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MicroRNA-155 Is an Epstein-Barr Virus-Induced Gene That Modulates Epstein-Barr Virus-Regulated Gene Expression Pathways▽†

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The cellular microRNA miR-155 has been shown to be involved in lymphocyte activation and is expressed in Epstein-Barr virus (EBV)-infected cells displaying type III latency gene expression but not type I latency gene expression. We show here that the elevated levels of miR-155 in type III latency cells is due to EBV gene expression and not epigenetic differences in cell lines tested, and we show that expression in EBV-infected cells requires a conserved AP-1 element in the miR-155 promoter. Gene expression analysis was carried out in a type I latency cell line transduced with an miR-155-expressing retrovirus. This analysis identified both miR-155-suppressed and -induced cellular mRNAs and suggested that in addition to direct targeting of 3′ untranslated regions (UTRs), miR-155 alters gene expression in part through the alteration of signal transduction pathways. 3′ UTR reporter analysis of predicted miR-155 target genes identified the transcriptional regulatory genes encoding BACH1, ZIC3, HIVEP2, CEBPB, ZNF652, ARID2, and SMAD5 as miR-155 targets. Western blot analysis of the most highly suppressed of these, BACH1, showed lower expression in cells transduced with a miR-155 retrovirus. Inspection of the promoters from genes regulated in EBV-infected cells and in cells infected with an miR-155 retrovirus identified potential binding sequences for BACH1 and ZIC3. Together, these experiments suggest that the induction of miR-155 by EBV contributes to EBV-mediated signaling in part through the modulation of transcriptional regulatory factors.

The Epstein-Barr virus (EBV) is a ubiquitous human oncogenic herpesvirus that establishes predominantly latent infection in B lymphocytes. In vivo, differential latency gene expression patterns are observed depending on the stage of infection and the differentiation state of the infected cell (21). EBV-associated Burkitt's lymphomas typically display a germinal center phenotype and express only one protein-coding gene, EBNA1 (latency type I gene expression), which is required for maintenance of the viral episome during cellular division. In contrast, EBV-associated B-cell lymphomas arising in immunocompromised individuals typically display a proliferating blast phenotype and express the full repertoire of protein-coding latency genes (type III latency; EBNA1, EBNA-LP, EBNA2, EBNA3A, EBNA3B, EBNA3C, LMP1 and LMP2). Whereas Burkitt's lymphomas typically exhibit a panoply of cellular genetic alterations that contribute to the malignant phenotype, lymphomas arising in immunocompromised individuals which express type III latency genes are often polyclonal, and fewer genetic alterations in the cellular genome are required to establish the malignant phenotype. In line with this, infection of peripheral blood lymphocytes with EBV in vitro leads to type III latency gene expression, which elicits an activated-B-lymphoblast phenotype.

MicroRNAs are a class of noncoding genes that appear to have broad influences on cellular signal transduction pathways (16, 45). MicroRNAs function through inhibiting translation of select groups of mRNA transcripts containing imperfect annealing sequences in their 3′ untranslated regions (3′ UTRs) and less frequently through other regions of the transcript (26, 27, 29). While the mechanisms through which translation inhibition occurs is currently under investigation by several groups, there is evidence for direct inhibition mechanisms (30) and for sequestration of microRNA-mRNA-protein complexes away from the ribosome to a perinuclear compartment referred to as the GW body (26, 27, 29). In this compartment, a subset of mRNAs appear to be targeted for degradation (26, 27). Therefore, translational inhibition by microRNAs occurs through multiple mechanisms, and the mechanism(s) utilized may depend on the specific microRNA and/or mRNA target.

A number of reports in the last few years have provided a considerable amount of support for a critical role of many microRNAs in cancer. Strong correlations between microRNA expression profiles and specific cancer lineages have been observed (for reviews, see references 7 and 13), and this may be due to their fundamental importance in regulatory processes involved in establishing and maintaining the tumor phenotype. Further support for their role in tumorigenesis comes from several lines of genetic evidence. Strikingly, Calin et al. showed that microRNAs are often encoded in fragile sites in the genome, where their expression can be altered by events such as genomic amplification, loss of heterozygosity, viral integration, or genomic rearrangement (2). Another study found that microRNAs display a high frequency of genomic alterations in human cancers (49). Array profiling studies have shown strong correlations with expression of specific microRNAs and the

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tumor phenotype (17, 28, 41, 47). Further evidence for an oncogenic role of certain microRNAs comes from transgenic mouse studies showing that overexpression of the miR-19 cluster or miR-155 results in increased frequency of tumor formation (5, 14). Original evidence for an oncogenic role of miR-155 came from studies describing its primary transcript, BIC, as a noncoding oncogenic transcript whose genomic locus is downstream from the avian leukosis virus integration site (4, 38).

Two recent mouse knockout papers identified a key role for miR-155 in immune function and demonstrated a critical role for miR-155 in the activation of B and T cells following immune stimulation (32, 34). The defect in immune-cell activation in miR-155 knockout mice appeared to be due at least in part to an inability to induce the transcription of genes required for immune cell activation.

Previous studies have shown high miR-155 expression in EBV-infected B cells displaying type III latency but not in EBV-infected cells displaying type I latency (19, 23). This suggests that, at a minimum, miR-155 may contribute to the activated phenotype of EBV (type III latency)-infected B cells. Interestingly, it was recently shown that Kaposi's sarcoma-associated herpesvirus (KSHV) encodes a microRNA with functional homology to miR-155, suggesting a critical role for miR-155-like microRNAs in herpesvirus biology (12, 36). Here, we investigated the activation of miR-155 by EBV and the possible roles that induced miR-155 may have in EBV signaling.

MATERIALS AND METHODS

Cell culture and treatments. All cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) plus 0.5% penicillin-streptomycin. For analysis of RNA from cells treated with demethylating agents, cells were treated with either 5 μM 5-aza-deoxycytidine (5-aza-C; Sigma catalog no. A3656) or 0.5 mM zebularine (Sigma catalog no. Z4775) for 3 days. Total RNA was isolated and analyzed as indicated below.

Real-time PCR. Total RNA was prepared using a Qiagen miRNeasy miniprep kit (catalog no. 217004) according to the vendor's protocol. For primary BIC transcript analysis, 2 μg of total RNA was reverse transcribed to make cDNA using a SuperScript III first-strand synthesis system (Invitrogen catalog no. 18080-051). PCR analysis of BIC expression was carried out using the following primers and conditions: BIC forward (BIC-b) (5′-CTC TAA TGG TGG CAC AAA-3′) and BIC reverse (BIC-c) (5′-TGA TAA AAA CAA ACA TGG GCT TGA C-3′) (6), 95°C for 3 min followed by 40 cycles of denaturation (95°C, 30 s), annealing (62°C 40 s), and extension (72°C 40 s). Analysis of EBV latency gene expression was carried out using previously described conditions (8, 39, 46). Primers and conditions were as follows: for Cp-initiated transcripts, Cpforward (5′-CAT CTA AAC CGA CTG AAG AA-3′) and Cpreverse (5′-CCC TGA AGG TGA ACC GCT TA-3′); for Wp-initiated transcripts, Wpforward (5′-GTC CAC ACA AAT CCT AG-3′) and Wpreverse (5′-CCC TGA AGG TGA ACC GCT TA-3′); for LMP1, LMP1 forward (5′-CTC CTA CTG ATG ATC ACC CT-3′) and LMP1 reverse (5′-AGA GAC CTA AGA CAA GTA AG-3′); for LMP2A, LMP2Aforward (5′-GAC TAT CAA CCA CTA GGA AC-3′) and LMP2Areverse (5′-CTG CCA AGA GTA GAA GTG AG-3′). PCR conditions for all EBV transcripts were 95°C for 3 min followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The reference RNA, glyceraldehyde 3-phosphate dehydrogenase (G3PDH) RNA, was analyzed using the primers G3PDH forward (5′-GCC AAG GTC ATC CAT GAC AAC TTT GG-3′) and G3PDH reverse (5′-GCC TGC TTC ACC ACC TTC TTG ATG TC-3′) (21) and the following conditions: 95°C for 3 min followed by 40 cycles of denaturation (95°C 30 s), annealing (62°C 40 s), and extension (72°C 40 s). Real-time PCR was conducted with 2 μl of 10-fold-diluted cDNA using iQSYBR Green Supermix (Bio-Rad catalog no. 170-8882) and an iQ5 multicolor real-time PCR detection system (Bio-Rad).

