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EPSTEIN-BARR VIRUS NUCLEAR ANTIGEN 1, OCT & GROUCHO/TLE IN CONTROL OF PROMOTER REGULATION

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ABSTRACT

The human herpes virus Epstein-Barr virus (EBV) is carried by approximately 95% of the adult population. It can form a lifelong latent infection in the B-cells by avoiding our immune system. A latent EBV infection is usually asymptomatic but a few EBV associated cancer forms has been described (e.g. Burkitt's lymphoma (BL)).

EBV can avoid the immunesystem by restricting expression of its latent genes. Based on variation in viral gene expression several different latency forms have been described, e.g. latency III where all latency genes are expressed and latency I (e.g. BL) where only the nuclear protein EBNA1 is expressed. EBNA1 has three important functions, replication and maintenance of the viral genome and to regulate transcription. In latency III cells EBNA1 is transcribed from the C promoter (Cp) together with six other proteins (EBNA1-6). The more restricted gene expression pattern in latency I cells is associated with a down regulation of the Cp and activity from the Q promoter (Qp) which leads to selective EBNA1 gene transcription. A switch between Cp and Qp usage might be instrumental in driving the host cell between latency forms. This thesis concerns the upstream control of Cp regulation with an emphasis on the interplay between cellular transcription factors and viral proteins.

An EBNA1 responsive element called Family of Repeats (FR) is situated upstream of the Cp. EBNA1 can bind to each of the 20 repeats found in FR, thereby activating transcription. We have used various methods to show that the two cellular transcription factors Oct-1 and Oct-2 can bind to the FR sequence in vivo and in vitro and that the binding varies between the different repeats. We also show that binding has an impact on promoter activity in which Oct-2 alone and Oct-1 together with Bob.1 can substitute for the effect of EBNA1 on FR or further enhance the effect of EBNA1.

We also describe the finding of a corepressor for Oct-proteins, namely the cellular protein Grg/TLE. The repression was shown to be highly dependent on the sequence to which Oct binds. This finding also applied to FR in EBV. All full-length Grg/TLE proteins as well as the truncated version Grg-5 can repress FR dependent Oct-2 activity. Binding of Grg/TLE to FR was also demonstrated both in vivo and in vitro. Repression by Grg/TLE could be cancelled by EBNA1, as well as the EBNA1 induced activity could be repressed by Oct-2 + Grg/TLE.

Keywords: *Epstein-Barr virus, EBNA1, Oct-1, Oct-2, Groucho, TLE, transcriptional regulation*

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

I.

Jenny Almqvist, Jiezhi Zou, Ylva Linderson, Cecilia Boreström, Endre Altiok, Henrik Zetterberg, Lars Rymo and Ingemar Ernberg. 2005. *Functional interactions of Oct-transcription factors with the family of repeats in Epstein-Barr virus oriP*. J Gen Virol. May;86 (Pt 5):1261-7.

II.

Stephen Malin, Ylva Linderson, **Jenny Almqvist**, Ingemar Ernberg, Tiziano Tallone and Sven Pettersson. 2005. *A DNA dependent conversion of Oct-1 and Oct-2 into transcriptional repressors by Groucho/TLE*. Nucleic Acids Res. Aug 15;33(14):4618-25.

III.

Jenny Almqvist, Jiezhi Zou, Sven Pettersson, Cecilia Boreström, Lars Rymo and Ingemar Ernberg. 2005. *Repression of Epstein-Barr virus enhancer family of repeats mediated transcription by Oct and Groucho/TLE transcriptional regulators, suggests an involvement in switching of latency programs*. Manuscript.

IV.

Jiezhi Zou, **Jenny Almqvist** and Ingemar Ernberg. 2005. *Mapping of the Oct-binding sites in Epstein-Barr virus enhancer FR and demonstration of binding of Oct and Groucho/TLE in vivo*. Manuscript.

ABBREVIATIONS

ab	Antibody
BL	Burkitt's lymphoma
bp	base pair
CBMI	CBMI-Ral-STO
ChIP	Chromatin immunoprecipitation
CTL	Cytotoxic T-cells
DNA	Deoxyribonucleic acid
ds	double stranded
DS	Dyad symetry
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
EMSA	Electro mobility shift assay
FR	Family of repeats
Grg	The mouse homologue to Groucho
HAT	Histon acetylases
HDAC	Histon deacetylases
HD	Hodgkin's disease
HIV	Human immunodeficiency virus
Ig	Immunoglobulin
IM	Infectious mononucleosis
IP	Immunoprecipitation
IR	Internal repeat
JAK	Janus Kinase
kb	Kilo base pair
LCL	Lymphoblastoid cell line
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NE	Nuclear extracts
NLS	Nuclear localisation signal
NPC	Nasopharyngeal carcinoma
Oct	Octamer binding factor
OHL	Oral hairy leukoplakia
ORF	Open reading frame
oriP	Origin of plasmid replication
PCR	Polymerase chain reaction
Pol II	RNA polymerase II
PTLD	Post-transplant lymphoproliferative disorder
RNA	Ribonucleic acid
Sp1	Stimulatory protein 1
STAT	Signal transducer and activator of transcription
TBP	TATA-box binding protein
TF	Transcription factor
TLE	Transducin like enhancer of split
TR	Terminal repeat

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OBJECTIVE

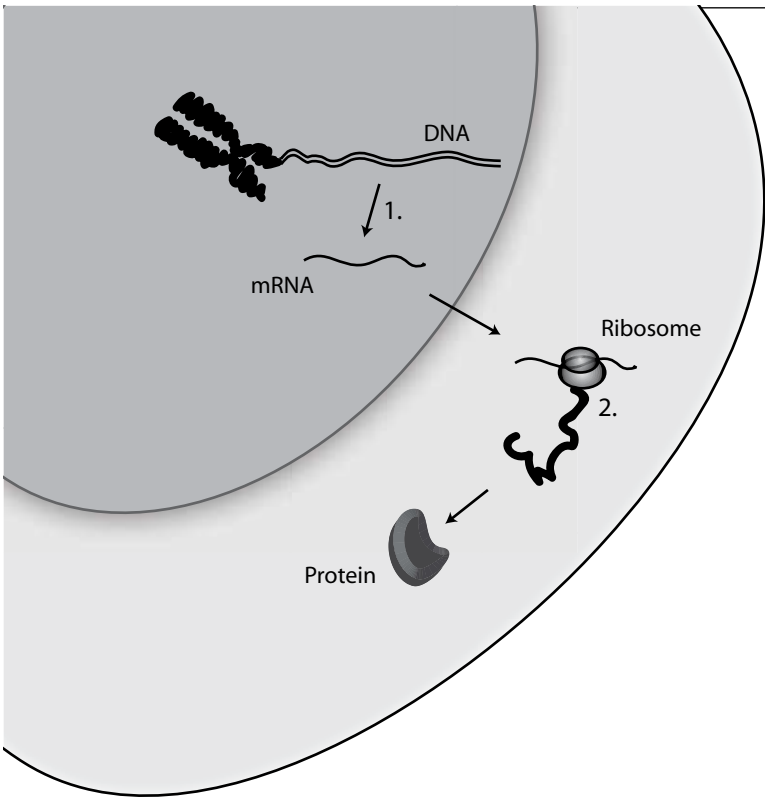
This study aims to elucidate the regulation of the C promoter (Cp) in the context of latency I-III-I switching in EBV. We focused on the Cp and its upstream regulatory element called family of repeats (FR) which is regarded to be one of the key players of the phenotypic difference between lat I and lat III cells. Even though the Cp has been studied for a long time and much is known about its upstream regulation, nothing is so far known about how it initially is turned off when a cell switches into latency I/II phenotype.

- In the first paper we wanted to see if a putative Oct-binding site in FR could attract Oct-proteins and if this binding would have any effect on the Cp activity (paper I).

- In the second paper we investigated if the Groucho/TLE family of co-repressors had any effect on Oct-induced promoter activity and if this effect was dependent on the DNA sequence to which Oct bind (paper II).

- In the third paper we combined the findings from paper I and paper II and analysed if Grg/TLE had any effect on Oct-FR induced promoter activity (paper III).

- In the fourth paper we wanted to further elucidate the binding of Oct and Groucho/TLE to FR (paper IV).



GENE REGULATION

Inside our ~2,85 billion base pairs long genome lies the information of 20.000 to 25.000 different protein encoding genes (127). The control of which of these genes should be translated into proteins at a precise moment has to be under extreme regulation in every single cell. The proteins might for example tell the cell if it should differentiate, or when it should divide and when not to divide, or if it should undergo cell growth or cell death. Disruption of gene regulation is therefore one of the most fundamental steps in the development of cancer (59). Regulation of gene expression occurs in many different levels, for example in the level of transcription, RNA processing and stability, translation and protein stability (Fig 1).

Fig 1. Schematic picture of gene expression including transcription (1) and translation (2).

Transcription

Transcription is the process where information stored in a gene is copied into messenger RNA (mRNA) that is translated into the building of a protein (fig 1) (for review see ref 3). This process is highly organised and tightly regulated. RNA polymerase (Pol) is the protein responsible for transcribing the DNA template into mRNA. RNA polymerases are enzymes that catalyse the formation of 3'-5' phosphodiester bond between the mRNA building blocks, the ribonucleotides. There are three different RNA polymerases in eukaryotic cells, RNA polymerase I-III. RNA polymerase I and III produce stable RNAs such as ribosomal RNA (rRNA) and transfer RNA (tRNA). RNA polymerase II (Pol II) produces cellular mRNA and in contrast to the product of Pol I and III that has to be abundant at all times, expression of mRNA by Pol II is tightly regulated. The majority of DNA viruses, including herpesviruses depend on the cellular DNA Pol II for transcription of their viral genes.

Transcription by Pol II is a three-step process including initiation, elongation and termination. It all starts with binding of the basal transcription machinery constituting Pol II, 40 polypeptides and the general transcription factors (TF) in an ordered fashion to the start site of the gene (the promoter). The DNA is then opened and unwound by the proteins and mRNA is formed by Pol II. Transcription proceeds along the DNA and the mRNA is elongated with the correct ribonucleotides until the transcription machinery reaches a termination signal. Transcription terminates and the transcription machinery and the mRNA are released from the DNA. The mRNA is bound by ribosome proteins and is transported out into the cytoplasm where it is translated into a chain of amino acids constituting the specific protein (130).

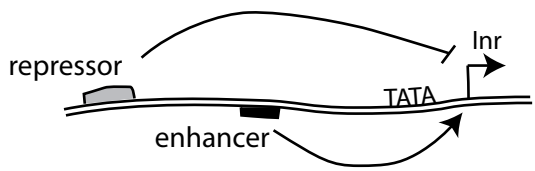


Fig 2. Schematic promoter structure. Arrow indicates the promoter.

Promoter structure

A typical promoter consists of an initiation site (Inr), the TATA box, the downstream promoter element and the regulatory regions upstream or downstream of the promoter (fig 2) (83). The TATA box is a well-conserved sequence rich in the tyrosine (T) and

adenosine (A) nucleotides situated around 20 to 35 bp upstream of the transcriptional start site. The DNA upstream of a gene normally contains regulatory regions in the DNA that can be recognized by transcription factors, which positively or negatively affects the level of transcription. These elements, located in a promoter-proximal position or many kb away in a promoter-distal position, are variable and highly gene-specific.

Transcriptional regulation

The basal transcription machinery is sufficient for low levels of transcription, but since expression of genes has to be tightly regulated in a cell, this control is not enough. In addition there are regulatory proteins (transcription factors, TF) in each cell that can bind to specific DNA sequences thereby regulating transcription positively or negatively. These DNA sequences are either called enhancers, if they are bound by proteins causing increased promoter activity, or silencers if bound by proteins that cause lowered promoter activity. The enhancer were first described in the genome of the SV40 virus by its ability to enhance transcription in an orientation independent manner. These regulatory sequenses can be situated close to the promoter or more distal. Distances up to 10.000 bp from the promoter have been observed. Proteins binding to these regulatory regions are called transcription factors and they can influence promoter activity, for example by interacting with components of the basal transcription machinery. The global architecture of transcription factors usually comprises discrete functional and structural domains, for example DNA or protein binding domains and domains activating or repressing transcription. An activator is a transcription factor that activates transcription while a repressor is a protein that represses it (56, 140).

Interaction between the basal transcription machinery and transcription factors could either be direct or mediated by cofactors (fig 3) (139). A cofactor is a protein that influences promoter

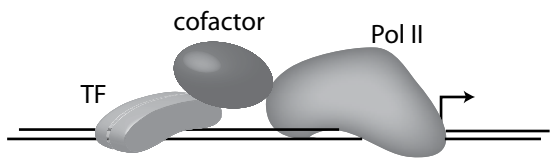


Fig 3. Simplified model of interaction between a transcription factor (TF), a cofactor and Pol II.

activity, but does not bind DNA directly. Instead they are recruited to the promoter region by DNA bound transcription factors (73). Like transcription factors these are called coactivators if they activate transcription and corepressors if they repress it.

Repression - acetylation

Each cell in the human body contains about two meters of DNA that needs to be packed very tightly to fit into the cell nucleus. The most compact form of DNA in the nucleus is called chromatin and its structural unit is called the nucleosome. The nucleosome consists of DNA wrapped around an octamer of histone proteins (fig 4). This tightly packed DNA is inaccessible for the DNA polymerase and has to be untightened for transcription to occur. Repression can be a result of hiding sequences necessary for transcription such as the promoter or the enhancer.

