Kaposi’s Sarcoma-Associated Herpesvirus Viral Interferon Regulatory Factor 4 (vIRF4) Targets Expression of Cellular IRF4 and the Myc Gene To Facilitate Lytic Replication

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Kaposi’s sarcoma-associated herpesvirus 8, or human herpesvirus 8, is a lymphotropic γ2-herpesvirus that is the etiologic agent of Kaposi’s sarcoma (KS) and two lymphoproliferative disorders: primary effusion lymphomas (PELs) and multicentric Castleman’s diseases (MCDs). KS and PEL cells are predominantly infected with the latent form of KSHV, expressing only a few latent genes whose proteins subvert various cellular pathways in order to increase the proliferation and survival of virus-infected tumor cells. Thus, understanding the KSHV-mediated regulation of host gene expression for its life cycle is the crux of this study.

The proto-oncogene c-Myc is a basic helix-loop-helix-leucine zipper transcriptional factor that regulates expression of more than 15% of all host genes, ultimately controlling proliferation, differentiation, and death. In particular, c-Myc expression is frequently deregulated in a large proportion of aggressive lymphomas, due to chromosomal translocation (e.g., Burkitt’s lymphoma), gene amplification (e.g., non-Hodgkin lymphomas), or abnormal stabilization (4–7). Interestingly, to maintain herpesvirus latency and oncogenesis, c-Myc protein is frequently stabilized and functionally activated in PELs by the KSHV-encoded latency-associated nuclear antigen (LANA) and viral interferon regulatory factor 3 (vIRF3), respectively (8–11). Thus, c-Myc is a key cellular factor coupling KSHV latency with growth transformation.

The cellular interferon regulatory factor (c-IRF) family of transcription factors, which are characterized by a unique tryptophan pentad repeat DNA-binding domain (DBD), is important in the regulation of interferons (IFNs) and IFN-inducible genes in response to viral infections. Among this family’s members, c-IRF4 is a lymphoid tissue-specific transcription factor that plays crucial roles in the development and functions of immune cells: it controls B-cell proliferation and differentiation and proliferation of mitogen-activated T cells. In addition, c-IRF4 binds to the IFN-stimulated response element (ISRE) of the major histocompatibility complex (MHC) class I promoter and the immunoglobulin lambda light chain enhancer together with PU.1, and it positively regulates the biosynthetic processes of interleukin-2 (IL-2), IL-4, IL-10, and IL-13. On the other hand, c-IRF4 negatively regulates Toll-like receptor signaling by competing with c-IRF5, and it inhibits proinflammatory cytokine production. Emerging evidence has indicated that c-IRF4 is also a pivotal factor that directly tar-
gets c-Myc gene expression, generating an autoregulatory feedback loop for cell growth in myeloma cells, as well as acting as a tumor suppressor in early B-cell development (12, 13). Notably, several studies have shown that c-IRF4 activation is critical for the Epstein-Barr virus (EBV)-mediated transformation of B lymphocytes (14, 15). On the other hand, it also acts as a negative regulator of KSHV replication and transcription activator (RTA) expression upon induction of KSHV lytic reactivation (16). Taken together, the data indicate that c-IRF4 plays multiple roles in the regulation of host and viral gene expression.

The KSHV genome carries at least 90 genes that are expressed during the latent and lytic phases of the viral life cycle. While KSHV shares the majority of its genes with other herpesviruses, KSHV also carries unique genes that are homologues of cellular genes involved in immune modulation, cell death, growth, and differentiation. These viral homologues play critical roles in subverting host antiviral immune responses (17). In particular, four interferon regulatory factors (vIRFs) bear significant homology with c-IRFs and counteract IFN- and tumor suppressor-mediated innate antiviral defenses by either subverting IFN production or deregulating p53 tumor suppressor function (17, 18). Specifically, vIRF4 has been shown to antagonize p53-mediated tumor suppressor activity by regulating two crucial components of the p53 pathway: the human double minute 2 (HDM2; also called MDM2) E3 ubiquitin ligase and the herpesvirus-associated ubiquitin-specific protease (HAUSP; also known as USP7) deubiquitylating enzyme (19, 20). In addition, vIRF4 was found to interact with CSL (RBP-Jk), a cellular transcriptional factor that acts as a critical coregulator of RTA, a master regulator of KSHV reactivation (21). While the biological consequences are still not clear, vIRF4 has been shown to compete with the Notch receptor for CSL binding to modulate Notch signaling (21). vIRF4 also interacts with the poly(A)-binding protein, potentially interfering with host translation (22). Finally, vIRF4 was recently found to be a positive regulator of RTA function itself (23). These observations indicate that vIRF4 potentially functions as a regulator by cooperation with either host or viral proteins to promote an efficient KSHV life cycle.

