The role of Epstein-Barr virus nuclear antigen 3C in the immortalisation process of human primary B-lymphocytes

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Madelaine Löfqvist

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First examiner: **Prof. Dr. D. Eick**

Second examiner: **Prof. Dr. J. Parsch**

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1. Introduction

1.1 Herpesviruses

The architecture of the virion is the criteria by which the members of the Herpesviridae family are classified. All herpesviruses consist of a core containing a large double stranded DNA, an icosadeltahedral capsid, tegument, and an envelope containing viral glycoproteins on the surface. So far, nine herpesviruses have been isolated from humans (HSV1, HSV2, HCMV, VZV, EBV, HHV 6A, HHV 6B, HHV 7 and HHV 8) (Kieff and Rickinson, 2001). Four biological properties are shared by the herpesviruses. (i) They all express a number of proteins involved in nucleic acid metabolism and processing of proteins (although the number of these enzymes may vary from one herpesvirus to another). (ii) The synthesis of viral DNAs and the assembly of the capsid take place in the nucleus. It is still unclear, however whether herpesvirions undergo internal cellular maturation or how they obtain their membraneous envelope (Enquist et al., 1998). (iii) All herpesviruses are able to remain in their natural host in an inactive state called latency. (iv) Production of infectious progeny virus is accompanied by the destruction of the infected cell.

Based on biological properties the herpesvirus family is been divided into three subfamilies, the alpha-, beta- and gamma-herpesviruses. Members of the alpha-herpesvirus subfamily are classified based on their variable host range, their relative short reproduction cycle, efficient spread to cells, and their capacity to establish latent infection primarily in sensory ganglia. Family members are human herpes simplex virus 1 and 2 (HSV 1, HSV 2), as well as Varicella Zoster virus (VZV). Classification of the beta-herpesvirus subfamily is based on their restricted host range, long reproduction cycle, and slow infection in culture. These viruses can establish latency in secretory glands, lymphoreticular cells, kidneys, and other tissue. Members of the beta-herpesvirus family are human cytomegalovirus (HCMV), murine cytomegalovirus (MCMV), and human herpes virus 6 and 7 (HHV 6, HHV 7). The gamma-herpesvirus are usually specific for either T or B-lymphocytes. All members replicate *in vitro* in lymphoblastoid cells, although some can cause lytic infection in epithelia cells and fibroblasts. Family members are Kaposi's sarcoma virus (KSV),

which was recently discovered and Epstein-Barr virus (EBV), which was identified approximately 40 years ago.

1.1.1 The Epstein-Barr virus (EBV)

The acute stage of EBV infection normally takes place in childhood without major clinical symptoms. The primary infection is characterised by lytic DNA replication, expression of almost all viral genes, virus production, and lysis of the infected cell. The host range for efficient EBV infection *in vitro* is restricted to primary B-lymphocytes (Henle et al., 1967; Pope et al., 1968). After the first acute phase of infection latency is established and several copies of the 172 kbp EBV genome is maintained in B-cells as an episome. EBV can also establish latent infection in other cell types, including T- or natural killer (NK) cells, although the efficiency is low. Latently infected primary B-lymphocytes become immortalized and yield growth-transformed lymphoblastoid cell lines (LCLs) (Henderson et al., 1977; Sugden and Mark, 1977). In this latent state, termed latency III, eleven of the approximately 90 genes of EBV are found to be expressed and induce an immune response in the host. B-cells, which present viral antigens, are eliminated and an EBV specific immunological memory develops. In some B-cells, probably memory B-cells, EBV substantially reduces the viral gene expression pattern to that of latency I or II. In latency I, only EBNA 1 is expressed, in contrast to latency II in which EBNA 1, LMP 1, LMP 2A as well as LMP 2B are expressed. As a consequence of switching to the latency state, the cell changes it´s phenotype indicated by altered surface marker expression and an arrest of proliferation. In latency I or II, EBV residing in B-cells evades the immune system and allows a persistent infection of the host. The frequency of EBV infected B-cells in the peripheral blood is 1 to 30 cells per $5x10^6$ B-cells. After receiving an appropriate stimulus virus can be reactivated from latency (Rowe, 1999). The cells containing reactivated virus are normally eliminated by the immune system. In immunocompromised persons, for example patients suffering from AIDS or undergoing organ transplantation, EBV can be responsible for the development of B-cell tumours.

1.1.2 Malignancies associated with EBV

Several malignancies are associated with EBV. Among these are Hodgkin's disease, Burkitt´s lymphoma, and Nasopharyngeal carcinoma. Despite the fact that all EBV positive individuals possess latently infected B-cells in their blood, they rarely develop tumours since the immune system prevents uncontrolled proliferation of infected B-cells in immunological healthy persons. Several factors contribute to the pathogenesis of monoclonal Burkitt´s lymphoma. One is malaria infection, which suppresses a T-cell response and stimulates B-cell proliferation. Other unknown factors can also contribute to chromosomal translocations of the c-*myc* gene into immunoglobulin encoded regions of different chromosomes, leading to constitutive active c-*myc* expression.

Nasopharyngeal carcinomas are nose and throat epithelial cell tumours, which are especially frequent in China, indicating that genetic or environmental components may play a role. Hodgkin's lymphoma is the most common malignant lymphoma in the Western world. It is characterised by an altered lymph node structure and the presence of mononuclear Hodgkin- and Reed-Sternberg cells. Reed-Sternberg cells are transformed B-cells that are less differentiated but very malignant. Typically more than 98% of the tumour mass in Hodgkin´s lymphoma consists of non malignant tumour-invading T-cells. The lymphomas are most frequently seen in immunocompromised persons and the expression pattern of viral genes among different tumours varies.

Lymphoblastic B-cell lymphomas are characterized by the expression of eleven viral genes, which is typical for latency III. Nasopharyngeal carcinoma, Hodgkin- and T-cell lymphomas all express EBNA 1 (EBV nuclear antigen 1), LMP 1, LMP 2A and B, which characterises latency II. In Burkitt´s lymphoma only EBNA 1 and two small non-coding RNAs are expressed, indicating latency I.

1.1.3 EBV genetics using BACs

In order to investigate the function of viral genes, virus mutants that possess a mutation of a specific gene of interest are of high value. The change in the phenotype of a virus mutant compared to wild-type virus gives hints for the function of the mutated gene. Two mutagenesis principles can be distinguished: (i) *reverse genetics*

in which a mutation is directed to the gene of interest and the resulting phenotype is investigated, and (ii) *forward genetics* in which the mutation is undirected and the responsible gene is only determined when the mutant shows an interesting phenotype (Wagner et al., 2002). Forward genetics has been performed using chemical mutagenesis, but the method is inefficient and it is time consuming to localize the mutated gene responsible for a distinct phenotype. Therefore, this approach is not very often used and reverse genetics is the method of choise. More than 10 years ago it was shown that reverse genetics can be performed with EBV using cosmid vectors in eukaryotic cells (Tomkinson and Kieff, 1992). A major disadvantage of this method is that eight recombination events between the cosmids must occur in the cell to reconstitute recombinant virus, with the recombination frequency being much lower in eukaryotic cells compared to e.g. yeast or bacteria. Another problem is unwanted recombination events within the virus genome during reconstitution (Spate et al., 1996). Finally, the establishment of revertants is very time consuming with the cosmid approach and therefore almost impossible/unfeasible.

Recently, genetic analysis of herpesviruses like EBV has been revolutionized by using bacterial artificial chromosomes (BACs). BACs are single copy F-factor-plasmid based vectors with a cloning capacity of more than 300 kb in size (Kim et al., 1992; Shizuya et al., 1992). The strict control of the F-factor replicon maintains a single copy of the BAC per bacteria cell, reducing the risk of recombination via homologous DNA stretches present in multiple copies of the viral DNA insert. The advantage of this method is that a gene can be studied in the context of the whole genome. The first viral genome to be cloned into a BAC was the mouse cytomegalovirus genome (Messerle et al., 1997). The EBV strain B95.8 was used for cloning the whole EBV genome into a F-factor plasmid for genetic manipulation in E.*coli* (Delecluse et al., 1998). For selection in prokaryotic as well as eukaryotic cells the chloramphenicol-acetyl transferase and hygromycin phospho-transferase genes were inserted. In addition, this EBV-BAC plasmid (p2089) (Fig.1.1), also called Maxi-EBV, additionally carries the green fluorescence protein (GFP) gene under the control of the CMV promoter, which makes identification of successfully transfected and infected cells possible. Infectious virus particles can be obtained from HEK293 cells by stable transfection with p2089 and induction of the lytic cycle. These p2089-derived virus particles infect and transform human primary B-lymphocytes and hence possess all the properties of wild-type EBV (Delecluse et al., 1998). The advantages of this

method is that any gene of interest can be mutated via homologous recombination in E.*coli* and the manipulated genome can be tested for successful mutagenesis before virus reconstitution by transfection of eukaryotic cells with the mutated Maxi-EBV. This method is a fast one step procedure that also allows establishment of revertants in a reasonably short time period.

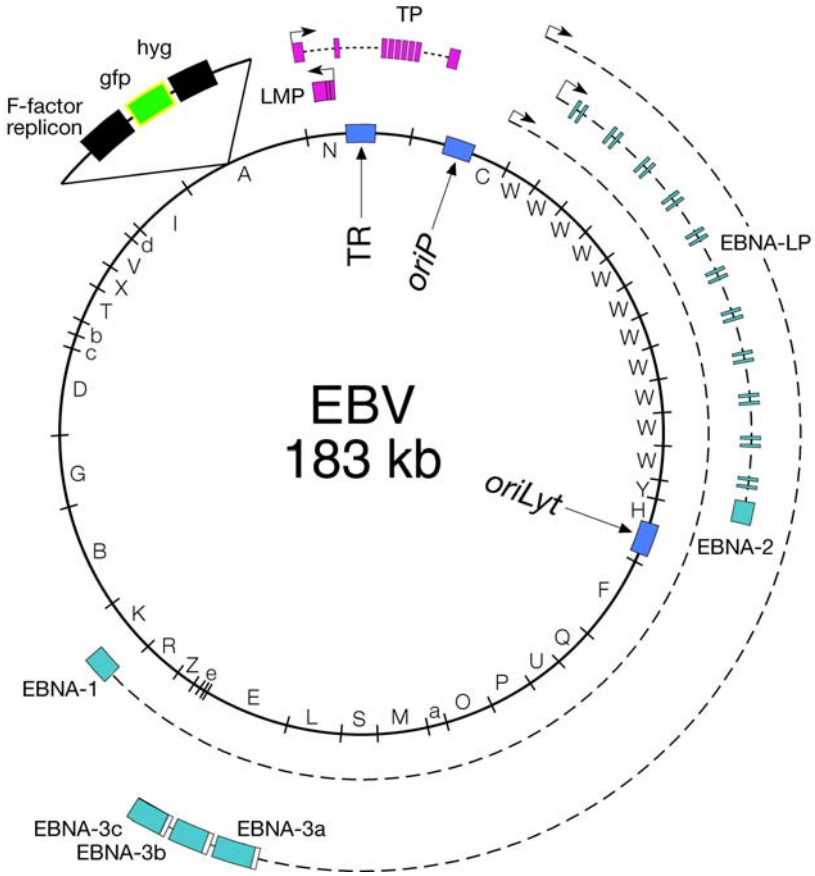


Fig 1.1 The Maxi-EBV p2089

The whole wild-type EBV genome was cloned into a F-factor plasmid, termed Maxi-EBV plasmid. The resulting plasmid is called p2089, which also carries the green fluorescence protein (GFP) and the hygromycin resistance gene (hyg) for selection in cell culture.

1.2 In vitro infection of primary human B-lymphocytes as a model system for EBV induced B-cell immortalisation

1.2.1 Formation of lymphoblastoid cell lines

For the development of therapeutics a better understanding of the molecular mechanisms by which EBV infects and immortalises B-lymphocytes is important. Knowledge of the proteins involved and their mechanism of action could be used for the development of specific inhibitors or vaccines against EBV.

In vitro, latent infection of B-lymphocytes with EBV is a commonly used model system for investigating the latent EBV infection as well as the pathogenic mechanisms by which tumours develop. *In vitro* EBV infection of primary B-lymphocytes results in type III latency, growth transformation of the infected cells and generation of immortalised lymphoblastoid cell lines (LCLs). Individually, EBV-infected primary B-cells undergo a dramatic change in morphology and proliferative behaviour (Rowe, 1999). The activated cells become large and irregular in shape and strongly adhere to each other to form large clumps (Bornkamm and Hammerschmidt, 2001). This phenotypic change is associated with the entry of the cells into the cell cycle and initiation of continuous proliferation. Proliferating cells have a doubling time of 20-30 h. The telomere length decreases continuously with each cell division until a critical length is reached. After approximately 100 divisions the cells undergo a crisis and the majority die. A few immortalised cells, which have up-regulated their telomerase activity and thereby stabilised their telomeres at a short length grow out (Counter et al., 1994; Kataoka et al., 1997). The presence of the virus in the latently infected cells can be detected using antibodies against any of the nine different virus proteins expressed in the LCLs. The nine viral proteins include six nuclear proteins and three integral membrane proteins. Two small non-polyadenylated RNAs (EBERs) are also expressed. Virus replication in LCLs is usually minimal or undetectable.

1.2.2 Viral proteins involved in B-cell immortalisation

Not all viral genes expressed in immortalized cells are required for the immortalisation process. The five proteins EBNA 1, 2, 3A, 3C, and LMP1 are required for B-cell immortalisation. EBNA 3B, as well as the two small RNAs, have been

shown to be dispensable (Tomkinson et al., 1993)(Swaminathan et al., 1991). Also EBNA LP (Hammerschmidt and Sugden, 1989) and LMP 2 (latent membrane protein 2) (Kim and Yates, 1993; Longnecker et al., 1993) are not absolutely required for immortalisation, but improve the out-growth of LCLs. EBNA 1 is necessary for maintenance of the viral genome as an episome in proliferating cells (Lee et al., 1999) (Humme et al., 2003). The genes that are thought to be essential for B-cell immortalisation are described in more detail below.

LMP 1 (latent membrane protein 1) is oncogenic *in vitro* (Kulwichit et al., 1998), and is considered essential for B-cell immortalisation (Kieff and Rickinson, 2001; Kilger et al., 1998). Nevertheless, a recent report has challenged this hypothesis (Dirmeier et al., 2003). Furthermore, LMP 1 is the only EBV protein that can mediate the characteristics of cell transformation in non lymphoid cell lines, for example fibroblasts (Baichwal and Sugden, 1988; Moorthy and Thorley-Lawson, 1993; Wang et al., 1985). LMP 1 induces a number of phenotypic and functional changes such as up-regulation of adhesion molecules like ICAM and LFA-3 (Khanna et al., 1993; Wang et al., 1990) and activation markers like CD21, CD23, CD40, and CD58 (Wang et al., 1985; Wang et al., 1990). LMP 1 also induces expression of anti-apoptotic genes such as Bcl-2, A20, and Bfl-1 (D'Souza et al., 2000; Henderson et al., 1991), cytokines like IL-6, IL-8, and IL-10 (Eliopoulos et al., 1999; Eliopoulos et al., 1997; Nakagomi et al., 1994) as well as the induction of the epidermal growth factor receptor EGFR (Miller et al., 1997).

EBNA 1 is a DNA binding nuclear protein that plays a central role in the maintenance of the latent state. It recruits the cellular origin of recognition complex (ORC) to the latent viral origin of replication oriP (Ritzi et al., 2003; Schepers et al., 2001). EBNA 1 binds to oriP, which possesses 20 EBNA 1 binding sites arranged in a tandem array (FR, family of repeats) and four sites arranged in dyad symmetry (DS element). Binding of EBNA 1 to the multiple binding sites is required and sufficient for episomal replication of the viral genome. Binding of EBNA 1 to the FR element is absolutely required for accurate separation of replicated episomes to the daughter cells (Aiyar et al., 1998). An array of glycine-alanine repeats, which was shown to prevent proteasomal degradation and peptide loading of MHC class I molecules, is located in the central N-terminal part of EBNA 1 (Levitskaya et al., 1995).

Like EBNA 1, EBNA 2 is a nuclear antigen, which also plays a major role in the immortalisation process. EBNA 2 is a transactivator of many cellular and viral genes, although it cannot bind directly to DNA. EBNA 2 induces the expression of the B-cell activation markers CD21 and CD23 (Calender et al., 1987; Wang et al., 1990), the cellular tyrosine kinase c-fgr (Knutson, 1990), and the viral genes LMP1, 2A and 2B and activates the viral C-promoter (Cp) (Abbot et al., 1990; Jin and Speck, 1992; Sung et al., 1991; Zimber-Strobl et al., 1991). The cellular protein mediating binding of EBNA 2 to its response element was identified as RBP-Jκ (Recombination signal-binding protein-Jκ) (Grossman et al., 1994; Waltzer et al., 1994; Zimber-Strobl et al., 1994). For the transactivation of LMP 1, EBNA 2 interacts with the transcription factor PU.1 (Johannsen et al., 1995; Laux et al., 1994). Recently it has been shown that *c-myc* is a direct target gene of EBNA 2 (Kaiser et al., 1999).

EBNA 3A, 3B and 3C are arranged in a tandem array in the viral genome. The role and significance of the EBNA 3C gene in the immortalisation process of human primary B-lymphocytes is the theme of this work, and therefore EBNA 3C domains and known functions will be described in more detail in the next part.

1.3 The Epstein-Barr nuclear antigen 3C (EBNA 3C) and its interaction with other proteins

The expression of EBNA 3A and EBNA 3C are essential for B-cell immortalisation but they cannot substitute for each other implying that their roles are different (Tomkinson et al., 1993). Despite the similarities within the EBNA 3 family, EBNA 3B is not essential for B-cell immortalisation.

Several EBNA 3C interacting proteins have been identified, including TBP, HDAC1, DP 103, prothymosin-alpha and Nm23-HI (Parker et al., 1996) (Grundhoff et al., 1999; Radkov et al., 1999; Subramanian et al., 2001). The biological significance of these interaction partners is yet not clear. In addition, EBNA 3C up-regulates the expression of CD21 (Kieff et al., 1990) and cooperates with EBNA 2 in the induction of the LMP 1 promoter through PU.1 (Zhao and Sample, 2000).

EBNA 3C is a 170 kDa large nuclear protein, containing a RBP-Jκ binding site in the N-terminal part of the protein (aa 181 to 257), a basic leucine zipper (bZip) (aa 239 to 285) and a domain (aa 181 to 365), that interacts with PU.1. The repression domain

stretches from aa 346 to 543 with a potential nuclear localisation signal (NLS) sequence in the middle. From aa 551 to 610 a poly-proline stretch is located, an activation domain stretches from aa 724 to 826 near the C-terminus, and aa 741 to 781 harbours a leucine-proline rich stretch and a second NLS sequence.

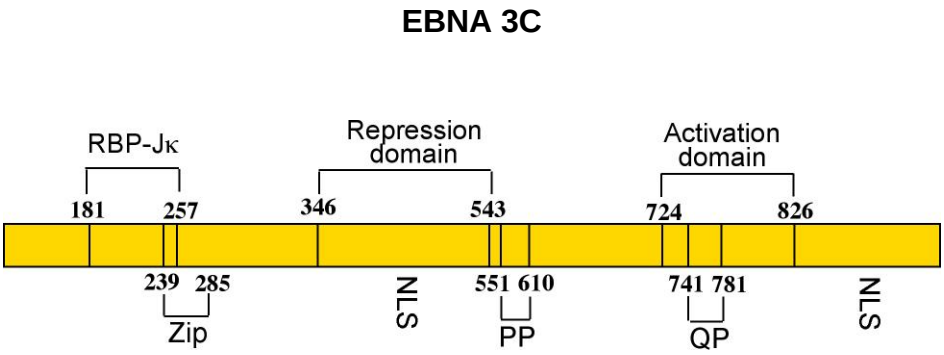


Fig. 1.2 Motifs and domains of the Epstein-Barr nuclear antigen 3C (EBNA 3C)

The EBNA 3C protein contains a RBP-Jκ binding site in the N-terminal part of the protein, between amino acid 181 and 257 and a basic leucine zipper between amino acid 239 to 285. Between amino acid 181 and 365 there are several PU.1 binding sites. A repressor domain has been located from amino acid 346 to 543, which also contains a nuclear localisation signal (NLS). In the C-terminal part between amino acid 551 and 610 a polyproline stretch is located, and an activation domain between amino acid 724 to 781. A leucine-arginine stretch is present between amino acid 741 to 781. A second NLS sequence is located near the C-terminus.

EBNA 3C inhibits transcriptional activation of EBNA 2 by preventing RBP-Jκ and EBNA 2-RBP-Jκ complexes from binding to their cognate RBP-Jκ-binding site (Bain et al., 1996; Johannsen et al., 1996; Radkov et al., 1997; Radkov et al., 1999; Waltzer et al., 1996; Zhao et al., 1996). EBNA 3C is believed to fine-tune and counterbalance the action of EBNA 2. This is achieved by binding of EBNA 2 to the PU.1-binding region. The EBNA 3C-EBNA 2 complex can activate the LMP 1 promoter (Zhao and Sample, 2000). The repression domain and the activation domain are both defined based on the effect on a Gal-4-responsive promoter when fused to the Gal4 DNA binding domain (Bain et al., 1996; Zhao et al., 1996). Moreover, wild-type EBNA 3C can specifically repress reporter plasmids containing the EBV Cp latency associated promoter. EBNA 3C also binds to a transcriptional repressor complex, thath includes the HDAC 1 enzyme, and targets this complex to the Cp promoter, which is mediated by the cellular DNA-binding protein RBP-Jκ (Radkov et al., 1997; Radkov et al., 1999). Since the Cp promoter also regulates

EBNA 3C mRNA transcription, EBNA 3C may contribute to a negative autoregulatory control loop of its own expression.

In addition to modulating transcription, EBNA 3C can substitute for the papillomavirus E7 and adenovirus E1A proteins in oncogenic transformation assays and enables activated (Ha-) Ras to transform primary rodent fibroblasts (REFs) (Parker et al., 1996). Similar to E7 and E1A, EBNA 3C can overcome the repressive effects of the cycline-dependent kinase inhibitor p16/INK4 in REF transformation assays (Parker et al., 1996). These reports indicate that EBNA 3C might override normal signals for growth arrest at the restriction point (R-point) in G1 of the cell cycle when Rb is primarily active. This finding was additionally confirmed by the finding that over-expressed EBNA 3C can induce cell cycle progression and suppresses the accumulation of the cyclin-dependent kinase inhibitor p27/KIP1, which is normally associated with exit from the cell cycle. Over-expression of EBNA 3C also leads to polyploidy and to cells with multiple nuclei, suggesting that it might influence additional cell cycle checkpoints (Parker et al., 2000). The PLDLS motif of EBNA perfectly matches the CtBP-binding site in E1A and it is essential and sufficient for the interaction of EBNA 3C with CtBP (Touitou et al., 2001). It has been reported that in some situations CtBP can recruit HDAC enzymes 1, 4, 5, 7, and Sin3A. The precise molecular mechanism by which CtBP inhibits transcription is unclear and may vary in different situations (Crook et al., 1998; Koipally and Georgopoulos, 2000; Meloni et al., 1999; Sundquist et al., 1998; Zhang et al., 2001). All the described motifs and potential interactions of EBNA 3C with other proteins reflect the complexity of this protein and the need to investigate the real importance of the various domains and motifs during EBV-induced growth-transformation of B-cells.

1.4 Aim of the project: Investigation of the importance of EBNA 3C in B-cell immortalisation in the context of an EBV infection

EBNA 3C is a viral gene essential for B-cell immortalisation (Tomkinson et al., 1993). Until now the genetic studies of the various domains in EBNA 3C have all been performed with ectopic transient expression systems. It is still unclear which of these domains or motifs play a major role in the immortalisation process during virus infection. Therefore, the goal of this work was to analyse the contribution of the different domains and motifs of EBNA 3C during the course of infection of primary resting B-cells. For this purpose, nine EBV-mutants with mutations in the EBNA 3C gene were established and the corresponding phenotypes were investigated by infection of human primary B-lymphocytes. The Maxi-EBV system (Delecluse et al., 1998) was used as the method of choice, which for the first time allowed EBNA 3C mutations to be analysed in the context of the whole EBV genome. In addition, to allow more rapid insertions of mutations into the Maxi-EBV, the red $\alpha\beta\gamma$ recombination system (Zhang et al., 1998) was adapted for EBV mutagenesis for the first time.

The second goal of this work was to establish a tight conditional system in which EBNA 3C could be switched off in LCLs in order to study the role of EBNA 3C during the initiation and/or maintenance of B-cell growth-transformation. The Cre/lox P system was chosen for this purpose. The first step was to flank the EBNA 3C gene with lox P sites within the whole EBV genome. The second step was to introduce the Cre protein into the EBV infected cells for excision of EBNA 3C and to confirm efficient excision. The last step was to sort the LCLs lacking EBNA 3C and to further cultivate them to study the importance of EBNA 3C during the initiation and/or maintenance of B-cell immortalisation.

