Supporting Information

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SI Results

A *BRLF1*-Promoter Mutant EBV Reveals a Block of Lytic Gene Expression Downstream of *BRLF1*. As published previously (1, 2), Zta can bind more efficiently to certain Zta-responsive elements (ZREs) in the *BRLF1* promoter when they are cytosine-phosphatidyl-guanosine (CpG)-methylated, a finding that was interpreted to mean that escape from viral latency and induction of the EBV lytic phase depends on the methylated status of this promoter. A corollary of this notion is that this promoter's being unmethylated might prevent the onset of the EBV lytic phase immediately after infection of primary B cells. To test this hypothesis, two of the three ZREs within the *BRLF1* promoter were replaced by CpG-free ZREs, which Zta binds strongly (Fig. S6*A*).

The EBV *BRLF1*-promoter mutant p4023 DNA was stably introduced into HEK293 cells, and clonal producer cells were selected as described (3). Transient ectopic expression of *BZLF1* readily induced the EBV lytic phase in the stably transfected HEK 293 cells even though the endogenous mutant EBV 4023 DNA did not carry CpG-methylated ZREs in the *BRLF1* promoter. Efficient de novo virus synthesis occurred, and progeny virus was titered on Raji cells as described (4, 5).

Primary human B cells were infected with this EBV *BRLF1*-promoter mutant (4023) or wild-type EBV (2089) with the same MOI. Expression of representative viral lytic genes was determined by semiquantitative RT-PCR 5 days postinfection (p.i.), when early *BZLF1* expression reached its peak (Fig. 1). The expression of *BRLF1* was not affected by the point mutations in its promoter. No detectable differences in the levels of an early (*BMRF1*) and two late (*BALF4*, *BLLF1*) viral genes were found when the primary cells were infected with either 4023 or 2089 virus (Fig. S6*B*).

Similar to experiments shown in Fig. 3 and summarized in Table 1 and Table S2, no progeny virus was detectable in supernatants from HEK293 cells transiently cotransfected with a *BZLF1* expression plasmid and genomic, unmethylated DNA of the *BRLF1*-promoter mutant EBV (p4023).

Together, these results indicate that the methylation of EBV DNA necessary for induction of EBV's lytic cycle occurs at ZRE sites other than those in the promoter of the *BRLF1* gene.

SI Materials and Methods

Plasmids. The maxi-EBV plasmid p2089 and the reconstituted wild-type EBV 2089 virus (6) encompass the complete genome of the EBV prototype B95.8 strain. The *BZLF1*-knockout mutant EBV (2809; BZLF1-KO EBV) is based on p2089 (7). General aspects of the establishment and genetic modifications of these two recombinant EBVs have been published (3). The *BRLF1* promoter mutant EBV (4023) and the CD2 reporter EBV (3875) were constructed in the course of our research as described in ref. 8. All details of the construction of the recombinant EBVs and their sequence compositions are available on request. The BZLF1 expression plasmid p509 is described in ref. 9. The coding region of the M.SssI de novo methyltransferase (a kind gift from New England Biolabs) was cloned into the plasmid pRK5 and is constitutively expressed from the CMV promoter (p3663). The BZLF1:GFP expression plasmid p3927 contains amino acids 149–245 of *BZLF1* cloned in phase with the *GFP* gene expressed from the CMV promoter in pEGFP-C1 (Clontech).

Construction of the CD2 Reporter EBV 3875. The rat *CD2* gene encompassing amino acid residues 1–233 of the rat CD2 coding region with the extracellular and transmembrane domains (10) was expressed from the viral *BMRF1* promoter and introduced into the backbone of the recombinant EBV plasmid p2089 (6) (schematically shown in Fig. 1*C*). This reporter gene can detect the onset of lytic gene expression in the context of an EBV-infected cell (11). Primary human B cells were infected with both 2089 EBV and the CD2 reporter virus 3875 at an MOI of 0.1.

Cells. The cell lines in this study have been described (5). HEK293/TR$^-$ and B95.8 cells served as controls (12). The CD40-stimulated B-blast cell line (LENL5) was used as described in ref. 13. Primary human B cells from adenoids (4, 5) were sorted according to their expression of IgD, CD38, and CD27 surface molecules with the antibodies α-IgD-FITC (Cat. 555778; Becton Dickinson [BD]), α-CD38-PE (Cat. 12–0389-73; eBiosciences), and α-CD27-APC (Cat. 337169; BD) and the aid of a FACS Aria instrument (BD). The sorted B cells were infected with an MOI of 0.01 for 24 h. The absolute number of B cells was ere measured by FACS with the aid of calibration beads purchased from Becton Dickinson (4, 5).

RT-PCR Analysis. Total RNA was extracted (RNeasy Mini Kit; Qiagen) according to the manufacturer's protocol. The integrity of the isolated RNA was examined with an Agilent Bioanalyzer. RNA was treated with DNaseI (Invitrogen) at 37 °C for 90 min reverse-transcribed at 55 °C with the SuperScript III First-Strand Synthesis Kit (Invitrogen) according to the manufacturer's protocol. One μL of the cDNA synthesis (final volume 20 μL) was used for PCR amplification. PCR reactions were performed as follows: denaturation step (95 °C for 4 min), amplification for 30–36 cycles [1.5 min at 95 °C, 1 min at 57 °C (range 52 °C–61° C), 1.5 min at 72 °C], elongation at 72 °C for 6 min. Oligonucleotide primer sequences are listed in Table S1.

