Epstein-Barr Virus Lytic Infection Induces Retinoic Acid-responsive Genes through Induction of a Retinol-metabolizing Enzyme, DHRS9*

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Lytic Epstein-Barr virus (EBV) replication occurs in differentiated, but not undifferentiated, epithelial cells. Retinoic acid (RA) induces epithelial cell differentiation. The conversion of retinol into its active form, retinoic acid, requires retinol dehydrogenase enzymes. Here we show that AGS gastric carcinoma cells containing the lytic form of EBV infection have enhanced expression of a gene (DHRS9) encoding an enzyme that mediates conversion of retinol into RA. DHRS9 expression is also increased following induction of lytic viral infection in EBV-positive Burkitt lymphoma cells. We demonstrate that the EBV immediate-early protein, BZLF1, activates the DHRS9 promoter through a direct DNA binding mechanism. Furthermore, BZLF1 expression in AGS cells is sufficient to activate DHRS9 gene expression and increases the ability of retinol to induce the RA-responsive gene, CYP26A1. Production of RA during the lytic form of EBV infection may enhance viral replication by promoting keratinocyte differentiation.

The switch between latent and lytic EBV infection is mediated by the two viral immediate-early (IE) proteins, BZLF1 and BRLF1. BZLF1 and BRLF1 are transcription factors that together activate expression of all early lytic viral genes (13). In this report, we have used microarray analysis to compare cellular gene expression in gastric (AGS) cells with latent, versus lytic, EBV infection. We show that transcription of the cellular gene, DHRS9, is highly induced by lytic EBV infection, and that this effect is mediated by the BZLF1 IE protein. The DHRS9 gene product is a short chain alcohol dehydrogenase that converts retinol to retinal. We demonstrate that BZLF1 binds RA in cells may be largely determined by the efficiency of retinol conversion within the cell (1).

Given the critical roles that RA plays in cell growth and differentiation, it is not surprising that a variety of viruses are regulated by RA and/or have developed mechanisms for regulating RA signaling (6). For example, treatment of undifferentiated human embryonal carcinoma cells with RA results in the cells becoming permissive for cytomegalovirus (CMV) infection (7, 8). In addition, RA activates expression of immediate-early and early lytic CMV genes in latently infected glioblastoma cells (9). The human papilloma virus (HPV) protein, E6, binds to the ADA3 protein and inhibits its ability to transactivate the retinoid target genes, retinoic acid-binding protein II and p21 (10). Treatment of HPV-16-infected cells with ATRA results in repression of the HPV 16 promoter, induces growth arrest and attenuates the growth promoting effects of EGF in HPV-immortalized cervical cells (11, 12).

Epstein-Barr virus (EBV) is a human herpesvirus that is associated with both epithelial and lymphoid malignancies (13). As a herpesvirus, EBV can infect cells in either a latent or lytic form. While EBV infection of B cells usually results in one of the latent forms of infection, EBV infection of oropharyngeal epithelial cells results in the lytic form of infection and allows the virus to be efficiently transmitted from host to host (14). In immunosuppressed patients, uncontrolled lytic EBV infection in tongue epithelial cells results in a lesion known as oral hairy leukoplakia (OHL) (15). Immunohistochemical analyses of OHL lesions indicate that lytic EBV infection occurs in the differentiated upper spinous layer of OHL lesions (16). In addition, differentiation of an epithelial cell line containing the latent type of EBV infection converts the virus to the lytic form of infection (17). Thus, the lytic form of EBV replication is tightly linked to epithelial cell differentiation.