To further delineate the role of vIRF4 in host-virus interaction, we examined the potential effects of vIRF4 on host gene expression in KSHV-infected PELs. This study reveals that the KSHV vIRF4 lytic protein specifically targets c-IRF4 in two independent ways: it suppresses c-IRF4 expression and competes with c-IRF4 for binding to the specific promoter region of the c-Myc gene, resulting in a drastic suppression of c-Myc gene expression. By utilizing recombinant KSHV, we also show that the loss of vIRF4 function in the down-regulation of c-IRF4 and c-Myc expression leads to a reduction of KSHV lytic replication. These results indicate that the KSHV vIRF4 lytic protein is a critical factor that robustly suppresses c-IRF4 and c-Myc expression, generating a favorable environment for viral lytic replication.

MATERIALS AND METHODS

Cell culture and transfection reagents. Tetracycline-inducible TREx-BCBL-1 and TRExBJAB cells (24) and H29 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) (Gibco-BRL). 293T and TREx293T cells (24) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and 1% P/S (Gibco-BRL). The iSLK cell line was a generous gift from Jinjong Myung (UCSF, San Francisco, CA) (25). iSLKBAC16, iSLK-vIRF4DBD_BAC16, and iSLK-KIRF4DBD_BAC16 cell lines were grown in DMEM supplemented with 10% FBS, 1% P/S, 1 μg/ml puromycin, 250 μg/ml G418, and 1.2 mg/ml hygromycin B. Transient transfections were performed with Lipofectamine 2000 (Invitrogen) and Effectin (Qiagen) according to the manufacturers’ instructions.

Plasmid construction. All constructs for transient and stable expression in mammalian cells were derived from the pcDNA5/FRT/To-Hygro and pcDH-CMV-Puro expression vectors. DNA fragments corresponding to the coding sequences of the wild-type (WT) vIRF4 gene were amplified from template DNA (kindly provided by Jürgen Hass) by PCR and then subcloned into pcDNA5/FRT/To-Hygro at the BamHI and NotI restriction sites and into pcDH-CMV-Puro vector at the EcoRI and BamHI restriction sites. C-terminally Flag-tagged c-IRF4 was expressed from a modified pcDH-CMV-Puro vector. The vIRF4DBD mutant was generated via PCR. All constructs were sequenced using an ABI Prism 377 automatic DNA sequencer to verify 100% correspondence with the original sequence.

Cell line construction. To establish KSHV-infected BCBL-1 cells expressing WT or mutant vIRF4 in a tetracycline-inducible manner, pcDNA/FRT/To-vIRF4/Au or pcDNA/FRT/To-vIRF4DBD/Au was transfected via electroporation along with the pOG44 Flp recombinase expression vector. Twenty-four hours after electroporation, cells were selected using 200 μg/ml of hygromycin B (Invitrogen) for 3 weeks. The detailed procedure has been described previously (20). BAC16, vIRF4DBD_BAC16, or R-vIRF4DBD_BAC16 DNA was transfected via Lipofectamine along with Effectin into iSLK lines. Twenty-four hours after transfection, cells were selected using 1 μg/ml puromycin, 250 μg/ml G418, and 1.2 mg/ml hygromycin B.

