Gene Therapy (2006) 13, 844–856

www.nature.com/gt

ORIGINAL ARTICLE

Genetic design of an optimized packaging cell line for gene vectors transducing human B cells

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Viral gene vectors often rely on packaging cell lines, which provide the necessary factors in trans *for the formation of virus-like particles. Previously, we reported on a first-generation packaging cell line for gene vectors, which are based on the B-lymphotropic Epstein–Barr virus (EBV), a human γ-herpesvirus. This 293HEK-derived packaging cell line harbors a helper virus genome with a genetic modification that prevents the release of helper virions, but efficiently packages vector plasmids into virus-like particles with transducing capacity for human B cells. Here, we extended this basic approach towards a non-transforming, virus-free packaging cell line, which harbors an EBV helper virus genome with seven genetic alterations. In addition, we constructed a novel gene vector plasmid, which is devoid of a prokaryotic antibiotic resistance gene, and thus more suitable for* in vivo *applications in human gene therapy. We demonstrate in this paper that EBV-based gene vectors can be efficiently generated with this much-improved packaging cell line to provide helper virus-free gene vector stocks with transducing capacity for established human B-cell lines and primary B cells.*
Gene Therapy (2006) **13,** 844–856. doi:10.1038/sj.gt.3302714; published online 19 January 2006

Keywords: *herpesvirus; Epstein–Barr virus; gene vector; virus-free packaging cell line*

Introduction

Vectors of viral origin are often used in gene therapy in order to introduce foreign genetic information into eukaryotic recipient cells. At present, no vector system possesses all characteristics necessary or desirable in an ideal gene delivery system, but Epstein–Barr virus (EBV)-derived vectors overcome several of the problems associated with other viral vector systems.[1,2] EBV is a human γ-herpesvirus with a DNA genome of about 170 kb pairs in size, which preferentially infects human resting or proliferating B cells. Infection is mediated via the cellular CD21 surface molecule, the major receptor for EBV,[3,4] resulting in EBV's characteristic B-cell tropism. Another hallmark of this virus is the establishment of latent infections in its target cells. In general, no virus is produced in infected cells, but EBV genomes are stably maintained as episomes and replicate as several extrachromosomal copies in synchrony with the cellular DNA.[5] Thus, latent infection sustains long-term persistence of the viral genomes *in vivo* and *in vitro*. Both latent infection and non-integrating, episomal maintenance are advantageous features for gene therapy. The large size of the EBV genome is another interesting aspect, because it offers the chance to construct gene vectors with the capacity to transduce complex, large gene loci[6,7] similar to adenovirus,[8] and herpes simplex virus.[9]

The majority of viral gene vectors are devoid of regulatory factors and structural proteins for autonomous encapsidation of the vector genome into a viral coat. These viral gene vectors rather rely on helper cell lines, which allow the exclusive packaging of viral vector sequences, but avoid the release of infectious helper virus. Such packaging cell lines often contain a helper virus genome that provides all the viral factors *in trans* necessary for the encapsidation of viral vectors, but in turn lacks *cis*-acting elements, mostly packaging signals, to prevent its own packaging and egress. Ideally, the helper virus contains only those subgenomic parts of the virus that are essential to provide the helper functions, embedded into an unrelated DNA context. The helper functions can also be separated and transiently provided as a set of expression plasmids. Alternatively, these expression plasmids can also be stably integrated into the host chromosomal DNA of the packaging cell at separate locations to minimize the risk of re-generating a replication-competent helper virus. Such systems have been successfully developed for viral vectors based on oncoretroviruses, lentiviruses and adeno-associated virus.[2] Little progress has been made towards packaging helper cell lines for herpesviruses, in spite of their promise as gene vectors owing to their persistence *in vivo* and tropism for resting as well as proliferating cells.[1]

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Received 17 May 2005; revised 6 November 2005; accepted 7 November 2005; published online 19 January 2006

The large sizes of herpesviral genomes ranging from about 100 to 250 kb pairs in length hindered their straightforward genetic manipulation to generate suitable helper virus genomes. With the cloning of a number of complete herpesvirus genomes in *Escherichia coli* with the aid of prokaryotic mini-F replicons, this limitation is overcome (see for a recent review Adler *et al*.[10]). In the prokaryotic host, genetic alterations of any type can now be introduced into the herpesvirus genomes by various techniques.[11–13] However, a stable packaging cell line is still difficult to establish with most herpesviruses because they generally evoke a lytic, productive mode of infection in the target cells *in vitro*, which results in the shut-off of cellular genes and the destruction of the virus-producing cells. Although the *trans*-acting factors involved in productive infection are generally identified, to our knowledge no successful attempt has been made to design a helper virus genome and a stable packaging cell line for members of the α- and β-herpesvirus families. Only transient packaging systems have been established as in the case of herpes simplex virus, where suitable helper virus genomes are concomitantly introduced along with the gene vector DNA.[14–17] This situation is very different from human γ-herpesviruses such as EBV and human herpesvirus 8, also termed Kaposi's sarcoma-associated virus. Both viruses readily establish an exclusively latent infection in human target cells *in vitro* and virus production can be subsequently induced by extrinsic signals or ectopic expression of viral key regulators.[3,18] These biological characteristics allowed the generation of a first-generation packaging cell line on the basis of EBV and the delivery of EBV-based gene vectors to transduce established cell lines and human primary B cells.[19,20]

This first-generation EBV packaging cell line relied on a modified viral genome derived from the prototype EBV strain B95-8.[21] The complete B95-8 EBV genome was cloned onto a prokaryotic mini-F replicon that also carries the genes for enhanced green fluorescent protein (GFP) and hygromycin resistance under the control of eukaryotic promoters. The terminal repeats (TR), *cis*-acting elements required for packaging of viral DNA, were then deleted from this construct in *E. coli*. This modified EBV genome was introduced as a helper virus genome into 293HEK cells and stable cell lines were selected.[19] Upon transient introduction of a gene vector DNA concomitantly with expression plasmids encoding the viral key regulator BZFL1 and the glycoprotein BALF4, which contributes to infectivity of virions,[22] gene vector stocks can be generated free of helper virus.[19]

This basic concept needed to be developed further because the EBV helper virus genome still encodes its full transformation potential. DNA recombination is known to take place during herpesvirus DNA replication, and therefore accidental recombinations between gene vector and helper virus DNAs are likely to occasionally reconstitute a replication- and transformation-competent parental virus (Wilkinson and Weller[23] and references therein). To overcome this obvious safety problem, we constructed a novel, non-transforming EBV genome by deleting or inactivating EBV genes involved in cellular transformation (*EBNA2*, *LMP1*, *EBNA3A*, *-B* and *-C*)[3] or transactivation of EBV's lytic phase (*BZLF1*).[24] In addition, the TRs were deleted to yield an EBV helper virus genome that cannot be packaged into herpesviral capsids.[25,26] This helper virus genome was introduced into 293HEK cells to establish a novel packaging cell line with improved efficiency and safety. For use with this cell line, we generated a new type of vector, which is efficiently packaged in this cell line and, as a safety feature for *in vivo* applications, carries the *supF* gene as a selection marker rather than an antibiotic resistance gene.

