Epstein-Barr Virus BHRF1 Micro- and Stable RNAs during Latency III and after Induction of Replication[▽]

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Epstein-Barr virus (EBV) microRNAs miR-BHRF1-1, -2, and -3 have been detected in latency III-infected lymphoblasts, where they are encoded within EBNA transcripts (X. Cai, A. Schafer, S. Lu, J. P. Bilello, R. C. Desrosiers, R. Edwards, N. Raab-Traub, and B. R. Cullen, *PLoS Pathog.* 2:e23, 2006). In latency III-infected lymphoblasts, we have also identified a stable 1.3-kb RNA, which begins 3' to miR-BHRF1-1, includes the BHRF1 open reading frame, and ends near miR-BHRF1-2. This 1.3-kb RNA is the residue of Drosha cleavage of the BHRF1 microRNAs from EBNA transcripts. Early after induction of EBV replication in latency I-infected Akata lymphoblasts, BHRF1 spliced 1.4-kb mRNA accumulated along with low levels of miR-BHRF1-2 and -3 and a 0.9-kb Drosha or miR-BHRF1-2 cleavage product of BHRF1 mRNA. The turning on of latency III infection at 48 to 72 h after induction of EBV replication was associated with higher miR-BHRF1-1, -2, and -3 levels; accumulation of the 1.3-kb RNA residue in the nucleus; abundant BHRF1 spliced 1.4-kb mRNA in the cytoplasm; and more abundant 0.9-kb mRNA cleavage product in the cytoplasm. These findings implicate miR-BHRF1-2 in 3' cleavage of BHRF1 mRNA in the cytoplasm and Drosha in cleavage of latency III EBNA and EBV replication-associated BHRF1 transcripts in the nucleus.

In primary human infection, Epstein-Barr virus (EBV) replicates in the oropharyngeal epithelium (60) and establishes latency III infection in B lymphocytes (48, 62, 67). During latency III infection, the EBV Cp or Wp EBNA promoters drive expression of six nuclear antigen proteins (EBNA2, EBNA1P, EBNA3A, EBNA3B, EBNA3C, and EBNA1) from a single alternatively spliced transcript (37, 54). The latency III primary EBNA transcripts include many open reading frames (ORFs) expressed in EBV replication and are the likely source of the 3 BHRF1 micro-RNAs (miRNAs), which are encoded in an intron of most EBNA RNAs (11, 50). In latency III infection, EBV also expresses three integral membrane protein (LMP1, LMP2A, and LMP2B)-encoding mRNAs, two small RNAs (EBER1 and -2), BamHI A rightward transcripts (BARTs) (7, 15, 22, 37, 54, 56, 58), and 24 BART miRNAs (11, 29, 50). Latency III EBV gene expression causes continuous cell proliferation, which results in vitro in lymphoblastoid cell lines (LCLs) and in vivo in lymphoproliferative diseases (37, 54). Only BART miRNAs are detected in latency I- or II-infected cells, in which EBNA1 is the only EBNA expressed, from a promoter downstream of BHRF1. However, latency III-associated proteins are also detected with EBV replication in epithelial cells in vivo (68) or late in EBV replication in latency I-infected Burkitt's lymphoma (BL) cells (72).

miRNAs are small non-protein-coding 20- to 25-nucleotide (nt) single-strand RNAs, which negatively control protein expression, by inhibiting translation or cleaving of mRNA (2, 6). Most miRNAs are processed in the cell nucleus from RNA polymerase II capped and polyadenylated RNAs by the RNase

III enzyme Drosha to release 70-nt RNA hairpin pre-miRNA (6, 10, 39, 40). Pre-miRNAs are exported to the cytoplasm by exportin 5 (44, 70). In the cytoplasm, pre-miRNA can be cleaved by the RNase III enzyme Dicer (33) in association with TRBP (17) to generate 22-nt mature miRNAs (21). Mature miRNAs can be incorporated into RNA-induced silencing complexes (RISC) and can direct RISC to complementary mRNA targets (6). The targets of the EBV miRNAs are not known, although miR-BART2 may cleave EBV DNA polymerase (BALF5) mRNA (11, 26, 50).

