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Murine Model System for EBV-related Diseases

submitted by

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STATEMENT

I declare that this is my first dissertation work to pursue the PhD degree.

I have written this thesis independently, without help of the others. The content of this thesis is based on experiments I performed myself.

Magdalena Zychlinska
Germany, Munich, 06.02.2007

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TABLE OF CONTENTS

ABBREVIATIONS

Δ - deletion

μ - micro

6-well - one well of 6-well plate

Amp – ampicillin

BAC - bacterial artificial chromosome

BCR – B-cell receptor

ca. - circa

CAM – chloramphenicol

CD – cluster of diifferentiation

chloro^{res}/amp^{sen} - chloramphenicol resistant and ampicillin sensitive

CsCl – cesium chloride-ethidium bromide centrifugation density gradient

DNA – deoxyribonucleic acid

dNTP – deoxyribonucleotide (dATP, dCTP, dGTP, dTTP)

E.coli – Escherichia coli

e.g. – for example

EBNA – EBV nuclear antigen

EBV – Epstein-Barr Virus

ECACC - European Collection of Animal Cell Cultures

EtBr – Ethidium Bromide

F - farad

FACS – flow cytometry

FBS – fetal bovine serum

FCS – fetal calf serum

G418 – antibiotic, resistance to it is encoded in *neomycin* gene

GC – germinal centre

GFP – green fluorescent protein

h(s) – hour(s)

HAT – selection medium containing Hypoxanthine/Aminopterin/Thymidine

HHV – Human Herpes Virus

HIV – Human Immunodeficiency Virus

HLA – human leukocyte antigen

Hprt – hypoxanthine phosphoribosyltransferase

hyg - hygromycin
IM – infectious mononucleosis
InvTarg – my final recombinant maxi-EBV targeting vector
Kac – potassium acetate
kb – kilo base pairs
KSHV – Kaposi's Sarcoma Herpesvirus
l - liter
L2000 – lipofectamine 2000
LB – Luria Broth
LCL – lymphoblastoid cell line
LCV - lymphocryptovirus
LIF – leukemia inhibitory factor
LMP – latent membrane protein
m - milli
M - molar
mES cells - murine embryonic stem cells
MHV-68 – murine herpesvirus 68
min. - minute
n - nano
NaAc – sodium acetate
NaAc – sodium acetate
neo – neomycin (G418) selection medium
No - number
nt - nucleotide
o/n – overnight
OD – optical density
OPTI – Opti-MEM transfection medium
OriLyt – lytic origin of viral DNA replication
OriP – origin of DNA replication
p - piko
PBS – phosphate-buffered saline
PCR – polymerase chain reaction
PFGE – pulse field gel electrophoresis
PGK – phosphoglycerol kinase

RNA – ribonucleic acid

Rnase - ribonuclease

rpm – rotations per minute

RT – room temperature

sec. – second(s)

SSR – site-specific recombinase

SV40 – simian virus 40

tet - tetracycline

TR – terminal repeats

U – unit(s)

UV - ultraviolet

V – volt

w/v – weight per volume

WHO – World Health Organisation

wt – wild-type

zeo - zeocin

1. INTRODUCTION

1.1. Epstein-Barr Virus (EBV) – an unusual human virus

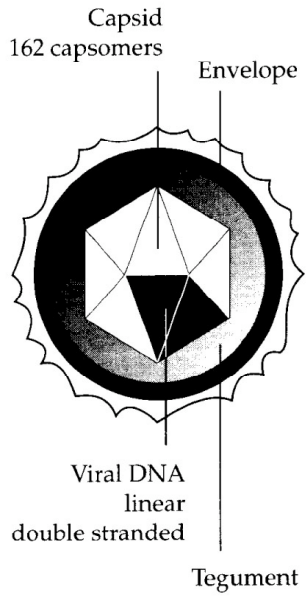
Epstein-Barr Virus (EBV), known also as Human Herpes Virus 4 (HHV4), belongs to the large family of herpesviruses and is categorized as a γ1-herpesvirus or Lymphocryptovirus subfamily (Tselis et al., 2004). Herpesviruses share common features of their structure and biology. They are enveloped viruses with large genomes of double-stranded DNA. Generally, these viruses can cause various diseases in humans and animals. '*Herpein*' from Greek denotes 'to creep', what refers to the 'sneaky' nature of the viruses and reflects their ability to remain silenced in the infected host and to re-appear. Several viruses belong to the three different subfamilies of Human Herpesviruses (HHV): Herpes Simplex Virus 1 and 2 (HSV-1 or HHV1, HVS-2 or HHV2) and Varicella-Zoster Virus (VZV or HHV3) form the subfamily of α-herpesviruses, Cytomegalovirus (hCMV or HHV5), Human Herpesviruses 6 and 7 (HHV6, HHV7) belong to β-herpesviruses, and Kaposi's Sarcoma Herpesvirus (KSHV or HHV8) are members of the γ-herpesviruses (McCance, 1998). A vast number of animal herpesviruses has been discovered. Murine γ-herpesvirus MHV-68 and different primates' lymphocryptoviruses deserve special attention in the context of EBV, because these related viruses show common characteristics.

EBV is an extremely successful virus with a peculiar life style. Although more than 95% of the human population is infected with EBV (WHO, 2006), very few individuals develop EBV-related diseases. They include Infectious Mononucleosis (IM), which can result from a primary infection with EBV as well as several EBV-associated tumours. The range of the disorders EBV provokes is considerably wide and the expression pattern of the viral genes differs from one disease to the next (Kuppers, 2003). It seems conclusive that a particular expression pattern of viral genes is the molecular link between their functions and the phenotype of the infected cell.

Epstein-Barr Virus (EBV) is a tumour herpesvirus with a very narrow host range infecting strictly humans (McCance, 1998). It infects preferentially human B cells, but it is also often detected in epithelial cells (Young and Rickinson, 2004) and

sporadically in other cells of the hematopoietic system, including T lymphocytes, monocytes, granulocytes and Natural Killer (NK) cells (Knecht et al., 2001).

EBV is a large enveloped herpersvirus with linear double-stranded DNA packaged in a protein capsid (Fig.1.1).



Glycoprotein spikes are inserted into the outer layer of the envelope (Klein, 1998). The linear virion genome circulates upon infection and persists in the nucleus of the host cell during its proliferation and resting state. Upon virus synthesis, the viral genome becomes linearized and is packaged into the virus capsid (Kuppers, 2003). In latently infected cells EBV DNA is mostly found in a multiepisomal form, only sporadically integrating into the host genome (Knecht et al., 1997; Miller, 1990).

Fig.1.1. Structure of the EBV herpesvirus. (modified from Klein, 1998) Envelope consists of a lipid bilayer with glycoprotein spikes. Protein tegument surrounds the nucleocapsid with 162 capsomeres and the double-stranded genomic DNA.

Two viral envelope glycoproteins are known to assist virus entrance into the host cell: gp350 and gp42. gp350 binds to the human CD21 receptor on the B cell surface, and the cell surface protein Human Leukocyte Antigen class II (HLAII) acts as a co-receptor for the viral glycoprotein gp42. The exact steps how EBV enters other cell types have not yet been well defined (Young and Rickinson, 2004). It seems that there are alternative ways since e.g., epithelial cells do not express CD21 receptor (Janz et al., 2000). After binding of the virus, it is endocytosed. Upon a fusion between the viral and cellular membranes the viral tegument and the nucleocapsid are released into the cytoplasm. The capsid is then transported to the nuclear pores via the cellular cytoskeleton and the EBV DNA is released into the nucleus (McCance, 1998).

As already mentioned EBV is an unusual virus. As such, its control over the host cell is not limited to virus production (Kuppers, 2003). EBV additionally exploits the host organism in its latent phase multiplying its genome further in the course of the virus-driven host cell proliferation. Thereby it is able to persist in its dormant state for the host's lifetime in resting B cells. In this silenced state EBV expresses a minimal set of

genes (Young and Rickinson, 2004). The resting form of the virus will be named a ‘silenced state’ in this work, and the term ‘latent’ will refer to the EBV ‘latent cycle’.

1.2. Gene expression of EBV

The EBV genome of about 184kb double-stranded DNA encodes nearly 100 genes. Around 90 of them are expressed during the lytic replication and are classified in three groups of immediate early, early and late proteins. Generally, the immediate early genes play a role in regulating gene expression of the virus, the early genes encode enzymes which are important for EBV DNA replication, and the late genes encode structural proteins of the virion. Some of those genes are also expected to participate in the viral immune evasion (Tselis et al., 2006). During its latent cycle EBV expresses only eleven genes, nine of which encode viral latent proteins: six EBV nuclear antigens (EBNAs): -LP, -1, -2, -3a, -3b, -3c, and three latent membrane proteins (LMPs): -1, -2a, -2b. The latent proteins are engaged in supporting B cell transformation *in vitro* by altering cellular signalling pathways or/and transcription (Young and Rickinson, 2004). Although the *in vivo* functions of the latent gene products are not clearly defined, the state of current knowledge is summarized in Tab.1.1. The viral proteins sustain the hidden state of viral infection, but can also determine activation and proliferation of the host cell (Young and Rickinson, 2004). Several of the latent gene products have been shown to retain similar functions as their cellular counterparts summarized in Tab.1.1. Although all latent genes are expressed in virus-induced proliferation of B cells during latency III, only EBNA2, EBNA3a and c, LMP1 and EBNA-LP are crucial for B cell outgrowth *in vitro*, along with EBNA1 for episomal genome maintenance. LMP2a and b (Young and Rickinson, 2004) and EBNA3b (Murray and Young, 2001) are dispensable. Transgenic mice with latent viral genes expressed from heterologous (immunoglobulin heavy chain or SV40) promoters were established. Transgenic mice with overexpressed LMP1 or EBNA2 develop tumours (Kulwichit et al., 1998, respectively; Tornell et al., 1996). EBNA1’s ability to induce tumourogenesis in transgenic mouse is controversial. First, EBNA1 had been found to stimulate B cell neoplasia (Wilson et al., 1996), but this finding could not be confirmed (Kang et al., 2005). LMP2a is not oncogenic but can bypass B cell development (Portis and Longnecker, 2004).

Name	Function	Mimicking cellular genes	Transformation potential *in vitro*	Tumour formation potential *in vivo*
EBNA1	- maintenance and replication of episomal EBV - transcriptional regulation of EBNAs and LMP1	High mobility group protein A1 (HMGA1a); (Thomae A., in preparation)	**-** (except of genome maintenance)	**+/-*** (o/ex)
EBNA2 (TF)	- transactivation of viral genes (LMP1, LMP2a) and certain cellular genes	Notch (binding to RBP-J□)	**+** (E)	**+** (o/ex)
EBNA-LP	- co-operation with EBNA2 in transactivation		**+*** (EG)	
EBNA3a	- repression of EBNA2-mediated transactivation		**+** (E)	
EBNA3b			**-**	
EBNA3c			**+** (E)	
LMP1	- promotion of B cell activation and proliferation	CD40 (activation of NF□B pathway)	**+** (E)	**+**
LMP2a	- role in B cell proliferation and survival in absence of BCR - role in immune evasion	B-cell receptor (BCR)	**+** (NE)	**-** (influences B cell development)
LMP2b	- possible regulation of LMP2a function		**-**	

Tab.1.1. Latent gene products of EBV. (adapted from Kuppers, 2003; Murray and Young, 2001; Young and Rickinson, 2004) (*): not consistent, controversial data; EBNA: EBV Nuclear Antigen, LMP: Latent Membrane Protein, TF: transcription factor, o/ex: overexpression from a heterologous promoter, BCR: B cell receptor, E: essential, NE: not essential, EG: required for efficient proliferation.

Diverse expression patterns of the latent EBV proteins were identified. Because conflicting views exist on the classification of the EBV latency programs, I present one version, only. This characterization includes five programs of EBV latent gene expression (Tab.1.2). The occurrence of those individual patterns is linked to the host cell tropism, stage of the cellular development (Fig.1.4a), or kind of EBV-positive tumour (Tab.1.3).

Latency program	Latent proteins expressed
0 (EBV latency state)	EBNA1, LMP2a
I	EBNA1
II	EBNA1, LMP1, LMP2a
I/II	EBNA1, LMP2a and b, LMP1*
III (EBV replicating state)	EBNA1, EBNA2, EBNA3a, b and c, EBNA-LP, LMP1, LMP2a and b

Tab.1.2. EBV latent proteins expression patterns. (based on Kuppers, 2003; Young and Rickinson, 2004)); (*): not always occurs; EBNA: EBV Nuclear Antigen, LMP: Latent Membrane Protein.

Beside the nine latent genes a number of non-coding viral RNA was also detected in all the latency programs. The transcripts comprise: BamHIA rightward transcript (BART), encoded by the BamHIA region, non-coding EBV-encoded RNAs (EBERs), EBER1 and EBER2 (Young and Rickinson, 2004), and several microRNAs (Pfeffer et al., 2004). The role of the non-coding transcripts is still under debate. Several potential open reading frames can be detected in the multiply spliced family of BART transcripts. One of them has been shown to have oncogenic properties *in vitro,* but their expression is questionable in EBV-infected cells. EBERs, which are expressed at more than 10^5 molecules per latently infected cell, are not essential for the transformation of human primary B cells (Young and Rickinson, 2004). They seem to negatively regulate antiviral effects of the host cytokine network (Nanbo et al., 2002).

1.3. B cells and their development

B cells are the main targets of an EBV infection *in vitro* and *in vivo* (Young and Rickinson, 2004). B cells, or B lymphocytes, are components of the immune system. They guard the peripheral tissues while circulating in the blood and in a specialized system of vessels, the lymphatic system. B cells, in cooperation with T lymphocytes, are able to mount specific immune responses against the wide range of pathogens encountered during lifetime. This is enabled by a high diversity of cell surface receptors, B-cell receptors (BCR) for B cells, which are unique for each mature lymphocyte. Upon activation B cells differentiate into plasma cells and release antibodies. All the cellular components of blood, red and white blood cells and platelets, develop from hematopoietic stem cells (HSC) in the bone marrow (BM). B and T lymphocytes originate from a common lymphoid progenitor, a stem cell of

more limited potential. The same progenitor gives rise to a third lineage of natural killer (NK) cells, which however, lack the antigen specificity, and as a part of the innate immunity can remove abnormal cells from the organism. Only B cells originate and mature in BM, T cells complete their maturation in thymus. Once the lymphocytes complete their maturation they enter the blood stream and migrate to the peripheral lymphoid tissues, such as spleen and lymph nodes, gut-associated lymphoid tissue (GALT), including tonsils and adenoids, bronchial-associated lymphoid tissue (BALT) and mucosal-associated lymphoid tissue (MALT). The circulating mature or naïve B cells are prepared to encounter and to be activated by the appropriate antigen (Janeway et al., 2005). Upon such activation B cells create a so-called germinal centres (GC) in the primary follicle of the secondary lymphoid organ (Fig.1.2). After the activated B cells enter the GC, they undergo rapid proliferation and differentiation. The extensively proliferating B cells in GC, called ‘centroblasts’, acquire somatic mutations in a process of ‘somatic hypermutation’ in order to increase the affinity of their BCR to the encountered antigen. The centroblasts then differentiate into resting centrocytes, which are verified in their ability to interact with the antigen presenting cells, such as helper T cells and dendritic cells, and thus, to recognize the antigen. B cells which survive this selection differentiate further into memory B cells or plasma cells and leave the GC.

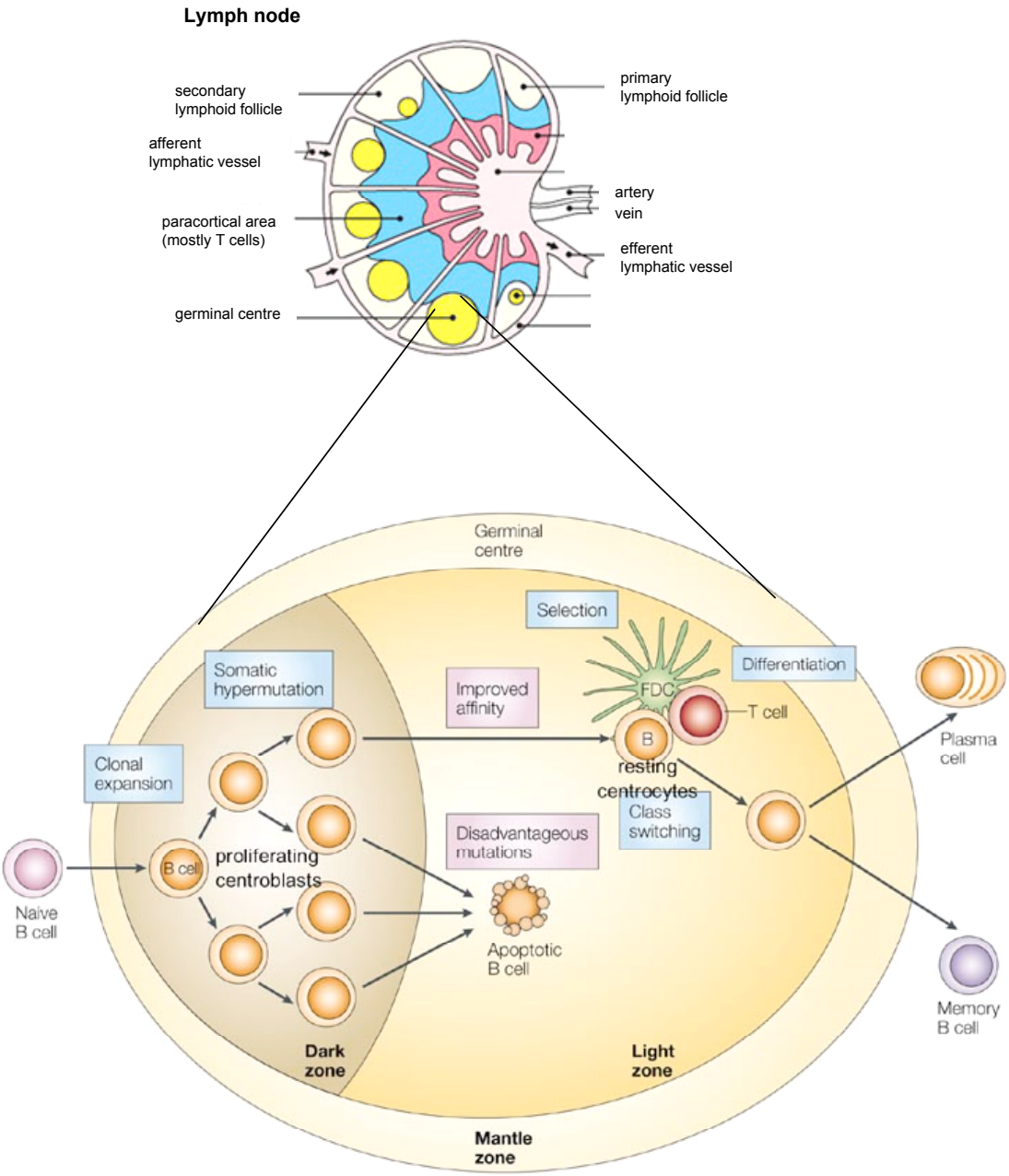


Fig.1.2. Antigen-activated B-cell development in the germinal centre. (modified from Kuppers, 2003 and Janeway et al., 2005) Mature B cells upon stimulation by antigen (interaction with Th and DC) migrate to the primary lymphoid follicles of the secondary lymphoid organs, such as lymph nodes. There, they reach the highly proliferative state of centroblasts, which form a germinal centre. In the outcome of somatic hypermutation (SHM) many antibody variants are generated. The resting centrocytes, which acquired higher affinity to the antigen upon SHM survive the selection process on the basis of an interaction with the Th and DC. The other GC B cells with disadvantageous mutations undergo apoptosis. The immunoglobulin genes of the positively selected centrocytes are then remodelled by class switching, which replaces the originally expressed immunoglobulin heavy-chain constant region genes by these of another class. Finally, the centrocytes, which are approved by the antigen verification, differentiate either into antibody-producing plasma cells or quiescent, resting memory B cells. Th: T helper cell, DC: dendritic cell, GC: germinal centre, FDC: follicular dendritic cell, SHM: somatic hypermutation.

1.4. Biology of EBV infection *in vivo*

The data on *in vivo* EBV infection stem from healthy carriers and patients with EBV-related diseases. EBV is orally transmitted and the infectious virus can be detected in oral secretions from Infectious Mononucleosis (IM) and immunosupressed patients, and at lower levels also from the virus carriers. Two different states of the virus - a lytic and a latent state - were distinguished in the experiments involving a viral replication inhibitor (acyclovir). Acyclovir eliminates virus excretion from the oropharynx (behind the oral cavity) but does not affect the level of latent infection in B cells. Moreover, only mature B cells can host the latent virus as the patients with X-linked agammaglobulinaemia (XLA) are not able to establish latent EBV infections (Murray and Young, 2001). Studies on the viral primary infection were performed with IM patients, and the following conclusions were made. EBV is shed from epithelial cells and B cells located in the oropharyngeal sites. The virus induces proliferation of infected naïve B cells in lymphoid tissues, and by expressing a full set of the viral latent genes it provokes specific cytotoxic T cell responses against EBV proteins (Young and Rickinson, 2004). Silenced EBV, non-replicating and expressing a limited gene set, persists in memory B cells of peripheral blood of IM patients (Murray and Young, 2001). Observations made with healthy virus carriers shed more light on persistent infections with EBV. Those people host also a silenced form of the virus in their memory B cells of the peripheral blood (Kuppers, 2003).

Several models of the EBV infection *in vivo* exist. Here, I refer to the infection model proposed by Young et al. (Fig.1.3).

After primary infection when EBV enters the human oropharynx where the lytic replication occurs, it infects B cells in the local area (Fig.1.3a). A full set of the viral latent proteins (latency III) is expressed in EBV-transformed B cells, whose outgrowth is controlled by the EBV-specific T cells. The virus becomes silenced (latency 0) and persist hidden in resting B cells for the host life-time. Most of the researchers agree that EBV in its silenced state persists in memory B cells, though, it is a matter of debate how the virus reaches this B cell compartment. In the presented model both ways of EBV entry into memory B cells are considered: direct infection of memory B cells, as well as an infection of naïve B cells and their passage through a germinal centre (GC).

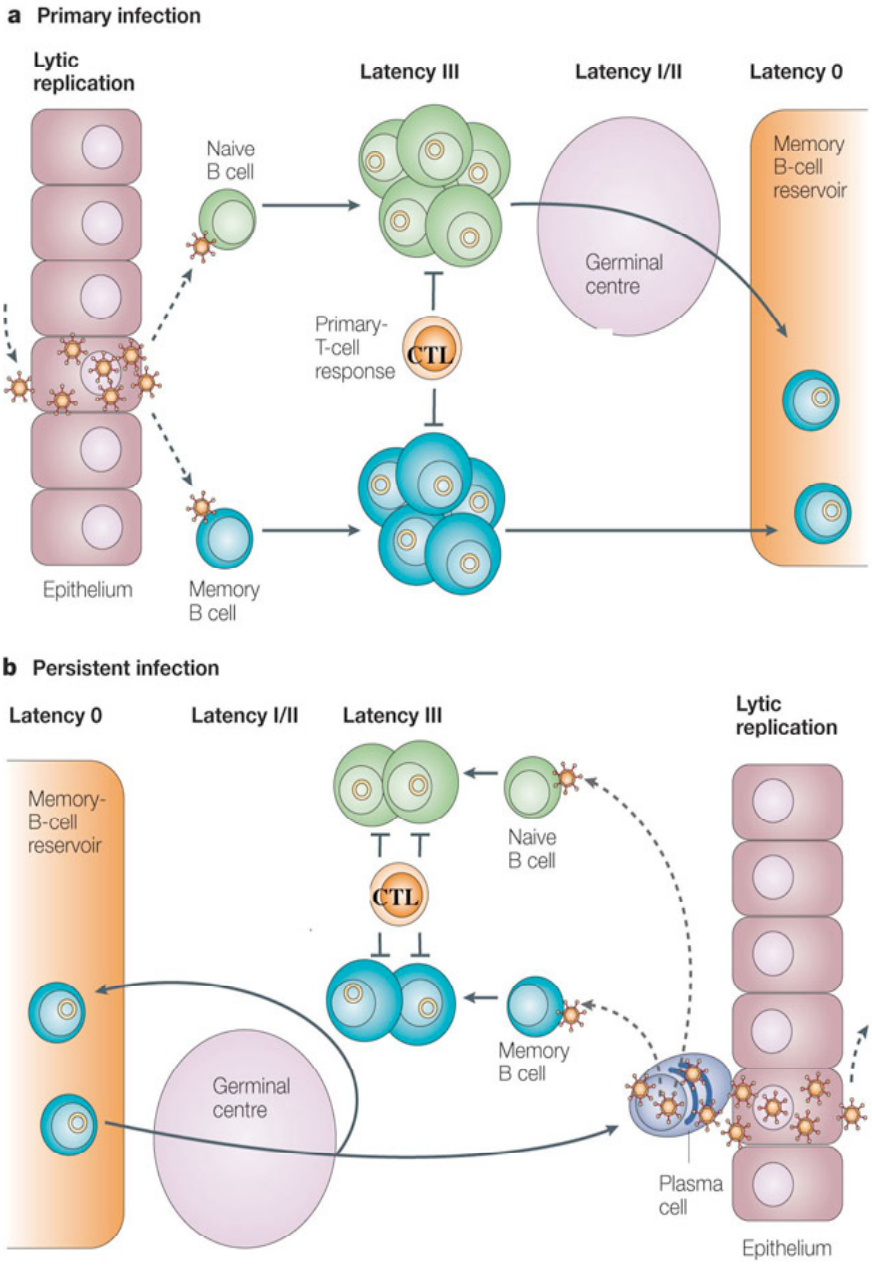


Fig.1.3. Model of EBV infection in a healthy individual. (modified from Young and Rickinson, 2004) (a) In the primary infection orally transmitted EBV enters the organism in the area of oropharynx and undergoes a lytic cycle in the local epithelium. EBV infects both naïve and memory B cells and stimulates their proliferation, which is negatively controlled by cytotoxic T cells abrogating uncontrolled B cell expansion. The proliferating B cells express the full latent EBV program (latency III). Naïve cells develop to memory cells in germinal centres, where the expression of EBV latent proteins is restricted to program I and II, and the virus ends up finally in the silenced state (latency 0) hiding in the memory B reservoir. The memory B cells infected directly in the proximity to epithelium do not need to traverse the germinal centre. Upon clonal expansion with expression of the full set of EBV latent genes (latency III) EBV in those cells becomes also silenced (latency 0). (b) The reactivation of the virus in the course of a persistent infection is initiated in the silenced memory B cells. They become recruited to the germinal centre, and they either reconstitute the memory B cell reservoir or differentiate to plasma cells. The latter give rise to virus progeny, which is then released through epithelium (oral transmission), or/and infects new B cell targets within the lymphoid tissue. CTL: cytotoxic T lymphocytes; EBV latency programs: I - EBNA1, II - EBNA1, LMP1, LMP2a, III - all latent genes.

The main argument in favour of the first scenario is the observation that EBV has an ability to infect not only naïve but also memory and GC B cells of IM patients. Additionally, it was reported that EBV-infected B cells of IM patients show no ongoing hypermutations within the expanding clones, and they were absent in the GC areas (Kurth et al., 2000). On the other hand, this scenario does not explain the disappearance of the infected naïve B cell population. The second theory is based on the similarities between EBV activation of B cells upon infection and the process of B cell antigen stimulation (Thorley-Lawson, 2001). These similarities are apparent and suggestive because different viral latent gene products mimic the basic antigen stimulation signals crucial for B cell survival and differentiation in the course of the germinal centre reaction (Fig.1.4). Upon activation by antigen the proliferating naïve B cells form germinal centres (GC), where the secondary contact with the antigen positively selects B cells with improved affinity. EBV seems to mimic the B cell activation process with its latent proteins (latency III program) and allow positive selection of B cells expressing a limited set of the viral proteins (latency II program) (Fig.1.4a). Naïve B cells become activated and later approved by binding of antigens to their B-cell receptors (BCR) and via interaction with activated helper T cells (Th) through the CD40 receptor on B cells (Fig.1.4b). EBV imitates the BCR- and CD40-derived signals with its latent proteins, LMP2A and LMP1, respectively (Thorley-Lawson, 2001).

During persistent EBV infection the virus-hosting memory B cells occasionally may become recruited to the GC and they either re-enter the memory B cell reservoir or differentiate into plasma cells to give rise to progeny virus (Fig.1.3b). The viral particles can be shed from the mucosal epithelium or infect new naïve and memory B cells.

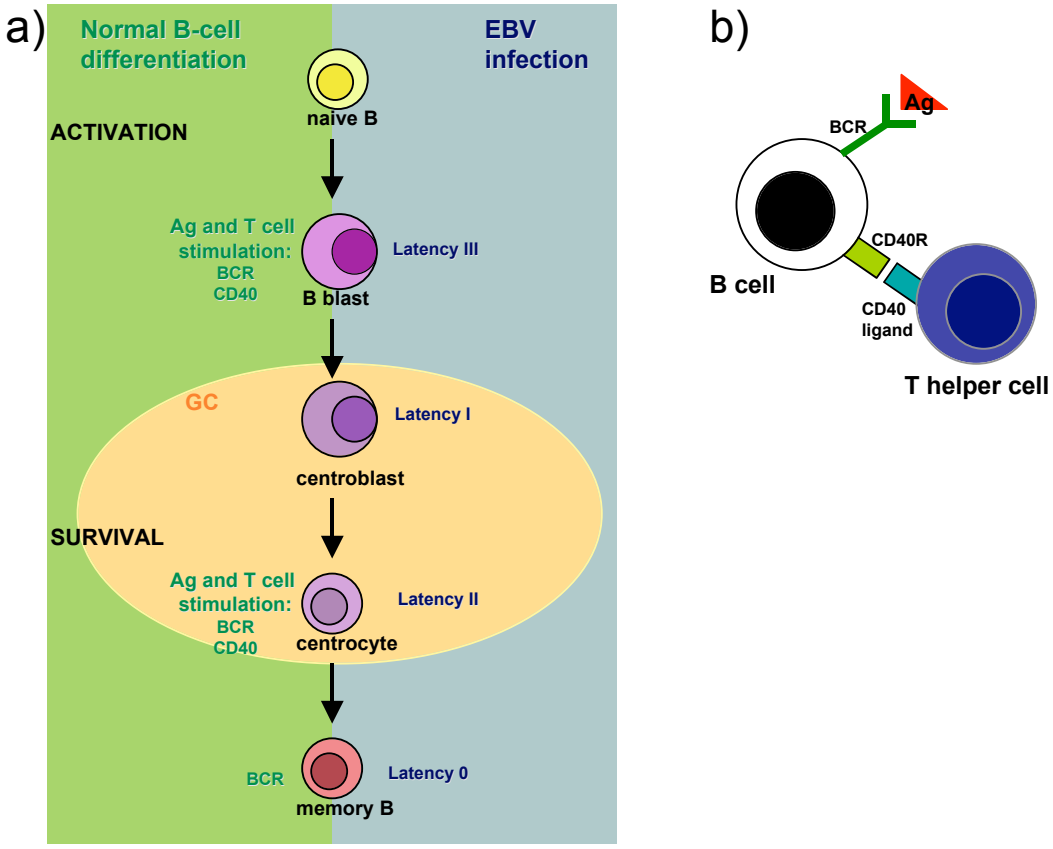


Fig.1.4. Similarities between B cell differentiation and EBV infection. (adapted from Thorley-Lawson, 2001; Thorley-Lawson and Babcock, 1999) (a) This model proposes an infection of naive B cells as a necessary step on the viral route to memory B cells. Mature, naive B cells undergo blast transformation both upon primary antigen stimulation and EBV infection. Those proliferating B cells establish a germinal centre as proliferating centroblasts, which differentiate further to centrocytes with improved affinity to the antigen. EBV latent expression is then limited to latency I and II programs. The secondary contact of the differentiating B cells with antigen and T helper cells decide about B cell survival by a verification of the affinity of their mutated B-cell receptors to the cognate antigen. Most likely EBV imitates signals from the BCR and CD40 surface proteins with its latent genes, LMP2a and LMP1, respectively. (b) B cells become stimulated by antigen via their B-cell receptor (BCR), and the interaction with activated T helper cells, which engage CD40 receptor on the B cell surface. GC: germinal centre, SHM: somatic hypermutation, Ag: antigen, BCR: B cell receptor; EBV latency programs: 0 - LMP2a, I - EBNA1, II - EBNA1, LMP1, LMP2a, III - all latent EBV genes expressed.

1.5. EBV-associated human diseases

Upon EBV infection the human host remains a carrier for a lifetime. If the first contact with the virus occurs early in childhood, the infection course is predominantly asymptomatic (Kuppers, 2003). Delayed contact with EBV leads in 30% of the cases to Infectious Mononucleosis (IM), so-called ‘kissing disease’, after oral transmission of the virus (WHO, 2006). Massive proliferation of B cells in IM is usually limited by the host immune system within few weeks (Kuppers, 2003). In some cases EBV leads to tumour development (malignancies associated with EBV infection are summarized in Tab.1.3).

Malignancy	**EBV association (%)**	**EBV latency**	**Cellular origin of malignant cells**	**Associated factors**	**Deregulation at molecular level**
Burkitt Lymphoma (BL)	95-100 (endemic) 20-30 (sporadic)	I	centroblasts (GC)	malaria, phorbol esters from plants, temperature and humidity	translocation, LOH, *myc*, *p53*, *Rb* involvement
Hodgkin Disease (HD)	40 (Western world) 90 (central America, children)	II	pre-apoptotic ('crippled') GC B cells		NFkB activation
Posttransplant lymphoprolife-rative disease (PTLD)	80	III (also I or II)	usually GC B cells (sometimes crippled)	immune suppression**	
AIDS-associated Lymphomas*	30-100	I or III	GC or post-GC B cells	immune suppression**, chronic antigenic stimulation	
Undifferentiated Nasopharyngeal Carcinoma (uNPC)	100	I/II	epithelial cells	fumes, smoke, salted fish, chemicals	
Gastric carcinoma	10	I/II	epithelial cells		
T lymphoma EBV(+)	5-50	I/II	helper and cytotoxic T cells		
Extranodal lymphoma	5-50	I/II	mostly NK cells		

Tab.1.3. EBV-associated diseases. (adapted from Kuppers, 2003), (supplemented with data from Young and Rickinson, 2004, Murray, 2001 #93, WHO) BL - Burkitt Lymphoma, GC- germinal centre; LOH- loss of heterozygosity; NK-Natural killer cells; (*): including PCNSL - Primary Central Nervous System Lymphomas, BL, PEL - Primary Effusion Lymphomas, DLCL - Diffuse Large-Cell Lymphoma (CB- centroblastic or IB- immunoblastic); (**): in the course of transplantation, radio- or chemotherapy.

EBV is linked mainly to lymphomas but it is also associated with epithelial cancers (Wensing and Farrell, 2000). EBV occurrence in tumour cells varies between the diseases. The virus is found with high frequency in B cell lymphomas (particularly in certain world regions), rather rarely in epithelial cancers and variably in T- and Natural Killer (NK)-cell lymphomas (Tab.1.3). The malignant cells infected with EBV exhibit distinct viral expression patterns in those various disorders, what might be

connected with their cellular origin. Several of the EBV-related tumours are meant to be associated with the environmental or/and genetic factors (Young and Rickinson, 2004). EBV-related diseases are encountered worldwide, however, some tumour types are more common in certain areas. For instance, 'endemic' Burkitt Lymphoma (BL) occurs mainly in New Guinea and Africa, with an incidence of 6-7 cases /100 000 in year (WHO, 2006). While the 'sporadic' BL is seen in the developed world with approximate 50-fold lower incidence (Murray and Young, 2001). Nasopharyngeal Carcinoma (NPC) is especially common in South-East Asia (1-10cases/100 000 in year) (WHO, 2006). T- and NK-cell lymphomas occur also most often in South-East Asia, and although the progenitor cells are rarely infected with EBV, they carry a high risk of lymphoma development. There is an incidence of 75 000 of new EBV-positive gastric carcinoma patients annually (Young and Rickinson, 2004). Hodgkin disease (HD) shows no geographical preference and is rather rare (1-3 cases /100 000 in year) (Murray and Young, 2001).

It has been observed that EBV-associated tumours, which are not directly linked to immune suppression involve additional environmental or genetic factors for their development (Young and Rickinson, 2004).