CD40L/IL-4 Stimulation of Primary B Cells. Human B cells from the peripheral blood of healthy donors can be activated efficiently and induced to proliferate in vitro by stimulating the CD40 receptors in the presence of IL-4, a combination of signals mimicking B-cell activation by T-helper cells (13, 14). We infected the three subpopulations of human B cells with either wild-type EBV (2089) or *BZFL1*-knockout EBV (2809) and cultivated the infected cells on CD40L murine feeder cells in the presence of IL-4 for 1 day. Cells were cultivated on plastic as a control. The cells were removed, and counted, and the number of GFP-positive B cells with characteristics of activated lymphocytes (by forward and sideward scatter criteria) was determined (4, 5). The remaining cells were cultivated further on plastic, and the number of GFP-positive activated B cells was determined at 5 and 9 days p.i.. The ratio between the number of cells infected with the *BZFL1*-knockout EBV (2809) and those infected with wild-type EBV (2089) was calculated.

De Novo CpG Methylation of EBV DNA. One microgram of p2089 EBV DNA was CpG-methylated in vitro with 5 units of the de novo methyltransferase M.SssI (New England Biolabs) and 0.6 μL SAM (32 mM stock concentration) in a final volume of 30 μL for 4 h according to the manufacturer's protocol. The extent of CpG methylation was checked with appropriate restriction enzymes, which are blocked by CpG methylation.

DNA Transfection and Generation of Infectious Viral Particles. DNA transfections into HEK293 cells were performed using polyethylenimine (PEI) (Sigma-Aldrich). The day before transfection, 5×10^5 cells per well were seeded into six-well cluster plates. During the preparation of the transfection mixture, cells were

switched to Optimem minimal medium (Invitrogen). For each experiment, two wells of a six-well cluster plate were transfected with 5 μg p2089 EBV-DNA and 1 μg p509 encoding *BZLF1*. The DNAs were mixed with 1 mL Optimem and combined with 1 mL Optimem containing 12 μL PEI dissolved in water (50%/50%; vol/vol). The mixture was incubated for 15 min at room temperature and was added to the cells for 4–5 h. For in vivo methylation of wild-type EBV, 5 μg p2089 EBV, 0.5 μg p509 encoding *BZLF1*, and 4 μg p3663 encoding *M.SssI* were transiently cotransfected into HEK293 cells.

Immunofluorescence Analysis. Transiently transfected HEK293 cells and the control cell lines HEK293/TR$^-$, B95.8 cells, or primary B cells infected with B95.8 or 2089 virus stocks were washed with PBS. Then 5×10^4 cells were fixed on slides with acetone (Zta, Rta and BALF4/VcAgp125), acetone:methanol (1:1) (BLLF1/gp350/220), or methanol:paraformaldehyde (1:2) (BMRF1/EA-D). After being washed for 10 min in PBS, cells were incubated for 60–75 min at room temperature with mouse monoclonal antibodies specific for Zta (BZ1) (15), Rta (clone 5A9) (16), BMRF1/EA-D (clone MAB919; Chemicon), BLLF1/gp350/220 (clone 72A1) (17), or BALF4/VcAgp125 (clone MAB184-L2; Chemicon). After several washes with PBS, cells were incubated with a secondary Cy3-coupled goat anti-mouse IgG antibody (Dianova). After repeated washings with PBS, cells were embedded and analyzed with an Axiovert inverted epifluorescence microscope (Zeiss).

Concentration of Viral Particles. B95.8 culture supernatants and supernatants from HEK293 producer cells lytically induced to synthesize progeny virus were harvested and centrifuged at 300 × *g* for 10 min followed by 15 min at 1,600 × *g* and applied to a Centricon tube (Millipore). Virus stocks were concentrated by centrifugation at 3,500 × *g* for 45 min in a JS 7.5 rotor, and then the concentrate was collected by centrifugation at 1,000 × *g* for 2 min.

Virus Release Assay. To monitor the onset of de novo synthesis of progeny virus, freshly isolated primary B lymphocytes (3.3×10^7) prepared from adenoids of three different donors were infected with wild-type EBV (2089) (MOI 0.1) in a total volume of 20 mL for 24 h. Cells were pelleted and washed twice in 15 mL RPMI-1640. The cells were resuspended in 5 mL RPMI-1640, and the remaining 2089 virus was neutralized with the α-gp350 monoclonal antibody 72A1 (17) at a final concentration of 6 μg/mL in RPMI-1640 cell culture medium for 3 h. At this concentration the neutralizing antibody reduced the infectivity of wild-type 2089 EBV to about 50%, as indicated in Fig. S2. After neutralization, the cells were washed twice in 15 mL RPMI-1640 and resupended in 20 mL cell-culture medium. After cultivation for another 24 h, residual virus was again neutralized with 6 μg/mL 72A1 antibody for 3 h. Cells were spun down, washed twice in a final volume of 15 mL RPMI-1640 to remove traces of the neutralizing 72A1 antibody, and resuspended at a final concentration of 1×10^6 cells/mL As early as 3 days p.i. and at the indicated later time points (Fig. 2*B*), 1×10^7 EBV-infected B cells were placed in a sieve (1-μm pore size) on top of a well with 5×10^6 indicator cells for 24 h, as shown schematically in Fig. 2*A*. For indicator cells, the human B-blast line (LENL5) was used, which depends on CD40L and IL-4 for its continuous survival and proliferation. Infection with EBV renders their proliferation independent of both exogenous activating signals (13). The indicator cells were harvested from the Transwell experiments and seeded in 48 wells of a 96-well cluster plate at a concentration of 1×10^5 cells per well. Medium was exchanged every week. To quantify infectious EBV released from the infected primary B cells, the number of wells with proliferating and GFP-positive B blasts was determined 5 weeks p.i. and is shown on the *y*-axis in Fig. 2*B*. Wild-type EBV (2089) and mock-infected B-blasts were included as controls.

