Epstein-Barr virus transcription activator R upregulates BARF1 expression by direct binding to its promoter, independent of methylation.

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Running title: R is the EBV lytic transcriptional activator of BARF1

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Epstein-Barr Virus (EBV) BamHI-A rightward frame 1 (BARF1) is considered a major viral oncogene in epithelial cells and has immune-modulating properties. However, in B cells and lymphomas BARF1 expression is restrained to the viral lytic replication cycle. In this study the transcriptional regulation of BARF1 during lytic replication is unraveled.

Bisulfite sequencing of various cell lines indicated a high level of methylation of the BARF1 gene control region. A BARF1 promoter Luciferase reporter construct was created using a CpG-free vector, enabling true assessment of promoter methylation. Induction of the EBV lytic cycle is mediated by the immediate-early proteins, BZLF1 (Z) and BRLF1 (R). R was found to activate the BARF1 promoter up to 250 fold, independent of Z and unaffected by BARF1 promoter methylation. Chromatin Immune Precipitation (ChIP), Electrophoretic Mobility Shift Assay (EMSA) and specific mutagenesis of the R responsive elements (RREs) demonstrated direct binding of R to RREs between -554 and -327 nucleotides relative to the BARF1 transcriptional ATG start site. The kinetics of BARF1 expression upon transactivation by R showed that BARF1 mRNA was expressed within 6 hours in context of the viral genome.

In conclusion, expression of the BARF1 protein during lytic replication is regulated by direct binding of R to multiple RREs in the gene control region, and is independent of the promoter methylation status. The early kinetics of BARF1 upon transactivation by R confirm its status as an early gene and emphasize the necessity of early immune modulation during lytic reactivation.

Keywords: Epstein-Barr Virus, BARF1, BRLF1, BZLF1, Rta, Zta, gene regulation
INTRODUCTION (962)

Epstein-Barr virus (EBV) infects 90% of the world population and persists in the host for life. It causes a relatively mild primary disease if acquired early in life and infectious mononucleosis if acquired after adolescence. EBV has dual tropism in vivo, infecting B lymphocytes and stratified epithelium (54;59), as reflected by its association with several lymphomas and carcinomas (35;44;52;68). Being a gamma herpesvirus, EBV can infect cells in either latent, or lytic form. Lytic replication, required for progeny virus production, occurs in epithelial cells and following differentiation of B cell into plasma cells (34;37;54). In latency, EBV expresses only a few genes necessary for viral genome persistence, altering signal transduction and cell cycle control, causing apoptosis inhibition, and modulation of immune recognition.

The BamHI-A rightward frame 1 (BARF1) protein is highly and selectively expressed in carcinomas such as nasopharyngeal carcinoma (NPC) and gastric carcinoma (GC) and is considered a major viral oncogene in epithelial cells (13;25;57;60;66). BARF1 may drive carcinogenesis by immortalizing and transforming epithelial cells of different origin and by up-regulating anti-apoptotic Bcl-2, enabling cell survival under inappropriate conditions (58;65). In addition, secreted hexameric BARF1 inhibits macrophage colony stimulating factor (M-CSF), thus manipulating myeloid cell growth and functions (12;28;61). However, in B cells and lymphomas BARF1 expression is restrained to the viral lytic replication cycle (24;49).

Lytic replication is mediated by the virally encoded DNA polymerase using the oriLyt replication origin, and results in the release of infectious viral particles (30;34). The switch from latent to lytic Epstein-Barr virus (EBV) infection is mediated by the viral immediate-early (IE) proteins, BZLF1 (Z, Zta, ZEBRA, EB1) and BRLF1 (R, Rta). Z and R are transcription factors which autostimulate their own expression, reciprocally activate each other, and cooperatively induce expression of all early lytic viral proteins, allowing the virus to replicate (30;34;41). Z is a 245 amino acid (aa) bZip family protein homologous to c-Jun and c-Fos, together forming the AP1 transcription factor. It contains three functional domains: a transactivation (TA) domain (aa1-167), a DNA binding domain (aa178-196), and a dimerization domain (aa197-221) (51). Z binds to the consensus AP1 motif as well as atypical AP1-like motifs known as Z-responsive elements (ZREs) (10;17;19;20).
R is a 605aa protein with homologues among the gamma herpesviruses (14;42;67). It contains a combined N terminal DNA binding and dimerization domain (aa1-232) and the TA (transactivator) domain is found in the C terminal region (43). Rta homodimerizes in the absence of DNA. R activates some promoters through a direct binding to specific DNA sequences, known as R response elements (RRE) (21;27;29;33), but other promoters are activated by indirect mechanisms (1;9;40;53). R activates the BZLF1 promoter indirectly through effects on cellular transcription factors (c-Jun and ATF-2) which bind to a CRE motif (1;11;22). RREs are GC-rich motifs of which the consensus recognition sequence is gNcc-N9-ggNg. The sequence of both the central nucleotides and, to a lesser extent, the flanking sequences contribute to the binding affinity and transcriptional activation by R (11). R promoter activation is inhibited by direct binding of EBV LF2 protein which mediates its translocation from the nucleus (26). R also directly interacts with the histone acetylase CREB-binding protein (CBP) (62), with Oct-1 (56), and with RanBPM, promoting R sumoylation (8).