1.6. Transgenic murine model systems

Transgenesis has become a very common approach to study genes functions and to establish animal models for human diseases. The mouse is a favourite small animal used worldwide in this field. Its short reproduction period, high homology of its genome to human sequences and an optimal size for organ and tissue investigations are the main advantages of this *in vivo* model system (Williams and Wagner, 2000). Two most common methods in mouse transgenic research comprise microinjection of the transgene DNA into a fertilized murine egg and development of a transgenic mouse from a genetically targeted embryonic stem (ES) cell clone (Fig.1.5). The latter method enables a specific integration of a transgene into the murine genome however, it is more work- and time-consuming (Williams and Wagner, 2000).

1.6.1. Microinjection of the transgene for random integration

The microinjection technique is based on the injection of the transgene directly into a fertilized mouse egg, where it integrates randomly into the mouse chromosome

(Fig.1.5A). The insertion takes place most often at one site, but it can also happen at two or more sites on different chromosomes (Vintersten et al., 2004). About 70% of the eggs survive this procedure (data from cooperating group of E.Wolf, Gene Centre, Munich) and can be transferred directly to a foster mother. As an outcome of this procedure the offspring is genetically homogenous straight away, unless recombination of the foreign DNA takes place after the first cell divisions. In that case, the offspring will be mosaic for the transgene (Williams and Wagner, 2000).

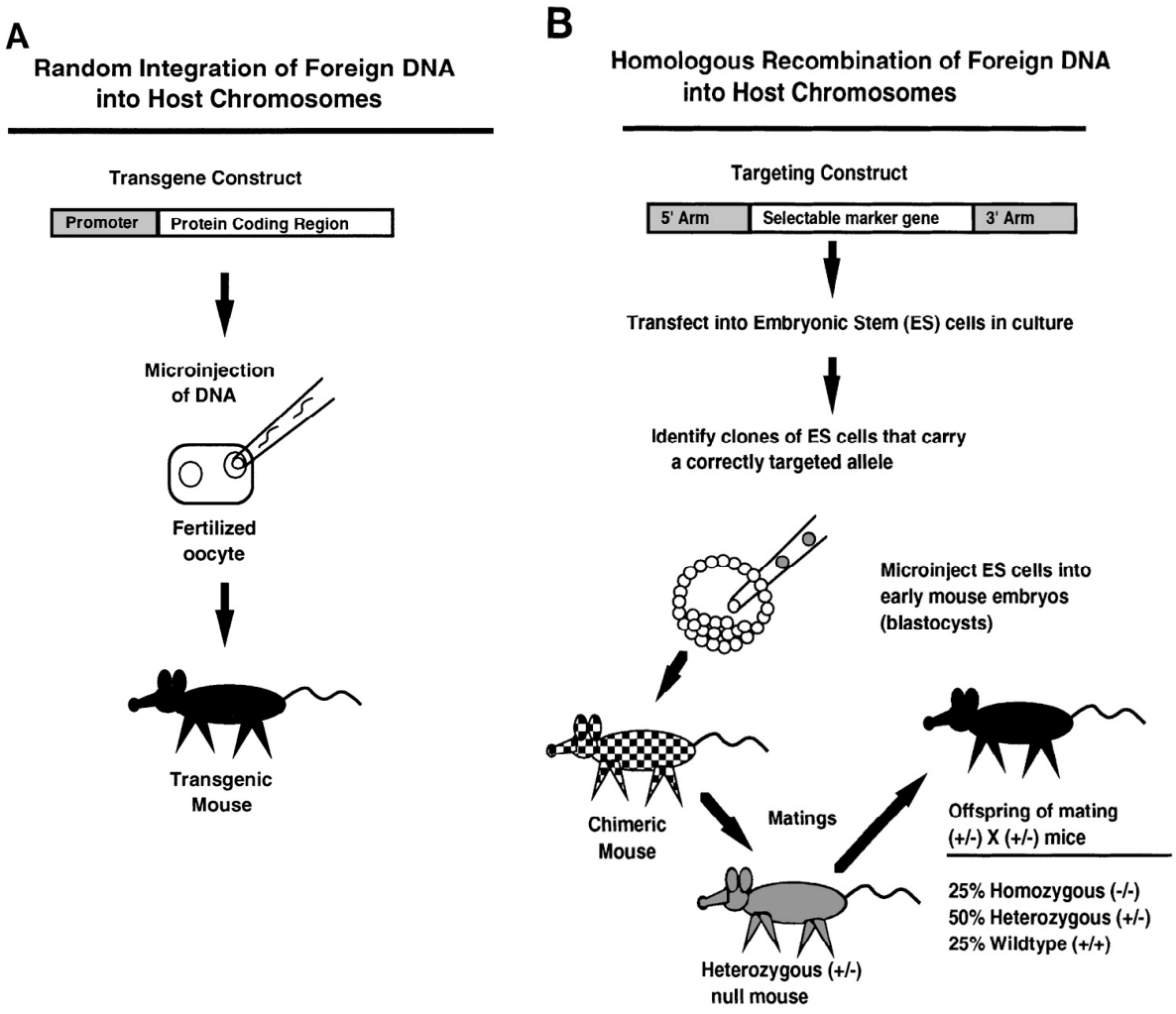


Fig.1.5. Random (A) and site-specific (B) transgene insertion into the murine genome. (Williams and Wagner, 2000) (A) Random integration takes place upon microinjection of a transgene construct into the male pronucleus of a fertilized mouse egg. The early embryo is transferred into a pseudopregnant foster mother, which gives birth to a transgenic mouse. (B) Targeting construct equipped with homologous flanks for the integration in the genome is transfected into embryonic stem (ES) cells, which are subsequently selected *in vitro* for the presence of the transgene. The desired ES cell clone with correctly integrated transgene is injected in the mouse blastocyte, which is then transferred into a foster mother. The chimeric offspring, which transmits the transgene in the germ line, gives rise to a heterozygous transgenic mouse upon cross-breeding. Further cross-breeding of the heterozygous mouse leads to a mouse homozygous for the inserted transgene.

During random transgene insertion the gene of interest is embedded in heterologous transcription regulatory sequences to direct its expression to a chosen cell type. This method is preferably applied when a recombinant protein is expressed to alter an animal biology and when studying transcriptional control (Williams and Wagner, 2000). Although random transgenesis is a very straightforward method, it also has some negative sides. Transgenes are often expressed under control of strong promoters, which may influence the expression of neighbouring genes. The transgene integration as such can interrupt expression of the genetic locus since the transgene can act as an insertional mutagene. Moreover, complete accidental inactivation of a gene necessary for embryonic development prevents further studies of such a 'knock-out' offspring. Lastly, uncontrolled expression of a given transgene in many different tissues may eventually lead to inexplicable results (Tronche et al., 2002).

1.6.2. Embryonic stem (ES) cell targeting

The pluripotent potential of the embryonic stem (ES) cells was discovered in the early eighties. ES cell lines, which maintain an undifferentiated state *in vitro*, were established upon ES isolation from murine and human embryos. After the implantation of the cultured ES cells back into an early blastocyste embryo they contributed to all cell types, including germ line in the resulting mouse chimera (reviewed in Wobus and Boheler, 2005). In parallel, it was observed that DNA fragment newly introduced into a mammalian cell can integrate into the genome via homologous flanks. This process is called homologous recombination (Folger et al., 1982). A combination of these two significant discoveries resulted in the creation of a transgenic mouse from ES cells targeted at a particular predetermined site through homologous recombination. Since then this approach is widely applied in transgenic research (Thomas and Capecchi, 1987).

ES cells targeting takes place at their undifferentiated state by transfection of a transgene, which integrates specifically into the ES cell genome (Fig.1.5B) via a homologous recombination event, or as a result of site-specific recombinase (SSR) action (Wobus and Boheler, 2005). Most often the transgene carries positive or negative selection markers what easies further analysis of the transfectants (Bockamp et al., 2002). ES cell clones with the desired genetic modification are then chosen on the basis of a genetic screen. Southern blotting and PCR are the most

standardized methods. The modified transgenic ES cells are then injected into a blastocyste originating usually in another mouse strain. The blastocyste is subsequently transferred into a pseudo-pregnant foster mother. The combination of cells from both strains within the blastocyte leads to a creation of a chimeric mouse, what is often reflected by the offspring's fur colour. After that, the chimeric offspring is crossed to obtain germline transmission of the targeted DNA to the next generation. Only then the outcome is successful and results in a mouse that hosts the transgene in all somatic and germ cells (Williams and Wagner, 2000).

1.6.3. Site-specific transgene insertion

Site-directed genome targeting of a transgene diminishes number of problems linked to the random approach as discussed in chapter 1.6.1. There are two means of introducing foreign genes at a specific site: homologous recombination and usage of site-specific recombinases (SSRs).

Homologous recombination

The mammalian cell is able to support homologous recombination between added exogenous DNA and the cellular genome on the basis of an exchange between identical DNA sequences. Selective pressure must be employed to select for this event as the frequency of homologous recombination is very low (Capecchi, 2005). Most preferred sites for the recombination of transgenes include housekeeping genes, whose expression remains constant throughout mouse development (Bronson et al., 1996).

Hprt, encoding hypoxanthine phosphoribosyl-transferase, is an example of a housekeeping gene. *Hprt* is probably the most commonly targeted site in the murine genome. The Hprt enzyme converts hypoxanthine and guanine into their respective nucleotides creating a salvage pathway for nucleotide synthesis. Targeting into this locus permits two modes of selection. Positive selection for a reconstructed, functional *hprt* gene involves cultivating the transfected cells in HAT medium consisting of Hypoxanthine, Aminopterin and Thymidine. While hypoxanthine and thymidine are sources of purine and pyrimidine respectively, aminopterin blocks the usual purine and thymidine synthesis routes enforcing the usage of the salvage hprt pathway. Negative selection against a functional *hprt* gene activity is based on the ability of the enzyme to convert purine analogues into toxic nucleotides. Additionally, localisation of the *hprt* gene on the X chromosome permits a manifestation of the

mouse phenotype already after single targeting of the male individual (Caskey and Kruh, 1979).

Site-specific recombinases (SSRs) (Cre and Flp systems)

SSRs recombine specific DNA sequences with high fidelity and they do not require any cofactors. Among many different recombinases Cre and Flp are employed in the genetic manipulation of cells. Cre (Cyclization REcombination) recombines DNA between two loxP sites in bacteriophage P1, wheras Flp inverts or 'FLiPs' sequences in between two FRT sites in *S.cerevisiae* (Branda and Dymecki, 2004). Recombinases mediate intramolecular reactions of deletion, insertion and inversion, as well as an intermolecular exchange between different DNA molecules such as chromosomes. The type of reaction depends on the orientation of the recombinase target sites loxP or FRT (Fig.1.6). If the target sites are oriented in the same direction, the recombinase will delete the flanked fragment. If the sites are oriented opposite to each other, the fragment in between becomes inverted by the recombinase.

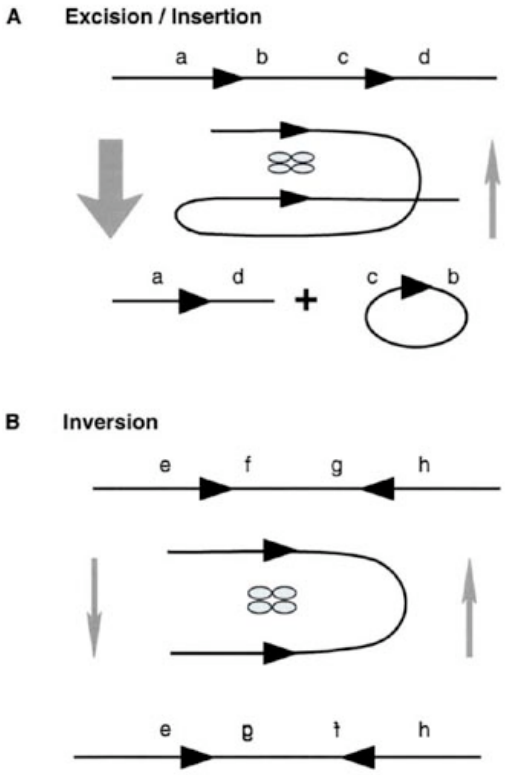


Fig.1.6. Reactions mediated by site-specific recombinases (SSRs). (modified from Branda and Dymecki, 2004) (A) Target sites (black arrows) oriented in the same direction enable deletion or insertion of the flanked fragment. (B) Two inverted target sites mediate SSR-mediated inversion.

In case of insertion or deletion, the latter is much more preferred by the enzyme, what makes it very improbable to introduce DNA fragment in such a way. Therefore, specific mutations in the target sequences were invented to favour the preferred

action (Fig.1.7). A recombination between two mutated sites results in the conformation that is no longer a substrate for the recombinase. The same approach is applied to 'freeze' the preferred conformation after SSR-mediated inversion.

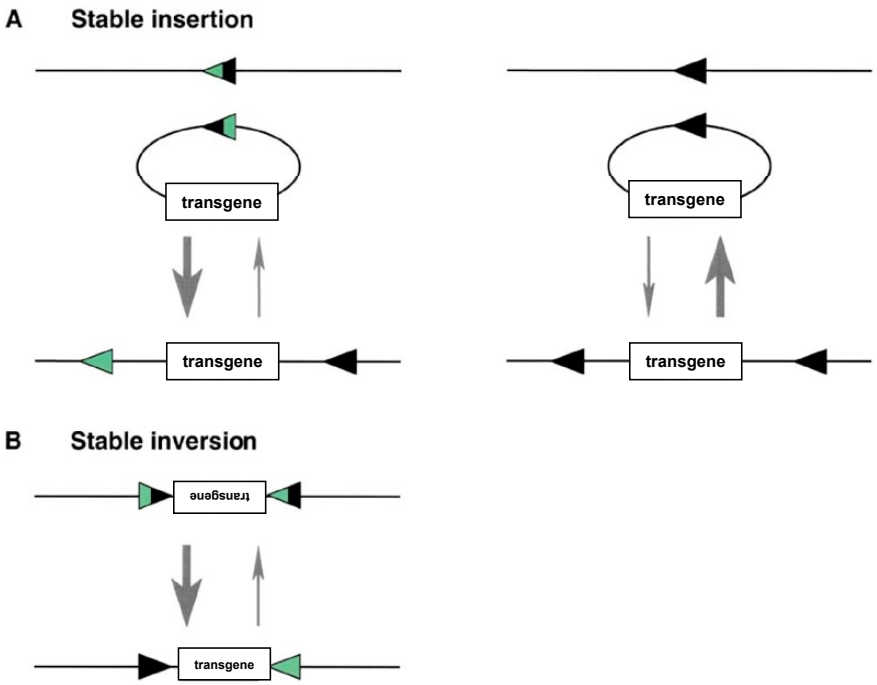


Fig.1.7. Application of mutated target sites for site-specific recombinases. (modified from Branda and Dymecki, 2004) (A) The modified sites are employed to control the outcome of the recombination. Upon a reaction between the mutated (two-colour) sites the resulting DNA structure cumulates the mutations in one of the resulting sites (green). The new conformation is resistant to the recombinase. The mutations may improve much the efficiency of the stable insertion. (B) The same approach proves useful for blocking the desired composition of the invertible fragment flanked by two inverted sites.

Site-specific recombinases are widely utilized in transgenesis. Their various applications comprise site-directed gene knock-out and knock-in, precisely set chromosomal translocation, as well as the removal of a selection cassette and intrachromosomal duplication. Recombinases are used to control genes in temporal and spacial manners. They could be either delivered to a desired tissue at a specific time point or their expression can be controlled by a promoter which is active in a certain tissue or during a certain developmental stage (Fig.1.8).

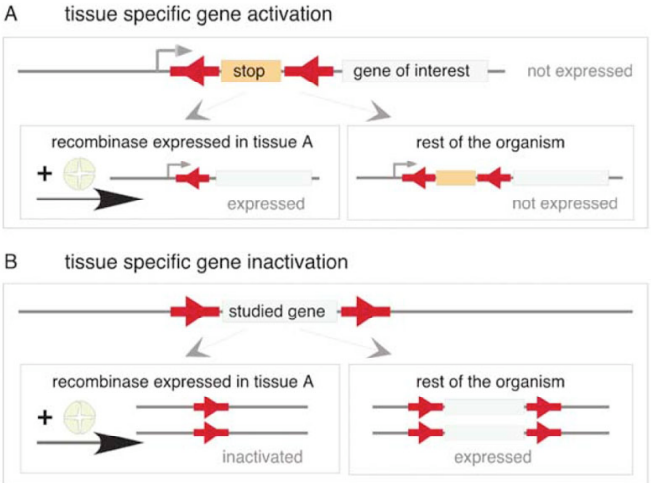


Fig.1.8. Application of site-specific recombinases (SSRs) in tissue-specific gene in-/activation. (modified from Tronche et al., 2002) (A) Activation of a blocked gene by removal of stop cassette from between promoter and coding gene, and (B) SSR-mediated knock-out (deletion of the gene in between two target sites) may be both controlled spatially by a tissue-specific promoter, which controls the expression of the recombinase.

1.7. Bacterial Artificial Chromosomes (BACs)

1.7.1. BACs as large vectors for DNA delivery

Up to date, there have been three kinds of DNA vectors with an unusual large size exceeding 30kb: Yeast Artificial Chromosomes (YACs), P1-based Artificial Chromosomes (PACs) and Bacterial Artificial Chromosomes (BACs). As there is much to be said about each vector type, I concentrate on BACs, only. All of them are reviewed in detail by Giraldo et al. (Giraldo and Montoliu, 2001), especially in the context of transgenic research. The discovery of BACs and PACs was the consequence of limitations in the application of YACs. Work with the latter requires special technical skills. Besides, the insert happens to be instable and can undergo rearrangements within the linear chromosomes. In the end, it can be difficult to purify them free of endogenous yeast chromosomes (Giraldo and Montoliu, 2001).

BACs are cloning vectors with a mini-F-factor-based replicon as a backbone. This bacterial replicon maintains a single copy of the recombinant BAC plasmid per cell. BAC plasmids are genetically more stable than YACs (Wagner et al., 2002). The cloning capacity of BACs reaches up to 300kb of foreign DNA (Shizuya et al., 1992). All these features make them especially suited to stably maintain an entire eukaryotic gene locus together with its regulatory elements. This is especially important for genes whose regulatory sequences are located tens of kilo base pairs away from their coding sequence. BACs in comparison to YACs and PACs are more convenient in handling and they can be easily separated from their *E.coli* host genome (Marshall et al., 2004).

1.7.2. Manipulations with BACs

A number of methods has been established to manipulate BAC plasmids in bacteria. Most commonly, an exchange between the BAC plasmid DNA and a linear DNA fragment is based on linear integration, while the allelic exchange between BAC and a shuttle plasmid employs the 'chromosomal building' technique (Wagner et al., 2002).

For linear integration an antibiotic resistance cassette is required to introduce a particular mutation into a BAC plasmid (Fig.1.9A). After the linear DNA fragment is transfected into *E.coli*, it recombines with the BAC plasmid via the homologous flanks in the presence of RecA. In the next step the selection marker, which is flanked by loxP or FRT sites, can be removed by Cre or Flp recombinase as described already in chapter 1.6.3.

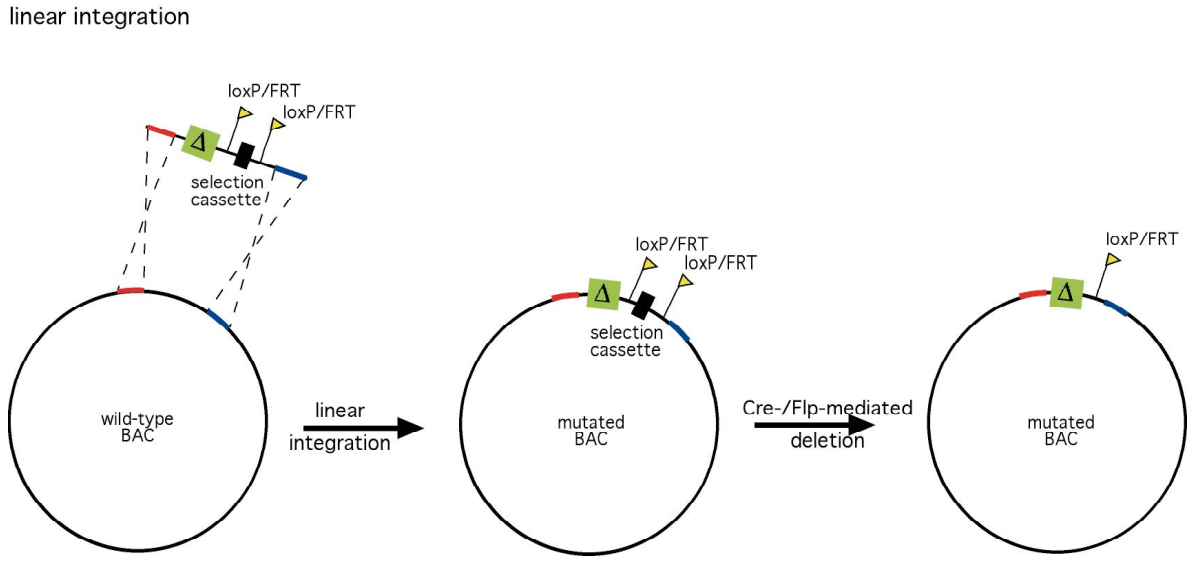


Fig.1.9A. Mutagenesis of BAC plasmids: linear integration. A linear fragment, which encompasses a mutant allele (Δ) recombines via homologous flanks (red and blue) with the wild-type BAC plasmid. In the second step, the selection marker gene by Cre or Flp recombinase-mediated deletion.

Application of the shuttle plasmid in the 'chromosomal building' technique allows introducing a specific mutation without leaving any operational traces in the recombinant BAC plasmid (Fig.1.9B).

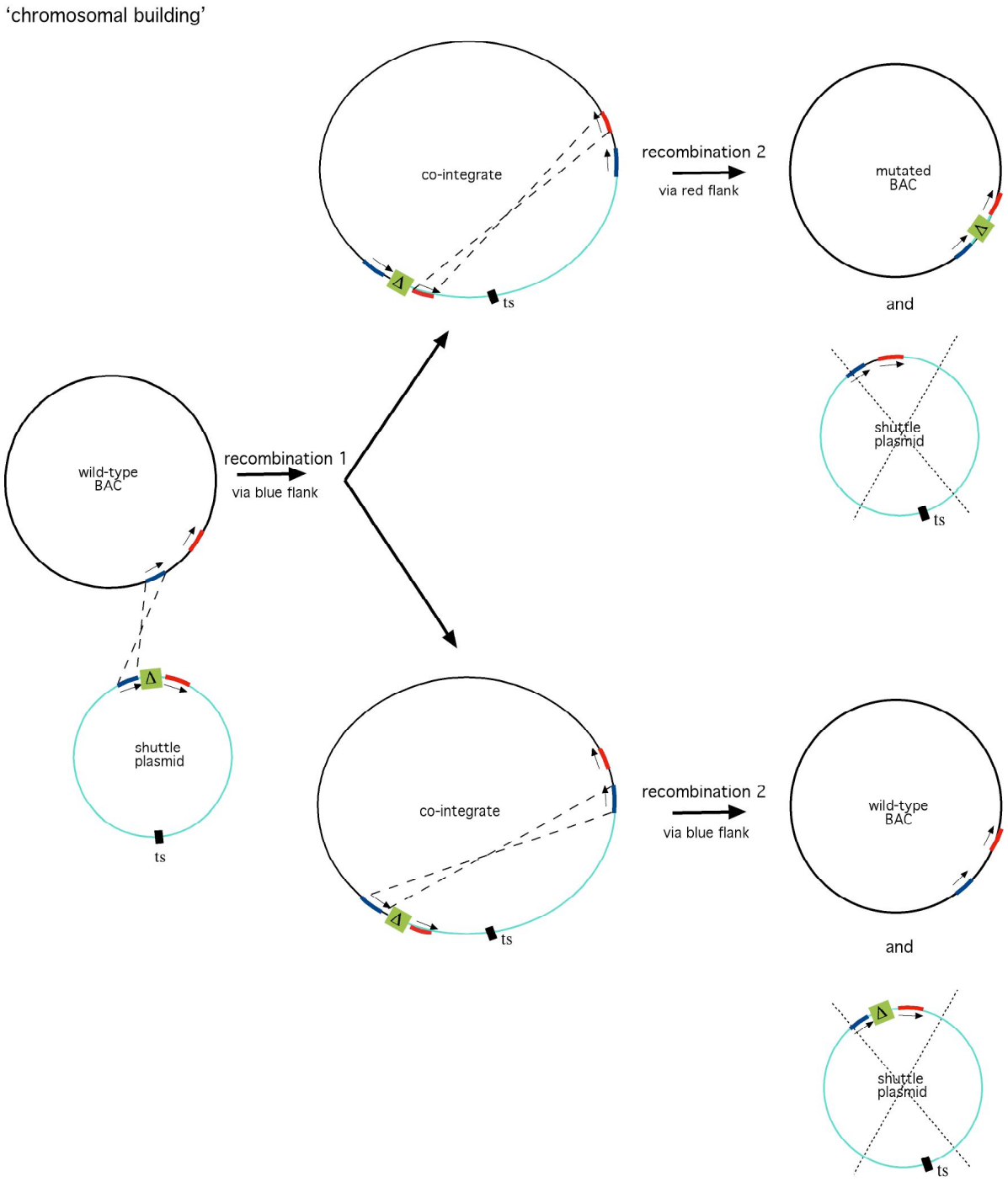


Fig.1.9B. Mutagenesis of BAC plasmids: 'chromosomal building'. Allelic exchange in a process called 'chromosomal building' is based on two steps of homologous recombination. In the first step, a shuttle plasmid carrying the mutation recombines with the wild-type BAC plasmid via the blue flank, and a co-integrate is created. The second recombination event takes place via the red flank resulting in a resolvation of the co-integrate maintaining the mutant allele. If the second recombination occurs via the blue flank again, the wild-type BAC plasmid is reconstituted. The temperature-sensitive shuttle plasmid is lost at the non-permissive temperature of 42°C.

First, a shuttle plasmid comprising the mutation is constructed, such that the mutation of the novel allele is bracketed by two DNA fragments homologous to the wild-type BAC plasmid sequences located at the site of the desired integration. Allelic

exchange between the shuttle and the BAC plasmids takes place by two independent rounds of homologous recombination in a RecA-assisted manner. In the first recombination step a co-integrate construct is created, which comprises sequences from both plasmids. In the second recombination step the co-integrate resolves. If the second recombination occurs via the homology arm of the first step, the wild-type BAC is reconstituted. If the second recombination takes place via the other arm, a mutant BAC is gained (Wagner et al., 2002).

1.7.3. BACs as transgenes

The idea of large DNA fragments as transgenes emerged from the need to overcome the 'position effect' (Giraldo and Montoliu, 2001). This term describes a phenomenon where host sequences flanking the insertion site of the transgene affect the transgene's expression (Wilson et al., 1996). The other advantage that artificial chromosomes brought into the transgenic field is the opportunity to operate with complete genes, including their regulatory elements, as described already above. Transgenic animals carrying large vectors (BACs, PACs, YACs) as transgenes are generated with efficiencies of 5-20% of newborns, and such efficiencies are comparable to the standard procedures using small constructs, of less than 30kb as transgenes (Giraldo and Montoliu, 2001). The copy number of the randomly integrated BACs and YACs is usually low, not exceeding 5 copies, however, it could reach as many as 13 copies (Nielsen et al., 1997). Presumably due to the large size of BAC transgenes a rearrangement or a fragmentation of their sequences can occur (Giraldo and Montoliu, 2001).

1.7.4. BACs and Herpesviruses

Herpesviruses have the largest genomes among mammalian viruses with up to 200 potential open reading frames (Wagner et al., 2002). Therefore, only the establishment of BAC technology enabled the universal genetic analysis of these complex viruses. Many herpesvirus genomes have been already cloned onto a BAC backbone, including EBV (mentioned already), Herpes Simplex Virus 1 (HSV1), human Cytomegalovirus (hCMV), Murine □ herpesvirus MHV-68 and Kaposi's Sarcoma Herpesvirus (KHSV) (Wagner et al., 2002). BAC-cloning of herpesvirus genomes requires several sequential steps and is described for EBV in Fig.1.10, as an example. In short, the sequences of the BAC replicon are introduced into a viral

genome via homologous recombination in the virus-infected cell line. Because the herpesvirus genome circularizes during replication, it can be isolated as a plasmid and transferred to *E.coli* cells, where it can be genetically manipulated (Wagner et al., 2002). Furthermore, the recombinant viral genome can be packaged into the virus particles in virus-negative cells upon spontaneous onset or induction of the viral lytic cycle. After infection with the recombinant virus its phenotype in the host cells can be analysed (Fig.1.10).

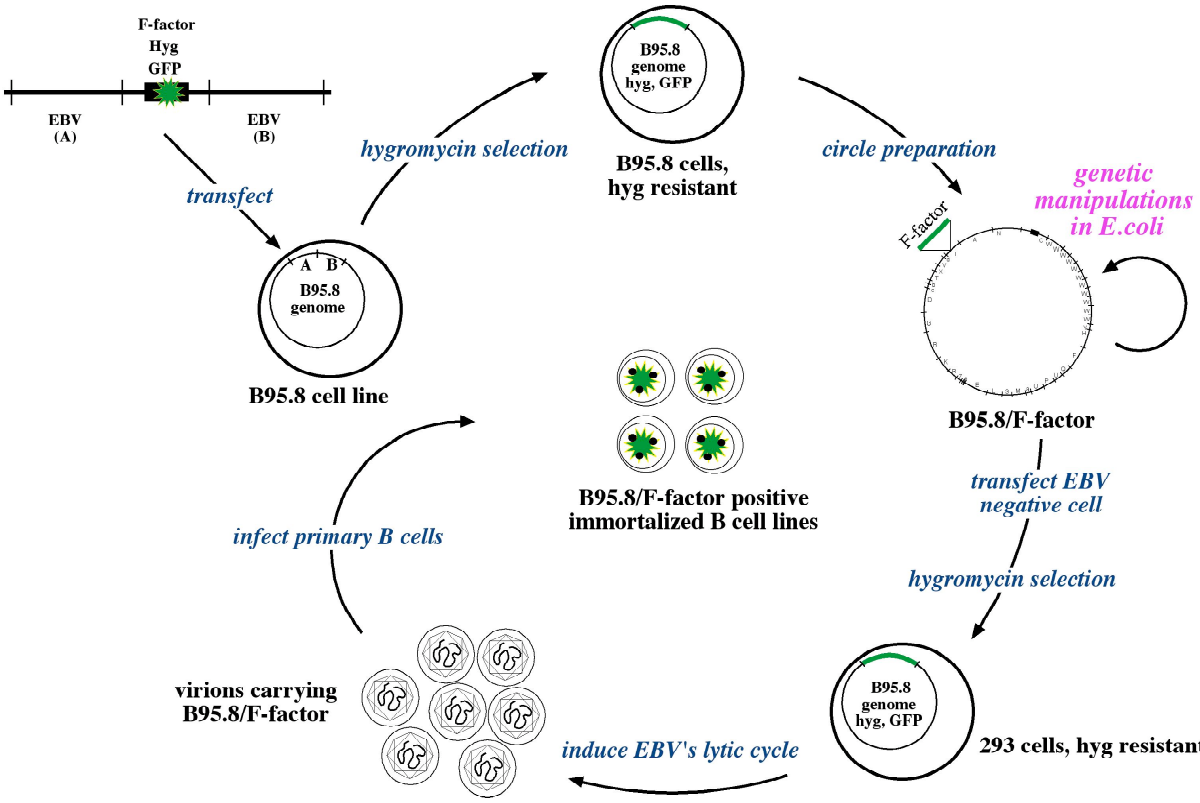


Fig.1.10. BAC-cloning of the EBV genome and its mutagenesis in bacteria. Schematic overview of the maxi-EBV system. (Delecluse and Hammerschmidt, 2000) After a fragment comprising F-factor bacterial replicon is transferred into a latently EBV-infected B95.8 B cell line, it recombines with the genome of EBV resulting in a B95.8/F-factor or maxi-EBV plasmid. The co-integrate is sustained in the cell line under selective pressure with hygromycin. Upon isolation of the maxi-EBV plasmid from the cell line and its transfection into *E.coli* cells, it can be genetically modified in the bacteria. Different genetic variants of maxi-EBV plasmid can be introduced into the EBV-negative cell line 293 for packaging into virus particles after initiation of the viral lytic cycle.

This approach opened the opportunity for a detailed genetic analysis of all viral genes. A combination of the bacterial F-factor replicon with the EBV genome resulted in an easy to manipulate viral EBV vector (Delecluse et al., 1998). Two different recombinant EBV vectors have been established: maxi-EBV covers the entire genome of EBV (Fig.1.11) and mini-EBV contains approximately 40% of the EBV genome, sufficient to transform primary B cells (Bornkamm and Hammerschmidt,

2001; Kempkes et al., 1995). Encapsidation of BAC-based EBV vectors has been improved with the invention of a virus-free 293 packaging cell line hosting an EBV mutant lacking its packaging signals (Delecluse et al., 1999).

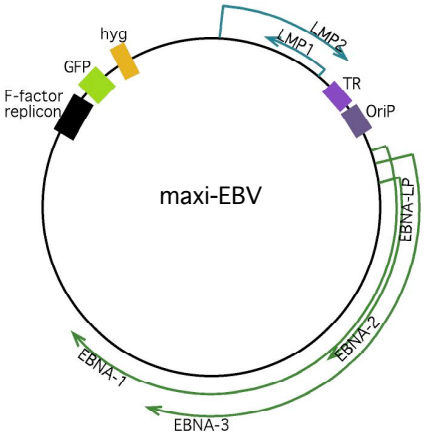


Fig.1.11. The EBV genome cloned onto F-factor bacterial replicon results in a BAC plasmid, termed maxi-EBV. The plasmid comprises an entire EBV genome, however, only latent genes, viral replicon (OriP) and viral terminal repeats (TR), necessary for virus packaging, are depicted here. Plasmid backbone includes the F-factor bacterial replicon, GFP and *hyg* resistance gene. GFP: green fluorescent protein, hyg: hygromycin, TR: terminal repeats, OriP: viral replicon, LMP: latent membrane protein, EBNA: EBV encoded nuclear antigen.

1.8. Current state of research on EBV

1.8.1. *In vitro* and *ex vivo* studies of EBV genes

The majority of the data on the molecular aspects of EBV infection originate from *in vitro* studies. Primary human B lymphocytes become growth-transformed by EBV's latent gene products and give rise to lymphoblastoid cell lines (LCL) *in vitro*. Cells can be either infected directly *in vitro* or infected cells can be isolated from an EBV-positive host. Research based on LCLs generated with wild-type or laboratory strains of EBV has generated a wealth of data on the characteristics of viral DNA and proteins (Kieff, 1991).

Murine cells expressing human receptors for EBV (CD21 and HLA class II) can be infected with the virus, although inefficiently leading to the expression of the EBV latent genes (latency II program) and a long-term persistence of the viral genome (Haan et al., 2001). Moreover, the characteristic and important feature of EBV to transform B cells was not shown in this approach. As a consequence this finding has not led to a murine model for EBV.

1.8.2. Existing *in vivo* models for EBV infection

Studying EBV *in vivo* is principally limited to human biopsies and therefore descriptive. As a result, many topics, in particular latency and oncogenesis, are hypothetically and controversially discussed (Young and Rickinson, 2004). EBV is a

strictly human virus and does not infect murine cells, what makes it extremely difficult to establish a model for its pathogenesis. There has been much progress for the last decades (reviewed in Kuppers, 2003; Young and Rickinson, 2004), which provided relevant insights into EBV's biology but many of the important and central questions remain unsolved.

Human-related species and EBV

Although New World primates are natural hosts for distantly EBV-related viruses, they are susceptible to EBV in an experimental setting. Cotton top tamarins, a certain species of New World monkey, develop a lymphoproliferative disease upon EBV infection, and some even die from lymphoma, but the virus is not transmitted orally between those animals, and it does not persist in the silenced state in their lymphatic compartment (Liebowitz, 1998).

EBV-related animal viruses and their natural hosts

EBV together with a related human herpesvirus – Kaposi sarcoma-associated herpesvirus (KSHV) - belongs to the subfamily of γherpesviruses. Among the animal herpesviruses from the same viral subfamily particularly two viruses reflect many aspects of the EBV-provoked diseases upon infection of their natural hosts: lymphocryptovirus (LCV) in Old World primates (e.g. rhesus monkey, orangutan) and murine gammaherpesvirus (MHV-68) in mouse.

Old World primates are not permissive to an EBV infection, but they are found infected with their own species-specific lymphocryptoviruses, which are closely related to EBV. The cross-reactivity could be responsible for their immunity to EBV. LCV genomes are highly homologous to EBV and encode a similar range of lytic and latent genes (Rivailler et al., 2002). Besides, the monkey-LCV model system reproduced many important features of an EBV infection, from oral transmission, through activation of peripheral B cells and antibodies production, to latent persistence of the virus in peripheral blood (Moghaddam et al., 1997). However, different aspects of disease progression and problems arising from work with such big animals make these models uncommon for studying EBV biology (Haan et al., 2001).

