Supporting Information

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SI Materials and Methods

Construction of EBER and BNLF2a Deficient Recombinant EBV. The EBV-encoded RNA (EBER)-1– and EBER-2–deficient recombinant EBV mutants ΔEBER and ΔEBER-TR^{-}2/293 were constructed by the targeted mutation of plasmid p2089 [encoding the B95.8 genome and giving rise to reproductive virus (1)], and p2114/TR^{-} [encoding the B95.8 genome with deleted terminal repeats and giving rise to EBV-like particles (2)], respectively. In both plasmids, the EBER genes were replaced by a prokaryotic expression cassette for the galK gene as described (3). The ΔBNLF2a mutant was generated by replacing the first codon (Met-1) of *BNLF2a* in p2089 by a stop codon.

In brief, a PCR fragment was generated that comprised the galK cassette and flanking arms homologous to the site of insertion. The product was inserted into the p2089 or p2114/TR– BAC, respectively, and SW105 clones were selected for competence in galactose metabolism. The EBER-mutant genomes were left with the introduced prokaryotic galK cassette serving as genomic spacer and termed ΔEBER/p4661 (derived from p2089) and TR–ΔEBER/p4662 (derived from p2114/TR–).

For traceless mutation of BNLF2a, we applied a two-step cloning procedure, first replacing the Met-1 codon by a prokaryotic galK cassette, as described above. For replacement of the GalK cassette in the second step, a DNA fragment was synthesized that comprised the mutated nucleotides (stop codon and analytic SpeI site) and 50 bp of sequences homologous to the neighboring regions of the previously inserted galK cassette. This fragment was introduced into a SW105 clone from the precedent round of mutation and bacteria were then counterselected for galactose metabolism by feeding deoxygalactose (Sigma). This mutant was termed ΔBNLF2a/p3912.

The intended recombination and overall integrity of the BACs were verified by multiple restriction digests and sequencing. High-quality BAC DNA was transfected into 293 cells, stable producer clones were selected, and virus stocks were generated as described previously (1). Additional information and cloning primers are available upon request.

Confirmation of Producer Genotypes by Southern Blot. Genomic DNA of producer clones was prepared using the QIAamp DNA Blood MiniKit (Qiagen). Three micrograms of DNA was cut with BamHI and BglII restriction enzymes (NEB) and resolved on a 0.8% agarose gel. Southern blotting was performed as described before (1). In brief, DNA was depurinated in 0.75% HCl in H_2O and denatured in 1.5 M NaCl/0.5 M NaOH and blotted to a nylon membrane (Millipore) by capillary transfer. The membrane was washed in 2× SSC buffer and prehybridized in 10 mL Church buffer at 65 °C for 30 min. The oriP probe was amplified by PCR using primers (5′-3′) GTCTTGGTCCCTGCCTGG and GGTTAGTAAAAGGGTCCTAAGGAAC and p2089 as PCR template. Fifty nanograms of PCR product were labeled with γ-P^{32} CTP using HighPrimeMix (Roche), purified on a Sephadex-50 column (GE Healthcare), and eluted in 400 μL Tris-EDTA buffer. The labeled probe was added to 25 mL Church buffer and incubated with the membrane at 65 °C overnight. Washing with 0.2× SSC/0.1 SDS decreased unspecific signals, and the membrane was exposed to Hyperfilm MP (GE Healthcare) at −80 °C.

Preparation of RNA, Reverse Transcription, and Quantitative PCR. Total RNA was prepared with the RNeasy MiniKit (Qiagen) and microRNA (miRNA) with the mirVana Kit (Ambion) according to the manufacturers' instructions. Total RNA from infected cells was extracted after thorough washing with PBS solution to eliminate free viruses. For the isolation of RNA from viral particles, supernatants were concentrated and pretreated with DNase and/or RNase (where indicated) and finally pelleted by ultracentrifugation at 100,000 × *g* at 4 °C for 1 h. RNA was eluted in RNase-free H_2O and digested with 2 U DNase I (amplification grade; Invitrogen) with addition of 1 μL RNAsin (Promega) for 30 min at 37 °C. RNA (0.5–2 μg) was used for reverse transcription with the SuperScriptIII Kit (Invitrogen) using random hexamer primers and miRNA-specific stem-loop primers, respectively. Details on stem-loop cDNA synthesis can be found elsewhere (4), Table S2 provides stem-loop–specific primers. Controls devoid of reverse transcriptase confirmed the absence of DNA in all samples, and controls devoid of RNA template excluded unspecific stem-loop cDNA synthesis. Quantitative PCR (qPCR) was performed in triplicates in a LightCycler 480 (Roche) by using SYBR Green Master I (Roche) and efficiency evaluated primers (Table S1). Standard qPCR was performed at 95 °C for 5 min, followed by 45 cycles of 60 °C for 10 s, 72 °C for 10 s, and 95 °C for 10 s. miRNA-specific signals were obtained in a touch-down qPCR with 95 °C for 5 min, followed by 5 cycles of 95 °C for 10 s and 70 °C for 1 min, 15 cycles of 95 °C for 10 s and an annealing temperature decreasing by 0.6 °C each cycle for 1 min, and a final 25 cycles with 95 °C for 10 s and 60 °C for 1 min. The product integrity was verified by a melting curve analysis. The calculation of crossing points was based on the second derivative maximum and relative transcript quantifications included PCR efficiencies. Values were calculated with LightCycler 480 Software 1.5 SP3 (Roche).