Viral gene expression is, in addition to regulation by transcription factors, controlled by epigenetic modulation. The linear EBV genome in virions is not methylated. However, in latently infected cells the majority of the EBV genome becomes highly methylated. EBV uses controlled methylation of its genome initially to prevent production of viral progeny after initial infection, which would kill its host, and to suppress the expression of immunodominant latent viral antigens shortly upon host cell immortalisation (2;5;16;34;48). DNA methylation, which plays a critical role in modulating the expression of both cellular and viral genes, induces transcriptional repression by multiple different mechanisms, including prevention of transcription factor binding to DNA and the recruitment of HDAC complexes (6). The patterns of EBV genome methylation are specific and selected viral promoters appear never to be methylated, such as Qp and both EBER genes and flanking sequence (47;55;64). For the (down-)regulation of these unmethylated regions, mechanisms other than methylation have been proposed (63;64). Z has enhanced ability to bind to methylated promoters (4;15), and methylation is required for the virus to enter the lytic phase (31;32). R preferentially activates unmethylated lytic promoters; however, methylation does not inhibit DNA binding (CK Wille, Herpes meeting Poland).
BARF1 is considered an early lytic gene (41) but detailed information about its transcriptional activation in the lytic cycle is absent. The control region of BARF1 is largely overlapping with that of BALF2. The BALF2 coding sequence is on the minus strand and its ATG start site only 734 nucleotides apart from BARF1. The methylation status of the BARF1 gene control region in various cell lines was investigated, showing a high level of methylated CpG islands. So far, it was unknown how BARF1 expression is regulated during the lytic stage. An independent study analysing R DNA binding using ChIP-sequencing indicated R to bind to the bidirectional BARF1/BALF2 promoter region, but no detailed mapping was provided and BARF1 gene responsiveness to R was not further analyzed (27). In this study the regulation of BARF1 in the lytic cycle by Z or R or in combination was investigated. We demonstrate that the BARF1 gene is transactivated by R and not by the major lytic switch protein Z, independent of methylation status, and show direct binding of R to multiple identified RRE sites. Site directed mutagenesis of RRE sites showed RRE 2 located between -516 and -498 and RRE 3 located between -426 and -409 relative to the BARF1 transcriptional ATG start site to be the major activating sites. These results revealed a new mechanism for the regulation of BARF1 expression.
MATERIALS AND METHODS (1330)

Cell culture

Hone-1, an EBV negative human nasopharyngeal carcinoma (NPC) cell line, SNU-719, a naturally derived EBV-infected gastric carcinoma cell line (50), and the P3HR1-derived cell line HH514 (a gift of G. Miller) were maintained in RPMI 1640 medium. Hela, 293 cells, and C666.1, a NPC cell line consistently harboring EBV (a gift from D. Thorley-Lawson), were maintained in DMEM. C666.1 cells were cultured in fibronectin-coated flasks (Sigma-Aldrich, Buchs, Switzerland). AGS cells were maintained in Ham's F-12 medium. EBV infected 293 cells, 293 cells infected with a Z knock-out EBV mutant, EBV-positive AGS (gifts from H.-J. Delecluse), and 293 infected with Z stop and R stop EBV have been described previously (18;56), and were maintained under 100 µg/ml hygromycin (Roche, Basel, Switzerland) selection. CNE-2 Akata cells (a gift from K. W. Lo), a NPC cell line superinfected with the Akata strain of EBV, was maintained in RPMI 1640 under 400 µg/ml G418 (Invitrogen, Carlsbad, CA, USA) selection. All media contained 10% FCS, 100 units/ml sodium penicillin, 100 µg/ml streptomycin sulphate and 2 mM L-glutamine. HH514 was induced by using 20 ng of 12-tetradecanoylphorbol-13-acetate (TPA) per ml and 3 mM sodium butyrate (NaB) as described previously (45).

Bisulfite sequencing PCR

Tumor material from C15 and C17 human NPC xenografted in mice were a kind gift from P. Busson (7). Genomic DNA was isolated from cells and tumor material using silica-based extraction (Basic kit, BioMerieux, Craponne, France). EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) was used for bisulfate treatment of 500 ng DNA, after which the area of interest was amplified using Amplitaq Gold (Roche). The following primer sequences designed to anneal to conversed DNA, and flanking the two largest methylation islands, were used [GeneBank accession No. NC007605]: region 1 (164414 – 164615) forward: AGTTAGTTAGGTTAGGTTTTTA and reverse: CTCACAAATACTATACACACACACATAATA, region 2 (164550 – 164792) forward: GCTTTGTGTTATTTAGGTAGTTT and reverse: CCTTTACCAACCCTATCTCTAC, and region 3 (164771 – 165045) forward:
AGAGGATTAGGGTTGGTAAAGGTAG and reverse: ACCATTACTCTAAACTCTCCTCACC. The PCR product was sent for direct sequencing to BaseClear (Leiden, The Netherlands).

**Plasmids**

Plasmid DNA was purified on maxiprep columns according to the manufacturer's protocol (Qiagen, Venlo, The Netherlands). pSG5 and pcDNA3.1 were obtained from Stratagene (La Jolla, CA, USA) and Invitrogen, respectively. The SG5-R and SG5-Z expression vectors (kindly provided by S.D. Hayward) containing the B95.8 BRLF1 and BZLF1 open reading frame respectively, and SG5-R (aa1-550) expressing R deleted for the transcriptional activation domain were previously described (23;56). The BARF1 promoter region from -678 to the ATG start site (164367 - 165045) [GeneBank accession No. NC007605] was cloned into the pCpG.LUC, a CpG-free luciferase reporter vector kindly provided by M. Rehli (36), using forward primer with SpeI-site: CTGACTAGTCTCATCACGCAACACCCACTGTTT, and reverse primer with BglII-site: AATAGATCTGCTGAGACTCTCTCTCACCAG. To construct deletion mutants the following forward primers with a SpeI-site were used:

- **ATG-633**: CTGACTAGTAAGTCAGTCAGGCTGGCCAGG,
- **ATG-582**: CTGACTAGTGATCTTGGCATGCCGCCCAGC,
- **ATG-468**: CTGACTAGTACCGCAAACACCACTGTGTAGC,
- **ATG-410**: CTGACTAGTGGCAGCTGGCTGTCCTGCAGG,
- **ATG-350**: CTGACTAGTCGATGCGGTGCCTGCAGG,
- **ATG-327**: CTGACTAGTGGCAGCTGGCTGTCCTGCAGG,
- **ATG-261**: CTGACTAGTGCCGAAAGGCAGGTCTTTGCTATCC,
- **ATG-220**: CTGACTAGTGCCGAAAGGCAGGTCTTTGCTATCC,
- **ATG-156**: CTGACTAGTCACGCTCGCGCCGGGC,
- **ATG-63**: CTGACTAGTTGATAAATGGGCAGTGCCAG.

