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ORIGINAL PAPER

BZLF1 transcript variants in Epstein–Barr virus-positive epithelial cell lines

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Abstract

Epstein–Barr virus (EBV) is a widely prevalent pathogen currently infecting over 90% of the human population and is associated with various lymphomas and carcinomas. Lytic replication of EBV is regulated by the expression of the immediate-early genes BZLF1 and BRLF1. In B lymphocytes, BZLF1 transcripts have been shown to be processed to a fully spliced form, as well as zDelta, a spliced variant containing only the first and third exons. While splice variants have been reported in nasopharyngeal carcinoma biopsies, alternative splicing of BZLF1 in EBV-positive epithelial cell lines has not yet been characterized. In this study, we identified the consistent expression of three distinct BZLF1 transcripts in the EBV-positive epithelial cell lines D98/HR1, AGS-BDneo, and AGS-BX1. These BZLF1 transcripts consisted of not only the normally spliced variant but also a completely unspliced and a spliced variant containing exons one and three only. In contrast, we detected only the normally spliced version of the BZLF1 transcript in B-cell lines (B95-8, IM-9, Raji and Daudi). Previous work has also demonstrated that inhibition of the mTOR pathway, via rapamycin, altered total levels of BZLF1 transcripts. We examined the production of specific transcript variants under rapamycin treatment and found that rapamycin alters the production of transcripts in a cell-type, as well as transcripts in variant-type, manners. The expression of these transcript variants may play a role in modulating the replication cycle of EBV within epithelial cells.

Keywords Epstein–Barr virus · BZLF1 · Transcript · mTOR · Splicing

Introduction

Epstein–Barr virus (EBV) is a human herpesvirus and is present in over 90% of human adults. Commonly associated with the disease infectious mononucleosis, EBV infection has also been associated with a variety of cancers such as Burkitt's lymphoma, nasopharyngeal carcinoma, and gastric carcinoma [1]. While the mechanism by which EBV promotes such cancers is still under investigation, it is thought to arise from the expression of lytic- and latent-associated proteins, and their interactions with host cell proteins [1, 2].

Upon initiation of lytic replication, expression of the two EBV immediate-early genes, BZLF1 and BRLF1, leads to a cascade of viral gene expression which culminates in the assembly and release of infectious virions [3]. The BZLF1 protein is known to bind and transactivate the BRLF1 promoter, as well as its own promoter [3, 4]. This has led to a wide array of research investigating how BZLF1 regulation alters both lytic replication as well as cellular processes. One such point of regulation is the alternative splicing of the BZLF1 transcript. EBV and other members of *Herpesviridae* are known to initiate splicing of both viral and cellular transcripts, resulting in alternate proteins [5].

The BZLF1 coding region consists of three exons and two introns [6]. Within B lymphocytes, a spliceoform of BZLF1 resulting from the removal of exon 2 has been reported and is thought to aid in the initiation of latency [6]. Additionally, a fusion of the BRLF1 and BZLF1 transcripts results in the expression of the protein RAZ, which is thought to downregulate BZFL1 expression through the heterodimerization and sequestration of BZLF1 protein [7]. Though these alternative splicing events are well-established in B-lymphocyte lines, little is known about the splicing of BZLF1 transcript within EBV-positive epithelial cells. Earlier work in nasopharyngeal carcinoma biopsies have shown that, in addition to the

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fully spliced transcript, EBV also produced two unspliced BZLF1 variants, as well as a variant containing exons one and three only [8].

The expression of BZLF1 and BRLF1 is dependent upon cellular transcription factors binding to and regulating their promoters. BZLF1 has binding sites for several cellular transcription factors, including Sp1/Sp3, MEF2D, CREB, SMADs, ATF family members, and YY1—a transcription factor whose activity can be regulated by mTOR kinase [3, 9–11].