2. Materials

2.1 Antibodies

Primary antibodies:

EBNA 3C, sheep	Novagene, polyclonal
LMP1, mouse	Dianova
EBNA 1, rat	E.Kremer, GSF
EBNA 2 (R3+1E6), rat	E.Kremer, GSF
gp350/220, mouse	ATCC
β-actin, Goat	Santa Cruz

Secondary antibodies:

Rabbit αsheep IgG, Horse-reddish-peroxidase conjugated	Promega
Rabbiț αmouse IgG, Horse-reddish-peroxidase conjugated	Promega
Rabbiț αrat IgG, Horse-reddish-peroxidase conjugated	Promega
Goạt αmouse IgG, Cy-5	Dianova
Donkey αgoat, Horse-reddish-peroxidase conjugated	Jakson Immuno research

2.2 Bacteria

E.*coli* DH5α	F-, *lac*l-, *rec*A1, *end*A1, *hsd*R17, Δ(*lac*ZYA-*arg*F), U169, F80d*lac*ZΔM15, *sup*E44, *thi*-1, *gyr*A96, *rel*A1 (Hanahan, 1985)
E.*coli* DH10B	F-, *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), Φ80d*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *end*A1, *ara*D139, Δ(*ara*, *leu*)7697, *gal*U, *gal*K, λ-, *rps*L, *nup*G (Life Technologies)

2.3 Plasmids

Commercially available plasmids

pUC19	NEB, USA
pCRII	Invitrogen, USA
pBluescriptII (SK-)	Stratagene, USA

From other groups, institutes or members of the lab

pCP15	(Cherepanov and Wackernagel, 1995)
p509	pCMV:BZLF1 (Hammerschmidt and Sugden, 1989)
p2670	pCMV:BALF4 (Neuhierl et al., 2002)
p2470.7	ER/EBNA 3C/ER in pUC19
p2701	Cre expresson vector
p2845	EBNA3C-ALDAS (Touitou et al., 2001)
Gag/pol	Expression vector (Beyer et al., 2001)
GALV	Expression vector (Beyer et al., 2001)
p2448.1	EBNA 3C knock-out (EBNA 3C replaced with tet)
p2956	pKan-oriR6K-2
p3054	Retroviral vector carrying IRES/mRFP
p3062	mRFP in pCRII

Plasmids constructed during this work

p2720

The *Hind*III/*Apo*I fragment corresponding from nt #2237 to #3718 (1482 bp) from pCP15 carrying the kanamycin resistance gene was treated with Klenow enzyme. The recipient vector p2719 was digested with *Bst*EII, treated with Klenow enzyme and then the two fragments were ligated together. This plasmid was used for creating all EBNA 3C deletion mutants, and it was used as a donor for creating the Maxi-EBV p2889 (see below).

p2721

The plasmid 2720 was digested with *Sna*BI/*Nsi*I and an oligonucleotide (primer 2721-F, 2721-R) was inserted in its place in between nt #6070 to #7116 to prevent a frame shift in the EBNA 3C gene. This plasmid was used for creating the Maxi-EBV p2837.

p2742

The plasmid 2720 was digested with *Apa*I/*Xho*I and an oligonucleotide (primer 2742-F, 2742-R) was inserted in its place in between nt #5558 to #7003 to prevent a frame shift in the EBNA 3C gene. This plasmid was used for creating the Maxi-EBV p2838.

p2743

The plasmid 2720 was digested with *Age*I/*Apa*I and an oligonucleotide (primer 2743-F, 2743-R) was inserted in its place to prevent a frame shift hereby nt #5322 to #6227 were deleted. This plasmid was used for creating the Maxi-EBV p2839.

p2744

Two digestions were made with the plasmid 2720. First it was digested with *Hind*III/*Eco*RV and the 5131 bp fragment, corresponding to nt # 10113 to # 4723, was isolated. The second digestion was performed with *Sna*BI/Hind *III* generating a 4046 bp corresponding to nt # 6067 to # 10113. The two fragments obtained were then ligated together, giving p2744, which was used for creating the Maxi-EBV p2840.

p2745

The plasmid p2720 was digested with *Age*I/*Age*I deleting nt #5318 to #5632 and an oligonucleotide (primer 2745-F, 2745-R) was inserted in its place to prevent a frame shift. This plasmid was used for creating the Maxi-EBV p2841.

p2864

The *Acc*I/*Xho*I fragment, from nt #1920 to #3619 of p2845 was ligated into the *Acc*I/*Xho*I digested vector 2720 resulting in an EBNA 3C gene with mutated PLDLS motif. This plasmid was used for creating the Maxi-EBV p2842.

p2957

The *Eco*RI/*Bam*HI fragment comprising the origin of replication (OriR6K) from p2956 and the *Afl*II/*Afl*II fragment from p2930 comprising the EBNA 3C gene, the kanamycin resistance gene and one lox P site, were both treated with Klenow enzyme and blunt ligated. This yielded p2957, which carries the OriR6K followed by the EBNA 3C gene, the kanamycin resistance gene, and one lox P site. This plasmid was used for creating the Maxi-EBV p2919.

p3067 retroviral control vector

The WPRE gene was amplified from the vector 3054 using primers with 5´-*Eco*RI and 3´-*Bgl*II overhangs. The mRFP ORF was cut out from the donor vector 3062 with *Nco*I/*Eco*RI. These both fragments were ligated in the Vector 3054 cut with *Nco*I/*Bam*HI.

p3087 Cre expressing retroviral vector

The Cre ORF was cut out from the plasmid 1891 with *Mlu*I/*Xho*I, this fragment was treated with T4 polymerase to obtain blunt ends. The vector 3067 was cut with *Not*I and treated with T4 DNA polymerase to obtain blunt ends and the two fragments were ligated to yield p3087.

Maxi-EBV plasmids generated during this work

All the mutants in the EBNA 3C gene were established according to conventional cloning techniques in the plasmid p2720 (see Fig. 4.2), that contains nucleotide coordinates from # 95240 to #102896 from B95.8 (Baer et al., 1984). The derivatives of p2720 that carry the different EBNA 3C mutant alleles were used as sub-constructs for the linear fragment transfer into the wild-type Maxi-EBV p2089. All the EBNA 3C mutants in the context of p2720 are under the control of the unmodified wild-type-EBNA 3C promoter. The following list describes the different cloning steps for construction of all Maxi-EBV mutants used in this study. The entire list of plasmids can be found in the database of the department Gene vectors.

p2837 (Δ aa 658-1013)

The linear fragment *Avr*II/*Nhe*I from p2721 was used for homologous recombination into p2089 resulting in p2837.

p2838 (Δ aa 489-970)

The linear fragment *Avr*II/*Nhe*I from p2742 was used for homologous recombination into p2089 resulting in p2838.

p2839 (Δ aa 410-710)

The linear fragment *Avr*II/*Nhe*I from p2743 was used for homologous recombination into p2089 resulting in p2839.

p2840 (Δ aa 212-658)

The linear fragment *Avr*II/*Nhe*I from p2744 was used for homologous recombination into p2089 resulting in p2840.

p2841 (Δ aa 408-510)

The linear fragment *Avr*II/*Nhe*I from p2745 was used for homologous recombination into p2089 resulting in p2841.

p2842 (PLDLS-ALDAS aa 728,731)

The linear fragment *Avr*II/*Nhe*I from p2864 was used for homologous recombination into p2089 resulting in p2842.

p2889 (reconstituted wt)

The linear fragment *Avr*II/*Nhe*I from p2720 was used for homologous recombination into the wild-type Maxi-EBV plasmid 2089 resulting in p2889.

p2686 (EBNA 3C knock-out)

The linear fragment *Avr*II/*Nhe*I from p2448.1 was used for homologous recombination into p2089 resulting in p2686.

p2919 (loxP-EBNA 3C-loxP)

The Maxi-EBV plasmid 2686 was modified using Cre/lox P site-specific recombination. The recombination was preformed in DH10B using the Cre expression vector p2701, upon its expression, the tet gene was excised and only one lox P site was left at the place of the EBNA 3C gene. The whole plasmid 2957 was

used for Cre/lox P site-directed reconstitution of the EBNA 3C wild-type gene into the already recombined 2686. The Cre protein was again expressed from the expression vector p2701. After site-directed recombination the plasmid 2957 had been integrated into the Maxi-EBV where the EBNA 3C gene is flanked by lox P sites. (The construction of p2919 is shown in Fig.4.12).

2.4 Cells and Cell lines

HEK293	human embryonal kidney epithelia cell line, transformed with the E1A and the E1B genes from Adenovirus type 5 (Graham et al., 1977)
WI38	human fibroblast cell-line (ATCC)
B95.8	lymphoblastoid marmoset B-cell line (Miller et al., 1972)
Raji	human EBV positive Burkitt´s lymphom cell line (Pulvertaft, 1964)
Phoenix	HEK293 cells, which has chromosomal integrated the GALV and the gag/pol genes.

HEK293-cell lines, stably transfected with Maxi-EBV plasmids

HEK/293-2089	p2089, wild type-EBNA 3C
HEK/293-2837	p2837, *Sna*BI/*Nsi*I deletion in EBNA 3C
HEK/293-2838	p2838, *Apa*I/*Xho*I deletion in EBNA 3C
HEK/293-2839	p2839, *Age*I/*Apa*I deletion in EBNA 3C
HEK/293-2840	p2840, *Eco*RV/*Sna*BI deletion in EBNA 3C
HEK/293-2841	p2841, *Age*I/*Age*I deletion in EBNA 3C
HEK/293-2842	p2842, ALDAS-PLDLS
HEK/293-2889	p2720, reconstituted EBNA 3C wild-type
HEK/293-2919	p2957, ori6K-loxP-EBNA 3C-loxP
HEK/293-2686	p2448.1 EBNA 3C knock-out

Lymphoblastoid cell lines established during this work

2089-LCL	LCL established with the Maxi-EBV plasmid 2089
2837-LCL	LCL established with the Maxi-EBV plasmid 2837
2838-LCL	LCL established with the Maxi-EBV plasmid 2838
2839-LCL	LCL established with the Maxi-EBV plasmid 2839
2840-LCL	LCL established with the Maxi-EBV plasmid 2840

2841-LCL	LCL established with the Maxi-EBV plasmid 2841
2842-LCL	LCL established with the Maxi-EBV plasmid 2842
2919-LCL	LCL established with the Maxi-EBV plasmid 2919
2889-LCL	LCL established with the Maxi-EBV plasmid 2889

2.5 Oligonucleotides

The oligonucleotides were synthesised by Metabion, Munich.

Primer pairs used for cloning deletion mutants and retroviral vectors

2721-F	5´-GTAAGACTGACTACGATGCA-3´
2721-R	5´-TCGTAGTCAGTCTTAC-3´
2742-F	5´-CTTCCGAACAATCATCAGT-3´
2742-R	5´-TCGAACTGATGATTGTTCGGAAGGGCC-3´
2743-F	5´-CCGGTAGACAGGGTTCGGACGTGGCC-3´
2743-R	5´-ACGTCCGAACCCTGTCTA-3´
2745-F	5´-CCGGTAGACAGGGTTCGGAA-3´
2745-R	5´-CCGGTTCCGAACCCTGTCTA-3´
*Nco*I-cre-F	5´-CCCCCCCCATGGCCTCCAATTTACTGACCG-3´
*Eco*RI-cre-R	5´-CCCCCCGAATTCTAATCGCCATCTTCCAGC-3´
*Eco*RI-WPRE-F	5´-GGGAATTCGAGCATCTTACCGCC-3´
*Bgl*II-WPRE-R	5´-GGGAGATCTCTTGGCATGCCAAGTTGA-3´

Primers used for confirmation of EBNA 3C excision

Rec A forward	5´-CGGAQGGAACTGCTAAACAGGAAAG-3´
Rec A reversed	5´-CCAAACCAAATGTAGAGGTCTGGC-3´
Rec B forward	5´-CAGTTTCTTCCCCTTCCTCTTCTG-3´
Rec B reversed	5´-ATGGGTGTTTCCAAGCCTGTGC-3´
wild-type forward	5´-CGGAGGAACTGCTAAACAGGAAAG-3´
wild-type reversed	5´-AAGCCAGGGATGTAACGCACTGAG-3´

Primers used for sequencing of the deletion mutants

EBNA 3C-A	5´-TCTTCAGGACATTCGACG-3´
EBNA 3C-B	5´-ATTACCCGTGGAATGCAC-3´
EBNA 3C-C	5´-TCAAGACGGCTTTCAACG-3´

2.6 Reagents

All reagents not listed here were provided by the company Merck, Darmstadt, in the form “pro analysis”.

Acrodisc syringe filter, 0,8μm	Pall Gelman Sciences, Germany
Acrylamide	Roth, Germany
Agar	Life Technologies, Germany
Agarose	Biozym, Germany
Alkaline Phosphatase	Roche, Germany
α[^{32}P]-dCTP (3000 Ci/mmol)	Amersham Pharmacia Biotech, Germany
Amido Black	Sigma, Germany
Ammoniumpersulphate (APS)	Bio-Rad, USA
Ampicillin	Roche, Germany
Bromphenolblue	Sigma, Germany
Chloramphenicol	Life Technologies, Germany
Dimethylsulfoxide	Merck, Germany
Dithiotheriol (DTT)	Sigma, Germany
DNA-purification kit (JetStar)	Genomed, Germany
DNA-1kb ladder	New England Biolabs, Germany
dNTPs	Roche, Germany
Dulbecco´s modified Eagle medium	Life Technologies, Germany
ECL Western blotting detection reagent-Kit	Roche, Germany
Ethanol	Merck-Eurolab GmbH, Germany
Ethylendiamintetraacetate-Na-salt (EDTA)	Roth, Germany
Films	Amersham Pharmacia Biotech, Germany
Fetal Bovine serum (FCS)	Gibco BRL, Germany

Glycerol	Merck-Eurolab GmbH, Germany
Glycine	Merck-Eurolab GmbH, Germany
HEPES	Gibco BRL, Germany
Hygromycin B	Calbiochem GmbH, Germany
Isopropanol	Merck-Eurolab GmbH, Germany
Klenow-polymerase	Roche diagnostics GmbH, Germany
L-Glutamine	Gibco BRL, Germany
Modified Eagle Medium	Gibco BRL, Germany
Monolaurat (Tween-20)	Sigma Chemie, Germany
N,N-Methylbisacrylamid	Roth, Germany
Nylonmembrane (Hydrobond^{TM}-N+-Nylonmembrane)	Amersham Pharmacia Biotech GmbH, Germany
Optimem	Gibco BRL, Germany
Penicillin	Gibco BRL, Germany
Phenol/Chloroform	Roth, Germany
Proteinase K	Roche, Germany
Poinceau Red	Roth, Germany
Potassiumacetate	Merck-Eurolab GmbH, Germany
Potassiumchloride	Merck-Eurolab GmbH, Germany
Restriction Enzymes	New England Biolabs, Germany
	MBI Fermentas, St. Leon-Rot
RNAse	Roche Diagnostics GmbH, Germany
RPMI 1640	Gibco BRL, Germany
SDS molecular weight marker (prestained)	Sigma Chemie, Germany
Sodium-di-hydrogensulphate	Merck Eurolab GmbH, Germany
Sodiumdodecylsulfate (SDS)	Merck-Eurolab GmbH, Germany
Tetracycline	Sigma Chemie, Germany
Triton X-100	Sigma Chemie, Germany
Trypsin	Life Technologies, Germany

3. Methods

Standard methods were performed according to the collection of protocols by Maniatis et al 1989. In the case of commercially available kits the specific instructions and protocol were used if not otherwise mentioned. For the composition of buffers etc. see the instructions for the corresponding kit.

3.1 Isolation and purification of nucleic acids

Plasmid DNA-isolation from bacteria for analytical purposes

For isolation of DNA for analytical purposes (*DNA-mini prep)* 5 ml LB medium was inoculated with a single bacterial colony over-night at either 37°C, 30°C or 42°C supplemented with appropriate antibiotics. The bacteria were spun down and the pellet was re-suspended in 200 µl lysis-buffer. The suspension was transferred to a 1.5 ml Eppendorf tube and boiled for 2 min at 95°C and then spun down at 14500 rpm. For screening of the preparations with restriction enzymes, master mix of enzymes and restriction enzyme buffers containing RNase A were prepared, such that 27 µl of the mix could be added to 3 µl of mini-prep supernatant to give 1 u of enzyme, 1x restriction enzyme buffer. Digests were incubated for 2h at the appropriate temperature before separating the DNA fragments on an agarose gel.

LB-media:	1% Trypton, 0.5% Yeast extract, 0.5% NaCl
LB-Agar-plates:	15g Bacto-Agar in 1 l LB-medium
TE buffer:	10 mM Tris/HCL, 1 mM EDTA, pH 8.0
Lysis-buffer:	50 mM Tris-HCl (pH 8.0), 62.5 mM EDTA, 0.4 % (v/v) Triton X-100, 2.5 M LiCl

Preparative plasmid DNA-isolation from bacteria with multicopy plasmids

Large quantities of plasmid DNA were purified using the Maxi-preparation kit from Jet Star. This procedure is based on the alkaline lysis method. The method is based on the fact that when lysing cells at high pH (12.0-12.6) high molecular linear chromosomal DNA is denaturated while low molecular weight supercoiled plasmid

DNA remains unaffected. The pH is neutralised at high salt concentrations hereby the chromosomal DNA precipitates and can therefore be separated. About 100 µl from a dense over-night culture was used to inoculate 400 ml LB medium with appropriate antibiotics and incubated over-night at either 37°C or 30°C in an orbital shaker. The DNA was extracted using the Jet Star kit as suggested by the manufacturer. The pellet was washed once with 70 % ethanol, air-dried and then re-suspended in 200 µl TE buffer.

Isolation of cellular DNA

Cellular DNA was prepared from about $1x10^7$ cells. The cells were spun down and washed once with PBS, re-suspended in 2.1 ml TE and lysed upon the addition of SDS (final concentration of 0.7%). The lysate was digested with proteinase K (50 µg/ml final concentration) for 2h at 50°C. After addition of 1 ml 5M NaCl the suspension was vigorously shaken and centrifuged for 30 min at 5000 rpm in a Sigma centrifuge, the supernatant was precipitated with the double volume of 100% ethanol, washed with 70% ethanol and re-suspended in a suitable volume TE buffer.

Concentration and measurement of DNA

Purification of DNA was achieved through extraction with phenol, followed by alcohol precipitation. The DNA was precipitated with two volumes of ethanol and a final concentration of 300 mM NaAc for 20 min on ice. The precipitate was centrifuged (14000 rpm, 20 min, 4°C), washed with 70 % ethanol shortly air-dried and then re-suspended in a suitable volume. The DNA concentration was determined with a photometer (Photometer, Eppendorf).

3.2 Cloning of DNA

Digestion using restriction enzymes

Restriction enzymes were used as suggested by the manufacturer. For the analysis of plasmid DNA, multiple digests were performed in a compatible buffer. For cloning purposes, DNA was precipitated between digests. After incubation the restriction enzyme was heat inactivated as suggested by the manufacturer.

Dephosphorylation of DNA 5´-ends

To prevent the re-ligation of linearised vector containing compatible ends, 5´dephosphorylation was performed. Per pmol linear DNA at least 1 unit CIP or SAP (CIP, calf alkaline phosphatase, or SAP, shrimp alkaline phosphatase) was added and incubated for 1 h at 37°C. Inactivation of the reaction was performed at 70°C for 20 min.

Fill-in of single-stranded 5´overhangs and removal of single-stranded 3´overhangs

To enable ligation of non-compatible ends, overhanging (sticky) ends were either filled in or removed with Klenow-DNA-polymerase or T4 DNA polymerase, respectively, to produce universally compatible (blunt) ends. Linearised DNA was treated with Klenow-DNA-polymerase or T4 DNA-polymerase for 1 h at 37°C, as recommended by the manufacturer. The reaction was stopped via inactivation for 20 min at 65°C or by separation on an agarose gel.

Isolation of DNA-fragments from agarose gels

After digesting DNA with restriction enzymes the resulting mixture of DNA fragments was loaded onto an appropriately concentrated agarose gel (0.6 to 1.8 %) and separated electrophoretically. The DNA was stained with ethidium bromide (at a concentration of 1 μg/ml) and made visible with UV-light. The desired band was cut out and the DNA was extracted using a Gel extraction Kit from Qiagen according to the manual of the manufacturer.

Ligation of DNA fragments

Vector fragment and insert fragment were mixed at a 1:10 ratio (in total not more than 100 ng in a volume of 10 μl). Ligase buffer and T4 ligase was added as recommended by the manufacturer, and incubated over-night at 14°C. The entire ligation reaction was used for transformation.

Design of double-stranded oligonucleotides and cloning of plasmids

In order to clone short double-stranded oligonucleotide sequences into plasmids the single stranded complementary sequences were synthesized with fitting over-hangs and annealed. The annealing was performed as follows: 100 pmol of each oligonucleotide, 1 µl 1M $MgCl_2$ and 10 µl 1M Tris/HCl pH 8,0 were pipetted into a vial and water was added to a final volume of 100 µl. The solution was boiled in a baker for 5 min and then let to cool down at 4°C (about 3 h).
From the annealed oligonucleotide 1 µl was used in conjunction with 100 ng of the plasmid vector and ligated in a total volume of 20 µl. After heat inactivation of the T4-ligase 2 µl were used for transformation of bacteria.

Preparation of chemically competent bacteria

To increase the efficiency of plasmid uptake (transformation), bacteria were treated with solutions of divalent cations. A single colony was used for inoculation of 5 ml LB medium, which was then incubated o/n at 37°C, while shaking. From the over-night culture 2 ml were used to inoculate 100 ml LB-medium with appropriate antibiotics, and grown at 37°C until an OD_{600} of about 0.5 was reached (approx. 3-5 h). The density was measured at a wavelength of 600 nm (OD_{600}). Bacteria were incubated for 15 min on ice and then pelleted (10 min, 3000 rpm, 4°C) in a Heraeus-centrifuge. After discharging the supernatant the bacteria pellet was re-suspended in 15 ml ice cold TFB I-buffer. The bacterial suspension was incubated for 50 min on wet ice, and then centrifuged as described before. The pellet was re-suspended in 2 ml ice cold TFB II-buffer and aliquots of 200 µl were snap-frozen in liquid nitrogen and stored at –80°C. NOTE: All vessels and pipettes must be pre-chilled: for best result work in a 4°C cold room.

TFB I-buffer: 100 mM $RbCl_2$, 50 mM $MnCl_2$, 30 mM KAc, 10 mM $CaCl_2$,
sterile filtered (0.2 µm filter)

TFB II-buffer: 10 mM MOPS pH 7.0, 10 mM $RbCl_2$, 75 mM $CaCl_2$, 15% glycerol,
sterile filtered (0.2 µm filter)

Production of electrocompetent bacteria

If nothing else is noted all steps were performed on ice or at 4°C. For the production of electrocompetent bacteria 200 ml LB-medium (with appropriate antibiotics) was inoculated with 2 ml from an over-night culture. When the bacteria had reached an OD_{600} of 0.5 to 0.6 the bacteria were incubated on ice for 15 min and then centrifuged (15 min, 4000 rpm, 2°C). The pellet was re-suspended in 200 ml PBS with 10% glycerol to discharge the salt and again centrifuged (15 min, 4000 rpm, 2°C). This step was repeated two more times. After the last centrifugation the pellet was re-suspended in 1.5 ml PBS with 10% glycerol and 70 µl aliquots were snap-frozen in liquid nitrogen and stored at –80°C.

Chemical transformation of bacteria

For the transformation of chemically competent bacteria 100 µl were thawed on ice. The DNA to be transformed was added to the bacteria together with 7 µl DMSO. The mixture was incubated on ice for 10 min and subsequently incubated at 42°C for 90 s (heat shock). After the heat shock, 5 ml LB-medium was added and incubated for 1 h at 30°C or 37°C in a shaking air incubator. After this step the bacteria were centrifuged (10 min, 3000 rpm, RT), the pellet was re-suspended in 150 µl LB-medium and plated on agar plates containing appropriate antibiotics. After over-night incubation at 30°C or 37°C bacteria colonies became visible.

Storage of bacteria

For long-term storage of bacteria 1 ml dense culture was mixed with glycerol at a final concentration of 20% (v/v), shortly mixed, and stored at –80°C.

3.3 DNA analysis

Agarose gel-electrophoresis

For analysis of the DNA-fragments obtained from digestion of the DNA with restriction enzymes the reaction was loaded onto an agarose gel, separated, and analysed. The DNA-fragment mixture was supplemented with 1/10 volume 10x

loading buffer (200 mM EDTA, 40% Ficoll 400, 0.02% (w/v) bromphenolblue). The agarose concentration used was between 0.6 to 1.5%, for Maxi-EBV-DNA separation always 0.8%. As electrolyte either a TAE-buffer or a TBE- buffer containing ethidiumbromide (final concentration 1 μg/ml) was used. The bands were separated on an agarose gel using 70 V for 1.5 h. Very large Maxi-EBV DNA fragments were separated at 70 V for 14-16h.

Southern Blot-analysis

The DNA-fragments separated using an agarose gel were transferred onto a nylon membrane (Maniatis et al., 1989), and hybridised with a probe labelled with $\alpha[^{32}P]$-dCTP. The labelled probe binds specifically to the complementary DNA sequences to make them visible by audiography.

Transfer from agarose gel to nylon membrane

The agarose gel was incubated for 30 min in 0.25 M HCl at RT. After washing the gel in water the agarose gel was incubated in denaturation buffer for 45 min at RT and then the DNA was transferred via capillary transfer to the nylon membrane (HybondN+). The transfer was completed after about 2-3 h and the membrane was washed for 30 min in 2×SSC. The prehybidisation was carried out with 15ml Church-hybridisation buffer for 1h at 65°C (Church and Gilbert, 1984).

1×TAE buffer:	40 mM Tris-HCl (pH 8,0); 5 mM NaAc; 1 mM EDTA
2×SSC:	300 mM NaCl, 30 mM sodium acetate
Church-buffer:	400 mM Na_2HPO_4, 100 mM NaH_2PO_4, 7% SDS, 1mM EDTA (pH 7.2)

Radioactive labelling of DNA

In order to make a probe, 50 ng of a plasmid or fragment containing the complementary sequence to the fragments to be detected was denaturated for 5 min at 95°C and then put on ice. 4 μl high-prime labelling kit containing dNTPs and Klenow-DNA-polymerase was added together with 50 μCi $\alpha[^{32}P]$-dCTP. The reaction was incubated at 37°C for 30 min, during this time the Klenow-DNA-polymerase uses the dNTPs to synthesise complementary strands. The reaction mixture was purified

using a pre-equilibrated Sephadex-column (G50). The radioactive labelled probe (4×10^7 dpm/ml) was added to 15 ml Church buffer and hybridised to homologous DNA-fragments over-night at 65°C. The hybridised nylon-membrane was washed in 0.1×SSC+0.1%SDS, 1×SSC+0.1% at 65°C and then autoradiographed at –80°C. The film was usually exposed over-night.

Sequencing

All sequencing was performed by the company SEQUISERVE Dr. Metzger (Vaterstetten, Germany). Maxi-EBV plasmids were sonificated prior to sequencing.

3.4 Polymerase chain reaction (PCR)

The standard PCR analysis was performed as suggested by the manufacturer. For analytical purposes *Taq* polymerase was used whereas for cloning purposes the proof-reading DNA polymerase *Pfu*I was used. Normally 50 ng DNA template to be amplified was mixed with 100 pg per primer, 0.5 mM dNTPs (dATP, dCTP, dGTP, dTTP), 2 units of DNA-polymerase and 25μM $MgCl_2$ in a total volume of 50 μl 1x reaction buffer.

The PCR was performed in a Robo-cycler Gradient 96 (Stratagene). If nothing else is given the standard PCR program was used. First the DNA was denaturated for 5 min at 95°C and then 30 cycles of the following sequence were performed: 1 min at 95°C denaturation, 1 min at 54°C annealing, and 2 min at 72°C or 73°C elongation (for the *Taq* polymerase 72°C and for *Pfu*I 73°C). After completing the 30 cycles the PCR products were elongated for 10 min at 72°C for completion of the synthesis.