Chemicals and antibodies. Selected cells were treated with 1 μg/ml of doxycycline (Dox) (Sigma) or 1 mM sodium butyrate (NaB) (Sigma) for the indicated periods. Primary antibodies were purchased from the following sources: IRF4 (D43H10) and β-casein antibodies from Cell Signaling, c-Myc (N-262) antibody from Santa Cruz Biotechnology, tubulin antibody from Sigma, Au antibody from Covance, histone H3 (ab1791) antibody from Abcam, and viral IL-6 (vIL-6) antibody from Advanced Biotechnologies. K8.1, LANA, and K5 antibodies were purchased previously (24). RTA rabbit polyclonal antibody was a generous gift from Yoshihiro Izumiy and Hsing-Jien Kung (University of California, Davis, Sacramento, CA). Polyclonal anti-vIRF4 antibody was generated against the region of vIRF4 encompassing amino acids 235 to 490.

RNA isolation and RT-qPCR. Total RNAs were extracted using TRI reagent (Sigma) and then reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad). The resultant cDNAs were measured by either conventional PCR or qPCR. Real-time PCR was performed using Sybr Green-based detection methods in a CFX96 real-time system (Bio-Rad) according to the manufacturer’s instructions. The 18S rRNA gene was used as a normalization control. RT-PCR graphs were made based on the averages for at least two independent experiments.

Microarray analysis. Total RNAs were isolated using Tri reagent (Sigma), and microarray analysis was performed using GeneChip Human Gene 2.0 ST arrays. Data were normalized between groups with Affymetrix Expression Console software, and the Benjamini and Hochberg algorithm was used to control the false discovery rate (FDR) of duplicate testing (FDR-adjusted P value of <0.01, selected for genes showing a >2-fold change; Partek Genomic Suite, Partek Incorporated). Heat maps for gene expression were created using Spotfire DecisionSite 9.1.1.

ChIP assays. Detailed descriptions of the ChIP assays have been published previously (26). Briefly, chromatin containing 10 μg of DNA was treated with 1 to 2 μg of antibodies overnight at 4°C for immunoprecipitation. On the next day, to pull down the DNA-protein complexes, pro-
tein A/G agarose was added for 4 h. Immunoprecipitation complexes were washed sequentially with RIPA buffer, LiCl buffer, and, finally, Tris-EDTA (TE) buffer. The DNA-protein-protein A/G agarose complex was resuspended in 100 μl of TE buffer containing 50 μg/ml RNase A and incubated for 30 min at 37°C. Proteinase K treatment, cross-link reversal, and DNA purification were done to prepare input DNA. Both input and ChIP DNAs were measured by qPCR. Based on the standard curve for each primer pair, the enrichment of proteins and histone modifications on specific genomic regions was calculated as the percentage of immuno-precipitated DNA compared to input DNA. Each data point in the ChIP figures is the average for at least three independent ChIP assays using three independent chromatin samples.

**Lentivirus transduction.** Gene expression constructs were prepared using the pcDH lentiviral vector. Supernatants from 293T cells transfected with either pcDH-vIRF4-Au or pcDH-IRF4-Flag together with packaging vectors were collected at 72 h posttransfection, followed by concentration of the viruses (2,400 rpm, 3 h, 4°C). One million TREx293, H929, or TREATBCBL-1 cells were used for spinning infections (1,800 rpm, 45 min, 30°C) in the presence of 10 μg/ml Polybrene. At 5 days postinfection, cells were harvested for immunoblotting and RT-qPCR analysis.

**Construction of vIRF4 DNA-binding domain-deficient KSHV (BAC16-vIRF4ΔDBD virus).** The vIRF4 DBD-deficient strain was generated by deleting the coding sequence of vIRF4 exon 1, resulting in vIRF4ΔDBD. Mutagenesis was performed in the *Esherichia coli* GS1783 strain by using “scarless” mutagenesis, as previously described (27). The vIRF4ΔDBD mutant was generated by amplifying a Kan’-I-SceI cassette from the pEP-Kan-S plasmid, using the following primers: forward primer, CAAACCTCACACCCCCTTCCCCGAGTTACATACCTAGTG TCACACTATGCTCAGCACAGAGAGAGCATgaggatacatggagg; and reverse primer, TGGTGGCTAAAAGCAAGGCGCATCGGTGTTCTGTT GTCGAGCCATGATGCACATGATGTAATCAGCattacgatatga ttagg. Upstream of the primers in CACC sequences indicate the KSHV genomic sequences that were used for homologous recombination, whereas the sequences given in lowercase letters were used to PCR amplify the Kan’-I-SceI cassette from the pEP-Kan-S plasmid. NheI and Asel restriction enzyme digestion of bacterial artificial chromosome (BAC) DNAs, followed by either conventional agarose gel electrophoresis or pulsed-field enzyme digestions of bacterial artificial chromosome (BAC) DNAs, followed by either conventional agarose gel electrophoresis or pulsed-field electrophoresis, were used to verify specific mutations without genetic rearrangements of the vIRF4ΔDBD mutant compared to WT BAC16 and revertant vIRF4ΔDBD BAC16.

