Original Paper

Intervirology

Intervirology 2009;52:8–16
DOI: 10.1159/000210833

Received: September 12, 2008
Accepted: February 18, 2009
Published online: April 7, 2009

Epstein-Barr Virus *WZhet* DNA Can Induce Lytic Replication in Epithelial Cells in vitro, although *WZhet* Is Not Detectable in Many Human Tissues in vivo

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Key Words
Epstein-Barr virus · *WZhet* · Recombination · Lymphoma · Carcinoma · Replication · Viral load

Abstract
Objective: *WZhet* is a rearranged and partially deleted form of the Epstein-Barr virus (EBV) genome in which the *BamH1W* region becomes juxtaposed with and activates *BZLF1*, resulting in constitutive viral replication. We tested whether *WZhet* induces viral replication in epithelial cells, and we studied its prevalence in a wide range of lesional tissues arising in vivo. ***Methods:*** A quantitative real-time PCR assay targeting EBV *WZhet* DNA was developed to measure this recombinant form of the EBV genome. ***Results:*** *WZhet* DNA was undetectable in any of 324 plasma or paraffin-embedded tissue samples from patients with EBV-associated and EBV-negative disorders. These included specimens from patients with Hodgkin or non-Hodgkin lymphoma, post-transplant lymphoproliferation, nasopharyngeal or gastric adenocarcinoma, and infectious mononucleosis. However, *WZhet* DNA was detected in vitro in EBV-infected AGS gastric cancer cells. Additionally, transient transfection of infected AGS gastric cancer cells showed that viral replication could be induced by a *WZhet* plasmid. ***Conclusion:*** This is the first evidence that *WZhet* induces the EBV lytic cycle in an epithelial cell line. Our negative findings in natural settings suggest that *WZhet* is a defective viral product that thrives in the absence of a host immune system but is rarely present in vivo.

Introduction

Several forms of cancer have been linked to Epstein-Barr virus (EBV) by virtue of the presence of viral DNA or gene products in tumor cells [1–6]. The gold-standard assay for defining EBV in a tumor is EBV-encoded RNA *(EBER)* in situ hybridization in paraffin-embedded tissue sections, because *EBER* stains are sensitive and specific

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for latent EBV infection, and the virus can be localized to specific cell types by microscopic interpretation. However, the sensitivity of *EBER* histochemical stains has been called into question by investigators who showed, by molecular and immunohistochemical assays, that EBV was present in some *EBER*-negative tumors [3, 5, 7–10]. These data are controversial because some laboratory assays for EBV are not designed to sensitively and specifically detect tumor-associated EBV or to distinguish it from the latent EBV normally carried by most humans in a subpopulation of benign B lymphocytes. In addition, the 'hit-and-run' hypothesis has been proposed to explain some of these discrepancies. This hypothesis states that segments of EBV DNA or EBV gene products are undetected in certain cells or entire tumors because portions of the EBV genome have been lost, rearranged, and/or integrated into host chromosomal DNA [3, 6]. For example, if the *EBER* gene were deleted during tumorigenesis, then the resulting tumor might be falsely diagnosed as EBV-negative if *EBER* hybridization were the only assay used to test for presence of the virus.

One well-known rearranged and partially deleted form of the EBV genome is called *WZhet*. This defective EBV genome forms self-contained replicons that multiply independently of the standard EBV genome and are capable of cell-to-cell spread [11]. In this abnormally rearranged form of EBV, large segments of viral DNA have been deleted and the remaining four portions, two from the terminal ends and two from the center of the genome, are rearranged, resulting in the juxtaposition of the otherwise distant *BamH1W* and *BamH1Z leftward reading frame 1 (BZLF1)* regions, thus the name *WZhet* [6]. This rearrangement results in constitutive expression of the immediate early gene protein, BZLF1, which triggers active viral replication of any intact wild-type EBV DNA that resides in the same cell [12]. Other retained sequences include the origin of viral replication, those sequences that are required for packaging viral DNA into virions, and open reading frames for *BZLF1*, *BMLF1*, and *BILF1* which encode proteins that transactivate lytic gene expression [6]. It is postulated that *WZhet* is not just an artifact but rather a novel mechanism by which the virus can switch, via recombinatorial events, from latent to lytic phases of its life cycle [13].

WZhet DNA was first identified in the Burkitt lymphoma cell line, P3HR-1 [14–17], and it has since been detected in oral hairy leukoplakia, thymic cancer, Burkitt lymphoma, Hodgkin lymphoma, and idiopathic pulmonary fibrosis [3, 6, 13, 18–20]. *WZhet* DNA is thought to be unstable in cells lacking wild-type EBV genome [13], but *WZhet* has also been detected in *EBER*-negative tumors suggesting that it could exist in cells lacking the gold-standard marker of latent EBV infection [3, 6, 13, 16, 18, 19].

