Transcriptional Regulation of Kaposi's Sarcoma-associated Herpesvirus-encoded Oncogene Viral Interferon Regulator Factor by a Novel Transcriptional Silencer, Tis*

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Viral interferon regulatory factor (vIRF) encoded by Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8, is the most recently identified human herpesvirus (1). Previous studies indicate that KSHV is etiologically associated with Kaposi's sarcoma and several other lymphoproliferative diseases including primary effusion/ body cavity-based lymphoma and a subset of multicentric Castleman's disease (1–14).

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† The abbreviations used are: KSHV, Kaposi's sarcoma-associated herpesvirus; vIRF, viral interferon regulatory factor; RT, reverse transcriptase; TPA, 12-O-tetradecanoylphorbol-13-acetate; DTT, dithiothreitol; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay.

The life cycle of herpesviruses consists of two phases: latent infection and lytic infection. Viral latent infection is generally associated with the development of herpesviruses-related tumors. Viral lytic replication often causes cell death; however, it also produces virions that are spread to other hosts (15). In Kaposi's sarcoma tumors, the majority of KSHV-infected cells express viral latent antigens; nonetheless, a small number of these cells also undergo viral lytic replication (16). KSHV encodes a unique set of nonstructural genes targeting at cellular signaling pathways, most of which are viral lytic genes (17). It has been suggested that KSHV lytic replication in a small number of Kaposi's sarcoma tumor cells is essential for sustaining Kaposi's sarcoma lesions through a paracrine mechanism (16). Thus, delineation of the molecular mechanism controlling the expression of KSHV lytic genes could help understand the pathogenesis of KSHV-related diseases.

One of the KSHV nonstructural regulatory lytic genes is the ORF-K9 that encodes the viral interferon regulatory factor (vIRF). vIRF is a 449-amino acid protein that shares sequence homology to cellular interferon regulatory factors (IRFs) (17). IRFs are a family of transcription factors that regulate interferon signal transduction through binding to interferon-stimulated response elements in the promoter of interferon-responsive genes (18–24). Early reports have demonstrated that vIRF causes cellular transformation of NIH3T3 and Rat-1 cells through the inhibition of interferon- and IRF-mediated signal transduction, prevents UV-induced apoptosis, and regulates the expression of KSHV genes (25–28). vIRF exerts these functions through direct binding to IRF-1, IRF-3, p300, cAMP response element-binding protein, and p53 tumor suppressor (29–32). These results indicate that vIRF is a genuine viral oncogene and a potent transcriptional regulator. Elucidation of the molecular mechanism controlling vIRF expression could lead to the understanding of its precise role in regulating the expression of cellular and KSHV genes.

Two ORF-K9-related transcripts, a major transcript of 1.7 kb and a minor transcript with additional 84 nt sequence upstream of the 3' end of the major transcript have been identified (33, 34). The expression of the minor transcript is weak, and can only be detected by nested-RT-PCR in uninduced KSHV-infected cells (33). We have previously demonstrated that the major transcript of vIRF gene is a viral early lytic transcript (34), whose expression can be induced by chemical stimuli such as 12-O-tetradecanoylphorbol-13-acetate (TPA) (34–37). We have also characterized the core promoter of vIRF gene, and defined a TPA-responsive region (34). In this study, we have further identified a novel transcriptional silencer Tis in the upstream regulatory sequence of the vIRF promoter.
TABLE I

| Primers | Sequence of the primers (5’-3’) |
|---------|---------------------------------|
| Reg1    | GATTGTGTATGTTTAGTTCTCCACAGTTTA |
| Reg2    | CTCACAGGTTAATGTTGATTAGT     |
| Reg3    | TAATAGGTAGTTGCTTTTCTGACATATCT |
| Reg4    | TCATTCTTGGATAGTTGTTGGGTTGGGGAAGC |

MATERIALS AND METHODS

**Cell Culture**—KSHV-infected cell lines BC-1, BC-2, and BCBL-1 (38, 39) were grown in RPMI 1640 medium (Sigma, St. Louis, MO), supplemented with 10% fetal bovine serum (Sigma), 10 μg/ml gentamycin, and 2 mM L-glutamine (Sigma). HeLa cells were obtained from America Type Culture Collection (Rockville, MD), and grown in Dulbecco’s modified Eagle’s medium (Sigma), supplemented with 10% fetal bovine serum, 10 μg/ml gentamycin, and 2 mM L-glutamine.