**smFISH.** Single-molecule fluorescence in situ hybridization (smFISH) experiments were performed according to the protocol of Raj et al. (28). TREATBCBL-1 pcDNA and TREATBCBL-1 vIRF4/Au cells were incubated with or without Doxy for 24 h, followed by RT-PCR analysis with human c-Myc-specific primers. Since the c-Myc gene is a target of β-catenin-mediated activation of TCF-dependent transcriptional factors, we also included primers specific for the Survivin, SIAH, and β-catenin genes and the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene, as a loading control. This showed that vIRF4 expression dramatically downregulated the c-Myc mRNA level, but not the Survivin, SIAH, or β-catenin mRNA level, in a time-dependent manner (Fig. 1B and C). These results were further confirmed by immunoblot analysis with specific antibodies. Endogenous c-Myc dramatically decreased in TREATBCBL-1 vIRF4/Au cells compared to that in TREATBCBL-1 pcDNA cells (Fig. 1D). In contrast, vIRF4 expression showed little or no change in tubulin and β-catenin protein levels under the same conditions, while a slow-migrating β-catenin protein was detected upon vIRF4 expression (Fig. 1D). Collectively, these results indicate that vIRF4 expression significantly affects the expression profiles of host genes, among which c-Myc expression is most affected.

**vIRF4 downregulates c-Myc gene expression in a c-IRF4-dependent manner.** c-IRF4 expression is restricted to the lymphoid and myeloid lineages and is frequently deregulated in lymphoma cells (13, 29). Additionally, a recent study showed that c-IRF4 is critical for the induction of c-Myc expression in multiple myeloma cells, playing a crucial role for their survival and expansion (13). Microarray analysis showed that c-IRF4 was also downregulated 3-fold upon vIRF4 expression. Given that c-IRF4 directly regulates c-Myc gene expression (13), we postulated that vIRF4 suppressed c-IRF4 expression, leading to the reduction of c-Myc expression. To investigate this, we utilized three different cell lines that harbor different c-Myc and c-IRF4 expression levels and promoter structures. First, H929 cells, which carry the authentic forms of the c-Myc and c-IRF4 genes (30), were transduced with a lentivirus carrying vIRF4 or GFP for 48 h and then subjected to RT-qPCR. Consistent with the microarray analysis data (Fig. 1A), we were able to observe the vIRF4-mediated downregulation of c-Myc and...
IRF4 gene expression (Fig. 2A). Next, we used BJAB Burkitt’s lymphoma cells, in which the c-IRF4 gene is intact but the c-Myc gene is translocated to the IgG promoter region (31). TRExBJAB cells expressing vector (pcDNA) or vIRF4 in a tetracycline-inducible manner were treated with or without Doxy and then subjected to RT-qPCR and immunoblot analyses (Fig. 2B). These analyses showed that while vIRF4 expression led to a significant reduction of the c-IRF4 mRNAs, it showed little or no effect on the c-Myc mRNA and protein levels (Fig. 2B, left panel). Third, when 293 cells, which carry an intact c-Myc gene but do not express c-IRF4 expression, were used, tetracycline-inducible vIRF4 expression led to no reduction of the c-Myc mRNAs (Fig. 2C). Finally, the c-Myc and c-IRF4 expression kinetics were compared in TRExBCBL-1 vIRF4/Au cells versus TRExBCBL-1 vIRF4/Au&Flag-c-IRF4 cells, carrying cytomegalovirus (CMV) promoter-mediated expression of the Flag-tagged c-IRF4 gene. These cells were harvested at various time points of Doxy treatment and used for RT-qPCR analysis. This showed that Doxy-induced vIRF4 expression in TRExBCBL-1 vIRF4/Au cells led to a rapid decrease of c-IRF4 mRNAs along with a marked reduction of c-Myc mRNAs (Fig. 2D, left panel). In contrast, Doxy-induced vIRF4 expression in TRExBCBL-1 vIRF4/Au&Flag-c-IRF4 cells showed a weak reduction of c-IRF4 mRNAs, with delayed kinetics, but little or no reduction of c-Myc mRNAs (Fig. 2D, right panel), indicating that ectopic expression of the c-IRF4 gene blocks the vIRF4-mediated suppression of c-Myc expression (Fig. 2D, right panel). These results collectively indicate that vIRF4 suppresses c-Myc expression in a c-IRF4-dependent manner.