In this study, a novel quantitative real-time PCR assay targeting EBV *WZhet* DNA was developed in order to measure levels of *WZhet* in human tissues including benign and malignant biopsy tissue and plasma from patients with various diseases, some of which are EBV-related. To show that *WZhet* could induce lytic viral replication, *WZhet* plasmid was transfected into an infected gastric epithelial cell line and levels of viral DNA were subsequently measured using Q-PCR.

Methods

Biopsy Tissue Samples

Paraffin-embedded tissue blocks (n = 287) were retrieved from the archives of our clinical and research laboratories under a protocol approved by our Institutional Review Board. Cases were selected to represent various normal and disease conditions, some of which were known to be EBV-related diseases. *EBER*-positive tissues included AIDS lymphoma (n = 5), AIDS-related Hodgkin lymphoma (n = 3), Hodgkin lymphoma (n = 5), non-Hodgkin lymphoma (n = 2), nasopharyngeal carcinoma (n = 2), and post-transplant lymphoproliferative disorder (PTLD; n = 3). *EBER*-negative tissues included AIDS lymphoma (n = 7), AIDS-related Hodgkin lymphoma (n = 1), and Hodgkin lymphoma (n = 4).

Other paraffin-embedded tissues included 113 gastric adenocarcinomas (11 *EBER*-positive and 102 *EBER*-negative); 89 gastritis samples from adults (n = 6) or from adults who also had gastric adenocarcinoma elsewhere in the stomach (n = 33) or from children ranging in age from 2 months to 20 years (n = 50); normal gastric mucosa from patients undergoing gastric bypass surgery (n = 5); normal colon (n = 9); meningioma (n = 11); Crohn's disease (n = 9), and ulcerative colitis (n = 11). Histological sectioning and DNA extraction was performed using a manual xylene-based protocol as previously described [21]. DNA from paraffin-embedded oral hairy leukoplakia tissue (n = 8) was provided by Dr. Jennifer Webster-Cyriaque at University of North Carolina at Chapel Hill.

Total EBV viral load was measured by Q-PCR using a TaqMan probe targeting the *BamH1W* viral sequence as previously described [21]. A Q-PCR for the human *APOB* gene served to insure that at least 50 cells were represented in each reaction. In addition, *APOB* levels served as a normalizer by which to compare viral concentrations across tissues of diverse size by reporting viral loads as a ratio of copies of EBV DNA per 100,000 cells as previously described [21, 22].

EBV-Encoded RNA in situ Hybridization

In every tumor tissue, *EBER* in situ hybridization was performed using either a manual method with fluorescein-labeled oligonulceotide *EBER* probe and the Super-Sensitive Poly-HRP ISH Non-Biotin Detection Kit (Biogenex, San Ramon, Calif.,

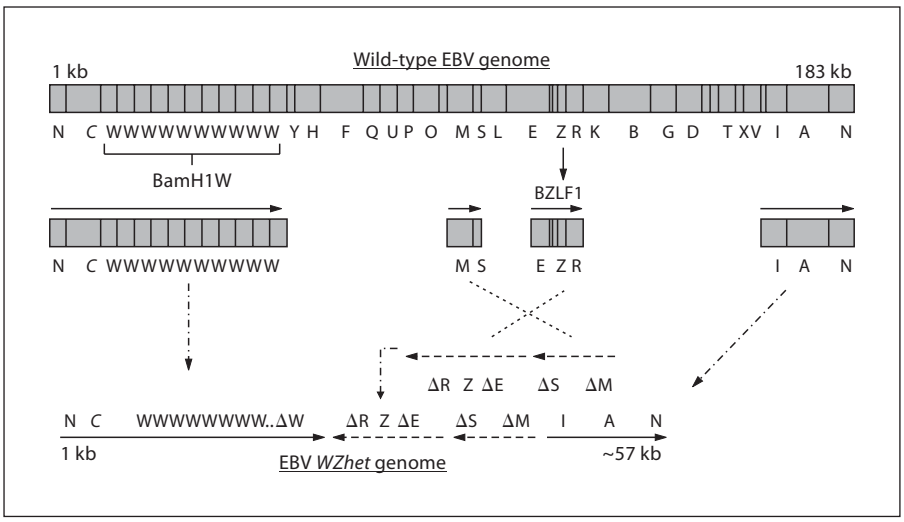

Fig. 1. Map of the *WZhet* partially deleted and rearranged EBV genome. *WZhet* is approximately 57 kb in length and is composed of four previously non-adjacent segments of EBV DNA. Two larger segments come from the terminal regions and two smaller segments from the center portion of the viral genome. The two smaller segments rearrange order with inversion of *BZLF1*. *BamH1W* and *BZLF1* regions, normally separated by about 55 kb, become juxtaposed. This structural change is thought to result in constitutive activation of the immediate early gene, *BZLF1*, which encodes the major viral protein transactivating lytic gene expression.