An infection with the murine MHV-68 virus is in certain aspects reminiscent of an EBV infection in humans because both MHV-68 and EBV can activate B cells evoking infectious mononucleosis syndrome and induce a viral silenced state.

Furthermore, the MHV-68 sequence is 80% homologous to the EBV genome. However, the disease phenotypes differ between both viruses (Stevenson and Efstathiou, 2005) and MHV-68 lacks all homologues for important EBV latent genes (Haan et al., 2001).

Other animal herpesviruses reconstruct certain aspects of EBV infection. For instance, rabbits infected with an EBV homologue, Cyno-EBV, develop T-cell lymphomas with chromosomal abnormalities (Hayashi and Akagi, 2000).
Herpesvirus saimiri (HSV) infects quite a wide range of hosts: from Old and New World primates to rabbits. In contrast to EBV it can initiate T-cell lymphoproliferative disease and certain subtypes can immortalize T-cells in culture. Its genome consists of genes similar to EBV genes but both herpesviruses differ completely in their set of latent genes (Jung et al., 1999).

Immunodeficient murine models
Intraperitoneal injection of *in vitro* EBV-infected human B cells into severe combined immunodeficient (SCID) mice causes lymphoproliferative disease seen in humans upon EBV infection. Tumours were also observed when the mice were injected with latently infected B cells isolated from human peripheral blood (Liebowitz, 1998). In a different model immunodeficient mice ($Rag2^{-/-}$, $\gamma_c^{-/-}$) can be engrafted with a human hematopoietic system and become a model system for EBV. This system can reconstruct EBV's entry into B cells, their subsequent proliferation, as well as limited EBV-specific T cell responses directed against the infected B cells (Traggiai et al., 2004).

Transgenic mice with single EBV latent genes
Several latent proteins of EBV have been introduced genetically into transgenic mice. The selected phenotypes have been already described in the chapter 1.2 and in Tab.1.1. Studying single EBV proteins in transgenic animals, although informative, cannot reflect the complexity of a natural viral infection.

2. AIM OF THE PROJECT

Little is known about the fate of the virally infected cells in the human host and many aspects of EBV's life cycle *in vivo* are not understood. What has also remained enigmatic is the occurrence of EBV-associated malignancies. To address these aspects a relevant small animal model system for EBV is required. The mouse is the most versatile small animal model for different human diseases (Williams and Wagner, 2000) and this study takes advantage of the well-established techniques of mouse genetics in order to introduce the complete EBV genome into the murine genome. This approach would overcome the inability of EBV to infect cells of non-human origin.

I undertook two routes to insert a genetically altered but otherwise complete EBV genome into murine cells to assure its stable integration. One route was based on the site-specific integration into the *hprt* locus of murine embryonic stem cells. The other route relied on pronucleus microinjection for random insertion of the EBV DNA into the genome of fertilized murine oocytes. In both approaches the undifferentiated, genetically modified mouse cells are transferred into foster mice to give rise to transgenic offspring. To engineer a recombinant EBV genome for its integration into the murine genome a special effort was made. This targeting construct (InvTarg) allows the conditional expression of EBV's latent genes via a Cre/loxP system. This approach prevents potentially adverse effects of EBV's latent genes on embryonic development but allows their expression in almost any chosen cellular compartment for which specific Cre-expressing mice are available.

This work is divided into three main parts. The first part (chapters 5.1-5.3) concentrates on the InvTarg targeting construct, its features, construction steps and functional analysis. The next section (chapter 5.4) describes handling methods applied in order to purify and prepare the large Bacterial Artificial Chromosome (BAC)-based targeting vector, InvTarg, for its introduction into the mouse cells. The last part (chapters 5.5 and 5.6) describes the many attempts to integrate the linear InvTarg transgene into the murine genome and the analysis of its integration.

3. MATERIALS

3.1. Bacterial strains

Strain	Genotype	Source
E.coli DH5	F-, lacI-, recA1, endA1, hsdR17, (lacZYA-argF), U169, F80dlacZ M15, supE44, thi-1, gyrA96, relA1	(Hanahan, 1985)
E.coli DH10B	F-, mcrA, (mrr-hsdRMS-mcrBC), 80dlacZ M15, lacX74, deoR, recA1, endA1, araD139, (ara, leu)7697, galU, galK, -rpsL, nupG)	(Life Technologies)

DH5 strain was a host for small DNA plasmids (up to 20kb), while DH10B was maintaining BAC constructs.

3.2. Cell lines

Human lines

18 aus 4	human B cell line immortalized with ΔEBNA1-EBV (p2828) integrated in the genome (donor 18)	(Humme et al., 2003), Fig.2, lane4
Raji	EBV positive Burkitt Lymphoma cell line	(Pulvertaft, 1964)
Wi38	primary embryonic fibroblast cell line	(ECACC)
HEK293 TR(-)	embryonic kidney epithelium cells transformed with adenovirus type 5 and hosting a maxi-EBV genome lacking its terminal repeats (EBV packaging cell line)	(Delecluse et al., 1999)

Murine lines

E14TG2a4	*hprt*-negative embryonic stem cell lines, derived from the 129/Ola mouse strain	(Hooper et al., 1987)
HM1		(Magin et al., 1992)

3.3. Media

Cell culture media

All human cell lines and primary human B cells isolated from patient samples were cultivated in RMPI 1640 medium with L-Glutamine (Life Technologies) complemented with 100µg/ml Streptomycin/100u/ml PenicilinG (Life Technologies), 10% FCS (Biochrom) and 1mM Sodium Pyruvate (Life Technologies). The maxi-EBV carrying stable line 293 TR(-) was cultured with selection medium with 80µg/ml Hygromycin B (Calbiochem).

Murine embryonic stem cells were grown in two cell culture media, which differed in two components:

Composition 1: DMEM (Invitrogen) with 15%FCS (PAN, South America);

Composition 2: Glasgow medium (BHK-21) (Invitrogen) with 10% HyClone FBS serum (Perbio).

The remaining components were used in common in both media: 1800u/ml ESGRO (Chemicon), 1.2mM sodium pyruvate (Invitrogen), 1.2mM L-glutamine (Invitrogen), 1.2x non-essential aminoacids (Invitrogen), 0.12mM β-Mercaptoethanol (Sigma).

Transfection media

Opti-MEM (Invitrogen) was used for lipofection and RPMI 1640 (without phenol red and L-glutamine; PAA Laboratories) for electroporation.

Bacterial media

LB (1% Trypton (Life Technologies) 0.5% yeast extract (Difco Laboratories), 0.5% NaCl)

LB agar plates (15g Bacto-agar (Difco Laboratories) per 1l LB)

SOB (LB with 10mM $MgCl_2$ (Merck) and 10mM $MgSO_4$ (Merck))

SOC (SOB with 20% glucose)

3.4. Oligonucleotides

All the oligonucleotides were synthesized by Metabion.

PCR primers

EES1F17*	5'- AAT ACA TCC AGA TTA AAA TCG CC -3'
EES1B2mod	5'- ATA ACT TCG TAT AAT GTA TGC TAT ACG AAC GGT AGA AGC CTA GGA ATT ATT TAC CAC ACC CC -3'

3.4.PCR1	5’- GCT TTC TAC TTC CCC TTT CTA CGC -3’
3.4.PCR2*	5'- TGG CTG GTG ATT GGA GGT TTG -3’
LMP1-F	5'- TCC ATC ATT TCC AGC AGA GTC G -3'
LMP1-B	5'- CTC CTT TGG CTC CTC CTG TTT C -3'
WW-F	5'- AAA GGG TAA CAG GAG AGG CAG G -3'
WW-B	5'- AAG AAG AGG AGG TGG TAA GCG G -3'
LMP2-F	5'- ACG ATG GCG GAA ACA ACT CC -3'
LMP2-B	5'- GCT GGG GGT GTC AAC AAA GAA C -3'
LOX71-F	5'- AAT CGC CAG AAA CAG GAG G -3'
LOX71-B	5'- TTT GTC CAG ATG TCC AGG G -3'
LOX66-F	5'- ATT ACA CCC TTT GCC CCA C -3'
LOX66-B	5'- TGC GTA GCA GCC ACT CTC TAA G -3'
I1-F	5'- CCT GGA CTG GTC TCT CAA TC -3'
I1-B	5'- CTC CTG TTA CCC TTT TAG AAC C -3'
EBNA1-F	5'- CGG GGT CGA GGA GGT AGT G -3’
EBNA1-B	5'- TTC GTC GGT AGT CCT TTC TAC G -3’

(*): used also for sequencing

Sequencing primers

M13rev (Invitrogen)	5´-CAG GAA ACA GCT ATG AC-3´
1.4seq1	5'- CCT TTG TTT TCA ACC TCT TCC G -3’
1.4seq2	5'- CAC CCC AAC TTG TTT ATT GC -3’
1.4seq3	5'- GAT GGA ACA CGA CCT TGA G -3’
2.5seq1	5'- CAG CGC GGG GAT CTC ATG -3’
2.5seq2	5'- GCC ATC CAA AGC ATT CG -3’
2.5seq3	5'- CAG CCT GGG ATA ACA CG -3’
3.4seq1.1	5'- GGT GTG GGC TGT GCG AGT G -3’
3.4seq2.1	5'- TAG CCA TCC AAA GCA TTC G -3’
3162seq1	5'- TGA ATC CTC AAC CCT ATA CC -3’
3162seq4	5'- TCT CCG CCT CAT TCA ATA G -3’
4.3seq-ORI_mut1	5'- TAG AGA ATA GGA ACT TCG GAA TAG -3’
p3299_HPRTpromoter	5'- GGC AGG AGA ATC ACT TGA AC -3’
p3299_ORI_lyt	5'- CAT AGA AAA TCA CCC CAA GTC -3’
p3299neoRflank	5'- AGC GAA ACA TCG CAT CG -3’

Synthetic oligos

EES101	5'-Pho-TCG AGA TAA CTT CGT ATA GCA TAC ATT ATA CGA AGT TAT GCT AGC GGA ATG TTA-3'
EES102	5'-Pho-GAT CTA ACA TTC CGC TAG CAT AAC TTC GTA TAA TGT ATG CTA TAC GAA GTT ATC-3'

3.5. Plasmids

Already established plasmids are listed below and constructs made in this project are summarized in Tab.1 of *Appendix*. The established plasmids stem from the collection of the Department of Gene Vectors, if not specified otherwise. Additionally, the description of all the plasmids mentioned in this work can be found in the laboratory database (File Maker Pro, CLONES.FP5).

pBluescript II sk (Stratagene)
pEGFP-C1 (enhanced Green Fluorescent Protein) (Clontech Laboratories)
p2266 (Cre recombinase)
p2670 (pCMV-BALF4)
p288.1 (SV40 early promoter-LMP1) – received from Mary Kay, Madison, USA
p509 (pCMV-BZLF1) (Hammerschmidt and Sugden, 1988)
p995 (disabled replication origin of EBV) (Schepers et al., 1993)
pCP20 (Flp recombinase) (Cherepanov and Wackernagel, 1995)
pCR2.1 (TA-cloning vector) (Invitrogen)
pKD46 (encoding red □□□proteins from phage □ (red□is arabinose-inducible))
pSV40/Zeo (Stratagene)

p2089 (wild type maxi-EBV) (Delecluse et al., 1998)
p3604 (mutant maxi-EBV plasmid modified at oriP and EBNA1)

3.6. Chemicals and biochemicals

1-Butanol (Roth)
Acetic acid (Merck)
Agarose (Invitrogen)
BSA (New England Biolabs)
Cesium chloride (Sigma)
KH_2PO_4 (Merck)
L-arabinose (Roth)
NaCl (Merck)
NaH_2PO_4 (monosodium phosphate) (Merck)

Chloroform (Merck)
CIP (Calf Intestine Phosphatase; alkaline phosphatase) (Promega)
DMSO (Dimethylsulfoxid) (Merck)
DNA Markers (New England Biolabs)
dNTPs (Promega)
EDTA (ethylenediaminetetraacetic acid) (Merck)
Ethanol (Merck)
Ethidium Bromide (if not specified Otherwise - Merck)
Glucose (Merck)
Glycerol (Merck)
Glycine (Merck)
HAT (5mM Hypoxanthine, 0.02mM Aminopterin, 0.8mM Thymidine) powder, 50x stock (Sigma)
HCl (Merck)
Isopropanol (Merck)
Isoamylalcohol (Merck)
KCl (Merck)
Na_2HPO_4 (disodium hydrogen phosphate) (Merck)
NaOH (sodium hydroxide) (Riedel-de Haen)
Neomycin (G418 disulfate salt) (A1720; batch: 200mg/ml, 71% active) (Sigma)
PBS for ES cell culture (Dulbecco's w/o Ca&Mg) (PAA Laboratories)
Phenol (Roth)
Potassium acetate (Merck)
SDS (Sodium dodecyl sulphate) (Merck)
Sodium acetate (Merck)
Spermidine (Sigma)
Spermine tetrahydrochloride (Sigma)
Sucrose (MP Biochemicals)
Tris(Tris-(hydroxymethyl)-aminomethan) (Roche)
Triton X-100 (Merck)
Trypsin-EDTA 1x (Life Technologies)

3.7. Enzymes

Lyzozyme (Sigma)
T4 ligase and 10x T4 DNA Ligase Buffer (New England Biolabs)
Restriction endonucleases (New England Biolabs and MBI Fermentas)
RNaseA (US Biological)
ProteinaseK (Roche)

3.8. Buffers

Church buffer	400mM Na_2HPO_4, 100mM NaH_2PO_4, 7%SDS, 1mM EDTA; pH 7.2
Dialysis buffer	10mM Tris-Hcl pH 7.5, 0.1-1mM EDTA, 100mM NaCl; filter-sterilized

Isopropanol saturated with CsCl and water	
Microinjection buffer	10mM Tris-Hcl pH7.5, 0.1-1mM EDTA, 100mM NaCl, 30□M spermine and 70□M spermidine; filter-sterilized
M-stat buffer	0.25mM EDTA, 0.5% Triton X-100, 50mM Tris-HCl pH8, 5% sucrose
PBS	137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.4mM KH_2PO_4; pH7.5
SSC 2x	300mM NaCl, 30mM sodium citrate
TAE 1x	40mM Tris-HCl pH8, 5mM NaAc, 1mMEDTA
TBE 1x	89mM Tris, 89mM Borate, 2mMEDTA
TE 1x	10mM Tris pH8, 1mM EDTA
TFB buffer	10mM K-Mes pH6.2, 50mM $CaCl_2$, 45mM $MnCl_2$, 100mM RbCl

3.9. Laboratory equipment

BioPhotometer (Eppendorf)

Cell culture equipment (hoods (BDK, Heraeus), incubators (Thermo Life Sciences))

Cetrifuges (Eppendorf, Hettich, Sorvall, Kontron), ultracentrifuge (Beckman) with 35ml and 11.5ml tubes (Sorvall)

Electrophoresis (horizontal) chambers: Model B3 (Owl Separation Systems), Model 41-2025-R (Peqlab)

Electroporation unit (Gene Pulser; BioRad) with cuvettes (Peqlab) (2mm - for bacteria, 4mm - for eukaryotic cells)

FACS machine (FACS Calibur; Becton-Dickinson)

Fluorimeter (DyNA Quant200; Hoefer),

Micropipettes (Gilson)

Microscope (phase contrast and fluorescent) (Zeiss)

3.10 Other materials

Acrodisc Siringe Filters (0.8□m) (Pall Gelman Sciences)

Biomax MS films (Eastman Kodak)

Folded filters (Schleicher&Schuell)

Human tissue samples (Kinderklinikum Dritter Orden)

QIAEX II and QIAquick Gel Extraction Kits (Qiagen)

Pasteur pipettes (glass) (Roth)

Plastic articles (plates, cryotubes, different tubes) (Falcon, Nunc, Eppendorf, Hartenstein)

Tooth picks (Forster)

4. METHODS

4.1. Bacteria culture

Bacteria were cultured either in suspension (LB medium) or on agar plates at the specified temperatures. Different antibiotic selection was applied at given final concentrations: 100□g/ml Ampicillin (Sigma), 20□g/ml Chloramphenicol (Merck), 12□g/ml Tetracycline (Merck), 25□g/ml Zeocin (Invitrogen). For long-term storage bacterial clones were kept in 50% glycerol stocks at –80°C. Bacteria were grown from the stock on a plate containing the appropriate antibiotics by dilution streaks.

4.2. Cell culture

All cells were cultivated in cell culture incubator at 37°C with 5% CO_2. Cell work was carried out in sterile conditions of a hood, especially important for antibiotic–free work with embryonic stem cells.
Cells were counted with a Neubauer chamber and living cells were distinguished from dead ones by a staining with Eosin (0.1% in PBS). Cells were collected by centrifugation for 5-10min. at 1200 rpm, RT.

Maintenance
293 TR(-) cells, not firmly adherent, were split every 3-4 days. After medium removal cells were stripped with a stream of a fresh medium, resuspended and plated (1:7). Adherent Wi38 cells were split twice a week with a 1:5 ratio by trypsynization. All B cells were cultured in suspension. Raji and EBV-transformed human B cells were split 1:5 every third day. ‘18 aus 4’ B cell clone was split 1:3 every five days.

Murine ES cell culture was carried-out exclusively with plastic pipettes (Cellstar). HM1 cells were cultured in ES medium of composition 1, while E14 cell line was maintained in ES medium of composition 2. The cells were split every second day with daily medium change. After washing the cells three times with PBS they were detached with 3min. incubation with Trypsin-EDTA (with 1:100 chicken serum). 10^6 cells were plated per 10cm dish in 10ml volume. Both ES cell lines were cultivated without feeder cells, directly on 0.1% gelatine (after equilibration to RT it was added

for 10-20min. to the dishes (5ml/10cm plate), and subsequently replaced with ES medium).

Storage
Long-term storage of the cells was carried-out in liquid nitrogen.

For cell freezing a pellet of 10^7 human cells was resuspended in 1ml RMPI 1640 medium with 10% DMSO and 30% FCS and placed in a 2ml Cryotube (NUNC).
Cells were thawed at 37°C in a water bath for few minutes. Upon adding of the vial content to 5ml medium, cells were pelleted (1200rpm, 5min., RT) and plated.

The murine ES cells were frozen at 3-5x10^6 cells per 2ml Cryotube (NUNC) in 1ml of ES medium complemented with 10% DMSO and 50% ES serum.
Thawing was carried by cryovial incubation at RT for few minutes and the cells were added to 30ml ES medium. Upon centrifugation (1200rpm, 10min., RT) cells were plated.

4.3. Molecular biology techniques

The common molecular biology techniques used in the current study were applied according to standard protocols (Sambrook et al., 1989). The standard methods included: phenol extraction of DNA and precipitation, measurement of DNA concentration, enzymatic restriction analysis, DNA dephosphorylation with alkaline phosphatase (CIP), ligation of DNA fragments, conventional cloning procedures.

4.3.1. Cre (Flp) - mediated deletion or inversion

Cre (Flp) was delivered by transient transformation of the *E.coli* cells hosting the given maxi-EBV plasmid with a Cre (Flp)-expressing plasmid, p2266 (pCP20). The deletion or inversion process was carried out on agar plates o/n at 30°C under double selection for the maxi-EBV plasmid construct and the Cre (Flp) helper plasmid. Single, uniform colonies were separated by a dilution streak at 42°C, which is the non-permissive temperature for the temperature-sensitive helper plasmids, such as p2266 and pCP20. Subsequent replica plating at 42°C overnight selected against the Cre (Flp)-helper plasmid and allowed the isolation of clones, which lacked the helper plasmid.

4.3.2. Dialysis of BAC DNA preparations

The BAC DNA preparations were dialysed for microinjection and for ES targeting separately, using microinjection and dialysis buffer, respectively. The DNA sample of up to 100μl volume was placed on a „V“ membrane (VMWP02500; Millipore) floating on 5ml of the dialysis (or microinjection) buffer in a 60mm petri dish. The process was carried for 0.5-2 hours.

4.3.3. Dilution streak of bacteria

Bacteria were streaked with a tooth-stick on one-third of surface of an agar plate. The next two streaks were done on the rest of the plate, each sourced in the previous one.

4.3.4. DNA isolation (small and BAC plasmids):

Mini-plasmid preparation

Small plasmids (up to 20kb) – ‘boiling minis’ protocol:

Bacteria were collected with a tooth-pick and transferred to the lysis buffer (200μl M-stat buffer and 20μl lyzozyme 10mg/ml). The bacteria were suspended by vortexing for about 30sec. Heating to 100°C in a sand bath for two minutes assured protein denaturation. Released DNA was found in a supernatant after centrifugation for 10min, at 13000rpm.

BACs (large plasmids exceeding 100kb) - ‘alkaline minis’ protocol:

Bacteria were scraped from an agar plate with a tooth-pick and added to 200μl of 1x TE with RNaseA (0.4μg/μl). Bacteria were resuspended by vortexing shortly. Lysis was carried by a mixture with 200μl of a freshly made lysis buffer (0.2M NaOH, 1% SDS) by sharp and short shaking followed by 3min. incubation on ice. SDS-protein complexes were precipitated by adding 200μl of Solution III (11.5% acetic acid, 3M potassium acetate) and mixing thoroughly, gradually increasing the force. After 10min. incubation on ice protein and the precipitate was pelleted at 13000rpm and 2°C for 15min. The supernatant was transferred into a new Eppendorf tube. 400μl isopropanol were added for precipitation of DNA, which was then pelleted at 13000rpm for 10min. at RT. The pellet was washed with 80% ethanol and centrifuged again with the same conditions for 5min. After short air-drying DNA was dissolved in 20μl 1xTE.

Maxi-plasmid preparation

Small plasmids (up to 20kb*)* – Jet Star DNA Isolation Kit (Genomed), according to the protocol provided by the company.

BACs (large plasmids exceeding 100kb) – Plasmid DNA was prepared with the Nucleobond DNA Isolation Kit (Macherey-Nagel), according to the manufacturer's protocol.

Highly pure DNA for transfection of eukaryotic cells was prepared with standard Cesium Chloride-ethidium bromide (CsCl) gradients (in detail below) or with a Qiagen DNA isolation kit (Large-Construct Kit, or EndoFree Plasmid Maxi Kit).

CsCl Gradient protocol (optimized):

6ml LB medium was inoculated with a single colony in and incubated with the appropriate antibiotic at 37°C for 6-8h ('overday culture'). A large size overnight culture consisted of six 2l flasks shaken at 30°C. Each flask contained 400ml LB, 1ml small 'overday culture', 24ml of 5M NaCl and antibiotic at the appropriate concentration. On the next day bacteria cells were pelleted at 4000rpm, 4°C for 15min. The six pellets were resuspended in 10ml Solution I each (50mM Glucose, 25mM Tris-HCl pH8, 10mM EDTA), and subsequently 35ml of Solution I was added per bottle. Next, 10mg of lyzozyme powder was added per bottle and the preparation was vigorously shaken several times and incubated for 10min. on ice. 58ml of Solution II (0.1% SDS, 0.2M NaOH) was added gently per bottle, the mixture was then mixed while turning up-side-down mildly but thoroughly six times, and immediately incubated on ice for a maximum of 5 min. To precipitate protein and cellular debris 70ml of Solution III (11.5% acetic acid, 3M potassium acetate) was added/bottle, which was then shaken ten times, first slowly and mildly, then fast and vigorously. Subsequently the bottles were incubated on wet ice for 30min. up to overnight. Protein and cell debris were pelleted for 20min. at 9000rpm, 4°C. The supernatant was filtered through gauze, then through paper filters and collected in 4 centrifuge bottles. At RT, the DNA was incubated for 20min. with isopropanol (0.7x of volume added). and collected by centrifugation at 9000rpm for 40min. DNA pellet was washed with 80% ethanol at 9000rpm for 30min. at RT. Once the pellets were air-dried they were dissolved in a total of 40ml 5xTE. 2mg proteinase K (10mg/ml) was added/bottle and the preparation was shaken at RT until the DNA precipitate dissolved. The solution was then incubated at 50°C for 1h, 100□g of RNaseA (1mg/ml) was added and the solution was shaken at RT for

1h. 1 gram of solid cesium chloride (CsCl) was added per 1ml of DNA solution and dissolved at 37°C (water bath). Ethidium bromide (EtBr) (Roth) was added to a concentration of around 5%. The DNA-CsCl-EtBr mixture was distributed into two 35ml ultracentrifugation tubes, which were filled to the top with 1.55 g/ml CsCl solution. Ultracentrifugation was carried for 3-5 days at 38000rpm at RT. DNA bands from both tubes of the first round of centrifugation were combined into a new 11.5ml ultracentrifugation tube and the second round was performed under the same conditions for 2 days. The lower DNA band was harvested and the EtBr was removed with a (CsCl and water)-saturated isopropanol in Corex glass tubes in multiple rounds of extraction. DNA was then precipitated with isopropanol and washed with 80% ethanol at 5000rpm and RT for 1h and 30min., respectively. The air-dried DNA pellet was dissolved in 100-600□l 1xTE (depending on the pellet size) and left at 4°C o/n.

4.3.5. Electroelution of BAC DNA fragment

Elution was carried out according to the protocol with Quik-Pik Electroelution (Stratagene) or GeneCAPSULE (Gbiosciences) capsules. DNA was eluted in Model B3 chamber (Owl Separation Systems) at 4°C for 1.5h or 20h in 1xTAE buffer.

4.3.6. Electrophoresis

Horizontal large size agarose gel electrophoresis

0.7% agarose gel was run overnight at 70V in 1xTAE buffer. 0.5-1□g DNA/lane was loaded for an analytical gel (model 41-2025-R electrophoresis chamber).

PFGE

0.6% to 1% agarose gels were run at the following settings: 120°, 6V/cm, 15h, 5-15sec.,14°C, in 0.5xTBE buffer. The loading was optimized at 0.5□g DNA/lane for an analytical gel and 3□g of well-dissolved DNA/lane for a preparative gel. (CHEF Mapper□ XA Pulsed Field Electrophoresis System; Bio-Rad)

Electrophoresis with other gel chambers was carried out according to standard protocols.

4.3.7. Electroporation of bacteria

Electrocompetent bacteria

A bacterial DH10B strain hosting both the appropriate BAC and a temperature sensitive helper pKD46 plasmid was expanded at 30°C from a single colony, in a volume of 5ml of an overnight LB culture, under antibiotic selection for both plasmids. 200ml LB culture culture was inoculated with 2ml of the overnight culture and 0.1%(w/v) L-arabinose was added for the induction of Rec proteins expression from the pKD46 plasmid. The bacteria were incubated for about 4h until OD_{600}=0.3-0.5 was reached. All the following steps of electrocompetent bacteria preparation were carried out at 0°C with ice-cold 10% glycerol. After the bacteria had been incubated for 15min. on ice, they were centrifuged at 2°C for 10min. at 7000rpm. After that, they were washed three times with the glycerol solution in the following steps: the bacteria pellet was resuspended in 10ml glycerol solution with a cooled pipette, 200ml more of the glycerol solution was added and bacteria suspension was pelleted at 7000rpm, at +2°C for 10min. After the last centrifugation step the cells were resuspended in 900□l glycerol solution, and frozen at –80°C for a long-term storage.

Electroporation of DNA fragments

The DNA fragment was generated with appropriate restriction enzymes, purified with phenol extraction and subsequent ethanol precipitation, and about 2□g was used for the electroporation. DNA and 60□l of electrocompetent bacteria were combined in a precooled 2mm wide electroporation cuvette on ice. Immediately after electroporation (1.9kV, 200□, 25□F) 1ml LB medium was added directly to the cuvette. Bacteria were transferred to a 15ml tube and incubated while shaking at 37°C for about 2h. The bacteria were pelleted at 6000rpm for 30sec., resuspended in the rests of liquid and plated at two different concentrations: 10□l and ‘the remaining volume’. The plates were incubated overnight at 42°C, the non-permissive temperature of the pKD46 plasmid, selecting for the recombined maxi-EBV plasmid bearing a double resistance as a result of homologous recombination.

4.3.8. Enzyme inactivation for BAC DNA preparation

Upon cleavage the restriction enzyme was inactivated for 20min. at 65°C in a heating block.

4.3.9. Genomic DNA isolation

Genomic DNA from ES cells and mouse tails was isolated with the Dneasy Tissue Kit (Qiagen). The procedure was carried according to the manufacture's protocol.

4.3.10. Homologous recombination in BAC-hosting bacteria

Prior to recombination the BAC-hosting bacteria were stably transformed with the pKD46 helper plasmid. This ampicillin-resistant plasmid expresses RecA and RecBCD proteins, which facilitate homologous recombination. Other small plasmids, cloned in the conventional way, served as a donor of the fragment for homologous recombination with the recipient maxi-EBV plasmid. The DNA fragment was electroporated into an appropriate BAC-hosting bacteria strain and selected for the recombined maxi-EBV plasmid (described in 'Electroporation'). Dilution streak of the recombinant bacteria was carried out at 42°C, the non-permissive temperature for the pKD46 plasmid, to obtain single clones. Subsequent replica plating (42°C, overnight) identified ampicillin-sensitive and pKD46-free recombinant clones.

4.3.11. Oligonucleotides annealing

The annealing mixture consisted of 1nmol of each oligo and 1x NEB2 buffer (New England Biolabs) in a total volume of 50□l. The mixture was heated to 75°C to melt DNA secondary structures and slowly cooled down to about 30°C to allow annealing and double-strand formation.

4.3.12. PCR

<table>
<tr><td colspan="2">PCR mixture consisted of: 0.5□g DNA template
dNTPs (10mM of each)
1x polymerase buffer (Promega)
primers (100pmol each)
0.25□l GoldTaq Polymerase (5u/□l) (Promega)
in 50□l final volume.</td></tr>
<tr><td><u>Program 1</u>: 95°C for 3min.
95°C for 45sec.
primers annealing - 53°C for 45sec.
72°C for 2min.</td><td><u>Program 2</u>: 94°C for 2min.
94°C for 20sec.
primers annealing - 56°C for 40sec.
72°C for 2min.</td></tr>
</table>

repeated for 35 cycles 72°C for 10min.	repeated for 30 cycles 72°C for 7min.
The PCR reactions were carried with a PCR robocycler (Gradient 96; Stratagene).	
Reaction 1 (part of Step1 of the cloning procedure) Plasmid template: p288.1 (350ng). Primers: EES1F17 and EES1B2mod. PCR program 1.	
Reaction 2 (part of Step 3 of the cloning procedure) Plasmid template: 50ng. Primers: 3.4.PCR1, 3.4.PCR2. PCR program 2.	
Reaction 3 (genotypic analysis of transgenic mice tails) Genomic DNA template: 0.5□g. PCR program 1 (with changes: step 1- 5min., step 3- 1min.15sec.). Annealing temperatures: LMP1 primers (59°C), LMP2 primers (57°C), WW primers (59°C). Two different positive controls were prepared, such that they mimic a single copy of the maxi-EBV vector (InvTarg) integrated into the murine genome. The control samples were composed of genomic DNA from both mES, and human B cell line with a known copy number of EBV DNA (’18 aus 4’ or Raji). The cell line ’18 aus 4’ carries 3 copies of an integrated EBV genome and Raji carries 50 copies of episomal EBV DNA. The control samples were named as follows: ‘E-18’ – combination of mES with ’18 aus 4’ DNA, and ‘E-R’ – combination of mES with Raji DNA.	
Reaction 4 (genotypic analysis of mES cells clones) Genomic DNA template: 0.5□g. PCR program 1 (with changes: step 1- 5min., step 3- 1min.15sec.). Annealing temperatures: LMP2 primers (57°C), I1 primers (59°C), LOX71 primers (54°C), LOX66 primers (55°C). One positive control, ‘E-18’, was the same as for the PCR *Reaction 3*, the other consisted of 30pg 3299.11 plasmid DNA.	

4.3.13. Plasmid clone verification

Recombinant plasmids were verified by standard restriction enzyme analysis. Detailed examination of the introduced features was carried with partial DNA sequencing.

Small plasmids (up to 20kb)

Plasmid DNA was purified from bacteria with the mini-plasmid preparation protocol (see 'DNA isolation'). The restriction analysis was conducted according to a standard protocol of DNA cleavage with endonucleases and analysed by a standard agarose electrophoresis. The parental plasmid served as a reference for the derived construct.

Once a plasmid clone was identified, it was propagated on a larger scale with a maxi-plasmid preparation (see 'DNA isolation'). The plasmid restriction pattern was again verified with a standard protocol.

BACs (large plasmids exceeding 100kb)

A similar two-step plasmid verification procedure was applied for large BAC plasmids. Firstly, after DNA was purified with the mini-plasmid preparation protocol (see 'DNA isolation'), its restriction pattern was investigated with a standard horizontal electrophoretical separation overnight. The total preparation obtained with the 'alkaline minis' protocol was digested for 3h with 30u restriction enzyme and 0.5□g RNaseA in 60□l of final volume. Either a wild type maxi-EBV (p2089), or a plasmid constructed in a previous step served as a reference for the complex restriction pattern.

Secondly, the correct BAC plasmid clone DNA was then prepared on a larger scale with the maxi-plasmid preparation protocol (see 'DNA isolation'). Verification of its restriction pattern was repeated with this DNA preparation with around 0.5□g DNA/sample. Critical parts of introduced genetic alterations were directly sequenced which required sonification of the BAC plasmid DNA.

Highly pure BAC DNA preparations used in transfection of eukaryotic cells were obtained with the Cesium Chloride Gradient protocol (see 'DNA isolation').

4.3.14. Precipitation of DNA (Protocol with 'FISHing')

The DNA solution was mixed with 1/10x DNA volume of 3M sodium acetate pH 5.2 solution (Sigma) and 2x DNA volume of 100% ethanol (-20°C cold). The precipitation mixture was stored at -20°C for a minimum of 30min. up to overnight. DNA precipitates could be then collected ('FISHed') with a flame-closed glass Pasteur pipette and washed by short immersion in 1ml of 70% ethanol. After air-drying for approximately 10min. at RT the DNA was dissolved by incubation of the Pasteur pipette tip in a given solution at RT overnight.

4.3.15. Replica plating of bacterial colonies

The purpose of this technique was to determine the phenotype of *E.coli* clones indicative of their antibiotic resistance. The clone was plated in parallel on two (or more) plates containing different antibiotics: one, selecting for the desired plasmid (positive control), and the other (or others), selecting for the unwanted plasmid (plasmids).

4.3.16. Sequencing

DNA sequencing was carried with given primers by a specialized company (Sequiserve). Considering BAC constructs, they were sonified and purified by phenol extraction and ethanol precipitation. About 5□g of the BAC DNA in 1xTE (0.5□g/□l) was provided for direct sequencing.

4.3.17. Sonification of BAC DNA

BAC DNA (5□g in 200□l TE) was sonified three times for 1sec., at 10% power and 20°C. Sonifier (250-D; Branson).

4.3.18. Southern blotting

Blotting

After separation with a horizontal electrophoresis gel apparatus the DNA bands were visualized with ethidium bromide agarose gel staining. The marker bands were marked with ink on the gel, and later the ink dots were transferred with pencil to the membrane. Prior to the transfer the gel was incubated in 0.25M HCl for 30min., and subsequently shaked in a Neutralization Buffer (1M NaCl, 0.5M NaOH) for 40min. The DNA was transferred to a Hybond N^+ hybridization membrane (Amersham Pharmacia) in a capillary manner for 2h (Southern, 1975).

Hybridization

Membrane was prehybridized by rolling at least for 1h up to overnight in Church Buffer at 60°C. The DNA for probes was stained with [□^{32}P] dCTP (Amersham Pharmacia) with the High Prime probe staining kit (Roche) and non-incorporated radioactive nucleotides were removed by gel filtration on Nick^{TM} equlibration column G-50 (Amersham Pharmacia). After heat denaturing of the probe at 95°C for 5min. followed by a quick cooling step on ice, it was combined with the Church buffer used

previously. The membrane was hybridized with the probe for 3h (or overnight) by rolling at 60°C. Unbound probe DNA was washed away from the blot with 0.2xSSC/1%SDS buffer at 60°C. Autoradiography was carried for 30min. up to overnight, at RT or at –80°C with intensifying screens.

4.3.19. Transformation

Competent bacteria:

Bacteria were cultured in SOB medium until the suspension reached the OD_{600}≈ 0.5. All the next steps of the preparation were conducted at 0-2°C. Bacteria were pelleted at 3000rpm for 10min. and the pellet resuspended in 10ml of ice-cold TFB buffer. Next, bacteria were pelleted again and resuspended in 2.5ml of the ice-cold TFB. 200µl of the competent bacteria were used/transformation sample.