Release from the tight association between DNA and the histones, is a prerequisite for transcription to take place and can be achieved by attaching acetyl groups to the histone proteins. Acetylation of lysine residues on the histone proteins leads to charge neutralization and a reduction in the affinity of histone-DNA interactions and this results in increased access of transcription factors and polymerase to the template (62).

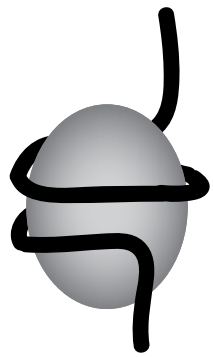


Fig 4.
DNA wrapped around an octamer of histone proteins

Histone acetyltransferases (HATs) are enzymes that acetylate histones and histone deacetylases (HDACs) are enzymes that deacetylate histones. HDACs are associated with transcriptional inhibition and are often found in corepressor complexes (19). Conversely, coactivator complexes often include HAT activity.

Repression - methylation

Another and much less dynamic way to influence transcription is to add methyl groups directly to the DNA (11). This usually occurs in the cell after DNA replication and preferentially to the cytosines of CpG motifs. This modification is done by methyltransferases and

often results in silencing of gene expression (17, 145). Repression of gene transcription by methylation could be a result of preventing transcription factors to bind their enhancer elements. A methylated CpG motif can also be bound by specific proteins (methyl-CpG binding proteins) that attract corepressors with HDAC activity. Due to the stable nature of methylation, it is most often used for long-term repression of gene expression during for example cell differentiation and stable silencing of genes.

The Oct-protein family

Octamer binding factor (Oct) is a family of transcription factors involved in regulation of a broad range of genes, from house keeping genes like the histone H2B to cell type specific gene expression, for example the immunoglobulin gene in B-lymphocytes (31, 87).

The Oct-proteins belongs to the POU domain family, all sharing a homologous DNA binding POU domain. It is a 150-160 amino acid long protein domain whose name is derived from the first four transcription factors in which it was found; the mammalian proteins Pit-1, Oct-1, Oct-2 and the nematode protein Unc86 (67, 179). Today the family includes more then 30 different proteins. The POU domain constitutes the DNA binding domain of the protein and is divided into two structurally different sub-domains separated by a variable linker (fig 5) (180). The C-terminal sub-domain resembles the well characterised DNA-binding motif called a homeodomain with a helix-turn-helix motif and was therefore named the POU homeodomain (POU_H). The N-terminal part also contains a helix-turn-helix motif but its folding is specific for the POU-family and hence it was named the POU specific domain (POU_S). The functional diversity in the POU family of transcription factors appears in part to be due to the structurally bipartite POU domain with its intrinsic conformational flexibility.

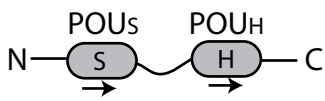


Fig 5
The two sub-domains POU_S and POU_H in the structural POU-domain.

The Oct-proteins were originally identified as monomeric transcriptional regulators bound to the octamer motif, ATGCAAAT. More recently they have been shown to be able to homo- and heterodimerize on specific DNA response elements. One newly found Oct-binding site is the palindromic Oct factor recognition element

(PORE), ATTTGAAATGCAAAT, found in the *osteopontin* (ONP) gene promoter (16). Another one is the MORE (More PORE) element, ATGCATATGCAT, found in various promoter sequences, to which all members of the Oct-family can bind cooperatively as homo- or heterodimers. The POU domain assembles in different ways on the PORE and MORE motifs (fig 6), which leads to differential recruitment of cofactors (189).

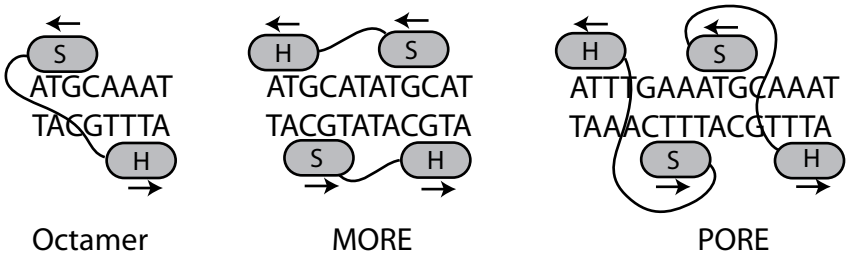


Fig 6. POU-domains bound to three different DNA motifs.

Oct-1

The gene of Oct-1 (*OTF1*) is encoded by at least 17 exons spanning over 200 kb. It is regulated by two distinct promoters; the 1U promoter (1Up) and the lymphoid specific 1L promoter (1Lp) (fig 7) (133). Expression is autoregulated by Oct-1 and Oct-2 (132, 210). The mRNA from *OTF-1* has a variable splicing pattern. The splice variants Oct-1A-E are ubiquitously expressed while Oct-1L, -1Rα and -1Rβ are lymphoid specific. Oct-1L has a tissue-specific expression pattern similar to Oct-2 (111). The ~100 kDa Oct-1 protein has a constant protein level through out the cell cycle, but the phosphorylation status of Oct-1 is cell cycle regulated (209). Oct-1 gets hyperphosphorylated as cells enter mitosis. This correlates with a strongly reduced DNA binding capacity and a concomitant lowering of transcription (150).

Fig 7. Schematic drawing of the Oct-1 gene.

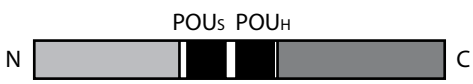


Fig 8. Schematic drawing of the Oct-1 protein.

Oct-1 influence transcription either alone or together with a variety of other transcriptional regulators. It activates transcription of for example small nuclear RNA (snRNA), Histone H2B and immunoglobulins, and it represses IL8 expression (31, 185, 201). Crystal structures of the Oct-1 POU-domain on MORE and PORE sequences show that the POU polypeptide can form different dimer arrangements which facilitate varying protein-protein interactions (fig 6) (148). Recruitment of the B-cell specific coactivator Bob.1 enhances the binding affinity of Oct-1 by "clamping" the two POU subdomains together. Interaction with Bob.1 is possible when Oct-1 is bound to PORE, but not to MORE where the interacting surface is inaccessible (189).

Oct-2

Oct-2 is tissue specific for B-lymphocytes and neuronal cells (61, 170). The primary RNA transcript of Oct-2 is subjected to alternative splicing yielding different variants (fig 9) that can either repress or activate transcription (105, 108, 198). The two splice forms Oct-2.1 and Oct-2.2 are most prevalent in B-cells, while Oct-2.4 and Oct-2.5 are more common in neurons. Oct-2 is expressed at low levels in early differentiation stages of B-cells and at higher level in more mature cells (e.g. in GC B-cells) (116, 160, 173). The Oct-2 protein level is constant through out the cell cycle (178).

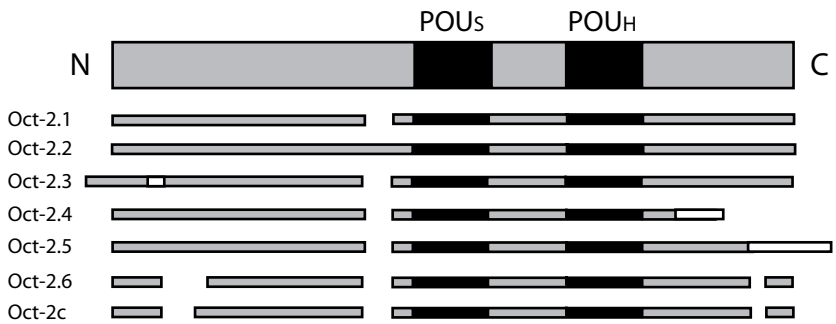


Fig 9.
A schematic drawing of the different Oct-2 splice variants.

Oct-2 is required for transcription of the CD36 gene and aid in activating the immunogloblin promoter (167). It represses transcription of the tyrosine hydroxylase gene in neuronal cells and is also responsible for repression of the herpes simplex virus (HSV) immediate early genes in neuronal cells (33, 104).

Bob.1

Bob.1 (OBF.1 or OCA-B) is a 256 aa long protein with an expression pattern largely restricted to B-cells. It has no intrinsic DNA-binding domain and functions as a coactivator being targeted to the DNA by other transcription factors. In the normal B-cell lineages the protein level of Bob.1 is higher in germinal center B-cells then in resting B-cells and the expression level in B-cell lymphomas is usually higher than normal (55). Bob.1 is necessary for the late B-cell development stages and induction of the formation of germinal centers (89, 122, 165).

Bob.1 is essential for octamer-dependent immunoglobulin gene transcription in B-cells and down-regulation of Bob.1 and/or Oct-2 in classical Hodgkin's disease is correlated with reduced immunoglobulin transcription (98, 174). Bob.1 interacts with the POU-domain of Oct-1 and Oct-2 bound to the octamer sequence (ATGCAAAT) and its activity is dependent on contact to the underlined adenine.

Groucho/TLE

The *Drosophila* protein Groucho is the founding member of the large Groucho/TLE family of corepressors which is involved in many important developmental processes, for example lateral inhibition, segmentation, sex determination and eye development (134). Groucho was identified in *Drosophila melanogaster* in 1968 by a viable mutation that resulted in clumps of extra bristles above the adult fly eye, reminiscent of the bushy eyebrows of Groucho Marx, hence the name (107). It has a calculated molecular mass of about 81 kDa, but the native molecular mass is 380 kDa suggesting that Groucho is a tetramer under native conditions (24).

The human variant of Groucho is named transducin-like enhancer of split (TLE) and the mouse homologue is called Groucho related gene (Grg). These proteins are very homologous and share a well conserved Q- and WD-domain (fig 10). The N-terminal Q-domain mediates tetramerization, while the WD-repeat domain has been assigned to mediate protein-protein interactions (77, 136,

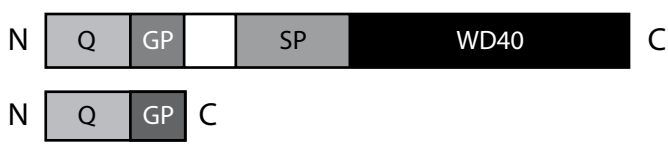


Fig 10. The full-length Groucho/TLE protein and the shorter Grg5/AES

137). The CCN-domain contains a putative casein kinase II/cdc2 phosphorylation site and a nuclear localization signal (176).

Groucho/TLE-1 can be divided into two groups depending on cellular compartmental localizations. One group is hyperphosphorylated and strongly associated with the nucleus, while the other group is less phosphorylated, has a lower repressing activity and shows reduced nucleus association (76). The degree of phosphorylation also varies throughout the cell cycle, where Groucho/TLE becomes hypophosphorylated at the G2/M transition (128).

Groucho/TLE functions as a corepressor for many DNA-binding transcription factors; e.g. Hairy-related proteins, Runt domain proteins, Pax-5 and NFκB (38, 45, 134). The mechanism of Groucho/TLE mediated repression has not been completely clarified yet but it seem as if different methods might be used at different occasions. Transcription can be repressed either directly by prevention of binding of activators to the DNA or by interaction with the basal transcriptional machinery. As an example Grg-5 can directly interact with TFIIE, a part of the basal transcriptional machinery, and thereby prevent elongation of transcription (205). Repression by Groucho/TLE could also be achieved by interaction with histone proteins (131) or by alteration of histone acetylation by recruitment of the *Drosophila* histone deacetylase Rpd3 or the human homologue HDAC1 (18).

There is a naturally truncated variant of Grg/TLE, which is called Grg-5 in mice and Amino terminal enhancer of split (AES) in human. It corresponds to the N-terminus of the full-length protein and have been shown to function both as a corepressors on its own (184) and as a dominant negative regulator of full-length Grg/TLE-proteins (fig 10) (153).

Groucho/TLE represses Oct-induced transcription (paper II)

Oct-proteins were originally identified as transcriptional activators, but was later also described to function as repressors (33, 104). This dual function is most likely due to recruitment of different co-factors such as the coactivator Bob.1. When looking for corepressor candidates for the Oct-proteins we noticed that many B-cell specific transcriptional activators, such as PU.1 and Pax5, interact with the Grg/TLE family of corepressors, which renders them from being

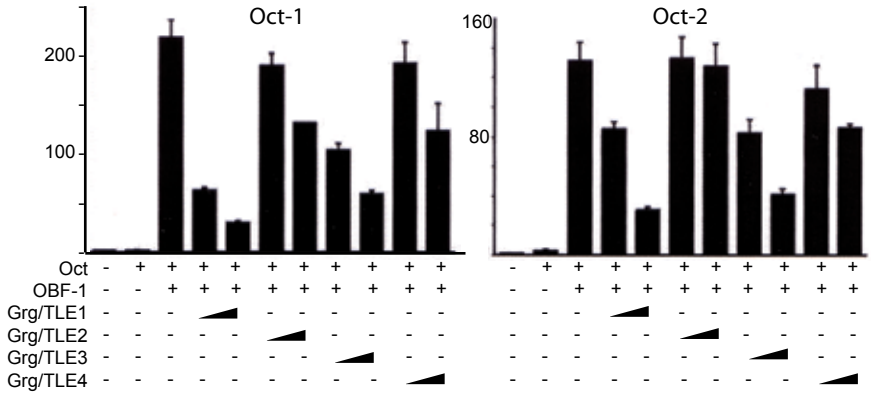


Fig 11. Represssion of Oct-1 or Oct-2 activity on the octamer motif with increasing concentration of Grg/TLE.

activators to repressors (38, 106). In paper II we sought out to see if the Grg/TLE family could function as corepressors of Oct-1 and Oct-2.