In Vitro Pulldown Assays with GFP:BZLF1. The DNA binding and dimerization domain of BZLF1 (amino acid residues 149–245) was cloned in phase downstream of the coding region of eGFP in pEGFP-C1 (Clontech) to generate the expression plasmid p3927.1. This plasmid was transiently transfected into HEK293 cells, which expressed the GFP:BZLF1 chimera at high levels as a nuclear protein. Nuclear proteins were isolated by high salt extraction as described (18), and 30 μg of protein was preincubated with 10 μg of sonicated competitor DNA (calf thymus DNA; Sigma) and 5 μg of poly-dIdC (Roche) in a final volume of 100 μL GFP-binding buffer (20 mM Hepes, pH 7.9/75 mM NaCl/2 mM MgCl2/10% glycerol/0.1 mg/mL BSA) on ice for 1 h. EBV genomic DNA was purified from *Escherichia coli* through two rounds of CsCl-ethidium bromide ultracentrifugation, precipitated, dissolved, and used directly as the CpG-unmethylated sample. Alternatively, the *E. coli*-derived EBV DNA was CpG-methylated in vitro with M.SssI as recommended by the manufacturer (New England Biolabs). The EBV DNAs were ultrasonicated to an average size of 500 bp. A 500-ng sample of EBV DNA was incubated with 30 μg preabsorbed nuclear extracts in 100 μL of GFP-binding buffer on ice for 1 h. After adding GFP-dilution buffer (20 mM Tris, HCl 7.5/150 mM NaCl/0.5 mM EDTA/protease inhibitor) to a final volume of 500 μL, DNA bound by GFP:BZLF1 was selected and immobilized with the GFP-Nanotrap reagent (Chromotek) at 4 °C overnight (19). The immunocomplex was washed twice with 1 mL GFP-dilution buffer and once with 1 mL GFP binding buffer containing 300 mM NaCl. The beads were resuspended in elution buffer (10 mM Tris/0.5 mM EDTA, pH 8.0/2% SDS/20 μg proteinase K) and incubated for 2 h at 65 °C. Protein-free DNA was phenol extracted, precipitated, and analyzed photometrically.

Microarray Hybridization. Labeling of selectively enriched DNA or input DNA (i.e., p2089 genomic EBV DNA purified from *E. coli*) was performed using the BioPrime Total Genomic Labeling System (Invitrogen) according to the manufacturer's instructions. Briefly, the DNAs were labeled with Alexa Fluor 3 or Alexa Fluor 5, and the efficiency of fluorochrome incorporation was determined at 555 nm and 647 nm, respectively. The two labeled probes of enriched and input DNA were combined, evaporated, and resuspended in 130 μL of hybridization buffer (Ocimum Biosolution). Hybridization to the tiling EBV-microarray was performed in a Tecan HS 4000 Pro Hybridization Station for 16 h at 60 °C with gentle agitation. After three washing steps at 30 °C for 1 min with 2× SSC and 0.1% SDS, 1× SSC, and 0.5% SSC, the slides were dried with nitrogen.

Imaging and Analysis of Microarray Data: In Vitro Pulldown Assays with GFP:BZLF1. One slide of our custom-made EBV microarray contains four copies of a complete set of 285 separate, partially overlapping PCR fragments with a length of 500–700 bp covering the complete genome of the prototype B95.8 EBV strain. Details of the PCR fragments, primer lists, control sample spots, and the overall design of the microarray are available upon request. We prepared the PCR fragments, but the slides were spotted by a commercial service provider (Ocimum Biosolutions). After hybridization and washing, imaging of the microarray slides was carried out with a GenePix Personal 4100A Scanner (Axon), and analysis of visual data was performed with the GenePix Pro-6 software. The ratio between signals obtained with enriched DNA fragments selected by the immobilized GFP:BZLF1 protein versus unselected input DNA was determined. For normalization, the calculated ratio median was set at 1. Ratios of the four copies of each fragment were averaged, and single spots with a standard error >0.15 were excluded from analysis. The plots of the ratios obtained with unmethylated or methylated recombinant EBV-DNAs are shown in Fig. S7.

Means and standard deviations were calculated from three independent experiments.

Methylated DNA Immunoprecipitation Assay. Primary B cells from adenoids were isolated and infected with B95.8 virus stocks at an MOI of 1.0. At various time points p.i., DNA was prepared using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions followed by RNaseA treatment. The methylated DNA immunoprecipitation (MeDIP) was performed as described previously (820). Briefly, DNA was sheared into fragments with an average size of 500 bp using a Branson Digital Sonifier model 250-D (maximum amplitude: 10%, 2 × 20 s, 1 s on/off). After purification with the NucleoSpin Extract II Kit (Machery-Nagel), 8 μg of this "input" DNA was denatured for 10 min at 95 °C and cooled on ice. Immunoselection of methylated DNA was performed in a final volume of 1 mL immunoprecipitation buffer (10 mM sodium phosphate, pH 7.0/140 mM NaCl/0.05% Triton X-100) by adding 20 μL of monoclonal 5-methylcytidine antibody (Diagenode). After incubation at 4 °C overnight, the DNA/antibody complexes were precipitated with 80 μL of Dynabeads M-280 sheep anti-Mouse IgG (Invitrogen) for 2 h at 4 °C and washed 3 times with 700 μL of IP buffer. DNA was recovered by proteinase K treatment, phenol/chloroform extraction, and ethanol precipitation.