vIRF4-mediated reduction of c-IRF4 and c-Myc mRNAs at single-cell level. We used the smFISH technique to quantify mRNA levels of the c-Myc, c-IRF4, and vIRF4 genes at the single-cell level (28), TRExBCBL-1 pcDNA cells (Doxyc-treated conditions) contained, on average, ~51 and ~22 copies of the c-IRF4 and c-Myc mRNAs per cell, respectively, and TRExBCBL-1 vIRF4/Au cells (Doxyc-untreated conditions) contained, on average, ~54 and ~18 copies of the c-IRF4 and c-Myc mRNAs per cell, respectively (Fig. 3A and B). In striking contrast, Doxy-induced
vIRF4 expression (average of ~11 mRNA copies per cell) led to a marked reduction of c-IRF4 and c-Myc mRNAs: on average, there were 28 and 4 copies of the c-IRF4 and c-Myc mRNAs per cell, respectively (Fig. 3A, yellow circles, and B). The smFISH experiments also revealed the heterogeneity of vIRF4 expression: upon Doxy treatment, a few TRExBCBL-1 vIRF4/Au cells showed nearly no vIRF4 expression, which led to no detectable reductions of the c-IRF4 and c-Myc mRNAs (Fig. 3A, white circles). By analyses of single cells, we obtained negative Spearman’s correlation coefficients for the c-Myc and vIRF4 transcripts (0.40) and for the c-IRF4 and vIRF4 transcripts (0.35), showing that vIRF4 expression suppresses c-IRF4 and c-Myc transcription at the single-cell level (Fig. 3C and D). In contrast, the c-Myc and c-IRF4 transcripts were positively correlated with each other in TRExBCBL-1 vIRF4 cells (Doxy-treated conditions) (0.37) (Fig. 3D). Overall, these results show that increased vIRF4 expression results in decreased c-IRF4 and c-Myc expression in individual cells, further supporting the hypothesis of vIRF4-mediated suppression of c-IRF4 and c-Myc expression.