A luciferase reporter assay was used to evaluate the function of Grg/TLE on Oct-induced transcription. Luciferase reporter vectors containing different Oct-binding sites were transiently cotransfected into cells together with Oct, Bob.1 and Grg/TLE. The results showed that Grg/TLE could infact repress Oct-induced promoter activity and that the repression of Oct-1 and Oct-2 was dependent on the DNA site to which they were bound.

A reporter vector carrying the classical octamer binding site Grg/TLE-1 and -3 was more potent in repressing both Oct-1+Bob.1 and Oct-2+Bob.1 induced transcription than Grg/TLE-2 and -4 (fig 11).

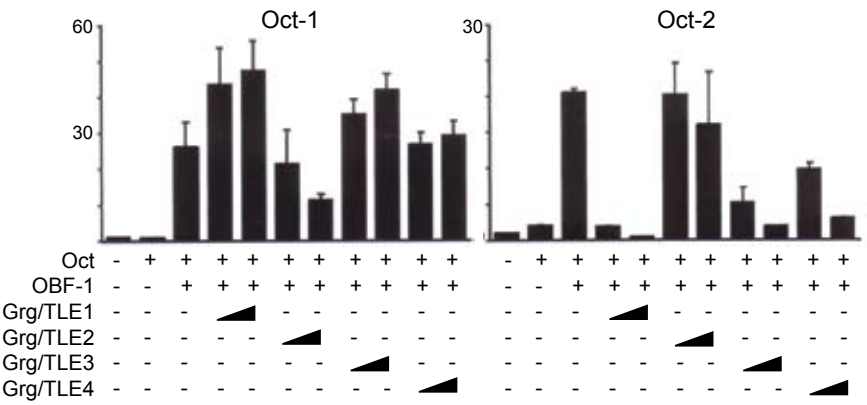


Fig 12. Repression of Oct-1 or Oct-2 activity on the PORE-D motif with increasing concentration of Grg/TLE.

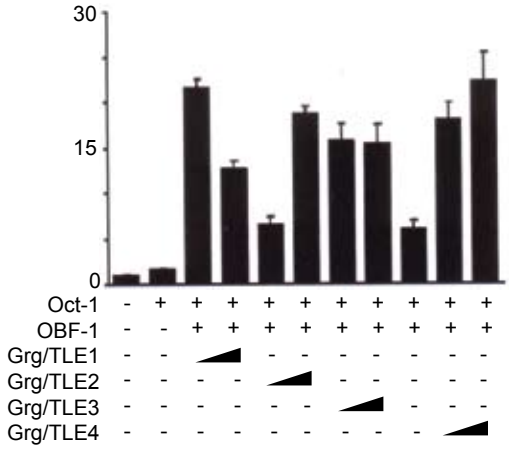
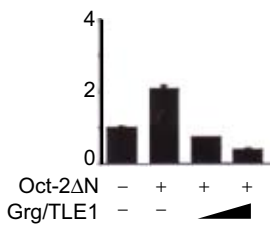


Fig 13. Repression of Oct-1 activity on the natural PORE motif with increasing concentration of Grg/TLE.

The PORE-D containing vector allowed Grg/TLE to discriminate between Oct-1 and Oct-2. There Grg/TLE-2 was the only variant that repressed Oct-1+Bob.1, while Oct-2+Bob.1 was repressed by Grg/TLE-1, -3 and -4 and not by Grg/TLE-2 (fig 12). Yet another result appeared when a luciferase vector carrying the natural PORE element from the immunoglobulin heavy chain promoter was used. With this vector only Grg/TLE-1 and -3 repressed Oct-1+Bob.1 induced transcription, much like the results we got with the octamer containing vector (fig 13). The results clearly show the complexity of Grg/TLE repression of Oct-proteins, where discrimination is due to the DNA-motif the proteins are binding.

To exclude that Bob.1 was responsible for the recruitment and repression by Grg/TLE we used a N-terminally deleted variant of Oct-2 (Oct-2ΔN) that has a stronger activating potential than full-length Oct-2 and therefore can be used without Bob.1. Results showed that Grg/TLE-1 could indeed repress Oct-2ΔN induced transcription in absence of Bob.1 (Fig 14).

Fig 14. Repression of Oct-2ΔN activity on the PORE-D motif with increasing concentration of Grg/TLE.



Interaction between the Grg/TLE-4 and Oct-2 was mapped to the serine and proline rich SP-domain in Grg/TLE-4 and to the POU domain in Oct-2.

Grg/TLE has been described to be able to repress transcription in a variety of different ways; one way is by recruitment of HDAC. This was not the case with Oct-proteins since repression still

occurred in presence of a HDAC inhibitor. Repression was also shown not to be due to disassembly of Oct-protein from DNA. This leaves the mechanism of repression to be a matter for future investigations.

The Grg/TLE proteins are extremely homologous both among each other and through out evolution. To get an idea about the importance of Grg/TLE repression of Oct-induced transcription we examined whether the Oct-proteins also could be repressed by the *Drosophila* variant, Groucho. With GST pull-down experiments we could demonstrate that Groucho can interact directly with the POU domain of Oct-2. Groucho could also repress Oct-2 induced promoter activity (fig 15). These two findings indicate that the interaction between Oct and Grg/TLE might be evolutionary conserved.

Since no other corepressors of Oct-proteins have yet been found it will be extremely interesting to find out if Grg/TLE are important for the repressing function that has been reported for Oct-proteins. Will Grg/TLE play any role in the repression of the IL8 gene, the tyrosine hydroxylase gene in neuronal cells or in the repression of the lytic cycle in HSV in neuronal cells (33, 104, 201)? Could it be Grg/TLE that renders Oct-2 to a repressor in neuronal cells? There is still a good chance that other proteins will prove to be of importance for repression of Oct-proteins; one example is that the N-terminus has been described to be involved in repression due to the finding that if it is removed the activating potential of Oct-2 increases drastically.

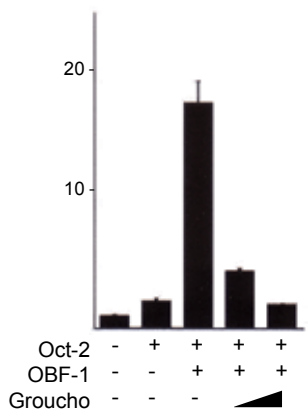


Fig 15. Repression of Oct-2 activity on the PORE-D motif with increasing concentration of the *Drosophila* Groucho.

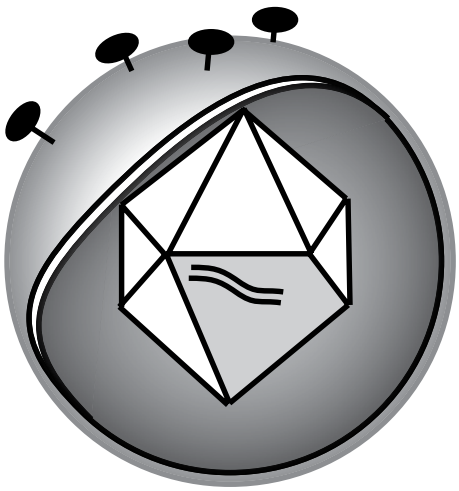


Fig 16.
The EBV particle showing the ds DNA, capsid, outer membrane and glycoprotein spikes.

EPSTEIN-BARR VIRUS

Epstein-Barr virus (EBV), also called human herpesvirus 4 (HHV4), is a gamma-herpes virus carried by more than 90% of the adult human population. It was discovered in 1964 by the two pathologists Tony Epstein and Yvonne Barr (40). Primary infection usually occurs asymptomaticly early in life, but if delayed, it might evolve into the benign self-limiting lymphoproliferative disease infectious mononucleosis (IM), also called glandular fever or "kissing disease". EBV forms a life long latent infection in the memory B-cell compartment, which is asymptomatic in the majority of individuals, but EBV is also associated with various malignancies, including Nasopharyngeal carcinoma, Burkitt's lymphoma (BL) and lymphomas in immunocompromised individuals (149).

The EBV genome

The EBV particle carries an over 172 000 bp long double stranded DNA and was the first herpesvirus genome to be completely sequenced (10). It contains 84 open reading frames (43). Accession number for the B95-8 model EBV strain in the genebank sequence library is NC 001345 and was recently corrected (34). Designations of the EBV genes are based upon in which fragment in the BamH1 restriction map of the B95-8 virus genome they are found. The fragments are listed in alphabetical order dependent on size. For example BKRF1 (EBNA1) is found in the BamH1 fragment K, right ward reading, open reading frame 1. The genome is enclosed in a complex, toroid-shaped capsid built by 162 capsomers and like all herpesviruses EBV carries a cell-derived envelope interspersed with viral glycoprotein spikes, gp350/220 (fig 16).

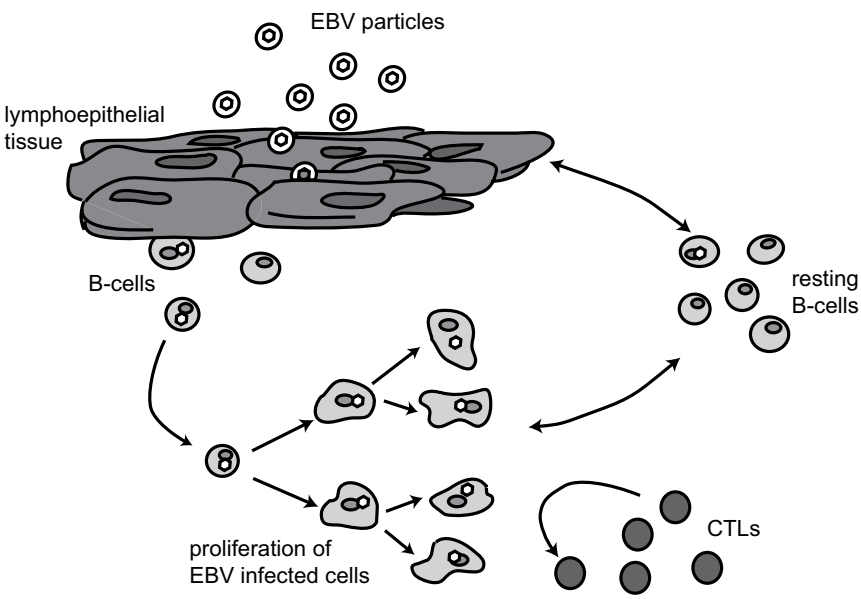


Fig 17.
An overview of the EBV life-cycle. EBV particles enter through the mucosal surface and infects naive B-cells (EBV is indicated by a white hexagon). EBV activates and drives proliferation of infected B-cells (blasts). CTLs kills infected cells. EBV infected cells avoid recognition by the CTLs by forming a more silent latent infection in the B-cell compartment.

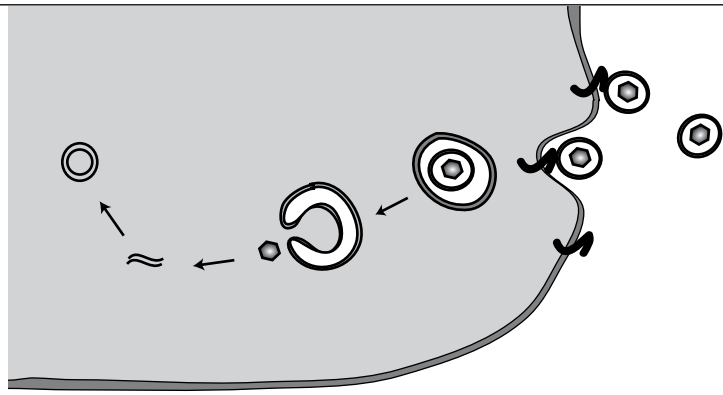


Fig 18.
Model of EBV infection.

The virus lifecycle

EBV is spread via saliva and enters the body by the mucosal surface in the oropharynx where it encounters infiltrating B-cells (fig 15). The glycoprotein gp350/220 in the viral envelope binds to CD21 (also called complement receptor type 2 = CR2) expressed on the B-cell surface (44). The binding is followed by aggregation of CD21 in the plasma membrane and the viral particle is internalised in cytosolic vesicles (fig 18). In the next step the nucleocapsid and tegument are released into the cytosol by fusion of the viral envelope and the vesicle membrane. The linear double stranded viral genome is circularised through homologous recombination of the terminal repeats and the DNA persists in the B-cell compartment as an extrachromosomal plasmid called an episome (fig 19) (75). A life-long latent infection is established in the B-cell compartment. In healthy carriers approximately one cell per million peripheral blood lymphocytes is positive for EBV DNA (23, 117). According to expression of surface markers the infected cells resemble memory B-cells (9). The viral load in the body is constant over time but varies between individuals (117). Lytic replication is activated in the oropharyngeal mucosa where virus particles are produced and secreted in the saliva, contributing to further spread of EBV.