For microarray analysis, DNA was amplified using the GenomePlex Complete Whole Genome Amplification (WGA) Kit (Sigma-Aldrich) according to the manufacturer's instructions with minor modifications. The fragmentation step in the protocol was omitted because MeDIP-DNA was already sheared. Before microarray hybridization, linear amplification of DNA was controlled using quantitative PCR.

Labeling of the probes, hybridization, and imaging of the microarray slides were carried out as described in earlier sections. The signal ratios of precipitated versus input DNAs of the four copies of each fragment were averaged, and single spots with a standard error >0.15 were excluded from analysis. For normalization, the calculated median ratio was set at 1. To analyze the kinetics of methylation pattern formation, the adjusted data were processed so that the ratio of a constant point of reference, Cp, was set at 1 in each experiment, because the Cp promoter region is free of methylated CpGs even in established lymphoblastoid cell lines (ref. 21 and references therein), in contrast to EBV genomes in the memory B cell reservoir in vivo (22). To validate the data, two biological repeats with two technical repeats of each were conducted. The plots of the ratios are shown in Fig. S3*B* covering five time points p.i. during a 12-week period.

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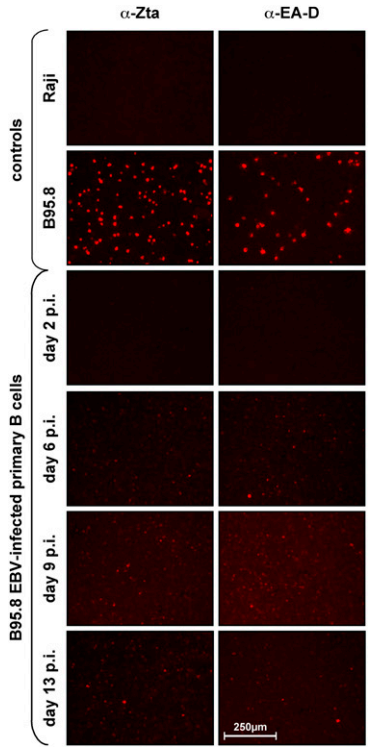

Fig. S1. Expression of BZLF1/Zta and BMRF1/EA-D in primary B cells infected with B95.8 virus. Primary adenoid B cells were infected with B95.8 virus with an estimated MOI of 1. The cells were harvested at different time points p.i., as indicated, and stained with antibodies directed against Zta and EA-D proteins in combination with a Cy3-coupled secondary antibody. The majority of infected B cells support low-level expression of Zta and EA-D, which become detectable about 6 days p.i. Raji cells, which do not support the spontaneous onset of the EBV lytic cycle, served as negative controls. The B95.8 cell line, in which ~10% of the cells support de novo EBV synthesis, served as a positive control.

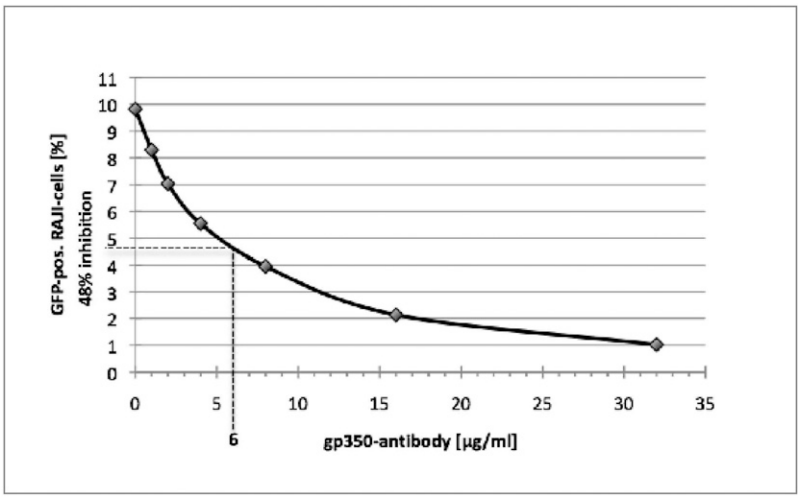

Fig. S2. Titration of the α-gp350/220–neutralizing antibody 72A1. Raji cells were infected with wild-type 2089 EBV encoding GFP in the absence (0 μg/mL) and increasing concentrations (up to 32 μg/mL) of 72A1. The infected Raji cells were analyzed for GFP expression by FACS 3 days p.i. A concentration of 6 μg/mL of 72A1 antibody neutralizes about half of this virus' infectivity, as indicated.