Transformation:

1-10ng of DNA in a maximum of 5µl volume was combined with 200µl of the competent bacteria. 7µl of DMSO was added/sample and vortexed briefly. Immediately after the heat shock (2min. at 42°C) 5ml of SOC medium was added/sample. Transformed bacteria were shaken for 1h at 37°C without antibiotics for bacteria recovery and phenotypic expression. The cells were pelleted at 3000rpm for 10min., plated with the appropriate antibiotic and incubated at the given temperature overnight. Single colonies of transformed bacteria were picked and expanded for further analysis.

10pg of Bluescript DNA was used as a positive control of the procedure. Negative controls comprised ligation reaction without ligase or without insert or without DNA.

4.4. Virological assays

4.4.1. EBV packaging into viral particles

Transfection of the EBV packaging cell line with maxi-EBV constructs

The 293 TR(-) packaging cells were seeded on 6-well-cluster plates and transfected with desired BAC construct. Transfection was carried with Lipofectamine (Invitrogen) in Opti-MEM medium (OPTI). The conventional cell culture medium was removed and 1ml of Opti-MEM was added/well and incubated for ca. 30min. BAC DNA was mixed with OPTI (1µg/0.5ml). Lipofectamine was combined with OPTI (4.5µl/1µg

BAC/0.5ml OPTI) and both solutions were mixed and incubated at RT for 45 min. To induce efficient virus production, two expression plasmids: p507 (BZLF1 expressing vector) and p2670 (BALF4 expressing vector) were co-transfected to the DNA samples in parallel. 0.5μg of each expression plasmid was added/50μl OPTI, and 6μl Lipofectamine/1μg DNA was separately mixed in 50μl OPTI. After combination of both mixtures the solution was incubated for 45min at RT. The OPTI had been removed from the cells before the DNA-lipofectamine complexes were added (1ml BAC and 100μl of the expression plasmid mix/each well). Cells were incubated with the complexes for a minimum of 4h, after which 2ml normal cell culture medium (without hygromycin)/well was added.

Viral supernatant collection

Three days after transfection supernatants from the transfected 293 cells were harvested. They were centrifuged at 2000rpm, RT for 1min., filtered with 0.8μm sterile filter, and stored in a 15ml falcon tube at +4°C.

Verification of the activity of the virus stocks

About 10^5 Raji cells were infected with 1ml of the virus supernatant, collected in the previous step, in a volume of about 2ml per one well of a 24-well-cluster plate. On the second day after infection GFP expressing cells (i.e. cells infected with recombinant EBV viruses comprising GFP) were counted under a fluorescent microscope, to estimate the concentration of infectious virions.

4.4.2. Infection of human primary B cells with EBV mutant viruses

Isolation of human B cells from lymphoid tissues (tonsils, adenoids)

The tissue samples soaked in PBS was cut into small pieces with sterile scissors in a 60mm plate, and subsequently strained through a sieve (Cell Strainer, 100μm, nylon; Falcon) into a 50ml falcon to obtain single cell suspensions. The cells were washed with 15ml PBS by centrifugation at 1200rpm for 5min. The cell pellet was resuspended in 30ml PBS and 1ml of defibrinated sheep blood erythrocytes (Oxoid) were added to deplete T cells in the subsequent step. Peripheral Blood Mononuclear Cells (PBMCs) were separated from the rest of cells with a Ficoll gradient. The cell mixture was placed on top of 15ml of Ficoll (GE Healthcare) and centrifuged for 30min. at 1850rpm and 10-15°C. PBMCs were collected from the interphase and

washed with 15ml PBS in three centrifugation steps: at 1500rpm, at 1300rpm and at 1200rpm. The cells were then resuspended in 2ml medium and counted.

B cell infection with EBV mutant viruses

$2x10^7$ isolated B lymphocytes in 5.5ml volume were infected with 5.5ml of the viral supernatant (1:1 B cells:virus volume ratio) and distributed on a 96-well-cluster plate (100µl/well) with Wi38 feeder cells (irradiated 5000 Rad/sample). The infected cultures were cultivated for 4-6 weeks to allow growth transformation of the B cells. The transformed cells were expanded on new plates without feeder cells.

4.5. Transgenic mouse development

4.5.1. Murine Embryonic Stem (ES) cells targeting

DNA electroporation into ES cells

Fresh medium was added to the ES cells approximately 3h prior to electroporation. Cells were trypsinized, counted and $2x10^7$ cells were combined with 30µg of plasmid DNA in 800µl of the transfection buffer (RPMI 1640) and transferred to 4mm transfection cuvettes. The electroporation conditions were set at 250V and 500µF, if not specified otherwise. Immediately after the shock 10ml ES cell medium was added to the transfected cells and they were seeded on 10cm plates.

DNA lipofection of ES cells with Lipofectamine 2000 – optimized protocol

Cells were seeded one day before transfection and plated at a density of $0.5x10^6$/well of a 6-well-cluster plate. On the day of transfection the medium was exchanged for fresh medium about 3h prior to transfection. Opti-MEM and Lipofectamine2000 (L2000) (Invitrogen) were equilibrated to RT. Both DNA and L2000 were separately diluted in 100µl (500µl for BAC) Opti-MEM each (2.5µl L2000/1µg DNA). After 5min. (max. 30min.) incubation at RT both diluted components were mixed resulting in a total volume of 200µl (1ml for BAC), and incubated for another 20min. at RT. In the meantime, the medium from the cells was exchanged for 1ml Opti-MEM/well. DNA-L2000 complexes were added to the cells in Opti-MEM and incubated overnight in a 37°C incubator (incubation volume – 1.2ml (2ml for BAC)). The transfection medium was replaced by fresh ES medium on the next day. Selection medium was applied

when the transfected cells reached about 80-90% density on the second day after transfection at the latest.

Selection for stably transfected ES cell clones

Selection was started on the second day after the transfection and was maintained for approximately 12 days. HAT selection medium with a final concentration of 100□M Hypoxanthine, 0.4□M Aminopterin and 16□M Thymidine was applied. For selection with G418 a final concentration of 350□l/ml was used.

Picking of the targeted ES cell clones

Selected ES cell clones were counted with a grid. Upon double rinse with PBS, they were picked in 15ml PBS with sterilized 200□l pipette tips set to a volume of 20□l under a dissecting microscope (Nikon SMZ645). The cell clumps were transferred to U-bottom-96-well-cluster plates for trypsinization to obtain single cell suspensions. They were transferred to gelatinized 96-well-cluster plates, one clone/well, and expanded in the appropriate medium.

4.5.2. Microinjection of BAC DNA into fertilized mouse eggs

The BAC DNA constructs at specified concentrations were handed over to the group of E.Wolf (Gene Centre, LMU, Munich) for injection into fertilized mouse oocytes (FVBxFVB). Between 20 to 30 injected oocytes were transferred per a recipient female. The offspring was kept in the animal facility at the Gene Centre, while the mouse tail genotypic analysis was conveyed by myself with PCR analysis (chapter 4.3.12).

5. RESULTS

5.1. Features of the maxi-EBV-based InvTarg targeting construct

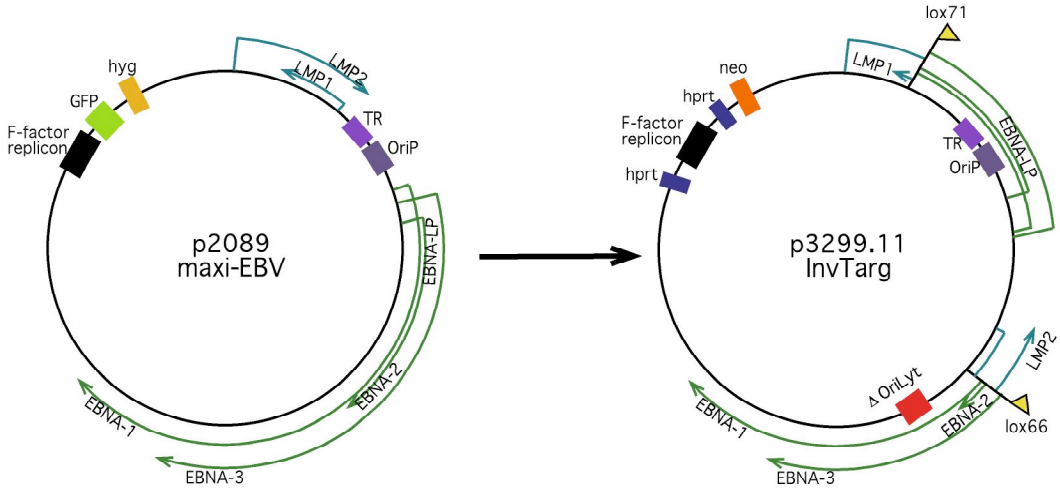


Fig.5.1. The InvTarg targeting construct (right) was constructed on the basis of the wild-type maxi-EBV plasmid p2089 (left). A part of the maxi-EBV genome was inverted and flanked by two variant loxP sites, which interrupted all latent genes and disabled their expression. The lytic origin of DNA replication, OriLyt, was inactivated to abrogate accidental release of infectious virion particles. To permit targeted integration of the linearized InvTarg construct, two sections of the *hprt* gene are present. hyg: hygromycin, neo: neomycin, TR: terminal repeats, OriP: viral latent replication origin, OriLyt: viral lytic origin.

The wild-type maxi-EBV plasmid p2089 was modified in a series of genetic steps to yield the InvTarg targeting construct p3299.11. The purpose of these genetic alterations was (i) to disable the expression of all EBV latent genes, (ii) to prevent formation of infectious virions and (iii) to allow locus-specific integration of the linearized targeting construct.

To disable latent gene expression, two variant loxP sites were introduced in opposite directions to allow Cre-mediated inversion of an approximately a 51kb segment (Fig.5.1). In this work the loxP site oriented in an opposite direction to the loxP wild-type site is named ‘loxPinv’. The two loxP sites are situated in the InvTarg construct such that they flank the inversion, which disrupts the transcription units of all latent EBV genes. A peculiar feature of the Cre/loxP system has to be considered. Cre-mediated deletion of a loxP-flanked DNA segment is a very efficient unidirectional process (Fig.5.2a), whereas Cre-mediated inversion is a continuous undirected ‘flip-flop’ action (Fig.5.2b).

To ‘freeze’ the state of a desired orientation of the inverting DNA segment, modified loxP sites, such as lox71 and lox66, are employed (Fig.5.3).

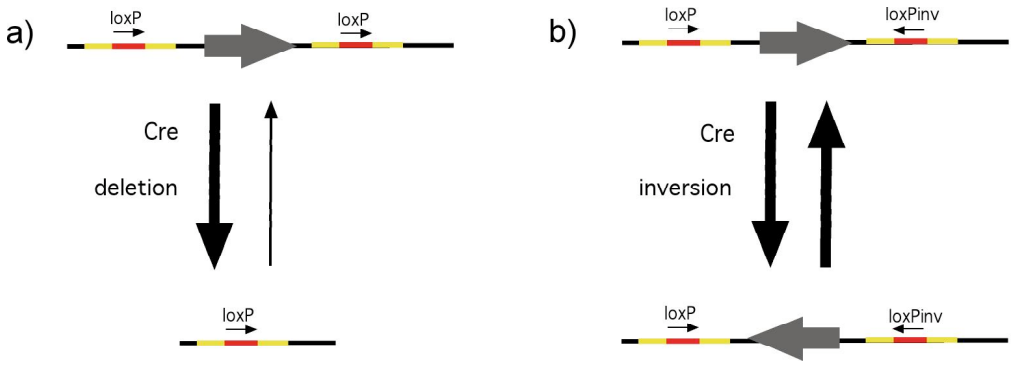


Fig.5.2. Intramolecular Cre/loxP system. Two different genetic configurations can be obtained as a result of Cre-mediated recombination. (a) Cre can delete a fragment flanked by two loxP sites oriented in the same direction. (b) Cre can invert a fragment in between two loxP sites oriented opposite to each other. thick grey arrow head- regulated gene, in yellow - inverted repeats, in red – spacer, small black arrow - loxP site orientation.

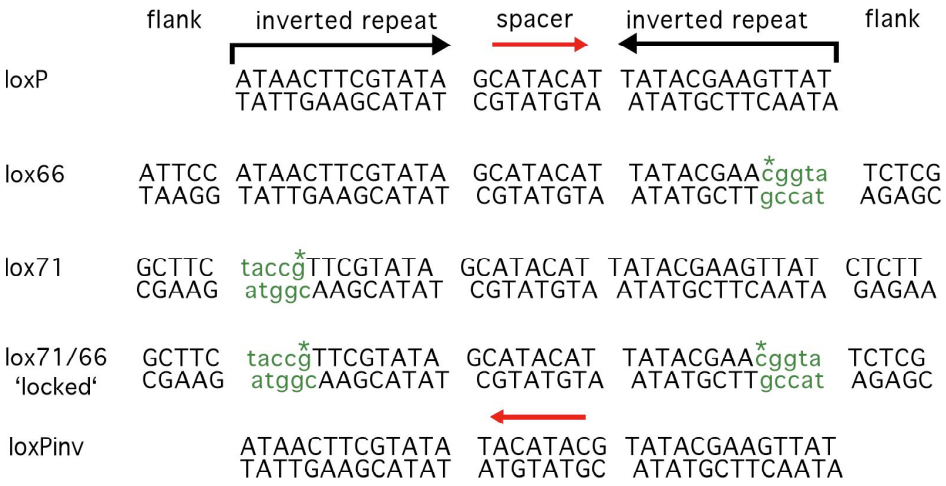


Fig.5.3. Structure of the wild type (loxP), inverted (loxPinv) and mutated (lox71, lox66) lox sites are shown, which are specific substrates for the Cre recombinase. The inverted repeats serve as recognition motifs for Cre binding. Lox71/66 ‘locked‘ and wild-type loxP sites are the result of the recombination of one lox71 and one lox66 site, and the resulting lox71/66 site is refractory to Cre-mediated reombination. spacer (red) - indicates the directionality of a lox site, flanks - show the DNA context for the lox sites; mutated nucleotides are shown in lower case and marked with green stars.

After an inversion in between one lox71 and one indirectly oriented lox66 site a ‘locked’ lox71/66 site is created, which is resistant to a subsequent Cre-mediated recombination, and one wild-type loxP site (Fig.5.4). This feature is a prerequisite for the one-step re-inversion of the lox71/lox66 bracketed segment in the InvTarg

targeting construct p3299.11 (Fig.5.1). Re-inversion leads to the activation of all latent EBV genes after transient expression of Cre.

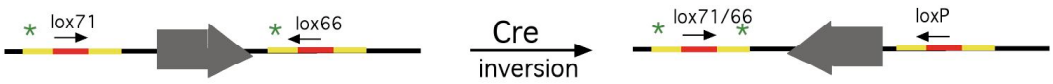


Fig.5.4. One-step Cre-mediated inversion of the fragment in between lox71 and lox66 sites. The conformation created upon Cre-mediated inversion is 'frozen' because the lox71/66 site is locked and thus no longer a substrate for the Cre recombinase. Mutations in the inverted repeats are marked with green stars.

To assure that there will be no infectious virus synthesized in a murine cell with the InvTarg construct integrated, the lytic origin of EBV is inactivated in this construct (Fig.5.1). This aspect is especially important to prevent accidental virus shedding from a transgenic animal, which would constitute a biohazard.

To permit germ line transmission of the InvTarg construct, it shall be stably integrated into the mouse genome. Site-specific incorporation into a housekeeping locus, such as *hprt*, will sustain faithful maintenance of the transgene in an open chromatin configuration during development and differentiation (Bronson et al., 1996). Two flanks, which encompass segments homologous to the murine *hprt* locus, are part of the InvTarg targeting construct (Fig.5.1) and shall support *hprt*-specific recombination. These two flanks can also complement and reconstitute the disabled *hprt* gene in the murine embryonic stem cell line (*hprt* mES). A functional *hprt* locus on the X-chromosome permits ES cell survival in HAT (Hypoxanthine/Aminopterin/Thymidine) selective medium (Szybalski and Szybalska, 1962). In addition, a G418/neomycin selection marker is located opposite to the reconstituting *hprt* flank in the InvTarg construct (Fig.5.1). The double selection for both selective marker genes shall facilitate the integration of the entire InvTarg construct.

The crucial steps, which were required to introduce numerous genetic alterations in the InvTarg targeting construct (p3299.11), are described in detail below.

5.2. Introduction of genetic modifications into the wild-type maxi-EBV plasmid leading to the InvTarg construct

To make this work more legible, only examples were chosen and presented on the next pages. Each new feature to be introduced into a maxi-EBV construct was first constructed in a small plasmid in a multi-step cloning procedure. An example of such a conventional cloning, provided for a maxi-EBV plasmid modified in the cloning Step 1 (chapter 5.2.1), is described in Steps 1.1-1.3 (chapters 5.2.1.1-5.2.1.3). All other small plasmids were generated in a similar way. The list of almost 30 plasmid constructs established during this work is provided in the *Appendix.*

The genetic characteristics of the InvTarg targeting construct described in the previous paragraph were introduced into the maxi-EBV wild-type plasmid p2089 in five consecutive steps (given below).

Each of these five steps encompasses several very specific work packages, which include the construction of complex features in conventional small plasmids for modifying maxi-EBV plasmids, introduction of these features into maxi-EBV plasmids, followed by careful analyses of the genetic modifications. All genetic manipulations were confirmed in restriction analysis assays (described in the clone verification protocol in *Methods*, chapter 4.3.13) and by partial sequencing of the modified loci. The restriction pattern of the parental wild-type maxi-EBV (p2089) or the maxi-EBV plasmid from the previous step served as a reference.

A hallmark of this chapter is homologous recombination in *E.coli*. Two different techniques were employed for the insertion of the different features into maxi-EBV plasmids: (1) the ‘chromosomal building’ technique, based on the allelic exchange of a genetic parental locus to a mutated one between two plasmids, and (2) a technique called ‘linear integration’, which allows site-specific recombination of a linear DNA fragment carrying a selectable marker gene. Both methods are described in the *Introduction* section (chapter 1.7.2).

The steps described in this work are listed below and the modifications of maxi-EBV plasmids are shown in grey:

Cloning step	Final plasmid
Introduction of the Cre/loxP system into wild-type maxi-EBV plasmid p2089 to control the expression of EBV latent genes (Fig.5.5A-C):	
Step 1: Insertion of the variant lox71 into into the 2nd intron of viral LMP1 gene.	p3101.31
Step1.1: PCR-mediated introduction of a lox71 site in the context of LMP1 gene.	p3060.10
Step1.2: Conventional cloning of a loxP site together with *zeo* cassette.	p3064.2
Step1.3: Construction of a floxed (lox71 and loxP) *zeo* resistance cassette in the context of the LMP1 gene.	p3071.1
Step 2: Insertion of an inverted loxP site (loxPinv) into the maxi-EBV plasmid p3101.31 and Cre-mediated inversion	p3125.5
Step 3: Exchange of the loxPinv site of p3125.5 plasmid for the variant lox66 site.	p3221.12
Introduction of the additional features into maxi-EBV plasmid (Fig.5.11A,B):	
Step 4: Inactivation of the EBV lytic origin of DNA replication, OriLyt within p3221.12 plasmid.	p3283.2
Step 5: Replacement of the *hyg* and *gfp* for *neo* and introduction of *hprt* flanks for site-specific integration within p3283.2 plasmid.	p3299.11

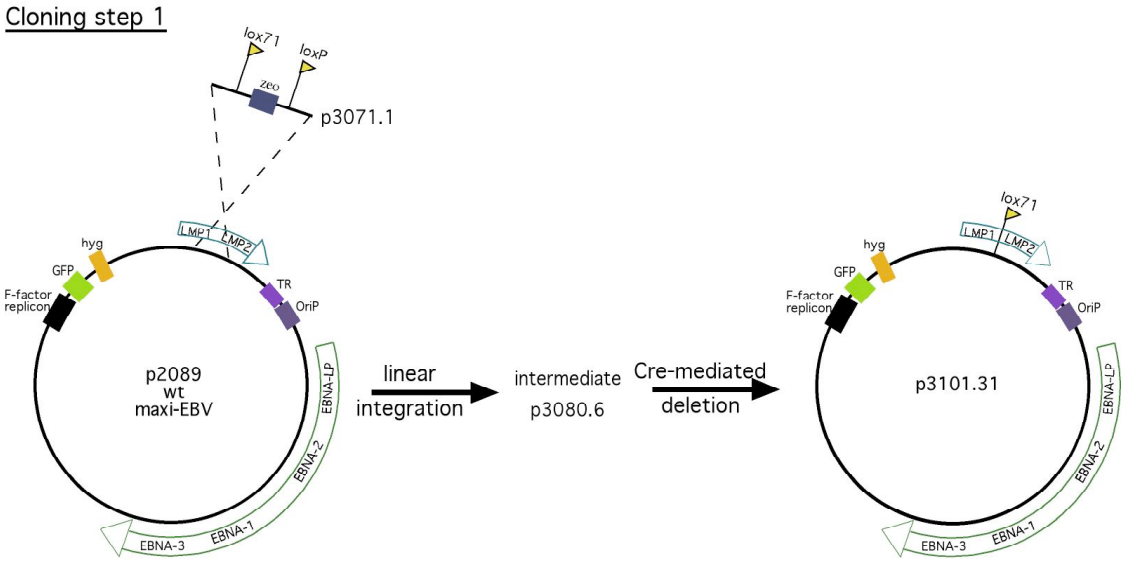


Fig.5.5.A Stepwise introduction of the Cre/loxP system into maxi-EBV plasmids: Step 1. Introduction of a *zeocin* resistance gene flanked by lox71 and loxP sites into the second intron of LMP1 gene by linear integration, followed by Cre-mediated deletion of the *zeocin* gene. zeo: zeocin.

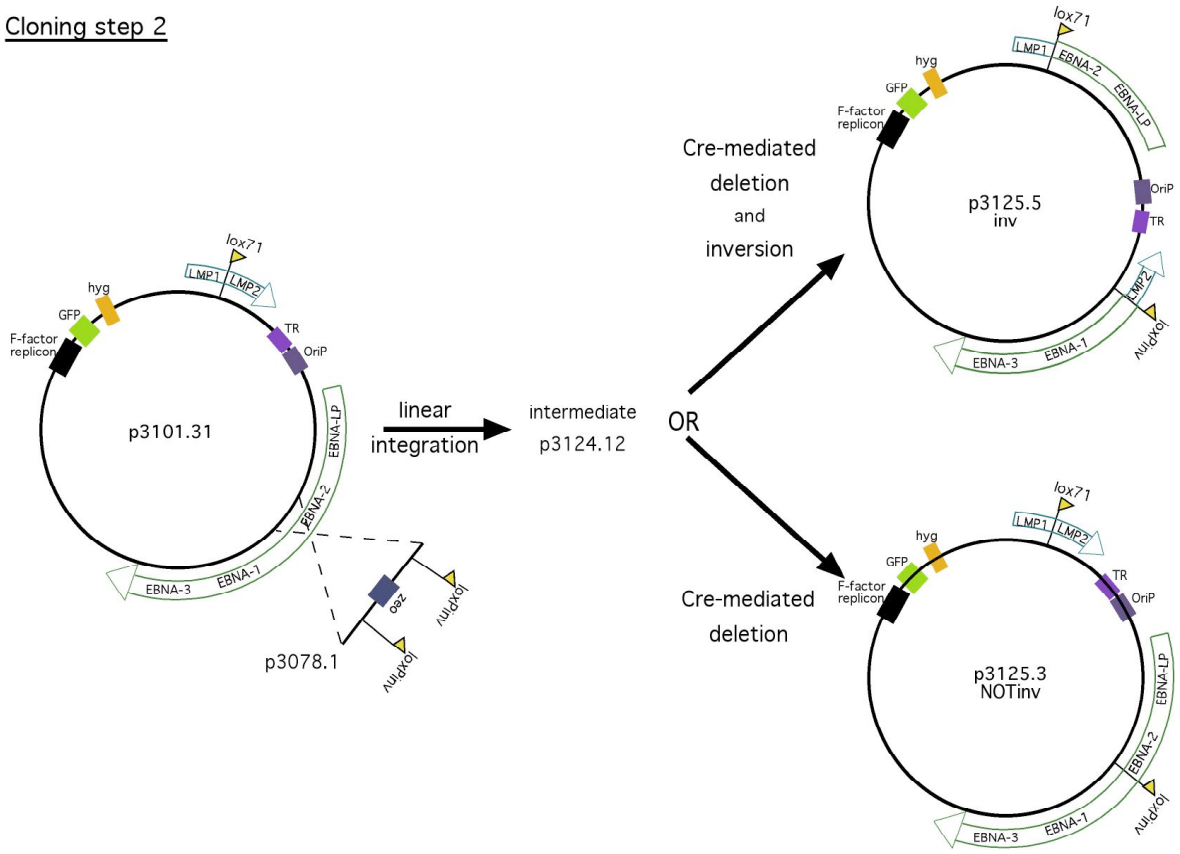


Fig.5.5B. Stepwise introduction of the Cre/loxP system into maxi-EBV plasmids: Step 2. Introduction of a *zeocin* resistance gene flanked by two loxPinv sites into the intron upstream to the Y1 exon of the EBNA-LP gene by linear integration, followed by Cre-mediated deletion of the zeocin gene and inversion of the lox71/loxPinv flanked EBV segment. zeo: zeocin, loxPinv: inverted loxP.

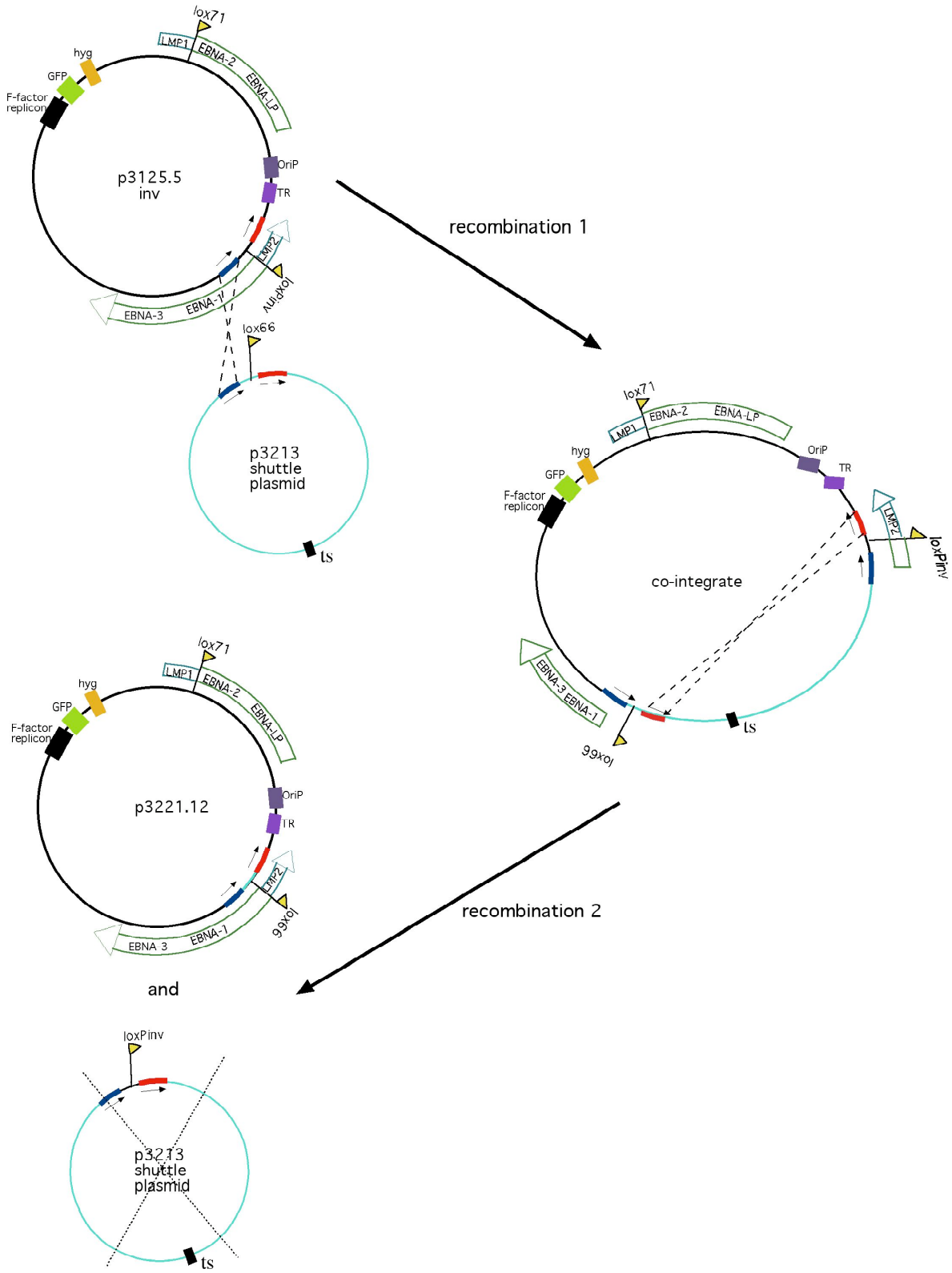


Fig.5.5C. Stepwise introduction of the Cre/loxP system into maxi-EBV plasmids: Step 3. Exchange of the loxPinv site for a lox66 site via 'chromosomal building' between p3125.5 maxi-EBV plasmid and the shuttle plasmid p3213. ts: temperature sensitive replication origin, loxPinv: inverted loxP.

5.2.1.1. Step 1.1: Introduction of a lox71 site into LMP1

A PCR product comprising the lox71 site and a part of LMP1 sequence was introduced into the backbone of the pCR2.1 plasmid. The lox71 sequence was incorporated at the 5'-end of the oligonucleotide primer EES1B2mod, which was used together with primer EES1F17 to amplify a part of the LMP1 gene by PCR (Fig.5.6a). The PCR reaction is described in *Methods* (*Reaction 1* in chapter 4.3.12). In the next step, the product was cloned into pCR2.1 on the basis of T/A overhangs (TA Cloning Kit, Invitrogen). This approach did not require a restriction digest of the PCR product, as the 3'-deoxyadenylate extensions added by the Taq polymerase during PCR reaction were subsequently combined with the 5'-deoxythymidylate extensions of the recipient vector. Therefore, after the PCR product was extracted from an agarose gel, it was directly ligated to the pCR2.1 plasmid DNA. DH5 competent bacteria were transformed with the ligation mixture and selected for ampicillin resistance at 37°C. The correct genetic composition of the final construct p3060.10 was confirmed by the verification procedure (*Methods,* chapter 4.3.13) and DNA sequencing with the M13rev primer.

5.2.1.2. Step 1.2: Conventional cloning of a loxP site together with the zeo cassette

An insert encompassing a wild-type loxP site was combined with a zeocin resistant pSV40/Zeo2 cloning vector (Fig.5.6b). The insert was constructed by the annealing of two oligonucleotides (*Methods*, chapter 4.3.11). pSV40/Zeo2 was cleaved with XhoI and BglII restriction enzymes, dephosphorylated and purified by phenol extraction and ethanol precipitation. 0.2pmol of the insert was ligated to 0.04pmol (100ng) of the cleaved and dephosphorylated pSV40/Zeo2 plasmid and transformed into DH5 competent bacteria, and selected for zeocin resistance at 37°C. The resulting construct p3064.2 was identified by restriction analysis as described in the verification procedure (*Methods,* chapter 4.3.13).

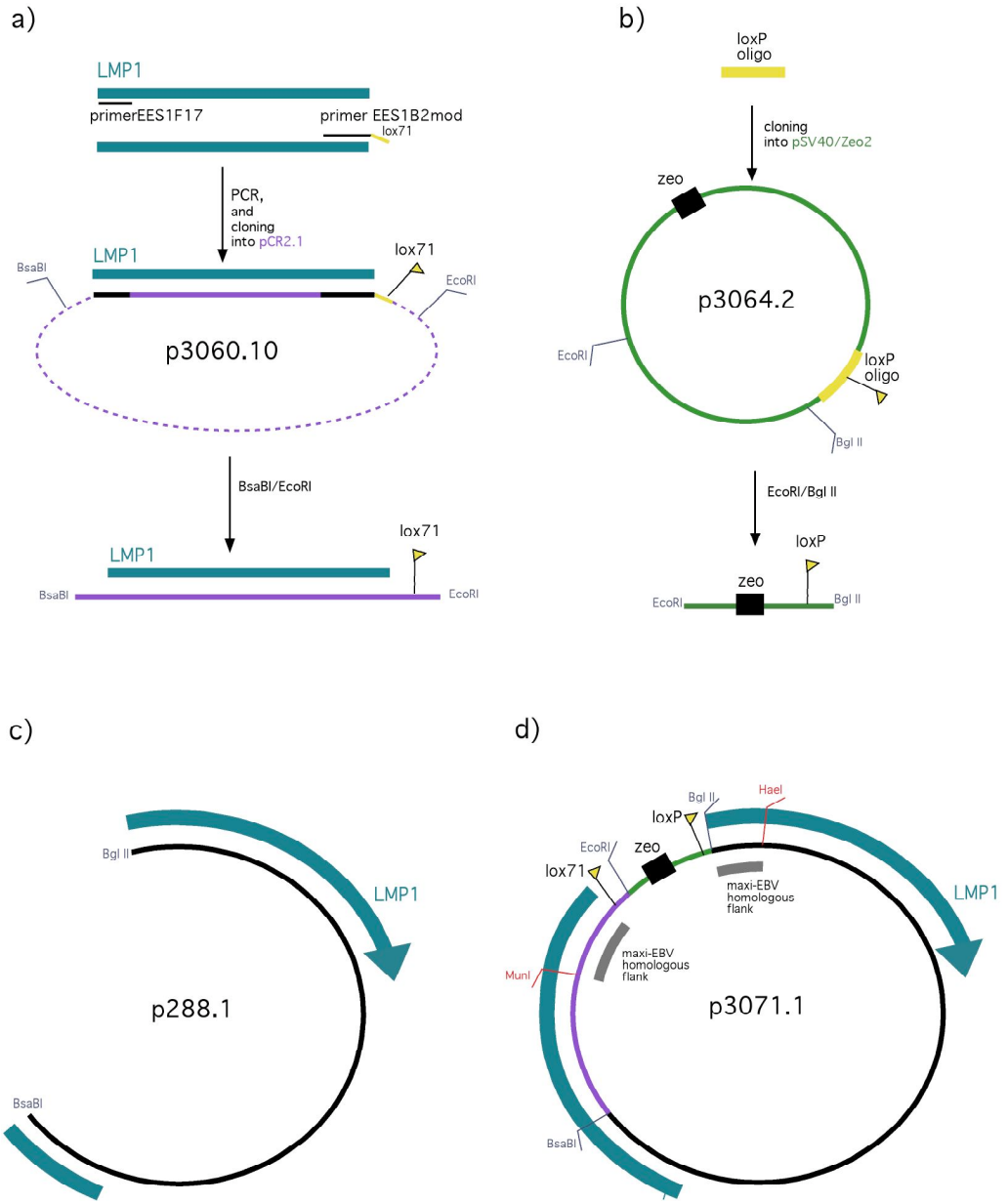


Fig.5.6. Conventional cloning of the small plasmid, which delivered a fragment for the recombination with maxi-EBV in the Step1 as shown in Fig.5.5. (a) PCR-mediated amplification of DNA fragment containing the lox71 site in the context of the viral LMP1 gene, and the PCR product introduction into the pCR2.1 cloning plasmid resulted in p3060.10 plasmid. The BsaBI/EcoRI fragment of the p3060.10 plasmid contained lox71 and a part of the LMP1 gene. (b) Arrangement of an oligo comprising the loxP site with a *zeo* gene in the SV40/Zeo2 vector. This step gave rise to p3064.2 plasmid, whose EcoRI/BglII fragment comprised both features. (c) BsaBI/BglII fragment of LMP1-containing p288.1 plasmid, which was used as a backbone for p3071.1 plasmid constructed in the next step. (d) The final plasmid p3070.1, encompassing the floxed zeocin cassette in the context of LMP1 gene, was composed of the fragments from both previous cloning steps: (a) and (b), linked to the segment of p288.1 plasmid (c). Depicted restriction enzyme sites: used in the conventional cloning (blue) and for producing a fragment for the recombination with the maxi-EBV in Step 1 (red). Flanks for this recombination are shown in grey. zeo: zeocin.