The experiments reported here investigate EBV miR-BHRF1-1, -2, and -3, which are encoded within introns of EBNA transcripts and are expressed in latency III-infected lymphoblasts, but not in latency I-infected BL or latency II-associated nasopharyngeal carcinoma (NPC) cells (Fig. 1A) (11, 50). miR-BHRF1-1, -2, and -3 are likely to be Drosha-cleaved products of EBNA introns. BHRF1 is an antiapoptotic Bcl-2 homologue, which is expressed early in EBV replication (31). Although RNAs that initiate upstream of the BHRF1 promoter and include the BHRF1 ORF are detected in latency III-infected lymphoblasts (3, 49, 52, 58), BHRF1 monoclonal antibody (MAb) rarely detects BHRF1 protein until early in EBV replication, when BHRF1 abundantly accumulates (49). miR-BHRF1-1 overlaps with the BHRF1 mRNA transcriptional start site and is therefore not encoded in BHRF1 mRNA, whereas miR-BHRF1-2 and -3 are potentially encoded in the BHRF1 mRNA 3'-untranslated sequence and may therefore be expressed from early times in EBV replication (3, 19, 50, 52).

MATERIALS AND METHODS

Cell culture and antibodies. B95-8 (46, 59), IB4 (65), recently derived LCLs, NPC C666-1 (18), and EBV-infected or uninfected BJAB (25), BL41 (14), and Akata (63, 64) cells were maintained in RPMI 1640 medium (Gibco-BRL) supplemented with 10% or 20% Fetal Plex animal serum complex (Gemini). C15

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FIG. 1. miR-BHRF1-1, -2, and -3 and an unspliced 1.3-kb BHRF1 RNA are putative Drosha cleavage products from a latency III EBNA promoter transcript intron. (A) Schematic diagram showing the early virus replication BHRF1 promoter (BHRF1p) and 1.83-kb primary transcript, the 1.4-kb spliced BHRF1 mRNA, the far upstream latency III EBNA promoters (EBNACp) that transcribe 110 kb of the 170-kb EBV genome during latency III infection, the probes used in Northern blots (5', intron, ORF, and 3'), the AATAA poly(A) addition signal, and the positions of the primary miR-BHRF1-1 (miR1), miR-BHRF1-2 (miR2), and miR-BHRF1-3 (miR3) genes. 5'-RACE-determined sequences of early replication-associated 1.4-kb BHRF1 mRNA and 1.3-kb latency III-associated RNA are shown. Two open arrows represent primers Racer1 (R1) and Racer2 (R2) used in 5'-RACE. (B) Northern analysis of selected EBV miRNAs in total RNA from indicated cell lines and tumors. Positions of miRNAs (miR) and pre-miRNAs (pre-miR) are indicated. EBV+, EBV infected. Ethidium bromide-stained tRNAs are included as loading controls. (C) Northern analysis of BHRF1 RNAs with the ³²P-labeled BHRF1 ORF probe detects the 1.3-kb EBNA transcript putative Drosha cleavage product. The sizes of Millennium RNA size makers (Ambion) are shown on the left in kb. (D) 5'-RACE analysis of BHRF1 RNA from indicated cells in latent infection or after induction of virus replication at 48 h (Repl., 48h). Reverse transcription was primed with Racer1 primer. The resulting cDNAs were poly(dC) tailed and amplified by PCR. PCR products from latency III IB4 (lane 2), latency I Akata (lane 3), latency III B95-8 (lane 4), and 48-h virus replication-induced Akata (lane 5) and B95-8 (lane 6) cells were separated in 1.5% agarose gel. PCR products were purified, cloned, and sequenced. Sequencing-confirmed EBV-specific PCR products are labeled with asterisks on the left of the lanes. The sizes of the 1-kb Plus ladder (Invitrogen) are shown on the left in bp.

and C17 nasopharyngeal tumors were passaged in nude mice as xenografts (9). MAbs PE2 (71), A10 (45), and 3E8 (42) are specific to EBNA2, EBNA3C, and BHRF1, respectively.

Western blot analysis. Cells were harvested at indicated times after virus replication induction and lysed in 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer. Cell extracts were separated by 10% sodium

dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad). Following incubation in blocking buffer (phosphate-buffered saline containing 0.1% Tween 20 and 5% nonfat milk), membranes were incubated with diluted primary MAbs overnight at 4°C with gentle shaking. Following washes and secondary antibody incubation, specific binding was visualized by ECL enhanced chemiluminescence (Amersham Biosciences).