**RNA Isolation and RT-PCR**—Total RNA was isolated from HeLa, BC-1, 293 cells, and human heart tissue using Promega’s RNAgent™ Total RNA Isolation System (Promega, Madison, WI). cDNAs were synthesized with total RNA using Moloney murine leukemia virus reverse transcriptase (Promega) in a reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 1 unit of RNasin, and random hexamer primers (Promega). Each PCR reaction mixture contained 5 μl of cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.25 mM dNTPs, 1 unit of Taq DNA polymerase (Sigma), and 1 μl of sense and antisense primers in a total volume of 50 μl. The primers used in PCR are as follows: P1, 5’-CTCACACCCACGCGCTTCAGTCA-3’; P2, 5’-GCTACACAAAGGCCCAGGATACACG-3’. The reaction was performed with 35 cycles at 94 °C for 40 s, 56 °C for 1 min, and 72 °C for 1 min.

**Deletion Analysis of the vIRF Promoter—vIRF promoter reporter constructs used in deletion analysis have been described previously (34). Briefly, a 1,052-kb DNA fragment spanning the region -991 to +62 relative to the vIRF gene transcriptional start site (+1) was inserted into the HindIII/XhoI sites of a promoter-less and enhancer-less chloramphenicol acetyltransferase (CAT) vector, pCAT-Basic to generate the reporter construct pCAT-991. The 5’-end sequence of pCAT-991 was then sequentially deleted to generate constructs pCAT-499 (~499 to +62), pCAT-337 (~337 to +62), pCAT-125 (~125 to +62), and pCAT-56 (~56 to +62) (Fig. 1, A and B).

**Construction of pBLCAT and pBLCATReg plasmids**—The CAT reporter plasmid pBLCATReg was constructed by inserting the PCR-amplified sequence from -337 to -125 upstream vIRF transcriptional start site into the HindIII/BamHI sites upstream of the HSV-TK promoter (pBLCAT2) fused to the CAT gene. pBLCATReg-rev was generated by inserting the sequence from -337 to -125 in a reverse orientation into pBLCAT2 (Fig. 2A). The pBLCATReg plasmids pBLCATRegI, pBLCATRegII, and pBLCATReg III were constructed by inserting the corresponding regulatory sequence of vIRF promoter into the HindIII/BamHI sites upstream of TK promoter in pBLCAT2. These plasmids contain the upstream regulatory sequences of vIRF promoter from -258 to -161, -323 to -234, and -499 to -334, respectively (Fig. 3, A and B).

To further dissect the Reg1 sequence (~258 to -161), overlapping oligonucleotides (~258 to -230, ~241 to -219, ~231 to -201, and -203 to -161) (Table I) were synthesized and cloned into the SalI site upstream of TK promoter in pBLCAT2 to generate CAT reporter plasmids pBLCATRegI, pBLCATRegII, and pBLCATRegIII. These constructs were D1pBLCATRegI, D2pBLCATRegII, and D3pBLCATRegIII were generated by inserting the oligomers with the target sequence motifs deleted into the SalI site upstream of the TK promoter in pBLCAT2 (Fig. 7, A and B). All constructs were confirmed by DNA sequencing with a Big Dye Terminator Cycle Sequencing Kit on an ABI 373-S sequencer (PE Bio-system, Foster City, CA). Transfection efficiencies were performed with LipofectAMINE™ 2000 reagent according to the instructions of the manufacturer (Invitrogen, Carlsbad, CA). CAT assay was performed as previously reported (25). Transfection efficiencies were normalized by DNA sequencing with a Big Dye Terminator Cycle Sequencing Kit on an ABI 373-S sequencer (PE Bio-system, Foster City, CA). CAT assay was performed as previously reported (25). Transfection efficiencies were normalized to co-transfection with a reporter plasmid pSV-β-galactosidase and β-galactosidase activity was determined following the instructions of the manufacturer (Promega). The conversion rate of the modified [3H]-labeled chloramphenicol was calculated with a Multi-Analysis Program (Bio-Rad, Hercules, CA).