The annealing temperature was calculated as follows.

$$\mathbf{T_H\ (°C) = 60 + (\ (\ G+C)\ x\ 41/\ Nt_{sum}\ )\ -\ (\ 600/Nt_{sum})}$$

Remark:

G+C are the number of cytosine and guanines in the primer sequence

Nt_{sum} is the total length of the primer sequence

3.5 Mutagenesis using Maxi-EBV plasmids

Production of electrocompetent arabinose-induced DH10B bacteria

About 5 μl from a glycerol culture of DH10B, which carries the mutated Maxi-EBV plasmid and the pKD46 recombination plasmid was used to inoculate 5 ml LB-medium with 100 μg/ml ampicillin. Since the recombination plasmid carries a temperature-sensitive origin of replication the inoculation was carried out over-night at 30°C. From this culture 2 ml were used to inoculate 200 ml LB-medium also with 100 μg/ml ampicillin. At an OD_{600} of about 0.15 to 0.18 the bacteria were induced with 2 ml of a newly prepared arabinose solution (10% w/v in LB-medium) to a final concentration of 0.1% (w/v) and shaken for another 30 min at 30°C for the expression of the proteins red α, red β, and red γ, which are needed for homologous recombination.

For making electrocompetent bacteria all material and solutions were pre-cooled to 0°C. The bacteria culture was put on ice for 15 min and then centrifuged (10 min, 7000 rpm, 0°C) in a GSA-rotor and a Sorval centrifuge pre-cooled to 0°C. The pellet was re-suspended in PBS with 10% glycerol and then centrifuged again. The process was repeated three times in total. After the last centrifugation step the supernatant was discharged and the remaining fluid was dried using a Kleenex tissue. The pellet was re-suspended in the small volume of fluid left in the centrifuge bottle and about 6-10 aliquots of 70 μl were pipetted into Eppendorf vials and frozen in liquid N_2, and stored at – 80°C.

Transformation of linear fragment into competent DH10B bacteria

EBV plasmid-DNA was transformed into DH10B E.*coli* by electroporation using a Gene-pulser (Bio-Rad). The competent DH10B bacteria (70 μl) were incubated for 5 min together with 1μg plasmid DNA, and then transferred to a pre-cooled electroporation cuvette (0.2 mm electrode distance). The electroporation was carried out at 2.0 kV, 200Ω and 25 μF, with a time constant of about 4-5 ms. The bacteria were transferred to 5 ml LB-media, incubated for 60 min at 37°C. When using a temperature sensitive recombination system the bacteria were incubated at 30°C. After incubation the bacteria were plated on agar plates with corresponding

antibiotics. The agar plates were incubated over-night at 37°C by which the temperature-sensitive recombination helper plasmid was lost. The analysis of the bacterial colony was performed as described in 3.1.

Isolation of single copy Maxi-EBV (BAC) DNA from bacteria for analytical purposes (*Mini-prep*)

Since the Maxi-EBV genome comprises of 184 kbp shearing of the DNA during the isolation should be prevented. 10 ml LB-medium was inoculated with a bacterial colony and grown over-night at 37°C. The bacteria were transferred to a 15 ml falcon tube and pelleted by centrifugation for 5 min at 3000 rpm in a Heraeus Centrifuge. The pellet was resolved in 300 µl solution I and transferred to an Eppendorf tube. For the alkaline lysis 300 µl solution II was added. SDS, chromosomal DNA and proteins were precipitated using 300 µl solution III. After 10 min centrifugation at 14000 rpm in the Eppendorf centrifuge 5415 the supernatant (about 1 ml) was extracted with 1 ml Phenol/Chlorophorm (1:1) and again centrifuged for 5 min. The upper phase being the water phase was put in a new tube and 1 ml isopropanol was added, this was then centrifuged at 14000 rpm for 15 min. After washing the pellet in 70% alcohol it was air dried and then re-suspended in 100 µl TE-buffer. The amount of DNA obtained with a Maxi-EBV mini-prep is enough for one digest

Solution I: (25 mM Tris/HCl and 10 mM EDTA, pH 8.0

Solution II: (0.2 M NaOH and 1% SDS)

Solution III: (3 M KAc, pH 4.8)

Isolation of Maxi-EBV DNA from bacteria for preparative purposes

For isolating Maxi-EBV DNA in large quantities the Nucleobond-Kit from Machery&Nagel was used. 400 ml LB-medium was inoculated with about 100 µl bacteria from an over-night culture or from frozen bacteria and incubated at 37°C. The bacteria were centrifuged at 4000 rpm in a Beckmann centrifuge for 10 min DNA was extracted according to the instruction protocol of the manufacturer. After precipitation using isopropanol the DNA-pellet was air-dried and re-suspended in 200 µl TE-buffer.

For digestion of the Maxi-EBV DNA, 1μg DNA was incubated with 40 u restriction enzyme for 3 h. The DNA fragments were separated on a 0.7% TAE gel and analysed using Southern blot analysis.

3.6 Cell culture and analysis of cells

3.6.1 Cell culture conditions

All cells were cultivated in an incubator at 37°C with an atmosphere of 5% CO_2. The culture media was complemented with 10% fetal calf serum (Seromed®), 2mM L-Glutamin, 100 μg/ml streptomycin, 100 μg/ml penicillin, 0.5 μg/ml amphotericin B, 1 mM sodium pyruvate and 5mM HEPES.

Adherent cell lines

The adherent fibroblast cell line WI 38 was cultivated in a RPMI media supplemented with 10% fetal calf serum and splitted 1:7 every 3 days. Trypsin was used to detach adherent cells, after 3 min incubation at RT the cells were re-suspended and transferred to larger flasks.

Determination of cell density

The cell density was determined in a Neubauer cell chamber. In order to distinguish dead from living cells the cell suspension was mixed one to one with a 0.1% Eosin solution dissolved in PBS. The dead cells absorb the read dye, and are therefore in the microscope easy to distinguish from the colourless living cells. Alternatively the cell density was determined using a Coulter cell counter.

Freezing and thawing of cells

About $1x10^7$ cells were spun down (1200 rpm, 5 min, 20°C) and re-suspended in freezing medium containing 40% RPMI medium, 50 % FCS and 10% DMSO, and pipetted into 2 ml-tube (NUNC-cryotube™). In order to prevent the cells from damage the cells were cooled slowly to –80°C by using layers of cotton. For long-term storage the cells were transferred to a standard liquid N_2-tank.

To reutilise the cells they were thawed rapidly at 37°C, carefully washed in pre-warmed medium centrifuged (7 min, 1200 rpm, RT) to remove all DMSO and then transferred to a culture flask or dish containing the appropriate medium.

3.6.2 Establishment of HEK293 stable cell lines carrying Maxi-EBV plasmid

The cells that were to be transfected were plated in a 6-well-cluster plate to a confluency of 70%. The transfection with lipofectamin™ in serum-free medium (Optimem I) was preformed according to the protocol of the manufacturer (Life Technologies), (6 µl lipofectamin™-reagent per 1 µg transfected DNA). For the establishment of EBV-positive cell lines HEK293 cells were used. These were grown in a 6-well-cluster plate and transfected with 1µg of the Maxi-EBV DNA. The next day the cells were transferred to a cell culture dish (140 mm diameter) and hygromycin was added to the medium to a final concentration of 100 µg/ml. Three to four weeks later the cell clones that were positive for GFP expression in the fluorescence microscope were picked and individually expanded.

3.6.3 Production of infectious virus particles and titer determination

Stable HEK293 cell clones that carried different Maxi-EBVs were grown in 6-well-cluster plates to a density of about 70%, and then the lytic cycle was induced. The cells were transfected with expression plasmids for BZLF 1 (p509; 0.5 µg/well) and BALF 4 (p2670; 0.5 µg/well) together 4 µl of the transfection agent lipofectamin, at a final volume of 100 µl with Optimem. The BZLF 1 gene is an immediate early gene, which induces lytic replication and upon co-transfection of BALF 4 the virus titers obtained have been shown to improve (Neuhierl et al., 2002). The transfection mix was incubated for 20 min at RT, and in the mean time the HEK293 cells were washed once with PBS and then 1 ml of Optimem was added to the cells. After the incubation the reaction mix was carefully dropped onto the cells. After incubation for 72 h at 37°C the supernatant was harvested and filtered through a 0.8 µm filter to remove cell debris. The different virus supernatants were quantified with the help of the GFP expression of infected Raji cells. Defined amounts of virus supernatant were added to $3x10^6$ Raji cells in a 24-well-cluster plate and incubated at 37°C for 72 h. On

the second day of infection the medium was exchanged. At the third day the number of GFP-positive cells was calculated in a UV-microscope. Relying on these data “green Raji units” per ml as an absolute number of infectious particles for the different virus stocks were defined.

3.6.4 Preparation of primary B-lymphocytes

The human primary B-lymphocytes were isolated from adenoids. The adenoid was cut into small pieces and a single cell suspension was mechanically compounded. To the cell suspension 0.5 ml sheep blood was added and incubated at 25°C for 15 min in order to bind to T-lymphocytes and then PBS was added to a final volume of 35 ml. The sample was purified over a 30% ficoll gradient (ficoll-density 1.077), by overlaying the cell-suspension on the ficoll gradient. The gradient was centrifuged at 1850 rpm for 45 min at 10°C, and the cells at the interface were isolated containing predominately primary B-lymphocytes. Ficoll and the remaining thrombocytes were removed by washing three times with PBS in a fractioned manner (centrifugation: 1700 rpm, 1500 rpm, 1200 rpm, for 7 min at 10°C).
After removing the PBS the B-lymphocytes were re-suspended in culture medium and counted. Most often the cells were used directly, but it is possible to store the cells over-night at 37°C in an incubator and use them the next day for infection experiments.

3.6.5 Infection of primary B-lymphocytes with EBV mutants and determination of the immortalisation frequency

Primary B-lymphocytes were extracted from adenoids and infected with different dilutions of adjusted virus supernatants. The infection was carried out in 96-well-cluster plates with γ-irradiated WI 38cells (feeder cells). The feeder cells were irradiated for 90 min with 100 rad/min. In each well 100 μl containing $1x10^5$ B-cells were pipetted and fed weekly. After 6 weeks, the number of growth transformed clones could be determined and about 20 wells were expanded. In order to determine the immortalisation frequency of the different Maxi-EBV mutants only dilutions were taken into account that fulfilled the statistic criteria of “single-hit”-

kinetics (Frisan et al., 2001). That was the case when 63% or fewer positive events were seen (out-grown wells per plate).

3.7 Immunofluorescence

By immunostaining, the late lytic protein gp125, a protein belonging to the VCA-complex, or the glycoprotein gp350 were detected in lytically induced HEK293-cells. The cells were washed with PBS and fixated with acetone for 20 min. The fixed cells were incubated with a specific monoclonal antibody for 30 min at 37°C. After washing the microscopic slide twice with PBS in a glass beaker the cells were incubated with a secondary antibody (Cy3-conjugated αMouse antibody) for 30 min at 37°C. Finally, the cells were washed three times with PBS and then embedded in a 10% glycerin/PBS solution. The cells were analysed at a wavelength of 546 nm using an inverted fluorescence microscope (Zeiss Axiovert 10) with an appropriate filter combination.

3.8 Retrovirus production and concentration

Calciumphosphate transfection of Phoenix cells

Phoenix cells were allowed to grow to a confluence of 70% in a 90 mm dish, and then transfected using calcium phosphate. In a 15 ml Falcon tube, 2 μg GALV expression vector, 10 μg gag/pol expression vector, 5 μg Cre expression vector (p3087) and 50 μl $CaCl_2$ 25 mM were combined and water was added to a final volume of 500 μl. Drop wise 500 μl 2x precipitation buffer was slowly added while pipetting in air into the mixture, this mixture was then incubated for 20 min at RT so that crystals could form. The cells were washed once with PBS and then 7 ml Modified Dulbecco´s medium with 2.5 μM chloroquine final concentration was added. After incubation, the DNA mixture was added drop-wise onto the cells and incubated for 8 h at 37°C. Subsequently the medium was changed and the cells were incubated over-night. After 24 h the first charge of retroviral supernatant was collected, and then again after 48 h.

2x precipitation buffer: 50 mM HEPES; 280 mM NaCl; 1.5 mM Na_2HPO_4; pH 7.05

Concentration

For concentrating the virus supernatant 31 ml of virus supernatant was transferred to an ultracentrifuge tube and centrifuged for 3 h at 35000 rpm (70.1 TI rotor, Beckmann ultracentrifuge L7-55). The supernatant was discharged and the virus pellet was re-suspended in 1 ml Modified Dulbecco´s medium.

Testing the virus supernatant

For testing the virus supernatant $2x10^5$ Raji cells were pipetted into a 24-well-cluster plate together with 100 µl of the concentrated virus supernatant and medium was added to a final volume of 1 ml. This mixture was then centrifuged for 1 h at 4000 rpm and further incubated for 48 h and analysed under the microscope. Since the retroviral vector expressing the Cre protein also encodes the mRFP gene successfully infected cells would become red indicating successful viral infection of individual cells.

Retroviral infection of LCLs

In a 24-well-cluster plate $2x10^5$ LCLs were transferred to together with 150 µl of concentrated virus supernatant and medium was added to a final volume of 1 ml. The plate was centrifuged at RT for 60 min at 4000 rpm. After centrifugation the plate was incubated at 37°C and the next day the medium was changed. Two days after infection the cells were observed under the microscope for expression of red fluorescence protein.

3.9 Protein analysis

SDS-polyacrylamide gel electrophoresis

For separation of the proteins a 7.5% polyacrylamidegel (*SDS-page*) was used. For gel casting a ready to use 30% acrylamide solution with 0.8% bisacrylamide was used. The stacking gel was made with 4x stacking-gel buffer and the running gel was made with 4x running gel buffer.

Preparation of protein extracts

For the analysis of total cellular protein about $1x10^8$ LCL cells were centrifuged (1200 rpm, 7 min, RT) and the pellet was washed once in PBS then re-suspended in 50 µl Ripa buffer and subsequently incubated for 30 min on ice. The concentration of the extracts were analysed with the Bradford method and measured in an Eppendorf photometer. Before loading the samples 12.5 µl 4x loading buffer was added and denaturated by boiling for 5 min at 95°C.

Lysis-buffer (Ripa):	150 mM NaCl; 20 mM Tris pH 7.5; 1% TRITON; 0.5% DOC; 0.1% SDS; 200 mM DTT.
SDS-loading-buffer (4x):	100 mM Tris-HCl (pH 6,8); 4 % SDS; 20 % glycerol; 0,2 % Bromphenol blue

SDS-polyacrylamide gel electrophoresis

Denaturating (SDS) polyacrylamide gel electrophoresis (PAGE) was performed essentially as described (Laemmli, 1970). Proteins were solubilised in Ripa buffer and denaturated at 95°C before separation on an SDS-PAGE gel. A pre-stained marker was loaded in parallel with the samples to be able to estimate the molecular weight. The acrylamide content of the gel varied according to the size of the proteins being examined. The samples were separated using a current of 30mA per gel in 1x SDS-running buffer.

10 % SDS-Gel (100 ml):	56.9 ml H_2O; 16.6 ml 2 M Tris-HCl (pH 8.9); 25 ml Acrylamide (30%)/Bisacrylamide (0.8%)-solution; 666.6 µl 0.5 M EDTA; 700 µl Ammoniumpersulfate (APS)-solution (10%) und 70 µl Tetramethylethylendiamin (TEMED)
Stacking-gel (10 ml):	5.62 ml H_2O; 2.5 ml 0.5 M Tris-HCl (pH 6.8); 100µl 10 % SDS; 1.68 ml Acrylamide (30%)/Bisacrylamide (0.8%)-solution; 100 µl Ammoniumpersulfate (APS)-solution (10%) und 6 µl Tetramethylethylendiamin (TEMED)
10x SDS-running buffer:	0.25 M Tris; 2.5 M Glycerin; 0.5% (v/v) SDS

Western blot analysis and immuno-detection of proteins

The Western-blotting technique is based on the separation of proteins by SDS-PAGE, immobilisation and detection by using specific antibodies. After separation of the proteins they were transferred to a Hybond C-membrane using a semi-dry transfer system. In order to transfer the proteins without air bubbles all layers were assembled in blotting buffer. First two layers of Whatman papers were added, then the membrane was added, the gel, and finally two additionally Whatman papers. The whole package was put in a semidry-blotting apparatus (Semi-Phor-electroblot system, Hoefer) and blotted for 1h at 80 mA. After this step the membrane was incubated for at least 1h in blocking-buffer in order to prevent unspecific binding followed by an incubation step with the primary antibody (diluted 1:4000) in PBST with 5% milk-powder w/v over-night at 4°C. Before incubation with the second antibody the membrane was washed five times with PBST for 7 min on a rocking platform at room temperature. The secondary antibody (diluted 1:4000) was incubated in PBST with 5% milk-powder w/v for 2h at RT, after this period the membrane was again washed as described as above. The secondary, peroxidase-conjugated antibody was visualized using the *ECL Western blot detection kit* (Amersham) according to the manufacturer's instruction. To detect the reaction a film was put on the membrane for up to 20 min.

Blotting-buffer:	48 mM Tris; 39 mM glycine; 0.0375% SDS; 20% methanol
Blocking-buffer:	PBST with 5 % Skim milk-powder
PBST:	PBS with 1% Tween-20

4. Results

For a general overview, the result section is divided into four parts. The first part gives an overview of the entire process of cloning a mutant EBV genome through to the establishment of recombinant Epstein-Barr viruses (4.1). The second part describes the cloning of the nine EBNA 3C mutants and the manipulation of the Maxi-EBV, the establishment of stably transfected HEK293 cell lines, the induction of the lytic cycle for virus production, and the quantification of the virus in the supernatants (4.2). The recombinant viruses were used in the third part of this work, in which the immortalisation efficiencies of the EBNA 3C mutants were investigated using primary human B-lymphocytes (4.3). From these experiments lymphoblastoid cell lines (LCLs) were established and analysed for expression of the viral proteins EBNA 3C, EBNA 2, EBNA 1 and LMP1. The last part describes the establishment of a conditional Cre/lox P EBNA 3C EBV mutant, the cloning of a retroviral vector expressing the Cre protein for Cre/lox P site-directed mutagenesis and the successful excision of the EBNA 3C gene from latent EBV genomes in LCLs (4.4).

4.1 Establishment of recombinant Epstein-Barr viruses

Fig.4.1 gives a schematic overview of all mutagenesis steps from cloning of the recombination fragments to production of recombinant EBVs. First, the recombination plasmid carrying the kanamycin resistance gene (Kan) encompassing the desired mutation and flanking viral homologies for homologous recombination was generated. Second the linear fragment containing the mutation, the kanamycin resistance gene and the flanking homologous regions was excised and electroporated into the DH10B E.*coli* strain carrying the recipient Maxi-EBV plasmid (p2089) and the helper plasmid pKD46 that expresses the recombinases redα and -β. Homologous recombination between the linear DNA recombination fragment and the targeted Maxi-EBV p2089 resulted in mutant Maxi-EBV genomes. To establish a stable cell line for virus production HEK293 cell were transfected with recombinant EBV DNA extracted from E.*coli.* Stable cell clones harbouring the mutant Maxi-EBV genome were selected using hygromycin selection. Subsequently, two plasmids encoding the viral genes BZLF1 and BALF 4 were transiently transfected into these cells to induce the lytic phase of the virus life cycle and to improve virus titers,

respectively. Quantification of the harvested virus supernatants was performed by infecting the human B-cell line Raji and quantitating cells expressing GFP.

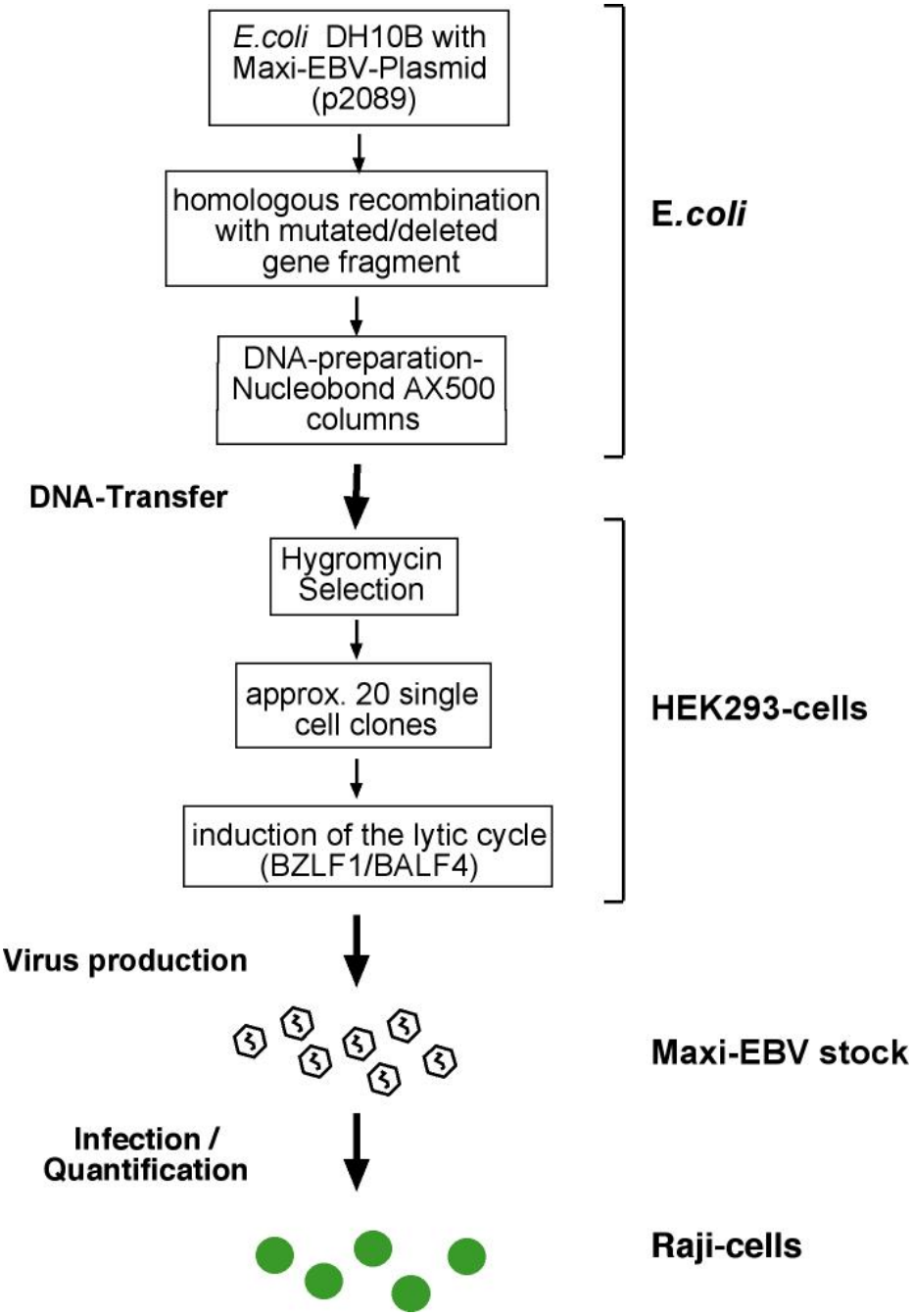


Fig.4.1 The process of production of recombinant Epstein-Barr viruses

An schematic overview of the generation of recombinant EBV mutant genes using homologous recombination in the E.*coli* DH10B strain, establishment of stable cell lines in HEK293 cells, and the induction and harvest of recombinant virus is shown. Quantification of infectious particles is done by infection of Raji cells, which express GFP when infected with recombinant EBVs.

4.2 Generation of nine recombinant Maxi-EBV genomes with partial deletions in EBNA 3C

To further understand the function of EBNA 3C, nine EBV mutants with deletions in EBNA 3C were generated to elucidate whether a particular domain or motif of EBNA 3C was critical for EBV induced B-cell immortalisation. In this chapter the different EBNA 3C Maxi-EBV mutants, the establishment of stable HEK293 producer cell lines, the induction of the lytic cycle, and the quantification of the virus stocks are described.

4.2.1 Cloning of the recombination plasmids

Nine different EBNA 3C deletion mutants were generated using different recombination plasmids derived from the parental plasmid p2720 (Fig. 4.2), carrying 7657 nucleotides from the B95.8 strain of EBV (nt position #95,239 to #102,896) including EBNA 3B, EBNA 3C, and the BZLF 1 genes. Between BZLF 1 and EBNA 3C the kanamycin resistance gene from plasmid pCP15 (Cherepanov and Wackernagel, 1995) was introduced.

p2720

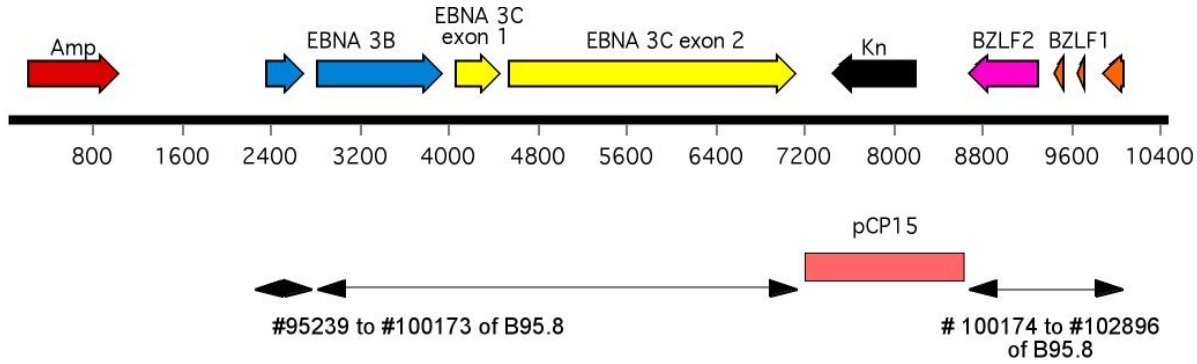


Fig.4.2 A schematic overview of the gene arrangement in plasmid 2720. This was used as a platform for mutagenesis of EBNA 3C in the Maxi-EBV 2089.

Plasmid 2720 contains the EBV genome sequence from nucleotides position #95,239 to #102,896 of the B95.8 strain of EBV. This fragment includes the two exons of EBNA 3B (blue arrows), the two exons of EBNA3C (yellow arrows), the BZLF1 (orange arrows) and the BZLF2 (purple arrows) genes. Downstream of the EBNA 3C gene the kanamycin resistance gene derived from plasmid pCP15 (black arrow) was inserted as a selectable marker in E.*coli* to select for successful recombination.