The plasmid was propagated in PIR expressing bacteria (Invitrogen). RRE mutants of the BARF1 promoter reporter construct were created using the Quickchange Lightning multi-site-directed mutagenesis according to manufacturer's instructions (Stratagene) to incorporate specific mutations in the pCpG.BARF1p(ATG-582).LUC.
In vitro DNA methylation

The use of a CpG-free reporter construct enables to study the effect of promoter methylation without silencing due to backbone methylation. In vitro DNA methylation of the luciferase constructs was accomplished with CpG methylase (SssI methyltransferase; New England Biolabs, Ipswich, MA, USA), by following the procedure recommended by the manufacturer. Completion of DNA methylation was confirmed by digestion with the restriction enzyme HpaII (New England Biolabs), which cleaves its recognition sequence only if the DNA is not methylated at the cytosine residue within the CpG motif.

Transfections

Cells were seeded the day prior to transfection. Transfections of HeLa cells for reporter assays were performed by use of Fugene 6 (Roche). Other cell lines were transfected by use of Lipofectamine 2000 (Invitrogen). Transfections were performed according to the manufacturer’s instructions, except that for reporter assays the reagent:DNA ratio was 1.5 µl:0.5 µg in 100 µl Opti-MEM for 2x10^5 cells plated in 1 ml medium in a twelve-wells plate.

Luciferase assays

Luciferase assays were performed 48 h after transfection by using extracts prepared by freeze-thawing the cell pellet in reporter lysis buffer according to the instructions of the manufacturer (Promega, Madison, WI, USA). Luciferase activity was assayed using the luciferase reporter assay system (Promega) as suggested by the manufacturer.

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation was performed as described previously (56). Briefly, 293 BRLF1-stop cells were transfected with pSG5 or pSG5-R, and cross-linked after 24h in EGS (ethylene glycolbis [succinimidyl succinate]) followed by fresh 1% paraformaldehyde. Following cell lysis and DNA fragmentation by sonication, DNA-protein complexes were immunoprecipitated with anti-BRLF1 (Argene, Shirley, NY, USA), and control anti-IgG (Santa Cruz, Santa Cruz, CA, USA) antibodies. Protein-DNA cross-linking was reversed at 65°C overnight, and DNA was purified using the Qiagen gel extraction kit. The presence of BARF1 promoter DNA fragments in each precipitate was detected using PCR using forward primer: GGCCCTGAACATGAGGTAGC and
reverse: TCTGGACTCTCCTCACCCAG (164829 – 165042), and primers for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were forward: TCACCACCAGGAGAAGGCT and reverse: GCCATCCACAGTCTTCTGGG.

EMSA
R550 protein extract and control extract were created as Chen et al. (10) by lysis of SG5-R550 and SG5 transfected Hela cells. Cells were harvested, centrifuged, and snap-frozen at -80°C. Frozen cell pellets were suspended in lysis buffer contained 0.42 M NaCl, 20 mM HEPES (pH 7.5), 25% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 1mM DTT, 1 mM PMSF and protease inhibitor cocktail (Roche), followed by 15 min maximum speed centrifugation at 4°C. Supernatants were stored at -80°C and protein concentrations were determined by the Bradford method (BioRad, Hercules, CA, USA). Annealed double-stranded oligonucleotides (Fig. 3C) were end-labeled with ³²P using T4 polynucleotide kinase (New England Biolabs) and desalted with G-25 sephadex columns (Roche). Binding reaction mixtures were performed in buffer containing 10 mM HEPES, (pH 7.5), 50 mM NaCl, 2 mM MgCl₂, 2.5 µM ZnSO₄, 0.5 M EDTA, 1 mM DTT, 15% glycerol, and 0.5 µg poly(dI-dC), using 15 µg total cell lysate followed, after 5 min at room temperature, by 11,000 cpm of labeled nucleotide in a total volume of 50 µl. For supershift reactions, anti-BRLF1 (Argene) was added 20 min following addition of the probe. The reactions were incubated for 40 min at RT before being loaded onto a 4% polyacrylamide gel in 0.5x Tris-borate-EDTA buffer at 35 mA. Gels were dried on Whatman paper under a vacuum and exposed to autoradiography film for 12 to 40 hours at -80°C.

Quantitative RT-PCR
Cells were plated in 6 well plates and transfected with either SG5-R or SG5 control. At designated time points cells were harvested in 1 ml Trizol (Invitrogen) and stored at -80°C until further processing. Guanidinium isothiocyanate-phenol-chloroform extraction was performed to isolate total cellular RNA, followed by DNase (Promega) treatment and ethanol-precipitation. cDNA was synthesized using AMV Reverse Transcriptase (Promega) and sequence specific primers: BARF1 forward: GCCCTCAACGCTGTCTGTC and reverse: GAGAGGCTCCCATCCTTTTC (165414 - 165433) [GeneBank accession No. NC007605], R...
RT-PCR was performed with SybrGreen (Roche) and aforementioned primers using the LightCycler® 480 system (Roche). After quantification to known concentration of the corresponding gene constructs, values were normalized to U1A.