TABLE 1. Primers used in this study

Primer ^a	Sequence
BHRF1-1 (forward).....	5'-CACCATGGCCTATTCAACAAG G-3'
PmiBH-2r (reverse).....	5'-TCAGGTTAATAAGGAGC-3'
BHRF intron-F.....	5'-GTTGGGATTAGGTTTA-3'
BHRF intron-R.....	5'-ATTCAAGTTAGCACCAC-3'
BHRF-3'-F.....	5'-CTGAGGTGAGTGTGGGA-3'
BHRF-3'-R.....	5'-CATCATTAAACATAGCGT-3'
BHRF-5'-F.....	5'-CTCACCATGACACACT-3'
BHRF-5'-R.....	5'-GGCAAACAGTTACCCGA-3'
BHRF1-1.....	5'-TAACCTGATCAGCCCCGGAGT TCCTGTCTC-3'
BHRF1-2.....	5'-TATCTTTTGGCGAGAAATTG AACCTGTCTC-3'
BHRF1-3.....	5'-TAACGGGAAGTGTGTAAGCAC ACCCTGTCTC-3'
BART1.....	5'-TCTTAGTGA AGTGACGTGCT CCTGTCTC-3'
BART2.....	5'-TATTTTCTGCATTCGCCCTTG CCTGTCTC-3'
MiR-16.....	5'-TAGCAGCACGTAATATTGGC GCCTGTCTC-3'
Racer1 (reverse).....	5'-GTCTCCACGGTGAAATATCT-3'
Racer2 (reverse).....	5'-CCTCAAGCAACACATGA-3'
PEBER1.....	5'-CCAGCTGGTACTTGACCGAAG AC-3'
PEBER2.....	5'-ATTAGAGAATCCTGACTTGCA AATGCTCT-3'

^a Primer pairs BHRF1-1/PmiBH-2r, BHRF intron-F/BHRF intron-R, BHRF-3'-F/BHRF-3'-R, and BHRF-5'-F/BHRF-5'-R were used in PCR to amplify EBV genomic DNA fragments from EBV-infected Akata cells for preparation of ³²P-labeled probes specific to the BHRF1 ORF, intron, and 3'- and 5'-untranslated regions, respectively. Oligonucleotides BHRF1-1, BHRF1-2, BHRF1-3, BART1, BART2, and MiR-16 were used as templates to produce ³²P-labeled RNA probes specific for miR-BHRF1-1, miR-BHRF1-2, miR-BHRF1-3, miR-BART1, miR-BART2, and miR-16, respectively.

RNA preparation and Northern analysis. Total RNA was prepared using TRIzol reagent (Invitrogen). Cytoplasmic and nuclear RNAs were fractionated from Akata cells at 48 h after virus replication induction by immunoglobulin G (IgG) cross-linking. Briefly, cells were resuspended in 4 volumes buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM MgCl₂) for 30 min on ice, Dounce homogenized, and spun at 2,000 rpm at 4°C for 5 min. The supernatant was extracted with TRIzol reagent to isolate cytoplasmic RNA. The buffer A-washed pellet was resuspended in 3 volumes of buffer B (20 mM HEPES [pH 7.9], 20% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA), incubated for 30 min on ice, and spun at 12,000 rpm for 20 min at 4°C. The separated supernatant was extracted with TRIzol reagent to isolate nuclear RNA. For miRNA or mRNA detection, 30 µg total RNA per lane was separated in 15% polyacrylamide gel or in denaturing 1% agarose-2.2 M formaldehyde gel, transferred onto GeneScreen Plus hybridization transfer membranes (Perkin-Elmer), immobilized by baking at 80°C for 2 h, and hybridized with ³²P-labeled RNA probes at 37°C overnight or ³²P-labeled DNA probes at 42°C overnight. ³²P-labeled RNA probes specific for each miRNA were prepared by *in vitro* transcription using the mirVana miRNA probe construction kit (Ambion) and primers (Table 1). ³²P-labeled 5', intron, ORF, and 3' EBV BHRF1 DNA probes were prepared by random primer DNA labeling (Invitrogen) using BHRF1 5'-untranslated, intron, ORF, and 3'-untranslated sequences as templates. Membranes were hybridized in Ultrahyb-Oligo hybridization buffer (Ambion). The signals in Northern blots were detected and quantified using PhosphorImager software (Amersham Biosciences).

Induction of EBV replication. Virus replication was induced in latency I Akata cells or latency III B95-8 cells. About 2×10^6 to 5×10^6 Akata cells were treated with 50 µg per ml mouse anti-human IgG (Dako Cyto, Japan) at 37°C for 4 h. Fresh medium was added to keep the cells at 5×10^5 cells per ml until harvest. EBV replication in B95-8 cells was induced by adding 4-hydroxytamoxifen to latency III-infected B95-8 LCL cells that stably express an RNA encoding a 4-hydroxytamoxifen-dependent fusion of the EBV immediately-early gene coding for BZLF1 (Zta) with a mutant estrogen receptor (34). Cell surface EBV

gp350 late gene expression was assayed with MAb 72A1 as an indicator of late virus replication (53, 66).