vIRF4 DBD is necessary to suppress c-Myc gene expression. To date, 9 IRF genes have been identified in the human genome and each IRF has a well-conserved N-terminal DBD containing a tryptophan pentad repeat and a central IRF association domain (IAD) (18). In line with this, vIRF4 also contains a potential DBD within its N-terminal region. To test whether the vIRF4 DBD plays roles in the suppression of c-IRF4 and c-Myc expression, we constructed TRExBCBL-1 cells expressing a vIRF4 mutant (vIRF4 DBD) lacking the N-terminal 151 residues. TRExBCBL-1 pcDNA, TRExBCBL-1 vIRF4/Au, and TRExBCBL-1 vIRF4 DBD/Au cells were treated with or without Doxy for 24 h, and levels of the c-Myc, Survivin, SIAH, β-catenin, and GAPDH mRNAs were then examined by RT-qPCRs using specific primers. This showed that unlike the WT, the vIRF4 DBD mutant did not suppress c-Myc expression (Fig. 4A, right panel). Immunoblotting also showed that WT vIRF4 led to a marked reduction of endogenous c-Myc expression, whereas the vIRF4 DBD mutant did not (Fig. 4A, left panel). Furthermore, RT-qPCR analysis confirmed that WT vIRF4 expression led to a marked reduction of the c-IRF4 and c-Myc mRNAs, whereas vIRF4 DBD mutant expression did not (Fig. 4B). These results suggest that the DBD of vIRF4 is necessary for its activity to suppress c-IRF4 and c-Myc expression.
vIRF4 competes with c-IRF4 by binding to the same c-Myc promoter region. To reveal the underlying mechanism of the vIRF4-mediated suppression of c-Myc expression, TRExBCBL-1 vIRF4/Au and TRExBCBL-1 vIRF4/H9004 DBD/Au cells were treated with or without 1 μg/ml of Doxy for 24 h and then subjected to a ChIP assay with anti-Au, anti-c-IRF4, or anti-histone 3 (H3) antibody, followed by elution of the immunoprecipitated chromatin. Subsequently, the ChIP DNAs were measured by qPCRs using specific primers corresponding to the promoter (P1 to P8) regions of the c-Myc gene (Fig. 5, top diagram). This showed that c-IRF4 was enriched on the P5 promoter region of the c-Myc gene, but its occupancy of the P5 promoter was dramatically reduced upon vIRF4 expression (Fig. 5, left panels). Remarkably, similar to c-IRF4, vIRF4 was also enriched on the P5 region of the c-Myc promoter (Fig. 5, left panels). In striking contrast, the vIRF4ΔDBD mutant did not bind to the P5 region of the c-Myc promoter, resulting in no effect on c-IRF4 enrichment on the P5 region of the c-Myc promoter (Fig. 5, right panels). ChIP and qPCR with anti-H3 antibody were included as controls (Fig. 5, bottom panels). These data collectively indicate that since both c-IRF4 and vIRF4 appear to bind to the same region of the c-Myc promoter, vIRF4 competes with c-IRF4 for binding to the c-Myc promoter.

vIRF4-mediated downregulation of c-Myc expression contributes to efficient KSHV lytic replication. Since depletion of c-Myc expression leads to the induction of KSHV reactivation in an RTA-dependent manner (32), we hypothesized that ectopic expression of vIRF4 contributes to efficient KSHV lytic replication through the downregulation of c-Myc expression. To assess this hypothesis, TRExBCBL-1 pcDNA, TRExBCBL-1 vIRF4/Au, and TRExBCBL-1 vIRF4ΔDBD/Au cells were treated with 1 μg/ml of Doxy together with sodium butyric acid (NaB; 1 mM) for various periods and subjected to RT-PCR analyses with specific primers against several KSHV genes (Fig. 6). RT-PCR analysis revealed that Doxy-induced exogenous vIRF4 expression greatly accelerated the induction of an immediate early gene (RTA), early genes (ORF36 and ORF57), late genes (ORF25 and ORF64), and latent genes (LANA and vIRF3), while vIRF4ΔDBD mutant expression did not (Fig. 6). Under these conditions, endogenous c-Myc protein levels were considerably reduced in TRExBCBL-1 vIRF4/Au cells, whereas this reduction was not observed in TRExBCBL-1 pcDNA and TRExBCBL-1 vIRF4ΔDBD/Au cells (Fig. 6, right panel). These results suggest that the vIRF4-mediated downregulation of c-Myc expression contributes to efficient KSHV lytic replication.

vIRF4ΔDBD KSHV mutant shows reduced lytic replication. In order to examine the effect of vIRF4-mediated c-Myc suppres-
sion on viral lytic replication in the context of the KSHV genome, we first generated the vIRF4/ΔDBD KSHV mutant by using BAC16 as a template for mutagenesis. A majority of the coding sequence of vIRF4 exon 1 was deleted from BAC16 by "scarless" mutagenesis (Fig. 7A) (27). To confirm that the recombinant BAC16 clones contained the vIRF4/ΔDBD mutation, the DNA fragments containing the mutated allele were directly sequenced, and the restriction endonuclease digestion patterns of the WT and vIRF4/ΔDBD BAC16 clones were also compared (Fig. 7B). Based on the DNA sequence results and the NheI and AseI enzyme digestion patterns, we selected an appropriate clone and designated it vIRF4/ΔDBD. In order to rule out the presence of a second-site mutation within the BAC16-vIRF4/ΔDBD genome, revertant clones were generated by homologous recombination with the PCR amplicon containing vIRF4 exon 1 and coding target sequences from the WTBAC16 and kanamycin resistance cassette portion. After confirming revertant clones via NheI and AseI enzyme digestion and DNA sequencing, we selected an appropriate clone and named it R-vIRF4/ΔDBD (Fig. 7B). Next, purified WTBAC16, vIRF4/ΔDBD, and R-vIRF4/ΔDBD DNAs from E. coli were transfected into the recombinant SLK cell line iSLK, which was engineered to express RTA in a tetracycline-inducible manner (25). Following selection for hygromycin resistance (Hyg′), cells were treated with 1 mM NaB together with Doxy for 24 h and then used for RT-qPCR analysis.