Mature miR-155 expression was assessed using a mirVana quantitative reverse-transcriptase PCR (qRT-PCR) microRNA detection kit (Ambion catalog no. 1558) with the mirVana qRT-PCR miR-155 primer set (Ambion catalog no. 30302) according to the manufacturer's protocol. PCR was carried out using the following conditions: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s.

The expression of BIC and miR-155 in experimental samples was determined by the comparative C_T method ($2^{-\Delta\Delta C_T}$), in which C_T is the threshold cycle and $\Delta\Delta C_T = [\Delta C_T \text{ BIC}] - [\Delta C_T \text{ reference RNA (G3PDH)}]$.

Plasmid cloning. To generate pPRIME-CMV-GFP-miR-155, a 368-bp region surrounding the miR-155 hairpin sequence was isolated from Mutu genomic DNA by PCR using the primers miR-155forward (5′-CAT AGT GCG GCC GCC TGT CAC TCC AGC TTT ATA ACC-3′) and miR-155reverse (5′-CAG GTC GAA TTC TAA GGT TGA ACA TCC CAG TGA C-3′) and cloned into the NotI and EcoRI sites of the plasmid pPRIME-CMV-GFP (37). pEhyg-miR-155 was generated by transferring the miR-155 sequence from pPRIME-CMV-GFP into the NotI and XhoI sites downstream from the hygromycin resistance gene in the plasmid pEhyg (35). The pMSCV-neo-LMP2A retroviral vector was generated by PCR amplification of the LMP2A reading frame from SG5-LMP2A (a generous gift from Richard Longnecker) and inserting into the EcoRI and BglII sites of pMSCV-neo. The pEhyg-FLAG-LMP1 retroviral vector was generated by PCR amplification of LMP1 from pcDNA3-FLAG-LMP1 (a generous gift from Nancy Raab-Traub) and inserting into pEhyg.

To attenuate the cytomegalovirus (CMV) enhancer in the construct pMIR-REPORT (Applied Biosystems), 341 bp of upstream CMV enhancer sequences were deleted by digesting pMIR-REPORT with BglII and SnaBI and religating with an adaptor. 3′ UTR sequences containing the putative miR-155 target sites were isolated from Mutu I genomic DNA by PCR and cloned immediately downstream from the luciferase reading frame in the plasmid pMIR-REPORT-dCMV. Primers used for PCR are as follows: for BACH1, BACH1sense (5′-AGT CAG ATC TTA AGC CAA TGG AAC CCT TGA TT-3′) and BACH1antisense (5′-AGT CAA GCT TAA AAT ATT GCC TTG AAA CAT TTT CTT AGA A-3′); for ZNF652, ZNF652sense (5′-GTC AAG ATC TCA AGG GAA TGA ACA AGA AGA CTA CC-3′) and ZNF652antisense (GTC AAA GCT TAC TAA CGA AGC CAA GCA GAA CAT-3′); for ZIC3, ZIC3sense (5′-GTC AAG ATC TCT TTA CAC TAC TTT TTC TTC CCC T-3′) and ZIC3antisense (5′-GTC AAA GCT TCC ATA TAA CCA CTG TAA AAG AAC TGT TTG TC-3′); for HIVEP2, HIVEP2sense (5′-GTC AAG ATC TGC ATG GGA TTT ACT GTT GCG T-3′) and HIVEP2antisense (5′-GTC AAC GCG TTG GGC AGA GCC TAG CAA TTA A-3′); for ARID2, ARID2sense (5′-AGT CAC GCG TGG ATT GTC AAC CAG CTT ATC TGC-3′) and ARID2antisense (5′-AGT CAA GCT TAA ATG AAG GAC TCA AGA AAA TGT TCA-3′); for CEBPB, CEBPBsense (5′-GTC AAG ATC TGA TGT TCC TAC GGG CTT GTT G-3′) and CEBPBantisense (5′-GTC AAA GCT TAG CCA ATT ACT GCC CCC AAA-3′); for SMAD5, SMAD5sense (5′-GAT CAG ATC TTC TTT TCA ATT ATA TTG TTA GTG GAC TTG T-3′) and SMAD5antisense (5′-GAT CAA GCT TGT AAG GAT AAG CAT CTA AGT CCA-3′); for SMAD4, SMAD4sense (5′-GTC AAG ATC TTC AGG CAT GGC TCA GAG CTT-3′) and SMAD4antisense (5′-GTC AAA GCT TTT ACT CTT GGT AAA ATT AAC TCA CCC ACA-3′), BCORL1, BCORL1sense (5′-GTC AAC GCG TTA CGA GCC AGA CCT ACT TCG G-3′) and BCORL1antisense (5′-GTC AAA GCT TAG GGA ATA GCT TTG TCA GAG CTC AT −3′); for CAB39, CAB39sense (5′-GAT CAC GCG TCT GGG TTC ATG AAG GCA AAT-3′) and CAB39antisense (5′-GAT CAA GCT TAT TAC AGG GCC AGC CAG ATA-3′); for ETS1, ETS1sense (5′-GTC AAG ATC TCC ATT CTG GAG AGG GAC TTC C-3′) and ETS1antisense (5′-GTC AAA GCT TTC GAC ACT TAC ATC GCT ACA TCT CTA A-3′); for MECP2, MECP2sense (5′-GTC CCA AAG ATC TGT CAG CTG TTT CTA GAG TTC CTA CCA T-3′) and MECP2antisense (5′-ATT AAC GCG TCA GCG GCA ACG GCC T-3′); for DET1, DET1sense (5′-GAC GAG ATC TAG CCA GAG AGA GGA GTG AGG-3′) and DET1antisense (5′-GAC GAA GCT TGC CGT GCT TAT GAG CTA AAT G-3′). The BACH1 10-mer wild-type and mutant oligonucleotide reporters were generated by cloning synthetic oligonucleotides into the BglII and HindIII sites of pMIR-REPORT-dCMV. Cloning oligonucleotides for these constructs were as follows: BACH1wt oligo sense (5′-AGC TTT GTG TCA TTA ATA GAA ACC CTT AAT GCT AAA CTG TTT ATA GGT AGA GAG AA-3′) and BACH1wt oligo antisense (5′-GAT CTT CTC TCT ACC TAT AAA CAG TTT AGC ATT AAG GGT TTC TAT TAA TGA CAC AA-3′); and for BACH1mut, BACH1mut oligo sense (5′-GAT CTT CTC TCT ACC TAT AAA CAG TTT ACA GAC GAG GGT TTC TAT TAA TGA CAC AA-3′) and BACH1mut oligo antisense (5′-AGC TTT GTG TCA TTA ATA GAA ACC CTC GTC TGT AAA CTG TTT ATA GGT AGA GAG AA-3′). All inserts were sequenced in their entirety to verify polymerase fidelity.

5′ RACE analysis. 5′ rapid amplification of cDNA ends (RACE) was carried out using total RNA isolated from the EBV type III latency cell line IB4 using

a Smart RACE cDNA amplification kit (Clontech) according to the manufacturer's protocol. The gene-specific primer 5′-CAG CCT ACA GCA AGC CTT CAG CAC TC-3′, which is complementary to the third exon of the human BIC/miR-155 primary transcript, was used. One major PCR product with a size of 350 bp was obtained and cloned into the PCR4-TOPO cloning vector by TA cloning. Eight positive clones from one PCR and one from a second PCR were sequenced to determine the 5′-most end in each case. The major start site of the human BIC transcript was found to be located at nucleotide 12596314 of human chromosome 21 (National Center for Biotechnology Information [NCBI], reference assembly).

Transfection and luciferase expression analysis. Mutant or wild-type miR-155 promoter-reporter vectors (1.25 μg) were cotransfected with 18 μg of carrier plasmid (pUHD10) into 5×10^6 JY cells using the reagent Lipofectamine (Invitrogen) according to the manufacturer's instructions. For 3′-UTR transfections, 3.75 μg of either the control (pPRIME-CMV-GFP) or miR-155 (pPRIME-CMV-GFP-miR-155) expression vector was cotransfected with 0.25 μg of the appropriate pMIR-REPORT-dCMV 3′ UTR reporter plasmid into 2×10^6 Mutu (EBV-negative) cells using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Cells were harvested 48 h posttransfection and analyzed for luciferase reporter activity using Promega firefly luciferase assay reagents according to the manufacturer's protocol.