USA) with methyl green counterstain, or by an automated method using fluorescein-labeled *EBER* probe on the Ventana Benchmark in situ hybridization system. Oligo(d)T probe served as an RNA preservation control in both assays. A tumor was considered *EBER*-negative if *EBER* staining was undetected or was expressed only in benign-appearing lymphoid cells, and *EBER*-positive if the signal was localized to neoplastic cells.

Plasma and Blood Samples

Frozen and fresh human plasma samples (n = 37) having high EBV viral loads were obtained from the archives of our clinical and research laboratories. EBV viral load had been measured by Q-PCR using a TaqMan probe targeting the *BamH1W* viral sequence as previously described [21]. These samples came from patients with histologically proven AIDS-related lymphoma (n = 10), post-transplant lymphoproliferative disorder (n = 6), or infectious mononucleosis (n = 12), and healthy blood donors (n = 9). Infectious mononucleosis was diagnosed by heterophile antibody-positivity among clinically ill patients seen by the University Student Health Service.

DNA was extracted as previously described [21]. Briefly, total DNA was purified from 200 μl of plasma using the QIAmp Blood Kit (Qiagen, Inc., Valencia, Calif., USA) following the manufacturer's protocol with elution into 50 μl of AE buffer (Qiagen). Prior to extraction, the plasma was spiked with 2 μl of IPC (TaqMan® Exogenous Internal Positive Control DNA, Applied Biosystems (ABI), Foster City, Calif., USA) and this was subsequently amplified to control for the efficacy of extraction and amplification.

WZhet *Quantitative Real-Time Polymerase Chain Reaction Assay*

The DNA sequence for EBV 'BamHI segment heterogeneous (het) 2.7 DNA' (*WZhet*; NCBI Accession No. M20820) served as the basis for designing PCR primers and a TaqMan probe spanning nucleotide numbers 1507–1655 using Primer Express 2.0 software (ABI): The forward primer targets *BZLF1*: 5′-GAC ATT CAT CAT TTA GAA ATG TAT CCA-3′; The reverse primer targets *BamH1W*: 5′-TTC TCA GTC CAG CGC GTT TA-3′; TaqMan probe targets *BamH1W*: 5′(6FAM)TGG TCC CCC TCC CTA GAA CTG AC(TAMRA)-3′. The Q-PCR assay targets a 149-bp region of the *WZhet* DNA sequence as depicted in figure 1.

The standard for quantitating *WZhet* was a pCMVneo/WZhet plasmid that was linearized using KpnI restriction enzyme (New England Biolabs, Beverly, Mass., USA) and purified by standard ethanol precipitation. Concentration was determined by UV spectrophotometry and a stock standard was prepared at a concentration of 1 ng/ml. Since each *WZhet* plasmid has one copy of the *BZLF1* gene, the copy number of *WZhet* DNA in the stock aliquots could be determined using *BZLF1* Q-PCR as previously described [21] on serial 10-fold dilutions ranging from 10^{-2} to 10^{-7} ng/μl of stock. Microsoft Excel was used to obtain a linear trend line for the average *BZLF1* viral loads for each serial dilution plotted against the concentration of *WZhet* plasmid. It was determined that the optimal standard curve for our assay relied on six serial dilutions ranging in concentration from 5×10^{-2} to 5×10^{-7} ng, which corresponds to 1,000,000 to 10 *WZhet* DNA copies per PCR. In any given run, this curve was considered acceptable if a difference of 3.3 ± 0.3 cycles was demonstrated between each of the 10-fold dilutions, and if the correlation coefficient was at least 0.99.

Q-PCR for *WZhet* was performed and products were detected using an ABI Prism 7900 or 7500 Real-Time PCR instrument and Sequence Detection System software. Thermocycling conditions were: 50° for 2 min, 95° for 10 min, 95° for 15 s, and 60° for 1 min for 40 cycles. Each 25-μl reaction contained: 1× TaqMan® Universal Master Mix, forward and reverse primer (15 pmol each), and TaqMan® probe (10 pmol). DNA template volume was 1 μl for paraffin-embedded tissues, and 5 μl for plasma samples. To check for amplicon contamination, every run contained at least two 'no template' controls in which nuclease-free water was substituted for template. Quantification results for experimental samples were extrapolated from the standard curve. Experimental samples were run in duplicate and a mean *WZhet* load was calculated.