Results

Construction of a non-transforming maxi-EBV helper virus genome

Previously, the complete EBV genome of the B95-8 strain of EBV had been cloned in *E. coli* with the aid of a prokaryotic mini-F replicon, which confers resistance against chloramphenicol and includes the genes for hygromycin resistance and *GFP*.[21] In *E. coli*, the cloned EBV genome, termed p2089/F factor, is easily amenable to genetic modifications including the generation of mutant EBV genomes, which are crippled for otherwise indispensable viral functions, that is, packaging signal sequences. With the deletion of these *cis*-acting signals from EBV DNA, a first-generation packaging cell line for EBV-derived vectors was established.[19] EBV encodes a number of genes, which are expressed in EBV-transformed and latently infected B cells as well as in EBV-associated malignancies. As these genes are presumably dispensable for virus synthesis, we wanted to construct a novel maxi-EBV helper genome that does not pose the risk of transducing viral oncogenes. With this idea in mind, we set out to remove a number of latent genes such as *EBNA2*, *LMP1*, *EBNA3A*, *-B -C* to avoid the expression of viral genes with transforming potential. The generation and accidental release of replication-competent helper virus during the packaging process of EBV gene vectors is an additional safety concern. In this study, we addressed this problem with the removal of the *BZLF1* gene, which is essential for productive infection and virus synthesis.

The genetic alterations of the p2089/F factor plasmid DNA, which comprises the prototype B95-8 EBV genome,[21] were performed sequentially (Table 1). Four consecutive rounds of homologous recombination were performed. The coding sequence of the latent gene *EBNA2*, which is essential for B-cell growth transformation *in vitro*,[26,27] was entirely deleted. The transmembrane spanning part of *LMP1* was also deleted, which resulted in an artificial and functionally inactivated LMP1 fusion protein with a dominant-negative phenotype for B-cell growth transformation (ΔTM-LMP1 in Dirmeier *et al*.[28]). Both genetic alterations were performed with the chromosomal building technique,[29] which allows the allelic exchange of genetic segments in a two-step procedure independent of a genetic marker or site-specific recombinase.[30,31] For alteration of the *EBNA2* and *LMP1* loci, two targeting plasmids were necessary (p2419 ΔEBNA2 and p2496 ΔLMP1 in Table 1), which carry the deleted alleles. The three alleles of the *EBNA3* family and the *BZLF1* gene are located in close vicinity. Therefore, a single targeting construct was established, in which the coding sequences of *EBNA3A*, *-B* and *-C* were deleted and a selectable marker gene for zeocin resistance was inserted into *BZLF1* to yield an

Table 1 Genealogy of maxi-EBV plasmids and genetic modifications

Maxi-EBV plasmid	*Genotype*	*Phenotype*	*Nucleotide coordinates*[a] *of genetic modification nos.*	*Targeting construct*	*Mode of genetic alteration*
p2089/F factor	Wild type	Cam^r	48 042–50 281	p2419 ΔEBNA2	Chromosomal building
p2491	*EBNA2*$^-$	Cam^r	168 686–169 397	p2496 ΔLMP1	Chromosomal building
p2510	*EBNA2*$^-$ *LMP1*$^-$	Cam^r	92 958–101 399 102 390–103 097	p2821 ΔEBNA3A–C ΔBZLF1	Linear integration
p2827	*EBNA2*$^-$ *LMP1*$^-$ *BZLF1*$^-$ *EBNA3A*$^-$ *-B*$^-$ *-C*$^-$	Cam^r, Zeo^r	169 929–172 281/1	p2068 ΔTR	Linear integration
p2831	*EBNA2*$^-$ *LMP1*$^-$ *BZLF1*$^-$ *EBNA3A*$^-$ *-B*$^-$ *-C*$^-$ *TR*$^-$	Cam^r, Zeo^r, Kan^r		None	

[a]Nucleotide coordinates of B95.8 strain of EBV.

inactivated and partially deleted gene (Table 1). The last modification was the removal of the TR packaging signals with a linear DNA fragment, in which the TR sequences were replaced by a selectable marker gene conferring kanamycin resistance (Table 1) as described.[19] Details of the different steps and the nucleotide composition of all targeting constructs and maxi-EBVs are available upon request.

After the final genetic modification, the resulting p2831 maxi-EBV plasmid was prepared from a single *E. coli* clone and the plasmid DNA was cleaved with *Bam*HI or *Bgl*II restriction enzymes to verify the computer prediction of fragment lengths. Restriction fragment analysis of p2831 plasmid DNA in comparison with p2089/F factor DNA confirmed the correct fragment pattern of p2831 in all details (Figure 1).

Establishment of the EBV packaging cell line 293-VII+

Similar to previous experiments,[19] the p2831 maxi-EBV DNA was stably introduced into 293HEK cells. After hygromycin selection, 33 out of 60 cell clones expressed GFP encoded by the p2831 helper virus genome. Single GFP-positive (GFP$^+$) cell clones were expanded and tested for supporting gene vector packaging as schematically shown in Figure 2. Briefly, the cells were transiently co-transfected with the gene vector plasmid DNA p1933/GFP_amp in combination with the two expression plasmids p509/BZLF1 and p2670/BALF4 to induce EBV's lytic phase and support virion production, respectively.[22,28] The concentration of *GFP*-transducing particles in the supernatants was determined by FACS analysis of vector-infected Raji cells. To select the cell clone with best packaging characteristics, all clones were tested at least three times and compared to the previously established TR$^-$2/293 packaging cell line. One clone, termed 293-VII, was selected for further analysis.

While screening the potential packaging cell lines, we noticed a consistently high GFP expression from the p2831 helper virus genome in those clones, which seemed to support efficient gene vector packaging. We therefore FACS-sorted the cell clone 293-VII and the original packaging cell line TR$^-$2/293 for the fraction of cells with the top 10% intensity of GFP. The sorted and unsorted cells of both cell lines were again analyzed for the efficient packaging of the p1933/GFP_amp gene vector. As shown in Figure 3, Raji cells infected with gene vector stocks generated from sorted cells showed a 1.5- to 2.1-fold higher transduction rate than stocks generated from the two unsorted packaging cell lines. The sorted cell population with superior packaging characteristics was termed 293-VII+ and chosen for a more detailed characterization.

Integrity of the helper EBV genome in the 293-VII+ packaging cell line

To confirm the composition and integrity of the p2831 helper virus genome in the 293-VII+ packaging cell line, the circular maxi-EBV plasmid DNA was isolated from the 293-VII+ cell line as described[32] and introduced into the *E. coli* DH10B strain by electroporation to permit a detailed restriction analysis. As shown in Figure 1, the plasmid DNA rescued from the 293-VII+ cell line showed the identical pattern with two restriction enzymes in direct comparison to the original p2831 plasmid DNA.

In a complementing approach, we undertook an intensive PCR analysis with total cellular DNA from the 293-VII+ cell line. PCR primers were selected that discriminate between wild-type and mutant alleles of *EBNA2*, *EBNA3A-C*, *BZLF1*, *LMP1* and TR (Figure 4). The *BLLF1* gene encoding gp350/220, *EBNA1*, and the *BZLF2* gene encoding gp42 served as positive controls. All PCR reactions confirmed and verified the genetic composition of the helper virus genome in the packaging cell line 293-VII+ in comparison to p2089/F factor and TR$^-$2/293 DNAs (Figure 4).