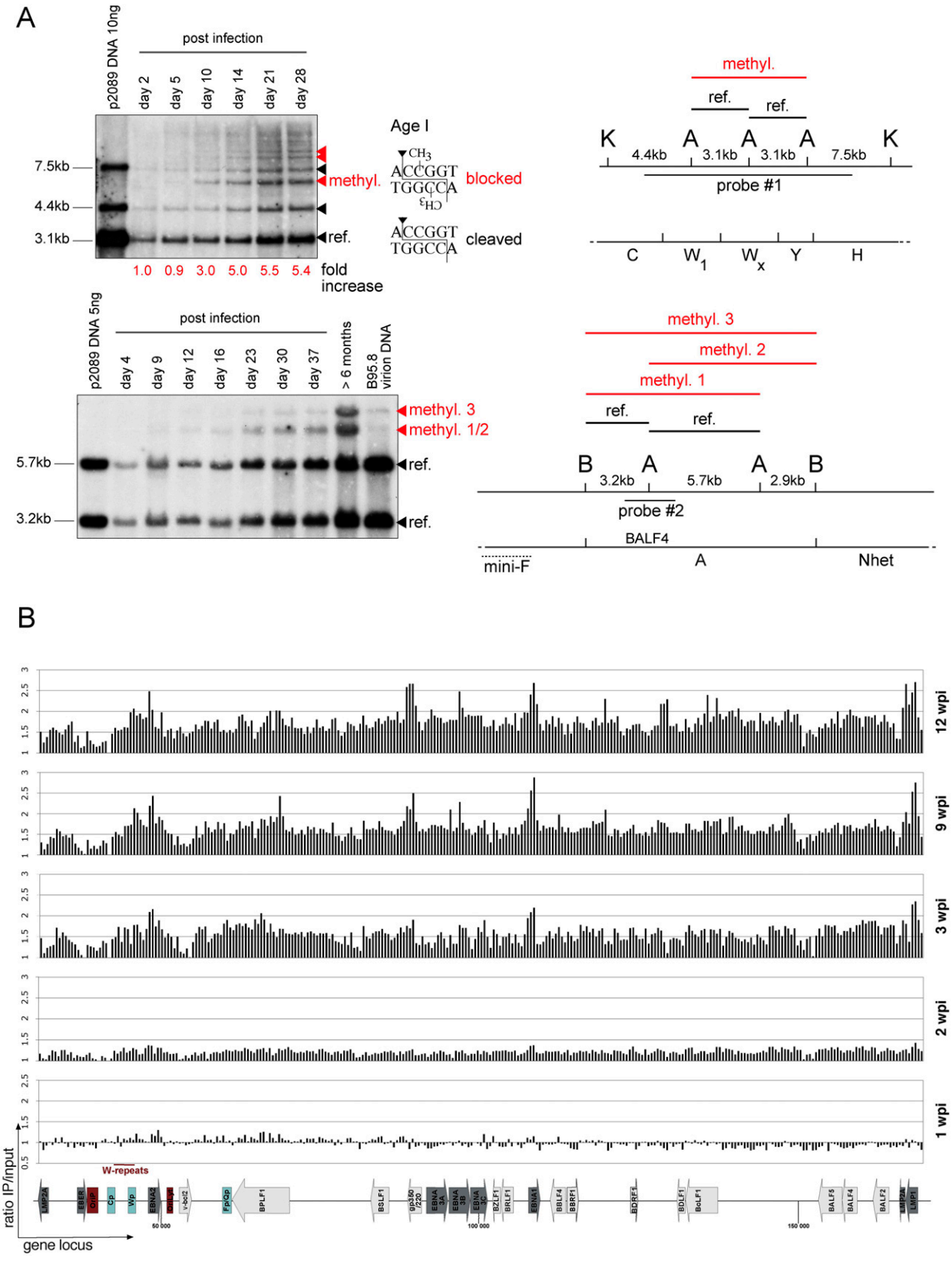

Fig. S3. continued.

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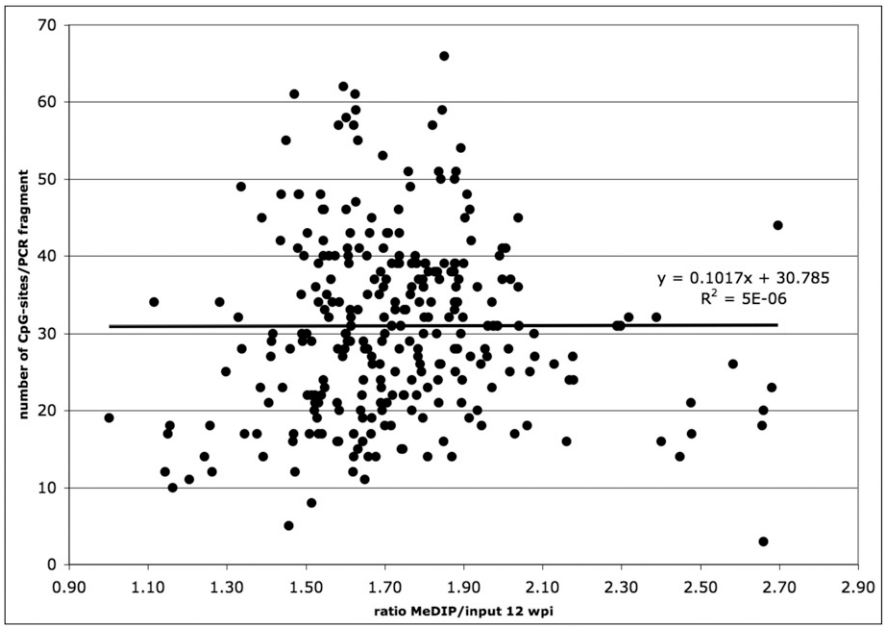