**Preparation of Nuclear Extracts**—Nuclear extracts from HeLa, BC-1, BC-2, and BCBL-1 cells were prepared as described previously (40). Briefly, 1 × 10⁶ cells were collected and washed three times with phosphate-buffered saline by centrifugation. Each cell pellet was suspended in 400 μl of low-salt buffer (1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 10 mM Hepes (pH 7.9)), and swollen on ice for 15 min. After the addition of 25 μl of 10% Nonidet P-40, the cell pellet was mixed and vortexed for 10 s. Following centrifugation at 13,000 × g for 30 s, the pellet containing the nuclei was resuspended by gentle stirring with a pipette tip in 50 μl of low-salt buffer (25% glycerol, 1.5 mM MgCl₂, 420 mM NaCl, 1 mM DTT, and 20 mM Hepes (pH 7.9)). The mixture was then incubated with rocking for another 20 min at 4 °C. After centrifugation at 16,000 × g for 45 min, the supernatant was collected, diluted with equal amount of dilution buffer (5 mM Hepes (pH 7.9), 20% glycerol, and 1 mM DTT), and stored at −70 °C for future use.

**Electrophoretic Mobility Shift Assay (EMSA)**—Double-stranded oligonucleotides corresponding to the Tis sequence (Reg1), D1Reg2, D2Reg2, D3Reg2, D6Reg2, D8Reg2, and D9Reg2 were commercially synthesized and purified by PAGE (Integrated DNA Technologies, Inc., Coralville, IA). The oligonucleotides were labeled with [γ-³²P]ATP using a 5’-end labeling kit (Promega), and used as probes in EMSA experiments. The oligonucleotide sequences (single strand) were as follows: Reg1-5’, CTCTTGGAGTTGACATATTCTCG-3’, D1Reg2-5’, CTCTTGAACATATTCTCG-3’, D2Reg2-5’, CTCTTGGAGTTGACATATTCTCG-3’, D3Reg2-5’, CTCTTGAACATATTCTCG-3’, M6-5’, GAGTTTCAGGTAGTAGG-3’, M9-5’, GAGTTTCAGGTAGTAGG-3’, EMSA was performed in 10 μl of reaction volume at room temperature. Each reaction mixture (10 μg of total nuclear extracts, 0.5 μg of double-stranded poly(dI-dC), 10 mM Tris-HCl (pH 8.0), 0.5 mM DTT, 1 mM MgCl₂, 50 mM NaCl, 0.5 mM EDTA, and 4% glycerol) was incubated at room temperature for 30 min, followed by addition of the labeled probe (30,000–50,000 cpm). For competition experiment, the reaction mixture was preincubated for 10 min at room temperature with 100-fold excess of competitor DNA before the addition of the radiolabeled probe. For antibody supershift assay, the radiolabeled probe was added after the reaction mixture was incubated for 45 min at room temperature with 1 μl of antibodies to either c-Myc or Nkx2.5 (Santa Cruz Biotechnology, Santa Cruz, CA), or 0.5 μl of an antibody to Stat1 (BD Bioscience, San Diego, CA). The samples were then separated in a 6% polyacrylamide gel in 0.5 × TBE at 320 V for 45 min.

To determine the core sequence motif(s) of Tis, the M0 oligomer, 5’, GAGTTTCAGGTAGTAGG-3’ (Fig. 8A), was used as wild type sequence. Sequential point mutations were introduced for each of the nucleotides to test their effects on DNA-protein interactions. The mutated oligomers were employed as competitors in EMSA with the labeled oligomer Tis as probe. The wild type and mutant oligomers were listed in Fig. 8A.

RESULTS

The Upstream Sequence of vIRF Gene Promoter Contains a cis-Element Repressing Its Promoter Activity—In a previous study, we have mapped the vIRF core promoter to a DNA fragment from +62 to +56 upstream of vIRF transcriptional start site (33). Reporter plasmids containing DNA fragments from +62 to +56 (pCAT-56) and −125 (pCAT-125) had strong CAT activities similar to pBLCAT2 (Fig. 1, B and C). In contrast, reporter plasmids containing DNA fragments from +62 to +337 (pCAT-337), −499 (pCAT-499), and −991 (pCAT-991) had 88, 91, and 93% reduced CAT activities, respectively, compared with pCAT-125 (Fig. 1C). These results suggest that the 5’-flanking region of vIRF gene contains a negative regulatory cis-element repressing its promoter activity and this element is...
located at the region from −337 to −125 upstream of *vIRF* gene transcriptional start site.

