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The Epstein-Barr virus miR-BHRF1 microRNAs regulate viral gene expression in *cis*

Brigid Chiyoko Poling, Alexander M. Price[1], Micah A. Luftig, Bryan R. Cullen*

Department of Molecular Genetics & Microbiology and Center for Virology, Duke University Medical Center, Durham, NC, USA

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ABSTRACT

The Epstein-Barr virus (EBV) miR-BHRF1 microRNA (miRNA) cluster has been shown to facilitate B-cell transformation and promote the rapid growth of the resultant lymphoblastoid cell lines (LCLs). However, we find that expression of physiological levels of the miR-BHRF1 miRNAs in LCLs transformed with a miR-BHRF1 null mutant (Δ123) fails to increase their growth rate. We demonstrate that the pri-miR-BHRF1-2 and 1–3 stem-loops are present in the 3'UTR of transcripts encoding EBNA-LP and that excision of pre-miR-BHRF1-2 and 1–3 by Drosha destabilizes these mRNAs and reduces expression of the encoded protein. Therefore, mutational inactivation of pri-miR-BHRF1-2 and 1–3 in the Δ123 mutant upregulates the expression of not only EBNA-LP but also EBNA-LP-regulated mRNAs and proteins, including LMP1. We hypothesize that this overexpression causes the reduced transformation capacity of the Δ123 EBV mutant. Thus, in addition to regulating cellular mRNAs in *trans*, miR-BHRF1-2 and 1–3 also regulate EBNA-LP mRNA expression in *cis*.

1. Introduction

Epstein Barr Virus (EBV) is a double-stranded DNA virus of the γ-herpes subfamily that infects > 90% of the global population. EBV is the causative agent of various malignancies including diffuse large B cell lymphoma (DLBCL), Burkitt lymphoma, post-transplant lymphoproliferative disorder (PTLD) and nasopharyngeal carcinoma. Additionally, EBV infection of naïve young adults results in infectious mononucleosis. During primary infection, EBV infects resting B cells and establishes a latency stage III infection, which is associated with DLBCL and PTLD. During latency III, EBV-infected B cells express the full repertoire of latency proteins (EBNA-LP, EBNA1, 2, 3A, 3B, 3C, LMP1 and 2A/B) as well as non-coding RNAs, including the EBV-encoded small RNAs (EBERs) and several microRNAs (miRNAs). In the laboratory, EBV latency III can be modeled by infection of primary human B cells to generate lymphoblastoid cell lines (LCLs) (Rickinson and Kieff, 2007).

EBNA-LP and EBNA2 are the first viral proteins expressed after B cell infection, through activation of the W promoter (Wp) by B cell specific transcription factors (Bell et al., 1998; Kirby et al., 2000; Tierney et al., 2000). EBNA2 and EBNA-LP then activate the Cp promoter, to drive expression of EBNA-LP, EBNA-1, 2, 3A, 3B and 3C, as well as the LMP1 promoter (LMP1p) (Harada and Kieff, 1997; Nitsche et al., 1997; Peng et al., 2005; Sung et al., 1991; Woisetschlaeger et al., 1990). Because the viral mRNAs encoding the EBNA proteins are all transcribed from the same promoters, alternative splicing is critical for their appropriate expression. EBNA-LP is the first open reading frame (ORF) after both the Cp and Wp promoters and EBNA-LP expression is dependent on a complex pattern of alternative splicing. First, EBNA-LP mRNAs require splicing from the first exon of the Wp transcripts (W0) early after infection, or the second exon of the Cp transcripts (C2) late in infection, to provide the initial AT dinucleotide in the ATG codon that initiates EBNA-LP translation. Second, EBNA-LP mRNAs must splice internally into the W1 exon, at the W1' 3' splice site, to incorporate the G in the ATG. EBNA-LP mRNAs then continue splicing to integrate multiple copies of the W1/W2 exon repeats in frame, which results in EBNA-LP proteins with a range of different molecular weights. (See Fig. 4 for the exonic structure of EBNA-LP mRNAs) The B95-8 strain of EBV encodes 11 W1/W2 repeats and it was previously shown that incorporation of a minimum of five repeats is necessary for full EBNA-LP function (Tierney et al., 2011). EBNA-LP mRNAs then splice from the terminal W2 exon to the Y1 and Y2 exons, where the translational stop codon for EBNA-LP is located in the Y2 exon. Y2 can then be spliced to several different 3' exons, including those that encode EBNA1, 2, 3A, 3B, or 3C. If the ATG initiation codon for EBNA-LP is not generated by appropriate splicing at the 5' end of these viral transcripts, then a long 5'UTR for the mRNAs encoding the 3' EBNA1, 2, 3A, 3B, or 3C open reading frames (ORF) is generated instead (Rogers et al.,

* Corresponding author.
E-mail addresses: Brigid.poling@duke.edu (B.C. Poling), price.alexander.m@gmail.com (A.M. Price), micah.luftig@duke.edu (M.A. Luftig), Bryan.cullen@duke.edu (B.R. Cullen).
[1] Present address: Pathology and Laboratory Medicine, The Children's Hospital of Philadelphia, 3501 Civic Center Blvd, Philadelphia, PA 19104.