1. Delecluse HJ, Hilsendegen T, Pich D, Zeidler R, Hammerschmidt W (1998) Propagation and recovery of intact, infectious Epstein-Barr virus from prokaryotic to human cells. *Proc Natl Acad Sci USA* 95:8245–8250.
2. Delecluse HJ, Pich D, Hilsendegen T, Baum C, Hammerschmidt W (1999) A first-generation packaging cell line for Epstein-Barr virus-derived vectors. *Proc Natl Acad Sci USA* 96:5188–5193.
3. Warming S, Costantino N, Court DL, Jenkins NA, Copeland NG (2005) Simple and highly efficient BAC recombineering using galK selection. *Nucleic Acids Res* 33:e36.
4. Varkonyi-Gasic E, Wu R, Wood M, Walton EF, Hellens RP (2007) Protocol: A highly sensitive RT-PCR method for detection and quantification of microRNAs. *Plant Methods* 3:12.

Fig. S1. VLPs from TR^{-}2/293 cells contain viral mRNAs and miRNAs. qRT-PCR revealed the presence of viral mRNAs (*A*) and miRNAs (*B*). BZLF1 mRNA levels were higher in TR^{-}2/293 VLPs compared with B95.8 particles (Fig. 2*B*), probably because this gene is being ectopically overexpressed in TR–2/293 cells to induce synthesis of VLPs. Error bars indicate the SD of three replicates.

Fig. S2. VLPs and gp350⁺/GFP⁺ exosomes contain comparable amounts of gp350 and GFP. (*A*) Raji cells were incubated overnight with VLPs or exosomes and analyzed by flow cytometry for GFP fluorescence and gp350 surface levels. (*B*) Five micrograms of protein from particle preparations were analyzed by immunoblotting for gp350 (antibody clone 72A1). TSG101 is incorporated into secreted particles and served as loading control.

Fig. S3. VLPs enhance the transformation potential of B95.8 virus stocks. Experiments were performed as described in Fig. 4. Significances of differences (*P* values) were calculated by Fisher exact test; n.s., not significant ($P > 0.05$). Shown are two of three independent experiments (the third experiment is shown in Fig. 4*B*).

Table S1. qPCR primers

Target	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product size, bp
BZLF1	CTGGTGTCCGGGGGATAAT	TCCGCAGGTGGCTGCT	107
BRLF1	CCTGTCTTGGACGAGACCAT	AAGGCCTCCTAAGCTCCAAG	100
BMRF1	CGTGCCAATCTTGAGGTTTT	CGGAGGCGTGGTTAAATAAA	116
BNLF2a	TGCTGACGTCTGGGTCCT	TGCTTTGCTAGAGCAGCAGT	98
BCRF1	ACCTTAGGTATGGAGCGAAG	GGGAAAATTGTCACATTGGT	110
BGLF5	TTCGGCCGCTATTAGCTTAG	GACGGGGGAATAATCAACCT	75
BHRF1	CATCTGGAACGGCTTACCTC	CCCTTGTTGAATAGGCCATC	82
BALF1	ACCGCAAACACCACTGTGTA	CGCAGTGTACAACGACCACT	75
LMP1	AGGCTAGGAAGAAGGCCAAA	CTGTTCATCTTCGGGTGCTT	109
LMP2AB	ATCGCTGGTGGCAGTATTTT	GAGTATGCCAGCGACAATCA	105
EBNA2	ACATGAACCGGAGTCCCATA	TGCGGGGTCTATAGATGGAG	82
EBER1	GACCTACGCTGCCCTAGAGGTTTTGC	CCAGCTGGTACTTGACCGAAGACG	150
EBER2	GGACAGCCGTTGCCCTAGTGG	AGCGGACAAGCCGAATACCCTTC	166
GUSB	CGCCCTGCCTATCTGTATTC	TCCCCACAGGGAGTGTGTAG	91

Table S2. miRNA-specific stem-loop primers and qPCR primers

Target	Specific first strand primer terminus (5′-3′)*	qPCR forward primer (5′-3′)	Product size, bp†
BHRF1-1	...CAACTCC	AGAGTAACCTGATCAGCCCC	59
BHRF1-2 3′	...CTCAATT	GGCGTATCTTTTGCGGCAGA	59
BHRF1-3	...CTGTGCT	GGCGTAACGGGAAGTGTGTA	59
BART1 5′	...CCACAGC	CGGTCTTAGTGGAAGTGACGT	60
BART2 5′	...CGCAAGG	GCCGGTATTTTCTGCATTCGC	60
BART3 5′	...CAGCACA	GGCGGACCTAGTGTTAGTGT	59
BART15	...CTCAAGG	GGCGGGTCAGTGGTTTTGTTT	60

Common first-strand stem-loop primer start sequence (5′-3′): GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACCTG-TAC.... Common miR-qPCR reverse primer (5′-3′): GTGCAGGGTCCGAGGT.

*First-strand synthesis was performed with miR specific stem-loop primers comprising a common part (5′ terminus) forming the stem-loop and a specific part (3′ terminus) annealing to the microRNA.

†Product sizes related to qPCR products generated with the combination of miR-specific forward primer (see row) and common qPCR reverse primer.