Furthermore, activation of NFκB is required for EBV-induced B cell transformation and its inhibition rapidly results in cell death (41, 42).

1.4 EBV-Associated diseases

EBV is associated with a still growing range of benign and malignant diseases the most common of which are listed below. For a detailed review of EBV-associated diseases, see reference (14).

Infectious Mononucleosis

Infectious mononucleosis (IM) is a lymphoproliferative disease that might arise if primary EBV infection is delayed through adolescence or adulthood. The usual clinical symptoms are high fever, headache, chills and sweats, fatigue and a severe soar throat. The disease is self-limiting, but the convalescence can be very long. Patients with acute IM shed high titers of infectious virus in the throat from lytic infection in the oropharynx (15).

Burkitt's lymphoma

Burkitt's lymphoma (BL) is the most common childhood cancer in equatorial Africa, found at an annual incidence of ~5-10 cases per 100'000 children. This high-incidence form of endemic BL also affects children in Papua New Guinea, the common denominator in both areas being that malaria is holoendemic. EBV is present in all endemic BLs and in up to 85% of cases in areas of intermediate incidence, such as Brazil and North Africa, but in only 15% of the low-incidence sporadic tumors of children in the developed world. Worth noting is that BL also is quite common among adult HIV carriers in the developed world, some 30-40% of these tumors are EBV-associated (14). All BL lymphomas carry one of three characteristic chromosomal translocations that place the MYC gene under the control of the Ig heavy chain or of one of the light chain loci. This MYC deregulation has been shown to be a key factor in the pathogenesis of BL (43-45).

Nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) is an epithelial tumor that is characterized by marked geographic and population differences in incidence. In western populations the incidence is as low as 0.25% of all cancers, occurring at a rate of 0.1 cases per 100'000 people per year (46), whereas in southern China and Southeast Asia it may represent 20% of all cancers, with an incidence of 100 cases per 100'000 people per year in some regions. The tumor is also quite

frequent in the Inuit population as well as the population of Mediterranean Africa (47). The EBV genome is consistently detected in NPC regardless of geographic distribution or racial background (48). The extreme geographical distribution of this malignancy suggests that environmental factors as well as disease susceptibility genes are involved in the oncogenic process. Several traditional foods such as Cantonese Chinese salted fish, and Harissa, a common ingredient in North African cooking, contain compounds that have been shown to reactivate latent EBV infected B cells in vitro (49).

Lymphoproliferative disease in immunodeficiency

T cell-immunocompromised individuals, such as transplant- and AIDS-patients are at high risk of developing B cell lymphomas. Most post-transplant lymphoproliferative disorders (PTLDs) arise in the first year after transplantation, when the immunosuppression is the most severe. Similar tumors are observed in patients with certain inherited forms of immunodeficiency syndromes such as X-linked lymphoproliferative syndrome and Wiskott-Aldrich syndrome. The lesions are mostly of B cell origin and range from atypical polyclonal B cell proliferations, which often regress following withdrawal or reduction of immunosuppression, to aggressive non-Hodgkin lymphomas, which generally do not resolve following immune reconstitution (16).

Hodgkin's disease

Hodgkin's disease (HD) is an unusual lymphoma of the human lymphatic system. The malignant Hodgkin/Reed Sternberg cells account for only 1-2% of the total tumor mass, in the middle of a non-neoplastic inflammatory infiltrate (50). In EBV-positive HD (30-50% of all cases) the virus is localized to the malignant cells and is clonal. The EBV association is more frequent in childhood, in older adults (>45 years) and in mixed cellularity cases. The true contribution of EBV to the pathogenesis of HD remains unknown, the presence of EBV might reflect a poor immune status, which in turn means that the patients might tolerate disease and its treatment less well (51). Increased knowledge of the virus' role in the basic biology of HD may generate novel therapeutic strategies for EBV-positive HD and the presence of EBV-latent antigens in the malignant HD cells may represent a target for cellular immunotherapy.

2

TRANSCRIPTIONAL REGULATION

2.1 Chromatin Remodeling and Gene Regulatory Sequences

Gene expression is a multistep process involving chromatin remodeling, transcription, RNA processing, RNA export, and translation in the cytoplasm. Each of these steps is carried out by a highly specialized, elaborate machinery that is tightly controlled in both time and space. These controls enable the various genes of the cell to be turned on or off at the exact time and place. In eukaryotes, genomic DNA is packaged into a structure called chromatin, compacting DNA more than 10,000-fold. Such condensation of DNA provides a considerable obstacle to the nuclear machinery that drives processes such as replication, transcription or DNA repair. Importantly, the structure of chromatin is dynamic, permitting localized decondensation and remodeling that facilitate the progress of the nuclear machinery. The basic structural subunit of chromatin is the nucleosome that is composed of 146 bp of DNA wound around a disc-shaped octamer of histone proteins (52).

Chromatin remodeling is a process that requires the activity of specific chromatin-remodeling complexes. There are two general classes; ATP-dependent and ATP-independent nucleosome remodeling complexes (53). The ATP-dependent complexes facilitate access of DNA-binding proteins to DNA by repositioning nucleosomes at the promoter or inducing conformational changes in the nucleosome. This is achieved by sliding the nucleosomes and exposing the DNA (54) or by inducing a continuous twist that allows access to DNA sites even in the absence of histone movement (55, 56). The ATP-dependent complexes catalyze post-translational modifications of the histone tails including acetylation, phosphorylation and methylation (52). Three different histone modifiers are implicated in these modifications; histone acetyltransferases (HATs), histone methyltransferases (HMTs) and histone kinases. Modification of histone tails by acetylation is known to increase the access of transcription factors to DNA through structural changes in

nucleosomes; therefore hyperacetylation of promoter sequences is considered the hallmark of a transcriptionally active gene. Conversely, recruitment of histone deacetylaces (HDACs) by transcriptional repressors leads to deacetylation of histone tails and transcriptional repression (57). In contrast to the vast amount of information available regarding the enzymes that acetylate and deacetylate histones, less is known about the enzymes that generate other histone modifications, such as methylation and phosphorylation. Histone methylation is generally associated with transcriptional repression. However, methylation of some lysine and arginine residues of histones results in transcriptional activation, such as methylation of lysine 4 of histone 3 (H3mK4), and arginine residues on H3 and H4. Phosphorylation of serine 10 on Histone 3 is also associated with transcriptional activation (58). It is not understood how phosphorylation contributes to transcriptional activation. The addition of negatively charged phosphate groups could neutralize the histone tails and could therefore reduce their affinity for DNA (52). The phosphorylated amino acids can also facilitate binding of transcription factors. Phosphorylation of H2A has been implicated in chromosome condensation and phosphorylation of H3 takes places at the signal from the DNA damage pathways (reviewed in (59)).