5.2.1.3. Step 1.3: Construction of a floxed (lox71 and loxP) zeo cassette in LMP1

The BsaBI/EcoRI fragment comprising the lox71 site (constructed in Step 1.1; Fig.5.6a) and the EcoRI/BglII fragment encompassing the loxP site assembled with the *zeo* gene (Step 1.2; Fig.5.6b) were both combined in a conventional cloning step with the LMP1 gene of the BsaBI/BglII-cleaved p288.1 plasmid (Fig.5.6c). All three fragments were extracted from preparative gels, ligated and transformed into DH5 competent bacteria. The selection was carried at 37°C for zeocin and ampicillin resistance. The restriction pattern of the resulting p3071.1 construct (Fig.5.6d) was confirmed in the verification procedure (*Methods,* chapter 4.3.13)

The MunI/HaeII fragment derived from the p3071.1 construct served for homologous recombination with the wild-type maxi-EBV plasmid p2089 in Step 1 of the maxi-EBV cloning procedure leading to the p3080.6 maxi-EBV construct (Fig.5.5A).

5.2.2. Step 2: Insertion of the second loxP site (loxPinv) into the upstream region of viral EBNA-LP's exon Y1 and Cre-mediated inversion

Individual steps of this cloning procedure were similar to the steps described in Step 1. A small plasmid p3078.1 was constructed by conventional cloning in *E.coli* DH5. Its *zeo* gene was bracketed by two inverted loxP sites (loxPinv) and flanked with two EBV segments (Fig.5.5B). The loxPinv-flanked *zeo* cassette was arranged upstream of the Y1 exon of EBNA-LP. The PstI/BbvCI fragment of the p3078.1 construct was electroporated into the DH10B bacterial cells bearing the maxi-EBV construct p3101.31 produced in Step 1 (chapter 4.3.10), and bacteria were selected with zeocin and chloramphenicol for the presence of the p3078.1- and p3101.31-derived fragments, respectively). The restriction pattern of the obtained maxi-EBV clones was analysed as described in the verification procedure (*Methods,* chapter 4.3.13). The correct composition of the introduced loxP sites in the identified clone p3124.12 was further confirmed by partial DNA sequencing (Step 2 of Fig.5.7).

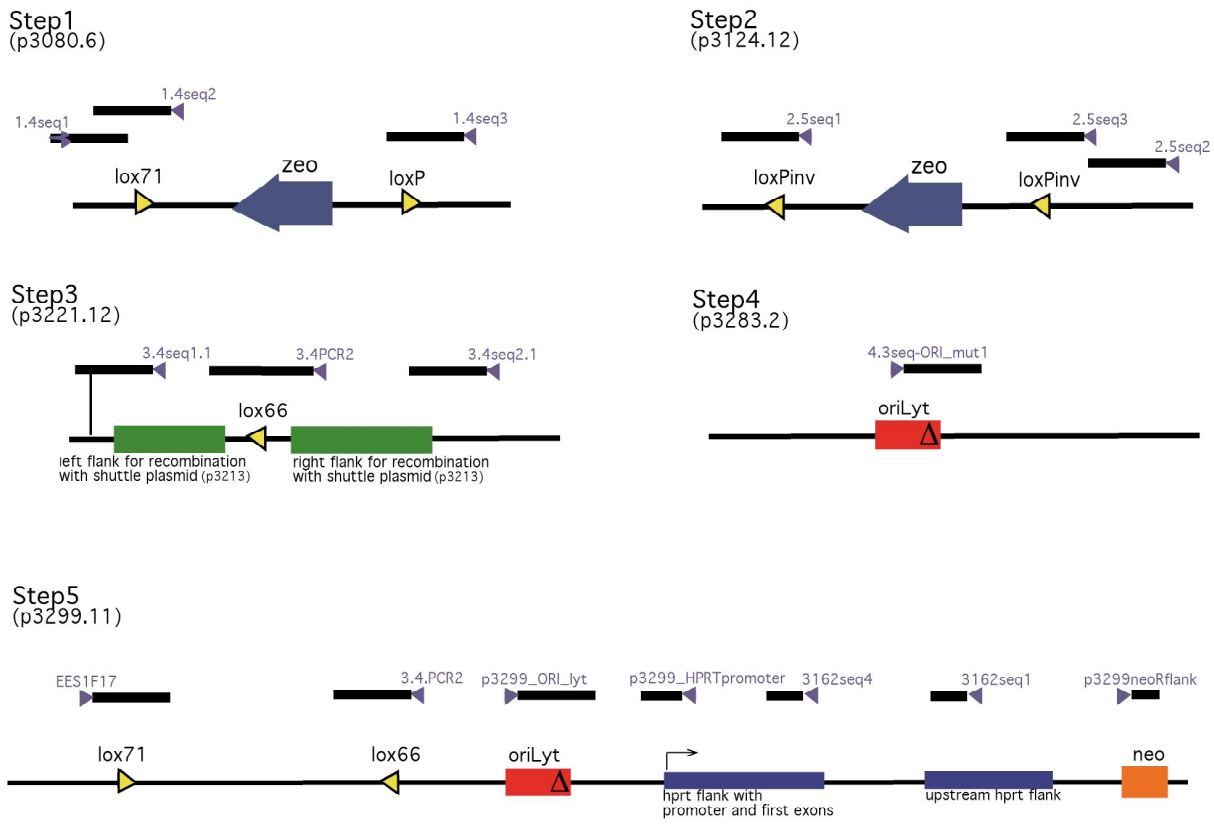


Fig.5.7. Confirmation of the genetic manipulations by DNA sequencing of different intermediate maxi-EBV plasmids and the final InvTarg targeting construct p3299.11. Each newly introduced feature was consecutively verified in each step: Step1: Both lox sites and the bordering sequences in p3080.6 (before Cre delivery), Step2: both inverted lox sites and their bordering sequences in p3124.12 (before Cre delivery), Step3: lox66 and its flanking regions in p3221.12, Step4: mutation in lytic origin in p3283.2, Step5: final InvTarg targeting construct was again confirmed for mutated lox sites (lox71, lox66), disabled lytic origin, and additionally for newly exchanged maxi-EBV's F-factor backbone (*hprt* flanks and *neo* introduced). primers depicted in violet, sequenced fragment - thick black line, zeo: zeocin, oriLyt: viral lytic origin, neo: neomycin.

Subsequently, Cre was delivered into the bacteria hosting the correctly recombined maxi-EBV construct p3124.12 (*Methods*, chapter 4.3.1), leading to the removal of the *zeo* cassette and to the inversion of the 51kb fragment in between the lox71 site, introduced in Step 1, and the newly introduced loxPinv. Replica plating allowed the identification of the clones, which had become zeocin-sensitive upon deletion of the *zeo* gene. Cre-mediated inversion in between the lox71 and the remaining loxPinv site was expected to give rise to two predicted compositions of the newly constructed maxi-EBV plasmids: p3125.5 - 'inverted' (inv) and p3125.3 - 'NOTinverted' (NOTinv) (Fig.5.5B). In the former the fragment in between the two lox sites lox71 and loxPinv was shown to be inverted as compared to the configuration in the wild-type maxi-EBV plasmid p2089. Identification of the correctly inverted clones – among them p3125.3 and p3125.5 – was confirmed by Southern blot hybridization (Fig.5.8). The designated probes A and B hybridized to sequences surrounding both loxP sites

(Fig.5.8a). Probe A was the complete p3071.1 plasmid and probe B was the complete p3078.1 construct (see *Appendix*, Tab.1). The blot with the hybridized probe A is presented as an example (Fig.5.8b). On the basis of the Southern blot experiments with both probes (A and B) the following conclusion was made. The 'inverted' clones constituted 15% of all the clones after transient expression of Cre recombinase, while the 'NOTinverted' ones represented 35%. The rest was composed of mixed (containing a mixture of both inv and NOTinv constructs) and incorrectly recombined clones. The following steps were pursued with the 'inverted' maxi-EBV plasmid p3125.5, after its overall restriction pattern was confirmed (*Methods*, chapter 4.3.13).

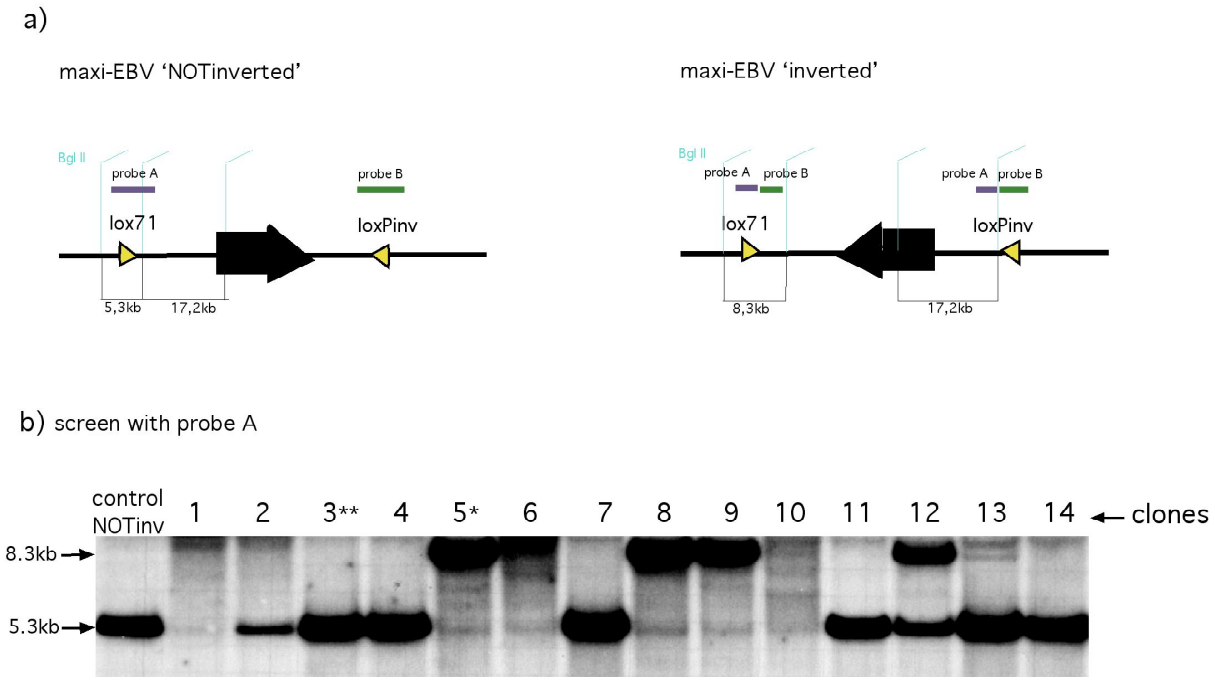


Fig.5.8. Genetic analysis of the Cre-mediated inversion. Identification of 'NOTinverted' and 'inverted' maxi-EBV constructs with Southern blot technique in the series of p3125 maxi-EBV plasmids. (a) Probe A hybridized to sequences around the lox71 site generating a pattern of 5.3kb and 17.2kb for the 'NOTinverted' construct, and 8.3kb and 17.2kb fragments for the 'inverted' construct. Fragments are obtained with BglII cleavage (in blue). (b) Southern blot analysis of the Cre-mediated inversion (probe A). Fourteen maxi-EBV-hosting bacterial clones were assessed for the inversion of the 51kb section in between loxPinv and lox71 sites. The maxi-EBV constructs were isolated with the mini-plasmid preparation protocol (chapter 4.3.4), cleaved with BglII and blotted. The 8.3kb fragment indicated the 'inverted' configuration as in the p3125.5 (*), while the 5.3kb fragment identified the 'NOTinverted' maxi-EBV version as in the p3125.3 (**). The 17.2kb fragments are not shown as they do not distinguish between both orientations. Bands of other sizes represent illegitimate recombinants as the clones 2,6 and 12.

The latent EBV genes of the p3125.5 maxi-EBV plasmid were disabled as planned by the inversion of the large DNA segment, which disrupts the coding sequences of the viral genes LMP1 and EBNA-LP, and abolishes transcription of all EBNA genes as well as LMP2A. Because this system lacked the means to predetermine the

outcome of an additional Cre-mediated inversion, the loxPinv site was exchanged for the variant lox66 site in the next step (Step 3).

5.2.3. Step 3: Exchange of the loxPinv site for the variant lox66 site

To accomplish the exchange of loxPinv *versus* lox66 the chromosomal building technique was applied, avoiding the introduction of a selectable marker gene into the maxi-EBV construct. Two sequential recombination events were required mediated via two different homologous flanks. First, the shuttle plasmid p3213 was constructed by conventional cloning and carried the lox66 site flanked by two regions (blue and red in Fig.5.5C) supporting homologous recombination with the loxPinv locus in the recipient maxi-EBV plasmid p3125.5. Second, recombination via the blue flank combined the maxi-EBV recipient plasmid p3125.5 with the shuttle plasmid p3213 to form a co-integrate of both plasmids (Fig.5.5C). The co-integrate was resolved in *E.coli* again through homologous recombination via the red flank. The genetic manipulations were assisted by expression of RecA from the p2975 helper plasmid in the recA-deficient DH10B *E.coli* strain. As an example, the details of the allelic exchange by the chromosomal building technique are outlined below.

DH10B *E.coli* cells hosting the p3125.5 maxi-EBV plasmid were transformed simultaneously with the shuttle plasmid p3213 and the helper plasmid p2975, and selected for chloramiphenicol, ampicillin and zeocin, (for p3125.5, p3213 and p2975, respectively) at 30°C. Both shuttle and helper plasmids replicated via a temperature-sensitive plasmid origin of DNA replication - at 30°C but not at the non-permissive temperature of 42°C. Plating the transformed bacteria at 42°C and selecting for chloramphenicol and ampicillin resistance favoured the outgrowth of colonies, which harboured the expected co-integrate, only (Fig.5.5C). The correct co-integrate was chosen on the basis of its genetic composition with the verification protocol (*Methods,* chapter 4.3.13), and was used in the second recombination step. Again, bacteria hosting the plasmid co-integrate were transformed with the RecA-helper plasmid (p2975) and selected for resistance against chloramphenicol and zeocin. The resolution of the co-integrate was performed overnight at 30°C. This step was repeated four to five times because spontaneous recombination is very infrequent (1-3%) even in presence of RecA protein, because no selection pressure could be used to select for the resolution of the co-integrate. Finally, the temperature was raised to 42°C in the presence of chloramphenicol selection, only, in order to counterselect the

temperature-sensitive helper plasmid p2975. By replica plating 200 clones were analysed for sensitivity towards ampicillin, indicating loss of the former shuttle plasmid p3213 (Fig.5.5C) and resolution of the co-integrate via homologous recombination. About 15 colonies, which were chloro^{res}/amp^{sen}, were verified by the verification protocol (*Methods,* chapter 4.3.13). The identification of maxi-EBV plasmid, in which the loxPinv site was replaced by lox66, was based on a diagnostic NheI site, which was only present in close vicinity of the loxPinv site but absent at the lox66. PCR amplification (*Methods,* chapter 4.3.12, *Reaction 2*) encompassing this region followed by NheI restriction analysis discriminated between both possibilities (Fig.5.9). The sequence of the newly incorporated lox66 site was confirmed by DNA sequencing, as shown in Fig.5.7 in Step 3, and the *E.coli* clone harbouring the modified maxi-EBV plasmid p3221.12 was chosen for further functional analyses.

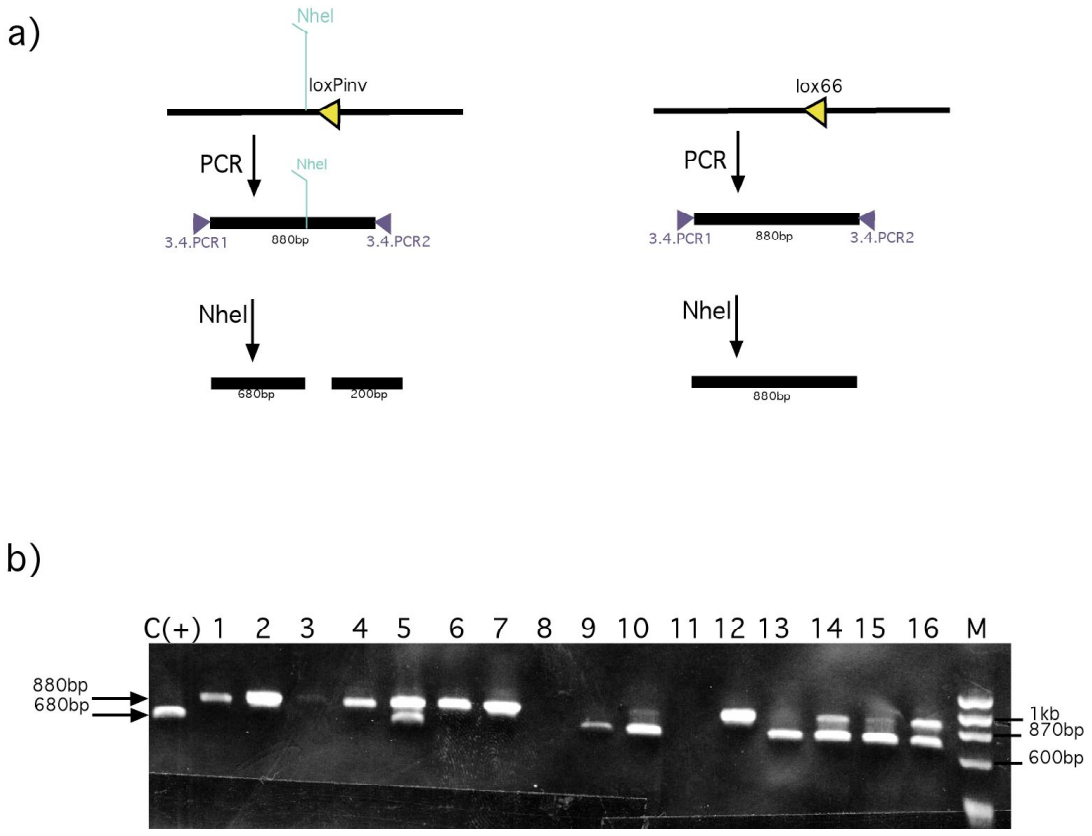


Fig.5.9. Verification of the exchange of the loxPinv site *versus* lox66 in maxi-EBV clones constructed in Step 3. (a) The NheI restriction cleavage site is situated next to the loxPinv site but absent at the lox66 site. PCR products, which amplified the relevant region encompassing the variant loxP sites, were analysed for the occurrence of the NheI site as indicated. (b) Restriction analysis of the PCR products obtained from sixteen maxi-EBV clones. M: molecular weight marker ΦX174HaeIII, C(+): positive control for NheI cleavage (p3125.5 hosting loxPinv); primers are marked in violet.

At this stage the functionality of the Cre/loxP system was evaluated in *E.coli*. Upon transient Cre expression in *E.coli* cells harbouring the maxi-EBV p3221.12, the wild-

type like conformation of the 're-inverted' p3301 maxi-EBV plasmids (Fig.5.14b) was predicted to be stabilized by the formation of the 'locked' lox71/66 site (Fig.5.4). The experimental procedure of Cre expression in *E.coli* cells is described in *Methods* (chapter 4.3.1). After Cre expression, the clones were analysed for the inversion event by restriction analysis (*Methods,* chapter 4.3.13) (Fig.5.10). Twelve out of twelve analysed p3301 plasmid clones were 're-inverted'; thus the efficiency of re-inverting the 51kb fragment to the initial, non-inverted orientation was very high, as expected. Clone 5 of the p3301 maxi-EBV plasmids was employed for the functional assay of the viral latent gene expression described in chapter 5.3.

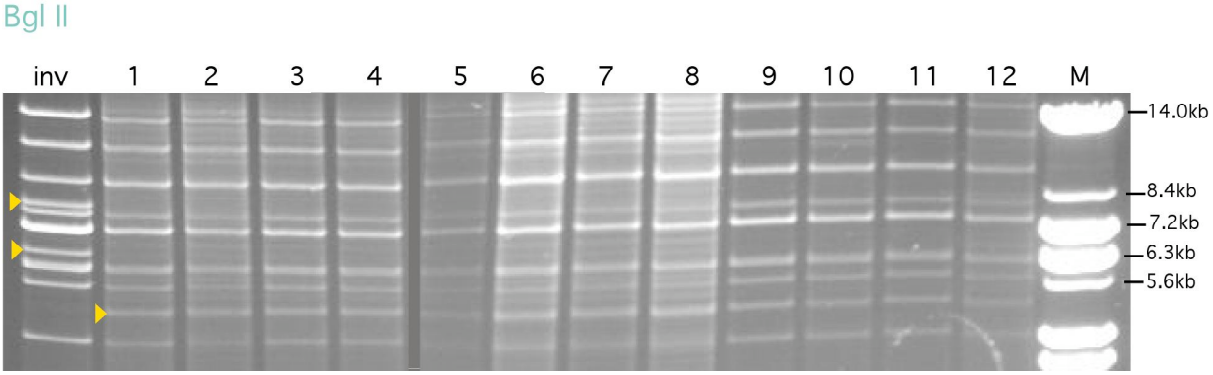


Fig.5.10. Cre-controlled re-inversion of the 51kb fragment flanked by lox71 and lox66 sites in the p3221.12 maxi-EBV plasmid. p3301 plasmid DNAs from twelve bacterial clones were cleaved with BglII and compared to a maxi-EBV plasmid with the inversion (p3283.2) as negative control (inv). Clones are numbered from 1 to 12. Yellow arrow heads mark bands indicative of 'inverted' and 're-inverted' wild-type like patterns; M: □ marker.

5.2.4. <u>Step 4:</u> Inactivation of the EBV lytic origin of DNA replication, OriLyt

The inactivated lytic replication origin of EBV, ΔOriLyt, was introduced by homologous recombination of a linear DNA fragment with the maxi-EBV plasmid p3221.12 established in Step 3. The specific mutation in ΔOriLyt is located in the downstream component of the lytic origin and leads to its complete inactivation, as in the plasmid p995 (Schepers et al., 1993). This mutation was transferred in a conventional cloning step from p995 to the small plasmid p3126.1. The NheI/SacI fragment derived from p3126.1 containing the inactivated lytic origin was used for homologous recombination (*Methods*; chapter 4.3.10) with the maxi-EBV plasmid p3221.12 as schematically shown in Fig.5.11A. The fragment also encompassed a tetracycline resistance cassette to promote the integration of the fragment into p3221.12. The resulting maxi-EBV plasmid p3274.7 was validated as described in the verification protocol in *Methods* (chapter 4.3.13).

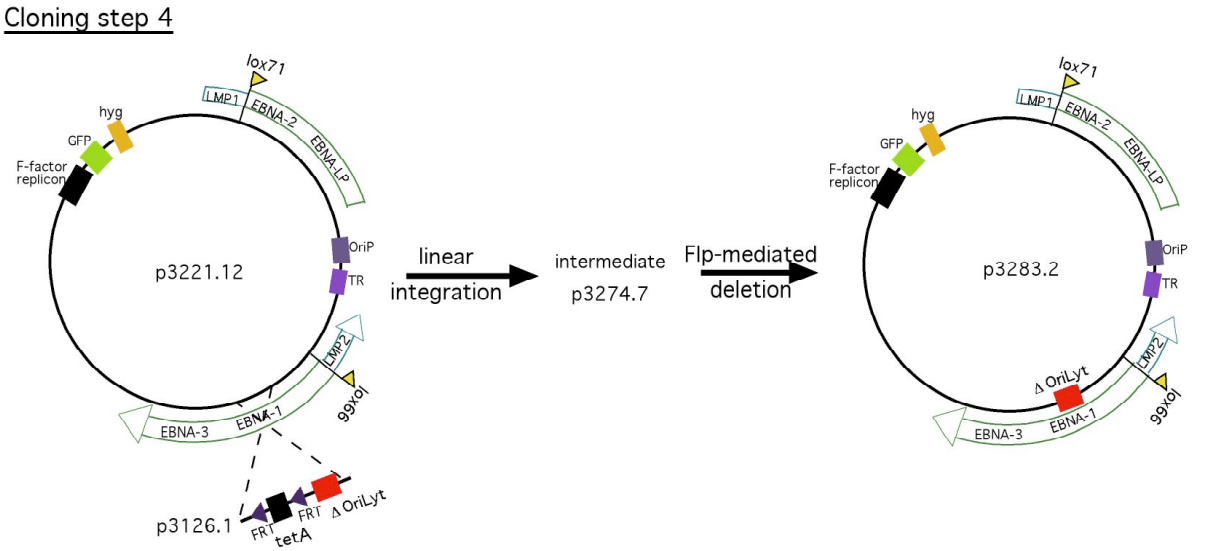


Fig.5.11A. Inactivation of OriLyt: Step 4. Inactivation of the EBV lytic origin of replication, OriLyt, by insertion of ΔOriLyt into the p3221.12 maxi-EBV construct. ΔOriLyt was introduced as a linear DNA fragment together with FRT-flanked *tetA* gene conferring resistance against tetracycline. Subsequent expression of Flp recombinase led to deletion of *tetA* from the p3274.7 intermediate maxi-EBV plasmid, to yield the maxi-EBV plasmid p3283.2.

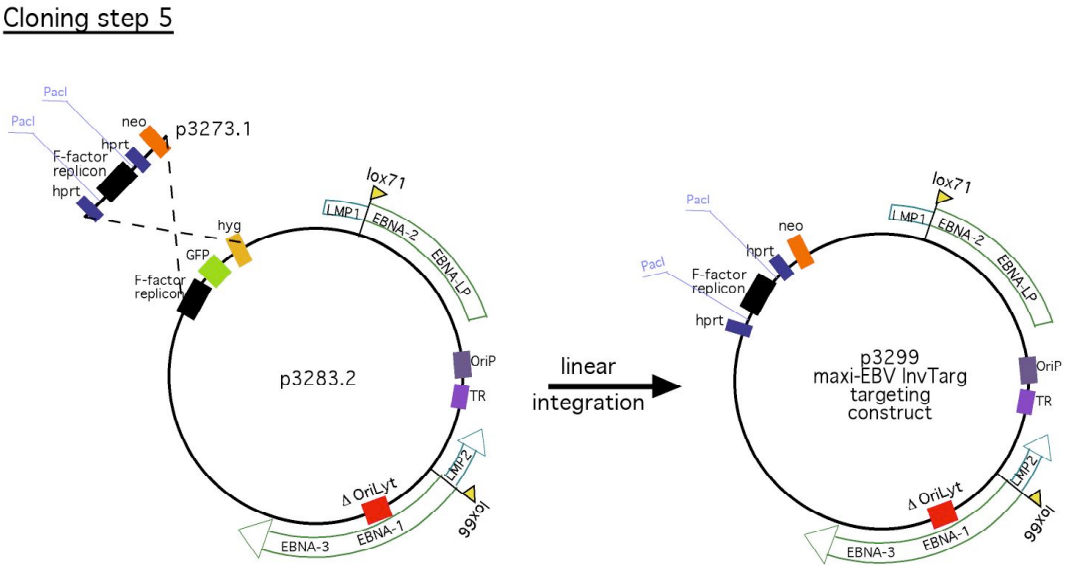
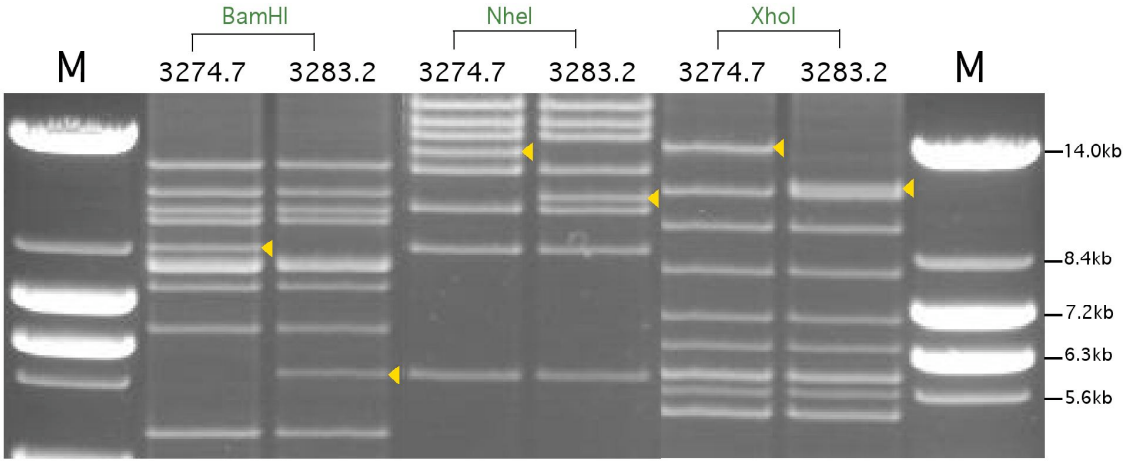


Fig.5.11B. Replacement of the F-factor backbone: Step 5. Exchange of the maxi-EBV backbone of p3283.2 plasmid to replace the selectable marker gene *hyg* for *neo* and to introduce two flanks of the *hprt* gene to yield the final targeting plasmid InvTarg p3299.11.

Subsequently, the tetracycline cassette in p3274.7 was deleted by Flp recombinase (Fig.5.11A), which was based on the same principles as the Cre/loxP system (Fig.5.2). The Flp recombinase removed the section in between two directly oriented FRT sites upon transient expression. The successive steps of the experimental

procedure are described in *Methods* (chapter 4.3.1). Replica plating allowed the identification of clones that had lost both the tetracycline resistance cassette and the Flp-expressing plasmid. The resulting recombinant maxi-EBV clones were analysed in the clone verification manner (*Methods,* chapter 4.3.13). Their restriction patterns were compared to that of the p3274.7 maxi-EBV plasmid previous to the Flp-mediated deletion (Fig.5.12). At this point, the mutation introduced into the lytic origin was assessed by DNA sequencing (Step 4 of Fig.5.7). The identified maxi-EBV construct p3283.2 harboured a non-functional viral lytic origin, ΔOriLyt, and reversibly blocked viral latent genes.

a)



b)

Prediction of the restriction patterns:

BamHI		NheI		XhoI	
p3274.7	p3283.2	p3274.7	p3283.2	p3274.7	p3283.2
11.867	11.867	18.080	18.080	13.942	11.417
10.477	10.477	15.591	15.591	10.720	10.720
9.693	9.693	14.318	14.318	9.353	9.353
9.221	9.221	13.019		7.947	7.947
8.567		11.693	11.693	6.897	6.897
8.020	8.020		10.494	6.374	6.374
7.914	7.914	9.840	9.840	5.952	5.952
7.910	7.910	8.313	8.313	5.926	5.926
7.396	7.396	5.782	5.782	5.668	5.668
6.535	6.535			5.393	5.393
5.055	6.042			5.372	5.372
5.053	5.055				
	5.053				

Fig.5.12. Restriction analysis of the p3283.2 maxi-EBV construct with an inactivated viral lytic origin (conducted in Step 4). (a) The pattern of the previous p3274.7 maxi-EBV construct (Fig.5.11A) served as a reference. Restriction patterns were verified with three different restriction enzymes (in blue). (b) List of the visible bands is provided (in kb). Brackets embrace the co-migrating fragments, which appear as a single band on the gel. M stands for marker; red colour depicts the bands, which differ between p3274.7 and p3283.2.

5.2.5. Step 5: Replacement of *hyg* and *gfp* for *neo* and introduction of *hprt* flanks for site-specific integration

The *neo* gene which carries resistance against G418/Neomycin is the gold standard in selecting murine embryonic stem cells (mES). Thus, this selectable marker gene was chosen for the InvTarg construct instead of *hyg*. In a single step, a series of events were achieved: the replacement of *neo* versus *hyg*, deletion of the gene encoding GFP and introduction of two *hprt* flanks (Fig.5.11B). The *hprt* flanks were introduced to promote the targeted site-specific integration of the InvTarg maxi-EBV plasmid in a linear form into the ∆*hprt* locus of the murine ES cell line. Towards this end, a conventional small plasmid was constructed in *E.coli* in a series of cloning steps (list of intermediate plasmids is provided in Step 5 of *Appendix*), shown in its linear form in Fig.5.13. The p3273.1 plasmid was linearized with SacI and subsequently electroporated into DH10B *E.coli* cells hosting the maxi-EBV p3283.2 (the construct of Step 4), and selected for tetracycline and chloramphenicol (for p3273.1 fragment and p3283.2 plasmid, respectively) as described in *Methods* (chapter 4.3.10). The resulting maxi-EBV recombinants were analysed for their genetic composition by the verification procedure (*Methods,* chapter 4.3.13).

The identified maxi-EBV plasmid p3299.11 (InvTarg) bore the Cre-controlled switch, the inactivated viral lytic origin and features for the targeted site-specific integration into the *hprt* murine locus via *hprt* flanks and the selectable marker *neo*. This InvTarg plasmid p3299.11 was sequenced at all the important, genetically manipulated sites (Step 5 of Fig.5.7).

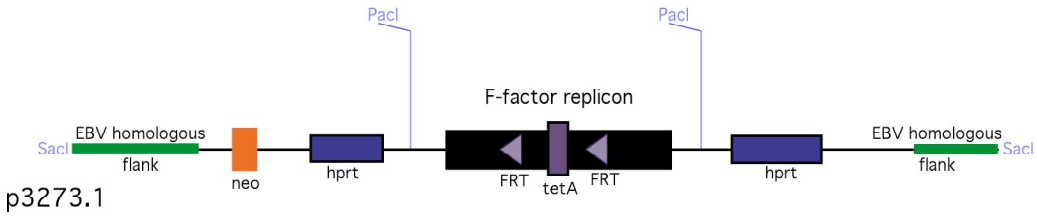
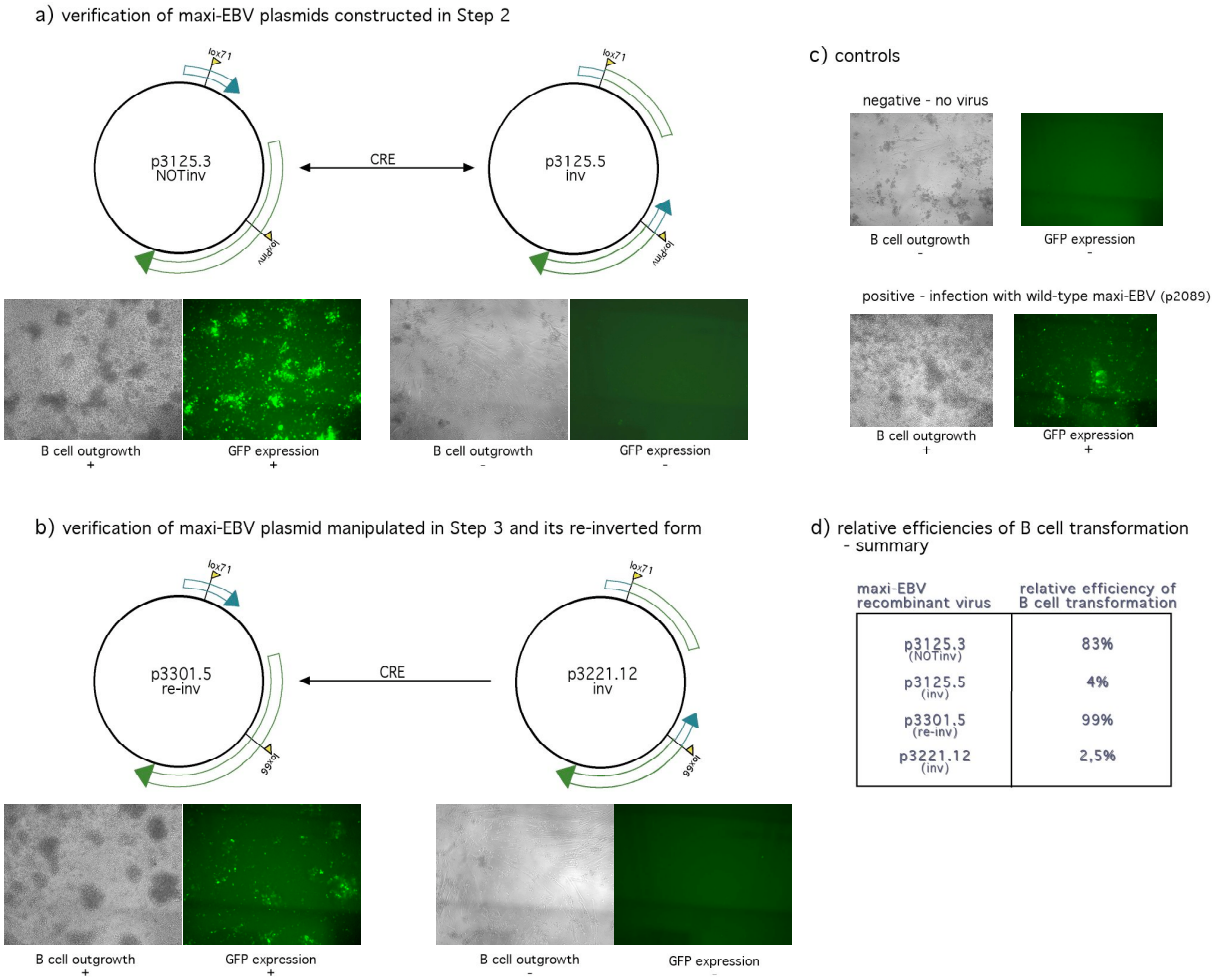


Fig.5.13. Structure of the conventional small plasmid used for recombination with the recipient maxi-EBV plasmid in Step 5. The small plasmid p3273.1 contained a number of features for introduction into the maxi-EBV construct p3283.2 via linear integration (through the EBV homologous flanks): *neo*, the *neomycin* resistance cassette, two *hprt* flanks, and the F-factor replicon containing FRT-flanked *tetA* resistance gene. PacI restriction sites allowed linearization of the final InvTarg targeting construct deleting the bacterial F-factor replicon.