5'-RACE. Rapid amplification of cDNA 5' ends (5'-RACE) was done as instructed by the manufacturer (Invitrogen). Briefly, the cDNAs were initially synthesized from 2 µg of total RNAs by SuperScript reverse transcriptase using a BHRF1 ORF-specific Racer1 primer (Table 1 and Fig. 1A). After RNase H treatment, polydeoxycytosines were added to the 3' ends of the cDNA by terminal deoxynucleotide transferase. PCR was done with nested Racer2 primer (Table 1 and Fig. 1A) and universal primer (Gibco-BRL) in a total volume of 50 µl with Taq DNA polymerase (Invitrogen) at 95°C for 2 min with 35 cycles at 94°C for 50 s, 50°C for 50 s, and 72°C for 1.5 min, followed by extension at 72°C for 10 min. The amplicons were analyzed by 1.5% agarose gel electrophoresis, gel purified, cloned into pCR4-TOPO (Invitrogen), and sequenced. The same sequence was obtained from at least four individual clones for each EBV specific PCR product.

Nucleotide sequence accession number. The DNA sequence of the EBV Akata strain BHRF1 gene has been deposited in GenBank under accession no. EF192979.

RESULTS

MiR-BHRF1-1, -2, and -3 are expressed at high level in latency III B cells, whereas miR-BART1 and -2 are expressed at high level in NPC cells. Northern hybridization was done with ³²P-labeled miRNA probes to validate our detection of EBV miRNAs. MiR-BHRF1-1, -2, and -3 were clearly detected in latency III EBV-infected BJAB, BL41, B95-8, IB4, and recently derived LCLs, but not in latency II NPC C666-1 (18), C15 cells (9), or latency I-infected Akata BL cells (Fig. 1B) (data not shown). Cell miR-16 blots are included as loading controls (38). In contrast, miR-BART1 and -2 were expressed at lower levels in latency III-infected B cells and latency I-infected Akata BL cells than in latency II-infected NPC C666-1 and C15 cells (Fig. 1B), in accord with previous results (11, 16, 27, 36, 50, 56, 61). EBV miRNAs were not detected in NPC C17 cells, presumably related to the low EBV genome presence and expression (9). Thus, high-level miR-BHRF1-1, -2, and -3 are tightly associated with latency III, as opposed to latency I and II (11) (Table 2).

Latency III is associated with expression of a stable, unspliced, 1.3-kb BHRF1 ORF-containing RNA. To further analyze BHRF1 latency III-associated RNAs, Northern hybridization was done with ³²P-labeled 5', intron, BHRF1 ORF, and 3' EBV DNA probes (Fig. 1A and Table 1). Northern analysis of total RNA detected an unspliced, 1.3-kb BHRF1 RNA in latency III-infected BJAB, BL41, and IB4 cells with BHRF1 ORF, 5', and intron probes, but not with the 3' probe (Fig. 1A and C and Table 2) (data not shown). This RNA was not detected in uninfected BJAB and BL41 BL cells, EBV latency I-infected Akata cells, or latency II-infected NPC C666-1, C15, or C17 cells (Fig. 1A and C) (data not shown), indicating that the 1.3-kb BHRF1 RNA is associated with miR-BHRF1-1, -2, and -3 expression and EBV latency III but not latency I or II infection.

To further characterize the BHRF1 1.3-kb RNA detected in Northern blots, the 5' sequence of the BHRF1 RNAs from IB4 and B95-8 cells was obtained by 5'-RACE analysis using latency I Akata cell RNA as a negative control. A 0.75-kbp PCR product was amplified from BHRF1 1.3-kb RNA from IB4 and B95-8 cells (Fig. 1D). Four 0.75-kbp PCR product clones were sequenced. The 1.3-kb BHRF1 RNA began with 5'-AAGAAGGG-3' (GenBank accession no. EF192979) (Fig. 1A and Table 2), which is the predicted 3' Droscha cutting site for