Accidental formation of growth-transforming EBV helper virus

Homologous recombination between the helper virus genome and gene vector DNA is likely to occur during herpesvirus replication[23] and has already been observed as a background phenomenon of our first-generation EBV packaging system.[19] In EBV vector systems, a central safety issue is the accidental formation of a reconstituted EBV genome that combines the genetic elements for B-cell growth transformation and virion

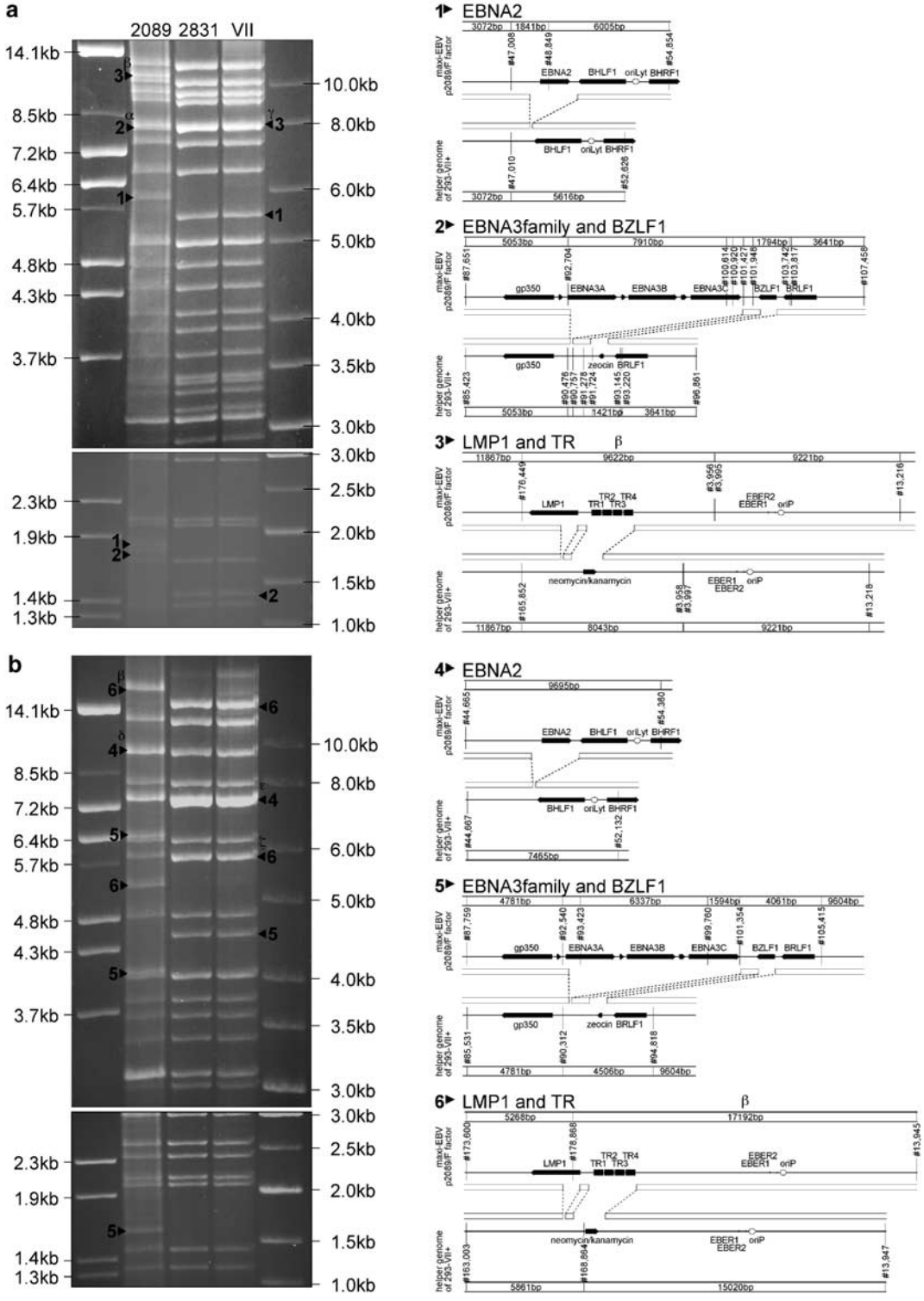

Figure 1 Comparative restriction analysis of the parental p2089 maxi-EBV plasmid and p2831 the helper virus genome. DNA of the maxi-EBV plasmid p2831 (indicated 2831), which carries genetic modifications in seven viral genes and *cis*-acting elements, was prepared from *E. coli*, digested with *Bam*HI (**a**) or *Bgl*II (**b**), and analyzed on ethidium bromide-stained agarose gels. Similarly, the p2831 plasmid was re-isolated from the packaging cell line 293-VII+ and rescued back into *E. coli* (indicated VII) to allow its restriction analysis in comparison with the original *E. coli* derived p2831 plasmid. As expected, the restriction patterns of both plasmid DNA preparations are identical but differ from the parental wild-type maxi-EBV genome (indicated 2089). All deviations between p2089 and p2831 are indicated together with detailed maps of the altered genetic loci. In detail: (α) two fragments of 7910 and 8020 bp; (β) the p2089/F factor plasmid carries six terminal repeats (TR) in contrast to only four TRs in the B98.5 prototype genome,[58] resulting in an increase of 1.1 kb in length; (γ) two fragments of 8043 and 8020 bp; (δ) two fragments of 9695 and 9604 bp; (ε) four fragments of 7481 bp (in p2831), 7457, 7455, and 7452 bp; and (ζ) two fragments of 5861 (in p2831) and 5822 bp.

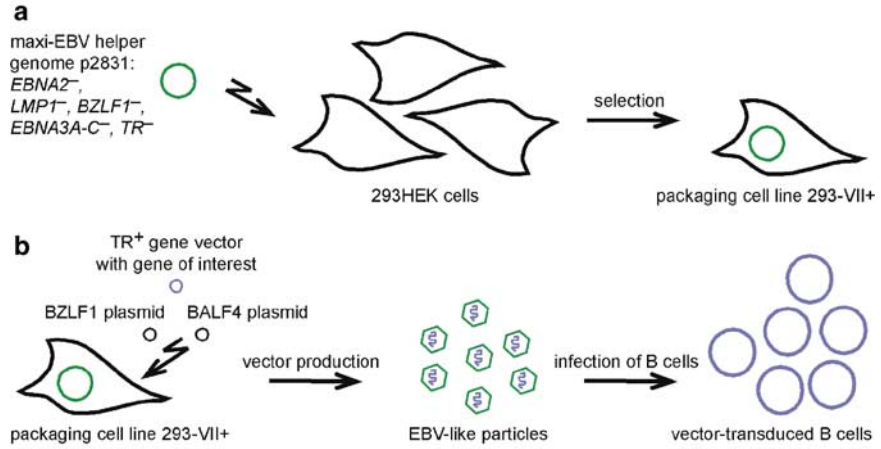

Figure 2 Schematic overview of the experimental design. Establishment of an EBV packaging cell line (**a**), and generation of EBV-derived gene vector stocks (**b**).

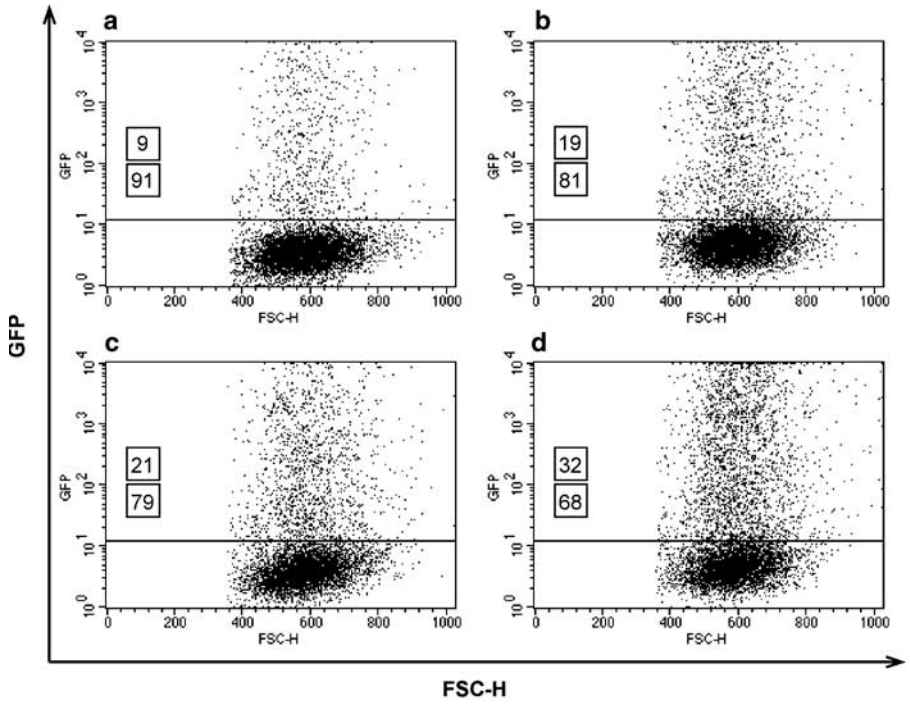

Figure 3 FACS analysis of Raji cells transduced with gene vector stocks generated with different packaging cell lines. 293-VII and TR⁻2/293 cells were sorted for the fraction of cells with the top 10% intensity of GFP expression. p1933/GFP_amp gene vector stocks were generated in the cell lines before and after sorting as described in Materials and methods. Vector stocks obtained with sorted cells (**b**, 293-VII+; **d**, TR⁻2/293) resulted in 1.5- to 2.1-fold higher transduction rates than stocks obtained with the two unsorted packaging cell lines (**a**, 293-VII; **c**, TR⁻2/293).