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1990).

In addition to proteins, EBV encodes two miRNA clusters named for their location in the EBV genome. The BamH1 fragment H rightward facing 1 (BHRF1) miRNA cluster consists of three pri-miRNA stem-loops, generating the four mature miR-BHRF1-1, 1–2, 1–2*, and 1–3 miRNAs (miR-BHRF1-2 gives rise to almost equal levels of two miRNAs derived from each arm of the pre-miRNA intermediate). The miR-BHRF1-1 miRNA is located in the promoter for the late BHRF1 mRNA while miR-BHRF1-2 and 1–3 are located in the BHRF1 mRNA 3' UTR. Additionally, the BamH1 fragment A rightward transcript (miR-BART) miRNA cluster is composed of 22 pri-miRNAs expressed from the various BART transcripts (Cai et al., 2006; Grundhoff et al., 2006; Pfeffer, 2004; Zhu et al., 2009). The miR-BHRF1 miRNAs are only expressed during latency III while the miR-BART miRNAs are expressed during all stages of latency, though at reduced levels in latency III (Cai et al., 2006; Zhu et al., 2009).

While the EBV-encoded miRNAs were discovered over a decade ago, and photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) has allowed for the identification of many of their mRNA target sites (Riley et al., 2012; Skalsky et al., 2012), elucidating their role during EBV-infection has remained challenging. So far, EBV-encoded miRNAs have been shown to play a role in immune evasion (Albanese et al., 2016; Tagawa et al., 2016; Xia et al., 2008), apoptosis (Kang et al., 2015; Lei et al., 2013), and inhibition of tumor suppressors (Bernhardt et al., 2016). Additionally, inactivation of the miR-BHRF1 miRNA cluster results in a reproducible and robust reduction in B cell transformation and LCL growth (Feederle et al., 2011a, 2011b; Haar et al., 2015; Seto et al., 2010), which suggested that the miR-BHRF1 miRNA cluster was promoting EBV transformation by downregulating the expression in *trans* of cellular mRNAs that inhibit this process. We tested this hypothesis by ectopically expressing the miR-BHRF1 miRNAs in LCLs generated using a previously described EBV mutant that lacks all these viral miRNAs (Δ123 LCLs). However, we were unable to rescue the slow growth rate of Δ123 LCLs, when compared to WT LCLs, even when all the miR-BHRF1 miRNAs were simultaneously expressed at or above physiological levels. Furthermore, we observed that the Δ123 LCLs expressed a greatly increased level of the EBV transcription factor EBNA-LP, when compared to WT LCLs derived from the same donor, that was again unaffected by ectopic expression of all three miR- BHRF1 miRNAs. Here, we present evidence in favor of the alternative hypothesis that the miR-BHRF1 miRNAs are in fact acting in *cis* to downregulate the expression of EBNA-LP to a level optimum for B cell transformation by inducing the cleavage of EBNA-LP mRNAs during miRNA excision.

2. Results

2.1. Trans-complementation does not rescue the growth of LCLs lacking the miR-BHRF1 microRNAs

Previous studies implicated the miR-BHRF1 miRNAs in enhancing the transformation of primary B cells into LCLs, as well as increasing their cell cycle progression, and this enhanced growth rate was maintained in established LCLs (Feederle et al., 2011a, b; Seto et al., 2010). Our original goal was to identify the individual miR-BHRF1 miRNA responsible for efficient growth of wildtype (WT) LCLs and we therefore first confirmed the slow growth phenotype of Δ123 LCLs. As shown in Fig. 1A, we indeed observed that WT LCLs grow approximately twice as fast as Δ123 LCLs derived from the same blood donor.