Remodeling of the chromatin in the promoter region of a gene is the first stage of transcriptional initiation. Promoters are normally located immediately adjacent to the gene in question and the positions in the promoter are designated relative to the transcriptional start site, where transcription of RNA begins. Positions upstream are negative numbers counting back from -1, for example -100 is a position 100 base pairs upstream. A typical promoter consists of a multitude of binding sites (cis-regulatory elements) for positive and/or negative transcription factors and a core promoter that is composed of a TATA-box or an initiator element (INR). There are also distant elements called enhancers that up-regulate promoter activity. They may be located from 200 bases to up to several tens of kb upstream or downstream of a promoter, within an intron, or downstream of the final exon of the gene. These enhancers are believed to come into contact with the promoter by looping out the intervening DNA sequences and forming a multiprotein complex that ultimately recruits the transcriptional machinery (25).

Modifications that repress transcription can also be achieved at the level of the DNA strands. DNA methylation, predominately found in cytosine bases in promoter CpG islands, seems to be responsible for the stable maintenance of a particular gene expression pattern through the mitotic cell division. Furthermore, highly regulated DNA methylation is essential during cell differentiation and embryonic development (60). Repression through methylation is executed in two ways: it inhibits binding of certain transcription factors to regulatory elements, and it attracts methyl CpG-binding proteins which in turn interact with

co-repressors to silence transcription. DNA methyltransferases are responsible for introducing methylations in unmethylated sites, as well as maintaining the pre-existing methylation patterns during DNA replication (reviewed in (61)).

2.2 The nucleus-where does transcription take place?

The nucleus is the largest organelle of animal cells and is organized in specific compartments that include proteinaceous nuclear bodies, chromatin, nucleolus, and the nuclear matrix. This matrix has been the subject of debate for years (62-65). However, with the evolution of laboratory and microscopy techniques the appearance and possible functions of the nuclear matrix have been intensely studied. The nuclear matrix consists of both nuclear proteins and RNA. Nuclear matrix proteins (NMPs) represent about 30% of the nuclear proteins, and are involved in organizing the nuclear DNA into loop domains through interactions between Matrix Association/Attachment Regions (MARs) and NMPs. In addition to serving a role in organizing the chromatin, the nuclear matrix provides a platform for the assembly of protein machines (e.g. replication and transcription factories) involved in the processing of the genetic information (66-68). It has previously been widely assumed that the protein complexes needed for transcriptional activation diffuse to their target promoters and move along the double helix as the genetic code is being transcribed. There is however increasing evidence that the situation might be the contrary and that the polymerases gather in discrete nuclear structures, called transcription factories. This clustering ensures high local concentration of the factors needed for transcriptional activation, and the promoter-region of a gene would therefore relocate to a factory before being transcribed (69). Notably, enzymes involved in chromatin remodeling such as SWI/SNF and HATs have been reported to be associated with the nuclear matrix (70, 71). Furthermore, MARs often flank promoters or enhancers, placing the regions in close proximity to the sites where the proteins necessary for transcription reside (72-74). Although the model of transcription factories fits with the observation that active genes can transiently cluster (75), one has to keep in mind that the biochemical evidence for these transcription factories is still missing. Future experiments are needed to validate this model and to prove the functional relevance of transcription factories (76).

2.3 Transcriptional regulation of the C promoter

Several studies have been conducted regarding the nuclear matrix association of the EBV genome, all of which identify the latent origin of replication *oriP* as the

MAR of the EBV genome during latent infection (77-79). The EBV MAR is located in the center of the locus control region (LCR) (fig. 3). The LCR concept has been developed mainly from research on the human β globin locus. LCRs and LCR-like elements have been found in many mammalian genome loci (80). They control gene expression and replication timing within their respective chromosomal domains. Usually they consist of open chromatin and shield their domain from the influence of regulatory elements of neighboring chromosomal loops. They are either associated with nuclear matrix attachment, transcriptional enhancer and replication elements, or are composed of such elements (81). Similar to cellular LCRs, the EBV LCR is associated with a MAR, a transcriptional enhancer (*oriPI*) and a replication element (*oriP*), and is characterized by open chromatin that extends from the 5′ end of the EBV-encoded small RNAs (EBERs) locus to the 3′ end of *oriP* (81-83).
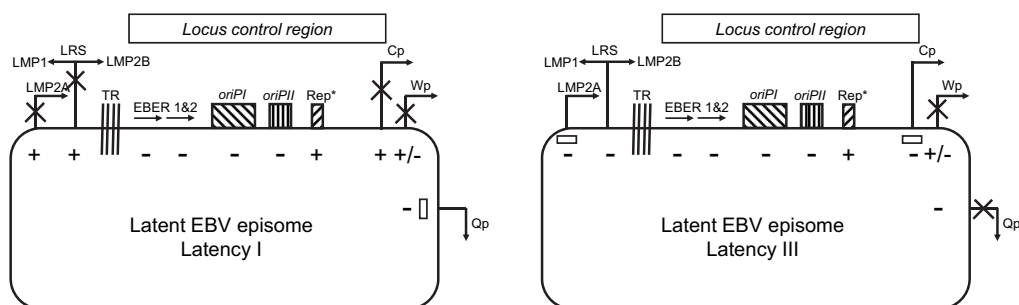

Figure 3. Histone modifications and epigenetic marks in latency type I and III Epstein–Barr virus genomes. The circular episomal genome is shown with the latent viral promoters (arrows) and their regulatory regions, including the latent origin of DNA replication *oriP* (not to scale). +, High level of regional CpG methylation; -, unmethylated or hypomethylated CpG dinucleotides. X designates a silent promoter. 'Acetylation islands', enriched in diacetylated histone H3 and tetraacetylated histone H4, are indicated by open boxes at latent promoters. (modified from (84))

The EBV genome is known to be generally associated with regularly spaced nucleosomes during latency, (83, 85). Histone tail modifications and higher-order chromatin structure may be one mechanism that regulates transcription patterns associated with different latency types (86, 87). Promoter selection plays a key role in determining latency type, and this selection depends on the interplay of host-cell-specific transcription factors and poorly defined epigenetic events that reinforce gene expression patterns over multiple generations (17, 21).

In LCLs (lat III), the EBV genome is mostly free of cytosine methylation at CpG dinucleotides, whereas in BL cells (lat I) the EBV genome is highly methylated and most of the viral chromatin can be found in a closed state, with the exception of the viral LCR and Qp (fig. 3). These two regions are unmethylated in latency I and Qp is transcriptionally active (82, 88, 89).