TABLE 2. BHRF1 RNAs in EBV-infected cells

Parameter	Result for RNA					
	BHRF1			miR-BHRF1-1	miR-BHRF1-2	miR-BHRF1-3
	1.4 kb	1.3 kb	0.9 kb			
Relative RNA distribution ^a						
Cytoplasm	150,000	43,000	144,000			
Nucleus	36,000	134,000	93,000			
Presence/absence of RNA in EBV ⁺ cell line						
BJAB (latency III)	—	+	—	+	+	+
BL41 (latency III)	—	+	—	+	+	+
IB4 (latency III)	—	+	—	+	+	+
B95-8 (latency III)	—	+	—	+	+	+
Akata (latency I)	—	—	—	—	—	—
Time course (h) of EBV replication induced ^b						
Akata (latency I)	12–144	72–144	72–144	72–144	72–144	72–144
B95-8 (latency III)	12–144	0–144	12–144	0–144	0–144	0–144
Northern analysis						
5' probe	+	+	+			
Intron probe	—	+	—			
BHRF1 ORF probe	+	+	+			
3' probe	+	—	—			
5'-end sequence	5'-TTTCATC	5'-AAGAAG	?			

^a Relative RNA distribution in cytoplasm and nucleus was determined from RNA in Fig. 3D. The EBV distributions in the mock and IgG cross-linked RNA preparations were 43,000 and 40,000 in cytoplasm and 577,000 and 608,000 in nucleus.

^b Time course of high-level expression after virus replication induction.

miR-BHRF1-1 (50). Since a Drosha cleavage 5' of miR-BHRF1-2 would result in a 1,312-nt RNA versus 1,430 nt for cleavage 5' of miR-BHRF1-3, the size of the RNA, detection with BHRF1 intron probe, and a specific inability to detect the 1.3-kb RNA with the BHRF1 3' probe are evidence that the 1.3-kb BHRF1 RNA extends from 3' of miR-BHRF1-1 to 5' of miR-BHRF1-2. No EBV-specific PCR product was detected with RNAs from latency I-infected Akata cells (Fig. 1D) (sequence data not shown). These data indicate that latency III EBV infection is associated with the accumulation of miR-BHRF1-1, -2, and -3 and of a stable unspliced BHRF1 1.3-kb RNA and support a model that these RNAs are Drosha cleavage products of EBNA transcripts or introns.

BHRF1 RNAs after induction of EBV replication in latency I-infected Akata cells. To test whether EBV BHRF1 miRNAs or 1.3-kb RNAs are also expressed as a consequence of early replication-associated BHRF1 mRNA expression or latency III EBNA expression late in EBV replication in latency I-infected Akata cells (72), EBV replication was induced in latency I-infected Akata cells (63, 64). Cell surface EBV gp350 late gene expression was assayed with MA b 72A1 as an indicator of late virus replication (53, 66). EBV gp350 expression was detected as early as 12 h, increased at 24, and peaked at 48 h, when 40% of Akata cells were gp350 positive by microscopy (data not shown).

Early EBV replication-associated BHRF1 protein (Fig. 2B) and spliced and polyadenylated BHRF1 1.4-kb mRNA (Fig. 3 and Table 2) were detected by 12 h after induction of EBV replication. BHRF1 1.4-kb mRNA [including 100-nt poly(A)

tail] increased somewhat at 24 to 72 h and persisted at high levels (Fig. 3, ORF and 3' probe). BHRF1 protein increased at 24 and 48 h and somewhat thereafter (Fig. 2B). As expected, BHRF1 mRNA hybridized to the ORF and 3' probes but not the intron probe (Fig. 1A and 3 and Table 2). An EBV-specific 0.32-kbp PCR product with the beginning sequence 5'-TTTCATC-3' (Fig. 1A and Table 2) was amplified by 5'-RACE analysis of RNA from induced Akata and B95-8 cells at 48 h (Fig. 1D, lanes 5 and 6). Similar products were not detected from latency I Akata or latency III B95-8 cell RNAs without induction of virus replication (Fig. 1D, lanes 3 and 4). Four clones of the 0.32-kbp 5'-RACE product were sequenced. The sequence start site was at nt 41501 in the EBV genome (GenBank accession no. AJ507799), 9 nt 5' to nt 41510, the previous start site determined by S1 analysis (19), and 25 nt 3' to the end of the BHRF1 TATTA sequence (TATA box).