Transient Transfection and Virion Measurements in a Cell Line Model

The AGS gastric cancer cell line (ATCC CRL-1739) was EBV-infected as previously described using a recombinant form of the EBV B95.8 strain [23–25]. This recombinant virus, courtesy of Dr. Henri-Jacques Delecluse of Heidelberg, encodes green fluorescence protein and a hygromycin B resistance gene to facilitate selection of infected cells [23]. The infected clone, AGS-B95-HygB, was grown in RPMI 1640 (Gibco, Invitrogen) supplemented with 100 μg/ml hygromycin B (HygB) as well as 10% fetal bovine serum and antibiotics. The level of infection was determined using a battery of six EBV Q-PCR assays performed as previously described on extracted DNA [21, 26], and by *EBER* in situ hybridization performed on formalin-fixed, paraffin-embedded cell pellets that were prepared from the cell line. Immunohistochemical stains for EBV lytic proteins (BZLF1 and BMRF1) were performed using a BioGenex Super Sensitive non-biotin horseradish peroxidase detection system and BMRF1 antibody (1:200 dilution; Research Diagnostics, Inc.) or BZLF1 antibody (1:20 dilution; Argene).

To evaluate the extent to which *WZhet* could induce lytic viral replication, the *WZhet* plasmid was transfected into AGS-B95-HygB gastric cancer cells and then several markers of viral replication were measured. To accomplish this, cells were plated at a density of 1.5×10^5 cells/well. Once they reached 60–80% confluence, transient transfection of *WZhet* plasmid was performed. The transfection mixture containing Lipofectamine™ Reagent (Invitrogen), 1.5 μg of *WZhet* plasmid DNA (not linearized), and 100 μl of OptiMEM (Gibco) was incubated for 30 min at room temperature. During preparation of the transfection mixture, the cells were starved in serum-free RPMI 1640 medium for 30 min at 37° in a humidified atmosphere with 5% CO_2. The starvation medium was replaced with serum-free medium and the Lipofectamin™/*WZhet* transfection mixture. The plates were incubated for 2 h at 37° in a humidified atmosphere with 5% CO_2. To show that hygromycin B did not affect the transfection rate or the level of viral replication in the AGS-B95-HygB cells, the transfection was performed again in the presence of 100 μg/ml hygromycin B. Following transfection, the cells were propagated in growth media for 2 days and DNA was harvested from supernatants and cells.

Before harvesting DNA from supernatant fractions, virions were separated from naked viral DNA based on differential degradation by DNase, as previously described [22]. In short, DNA unprotected by a capsid was targeted for degradation by treating the supernatant (200 μl) with 25 μl DNase (DNase RQ1, Promega, Madison, Wisc., USA) for 1 h followed by an additional 25 μl DNase for a second hour at 37°. Stop Buffer (Promega) and incubation at 56° served to inactivate the enzyme. DNA was isolated from cell pellets and from supernatants (DNase-treated and untreated) using the QiaAmp Mini DNA Kit (Qiagen). *WZhet* Q-PCR was used to verify that *WZhet* plasmid DNA was transfected into the cells. *BamH1W* Q-PCR assay was used to measure EBV DNA, and the percentage of EBV DNA remaining post-DNase treatment (e.g. virion DNA) was calculated. The human *APOB* Q-PCR assay was used as a control to ensure that the DNase enzyme was working efficiently.

Results

Assay Linearity, Sensitivity, and Reproducibility

A quantitative real-time PCR assay targeting EBV *WZhet* DNA was developed for sensitive and precise measurement of *WZhet* DNA. Using linearized plasmid as the standard, the assay was efficient as shown by a cycle difference of 3.3 or 3.4 for each serial 10-fold dilution (representing slope of the standard curve). In addition, the assay was linear across 5 orders of magnitude as shown by a correlation coefficient >0.99 (representing the linearity of the standard curve). The *WZhet* Q-PCR assay was sensitive, detecting as few as 10 copies per PCR of this defective form of the EBV genome. Reproducibility of the assay was examined by replicate testing of the linearized *WZhet* DNA standards at each of the six serial dilution standards in duplicate on 10 different days, and the average cycle threshold value was used to calculate a coefficient of variance of 2%, suggesting that the assay is quite precise and reproducible.