SDS-page and Western blot. Cells were lysed in RIPA buffer containing Protease Inhibitor Cocktail (Roche) and sonicated, after which cell debris was removed by centrifugation. Supernatants were diluted in 2x loading buffer (Biorad) with β-mercaptoethanol, denatured for 5 min at 95°C and separated on a 10% SDS-page gel. After transferring to Hybond ECL nitrocellulose membrane (GE Healthcare, Little Chalfont, UK), the membrane was blocked in PBS with 0.05% Tween-20 (PBST) containing 3% non-fat dried milk for 1 h at RT, after which the following antibodies were incubated for 2 hours at RT in PBST containing 5% BSA: anti-BRLF1 1:250 (Argene), Anti-BZLF1 1:250 (Santa Cruz Biotechnology, sc-53904) or Anti-β-actin peroxidase 1:10,000 (Sigma-Aldrich). After incubation with peroxidase labeled secondary antibody rabbit anti-mouse (Dako, Glostrup, Denmark) bands were visualized with ECL+ (GE Healthcare).
The BARF1 promoter is highly methylated in cell lines and carcinomas

The methylation status of EBV-promoters influences the regulation by various transcription factors. Analysis of the number of CpGs in the BARF1 promoter by using Methprimer (38) showed two large CpG islands spanning the whole putative promoter region up to -607 relative to the BARF1 ATG start site (Fig. 1A). Bisulfite sequencing PCR (BSP) primers were developed to completely analyze the region spanning from ATG-496 to the ATG start site comprising the CpG regulatory elements. Two cell lines, HH514 Burkitt’s lymphoma cells, and C666.1 cells consistently harboring EBV were studied, next to C15 and C17 mouse xenograft NPC tumor material (7). BARF1 mRNA was expressed in the C666.1 NPC cell line and in C15 and C17 NPC mouse tumor (Fig. 1B). After bisulfite treatment, the two CpG rich regions were amplified for sequence analysis. Almost all CpGs in the investigated samples were methylated (Fig. 1C, black dots), both in carcinoma and B cell lines, indicating that BARF1 is expressed, despite methylation of its promoter. CpG site I was (partly) unmethylated (white dot) in C666.1 cells and C17 tumor material. CpG site II was unmethylated in HH514 cells. Treatment of HH514 cells with the histone deacetylase inhibitor sodium butyrate (NaB) combined with TPA for 24h induced the early lytic cycle, which is associated with hypomethylation of the viral DNA (46). BSP methylation analysis of NaB-TPA treated HH514 cells, demonstrated that the vast majority of the BARF1 promoter was demethylated (Fig. 1D). Quantitative analysis confirmed that the lytic cycle immediate early gene R and the early gene BARF1 mRNA were induced by NaB-TPA treatment (Fig. 1B).

R, but not Z, activates the BARF1 promoter, independent of methylation status

To examine if and which of the immediate early proteins were responsible for the induction of the BARF1 gene in the lytic cycle the BARF1 promoter sequence (up to -679 nucleotides from the ATG start site) was inserted upstream of the luciferase gene in a pCpG-free reporter construct. EBV positive cells have always a certain amount of baseline lytic activity, 1 to 5 % of the cells express Z and/or R. The constitutive activity of the BARF1 promoter was higher in EBV positive cells (Fig. 2A), indicating that an EBV gene is responsible for the activity. The reporter construct
was cotransfected with an expression vector containing the R gene, in multiple EBV positive and negative cell lines. Induction of luciferase activity was evaluated 48 hours after transfection. Equal R expression levels were confirmed by Western blot (data not shown). R induced 50 to 250 fold upregulation of luciferase activity in both EBV positive and negative cell lines (Fig 2B). The BARF1 promoter region was found to be highly methylated. To examine how methylation affects its ability to be activated, the pCpG luciferase construct containing the BARF1 promoter was ex vivo methylated using methyltransferase or mock treated. Both the constitutive as the R induced activity of the BARF1 promoter driven luciferase construct was weaker in the methylated construct, leaving the fold induction mostly unaffected by methylation (Fig. 2C and 2D). To some promoters, Z preferentially binds to the methylated versus the unmethylated Z response elements (4;15). A minor induction of the methylated construct was seen when cotransfected with the Z expression vector (Fig. 2D), and depending on the cell line, Z induced an average of 45 fold induction of luciferase activity of the methylated construct (data not shown). Cotransfection with both Z and R resulted in a lower induction of luciferase activity then when R alone was expressed (data not shown).

R alone can induce BARF1 expression in EBV infected epithelial cells.

To obtain conclusive evidence that R activates BARF1 RNA expression in the context of EBV infection, EBV positive cells were transfected with R expression vector and quantitative RT-PCR was performed. CNE Akata and AGS B95.8 (not shown), both carrying recombinant EBV, and C666.1 cells, a NPC cell line consistently harboring EBV, show induction of BARF1 mRNA above basal expression levels when R was transfected (Fig. 2E). To rule out that this induction is independent of viral replication, cells with a Z defective EBV, 293 Zstop and AGS ZKO (not shown), incapable of lytic induction were transfected with the R expression vector. The strong induction of BARF1 mRNA confirms that R is capable of transactivating the BARF1 promoter in the context of the viral genome, independent of Z or viral replication (Fig. 2F). The increase of BARF1 RNA expression was R dosage dependent.
Multiple R responsive elements are mapped between -544 and -327 nucleotides relative to the ATG start site.