The regulation of Cp has been the subject of several investigations, and a number of positive cis-acting transcription regulatory elements for Cp activity have been identified (90-97). EBNA1 homo-dimers activate the Cp by binding to a sub element of the latency origin of replication (*oriPI* or FR), which functions as an EBNA1-dependent enhancer of the Cp (90, 92, 93, 98). A glucocorticoid-response element (GRE) has been identified in the Cp upstream region (94). A third cis element identified upstream of the Cp is the EBNA2 responsive enhancer/element (E2RE) (18, 95, 96). EBNA2 is unable to bind DNA on its own, and therefore activates transcription through two adaptor proteins RBPJκ, alternately referred to as C promoter binding protein 1 (CBF1) (99-103) and AUF1 (CBF2) (104, 105).
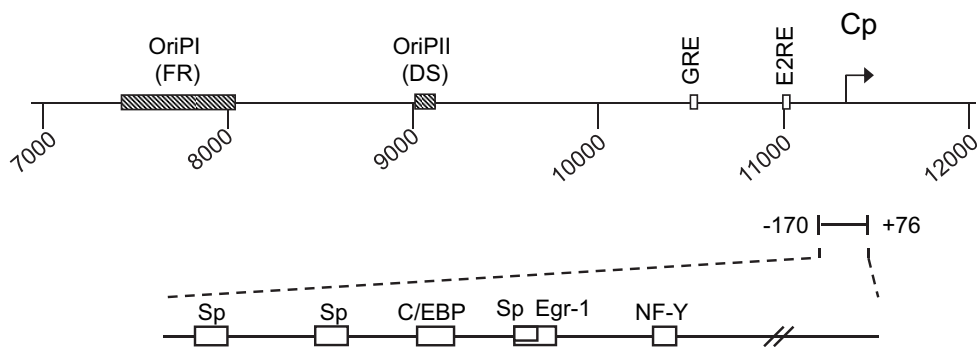

Figure 4. Sequences involved in the regulation of the EBV Cp.

The sequence coordinates are from the DNA sequence of the B95-8 EBV genome. The arrow indicates the Cp transcription start site at position 11336 (+1).

In a previous investigation, our group showed that Cp activation is highly dependent on several promoter-proximal regulatory elements (106). A central finding was the identification of a GC-rich sequence in the -99/-91 Cp region essential for promoter activity, which contains overlapping binding sites for Sp1 and Egr-1. Moreover, we demonstrated that the transcription factor Nuclear Factor Y (NF-Y) interacts with the previously identified CCAAT box in the -71/-63 Cp region, which also appears to be essential for promoter activity (93).

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A recently published study, by Chau et al., focused on the region surrounding the E2RE to identify additional transactivators. They found several new proteins interacting with this region, including E2F1, Rb and the histone demethylase LSD1 (107). Chau et al. have also reported that CTCF, a chromatin boundary factor with well-established enhancer-blocking activity, binds to EBV sequences between the *oriP* and the EBNA2 response elements (CBF1) of the Cp. Binding is associated with a latency I expression pattern, and has been implicated in decreasing transcription levels of EBNA2 mRNA (108).

3

THE PRESENT INVESTIGATION

3.1 Aims of the study

Overall aim

The Epstein-Barr virus C promoter (Cp) regulates transcription of a multicistronic RNA encoding EBNAs1-6 in proliferating lymphoblastoid cells during EBV latency type III. It is a well-established fact that the EBV origin of plasmid replication (*oriP)*, together with EBNA1, functions as a transcriptional enhancer of the Cp. The mechanism for this interaction is however not completely understood at the molecular level. EBNA1 has no apparent enzymatic activity and is thought to fulfill its functions by mediating interactions with specific host cellular proteins. This project has been focused on the study of factors affecting *oriPI*-dependent Cp activation, the primary objective being to identify novel interactions that could mediate the link between the promoter and the enhancer.

Paper I
- To determine the functional importance of the transcription factor NF-Y for Cp activity and *oriPI*-EBNA1-induced enhancement of Cp activity

Paper II
- To determine the number of EBNA1-binding repeats in the *oriPI* necessary for the generation of a functional preinitiation complex and for efficient Cp activation

Paper III
- To investigate putative Oct-binding sites in the *oriPI* and their functional effect on the enhancer activity of the *oriPI*

Paper IV
- To identify factors that could bind to Cp as well as to *oriPI*, and possibly mediate the interaction between the promoter and the enhancer, using DNA affinity purification and mass spectrometry

3.2 Materials and Methods

The purpose of this section is to provide an overview of the materials and methods used in the work of the thesis. Detailed descriptions are available in the papers and manuscripts at the end of the thesis.

Cell lines

The cell lines used in this study were chosen based on their phenotype, transfectability and their endogenous expression of various transcription factors (see table 2). The optimal environment to test the transcriptional regulation of a promoter or promoter fragment is in most cases a cell line where the promoter itself is active. When working with the EBV C promoter this often poses a problem as the lymphoblastoid cell lines, where the Cp is transcriptionally active, have very low transfection efficiency.

Table 2 Cell lines used in this thesis

Cell line	EBV	Latency	Comment
BJAB	No	-	BL cell line
DG75	No	-	BL cell line
Rael	Yes	I	BL cell line
P3HR1	Yes	II/III	EBNA2 deficient BL cell line
Raji	Yes	II/III	Expresses EBNA2, but lacks EBNA3C and is defective in lytic replication
B95-8	Yes	III	Marmoset derived, infected with B95-8 strain
Cbc-Rael	Yes	III	Cord blood cells infected with the Rael strain
IB4	Yes	III	Placental lymphocytes infected with the B95-8 strain
WW1-LCL	Yes	III	B cells infected with the QIMR-Wil virus isolate
B95-8-LCL	Yes	III	B cells infected with B95-8 strain
Molt 4	No	-	T cell line from acute lymphoblastic leukemia
EREB2-5	Yes	III[1]	B cells infected with the EBNA2-deficient P3HR1 strain complemented with an EBNA2-oestrogen receptor fusion construct (ER-EBNA2) that requires 1 μM estrogen in the medium for the activation of the EBNA2 function
Schneider's Drosophila line 2 (SL2)	No	-	Embryonic Drosophila melanogaster cell line Lacks endogenous expression of many transcription factors such as NF-Y and Sp1.
HEK293A	No	-	Human embryonic kidney monolayer epithelial cell line

[1] in the presence of 1 μM estrogen in the medium, estrogen withdrawal leads to inactivation of EBNA2 and entrance of the cells into the resting phase of the cell cycle.

The lymphoid B- and T-cell lines were maintained in RPMI 1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma), and penicillin-streptomycin (Sigma). HEK293A cells were maintained in minimal essential medium (Gibco Life Technologies Inc.) supplemented with 8% fetal bovine serum (Sigma), and penicillin-streptomycin. All cell lines except SL2 were kept in 37 °C in a humidified atmosphere containing 5% CO_2. The SL2 cells were grown in room temperature in Schneider's *Drosophila* medium (Gibco) supplemented with 10% fetal bovine serum (Sigma), and penicillin-streptomycin (Sigma).

Transfection

Transfection of DNA into mammalian cells is a routine tool for studying gene structure and function. During a period of 2 to 4 days after introduction of the DNA a transfected gene can be expressed and analyzed. In this thesis three different techniques have been used to introduce DNA into the cells: calcium-phosphate-precipitation mediated transfection (SL2-cells), electroporation (all lymphoid cells) and lipid-mediated transfection (HEK293A). The reason for using three different transfection techniques is that the cell types respond very differently to various transfection techniques and the system often has to be optimized.

In order to identify elements in a promoter region reporter genes are widely used. The promoter region is fused in front of the reporter gene and the construct is transfected into the cell types of choice. The promoter region and the transcription factors in the cell will regulate the expression of the reporter gene. The assay of the reporter protein generates an indirect measurement of promoter activity. In this thesis we have used cloned wild-type, truncated and/or mutated promoter fragments, sometimes in combination with over-expression of wild-type transcription factors or dominant negative transcription factor analogues.