Vitamin A (retinol), and its metabolites, all-trans-retinoic acid (ATRA) and 9-cis-retinoic acid, control a variety of essential biological functions including fetal development, reproduction, growth, and differentiation (1). The effects of retinoic acid (RA) on the cell are mediated through retinoid X receptors (RXRs) and retinoic acid receptors (RARs) (2). Most cellular retinoic acid is derived from the inactive precursor, retinol. A series of enzymes are required to convert retinol into RA. Retinol is initially converted to retinal by alcohol dehydrogenase and short chain dehydrogenase/reductase enzymes (3). Retinal is then converted to retinoid acid by aldehyde dehydrogenases (4, 5). In the context of the intact organism, the availability of retinoic acid may be largely determined by the efficiency of retinol conversion within the cell (1).

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‡ The abbreviations used are: ATRA, all-trans-retinoic acid; RA, retinoic acid; RXR, retinoid X receptor; RAR, retinoic acid receptor; CMV, cytomegalovirus; HPV, human papilloma virus; EBV, Epstein-Barr virus; OHL, oral hairy leukoplakia; IE, immediate-early; TIK, telomerase-immortalized human keratinocyte; RT, reverse transcription; VCA, viral capsid antigen; EMSA, electrophoretic mobility shift assay; ZRE, Z-responsive element.
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directly to two sites in the DHRS9 promoter and that one of these binding sites is required for efficient BZLF1 activation of the DHRS9 promoter. Most importantly, we show that BZLF1 dramatically enhances the ability of retinol to activate the RA-dependent cellular gene, CYP26A1, in cells. These results suggest that EBV has hijacked the retinol metabolizing machinery to promote cellular differentiation and thus favor lytic viral replication.

EXPERIMENTAL PROCEDURES

Cell Lines—AGS (a gastric carcinoma cell line), HeLa (a cervical carcinoma cell line), and HT-29 (a colorectal adenocarcinoma cell line) were obtained from American Type Culture Collection. Wild-type EBV (B95.8 strain) expressing the green fluorescence (GFP) and hygromycin B resistance genes, and BZLF1- and BMRF1-deleted mutant viruses, were constructed as described previously (18) using bacterial artificial chromosome technology (19, 20). The wild-type, BZLF1-deleted (Z-KO), and BMRF1-deleted (BMRF1-KO) viruses were used to establish stable EBV-positive AGS lines as described previously (21, 22). Akata is an EBV-positive Burkitt’s lymphoma cell line, which was a gift from K. Takada at Hokkaido University, Sapporo, Japan and has previously been described (23). The telomerase-immortalized human keratinocyte (TIK) line was a gift from A. J. Klingelhutz at the University of Iowa and originally derived from human neonatal foreskin as described previously (24). AGS and AGS-EBV cell lines were cultured in Ham’s F-12 medium, and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium and Akata cells in RPMI 1640 medium. HT-29 was cultured in McCoy’s 5a medium (modified). All lines were cultured with 10% fetal bovine serum at 37 °C with 5% CO₂ and 100% humidity, except for TIK cells, which were maintained in keratinocyte-serum-free medium (Invitrogen) with epidermal growth factor and bovine pituitary extract added.

Retinol Gene Induction Assays—For retinol gene induction assays, AGS cells were grown in Ham’s F-12 medium supplemented with charcoal/dextran-stripped serum (Gemini Bio-Products) for 2 days prior to transfection with FuGENE 6 (Roche Applied Science). AGS cells were transfected with either 0.5 μg of BZLF1 or pSG5, together with 0.75 μg of the RARα and RXRα receptors, respectively. Twenty-four hours following transfection cells were treated with medium alone (with 0.1% ethanol), 10 μM retinol (Sigma), or 10 μM ATRA (Sigma). Cells were then harvested for cDNA synthesis at 24 h post-addition of retinol or ATRA. Retinol and ATRA were dissolved in absolute ethanol under reduced light conditions and added to cells daily.

IgG Cross-linking—The B cell receptor of EBV-positive Akata cells was cross-linked with 50 μg/ml of anti-human IgG (Sigma) 48 h prior to harvesting of RNA for cDNA synthesis.