Fig. S3. Analysis of CpG methylation of virion and genomic EBV DNA after various time points p.i. (*A*) EBV genomic DNA is not detectably CpG-methylated shortly after infection but slowly acquires cytidine methyl groups as indicated by Southern blot hybridization of primary B cells infected with wild-type EBV 2089 at different time points p.i.. Virion DNA derived from supernatants of the B95.8 cell line and recombinant EBV DNA isolated from *E. coli* (p2089) free of methylated CpGs were included as well as genomic DNA from a lymphoblastoid cell line infected with wild-type EBV 2089 which had been in culture for more than 6 months p.i. The DNAs were cleaved with KpnI (K) or BamHI (B) and the methylation-sensitive restriction enzyme AgeI (A), separated by electrophoresis, and hybridized with two different probes as indicated. Probe #1 detects the fully cleaved EBV reference fragments W_1 and W_x (indicated as "ref."), which are the EBV Bam-W-repeats. Probe #2 detects the genomic locus of the *BALF4* gene. Bands labeled "methyl." are indicative of AgeI sites blocked by CpG methylation that otherwise are cleaved and detected as "ref." fragments. Likewise, black arrowheads point to detected fragments cleaved to completion by AgeI and KpnI or AgeI and BamHI; red arrowheads point to fragments originating from incompletely cleaved DNA caused by the blocking of AgeI by CpG methylation. The relative ratios of the signal strengths "methyl."/"ref." as measured by an phosphoimager scan are indicated in red below the individual lanes in the top panel and are termed "x-fold"; the situation on day 2 p.i. is set as 1.0. The fragments labeled A, C, W_1, W_x, Y, Nhet, and H indicate the localization of typical BamHI cleavage products of the EBV genome of the strain B95.8 (23); mini-F indicates the position of the prokaryotic backbone in the genome of wild-type EBV 2089 (6). Up to 10 copies of the W repeats (W_1, W_x) give rise to a ladder of bands indicated by red arrowheads because of partial AgeI cleavage (*Top*). (*B*) MeDIP and microarray analysis. DNA isolated from primary B cells infected with B95.8 virus at an MOI of 1.0 was prepared after different time points p.i. The DNA samples were enriched for 5′-methylcytidine nucleotides with a monoclonal antibody, as described (20) (details can be found in *SI Material and Methods*) and were hybridized to our custom-made EBV tiling microarray. The array contains four identical sets of 285 PCR fragments, each covering the entire genome of the B85.8 strain of EBV. To analyze the kinetics of methylation pattern formation, the adjusted data were processed so that the ratio of a constant point of reference, Cp, was set to 1 in each experiment, because the Cp promoter region is free of methylated CpGs even in established lymphoblastoid cell lines (21 and references therein), in contrast to EBV genomes in the memory B cell reservoir in vivo (22). The results show that CpGs in EBV genomic DNA are entirely or mostly unmethylated early after infection but become increasingly methylated in resident EBV DNA of latently infected lymphoblastoid cell lines over time. The results of one of two biological repeats are shown. (*C*) CpG methylation does not correlate with the occurrence of CpG dinucleotide motifs. The *x*-axis shows the ratio of MeDIP-enriched EBV DNA versus input DNA of cells 12 weeks p.i. as in (*B*, *Top*). The *y*-axis shows the number of CpG dinucleotide pairs that occur in each of the 285 tiling PCR fragments of the EBV microarray. The regression curve was flat, its coefficient of determination was extremely low ($R^2 = 5 \times 10^{-6}$), and the correlation coefficient was <0.003. The results indicate no correlation between the degree of CpG methylation in latently infected B cells and the occurrence of CpGs in EBV DNA.

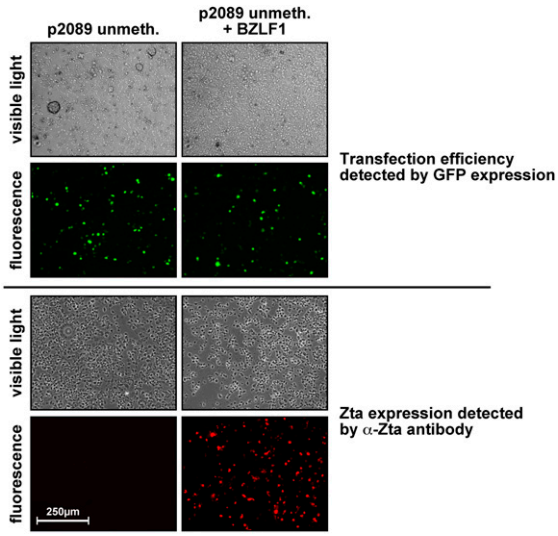

Fig. S4. DNA transfection controls of maxi-EBV DNA in HEK293 cells. Wild-type EBV DNA p2089 purified from *E. coli* (~180 kbp in size) encoding *GFP* was transfected into HEK293 cells. About 10% of the cells are transfected, as indicated by the appearance of GFP-positive cells. The transfection efficiency of p509 plasmid DNA (~7.8 kbp in size) encoding *BZLF1* is superior in transient transfection experiments, as expected. Immunofluorescence staining indicates that p2089 DNA does not express its endogenous *BZLF1* gene efficiently upon transient transfection, but cotransfection of the BZLF1 expression plasmid p509 leads to its detectable expression.