At 12 h to 48 h, a small amount of 0.9-kb BHRF1 3'-cleaved mRNA was detected by BHRF1 ORF and 5' probes but not by intron or 3' probes (Fig. 3B and Table 2) (data not shown). Intron probe detected the 1.3-kb latency III RNA and a non-specific 1.0-kb RNA (Fig. 3A). The 0.9-kb BHRF1 RNA significantly increased in abundance at 48 to 72 h (Fig. 3B). As previously described (72), late-replication-onset latency III-associated EBNA2, EBNA3C, and EBNA1 expression were detected at 24 to 48 h and were abundant at 72 h (Fig. 2B). In parallel with latency III EBNA expression, pre-miR-BHRF1-1, -2, and -3 were readily detected at 48 h and were abundant by 72 h (Fig. 2A). By 72 h, miR-BHRF1-1, -2, and -3 (Fig. 2A) and 1.3-kb Drosha-cleaved EBNA intron RNA were abundant

FIG. 2. Induction of EBV replication in latency I-infected Akata cells results in late replication-associated latency III EBNA proteins and miR-BHRF1-1, -2, and -3. (A) Northern analysis of EBV miRNAs using total RNAs extracted at the indicated time points after EBV-infected Akata cell-IgG cross-linking. miRNA (miR) and pre-miRNA (pre-miR) are indicated. (B) Time course of latency III EBNA and BHRF1 protein expression after EBV-infected Akata cell IgG cross-linking. At the indicated times after induction of virus replication, Western blot analysis was done with MAbs specific to the indicated proteins.

(Fig. 3). The 1.3-kb RNA hybridized to 5', intron, and ORF probes but not to the 3' probe, consistent with cleavage 5' to miR-BHRF1-2 or -3 (Fig. 3) (data not shown). The 1.3-kb RNA was identical in size to the latency III-associated EBNA intron RNA, which is Drosha cleaved 3' to miR-BHRF1-1 and 5' to miR-BHRF1-2, versus the 1.43 kb expected from Drosha cleavage 5' to miR-BHRF1-3. Thus, activation of latency III EBNA transcription is the likely basis for the high-level miR-BHRF1-1, -2, and -3 and 1.3-kb residual EBNA intron RNA late in Akata cells (Fig. 3 and Table 2).

As miR-BHRF1-1, -2, and -3 became abundant at 48 to 72 h (Fig. 2A), the 0.9-kb ORF-positive and 3'-truncated RNA also became more abundant (Fig. 3B and Table 2). Thus, the 0.9-kb RNA has the size and sequence content consistent with cleavage of BHRF1 mRNA 3' to miR-BHRF1-2 by Drosha in the nucleus or by miR-BHRF1-2 in the cytoplasm.

To further clarify the genesis of 0.9-kb BHRF1 3'-truncated RNA, cytoplasmic and nuclear RNAs were isolated from

EBV-infected Akata cells at 48 h after virus replication induction, and RNAs were analyzed by Northern hybridization with 32 P-labeled BHRF1 ORF probe. As a control, the isolated RNAs were separated in 5% polyacrylamide gel and probed with 32 P-labeled DNA oligonucleotide probes specific for EBV-encoded EBER1 and EBER2 small nuclear RNAs (Table 1) (5, 24, 32, 41). As shown in Fig. 3D, EBERs were in the nuclear RNA fraction from Akata cells before and 48 h after induction of virus replication. Furthermore, the latency III EBNA-associated 1.3-kb unspliced and putatively Drosha-cleaved BHRF1 RNA was threefold more abundant in the 48-h nuclear versus cytoplasmic RNA (Fig. 3D and Table 2). Moreover, BHRF1 1.4-kb mRNA was fourfold more abundant in 48-h cytoplasmic versus nuclear RNA. Importantly, the 0.9-kb spliced and 3'-truncated BHRF1 RNA was 1.6-fold more abundant in the cytoplasm than in the nucleus, consistent with this RNA being mostly a product of miR-BHRF1-2 cleavage of BHRF1 mRNA in the cytoplasm, as postulated (38).

miR-BART1 and -2 expression did not change following induction of EBV replication in latency I-infected Akata cells (Fig. 2B).

EBV replication in latency III-infected B95-8 cells does not result in significantly increased miR-BHRF1-1, -2, or -3 or 1.3-kb RNA levels. To evaluate the effects of preexistent latency III EBV infection on EBV replication-associated RNA levels, EBV replication was induced in B95-8 cells (34). Surface gp350 expression increased at 12 to 24 h and peaked at 48 h, when 50% of B95-8 cells were gp350 positive by microscopic evaluation (data not shown). In contrast to latency I-infected Akata cells, EBNA proteins (Fig. 4B), pre-miR- and miR-BHRF1-1, -2, and -3, and 1.3-kb residual Drosha-cleaved RNA barely changed at any time following induction of replication in latency III-infected B95-8 cells (Fig. 4A) (data not shown). BHRF1 mRNA and protein increased significantly by 12 h and were at high levels thereafter (Fig. 4B and C). BHRF1 0.9-kb RNA was also detected at a constant level by BHRF1 ORF and 5' probes but not by intron and 3' probes from 12 h onward (Fig. 4C and Table 1) (data not shown). These data indicate that EBV replication in preexisting latency III-infected cells is associated with little change in EBNA, miR-BHRF1-1, -2, or -3, or 1.3-kb RNA expression, early turn on of BHRF1 1.4-kb mRNA and 0.9-kb cleavage product, and a constant ratio of BHRF1 mRNA and cleavage product throughout replication.