propagation. With our first-generation helper genome, a single recombination event, which reintroduces TR sequences into the helper genome, will result in a transforming, wild-type maxi-EBV genome. With the optimized packaging cell line 293-VII+, however, such an event will be close to impossible because the p2831 helper virus genome lacks four of the five latent genes, each of which is known to be essential for the transformation process (for a review Kieff and Rickinson[3]). Single EBV mutants negative for *EBNA2*,[26] *LMP1*,[28,33] *EBNA3A* or *EBNA3C*[34,35] are non-transforming or have a severely impaired transformation phenotype.

The probability of an accidental homologous recombination event depends on the extent of sequence homology and the length of homologous sequences shared by the DNA molecules. To detect recombination products, which might give rise to growth transformed B cells and originate from the two EBV packaging cell lines, we used two plasmids with the same TR sequences in a different context. The gene vector plasmid p1933/GFP_amp encompasses the TR region plus 1.3 kb of authentic EBV sequences, which flank the TR. This plasmid also contains the lytic origin of DNA replication, *oriLyt*, which considerably increases the frequency of

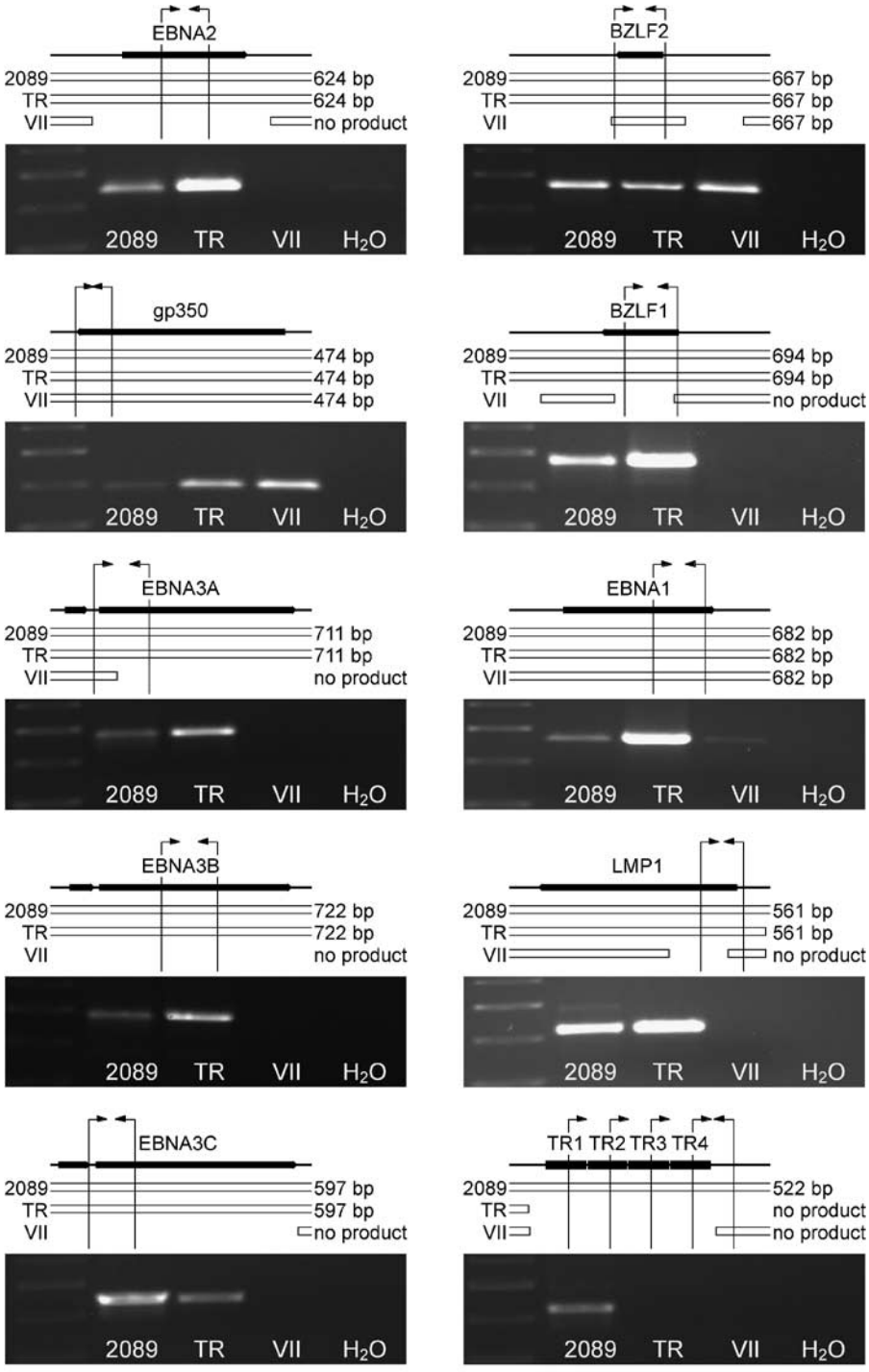

Figure 4 Comparative PCR analysis of selected genetic loci in the packaging cell line 293-VII+. Total cellular DNA was prepared from three cell lines 293–2089 (indicated 2089), TR$^-$2/293 (indicated TR) and 293-VII+ (indicated VII), and subjected to PCR amplifications with primer pairs indicative of the genetic alterations in the p2831 helper virus genome. PCR products obtained from the coding sequences of the *gp350*, *EBNA1* and *gp42* genes served as positive controls.

DNA recombination.[36] In contrast, p925 provides TR plus two TR-flanking region of 6.5 and 4.0 kb in length for homologous recombination with both helper virus genomes, but lacks other EBV-derived sequence elements including *oriLyt*. These plasmids were separately introduced into the first-generation packaging cell line TR$^-$2/293 or the optimized cell line 293-VII+ along with p509/BZLF1 and p2670/BALF4 according to the standard packaging procedure. Supernatants were collected and used to infect primary B cells in 96-well cluster plates and growth transformation was assessed by visual examination 6 weeks post-infection. Supernatants from the TR$^-$2/293 packaging cell line transfected with the plasmid p925 contained up to five transforming events per ml (Table 2); transforming events were rare with the vector plasmid p1933/GFP_amp, which shares short

Table 2 Release of growth-transforming helper virus from two EBV packaging cell lines

Virus supernatants			*Numbers of LCLs*[c]
Helper cell line	*Transfected plasmids*[a]	*Titers/ml*[b]	
TR$^-$2/293	None	ND	0/0/0
	p1933/eGFP_amp	$\sim 1 \times 10^6$	1/1/1
	p925	ND	5/0/1
293-VII+	None	ND	0/0/0
	p1933/eGFP_amp	$\sim 3 \times 10^5$	0/0/0
	p925	ND	0/0/0
293–2098	None	$\sim 1 \times 10^3$	37/96/78