Immunoblot analysis

Immunoblot (western blot) is a method of detecting specific proteins in a given cell extract or tissue homogenate. Gel electrophoresis is used to separate native or denatured proteins by the size of the polypeptide (denaturing conditions) or by the 3D structure of the protein (native/non-denaturing conditions). The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are detected using antibodies specific to the target protein. In this thesis we have used whole cell extracts from continually growing cells or the lysates from the reporter assays of the transfected cells to study the levels of endogenously expressed proteins and to verify the expression of transiently

expressed proteins, respectively. Immunoblotting is at best *semi*-quantitative, it is therefore necessary to ensure equal loading of all samples on the gel. We have confirmed equal loading by measuring total protein expression using Bradford reagent, Ponceau S staining of the blotted membrane and/or probing for protein levels of β-actin or the housekeeping gene GAPDH.

Isolation of B cells

EBV-negative B cells were purified from buffy coat from an EBV-negative donor, using the B Cell Negative Isolation Kit (Dynal Biotech ASA, Oslo, Norway). The final B cell preparation was 70% pure, as determined by flow cytometric analysis in a FACScan apparatus (BD Biosciences, San José, Ca) using phycoerythrin-conjugated anti-CD19, anti-CD14 and anti-CD45 monoclonal antibodies (BD Biosciences).

Cloning and sequencing

All manipulations involved in vector constructions were carried out by standard procedure (109). The correct sequences of all constructs were verified using the ABI Prism®Big Dye™Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems). Sequences were compared with the published EBV-genome strain B95-8 (GenBank V01555).

RNA analysis

S1 endonuclease protection assay is used to identify specific RNA molecules in a heterogeneous RNA sample extracted from cells. The technique can identify one or more RNA molecules of known sequence even at low total concentration. The extracted RNA is first mixed with ^{32}P-labeled antisense DNA probes that are complementary to the sequence or sequences of interest and the complementary strands are hybridized to form a DNA-RNA hybrid. The mixture is then exposed to S1 endonuclease that specifically cleaves *single*-stranded RNA but has no activity against the DNA-RNA hybrid. As the reaction is completed the only remaining RNA molecules are the ones that are hybridized and complementary to the radioactively labeled DNA probe. The protected fragments are analyzed by polyacrylamide gel electrophoresis (PAGE) and the bands are visualized on the dried gels by exposure of storage phosphor screens.

DNA affinity purification and MS identification

In DNA affinity purification biotinylated double stranded DNA is coupled to streptavidine-coated magnetic beads. The DNA-beads are incubated with nuclear extracts or purified proteins. The resulting DNA-protein-complexes are extensively washed and the released proteins are analyzed using mass spectrometry to identify unknown interactors, or immunoblot to verify binding of known interactors. Histones and other DNA-binding proteins can give rise to background problems. Using DNA baits no longer than 500 bases and preclearing the nuclear extract with competitor DNA, such as sonicated salmon sperm, can reduce these problems. It is crucial to use a control DNA bait of the same length as the target DNA sequence to find proteins that bind your bait DNA specifically.

Mass spectrometry (MS) has emerged during the last decade as the core method of choice when trying to identify constituents of multiprotein complexes. To identify individual proteins in a complex mixture the enzyme trypsin is often used to generate smaller peptides that are more suitable for mass spectrometric analysis. Trypsin has a very well defined specificity, as it hydrolyzes only the peptide bonds in which the carbonyl group is contributed either by an arginine or lysine residue. Using tandem mass spectrometry it is possible to obtain the amino acid sequence of the peptides in a sample.

In paper IV, we performed a fractionation of the tryptic peptides using liquid chromatography, followed by mass determination of the intact peptides. Separate peptide masses were isolated and collided with an inert gas, causing them to dissociate into fragment ions. The dissociation takes place at the weakest bond in the peptide, i.e. the peptide bond, and the mass-to-charge ratio (m/z) of these fragment ions is subsequently used to identify the amino acid sequence of the peptide. As the MS technique is very sensitive the isolation and purification of the complex in question, in combination with appropriate controls, is of outmost importance

In paper IV the promoter-proximal region of the Cp (-170Cp), spanning EBV coordinates 11156-11374 was amplified from the reporter plasmid pg(-170Cp)CAT by PCR using biotinylated primers in order to create the bait for the DNA affinity purification. Control biotinylated fragments were obtained by PCR amplification of the ampicillin coding region (Amp), using the above-mentioned plasmid as template, yielding a bait of the equal size. Nuclear extracts from the latency III cell lines cbc-Rael and WW1-LCL were used. The purified protein complexes were released from the magnetic beads and concentrated by ultrafiltration. Subsequently, the proteins were separated on a polyacrylamide gel and subjected to in-gel digestion with trypsin. The tryptic peptides were separated on a reversed phase column using a linear gradient prior

to MS analysis. Mass spectrometric analysis of the extracted tryptic peptides was performed by LC-MS/MS analysis on a Hybrid Linear Ion Trap (LTQ)-Fourier transform (FT-ICR) mass spectrometer (Thermo Electron, Bremen, Germany) coupled on-line to a nano-LC instrument (MDLC, GE Health Care). The identity of the isolated proteins was established by database searches using an in-house copy of the Mascot software (Version 2.2.01, Matrix Science, http://www.matrix-science.com). The results from Mascot database searches were imported into the ProteinCenter software and the proteins purified using the Amp-bait were subtracted from the protein hits identified in the Cp-bait. The resulting list of proteins was further filtered to contain only proteins annotated as transcription factors.

After identification of proteins binding the Cp, we repeated the DNA affinity purification assay using PCR-amplified *oriPI*-fragments as baits. The purified proteins from these experiments were analyzed by immunoblot using antibodies against the proteins identified in the MS analysis using the Cp as bait. The length of the bait in DNA affinity purification is important, since the longer the bait the more unspecific pull-down is generated. We therefore chose the *oriPI*-fragment containing eight EBNA1-binding repeats (Δ*oriPI* 7784-8190) in these subsequent affinity purifications. This fragment is able to transactivate the Cp in transient transfection experiments. The Δ*oriPI*-fragment containing three EBNA1-binding repeats is unable to transactivate the Cp and was used as a background control. The Δ*oriPI* templates used in these purifications were generated and studied in our lab (110). To avoid differences in protein pull-down due to different lengths of bait-DNA, the binding site for the unbiotinylated primer used to amplify the Δ*oriPI*-control bait was located in the pGem-zf(+) plasmid, 300 bp upstream of the Δ*oriPI*-fragment. To further map the region of the -170Cp that binds the identified proteins we split the original -170Cp-bait in halves (baits A and B). We also used a number of mutated -170Cp fragments as baits to further characterize the binding sites. Primers were designed using MIT Primer3 software (frodo.wi.mit.edu/cgi-bin/primer3_www.cgi) and acquired from Invitrogen Corporation.