**Regression of Heterologous HSV-TK Promoter Activity by the Upstream Regulatory cis-Element of vIRF Promoter**—To determine whether the upstream negative regulatory cis-element of the *vIRF* gene promoter can exert its effect on a heterologous promoter, a single copy of the DNA fragment (−337 to −125) was inserted into the upstream of the TK promoter in pBLCAT2 (Fig. 2A) to generate pBLCATReg plasmid. Compared with that of pBLCAT2, the TK promoter activity (pBLCATReg) was reduced 87% in HeLa cells (Fig. 2B), indicating that the cis-element also has a repression effect on the heterologous TK promoter. Similarly, the TK promoter activity was reduced 85% when the cis-element was inserted in a reverse orientation into pBLCAT2 (pBLCATReg-rev and Fig. 2B), indicating that the repression function of the cis-element is orientation-independent.

**Mapping of the Negative Regulatory cis-Element in vIRF Promoter**—To map the region involved in the repression of promoter activity, the sequence region from −337 to −125 was dissected into two fragments (−161 to −258 and −234 to −323), and their corresponding CAT reporter plasmids pBLCATRegI and pBLCATRegII were constructed by inserting these fragments into the upstream of TK promoter in pBLCAT2 (Fig. 3, A and B). Since the sequence region from −499 to −334 had no repression effect (Fig. 1), its corresponding construct pBLCATRegII was used as a control. Transiently transfected into HeLa cells, pBLCATRegII showed promoter activity similar to pBLCATRegIII, suggesting the region from −323 to −234 is not responsible for the repression of promoter activity. In contrast, pBLCATRegI had 85% lower promoter activity than that of the parental plasmid pBLCAT2 (Fig. 3C), indicating that the region between −258 and −161 in the *vIRF* gene promoter is involved in the transcriptional repression of the promoter activity.

To further map the negative regulatory cis-element, 4 overlapping oligonucleotides within the region from −258 to −161 (RegI) were synthesized and used to construct plasmids pBLCATRegI-1, pBLCATRegI-2, pBLCATRegI-3, and pBLCATRegI-4 (Fig. 4, A and B). As shown in Fig. 4C, pBLCATRegI-1, pBLCATRegI-3, and pBLCATRegI-4 had CAT activities similar to that of pBLCAT2. In contrast, pBLCATRegI-2 had 85% lower CAT activities than that of the parental plasmid pBLCAT2. These results indicate that the RegI-2 sequence contains the negative regulatory cis-element, whose negative repression function is independent on its position to the TATA box.
To further confirm the effect of RegI-2 on the repression of vIRF promoter activity, RegI-2 sequence was deleted in the reporter plasmid pCAT-991 by site-directed mutagenesis. As shown in Fig. 5, deletion of the RegI-2 sequence (ΔpCAT-991) relieved its repression effect on wild type pCAT-991. The CAT activity of ΔpCAT-991 reached 78% of pCAT-125 (100%). These results demonstrated the critical role of the RegI-2 sequence on the repression of vIRF promoter activity. Taken together, the above results indicate that the negative regulatory cis-element (−241 to −219) in vIRF promoter is a transcriptional silencer, and is named Tis.

**Binding of Transcriptional Regulators to Tis**—To determine whether Tis has any potential binding site(s) for transcriptional repressor(s), its sequence, RegI-2 (5′-CTCCACGAGTTA-ATAGGTAAGGT-3′), was used as a probe in an EMSA. As shown in Fig. 6A, when crude nuclear extract from HeLa cells was used, two major shifted complexes were observed (lane 2). Treatment of the nuclear extracts with unlabeled RegI-2 strongly inhibited the intensities of the shifted complexes (lane 3), indicating that both shifted complexes were RegI-2-specific DNA-protein interaction complexes. The protein complexes bound to the upper and lower bands were named as vIRF regulatory sequence-binding protein complex 1 and 2 (vR1 and vR2), respectively.