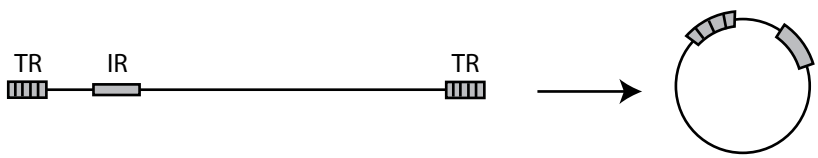


Fig 19. Linear and circularized (episomal) EBV DNA showing terminal repeats (TR) and internal repeats (IR)

There are two kinds of cells that are regarded to be the main cell types infected by EBV; B-lymphocytes and epithelial cells. Epithelial cells do not express CD21 on their surface and it is still controversial how EBV enters these cells. But there is clear evidence that epithelial

cells are permissive for viral infection *in vivo* since EBV is discovered in epithelial cells both in the AIDS related disease called oral hairy leukoplakia (OHL) and in nasopharyngeal carcinoma (NPC) (53, 119, 121). EBV is also able to enter other subsets of lymphocytes, for example macrophages, T cells and NK cells (82).

Transformation

The normal fate of a cell is to divide a certain number of times, differentiate and then undergo programmed cell death. This is a tightly regulated process where cellular proteins and external factors influence each other to form a perfect balance in the body. Cancer is one example where this regulation has gone wrong and the cancer cell is referred to as being immortal or transformed (59).

The characteristic properties that distinguish transformed from normal cells are their subvertions of signals that control cell growth, DNA replication and cell division. A few viruses have the ability to transform cells. EBV is one of them and it is capable of immortalizing primary human B-lymphocytes *in vitro* (123). The EBV transformed cells, called a lymphoblastoid cell line (LCL), acquire unlimited proliferation and the morphology and growth behaviour of the cells change dramatically (123). They become large and irregularly shaped, develop villipodia and strongly adhere to each other forming large clumps. An LCL is characterized by upregulated expression of B-cell activation markers such as CD21, CD23 and absence of germinal center markers like CD38.

Tumour association

Even though EBV is a common virus with a high prevalence in the adult human population, the majority of infected persons never notice that they harbour this virus. But in a few individuals EBV might be a risk factor for disease. Several different malignancies have been associated with EBV such as Burkitt's lymphoma (BL), Nasopharyngeal carcinoma (NPC), Hodgkin's disease and lymphomas in immunocompromised individuals (120).

Burkitt's lymphoma

Burkitt's lymphoma (BL) is a B-cell tumour appearing in the jaw. It was first described in the late 1950-ies by the missionary surgeon

Denis Burkitt working in Afrika (21). He discovered what today is thought to be the most common childhood cancer in equatorial Africa and Papua New Guinea. These constitute the endemic form of BL out of which 95% are EBV positive. BL also occurs sporadic worldwide with a lower incidence and out of those only 10-20% are EBV positive (211).The only EBV protein expressed in BL is EBNA1, categorizing the lymphoma into latency I. Some BL cell lines tend to drift to a more LCL-like phenotype (latency III) when they are propagated in vitro for a long time, leading to expression of all EBNA and LMP1 proteins (54, 157, 158).

A hallmark of BL is chromosomal translocation resulting in deregulation of the proto-oncogene *c-myc*. The BL specific translocation involve movement of the long arm of chromosome 8 near the site of the c-myc locus (8q24) to either the proximity of the gene for the immunoglobulin heavy chain at chromosome 14 (t[8,14]), or to the kappa light chain locus on chromosome 2 (t[2;8]), or to the lambda light chain locus on chromosome 22 (t[22;8]). This results in constitutive transcription of *c-myc* (112, 135).

Nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) is a malignant neoplasm of epithelial cells in the nasopharynx. NPC is rare in caucasian but has a very high incidence in Southeast Asia, especially southern China (over $30/10^5$) and also in the arctic regions, for example among the Eskimos in Greenland and Alaska. This uneven geographical and ethnic distribution has led to the idea about three main ethiological factors, genetic, environmental and virological. Patients with NPC usually have elevated titers of specific IgA antibodies to EBV and 100% of the undifferentiated cases of NPC are EBV positive with a latency II phenotype (35, 211).

Hodgkins lymphoma

Hodgkins lymphoma (HD) is characterized by large atypical tumour cells called Reed-Sternberg (RS) cells. These usually represent less than 1% of the tumour tissue and most other cells are non-malignant. Nearly all RS cells derive from pre-apoptotic, crippled germinal-center B-cells and it is thought that EBV might have a role in the rescue of these cells from apoptosis. Of the classical HL in the western world 40% of the RS cells harbour EBV. These show a latency II gene expression pattern with a silenced Cp. The RS cells

have a silenced immunoglobulin gene due to crippled octamer-dependent transcription caused by down regulation of PU.1, Bob.1 and/or Oct-2 (174, 186).

Other EBV associated lymphoproliferative disorders

Immunosuppressed individuals, like for example AIDS patients or transplant recipients, that have an impaired immune system are at high risk of developing EBV associated lymphomas. The EBV-driven B-cell lymphomas that may arise in this group are very heterogeneous and usually have a latency III phenotype (187). They can partly be explained by an inability of the patient to mount an effective cytotoxic T-cell response against EBV.

AIDS patients can develop a wart-like lesion, called oral hairy leukoplakia (OHL), on the tongue in which EBV replicate lytically (53).

Latency

Like all herpes viruses EBV is capable of establishing a latent infection which is characterized by a silent infection without production of new viral particles. EBV also has the remarkable ability to vary the range of proteins expressed during latency and thereby influence the phenotype of the latently infected cell. Until now 12 latent genes, out of which 10 are translated into proteins, have been identified in the EBV genome. These genes include six nuclear proteins (EBNA1 to EBNA6) three membrane proteins (LMP1, LMP2A and LMP2B), two small non-polyadenylated RNAs (EBER1 and EBER2) and BART/BARF0. At least four different viral gene expression patterns have been described in latently infected B-cells and these make the criteria for the latency programs called latency 0, latency I, latency II and latency III.

A switch between the different latency forms (latency III to latency I) has been described in some BL cell lines that have a tendency to drift into a latency III phenotype during cell culture. A shift is also thought to occur in vivo after primary infection (fig 17) (157, 158).

Latency 0

Latency 0 is the latency form with the most restricted pattern of gene expression. It is found in the EBV-infected resting memory B-

cells of healthy carriers and is thought to represent the way for EBV to escape the immune system. LMP2A and possibly the EBERs and BART RNAs are the only genes expressed (27, 154). Earlier studies have also found EBNA1 in this latency type but in retrospect this could be due to induction of EBNA1 expression during the complicated experimental procedure (23, 188a). There have also been reports that no EBV genes are expressed in these cells (70).

Latency I

Latency I is represented by cells found in EBV positive Burkitt's lymphoma (BL) biopsies and BL cell lines. Expression of EBERs and EBNA1 from the Q promoter are found in these cells (149).

Latency II

The latency II cell is characterised by expression of EBERs, BARTs, EBNA1 from the Q promoter and all three LMP proteins. It has been found in NPC, HD and nasal NK/T-cell lymphoma (149).

Latency III

In latency III all 12 latency genes are expressed; the EBERs, BARTs, all six EBNA proteins from the Wp or Cp and all three LMPs (149). This group was first defined in LCLs and in group III BL cell lines, but has also been found in IM patients and in lymphoproliferative disorders in immunocompromiced individuals (118). Latency III is sometimes referred to as the growth program (188). It has a characteristic phenotype with expression of activation markers (CD23), high levels of adhesion MHC class I molecules (CD11a, CD54, CD58) and the cells tend to grow in large aggregates.

The EBV latency genes

The EBNA proteins

The six EBNA proteins (Epstein-Barr virus nuclear antigen) are all expressed from the same over 100 kbp long transcript, spliced into bicistronic mRNAs. The EBNA5 mRNA is constructed through alternative splicing of all other EBNA transcripts (14). An alternative nomenclature excists for EBNA3 (EBNA3A), EBNA4 (EBNA3B), EBNA6 (EBNA3C) and EBNA5 (EBNA-LP) .

EBNA1

The nuclear protein EBNA1 is a sequence specific DNA binding protein with three important functions: to regulate expression of viral proteins, initiate DNA replication and partitioning the viral episomes during cell division. The protein is more than 600 aa long (66-95 kDa), and it shows strain specific size variation due to an internal variable glycin-alanin repeat (GAr) domain (52, 65, 66). EBNA1 has a basic N-terminus, a nuclear localization signal (NLS) and a C-terminal DNA-binding and dimerisation domain (fig 20) (7, 28).

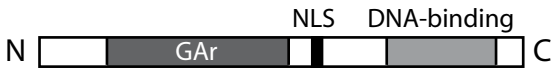


Fig 20. The EBNA1 protein.

EBNA1 binds as a dimer to the 16 bases long palindromic consensus sequence 5'-G(A/G)TATCAT-ATGCTA(C/T)C-3' that is situated in three different areas of the viral genome; in the Q promoter and at two sites in *oriP* upstream of the Cp called dyad symmetry (DS) and family of repeats (FR) (144). (fig 21) The binding affinity between EBNA1 and these three sites differs; FR is bound with the highest affinity and Qp with the lowest. This is due to sequence variation in the target DNA (8, 81).

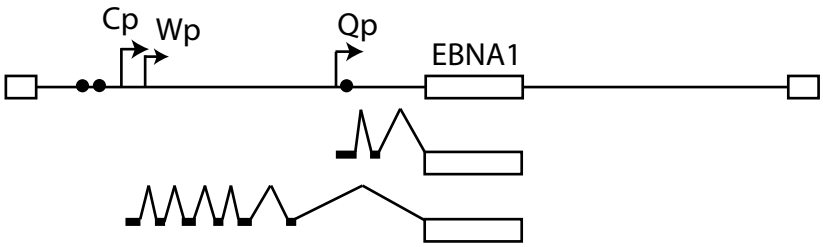


Fig 21. Schematic drawing of the EBV genome with the three EBNA1 binding sites marked with black dots. Arrows indicate the three promoters of the EBNA1 gene.

EBNA1 is an extremely stable protein with a very long half-life (90). The GAr contributes to its stability by blocking proteasomal degradation of EBNA1. This means that even though EBNA1 is expressed in virtually all different forms of EBV associated malignancies it is seldom discovered by the immune system (101, 102). The GAr has more functions than preventing proteasomal degradation. GAr keeps the EBNA1 level in an EBV infected B-lymphocyte stable over time by inhibiting translation of the EBNA1 mRNA in cis (175, 204). Both of these functions contribute in preventing presentation of EBNA1 on MHC class I molecules

and thereby avoiding cytotoxic T-cell (CTL) recognition. Despite prevention of proteasomal degradation CD8+ responses to EBNA1 do exist (88). This is most likely a result of stimulation by antigen presenting cells processing extracellular EBNA1 and presentation on MHC class I to CTLs. These CTLs have been found in isolates from healthy EBV positive persons, but they fail to recognize virus-infected cells and are thought to be biologically ineffective (12).

EBNA1 regulates transcription of all EBNA proteins including its own from the Cp. It activates transcription from the Cp through binding to the upstream FR element in *oriP* resulting in positive autoregulation of its own expression during latency III when the Cp is active (144, 146, 147, 181). During latency I the Cp is inactive and expression of EBNA1 is regulated from the Qp downstream of the Cp. The only explanation for this silencing has for a long time been methylation of the promoter area (6, 114, 152). Contrary to the FR-Cp situation EBNA1 repress its own transcription when bound to its binding sites in Qp (164, 183, 190).

EBNA1 is the only viral protein required in trans for plasmid maintenance (203). It initiates replication by binding to *oriP* and during cell division (mitosis) it attaches the viral genome to cellular DNA through binding to the cellular protein EBP2 (EBNA1 binding protein 2), which is a component in the mitotic chromosome, thus assuring an even distribution of the viral copies into the two daughter cells (85, 166).

Many cellular proteins have been identified to interact with EBNA1, for example the nuclear transport proteins Importin-α and karyopherin-α1. USP7, also called herpesvirus-associated ubiquitin-specific protease (HAUSP), is another example. USP7 has no effect on turnover or cell surface presentation of the EBNA1 protein, but it does cause an increase in EBNA1 replication activity and it cleaves ubiquitin from EBNA1 (71, 72).

EBNA2

EBNA2 is a viral transcription factor. It is one of the first viral proteins expressed from the Wp after infection and it is required for the following shift in promoter activity from Wp to Cp (4, 182, 200). EBNA2 is also essential for immortalisation of EBV infected B-lymphocytes *in vitro* (29, 58).

Although EBNA2 functions as a transcriptional activator it lacks the ability to bind DNA, instead EBNA2 is targeted to its specific

sequence (GTGGGAA) by the DNA-bound cellular protein RBP-Jκ (CBF1) (64, 78, 182). RBP-Jκ, is a cellular transcription factor targeted by the Notch signalling pathway. Once activated, Notch goes into the nucleus and replaces the transcriptional corepressor (e.g. TLE) in complex with RBP-Jκ to initiate transcription. Some of the EBNA2 functions have been reported to be replaceable by the Notch protein (51). Two additional viral promoters are activated by EBNA2, the LMP2A promoter and the bi-directional promoter for LMP1 and LMP2B (1, 49, 79, 171). EBNA2 also activates cellular genes including CD21, CD23 and c-myc (30, 84, 194).