Electrophoretic Mobility Shift Assay (EMSA)

The electrophoretic mobility shift assay is a sensitive method for determining interactions between protein and DNA. The binding reaction takes place *in vitro* by mixing nuclear extracts or *in vitro* translated proteins with a labeled double-stranded DNA probe. The protein-DNA complexes are separated from free DNA by non-denaturing polyacrylamide gel electrophoresis (PAGE), based on the differences in charge, conformation and size. Binding of a nuclear protein to the labeled DNA causes it to move more slowly during gel electrophoresis and

results in the appearance of a shifted band. An antibody that recognizes the protein can be added to this mixture to create an even larger complex with a greater shift. This is called a supershift assay, and is used to identify a protein present in the protein-nucleic acid complex. Supershifting is not always possible, as this requires specific antibodies to the target proteins that are able to bind the protein when it is bound to the DNA. Another way to try to deduce the identity of the protein is by competition experiments with cold consensus sequences of the target-protein binding site. An excess of the unlabeled consensus sequence is added to the binding reaction, if any of the shifted bands are reduced or disappear, the band is most likely caused by binding of the target protein.

In paper III EMSA was used to verify binding of several Oct-proteins to the EBNA1-binding repeats of the *oriPI* (FR). A single repeat was ^{32}P end-labeled and used as a probe together with nuclear extracts from the latency I cell line Rael. Complexes were supershifted by using specific antibodies, and competition experiments were performed with a cold probe (FR) and octamer sequence (OCTA).

In paper IV we used EMSA to narrow down the region of the Cp where the proteins found in the DNA affinity purification bound. Using DNA affinity and immunoblot we had verified that all three proteins of interest bound in a 90 bp region of the Cp. This sequence was split into three probes; probe 1 (-168/-128)Cp (nucleotides 11168 to 11208), probe 2 (-126/-87)Cp (nucleotides 11210 to 11249) and probe 3 (-149/-109)Cp (nucleotides 11187 to 11227). One strand of each probe was end-labeled with ^{32}P. The binding sites of all three proteins were verified through a series of competition experiments, where unlabeled consensus oligos for each of the proteins of interest were used. In previous studies we have found that Sp-factors bind very strongly to this region, therefore we also added cold Sp-consensus to the mix, in order to remove the Sp-bands that were co-migrating with the complexes of interest.

Chromatin Immunoprecipitation (ChIP) assay

ChIP (chromatin immunoprecipitation) is a powerful tool for the spatial and temporal mapping of chromatin-bound factors *in vivo*. The DNA-binding proteins, such as histones and transcription factors, are chemically cross-linked to the DNA inside the nuclei of living cells or tissues. The cross-linked cells are lysed and the chromatin is sheared by sonication to an average size of 200-600 bp. The protein-DNA complex can thereafter be immunoprecipitated out of the lysate by using an antibody that is specific to a putative DNA-binding protein. To verify if your sequence of interest is pulled down with the antibody, the

chromatin is de-crosslinked, the proteins are degraded by proteinase K and the resulting "naked DNA" is analyzed by quantitative PCR (Q PCR). The amount of DNA specifically pulled down is compared to the amount pulled down by a non-related antibody or pre-immune serum (normal IgG). It is also important to monitor the amount of DNA that is pulled down unspecifically by the antibody of interest. This is achieved by running a Q PCR of an unrelated region of the genome.

In paper IV, the ChIP assay was carried out according to the protocol provided by Nelson et al (111), with the following modifications: after cross-linking, glycine treatment and wash with ice cold PBS, the cells were lysed in Buffer A for 10 min on ice (Buffer A: 10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl), the cell suspension was vortexed for 10 s and the nuclei were recovered by centrifugation. Chromatin was extracted in 100 μl SDS lysis buffer per $2x10^6$ cells for 10 min on ice (SDS lysis buffer: 1% SDS, 10 mM EDTA, 50 mM Tris pH 8.0, supplemented with protease inhibitors (Complete EDTA-free, Roche)). This extract was sonicated to shear the chromatin to an average size of 200-600 bp using a Diagenode Biorupture, according to the manufacturer's instructions. The sheared chromatin was incubated with primary antibody overnight at 4 °C. The immunocomplexes were captured with Protein A Sepharose 4 Fast Flow (GE Life sciences), washed, and precipitated DNA fragments were isolated with 10% Chelex-100 (Bio-Rad). Isolated DNA fragments were quantified by real time PCR using QuantiTect SYBR Green RT-PCR Kit (Qiagen) on a Rotor-Gene real time rotary analyzer (Corbett Life Sciences). Primers were designed using MIT Primer3 software (frodo.wi.mit.edu/cgi-bin/primer3_www.cgi) and acquired from Invitrogen Corporation. A primer pair covering the *oriPII-region* (DS) of the EBV was used as a negative control. The antibodies used for ChIP were: rabbit-anti Oct-2 (sc-233x, Santa Cruz), rabbt-anti-E2F1 (sc-193x, Santa Cruz), rabbit-anti DrilI (A300-228A, Bethyl Laboratories Inc.) and normal rabbit IgG (Upstate).

3.3 Results and Discussion

NF-Y and Sp1 co-regulate the Epstein-Barr virus C promoter (paper I)

It is a well-established fact that *oriPI* in conjunction with EBNA1 functions as a transcriptional enhancer of Cp (90, 98). The mechanism for the interaction between the *oriPI*-EBNA1 complex and Cp is, however, not understood at the molecular level. In a previous study, we showed that the minimal *oriPI*-EBNA1-responsive Cp region comprises sequences between positions –111 and +76 relative to the Cp transcription start site (106). This region contains one Sp site

and the previously identified CCAAT box (93) and mutation of either site completely eliminated Cp activity (106). The CCAAT box is a common eukaryotic promoter element and our previous study also showed that NF-Y can bind the Cp CCAAT box *in vitro* (106). NF-Y is a ubiquitous multimeric transcription factor consisting of three subunits A, B and C that are all required for DNA binding and transcriptional activation (112-115). To further investigate the role of NF-Y on Cp transcriptional regulation, we used a mutated NF-YA analogue that forms a dysfunctional trimeric complex with the B and C subunits unable to bind the cognate DNA motif. This dominant negative mutant has been widely used to characterize NF-Y-dependent promoters (116-119). In transient transfections using EBV-positive B cells of latency I and III phenotype pattern, over-expression of dnNF-YA almost abolished *oriPI*-EBNA1-independent and -dependent Cp activity. These results demonstrated that NF-Y is indeed important for Cp activity and that other CCAAT box-binding transcription factors could not compensate for the loss of NF-Y function.

To investigate the requirement of NF-Y and Sp1 for the *oriPI*-EBNA1-enhancer function, we co-transfected SL2 cells with *Drosophila* expression vectors for Sp1, NF-Y and EBNA1. The results revealed an absolute requirement of both Sp1 and NF-Y for the *oriPI*-EBNA1-mediated up-regulation of Cp. In fact, NF-Y seemed to operate in conjunction with Sp1 to regulate Cp. Expression of both Sp1 and NF-Y in *Drosophila* cells (SL2) led to amplification of Cp activity far beyond that seen with either transcription factor alone, implying a direct or indirect functional interaction. Interestingly, previous investigations demonstrated a physical interaction between these two transcription factors both *in vitro* and *in vivo* (120, 121). The EBNA1-mediated transactivation of Cp in SL2 cells was however relatively low (2-4-fold) as compared to what has been reported from co-transfection studies in EBV-negative lymphoid and non-lymphoid cells (generally >10-fold) (92, 98, 122). This difference in enhancement is most likely due to factors not present in the SL2 environment.