**DISCUSSION**

c-Myc is a major transcriptional factor that is involved in cell growth and proliferation and also plays an important role in the regulation of cellular Myc gene expression by vIRF4.
development of B-cell lymphomas (33, 34). For instance, c-Myc overexpression as a consequence of reciprocal translocation to the immunoglobulin loci has been found in various B-cell lymphomas (35). Although there are currently no reports of any c-Myc mutations or its locus rearrangements in KSHV-associated KS and PEL, growing evidence indicates that c-Myc is an important host factor in the development of KSHV-associated malignancy. Hence, it is not surprising that KSHV has evolved various mechanisms to modulate c-Myc expression to promote and regulate its own life cycle. Specifically, two KSHV latent proteins, LANA and vIRF3, have been shown to enhance the stability and function of c-Myc to enhance its transactivation activity, thereby inducing target gene expression (8–10, 36, 37). Furthermore, short hairpin RNA (shRNA)-mediated depletion of c-Myc gene expression breaks the KSHV latency program and facilitates progression into the lytic phase in an RTA-dependent manner, suggesting that c-Myc plays an important role in maintaining KSHV latency (32). Forero et al. recently reported that c-IRF4 also participates in the maintenance of KSHV latency by acting as a negative regulator of KSHV lytic gene expression (16). In this study, we identified KSHV vIRF4 as a negative regulator of c-IRF4 and c-Myc gene expression, which ultimately contributes to efficient KSHV lytic reactivation. Specifically, KSHV vIRF4 not only suppresses c-IRF4 expression but also competes with c-IRF4 for binding to the c-Myc promoter, resulting in the comprehensive suppression of c-Myc expression. These results indicate that the KSHV vIRF4 lytic protein specifically targets the expression and function of c-IRF4 to downregulate c-Myc gene expression, generating a favorable genetic environment for viral lytic replication. Thus, KSHV vIRF4 is included in the ever-growing list of viral genes that affect c-Myc expression and function, further emphasizing the important role of the c-Myc gene in the herpesviral life cycle.

Despite various approaches, including microarray, RT-qPCR, smFISH, ChIP, and immunoblot analyses, the details of how vIRF4 suppresses c-IRF4 expression are still elusive. There are several potential mechanisms that may explain how vIRF4 contributes to the deregulation of c-IRF4. c-IRF4 has been shown to bind to its promoter element, providing a positive-feedback signal to its own gene expression (38). Since KSHV vIRF4 exhibits the highest homology with c-IRF4 among nine c-IRFs, we hypothesize that vIRF4 may function as a decoy of c-IRF4 by disturbing c-IRF4’s positive-feedback signal, which ultimately suppresses c-IRF4 gene expression. As shown in Fig. 1 and in Table S1 in the supplemental material, vIRF4 expression grossly affects host gene expression profiles, further supporting the role of vIRF4 as a potential transcriptional regulator. Indeed, we attempted to test whether vIRF4 competes with c-IRF4 for binding to the c-IRF4 promoter as seen with the c-Myc promoter competition. However, due to an extremely weak binding ability of c-IRF4 to its own promoter, we were not able to test this hypothesis in detail. Furthermore, we did not detect a specific interaction between vIRF4 and c-IRF4, suggesting that vIRF4 may not directly affect the interactions of c-IRF4 with other transcription factors (unpublished data). Since c-IRF4 expression is under the control of various transcription factors, such as STAT, MITF, and NF-κB (39–42), vIRF4 may suppress c-IRF4 expression indirectly, by affecting functions of other host genes, including those encoding the STAT, MITF, or NF-κB transcription factor. Additional studies should be directed toward identifying the particular mode(s) of vIRF4-mediated suppression of c-IRF4 expression.