The sequences up- and downstream of the EBNA 3C gene served as flanking donor regions for homologous recombination with the recipient Maxi-EBV genome. In Fig.4.3 the generated deletion mutants are schematically shown. On the left side the name of the donor plasmid, based on p2720 is shown and on the right side the corresponding Maxi-EBV-plasmid name is given. For reconstituting a wild-type Maxi-EBV by homologous recombination the whole EBNA 3C gene from the recombination plasmid 2720 was used. This Maxi-EBV mutant (p2889) was generated to confirm that all the effects observed are the consequence of modifications within the EBNA 3C gene and not caused by unwanted recombination events or an epiphenomenon in the EBV genome. The establishment of the lox P flanked EBNA 3C Maxi-EBV mutant (p2919) is described in detail in section 4.4.2.1. For the remaining mutants, specific motifs or domains of EBNA 3C were mutated. In p2864 the PLDLS motif in EBNA 3C was mutated to ALDAS. In plasmid 2745 the sequence between nt position 5318 to 5632 which corresponds to amino acids 408-510, were deleted and hence EBNA 3C lacks about half of its repressor domain. The plasmid 2743 has a deletion between nt position 5322 to 6227 corresponding to amino acid 410 to 710 and therefore lacking a larger part of the repressor domain, one NLS sequence and the polyproline stretch. Mutant plasmid 2744 with a deletion from nt position 4729 to 6066, corresponding to amino acid 212 to 658 of EBNA 3C -lacks half of its RBP-Jκ binding site, the basic leucin zipper sequence and the whole repressor domain. In the plasmid 2721 the sequence from nt 6066 to 7134, corresponding to amino acids 658 to 1013 and comprising the whole activation domain and one NLS sequence were deleted. The plasmid 2742 has a deletion from nt 5558 to 7003 which corresponds to amino acids 489 to 970 containing a large part of the repressor domain, one NLS sequence, a poly-proline stretch and the activation domain. From all recombination plasmids a linear fragment carrying the mutated EBNA 3C gene, the kanamycin resistance gene and flanking viral homologies was cut out and introduced into the wild-type Maxi-EBV 2089 by homologous recombination. This mutagenesis step is described in detail in the next section. Only the conditional lox P flanked EBNA 3C Maxi-EBV mutant (2919) was established differently, which is described under 4.4.1.2.

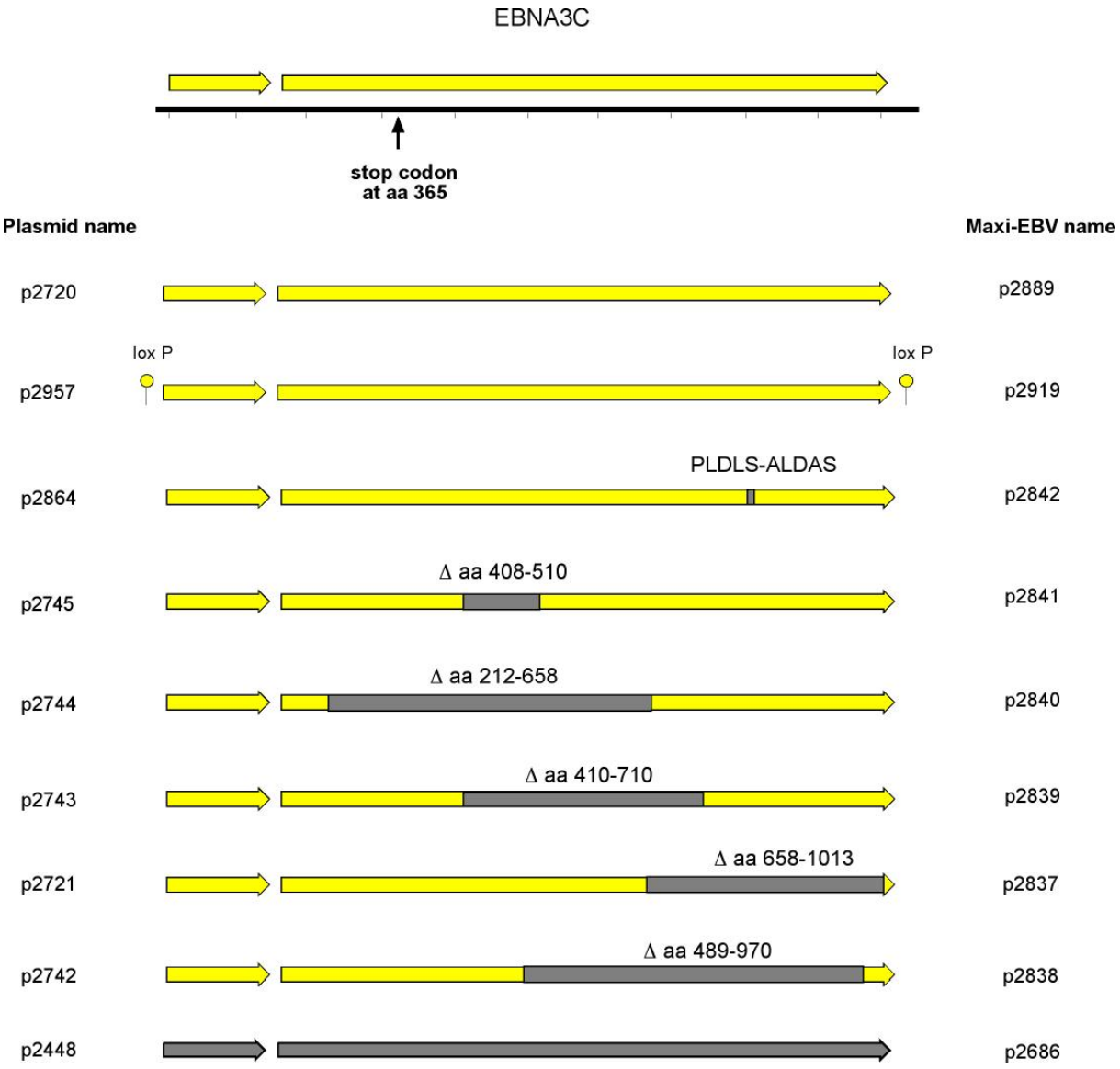


Fig.4.3 Schematic overview of the different EBNA 3C mutations in the recombination plasmid 2720.

The name of the recombination plasmid and the corresponding Maxi-EBV name is given on the left and right sides, respectively. The wild-type EBNA 3C gene in the recombination plasmid p2720 is shown at the top of the figure. The deletion mutants are arranged below and the deletions are marked with grey bars. To rule out any unwanted effects of the mutagenesis procedure itself, the wild-type EBNA 3C gene of p2720 was used to reconstitute a recombinant wild-type EBV genome by homologous recombination in E.*coli* generating the Maxi-EBV 2889. In the recombination plasmid p2864 two amino acids within the PLDLS motif were changed to ALDAS, in p2745 nt position 5318 to 5632, corresponding to aa 408 to 510, were deleted. In p2744 nt position 4729 to 6066, corresponding to aa 212-658, are lacking. Plasmid 2743 has deleted nt. 5322 to 6227, corresponding to aa 410-710, in p2721 nt position 6070 to 7116 corresponding to aa 658-1013, were deleted. In p2742 nt position 5558 to 7003, corresponding to aa 489-970, were deleted. In p2448 the EBNA 3C is replaced by the tetracycline resistance gene, which is flanked by two lox P sites. The black arrow in amino acid position 365 indicates the stop codon introduced by Tomkinson for investigating the EBNA 3C phenotype (Tomkinson et al., 1993).

4.2.2 Red αβγ mediated mutagenesis of EBNA 3C in the Maxi-EBV

Until now, mutagenesis of the Maxi-EBV (p2089) has been performed in the recA$^-$, recBCD$^+$ DH10B E.*coli* strain. This has the advantage that the EBV genome is genetically stable since the recombination protein rec A is not expressed. One disadvantage of this strain is that it expresses the rec BCD genes, which forms a trimeric complex known as exonuclease V, which readily degrades linear DNA fragments. Therefore, an exonuclease inhibitor and the rec A recombinases had to be expressed from a plasmid for mutagenesis in the E.*coli* strain. Since this system is not very efficient, the newly described redαβγ recombination system from bacteriophage λ (Zhang et al., 1998), which is expressed from plasmid pKD46, was established for EBV mutagenesis in E.*coli.*

The novel red recombination system uses an inducible expression of the bacteriophage λ genes. Into the DH10B the Maxi-EBV genome together with the temperature sensitive plasmid pKD46, was introduced (see Fig 4.4 left part). In addition to the open reading frames for the recombination proteins redα, redβ, and the exonuclease inhibitor redγ pKD46 contains the ampicillin resistance gene for selection in bacteria. Replication of plasmid pKD46 occurs at the permissive temperature of 30°C, only, since the plasmid carries a temperature-sensitive (ts) origin of replication. The homologous recombination using a linear fragment is schematically depicted and described in Fig.4.4. After electroporation of the linear fragment, carrying the mutated EBNA 3C, the kanamycin resistance gene, and flanking homologous regions, recombination occurs at 30°C. Following an increase in temperature to the non-permissive temperature of 42°C and co-selection with chloramphenicol and kanamycin to select for the antibiotic resistance genes of the Maxi-EBV plasmid and the linear fragment, respectively, only colonies which have integrated the linear fragment and thereby gained resistance against both antibiotics develop. At the same time the plasmid pKD46 is lost upon the raise in temperature (Fig.4.4 right part). The phenotype of the E.*coli* colonies is easily tested using replica plating.

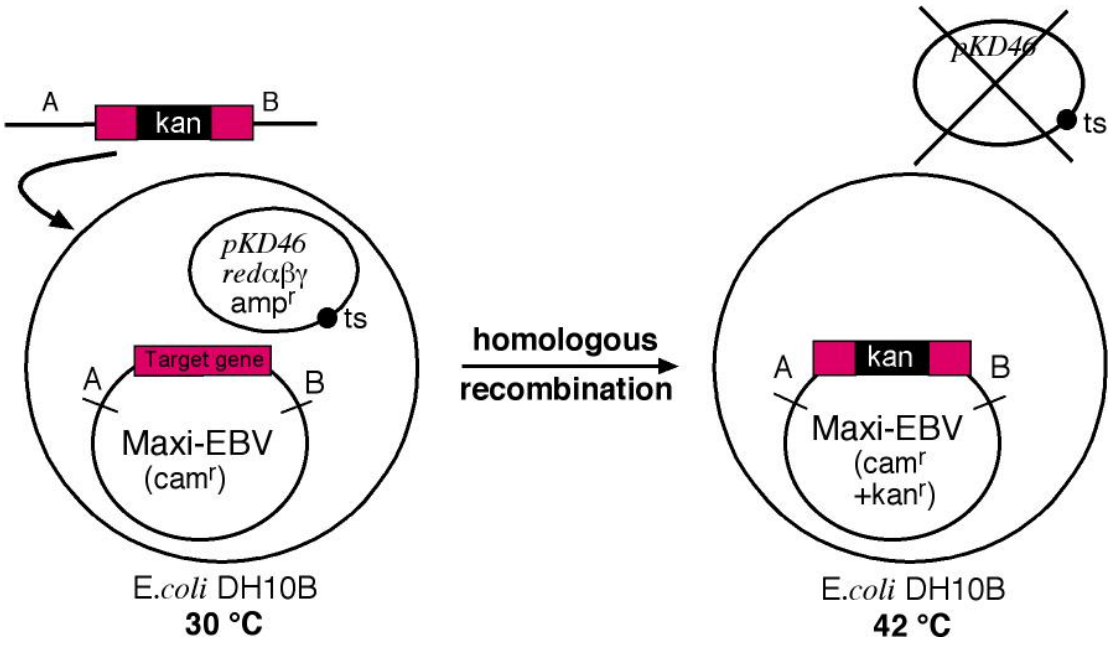


Fig.4.4 Mutagenesis of the Maxi-EBV genome in E.*coli* DH10B using homologous recombination.

This figure shows the temperature-sensitive (ts) plasmid pKD46 and the Maxi-EBV plasmid in DH10B. The temperature-sensitive plasmid carries the ampicillin resistance gene (amp^r), a temperature-sensitive origin of replication (ts), and expresses the recombinases red$\alpha\beta\gamma$ from phage λ. The Maxi-EBV plasmid carries a chloramphenicol resistance gene (cam^r) and the target gene to be mutated.

The linear fragment containing the mutated gene flanked with homologous sequence stretches A/B (at least 35 nucleotides) is electroporated into the E.*coli* strain DH10B, where homologous recombination takes place. The degradation of the linear fragment is prevented by expression of redγ. After two hours incubation at 30°C and over-night incubation at 42°C in the presence of chloramphenicol and kanamycin only those bacterial clones grow in which homologous recombination between the linear DNA fragment and the Maxi-EBV occured. At 42°C, the temperature-sensitive recombination plasmid pKD46 is lost.

In addition, colonies were analysed for ampicillin sensitivity (Amp^s) indicating the loss of plasmid pKD46. After successful homologous recombination in E.*coli* the correct genotype was confirmed for each mutant using Southern blot analysis (see Fig.4.5). As a radioactively labelled probe the plasmid 2470.7, harbouring the whole EBNA 3C gene, was used.

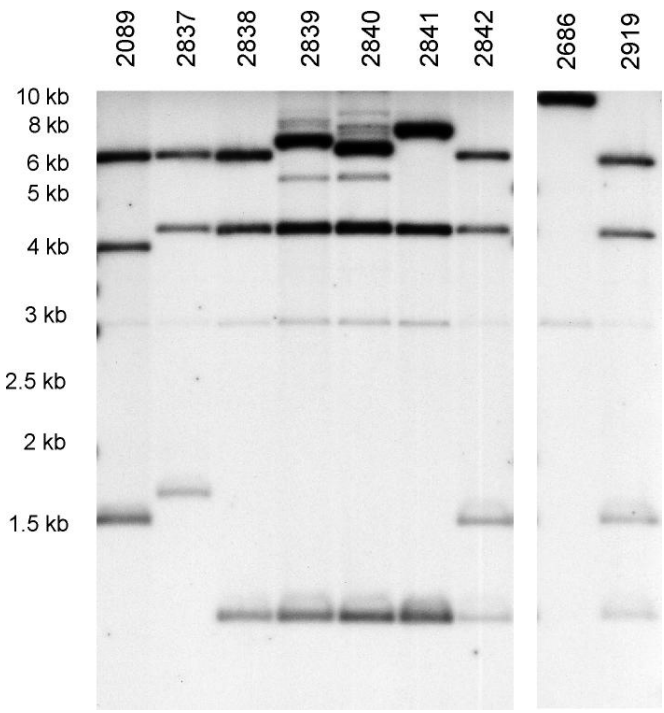


Fig.4.5 Confirmation of correct mutagenesis of EBNA 3C within the Maxi-EBVs in E.*coli.*

Shown is a Southern blot analysis performed with the EBNA 3C deletion mutant EBV plasmids. The E.*coli* DNA was digested with *Bgl*II and detected with a probe directed against the whole EBNA 3C gene (2470.7). The fragment patterns were compared to that of the wild-type EBV plasmid (2089) and correspond to the anticipated, correct fragment sizes of individual EBV mutant genes as they can be seen schematically in Fig.4.9 A through D.

For the Maxi-EBV mutant p2837 the expected changes in the restriction fragments could be confirmed (shift from 4061 bp to 4375 bp, and from 1594 bp to 1703 bp). In the p2838 Maxi-EBV mutant a shift from 4061 bp to 4374 bp confirmed correct mutagenesis. In p2839 the expected shift from 6337bp to 7028bp as well as the shifts from 4061 bp to 4375 bp and 1594 bp to 1174 bp were observed. For p2840 the shifts from 6337 bp to 6593 bp, 4061 bp to 4375 bp and 1594 bp to 1174 bp confirmed correct mutagenesis. For p2841 the shift from 6337 bp to 7616 bp, 4061 bp to 4374 bp and 1594bp to 1174bp were confirmed. For the p2842 the expected shift from 4061 bp to 4375 bp and an additional band at 1174 bp confirmed correct mutagenesis. For p2686 the expected band at 10648 bp was detected and for the re-constituted wild-type p2889 the expected shift from 4061 bp to 4716 bp was detected. After Southern blot confirmation, Maxi-EBV plasmid DNA was extracted from the individual E.*coli* strains. The flow chart of the different steps described in this chapter can be seen in the schematic drawing of Fig. 4.1 (E.*coli*).

4.2.3 Establishment of twelve producer cell lines and generation of virus stocks

All the steps described here are schematically shown in Fig.4.1 (HEK293 cells). The purified plasmid DNAs from the twelve Maxi-EBVs were transfected into HEK293 cells. After two to four weeks of selection with hygromycin in the cell culture medium (100 μg/ml final concentration) it was possible to identify GFP-positive single cell clones, that were picked and individually expanded.

All the established HEK293 single cell clones were tested with Southern blot analysis in order to confirm correct genotype. Genomic DNA was extracted from approximately ten cell lines per mutant as described in 3.1 and digested with *Bgl* II for three hours. For detection of the mutated EBNA 3C genomes a probe (p2470.7) that carries the entire EBNA 3C gene was used. The expected correct restriction fragment hybridization signal and Southern blot patterns were confirmed for all mutants. For all mutants the same patterns were expected as described in detail in chapter 4.2.2.

After conformation of the genetically altered genomes the established HEK293 Maxi-EBV EBNA 3C mutants were tested for virus production. The switch from the latent to lytic cycle of EBV can be induced by transient expression of the transactivator gene BZLF1 (Hammerschmidt and Sugden, 1988). Cotransfecting the glycoprotein BALF4 raises the virus production efficiency by at least a factor of ten (Neuhierl et al., 2002). Three days after cotransfection of BZLF1 and BALF4 the induction of the lytic cycle was confirmed by immunostaining of the transfected cells for the expression of gp350, a latent viral gene, the binding partner of CD21 and the primary receptor for EBV on primary B-lymphocytes (Janz et al., 2000). For the quantification of the virus stocks the Raji cell line was used, which is a Burkitt´s-Lymphoma B-cell line. Dilutions of the virus stocks were incubated with $3x10^5$ Raji cells in a 24-well-cluster plate in a final volume of 1 ml. After 24h the medium was changed and after another 72h the virus titers were determined. The titer was calculated using an inverted fluorescence microscope in which the GFP positive cells were easily detected. For this purpose three times 10 μl from every dilution were transferred to a Terasaki-plate, the absolute number of green Raji cells was counted and a mean value for every dilution was calculated. In Fig 4.6 an example is shown with 2089 virus supernatant in three dilutions. The figure displays only a part of a single Terasaki-well.

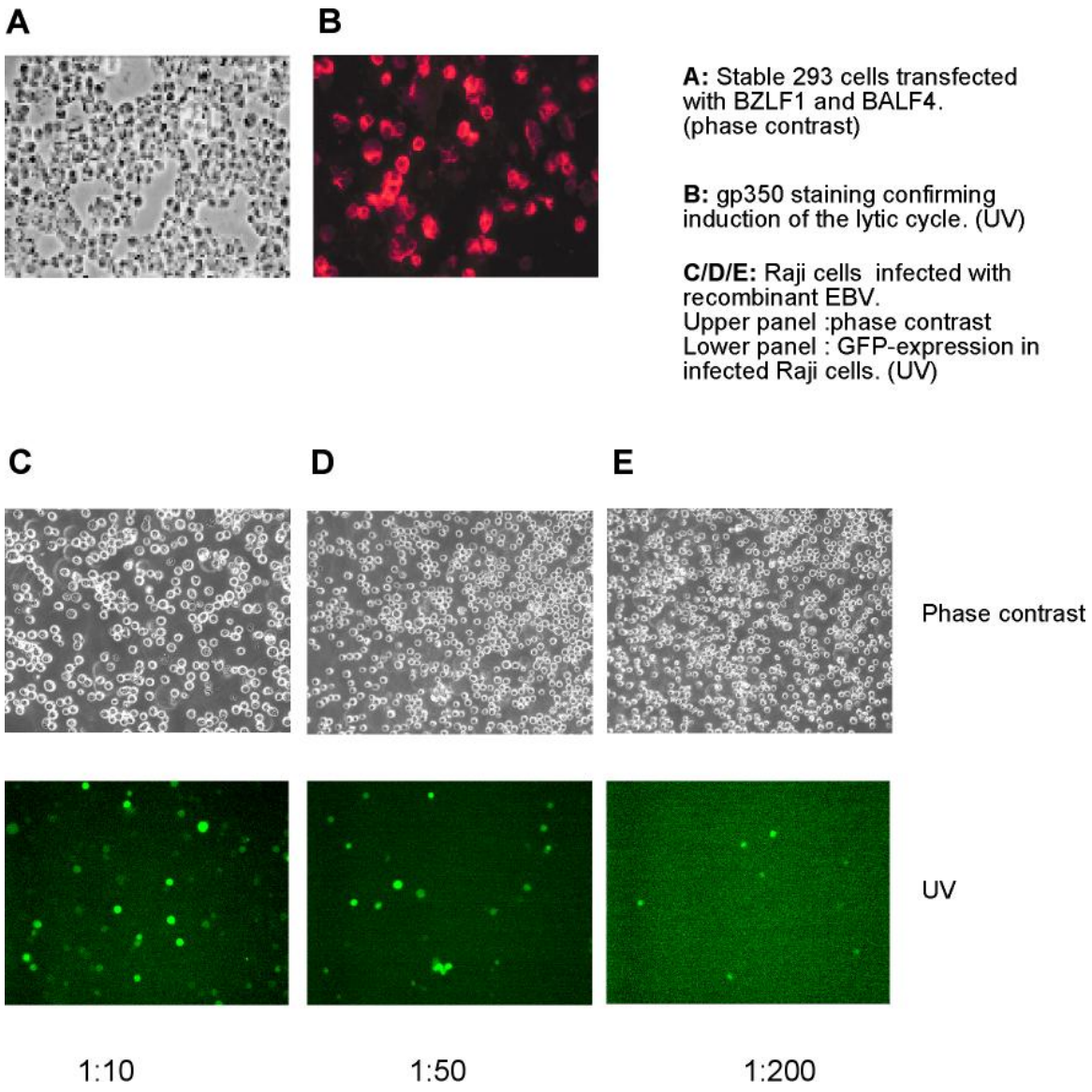


Fig.4.6 Induction of the lytic cycle in 293 cells stably transfected with Maxi-EBV, and GFP expression of infected Raji cells.

(A/B) In stably transfected HEK293 cell line carrying a Maxi-EBV the lytic cycle was induced by transient cotransfection of expression plasmids encoding the genes BZLF1 and BALF 4. After three days, the virus supernatant was harvested, and the cells were fixed on a glass slide and permabilized with acetone. In order to confirm induction of the lytic cycle cells were immunostained with a monoclonal antibody directed against the late viral glycoprotein gp350/220. For visualisation a Cy-3 conjugated secondary antibody was used. The binding of the secondary antibody was detected by an inverted UV fluorescence microscope. The virus supernatant was harvested after three days, and 1ml virus supernatant was incubated with $3\tilde{\times}10^5$ Raji cells at 37°C for 72h.
(C/D/E) The successful infection of Raji cells was visualised by an inverted fluorescence microscope. The number of infectious particles was determined by calculating the number of GFP-positive Raji cells in each dilution, and from this a mean value was calculated. One green Raji unit (GRU) was defined as one green Raji unit pro ml in order to compare the titer of virus supernatants from different Maxi-EBV mutants.

Between $5x10^3$-$1x10^5$ infectious particles /ml were obtained for the different mutants. One green Raji cell was identified as “green-Raji-unit” (GRU), which was defined to equal one infectious particle. These quantified virus supernatants were adjusted relative to one another and used in B-cell immortalisation assays as described below.

4.3 Infection of primary human B-lymphocytes with recombinant EBNA 3C EBVs

4.3.1 Reduced immortalisation efficiency with EBNA 3C mutants compared to wild-type EBV

In three independent experiments human primary B-lymphocytes were extracted from adenoids (described in detail in 3.6.4) and infected with recombinant virus or wild-type virus, obtained from virus producing HEK293 stable cell lines. This procedure is described in detail in 3.6.3. To determine the immortalisation efficiency $1x10^5$ B-lymphocytes were infected with dilutions of the virus supernatants in 96-well-cluster plates (half a plate per dilution step). In order to improve the out-growth of immortalised B-lymphocytes these were cultivated on γ-irradiated human fibroblasts (WI38) also called “feeder cells”. The feeder cells produce unidentified soluble factors that improve the clonal out-growth of EBV infected lymphoblastoid cells (Sugden and Mark, 1977). After six weeks the wells containing proliferating cells were calculated and compared with those that did not show proliferation. When less than 63% positive single events (30 out of 48 wells) containing proliferating B-cells were detected, the theoretical value for calculating the number of GRU per “one hit” was obtained. One hit means one virus particle being responsible for the out-growth of transformed B-cells in one well. In order to determine the immortalisation efficiency, three independent experiments were performed and the amount of virus particles needed for establishing single cell clones was calculated, expressed in GRU per clone needed for “one hit” (Fig.4.6). The virus dilutions were taken into consideration for the statistical calculation of the “one hit” kinetics (Frisan et al., 2001). The immortalisation efficiency was also expressed in percent, where the amount of GRUs needed for immortalisation using wild-type EBV was normalized to 100% and the mutants were placed in relation to this value (Tab.4.1).

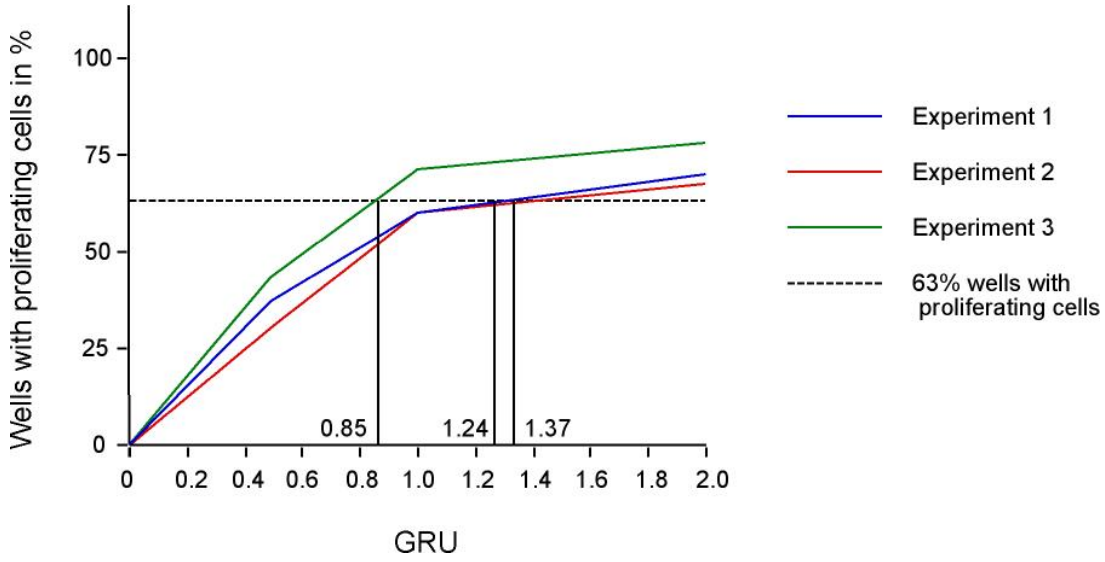


Fig.4.7 Determination of the immortalisation efficiency of the recombinant wild-type virus 2089.