We previously demonstrated that inhibition of mTORC1 (the mechanistic target of rapamycin complex 1) by rapamycin treatment suppressed lytic replication of EBV in B cells, while promoting EBV lytic replication in epithelial cells [12]. Each correlated with a loss or gain of BZLF1 and BRLF1 proteins, respectively, in a cell-type dependent manner. Under rapamycin conditions, BZLF1 and BRLF1 protein levels decreased in B cells, while they increased in epithelial cells. These protein quantities also correlated with a reduction of BZLF1 and BRLF1 transcripts in B cells, and an increase in these transcripts in epithelial cells, after rapamycin treatment [12].

The protein levels of the immediate-early proteins BZLF1 and BRLF1 are important for regulating EBV lytic replication. We previously determined that overall BZLF1 and BRLF1 transcript and subsequent protein levels are dependent, at least in part, upon the mTOR pathway [12]. However, we did not know if alternative splicing of transcripts played a role in how mTOR inhibition altered overall BZLF1 transcript levels.

Here we demonstrate that the EBV-positive epithelial cells D98/HR1, AGS-BDneo, and AGS-BX1 produced three transcript variants of BZLF1. The relative abundance of each form was dependent upon the induction status of the cells, as well as upon treatment with the mTORC1 inhibitor rapamycin. These results differed from the single BZLF1 transcript identified in EBV-positive B cell lines. This report describes the first analysis of transcript variants in commonly used EBV-positive epithelial cell lines. Differentially expressed spliceoforms may subsequently play a role in the regulation of BZLF1 activity and consequently the EBV lytic cycle.

Materials and methods

Cell culture, induction, and rapamycin treatment

AGS-BX1 and AGS-BDneo gastric carcinoma cells (both EBV-positive) were grown in supplemented Ham's F12 medium (Ham's F12, 10% fetal bovine serum, fungicide, streptomycin, and penicillin) under G418 selection (500 µg/mL). D98/HR1 cells were grown in supplemented DMEM medium (DMEM, 10% fetal bovine serum, fungicide, streptomycin, and penicillin). Viral induction was achieved by treating cells with 5 ng/mL TPA and 0.75 mM sodium butyrate for 24 h. EBV-positive B lymphocytes (B95-8, IM-9, Raji and Daudi) were grown in supplemented RPMI media (RPMI, 10% fetal bovine serum, fungicide, streptomycin, and penicillin). Viral induction was achieved by treating cells with 20 ng/mL TPA and 3 mM sodium butyrate for 24 h, and was confirmed by monitoring BZLF1 expression. Cells were treated with rapamycin (Sigma-Aldrich) or the vehicle DMSO for 24 h prior to induction of cells.

RNA isolation, RT-PCR, qRT-PCR

Total RNA was isolated with a QIAGEN RNeasy® kit 24 h post-induction. Cytoplasmic RNA was isolated with a modified QIAGEN protocol, by first lysing cells with buffer cold RLN buffer (50 mM Tris·Cl, pH 8.0, 140 mM NaCl, 1.5 mM MgCl2, 0.5% (v/v) Nonidet P-40), centrifuging the lysate at 300 g for 2 min at 4 °C, and subjecting the supernatant (cytoplasmic fraction) to the remaining QIAGEN RNeasy kit protocol. 50 ng of the purified RNA then underwent RT-PCR using 50 pmol each of forward and reverse primers (see Table 1) (MWG-Biotech) and the Promega Access RT-PCR Introductory System. RT-PCR products were separated on 0.8% agarose gels containing 0.01% ethidium bromide and visualized with a ChemiDoc-XRS. Quantitative RT-PCR was performed using the Applied Biosystems Power SYBR Green RNA-to-C_T 1-Step kit. RNA harvested from Raji or AGS-BX1 cells was analyzed with variant-specific primers (Table 1), using both experimental and technical triplicates,

Table 1 Primer sequences

Primer name	Sequence (5′→3′)	For:
Z-forward	CACCATGATGGACCCAAACTCGACT	All BZLF1 transcripts
Z-reverse	GAAATTTAAGAGATCCTCGTCTAA	All BZLF1 transcripts
ZTop-forward	TTTTTGTCCGCAGGTGGCTTCTGCC	Exons 1, 2, 3/unspliced only
ZMiddle-forward	AGCACTACCGTGAGGTGGCTTCTGC	Exons 1, 2, 3/spliced only
ZBottom-forward	ACAGCCAGAATCGGTGGCTTCTGCC	Exons 1, 3/spliced only
GAPDH-forward	CTCCTCCTGTTCGACAGTCAGC	Control
GAPDH-reverse	CCCAATACGACCAAATCCGTT	Control

and the levels held relative to a GAPDH-positive control (primers listed in Table 1).