5.3. Functional analysis of the conditional EBV latent genes switch, a core feature of InvTarg construct

The InvTarg maxi-EBV plasmid p3299.11 I constructed (chapters 5.1 and 5.2) bears a unique switch, which allows the activation of almost all viral latent genes upon expression of Cre. The construct is 'off' meaning that the maxi-EBV cannot express its latent genes and is therefore incapable of growth transforming primary human B cells. On the basis of this read-out the function of Cre-recombinase to activate the latent genes by re-inversion was verified. Four different maxi-EBV constructs were tested: p3125.3, p3125.5, p3221.12 and p3301.5. The maxi-EBV plasmids p3125.3 and p3125.5 were obtained in Step 2 during the construction of the InvTarg plasmid (Fig.5.5B). They constitute an isogenic pair, which differs only in the orientation of the loxP-flanked EBV segment. In p3125.3 the segment is oriented as in wild-type maxi-EBV (Fig.5.5B, p3125.3 NOTinv), but the segment is inverted in p3125.5 (Fig.5.5B, p3125.5 inv) inactivating EBV's latent genes. Both plasmids carry one loxP site (loxPinv) and one variant lox71 site. The remaining two maxi-EBV plasmids, p3221.12 (Fig.5.5C) and p3301.5 (not shown) also form a pair which differs with respect to the orientation of the EBV segment: p3221.12 has its latent genes disabled but p3301.5 corresponds to the situation in wild-type maxi-EBV. The latter was derived from the former by transient expression of Cre in *E.coli* as described in chapter 5.2.3.

All four maxi-EBV plasmids were packaged into EBV virions as described in *Methods* (chapter 4.4.1) and human primary B cells were infected (*Methods*, chapter 4.4.2). Four to six weeks post infection the infected B cells were assayed for their capacity to yield lymphoblastic cell lines transformed by EBV. The data provided in Fig.5.14 proved that only maxi-EBV plasmids, which had the loxP-flanked segment in wild-type conformation gave rise to transformed B cells, as expected. The data also document that the genetic alterations in particular the introduced loxP sites did not interfere with the transforming capability of the maxi-EBVs.



F**ig.5.14. Functional analysis of the Cre-mediated switch of EBV latent genes with four maxi-EBV plasmids.** The maxi-EBV plasmids were packaged into EBV viral particles and primary human B cells were infected with these viruses. (a) The loxP sites introduced in plasmid 3125.3 (NOTinv) did not interfere with B-cell transformation process, while the inversion in p3125.5 (inv) interrupted viral latent genes expression. (b) The same held true for the constructs hosting lox66 instead of loxPinv. The 'inverted' construct p3221.12 (inv) did not transform B cells, while the p3301.5 (re-inv) plasmid after re-inversion supported B cell proliferation. (c) Wild-type maxi-EBV served for a positive control in the experiment. It is capable of transforming B cells and does sustain their proliferation. The negative control comprised uninfected B cells, which died within 1-2 weeks post infection. (d) Relative transformation efficiency of all four plasmids was calculated from four independent experiments carried for each plasmid. The unexpected but low ability of the 'inverted' constructs to stimulate B cell proliferation was most probably linked to contaminating EBV field strains present in the primary human B cell preparations, because the transformed B cells lacked the GFP expression. B cell proliferation was assessed with light microscopy, GFP expression was visualized with UV fluorescent microscope.

5.4. Linearization and further processing of the InvTarg construct, prior to introduction into murine cells

5.4.1. Preparation and purification of the InvTarg plasmid DNA from *E.coli*

In order to introduce large maxi-EBV DNA into murine cells the quality of the DNA preparation must be improved, in terms of purity and intactness, as compared to the procedure of genetic manipulations in *E.coli*. Three different methods of maxi-EBV

plasmid DNA purification were tested: alkaline lysis followed by cesium chloride/ethidium bromide (CsCl)-gradient preparation (detailed protocol in *Methods*, chapter 4.3.4) and two Qiagen kits: EndoFree Maxi Plasmid and Large-Costruct kit. The DNA quality was assessed by PFGE. Two different maxi-EBV constructs, of about 185kb in size, were employed in the assay: p2089, a wild-type maxi-EBV, and p3299.11, the InvTarg targeting construct (Fig.5.15).

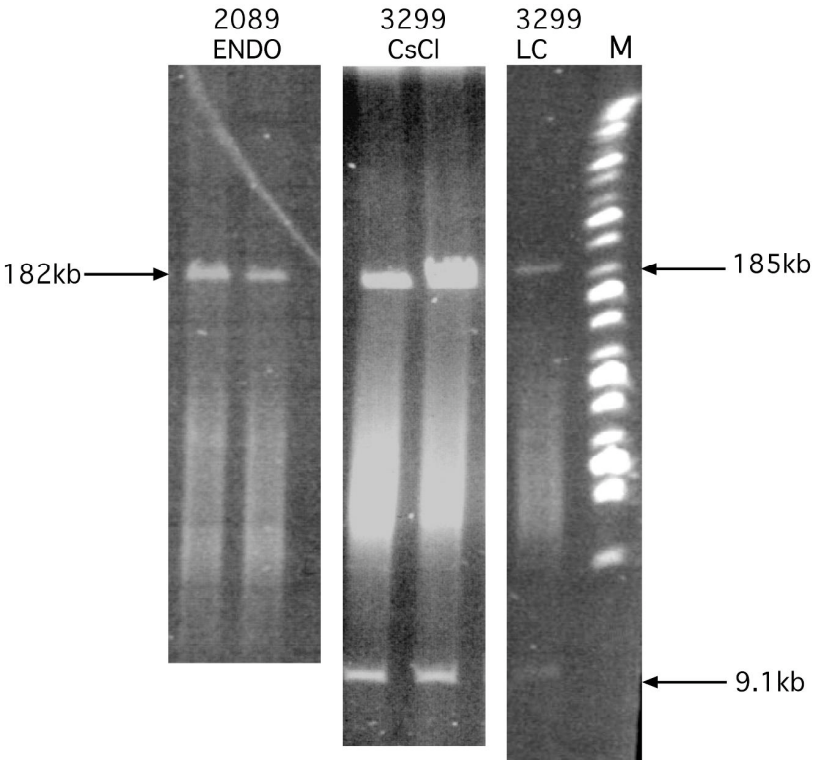


Fig.5.15. Comparison of different methods for large maxi-EBV DNA preparation. Two different maxi-EBV plasmids were purified and assessed by PFGE: p2089, a wild-type maxi-EBV, and p3299.11, InvTarg targeting construct. Maxi-EBV DNAs were cleaved with a given enzyme and 0.5□g of each digestion was loaded per lane of a pulse field gel (although it was calculated to load the same amount of linearized DNA per one lane, the amounts differ between preparations as the DNA concentration was not always accurate). p2089 DNA was purified with Qiagen EndoFree Maxi Plasmid Kit and linearized with SgfI (ENDO). p3299.11 was isolated either with the CsCl-gradient protocol (CsCl) or with Qiagen Large-Costruct kit (LC) and cleaved with PacI. M: MidRange marker.

The constructs were linearized with restriction enzymes as indicated. According to evaluation by PFGE, the quality of the maxi-EBV DNA preparations from all the protocols was comparable, but DNA obtained via CsCl-gradient was superior in terms of quantity.

5.4.2. DNA integrity of the InvTarg construct

Standard restriction analysis of large maxi-EBV plasmids cannot assess their molecular integrity because conventional vertical electrophoresis does not allow proper verification of DNA fragments exceeding 30kb but a pulse field gel electrophoresis (PFGE) allows separation of very large DNA molecules (Fig.5.16a).

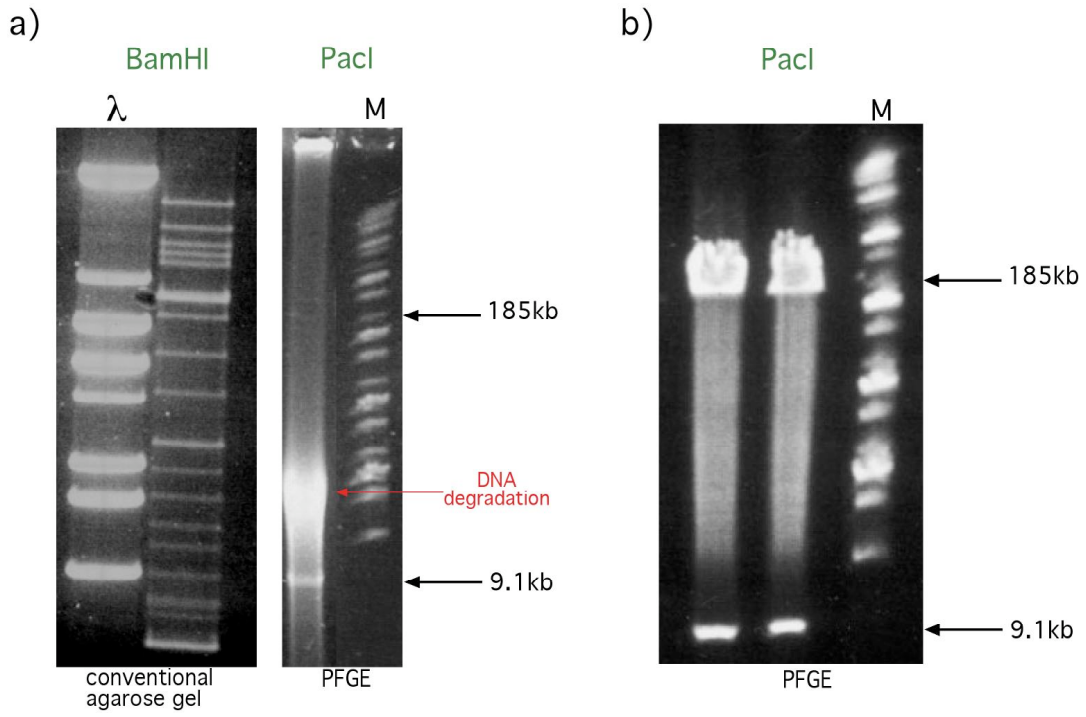


Fig.5.16. Electrophoretic analysis of digested InvTarg p3299.11 DNA. (a) The same preparation of p3299.11 BAC plasmid was either digested with the frequently cleaving BamHI enzyme and run on a conventional horizontal agarose gel (0.5□g/lane), or cleaved with PacI (two fragments of 185kb and 9.1kb are expected) and loaded on PFGE gel (1□g/lane). Only the latter method revealed the poor quality of the 3299.11 DNA preparation. (b) The optimal loading capacity per one lane of PFGE gel was 3□g/lane of PacI-digested 3299.11 DNA. M: MidRange marker, □: □-BstEII marker, PFGE: Pulse Field Gel Electrophoresis.

To aid the isolation of the 185kb band of the linearized InvTarg plasmid, the practical loading capacity of a single lane on a PFGE gel was experimentally determined. Although it was possible to load as much as 15□g of 3299.11 PacI-cleaved DNA per one lane, a characteristic smear appeared indicating overloading (Fig.5.17a, lane1). Therefore, 3□g of well-dissolved 3299.11-PacI DNA per lane was found to be optimal (Fig.5.16b).

5.4.3. Linearization of InvTarg plasmid DNA

In the following step the InvTarg targeting construct was linearized, which was mandatory to allow its chromosomal integration, because linear DNA molecules are

preferred in both random (Giraldo and Montoliu, 2001) and site-specific (Capecchi, 2005) transgene integrations. After digestion of the InvTarg plasmid p3299.11 with the PacI restriction enzyme, the 185kb targeting fragment was purified by PFGE. To test the excision of this fragment from a single lane of PFGE gel (for its subsequent electroelution with Quik-Pik kit), cleavage of different amounts of DNA in small volume was evaluated. Different 3299.11 DNA amounts were cleaved overnight with PacI in 80μl volume, which was then separately loaded on a PFGE gel (Fig.5.17a).

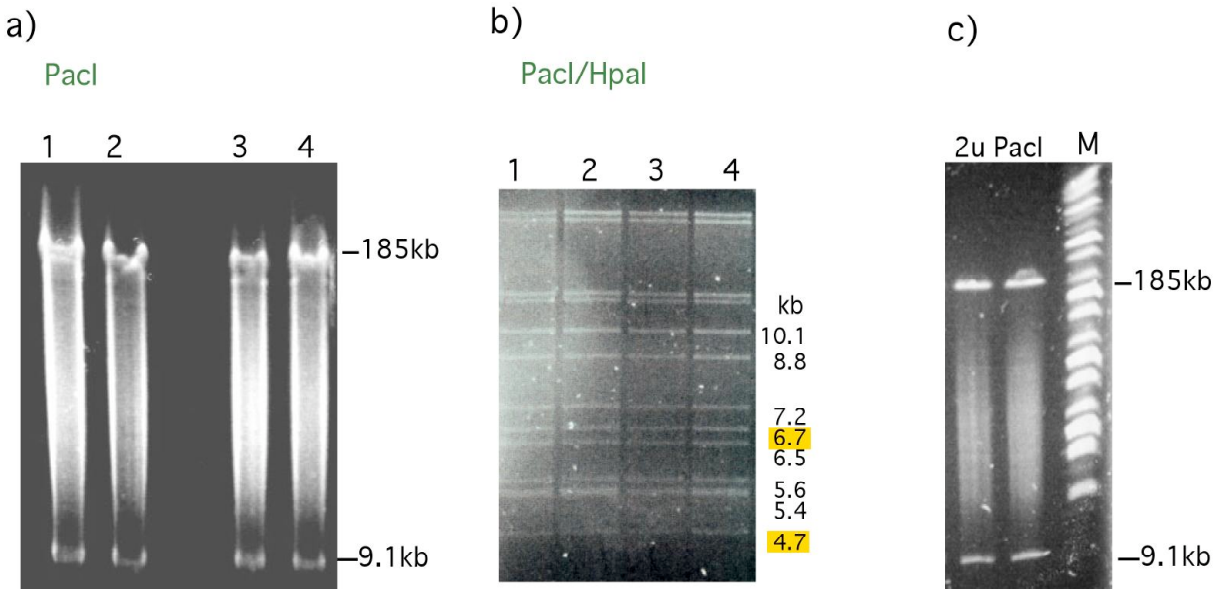


Fig.5.17. Optimization of long-term PacI digestion of p3299.11 InvTarg plasmid. (a) p3299.11 maxi-EBV DNA was cleaved with 5u PacI in 80μl overnight and the whole digest was loaded on single lanes of PFGE gel. Samples differed in DNA amount taken for each cleavage: 1 - 15μg, 2 - 12μg, 3 - 10μg, 4 – 8μg. (b) 0.5μg from each of the four PacI-cleaved samples was digested with HpaI and run on large conventional horizontal agarose gel. Presence of 6.7kb and 4.7kb bands in PacI/HpaI cleavage indicated complete cleavage with PacI. (c) Overnight cleavage of 1μg 3299.11 DNA in 80μl volume was carried with 2u of PacI enzyme, and subsequently loaded on a PFGE gel (two samples cleaved in the same conditions). M: MidRange marker.

Complete digestion with PacI was verified with the subsequent cleavage with HpaI (Fig.5.17b). As indicated by the presence of the PacI/HpaI specific fragments in this double cleavage, all the PacI digestions were complete. These results indicated that as much DNA as 15μg could be cleaved to completion with as little enzyme as 5u PacI (0.3u/μg DNA), but very high amounts of DNA loaded on single lanes of the PFGE gel were not optimally separated during electrophoresis (smear along the lanes; Fig.5.17a).

The effect of increased PacI concentration during overnight digestion on DNA quality was also investigated (Fig.5.17c). After 1μg of 3299.11 DNA was cleaved with 2u PacI (2u/μg DNA) in 80μl, its linearization was estimated with an analytic PFGE gel.

The higher enzyme concentration did not increase the usual level of DNA degradation (smear between 185kb and 9.1kb bands).

Shortening of the digestion time was also investigated for the 3299.11 cleavage with PacI. 1□g of 3299.11 maxi-EBV DNA was digested for 4h in 80□l with 8-15u (8-15u/□g DNA) of PacI, and the DNA quality was estimated with a PFGE gel (Fig.5.18). The cleavage conditions did not reveal an increase in DNA degradation.
In the next experiment, lowered enzyme concentration was verified in the same cleavage reaction. As a result, 3□g of 3299.11 DNA was cleaved to completion in 80□l within 4h with 10uPacI (3.3u/□g DNA), only (Fig.5.19, lanes 3 and 4).

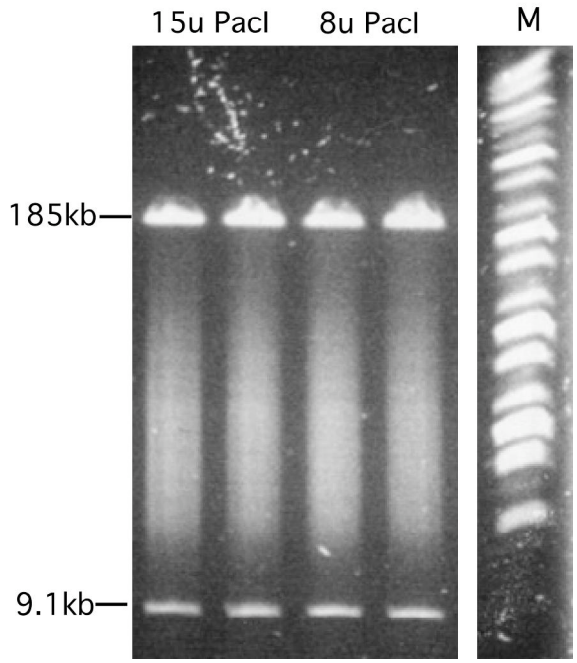


Fig.5.18. Optimization of sort-term PacI digestion of p3299.11 InvTarg plasmid DNA. Different amounts of enzyme were compared in terms of DNA quality (two samples/condition). 1□g of p3299.11 DNA was digested for 4h with 8u or 15u of PacI in 80□l volume. DNA was subsequently separated on a PFGE gel. M: MidRange marker.

In the final conclusion from the digestion experiments, optimal conditions for the complete cleavage of the 3299.11 DNA with PacI, without overdigestion, were set. They differed between the long-term overnight digestion (in average 16h) and the short-term digestion for 4h, and are summed up in Tab.5.1. The ratios of the PacI concentration to DNA concentration were calculated to set the final range of the border-line concentrations of the enzyme that can cleave DNA completely without causing its degradation.

Cleavage time	Overnight		4h	
Enzyme concentration	***Minimum PacI***	***Maximum PacI***	***Minimum PacI***	***Maximum PacI***
Cleavage conditions in 80µl volume	5u PacI for 15µg DNA	2u PacI for 1µg DNA	10u PacI for 3µg DNA	8 to15u PacI for 1µg DNA
Ratio of PacI concentration (u/µg DNA)/ DNA concentration (µg/µl)	0.3 Pac/ 187 DNA = 0.0016	2 Pac/ 12.5 DNA = 0.16	3.3 Pac/ 37.5 DNA = 0.088	8 to 15 Pac/ 12.5 DNA = 0.64 to 1.2
Range of optimal cleavage conditions	**0.0016-0.16**		**0.088-1.2**	

Tab.5.1. Optimal cleavage conditions for the InvTarg plasmid DNA. The border-line concentrations of PacI enzyme for p3299.11 DNA cleavage, which led to the complete cleavage without overdigestion, are specified for long-term (overnight) cleavage and short-term digestion (4h). Ratios of the enzyme concentration to the DNA concentration set for both border-line settings delimit the range of the optimal cleavage conditions, allowing easy comparison of each next digestion to the optimized reactions.

5.4.4. Further processing of the linearized InvTarg DNA

In order to purify transgene DNA prior to introduction into murine cells, after digestion with PacI the 3299.11 the InvTarg construct was processed in four different ways: (i) the enzyme was inactivated (ii) followed by dialysis, (iii) the DNA was directly precipitated or (iv) the 185kb fragment was electroeluted from a preparative PFGE gel. The quality of the DNA upon each procedure was assessed on an analytical PFGE gel.

(i)and (ii): Enzyme inactivation and dialysis of the InvTarg plasmid preparation

In order to prevent an influence of an active PacI restriction enzyme on the subsequent transfection and injection procedures, the enzyme was heat-inactivated according to the procedure described in *Methods* (chapter 4.3.8). In the second approach the PacI-digested DNA was dialysed after the enzyme inactivation with the intention of adjusting salt concentration of the DNA preparation prior to its introduction into mouse cells. Dialysis was conducted against a microinjection buffer for the microinjection purpose, or against a buffer suitable for the transfection into ES cells. PFGE analysis revealed that the linearized maxi-EBV 3299.11 DNA did not become degraded upon enzyme inactivation and/or subsequent dialysis in comparison to DNA, which was ‘simply’ cleaved (without further processing) (Fig.5.19).

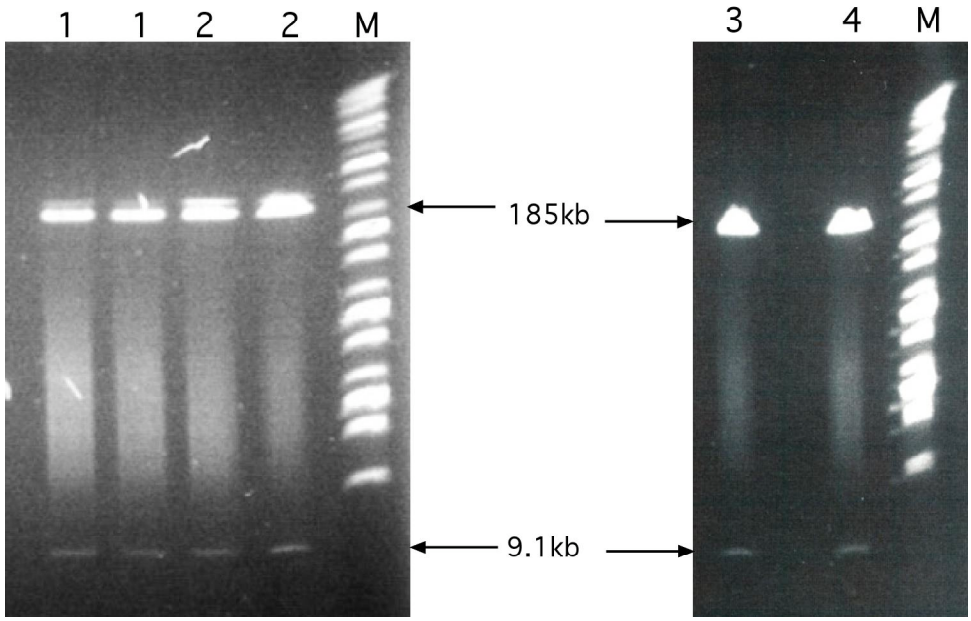


Fig.5.19. Enzyme inactivation and dialysis of the linearized InvTarg p3299.11 targeting construct. 1□g of p3299.11 DNA was cleaved with PacI and run on a pulse field gel. DNA was loaded directly after cleavage (1), after enzyme inactivation (2, 3), and after enzyme inactivation with subsequent dialysis (4). Samples 1 and 2, as well as samples 3 and 4 were prepared in parallel. M: MidRange marker.

(iii) Precipitation of InvTarg construct DNA

Prior to chromosomal integration targeting vectors are most commonly precipitated after linearization in the first step towards establishing a transgenic mouse. Linear DNA of the size of the InvTarg maxi-EBV plasmid is very fragile and therefore sensitive to this treatment. Particularly, once linearized, it is vulnerable to mechanical breakage due to its large size. Two different approaches of DNA precipitation following the linearization were tested: the standard protocol and precipitation 'with FISHing' (described in *Methods*, chapter 4.3.14). The DNA pellet was resuspended either in the appropriate buffer for microinjection or in 1xTE for ES transfection. The standard precipitation most often led to DNA degradation (Fig.5.20a), while the alternative precipitation protocol 'with FISHing' predominantly resulted in mostly intact linear DNA molecules (Fig.5.20b).

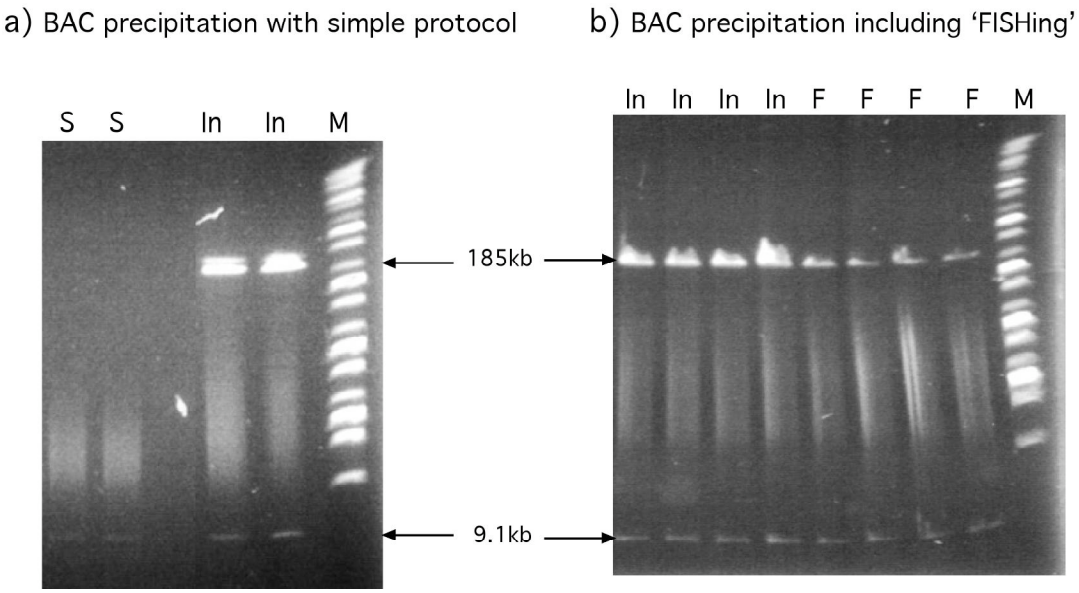


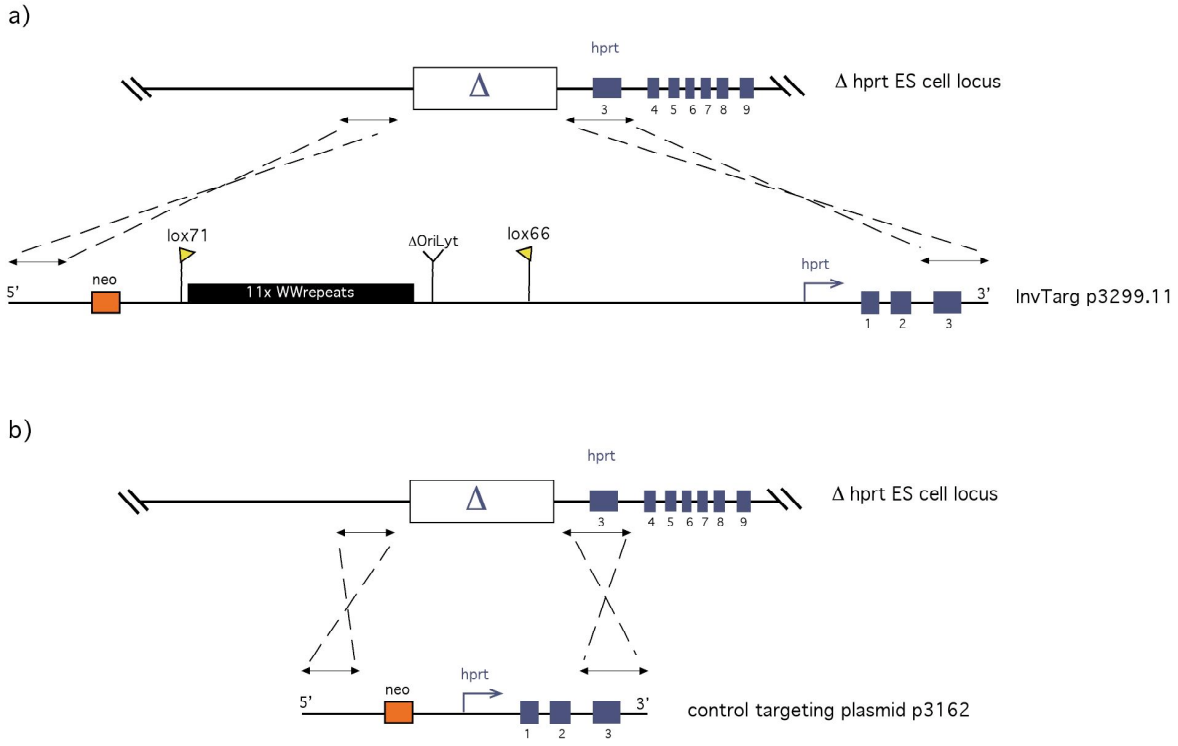
Fig.5.20. DNA precipitation of the linearized InvTarg p3299.11 targeting construct. p3299.11 DNA digested with PacI was directly precipitated with either the standard precipitation protocol (S) or the precipitation protocol 'with FISHing' (F). The PacI-digested 3299.11 DNA treated with an enzyme inactivation (In) served as a positive control for the DNA quality. Loading: 1□g/lane (a), 300ng/lane (b). M: MidRange marker.

(iv) Electroelution of the 185kb InvTarg targeting fragment after PFGE

Isolation of a targeting fragment from a transgene construct is advantageous because this fragment can be directly enriched. Moreover, contaminating bacterial DNA, the plasmid backbone and degraded plasmid DNA can be all omitted. Following linearization with PacI the 3299.11 InvTarg DNA was run on a preparative PFGE gel. The fragment of 185kb was excised and subsequently electroeluted from the gel according to the Quik-Pik protocol (*Methods,* chapter 4.3.5). The eluted fragment was dialysed against the appropriate buffer for microinjection. Electroelution was successful, high quality of the linear targeting maxi-EBV vector was produced (Fig.5.26, samples 1-4). However, recovery of the linear maxi-EBV DNA in this approach was very low as expected, and it underwent degradation within around two weeks of storage at +4°C. Therefore, it was impossible to exploit this method for DNA transfection into murine ES cells.

5.5. Site-specific insertion of InvTarg construct into murine ES cells

The murine Δ*hprt* embryonic stem (ES) cell lines (HM1 and E14) are characterized by an inactivated *hprt* gene on the Y chromosome. *hprt* can serve as a selection marker because *hprt*-negative cells cannot survive in HAT (Hypoxanthine, Aminopterin and Thymidine) medium as described in chapter 1.6.4 of *Introduction*. The *hprt* locus of Δ*hprt* ES cells can be restored with an appropriate targeting construct, which promotes homologous recombination with the *hprt*$^-$ allele and introduces a suitable promoter with first two exons of *hprt* (Fig.5.21).



5.21. Reconstruction of the disabled *hprt* locus in Δ*hprt* murine ES cells. The DNA targeting construct complements the deleted *hprt* locus with an *hprt* human promoter, human first exon and murine exon two located all on the right flank of the linearized construct. The left flank of the transgene recombines with the upstream region of the *hprt* locus providing the second selection marker gene, *neomycin (neo)*. The construct can recombine with the *hprt* locus via homologous recombination (dashed lines). (a) PacI-linearized InvTarg maxi-EBV construct p3299.11 (185kb) introduces a recombinant EBV genome, with conditional viral latent gene expression, together with the *neo* selection cassette. (b) 20kb control targeting construct carries the *neo* gene. neo: neomycin, hprt: hypoxanthine phosphoribosyl-transferase, WW: viral repeats, ΔOriLyt: disabled viral lytic origin of DNA replication; hprt promoter and its nine exons are shown in blue, bidirectional arrow heads indicate homologous regions. Figure not drawn to scale.

The InvTarg maxi-EBV targeting construct, when cleaved with PacI, provides the missing *hprt* parts with its right flank. The left flank of InvTarg carries sequences homologous to the upstream *hprt* locus in conjunction with a second selectable

marker gene – *neo,* encoding resistance against G418 antibiotic. Both flanks bracket the genetically altered but complete EBV genome. Introducing this DNA transgene into ∆*hprt* ES cells should promote its homologous recombination and result in cells, which can survive selection with both HAT and G418.

5.5.1. Optimization of murine ES cells transfection

Because transfection of such a large linear DNA molecule as the InvTarg maxi-EBV plasmid constitutes a challenging task, the conditions of the procedure were first assessed with a smaller (20kb) control plasmid – p3162, equipped with *neo* resistance and *hprt* flanks for integration into the *hprt* locus of □*hprt* murine ES cells (Fig.5.21b). As a prelude, transfection conditions for HM1 cell line were assayed with a small GFP plasmid and the most favouring conditions were chosen in transient transfection experiments. Two different electroporation conditions were tested in this experiment with the pEGFP-C1 plasmid: 250V, 500□F (E-1), 800V, 3□F (E-2) (Tab.5.2).

Cell culture conditions	Electroporation conditions	
	E-1	E-2
HM1-1	1.1% survival 0.4% GFP	2.7% survival 0.08% GFP
HM1-2	3% survival 0.6% GFP	4% survival 0.08% GFP

Tab.5.2 Initial optimization of the transfection conditions for the HM1 □*hprt* ES cell line. This experiment was based on the transfection of the small GFP plasmid, pEGFP-C1, into two samples of HM1 cells cultured under different conditions and transfected with two different electroporation settings: HM1-1- in DMEM medium, HM1-2- in Glasgow medium, E-1- 250V and 500□F, E-2- 800V and 3□F. The readout was evaluated under light (survival) and fluorescent (GFP) microscope. The results shown represented the percentage of GFP-positive cells of 10^7 cells plated.

Additionally, the HM1 cells were cultured in two different media, DMEM (HM1-1) and Glasgow (HM1-2), both complemented with FCS (PAN), to test which culture conditions favour successful DNA uptake and cell survival. $2x10^7$ cells were transfected with 30□g of the GFP-encoding plasmid, pEGFP-C1, and half of the transfected cells were plated on 3 (for E-1) or 4 (for E-2) 10cm plates. The efficiency of DNA uptake was evaluated on the second day after transfection. HM1 ES cells cultured in Glasgow medium (HM1-2) showed better survival and transfection efficiency than cells cultured in DMEM medium (HM1-1) (Tab.5.2). Milder electroporation conditions (E-2) led to improved cell survival but resulted also in a much lower transfection efficiency: 0.6% of the transfected cells expressed GFP after

E-1 transfection, in contrast to 0.08% after E-2 transfection for HM1-2 culture conditions.

In order to further improve the efficiency of the ES transfection procedure, different □*hprt* ES cell lines, HM1 and E14, and two distinct transfection and selection methods were tested in a single experiment targeting the *hprt* locus with the control plasmid p3162 (Tab.5.3).

Tab.5.3 Further optimization of □*hprt* ES cells targeting with the small targeting plasmid p3162. Three transfection conditions were tested with the p3162 plasmid carrying *neo* resistance and *hprt* homologous flanks. Two different □*hprt* ES cell lines were investigated: E14 and HM1. The cells were either electroporated (250V, 500□F) or transfected with L2000 (Invitrogen protocol), and subsequently selected with HAT alone, or first with G418 followed by HAT medium (G418 □ HAT) medium. The readout is presented in $x10^{-4}$% of the number of colonies with respect to cells transfected initially (10^6 for lipofection, 10^7 for electroporation), and represents the average from two experiments.

Transfection method	Selection	Cell line	
		E14	HM1
Electroporation	HAT	0.35	0.1
	G418 → HAT	0.95	0.5
Lipofection (L2000)	HAT	2	0.5
	G418 → HAT	18	3

In a first approach, $2x10^7$ cells were electroporated with 30□g PacI-linearized p3162 plasmid DNA and seeded on four 10cm plates, which were selected differently. One half was selected with HAT medium and the other half was selected first with G418 for one week and subsequently with HAT medium alone. In a second approach, the linearized p3162 plasmid was transfected with lipofectamine 2000 (L2000) according to the manufacture's protocol (Invitrogen). 4□g of this DNA was transfected per 10^6 cells (in one well of a 6-well cluster plate). Four ES cell samples were transfected in a single experiment. Two samples were put under selection with HAT medium and the other two were selected first with G418 and then with HAT medium alone. The results of these experiments are summarized in Tab.5.3. The E14 ES cell line showed a 2-6 fold better targeting efficiency than the HM1 cell line. Transfection with L2000 appeared to yield improved results in comparison to electroporation by a

factor of 5-18. Sequential selection with G418 followed by HAT medium alone (G418 → HAT) resulted in a 3-9 fold higher efficiency than selection with HAT medium, only. In conclusion, lipofection of the E14 ES cell line and sequential G418 → HAT selection were employed in the following transfections.