Retroviral infections. Transient transfections were performed by using a modified version of the calcium phosphate precipitation procedure (a detailed protocol is available at http://www.flemingtonlab.com). Briefly, 10^6 HEK293 cells were plated onto 100-mm-diameter tissue culture dishes. The following day, the medium was replaced with 8 ml of fresh supplemented Dulbecco's modified Eagle medium; 4 h later, DNA precipitates were generated by mixing 0.5 ml of 1× HEPES-buffered saline (0.5% HEPES, 0.8% NaCl, 0.1% dextrose, 0.01% anhydrous Na_2HPO_4, 0.37% KCl [pH 7.10]) with a total of 30 μg of plasmid DNA (10 μg retroviral vector, 10 μg vesicular stomatitis virus G protein expression vector, plus 10 μg pVPACK dGI packaging vector). A total of 30 μl of 2.5 M $CaCl_2$ was added, and samples were mixed immediately. Precipitates were allowed to form at room temperature for 20 min before being added dropwise to cells. Cells were incubated at 37°C with 5% CO_2 for 16 h before the medium was replaced with 10 ml of fresh Dulbecco's modified Eagle medium (plus 10% fetal bovine serum).

Forty-eight hours later, viral supernatants were collected and subjected to one round of centrifugation followed by filtration through a 0.45-μm surfactant-free cellulose acetate filter. Infections were carried out in six-well plates with 1 ml virus plus 10^6 appropriate B cells suspended in 1 ml Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. Polybrene was added to a final concentration of 12 mg/ml, and the mixture was mixed by gentle rocking. Cells were spun in six-well plates at $1{,}000 \times g$ for 1 h followed by a 4-h incubation at 37°C in 5% CO_2. Cells were then collected, spun down, and resuspended in 2 ml RPMI (plus 10% fetal bovine serum, penicillin, streptomycin, and glutamine) per well. Cells were cultured for 2 days prior to antibiotic selection.

Western blot analysis. pEhyg-miRcntl- and pEhyg-miR-155-infected Akata and Mutu (EBV-negative) cells were harvested, and nuclear extracts were generated. From each cell line, 7×10^7 cells were suspended in 300 μl of hypotonic buffer (10 mM HEPES [pH 7.9] 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, plus protease and phosphatase inhibitor cocktails), 18 μl of 10% NP-40 was added, and the mixture was vortexed on high for 10 s. The tubes were transferred to a Microfuge and spun for 2 min on high. The supernatant was taken off, 100 μl of nuclear extraction buffer (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, plus 1 mM dithiothreitol and protease and phosphatase inhibitor cocktails) was added, and the pellet was suspended immediately by vigorous pipetting. The tubes were put on a rotator for 15 min at 4°C and spun for 5 min in a Microfuge (14,000 rpm). The supernatant was transferred to new tubes and quantitated for use in Western blot analysis. Antibodies recognizing BACH1 (Santa Cruz Biotechnology; C-20, catalog no. sc-14700) and actin (Sigma catalog no. A4700) were used for Western blot analysis. Western blotting was carried out with 25 μg of total cell lysate loaded in each lane. Signal detection was carried out with an Odyssey infrared imaging system (Li-Cor Biosciences).

Cellular mRNA microarray analysis. RNAs prepared using Qiagen RNeasy were obtained from two separate pEhyg infections and two separate pEhyg-miR-155 infections. Each sample was labeled with Cy3 and with Cy5 to allow for dye swaps for each pair of pEhyg- and pEhyg-miR-146a-infected cells and hybridized to Agilent human 4-by-44,000 60-mer oligonucleotide arrays (Miltenyi Biotech), resulting in a total of four array hybridizations. For BL30 versus BL30(B95-8) arrays, two separate RNA preparations were generated for each cell line using a Qiagen RNeasy kit. Each pair of RNAs was subjected to two separate array hybridizations with Cy3/Cy5 labeling and two separate array hybridizations in a dye swap configuration (Cy5/Cy3), for a total of eight array hybridizations.

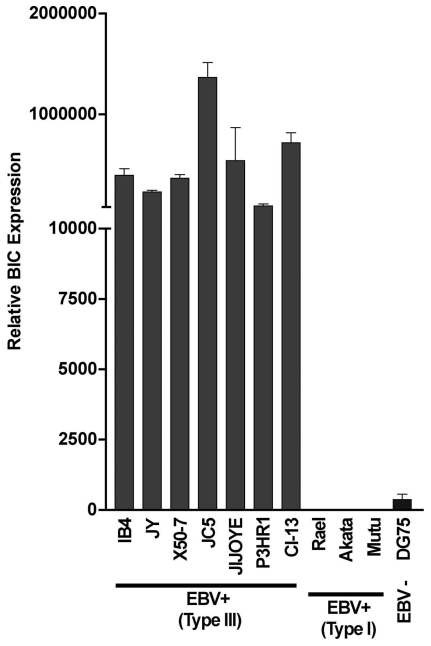


FIG. 1. BIC expression in B-cell lines. Real-time RT-PCR was used to assess BIC expression in a panel of EBV-positive B lymphocytes expressing type III and type I latency genes and in the EBV-negative cell line DG75. Values are normalized to G3PDH.

Microarray analyses were performed using the "marray" and "limma" packages in Bioconductor (11). The pEhyg versus pEhyg-miR-155a arrays were considered independently of the BL30-versus-BL30(B95-8) arrays. In both cases, array images were inspected for scratches and defects, and loess normalization was performed on each array to adjust for intensity-related biases. Normalization was also performed across each set of arrays to achieve consistent intensity distributions. Following array normalization, linear models for differential expression were fitted to the $\log_2$ ratios of the noncontrol spots for each experiment, employing model designs accounting for technical replication and dye swap. Fitted values from these models represented average $\log_2$ ratios for the full set of arrays. Standard errors and corresponding *P* values for each spot were estimated from the data according to the model design, and the set of *P* values was adjusted for multiple testing.

Microarray data accession numbers. Accession numbers for newly reported microarray data are NCBI GEO series accession number GSE10868 and NCBI GEO array accession numbers GSM274844 to GSM274849, GSM274851, GSM274875, GSM275310, and GSM275359 to GSM275361.

RESULTS

Analysis of BIC in EBV-infected cell lines. Previous studies correlated BIC/miR-155 expression with EBV latency type III. We further investigated this issue with a panel of additional type III and type I cell lines by quantitative real time RT-PCR. As shown in Fig. 1, BIC is robustly expressed in all type III cell lines tested, and minimal expression was observed in each of the three type I cell lines tested. High expression was observed in the cell line P3HR1 and its derivative clone, CL13, which are deleted for EBNA-LP and EBNA2.

BIC and miR-155 are induced by EBV. We next examined whether the differential expression of BIC/miR-155 in type III versus type I latency cell lines is specifically due to EBV gene expression and not epigenetic differences in these cell types. This is a relevant issue, because the BIC promoter contains a CpG island which is methylated in type I cells (40; Q. Yin and