EBNA3, 4 and 6

EBNA3, 4 and 6 (EBNA3A, 3B and 3C) are arranged in tandem in the EBV genome and share a similar exon-intron structure. They have all been shown to interact with the cellular protein RBP-Jκ (151).

EBNA3 and its association with RBP-Jκ is important for conversion of infected B-cells into LCLs (113). EBNA3 and 6 are both transcriptional repressors that are able to bind to DNA through RBP-Jκ regulating both viral and cellular promoters (80, 151, 192). Both proteins repress EBNA2 induced promoter activity and EBNA6 can directly repress EBNA2 induced Cp activity through recruitment of HDACs (142, 143).

EBNA6 functions as an oncoprotein directing cell cycle progression through the G1 restriction point into S phase through interactions with Cyclin A. This makes EBNA6 essential for immortalization of B-lymphocytes (92, 93).

EBNA4 can protect BL cells from apoptosis induced by serum starvation. Survival could be attributed to increased expression of the oncoprotein bcl-2, a well known repressor of cell death (168).

EBNA5

EBNA5 (EBNA-LP) together with EBNA2 are the first proteins expressed after EBV infection of B-lymphocytes *in vitro* and EBNA5 is required for efficient immortalization of the cells (4, 169). The size of EBNA5 varies between isolates due to different splicing of the repeated BamH1 W fragments in the EBNA5 gene (37).

EBNA5 is a coactivator of EBNA2-activated transcription (60,

126). Together they can up-regulate cyclin D2 thus inducing G0 to G1 transition in resting B-lymphocytes (169).

LMP1

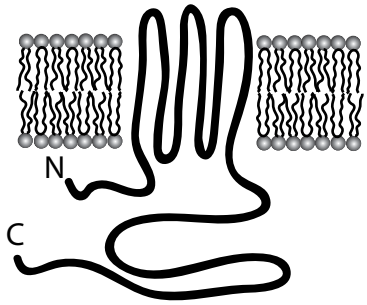


Fig 22. schematic drawing of LMP1

Latent membrane protein 1 (LMP1) is found in the cytoplasmic membrane of the cell and is essential for EBV-mediated growth transformation by B-lymphocytes (86). Transformation, metastasis and protection against apoptosis are the three main functions conferred by LMP1. It has six transmembrane segments, a short cytoplasmic N-terminal tail and long cytoplasmid C-terminus (fig 22). The function of LMP1 resembles a constitutively active receptor and is described as a viral homolog of the cellular CD40 protein, a member of the tumour necrosis factor receptor (TNFR) superfamily. LMP1 signalling resembles constitutive, amplified and abnormal CD40 signalling (103). Signalling through the two C-terminal domains called CTAR1 and CTAR2 result in activation of five different signalling pathways, the PI3k-, JNK-, NFκB-, p38- and the JAK/STAT-pathway, leading to upregulation of several cellular genes, e.g. CD23, CD44, Bcl-2 and A20 (39, 57, 68, 96, 156, 191, 193). Upregulation of the anti apoptotic protein Bcl-2 confers resistance to apoptosis. Constitutive activation of NFκB is the hallmark of the biological activities of LMP1 (50, 63).

LMP2A & LMP2B

LMP2A and LMP2B are two membrane proteins transcribed from the same gene except the first exon, which is lacking in LMP2B. Their genes are situated on both ends of the EBV genome and cannot be transcribed unless the genome is circularised (100). LMP2B is transcribed from a bi-directional virally regulated promoter shared with LMP1, while LMP2A has a promoter of its own (99, 177). Both proteins are predicted to contain 12 transmembrane domains and a short intracellular C-terminus, but only LMP2A has the long intracellular N-terminus (fig 23) (109, 155). The N-terminus of LMP2A is responsible for signal transduction (2, 115).

LMP2A can inhibit BCR signalling by sequestering BCR-associated tyrosine kinases, e.g. Syk and Lyn (110, 149). This prevents unwanted antigen-triggered activation of EBV-positive B-cells that otherwise would cause untimely entry into lytic cycle. LMP2A has also been shown to bind cellular E3 ubiquitin-ligases that could ubiquitinate LMP2A-bound tyrosine kinases causing their proteasomal degradation (197). LMP2A have also been found to have the oposite effect in cells lacking a functional BCR where it mimics BCR-signalling hence giving the cell survival signals (22). Despite this neither LMP2A or LMP2B are needed for EBV-induced immortalization of primary B-lymphocytes *in vitro*.

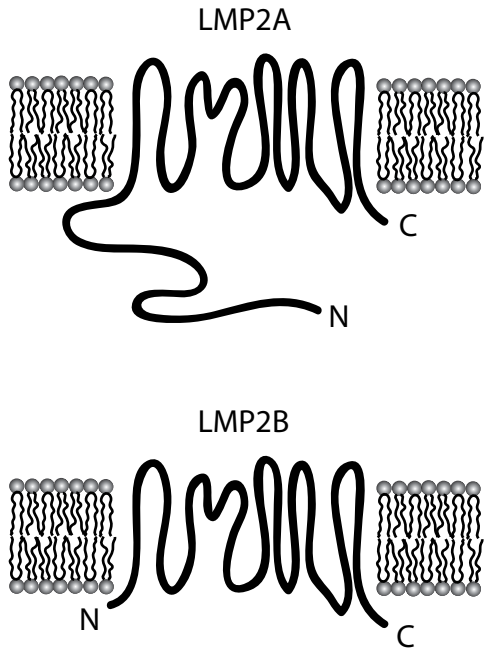


Fig 23. schematic drawing of LMP2A and LMP2B

EBER1 & 2

EBER1 & EBER2 are two short non-translated EBV-encoded RNAs. They are transcribed by RNA polymeras III and are detected in virtually all EBV associated tumours (149). The EBERs have been reported to induce secretion of interleukin-10 (IL-10) which might result in stimulated growth of infected B-cells and suppression of cytotoxic T-cells (91).

BART/BARF0

The BamHI-A rightward transcripts (BART) is a family of mRNAs expressed in all EBV latency programs, including EBV-infected B cells in healthy carriers. The BART transcript contains at least two ORFs and BARF0 refers to one putative protein. It was originally found in NPC samples and later in almost all EBV associated malignancies (20, 69). It is still under debate whether any of the BARTs are translated into to proteins or not.

Another ORF in the BARTs, called RK-BARF0 encodes a transcript with a complex pattern of alternate splicing (159). In *in vitro* experiments it has been reported to be involved in the Notch signalling pathway possibly through inhibiting transcriptional activation induced through RBP-Jκ by Notch or EBNA2 (172).

W promoter

After *in vitro* infection of B-lymphocytes, EBNA2 and EBNA5 are the first EBV encoded genes to be expressed from the viral W promoter (Wp). Within 36 hours EBNA2 drives a switch in promoter usage from Wp to the downstream C promoter (Cp) (199).

Q promoter

During latency I and latency II the EBNA1 gene is transcribed from the Q promoter (Qp). These cells exclusively express EBNA1 as compared to latency III cells where all six EBNAs are expressed. The Qp is a TATA less promoter resembling cellular promoters to house holding genes, and like those the Qp can be active in virtually every cell type (163).

No B-cell specific or viral proteins are necessary for Qp activity, but many proteins are involved in its regulation. An interferon-stimulatory response element (ISRE) is situated upstream of the promoter start site and when bound by the cellular interferon response factors; IRF1, 2 and 7 transcription is repressed (162, 208). In concordance with Qp activity is the finding that IRF-2 has a higher expression level in latency III then in latency I cells (207). The JAK/STAT pathway positively regulates Qp trough binding of STAT3 to two STAT binding sites on both sites of the ISRE, thereby opposing the effect of the IRFs (25, 26). Many of the STAT proteins are constitutively activated in EBV positive cells.

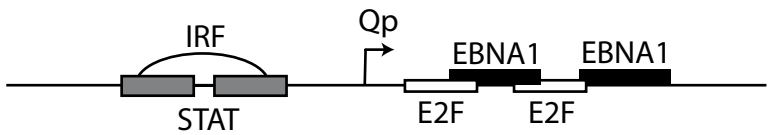


Fig 24. Schematic map of the Qp with protein binding motifs.

EBNA1 negatively autoregulates its own transcription by binding the two EBNA1 binding sites downstream of Qp (fig 24) (161). As a result the EBNA1 protein level in latency I is low compared to latency III. Upstream of and interspersed between the EBNA1 sites are two E2F binding sites and binding of E2F to the Qp can displace the EBNA1 proteins (183). E2F is a cellular transcription factor that regulates cellular proteins important in cell cycle progression and it activates transcription from the Qp in a cell cycle dependent manner (32). In early G1, E2F exists in a complex with unphosphorylated

Rb, a tumour suppressor protein. As G1 proceeds Rb becomes hyperphosphorylated and releases E2F that now is able to activate transcription of cellular genes including the Qp. (183).

C promoter

The C promoter drives the transcription of an over 100 kbp long polycistronic RNA that is spliced to produce all six EBNA proteins (13, 14).

Many transcription regulatory elements have been identified in the regions upstream of the Cp (fig 25). The major enhancer element called family of repeats (FR) is situated about 3000 bp upstream of the promoter in an area designated *oriP*. Binding of EBNA1 is essential for activation of Cp activity (97, 141, 146). The minimal promoter sequence necessary for FR-EBNA1-induced Cp activation is the -111/+76 region (125).

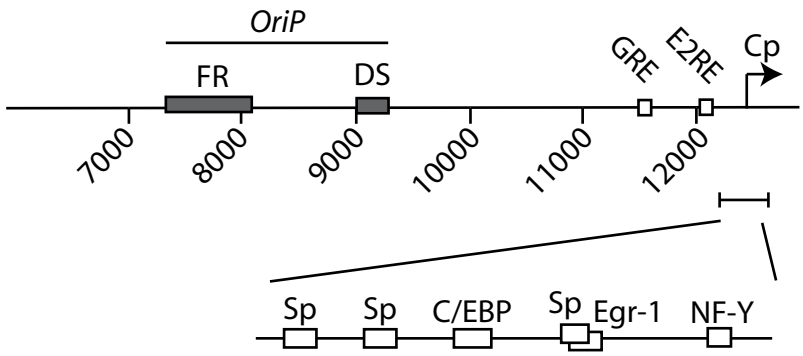


Fig 25. A detailed map of the Cp regulatory region. The lower part represents the area between -170 and 0 from the promoter start site (arrow).

In paper I we showed that there are motifs bound by the cellular transcription factors Oct-1 and Oct-2 in-between the EBNA1-binding sites in FR. Binding to FR was experimentally shown in an EMSA and supershifting with Oct-specific antibody. In a Luciferase assay Oct-2 alone and Oct-1 together with the B-cell specific coactivator Bob.1 was shown to activate transcription (5).

A glucocorticoid-responsive element (GRE) has been identified upstream of the Cp and glucocorticoid treatment of cells led to increased Cp-derived mRNA levels (94).

EBNA2 binds to the EBNA2-responsive enhancer (E2RE) through the cellular protein RBP-Jκ (CBF1). RBP-Jκ repress

transcription from Cp but when EBNA2 is bound the Cp is activated (78, 182). This site is also bound by the cellular transcription factor AUF (CBF2) that activates the Cp in presence of cAMP (48).

The –170/-55 region of the Cp contains several transcription regulatory elements, for example three Sp binding sites, a C/EBP binding site and an Egr-1 binding site (124). Of all different cellular Sp proteins only the transcriptional activator Sp1 could bind to and activate transcription from the Cp. EBNA1 in conjunction with Sp1 generated a significant increase in Cp activity compared to the effect of either protein alone (125).

The two cellular proteins C/EBPγ and β bind to the C/EBP site. Both are present in the EBV positive BL cell line Rael (latency I), in contrast to the LCL CBMI-Ral-STO (latency III) that only express C/EBPγ (125).

Egr-1 is normally not expressed in mature resting B-cells in vivo but is upregulated after EBV infection and immortalisation *in vitro*. The protein is invariably associated with a latency III phenotype and its expression is abolished in latency I cells. The Egr-1 site is overlapping with one of the Sp sites and contrary to Sp1 that activate transcription Egr-1 represses Cp activity (125).

NF-Y is a multimeric protein that consists of three highly conserved subunits; NF-YA, -YB and -YC that all are required for binding to the CCAAT-box upstream of the Cp. Its function is to recruit upstream regulatory transcription factors to the proximal promoter complex. NF-Y has a low expression level in resting B-cells and high in proliferating cells. NF-Y is present both in Rael and CBMI-Ral-STO. NF-YA and Sp1 interacts physically and EBNA1 can only activate the Cp in the presence of these two proteins (15, 125).

The Cp is only active in latency III cells and not in any other latency form. How the promoter is turned off is still not clear but once the promoter is silenced, methylation seems to be the way to keep it turned off. A 4,5 kbp region around *oriP* is unmethylated in latency I cell lines, and EBNA1 binding to *oriP* seem to be protective (41, 42, 74).

Variation in FR influence protein binding (paper I and IV)

FR upstream of *OriP* consists of a 30 bp long sequence, which in the model virus strain B95-8 is repeated 20 times. Different cell lines vary in the number of repeats and there is also sequence variation between the different repeats within FR (47). EBNA1 can bind to each repeat as a dimer and its binding is essential for Cp activity (97, 141, 146). Seven to nine repeats are required for full EBNA1 dependent enhancement of transcription from the Cp (202, 206). In a footprinting analysis 25 of the 30 bp, centred over the EBNA1 binding site, are protected by EBNA1 (74, 144).