The original lipofection protocol from Invitrogen was altered in an attempt to further improve ES cell targeting with the linearized p3162 construct. Minor changes in the original Invitrogen protocol did not alter the targeting efficiencies as detailed in Tab.5.4, but further modification of several parameters resulted in an improvement (Tab.5.5).

Changing condition	Colony number/6-well (G418/HAT double resistant)
-	16
$0.2x10^6$ cells plated for transfection	3
cells splited on day 1 a.t.	7
30min. incubation of DNA-L2000 complexes	16

Initial protocol from Invitrogen - 6-well format

- cells plated at $0.5x10^6$/well 1 day before transfection

transfection:

- medium change - 2.5ml/well
- DNA and L2000 diluted separately in 0.25ml Opti-MEM (2.5μlL2000/1μg DNA)
- DNA and L2000 solutions combined and 0.5ml of DNA-L2000 complexes incubated for 20min. at RT
- DNA-L2000 complexes added to cells and incubated o/n (in 3ml final volume)
- medium change on next day (day1), start selection on day 2

Tab.5.4. Initial optimization of the E14 ES cells transfection with lipofectamine 2000 for the PacI-linearized p3162 control plasmid. Several transfection conditions (listed on the left side) were changed in comparison to the original protocol for transfection with Lipofectamine2000 (L2000) provided by Invitrogen (shown on the right side). 4μg of PacI-linearized p3162 was lipofected per 10^6 E14 cells with the Invitrogen protocol, which were then selected first with G418 then in HAT medium (G418 → HAT). Transfection results are given in colony number per 10^6 cells transfected, and are the average from three separate samples. 6-well - one well of the 6-well plate, RT – room temperature, Opti-MEM – serum free medium, a.t.: after transfection.

Conditions, which were expected to increase the transfection and targeting efficiencies included higher concentration of DNA with Lipofectamine2000 (DNA-L2000) complexes and a lower volume of the serum-free medium for the incubation of cells with the DNA-L2000 complexes. Time for incubation of cells with the DNA-L2000 complexes also affected ES colony formation (Tab.5.5). In conclusion, the final conditions were: 10^6 E14 ES cells, 1μg PacI-cleaved p3162 DNA, DNA-L2000 complexes incubated in 0.2ml of serum-free medium (Opti-MEM), and the incubation of cells with the complexes in 1.2ml of Opti-MEM overnight ('optimized protocol' specified in detail in *Methods*, chapter 4.5.1).

Incubation of cells with DNA-L2000 complexes	Colony number/6-well (G418/HAT double resistant)
+1ml medium after 4h	28
medium change after 4h	12
o/n	32

Optimized lipofection protocol - 6-well format

- cells plated at $0.5x10^6$/well 1 day before transfection

transfection:

- medium exchange
- DNA and L2000 diluted separately in 0.1ml Opti-MEM (2.5μlL2000/1μg DNA)
- DNA and L2000 solutions combined and the 0.2ml of DNA-L2000 complexes incubated for 20min. at RT
- in meantime cells prepared for transfection - medium replaced by 1ml Opti-MEM/well
- the DNA-L2000 complexes added to cells and incubated o/n (in 1.2ml final volume)
- medium change on next day (day1), start selection on day 2

Tab.5.5 Standarization of the transfection protocol with lipofectamine 2000 for the PacI-linearized p3162 control plasmid. The optimized lipofection protocol (shown on the right side) originated in the initial Invitrogen protocol. One more transfection condition was additionally tested and its variations are given on the left side of the table. 10^6 E14 ES cells in one 6-well were lipofected with Lipofectamine2000 (L2000) and with 1□g of PacI-linearized p3162 plasmid following the optimized protocol (detailed in *Methods*, chapter 4.5.1). Variations in the incubation time of cells with the DNA-L2000 complexes led to different results in terms of colony formation under G418 selection followed by HAT selection (G418 □ HAT). Transfection results are given in colony number per 10^6 cells transfected and as average from 2-5 separate samples. 6-well - one well of 6-well plate, RT – room temperature, Opti-MEM – serum free medium.

Summing up the experiments aiming at optimization of the ES cells transfection procedure the following conclusions were made. Although transfection of HM1 cells was optimized (Tab.5.2), it did not reach the targeting efficiencies achieved with the other Δ*hprt* ES line, E14 (Tab.5.3). Most favourable targeting conditions for the E14 cell line were chosen: transfection with Lipofectamine2000 (L2000) and selection with first G418 for a week followed by HAT containing selective medium (Tab.5.3). An improved protocol for E14 transfection with L2000 was established in a series of optimization experiments (Tab.5.4 and Tab.5.5).

5.5.2. Transfection of the linearized InvTarg construct into murine ES cells

Because the size of the linearized maxi-EBV targeting plasmid InvTarg is 185kb as compared to the much smaller test plasmid p3162, uptake of DNA of that size was investigated in transient transfection experiments with murine ES cells. The InvTarg maxi-EBV plasmid p3299.11 lacks a simple phenotypic marker such as *gfp*, therefore, I used a different maxi-EBV plasmid, p3604, for the initial transient DNA transfections. This plasmid was digested with either SgfI or AscI to cleave it once or twice in the following conditions: 10□g DNA in 200□l with 5u AscI or SgfI for 15h. The

quality of the cleaved DNAs was assessed with PFGE (Fig.5.22a). 3□g of the linear DNA molecules, upon cleavage with SgfI or AscI, were transfected with the developed protocol with Lipofectamine2000 (L2000) into 10^6 E14 ES cells (chapter 5.5.1) with adjustments for the large BAC plasmids ('optimized protocol' for BACs, in *Methods*, chapter 4.5.1). The adjustments were made to adopt transfection conditions for large BAC plasmids established in my laboratory (D.Pich, personal communication). Two different methods, UV-fluorescent microscopy and FACS analysis, were applied to assess the transfection efficiencies in ES cells. The ES cells were successfully transfected with the GFP-encoding maxi-EBV plasmid (Fig.5.22b) and the transfection efficiency was estimated to be about 15% on average (Fig.5.22c). The largest 185kb fragment produced by SgfI cleavage of p3604 entered the cells with 2% reduced efficiency as compared to the 110kb fragment obtained after digestion with AscI (Fig.5.22c). However, the efficiencies were most probably overestimated for the linearized large BAC plasmid, as cellular uptake of smaller DNA fragments containing GFP was also recorded. Therefore, the efficiencies of BAC transfections are further called 'relative', and the aim of these experiments was a preliminary optimization of transfection of large linearized DNA plasmids with a simple visual assessment.

Several transfection experiments with the test plasmid p3162 suggested a correlation between the number of successfully targeted ES cells and the amount of DNA transfected or concentration of L2000 used (data not shown). This observed correlation for large maxi-EBV plasmids was verified separately with the linear p3604 plasmid, as the size of the DNA molecules could have an influence on the outcome. The p3604 maxi-EBV plasmid was cleaved with AscI (10□g DNA in 200□l with 5u AscI for 15h), 'enzyme inactivated' (*Methods*, chapter 4.3.8) and verified with PFGE (Fig.5.22a). Different amounts of DNA were transfected with different amounts of L2000 into E14 ES cells ('optimized protocol' for BACs, *Methods,* chapter 4.5.1). The results are shown in Fig.5.23, indicating that an increase in DNA or L2000 concentration slightly improved the relative DNA uptake but the relative transfection efficiency could not be further improved with up to 9□g DNA (Fig.5.23b). Within single experiment the same DNA preparation was used for different conditions of transfection.

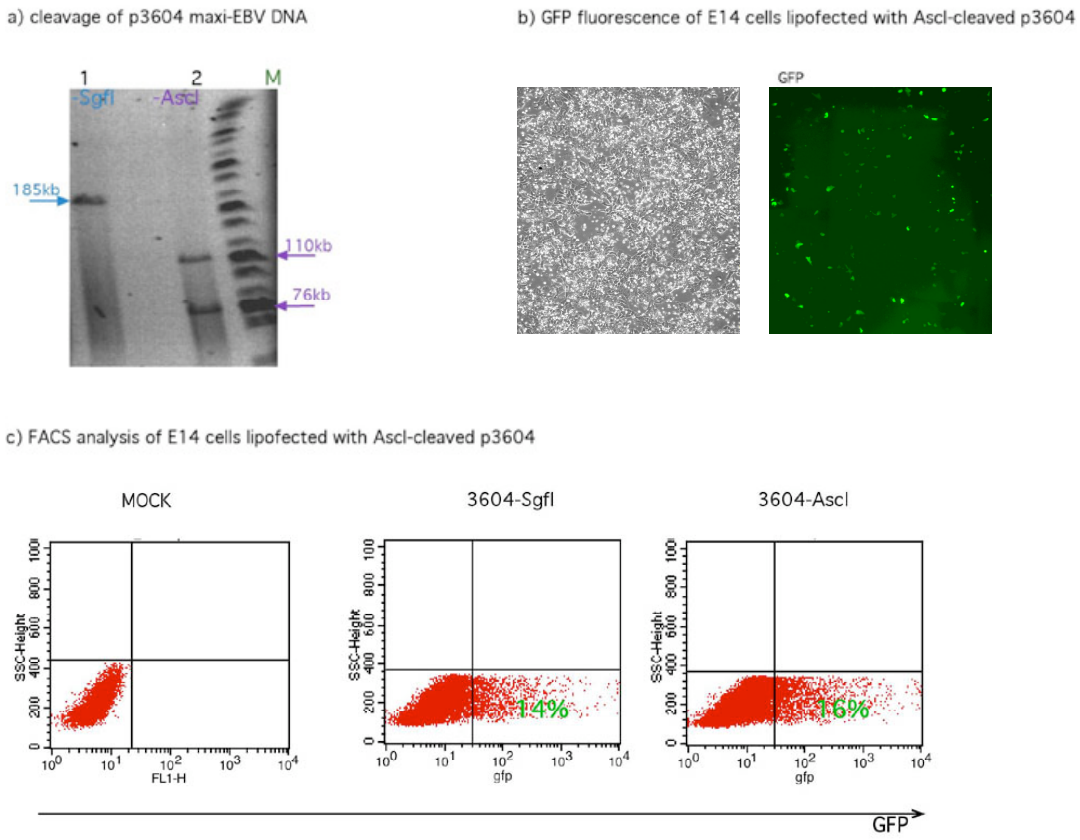


Fig.5.22. ES cells are transduced with large maxi-EBV DNA plasmids. (a) Quality of the digested and 'enzyme inactivated' p3604 DNA was assessed on a PFGE gel; 0.5□g DNA was loaded per lane. Each cleavage produced fragments of discrete lengths: AscI - 110kb and 76kb, SgfI - 185kb. M - MidRange marker. (b) 3□g of p3604-AscI (a, lane2) was lipofected into E14 ES cells with Lipofectamine2000. (c) FACS readout of E14 cells lipofected with 2.5□l/□g DNA of L2000, and with 3□g of p3604 cleaved with SgfI (180kb fragment; a, lane1) or 3□g of AscI-cleaved p3604 (110kb and 76 kb fragments; a, lane2). Untransfected cells served for a mock control. Results are shown in percentage of GFP-expressing cells in gated ES cell population. Detection of GFP with fluorescent microscope and FACS ($2x10^4$cells/sample) were performed about 24h post transfection (b, c). PFGE: Pulse Field Gel Electrophoresis, GFP: green fluorescent protein.

In summary, transient transfection experiments with E14 ES cells and large maxi-EBV plasmids were assessed with two independent methods. Higher concentration of the transfecting agent (L2000) was not toxic for the transfected cells with 15□l L2000 per 10^6 cells and 6□g DNA transfected. The efficiency of transfecting 10^6 cells with 3□g DNA and 7.5□l L2000 differed between 9% and 16% in distinct experiments, which was probably related to different DNA preparations. In the final conclusion, the amount of 180kb long DNA fragment for transfection into 10^6 E14 cells was set 3-6□g with 2.5□l L2000/□g DNA.

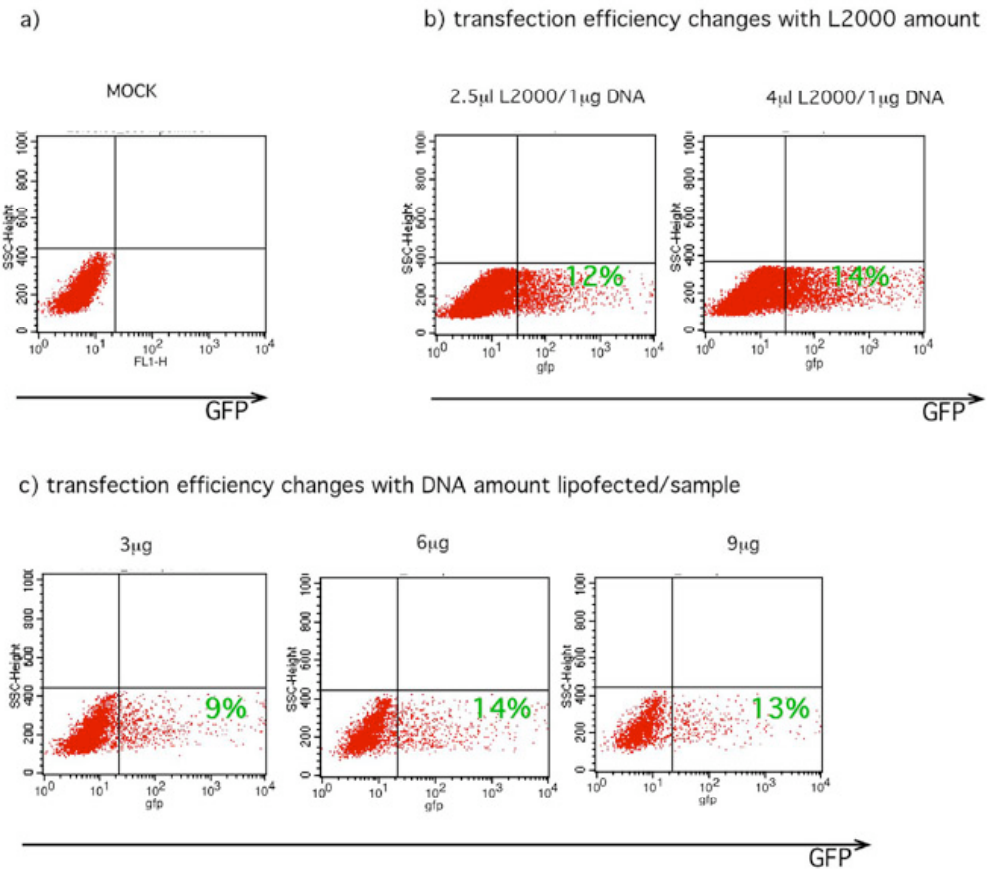


Fig.5.23. Conditions for optimal transfection of ES cells with linearized large maxi-EBV plasmid DNA. AscI-cleaved p3604 DNA (Fig.5.22a, lane2) was transfected into 10^6 E14 ES cells with Lipofectamine2000. (a) Untransfected cells were used as a negative control (MOCK). (b) Different amounts of L2000/µg DNA (3µg DNA transfected/sample) (c) and various DNA amounts/sample (2.5µl L2000/µg DNA) were examined. FACS analysis was conducted about 24h post transfection ($2x10^4$cells/sample). Transfection efficiency is given in percentage of GFP-expressing cells in the gated ES population. L2000: Lipofectamine2000, GFP: green fluorescent protein.

Finally, the maxi-EBV targeting construct p3299.11 was cleaved with PacI, purified and transfected into E14 ES cells under optimal conditions (*Methods*, chapter 4.5.1, 'optimized protocol' for BACs). The cleavage conditions for the InvTarg plasmid were set within the optimized range (Tab.5.1), and additionally, the quality of the linear DNA preparation in each sample was verified on a PFGE gel as exemplified in Fig.5.19 for enzyme inactivation and dialysis, and Fig.5.20 for precipitation of DNA. Transfections were done many times, as summarized in Tab.5.6. The amount of DNA transfected ranged in between 3-6µg/10^6 cells, as optimized in transient experiments with maxi-EBV DNA (Fig.5.22 and 5.23). The transfection protocol differed slightly between the trials in reference to the 'optimized protocol' in order to increase the precentage of surviving cells; either the incubation time of cells with DNA-L2000 complexes was shortened from overnight to 4h or the amount of

transfected cells was doubled. Two different selection modes were applied: HAT and a combination of HAT and G418.

Unfortunately only one round of lipofections yielded a single ES cell colony that survived selection with HAT, only (Tab.5.6). The single ES cell clone, which arose from this experiment, was subjected to further analysis described in the next chapter (5.5.3).

No	Transfection		Selection	No of samples (10^6cells/ sample)
	Protocol	*DNA* (□g/10^6 cells)		
Enzyme inactivation:				
Reference	A	6	HAT	6 (1 colony out of 6x10^6 transfected cells)
1	A	3	HAT	16
2	A	6	HAT	12
3	A	6	HAT+neo	18
Enzyme inactivation+Dialysis:				
4	B	3	HAT	10
5	B	5	HAT	16
Ph FISH:				
6	A	6	HAT	6
7	A	6	HAT+neo	6
8	C	6	HAT	34
9	C	6	HAT+neo	48

Tab.5.6 Summary of ES cell transfections with the PacI-linearized maxi-EBV targeting plasmid, InvTarg. Successful targeting conditions of a single HAT-resistant clone are provided in the first lane (Reference). Overview of 9 different transfections with the variations in different relevant parameters is given below, with the similarities to the reference conditions depicted in green. E14 cells were transfected with Lipofectamine2000 (L2000) with slightly different transfection protocols: A – optimized (*Methods*, chapter 4.5.1, protocol for BACs), B - incubation time of cells with DNA-L2000 complexes was shortened to 4h, and followed by medium change, C – 2x10^6 ES cells transfected/sample. Selection medium with HAT, or with HAT and G418 combined (HAT+G418). Each transfection conditions were tested in 1-6 separate experiments.

5.5.3. PCR analysis of the hprt$^+$ ES cell clone targeted with the InvTarg construct

The analysis was based on PCR verification of the targeting construct InvTarg in the *hprt* locus of the ES cell clone, which survived selection with HAT medium. Different PCR primer pairs were designed to detect relevant parts of the integrated linear maxi-EBV p3299.11 DNA (Fig.5.24, in blue). The region containing the two loxP sites and viral WW repeats was especially important for verification, because it was functionally relevant for the Cre-mediated activation of the EBV latent genes by re-inversion of the loxP-flanked segment. Therefore, three primer pairs were set in this region: lox71, WW and lox66. PCR products from the other two primer pairs (LMP2 and EBNA1) shall indicate whether left and right end of the linear 3299.11 transgene, respectively, were integrated in the ES cell genome.

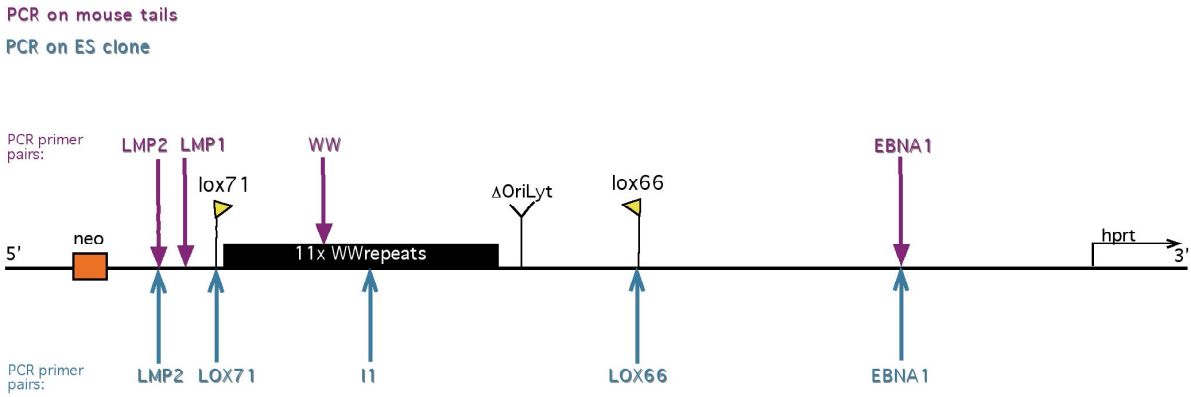


Fig.5.24. Schematic presentation of the InvTarg maxi-EBV targeting construct p3299.11 in the mouse *hprt* gene locus and its detection by PCR. Horizontal line represents the PacI-linearized intact p3299.11 construct integrated into the genome of the Δ*hprt* ES cell line, E14. PCR-mediated amplifications of relevant genetic loci in transgenic mice (upon microinjection, described in chapter 5.6) are depicted in violet, while the ones used in PCR with ES cell template DNA are shown in blue. ΔOriLyt: mutated lytic origin of EBV, neo: neomycin, gene encoding resistance against G418, ES: embryonic stem cells;

The one HAT-resistant ES cell clone targeted with the InvTarg maxi-EBV construct was examined for the presence of the EBV sequences. On the basis of the extensive PCR analysis I found that only the region of WW repeats was integrated into the ES genome (Fig.5.25). The selection conditions revealed the presence of a functional *hprt* gene but no resistance against G418 selection, suggesting that the *neo* resistance cassette was also absent. Apparently, only fragments of the InvTarg maxi-EBV targeting construct were integrated resulting in the functional restoration of the *hprt* gene.

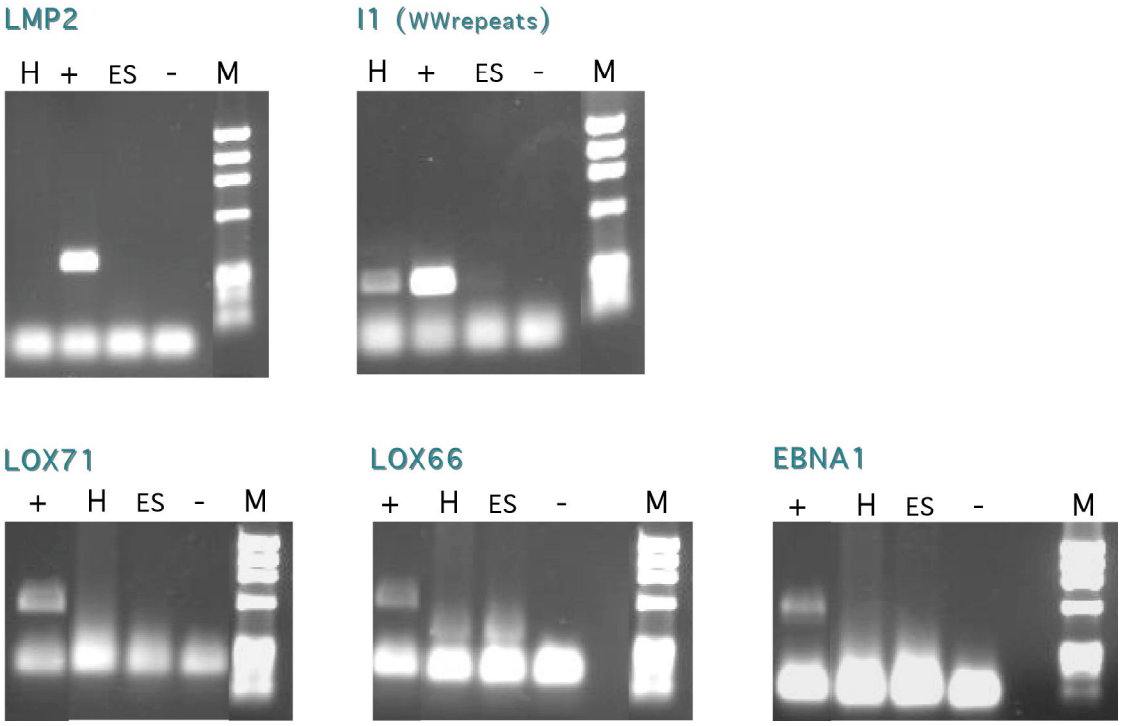


Fig.5.25. PCR analysis of the integrated InvTarg targeting construct p3299.11 in the HAT-resistant ES cell clone. DNA from targeted E14 ES cells was used as template for PCR reaction with different primers (schematically shown in Fig.5.24). M: □ marker, H: HAT resistant ES clone, (-): negative, water control, ES: E14 genomic DNA, negative control, (+): positive control: genomic DNA containing EBV (E-18) - for LMP2 and I1 PCR, and 30pg 3299.11 DNA - for LOX71, 66 and EBNA1 primers. PCR conditions and the positive controls are described in *Methods* (chapter 4.3.12, *Reaction 4*).

5.6. Random integration of linearized InvTarg construct upon microinjection

Microinjection of the large BAC DNA into fertilized mouse eggs is an alternative method to establish transgenic mice. In contrast to the targeted integration into a certain predetermined locus, chromosomal integration of foreign microinjected DNA molecules is a random and uncontrolled process.

5.6.1. Microinjection of the linearized InvTarg construct into fertilized murine oocytes

PacI-linearized InvTarg p3299.11 DNA was prepared with different protocols and its quality was assessed with analytical PFGE gels (Fig.5.26). The digestion conditions were set within the optimized range (Tab.5.1).

The linear 3299.11 DNA was electroeluted, 'enzyme inactivated' or precipitated (*Methods,* chapter 4.3.5, 4.3.8. and 4.3.14, respectively). DNA concentrations of the eluted samples were estimated on a pulse field gel by comparison with a quantitative molecular weight markers. The concentrations of 3299.11 DNA in the 'enzyme inactivated' and precipitated samples were calculated from the input DNA used for the cleavage or by employing spectrophotomeric measurements. The prepared InvTarg targeting construct was microinjected into fertilized oocytes of the FVBxFVB mice strain (*Methods*). A total of eight injection trials led to surviving offsprings in only one round. All results are summarized in Tab.5.7.

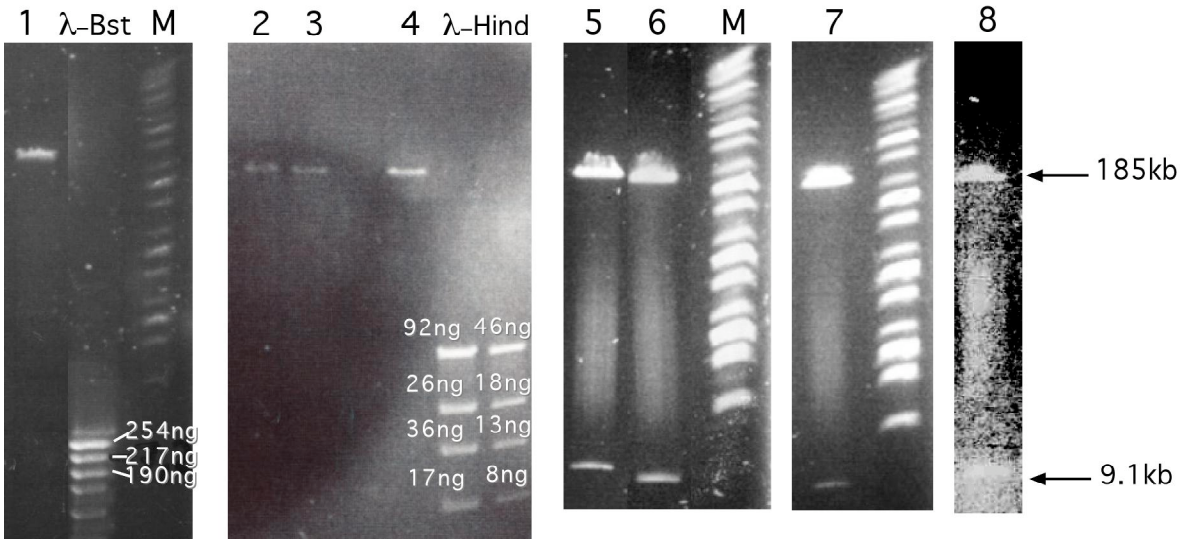


Fig.5.26. Preparation of the PacI-linearized p3299.11 InvTarg DNA for microinjection. Preparations of 3299.11 DNA used in the procedure are numbered according to microinjection dates. The same numbering system was applied in Tab.5.7. DNA was digested with PacI, treated in the indicated way (Tab.5.7) and run on a pulse field gel. Loading: 1/10 to 1/2 of the eluted sample/lane - samples 1-4, 250ng/lane – samples 5 and 6, 1□g/lane – sample 7, 150ng/lane - sample 8. M: MidRange marker, quantitative markers: □-Bst: □-BstEII, □-Hind: □-HindIII.

No	DNA			Foster mothers transferred (with injected embryos)	RESULT		
	Time between cleavage and injection (days)	*Further processing*	*Injected at concentration (ng/□l):*		*Embryos did not develop in uterus*	*Embryos died in uterus*	*Born mice*
1	14	Electroelution	2	2fm	-	-	1fm - 5 alive (partly transgenic), 1fm - 1 dead (transgenic);
2	5	Electroelution	0.25	2fm	2fm	-	-
3	7	Electroelution	0.25	1fm	1fm	-	-
4	12	Electroelution	0.25	1fm	1fm	-	-
5	4	Enzyme inactivation	0.25	1fm	-	-	1 died after birth (from 1fm);
6	5	Enzyme inactivation	0.25	1fm	1fm	-	-
7	5	Enzyme inactivation	0.5	2fm & 1fm	2fm & 1fm	- -	- -
8	2	Ph FISH	0.25	4fm	2fm	2 from 1fm, 1 from 1fm	-

Tab.5.7. Overview of all microinjection trials of the InvTarg targeting construct p3299.11-PacI into fertilized oocytes. Summary of all the injections provides information on how and when p3299.11 DNA was prepared, at which concentration it was injected and what the result was in terms of trangenic mice delivery. DNA preparations used in the procedure are numbered according to microinjection dates and refer to the numbers of DNA samples shown in Fig.5.26. overnight: overnight, fm: foster mother, Ph FISH: DNA precipitation with 'FISHing'.

5.6.2. PCR analysis of mice born from oocytes microinjected with the InvTarg construct

The five mice, which successfully survived oocyte injections and the one still-born mouse obtained in the course of microinjection 1 (Tab.5.7) were analysed by PCR for the presence of the integrated maxi-EBV DNA p3299.11. The localization of the PCR primer pairs is shown in Fig.5.24. Only parts of the viral WW repeats region were detected in the five mice alive (Fig.5.27, lanes 1-5), while unfortunately only the still-born mouse had all the investigated regions inserted (Fig.5.27, lane 6).

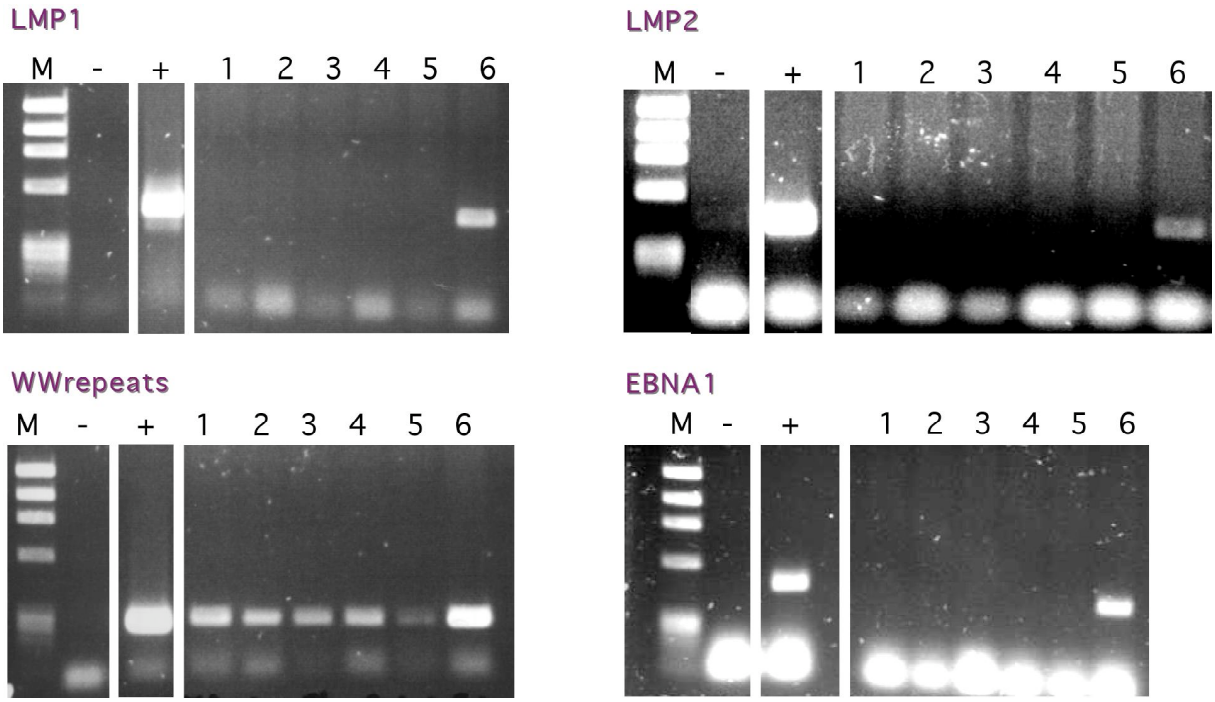


Fig.5.27 Detection of microinjected linear p3299.11 DNA in mouse genomic DNA by PCR. DNA from mouse tails was employed as template for PCR reactions with different primers (schematically shown in Fig.5.24). 1 - 6 - DNA from six mice; M: □ marker, (-): negative, water control, (+): positive control: genomic DNA containing EBV (E-R for PCR on EBNA1, E-18 - for the rest of samples). PCR conditions and the positive controls are described in *Methods* (chapter 4.3.12, *Reaction 3*).

Summing up the *Results* section, the p3299.11 maxi-EBV conditional plasmid, InvTarg, has been constructed, and its Cre-mediated switch regulating EBV's latent gene expression was functionally tested in primary human B cells. The optimal conditions for linearization and further purification of the InvTarg plasmid DNA were chosen, and the experimental settings for the targeting of the *hprt* locus of the murine ES cells with the small control plasmid p3162 were optimized. However, both the transgenic ES cell lines targeted with InvTarg targeting vector, and the transgenic mice obtained upon microinjection with the same construct had only subgenomic pieces of the InvTarg transgene integrated.

6. DISCUSSION

Much information has been already gathered on EBV *in vitro* and different *in vivo* models for EBV exist but a complex animal model for the EBV-related diseases is still lacking. The experimental work of my thesis identified critical aspects of the steps towards a murine model system for EBV. The experience gathered here creates the basis for the next steps to the final goal. The proposed model would provide the complex environment of a living organism for studying the function of EBV. Additionally, the application of a sophisticated molecular switch governing EBV's latent genes can open more possibilities to study the biology of this virus.

6.1. InvTarg maxi-EBV targeting construct

The maxi-EBV InvTarg vector has been constructed on the basis of the established wild-type maxi-EBV plasmid p2089, a large Bacterial Artificial Chromosome (BAC) vector comprising the complete genome of the prototype EBV strain B95.8 (Delecluse et al., 1998). In my project, I first constructed the targeting construct InvTarg (p3299.11) in *E.coli*. The many necessary genetic alterations to construct the final maxi-EBV plasmid, InvTarg, were based on common genetic techniques developed in *E.coli* (reviewed in Wagner et al., 2002). A series of recombinant conventional and maxi-EBV plasmids had to be established and many different steps were needed to precisely introduce the planned genetic modifications. The techniques utilized in the construction of the large BAC plasmids are described in *Introduction* (chapter 1.7.2).

Two variant loxP sites were introduced into the wild-type maxi-EBV vector to control viral latent genes expression indirectly via a Cre recombinase-mediated genetic switch. The Cre/loxP system is widely applied in genetic research as described already in chapter 1.6.3 of *Introduction.* Furthermore, Cre was reported to invert large DNA fragments (Campo et al., 2002). The main feature of the InvTarg targeting vector is an inversion of a segment of EBV's genomic DNA with respect to the parental wild-type plasmid p2089, which disables the expression of almost all the latent EBV genes. The inversion of 51kb was made possible by two loxP sites, which bracket the inverted segment. Upon transient expression of Cre recombinase latent gene expression can be activated in that Cre mediates re-inversion of the loxP-

flanked EBV segment. Because Cre functions in every cell, latent EBV genes can be switched on at will. A stabilization of the ‘re-inverted’, activated conformation of EBV’s latent genes is possible due to two variant and asymmetric loxP sites: lox71 and lox66 (Fig.5.4). Upon Cre-mediated re-inversion, a mutated loxP site is created, lox71/66 ’locked’, which is no longer a substrate for the recombinase. A similar Cre system, which is based on these two variant loxP sites was already applied for one-directional inversions (Oberdoerffer et al., 2003; Zhang and Lutz, 2002).