NF-Y has been shown to recognize CCAAT boxes in several genes involved in regulation of cell growth, including murine ribonucleotide reductase R2 (123), murine E2F-1 (124), cyclin B1 (125), cdc2, cyclin A, cdc25C (126) and human thymidine kinase (127). Recent studies also demonstrated that stable expression of a dominant negative mutant of NF-Y in mouse fibroblast cells resulted in retardation of cell growth and inhibition of transcription of various cellular genes (128), implying that NF-Y may be crucial for cell cycle progression. These observations led us to hypothesize that there may be a difference in NF-Y expression in resting as compared to proliferating B cells. EREB2-5 cells provide a useful system to study this hypothesis, since estrogen withdrawal from the cell culture medium arrests these cells in the cell cycle and induces a phenotype, which resembles normal, resting B cells. Activation of EBNA2 in

EREB2-5 cells with estrogen induces cell proliferation and an LCL-like phenotype (29). Previously, Yoo *et al.* investigated Cp and Wp activity in EREB2-5 cells grown in the presence of various concentrations of estrogen (18). Estrogen-depleted resting cells only used Wp, whereas Cp was quiescent. This finding was attributed to the lack of intranuclear EBNA2 function. In our study, we measured the levels of the NF-YA subunit at different times after withdrawal or addition of estrogen to the cell medium and related NF-YA expression to Cp and Wp activity, measured as steady-state levels of Cp- and Wp-initiated transcripts by S1 nuclease mapping. The experiments revealed that resting EREB2-5 cells expressed low levels of NF-YA, whereas proliferating EREB2-5 cells expressed high levels. After withdrawal of estrogen, Cp usage declined rapidly before the level of NF-YA had decreased, most likely reflecting loss of EBNA2 function. These data corroborate the importance of EBNA2 for regulating Cp in the viral context. However, upon addition of estrogen and reconstitution of EBNA2 function, the Cp usage was not fully up-regulated until the levels of NF-YA were restored. These data indicate that NF-Y may be an important factor for the Wp-to-Cp switch. In agreement with this hypothesis, primary B cells isolated from an EBV-negative donor expressed low levels of NF-Y. This might explain why the first EBV promoter to be activated upon infection is Wp, not Cp, and why the Wp-to-Cp switch coincides with triggering of the cellular growth program.

Multiple EBNA1-binding sites within *oriPI* are required for EBNA1-dependent transactivation of the Epstein-Barr virus C promoter (paper II)

The *oriPI*/FR is the EBNA1-dependent enhancer of the Cp that controls transcription of six different latent nuclear proteins. EBNA1 binds as a dimer to the palindromic core consensus 16 bp sequence, which can be found in several copies at three different sites in the EBV genome: (i) downstream of the Q promoter, (ii) in the dyad symmetry element (DS or *oriPII*) and (iii) the family of repeats of *oriP* (FR or *oriPI*) upstream of the Cp (90, 91, 129, 130). In the prototype B95-8 virus, the *oriPI* consists of 20 copies of the 30 bp repeat domain, containing the EBNA1 core binding site (92, 98, 122). To further unravel the mystery of the transcriptional activation complex that is composed of the *oriPI*, EBNA1 and Cp, we constructed a series of *oriPI*-deletions in *oriPI*-CpCAT reporter plasmids. Finding out the minimal number of EBNA1 binding site repeats required for enhancement of Cp transcriptional activity would be helpful for the future identification of factors that could link the *oriPI* to the Cp. Transient transfection studies on *oriPI*-EBNA1-induced activation of promoters must be carried out using cell lines that do not express EBNA1 due to the fact that presynthesized EBNA1 binds to *oriP*-sequences and enhances nuclear import of *oriP*-containing plasmids in transient transfection experiments through

its nuclear localization signal (122). In the case of *oriP*-containing reporter plasmids, the transfection efficiency and thus the measured reporter activity is increased by an enhanced *oriP*-EBNA1-mediated nuclear import of the plasmids, giving rise to a spurious impression of an enhanced intranuclear promoter activity. Importantly, this effect is not seen in EBNA1-negative cells (e.g. DG75) in which the *oriP*-carrying reporter plasmids are co-transfected with EBNA1-expression plasmid (122).

The study revealed that 20 EBNA1-binding sites within *oriP*, i.e. the full-length *oriPI*, resulted in a 280-fold activation of Cp by EBNA1. When the number of EBNA1-binding sites was reduced to eight, *oriPI*-EBNA1-induced activation of Cp showed a relatively minor decrease to a level of about 100-fold activation. However, reporter constructs with four to six EBNA1-binding sites in *oriPI* still responded to EBNA1 but with only an approximately 10-20-fold up-regulation of promoter activity by EBNA1, and constructs that contained three or fewer EBNA1-binding sites were not significantly activated by EBNA1. Previously, we reported that *oriPI* acts as a negative regulatory element on viral promoters in the absence of EBNA1 (92). Our present data indicated that this repressive effect is especially strong in the constructs that contain eight or more EBNA1-binding sites, as these have the lowest basal activity in the absence of EBNA1. The fact that eight or more EBNA1-binding sites are required for full EBNA1-induced Cp activity underscores the complexity of the process. It suggests that EBNA1 may activate transcription via weak heterotypic interactions between multiple EBNA1 homodimers and cellular transcription factors at Cp. Moreover, EBNA1-induced remodeling of the EBV chromatin structure including DNA looping and perturbation of the structure of specific nucleosomes most likely is a prerequisite for the subsequent formation of a transcriptionally active promoter (131, 132).

Oct regulation through EBV *oriPI* (paper III)

The members of the Oct family of transcription factors were originally identified as transcriptional regulators when bound to the octamer motif ATGCAAAT. Oct-1 is expressed ubiquitously in all types of tissues (133, 134). It interacts with a variety of other transcriptional regulators and activates transcription of small nuclear RNA, histone H2B and immunoglobulin genes (135-137). Oct-2 is tissue-specific for neuronal cells and B cells. It is involved in activating transcription from promoters of the immunoglobulin genes (136). In paper III, a putative Oct-binding site was identified in the *oriPI* sequence, slightly overlapping the 25 bp fragment that is protected in DNase protection assays in EBV-positive cells (138). Using an *oriPI*-derived probe in EMSA, with nuclear extracts from an EBV-positive B cell line we could confirm binding of Oct-1

and Oct-2 to the *oriPI*. In an attempt to further map the Oct-binding site in the *oriPI*, the probe was divided in halves, separating the putative Oct-site from the EBNA1-site. The EMSA showed that the EBNA1 and Oct-2 bound to different parts of the *oriPI*-probe.