DISCUSSION

In sum, these data confirm that high-level miR-BHRF1-1, -2, and -3 are a characteristic of EBV latency III infection and EBNA transcription (11) and further indicate that EBNA intron cleavage by Drosha to produce miR-BHRF1-1, -2, and -3 also results in a residual stable 1.3-kb RNA. This RNA begins with 5'-AAGAAGGG-3', which is the predicted sequence (50) 3' to the miR-BHRF1-1 cleavage site and therefore differs from the early replication BHRF1 1.4-kb mRNA, which we now find begins 35 nt 5' to the 1.3-kb RNA. Both RNAs hybridized to the BHRF1 5' and ORF probes indicated in Fig. 1 and differ in that the 1.3-kb RNA hybridizes to the intron probe but not to the 3' probe, whereas the 1.4-kb mRNA hybridizes to the 3' probe but not to the intron probe. By size,

FIG. 3. Time course of BHRF1 RNA expression in EBV-infected Akata cells after IgG cross-linking. Shown are the BHRF1 intron probe (A), ORF probe (B), and 3'-untranslated region probe (C), as depicted in Fig. 1. (D) Subcellular localization of 1.4-kb BHRF1 mRNA, 1.3-kb unspliced, cleaved BHRF1 RNA, and 0.9-kb spliced, cleaved BHRF1 RNA. Cytoplasmic (Cyto) and nuclear (Nuc) RNAs were separately isolated from EBV-infected Akata cells at 48 h after mock (–) or IgG (+) cross-linking. The sizes of Millennium RNA markers (Ambion) are shown on the right in kb. EBV BHRF1 RNAs and EBERs are indicated by size in kb and bp, respectively. NS, nonspecific 1.0-kb cell RNA.

the 1.3-kb RNA is most likely Drosha cleaved at the beginning of miR-BHRF1-2, since that would yield a 1,312-nt RNA versus 1,430 nt for Drosha cleavage at the beginning of miR-BHRF1-3. The defined length of the 1.3-kb RNA is also evidence that at least two miRNAs are cleaved from the primary EBNA transcript, which is much longer and variable in length (37). Moreover, latency III RNAs and proteins and high-level miR-BHRF1-1, -2, and -3 and 1.3-kb RNAs accumulate late in replication in latency I-infected Akata cells (72) and the 1.3-kb RNA localizes to the nucleus (Fig. 3D and Table 2), as expected for a stable product of Drosha cleavage. In stability, the 1.3-kb BHRF1 RNA is reminiscent of the stable truncated RNA fragment identified from Drosha processing of cell primary miR-30 (10).

At 12 h after induction of EBV replication in latency I-infected Akata cells, miR-BHRF1, -2, and -3 were detected along with moderately high levels of 1.4-kb BHRF1 mRNA (43, 72) and small amounts of 0.9-kb BHRF1 ORF-positive, intron-negative, 3'-truncated mRNA. Since the BHRF1 early promoter initiates transcription within miR-BHRF1-1-encoding DNA, the expression of miR-BHRF1-2 and -3 in the absence of miR-BHRF1-1 indicates that miR-BHRF1-2 and -3 are processed by Drosha cleavage of an RNA initiated by the BHRF1 early promoter. Since spliced RNAs are usually exported, Drosha cleavage of a nonspliced BHRF1 transcript might have been anticipated and would have resulted in a 1.3-kb intron probe-positive RNA. The absence of 1.3-kb RNA and the presence of a 0.9-kb ORF-positive, spliced, and 3'-truncated RNA at 12 to 24 h (Fig. 3 and Table 2) are consistent

with the possibility that the 0.9-kb RNA is a miR-BHRF1-2 cytoplasmic cleavage product of the 1.4-kb BHRF1 spliced mRNA.