Two additional genetic features were introduced into the InvTarg targeting construct. In order to prevent accidental production of the infectious virus in murine cells or transgenic mice, EBV’s lytic origin of DNA replication was disabled. Because spontaneous virus production is possible in EBV-infected cells (Bornkamm and Hammerschmidt, 2001). The housekeeping locus of *hprt* murine gene assures ubiquitous and constitutive expression of the integrated transgene (Bronson et al., 1996). Therefore, flanks for a site-specific homologous integration into the murine *hprt* locus were added, to insert the maxi-EBV targeting vector into an active gene locus.

It was very important to assess the function of the Cre-mediated switch and to confirm that the introduction of two loxP sites did not interfere with the expression of crucial latent EBV genes. The latent genes of EBV are engaged in B cell transformation *in vitro* (Murray and Young, 2001). The Cre-mediated switch was functionally assessed in primary human B cells (chapter 5.3). Precursors of the InvTarg targeting plasmid, i.e. maxi-EBV plasmids with a functional viral origin of lytic replication were used. Four maxi-EBV plasmids, which constitute two pairs, were engineered in Step 2 and 3 as described in chapter 5.2 and Fig.5.5B,C. Two plasmids, p3125.3 and p3125.5, carry lox71 and loxPinv sites, and the remaining two constructs, p3221.12 and p3301.5, bear lox71 and lox66 sites. EBV latent gene expression is blocked in p3125.5 and p3221.12 because these maxi-EBVs encompass the ‘inverted’ conformation, whereas latent genes are active and in wild-type, i.e. ‘re-inverted’ conformation in p3125.3 and p3301.5. All maxi-EBV precursor plasmids were separately packaged into EBV viral particles in a helper packaging cell line and the virions were subsequently used to infect B cells. Upon infection with the wild-type EBV B cells become transformed, only when the essential set of viral latent genes are expressed. Thus, the wild-type and ‘re-inverted’ constructs growth-transformed B cells as expected, while maxi-EBVs with the ‘inverted’ configuration

did not lead to an outgrowth of B cells (Fig.5.14, chapter 5.3). Thus, expression of Cre recombinase permits the spatial and temporal control over crucial viral latent genes.

The Cre-mediated switch of the EBV latent gene expression was introduced into the InvTarg targeting vector in order to avoid a potential adverse impact of the viral latent gene products on mouse development. Additionally, this approach enables activating all latent genes in different cell compartments and at distinct time points by cross-breeding with specific Cre-expressing mice, so-called deleter mice. Cre-deleter mice, which express Cre in a certain cellular compartment, only, are available now in many different cell types and even different stages of cellular differentiation (Metzger and Feil, 1999). Crossing these mice with an EBV-transgenic mouse would allow the exclusive expression of EBV's latent genes in T cells, epithelial cells of the oropharyngeal or gastric epithelium, for example.

Because EBV's impact on other cell types but B lymphocytes has not been well defined (Young and Rickinson, 2004), the controlled expression of the EBV latent genes in those other cells could possibly show their role in the cell transformation.

It is uncertain whether EBV provokes tumours in humans directly through the expression of its gene products, its integration into the host genome or indirectly initiating cellular genetic lesions (Kuppers, 2003). This issue arose from the observation that EBV is absent in certain percentage of the tumour cells in many EBV-associated diseases (Tab.1.3). A modified version of the InvTarg vector, in which the viral latent genes become inactivated upon Cre expression, might indicate whether EBV genes have a role in maintaining the state of a transformed cell.

6.2. Introduction of the InvTarg targeting vector into murine cells

Two technical approaches were used to stably introduce the InvTarg targeting construct p3299.11 into murine cells as a prelude to an EBV-transgenic mouse: (1) pronucleus microinjection of the EBV construct into a fertilized oocyte, and (2) ES cell genome targeting. In both cases, the manipulated mouse cells in their undifferentiated state shall give rise to transgenic animals upon their implantation into the uterus of a foster mother. The site-specific approach of the ES cells-based technique has the advantage of circumventing random insertional mutagenesis, which is common in microinjected oocytes (Williams and Wagner, 2000). Problems can arise from neighbouring sequences, which might have an unforeseeable impact

on the accidentally inserted transgene (reviewed in *Introduction*, chapter 1.6.1). The ES cell-based transgenic mouse development is considered to be 'more sophisticated and demanding' (Williams and Wagner, 2000). Both transgenic techniques were applied in the current study in order to complement each other to increase the chance of success in this ambitious project.

Considering the targeting vector, conditions for both locus-specific insertion into the mouse genome and subsequent expression of the vector-encoded genes have to be fulfilled. Hasty et al. demonstrated up to nine-fold better ES targeting efficiency with so-called, 'insertion vectors' in comparison to 'replacement vectors' (Hasty et al., 1991b). The ends of the linearized 'insertion vectors' are homologous to the sequence at the targeted site, while 'replacement vectors' are linearized outside of the region of homology. My InvTarg targeting vector p3299.11 is an 'insertion vector'; thus, the double-strand break within the region of homology should increase the rate of insertion considerably (Orr-Weaver et al., 1981). The length of homologous flanks of the p3299.11 construct is also within the optimal range: 3.4kb and 7kb. Both flanks should assure efficient homologous recombination, as fragments of more than 2kb suffice and longer ones (up to 7kb) increase the efficiency of the insertion (Hasty et al., 1991a). The original EBV promoters included in the integrated maxi-EBV InvTarg construct are expected to control EBV gene expression after its integration into the murine chromosome. There are two indications, which presume the active state of EBV promoters in mouse cells. Two EBV's latent genes, LMP1 and LMP2, were reported to be expressed upon introduction of an episomal maxi-EBV vector comprising all EBV genes into murine ES cells (H.Herrmann, personal communication). However, only three latent genes were analysed in total. Besides, the EBV latency II program was shown to be expressed in EBV-infected murine B cells carrying the human surface receptors for EBV (Haan et al., 2001) but here again expression of the latent EBV genes was restricted.

6.2.1. Bacterial Artificial Chromosome (BAC) as a transgene

My maxi-EBV targeting construct, InvTarg, which is based on a bacterial mini-F backbone and contains the entire EBV genome, belongs to the family of large Bacterial Artificial Chromosomes (BACs). There are several reports, which used BAC vectors as transgenes to establish transgenic mice (summarized in Tab.6.1).

BAC PREPARATION prior to INTRODUCTION into MURINE CELLS		*REFERENCES*
BAC FORM	**DNA PROCESSING AFTER CLEAVAGE**	
Microinjection		
episomal	-	(Abe et al., 2004) (Antoch et al., 1997)
linearized	precipitated	(Jessen et al., 1998)
linear fragment (*)		(Mata et al., 2003) (Yang et al., 1997) (Antoch et al., 1997)
	fragment isolated with gelase	(Nielsen et al., 1997)
	fragment isolated by electroelution	(Chrast et al., 1999) (Jiang et al., 2005) (Al-Hasani et al., 2004)
	fragment isolated with agarase	(Takahashi et al., 2000) (Mullins et al., 1997) (Vadolas et al., 2005) (Chrast et al., 1999)
ES targeting		
ES Electroporation		
linearized	diluted with PBS	(Heaney et al., 2004)
	precipitated	(Testa et al., 2003) (Valenzuela et al., 2003)
	extracted with phenol/chloroform	(Yang and Seed, 2003)
ES Lipofection		
episomal	-	(Ma et al., 2004)

Tab.6.1. Preparation of BAC-based transgenes for establishing transgenic mice. Both episomal and linearized forms of BAC constructs were inserted into mouse genomes to give rise to transgenic mice. BAC vector size ranges from 100 to 250kb. (*): BAC DNA was separated on PFGE and isolated from the gel subsequently.

BAC constructs can encompass large mammalian gene loci or complete viral genomes, such as EBV, but because of their large size they are more prone to physical fragmentation than small plasmids (Giraldo and Montoliu, 2001). As a consequence, they are more difficult to handle. More than fifteen years of extensive research on BAC vectors, which were first introduced in 1989 (O'Connor et al., 1989), have accumulated knowledge how to handle such large DNA molecules (reviewed in

Giraldo and Montoliu, 2001). Publically available protocols describe BAC manipulation in bacteria, DNA purification, linearization and preparation for introduction into eukaryotic cells.

During my experiments two protocols were employed for BAC DNA purification from bacteria: alkaline lysis followed by cesium chloride-ethidium bromide density (CsCl) gradient centrifugation and Qiagen DNA isolation kits. Both techniques provided BAC DNA of good quality, what was assessed by PFGE (Fig.5.15). Similar DNA purification methods were employed in successful trials leading to BAC-transgenic mice (Heaney et al., 2004; Jiang et al., 2005; Takahashi et al., 2000).

The maxi-EBV InvTarg targeting plasmid p3299.11 was introduced as a transgene into murine cells in its linearized form upon digestion with PacI. Both unseparated digested DNA fragments or individually prepared targeting fragments were transduced into fertilized oocytes or ES cells. BAC DNA can be delivered into mammalian cells in three different forms: episomal, linearized or as an isolated DNA subfragment. All versions have been applied successfully while generating transgenic mice (summarized in Tab.6.1). The episomal form is the easiest to handle especially in its supercoiled form but linearization can assure transgene interruption at the preferred site preventing accidental genetic instability of the recipient chromosome (Giraldo and Montoliu, 2001). Moreover, linear fragments have been observed to integrate more easily into mouse chromosomal DNA than intact plasmids (Abe et al., 2004; Brinster et al., 1985). Separation of the targeting fragment from the prokaryotic plasmid backbone is the most accurate and clean way of preparing the transgene but also the most technically demanding approach (Giraldo and Montoliu, 2001).

Pulse Field Gel Electrophoresis (PFGE) was employed for the separation of the large BAC DNA molecules. This method allowed estimation of the quality of the DNA preparation after digestion and separation of the large targeting fragment from the much smaller BAC plasmid backbone. In my work PFGE had an apparent advantage as compared to standard horizontal electrophoresis (Fig.5.16a). The PFGE technique was the basis for optimizing the cleavage conditions of BAC plasmid DNA. Although PFGE cannot distinguish between intact DNA molecules and those with single strand breaks, it can monitor overall DNA quality. On this basis, I could demonstrate the range of cleavage conditions, which assured complete BAC DNA cleavage without overdigestion (Tab.5.1).

After the 'simple cleavage' with the restriction enzyme PacI, the InvTarg DNA was purified prior to introduction into murine cells in order to minimize a potential harmful influence on cell survival. The purification of the linear InvTarg vector was done with a great care in order to avoid mechanical shearing of the DNA molecules. Four different protocols were developed, which are described in chapter 5.4.4 of the *Results* section: (i) enzyme inactivation (ii) followed by dialysis, (iii) DNA precipitation and (iv) DNA fragment electroelution after PFGE.

Large BAC plasmids easily shear upon each physical manipulation, in particular as linear DNA molecules. Therefore, treatment of the cleaved InvTarg construct was limited to (i) heat-inactivation of the enzyme in 'simply cleaved' DNA preparation. The subsequent dialysis (ii) was done to adjust the salt concentration of the DNA samples prior to DNA injection or transfection. (iii) Precipitation of the transgenic DNA is a standard method for a transgene preparation, and (iv) electroelution allows the preparation of the targeting fragment and removal of other components of the digestion mixture. Different sets of these purification protocols were chosen for each procedure of DNA delivery into murine cells as described in detail in the *Results* section (Tab.5.6 and 5.7).

The protocols chosen for the purification of cleaved BAC DNA reflect the procedures adopted from successful projects leading to BAC-transgenic mice (summarized in Tab.6.1). 'Simple' cleavage without subsequent enzyme inactivation, where the digested preparation was either diluted with PBS (Heaney et al., 2004), or extracted with phenol/chloroform (Yang and Seed, 2003), was successfully employed for BAC transgenic mice derived from targeted ES cells. Exactly the same conditions for enzyme inactivation are proposed by the Stanford School of Medicine (http://med.stanford.edu/transgenic/dnamicro.html) for BAC DNA microinjection. DNA dialysis was combined with electroelution (Chrast et al., 1999; Jiang et al., 2005) or agarase treatment (Chrast et al., 1999; Mullins et al., 1997) of the PFGE separated targeting fragment prior to microinjection. DNA precipitation (Jessen et al., 1998) and electroelution (Al-Hasani et al., 2004; Chrast et al., 1999; Jiang et al., 2005) have been already described as methods for BAC transgenic mice by microinjection. Transgenic and Chimeric Facility from the University of Pennsylvania School of Medicine also recommends BAC ethanol precipitation prior to microinjection (www.med.upenn.edu/tcmf). The precipitation of large transgenes before their transfection into ES cells is one of the preferred BAC processing methods employed

(Testa et al., 2003; Valenzuela et al., 2003). In this study, the protocol for BAC DNA precipitation ('with FISHing', chapter 4.3.14) was adopted from the collaborating laboratory (THP, Bernried, Germany), which uses this method as a routine for establishing BAC-transgenic animals.

6.2.2. Microinjection of InvTarg maxi-EBV targeting construct

The prepared linearized InvTarg targeting construct was microinjected into fertilized murine oocytes in several attempts. The injected early embryos were transferred into the foster mothers and gave rise to transgenic mice, which were analysed with PCR for the presence of the randomly integrated InvTarg transgene.

Although the microinjection procedure was successful for all oocyte injections, it led to transgenic litter in one case only. The other seven attempts led to early embryo absorption in uteri and no offspring (Tab.5.7).

Parameters, which might have lowered the efficiency of establishing transgenic mice by microinjection might include the quality of BAC targeting construct, its concentration and the conditions of the injection.
The quality of each InvTarg DNA preparation was first approved with PFGE prior to its injection. All the microinjected DNA samples are shown in Fig.5.26.
Any substance other than DNA or microinjection buffer could be lethal for the early embryos (Vintersten et al., 2004). In my attempts with 'simply' PacI-cleaved vector DNA without any further purification the inactivated enzyme could potentially be toxic to the early embryos but one mouse was born alive after injection of the transgene, which had been prepared in this way (Tab.5.7, No 5). In precipitated or eluted DNA preparations the components of the restriction reaction had been removed. Polyamines, such as spermidine and spermine, are components of the microinjection buffer to stabilize the integrity of the transgene (Montoliu et al., 1995). Spermidine and spermine are unstable during prolonged storage at non-optimal temperature (Vintersten et al., 2004), but even if they had been degraded, there is no scientific data demonstrating their toxicity to fertilized murine oocytes or mouse embryos. Moreover, polyamines were found to be dispensable for the stability of BAC vector DNA during oocyte microinjection (Giraldo and Montoliu, 2001). Considering the careful and considerate DNA preparation in this work, there should be no toxic ingredients contaminating the injected DNA solutions, but such a risk cannot be completely excluded.

The concentration of the microinjected InvTarg targeting construct did not exceed 2ng/μl, what is in line with publications dealing with establishing BAC transgenic mice (Chrast et al., 1999; Nielsen et al., 1997; Takahashi et al., 2000). Higher concentration increases the efficiency of transgene insertion but decreases the viability of the embryos (Vintersten et al., 2004) and can result in multiple transgene integrations. Based on the experience of our collaborators the DNA concentration was even decreased to 0.25ng/μl. Thus, the concentration of the injected InvTarg preparations was not too high in comparison to the references. In fact, the first successful series used the InvTarg transgene concentration of 2ng/μl injection and led to living offspring (Tab.5.7, No1). On the other hand, it is very unlikely that the reduced BAC DNA concentration used in further attempts was responsible for no offspring. If the transgene concentration was too low, non-transgenic wild-type mice would have been born instead. Other groups, which had their BAC constructs injected at the same facility, were successful with this low DNA concentration.

The microinjection procedure was conducted in the collaborating laboratory of E.Wolf (Gene Centre, LMU, Munich) according to their standard protocol.

The analysis of born transgenic mice was based on PCR detection of selected loci within the InvTarg maxi-EBV vector with four primer pairs as shown schematically in Fig.5.24. Among six transgenic mice only one possibly had the entire InvTarg construct integrated (Fig.5.27), but it was still-born. It is unclear whether its decreased viability was due to insertional mutagenesis, which is a common event upon random insertion (Williams and Wagner, 2000). This single event does not support the general conclusion that integration of EBV genes could interfere with mouse embryonic development. The other alive transgenic mice had only pieces of the InvTarg targeting construct integrated. Namely, a fragment of viral ‘WW repeats’ was detected in all five mice (Fig.5.27). The ‘WW repeats’ region of the EBV genome consists of up to 11 direct repeats (Bornkamm and Hammerschmidt, 2001). As a consequence, it was impossible to determine the exact size of the integrated fragment by PCR. The failure to detect the remaining parts of the maxi-EBV vector in the chromosomal DNAs of the surviving mice was not due to insufficient sensitivity of the PCR analysis. In reconstruction experiments I could unequivocally detect a single copy of EBV DNA spiked into mouse cellular DNA samples (Fig.5.27, positive control). Because the aim of my project was the chromosomal integration of the

entire recombinant maxi-EBV targeting construct, the negative result with three out of four primer pairs precluded further investigation of these transgenic animals.

In summary, several unidentified factors could have led to the failure to obtain a transgenic EBV mouse upon microinjection. Many different preparations of the linear InvTarg DNA were tested and the integrity of the large linear DNA molecules was assessed with PFGE analysis, but single strand DNA breaks and DNA shearing during transfer to the oocytes cannot be absolutely excluded. There was no hint to a toxic compound contaminating the InvTarg DNA preparations and the concentration of the injected samples rather did not constitute a problem either. Because one EBV-transgenic mouse was still-born, EBV genes might have a detrimental effect on mouse development.

6.2.3. Targeting of the linearized InvTarg maxi-EBV construct into the *hprt* locus of Embryonic Stem (ES) cells

Murine Embryonic Stem (ES) cells were transfected with the linearized targeting construct, InvTarg, and selected for its chromosomal integration. Targeted ES cells were tested for the presence of the integrated transgene by PCR analysis.

The conditions for relative transduction of the InvTarg targeting construct into murine ES cells were optimized in transient transfection experiments (Fig.5.22) but the integration of the InvTarg vector into the Δ*hprt* locus of ES cells was observed only once (Tab.5.6, 'Reference'). The rest of the transfection trials are summarized in the same table in relation to the single successful experiment.

Different parameters, which might have influenced the outcome, are discussed below.

It has been shown by different groups that Δ*hprt* ES cell lines similar to the ones used in my approach (HM1, E14) can give rise to transgenic mice with BAC DNA as a transgene (Heaney et al., 2004; Testa et al., 2003). Moreover, the E14 cells were successfully targeted with the linearized small control plasmid (Tab.5.3).

Among different ES cell targeting methods electroporation is the preferred one for transfection of ES cells (projects summarized in Tab.6.1). In the initial transfection attempts of my PhD work electroporation of the targeting construct was applied but in the end it proved to be less efficient than lipofection (Tab.5.3). Murine ES cells were shown to be effectively transfected by lipofection (Kobayashi et al., 2005; Wobus and Boheler, 2005), also with lipofectamine2000 (L2000) (B.P. Murphy, personal

communication). Unfortunately, no report exists on L2000-mediated transfection of murine ES cells, particularly with BAC DNA. However, L2000 was used for efficient transfections of different mammalian cell types (Ali et al., 2004; Dalby et al., 2004), including delivery of BAC DNA (Kotzamanis et al., 2005; Magin-Lachmann et al., 2004). Starting from the original Invitrogen protocol, lipofection with L2000 was optimized with a small (20kb) plasmid (p3162) equipped with neomycin resistance and *hprt* flanks (Tab.5.5). The main modifications addressed DNA concentration, volume and cell culture medium used for incubation of the transfected cells with the L2000-DNA complexes. The volume was reduced and a serum-free cell culture medium was found superior. Further alterations of the protocol were necessary for lipofections of the large maxi-EBV vector InvTarg DNA. The experiments were transient transfections with maxi-EBV DNA carrying Green Fluorescent Protein (GFP) and subsequent analysis of the number of GFP-expressing cells. The optimal conditions found in these experiments were: 10^6 ES cells per well, 6□g BAC DNA and 2.5□l L2000 per 1□g BAC DNA (Fig.5.23). These experimental settings are close to the parameters used in similar approaches (Kotzamanis et al., 2005; Magin-Lachmann et al., 2004). In these reports also serum-free media and reduced volume were applied for incubation of the cells with transfecting agents.

A single approach was published, where BAC DNA was successfully integrated into the *hprt* locus of ∆*hprt* ES cells in a site-specific manner, complementing a mutation in murine *hprt* gene upon HAT selection (Heaney et al., 2004). A combination of both G418 and HAT selection should allow identification of those ES cells that had the entire InvTarg targeting construct integrated into the murine *hprt* locus, as the *hprt* segment restoring the non-functional *hprt* gene and the *neomycin* resistance gene *neo* were placed on both distal flanks of the targeting vector DNA molecule. The selection mode optimized for the small control plasmid (p3162) comprised selection with G418 for approximately one week followed by selection with a cell culture medium with Hypoxanthine, Aminopterin and Thymidine (HAT) for another week (‘neo□ HAT’; Tab.5.3). The average efficiency of the procedure reached 18 ES colonies/10^6 cells. Under ‘HAT only’ selection the efficiency dropped to 2 colonies/10^6 cells, what was still comparable with related protocols [Heaney, 2004 #296; U.Strobl, personal communication]. In addition, emergence of double (HAT and G418) resistant cells proved that none of the compounds of selection medium was toxic to the transfected cells. The most probable reason for the advantage of the ‘neo□ HAT’

selection over the HAT medium alone was an improved initial survival of the transfected cells under G418 selection in comparison to HAT. Successful chromosomal integration of an intact BAC plasmid is a rare event compared with the much smaller control plasmid p3162. The ES cells transfected with the InvTarg construct were mainly selected with HAT selective medium alone, similar to the published protocol (Heaney et al., 2004), because it selected for the specific restoration of the murine *hprt* gene.

Targeting vector preparation, transfection and selection conditions varied slightly between the many transfections into ES cells (Tab.5.6). The effects of these alterations were examined in parallel to encounter hints for targeting ES cells more efficiently. According to the one publication, the efficiency of BAC targeting into the *hprt* locus in ES cells is one in 2-6x10^6 transfected ES cells (Heaney et al., 2004). Because of the similarities between this published protocol and my attempts with InvTarg targeting, the efficiency of the InvTarg plasmid integration was estimated to be comparable. The different conditions were tested between 6 and 48 times, which should result in the theoretical probability of at least two to around hundred successful targeting events.

The single HAT-resistant ES cell clone was investigated for the presence of the integrated InvTarg construct with PCR primers that amplify different parts of the targeting (Fig.5.24). Three primer pairs (LOX71, I1 and LOX66) were designed to detect a fragment placed in between and covering two loxP sites. The initial intention was to verify the Cre-mediated re-inversion in transgenic ES cells. Unfortunately, the single HAT-resistant ES clone had only fragments of EBV's WW repeats integrated (Fig.5.25). Again, as in the case of the microinjected InvTarg targeting vector (chapter 6.2.2), it was impossible to determine the size of the integrated fragment because the I1 primer pair did not discriminate between the eleven WW repeats present in the InvTarg vector. The positive controls in the PCR reactions (Fig.5.25) were also designed to simulate integration of a single copy EBV genome per cell. No further attempt was made since this ES cell clone did not fulfil the expectations. Because this ES cell clone survived in HAT medium, the partial insertion of the InvTarg vector had restored the *hprt* locus but the *neomycin* resistance cassette was non-functional or absent in this clone as the cells did not survive selection with G418.

In the conclusion, the developed targeting protocol of the small control plasmid resulted in double resistant (against G418 and HAT) murine ES cells with a

reasonable efficiency. The result of one positively targeted ES cell clone with subgenomic fragments of the recombinant EBV vector integrated and a restored *hprt* locus could be due to inadequate intactness of the transfected DNA molecules and/or still insufficient transfection efficiency with the large InvTarg plasmid.

6.3. Outlook

DNA fragmentation might constitute a common problem for both approaches of introducing BAC DNA into murine cells. Therefore, alternative approaches need to be considered to overcome this problem.

6.3.1. Alternatives to the applied methods.

Optional transgene delivery and site-specific insertion

Covalently closed, circular forms of BAC vector DNAs are more stable than the linear molecules of similar size (Vintersten et al., 2004). Thus, it would be technically much less demanding if BAC episoms can be introduced into recipient cells and then become integrated into the cellular genome. Site-specific recombinases (SSRs) are able to stably insert circular vectors into a linear genome (reviewed in chapter 1.6.3, and Fig.1.7). SSRs could be also used in this project. Because the Cre/loxP system has been already employed as a switch in the InvTarg targeting vector, it cannot be used here, but the Flp/FRT system is a realistic alternative as demonstrated already (Dymecki, 1996). To improve the efficiency of the insertion, one could use mutated FRT sites, which favour the unidirectional Flp-mediated integration (Branda and Dymecki, 2004), similar to the lox71/lox66 sites used in my work. Optionally, □C31 integrase could be used for the site-specific vector insertion (Hollis et al., 2003). Integrase-mediated recombination through the att sites also assures one-directional reaction. Both Cre recombinase (Araki et al., 1995) and □C31 integrase (Hollis et al., 2003) were reported to mediate recombination in the fertilized oocytes upon microinjection of Cre-expressing plasmid or mRNA encoding integrase. Flp recombinase was also employed for transgene recombination in fertilized oocytes upon microinjection (Schaft et al., 2001).

Alternative ways of DNA delivery to ES

Nucleofection by Amaxa combines chemical transducers with a milder version of electroporation and targets DNA molecules directly to the nucleus of transfected cell.

This technique was used in delivering DNA to murine ES cells in both transient and stable transfections (Kobayashi et al., 2005; Lakshmipathy et al., 2004). Besides, Amaxa provides a kit with conditions adjusted to murine ES cells. Large BAC DNA has been transduced with this technique to different cells, including human primary B cells (C.Mancao, personal communication). This result indicates that the Amaxa technology might be superior compared to chemical transfection procedure.

6.3.2. Inherent opportunities of the project

The construction of a Cre-regulated maxi-EBV vector is a novelty in the EBV field and opens new opportunities for studying latent EBV genes in other cells but B lymphocytes or during *in vitro* cell differentiation.

Transgenic mice harbouring the stably integrated EBV genome in all cells could be manipulated by cross-breeding with different Cre-mice, to activate latent EBV gene expression in different tissues or at distinct stages of organ development.

To my knowledge there is only one report in the literature, which succeeded in introducing the entire viral genome - Human Immunodeficiency Virus (HIV) - into a mouse gene locus (Hanna et al., 1998). Because this retroviral genome does not exceed 10kb, the realisation of a transgenic EBV-mouse project would be a huge step forward. Furthermore, it could become an example for the generation of animal models with DNA viruses of similar sizes (100-200kb), like KSHV, which also lacks many research options to study this virus thoroughly.

7. SUMMARY

Epstein-Barr Virus (EBV) is involved in several human malignancies via its latent gene products, which interact with cellular proteins and mimic discrete functions of cellular signalling pathways. Enigmatically, more than 90% of the human population carries this human tumour virus but virus-associated tumours are relatively rare.

Most studies on EBV have been carried out *in vitro* and *ex vivo* on EBV-transformed human B cells or on human biopsies. Established *in vivo* model systems do not reflect the main aspects of EBV-associated diseases in humans. This limited tool set is the result of EBV's inability to infect cells of non-human origin, which lack the surface receptor for EBV.

My PhD work aimed at engineering a transgenic mouse, which carries a conditionally inactivated EBV genome.

This study took advantage of the well-established techniques of mouse genetics in order to stably integrate the entire EBV genome into the murine genome. This approach would not only overcome the inability of EBV to infect animal cells but it would also permit to study the complete virus in an immunocompetent and easy-to-handle living organism.

I undertook two routes to establish a transgenic mouse with the complete EBV genome inserted. One route was based on the site-specific integration into the *hprt* locus of murine embryonic stem cells. The other route engaged pronucleus microinjection of the EBV DNA into fertilized murine oocytes. In addition, the EBV genome was genetically manipulated prior to its introduction into murine cells. On the basis of the *E.coli* cloned EBV strain B95.8, I constructed a novel EBV mutant with unique features. This EBV targeting construct (InvTarg) allows conditional expression of EBV's latent genes via a Cre/loxP system. Such approach prevents potentially adverse effects of EBV's latent genes on embryonic development but allows their expression in almost any chosen cellular compartment for which specific Cre-expressing mice are available.

The InvTarg recombinant EBV genome is 185 kb in size, based on a bacterial replicon, and therefore belonging to Bacterial Artificial Chromosomes (BACs). Two genetically modified and inversely oriented loxP sites were introduced in *E.coli* cells

at the predetermined sites of the InvTarg, and the bracketed segment was inverted by Cre recombinase, disrupting transcription of almost all viral latent genes. In transgenic animals this inversion can be reverted and the latent genes can be re-activated at will by cross-breeding with Cre-expressing mouse (re-inversion). The ability of Cre to invert the big fragment was verified in infection experiments with human primary B cells. As expected, the 'inverted' EBV construct, such as InvTarg, failed to transform primary B cells, when the viral latent genes were not expressed. Despite sustained efforts, both gene delivery techniques did not lead to a transgenic mouse with the entire EBV genome inserted, but resulted in the integration of only subgenomic segments of the InvTarg recombinant EBV DNA.

A number of technical problems were identified during this work, indicating more specific direction for further research. On the basis of the experience gained here, the project of an EBV transgenic mouse can be carried on. In addition, the InvTarg maxi-EBV conditional vector might be employed in other experimental conditions, like different cell types or distinct stages of cell differentiation, for studies on latent EBV genes.

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Education

Since 03/2003	**Ludwig Maximilian University, Faculty of Biology**, Munich, Germany PhD Thesis Expected termination: December 2006 (submission to university) Subject of the thesis: *A Mouse model for EBV-related diseases.*
10/1997-06/2002	**Intercollegiate Faculty of Biotechnology at University and Medical University,** Gdansk, Poland Graduated with M.Sc.; Subject of Master's thesis: *Characterisation of a reverse signalling pathway through mTNF (membrane Tumour Necrosis Factor) as a receptor.*
09/1993-05/1997	**Bohaterow Westerplatte Secondary School no.3**, Gdansk, Poland Graduated with Maturity Exam, which entitles to seek admission to higher education.

APPENDIX

Tab.1. Complete list of DNA constructs generated in order to generate the final maxi-EBV targeting vector (InvTarg) with the necessary features. Part of these plasmids were described in the thesis, only.

Bacterial Artificial Chromosome (BAC) constructs, originated in wild type maxi-EBV (p2089) are marked with grey. Maxi-EBV plasmids contain entire genome of EBV and GFP. H.H.-Heidrun Herrmann, W.H.-Wolfgang Hammerschmidt, D.P.-Dagmar Pich, M.Z.-Magdalena Zychlinska. Zeo-zeocin, loxPinv-inverted loxP, tet–tetracycline, neo-neomycin. (*): 'locked' conformation, when loxP sites are no longer substrates for Cre-recombinase, what stabilizes actual conformation created by Cre.

Cloning Step		Construct Name	Features	Made by
1	1.1	3060	pCR2.1 based; lox71site	H.H.
	1.2	3064	pSV40/Zeo2 based; loxP site	H.H./M.Z.
	1.3	3071	p288.1 based; floxed (with unidirectional lox71 and loxP sites) *zeo* cassette set in context of LMP1	M.Z.
	1.4	3080	p2089 based; floxed (with unidirectional lox71 and loxP sites) *zeo* cassette in entire EBV genome context	M.Z.
	1.5	3101	p3080 based; lox71 site	M.Z.
2	2.1	3061	pCR2.1 based; loxPinv site	H.H.
	2.2	3068	pSV40/Zeo2 based; loxPinv site	H.H./M.Z.
	2.3	3072	p3068 based; floxed (with two loxPinv) *zeo* cassette	M.Z.
	2.4	3078	p3072 based; floxed (with two loxPinv) *zeo* cassette set in context of Y 1, Y2 and EBNA2	M.Z.
	2.5	3124	p3101 based; floxed (with two loxPinv) *zeo* cassette in a ca.50kb distance to lox71	M.Z.
	2.6	3125.3	p3124 based; region in between lox71 and loxPinv is NOTinverted (EBV latent genes active)	M.Z.
		3125.5	p3124 based; region in between lox71 and loxPinv is inverted (EBV latent genes inactivated by inversion)	M.Z.
3	3.1	3108	p3078 based; inverted lox66 set in context of Y 1, Y2 and EBNA2	H.H.
	3.2	3165	p3108 based; inverted lox66 set in context of Y 1, Y2 and EBNA2, and LMP1	H.H.
	3.3	3213	pST76-amp based; shuttle plasmid (for chromosomal buiding) with inverted lox66 set in context of Y 1, Y2 and EBNA2, and LMP1	H.H.
	3.4	3221	p3125.5 based; region in between lox71 and inverted lox66 is inverted (EBV latent genes inactivated by inversion)	M.Z.

	3.5	3301	p3221 based; region in between lox71 and inverted lox66 is re-inverted (EBV latent genes active; 'locked'* conformation)	M.Z.
4	4.1	3126	p995 based; disabled lytic origin of EBV, *tet* resistance cassette (between two unidirectional FRT sites)	M.Z.
	4.2	3274	p3221 based; region between lox71 and lox66 inverted, disabled EBV lytic origin, *tet* cassette (between two unidirectional FRT sites)	M.Z.
	4.3	3283	p3274 based; region between lox71 and lox66 inverted, disabled EBV lytic origin, single FRT site	M.Z.
5	5.1	2960	p2949 based; murine *hprt* flanks and human *hprt* promoter, PacI site next to one *hprt* flank	D.P./W.H.
	5.2	2996	p2960 based; murine *hprt* flanks and human *hprt* promoter, PacI site next to one *hprt* flank, restriction sites between both *hprt* flanks	W.H.
	5.3	2997	p2996 based; murine *hprt* flanks and human *hprt* promoter, restriction sites between both *hprt* flanks, two PacI sites next to each of *hprt* flanks	W.H.
	5.4	2998	p2997 based; murine *hprt* flanks and human *hprt* promoter, restriction sites between both *hprt* flanks, two PacI sites next to each of *hprt* flanks, a long flank for recombination with maxi-EBV plasmid	W.H.
	5.5	3033	p2998 based; murine *hprt* flanks and human *hprt* promoter, restriction sites between both *hprt* flanks, two PacI sites next to each of *hprt* flanks, both a long and a short flank for recombination with maxi-EBV plasmid	H.H./W.H..
	5.6	3162	p3033 based; murine *hprt* flanks and human *hprt* promoter, restriction sites between both *hprt* flanks, two PacI sites next to each of *hprt* flanks, both a long and a short flank for recombination with maxi-EBV plasmid, PGK-*neo* cassette	H.H.
	5.7	3260	p3162 based; murine *hprt* flanks and human *hprt* promoter, restriction sites between both *hprt* flanks, two PacI sites next to each of *hprt* flanks, both a long and a short flank for recombination with maxi-EBV plasmid, PGK-*neo* cassette, *zeo* resistance cassette	M.Z.
	5.8	3164	pMBO131(MCS) based; combination of *tet* cassette (between two unidirectional FRT sites) with F-factor backbone (bacterial replicon for BACs)	M.Z.
	5.9	3273	p3164 based; combination of *tet* and F-factor of p3164 with *hprt* and EBV flanks, *neo* and *zeo* of p3260	M.Z.
	5.10	3299 (InvTarg)	p3283 based; final targeting construct with inverted region between lox71 and lox66 (inactive EBV latent genes), disabled EBV lytic origin, *neo* resistance and *hprt* flanks, *tet* between two unidirectional FRT sites in plasmid backbone and an additional single FRT site, (no GPF)	M.Z.

Tab.2. List of the ready plasmids used in the complete project of InvTarg vector cloning procedure, and not mentioned in this thesis.

Plasmid name	Features	Source
pST76-amp	Shuttle plasmid for chromosomal building	[Posfai, 1997 #402]
p2949	promoter and exon 1 of *hprt* are of human origin, next two *hprt* exons – of murine origin	[Bronson, 1996 #5c] received from Ursula Strobl (pMP9)
pMBO131(MCS)	F-factor plasmid	[O'Connor, 1989 #403]