To calculate the immortalisation efficiency of a given adjusted virus stock at least three independent immortalisation experiments were performed. For the evaluation of the experiments dilutions were used and taken into consideration when calculating the theoretical value for the "one hit" kinetics (Frisan et al., 2001). This is the case when 63% of the wells (30 out of 48 wells in half a 96-well-cluster plate) contain proliferating cells. The wells containing proliferating cells were calculated for every dilution step and expressed in percent of total number of wells. These values were plotted graphically versus virus dose (GRU) in a diagram as shown here. The immortalisation efficiency for every single experiment was determined at the intersection point of the curve with the line at 63%. In these three experiments 0.85 GRUs, 1.24 GRUs and 1.37 GRUs were needed to obtain a lymphoblastoid cell clone. The immortalisation efficiency for the wild-type virus 2089 was determined as the mean of these values to yield the value of 1.2 GRUs based on the three experiments shown here.

As expected the reconstituted wild-type Maxi-EBV (p2889) immortalises B-lymphocytes as efficiently as wild-type EBV (p2089) (see Fig.4.7). This finding indicates that the effects seen with the mutants are due to the deletions in the EBNA 3C gene and not an artifact of the manipulation of the EBV genome. Surprisingly, all mutants showed a drastic impairment in their immortalisation efficiencies compared to wild-type EBV. Even the mutant with the two point mutations where the PLDLS motif was changed to ALDAS showed a reduction in immortalisation efficiency to 11.8% suggesting that the PLDLS motif plays an important role in the context of the EBNA 3C gene product.

Maxi-EBV constructs	Green Raji units (GRU) /mutant	Immortalisation efficiency in %	Standard deviation
2089 (wild-type)	2.1	100	-
2889 (reconstituted wild-type)	0.77	119.0	36.5
2919 (lox P flanked EBNA 3C)	1.9	52.6	*) -
2842 (PLDLS-ALDAS)	8.3	11.8	2.7
2841 (Δ aa 408-510)	14.5	6.1	3.2
2840 (Δ aa 212-658)	22.8	4.7	2.6
2839 (Δ aa 410-710)	37.1	3.5	1.2
2837 (Δ aa 658-1013)	75.0	1.8	0.7
2838 (Δ aa 489-970)	92.9	1.5	0.3
2686 (EBNA 3C knock-out)	1000	0	0

Tab.4.1 Immortalisation efficiencies of EBNA 3C Maxi-EBV mutants.

After the establishment of stable HEK 293 cell lines producing recombinant EBNA 3C Maxi-EBVs the virus titers from the different Maxi-EBVs were quantified and adjusted on the basis of the GFP-expression of infected Raji cells. In order to evaluate and adjust the virus supernatants a green Raji-cell was defined as “green Raji unit” (GRU), and also defined as one infectious particle. The first row shows the Maxi-EBV mutants, the second column the number of required GRUs (infectious particles) needed per growth-transformed B-cell clone. These values represent the mean value of at least three independent experiments, except for 2919 for which only one experiment was performed as indicated by (*). In each independent set of experiments the wild-type Maxi-EBV 2089 was used as a reference standard to normalise the data to 100%. In column three the immortalisation efficiency of the EBNA 3C Maxi-EBV mutants are compared to wild-type. Standard deviation for the EBNA 3C mutants are seen in column four.

The mutant 2841 with only a small deletion in the repressor domain and an intact PLDLS motif, growth-transformed B-cells with an efficiency of only 6.1%. The mutant 2839, lacking a part of the repressor domain was characterised by an immortalisation efficiency of 4.7% whereas the mutant 2840 with the complete deletion of the repression domain, showed an efficiency of only 3.5%. The two mutants 2837 and 2838, both lacking the whole activation domain showed the lowest immortalisation efficiencies with 1.5% and 1.8%, respectively. The EBNA 3C knock-out mutant did not yield growth-transformed B-cells at all. These data suggest that the entire gene product is important for the process of B-cell immortalisation.

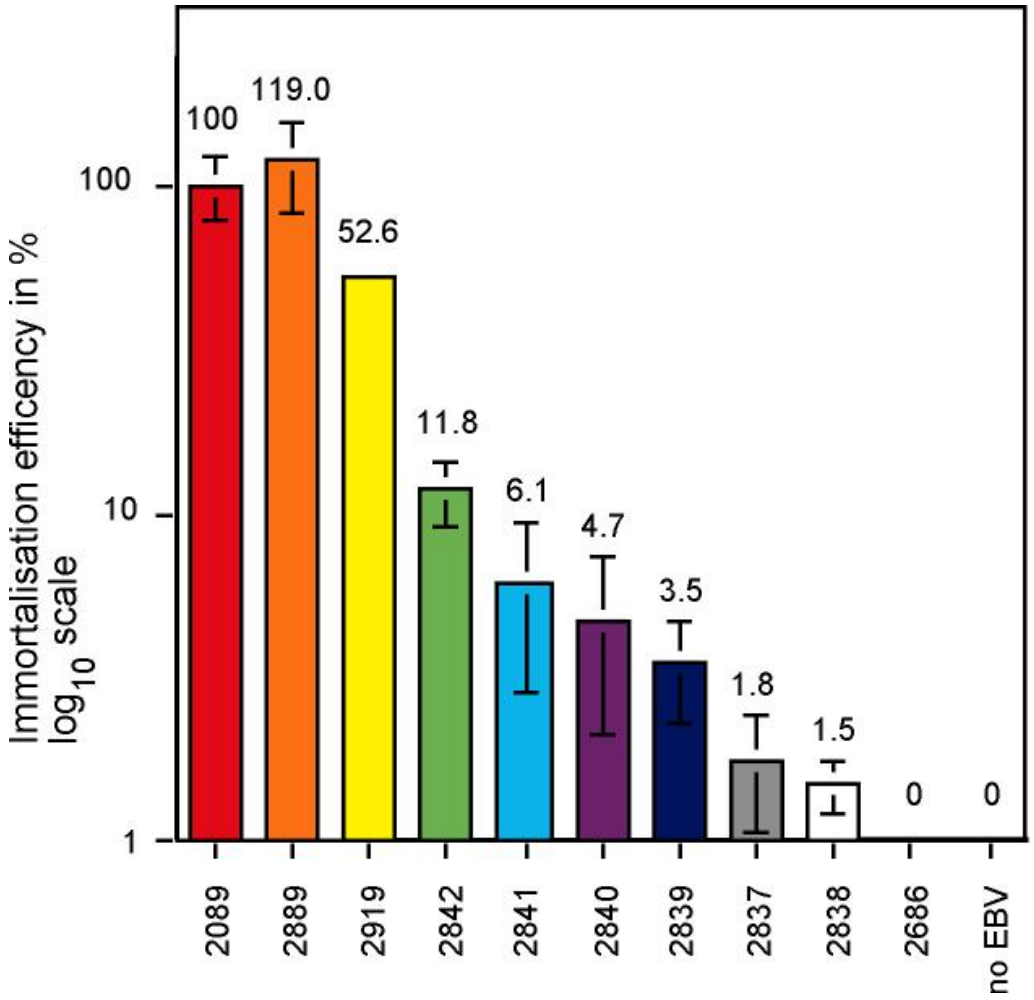


Fig.4.8 Immortalisation efficiency of EBNA 3C Maxi-EBV mutants.

The immortalisation efficiency of the EBNA 3C Maxi-EBV mutants as described in Table.4.1 are shown in this diagram. The y-axis shows the immortalisation efficiency in %. For all mutants three independent experiments were performed except for the Maxi-EBV mutant 2919 where only one was undertaken. The wild-type EBNA 3C virus stock was set to 100%. All the EBNA 3C deletion mutants were heavily compromised in their immortalisation efficiency. Even the two point mutations (PLDLS-ALDAS) in the PLDLS motif led to a drastic decrease in immortalisation efficiency (12%, green bar). As expected the complete knock-out is not able to immortalise B-cells at all. The re-established wild-type Maxi-EBV 2889 immortalises similar to the wild-type 2089. Therefore it could be assumed that the observed effects are due to the deletions in EBNA 3C and not an effect of the homologous recombination event.

4.3.2 Establishment of mutant EBNA 3C LCL clones

After 6 weeks the immortalisation experiments were evaluated. Approximately 20 clones from each mutant were picked from the 96-well-cluster plates and further expanded in the absence of WI38 feeder cells. The genomic phenotype of the mutants could be confirmed by Southern blot analysis after the clones reached a density of $1x10^6$ cells/ml. As described in 3.1 genomic DNA was extracted and digested with *Bgl*II for three hours then a Southern blot analysis was performed using the entire plasmid 2470.7 as a probe encompassing the entire EBNA 3C gene (Fig.4.9A-D). For the mutants 2889, 2919, 2842, and 2841 *in vitro* growth-transformed B-cell lines, also termed lymphoblastoid cell lines (LCLs) could be established. For the both mutants 2889 and 2919 LCLs reached a cell density by which they could be tested in Southern blot analysis after approximately 4 weeks like wild-type. The mutants 2841 and 2842 needed approximately 5 to 6 weeks to obtain the same cell density. For the mutants 2840, 2839, 2838 and 2837, which comprise larger deletions of the EBNA 3C gene, only very few LCLs could be established. In case of the Maxi-EBV mutant 2840 two LCLs were established, the Maxi-EBV mutant 2839 yielded five, 2838 only one, and 2837 two LCLs. After approximately 10 weeks the 2837, 2839 and 2840 LCLs reached a cell density by which the LCLs could be tested, whereas the mutant 2838 needed 13 weeks to obtain the same cell density.

A

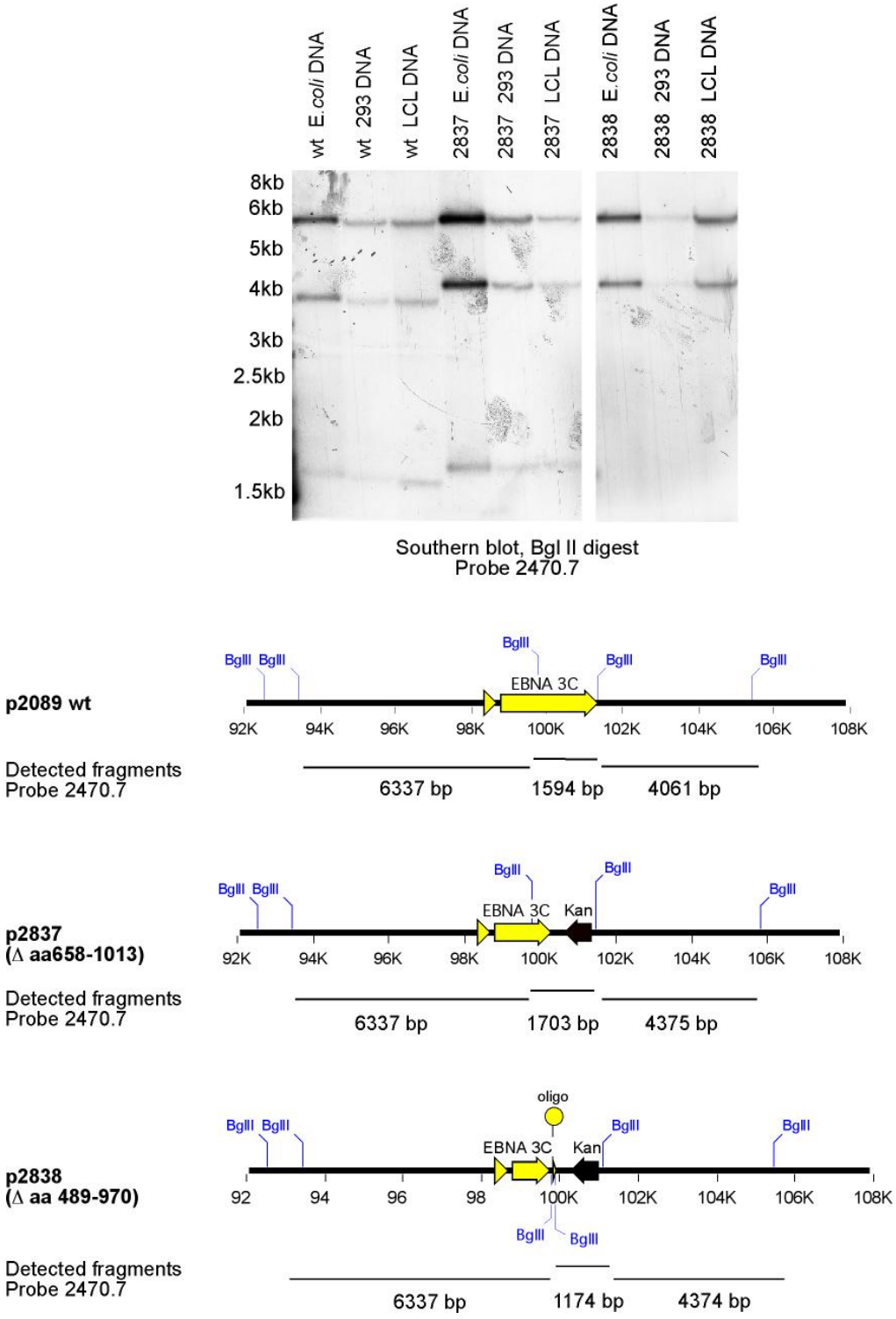


Fig.4.9A Confirmation of the correct genotype of the deletion mutants (2837 and 2838) in LCLs

Fig.4.9A shows the Southern blot analysis of both the mutants 2837 (Δ aa 658-1013) and 2838 (Δ aa 489-970). Recombinant DNA was extracted from E.*coli*, HEK293 producer cell lines and LCLs. As a control wild-type (2089) DNA was used. All DNAs were digested with *Bgl*II, and visualized with a probe detecting the whole EBNA 3C gene (2470.7). The expected fragments for the wild-type and both the mutants 2837 (Δ aa 658-1013) and 2838 (Δ aa 489-970) are shown.

B

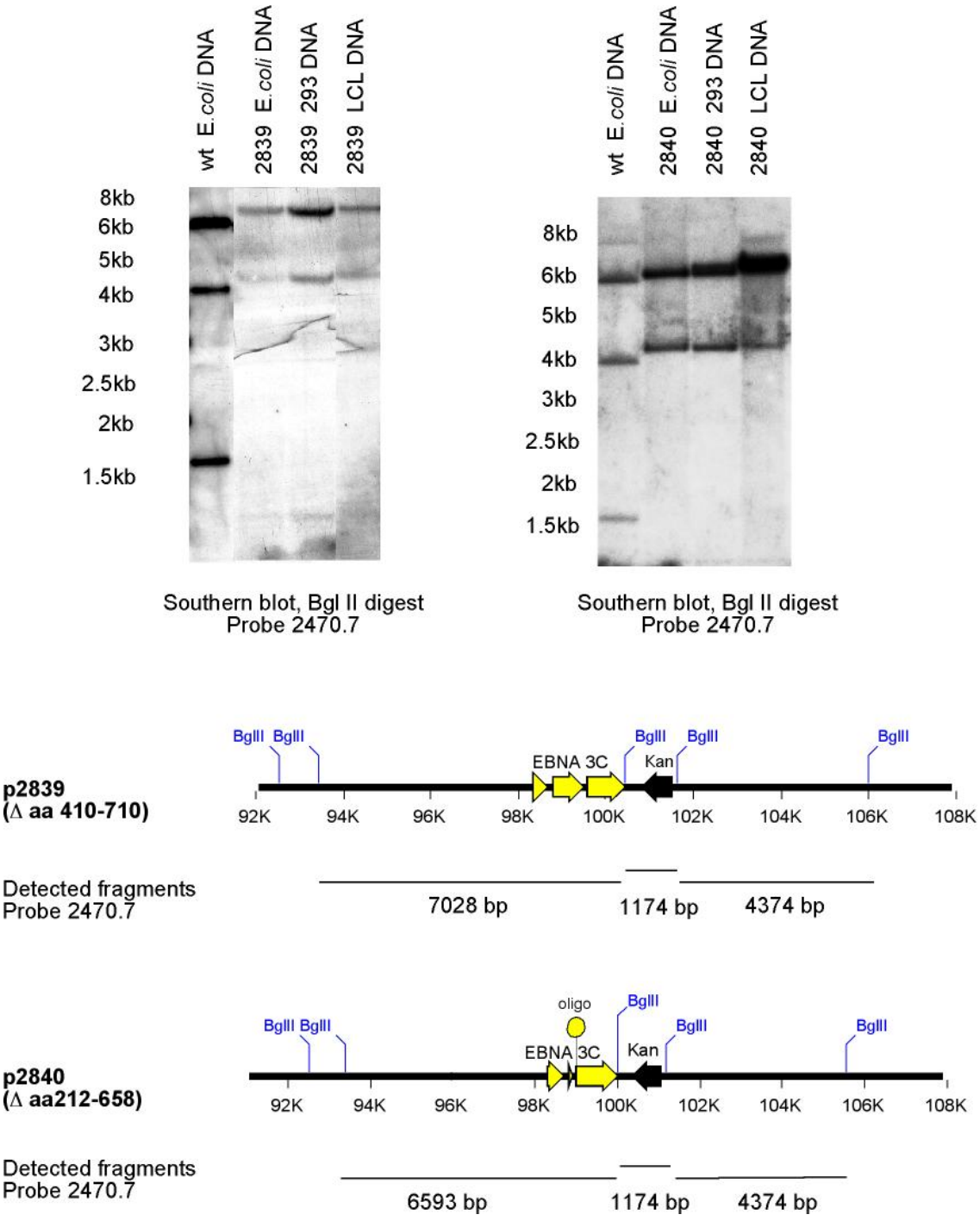


Fig.4.9B Confirmation of the correct genotype of the deletion mutants (2839 and 2840) in LCLs

Fig.4.9B shows the Southern blot analysis of both the mutants 2839 (Δ aa 410-710) and 2840 (Δ aa 212-658). Recombinant DNA was extracted from E.*coli*, HEK293 producer cell lines and LCLs. As a control wild-type (2089) DNA was used. All DNAs were digested with *Bgl*II, and visualized with a probe detecting the whole EBNA 3C gene (2470.7). The expected fragments for the mutants 2839 (Δ aa 410-710) and 2840 (Δ aa 212-658) are shown.

C

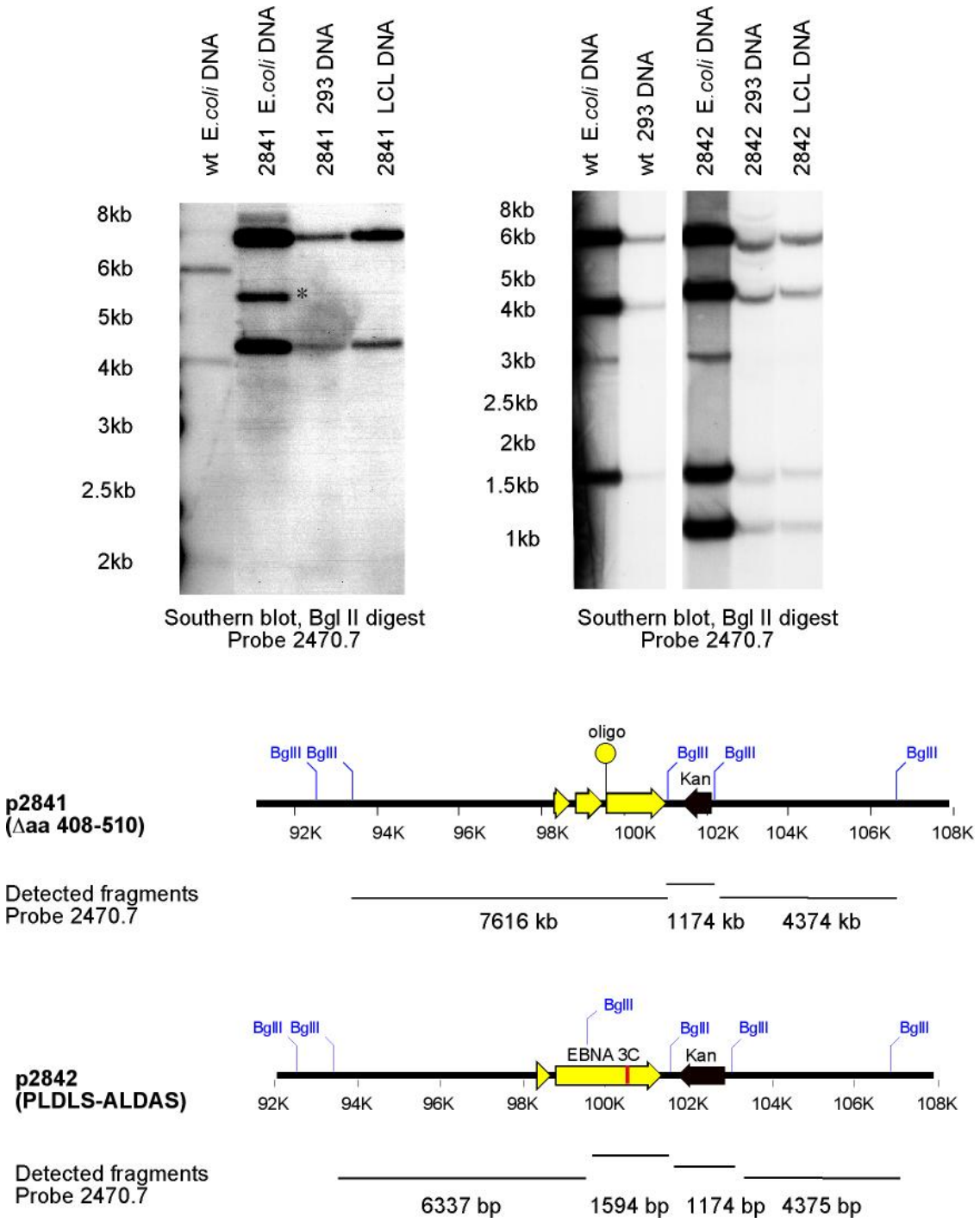


Fig.4.9C Confirmation of the correct genotype of the deletion mutants (2841 and 2842) in LCLs

Fig.4.9C shows the Southern blot analysis of the both mutants 2841 (Δ aa 408-510) and 2842 (PLDLS-ALDAS). Recombinant DNA was extracted from E.*coli*, HEK293 producer cell lines and LCLs, as a control wild-type (2089) DNA was used. All DNAs were digested with *Bgl*II, and visualized with a probe detecting the whole EBNA 3C gene (2470.7). Below the expected fragments for the mutants 2841 (Δ aa 408-510) and 2842 (PLDLS-ALDAS) are shown. (*) this is most likely the result of a partial *Bgl*II digestion.

D

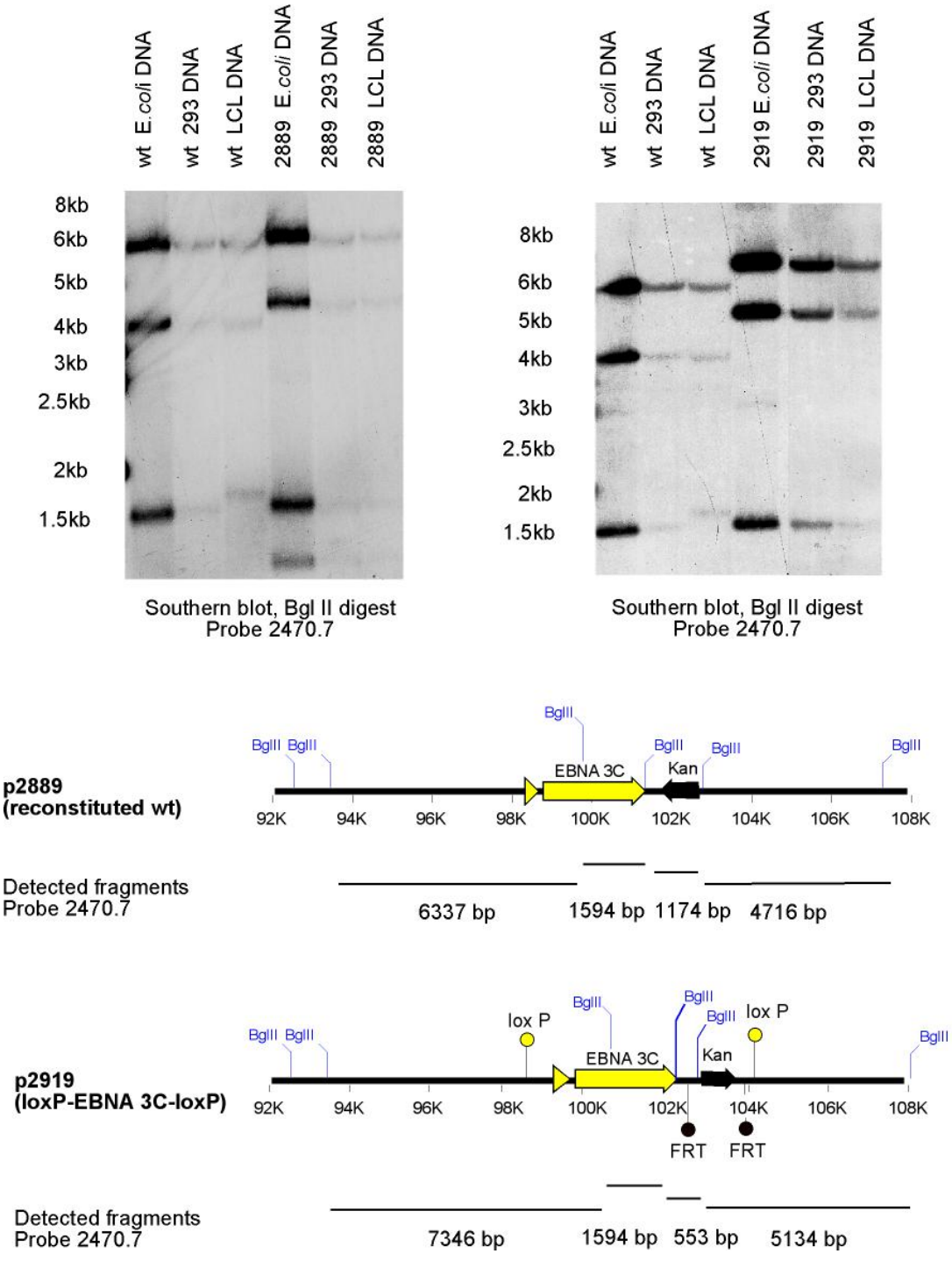


Fig.4.9D Confirmation of the correct genotype of the deletion mutants (2889 and 2919) in LCLs

Fig.4.9D shows the Southern blot analysis of both the mutants 2889 (reconstituted wild-type) and 2919 (lox P flanked EBNA 3C). Recombinant DNA was extracted from E.*coli*, HEK293 producer cell lines and LCLs. As a control wild-type (2089) DNA was used. All DNA were digested with *Bgl*II, and visualized with a probe detecting the whole EBNA 3C gene (2470.7). The expected fragments for the mutants 2889 (reconstituted wild-type) and 2919 (lox P flanked EBNA 3C) are shown.