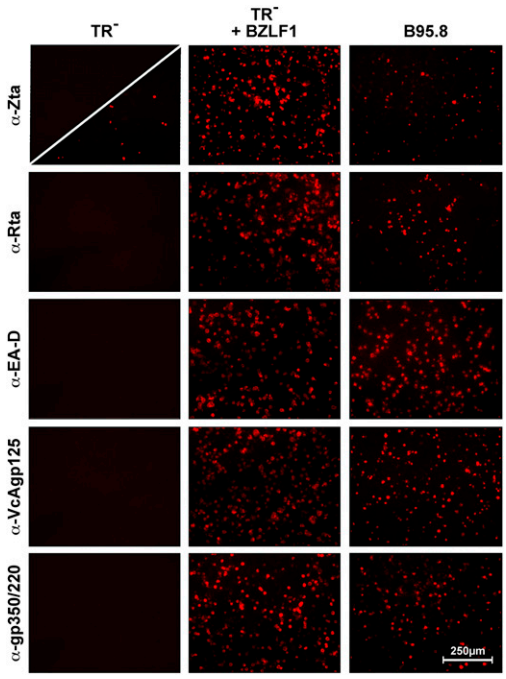

Fig. S5. Positive and negative controls of the immunostaining shown in Fig. 3*B.* The panel on the top left indicates two different levels of spontaneous expression of *BZLF1*, which depends on the culture conditions of the TR^- cell line.

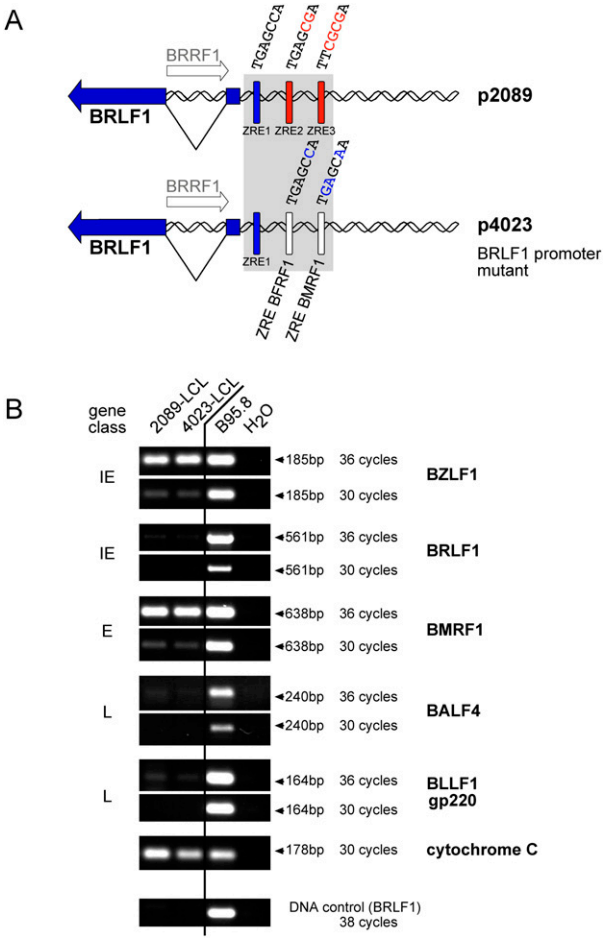

Fig. S6. A *BRLF1*-promoter mutant EBV reveals a block of lytic gene expression downstream of *BRLF1*. (*A*) The *BRLF1* promoter (shaded area) and its three ZREs are shown together with the two exons of *BRLF1*. ZRE2 and -3 indicated in red encompass CpG motifs and were mutated at single-nucleotide residues to obtain CpG-free ZREs as present in the promoters of *BFRF1* (24) and *BMRF1* (25, 26), respectively. (*B*) Two of three ZREs within the *BRLF1* promoter were replaced by CpG-free ZREs, which are strongly bound by Zta. Primary human B cells were infected with this EBV *BRLF1*-promoter mutant (4023) or wild-type EBV (2089) with the same MOI. Expression of representative viral lytic genes was determined by semiquantitative RT-PCR 5 days p.i., when early *BZLF1* expression reached its peak (Fig. 1). The expression of *BRLF1* was not affected by the point mutations in its promoter. No detectable differences in the levels of an early (*BMRF1*) and two late (*BALF4*, *BLLF1*) viral genes were found when primary cells were infected with either 4023 or 2089 virus. This result, together with others described in detail in the *SI Results*, indicate that the methylation of EBV's DNA necessary for induction of EBV's lytic cycle is at ZRE sites other than those in the promoter of the *BRLF1* gene.