The sequence variation within FR in B95-8 was analysed to contain five different variants of the EBNA1 binding site (table 1). When different repeats were used as probes in an Electro mobility shift assay (EMSA) the result showed quite large variation in the protein-DNA complex formation between the different probes (fig 26). EBNA1 was shown binding to all probes by antibody

Table 1. categorization of the FR sequence

1)	**G**AG	GATAGCATATGCTACC	C**G**G ATA**C**AGAT	Ea:Oa
2)	TAG	GATAGCATAT**A**CTACC	CAG ATATAGAT	Eb:Ob
3)	TAG	GATAGCATATGCTACC	CAG ATATAGAT	Ea:Ob
4)	TAG	GATAGC**C**TATGCTACC	CAG ATATA**A**AT	Ec:Oc
5)	TAG	GATAGCATAT**A**CTACC	CAG ATATAGAT	Eb:Ob
6)	TAG	GATAGCATATGCTACC	CAG ATATAGAT	Ea:Ob
7)	TAG	GATAGC**C**TATGCTACC	CAG ATATAGAT	Ec:Ob
8)	TAG	GATAGCATATGCTACC	CAG ATATAGAT	Ea:Ob
9)	TAG	GATAGCATATGCTA**T**C	CAG ATAT---T	Ed:Od
10)	T**G**G	G-TAG**T**ATATGCTACC	CAG ATATA**A**AT	Ee:Oc
11)	TAG	GATAGCATAT**A**CTACC	C**TA** AT**C**T**CT**AT	Eb:Oe
12)	TAG	GATAGCATATGCTACC	C**G**G ATA**C**AGAT	Ea:Oa
13)	TAG	GATAGCATAT**A**CTACC	CAG ATATAGAT	Eb:Ob
14)	TAG	GATAGCATATGCTACC	CAG ATATAGAT	Ea:Ob
15)	TAG	GATAGC**C**TATGCTACC	CAG ATATA**A**AT	Ec:Oc
16)	TAG	GATAGCATAT**A**CTACC	CAG ATATAGAT	Eb:Ob
17)	TAG	GATAGCATATGCTACC	CAG ATATAGAT	Ea:Ob
18)	TAG	GATAGC**C**TATGCTACC	CAG ATATAGAT	Ec:Ob
19)	TAG	GATAGCATATGCTA**T**C	CAG ATAT---T	Ed:Od
20)	TGG	G-TAG**T**ATATGCTACC	CAT GGCAACAT	Ee:Of
		EBNA1 (E)	Oct (O)	

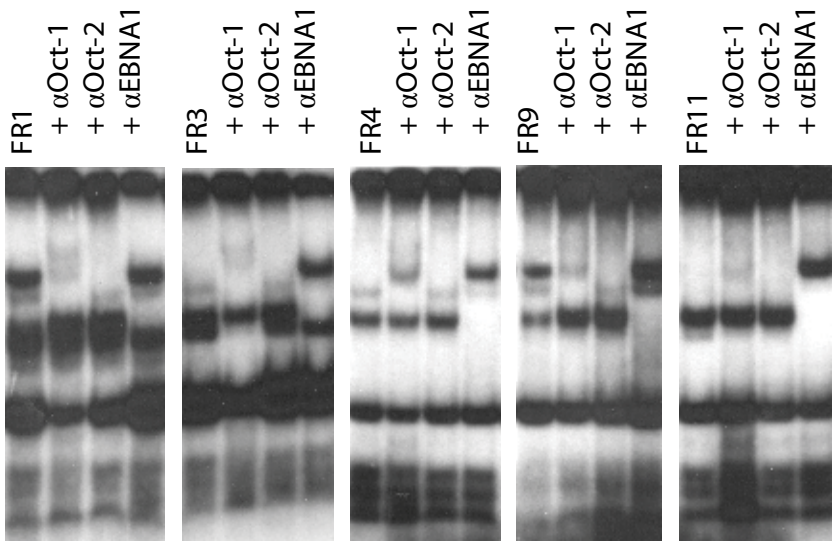
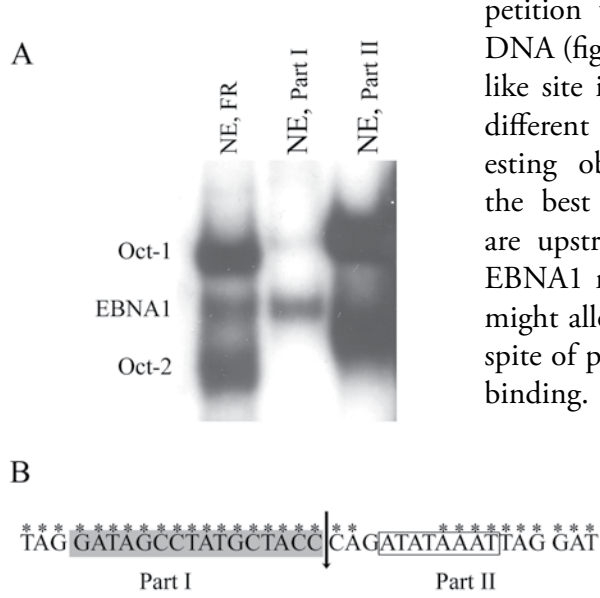


Fig 26.
EMSA on NE from CBMI-Ral-STO. Supershifting with specific antibodies as indicated in figure. Probe names (e.g. FR1) referes to respective repeat in table 1.

supershifting. Despite the fact that foot-printing have not given any strong indication that any other proteins except EBNA1 bind to FR the EMSA clearly shows many FR-specific complexes that do not react with the EBNA1 ab. Some of these bands could however be demonstrated to contain the cellular Oct-transcription factors Oct-1 and Oct-2.

Further studies of the putative Oct-binding site in the FR sequence revealed that EBNA1 and Oct-proteins bound to different parts of the repeat. This was demonstrated in an EMSA using competition with cold specific DNA (fig 27). The octamer-like site in FR exists in six different variants. An interesting observation is that the best Oct-binding sites are upstream of the worst EBNA1 motifs in FR. This might allow Oct binding in spite of presence of EBNA1 binding.

Fig 27.
A) EMSA on NE from Rael and cold competition with part I or Part II of the FR probeas indicated in figure.
B) Probe sequence



Novel cellular proteins influencing the Cp activity (paper I, III & IV)

Binding of other proteins than EBNA1 to FR has long been debated partly due to the results from foot-printing experiments (74, 144). These analyses does not exclude that other protein can bind to FR with a similar protection pattern as EBNA1, and also the results from the different repeats show variation in sensitivity to cleavage. Despite to the common idea that EBNA1 is the only protein binding to FR during all forms of latency there have been a few reports of cellular proteins binding to *oriP* both in presence of EBNA1 as well as in its absence (95, 129, 195, 196). One report even showed that a novel FR-binding protein could displace EBNA1 from FR (196).

There are reasons to believe that more proteins then EBNA1 can bind to FR and that there still are unidentified proteins involved in Cp regulation. Boreström et al saw a difference in EBNA1 induced transcription between different cell lines indicating that a cell specific protein might be involved (15). In our experiments we could see that a higher amount of EBNA1 was needed for the same promoter activity in the epithelial cell line 293 as in the EBV negative BL cell line DG75. Another reason is that we still to date don't know how the Cp is turned off when the virus switch to use the Qp.

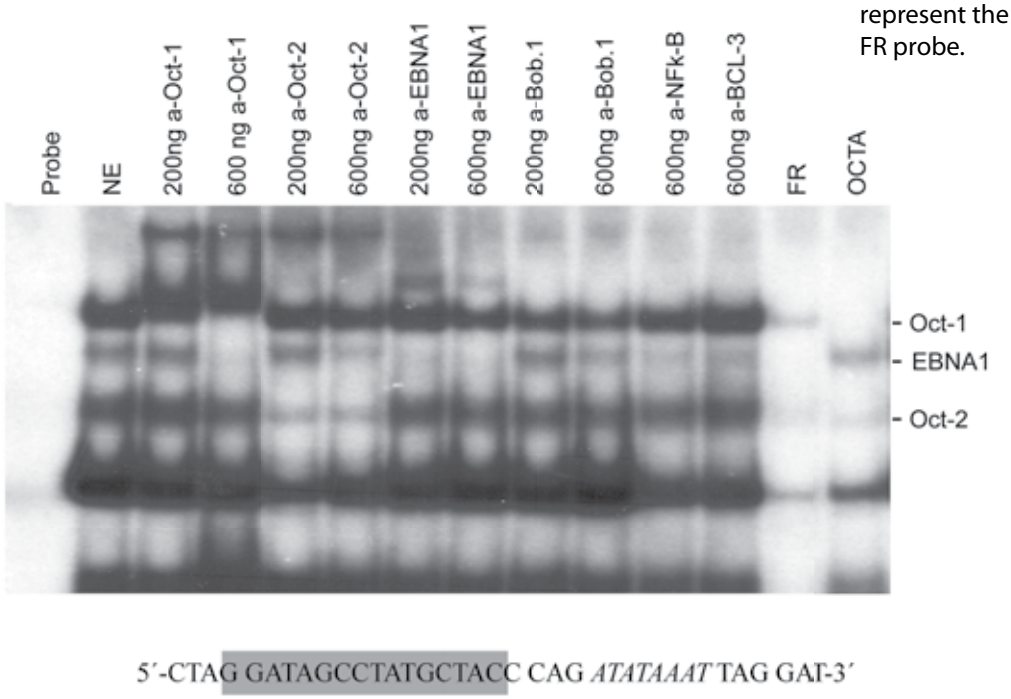


Fig 28. EMSA on NE from Rael. Supershifting with specific ab as indicated in figure. Sequence represent the FR probe.

In paper I we describe how we in the FR sequence found motifs resembling the Oct-binding site (ATGCAAAT). Since Oct-2 has an almost exclusive B-cell specific expression pattern we thought it was a god candidate for proteins binding to FR and since it is a transcription factor it might also be able to affect the Cp activity. Another interesting fact is that both Oct-1 and Oct-2 are involved in regulating the lytic/latent switch in HSV belonging to the same virus family as EBV (104). The best Oct-sites in FR also coinside with the least hypersensitive repeats in the foot-printing analyses (144).

We were able to show that the octamer-like DNA sequences found in FR could indeed bind both Oct-1 and Oct-2 as shown with EMSA using one FR repeat as a probe (fig 28). The pattern in the EMSA gave several bands and some were identified to contain either Oct-1 or Oct-2, but there were also FR specific complexes that did not contain Oct-proteins or that might include other proteins in addition to Oct-proteins. Further studies of the Oct-binding site in the FR sequence revealed that EBNA1 and Oct-proteins bound to different parts of the repeat (fig 27). The putative Oct-binding site was further evaluated using base-pair mutations (fig 29). Mutations destroying the entire octamer sequence abolished all binding of Oct-proteins (FRm5) while mutating the site into a perfect octamer enhanced binding (FRm1). Also worth noting is that when the Oct-binding site was destroyed the EBNA1 complex became more intense, and the opposite when the EBNA1 binding site was destroyed (FRm8) the Oct-complexes were intensified.

To further explore the binding of Oct-proteins to FR we applied our newly developed affinity-based method for identifying FR-binding proteins *in vitro*. The full-length FR was coupled to magnetic beads that were used to extract FR-binding proteins from

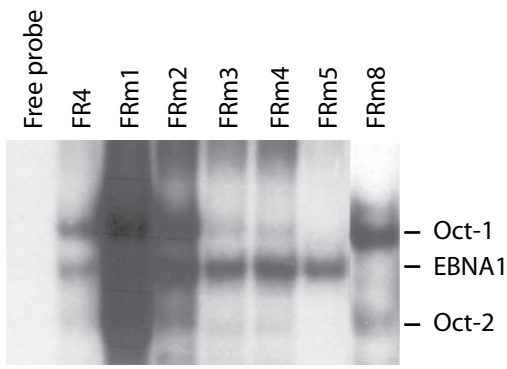


Fig 29. EMSA on NE from CBMI-Ral-STO with different mutatied FR-probes as indicated in figure. (for sequence info see paper IV)

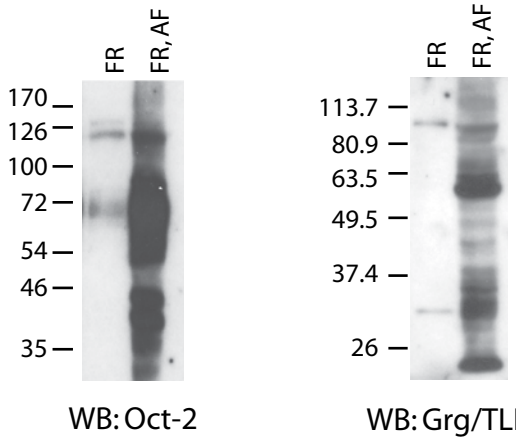


Fig 30. Extraction of FR-bound proteins. Lanes correspond to proteins bound to FR or proteins left in the NE after extraction (AF).