We next wanted to establish that the miR-BHRF1 miRNAs were indeed necessary and sufficient to rescue the slow growth phenotype of Δ123 LCLs by ectopic expression of the entire repertoire of miR-BHRF1 miRNAs. To this end, the miR-BHRF1-1, 1–2 and 1–3 pri-miRNAs were cloned into the antisense-oriented intron present in the Tet-inducible lentiviral miRNA expression vector pTREX, as this vector allows a consistently higher level of pri-miRNA expression when compared to conventional lentiviral vectors (Fig. 1B) (Poling et al., 2017). miR-BHRF1-1 and 1–3 are not well expressed from their natural pri-miRNA stem-loops, isolated from the EBV genome (Feederle et al., 2011a; Haar et al., 2015), so they were expressed from expression cassettes based on the human pri-miR-30 precursor, as previously described (Zeng et al., 2002). Additionally, miR-BHRF1-2/2* was cloned into pTREX using its natural stem-loop and flanking sequences. After selection of transduced cells by puromycin selection, and induction of miRNA expression by addition of doxycycline (Dox), we observed levels of expression of miR-BHRF1-1, 1–2, 1–2* and 1–3 in Δ123 LCLs that were similar to the expression levels observed in WT LCLs (Fig. 1C). Since miRNAs are thought to regulate mRNA expression in *trans* (Chong et al., 2010; Guo et al., 2010; Zeng et al., 2002), we hypothesized that ectopic expression of the miR-BHRF1 miRNAs in Δ123 LCLs would increase the growth of these mutant LCLs to WT levels. However, we did not observe any rescue of the slow growth phenotype ($p < 0.05$) (Fig. 1D). This suggested that the miR-BHRF1 miRNAs do not enhance LCL growth via their canonical role of mRNA downregulation in *trans* by translational inhibition or degradation of targeted mRNAs (Chendrimada et al., 2005; Huntzinger and Izaurralde, 2011; Liu et al., 2004; Meister et al., 2004).

2.2. The EBNA-LP protein and its co-transcriptional activity are upregulated in Δ123 LCLs

Because the slower growth of the Δ123 LCLs was not rescued by expression of the EBV miR-BHRF1 miRNAs in *trans*, we sought an alternative hypothesis to explain this phenotype. Previously, we reported that Δ123 LCLs display a marked upregulation in the expression of the EBNA-LP protein (Feederle et al., 2011a, b) and we confirmed that EBNA-LP levels in Δ123 LCLs are indeed between 5- and 20-fold higher than seen in control WT LCLs (Fig. 2A and B). This latter result cannot be explained based on the model that the miR-BHRF1 miRNAs are acting to downregulate EBNA-LP mRNA expression in *trans,* as there are no predicted target sites for these viral miRNAs in the EBNA-LP mRNAs, and moreover we and others failed to detect the binding of miR-BHRF1 miRNAs to EBNA-LP mRNAs using high throughput CLIP techniques (Riley et al., 2012; Skalsky et al., 2012). Indeed, we did not observe any reduction in EBNA-LP protein expression when the miR-BHRF1 miRNAs were ectopically expressed at physiological levels in Δ123 LCLs (Fig. 2C).

To determine if the increased expression of the EBNA-LP protein correlated with an upregulation of the Cp and LMP1 promoters, which are transcriptionally activated by EBNA-LP and EBNA2 (Harada and Kieff, 1997; Nitsche et al., 1997; Peng et al., 2005; Sung et al., 1991; Wang et al., 1990; Woisetschlaeger et al., 1990), we used 4-thiouridine (4SU) to label nascent transcripts for 60 min. Nascent transcripts were purified and their expression levels were then compared to total transcript levels to determine half-life, nascent transcript expression, and total transcript expression (Fig. 3A–C). As expected, we saw a significant increase in the level of nascent and total Cp driven and LMP1 transcripts in the Δ123 LCLs, when compared to control mRNAs encoding GAPDH, β-Actin, and SETDB1 (Fig. 3A and B), indicating that overexpression of EBNA-LP leads to a specific increase in EBNA-LP-mediated transcriptional activation of these viral mRNAs. In contrast, we did not see any major change in viral mRNA half-life when WT LCLs were compared to Δ123 LCLs (Fig. 3C). In addition to the upregulation of EBNA-LP regulated transcripts, we also saw a significant increase in both total and nascent Wp driven transcripts for some of our donors, which corroborates published findings (Feederle et al., 2011a, b).

In addition to an increase in EBNA-LP regulated viral transcripts, the encoded viral proteins were also upregulated in Δ123 LCLs. Thus, we saw an upregulation of LMP1 expression in all six donors tested and upregulation of EBNA2 in six out of eight donors tested (Fig. 3D and E). The EBNA2 exon (YH) is one of many exons that can be alternatively spliced in Cp and Wp-driven pre-mRNAs; however, during the