Affymetrix Microarray Analysis—Total RNA was extracted from EBV-negative AGS cells, AGS wild-type EBV, AGS Z-KO, and AGS BMRF1-KO cells using the RNaseasy kit (Qiagen). Affymetrix microarray analysis was then performed at the UNC Functional Genomics Core using Affymetrix human genome U133 plus 2.0 arrays. Data sets were extracted and analyzed using Genespring software comparing the panel of EBV-positive AGS cells to EBV-negative AGS cells.

Plasmids—The BRLF1 and BZLF1 expression vectors contain the BRLF1 and BZLF1 genomic sequences, respectively, inserted in the pSG5 expression vector (Stratagene) under the control of the simian virus 40 promoter (a gift from Diane Hayward) (25). pSG5-ZA185K contains the cDNA of a mutant BZLF1 cDNA (containing a point mutation that alters amino acid 185 from alanine to lysine) (26–28). The RARα and RXRα receptor expression vectors contain the respective cDNA sequences cloned into the pFLAG-CMV vector (a gift from David Jones, University of Utah). The DHRS9 promoter constructs contain the DHRS9 promoter sequences from positions −2229/+79, −209/+415, +161/+415 (in reference to the translational start site) and cloned into the pGL3 luciferase reporter construct (a gift from David Jones, University of Utah) (29). The DHRS9 promoter construct containing promoter sequences −150/+415 was generated by PCR using site specific primers, 5′-GGGCTCGAGCACAATGGGAGTGACTCACAAGAGCA-3′ (sense primer; the XHOI site is underlined) and 5′-GGGAGCTTGGGAGACCTTCCCCCTCAAGTTTACA-3′ (antisense primer; the HindIII site is underlined) and cloned into the pGL3 luciferase vector (Promega).

Semiquantitative RT-PCR—Total RNA was isolated from cell pellets using an RNasey Plus kit (Qiagen). cDNA was synthesized from 2 μg of total RNA using Superscript II (Invitrogen). PCR was performed for the following genes for 35 cycles unless otherwise stated. Primers and conditions were as follows for the DHRS9 gene (GenBank™ accession number: NM_005771), 5′-CTCTGTGTGGTTCTGGAGCTGTA-3′ (sense) and 5′-CCTCCTGACTGCAATTTGTAGGGA-3′ (antisense). The colon-associated DHRS9 promoter primers were 5′-GTCTCTCTGAGGTTCCTGCC-3′ (sense) and 5′-GAAGACACCCGATTCTTGTCA-3′ (antisense) (based on 5′-rapid amplification of cDNA ends and cDNA from colon epithelial cells (29)). PCR conditions were as follows: initial denaturation at 94 °C for 2 min, followed by denaturation at 94 °C for 30 s, with annealing at 56 °C (DHRS9 gene) or 54.8 °C (DHRS9 promoter) for 30 s and extension at 72 °C for 30 s; a final extension at 72 °C for 5 min was also performed. CYP26A1 primers and conditions were as described previously (48). BZLF1 and β₂-microglobulin primers and conditions were as previously described (30), with 25 cycles used to detect BZLF1.

Immunoblot—Protein expression in AGS cells was measured by immunoblot analysis performed as described previously (31), using anti-EBNA1 (1:1000) (a gift from Lori Frappier, University of Toronto), anti-BMRF1 (1:250) (Capricorn), anti-BZLF1 (1:250) (Argene), anti-BRLF1 (1:250) (Argene), and β-actin (1:5,000) (Sigma) antibodies and an ECL detection kit (Amersham Biosciences).