The 0.9-kb BHRF1 spliced and 3'-truncated RNA increased in abundance at 48 to 72 h (Fig. 3B), concomitant with latency III protein expression, miR-BHRF1-1 expression, increased miR-BHRF1-2 and -3 expression, and unspliced 1.3-kb residual Drosha-cleaved RNA expression. miR-BHRF1-1 expression implicates latency III EBNA transcripts for the increased miR-BHRF1 RNAs. Latency III EBNA transcription and Drosha cleavage of EBNA RNA late in virus replication in Akata cells are the likely origin of high-level miR-BHRF1-1, -2, and -3 and unspliced 1.3-kb BHRF1 RNA. The concomitant increase in latency III miR-BHRF1-2 and 0.9-kb spliced and 3'-truncated RNA and the cytoplasmic localization of the 0.9-kb RNA at 48 h (Fig. 3 and Table 2) clearly implicate miR-BHRF1-2 cleavage in the cytoplasm in the genesis of the 0.9-kb RNA, although increased Drosha cleavage of spliced BHRF1 mRNA in the nucleus has not been fully excluded.

miRNAs can mediate target mRNA cleavage of fully or partially complementary target sequences (4, 73). EBV miR-BART2 is implicated in cleaving its complement in the BALF5 mRNA 3'-untranslated sequence (26, 50). In *Caenorhabditis elegans* development, *let-7* miRNA cleaves partially complementary *lin-41* target mRNA and *lin-4* miRNA cleaves partially complementary *lin-14* and *lin-28* (4). Cytoplasmic helicases such as Dicer (28) and Gemin3 (69), a component of RISC (47), may unwind target RNAs and enable miRNA binding to fully or partially complementary targets. In stable latency

FIG. 4. Induction of EBV replication in latency III-infected B95-8 cells does not significantly affect miR-BHRF1-1, -2, or -3 or latency III EBNA protein expression but results in virus replication-associated 1.4-kb BHRF1 mRNA and spliced, 3'-truncated 0.9-kb BHRF1 RNA expression. (A) Northern analysis of selected EBV miRNAs using total RNA from B95-8 cells following induction of virus replication. miRNA (miR) and pre-miRNA (pre-miR) are indicated. (B) EBNA and BHRF1 protein expression in B95-8 cells following induction of virus replication. Western blot analysis was done with indicated antibodies and whole-cell extracts prepared at the indicated time points after induction of virus replication. (C) Time course of BHRF1 RNA expression in latency III-infected B95-8 cells after EBV replication induction. Ethidium bromide-stained rRNAs are included as an RNA loading control. The 0.9-kb and 1.4-kb RNA positions are indicated. The sizes of Millennium RNA markers (Ambion) are shown on the right in kb.

III infection in LCLs, Drosha cleavage of EBNA transcript derived potential BHRF1-encoding RNA within the nucleus and miR-BHRF1-2-mediated cleavage of BHRF1 RNA in the cytoplasm may protect against the potential excessive survival effects of BHRF1 expression.

The high-level expression of miR-BHRF1-1, -2, and -3 and of intron-containing 1.3-kb BHRF1 RNA in latency III in LCLs and late in replication suggests that these RNAs may be important for B-lymphocyte survival during stable latency III infection (1, 35), during EBV replication, or in the face of T-cell immune attack, in vivo.

Other herpesviruses such as Kaposi's sarcoma-associated herpesvirus (KSHV) (13, 29, 51, 57), mouse gammaherpesvirus 68 (MHV68) (51), human cytomegalovirus (51), herpes simplex virus type 1 (HSV-1) (20, 30), and Marek's disease virus (MDV) (8) also encode miRNAs. KSHV encodes 12 distinct miRNAs from a 4.5-kb genome segment, directly upstream of KSHV latency-associated ORFs 71, 72, and 73 (12, 55). These miRNAs are expressed in latently infected cells and are largely unaffected by induction of virus replication (13). MHV68 encodes nine miRNAs within a 6-kb region that encodes eight

small RNAs. These miRNAs may be processed from RNA polymerase III transcripts (51). Human cytomegalovirus miRNA genes are spread through the genome (51). MDV encodes eight miRNAs, five of which flank the *meq* oncogene. Three map to the latency-associated transcript (LAT) and may contribute to MDV-induced cell transformation (8). HSV-1 LAT miRNA is surprisingly similar to EBV BHRF1 miRNAs in being produced from a stable intron in latent infection and late in replication (23). HSV-1 miR-LAT down-regulates transforming growth factor β and SMAD3 expression and has antiapoptotic effects (30). EBV miR-BHRF1-2 and -3 RNAs may have opposite effects in down-regulating BHRF1 expression.

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