ND = not determined.
[a]Co-transfected with p509/BZLF1 and p2670/BALF4.
[b]GFP$^+$ Raji cells per 1 ml supernatant determined by FACS analysis; $\sim$, the mean of three independent experiments.
[c]Numbers of wells in a 96-well cluster plate with GFP$^+$, proliferating B cells; the results of three independent experiments are shown.

stretches of sequence homology with both helper genomes, only. With the optimized packaging cell line 293-VII+, not a single transformation event was ever observed, confirming our hypothesis that the optimized helper virus genome should be transformation incompetent, which is consistent with a maxi-EBV gene vector that lacks the EBV genes *EBNA2*, *LMP1*, *EBNA3A* and *EBNAC*.[37]

Estimation of the frequency of recombination between gene vector and helper virus DNAs

As experiments described in the previous paragraph are difficult to quantify, we designed a different setup to determine the frequency of DNA recombination directly. We used the gene vector plasmid p588,[19,26] which can be efficiently packaged but lacks a phenotypic marker gene in contrast to p1933/GFP_amp. p588 and p1933/GFP_amp were separately introduced into the first-generation packaging cell line TR$^-$2/293 or the cell line 293-VII+ along with p509/BZLF1 and p2670/BALF4. Supernatants were collected and used to infect Raji cells according to our standard quantification method.[22,28,37] With the p1933/GFP_amp gene vector, we obtained approximately 1.3×10^5 and 1×10^5 'green Raji units' per ml supernatant with the TR$^-$2/293 and 293-VII+ packaging cell lines, respectively, in two experiments. Similarly, we investigated the supernatant containing the p588 gene vector for the occurrence of GFP$^+$ Raji cells. GFP-transduced Raji cells provide a measure for the release of GFP$^+$ helper virus, presumably through recombination of p588 plasmid DNA and genomic helper virus DNA. We obtained about 30 and 12 GFP$^+$ Raji cells on average with the supernatants of the TR$^-$2/293 and 293-VII+ packaging cell lines in two independent experiments. Assuming that the concentration of p588 gene vector was similar to p1933/GFP_amp gene vector units, these numbers indicate a recombination frequency of 0.03 and 0.01% with supernatants generated with the TR$^-$2/293 or 293-VII+ packaging cell lines, respectively. Very few GFP$^+$ cells ($<0.002\%$) could be found with supernatants generated with the TR$^-$2/293 or 293-VII+ packaging cell lines transiently transfected with p509/BZLF1 and p2670/BALF4, only, which is consistent with a recent report.[38]

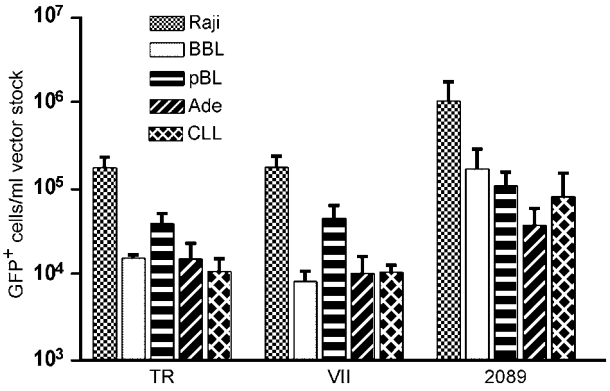

Figure 5 FACS analysis of different human B cells infected with GFP$^+$ wild-type EBV or transduced with p1933/GFP_amp gene vector stocks. A total of 10^6 primary peripheral B cells (pBL), 10^5 B cells isolated from adenoids (Ade), from patients with B-cell chronic lymphocytic leukemia (CLL), activated B blasts (BBL), and Raji cells were infected with wild-type 2089 maxi-EBV (indicated 2089) or transduced with 10-fold concentrated p1933/GFP_amp gene vector stocks obtained from the packaging cell line 293-VII+ (indicated VII) or TR$^-$2/293 (indicated TR). GFP$^+$ cells were quantified by FACS analysis with calibration beads as a volume standard as described in Materials and methods 3 days post-infection. The concentration of GFP-expressing transduced B cells per ml of virus or gene vector stock are shown. The data are the mean and standard deviation of three independent experiments.

Infection of primary B cells and B-cell lines

Wild-type EBV has a tropism for B cells, which is shared by gene vectors generated with the TR$^-$2/293 packaging cell line.[19,20] To analyze the tropism of gene vectors generated with the 293-VII+ packaging cell line, we transduced primary resting B cells prepared from adenoids or peripheral blood, activated B blasts[39] and B cells from patients with chronic lymphocytic leukemia. The numbers of GFP-expressing CD19+ B cells were determined by FACS analysis. As shown in Figure 5, human B cells were readily transduced with 293-VII+ derived gene vectors, and transduction efficiencies were comparable to TR$^-$2/293-derived gene vectors produced under the same conditions but lower than with wild-type 2089 EBV.

Construction of a gene vector without antibiotic resistance gene

With regard to a potential therapeutic application of our EBV-based gene vectors, we decided to eliminate the antibiotic resistance gene needed for DNA propagation of the gene vector plasmids in *E. coli*. An alternative is the *supF* gene, which encodes a tyrosine amber suppressor tRNA able to read through amber codon mutations. To our knowledge, all available *E. coli* strains suitable for the propagation of *supF* plasmids carry additional helper plasmids such as P3, which is based on the RP1 plasmid of the IncP group,[40,41] and encodes two antibiotic resistance genes with amber mutations. As a consequence, DNA of *supF* plasmids cannot be prepared from *E. coli* without contaminating P3 helper plasmid DNA.