Viral Capsid Antigen (VCA) Immunofluorescence—EBV-negative AGS cells and AGS cells infected with wild-type B95.8 EBV were grown on glass coverslips and fixed in 100% ice-cold acetone. Cells were incubated with VCA-specific mouse monoclonal antibody (Argene) at a 1:50 dilution and then incubated with a 1:1000 dilution of Alexa Fluor 594-labeled anti-mouse specific antibody conjugate (Molecular Probes). Cell nuclei were then counterstained with 4′,6-diamidino-2-phenylindole (Sigma) and visualized using a fluorescence microscope.
Luciferase Reporter Assays—AGS and HeLa cells were transfected with FUGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. Cells were seeded at a density of 200,000 cells per well in 6-well plates and transfected the following day. Transfections were performed using 1 μg of total DNA (including 0.5 μg of expression vector and 0.5 μg of reporter vector) and cells harvested 48 h post-transfection. Luciferase activity was assayed using the luciferase reporter assay system (Promega) as suggested by the manufacturer.

Probes for Electrophoretic Mobility Shift Assay (EMSA)—19-mer oligonucleotides representing potential BZLF1 binding sites (Z-responsive element (ZRE)) within the DHR59 promoter were annealed and 5′-end labeled with 32P using the Klenow reaction (New England Biolabs). 19-mer oligonucleotides were as follows (potential ZRE sites are underlined): ZRE1 (5′-GATCCGTTGTGTCACAATGG-3′ (sense) and 5′-CCA-TTGTGACACAAAGTGA-3′ (antisense)), ZRE2 (5′-GATCT-GGGAGTGACTCACAGAGCAA-3′ (sense) and 5′-TGCTCTTGTGAGTCATCACTCCCA-3′ (antisense)), and ZRE3 (5′-GATCTGAGGAGTTACC-3′ (sense) and 5′-GAG-GAATCCATAGTCTCC-3′ (antisense)). A positive control of the methylated BRLF1 promoter oligonucleotide (BRLF1p) was used as described previously (32).

EMSA—Radioactively labeled probes were incubated with in vitro translated BZLF1 (or pSG5 empty vector), generated using the TNT T7 Quick Coupled Transcription/Translation System (Promega) in accordance with the manufacturer’s instructions. EMSA assays were performed as described previously (32).

Site-directed Mutagenesis—Site-directed mutants of the ZRE-2 BZLF1 binding site in the DHR59 promoter were generated in the context of both the −209/+415 and −150/+415 luciferase reporter constructs using the QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by sequencing. Sequences of the oligonucleotides used to create the mutant ZRE2 constructs were as follows, ZRE2 (5′-GACGTCA-CAATGGAGTGCTCACAGCAAGGAGGAG-3′ (sense) and 5′-CTTCTGTGTCTCTGTGACTCAGCTCCTGACAC-3′ (antisense) (the mutated ZRE2 site is underlined)).

RESULTS

AGS Cells Infected with Wild-type, BZLF1-KO and BMRF1-KO EBV Have Lytic, Latent, or Abortively Lytic Infection, Respectively—To compare cellular gene expression in cells with latent, abortively lytic, or fully lytic EBV infection, we created a panel of gastric carcinoma (AGS) cell lines by infecting the cells with various wild-type or mutant recombinant viruses (expressing GFP and hygromycin B resistance genes in the viral genome) and selecting for cell lines which were GFP+ and resistant to hygromycin B. AGS cells infected with the wild-type control virus, designated B95.8-WT (18), expressed the two immediate-early proteins, BZLF1 and BRLF1, as well as the early lytic protein BMRF1 (Fig. 1A) and the late viral protein, VCA (Fig. 1B). Thus, AGS cells infected with wild-type virus are capable of supporting persistent, highly lytic EBV infection. In contrast, AGS cells infected with the BZLF1-KO virus (in which the IE BZLF1 gene is deleted) (19) as expected showed no expression of the lytic viral proteins, BZLF1, BRLF1, and BMRF1, but did express the latent viral protein, EBNA-1 (Fig. 1A). AGS cells infected with the BMRF1-KO virus (20) had an abortively lytic infection; they expressed both of the two IE proteins (BZLF1 and BRLF1) but did not express BMRF1 and could not support lytic viral replication, since the BMRF1 gene (which encodes the viral DNA polymerase processivity factor) is required for lytic EBV replication (20).