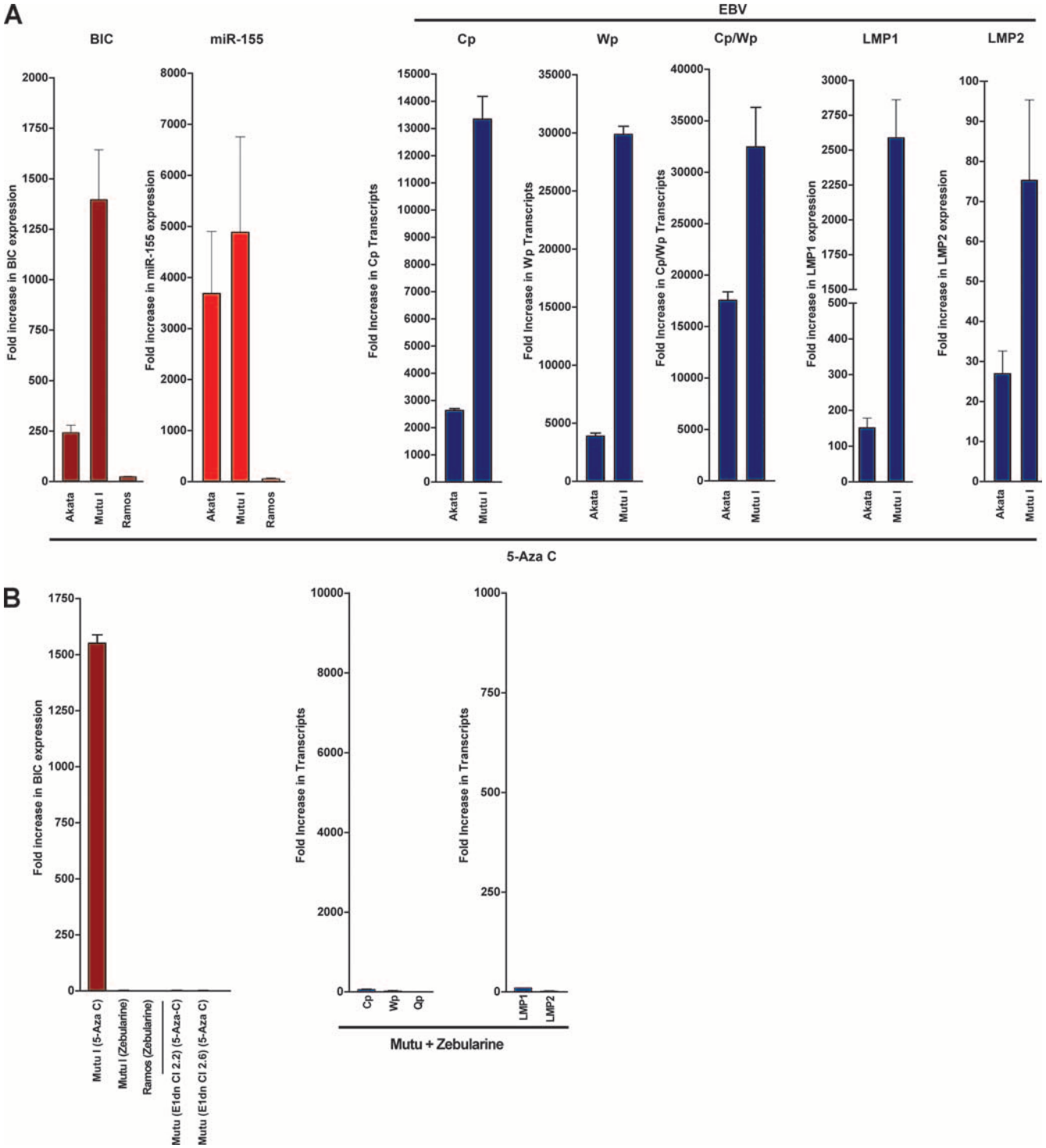


FIG. 2. Activation of BIC and miR-155 expression in type I latency cell lines treated with 5-aza-C. (A) BIC and miR-155 expression are induced in EBV-positive type I latency cell lines but not an EBV-negative cell line treated with 5-aza-C. Values are the increase in BIC and miR-155 expression in cells treated with 5 μM 5-aza-C relative to untreated cells. Graphs on the right show increases in latency gene expression in Akata and Mutu I cells following exposure to 5 μM 5-aza-C. All values are relative to G3PDH expression. (B) The demethylating agent zebularine does not induce BIC expression in Mutu I cells, and 5-aza-C does not induce BIC expression in EBV-negative derivatives of Mutu I cells. Values were normalized to those for G3PDH. Graphs on the right show that zebularine does not substantially induce EBV latency gene expression.

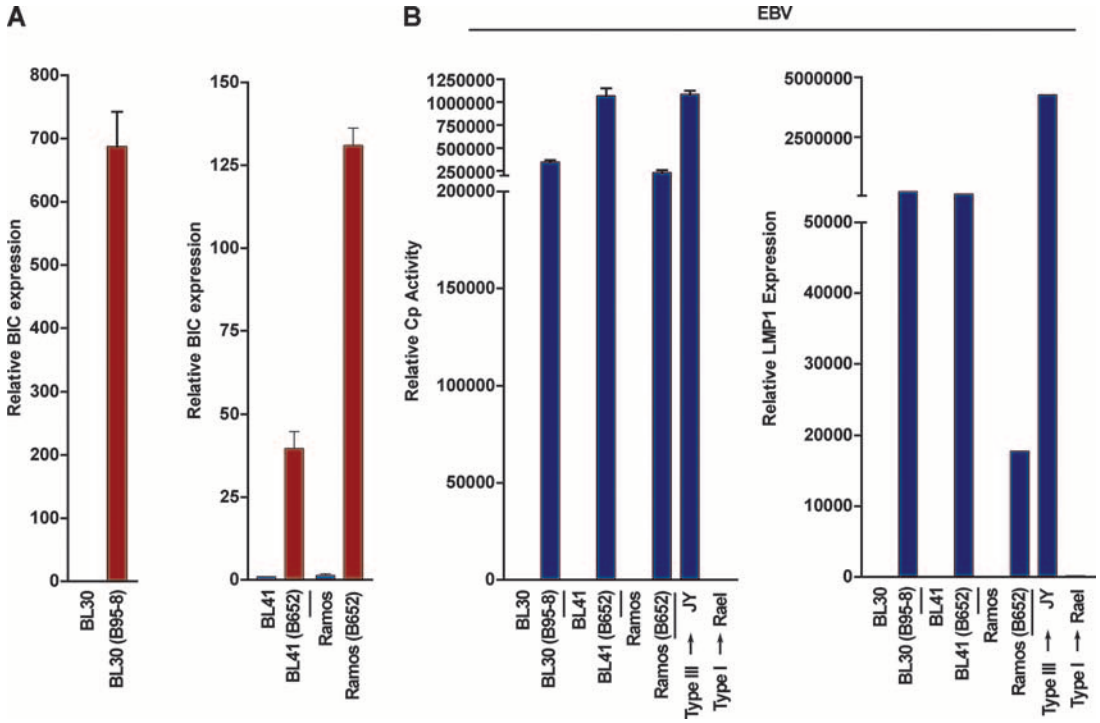


FIG. 3. Infection of EBV-negative cells induces BIC expression. (A) IARC BL30 and IARC BL30 (B95-8) cells were analyzed for BIC expression. The EBV-negative BL41 and Ramos cells were infected with EBV B652 (9), which contains a neomycin resistance marker, and selected for 2 weeks. (B) EBV-infected BL30, BL41, and Ramos show usage of the type III latency promoters Cp and LMP1p. JY is a positive control cell line expressing type III latency genes, and Rael is a type I latency cell line. All values were normalized to those for G3PDH mRNA.

E. Flemington, unpublished data). To begin to address this issue, we first treated two type I latency cell lines and an EBV-negative cell line with 5-aza-C, to induce type III latency gene expression, and analyzed BIC and miR-155 expression. As shown in Fig. 2A, 5-aza-C induces type III latency gene expression in the type I cell lines, Akata and Mutu I. Both BIC and miR-155 are induced by 5-aza-C in Akata and Mutu cells but not in the EBV-negative cell line Ramos. To further establish that induction in Akata and Mutu is due to EBV gene expression and not a defect in the ability of BIC/miR-155 to be induced by 5-aza-C in Ramos cells, two further experiments were carried out. First, induction of BIC was assessed in Mutu I cells treated with the demethylating agent zebularine, which has been shown to be defective for induction of EBV latency (33). Figure 2B shows that zebularine is unable to influence BIC expression. Secondly, EBV-negative derivatives of Mutu I cells were generated by retroviral transduction of a dominant negative form of the EBV episomal replication factor EBNA1 (3), and BIC expression was assessed in these cells following 5-aza-C treatment. While induction of the EBV-positive parental Mutu I cells showed induction of BIC following 5-aza-C treatment, BIC expression was not induced in either of the two EBV-negative derivatives of Mutu I cells (Fig. 2B). Lastly, we analyzed BIC and miR-155 expression in matched EBV-positive type III latency versus EBV-negative Burkitt's lymphoma cells. As shown in Fig. 3, infection of Ramos, BL30, and BL41 with EBV resulted in type III promoter usage and led to induction of BIC and miR-155. Together, these results demonstrate that the high expression of BIC/miR-155 in type III latency cells is likely due to EBV latency gene expression and not clonal or epigenetic differences.

Expression of BIC/miR-155 in type III latency cells requires a conserved AP-1 element proximal to the transcription initiation site. We have recently demonstrated that induction of the BIC promoter by B cell receptor activation requires a conserved AP-1 element located 40 bp upstream from the start site (48). We also noted possible alternative promoter usage for BIC (48). We therefore carried out 5′ RACE in a type III latency cell line to determine whether the promoter used in cell lines expressing type III latency genes is the same as that used following B-cell receptor activation. This analysis demonstrated that transcription initiation in type III latency cell lines occurs at the same location as transcription initiation following B-cell receptor activation (Fig. 4A; also see Fig. S1 in the supplemental material). To assess whether the conserved AP-1 element or the upstream NF-κB site contributes to promoter activity in type III latency cells, JY cells were transfected with either wild-type, NF-κB mutant, or AP-1 mutant BIC/miR-155 reporter plasmids. The NF-κB mutant is approximately 45% less active than the wild-type promoter in this system, suggesting the possible contribution of NF-κB signaling to BIC/miR-155 promoter activity in type III latency cells (Fig. 4B). The AP-1 mutant showed 18-fold less activity than the wild-type promoter, indicating a critical role for the AP-1 site in facilitating BIC promoter activity in EBV-infected type III latency cells.