DNA sequencing

In order to sequence the RT-PCR products, DNA bands were purified from agarose gels using a QIAGEN Minielute® gel extraction kit and sent to Eurofins for sequencing. The resulting DNA sequences were analyzed using BLAST nucleotide and compared to the published EBV genome sequence. Genomic BZLF1 sequences for Raji and AGS-BX1 cells were obtained by first isolating the EBV genome using a modified Hirt procedure [13] and purifying the genomes using a QIAGEN® Plasmid Maxi prep column. The extracted genomes were used as a template for PCR with the same forward and reverse primers as used for RT-PCR of the BZLF1 transcripts (Table 1). The resulting PCR products were similarly separated, isolated, and sequenced using the same protocol as used for the RT-PCR products.

Statistical methods

Error was calculated using standard error and significance was tested using a two-tailed, homoscedastic student *t* test. *P* values ≤ 0.05 were considered significant. Each data point represents at least $n = 3$.

Results

EBV-positive epithelial cells contain alternatively spliced transcripts of BZLF1

To determine whether BZLF1 transcript splice variants existed in EBV-positive cell lines, we performed RT-PCR with primers designed to amplify BZLF1 from the 5′ end of exon 1 to the 3′ end of exon 3. In analyzing the RT-PCR products within lytic EBV-positive epithelial cells lines (AGS-BX1, AGS-BDneo, and D98/HR1), three distinct BZLF1 bands were consistently observed. These bands were measured at approximately 945 bp, 738 bp, and 633 bp in length [AGS-BX1 results, representative of all three cell lines examined, are shown in Fig. 1a, lanes 1, 2, 3, and 4 (diamonds)]. Upon sequencing of the three resulting cDNAs, it was found that these sequences corresponded to an unspliced BZLF1 transcript, a fully spliced BZLF1 transcript, and a fully spliced BZLF1 transcript missing exon 2 (100% identity for each). Additionally, a fourth band was periodically found, at approximately 861 bp, representing an intermediate transcript between the unspliced and fully spliced transcripts (Fig. 1a, lane 4, denoted with a square). As only properly spliced mRNAs are exported from the nucleus into the cytosol for translation, we sought to examine which transcript variants were present in the cytoplasm, and would thus be available for translation. We isolated cytoplasmic RNA from uninduced or induced EBV-positive epithelial cells (AGS-BX1 shown) and performed RT-PCR as above. Figure 1c shows that the two forms present in the cytoplasm are the fully spliced transcript and the spliced version missing exon 2, as expected.

Analysis of the RT-PCR products from the B-lymphocyte cell lines (Raji, B95-8, Daudi and IM9 cells), however, revealed only a single, fully spliced BZLF1 transcript, as previously reported (Raji results, representative of all four cell lines examined, are shown in Fig. 1b) [6]. This transcript corresponded to the sequences found in the published EBV genome, and contained exons 1, 2, and 3 (Fig. 1b).

General increases in BZLF1 spliceoforms following induction of lytic replication

To determine how induction of lytic replication affected transcript levels, BZLF1 transcripts from uninduced and induced EBV-positive epithelial cells (AGS-BX1) were analyzed by qRT-PCR to examine the abundance of each transcript variant during lytic replication. Uninduced AGS-BX1 cells exhibit a low level of lytic replication. Upon induction of lytic replication, the three transcript variants (unspliced, spliced, and alternatively spliced) displayed + 14.8-, + 18.8-, and + 7.32-fold increases, respectively, relative to uninduced levels (Fig. 2a).