Lytic EBV Infection in AGS Cells Activates Expression of the Cellular DHR59 Gene—Using the panel of cell lines described above, we compared cellular gene expression in EBV-negative AGS cells versus AGS cells with fully lytic (wild-type virus), abortively lytic (BMRF1-KO virus), or latent (BZLF1-KO virus) EBV infection. Microarray analysis was performed with an Affymetrix whole genome microarray. This analysis indicated that the DHR59 gene was the most highly activated cellular gene (increased 10-fold in comparison with uninfected AGS cells) in cells infected with either wild-type virus or the

FIGURE 1. Infection of AGS cells with wild-type EBV and lytic-defective EBV mutants. A, AGS cell were infected with wild-type B95.8 EBV (Wt-EBV), or viruses deleted in the lytic genes BZLF1 (BZLF1-KO; lytic-defective) or BMRF1 (BMRF1-KO; abortively lytic). Stable EBV-positive cell lines were selected using hygromycin B resistance. Expression of the latent protein EBNA1 and the lytic viral proteins BZLF1, BRLF1, and BMRF1 in the stable hygromycin-selected cell lines was examined by immunoblot analysis. β-Actin is also shown as a loading control. B, expression of the late viral protein, VCA, was examined by immunofluorescence in AGS cells infected with wild-type B95.8 EBV and EBV-negative AGS cells. 4′,6-Diamidino-2-phenylindole staining was performed on the same cells.
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Virus

|     | WT-EBV | BZLF1-KO | BMRF1-KO | +RT | -RT |
|-----|--------|----------|----------|-----|-----|
|     |        |          |          | +RT | -RT |

FIGURE 2. Lytic EBV infection induces expression of the cellular DHRS9 gene in AGS cells. CDNA was synthesized from RNA harvested from AGS cells stably infected with wild-type or mutant EBV. DHRS9 expression and BZLF1 expression was quantitated by RT-PCR analysis using undiluted or diluted (1:10) CDNA or no reverse transcriptase (-RT). β2-Microglobulin levels are also shown as a loading control.

BMRF1-KO virus (data not shown). In contrast, AGS cells infected with lytic-defective BZLF1-KO virus showed no change in the level of DHRS9 expression compared with EBV-negative AGS cells.

To confirm that DHRS9 expression is increased in AGS cells infected with wild-type EBV and BMRF1-KO viruses, we performed semiquantitative RT-PCR using cDNA from EBV-positive and EBV-negative AGS cells. AGS cells infected with either wild-type virus, or BMRF1-KO virus, expressed considerably more DHRS9 than EBV-negative AGS cells or AGS cells infected with the BZLF1-KO virus (Fig. 2). These results indicate that lytic EBV infection induces DHRS9 expression in AGS cells and that this effect is likely mediated by one of the two EBV IE proteins, since it also occurs in cells infected with the replication-defective BMRF1-KO virus.

BZLF1 Induces DHRS9 Expression—To determine whether the ability of lytic EBV infection to induce DHRS9 expression in AGS cells is mediated by BZLF1 or BRLF1, EBV-negative AGS cells were transfected with the SG5 control vector, a BRLF1 expression vector, or a BZLF1 expression vector, and DHRS9 expression was measured 2 days later by RT-PCR. AGS cells transfected with the BZLF1 expression vector expressed considerably more DHRS9 than cells transfected with the SG5 control vector or the BRLF1 expression vector (Fig. 3A). The results show that BZLF1 alone is sufficient to induce DHRS9 expression in AGS cells.