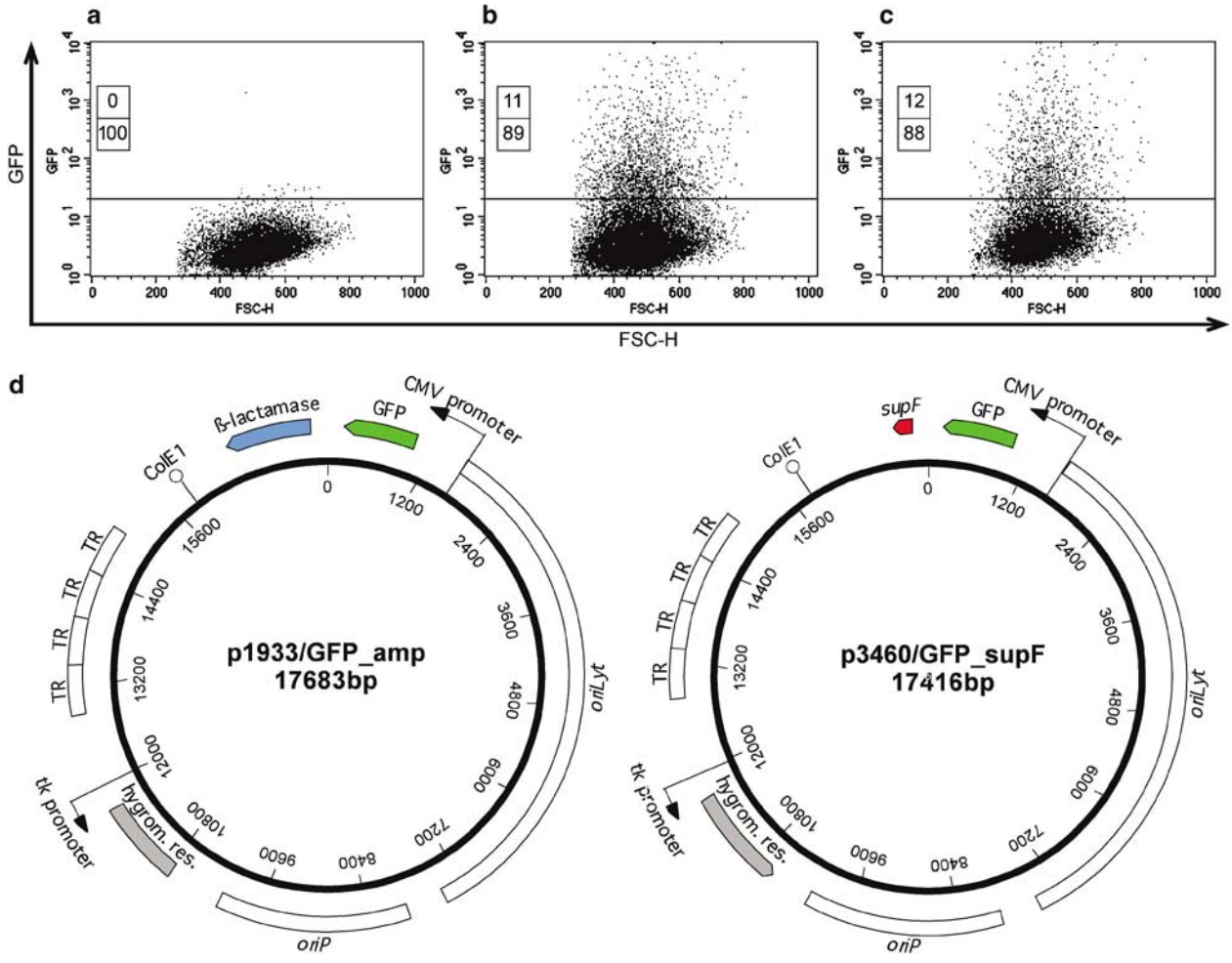

Figure 6 FACS analysis of Raji cells transduced with p1933/GFP_amp or p1933/GFP_supF gene vector stocks. The transduction rates of two gene vectors were compared, which differ with respect to their prokaryotic marker genes. p1933/GFP-amp- (**b**) and p3460/GFP_supF- (**c**) containing vector stocks were generated in 293-VII+ packaging cells and used to transduce Raji cells. (**a**) As a negative control, Raji cells were incubated with supernatant from 293-VII+ cells, which had been incubated with p509/BZLF1 and p2670/BALF4, only. (**d**) The plasmid maps of the gene vector plasmids p1933/GFP-amp and p3460/GFP_supF are shown together with relevant features. ColE1, prokaryotic origin of DNA replication; tk promoter, promoter of the herpesvirus 1 thymidine kinase gene; hygrom. res., hygromycin phosphotransferase gene; TR, terminal repeat, *oriP*, plasmid of DNA replication; *oriLyt*, lytic origin of DNA replication.

We wished to modify our gene vector plasmid and replaced the antibiotic resistance marker with *supF* to generate p3460/GFP_supF (Figure 6). Next, we constructed a novel *E. coli* strain on the basis of DH10B (Invitrogen), which carries the amber *Tet*^{r} and amber *Amp*^{r} genes from P3 stably integrated into the *recA*1 locus of DH10B to yield the strain DH10B Amp^r/Tet^r (amber). Details of its construction can be found in the Materials and methods section. The correct phenotype of DH10B Amp^r/Tet^r (amber) was confirmed by transformation of *supF* encoding plasmids such as p3460/GFP_supF, pcDNAI or pCDM8 derivatives (Invitrogen) and subsequent selection for ampicillin and tetracycline resistance. Control plasmids that do not carry a *supF* gene did not confer this phenotype in DH10B Amp^r/Tet^r (amber) as expected (data not shown).

We compared gene vector stocks produced with p1933/GFP_amp or p3460/GFP_supF gene vector plasmids in 293-VII+ cells. Recombinant EBV particles generated with both gene vectors are equally efficient to transduce human B cells with the phenotypic marker GFP (Figure 6).

Discussion

The field of gene and immune therapy is sensitized to vector-mediated oncogenesis and EBV is a known oncogenic virus. It is therefore obvious that the development of viral vectors based on EBV has to be further advanced to eliminate potential risks in their future *in vivo* application in gene therapy trials. Interest in an EBV vector for clinical use requires solid answers to basic questions on EBV vector biology. Therefore, we wanted to address some of the main issues, which include the transformation potential of EBV-derived gene vectors. Much is known about the molecular and cellular biology of a number of latent EBV genes, which, in general, mimic cellular functions.[3,42,43] It is clear from a vast number of publications that *EBNA2*, *LMP1* and *EBNA3*

family members play essential roles in EBV-related transformation processes, but EBV's latent genes such as *LMP2A* and *EBNA1* also contribute to a stable virus–host relationship.[3,43] The distinct group of latent EBV genes is functionally independent from the majority of lytic genes, which are believed to support virus production, only. We therefore assumed that latent EBV genes would be dispensable for virion synthesis.

Gene vector preparations, which are contaminated with infectious and replication-competent helper virions pose an additional risk in gene therapy with viral vectors, which might cause the spread of a recombinant human virus. We wanted to minimize this risk with a *BZLF1*$^-$ helper virus genome, which does not support spontaneous virus production because the *BZFL1* gene product is essential for the transcriptional activation of early lytic genes as well as the onset of lytic DNA replication.[3,24,44] As a consequence, an accidental release of a *BZFL1*$^-$ EBV helper virus would be restricted to a single round of infection, only.

Very much in contrast to the majority of other members of the herpesvirus family, EBV can adopt a latent as well as a lytic state in certain cell lines *in vitro*, which is a unique feature and a prerequisite for the construction of stable herpesviral packaging cell lines, in which EBV resides in its latent state before the induction of its lytic phase. Unlimited genetic alteration of the EBV genome is a rather recent option, which now allows the construction of any type of viral mutant.[11,28,45,46] We made use of these biological and technical aspects and report here on a novel packaging cell line with an EBV-based TR$^-$ helper virus genome with modifications in six viral genes. The 293-VII+ packaging cell line is superior to the first-generation helper cell line, TR$^-$2/293, in that the release of infectious and growth-transforming viral particles can be excluded (Table 2). It will still be possible in a future extension of this work to improve on the safety aspects of an even further developed EBV-based packaging cell line or gene vectors. As an example, *EBNA1*, which is essential for the episomal maintenance of the EBV genome or EBV-derived gene vectors, is controversial with respect to virus-associated diseases.[47–50] The *EBNA1* gene could be deleted from the helper virus genome and stably integrated in the cellular chromosome of the packaging cell line. Moreover, EBNA1 can be functionally replaced with proteins, which consist of EBNA1's DNA-binding domain fused to cellular chromatin components,[51] which might be less problematic than wild-type EBNA1. Equally, EBV-derived gene vectors, which rely on *oriP* in conjunction with EBNA1 for their extrachromosomal maintenance in the recipient cell, might also benefit from this modification. Moreover, the exchange of an antibiotic resistance gene for a prokaryotic marker such as *supF* is a favorable step towards safer gene vectors because the accidental transfer of resistance genes to prokaryotic organisms is a potential risk and can be easily avoided as described here.

DNA recombination between the helper virus genome and gene vector DNAs in the packaging cell line 293-VII+ occurs with similar frequencies as in our previously described basic system[19] given the inherent mechanics of herpesviral DNA replication.[23,36] The frequency of helper virus regeneration, which was found to be about 0.02% in this paper, is most likely an underestimation. For technical reasons, we used the gene vector plasmid p588, which lacks the expression cassette encoding *GFP*. The inclusion of *GFP* on both the helper virus genome and the gene vector, which is a more likely constellation, will lead to an increase of the frequency of helper virus regeneration owing to DNA sequence elements, which are targets for homologous recombination. In addition, there will be helper viruses with GFP deleted in the course of DNA recombination, which were not detected in our experimental setting. Taking both aspects into account, the frequency of helper virus regeneration is expected to be considerably higher than 1/10 000 even with the newly developed packaging cell line. However, with our new packaging cell line, the resulting virions are not expected to transduce viral oncogenes (Table 2); therefore, a potential risk with human wild-type γ-herpesviruses is eliminated.