Since both LMP1 and LMP2A have previously been shown to stimulate transcription through AP-1 signaling, we assessed

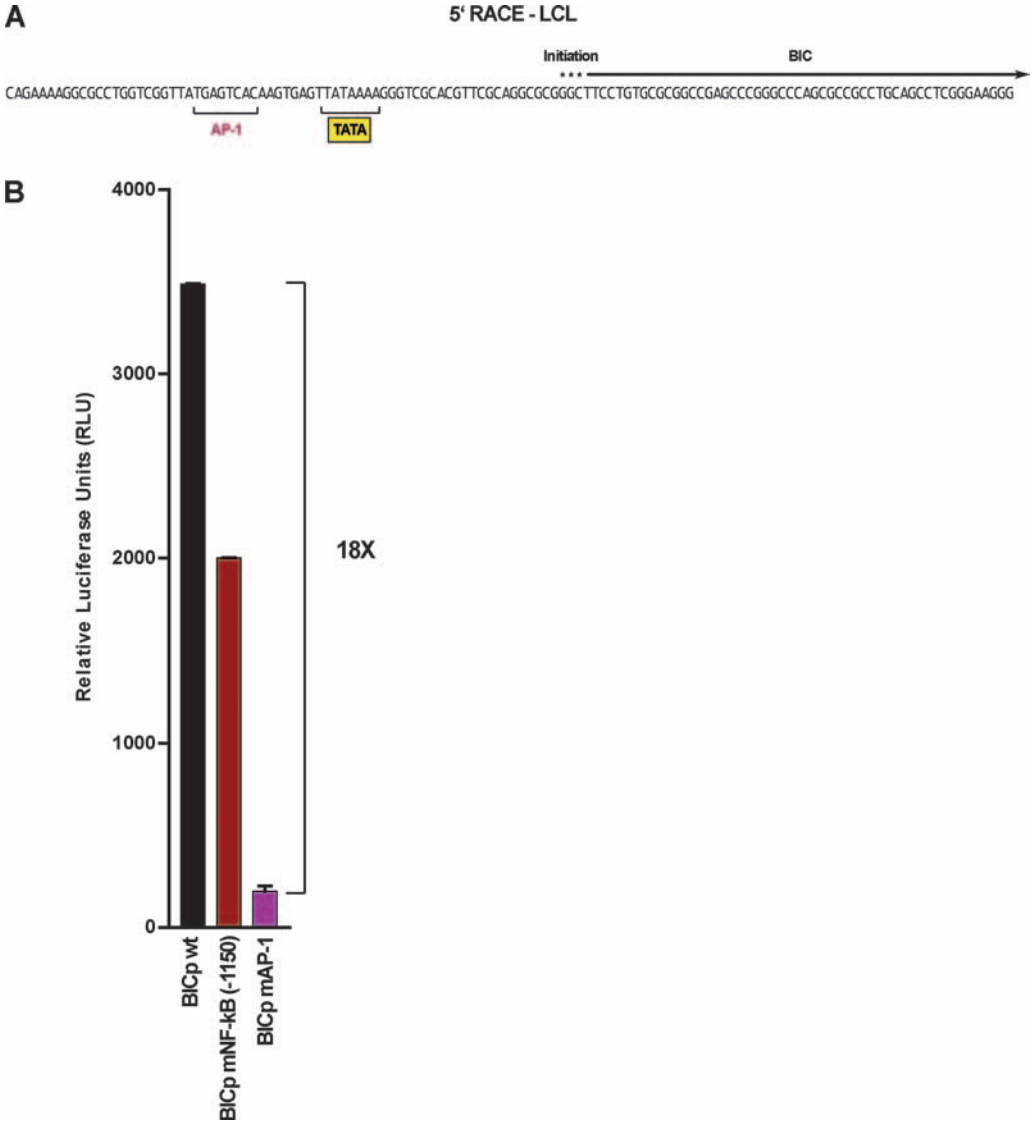


FIG. 4. (A) 5′ RACE was carried out to assess the site of transcription initiation in the type III latency cell line IB4. (B) Reporter analysis of the wild type and NF-κB and AP-1 site mutants in the type III cell line JY. Error bars represent standard deviations of triplicate transfections.

whether LMP1 or LMP2A could induce BIC/miR-155. Duplicate control and duplicate LMP1 (strain B95-8) retroviral infections were carried out in the cell lines Mutu-E1dn-Cl.3 (an EBV-negative derivative of the cell line Mutu I), Akata, Ramos, A549, and BL30, the cells were selected for 14 days, and RNAs were analyzed for BIC and/or miR-155 expression. Although in most cases LMP1 was expressed at levels similar to that observed in type III latency cells, little more than twofold increases in BIC/miR-155 were observed in any of these cell lines (data not shown). Similarly, duplicate infections of Ramos and BL30 cells with a control or an LMP2A retrovirus did not yield substantial increases in BIC/miR-155 expression. Lastly, infection of Ramos and BL30 with LMP1 plus LMP2A did not induce substantial BIC/miR-155 expression (data not shown). These results suggest that although expression of BIC/miR-155 is dependent on AP-1 signaling in type III latency cells, activation of BIC/miR-155 is likely controlled through an unexpectedly complex mechanism, possibly involving additional type III latency genes which may cooperate with LMP1 and/or LMP2A to yield robust BIC/miR-155 expression.

Gene expression analysis in miR-155 expressing cells. To begin to gain insight into how EBV-induced miR-155 expression may influence EBV-mediated signal transduction, gene array analysis was carried out in Akata cells transduced with an miR-155 retroviral expression vector. Akata cells were infected in duplicate with a control retrovirus (pEhyg-miR-cntl) or an miR-155 retrovirus (pEhyg-miR-155), and total RNAs were prepared 2 weeks after selection with hygromycin. The pEhyg-miR-155-infected but not the pEhyg-miR-cntl-infected cells expressed the mature form of miR-155 at levels similar to that of type III latency cell lines (Fig. 5), indicating that the miR-155 transcript generated from this retrovirus mediates appropriate microRNA processing. RNA preparations were then subjected to mRNA array analysis using a two-color Agilent

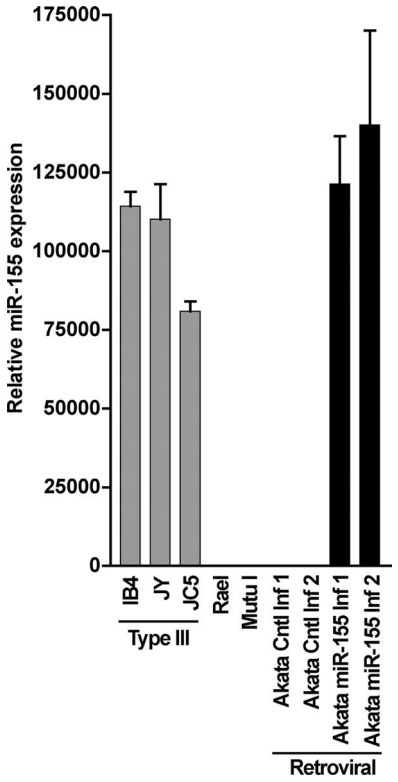


FIG. 5. Mature miR-155 expression in Akata cells transduced with an miR-155-expressing retrovirus (pEhyg-miR-155). Real-time PCR analysis of miR-155 expression was carried out with the type III cell lines IB4, JY, and JC5 and in the type I cell lines Rael and Mutu I as a reference. Values are relative to expression in Mutu I cells.

60-mer oligonucleotide 4-by-44,000 platform. Arrays were carried out with duplicate infections, and dye swaps were analyzed for each. Genes showing >2-fold differences in both sets of infections with *P* values of less than 0.01 were then compiled (see Table S2 in the supplemental material for gene lists). By this criterion, 84 induced and 78 suppressed genes were identified, with most genes having adjusted *P* values of <0.001. Of the suppressed genes, 17 have potential seed sequences of 7-mer or better (Table 1), and many of these may be direct targets of miR-155. In contrast, the induced genes and many of the remaining suppressed genes that do not contain potential miR-155 seed sequences are likely regulated through indirect mechanisms.