We next investigated whether BZLF1 can induce DHRS9 expression in other cell lines. The cervical carcinoma cell line, HeLa, and telomerase-immortalized neonatal foreskin keratinocytes (TIK), were transfected with the SG5 control vector or the BZLF1 expression vector, and DHRS9 expression was examined by RT-PCR 2 days later. As shown in Fig. 3B, BZLF1 clearly induced DHRS9 expression in HeLa cells, as well as TIK cells. The lower level of DHRS9 activation in TIK cells versus HeLa cells likely reflects the much lower transfection efficiency in TIK cells, as indicated by the lower expression of transfected BZLF1. These results indicate that the EBV IE protein, BZLF1, activates DHRS9 expression in a variety of different epithelial cell lines.

DHRS9 Expression Is Activated by Lytic EBV Infection in B Cells—To determine whether lytic EBV infection also induces DHRS9 expression in a B cell environment, the lytic form of EBV infection was induced in the EBV-positive Burkitt’s lymphoma line, Akata, by cross-linking the cell surface IgG with anti-IgG antibodies as previously described (33). EBV-negative Akata cells showed no expression of DHRS9 (Fig. 4). In contrast, EBV-positive Akata cells had a small level of DHRS9 expression in the absence of anti-IgG treatment (consistent with the low level of BZLF1 expression observed in EBV-positive Akata cells even in the absence of anti-IgG treatment) and had a much higher level of DHRS9 expression (as well as BZLF1 expression) following anti-IgG treatment (Fig. 4). These results demonstrate that lytic EBV infection also induces DHRS9 expression in a B cell environment.
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**FIGURE 5.** **BZLF1 strongly activates the **DHRS9** promoter.** A, AGS cells were transfected with the control vector pSG5, wild-type BZLF1, or a mutant BZLF1 (ZA185K), which is defective in DNA binding. Cellular RNA was extracted 48 h post-transfection and cDNA synthesized. The level of a **DHRS9** transcript variant previously shown to be highly expressed in colon cells (29) was measured by RT-PCR. The colorectal cell line HT-29 was included as a positive control for the **DHRS9** colon specific transcript. B, HeLa and AGS cells were transfected with the full-length **DHRS9** promoter luciferase construct (containing promoter sequences from −2229/+1073 relative to the mRNA start site) or a promoterless luciferase construct (containing sequences from −161/+415 relative to the **DHRS9** mRNA start site) plus control vector pSG5 or the BZLF1 expression vector. Cell lysates were harvested 48 h after transfection and luciferase assays performed; fold induction in luciferase activity in the BZLF1-transfected cells (relative to the SG5 control vector transfected cells) is indicated. C, HeLa cells were transfected with the full-length **DHRS9** promoter, a construct containing promoter sequences from −209 to +415 (relative to the mRNA start site), or the promoterless luciferase construct (containing sequences from +161/+415 relative to the **DHRS9** mRNA start site). Cell lysates were harvested 48 h after transfection and luciferase assays performed.

**BZLF1 Activates the **DHRS9** Promoter**—Several different messages are derived from the **DHRS9** gene and there are at least two different promoters driving **DHRS9** expression (29, 34). Expression of **DHRS9** is found in varying epithelial tissues including, lung, colon, and testis, and different promoters are used to generate **DHRS9** depending on the tissue (29, 34, 35). To determine which of the various **DHRS9** messages is activated by BZLF1, we used a series of different primers capable of distinguishing between the various **DHRS9** messages to PCR amplify cDNA in AGS cells with or without transfected BZLF1 (Fig. 5A and data not shown). The results of these experiments indicated that BZLF1 induces a **DHRS9** message in AGS cells, which was previously shown to be highly expressed in colon cells (29) (Fig. 5A). As expected, this transcript was also highly expressed in the colorectal cell line, HT-29 (Fig. 5A).