The BARF1 promoter region harbours multiple potential R responsive elements (RREs) based on consensus sequence gNcc[9]ggNg (11) as depicted in Fig. 3A. To determine if R directly binds to the BARF1 promoter region in EBV harboring cells, Chromatin Immune Precipitation (ChIP) assays were performed using extracts from BRLF1-stop cells transfected with a R expression vector or a control vector. R was precipitated by anti-R antibody, and cross-linked DNA was PCR amplified. As shown in Fig. 3B, the ChIP assays demonstrate that R binds to the BARF1 promoter region or in its immediate proximity.

To map the precise locations of the RREs in the BARF1 promoter, a series of probes was designed spanning each of the various RREs and surrounding nucleotides in the BARF1 promoter (Fig 3C). The ability of R to bind to these RRE probes was evaluated in an Electro Mobility Shift Assay (EMSA), using lysate of cells transfected with a R550 (aa1-550) expression vector, which lacks the activation domain and was previously shown to bind with higher affinity than full length R in EMSA (11). A probe containing the EBV BMLF1 (SM) promoter RRE, previously found to be a strong RRE (11), served as a positive control. The probes that showed detectable R binding contained BARF1 promoter sequences from -544 to -327 nucleotides relative to the BARF1 transcriptional ATG start site (Fig. 3D). Specific interaction between the oligos and R550 was confirmed by supershift (S) with antibody to R. Remarkably, the probe containing a predicted RRE sequence with the best homology to the optimal R binding element (R7) (11) did not show detectable binding in EMSA (not shown). The BARF1 RRE probes also demonstrate binding to unknown cellular factors, some of which have the same migration properties as R. Therefore, the supershift bands with antibody against R are better visible. These unknown factors are not further described in this study. The BMLF1 oligo forms, apart from R, a complex with YY1 (11).

To further identify the RRE responsible for BARF1 promoter activation by R, deletion mutants of the reporter construct were made, shortening the BARF1 promoter sequence from the original -679 to -63 nucleic acids relative to the ATG start site in small, 23 to 114 nucleotide steps (Fig.
After removing the region between ATG-679 and -582 an initial three-fold increase of luciferase up to 900 can be seen (Fig. 3E), which can be explained by loss of interfering proteins or suppressors. When R1 and R2 (between 5’ deletion ATG-582 and -468) were deleted, luciferase activity showed a 14 fold decrease and continued to slowly decrease down to ATG-327 (Fig. 3E), indicating that multiple RREs could work in synergism.

RREs are required for R dependent BARF1 promoter activation.

To illustrate the importance of the RREs for BARF1 promoter activation by R, mutants of the reporter construct were made in which single RRE’s or the combination of all four were mutated in the CG-rich motif (Fig. 4A). These constructs were transfected into AGS cells together with the expression vector for R and luciferase activity was measured. Mutation of RRE 2 and RRE 3 showed a decrease in luciferase activity compared to control (Fig. 4B). Mutation of RRE 1 and 4 showed an increase in luciferase activity. It is possible these single mutations allowed more R binding space for RRE 2 and 3, or new cellular transcription factor binding sites were created. When all four RREs were mutated, luciferase activity was reduced by 84%.

BARF1 expression kinetics in response to R

The kinetics of R responsive genes varies among cell types, and depends on the type of lytic stimuli used (11;33;41). The abundance of BARF1 RNA was examined at various time points after transfection with a R expression vector in AGS B95.8 wt cells, and compared to cells that were transfected with control vector. BARF1 mRNA in these cells was induced 15 hours after transfection with R expression vector and fold induction was low (~35) compared to empty vector control (Fig. 5A). AGS B95.8 wt cells have some lytic cells in the pool (39), expressing R protein (Fig. 5B) and expressing BARF1, which could shield new induction of BARF1 RNA by R. To obtain a more accurate and sensitive readout of BARF1 expression kinetics by R, AGS Z knock out cells were used, which do not express Z and have no background expression of R protein (Fig. 5D). Baseline levels of R mRNA were 630 fold lower in AGS ZKO cells compared to AGS B95.8 wt cells (Fig. 5E) and baseline levels of BARF1 RNA were 38 fold lower in AGS ZKO cells.
compared to AGS B95.8 wt (Fig. 5F). In AGS ZKO cells, induction of BARF1 RNA can be detected as early as 6 hours after transfection with R expression vector (Fig. 5C), confirming that BARF1 expression as an early gene during lytic reactivation is activated by immediate early R transactivating protein.

DISCUSSION (1465)

Secreted BARF1 protein is a potent immune modulator and intracellularly, BARF1 may drive carcinogenesis. BARF1 is considered an early lytic gene but detailed information about its epigenetic regulation and its transcriptional activation in the lytic cycle is absent (41). Latency of EBV is enabled by dense methylation of the viral episome. The methylation status of an EBV promoter influences its regulation by various transcription factors, and it generally induces transcriptional repression. In latency BARF1 is expressed, however this is only observed in EBV-related carcinomas and not in lymphomas (25). In carcinomas BARF1 expression is not related to lytic cycle activation (57). The transcription of BARF1 in latent carcinomas might be explained by different levels of epigenetic regulation and/or involvement of different host cell transcription factors. The methylation status of the BARF1 gene control region in various cell lines was investigated and almost all CpGs were methylated, both in carcinoma and B cell lines, indicating that BARF1 transcription factor(s) must be able to overcome methylation-induced repression. When NaB and TPA were use to induce the lytic cycle, virtually all CpGs were demethylated. However, this can be a combined effect of the histone deacetylase inhibiting function of NaB in parallel with lytic cycle induced demethylation.