At present stage, there are obvious limitations, which need to be addressed in the future to make our vector system practically useful. In comparison to other herpesvirus vectors, the vector titers are relatively low (similar to the titers of EBV itself) and the tropism is restricted to human B cells, only. This restriction is beneficial when dealing with B cells as target, but is an obstacle for a wider application. Moreover, our transient packaging system requires the co-transfection of the gene vector plasmid to be packaged along with two 'starter' plasmids. It will be feasible to stably introduce both BZLF1 and BALF1 into the genome of the helper cell and induce their concomitant expression in a conditional manner. Similarly, the gene vector plasmids could be stably introduced into the helper cell and maintained as replicating episomes by incorporating *oriP* sequence elements. All these steps should lead to a considerable improvement of our EBV packaging system.

Clearly, gene therapy is a safety issue for patients, who are infected with wild-type EBV strains and receive EBV-derived gene vectors. This scenario is not restricted to EBV, but is controversial with most viral vectors. Therefore, it will be a challenging task to evaluate the overall risk of EBV-derived gene vector in a therapeutic situation.

Materials and methods

Cell lines

The 293HEK cell line is derived from primary human embryonal kidney cells transformed with human adenovirus type 5 DNA and was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig).[52] The 293–2089 are 293HEK cells stably transfected with p2089/F factor plasmid DNA, which comprises the prototype B95-8 EBV genome cloned in *E. coli* as described earlier.[21] The TR$^-$2/293 cell line is derived from 293HEK cells stably transfected with a derivative of the p2089/F factor plasmid lacking the TRs of EBV as described.[19] All 293HEK derived cell lines were maintained in ISCOVE medium with 10% fetal calf serum, 100 U of penicillin per ml and 100 μg of streptomycin per ml at 37°C in a 5% CO_2 atmosphere. B blasts are EBV negative, proliferating B cells generated by co-cultivation of primary peripheral B-lymphocytes on irradiated CD40L expressing feeder cells[53] in the presence of 2 ng/ml IL-4 (PAN Biotech) and 1 mg/ml

cyclosporin A (Novartis). The Raji line is derived from an EBV-positive Burkitt's lymphoma.[54] B95-8 is a lymphoblastoid cell line obtained by infection of marmoset monkey peripheral leukocytes with EBV.[55] The primary human embryonic lung fibroblast cell line Wi38 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and used as feeders for the infection of primary B cells. Primary human blood B cells were obtained from healthy donors or from patients with chronic lymphocytic leukemia. B cells were also isolated from adenoidectomy samples. All materials were obtained after informed consent and approval by the IRB. All B cells were purified through Ficoll density centrifugation. For infection experiments, B cells with the exception of Raji cells were infected on an irradiated CD40L feeder cell layer[53] in the presence of 4 ng/ml IL-4 as described. To determine the capacity of different gene vector stocks to transform primary B cells, EBV-negative-purified B-lymphocytes were infected on irradiated (75 Gy) Wi38 feeder cells (1×10^4 cells/well of a 96-well cluster plate). All B cells and both feeder cell lines were cultivated in RPMI (Invitrogen) supplemented with 10% fetal bovine serum, 100 U of penicillin per ml and 100 μg of streptomycin per ml at 37°C in 5% CO_2 atmosphere.

Construction of viral mutants

The consecutive genetic alterations that led to the helper virus maxi-EBV plasmid p2831 (Table 1) were constructed on the basis of the wild-type B95.8 EBV strain cloned onto the F-factor plasmid p2089.[21] The deletion or inactivation of the different EBV genes and TR in the EBV genome was carried out with two different genetic procedures, chromosomal building and linear integration, as described in a recent review.[11] In order to delete *EBNA2* and *LMP1*, co-integrates with appropriately constructed shuttle plasmids (Table 1) and the two EBV plasmids p2089/F factor and p2491 (Table 1) were formed with the 'chromosomal building' technique[29] and the conditional *recA* expression plasmid p2994 (pST76-hyg), which led to the maxi-EBV constructs p2510 after routine genetic manipulations (Table 1). To promote the integration of linear DNA fragments in the DH10B *E. coli* host, the conditional *recA*/*redγ* expression plasmid p2650 based on the pST76-amp plasmid was employed together with suitable targeting constructs and prokaryotic resistance marker genes (Table 1). The mutation in the *BZLF1* gene is described[24] as well as the deletion of TR.[19] The genetic compositions of the modified EBV genomes were verified by restriction enzyme analysis, Southern blot hybridization and partial DNA sequencing. Details for the generation of all EBV mutants are available upon request.

Plasmids

p509/BZLF1 is an expression plasmid encoding the viral transactivator *BZLF1*, which acts as a molecular switch to induce the lytic phase of EBV's life cycle.[56] p2670/BALF4 is based on the pRK5 expression plasmid[57] encoding the *BALF4* gene of the B95-8 strain as described.[22] The plasmid p925 contains an 12.7 kb EBV segment encompassing four TRs of the B95-8 EBV strain[58] from nucleotide coordinate nos. 163 473 to 172 281/1 to 3955 cloned in pUC19. All plasmid DNAs were prepared with Endofree Maxi-Prep (Qiagen) and the plasmid sequences are available upon request.

Two gene vector plasmids were used in this study. p1933/GFP_amp is 17.7 kb pairs in size and is a derivative of p588[19,26] and harbors three segments of the B95-8 EBV strain[58] with nucleotide coordinate nos. 7333 to 9521, 48 848 to 54 713, and 169 424 to 172 281/1 to 648 encompassing the plasmid origin of DNA replication, *oriP*, the lytic origin of DNA replication, *oriLyt*, and the terminal repeats, TR, respectively. The plasmid also encodes the *GFP* gene (enhanced green fluorescent protein) from pEGFP-C1 (Clontech). p3460/GFP_supF is based on p1933/GFP_amp; the β-lactamase has been replaced by a *supF* gene, which enables read-through translation of amber stop codons and confers resistance against ampicillin and tetracycline in the *E. coli* strain MC1061/P3 (*ara*D139 Δ(*ara*ABC-*leu*)7679 *gal*U *gal*K Δ*lac*X74 *hsd*R2(r_k^-,m_k^+) *rps*L(Str^r) *thi*-1 *mcr*B {P3: Amp^r (amber), Tet^r (amber), Km^r}) and the novel *E. coli* strain DH10B Amp^r/Tet^r (amber) (F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1::p3229[Amp^r (amber), Tet^r (amber), Kan^r] *end*A1 *ara*D139 Δ(*ara*,*leu*)7697 *gal*U, *gal*K,λ^- *rps*L *nup*G). To construct DH10B Amp^r/Tet^r (amber), the plasmid p3229 was cloned. p3229 is based on pST76-kan[59] and carries the *Amp^r* (amber) and *Tet^r* (amber) genes from P3 flanked by *rec*A sequences. In order to construct this targeting plasmid, the *Amp^r* and *Tet^r* genes were amplified by PCR from DNA of the *E. coli* strain MC1061/P3 with gene-specific primers (amberamp5′: 5′-TTACCAATGCTTAATCAGTGA, amberam p3′: 5′-ATGAGTATTCAACATTTCCGT; ambertet5′: 5′-GTGAAACCCAACATACCCCTG, ambertet3′: 5′-TCAG CGATCGGCTCGTTGCCC) and cloned into the *Pme*I site of the *recA* gene derived from pKY102[60] in the context of pST76-kan.[59] The entire plasmid p3229 was chromosomally integrated into the *recA*1 gene of DH10B by homologous recombination with the aid of the *recA* expression plasmid p2650[61] at 42°C under concomitant kanamycin selection. The chromosomal integration was verified with a detailed PCR analysis (data not shown).