Undetectable EBV WZhet *DNA in vivo*

A series of 287 paraffin-embedded tissue samples (39 *EBER*-positive lesions and 248 *EBER*-negative tissues) were tested for EBV *WZhet* DNA by Q-PCR. These included a wide variety of lesions whose histopathologic classification and *EBER* status are listed in tables 1 and 2. For simplicity, only a sampling of *EBER*-negative tumors and various benign tissues are shown in table 2. Surprisingly, *WZhet* DNA was not detected by the *WZhet* Q-PCR assay in any of the tissues examined in this study. A control Q-PCR targeting the human *APOB* gene verified that DNA had been extracted from each sample and was available for amplification. The *APOB* assay also serves as a gauge of the number of cells assayed from each tissue block, and in this series there was a range from 94 to 69,917 cells (mean 12,800) per tissue that was represented in the amplification reactions. These findings suggest that *WZhet* is not commonly found in the spectrum of lesions that was examined in vivo regardless of whether the lesion contains latent EBV (e.g. various *EBER*-positive lymphomas and carcinomas) or lytic EBV (e.g. oral hairy leukoplakia).

To evaluate whether *WZhet* DNA was circulating in the bloodstream as cell-free DNA, a series of 37 plasma samples were assayed for *WZhet* by Q-PCR. None of the 37 plasmas had detectable *WZhet* DNA to a sensitivity of 10 copies per PCR. These plasma samples were from patients with various EBV-related disorders (n = 28) and from healthy blood donors (n = 9). These samples were

Table 1. *WZhet* and total EBV (BamH1W) levels in *EBER*-positive paraffin-embedded tissues

Case No.	Diagnosis	Control *APOB*	EBV DNA copies/100,000 cells	
			BamH1W	*WZhet*
1	Oral hairy leukoplakia	1,243	2,624,346	0
2	Oral hairy leukoplakia	18,382	1,142	0
3	Oral hairy leukoplakia	5,782	2,292	0
4	Oral hairy leukoplakia	2,433	15,231	0
5	Oral hairy leukoplakia	3,635	1,018	0
6	Oral hairy leukoplakia	3,128	3,608,042	0
7	Oral hairy leukoplakia	1,180	297	0
8	Oral hairy leukoplakia	14,732	6,889,764	0
9	AIDS lymphoma	915	125	0
10	AIDS lymphoma	11,409	48,540	0
11	AIDS lymphoma	2,228	631,239	0
12	AIDS lymphoma	209	2,392	0
13	AIDS lymphoma	592	349,493	0
14	Hodgkin, AIDS-related	3,633	2,697	0
15	Hodgkin, AIDS-related	979	3,575	0
16	Hodgkin, AIDS-related	4,032	397	0
17	Hodgkin lymphoma	7,666	75	0
18	Hodgkin lymphoma	467	14	0
19	Hodgkin lymphoma	1,803	86	0
20	Hodgkin lymphoma	18,058	2,226	0
21	Hodgkin lymphoma	94	0	0
22	Non-Hodgkin lymphoma	1,501	41,173	0
23	Non-Hodgkin lymphoma	6,178	58,514	0
24	Nasopharyngeal carcinoma	6,935	250,397	0
25	Nasopharyngeal carcinoma	69,917	23,047	0
26	Post-transplant LPD	6,559	15,612	0
27	Post-transplant LPD	2,733	5,269	0
28	Post-transplant LPD	2,778	25,054	0
29	Gastric adenocarcinoma	2,974	44,956	0
30	Gastric adenocarcinoma	271	200,000	0
31	Gastric adenocarcinoma	2,896	90,919	0
32	Gastric adenocarcinoma	1,198	193,823	0
33	Gastric adenocarcinoma	2,734	144,221	0
34	Gastric adenocarcinoma	80,033	117,755	0
35	Gastric adenocarcinoma	8,770	130,935	0
36	Gastric adenocarcinoma	23,209	70,757	0
37	Gastric adenocarcinoma	38,723	112,875	0
38	Gastric adenocarcinoma	5,623	44,496	0
39	Gastric adenocarcinoma	2,812	64,047	0

Table 2. *WZhet* and total EBV *(BamH1W)* levels in selected paraffin-embedded *EBER*-negative tumors and normal/benign tissues