The switch from latent to lytic EBV infection is mediated by the viral immediate-early proteins Z and R inducing a series of non-structural viral genes preparing the cell for survival and viral DNA replication, finally resulting in the production and release of infectious viral particles (30;34).

BARF1 belongs to the group of non-structural EBV genes, functioning in apoptosis resistance and immune modulation (28;58;61;65). This study reveals that R, and not Z, is the BARF1 transcriptional activator. R transactivates BARF1 by direct binding to its promoter, and methylation does not influence R transactivating activity of the BARF1 promoter. EBV viral genome
methylation differentially affects BZLF1 (Z) versus BRLF1 (R) activation of lytic viral promoters. Z has enhanced ability to bind to methylated, versus unmethylated ZREs of the Na and R promoters, but not other EBV promoters (4;15), while R activation of lytic promoters can be inhibited by methylation (BMRF1) or even put to a stop (BALF2, BLRF2) (CK Wille, presented at the 36th International Herpesvirus Workshop, Gdansk Poland, 24 to 28 July 2011). Assays using a BARF1 promoter reporter construct, either methylated or unmethylated, showed R, and not Z, to be responsible for the induction of BARF1 transcription. Z was only moderately capable of inducing the methylated reporter construct, and the Z responsive elements found in the BARF1 promoter area are reversed oriented (3). Methylation reduced both the constitutive activity (approximately 4 fold lower, data not shown) as the R induced activity of the BARF1 promoter reporter construct, leaving the fold induction by R mostly unaffected by methylation, and indicating that other cellular transcription factors are affected by the methylation. In induced HH514 BL cells, C666.1 NPC cells, and C17 xenografts BARF1 as well as R mRNA was present (Fig. 1B). However, in the C15 xenograft only BARF1 mRNA could be detected. BARF1 is highly expressed in NPC and GC in which EBV displays a latency phenotype with no expression of lytic cycle genes (13;25;57;60;66). In carcinomas BARF1 expression is likely regulated by a cell type specific transcription factors other than the R early gene, explaining the presence of R independent BARF1 mRNA in the C15 xenograft. Many RREs in the BARF1 promoter as well as other gene promoters contain CpG motifs in both the core and linker regions and R binding to these RREs is not affected by methylation. Differences in level of epigenetic modification in gene control regions, ranging from the RRE to the TATA-box and polymerase binding site, might explain why methylation is not inhibitory for BARF1 transcriptional activation while it is for other R responsive genes. The relative distance to the TATA-box and the quality and quantity of RREs combined with epigenetic modifications all together influence the interaction with other factors of the transcriptional apparatus. Expression of R in cells harboring full or Z knock out EBV episomes, demonstrated that R is capable of transactivating the BARF1 promoter in the context of the viral genome and independent of Z or viral replication. Chromatin immune precipitation further demonstrated that R
binds to the BARF1 promoter region. The R transcriptional activator binds to specific DNA sequence. Chen et al. previously published the consensus and optimal RRE sequences, based on mutagenesis of the core and 9 nucleotide linker [N9] sequences in the RRE in the BMLF1 promoter (11). Based on this consensus sequence, leaving room for error, we found that the BARF1 promoter region harbours seven potential RREs (Fig. 3B). EMSA showed R binding to four probes containing BARF1 promoter sequences from -544 to -327 relative to the BARF1 transcriptional ATG start site. Experiments with 5’ deletion mutants of the reporter construct showed a 14 fold decrease of activity when RRE1 and RRE2 were lost and additional 4 fold decrease after loss of RRE3 and RRE4, indicating that the RREs work in synergism. Mutants of the RREs in the BARF1 promoter reporter construct illustrate the importance of the RREs in promoter activation by R. Single mutation of RRE2 or RRE3 results in loss of transactivating activity and mutation of all four RREs reduces luciferase activity to 16% of the unmutated control. We found the major RREs responsible for R activation of the BARF1 promoter were RRE 2, located between -516 and -498, and RRE 3 located between -426 and -409 relative to the BARF1 transcriptional ATG start site. Independently, Heilmann et al. (27) indicated that R binds to the bidirectional BALF2/BARF1 promoter. The BALF2 primer set used to determine R binding by ChIP analysis is flanking RRE2 and overlapping RRE3, with the high confidence ChIP-sequence peak in the middle as indicated by the asterisk in Figure 3A. Although cell type differences and limitations of ChIP have to be considered, our findings largely agree with their data. Since RRE7 has the best overlap with the optimal RRE it is remarkable it does not show binding with EMSA. Also the 5’deletion mutants of the BARF1 promoter reporter construct indicate RRE7 to be non functional as a R binding site. On the other hand, our group identified in the BARF1 promoter four RREs which all differ from the consensus RRE, but did show binding in EMSA and were together capable of a strong promoter induction. RRE1 and RRE2, although having consensus core sequences, enclose internal spaces which deviate from the consensus in length. RRE3’s second core sequence is acgt instead of the consensus ggNg, and also RRE4’s second core sequence (acgt) differs from the consensus. Our four RRE oligos showed a low binding affinity in EMSA compared to the BMLF1 positive control oligo. Nevertheless, R was found to
induce a 50 to 250 fold upregulation of luciferase activity. Most likely, the four RREs in the BARF1 promoter region work in synergism, with RRE2 and RRE3 as the dominant responsible sites. Furthermore, the EMSA radiographs of all four BARF1 RRE oligos showed additional complexes of various sizes with other cellular factors (Fig. 3D). Future studies utilizing DNA pull down assays combined with mass spectrometry proteomics analysis might elucidate the identity and importance of the unknown factors in BARF1 gene regulation.