4.3.2.1 Proliferation phenotype of LCLs carrying the different EBV mutants with deletions in EBNA 3C

During the establishment of the LCL clones infected with the various EBNA 3C Maxi-EBV mutants, differences in the growth behaviour could be observed. The 2842 (PLDLS-ALDAS) mutant and the 2841 (Δ aa 408-510) needed only between 5 and 6 weeks to reach a sufficient cell density, which allowed confirmation of their genome integrity by Southern blot analysis. In contrast, the mutants 2837, 2838 and 2840 needed approximately 10 weeks, and the 2838 (Δ aa 489–970) mutant required thirteen weeks to achieve the same cell density. In order to clarify whether these differences in growth behaviour were maintained after the establishment of LCLs, their proliferative phenotype were observed over a period of 12 days. For this purpose $3x10^5$ cells from every mutant were plated in a 6-well-cluster in a total volume of 3 ml and incubated at 37°C. The cell density was measured regularly, and wild-type LCLs (2089) were used for comparison. As seen in Fig.4.10, all mutants grew much slower than the wild-type control 2089. Small differences in proliferative behaviour could be seen between the mutants after twelve days. The differences in time needed for establishing LCLs was not reflected in their proliferative growth behaviour.

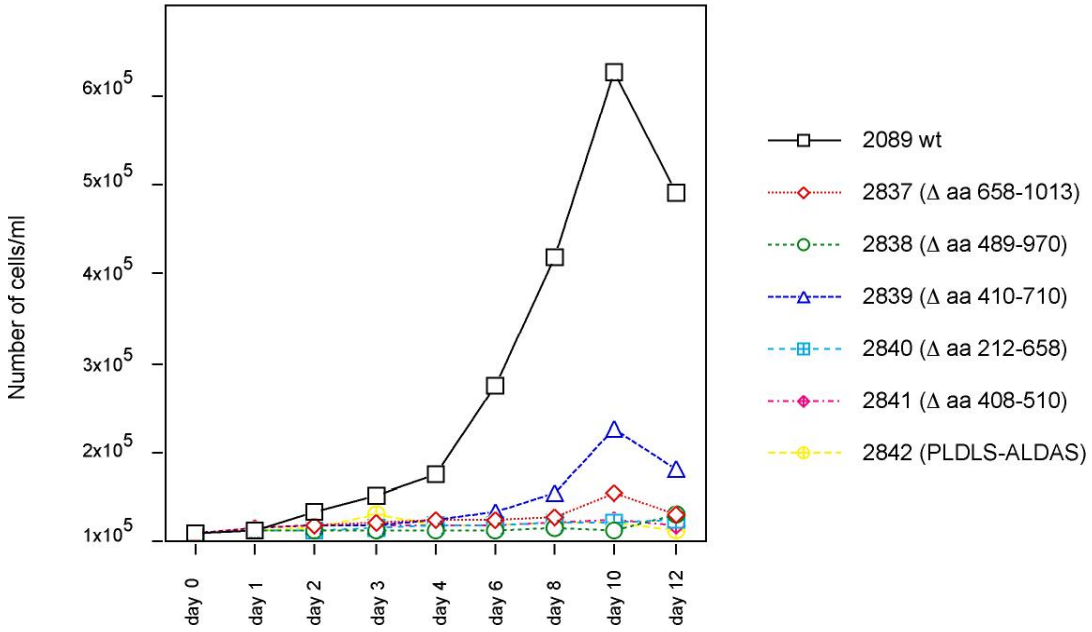


Fig.4.10 Growth curve of established LCL mutants

This figure shows the growth of the different LCLs carrying recombinant EBVs with the EBNA 3C deletion mutants. The wild-type LCL clone 2089 was used as a control. From each cell line $1x10^5$ cells/ml were plated and their density was measured at a regularly basis.

4.3.3 Expression of EBNA 3C in the established LCLs carrying mutant EBNA 3C

To confirm expression of the mutant EBNA 3C proteins the established LCLs were investigated by Western blot immuno detection of EBNA 3C. The protein extracts were measured using the Bradford method and equal amounts of total protein extracts (100μg/lane) were loaded. The EBNA 3C deletion mutants were detected with a polyclonal antibody directed against EBNA 3C and β-actin was used as a loading control. For each mutant the expected molecular weight of the recombinant protein was theoretically calculated. For the 2837 mutant (Δ aa 658-1013) and the 2838 mutant (Δ aa 489-970) the molecular weights of the recombinant proteins were calculated as 73.3 kDa and 60.7 kDa, respectively. For both mutants it was not possible to detect any recombinant protein although the same amount of protein was loaded as for the other mutants. Most likely the major epitopes, which the polyclonal antibody recognizes have been deleted in these two mutants. For the 2839 mutant (Δ aa 410-710) the molecular weight was calculated as 79.9 kDa and a band is seen at approximately 80 kDa. For the 2840 mutant (Δ aa 212-658) the molecular weight was calculated as 63.5 kDa, and indeed a band at about 65 kDa could be detected. Since a weak band at the same position is also seen with the other mutants, it is not clear whether this band is specific for mutant 2840 or not. Since the band seen for 2840 is more pronounced compared to other lanes and equal amounts of total protein were loaded, I conclude that this band is EBNA 3C specific. There is also an additional band seen at about 55 kDa for the 2840 mutant, this may be a degradation product. The molecular weight of mutant EBNA 3C in 2841 (Δ aa 408-510) was calculated as 100.5kDa. And indeed, a week band at approximately 100 kDa was detected, together with a strong signal at approximately 78 kDa. The band at 100 kDa is thought to be the recombinant protein, and the band at 78 kDa may be a degradation product. The mutant 2842 with the point mutation of two amino acid residues within the PLDLS motif expresses recombinant EBNA 3C with a molecular weight similar to that of the wild-type protein (112.2 kDa). The upper of the two bands is thought to be EBNA 3C and the lower of the two may again be a degradation product.

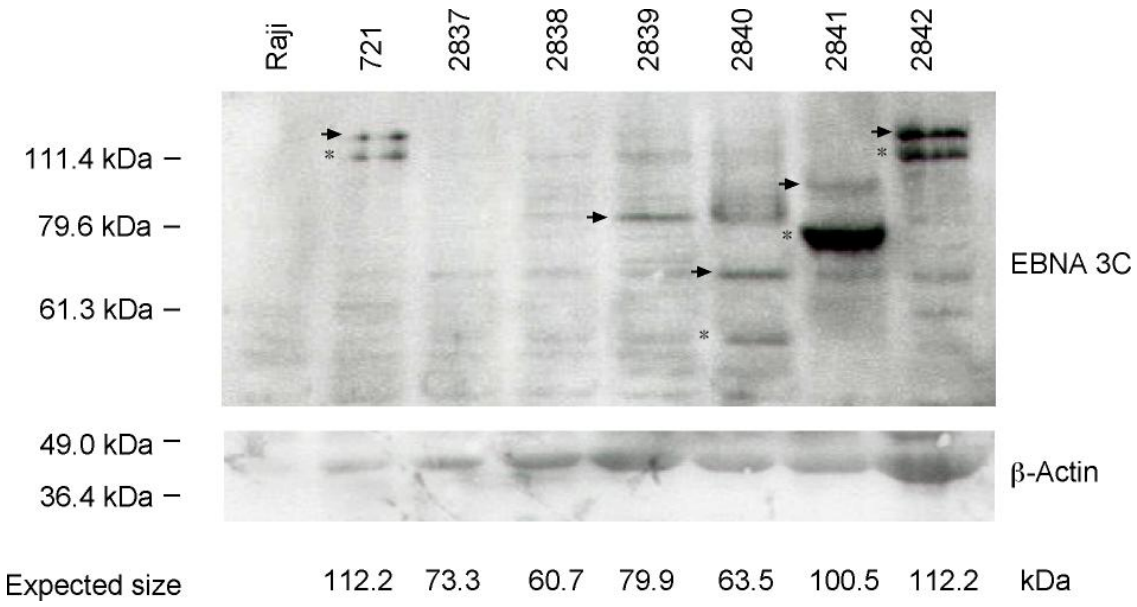


Fig.4.8 Confirmation of mutant EBNA 3C protein expression in established LCLs.

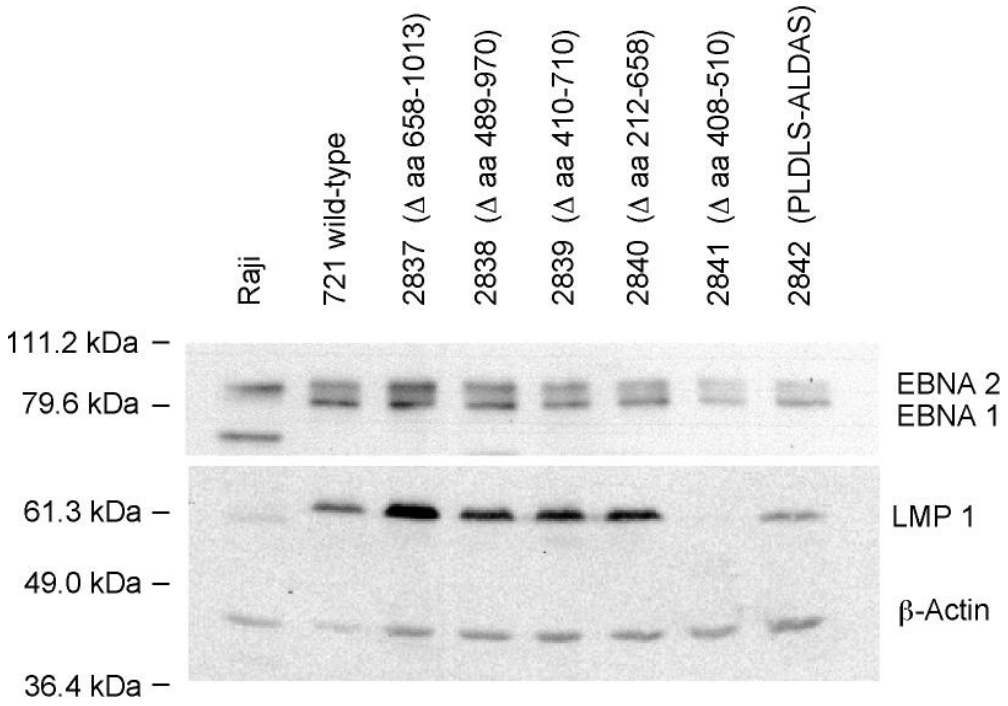
The upper panel of the figure shows the protein expression of mutant EBNA 3C in LCLs compared to an LCL infected with BGT.P wild-type EBV (721). The lower panel shows the detection of β-actin as a loading control. As a negative control the Raji cell line, which does not express EBNA 3C, was used. The 721 cell line was used as a positive control for EBNA 3C expression. For the two mutants 2837 (Δ aa 658-1013) and 2838 (Δ aa 489-970) no specific bands could be observed. For the mutant 2839 (Δ aa 410-710) a band was seen at about 80 kDa and for the 2840 mutant (Δ aa 212-658) a band was detected at about 65 kDa. The 2841 mutant (Δ aa 408-510) showed a weak band at approximately 100 kDa, which is probably the recombinant protein and a strong band at 78 kDa, which is probably a degradation product. The 2842 mutant (PLDLS-ALDAS) showed a band migrating at approximately 112 kDa, as expected. Bands marked with a (*) are probably degradation products.

4.3.4 EBNA 3C deletion mutants alter the expression of EBNA 1, EBNA 2 and LMP 1

EBNA 3C has been reported to act as a transcriptional activator as well as repressor. In addition EBNA 3C was found to cooperate with EBNA 2 in the induction of the LMP1 promoter through PU.1 (Zhao and Sample, 2000). Therefore it was of interest to analyse the influence of the EBNA 3C deletion mutants on the expression of EBNA 2 and LMP 1 by Western blot analysis. The number of EBNA 1 molecules in any LCL line is very stable and independent of the EBV genome copies present per cell (Sternas et al., 1990). Therefore, the expression of EBNA 1 was also investigated and used as a second control. From each LCL clone carrying the different EBNA 3C mutants, protein extracts were prepared and measured using the Bradford method. From each mutant LCL 50 μg protein extract was loaded on a gel. As a standard

loading control the cellular protein β-actin was detected. For the analysis of the LMP 1 protein a monoclonal mouse antibody was used, and for the detection of EBNA 1 and EBNA 2 two monoclonal rat antibodies were used.

A



B

	721	2837	2838	2839	2840	2841	2842
EBNA 2	100 %	50%	40%	30%	30%	20%	10%
EBNA 1	100%	40%	40%	30%	30%	10%	10%
LMP 1	100%	90%	60%	60%	60%	‹5%	20%

Fig.4.12 Investigation of EBNA 1, EBNA 2, and LMP 1 expression in established LCLs carrying the Maxi-EBV EBNA3C deletion mutants.

(A) The established LCLs with mutant EBNA 3Cs were tested for protein expression of EBNA 1, EBNA 2, and LMP 1. As wild-type control the 721 LCL was used. Detection of β-actin was used as a loading control. EBNA 2 migrates with an approximate weight of 85 kDa, EBNA 1 at 80 kDa and LMP 1 at 63 kDa. (B) Phosphor-imager evaluation of the bands for the approximation of protein signals. All values were normalised to β-actin, and the value for wild-type (721) was set to 100%.

In Fig.4.12A the protein expression of EBNA 2, EBNA 1, and LMP 1 is shown for the LCLs with the different mutant EBNA 3Cs. As a loading control β-actin was used, and 721 served as a wild-type control. The Western blot analysis was carried out several times, and here one experiment is shown. In Fig.4.12B the strength of the protein signal in the Western blots was evaluated using a phosphor-imager taking all the experiments in consideration. The values obtained were all normalised to β-actin, which was used as a loading control. The calculated values for each mutant (approximated with the phosphor-imager) were compared to a reference LCL infected with wild-type EBV (721). The value for 721 was set to 100%. The mutants 2837 (Δ aa 658-1013), 2838 (Δ aa 489-970), 2839 (Δ aa 410-710), and 2840 (Δ aa 212-658) all showed a comparable expression pattern, expressing only between 30-50% of EBNA 2 and 30-40% of EBNA 1 compared to the wild-type situation (721). Furthermore, these mutants expressed between 60-90% of LMP 1 compared to wild-type. More interesting are the mutants 2841 (Δ aa 408-510) and 2842 (PLDLS-ALDAS), which expressed only 20% and 10% of EBNA 2, respectively, as compared to wild-type. Mutant 2842 expressed 20% of LMP 1 and the 2841 mutant only to ‹5% compared to wild-type. A surprising observation was the fact that the expression of EBNA 1 is also altered since it is normally expressed at the same level independent of the number of EBV copies in the LCL clone (Sternas et al., 1990). For the 2841 and 2842 mutants EBNA 1 is only expressed at a level of approximately 10% compared to wild-type. This suggests that EBNA 3C influences the transcription of EBNA 1 as well as EBNA 2 and LMP 1.

4.3.5 EBNA 3C knock-out mutant EBV does not yield LCLs.

It was reported that EBNA 3C is essential for the B-cell immortalisation process with a mutant which has a stop codon at amino acid coordinate 365 of EBNA 3C (Tomkinson et al., 1993). One disadvantage of their approach is that it is unknown what effect the introduction of a stop codon may have on the phenotype since it cannot be estimated what influence the truncated protein may have. The advantage of using the Maxi-EBV approach to establish an EBNA 3C complete knock-out is that no manipulation has been made within the gene. Using the EBNA 3C knock-out EBV mutant the finding of Tomkinson et al. was confirmed since it was not possible to obtain a single LCL. Infection of primary B-cells with EBV is associated with a

dramatic change in their morphology and growth behaviour. The cells become activated, loose their round small size, become large, irregular in shape and strongly adhere to each other forming large clumps.

Immortalisation experiments were performed as described in 3.6.5 using the complete EBNA 3C knock-out mutant (2686). These infection experiments were carried out with different dilutions of virus supernatant, using 100 GRUs (Green Raji Units), 500 GRUs and 1000 GRUs per 96-well to infect $1x10^5$ B-cells, respectively.

After 15 days the B-cells infected with wild-type virus show a change in their morphology and formed large clumps indicating proliferation. In addition, the cells in these clumps are expressing the GFP gene indicating successful infection with the wild-type Maxi-EBV 2089. For the wild-type infected B-cells GFP positive cells could for the first time be detected after approximately 7 days, and the first small clumps were observed after 10 days. In contrast to these observations B-cell infected with EBNA 3C knock-out virus does not show any change in their morphology and proliferation until day 15, although single GFP expressing cells were observed for the first time indicating successful infection with the EBNA 3C knock-out virus. After 20 days post-infection the B-cells infected with the EBNA 3C knock-out virus now showed a similar clumping phenotype as cells infected with wild-type EBV. Although the clumps are growing much slower they are also expressing the GFP gene indicating that they are successfully infected with EBNA 3C knock-out virus. In nearly all the wells infected with 1000 GRUs this phenotype was observed. For the B-cells infected with 500 GRUs per well approximately half of the wells showed this phenotype, and with 100 GRUs per well only a few wells could be found. A dose dependency was observed indicating a lower efficiency. In contrast only 1 GRU wild-type virus per well was needed to obtain proliferation in all wells. Three to six weeks after infection the EBNA 3C knock-out cells arrested and died, any attempt to expand the individual proliferating B-cells failed. This initial proliferation raised the question, whether the immortalisation process can be divided into two consecutive phases: an initial phase and a stable maintenance phase. Hence it could be possible that EBNA 3C is essential for the maintenance of the growth-transformed phenotype of infected B-cells, but not for their initial proliferative phase during the first few cell

doublings. This model would explain the initial proliferation seen in Fig 4.13 and the subsequent failure to expand the infected cells any further.

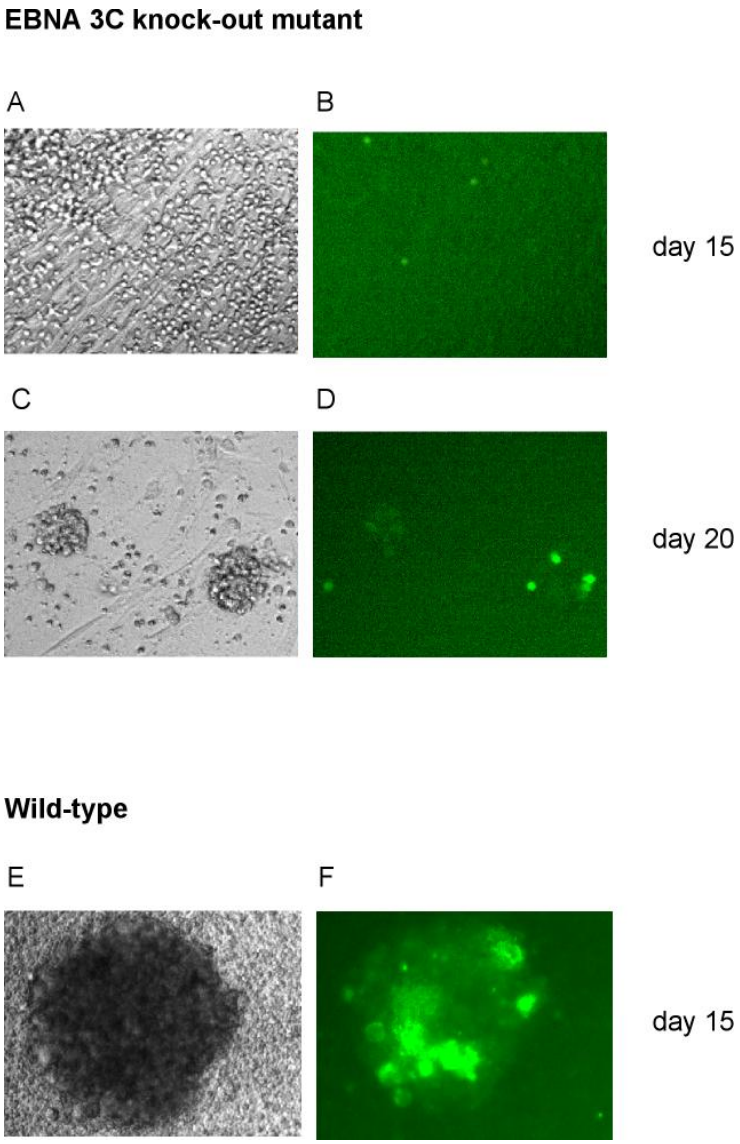


Fig.4.13 Infection of primary B-lymphocytes with the EBNA 3C knock-out Maxi-EBV.

This figure shows one well of a 96-well-cluster plate of an immortalisation experiment (as explained in 3.6.5) with the EBNA 3C knock-out mutant, and one well of the wt control. (A) shows one well of a 96-well cluster plate 15 days post-infection with the EBNA 3C knock-out Maxi-EBV. (B) The same well is illuminated by UV light, B-cells infected with recombinant EBV are green due to the expression of GFP. (C) Shown is the same well as in (A) now 20 days after infection. The B-cells displayed the typical phenotype of proliferating B-cells, but after this initial growth they arrested and died within 6 weeks. In (D) the UV picture of the same well as in (C) is seen. (E) shows the B-cells infected with wild-type virus 15 days post-infection, and (F) is the same well visualised by UV illumination.

4.4 Generation of an inducible system for the investigation of the importance of EBNA 3C in the initiation or maintenance phase of B-cell immortalisation

4.4.1 Generation of an inducible EBNA 3C knock-out system

For investigation of the role of EBNA 3C in the initiation or maintenance phase of B-cell immortalisation, a Cre/lox P conditional on/off system was employed. Described in the following part is the Cre/lox P system, the generation of the lox P flanked EBNA 3C gene, and the retroviral vector expressing the Cre protein.

4.4.1.1 The Cre/loxP system

In Fig 4.14 the lox P sequence is shown. This sequence contains an eight base pair long core-sequence flanked with the 13 base pair inverted repeats, which are used by the Cre recombinase as recognition sequence (Hoess et al., 1990; Mack et al., 1992).

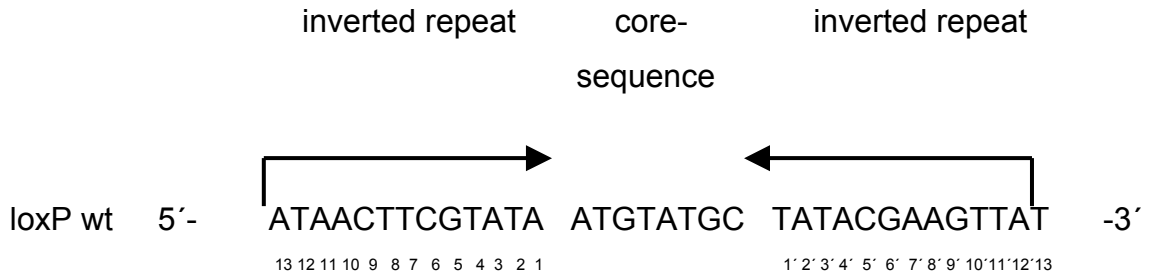


Fig.4.14 The lox P sequence used in the 2919 mutant.

This figure shows one of the two lox P sequence motives used in the 2919 Maxi-EBV mutant. A lox P sequence consists of a core sequence, which is flanked by two inverted repeats. The different parts are separated through a blank space. The arrows indicate the direction of the inverted repeats, and the numbers indicate the nucleotide position starting from the core sequence.

The Cre/lox P recombinase system from bacteriophage P1 can be used in prokaryotic as well as eukaryotic cells and is frequently employed to generate conditional mutants (Dymecki, 2000; Torres and Kühn, 1997). The recombination is only dependent on the Cre protein and the presence of lox P sequence motifs and is absolutely reliable. When two lox P sites are orientated in the same direction the

nucleotide base pairs in between are excised and deleted to form a circle, which is eventually lost. Only one functional lox P site is left at the location where the site-specific recombination took place. The reverse reaction is also feasible. When one lox P site is located on one plasmid and one lox P site on another, the two plasmids merge into one. Both systems were used in my work.

4.4.1.2 The conditional Lox P flanked EBNA 3C Maxi-EBV mutant

In Fig.4.15 the steps leading to the conditional lox P flanked mutant are shown. The already existing Maxi-EBV EBNA 3C knock-out mutant where the EBNA 3C gene was replaced with the Lox P flanked tetracycline gene was transformed into DH10B together with the Cre expression plasmid 2701. In E.*coli* the tetracycline gene was deleted upon Cre expression resulting in a single lox P site in the Maxi-EBV genome. This single lox P site served as the target sequence to incorporate the entire plasmid 2957 via site-specific Cre recombination. The plasmid 2957 carries upstream of the EBNA 3C gene the origin of DNA replication from the OriR6K-2 plasmid and downstream of the EBNA 3C gene the kanamycin resistance gene and a single lox P site. Since the OriR6K origin of DNA replication only replicates in bacteria that express the PIR protein, this plasmid is replication deficient in DH10B. Only clones can grow in the presence of kanamycin, which carry the p2957 plasmid integrated into the context of the Maxi-EBV via the single lox P site to confer double resistance against chloramphenicol and kanamycin. After site-specific recombination a Maxi-EBV plasmid was obtained which carries the lox P site, the OriR6K plasmid upstream the EBNA 3C gene followed by the kanamycin resistance gene and a second lox P site (Fig.4.15). The clones were tested with Southern blot analysis for the correct genotype as seen inFig.4.9A-D. DNA was prepared and transfected into HEK293 cells to establish single cell lines. Genomic DNA was tested with Southern blot analysis. After confirmation of the correct genotype, the EBV lytic cycle was induced to allow virus production and the virus stock was used to infect human B-lymphocytes as described in 4.2.1, from which LCLs could be established.