Case No.	Diagnosis	Control *APOB*	EBV DNA copies/100,000 cells	
			BamHIW	*WZhet*
40	AIDS lymphoma	32,544	34	0
41	AIDS lymphoma	12,626	8	0
42	AIDS lymphoma	50,966	57	0
43	AIDS lymphoma	41,928	0	0
44	AIDS lymphoma	14,180	21	0
45	AIDS lymphoma	18,094	6	0
46	AIDS lymphoma	60,348	5	0
47	Hodgkin, AIDS-related	11,660	17	0
48	Hodgkin lymphoma	1,551	0	0
49	Hodgkin lymphoma	643	0	0
50	Hodgkin lymphoma	4,203	0	0
51	Hodgkin lymphoma	1,182	0	0
52	Gastric adenocarcinoma	4,109	0	0
53	Gastric adenocarcinoma	3,289	9	0
54	Gastric adenocarcinoma	1,875	0	0
55	Gastric adenocarcinoma	11,655	17	0
56	Gastric adenocarcinoma	2,148	93	0
57	Gastric adenocarcinoma	45,494	4	0
58	Gastric adenocarcinoma	10,035	0	0
59	Gastric adenocarcinoma	13,933	1	0
60	Gastric adenocarcinoma	1,744	0	0
61	Gastric adenocarcinoma	225	222	0
62	Gastric adenocarcinoma	19,857	29	0
63	Gastric adenocarcinoma	23,224	142	0
64	Adult gastritis	5,726	271	0
65	Adult gastritis	7,612	66	0
66	Adult gastritis	1,445	0	0
67	Adult gastritis	6,529	3	0
68	Adult gastritis	1,299	108	0
69	Pediatric gastritis	401	0	0
70	Pediatric gastritis	558	18	0
71	Pediatric gastritis	136	37	0
72	Pediatric gastritis	317	0	0
73	Crohn's disease	1,238	0	0
74	Crohn's disease	9,278	10	0
75	Crohn's disease	277	0	0
76	Crohn's disease	5,568	0	0
77	Ulcerative colitis	7,035	71	0
78	Ulcerative colitis	748	2,406	0
79	Ulcerative colitis	33,311	0	0
80	Ulcerative colitis	6,488	77	0
81	Meningioma	27,956	0.4	0
82	Meningioma	54,614	0	0
83	Normal gastric mucosa	5,150	0	0
84	Normal gastric mucosa	11,326	0	0
85	Normal colon mucosa	7,686	3	0
86	Normal colon mucosa	9,853	0	0

Table 3. EBV DNA and human DNA control levels in the AGS-B95-HygB gastric cancer cell line before and after *WZhet* plasmid transfection

	APOB control (cellular genomes, n)	*BamH1W* Q-PCR (EBV genomes, n)	*WZhet* Q-PCR (rearranged EBV genomes, n)
Before *WZhet* plasmid transfection			
Cells	90,359	385,904	1,476
Supernatant	987	86,460	2
After *WZhet* plasmid transfection			
Cells	67,602	460,256	4,182,500
Supernatant	791	207,984	1,020,427

All values represent the number of genomes (i.e., cellular, EBV, or rearranged EBV) per PCR.

assayed for *WZhet* DNA and were also tested for traditional EBV viral load by *BamH1W* Q-PCR. The EBV-related disorders included AIDS lymphoma (n = 10), EBV-positive post-transplant lymphoproliferative disorder (n = 6), and infectious mononucleosis (n = 12) cases having plasma *BamH1W* viral loads ranging from 0 to 81,000 copies/ml for the AIDS lymphoma cases, from 308 to 119,000 copies/ml for post-transplant lymphoproliferative disorders, and from 49 to 13,000 copies/ml for infectious mononucleosis cases. In contrast, the healthy blood donors (n = 9) had no detectable plasma *BamH1W* DNA to a sensitivity of 5 copies of EBV DNA per PCR, which translates to no *BamH1W* DNA to a sensitivity of 250 copies/ml of plasma. To show that DNA was extracted from plasma and that no significant inhibitors were present, each plasma sample had been spiked with an exogenous non-human DNA prior to extraction, and this spiked DNA was consistently amplified among the 37 plasma samples. The absence of *WZhet* in all 37 plasma samples as well as all 287 biopsy tissues implies that *WZhet* is not commonly present in the spectrum of lesions evaluated in vivo even when EBV infection is abundantly present as shown by traditional *EBER* or EBV viral load assays.

Detection of WZhet *DNA in vitro*

The failure to detect *WZhet* DNA in any of the biopsy or plasma samples examined suggests that this defective form of EBV is rare in vivo. An alternative explanation for our failure to detect *WZhet* could be technical failure of our assay. In particular, the plasmid insert used for design and control of our assay may not represent the typical *WZhet* structure that is present in vivo even though it was cloned from the prototypic P3HR1 cell line which, in turn, was derived from a human lymphoma. It should be noted, however, that this plasmid amplified with all three of our Q-PCR assays (targeting *BZLF1*, *BamH1W*, and *WZhet)*, confirming these sequences were present.