To determine whether the DNA-protein binding pattern could be reproduced in KSHV-infected cell lines, the nuclear extracts from BC-1, BC-2, and BCBL-1 cells were employed in EMSA. As shown in Fig. 6B, the RegI-2-specific DNA-protein interaction complexes were also observed in BC-1, BC-2, and BCBL-1 cell lines, indicating that the RegI-2-binding proteins are present in both KSHV-negative and -positive cell lines.

**Mapping of the Minimal Binding Site(s) of Transcriptional Repressor(s)**—To define the binding site(s) of transcriptional repressors involved in the transcriptional repression, the MatInspector2.2 program was used to analyze the potential binding sites of transcriptional factors (41). Comparative analysis of RegI-2 sequence with the GenBankTM data base did not identify any identical binding sequence of known transcriptional repressor(s), including a Myc/Max motif, a Nkx2.5 motif, and an 8EF1 motif (Fig. 7A).

To determine whether these sequence motifs were related to the repression of promoter activity, deletions corresponding to these motifs were introduced into the wild type RegI-2 sequence (23 nucleotides) (Fig. 7B). The resulting oligonucleotide D1RegI-2 (5′-CTCCTTAATAGGTAAGGT-3′), D2RegI-2 (5′-CTCCACGAGTTAATAGGTAAGGT-3′), and D3RegI-2 (5′-CTCCACGAGTTAAGGT-3′) were used to generate reporter constructs D1pBLCATRegI-2, D2pBLCATRegI-2, and D3pBLCATRegI-2.
suggested that the sequence motifs TTAATAGG and ATAGGTAG are critical for the repression of promoter activity.

To further determine whether these sequence motifs are critical for the binding of vR1 and vR2, the oligonucleotides D1RegI-2, D2RegI-2, and D3RegI-2 were used as competitors of RegI-2 probe in an EMSA. As shown in Fig. 7D, D1RegI-2 with the sequence motif CACGAG deleted strongly competed with RegI-2 for the formation of both vR1 and vR2 (lane 6). This pattern is similar to that when RegI-2 itself is used as a competitor (lane 3), suggesting that the sequence motif CACGAG is not essential for the binding of the repressor(s). When the oligomers D2RegI-2 (lane 4) and D3RegI-2 (lane 5) were used as competitors, the DNA-protein binding patterns remained unchanged, indicating that both sequence motifs TTAATAGG and ATAGGTAG were essential for the DNA-protein interactions. Indeed, the oligomer D1RegI-2 produced the same shifted pattern as RegI-2 did (Fig. 7E). In contrast, no shifted complex could be detected when labeled oligomers D2RegI-2 and D3RegI-2 were used as probes (Fig. 7, F and G). Taken together, these findings suggest that both sequence motifs TTAATAGG and ATAGGTAG are required for the binding of the repressor(s).

**FIG. 7. Deletion analysis of the binding sites of transcriptional repressor(s).** A, potential binding sites of known transcriptional factors in RegI-2 sequence. B, RegI-2 was used as a wild type in deletion analysis to define the motifs involved in Tis transcriptional repression. The sequence motifs CACGAG, TTAATAGG, and ATAGGTAG within RegI-2 were independently deleted to generate the oligonucleotides D1RegI-2, D2RegI-2, and D3RegI-2. C, the plasmids D1pBLCATRegI-2, D2pBLCATRegI-2, and D3pBLCATRegI-2 were constructed by inserting the oligomers D1RegI-2, D2RegI-2, and D3RegI-2 into the upstream of the TK promoter in pBLCAT2, respectively. The constructs were transiently transfected into HeLa cells and the CAT assay was performed. The result is the average relative CAT activity with standard deviation from three independent experiments compared with that of pBLCAT2. D, EMSA with RegI-2 as a probe and various oligomers as competitors. Nuclear extract from HeLa cells was added to all reactions except that of lane 1. Both vR1 and vR2 complexes were observed (lane 2), which were competed by unlabelled RegI-2 (lane 3) and D1RegI-2 (lane 6), but not by D2RegI-2 and D3RegI-2 (lanes 4 and 5). All competitors were 100-fold excess of the probe. E–G, EMSA with oligomers D1RegI-2, D2RegI-2, and D3RegI-2 as probes. vR1 and vR2 complexes were observed using probe D1RegI-2 (E), but not detected using probes D2RegI-2 (F) and D3RegI-2 (G). Lanes 1, 4, and 7 are reactions without nuclear extract; lanes 2, 5, and 8 are reactions with nuclear extract; lanes 3, 6, and 9 are reactions competed by unlabelled D1RegI-2, D2RegI-2, and D3RegI-2, respectively.