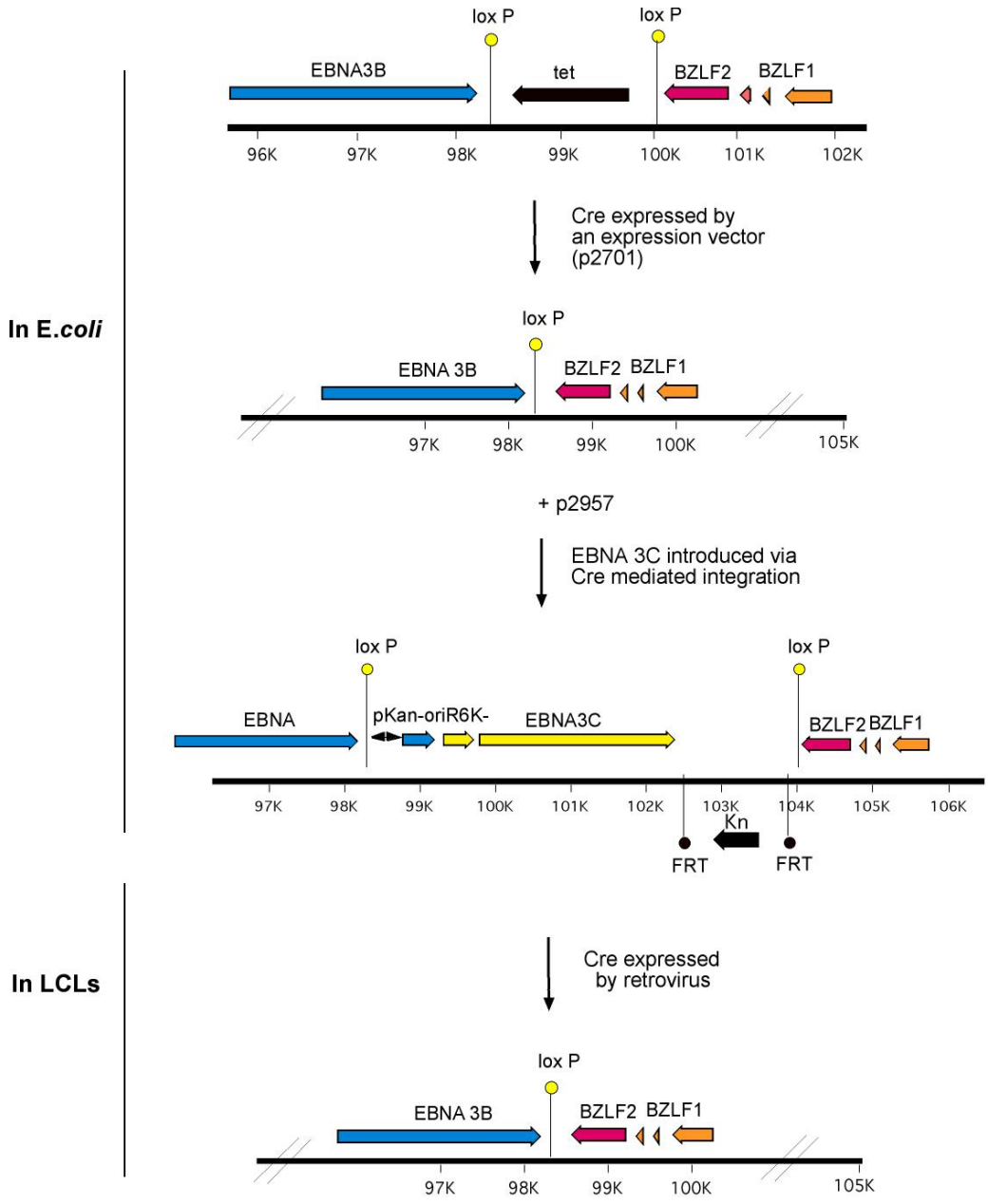


Fig.4.15 Establishment of a conditional EBNA 3C knock-out mutant in LCLs via Cre/lox P recombination.

In this figure the process is described in which the conditional EBNA 3C knock-out Maxi-EBV in LCLs was established. In the E.*coli* strain DH10B the EBNA 3C knockout Maxi-EBV mutant p2686 was introduced together with the Cre expression plasmid p2701 whereby the tetracycline resistance gene was lost upon Cre/lox P recombination leaving one lox P site. A plasmid (p2957) carrying the origin of replication from the plasmid oriR6K, the EBNA 3C gene, the kanamycin resistance gene, and one lox P site was introduced into bacteria carrying the Maxi-EBV mutant with the EBNA 3C knock-out. As expected the whole plasmid (p2957) was inserted into the Maxi-EBV genome through Cre/lox P-mediated recombination. The plasmid p2957 carries the OriR6K origin of replication that only replicates in the presence of the PIR protein, and hence does not replicate in the DH10B E.*coli* strain. From the Maxi-EBV plasmid containing the lox P flanked EBNA 3C gene a stable HEK293 producer cell line was established, virus production was induced, and LCLs were established as described in the chapter 4.2.3 through 4.3.2. The established LCLs were then infected with a retroviral vector expressing the Cre protein, to establish a conditional knock-out mutant of EBNA 3C in LCLs.

These LCLs were also tested in Southern blot analysis for the correct genotype (see Fig.4.9D). To excise the EBNA 3C gene a Cre encoding plasmid had to be introduced into the LCLs to express Cre. Since the LCLs are not easily transfected an alternative approach had to be found.

4.4.1.3 Expression of the Cre protein in LCLs using a retroviral vector

LCLs are difficult to transfect, since electroportation or other standard methods result in only a few percent successfully transfected cells. As an alternative method infection with a retroviral vector could be used instead. Retroviruses can infect several types of mammalian cells, but it was unclear if they infect LCLs efficiently. A retroviral vector (3087) was generated for retroviral vector production and infection of LCLs to express the Cre protein. The retroviral gene vector contains two long terminal repeats (LTRs), the Cre gene, an IRES cassette, which was followed by the monomeric red fluorescence protein (mRFP) ORF (Campbell et al., 2002) for detection and selection in cell culture (Fig 4.16). As a control, the vector p3067 was generated, being identical to p3087 but lacking the Cre ORF (Fig 4.16).

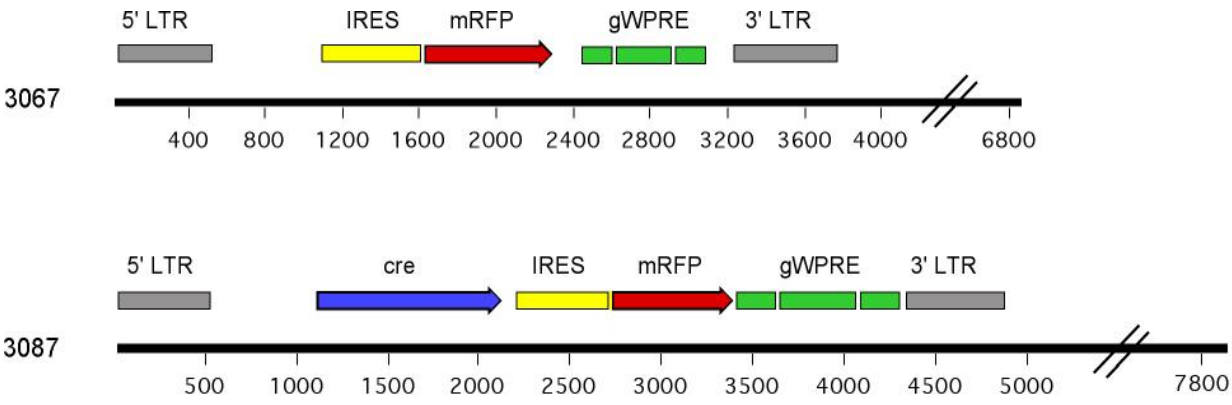


Fig.4.16 Design of the retroviral vector for Cre expression after infection of LCLs.

The Cre expressing retroviral vector 3087 was used to infect and express the Cre protein in LCLs containing the lox P flanked EBNA 3C Maxi-EBV genome (2919). The 3087 proviral vector genome bracketed by modified Moloney leukemia virus long terminal repeats (LTR) (grey bars) contains the coding sequence of the Cre protein (blue arrow), an IRES cassette (yellow bar), the ORF for mRFP (red arrow), and the woodchuck hepatitis virus posttranscriptional regulatory element WPRE (green bars). The control vector 3067 depicted above is identical to 3087 with the exception of the Cre ORF, which is missing in 3067.

Both constructs were separately transfected into Phoenix cells together with expression vectors for gag/pol and GALV. For packaging of the retroviral RNA, the capsid protein and the reverse transcriptase expressed by gag/pol are needed. The GALV glycoprotein is incorporated into the viral envelope and enables the infection of human cells. After two days, the virus supernatant was collected from Phoenix cells and concentrated by ultracentrifugation (1:30). These virus stocks were used to infect LCLs carrying the lox P flanked EBNA 3C Maxi-EBV (2919).

4.4.1.4 Infection of B-cells with recombinant retrovirus

First it was analysed if the retroviral vectors 3067 and 3087 can infect B-cells. For this purpose $2x10^5$ Raji cells were infected with 150 μl retrovirus supernatant, which had been concentrated 1:30 by ultracentrifugation. Shown in Fig.4.17 are the retrovirus-infected B-cells two days post infection, the left panel shows mock infected cells, the middle panel B-cells infected with the retroviral control vector (3067), in the right panel are the B-cells infected with the Cre expressing retroviral vector (3087). Top panels are seen with phase contrast, and below the corresponding cells seen through UV light. The expression of mRFP was indicative of infected cells with the different retroviral vectors. The Cre expressing vector 3087 infects the Raji cells to approximately 10% whereas the control vector (3067) infects approximately 40% (Fig.4.17 lower panel). The difference in infection efficiency could be caused by a detection problem of the mRFP. The Cre gene upstream the IRES cassette could influence the expression levels of mRFP. Another possibility is that the genome containing the Cre ORF is not as efficiently packaged as the genome for the control vector due to its larger size.

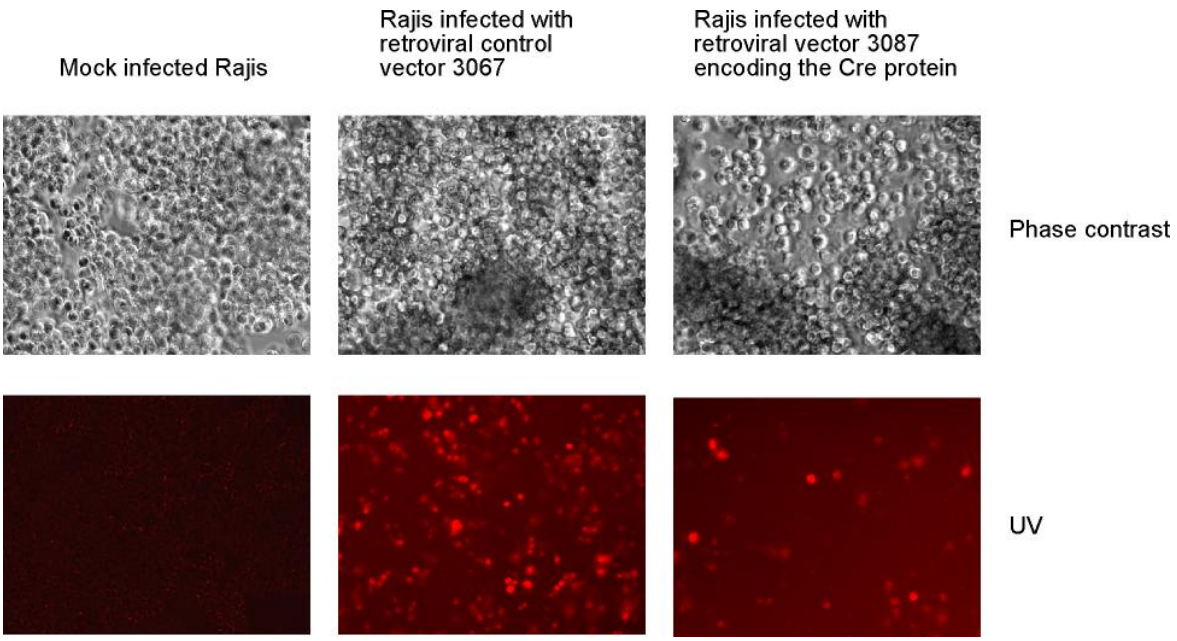


Fig.4.17 Recombinant retroviruses successfully infect Raji cells.

Raji cells were incubated with recombinant retroviruses to determine if these vectors can infect B-cells. To produce recombinant retrovirus supernatant Phoenix cells were transiently transfected with the retroviral vector plasmid together with the expression plasmids for the retroviral gag/pol and GALV proteins. After 12h and 24h the supernatant was collected and concentrated (1:30) by ultracentrifugation. On the left Raji cells are shown which are mock infected, in the middle panel Rajis were infected with the control vector 3067, which only contains the IRES cassette and the coding mRFP sequence, on the right Rajis are seen that were infected with the 3087 construct containing the Cre gene followed by the IRES cassette and the mRFP ORF shown in Fig.4.16.

The top panels show photo micrographs taken with phase contrast with visible light and the lower panels show the same frames taken under UV light with an inverted fluorescence microscope. Successfully infected Raji cells are seen in red in the lower panel. Infection efficiency drops to about 10% from about 40% infected Raji cells when the retroviral vector contains the Cre coding sequence upstream of the IRES cassette.

4.4.1.5 Confirmation of Cre expression and EBNA 3C deletion

EBV genomes in which the EBNA 3C gene was excised after Cre recombination were detected with PCR amplification. Three sets of primers were designed, the Rec A and B primer pairs and the wild-type primer pair seen in Fig.4.18. In case of Cre-mediated recombination and hence loss of the EBNA 3C gene a 803 bp fragment would be detected with the Rec A primer pair and a 510 bp fragment with the Rec B primer pair, and for the wild-type primer pair a 309 bp fragment indicative of non-recombined EBV genomes would be generated by PCR amplification.

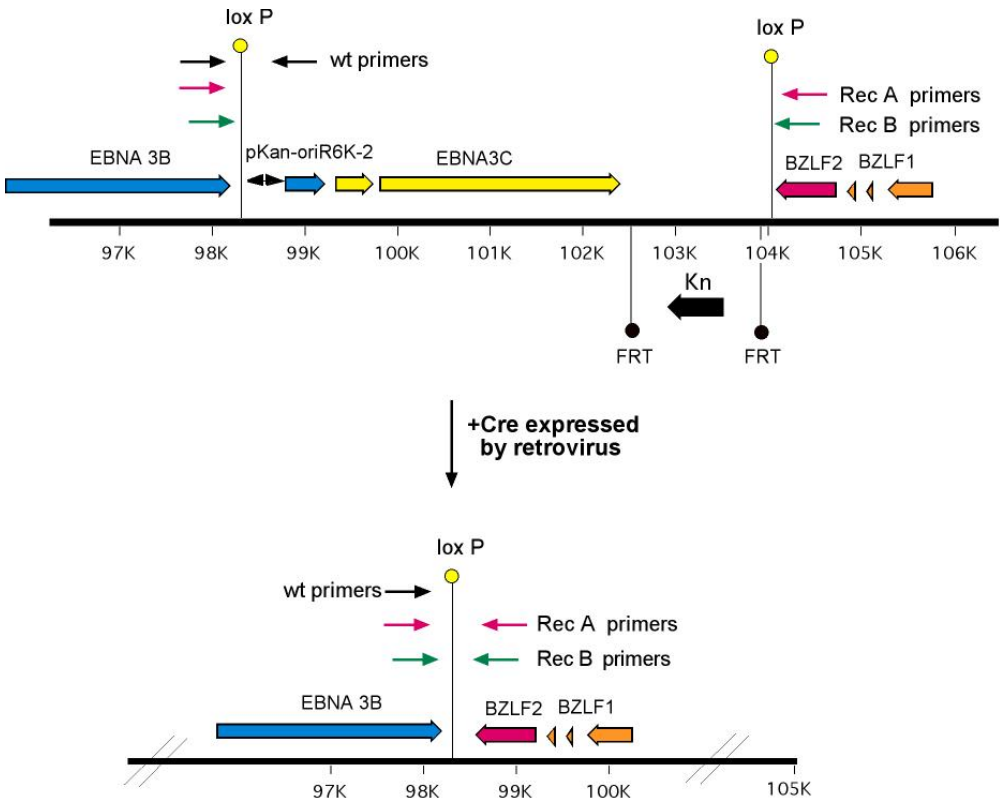


Fig.4.18 Primers used for identification of successfully Cre-mediated deletion of EBNA 3C in Maxi-EBVs.

In this figure the primer pairs Rec A and Rec B for detection of Cre-deleted EBNA 3C in the Maxi-EBV genomes are seen. The wild-type primer pair for detection of non-recombined parental Maxi-EBV genomes are also shown. The top of the figure shows a wild-type Maxi-EBV genome and the wild-type primer pair (black arrows), that will generate a PCR product of approximately 309bp. Below a Maxi-EBV genome is seen that has undergone Cre-mediated recombination, the Rec A primer pair (red arrows) will generate a 803bp fragment and the Rec B primer pair (green arrows) will generate a 510bp fragment in a PCR amplification indicative of successful Cre recombination and establishment of an EBNA 3C knock-out mutant.

First, the detection limit of the primer pairs Rec A and Rec B was tested. To make the experiment as realistic as possible 50 ng genomic wild-type LCL DNA was mixed in each reaction, together with recombined DNA extracted from E.*coli* in dilutions (3 ng, 30 pg, 3 pg, 0.3 pg, 30 fg). In Fig.4.19 the PCR amplification performed with the template mixture described above are seen in lane 1 to 5 for each primer pair. With both the Rec A and Rec B primer pairs it was possible to detect EBV genomes, that had undergone Cre mediated recombination. Since the Rec A primer pair was more sensitive it was used in further PCR amplifications.

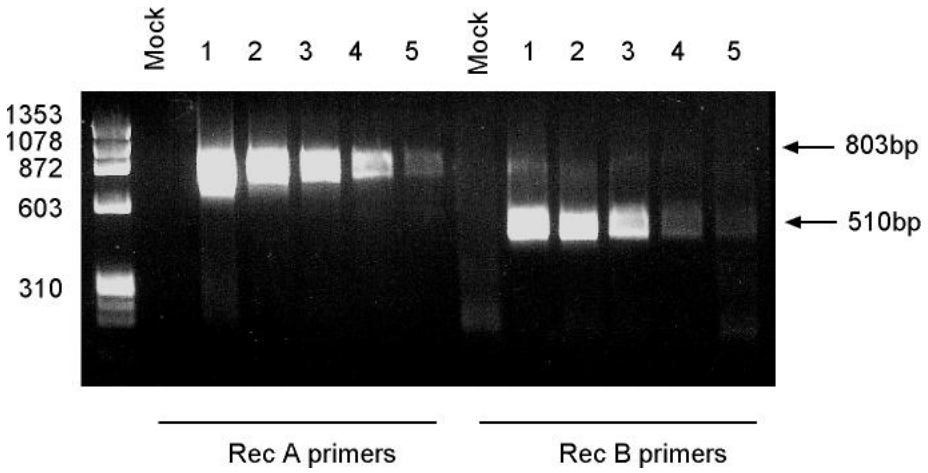


Fig.4.19 The Rec A/B primers can detect as little as 30 fg recombined Maxi-EBV genome.

In order to determine the detection limit of primer pairs that recognise the EBNA 3C deleted Maxi-EBV genome after Cre expression, a PCR analysis was performed with the two primer pairs using decreasing amounts of recombined plasmid DNA as template in a reconstitution assay. To make the experiment as realistic as possible 50 ng unmodified cellular genomic DNA was added to each reaction prior to PCR amplification. In lane 1 (3 ng) recombined DNA was added, in lane 2 (30 pg), in lane 3 (3 pg), in lane 4 (0.3 pg) and in lane 5 (30 fg). As can be seen from this assay the Rec A primer pair is more sensitive than the Rec B primer pair and hence detect as little as 30 fg recombined DNA template.

LCLs containing the lox P flanked EBNA 3C Maxi-EBV (2919) were mock infected, infected with the retrovirus (3067) control vector, or the retrovirus vector expressing the Cre protein (3087), respectively. After four and eight days post infection, genomic DNA from $2x10^5$ cells was extracted and used as a template in PCR amplification with the Rec A and the wild-type primer pairs. Four days post infection in cells infected with the Cre expressing retroviral vector (3087) a band at 803bp was detected indicative of successfully recombined EBV genomes (Fig.4.20A, lane 3). For the mock infected LCLs as well as the LCLs infected with retroviral control vector (3067), only a band at 309 bp was detected indicating non-recombined EBV genomes (Fig.4.20A, lane 4 and 5) as expected. The 309bp band in lane 6 indicated that the LCLs infected with the Cre expressing retrovirus (3087) was a mixed population.

PCR amplification performed after eight days is seen in Fig.4.20B. In lane 3, an 803bp band was detected indicating that the LCLs still contained recombined EBV genomes, and the 309bp band in lane 6 indicated that even after eight days post infection the population was mixed. For the mock infected LCLs and the LCLs

infected with the retroviral control vector (3067) a 309bp band was detected. In conclusion, it was possible to infect LCLs with the retroviral constructs (3067) and (3087), and as seen in Fig.4.20 A and B the EBNA 3C gene was successfully excised using the Cre/lox P system.

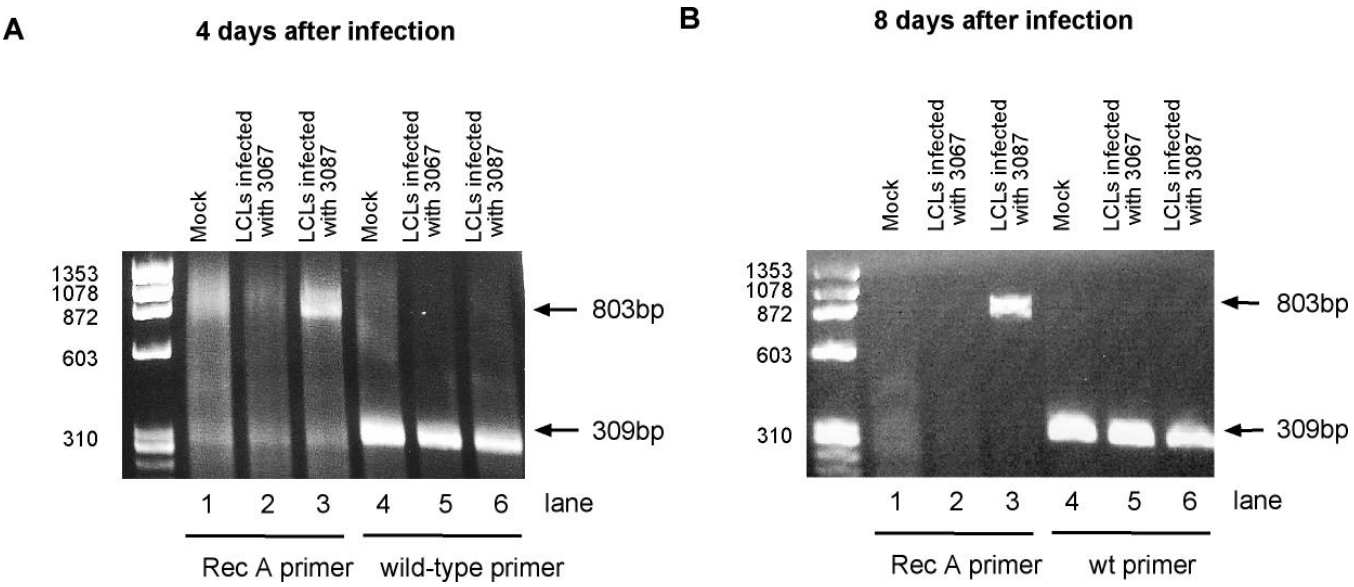


Fig.4.20 Detection of the deleted EBNA 3C gene within the Maxi-EBV genome using Cre recombination.

In this figure the PCR amplification of the lymphoblastoid cell line infected with the Cre expressing retroviral vector is shown. LCLs carrying the lox P flanked EBNA 3C Maxi-EBV were infected with the retrovirus 3087 expressing the Cre protein. (A) Shows the PCR amplification performed with genomic DNA extracted 2 days after infection. First lane are mock infected LCLs and as expected no recombined EBV molecules were detected with the Rec A primer pair. Second lane is DNA from LCLs infected with the Cre expressing retroviral vector 3087. A band of 803bp is seen indicating that Cre is functional and when expressed the Cre/lox P recombination was taking place. In lane 3 and 4 the same template DNA as in 1 and 2 was used, the primer pair used in PCR analysis detects wt EBV molecules. Lane 3 is a PCR positive control for lane 1. (B) Genomic DNA was extracted 8 days after infection and used as template. Lane 1 and 4 were performed with genomic DNA extracted from mock infected LCLs and as expected no fragment was detected in lane 1 indicative of no Cre-mediated recombination. In lane 4 a band characteristic to wt EBV molecules was seen at 309 bp. In lane 2 and 5, genomic DNA from LCLs infected with the control vector 3067 was used as template and, as expected no band was seen in lane 2 since no recombination took place. In lane 4 the expected band at 309 bp was detected indicating that the DNA used as template in lane 2 was intact. In lane 3 and 6 genomic DNA from LCLs infected with the retroviral vector 3087 expressing the CRE protein was used as template. EBV molecules, which have undergone Cre recombination and carry a deleted EBNA 3C gene locus were detected in lane 3 (803 bp band). LCLs infected with the Cre-expressing retroviral vector generated a mixed population in that PCR signals indicative of Cre-mediated EBNA 3C deletion in lane 3 and PCR signals indicative of the presence of EBNA 3C in lane 6 coexist.

This is the first time a conditional system for EBNA 3C LCLs could be established. In one LCL cell a few to several hundred copies of EBV can be present. At this time point it is not possible to determine whether or not excision of EBNA 3C is performed in all copies of EBV in the cell. In order to elucidate if excision is complete a pure population of retroviral infected LCLs is necessary. For this purpose the cells were sorted using FACS analysis. Sorting the retroviral infected Rajis seen in Fig.4.17 was possible (data not shown), but sorting the retroviral infected LCLs was not possible. Several attempts were made to sort the cells but the weak mRFP expression in the LCLs made it impossible.

5. Discussion

For the genetic investigation of EBV genes different methods have been applied. Due to the large EBV genome of more than 170 kb it has not been possible to analyse EBV genetically for a long time. Therefore, most EBV genes were investigated individually by gene expression analysis using different mostly transient expression systems. A first approach for the generation of mutants in the EBV context was reported by Hammerschmidt and Sugden in 1989 (Hammerschmidt and Sugden, 1989). In this approach the EBV positive P3HR-1 cells were used; a cell line, which harbours a strain of EBV that is unable to immortalise B-lymphocytes. This defect is due to a deletion comprising the EBNA 2 gene and the last two exons of EBNA LP (Cohen et al., 1989; Hammerschmidt and Sugden, 1989). In the first genetic study the two missing genes were introduced via an EBV-based vector (p544-4) that can be packaged into a viral coat. The p544-4 vector was transfected into the P3HR-1 cells, the lytic cycle was induced and a mixture of P3HR-1 and p544-4 virions was obtained. Human primary B-lymphocytes could only be immortalised if they were co-infected with both viruses due to the trans-complementation of the EBNA LP and EBNA 2 genes (Hammerschmidt and Sugden, 1989). In a few cases, a homologous recombination event led to the incorporation of the EBNA 2 gene in the P3HR-1 virus genome and also yielded immortalisation-competent EBV.