Previously, Chen et al. indicated that the kinetics of expression of R transactivated genes did not correlate with RRE affinity, and that other components might interfere with transcriptional activation (11). Although the affinity of R to the RREs in the BARF1 promoter region were not strong, BARF1 mRNA expression was detected rapidly at 6 hours after transfection with a R expression vector in the AGS ZKO cell line. The maximum levels of BARF1 RNA were observed at 15 hours. The BARF1 expression kinetics induced by R were similar as observed by a microarray analysis of EBV lytic gene transcription induced by IgG crosslinking of Akata cells which resulted in BARF1 mRNA expression starting from 6 hours after induction and reaching maximum levels after 12 hours (41). The classification of BARF1 to be an early gene indicates that the action of BARF1 protein in a biological context, inhibiting apoptosis and acting as an immune modulator, is necessary in the early hours of lytic replication.

In conclusion, expression of the BARF1 protein during lytic replication is directly regulated by R, and not by the major IE protein Z, independently of BARF1 promoter methylation. The R responsive elements were mapped between -544 and -327 relative to the BARF1 transcriptional ATG start site. The fast kinetics of BARF1 expression induced by R indicated that BARF1 protein expression is necessary early during the viral replication cycle. Future experiments are necessary to unravel the role of cellular factors in BARF1 transcriptional regulation, both during lytic reactivation by R and in latent EBV positive carcinoma.


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Figure 1. Methylation of the BARF1 promoter region [A] Methprimer analysis of CpG islands in the BARF1 promoter showed two large CpG islands spanning the whole promoter up to -607 nucleotides relative to the BARF1 ATG start site. [B] Quantitative PCR of BARF1 and R mRNA normalized to U1A. BARF1 mRNA was detected in both C15 and C17 NPC mouse tumor material and in C666.1 NPC cells. R levels in C15 mouse tumor material were below detection limit. BARF1 mRNA was low in HH514 cells but could be expressed upon treatment with NaB and TPA. [C] Bisulfite sequencing PCR (BSP) primers (arrows) was performed to analyze the CpG islands on the BARF1 promoter from ATG-496 to the ATG start site. Almost all CpGs in the investigated samples were methylated (black dots), both in HH514 Burkitt’s lymphoma cells and C666.1 cells consistently harboring EBV and C15 and C17 mouse xenograft NPC tumor material. CpG site I was unmethylated (white dot) in C666.1 cells and C17 tumor material. CpG site II was unmethylated in HH514 cells. [D] BSP analysis of 24h NaB-TPA treated HH514 cells, shows that the BARF1 promoter was partially (grey dots) or completely (white dots) demethylated.

Figure 2. R, but not Z, activates the BARF1 promoter, independent of methylation status and in the context of the viral genome. A BARF1 promoter reporter construct was created by inserting the promoter sequence, up to -679 nucleotides from the ATG start site upstream of the luciferase gene in a CpG-free reporter construct. [A] Luciferase assays showed that the constitutive activity of the BARF1 promoter in pCpG.LUC versus pCpG.LUC empty was highest in EBV positive C666.1 and SNU-719 cells. [B] Cotransfection of the reporter construct with an R expression vector induced a 50 to 250 fold upregulation of luciferase activity in most cell lines and up to 3000 fold in Hela cells. Hela, AGS and SNU-719 (n = 2), and C666.1 and Hone1 (n = 3), SEM is shown. [C] Ex vitro methylated reporter construct maintained its activation when cotransfected with a R expression vector. [D] Representative example of unmethylated or methylated promoter construct cotransfected either with a R or Z expression vector in AGS cells. Both empty vector (-) and R vector RLU values were lower when the promoter luciferase construct was methylated, leaving the fold activation mostly unaffected. SDS-page westernblot
confirmed equal expression levels of Z and R protein. [E] EBV positive C666.1 cells expressed more BARF1 mRNA 48h after transfection with R, indicating that R upregulates BARF1 in context of EBV infection. [F] Parallel experiments using Z defective 293HEK cells demonstrate autonomous R transactivating activity of BARF1 independent of the lytic cycle. When only 1% of the transfected DNA consisted of R expression vector, induction of BARF1 was reduced, indicating that BARF1 induction was R dosage dependent.

**Figure 3. R binds to RREs on the BARF1 promoter.** [A] Potential R responsive elements (R1 to R7) are depicted on the BARF1 promoter region. Black vertical lines represent methylation sites. Rounded grey indicators point to the deletion mutants made from the BARF1 reporter construct, shortening the BARF1 promoter sequence from the original -679 to -63 relative to the ATG start site. The asterisk indicates the R ChIP-sequence peak as determined by Heilman et al. (27). [B] ChIP assays were performed using extracts from 293HEK BRLF1-stop cells transfected with a R expression vector (R) or a control vector (-). R was immunoprecipitated by a control antibody or anti-R antibody, and co-immunoprecipitated DNA was PCR amplified. The band in lane 6, indicates that BARF1 promoter region DNA is specifically precipitated with R. [C] The RRE optimal sequence and the consensus sequence according to Chen et al (11) are shown. Oligos of the potential RREs in the BARF1 promoter with surrounding nucleotides used in EMSA. To create double stranded probes, oligos were hybridized with their respective opposite strand. The oligo of the BMLF1 promoter RRE serving as positive control. CpG sites are accentuated by stars. [D] The ability of in vitro translated C terminal truncated R (R550) to bind to ²³P end-labeled probes of the potential RREs in the BARF1 promoter was examined by EMSA. Extracts were made of Hela cells transfected with control (-) or R550 (+). A probe containing the BMLF1 promoter RRE served as a positive control. Four of the seven RREs (RRE1 to 4) on the BARF1 promoter showed binding (R). Supershift: (S), unbound oligo: (F). [E] Deletion mutants of the BARF1 promoter reporter construct were made as indicated in Fig. 3B. AGS cells were transfected with the deletion mutant luciferase constructs and with or without R expression vector.
The R induced luciferase activity (fold) was measured 48 hours after transfection. A representative experiment is shown.