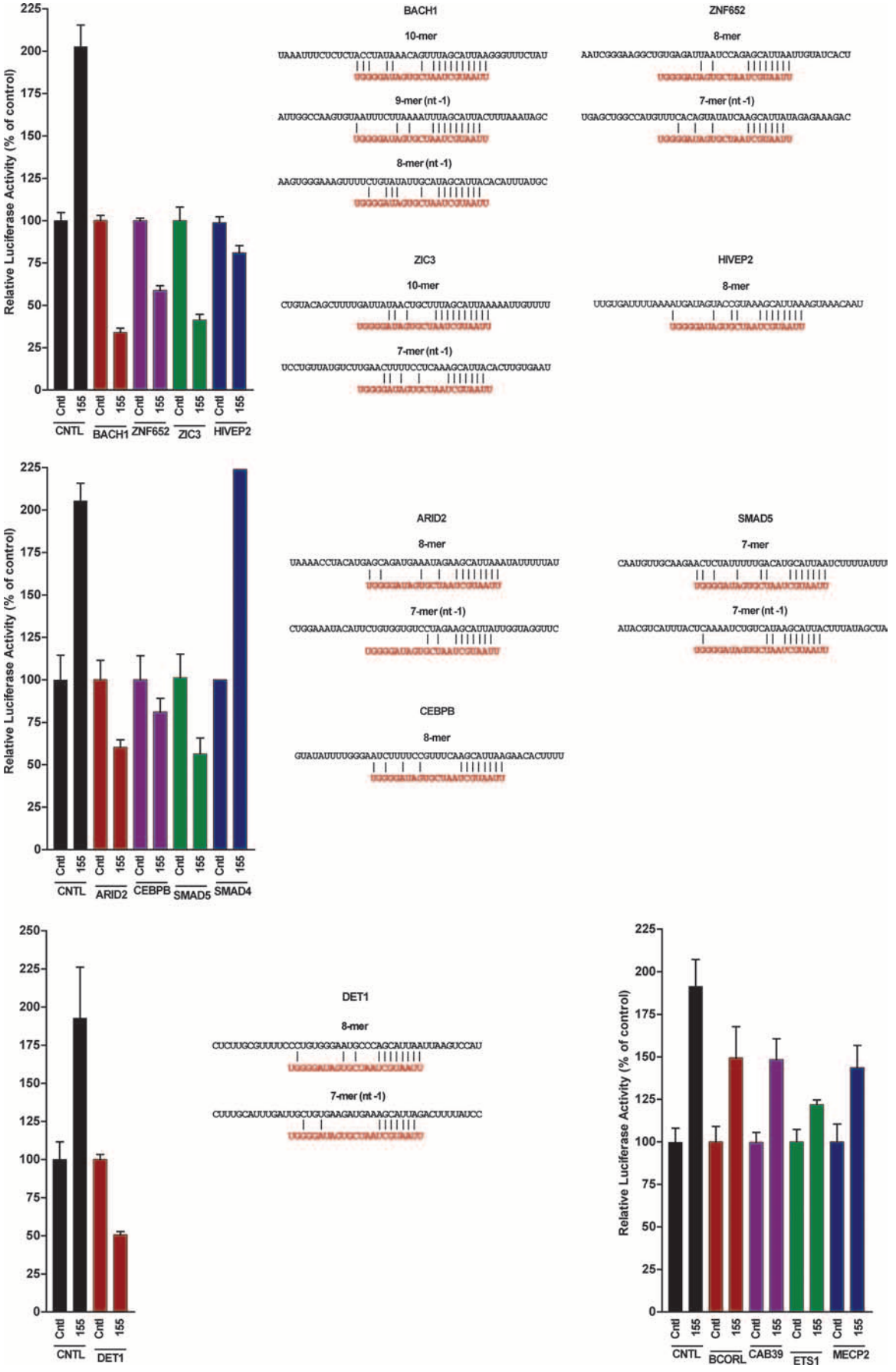
miR-155 targets cellular regulatory genes. Many of the miR-155-suppressed genes with potential miR-155 target sites (Table 1) are genes with known regulatory functions. For example, DET1 has been shown to regulate transcription and promote ubiquitin-mediated degradation of transcription factors such as c-Jun (44), and BACH1, MYB, and CEBPB are known transcription factors. To explore the possibility that miR-155 regulates some of these as well as other regulatory genes predicted by microRNA targeting prediction algorithms (Targetscan [25] and a Bayesian method developed by John et al. [20], the 3′ UTRs of the regulatory genes for BACH1, DET1, CEBP, ZNF652, ZIC3, HIVEP2, ARID2, SMAD5, BCORL1, CAB39, ETS1, and MECP2 were cloned between the luciferase open reading frame and the polyadenylation signal of a luciferase reporter plasmid (pMIR-REPORT-dCMV). EBV-negative Mutu cells (Mutu-E1dn-Cl.3) were cotransfected with this panel of 3′ UTR reporters and either a control or an miR-155 expression vector (Fig. 6). Notably, miR-155 reproducibly induced the reporter plasmid containing no inserted 3′ UTR about twofold. This effect is not due to an absence of an inserted 3′ UTR, since a panel of other 3′ UTRs including the putative miR-146a target 3′ UTR SMAD4 are similarly induced by miR-155 (Fig. 6 and data not shown). This analysis indicated that the 3′ UTRs from the transcriptional regulatory genes BACH1, ZIC3, ZNF652, ARID2, SMAD5, HIVEP2, and CEBPB as well as DET1 are modulated by miR-155. Of these, we further explored suppression of BACH1. A 50-bp oligonucleotide spanning the 10-mer BACH1 site or a mutant derivative in which the miR-155 seed sequence was mutated was cloned into pMIR-REPORT-dCMV. As shown in Fig. 7, the wild-type but not the mutant BACH1 10-mer was suppressed by miR-155. We next examined expression of endogenous BACH1 in cells transduced with a miR-155 retrovirus. As shown in Fig. 7, BACH1 expression is lower in Akata and Mutu (EBV-negative) cells transduced with pEhyg-miR-155 compared to pEhyg-miR-cntl retroviruses.

Coordinately regulated genes in miR-155-transduced and EBV-infected cells. To gain further insight into how induction of miR-155 may contribute to EBV-mediated signal transduction, gene array analysis was carried out in EBV-negative BL30 cells and EBV-infected BL30 cells (which display type III latency [data not shown]). Two separate RNA preparations were made from the EBV-positive and EBV-negative BL30 cells. Each preparation was used for duplicate hybridizations and

TABLE 1. Potential seed sequences identified in genes showing >2 fold-lower expression ($P < 0.01$) in pEhyg-miR-155-transduced Akata cells relative to pEhyg (control) transduced cells[a]

Gene	Seed	Location
AFF2	7-mer	3′ UTR
BACH1	10-mer	3′ UTR
	9-mer	3′ UTR
	8-mer	3′ UTR
	7-mer	3′ UTR
BCL2	7-mer	3′ UTR
CDKN1B	8-mer (nt −1)	3′ UTR
CEBPB	8-mer	3′ UTR
DET1	8-mer	3′ UTR
	7-mer (nt −1)	3′ UTR
LILRA1	9-mer	3′ UTR
ZNF275	8-mer	3′ UTR
FUT1	7-mer	ORF
KLHL5	8-mer	3′ UTR
	7-mer	3′ UTR
LHFPL2	7-mer	3′ UTR
MYB	7-mer (nt −1)	3′ UTR
	7-mer (nt −1)	3′ UTR
MYO10	8-mer	3′ UTR
RFK	7-mer	3′ UTR
	7-mer	3′ UTR
	7-mer (nt −1)	3′ UTR
STS	8-mer (nt −1)	3′ UTR
	7-mer	3′ UTR
TNFAIP2	7-mer (nt −1)	3′ UTR
ZNF179	7-mer (nt −1)	ORF

[a] nt, nucleotide; ORF, open reading frame.