To analyze the functional significance of this finding, transient transfections were conducted in HEK293A and DG75 cells, with a luciferase reporter construct consisting of the *oriPI* upstream of a thymidine kinase (TK) promoter. Expression plasmids for EBNA1, Oct-2, Oct-1 and Bob.1 were used in co-transfections. In summary, over-expression of Oct-2, but not Oct-1, up-regulated the *oriPI*-dependent TK promoter activity in HEK293A cells, to the same extent as EBNA1. However, Oct-1 could only activate transcription in combination with the B cell-specific co-activator Bob.1. We also detected a small additive effect of co-transfection of EBNA1 with Oct-2, or with Oct-1 in combination with Bob.1. The same experiments were conducted in the EBV-negative B cell line DG75, to obtain a more biologically relevant background. EBNA1 activated transcription more efficiently in this cell line in comparison to HEK293A, which could be a result of background levels of Oct-proteins, or other proteins necessary for the EBNA1 transactivation. Oct-1 together with Bob.1 and Oct-2 alone both activated transcription in the *oriPI*-luciferase reporter system. Oct-1 also showed a slight activation on its own without Bob.1, probably due to the presence of endogenous Bob.1 in DG75. Similar results were obtained when combining the *oriPI* with the minimal Cp fragment (-170Cp), in both HEK293A and DG75. In the physiological setting, EBNA1 and Oct-proteins may cooperate in the regulation of the Cp. This may have direct implications for B cell-specific regulation of Cp.

Bright, E2F1 and Oct-2 binds the Epstein-Barr virus C promoter and the *oriPI*, linking the promoter to the enhancer (paper IV)

In papers I and III we studied the *oriPI*-Cp interaction in cells from different lineages and realized that the EBNA1-dependent *oriPI*-enhancement on Cp activity was far greater in cells of the B cell lineage. These observations led us to the hypothesis that there must be additional proteins, possibly of B cell origin that are needed for an efficient transcriptional activation. In paper IV we set out to find additional proteins in this presumably large macromolecular complex that initiates transcription from the Cp, using DNA affinity purification and mass spectrometry. In DNA affinity purification it is vital not to use to long baits, since this causes massive background problems. We therefore decided to use baits covering the -170Cp region or the *oriPI* fragment with eight EBNA1 repeats, in the initial purifications. Baits amplified from the ampicillin coding region or containing three EBNA1 repeats were used as negative controls. The

purified proteins from the Cp and Amp-baits were analyzed by MS, of the 30 transcription factors identified as putative with Cp interactors, we selected Bright, E2F1 and Oct-2 for further characterization. Interestingly, E2F1 as well as Oct-2 have previously been shown to bind the *oriP*-region (139, 140). The protein eluates of *oriPI*-purifications were analyzed by immunoblot, and the results showed that all three factors also bound to Δ*oriPI*. Notably, Bright-binding was highly enriched in the activation-competent Δ*oriPI* with eight EBNA1 binding repeats, in comparison to the activation-incompetent Δ*oriPI*-construct with three EBNA1 repeats. Using a series of competition EMSAs and additional DNA affinity purifications with mutated Cp baits, we were able to map the binding sites of all three proteins to a short segment of Cp in close proximity of each other. This region has previously been shown to be required for both *oriPI*-dependent and -independent transcriptional activation indicating that the interactions are important for Cp activity (106). *In vivo* binding of the proteins to both the -170Cp- and the *oriPI* regions was determined using ChIP (Chromatin Immunoprecipitation).

E2F1 is a ubiquitously expressed protein that belongs to a family of transcription factors that play a crucial role in cell cycle progression and regulates genes required for G1/S transition. Chau et al. showed in recent, detailed ChIP studies with synchronized EBV positive cells, that the binding of E2F1 and Rb to a second E2F1 binding site in the Cp, vary in a cell cycle dependent way, indicating that the Cp is cell cycle regulated (107). We reached a similar conclusion in our study of the interaction of NF-Y with the CCAAT-box of the Cp in paper I. Notably, many cell cycle promoters typically contain both E2F sites and CCAAT boxes (116, 117, 141-145). Genes that are regulated during the cell cycle are typically active in one phase and inactive in the others. E2F1 has long been considered to be oncogenic because of its ability to promote cell cycle progression. When E2F1 dissociates from hyper-phosphorylated Rb during the G0/early G1 phase, it activates the transcription of target genes, committing cells in late G1 phase to initiate cell cycle progression. E2F1 has the capability to promote quiescent cells to enter S phase and acts as an oncogene in transforming assays (reviewed in (146)). The protein has been shown to be essential for c-Myc-induced carcinogenesis both *in vitro* and *in vivo* (147). In an EBV context the interaction with c-Myc is particularly interesting as this protein is up-regulated by EBNA2 in early EBV infection and is essential for the transformation of B cells (28).

Bright (aka DrilI/ARID3a/E2FBP1) is a novel player in the EBV field. It is a member of the ARID family of transcription factors and is expressed in a highly regulated fashion in B cells where it enhances immunoglobulin transcription. Its expression is induced by stimulation of B cells with antigen or polyclonal mitogens (148). Bright binds to A+T-rich regions of the heavy-chain enhancer

(Eμ) previously identified as a MAR, and interacts with DNA as a multimeric complex that includes multiple copies of the protein. Previous studies have shown that Bruton's tyrosine kinase (Btk) (149), as well as the ubiquitously expressed transcription factor TFII-I (150), are components of the Bright Eμ DNA-binding complex. TFII-I is a multifunctional transcription factor that is induced by a variety of external signals (151-155). In normal resting B cells, TFII-I is largely found in the cytoplasm, a significant portion of which is tethered to Btk. Upon cross-linking of the surface B cell receptor, TFII-I is tyrosine-phosphorylated in a Btk-dependent fashion and translocates from the cytoplasm to the nucleus (151). It has been demonstrated that induction of immunoglobulin heavy-chain transcription through Bright requires TFII-I (150). Importantly, TFII-I is proposed to function both as a basal transcription factor facilitating communication between basal machinery at the core promoter and as a transcription activator contributing to protein complexes assembled at distant upstream sites (156, 157). Altogether, these observations make Bright a very interesting candidate for linking the *oriPI*-EBNA1-complex to the Cp. To further study the importance of Bright on Cp activity, we conducted transient transfections in DG75, an EBV-negative B cell line that does not express detectable levels of Bright endogenously. We show that exogenous Bright expression up-regulated *oriPI*-dependent Cp activation in the absence of EBNA1. Furthermore, endogenous Bright expression was shown to correlate with latency III but not latency I and II expression patterns in EBV positive cell lines, suggesting that Bright expression is important for Cp transcriptional activity *in vivo*. In the EBV context Bright binds Cp, as well as *oriPI*, and might conceivably assist in the targeting of the promoter to the MAR of the *oriPI*. The sequence between the Cp and *oriPI* could then loop out and place the enhancer in close proximity of the promoter.

The activity of most genes in higher eukaryotes is regulated by promoter-proximal elements as well as distal enhancers. The former are located within 100-200 bp from the transcription start site, whereas the latter can be located 1-100 kbps from the promoter. One or more promoter proximal elements are required for mediating enhancer action at a distance (158-160). As the chromatin structure unravels, the assembly of the preinitiation complex at the enhancer and the promoter can take place.