NE of Rael and CBMI cell lines. Proteins that bound to FR were eluated, separated on PAGE and blotted with specific ab. EBNA1 was found in the eluate from both Rael and CBMI and so was Oct-2 (fig 30). Oct binding to FR was also shown *in vivo* by chromatin immunoprecipitation (ChIP) on NE from both cell lines (fig 31). The finding that Oct protein bound to FR both in fishing (*in vitro*) and in the ChIP assay (*in vivo*) strengthen the finding that Oct-2 is in fact binding to FR *in vivo* both in latency I as well as in latency III cells.

With the affinity based method and the ChIP assay we could also detect the novel corepressor for Oct-proteins, Grg/TLE, binding to FR in NE from CBMI (fig 30 and 31).

To evaluate the effect on transcription of Oct-proteins binding to FR we constructed a FR-luciferase reporter vector carrying a heterologous promoter. This vector (pT81luc-FR) was transiently cotransfected together with Oct-proteins, EBNA1 and Bob-1. Binding of Oct-proteins had a clear impact on promoter activity. Oct-2 activate transcription on its own, while Oct-1 only could do so

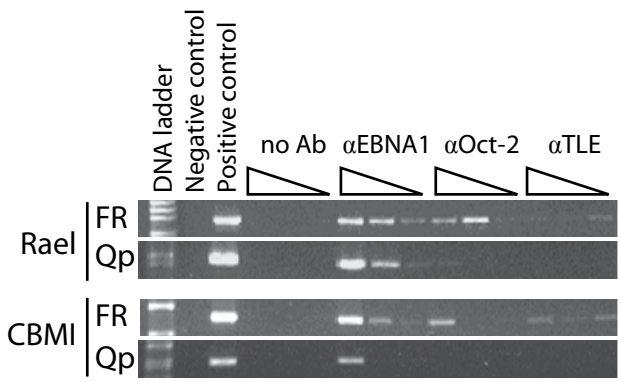


Fig 31. ChIP assay of proteins bound to FR. Triangles indicate decreasing amount of immuno-precipitated DNA.

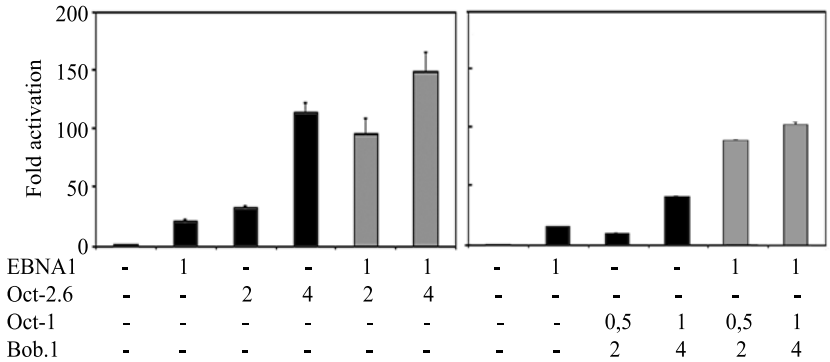


Fig 32. luciferase activity obtained in 293A cells transfected with a FR-lucifease vector and expression plasmids as indicated in the figure.

together with is coactivator Bob.1 (fig 32). Oct-2/Oct-1+Bob.1 cotransfected with EBNA1 gave a strong additive effect on transcription indicating that both proteins can bind at the same time to FR and still functionally affect transcription. To evaluate the effect of Oct-proteins on the natural EBV C promoter we used another Luciferase vector containing FR and the Cp (p(oriPI/-170Cp). Resuts showed that Oct-2 could activate the promoter on its own (fig 33). Experiments with constructs carrying the natural Cp were much more problematic than those containing the heterologous promoter.

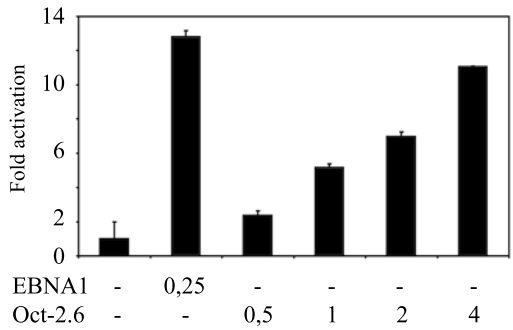


Fig 33. luciferase activity obtained in 293A cells transfected with a FR-Cp-lucifease vector and EBNA1 and Oct-2 as indicated in the figure.

When we earlier showed that Grg/TLE can repress Oct-2 induced activity with varying effect depending on what DNA sequence Oct bound. We brought Grg/TLE into our FR-experiments to evaluate its effect on FR-bound Oct activity. All different forms of Grg/TLE had a repressive effect on FR-bound Oct-2 induced transcription but its effect on Oct-1 + Bob.1 was unexpected (fig 35A and C). Grg/TLE-2 and -3 had no effect on Oct-1+Bob.1 activity, while Grg/TLE-1 and -4 strongly activated transcription. Activation was also seen when Grg/TLE-3 and -4 was cotransfected with EBNA1, while Grg/TLE-1 and -2 had no influence on promoter activity (fig

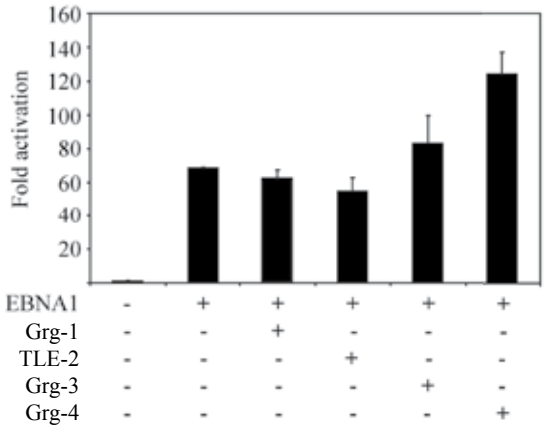


Fig 34. Effect of Grg/TLE proteins on EBNA1-induced transcription.

34), however EBNA1 activity was repressed when Grg/TLE-3 was cotransfected together with Oct-2.

Since Oct-2 alone is such a strong activator in the FR-luciferase system we were given the opportunity to look at the effect on full-length Oct-2 with all different Grg/TLE variants in absence of Bob.1. In paper II we had already shown that Bob.1 was not necessary for repression with Grg/TLE-1 and we could now show that this held true for all four full-length Grg/TLE proteins on FR (fig 35B). When we compared with the results from the different luciferase constructs in paper II the FR-results was not directly comparable with any of the tested Oct-binding sites. Repression of Oct-2 activity with all Grg/TLE variants was not seen on any of the constructs in paper II and the results with Oct-1 + Bob.1 was also very different with no repression and activation with Grg/TLE-1, -3 and -4.

Beside the full-length variants of Grg/TLE a short C-terminally truncated variant excists which is called Grg-5 in mice and

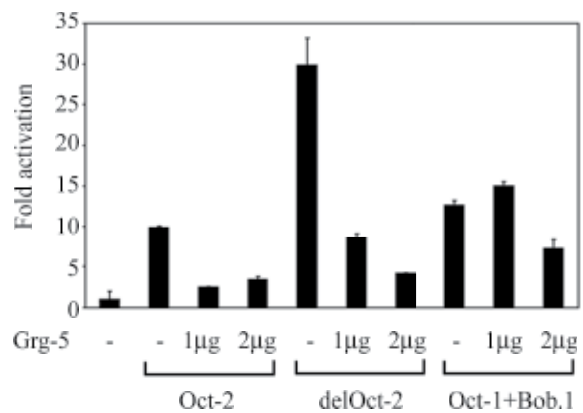


Fig 36. Effect of Grg-5 on Oct-induced transcription.

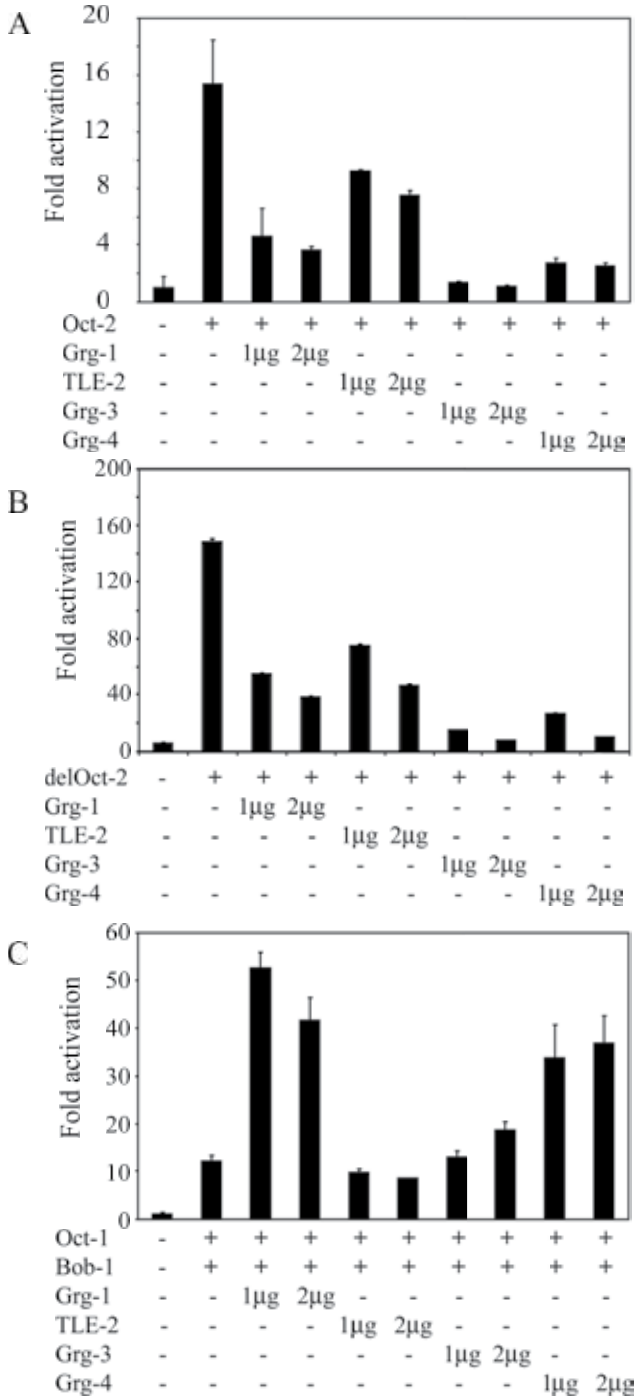


Fig 35.
Effect of Grg/ TLE proteins on Oct-induced transcription.
A) Oct-2
B) delOct-2
C) Oct-1.

Aminoterminal enhancer of split (AES) in humans. Grg-5 has both been described to function as a repressor on its own but also to function as a dominant negative repressor of full-length Grg/TLEs by sequestering them in a non-functional form. In our FR-luciferase system we showed that Grg/TLE could repress Oct-2 induced repression on its own (fig 36), and that it had no repressive effect on Oct-1+Bob.1 activation.

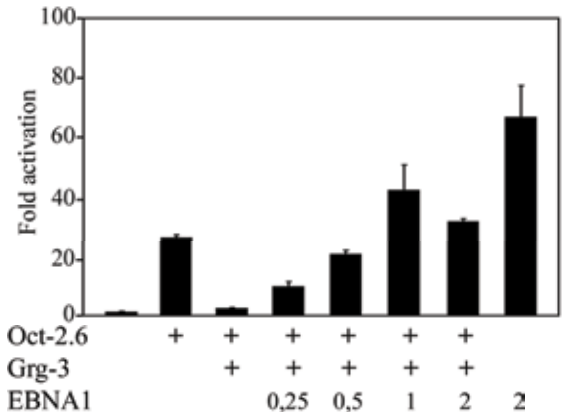


Fig 37. Effect of EBNA1 on Oct-2+Grg/TLE repressed transcription.

Since EBNA1 is activating transcription from the Cp through FR and the effect of Grg/TLE + Oct-2 is repressive we wanted to see if these two states were interchangeable. By introducing increasing amount of EBNA1 to a transfection with stable amounts of Oct-2 and Grg/TLE-3, EBNA1 could override the repression and activate transcription (fig 37). The opposite experiment was done with stable amount of EBNA1 and increasing amount of Grg/TLE-3 and Oct-2 (fig 38).

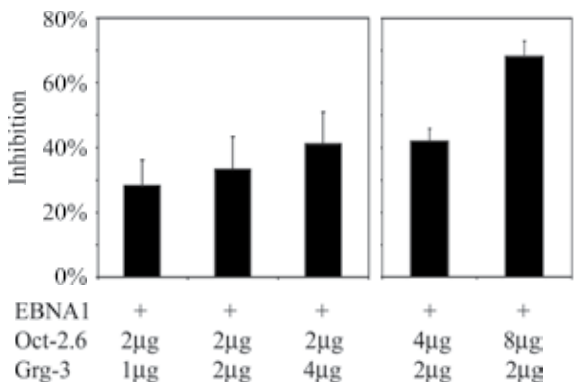


Fig 38. Effect of Oct-2+Grg/TLE on EBNA1 induced transcription.

Switch between latency forms

It is plausible that the transition from latency III to latency I occurs *in vivo* after primary infection. During primary infection there are no ab or CTLs directed against EBV and the infected cells can acquire latency III represented by the full growth program and expression of all latency genes. As a result cell proliferation is induced leading to an increased number of infected cells in the body. These cells are extremely immunogenic and within a few weeks the host has developed a strong immune response against EBV. To survive eradication EBV has to switch into a more restricted form of latency, latency I or latency 0.