A similar approach used by the group of Elliot Kieff is based on these spontaneous recombination events. They also used P3HR-1 cells, which were electroporated with an expression vector encoding the immediately early activator of EBV replication (BZLF1), a DNA fragment for the restoration of the deleted EBNA 2 and EBNA LP genes and a fragment containing the mutated gene of interest (Tomkinson et al., 1993). A major disadvantage is that only a very small part of the EBV genomes undergo homologous recombination with both fragments. Nevertheless, B-cells were then infected with the mixed virus gained from the P3HR-1 cells. In consequence, the B-cells were infected by a mixture of P3HR-1 virus, wild-type virus and recombinant virus carrying the desired mutation. A second round of virus production, B-cell infection, and limiting dilution assays aimed at obtaining singly infected primary B-cells was necessary to separate the unwanted helpervirus from the mutant. Clearly,

only mutant viruses capable of immortalising primary B-cells can be analysed and characterised by this approach. With this method, which is time consuming and laborious, it is also not possible to investigate genes important for virus production and maturation.

Therefore, new methods had to be developed for faster and more efficient mutagenesis of large DNA fragments. The first cloning vectors for large DNA fragments were yeast artificial chromosomes (YACs), which are maintained in yeast cells. Unfortunately, this method causes difficulties such as frequent rearrangements, insert instability, and contamination of purified YACs with yeast DNA (Ramsay, 1994; Schalkwyk et al., 1995). A new method was based on bacterial artificial chromosomes (BACs), which are propagated in bacteria and have a cloning capacity of up to 300 kb. The first herpesvirus genome to be cloned as a BAC was the 230 kb mouse CMV genome (Messerle et al., 1997). Shortly thereafter the EBV genome was cloned as a BAC, which enables faster and more efficient establishment of EBV mutants (Delecluse et al., 1998). This technique has been used in my work for the establishment of nine EBNA 3C Maxi-EBV mutants. Initially, the bacteria Rec ABC recombination system was used, but after the publication of the more efficient arabinose-inducible recombination system red from bacteriophage lambda (Stewart et al., 1998) this system was established within this work for the mutagenesis of the Maxi-EBV genome for the first time. The inducible red based recombination system allowed a more efficient and reliable introduction of mutations into the Maxi-EBV genome. As a consequence, the number of successfully mutated genomes was considerably increased with the new system.

5.1 The role of EBNA 3C in the immortalisation process of B-lymphocytes

EBNA 3A, -3B and -3C ORFs are arranged in a tandem in the EBV genome where EBNA 3A and EBNA 3C are considered essential for immortalisation of human primary B-lymphocytes (Tomkinson and Kieff, 1992; Tomkinson et al., 1993). These findings were obtained 10 years ago with mutants established using the system of Elliot Kieff´s group. As discussed earlier, this system is not only time consuming and laborious but also produces virus supernatant with a very low titer of recombinant

virus. It is unclear what role the low virus titer might have played when determining EBNA 3C´s role in the process of B-cell immortalisation. The exact mechanism through which EBNA 3C contributes to the immortalisation was not yet clear since establishment of mutants using this system would be too tedious.

Therefore, the Maxi-EBV system was used in this work to generate and functionally analyse EBNA 3C deletion mutants. With this system it was possible to generate genetically pure and quantified virus stocks with a titer of $5x10^4$-$1x10^5$ infectious particles/ml. The contribution of individual viral genes in the immortalisation process can hereby be quantitatively determined, and the effect of different mutants can be compared directly. In my PhD work nine EBNA 3C mutants have been constructed: one with a mutated PLDLS motif, three lacking more or less the whole repressor domain and two lacking the activation domain. Also one revertant, one entire EBNA 3C knock-out and one lox P flanked EBNA 3C mutant were generated. The mutants were established in E.coli and all steps from homologous recombination in E.coli to the establishment of EBNA 3C mutant LCLs were verified using Southern blot analysis.

Primary human B-lymphocytes infected with EBV are normally cultivated on embryonic fibroblasts "feeder layer". The feeder cells improve the out-growth of immortalised B-cell clones through the expression of yet to be identified soluble factors (Sugden and Mark, 1977). Three phenotypes of growth-transformed B-cells have been defined (Dirmeier et al., 2003). Phenotype I was characterised by B-cells with an indefinite life span. Phenotype II includes B-cells, which are growth-transformed and have a finite life span. Only every fourth LCL becomes immortal after the proliferative crisis and continues to proliferate indefinitely to yield phenotype I cell lines. The LCLs belonging to phenotype III are growth-transformed but show a sustained proliferation only in the presence of feeder cells.

After the establishment of LCLs carrying EBNA 3C Maxi-EBV mutants, feeder cells were no longer needed for further cultivation. The LCLs established with the Maxi-EBV carrying the EBNA 3C deletion mutants could be classified as phenotype II. For the first time EBNA 3C deletion mutants in the context of the whole EBV genome were established and investigated for immortalisation efficiency. It was very surprising to observe that all the mutants were severely impaired in their ability to

growth-transform primary human B-lymphocytes. However, it was possible to establish LCLs from all deletion mutants except the mutant lacking the entire EBNA 3C gene.

There are a limited number of publications, which have identified domains and motifs in EBNA 3C. All these studies have used artificial heterologous systems. As a consequence it was unclear what role domains and motifs of EBNA 3C play in the immortalisation process of primary human B-lymphocytes. In contrast to artificial ectopic expression systems, which have been solely used by others to functionally characterise EBNA 3C, I concentrated on studying EBNA 3C in the context of the complete EBV genome. For this purpose, the nine EBNA 3C mutants were investigated in a quantitative manner for their ability to growth-transform resting primary B-cells.

In the C-terminal part of the protein, a PLDLS binding motif was identified, which was also found in the adenovirus protein E1A and papillomavirus protein E7. The PLDLS motif was necessary and sufficient for interaction with CtBP (E1A C-terminal binding protein) (Subramanian et al., 2001). It is further known that CtBP belongs to a highly conserved family of transcriptional co-repressors, and that human CtBP acts as a co-repressor for the ZEB transcription factor, which is involved in the regulation of lymphocyte and muscle differentiation (Quinlan et al., 1988). Furthermore, CtBP is in some situations capable of recruiting chromatin-modifying histone deacetylase (HDAC) enzymes 1,4,5, and 7 and binding to Sin3A. The precise molecular mechanisms by which CtBP inhibits transcription is dependent on the particular situation (Criqui-Filipe et al., 1999; Koipally and Georgopoulos, 2000; Meloni et al., 1999; Sundquist et al., 1998; Zhang et al., 2001). It is thought that EBNA 3C binds a transcriptional repressor complex, which includes HDAC1. The complex is then targeted to the Cp promoter by the cellular DNA-binding protein RBP-Jκ (Crook et al., 1998; Radkov et al., 1997). The Cp promoter is the main promoter for the EBNA 3C encoding transcript, and might hence constitute a negative autoregulatory control loop. In order to investigate the significance of this motif in the B-cell growth-transformation process, an EBNA 3C-ALDAS mutant was established. The PLDLS motif in EBNA 3C was mutated, the proline in position 728 and the leucine in position 732 were mutated to alanines. Surprisingly, the 2842 (PLDLS-ALDAS) mutant showed a very strong impairment in B-cell growth-transformation since it only

immortalised human B-cells with a relative efficiency of 11.8% compared to wild-type. It seems as if the binding of CtBP to EBNA 3C and thereby the formation of the Cp repressor complex is essential for the immortalisation process. Another explanation could be that EBNA 3C´s structure is partially disturbed due to the mutation and as consequence the protein is no longer fully functional leading to the low immortalisation efficiency observed. This assumption is rather unlikely since the conservative exchange of two amino acids to alanine, being an uncharged amino acid with a small hydrophobic side-chain, is the least invasive mutation possible.

Deletion mutants affecting the repressor domain

There are conflicting data concerning the domain between amino acid 346 and 543 of EBNA 3C which has been identified as a repressor domain when fused to the DNA-binding domain (DBD) of GAL4 (Bain et al., 1996; Marshall and Sample, 1995). This domain was also found to repress the Cp promoter, which is the main promoter for EBNA mRNA transcription (Radkov et al., 1997). Since the Cp promoter is the major promoter for EBNA expression, the repressor domain of EBNA 3C can negatively regulate the expression of EBNA 3C. On the other hand it was shown that the domain comprising amino acids 346 to 543 of EBNA 3C was essential and sufficient for co-activation of the LMP 1 promoter with EBNA 2 (Lin et al., 2002). EBNA 3C and the acidic domain of EBNA 2 can also co-activate the LMP 1 promoter. The co-activation is independent of the repressor domain of EBNA 3C indicating at least two ways for EBNA 3C to regulate the LMP 1 promoter (Lin et al., 2002). In order to investigate the role of the repressor domain in immortalisation assays, three deletion mutants were established. The 2841 mutant was an internal deletion mutant lacking amino acid 408 to 510, which only immortalises B-cells to 6.1% compared to wild-type. The 2839 mutant immortalised B-cells with 3.5% compared to wild-type and lacks amino acid 410 to 710, which includes most of the repressor domain but also the proline rich stretch from amino acid 551 to 610. The mutant 2840 carries a deletion between amino acid 212 and 658 and hence the whole repressor domain is deleted. In addition, the basic leucin zipper as well as more than half of the RBP-Jκ binding domain is lacking in this mutant. The 2840 mutant immortalises B-lymphocytes with an efficiency of 4.7% compared to wild-type. If the severe impairment in B-cell immortalisation efficiency is due to the loss of repression of the

Cp promoter, the repressor domain and the PLDLS motif are not redundant in this function. It is more likely that due to the large deletions the structure of the protein is partially lost and thereby the protein is no longer fully functional.

Deletion in the activation domain

The region in between amino acids 724 to 826 of EBNA 3C was characterised as a transcriptional activator domain when fused to the DNA-binding domain of GAL4 (Marshall and Sample, 1995). Two mutants 2837 and 2838 were constructed to investigate the role of the activation domain. The mutant 2837 carries a deletion in EBNA 3C that stretches from amino acid 658 to 1013 and hence lacks the activation domain and the leucin-proline-rich stretch. This mutant growth-transforms primary human B-cells to only 1.8% compared to wild-type. The establishment of LCLs with the 2837 mutant took seven weeks and only two cell lines could be established. The 2838 mutant carries a deletion from amino acid 489 to 970, which comprises about half of the repressor domain, the proline-rich stretch, the whole activation domain and the leucin-proline-rich stretch. The immortalisation efficiency of this mutant was only 1.5% compared to wild-type, and it took 13 weeks for the establishment of LCLs. From the three independent immortalisation experiments it was only possible to expand two lymphoblastoid cell lines. The reason for the strong reduction in immortalisation efficiency could be due to the loss of activation of genes needed for the immortalisation through the activation domain. One other possibility is that the large deletions at least partially leads to loss of structure and hence function.

The effects seen in the immortalisation assays with the EBNA 3C deletion mutants are relevant since the reconstituted revertant (2889) showed the same phenotype as the "normal" wild-type (2089). This observation indicated that the effects seen are due to the EBNA 3C deletions and not due to the homologous recombination events or adverse effects due to the integration of a prokaryotic antibiotic selection marker. The severely impaired immortalisation efficiency seen for all the deletion mutants can be attributed to the fact that in order to regulate protein expression EBNA 3C needs all the reported and investigated domains and motifs. It could be shown that the PLDLS motif of EBNA 3C and thereby the binding of CtBP is important for B-cell immortalisation. The effects of the deletion mutants lacking the repressor domain (2841, 2839, 2840) and the mutants lacking the activation domain (2837, 2838) in the

immortalisation process of B-cells are more likely to depend on structure than on a specific domain. The surprising results showing that all investigated domains and motifs are essential for B-cell immortalisation lead to the speculation that EBNA 3C might be part of a larger transcriptional regulation complex, in which the structure is important for the coordinated recruitment and/or binding of transcription factors to the complex.

5.1.1 EBNA 3C deletion mutants alter the expression of EBNA 1, EBNA 2 and LMP 1

It has been reported that EBNA 3C together with EBNA 2 can activate the LMP1 promoter (Lin et al., 2002; Zhao and Sample, 2000). It is further known that EBNA 3C co-activation in conjunction with EBNA 2 requires a SUMO homology domain (Rosendorff et al., 2004). LMP 1 has been shown to be essential in the process of B-cell immortalisation (Kieff and Rickinson, 2001; Kilger et al., 1998). This finding was challenged since with a complete LMP 1 knock-out in the Maxi-EBV context it was possible to obtain LCLs, although with a very low frequency (Dirmeier et al., 2003). LMP 1 has also been shown to act as a viral oncogene in vivo (Kulwichit et al., 1998; Wang et al., 1985; Wilson et al., 1990). Precise regulation of LMP 1 expression is important since the level of LMP 1 is critical for cell activation, adhesion, growth factor secretion and survival (Baichwal and Sugden, 1989; Kaykas and Sugden, 2000; Kilger et al., 1998; Sandberg et al., 2000; Wang et al., 1985; Wang et al., 1988). Since EBNA 3C interacts with and regulates other viral proteins the expression of EBNA 1, EBNA 2 and LMP 1 was investigated in Western blot analyses. The concentration of protein in crude total extracts was measured and equal amounts were used for the immuno-blots. The mutants 2837, 2838, 2839 and 2840 all showed somewhat lower expression of EBNA 1, EBNA 2 as well as LMP 1 compared to wild-type (721). Surprisingly, the 2841 mutant expressed LMP 1 to only 5% and the 2842 mutant only to 20%, compared to 721. Furthermore the 2841 mutant expressed EBNA 2 to only 20% and the 2842 mutant only 10% compared to 721. Moreover the 2841 and 2842 mutants expressed EBNA 1 to only about 10% compared to 721. This was unexpected since it has been reported that the number of EBNA 1 molecules is constant and does not depend on the number of EBV copies in the cell (Sternas et al., 1990). These findings indicate that EBNA 3C is involved in

transcriptional regulation of not only EBNA 2 and LMP 1 but also EBNA 1. As hypothesised before EBNA 3C might be part of a transcriptional regulatory complex, which is functionally disturbed when the structure of EBNA 3C is impaired. One can postulate that the down-regulation seen in the EBNA 3C-ALDAS mutant could be due to the fact that the binding of CtBP and HDAC 1 might be important for the transcriptional regulatory complex. The reason why the 2841 mutant showed such low expression of EBNA 1, EBNA 2, and LMP 1, could be due to the fact that the recombinant EBNA 3C protein is degraded very fast as indicated in the Western blot analysis of EBNA 3C. Because of the fast degradation of the protein, the protein is unable to activate transcription and hence the low expression of EBNA 1, EBNA 2 and LMP 1 is observed. The mechanism by which EBNA 3C activate transcription can only be speculated to-date, but the observation remains that EBNA 3C regulates viral transcription of EBNA 1, EBNA 2 and LMP 1, which are the most important proteins in the process of B-cell growth-transformation.

5.2 The EBNA 3C knock-out phenotype

The data that EBNA 3C is essential for B-cell immortalisation was obtained using the obsolete P3HR-1 system (Tomkinson et al., 1993). It is known that it is possible to obtain much higher virus titer using the BAC technology. As a consequence it has been shown for LMP 1 that a highly concentrated virus stock with the complete LMP 1 knock-out is still able to growth-transform primary human B-lymphocytes, although to a very low extent (Dirmeier et al., 2003). Since the same might be the case for EBNA 3C an EBNA 3C knock-out mutant was established where the EBNA 3C gene was replaced with a tetracycline resistance gene. The publication by Tomkinson was verified since even with high titer of EBNA 3C knock-out mutant virus it was not possible to immortalise human B-cells. Surprisingly, within the first three weeks after infection an initial proliferative out-growth was observed, where the infected primary human B-lymphocytes showed the phenotype of transformed B-cells. However, sustained B-cell proliferation was not observed (see Fig 4.12). The reason for this initial proliferation is unclear. A severe deregulation of EBNA 1, EBNA 2 and LMP 1 might be the reason why the EBNA 3C knock-out mutant cannot growth-transform B-cells stably. The initially observed proliferation might reflect early conditions that are different from conditions needed than for maintaining the

proliferation status. All the mutants of the different domains and motifs lie between amino acid 212 and 1013 (see Fig.4.3), and no mutant affects the first 212 amino acids of EBNA 3C. Since it was possible to obtain LCLs from all deletion mutants but not from the complete EBNA 3C knock-out, it might be possible that an interaction with the first 200 amino acids is important for B-cell immortalisation. Another possibility would be that multiple interactions are needed and by deleting one or two interaction domains of EBNA 3C the immortalisation efficiency is severely reduced.

5.3 A conditional EBNA 3C system

The second part of this work was to establish a conditional system in primary human B-lymphocytes in which the EBNA 3C expression could be switched off. With the conditional system it should be possible to investigate the importance of EBNA 3C in the initiation and/or maintenance phase of B-cell immortalisation. It should also be possible to identify cellular target genes of EBNA 3C. There are several conditional systems available (e.g. fusion of the protein of interest with the hormone binding domain of the estrogen receptor, the transcriptional tet repressor/activator system, or Flp, and Cre/lox P recombination systems).

One way to obtain a conditional system would be to fuse the gene of interest with the estrogen binding domain (Kempkes et al., 1995). The fusion protein is retained in the cytoplasm by heat shock protein 90, which binds to the estrogen binding domain and hereby prevents the protein from being translocated into the nucleus. Upon addition of estrogen, the complex dissociates and the protein is transported into the nucleus and hence can be active. For creating a conditional EBNA 3C system, three Maxi-EBV estrogen-EBNA 3C fusion mutants were initially constructed at the beginning of this work. Since it was not known if the fusion to either the C-terminus or the N-terminus would effect the function of the protein both mutants were established as well as a mutant where the estrogen binding domain was fused to both the N- and the C-terminus of the gene. B-cells were infected with the different viruses obtained with the EBNA 3C estrogen binding protein fusion proteins. Unfortunately, the mutants showed no significant differences in immortalisation efficiency with or without estrogen. Similar problems have been reported for EBNA 1 (unpublished data of the Hammerschmidt lab) and EBNA 3A (unpublished data, Bettina Kempkes). For the EBNA 1 estrogen fusion protein mutant it was not possible to obtain stable HEK-293

cell lines, and for the EBNA 3A estrogen fusion protein mutant the spontaneous cleavage of the estrogen binding domain was observed in Western blot analysis (personal communication, Bettina Kempkes). This could also be the case for the EBNA 3C-estrogen fusion proteins.

Another way to regulate EBNA 3C expression would be the use of the Cre/lox P system or the tet on/off system. The tet on/off system is based on the transcriptional regulation of the promoter of the gene of interest (Gossen et al., 1995; Weinmann et al., 1994) and therefore in this case not feasible since the promoter regulating EBNA 3C expression also regulates the genes EBNA 2, EBNA LP, EBNA 3A and EBNA 3B. To circumvent this problem a conditional system based on Cre/lox P site-specific recombination was established within this work. A disadvantage of this system compared to the estrogen or tet systems is that the Cre/lox P system only allows just one unique switch, which is irreversible. This is achieved by the excision of a stop-spacer (turn-on) or excision of the gene (turn-off). The advantage of the Cre/lox P system is the possibility of a complete inactivation of the gene of interest without residual activity.

To apply the Cre/lox P system for the EBNA 3C shut off a Maxi-EBV carrying the lox P flanked EBNA 3C gene was cloned, a stable producer cell line established and virus supernatant harvested, which was used for the establishment of LCLs. Since LCLs are not easily transfected with DNA a retroviral vector was used for delivering the Cre protein into the established LCLs. It was known that retroviral vectors successfully infect an array of different cells. In this work, it was also shown that these retroviral vectors could successfully infect LCLs. The design of the retroviral vectors is based on the vector constructs published by Hildinger et al. (Hildinger et al., 1999). In the retroviral back-bone the open reading frame of the Cre protein without promoter and poly-A sequence was cloned followed by an internal ribosomal entry site (IRES) and the mRFP gene for detection of successfully infected cells. In infected cells the construct is transcribed as a bicistronic mRNA from the retroviral promotor of the LTR. Due to the gene arrangement and the IRES cassette one can assume that the cells expressing the mRFP also express the Cre protein. Infectious particles were obtained using a packaging helper cell line. In parallel to the Cre expressing retoviral vector (p3087) the same vector without the Cre protein gene (p3067) was used as a control vector.

First, it was tested whether the retroviral vectors could infect the EBV positive B-cell line Rajis. The infected Raji cells were detected by expression of mRFP using a fluorescence microscope indicating an efficient infection. More important was the fact that the retroviral vectors could infect the established lox P flanked EBNA 3C LCLs, although with a lower efficiency than the Raji-cells. The next step was to prove that the retroviral vector expressing the Cre protein was functional in LCLs. If so EBNA 3C should be excised via Cre/lox P site-directed recombination. Indeed, EBV genomes without EBNA 3C could be detected by PCR analysis. Thus, a functional conditional system was established for the investigation of the importance of EBNA 3C in the initiation and/or maintenance phase of B-cell immortalisation. To investigate the importance of EBNA 3C during B-cell immortalisation it is essential to obtain a pure population of LCLs infected with the retroviral vector. For this purpose the infected cells were sorted using FACS. Two distinct populations of LCLs, one infected with control vector and one infected with Cre expressing vector, would allow to analyse the putative phenotype of an EBNA 3C knock-out in comparison to the non-infected population as negative control. If this is the case these cells could be studied concerning their proliferative characteristics with and without EBNA 3C. This was not possible since the expression of the mRFP gene was unfortunately not strong enough for sorting the cells using FACS analysis. To improve this situation in the future the expression of mRFP has to be increased. One possibility would be to clone the mRFP gene upstream of the IRES cassette in the retroviral vector for stronger expression. Alternatively one could also use a surface marker such as CD34 or NGF-R for sorting of the cells using FACS (Fehse et al., 2002).

By establishing this conditional system EBNA 3C can be knocked out at any chosen time during the process of B-cell immortalisation and hence it should now be possible to investigate the role of EBNA 3C in the initiation and/or maintenance of B-cell immortalisation. Furthermore the effects of EBNA 3C on the expression of EBNA 1, EBNA 2 and LMP1 can be investigated. Using the established conditional EBNA 3C mutant, it should be possible to identify target genes of EBNA 3C in further experiments.

6. Summary

Epstein-Barr virus (EBV) is known to infect primary B-lymphocytes and hereby induce proliferation. The *in vitro* immortalisation of B-cells is a model system that reflects the mechanisms by which a tumour develops. *In vitro* the Epstein-Barr nuclear antigen 3C (EBNA 3C) is considered to be essential for the immortalisation of B-cells. The aim of this work was to clarify the influences of the domains and motifs of EBNA 3C in the immortalisation process of primary human B-lymphocytes.

The establishment of EBNA 3C mutants in the context of the whole EBV genome was achieved by using the Maxi-EBV. For a more efficient and reliable mutagenesis of the Maxi-EBV in E.*coli* a new inducible recombination system was established using the red $\alpha\beta\gamma$ functions from bacteriophage lambda. Nine recombinant EBVs with different mutations in EBNA 3C were established and their phenotype was investigated. In addition, an EBNA 3C revertant virus was established which revealed the same phenotype as the original wild-type EBV indicating that the described effects for the mutants were significant and not an artefact caused by the mutagenesis itself. All mutations within the open reading frame of EBNA 3C showed a substantial impairment in immortalisation efficiency compared to wild-type. For the EBNA 3C complete knock-out it was not possible to establish any LCLs confirming already published data. Surprising was that the primary B-cells infected with the EBNA 3C knock-out virus showed an initial proliferative-like growth after three weeks, but then ceases to proliferate and died.

To further investigate the phenotype seen with the EBNA 3C knock-out an inducible Cre/lox P system was established. This system is a first step to elucidate the role of EBNA 3C in the initiation and/or maintenance of B-cell immortalisation. A lox P flanked EBNA 3C Maxi-EBV was generated and LCLs were established. Since it is known that LCLs are difficult to transfect, a retroviral vector was chosen for the expression of the Cre protein in the LCLs. This vector carries the Cre gene followed by an IRES cassette and the monomeric red fluorescence protein (mRFP) gene. It was possible to infect LCLs with the retroviral vector and the excision of EBNA 3C could successfully be determined using PCR analysis. But for the investigation of the

role of EBNA 3C in the initiation and/or maintenance of B-cell immortalisation a pure population of LCLs carrying the lox P flanked Maxi-EBV infected with the retroviral Cre expression vector would be needed. Despite attempts to sort the LCLs it was not possible to enrich a homogenous population that lacks EBNA 3C. This was mainly due to the very low mRFP expression levels from the retroviral vector. In future, this problem could be overcome by using another marker or cloning the mRFP gene upstream of the IRES cassette since only very low concentrations of Cre protein is needed for Cre/lox P site-directed mutagenesis.

7. Abbreviations

aa	Amino acid
APS	Ammonium peroxodisulphate
ATP	Adenosin-5-triphosphate
bp	Base pair
BSA	Bovine serum albumin
bZIP	Basic leucine zipper
CD	Cell differentiation marker
Ci	Curie
CMV	Cytomegalovirus
CtBP	C-terminal binding protein of E1A
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
DTT	Dithiotheriol
EBNA	Epstein-Barr virus nuclear Antigen
EBNA LP	Epstein-Barr virus nuclear Leader-protein
EBV	Epstein-Barr virus
E.coli	Escherichia coli
EDTA	Ethylendiamintetraacetic acid
EGF	Epidermal growth factor
FCS	Fetal calf serum
Fig.	Figure
h	Hour
HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazinethan-sulphoacid
ICAM-1	Intracellular adhesion molecule
IL	Interleukin
Kb	Kilobase pair
kDa	Kilodalton
l	Liter
LB	Laura-Bertani
LCL	Lymphoblastoid cell line

LMP	Latent membrane protein
mA	Milliampere
min	Minute
mRNA	Messenger RNA
nt	Nucleotide
PAGE	Polyacrylamidgel-electrophoresis
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonylfluorid
RBP-Jκ	Recombination signal sequence binding protein J
SDS	Sodiumdodecylsulphate
SV40	Simian virus 40
TMED	N,N,N,N-Tetramethyletylendiamin
Tris	Tris-hydroxymethyl-aminomethan
rpm	Rotations per minute
UV	Ultra violet
V	Volt
v	Volume
w	Weigt

8. Literature

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Curriculum Vitae

Personal data

Name	Madelaine Löfqvist
Date of birth	17.06.1974
Place of birth	Lomma, Sweden
Nationality	Swedish

Education

1981-1990	Shool, Löddeköpinge
1990-1993	Gymnasium, Landskrona and Malmö
June 1993	Diploma for University-entrance
1993-1995	University; Teacher 4-7, Ma/NO, Malmö
1995-1996	Astra Pharmaceuticals, Södertälje
1996-2000	University, Biotechnology, Eskilstuna
June 1999-December 1999	Diploma thesis in the group of Dr. Hans Burgert Genecenter, Ludwig-Maximilians-University, Munich
27/10/2000	Master of Science, Biotechnology
June 2000-July 2004	PhD in the group of Prof. Dr. Wolfgang Hammerschmidt at GSF, Department of Gene Vectors, Munich