Figure 4. RREs are required for R dependent BARF1 promoter activation  

[A] Site-directed mutations of the RREs in BARF1 promoter ATG-582 luciferase reporter construct were made. Seven nucleotides, predominantly in the core sequences were selected for mutation. Original and their respective mutants are shown.  

[B] AGS cells were transfected with the mutated reporter constructs in combination with either the empty vector or the R expression vector. The amount of luciferase activity was determined 48 hours after transfection. The induction of luciferase activity of 3 experiments is shown.

Figure 5. R transactivates BARF1 mRNA with early kinetics  

[A] AGS B95.8 cells were transfected with R expression vector or empty vector. Cells were collected at different time points after transfection and RNA was isolated and converted to cDNA using gene specific primers. Quantitative RT-PCR was performed and BARF1 values were normalised to U1A. The graph represents the fold induction of BARF1 mRNA by R, average of two experiments.  

[B] SDS-page Western blot analysis of R expression levels in AGS B95.8 cells after transfection with empty vector (-) or various time points after transfection with R expression vector. 

[C] AGS ZKO cells were transfected with R expression vector or empty vector and RT-PCR was performed on samples harvested at various time points as in Fig. 5A. The fold induction of BARF1 mRNA by R, average of three experiments, is shown.  

[D] SDS-page Western blot analysis of R expression levels in AGS ZKO cells after transfection with empty vector (-) or various time points after transfection with R expression vector. R protein was detectable 6 hours after transfection.  

[E] R mRNA levels indicated a 630 fold difference in R baseline expression between AGS ZKO and AGS B95.8 cells.  

[F] BARF1 mRNA levels indicated a 38 fold difference in BARF1 baseline expression between AGS ZKO and AGS B95.8 cells.
Figure 1

A. Obs / Exp

B.

C15 C17 C666.1 HH514 HH514 NaB/TPA

D. unknown

Note: The figure includes a graph and bar charts with various data points and annotations.
Figure 2

A.  AGS 264 fold  311 fold

B.  Increase of luc activity with R

C.  Increase of luc activity with R methylated promoter

D.  AGS

E.  C666.1

F.  AGS ZKO

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Figure 3

A. D.
BMLF1              R1               R2              R3               R4
--+  +
-+  -+
--+  +
-+  -+
--+  +
-+  -+
--+  +
-+  -+

Rα - R 

B. Input control Ab anti-R

BARF*tp

GAPDH

C.

Optimal: GTCC7 N NC7 GGCG
Consensus: GTCC7 NNC7 GGNG (n=9)

Oligo 1: GCCATAGAC GCCA ATG AAT CGA ATGCCCTCCAG (n=8)
Oligo 2: TGGGCGCAT GCCA CCAGCAGAA GCGC GCCTCTTGTG (n=10)
Oligo 3: CTGAGCCGAC GCCA ATGTTGCTGT AGC STGTGTGAC
Oligo 4: CTGCTGTCTT GCCA CGATCTGGC GCCTCTTGCCCG
Oligo 5: GCCGGACAT GCCG TCACGCTGCA GCG CTCTGGGGGAC (Rev)
Oligo 6: GCCGCAAGAT GCCC TGAACATGAC GTG GTCGGCTACA
Oligo 7: GCCGCCAT GCCC CTGATGACG GCCA CAGCATCTCC

BMLF1 (SM): GCCCCAGAT GCCC CTGATCAT GCCA CAGCATCTCC

D. BMLF1

R1 R2 R3 R4 R5 R6 R7

S R S R S R S R

YY1

E. FOLD values activity

Figure 4

A. 

Consensus: GNCC (n=9) GGNG

RRE 1: GCGATAGAG GGCC AGGTAGTG GGCG ATTGCCCTCAG
RRE mutant 1: GCGATAGAG ACTT AGGTAGTG AATA ATTGCCCTCAG
RRE 2: TGGGCGATT GCCC CCAGCACGAA GGCG GCGCTCTTGTG
RRE mutant 2: TGGGCGATT ACTT CGAGCAAGAA AATA GCGCTCTTGTG
RRE 3: CTGGCCGGCC GACC AGTAGTCGT AATA GTCGTTGTACA
RRE mutant 3: CTGGCCGGCC ACTT AGTAGTCGT AATA GTCTTTGTACA
RRE 4: CCGTGTCTTT GCCC CCGATGTCG GCTG TCCTGCAGGCC
RRE mutant 4: CCGTGTCTTT ACTT CCGATGTCG AATA TCCTGCAGGCC

B. 

![Graph showing % reduction of luciferase activity](image)
Figure 5

A. C.  
R $\rightarrow$ $\beta$-actin $\rightarrow$ R $\rightarrow$ $\beta$-actin $\rightarrow$

B. D.  
- 1 2 4 8 15 24 hours - 1 2 4 8 15 24 hours

E.  
$10^{-2}$ $10^{-3}$ $10^{-4}$ $10^{-5}$ $10^{-6}$ $10^{-7}$ $10^{-8}$  
R / U1A

F.  
$10^{-2}$ $10^{-3}$ $10^{-4}$ $10^{-5}$ $10^{-6}$ $10^{-7}$ $10^{-8}$  
BARF1 / U1A

AGS ZKO AGS B95.8

AGS ZKO norm BARF1 +R/-R

AGS ZKO norm $1E-03$ $1E-02$

$1E-05$ $1E-04$

$1E-05$ $1E-04$

$1E-06$ $1E-05$

$1E-06$ $1E-05$