In a transient transfection assay, deletions of the sequence motifs TTAATAGG (D2pBLCATRegI-2) and ATAGGTAG (D3pBLCATRegI-2) relieved the RegI-2 (pBLCATRegI-2) repression of TK promoter activity by 80 and 50%, respectively. In contrast, deletion of the sequence motif CACGAG (D1pBLCATRegI-2) had no effect on relieving the RegI-2 repression of TK promoter activity of pBLCATRegI-2 (Fig. 7C),
FIG. 8. Determination of the core sequence motifs for the binding of transcriptional repressor(s). A, wild type (M0) and a panel of mutant (M1-M15) oligomers used for the identification of the binding sequence motif(s). The mutated nucleotide in each mutant oligomer was shown underlined. B, competition of vR1 and vR2 complexes by unlabeled oligomer (M0) and mutant oligomers (M1-M15). Representative figure of competitive EMSA using wild type oligomer M0 and a panel of mutants M1-M15 to map the nucleotides critical for the interaction of transcriptional repressor(s) with double-stranded Regl-2 probe. The intensities of the bands were quantitated with a Molecular Imager for vR1 band (C) and vR2 band (D), and expressed as percentage of relative binding intensities using the formula [bound/(bound + free)] × 100%, and further calibrated with those obtained without competitor (lane N2 as 100%). N1 is the control without nuclear extract, and N3 is the control with Regl-2 as competitor. Results are the averages with standard deviations from three independent experiments. E, EMSA with mutants M6 (lanes 1–3) and M9 (lanes 4–6) as probes to confirm the effect of mutated nucleotides on the binding of the repressor(s). Labeled M6 (lanes 1–3) and M9 (lanes 4–6) were used as probes in the presence (lanes 2, 3, 5, and 6) and absence (lanes 1 and 4) of nuclear extract, and with unlabeled M6 (lane 3) and M9 (lane 6) as competitors. Lane 7 is the control using Regl-2 as a probe.
Novel Transcriptional Silencer in vIRF Gene Promoter

We have previously observed that a region in vIRF promoter has a transcriptional repression effect (34). In this study, we have employed deletion analysis to further examine a 1.052-kb 5'-flanking region of the vIRF gene in an attempt to identify the functional regulatory element that is responsible for such repression. Our results demonstrated that the sequence region from −337 to −125 had a strong repression effect on the CAT reporter gene driven by both vIRF promoter and heterologous TK promoter (Figs. 1 and 2). Dissection of this region showed that Regl (−258 to −161) is required for the repression activity (Fig. 3). The repression function of this cis-element is position- and orientation-independent, indicating the presence of a transcriptional silencer within this region. Further dissection of the Regl sequence identified a 23-bp DNA fragment from −241 to −219 (Regl-2) as the transcriptional silencer Tis (Fig. 4). Deletion of Tis in wild type pCAT-991 strongly relieved its repression effect on vIRF promoter (Fig. 5). EMSA identified two major shifted complexes, vR1 and vR2, in this region, which can be competed by unlabeled Regl-2 (Fig. 6), indicating that both vR1 and vR2 were specific. These results point to the presence of binding motif(s) of transcriptional repressor(s) in Tis. Since Tis is capable of repressing the promoter activities of both vIRF gene and heterologous HSV-TK gene, the repression function of Regl-2 may be universal rather than vIRF promoter specific.