Relative Luciferase Activity (% of control)
225
200
175
150
125
100
75
50
25
0
Cntl
155
CNTL
BACH1
ZNF652
ZIC3
HIVEP2
ARID2
CEBPB
SMAD5
SMAD4
DET1
BCORL
CAB39
ETS1
MECP2
BACH1
10-mer
UAAAUUUCUCUCUACCUAUAAACAGUUUAGCAUUAAGGGUUUCUAU
9-mer (nt -1)
AUUGGCCAAGUGUAAUUUCUUAAAAUUUAGCAUUACUUUAAAUAGC
8-mer (nt -1)
AAGUGGGAAAGUUUUCUGUAUAUUGCAUAGCAUUACACAUUUAUGC
ZNF652
8-mer
AAUCGGGAAGGCUGUGAGAUUAAUCCAGAGCAUUAAUUGUAUCACU
7-mer (nt -1)
UGAGCUGGCCAUGUUUCACAGUAUAUCAAGCAUUAUAGAGAAAGAC
ZIC3
10-mer
CUGUACAGCUUUUGAUUAUAACUGCUUUAGCAUUAAAAAUUGUUUU
7-mer (nt -1)
UCCUGUUAUGUCUUGAACUUUUCCUCAAAGCAUUACACUUGUGAAU
HIVEP2
8-mer
UUGUGAUUUUAAAAUGAUAGUACCGUAAAGCAUUAAAGUAAACAAU
ARID2
8-mer
UAAAACCUACAUGAGCAGAUGAAAUAGAAGCAUUAAAUAUUUUUAU
7-mer (nt -1)
CUGGAAAUACAUUCUGUGGUGUCCUAGAAGCAUUAUUGGUAGGUUC
SMAD5
7-mer
CAAUGUUGCAAGAACUCUAUUUUUGACAUGCAUUAAUCUUUUAUUU
7-mer (nt -1)
AUACGUCAUUUACUCAAAAUCUGUCAUAAGCAUUACUUUAUAGCUA
CEBPB
8-mer
GUAUAUUUUGGGAAUCUUUUCCGUUUCAAGCAUUAAGAACACUUUU
DET1
8-mer
CUCUUGCGUUUUCCCUGUGGGAAUGCCCAGCAUUAAUUAAGUCCAU
7-mer (nt -1)
CUUUGCAUUUGAUUGCUGUGAAGAUGAAAGCAUUAGACUUUUAUCC

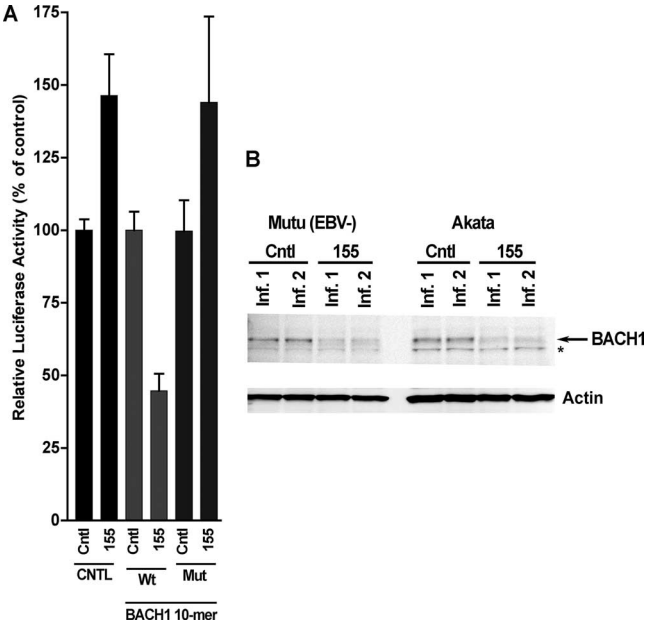


FIG. 7. Seed sequence specificity of miR-155 and Western blot analysis of BACH1 expression in miR-155 expressing cells. (A) 3′ UTR experiments were carried out as for Fig. 6. "Wt" represents a 3′ UTR reporter containing 40 bp of wild-type sequences including the BACH1 10-mer miR-155 site, and "Mut" represents the 3′ UTR reporter containing the same sequences with a 6-bp mutation in the miR-155 seed sequence. (B) Nuclear extracts were generated from the indicated cells, and equal amounts of total protein were loaded in all lanes. Western blots were probed with the BACH1 and actin antibodies.

duplicate dye swap hybridizations, and genes showing a >2-fold change in EBV-positive versus EBV-negative cells with *P* values of less than 0.01 were selected (see Table S3 in the supplemental material). Genes induced or suppressed >2-fold by miR-155 (in Akata cells) and by EBV infection were identified (Fig. 8). Of the genes commonly suppressed at this level, CEBPB, DET1, KLHL5, STS, and ZNF179 have potential miR-155 target sites in their 3′ UTRs (Table 1). Also notable in the downregulated list is the gene for the cyclin-dependent kinase inhibitor CDKN1C (also referred to as p57KIP2), the downregulation of which may contribute to the proliferation phenotype of EBV-activated blasts. Induced genes include that encoding the transferrin receptor (TFRC), which is a classic marker for EBV infection. (BIC appears in this list but may be detected in miR-155-transduced Akata cells by hybridization to the region of the BIC gene cloned into the pEhyg retroviral vector.)

To gain insight into possible mechanisms through which miR-155 induces gene expression, the promoter sequences for each of the miR-155 and EBV induced genes were extracted from the Database of Transcriptional Start Sites (42), a database of experimentally determined transcriptional start sites. Sequences from −2 kb to +200 bp were extracted and scanned for potential transcription factor binding sites using TFSEARCH (15). Although the bulk of EBV-induced genes have been shown to be induced through the activation of NF-κB (1), none of the miR-155/EBV-induced genes contained predicted NF-κB sites with a prediction score greater than 90%. However, five of the seven induced genes contained at least one predicted AP-1 element with a >90% prediction score, and the transferrin receptor promoter contained an AP-1 site with a prediction score of 89.7% (Table 2). This raises the possibility that one of the transcription factor pathways that miR-155 signals through during EBV infection is the AP-1 pathway. Although there are a number of ways that this pathway could be regulated in miR-155-expressing cells, it is notable that BACH1 is a transcriptional repressor that binds directly to AP-1 promoter elements (22). This suggests that suppression of BACH1 by miR-155 may help relieve BACH1-mediated inhibition of at least a subset of promoters containing AP-1 elements.

DISCUSSION

Regulation of BIC by EBV. We have demonstrated that the previous correlation between type III latency and expression of BIC/miR-155 is the result of EBV gene expression and not clonal and/or epigenetic differences between type III and type I cell lines. This is a germane issue, since the BIC promoter/exon 1 contains a CpG island that is highly methylated in type I latency cells and hypomethylated in type I infected cells (40; Yin and Flemington, unpublished). In a reporter study, we show that expression of the BIC promoter requires a conserved AP-1 promoter element. Nevertheless, we have thus far been unable to observe substantial induction of BIC by LMP1, LMP2A, or the combination of LMP1 and LMP2. We also considered the possibility that the lytic transactivator Zta, which binds to AP-1 promoter elements, may contribute to induction of BIC. Nevertheless, induction of the lytic cycle in Akata cells by treatment with anti-immunoglobulin G resulted in only a minor induction of BIC expression (data not shown), and induction of Zta in conditionally expressing HeLa cells similarly did not result in a substantial increase in miR-155 expression. It is possible that Zta may in fact induce BIC/miR-155 under certain circumstances, but a lack of correlation in uninduced cells with the degree of spontaneous reactivation does not support the idea that this is a major factor in EBV-mediated induction of BIC/miR-155.

Our previous studies showed that induction of BIC by B-cell receptor cross-linking is mediated at least in part by the binding of JunB and FosB to the BIC AP-1 promoter element (48). Cahir-McFarland et al. (1) showed that EBV induces the expression of JunB, and our array analysis in EBV-positive versus EBV-negative BL30 cells similarly revealed induction of JunB by EBV (see Table S3 in the supplemental material). In con-

FIG. 6. 3′ UTR analysis of candidate miR-155 target genes. All values are relative to the respective reporter cotransfected with the control expression vector, pPRIME-CMV-GFP-miRcntl. All values are derived from triplicate transfections, and error bars show standard deviations. Potential seed sequences contained in the 3′ UTRs of regulated transcripts cloned into reporter plasmid are displayed to the right of the respective 3′ UTR experiment results. The 3′ UTRs shown in the bottom right panel were considered to be inhibited weakly.