Several recent reports have described a network of transcriptional promiscuity between c-IRF4 and c-Myc in different lineage cells or cancer cells (13, 43). In line with this, our ChIP assay identified a novel function of c-IRF4 as a transcriptional regulator
of c-Myc expression in PELs. Under normal conditions, c-IRF4 was enriched on the P5 region of the c-Myc promoter, likely serving as a transcriptional activator of c-Myc expression (Fig. 5). Remarkably, vIRF4 effectively competed with c-IRF4 for occupancy on the P5 region of the c-Myc promoter (Fig. 5). This suggests that KSHV vIRF4 employs two complementary means to robustly block c-Myc gene expression: vIRF4 lowers the c-IRF4 protein level and also inhibits c-IRF4 binding to the c-Myc promoter. Interestingly, the vIRF4ΔDBD mutant demonstrated loss of function in the downregulation of c-Myc expression, suggesting that the DNA-binding activity of vIRF4 is important for these functions, although, to date, none of the KSHV vIRF1 to -4 proteins has been shown to possess a functional DNA-binding activity. However, Hew et al. recently showed the crystal structure of the vIRF1 DBD in complex with DNA, establishing vIRF1 as a potential DNA-binding protein (44). This also shows that the vIRF1 DBD binds DNA, whereas full-length vIRF1 does not, suggesting a potential cis-acting regulatory mechanism similar to that of host IRFs (44). Thus, it is worth considering that vIRF4 may directly bind unique DNA elements in a certain context of transcriptional regulation, ultimately orchestrating cellular and viral gene expression for KSHV lytic reactivation.

We showed that WT vIRF4 greatly accelerated the induction of an immediate early gene (RTA), early genes (ORF36 and ORF57), late genes (ORF25 and ORF64), and latent genes (LANA and vIRF3), while the vIRF4ΔDBD mutant did not (Fig. 6). This observation was further confirmed by using recombinant KSHV strains, i.e., WT_RAC16, vIRF4ΔDBD_RAC16, and R-vIRF4ΔDBD_RAC16 in iSLK cells, where expression of the immediate early, early, and late genes was considerably lower with vIRF4ΔDBD_RAC16 than with WT_RAC16 and R-vIRF4ΔDBD_RAC16 (Fig. 7). Furthermore, endogenous c-Myc protein levels were considerably reduced upon WT
vIRF4 expression but not upon vIRF4ΔDBD mutant expression (Fig. 6). These data suggest a reverse correlation between the vIRF4-mediated downregulation of c-Myc expression and the vIRF4-mediated upregulation of KSHV lytic gene expression. However, it should be noted that the ΔDBD mutant loses functions other than its activity to downregulate c-Myc expression, which may also affect KSHV reactivation. Further detailed analysis is necessary to elucidate the vIRF4-mediated regulation of the KSHV life cycle. In summary, our findings presented here strongly indicate that similar to its cellular counterpart, vIRF4 works as a potential viral transcription factor to modulate host gene expression to build favorable environments for the KSHV lytic life cycle. This also suggests that KSHV has evolved novel mechanisms to maintain the fine balance between latency and viral reactivation by regulating two host factors, namely, c-Myc and c-IRF4.

ACKNOWLEDGMENTS

This project was supported in part by NIH award CA014089 to H.-R.L.; by awards CA082057, CA31363, CA115284, CA115284, AI073099, AI105809, DE023926, and HL110609 and grants from the Hastings Foundation and the Fletcher Jones Foundation to J.U.J.; by awards DE021445 and CA134421 to P.F.; and by a grant from the Howard Hughes Medical Institute to T.H.

We thank Y. Izumiya, H. Kung, J. Myung, and J. Hass for providing reagents and also thank the Jung lab members for their support and discussions.

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February 2014 Volume 88 Number 4 jvlas.org