As previous work demonstrated that inhibition of mTORC1 via rapamycin treatment increased overall BZLF1 transcript levels in epithelial cells [12], we next treated AGS-BX1 cells with 5 nM rapamycin prior to induction and measured the transcript variants via qRT-PCR. The fold-changes due to induction alone were further increased by rapamycin treatment, leading to + 28.2- (unspliced), + 29.6- (spliced) and + 15.3-fold (alternatively spliced) increases (Fig. 2b). Taken together, these three variants all displayed increases in transcript levels upon lytic reactivation. Furthermore, inhibition of the mTOR pathway led to an even greater increase in abundance for all three BZLF1 transcript forms. This result concurs with our previous report that demonstrated that rapamycin treatment of EBV-positive epithelial cells caused an overall increase in BZLF1 transcript levels [12].

The same analysis was performed for the Raji B-cell line, although only the fully spliced BZLF1 variant produced a consistent threshold value during qRT-PCR, supporting our earlier RT-PCR analysis (seen in Fig. 1b). This transcript increased in abundance as expected following lytic induction (a 10.3-fold change) (Fig. 2c). This increase was suppressed by rapamycin treatment, yielding only a 5.51-fold increase after lytic induction, a 47% decrease as compared to induction only (Fig. 2c). This result supports earlier studies