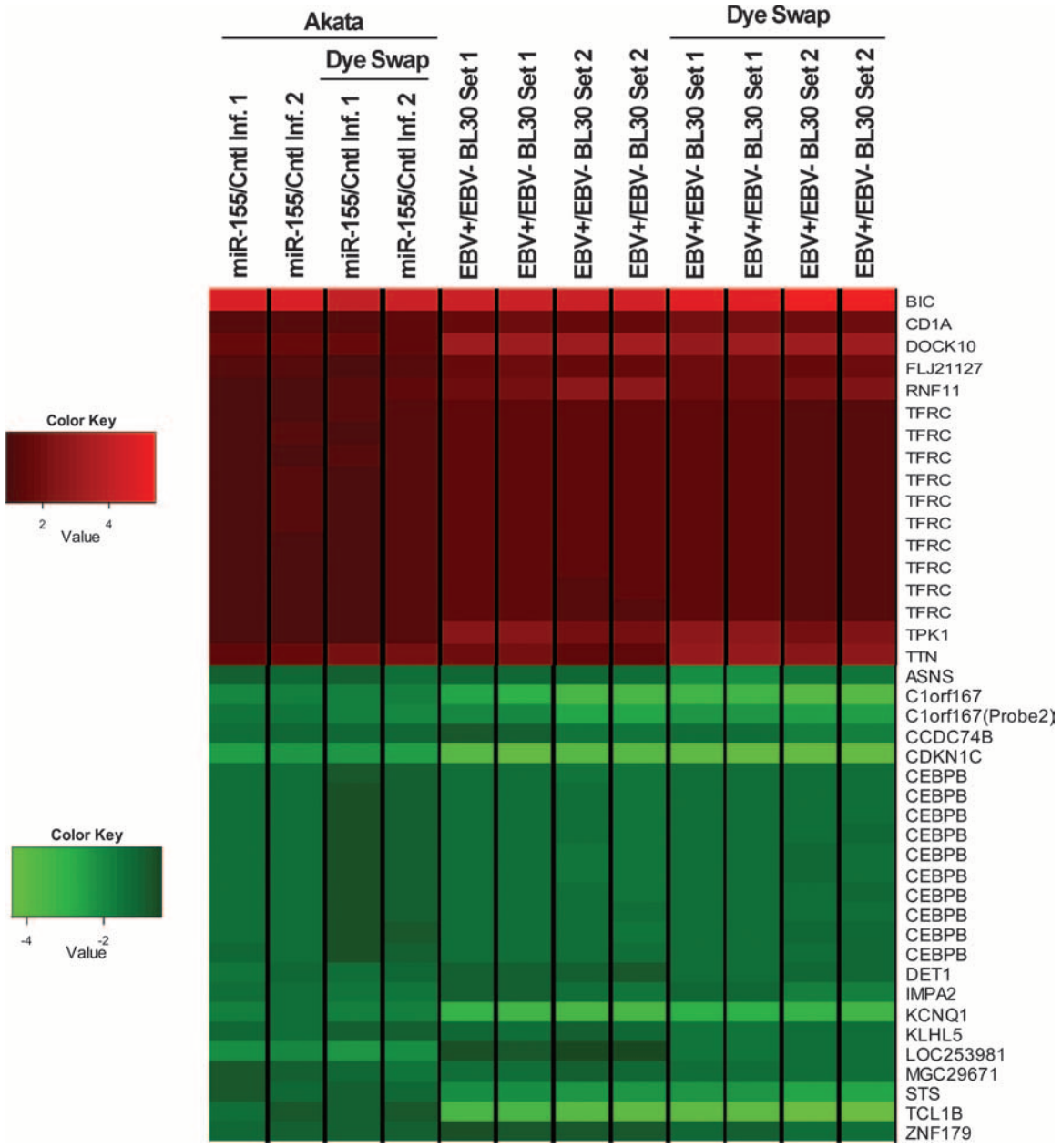


FIG. 8. Heat map representing common differentially expressed genes in miR-155-expressing Akata cells and EBV-infected BL30 cells. Genes showing >2-fold differential expression with P values of <0.01 in both pEhyg-miR-155-transduced Akata cells and EBV-positive BL30 cells are shown. Dye swap experiments were carried out to filter out differences due to dye bias.

trast, at least at the RNA level, no significant induction of FosB was observed in EBV-infected BL30 cells, although an approximately twofold increase in c-Fos RNA levels was observed. This raises the possibility that induction of BIC/miR-155 by EBV may be distinct from induction mediated by B-cell receptor activation. We hypothesize that LMP1 contributes to the induction of BIC/miR-155 in part through the induction of JunB but that another EBV latency gene(s) provides unique and essential cooperative functions necessary to sustain the robust BIC/miR-155 expression observed in type III latency cells.

TABLE 2. Sequence, TFSEARCH score, and position of AP-1 sites in the promoters of miR-155- and EBV-induced genes

Gene	Sequence	Score (%)	Position
CD1A	None		
DOCK10	ATGACTCAA	92.1	+16 to +24
FLJ21127 (TECT-1)	CTGACTCAG	97	−463 to −455
RNF11	AGTGACTTCGT	90.7	−1505 to −1495
	ATGATTCAG	92.6	−1039 to −1031
	GATGACGCAGT	89.7	−33 to −23
TFRC	ATTAAGTCACT	89.7	−623 to −613
TPK1	GTGAGTCAC	92.3	−368 to −360
TTN	ATGACTCAG	98.3	−927 to −919
	CTGACTCAG	97	−356 to −348

miR-155 regulated transcriptional regulatory genes. We propose that at least some of the pathways that are influenced by miR-155 are influenced by the direct targeting of transcriptional regulatory factors. Figure 6 shows that miR-155 can inhibit expression through the 3′ UTRs of a number of transcription factors. Notably, two recent papers similarly demonstrated the direct targeting of BACH1 by miR-155 and a KSHV-encoded functionally homologous microRNA, miR-K12-11 (12, 36). This indicates that suppression of BACH1 as well as other miR-155 targets is a conserved activity in the herpesviruses EBV and KSHV (and likely other members of the herpesvirus family). BACH1 is a transcriptional repressor that has been shown to bind consensus AP-1 promoter elements (22). In light of the known activation of AP-1 by EBV as well as the role of AP-1 in the activation of lymphocytes during immune responses, it is reasonable that suppressing the expression of AP-1 inhibitors such as BACH1 may facilitate derepression of AP-1 promoter elements. Inhibiting the expression of BACH1 may decrease occupancy of AP-1 sites to allow the binding of activating AP-1 factors. The array analysis of miR-155-expressing Akata cells and EBV infection of BL30 cells identified DET1 as a common down-regulated gene containing a potential miR-155 target site. We have also shown that the 3′ UTR of DET1 is suppressed by miR-155. The down-modulation of DET1 by miR-155 and EBV is also interesting as a possible candidate for the regulation of AP-1 signaling, since it has been shown to mediate degradation of c-Jun (44).

Like BACH1, ZNF652 and HIVEP2 are also DNA binding transcriptional repressors (18, 24). Although inhibition of the HIVEP2 3′ UTR is less robust than that of the other 3′ UTRs tested here, it is interesting because it has been shown to bind to and inhibit the c-Myc intron 1 regulatory element through an NF-κB binding sequence (18) and has been shown to be downregulated in breast cancers (10). It is possible that miR-155 may cooperate with other factors, such as other microRNAs, to facilitate more robust suppression of HIVEP2 in EBV-infected cells. In this context, suppression of HIVEP2 may be robust enough to facilitate derepression of NF-κB sites. ZNF652 is a transcriptional repressor that has been shown to bind the ETO family member CBFA2T3, a putative breast tumor suppressor that plays a role in lymphocyte differentiation and leukemia (24).

As shown in Fig. 6, miR-155 reproducibly induces the expression of control reporter plasmids (that do not contain miR-155 3′ UTR targeting sequences) about twofold. In this reporter plasmid, luciferase activity is driven by an enhancer-truncated CMV promoter. However, this promoter still contains binding sites for a number of transcription factors, including a perfect consensus AP-1 site as well as two NF-κB sites. It is possible that even in the context of numerous binding sites for transcriptional activators, miR-155 may impart derepression of this promoter through the inhibition of associated repressors.

We also note a number of transcriptional activators whose 3′ UTRs can be inhibited by miR-155. The downregulation of one or more of these could play a role in inhibiting miR-155-regulated genes shown in Table S2 in the supplemental material and in Fig. 8. Of the transcriptional activator genes that are suppressed by miR-155 in 3′ UTR reporter assays, the most strongly inhibited is ZIC3. ZIC3 is a zinc finger protein that plays a critical role in the development of embryonic patterning (43). Based on the current literature, a possible role for ZIC3 in lymphocytes is less clear, but our array analysis shows that it is expressed in B lymphocytes. Mizugishi et al. (31) determined the optimal binding sequences for ZIC3 as GGGTGGTC, and mutagenesis studies found some flexibility with respect to the last C residue. The promoter of the miR-155/EBV-downregulated gene C1orf167 contains a perfect match to the 8-mer consensus located 345 bp upstream from the transcriptional start site. It is therefore possible that inhibition of ZIC3 expression by miR-155 may contribute to the downregulation of C1orf167 transcription. It is also notable that four other miR-155/EBV downregulated genes, CCDC74B, CEBPB, KCNQ1, and KLHL5, contain GGGTGGT sequences within −2 kb to +200 bp of their promoters which could similarly play a role in modulating promoter function. Further studies will be required to ascertain the involvement of ZIC3 and/or other miR-155 regulated transcription factors in the regulation of miR-155-inhibited genes.

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