To enter the pool of memory B-cells it is thought that the EBV infected cell has to go through a germinal center (GC) where CD40 signalling will rescue the B-cell from undergoing apoptosis (ref). In EBV transformed cells LMP1 is responsible for delivering survival signals to the cell by mimicking CD40 stimulation. The latency switch can be shown experimentally in vitro by CD40 ligation of LCLs (138). CD40 cross-linking leads to down regulation of EBNA2, 3, 4, 6 and LMP1 together with reduced Cp activity. In addition the GC-restricted surface marker CD77 was upregulated. To do a switch between latency forms experimentally *in vitro* is very hard and so far this has been the only successful attempt.

When grown in culture some BL cells tend to drift into an LCL like phenotype, with activation of the Cp and down regulation of the Qp. This results in expression of all latent EBV genes and the growth program is turned on (157, 158). This could hypothetically be explained by the removal of the suppressing force the immune system had on the EBV infection in the body, which is missing when the cells are grown *in vitro*. But this does not explain why some BL keep the latency I phenotype *in vitro*. In cells with a latency I gene expression pattern the Cp is methylated while it is unmethylated in latency III. Activation of Cp and expression of the EBNAs and LMPs can be mimicked by treatment of a BL cell with the demethylating agent 5-Azacytidine (41, 114).

The latency switch model (paper III)

In western blot experiments we could see that the level of EBNA1, Oct-2 and Bob.1 varied drastically between latency I and latency III cells (fig 39) and that this also reflected on the intensity of the different DNA-protein complexes in the EMSAs. The concentration of Oct-2 and Bob.1 is much lower in latency III than in latency I cell lines. The opposite is true for EBNA1 whose level is higher in latency III cells than in latency I. We also showed that EBNA1 induced promoter activity could be repressed by Oct-2 + Grg/TLE-3, and that repression by Oct-2 and Grg/TLE-3 could be released by cotransfection of EBNA1 (fig 37 and 38). We expect that EBNA1 would bind with a higher affinity to FR than Oct. But the finding of Oct protein bound to FR both in fishing (*in vitro*) and in the ChIP assay (*in vivo*) suggests that Oct-2 can in fact binding to FR both in latency I as in latency III cells. We also showed that the best Oct-binding sites were followed by the worst EBNA1 motifs in FR. This might allow Oct binding in spite of presence of EBNA1 protein. Based on these data and the finding that Grg/TLE can repress Oct-2 we postulated a model of how latency switching could occur (fig 40):

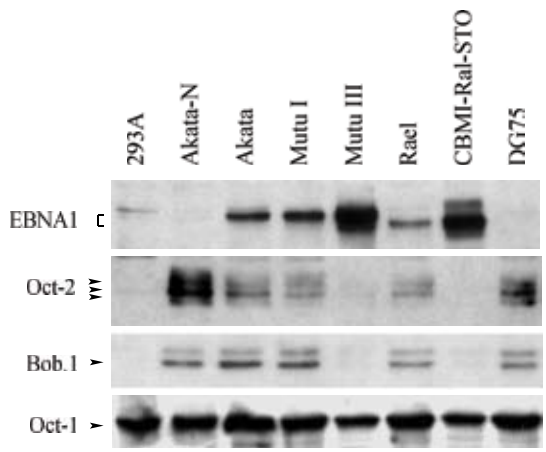


Fig 39. Protein levels in different cell lines as shown by Western blott.

In a latency III cell the level of EBNA1 molecules is higher then the Oct-2 level, which make the probability of Oct-2 binding to FR less than the proteins that EBNA1 would bind to it. Hence no recruitment of Groucho/TLE would occur and the Cp would be active. In a latency I cell the EBNA1 level is much lower, this would allow Oct-2 to bind FR, attract Groucho/TLE and turn the

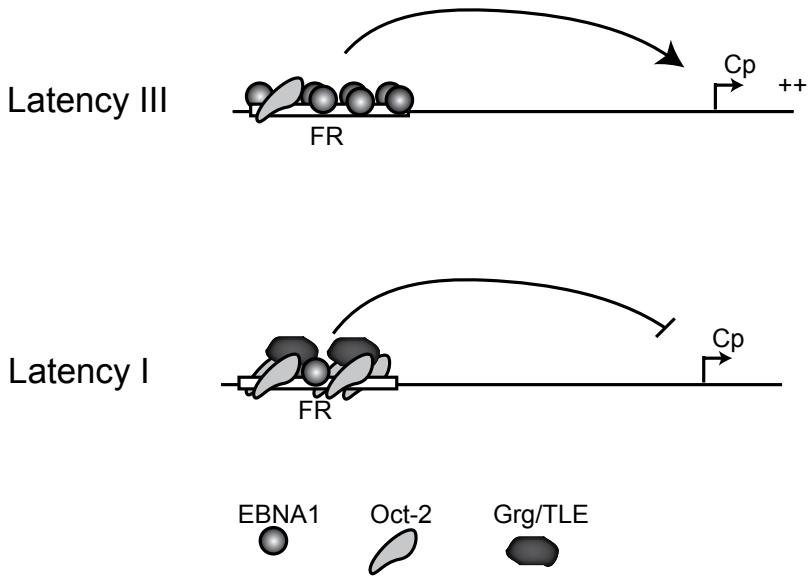


Fig 40.
A suggested model of how EBNA1, Oct-2 and Grg/TLE might regulate the Cp via FR.

promoter off. This hypothesis has many drawbacks; one is that the level of EBNA1 and Oct-2 has to shift before the switching occurs. Information about regulation of the Oct-2 gene is very scarce and the only way of lowering the EBNA1 level that is known today is to turn the Cp off. Another problem with the model is that there still is a chance that the Cp-Qp switch never occurs in the real lifecycle of EBV because the Qp is directly turned on via Wp or directly at primary infection. Further work is definitely needed to verify or reject this hypothesis, and due to the problematics of imitating the switch in vitro the results might even show that Oct and Grg/TLE have a completely different role in the EBV lifecycle than we think today.

METHODS

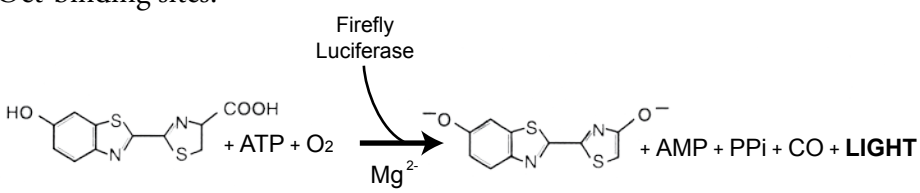
Electro mobility shift assay

An electro mobility shift assay (EMSA) was used in these studies to explore if the EBNA1, Oct-1, Oct-2 and Grg/TLE proteins could physically bind to the promoter regulatory region FR in EBV.

In an EMSA a probe consisting of short (20-30 bp) ds DNA labelled with radioactively marked nucleotides is incubated with cellular nuclear extracts. Proteins bound to the probe cause a shift in the DNA-protein complex as compared to the unbound free probe when analysed by electrophoresis in a non-denaturing polyacrylamide gel. The complexes was then analysed either by super shifting with protein specific antibodies or by competition with unlabeled DNA. Antibodies bound to the protein-DNA complex cause a shift in size or completely abolish the protein-DNA interaction making the complex disappear. Competition is conducted with a large excess of unlabelled DNA and aims at specifically compete out protein binding to the probe.

Luciferase assay

To evaluate if the Oct and Groucho/TLE proteins had any effect on transcription we constructed different luciferase reporter vectors that contained the putative Oct-binding sites and measured their effect on transcription of the luciferase gene. pT81luc-FR contained the FR element from EBV and a heterologous thymidin kinase promoter, while p(oriPI/-170Cp)luc contained FR and the natural Cp. In paper II we used numerous luciferase vectors containing different Oct-binding sites.



The luciferase assay is a fast and sensitive reporter system that can be used for studies of gene regulation and coupled events. In the assay produced Firefly Luciferase is measured by adding Beetle luciferin. Light is produced in the reaction where luciferase converts luciferin into oxyluciferin that can be quantified in a luminometer.

Cell lines

Cell lines used in these studies were chosen based on their phenotype and transfectability. We also wanted to control presence and expression of proteins involved in the experiments as much as possible. The human kidney epithelial cell line 293A was chosen for the transient transfection experiments because it is an easily transfectable model cell line that does not contain any Oct-2 or Bob.1. Since Oct-1 is ubiquitously expressed 293A cells do however contain Oct-1. DG75 was used because it is a well-characterized and easily transfectable B-lymphoid cell line. It is of Burkitt's lymphoma origin and resembles a latency I cell even though it is EBV negative. This cell contains all proteins used in these studies except EBNA1 and thereby offers an environment resembling the natural surroundings of the EBV C-promoter region.

Transfection

Transfection is to introduce foreign DNA into a cell by means of using chemicals or electricity. For transfection of monolayer cells attached to a surface, e.g. 293A we used a lipofectine-based product (FuGENE 6, Roche). When mixed with DNA the DNA gets trapped in small lipid-spheres which, when mixed with the cells, fuses with the cellular membrane bringing the DNA inside the cell. When transfecting "floating" cells that grow in suspension the lipofectin method does not work to satisfaction because the lipid spheres cannot fall down on the cells for its action to take place, they rather falls down to the bottom of the flask. For these kinds of cells (e.g. the BL cell line DG75) we used electroporation. DNA is mixed directly with the cells and a small current is send through the medium this is thought either to form pores through which the DNA is taken up, or it stresses the cells leading to uptake of the DNA. Electroporation is much less efficient then lipofectin with the result that you need more DNA for electroporation.

The efficiency of DNA transfection is dependent on several factors, the condition of the cells, the pureness of the DNA, the amount of DNA. This makes transfection a sensitive method to work with and it is necessary to have good controls. For valuation of the transfection efficiency we cotransfected the cells with the same amount of an expression vector containing the β-galactosidase gene.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) is a technique for looking at *in vivo* interaction between known protein and DNA. Cells are treated with formaldehyde that covalently crosslink DNA bound proteins to the DNA. The DNA-protein complex is extracted from the cells and the DNA is fragmented by sonication until an average length of 600 bp. The complexes are incubated with specific antibodies to the proteins in question and then pelleted by beads coated with Protein A. The antibody-bound complex is washed and the protein-DNA crosslinking is reversed, releasing the DNA, which is analysed by PCR of the analysed DNA fragment. This method only applies to the cases where the protein and DNA is known but there still is a question about the DNA-protein interaction.

Making ChIP with FR is tricky since the FR fragment is a repeated sequence on which it is very hard to perform a specific PCR. This problem was solved by using a non-repetitive sequence just outside of FR for the PCR and was controlled by EBNA1 as described in other studies (36).

Affinity-based extraction of FR-binding proteins

This is a novel affinity based method of finding proteins binding to FR. The FR-sequence functions as a DNA-bait that is attached to magnetic beads. The bait is incubated with NE from the cells of interest. DNA (FR) binding proteins is then eluated, separated on PAGE and blotted with ab specific for the protein in question.

CONCLUDING REMARKS & FUTURE PERSPECTIVE

We have used various methods to show that Oct-1 and Oct-2 can bind to the FR sequence *in vivo* and *in vitro* and that the binding varies between the different repeats. We were also able to show that binding have an impact on promoter activity. Oct-2 or Oct-1 + Bob.1 can substitute the effect of EBNA1 on FR or further enhance the effect of EBNA1.

We also describe the finding of a corepressor for Oct-proteins, namely Grg/TLE. How Oct-proteins repressed transcription was earlier a mystery and this novel finding might explain some of it. The repression was shown to be highly dependent on the sequence to which Oct binds. This sequence specificity was however not a new finding because Oct recruitment of Bob.1 is also dependent on

the sequence to which Oct bind. We were not able to demonstrate how repression is achieved, but we excluded that it is due to HDAC recruitment or removal of Oct from the DNA. There are still many questions to solve in this complex story and one of the most interesting will be to see whether any of the Grg/TLEs are responsible for the repressing effects assigned to Oct-1 and Oct-2.

In paper III the findings about Oct and Grg/TLE were applied to the Cp in EBV where we show that all full-length Grg/TLE proteins as well as the truncated version Grg-5 can repress FR dependent Oct-2 activity. To our surprise we could also demonstrate binding of Grg/TLE to FR both *in vivo* and *in vitro*. Repression by Grg/TLE could be cancelled by EBNA1, as well as the EBNA1 induced activity could be repressed by Oct-2 + Grg/TLE. These experiments were all done on a heterologous promoter and to make certain that the effect is not only an artefact we are planning to study these effects on a Luciferase constructs containing the natural Cp.

Our findings could be of importance for the understanding of the natural EBV lifecycle. After primary infection, infected cells are thought to acquire latency III with an active Cp, but the cells that are found circulating in the blood in healthy individuals is rather latency I/0 with a silenced Cp. Still nobody knows how the Cp is turned off when the cell turns from latency III to latency I. When the shift is already done methylation keeps the promoter in check, but methylation in itself does not cause a shift in promoter activity. Our findings about Grg/TLE and Oct-2 might give some insight into how this switch in promoter usage might occur, but further investigations are still needed.

The understanding of how the Cp is regulated might in the future also be used to manipulate latency I EBV positive cancer cells so that the Cp is turned on. This cell would then be more potent in evoking an immune response and hopefully be killed.

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