BZLF1 activates cellular and viral genes through direct, as well as indirect, mechanisms (36, 37). To determine whether BZLF1 must bind directly to DNA to activate **DHRS9** transcription, we examined the effect of a BZLF1 mutant (ZA185K) that contains a point mutation altering amino acid 185 from alanine to lysine and is unable to bind DNA (26–28). The BZLF1 mutant (ZA185K) was incapable of activating **DHRS9** transcription (Fig. 5A). Thus, direct binding of BZLF1 to the **DHRS9** promoter may be involved in the activation of **DHRS9** transcription.

To determine whether BZLF1 enhances the activity of the promoter driving the colon-associated **DHRS9** message, a reporter gene construct containing the **DHRS9** promoter sequences from −2229 to +1073 linked to the luciferase gene (29) was cotransfected into HeLa and AGS cells in the presence or absence of BZLF1 (Fig. 5B). BZLF1 strongly increased the activity of the **DHRS9** promoter (in comparison with the control SG5 vector) in both HeLa and AGS cells. In contrast, BZLF1 did not increase the luciferase activity of a luciferase vector containing **DHRS9** sequences from +161 to +415 (relative to the mRNA start site) linked upstream of the luciferase vector (Fig. 5B). These results indicate that BZLF1 activates the intact **DHRS9** promoter in both HeLa and AGS cells. To further identify the **DHRS9** promoter sequences required for BZLF1 activation, we constructed another **DHRS9**-luciferase vector containing the **DHRS9** promoter sequences between −209 and +161. As shown in Fig. 5C, **DHRS9** promoter sequences between −209 and +161 were sufficient for efficient activation by BZLF1.
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more retinoic acid production, and hence more efficient activation of retinoic acid-responsive genes, in cells expressing BZLF1 than in vector-transfected cells. To determine whether this is indeed the case, we transfected AGS cells with vector control, or the BZLF1 expression vector, in the presence or absence of the retinoic acid receptors (RARα and RXRα) and treated the transfected cells with or without retinol. RT-PCR analysis was then performed to examine the ability of retinol to induce expression of the retinoic acid-responsive cellular gene, CYP26A1, in the various conditions. AGS cells have very low endogenous levels of the retinoid receptors RARα and RXRα and thus do not respond to the effects of retinoic acid unless transfected with the RA receptors (38). Expression of the cellular CYP26A1 gene is known to be highly induced by retinoic acid (39).

As shown in Fig. 8, AGS cells that were transfected with the RA receptors and treated with ATRA had a greatly increased level of CYP26A1 expression, as expected. Retinol plus retinoic acid receptors induced only low level expression of CYP26A1. BZLF1 plus RA receptors in the absence of retinol did not induce CYP26A1 expression, indicating that BZLF1 does not directly activate CYP26A1 expression. Most importantly, AGS cells transfected with the BZLF1 expression vector in the presence of retinol had high level CYP26A1 expression (Fig. 8). These results illustrate that BZLF1 expression enhances the ability of retinol to activate retinoic acid-responsive genes.

**DISCUSSION**

Retinoic acid plays an essential role in regulating the differentiation of epithelial cells. Cellular retinoic acid is derived from the metabolism of retinol, and the ability of cells to metabolize retinol is a major rate-limiting step in regard to retinoic acid production. The Dhrs9 gene encodes a retinol-metabolizing enzyme and is expressed in a variety of ciliated epithelial cell types. It has previously been shown that certain colon carcinomas down-regulate expression of the Dhrs9 gene product, thereby reducing the production of retinoic acid and limiting the inhibitory effects of RA on tumor cell growth (29, 40). In this report, we demonstrate that an Epstein-Barr virus lytic protein, BZLF1, enhances expression of Dhrs9 and increases the ability of retinol to activate RA-responsive cellular genes. As EBV lytic replication is induced by epithelial cell differentiation (17), this ability of the virus to stimulate RA production presumably helps the virus to promote its own replication.