To show that the *WZhet* plasmid could induce EBV replication in vitro, the plasmid was transfected into an EBV-infected gastric cancer cell line, AGS-B95-HygB, which was then examined for the effect on expression of replicative viral genes *(BZLF1 and BMRF1)* and on the level of intact virions in the supernatant. Before transfection was performed, baseline tests were performed on the AGS-B95-HygB using six EBV Q-PCR assays that target six disparate segments of the viral genome. The AGS-B95-HygB line had high viral load by all six Q-PCR assays. *WZhet* DNA was also detected by Q-PCR, suggesting that *WZhet* is produced in infected cells that are cultured in vitro where the human immune response is absent (table 3). Furthermore, *EBER* in situ hybridization showed *EBER* expression in 90% of the AGS-B95-HygB confirming that these cells were indeed infected. BZLF1 and BMRF1 histochemical stains showed that approximately 35% of cells expressed these replicative viral proteins at baseline.

Despite the baseline presence of *WZhet* DNA, a transient transfection was performed to determine if viral replication could be further induced by the *WZhet* plasmid. As shown in table 3, successful transfection of the *WZhet* plasmid occurred as shown by increased levels of *WZhet* by Q-PCR of DNA extracted from cell pellets. Total EBV genomes, as measured by *BamH1W* levels, likewise rose upon transfection, particularly in the supernatant where EBV levels rose more than 2-fold.

To determine if the viral DNA in the supernatant represented encapsidated virions, virion production was determined by measuring *BamH1W* load by Q-PCR in the supernatant before and after treating with DNase I en-

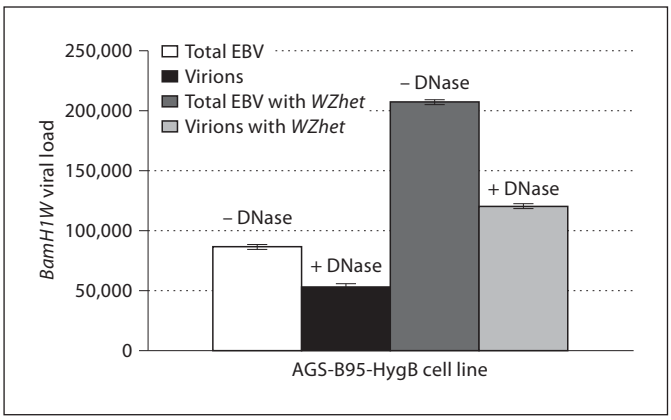

Fig. 2. EBV *WZhet* plasmid induces viral replication in AGS-B95-HygB gastric cancer cells. A large percentage of EBV DNA was not digested by DNase I in AGS-B95-HygB cell supernatant, suggesting active viral replication with virion production. *WZhet* plasmid transfection more than doubled the number of virions produced by AGS-B95-HygB cells.

zyme. Our prior validation work showed that the capsid protects virions from digestion by DNase I enzyme, so any detectable EBV DNA remaining in the supernatant post-digestion represents virion DNA. A substantial proportion of the viral DNA (57%) was DNase I-resistant, suggesting that virion production was induced by AGS-B95-HygB cells upon *WZhet* transfection (fig. 2). Interestingly, both naked EBV DNA and encapsidated EBV DNA rose in equal proportions after transfection, whereas human *APOB* levels did not rise, implying that the naked DNA is not derived from dying cells rather it appears to represent EBV DNA that failed to acquire a capsid. These findings demonstrate that the *WZhet* plasmid can induce viral replication when coexpressed with the wild-type EBV genome.

Discussion

In this study, there was no evidence that human tissues contain the *WZhet* form of the EBV genome when tested for by a novel Q-PCR assay. No *WZhet* DNA was detected in any of the benign (n = 134) or malignant (n = 153) paraffin-embedded tissues screened in our study, nor was it found in plasma samples from patients with EBV-related disorders (n = 28) or healthy controls (n = 9). This result implies that the rearranged, partially deleted *WZhet* DNA is rarely present in human tissues. This finding is important because it refutes the postulate that this atypical form of EBV DNA is commonly present in human tissues that were negative for EBV by more traditional laboratory tests. Furthermore, it implies that *WZhet* does not commonly coexist with the more traditional *EBER*-expressing viral genome in several types of lesions that have not been previously examined for presence of *WZhet*. Furthermore, it implies that *WZhet* is not commonly present in several types of EBV-related tumors that have not been previously examined for coexistence of *WZhet*.

Other novel aspects of this study were (1) the development and application of a real-time PCR assay to detect and quantify *WZhet*, and (2) the first demonstration that epithelial cells are susceptible to induction of lytic viral replication upon transfection by *WZhet*, whereas prior studies had shown this effect only in lymphoid cells. The rather modest extent of viral induction in AGS epithelial cells was similar to the modest degree of rise in EBV DNA reported when B95.8 lymphoid cell lines, which also harbor baseline viral replication, are induced to replicate further by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [27].