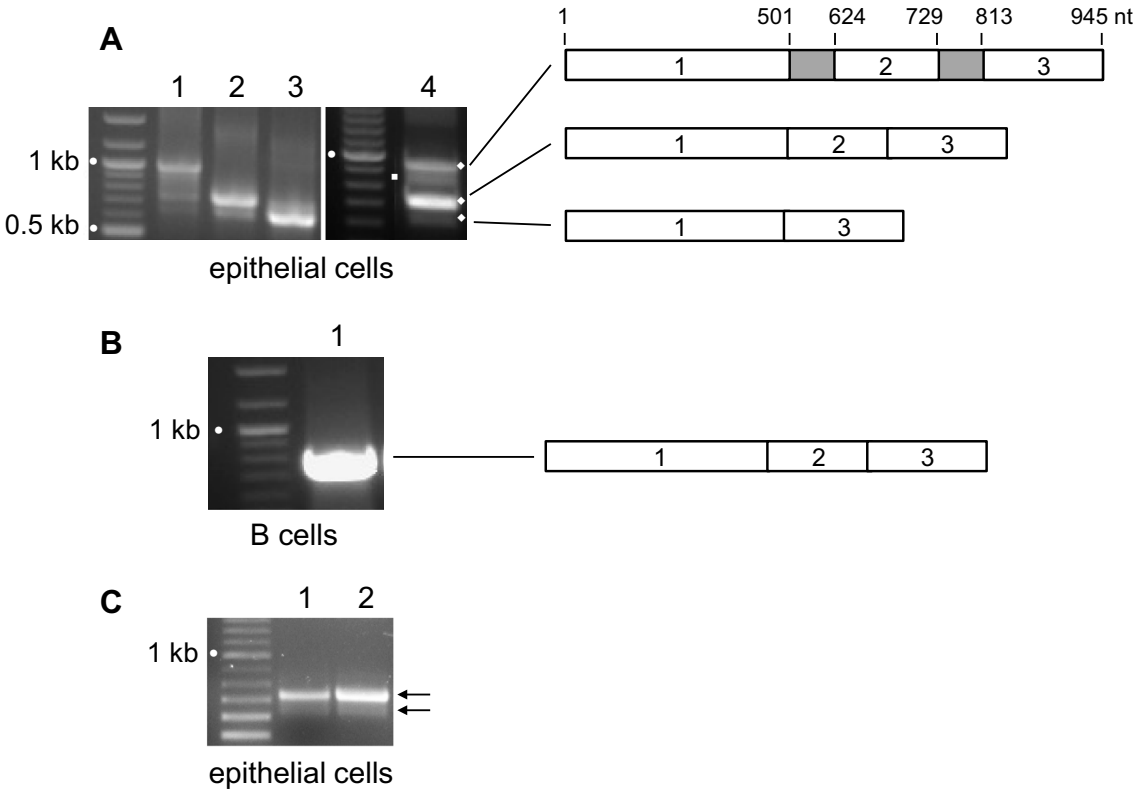

Fig. 1 EBV-positive epithelial cells produce BZLF1 transcripts variants not detected in EBV-positive B cells. **a** EBV-positive epithelial cells (AGS-BX1 shown) were induced into lytic replication and RT-PCR performed with primers (Z-forward and Z-reverse, see Table 1) that detected three BZLF1 reproducible transcript variants (lane 4, bands denoted with diamonds on right side). Each variant could be amplified separately with primers specific to each variant [ZTop-forward (lane 1), ZMiddle-forward (lane 2), or ZBottom-forward (lane 3) primers, each with the Z-reverse primer, see Table 1]. A partially spliced variant also can be seen in lane 4, denoted by a square to the left side of lane 4. The exon structure for each variant is also presented (not to scale). **b** EBV-positive B cells (Raji shown) were induced into lytic replication and RT-PCR performed with primers (Z-forward and Z-reverse) that detected one BZLF1 splice form (lane 1). The exon structure for this variant is presented and represents the fully spliced variant. **c** AGS-BX1 cells were either uninduced (lane 1) or induced (lane 2) into lytic replication and RT-PCR performed on cytoplasmic RNA with the same primers as in A. Arrows refer to the two spliced forms present

which demonstrated that BZLF1 transcript levels decreased in rapamycin-treated B cells, relative to untreated cells [12].

To determine if mTORC1 inhibition led to variant-specific differences, we performed RT-PCR on RNA isolated from uninduced, induced, or rapamycin-treated and induced AGS-BX1 cells and examined the abundance of each splice variant. Figure 3 shows each variant in terms of its percentage in relation to total transcript present per condition. While induction or rapamycin treatment did not lead to percentage changes of the unspliced variant, induction did cause a statistically significant decrease in the percentage of the alternatively spliced variant (compared to what was present in uninduced cells), thus generating more of the normally spliced transcript. Rapamycin treatment of induced cells also caused a statistically significant increase in the normally spliced transcript abundance; however, the decreased abundance of the alternatively spliced version was not as significant, compared to uninduced. Taken together, these results suggest that induction favors a decrease in production of the alternatively spliced transcript and that the addition of rapamycin more strongly favors production of the full-length spliced transcript, both would presumably lead to the enhanced production of functional BZLF1 protein.

Discussion

It was observed that expression of the immediate-early gene BZLF1 in EBV-positive epithelial cells resulted in the generation of three transcript variants (an unspliced variant, a spliced variant with all three exons, and a spliced variant with exons one and three only). The same transcript forms were found in three different EBV-positive epithelial cell lines. The same analysis within EBV-positive B cells yielded only one transcript (the spliced variant containing all three exons). It is important to note that