The effect of epithelial cell differentiation on EBV infection has been studied in vivo by analyzing lesions known as OHL, which are found only in immunocompromised patients and are caused by completely lytic EBV infection in tongue epithelial cells. In OHL lesions, EBV infection is restricted to the upper spinous differentiated layers of the tongue, with no viral infection or replication observed in the undifferentiated layers of the epithelium (16). Whether the apparent inability of EBV to infect less differentiated epithelial cells in vivo is due to the inability of the virus to enter such cells (perhaps because the as yet unknown epithelial cell receptor is only expressed on more differentiated cells), versus some later step of viral infection, is not entirely clear. However, in vitro evidence suggests that epithelial cell differentiation may be required for the lytic form of
viral replication. Treatment of latently infected EBV-positive epithelial cell lines with differentiating agents converts the virus to the lytic form of viral infection (17). In addition, the BZLF1 promoter is activated by epithelial cell differentiation (41). In contrast to EBV infection of normal epithelial cells, which is completely lytic and limited to differentiated cells, the major epithelial cell malignancy associated with EBV infection, nasopharyngeal carcinoma, is composed of poorly differentiated cells in which the virus is mainly restricted to a latent form of infection.

Given the close association between efficient lytic EBV infection and epithelial cell differentiation, our finding that the EBV IE viral protein, BZLF1, enhances expression of a retinol metabolizing enzyme is particularly interesting and relevant to viral pathogenesis. By increasing DHRS9 expression, the virus likely increases the amount of cellular retinoic acid and thus enhances transcription of retinoic acid-responsive genes. In support of this, our results in AGS cells showed that BZLF1 greatly increases the ability of retinol to induce a well known RA target gene, CYP26A1. Enhanced production of RA in virally infected cells may allow EBV to replicate more efficiently by promoting epithelial cell differentiation. In addition, RA released from virally infected cells could potentially affect neighboring cells through a paracrine mechanism, promoting their differentiation and enhancing cell to cell spread of virus.

In addition to promoting lytic viral replication in epithelial cells, BZLF1 induction of DHRS9 gene expression may also promote lytic EBV replication in B cells. Increasing evidence suggests that the lytic form of viral infection in B cells is tightly correlated with terminal differentiation of memory B cells into plasma cells (42). Importantly, RA was recently shown to induce differentiation of resting B cells into plasma cells (43). As our results indicate that lytic EBV infection in B cells (Akata) activates DHRS9 gene expression, this suggests that lytic EBV infection could promote plasma cell differentiation through enhanced RA production.

The ability of BZLF1 to enhance retinol-mediated expression of the cytochrome P450 enzyme, CYP26A1, may also benefit the virus by inhibiting cellular apoptosis and preventing early death of the host cell before infectious viral particles can be released. CYP26A1 has been shown to protect cells from an array of apoptotic stimuli including TRAIL, oxidative stress, genotoxic drugs, and γ-irradiation (44). Furthermore, elevated levels of CYP26A1 are found in cancers such as acute promyelocytic leukemia (45) and squamous cell carcinomas of the head and neck (46).

Although we show here that BZLF1 enhances expression of a retinol metabolizing protein, and by this mechanism presumably increases transcription of RA-responsive cellular genes, BZLF1 was previously shown to directly interact with RAR and RXR receptors and inhibit their ability to activate RA-responsive genes (47). These results suggest that BZLF1 may function as either a positive, or negative, regulator of RA signaling, possibly depending upon the amount of BZLF1 expressed, the cell type, or the particular cellular gene target. In the case of the RA target gene CYP26A1, our results in AGS cells clearly indicate that the positive effects of BZLF1 on retinol metabolism out-
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weigh any negative effects on RA signaling mediated by the direct interaction of BZLF1 with the RAR/RXR receptors. Interestingly, as the direct interaction between BZLF1 and RAR/RXR receptors has been shown to inhibit the ability of BZLF1 to activate early lytic genes (47), induction of RA metabolism by BZLF1 could serve as a mechanism for inhibiting early lytic viral gene transcription, allowing the virus to advance to late lytic gene transcription.

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