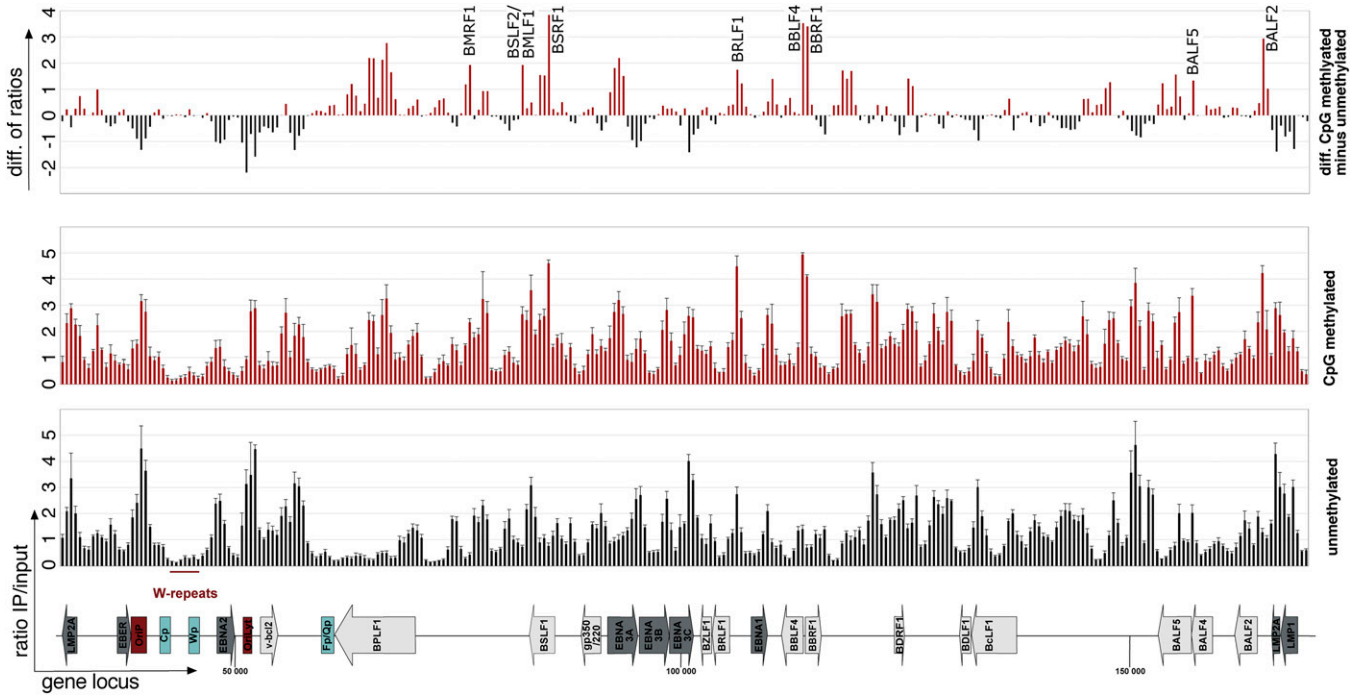

Fig. S7. Microarray analysis of in vitro BZLF1-selected EBV DNA. Results of microarray hybridizations with in vitro enriched unmethylated (*Bottom*) and CpG-methylated (*Middle*) wild-type p2089 EBV DNA by GFP:BZLF1. The combined information of both results (*Top*) is expressed as the difference between methylated and unmethylated EBV DNA for each probe. Mean and SD are shown. Acronyms indicate the location of selected EBV genes and their promoters. The graphic below the microarray results shows the blueprint of the EBV genome and selected genes for orientation together with the nucleotide coordinates of the B95.8 strain.

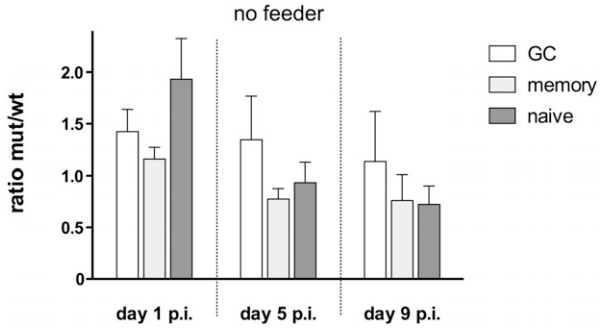

Fig. S8. Expression of *BRLF1* provides no proliferative advantage to naïve, memory, or germinal center (GC) B cells. Subpopulations of primary B cells from three different donors were sorted and infected with wild-type (wt) EBV or the *BRLF1*-knockout (mut) EBV (7). Proliferation of infected cells was measured by FACS as described (4), and the ratios of the number of viable, GFP-positive cells infected with the *BRLF1*-knockout EBV (mut) versus the number of cells infected with wild-type EBV (wt) were calculated as indicated. Means and standard deviations of three independent experiments are shown. In a two-way ANOVA analysis no statistical significance was found in any B-cell subpopulations.

Table S1. PCR primers and their characteristics

Name of primer pair	Gene	Product size (nt)	Forward primer sequence (5–3′) backward primer sequence (5′–3′)	Nucleotides	Temperature (°C)	GC (%)
b-act	*β-actin*	900/463*	CACCCTGTGCTGCTCACCGAGGCC ACCGCTCGTTGCCAATAGTGATGA	24 24	69.3 62.4	70.8 50.0
BRLF1/GI	*BRLF1*	561	CAGGGGGGAGTCCAGATTC ATAGCAGCGGTCCACCAAG	19 19	53.0 52.5	63.2 57.9
EA-D/EG	*BMRF1*	638	AGCGACAGGCTGAGGAACG CTGGAGTGGCTGGGAATGG	19 19	55.2 55.1	63.2 63.2
gp220/ F23B25	*BLLF1*	164	GTCACCTGTGTTATATTTTCACCACTTTC CCTACCAACCTCACCGCACC	29 20	56.7 56.6	37.9 65.0
ICYC1/F1B1	Cytochrome C-1	450/178*	CCTGGTGGGCGTGTGCTAC CAATGCTCCGTTGTTGGCAG	19 20	56.1 56.4	68.4 55.0
VcA/F8B11	*BALF4*	240	CTGGGGGGTGAGGAAGTCG CAACACAACCGTGGGCATAGAG	19 22	56.5 56.8	68.4 54.5
Z/F2B2	*BZLF1*	310/185*	AAGCCACCCGATTCTTGTATCG CAGCAGCAGCAGTGGTGTTTG	22 21	56.9 56.7	50.0 57.1

*Lengths of the PCR products differ depending whether unspliced or spliced transcripts were detected.

Table S2. Ectopic expression of the de novo methyltransferase *M.SssI* and *BZLF1* supports synthesis of EBV progeny in HEK293 cells transiently transfected with wild-type EBV DNA (p2089)

p2089 maxi-EBV DNA	BZLF1 expression plasmid	M.SssI expression plasmid	Median # wells (range)
Unmethylated	+	–	0 (0/0)
	+	+	45 (18/48)

Data are from seven independent experiments.