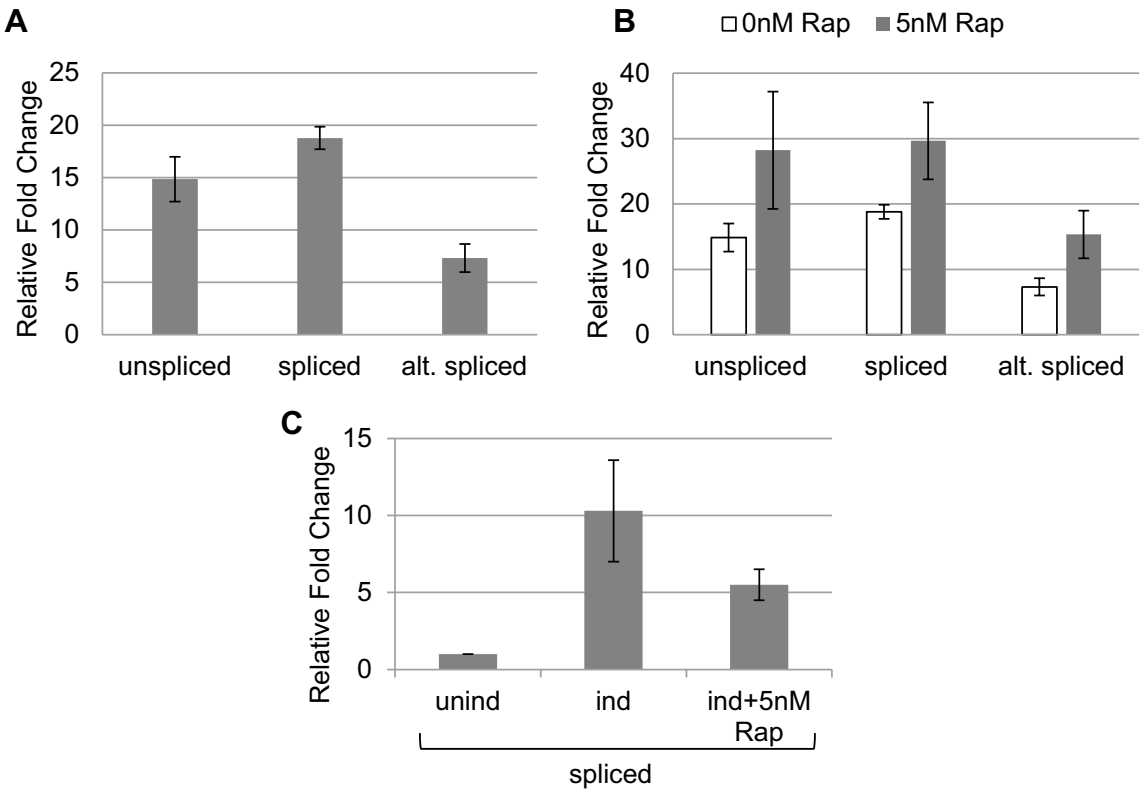

Fig. 2 Abundance of BZLF1 splice variants changes following lytic induction and rapamycin treatment. **a** qRT-PCR was performed on RNA isolated from uninduced and induced AGS-BX1 cells, with primers designed to specifically detect the unspliced, spliced, and alternatively spliced variants (see Table 1). The fold-changes for each variant, relative to uninduced, are presented. **b** qRT-PCR was performed on RNA isolated from uninduced, induced, and rapamycin-treated/induced AGS-BX1 cells, with the variant-specific primers as described for A. The fold-changes for each variant, relative to uninduced, are presented. **c** qRT-PCR was performed on RNA isolated from uninduced, induced, and rapamycin-treated/induced Raji cells, with the variant-specific primers as described for A. The fold-changes for each variant, relative to uninduced, are presented (only the fully spliced variant amplified). Results are based on three biological replicates, each with three technical replicates. *Rap* rapamycin, *ind* induced, *alt. spliced* alternatively spliced

the original primers we used to find these variants corresponded to the 5′ end of exon one and the 3′ end of exon three; thus, any variants that do not share both of these ends would not have been identified in our analysis.

The transcript identified by our analysis in B cells, after treating cells with TPA and sodium butyrate for 24 h, was also found to be the predominant transcript by Lau et al. [6], when they treated B95-8 cells with TPA and phosphonoacetic acid (PAA, which blocks viral DNA replication), or when they treated Akata cells with anti-immunoglobulin for 4 h. Lau et al. found that longer treatments with TPA alone (3 days) or anti-immunoglobulin (20 h) led to the production of ZΔ [6], the variant that we referred to as "alternatively spliced" in our epithelial cells. Our shorter induction time may account for the fact that we only saw one transcript variant in B cells; however, the same induction time was sufficient to produce the transcript variants, including alternatively spliced/ZΔ, in epithelial cells. The transcript variants we found in epithelial cells are similar to those found in NPC biopsies [8]; our findings represent the first such transcript variants found in stable EBV-positive epithelial cell lines.

EBV-positive B cells are tightly latent, with induction causing a large increase in BZLF1 transcript levels (as seen here) and protein levels. The EBV-positive epithelial cells used in this study are not so tightly latent, however, with a low level of lytic activity (also seen with low levels of lytic proteins produced in [12]). Here we see that induction caused a large increase in transcription of BZLF1 within these cells, for all transcript variants; the largest increase after induction was for the spliced variant containing all three exons.