Our clinical findings are somewhat at odds with several previous studies that reported finding *WZhet* DNA in Burkitt lymphoma, oropharyngeal lesions, *EBER*-negative and *EBER*-positive Hodgkin lymphoma, thymic carcinoma, and idiopathic pulmonary fibrosis [6, 13, 15, 16, 19, 20]. In support of previous findings, Kelly et al. [13, 28] detected *WZhet* DNA in the peripheral blood and lung tissue of patients suffering from idiopathic pulmonary fibrosis, although the role of EBV in idiopathic pulmonary fibrosis remains uncertain [29]. Kelly et al. [13] also demonstrated that *WZhet* DNA was always associated with the presence of other EBV DNA, suggesting that the defective viral genome cannot exist without the standard EBV genome. It should be noted that the current study did not include pulmonary fibrosis and several other disease entities with which WZhet was previously associated.

In agreement with our study, Knecht and Odermatt [30] also found no detectable *WZhet* DNA in EBV-positive Hodgkin lymphoma, and the discrepancy with prior work was explained, at least in part, by a difference in the age of patients, since Gan et al. [6] had shown *WZhet* DNA on patients who were less than 21 years old at diagnosis whereas Knecht and Odermatt [30] studied adults. Loss of defective genomes was hypothesized to correlate with differences in immunity between children and adults [30].

Technical factors could also explain the observed discrepancies. Prior studies generally used PCR followed by Southern blot analysis, and ours is the first study to use quantitative PCR [14, 15, 17, 20]. One cannot exclude the possibility that naturally occurring WZhet has alternative recombination junctions or polymorphisms that render it non-amplifiable by our assay, particularly since our assay amplifies a segment of only 149 bp flanking the recombination site within the P3HR1 standard. In support of the validity of our new assay is the detection of *WZhet* in infected AGS cells as well as detection of prototypic WZhet derived from P3HR1 cells. Assay specificity was assured by application of an internal TaqMan probe that simultaneously permits quantification of accumulating amplicons against a standard prepared from a plasmid insert of the prototypic P3HR1 Burkitt lymphoma cell line. Our assay was sensitive to as few as 10 copies of *WZhet* per PCR, which is probably similar to the sensitivity levels achieved in prior studies in which PCR followed by Southern blot analysis was used, although prior work was done on fresh or frozen tissue as opposed to the paraffin-embedded tissue that we targeted. To assure that amplifiable DNA was recovered, we applied a second PCR of the human *APOB* gene that verified the efficacy of DNA extraction and amplification from each paraffin-embedded tissue.

WZhet has been shown to be unstable in uninfected cell lines as well as in the P3HR1 Burkitt cell line. So, even if *WZhet* were formed in vivo, it could be lost, particularly if it activates viral replication and thereby elicits a host immune response [31–35]. In this regard, one might expect to see it more commonly in immunocompromised individuals where uncontested survival of infected cells appears to be more frequent. However, *WZhet* was not detectable in AIDS or allogenic transplant patients, even those who harbored EBV-related malignancies and who were clearly unable to control the proliferation of latently infected cells. Furthermore, *WZhet* was not detected in our post-transplant lymphoproliferations or in two ulcerative colitis tissues focally expressing the lytic viral proteins BZLF1 and BMRF1 (data not shown), suggesting that *WZhet* is not the explanation for activated infection in these lesions. Finally, *WZhet* was not detected in primary EBV infection even though infectious mononucleosis patients are only just developing the antibody and cell-mediated immune reactions that will protect them from rampant latent and lytic EBV infection for the duration of their lives. In contrast to the in vivo samples, *WZhet* DNA was detected in cultured AGS-B95-HygB gastric cancer cells. This supports the hypothesis that *WZhet* DNA occurs in the absence of selective pressures enforced by a host immune system.

In summary, our findings support a role for EBV *WZhet* DNA in inducing viral replication in vitro. However, our inability to detect EBV *WZhet* DNA in any of 324 plasma or tissue samples, including those with latent or lytic EBV infection, suggests that either *WZhet* is not formed in the spectrum of lesions examined in vivo or that it exists only transiently. The findings serve to emphasize that in vitro EBV infection in cell culture does not necessarily mimic in vivo EBV infection in a living host.

Acknowledgements

This study was sponsored by grants from the National Cancer Institute (CA107966) and from the NCI Environmental Pathology Training Program (T32-ES07017). The authors thank our colleagues who provided samples for this study, including Jennifer Webster-Cyriaque, DDS, Bruce Israel, MD, Douglas Morgan, MD, MPH, Ricardo Dominguez, MD, Leigh B. Thorne, MD, You-Jun Shen, MD, Mari Mino-Kenudson, MD, and Gregory Lauwers, MD. We also thank the Anatomic Pathology Core Laboratory of the UNC Department of Pathology and Laboratory Medicine for technical support.

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