Several factors studied during the course of this thesis are implicated in the relief of nucleosome-induced repression, through their interactions with coactivators possessing ATP-dependent chromatin remodeling (SWI/SNF) and intrinsic HAT activities (p300, CBP and P/CAF). p300 and CBP are closely related and are also involved in recruiting the basal transcriptional machinery including RNA polymerase II to the promoter and can act as adaptor molecules (reviewed in (161)). EBNA2 is able to recruit all of these co-activators to the Cp (162-164), possibly setting the stage for the recruitment of NF-Y, Sp1, E2F1, Bright, Oct-2, the *oriPI*-EBNA1 complex and additional factors that lead to the subsequent activation of transcription. Bright and EBNA1 may also play a part in this stage-setting process. Binding of Bright has been reported to contribute to chromatin accessibility in the case of the immunoglobulin heavy chain enhancer (165) and EBNA1-induced chromatin remodeling includes DNA looping and perturbation of the nucleosome structure (131, 132).

Interestingly, many of the factors identified in this project have also previously been reported to interact with each other, as well as linking promoters to enhancers. In summary all of the identified factors fall into a complex meshwork that could be the complex that links the *oriPI* to the Cp (See Fig. 5).

In a previous study EBNA1 was shown to interact with the cellular protein p32/TAP and the interaction was shown to be essential for *oriPI*-EBNA1-transactivation of a thymidine kinase promoter. The protein binds two regions of EBNA1, and derivatives lacking either region are defective for transcriptional activation (166). Notably, p32/TAP interacts with EBNA1 independently of DNA-binding or other proteins and also associates with the *oriP in vivo* (167). Additionally, p32/TAP has been reported to interact with the general transcription factor TFIIB and presumably contributes to the efficient recruitment of this factor to the RNA pol II preinitiation complex (168). These observations suggest that p32/TAP has a role in the activation of transcription by EBNA1. Interestingly, a more recent study reports a physical interaction between NF-Y and p32/TAP, in this case however, the interaction leads to transcriptional repression in *in vitro* studies (169). The question is, how would this protein function in a context where it is able to form a bridge between

EBNA1 and NF-Y? It is very tempting to hypothesize that p32/TAP could link the *oriPI* to the Cp through protein-protein interactions and help recruit the RNA pol II preinitiation complex.
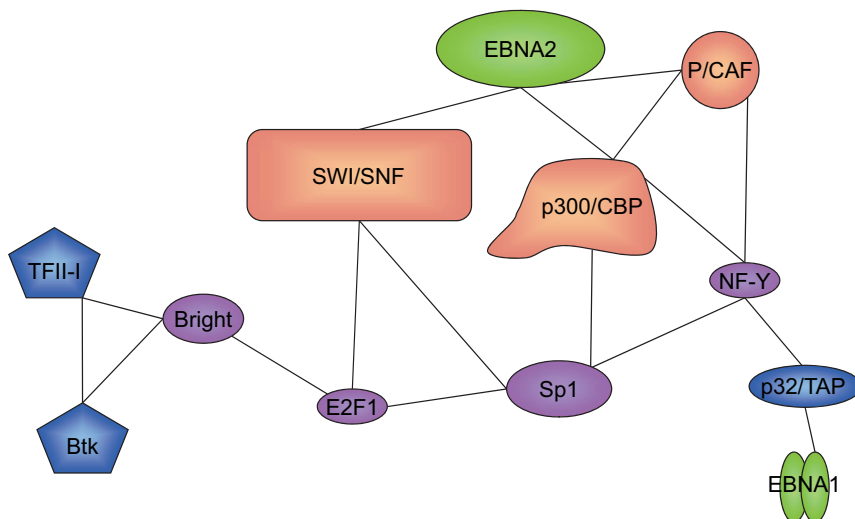

Figure 5. Protein interaction map of some of the proteins interacting with the EBV Cp and *oriPI*
Bright (149, 150, 170), EBNA1 (166), EBNA2 (162, 163), E2F1 (170-174), NF-Y (120, 121, 169, 175), Sp1 (120, 121, 171, 172, 174, 176, 177).

Throughout this thesis, the work has been focused on finding cellular proteins that are involved in the regulation of the Cp, but the importance of EBNA2 in this process cannot be overlooked. In transient transfections Cp can be activated independently of EBNA2, however in the endogenous genome, this is not the case. Cp activity *in vivo* correlates completely with EBNA2 expression (18, 178) and a correlation between EBNA2 recruitment and histone acetylation of the promoter has been reported (179). Trying to put together the pieces of this macromolecular complex is like doing a 3D jigsaw puzzle whose components vary over time. In summary, a model can be put forward where the expression of EBNA2 and EBNA5 from the Wp pushes the infected B cell into the cell cycle through up-regulation of c-Myc. The cycling of the cell induces the expression of E2F1, Bright and NF-Y. As the EBNA2 levels rise, and EBNA2 binds to the Cp, the chromatin remodeling complexes are recruited, inducing a more relaxed chromatin structure. This change in structure enables the binding of E2F1, Bright, Sp1, NF-Y, Oct-2 and possibly other transcription factors that ultimately recruit the RNA pol II preinitiation complex and transcription is initiated.
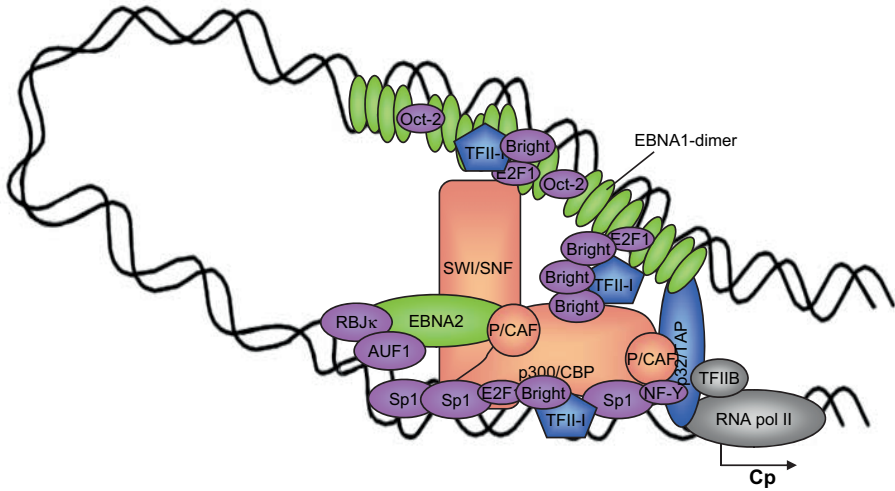
Figure 6. A proposed model for the *oriPI*-EBNA1-Cp transcriptional activation complex

Another interesting theory is that the Cp bound by transcription factors relocate to come into contact with the matrix-associated EBNA1-bound *oriP*, looping out the intervening sequences and consequently comes into contact with a matrix associated RNA pol II transcription factory.

Naturally, the *in vivo* situation is most likely even more complex than the one depicted in Fig. 6 and more studies are needed to confirm the findings of this thesis. Many new roads have opened and exploring them one by one will be an extremely interesting challenge.

4

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