Our previous work demonstrated that inhibition of mTOR via rapamycin treatment affected lytic replication in EBV-positive cells by either causing a decrease in overall BZLF1 transcript levels in B cells (thus decreasing lytic replication in these cells) while simultaneously increasing overall BZLF1 transcript levels in epithelial cells (thus increasing lytic replication in these cells) [12]. These results were confirmed in the present study, at

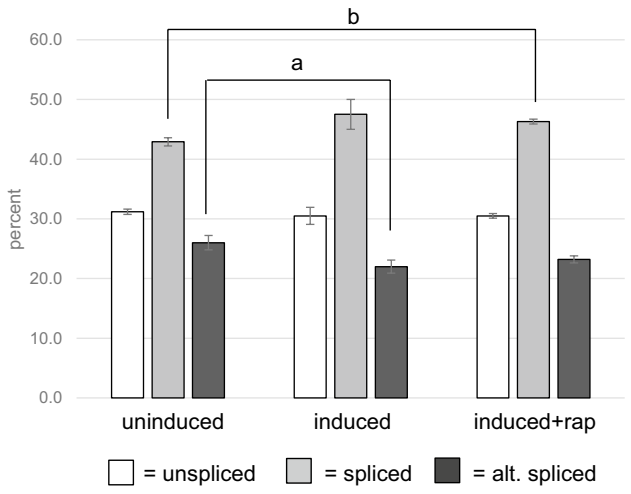

Fig. 3 Induction and rapamycin treatment of EBV-positive epithelial cells alter the stoichiometry of transcript variants. RT-PCR was performed on RNA isolated from uninduced, induced, or rapamycin-treated/induced AGS-BX1 cells with primers (Z-forward and Z-reverse, see Table 1) that detected all three BZLF1 transcript variants. After gel electrophoresis, Image J was used to quantify the resulting DNA bands. The percentage of each variant was calculated and is presented. $N=3$. a indicates statistical significance ($P<0.05$) between the uninduced and induced alternatively spliced variants; b indicates statistical significance ($P<0.05$) between the uninduced and rapamycin-treated/induced normally spliced variants. *Rap* rapamycin, *alt. spliced* alternatively spliced

the individual transcript variant level. As expected, the B-cell-specific BZLF1 transcript decreased in abundance by rapamycin treatment, while the levels of each variant increased by rapamycin treatment in epithelial cells. Of note, induction and rapamycin treatment both resulted in a greater presence of the full-length spliced transcript, in relation to the other two transcript types.

In terms of functionality for each transcript variant, the spliced transcript containing all three exons, found in all cell lines tested, would provide the mRNA template for production of the wild-type BZLF1 protein. This protein, which has domains for transactivation from exon 1, DNA binding from exon 2, and dimerization from exons 2/3, would provide full BZLF1 protein function. At present, the roles of both the unspliced variant and the alternatively spliced variant (missing the DNA binding domain completely) are unknown, but likely act to regulate the lytic cycle. Splicing introns out of the transcript, which is necessary for mRNA export from the nucleus, may be a point of regulation in terms of BZLF1 gene expression and protein production. The unspliced variant may function as a repressor of latency in epithelial cells, by inhibiting latency-associated proteins or by promoting the constitutive activation of lytic machinery. The spliced variant missing the DNA binding domain may make a protein that still forms a heterodimer with wild-type BZLF1 protein, thus regulating BZLF1 protein activity; this was also speculated by Lau et al. [6].

Overall, we have identified splice variants of BZLF1 that occur in three different stable EBV-positive epithelial cell lines. These variants differ from what has been observed in stable EBV-positive B cells (especially under short induction times), and likely tailor lytic replication within epithelial cells.

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Author contributions JN conceived of experiments; JN and AA carried out experiments; AA and JN wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent There were no human participants in this study.

Research involving human and animal participants This article does not contain any studies with human participants or animals performed by any of the authors.

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