The p2089/F factor plasmid, also termed p2089 maxi-EBV, carries the wild-type EBV genome of the B95-8 EBV strain as described.[21] The TR$^-$2/293 cell line is stably transfected with the maxi-EBV genome p2114/ΔTR, which is p2089/F factor with its TR packaging signals deleted by insertion of a kanamycin resistance gene.[19]

DNA transfections

DNA of the EBV helper plasmid p2831 was prepared with ethidium bromide–CsCl gradients as described,[19] and transfections into 293HEK cells were performed with Lipofectamine (Invitrogen). Cells were seeded at about 50% confluence into six-well cluster plates 1 day before transfection. For transfection, cells were placed in OptiMEM medium (Invitrogen) for 1 h and incubated with gene vector plasmid DNA embedded in lipid micelles (1 μg DNA, 6 μl Lipofectamine per well; preincubation for 45 min at 32°C) for 4 h. After 3 days, the cells were transferred onto a 13 cm cell culture dish and cultivated in the presence of 100 μg/ml hygromycin to obtain single colonies. After 18 days, single GFP$^+$ and hygromycin-resistant colonies were expanded and analyzed for gene vector packaging. The cell clone 293-VII+, which showed the highest concentration of vector particles in the supernatant, was chosen for subsequent experiments.

To generate gene vector stocks, the helper cell line 293-VII+ was transiently transfected with Polyfect (Qiagen) or Metafectene (Biontexx) and a mixture of three different plasmids. A total of $2–6 \times 10^5$ 293-VII+ cells per well were seeded into six-well cluster plates 1 day before transfection in 2 ml medium without antibiotics. The cells were cotransfected with 0.5–1 μg gene vector plasmid DNA (p1933/GFP_amp or p3460/GFP_supF) and 0.25–0.5 μg each of the expression plasmids p509/BZLF1 and p2670/BALF4 to induce EBV's lytic phase and support virion production, respectively.[22,28] Plasmid DNAs were incubated at room temperature with 3–5 μl Polyfect or Metafectene per μg DNA in a volume of 200 μl for 15–20 min before transfection. (We now prefer Metafectene, which consistently yielded higher concentrations of gene vector preparations.) After approximately 12 h, the medium was removed, and 5 ml fresh medium was added per well. The supernatant of the transfected 293-VII+ cells was harvested after 3 days, cell debris was removed by low-speed centrifugation and filtration (0.8 μm pore size filter), and the supernatant was stored at 4°C. For the experiments shown in Figure 5, vectors stocks were also concentrated 10-fold by ultracentrifugation (SW28 Beckman rotor 12 500 r.p.m., 10°C, 3 h).

The concentrations of the different gene vector preparations were also quantified by infection of Raji cells as described.[22,28,37] Briefly, 3×10^5 Raji cells were incubated at 37°C in 24-well cluster plates with different dilutions of the gene vector stocks to be analyzed. The absolute number of GFP$^+$ cells was determined by UV microscopy in a defined fraction of the total Raji cell population 3 days after infection. On the basis of these data, 'green Raji units' per ml were calculated as a measure of the concentration of transducing gene vector particles in different gene vector preparations. Infection and evaluation of GFP$^+$ Raji cells underestimates the concentration of infectious EBV virions by a factor of at least ten.[45]

Plasmid Rescue in E. coli

To confirm the genetic composition of the helper virus genome, circular maxi-EBV plasmid DNA was isolated from the 293-VII+ helper cell line as described.[32] After extraction, DNA was introduced into the *E. coli* DH10B strain by electroporation (1800 V, 25 μF, 100 Ω, 1 mm gap cuvettes, Genepulser, Biorad). Transformants were selected on agar plates containing 50 μg/ml of kanamycin. Several colonies were picked and the DNA composition of the rescued maxi-EBV plasmid was analyzed with *Bam*HI and *Bgl*II restriction, gel electrophoresis, Southern blot hybridization and PCR.

PCR analyses

To analyze the EBV status in primary B cells from different donors, genomic DNA was isolated from purified B cells and analyzed by PCR (35 cycles, 95°C, 1 min; 57°C, 45 s; and 72°C, 1 min) with EBNA1-specific primers (5′-CCAGTAGTCAGTCATCATCATCCG and 5′-TGGAAACCAGGGAGGCAAATC) and *Pfu* polymerase (MBI). The integrity of the helper virus maxi-EBV p2831 in the packaging cell line 293-VII+ was analyzed by PCR (35 cycles, 95°C, 1 min; 50–60°C, 45 s; and 72°C, 1 min) and *Pfu* polymerase (MBI) with the following primer pairs: EBNA2, 5′-TGCTATGCGAATGCTTTGGATG and 5′-TTGAGTCTTAGAGGGTTGCGGG; gp350/BLLF1, 5′-(GATATC)TTATACATAGGTCTCGGCGTCATC and 5′-(GATAT)CACGCCCCCCCAAAATGCAACGTCG; EBNA3A, 5′-CCCATCACACAACAACAAGGTAAG and 5′-AAGTCTATCCCATACGCACGACC; EBNA3B, 5′-GAGGAGGAAGACAAGAGTGGAATG and 5′-TTTCGTTGGGTCATCTGGAGTC; and EBNA3C, 5′-CGGGCTGTCAAGGTGAGTATG and 5′-ATGAAACGCACGAAATCTAAAAG in the presence of 3% DMSO; gp42/BZLF2, 5′-(CAATT)GCTATTTGATCTTTGA and 5′-TGGTTTCATTTAAGCAGGTG; BZLF1, 5′-CATTTTCTGGAAGCCACCCG and 5′-TGAAGATGATGGACCCAAACTCG; EBNA1, 5′-CCAGTAGTCAGTCATCATCATCCG and 5′-TGGAAACCAGGGAGGCAAATC; LMP1, 5′-AGTAAGCACCCGAAGATGAAC and 5′-CCGCAAATCCCCCCG; and TR, 5′-CGCCGTTGGAGGGTAGAATG and 5′-TGTCAGCAGTTTCCTTTGTGC in the presence of 6% DMSO.

Infections and flow cytometry

GFP-encoding gene vector stocks were quantified by infection of Raji cells. For direct FACS analysis of GFP expression, gene vector-transduced Raji cells were washed once with phosphate-buffered saline (PBS) 3 days after transduction and analyzed with a defined amount of APC beads (BD Pharmingen, Germany) as a volume standard to determine the absolute number of GFP$^+$ Raji cells. On the basis of these data, transducing units per ml were calculated as a measure of the concentration of gene vectors in the different vector preparations. For FACS analysis of CD19 surface expression, the cells were incubated with a CD19-specific, directly conjugated antibody (BD Pharmingen) for 30 min in PBS and 5% FCS on ice. Nonspecific binding was evaluated by incubation with a human isotype IgG1 mAb (BD Pharmingen). Cytometric analysis was performed with a FACScalibur cytometer.

Growth transformation of primary B cells

Primary B cells (1×10^5/well of a 96-well cluster plate) were infected on Wi38 feeder cells with 200 μl of filtered (0.8 μm pore size) supernatants generated with the packaging cell lines 293-VII+ and TR$^-$2/293 as described in the Result section. The cells were fed once a week as described[28] and proliferating clones were expanded and analyzed after 6 to 8 weeks.

Acknowledgements

We thank Joachim Ellwart and Karin Nispel for support with the cell sorting device. Our work was supported by SFB455 of the Deutsche Forschungsgemeinschaft, by the Bayerische Forschungsstiftung and by Public Health Service Grant CA70723 to WH.

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