Our data demonstrated that vR1 and vR2 complexes observed in EMSA with Regl-2 as probes are specific and responsible for the transcriptional repression of vIRF promoter. Comparative analysis with the database of transcriptional factors did not match Regl-2 with any identical sequences of known transcriptional silencers. However, the Regl-2 sequence was found to contain the potential core sequence motifs corresponding to the binding sites of transcriptional factors Myc/Max, Nkx2.5, and δEF1 (Fig. 7). Deletion of the sequence motifs CACGAG, TTAATAGG, and ATAGGTTAG corresponding to these transcriptional factors relieved the repression effect of Regl-2 on promoter by 2, 80, and 50%, respectively, indicating that the sequence motif TTAATAGTAG is critical for the repression of the vIRF promoter activity. When D1Regl-2, D2Regl-2, and D3Regl-2 were used as competitors in EMSA, D1Regl-2 inhibited the formation of the shifted complexes, while D2Regl-2 and D3Regl-2 had no effect. Meanwhile, when D1Regl-2, D2Regl-2, and D3Regl-2 were used as probes for EMSA, D1Regl-2 had the shifted pattern similar to that of Regl-2, while both of D2Regl-2 and D3Regl-2 formed no shifted complexes (Fig. 7). Point mutation analysis demonstrated that the sequence motif TTAAT is critical for the binding of the repressor(s) in vR1, and the sequence motif GTTAATAG is critical for the binding of the repressor(s) in vR2 (Fig. 8). This sequence motif is very similar to the binding motif of Nkx2.5. However, antibodies to Nkx2.5, δEF1, and Myc/Max did not supershift vR1 and vR2 complexes or affect gel shift patterns in EMSA, indicating that all three transcriptional factors are not involved in Tis DNA-protein interactions (Fig. 9A). Furthermore, Nkx2.5 is a known heart tissue-specific factor whose expression is absent in cell lines used in this study, thus excluding its role in the formation of vR1 and vR2 complexes (Fig. 9B).

FIG. 9. Determination of transcriptional factors required for Tis DNA-protein interactions. A, gel supershift assay. Antibodies to transcriptional factors Nkx2.5 (lane 4), δEF1 (lane 5), and c-Myc (lane 6) were preincubated with reaction mixtures before the addition of the radioabeled Regl-2 probe. None of the antibodies affected the gel shift patterns. Lane 1, without nuclear extract; lane 2, with nuclear extract; and lane 3, with nuclear extract plus competitor. B, Nkx2.5 is not expressed in HeLa (lane 1), BC-1 (lane 2), and 293 cells (lane 3) as determined by RT-PCR. RNA sample from heart tissue was used as a positive control for the RT-PCR (lane 4).

Regulation of eukaryotic gene expression is usually involved with multiple transcriptional factors, which function to activate or repress transcription via binding to cis-regulatory elements. Transcriptional repressors, in particular, play a central role in vital biological processes, such as the development and regulation of cell growths. Our results have demonstrated that the Tis region from −241 to −219 in the vIRF gene promoter is a transcriptional silencer. This region interacts with a yet unidentified transcriptional repressor(s) that represses vIRF promoter activity. Negative regulation has been suggested to play a critical role in modulating the expression of eukaryotic genes (42–45). A large number of studies performed in the last decade have led to a better understanding of the mechanism of negative regulation in gene expression (46–54). Although there are some other potential binding sites such as AP1 and SP1 for the binding of positive regulators in the upstream regulatory sequence of the vIRF promoter, our data suggest that negative regulation is predominant in the regulation of vIRF gene expression in latent steady-state.

Transcriptional repressors are generally divided into two categories: passive repressors and active repressors. Passive repressors function by competing with activators or basal transcriptional factors for the access to their binding DNA sequences, and the recruitment of inhibitory chromatin components to the promoter. The active repressors inhibit gene transcription by directly interacting with positive regulators or transcriptional factors of basal transcriptional machinery (44, 45). Previous studies have demonstrated that vIRF is a viral early gene whose expression is minimal in KSHV latent replication but could be increased to high level during KSHV lytic replication (34–37). Based on the previous reports and our
current study, we propose the following model for the role of Tis in the regulation of vIRF gene expression (Fig. 10). In KSHV latent replication, the expression of vIRF gene is repressed by the transcriptional repressor(s), vR1 and vR2, which bind to Tis and interact with the basal transcriptional complex to repress vIRF gene transcription. In KSHV lytic replication or after TPA induction, the positive regulator(s) interact with the transcriptional repressors vR1 and vR2 or basal transcriptional machinery to dissociate the binding of vR1 and vR2 to the basal transcription complex of vIRF promoter, and abolish the inhibitory effect of vR1 and vR2 (see “Discussion” for a more detailed interpretation). TATA refers to the TATA box in the vIRF promoter region; TBP stands for the TATA box-binding protein; TAFs stands for the TBP-associated factors; TPA-RE refers to the TPA-responsive element.

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