Transcriptional Regulation of the Kaposi’s Sarcoma-Associated Herpesvirus K15 Gene

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The K15 gene product of Kaposi’s sarcoma-associated herpesvirus (KSHV) is a transmembrane protein that is encoded by the last open reading frame of the KSHV genome. The K15 protein has been implicated in modulation of B-cell signal transduction and activation of the Ras/mitogen-activated protein kinase and NF-κB signal transduction pathways. Here we report the identification of the transcriptional start site of the full-length K15 gene in KSHV-positive BCBL-1 cells. We have mapped the K15 transcriptional start site to a position 152 nucleotides upstream from the translation start site by rapid amplification of cDNA ends and RNase protection assays. We have also characterized the K15 promoter element. To analyze the cis-acting elements necessary to regulate K15 gene expression, a series of 5’ promoter deletion constructs were generated and subcloned upstream of the luciferase reporter gene. Transcriptional assays with these mutant promoters demonstrated that chemical induction in latently infected KSHV-positive BCBL-1 cells activated K15 transcription. In addition, K15 promoter transactivation was also mediated by the viral immediate-early protein Orf50/Rta, suggesting that the K15 gene is actively transcribed during lytic replication.

Kaposi’s sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8, was first discovered in 1994 by Chang et al. in Kaposi’s sarcoma (KS) lesions procured from patients with AIDS (7). Subsequently, KSHV DNA sequences were detected in all four types of KS (1): (i) classic KS, which affects elderly men of Mediterranean and eastern European descent; (ii) endemic KS, which is highly prevalent in sub-Saharan Africa (28); (iii) iatrogenic or immunosuppressive KS, which develops in solid-organ transplant recipients (4, 26); and (iv) AIDS KS. In addition, KSHV is implicated as the etiologic agent of two lymphoproliferative disorders in both human immunodeficiency virus-positive and human immunodeficiency virus-negative individuals, body cavity-based lymphoma (BCBL) or primary effusion lymphoma (PEL) (5, 14, 21), and the plasmablastic variant of multicentric Castleman’s disease (32). PEL is a form of non-Hodgkin’s lymphoma that presents as malignant effusions in pleural, pericardial, or peritoneal cavities without a detectable tumor mass or, alternatively, manifests as a solid mass in lymph nodes, lungs, or the gastrointestinal tract (35). KSHV exhibits cell tropism for vascular endothelial cells, epithelial cells, and B cells (20). KSHV is classified as a member of the herpesvirus family, a group of enveloped viruses containing double-stranded DNA (1). KSHV is a member of the gamma subgroup of herpesviruses, which is further subdivided into two genera: lymphohemotrophic viruses (gamma-1) and rhadinoviruses (gamma-2). Members of the gamma-1 herpesvirus subfamily include Epstein-Barr virus, Lymphohemotrophicvirus of rhesus monkeys, and Herpesvirus papio of baboons. The gamma-2 herpesvirus subfamily is comprised of KSHV, Herpesvirus saimiri, Rhesus monkey rhadinovirus, and Marve herpesvirus 68 (9). There are 81 open reading frames (ORFs) in the long unique region of the KSHV genome flanked by terminal repeats (24).

Similar to Epstein-Barr virus LMP2A, K15 is able to block B-cell receptor signal transduction (8). The cytoplasmic tail of K15 contains conserved SH2 binding motifs which are known to interact with the Src and Syk families of protein tyrosine kinases (8). The Src protein tyrosine kinase family members, Src, Fyn, Lck, Hck, and Yes, bind to the C terminus of K15 between amino acids 355 and 373 and phosphorylate the tyrosine residue at position 481 in the SH2 binding motif (3).
Recent studies demonstrated that the largest (45-kDa) K15 gene product, which is a protein with 12 membrane-spanning domains, activates the Ras/mitogen-activated protein kinase and NF-κB signal transduction pathways (3). A tumor necrosis factor receptor-associated factor binding site is also present in the carboxy terminus of K15. Tumor necrosis factor receptor-associated factors 1, 2, and 3 bind to the YEEVL motif in the cytoplasmic tail of K15, an interaction required for the activation of NF-κB (13). A point mutation at tyrosine residue position 481 (Y481) within the Y481EEVL motif abolished K15-mediated activation of the NF-κB and Ras/mitogen-activated protein kinase pathways, suggesting that phosphorylation of YEEVL is necessary for signaling (3). In addition, K15 interacts with antiapoptotic protein HAX-1 in vitro and in vivo (27).

K15 is likely to play a significant role in KSHV-induced pathogenesis. Hence, understanding the mechanism by which K15 expression is regulated may shed light on its role in the KSHV life cycle. In the present study, we have identified the K15-P transcription initiation site by 5′ rapid amplification of cDNA ends (RACE) and RNase protection assay (RPA) and have mapped the K15-P promoter element in a number of different cell lines. We also show that the KSHV immediate-early protein Orf50/Rta transactivates the K15 promoter element.

MATERIALS AND METHODS

Cell culture. Suspension BCBL-1 cells and BJAB cells were cultured in RPMI 1640 medium (Cellgro) containing 1-glutamine, 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (50 μg/ml). For BCBL-1 cells, the medium was further supplemented with 5 μM 2-mercaptoethanol and 0.0075% sodium bicarbonate. Adherent 293 epithelial cells were maintained in Dulbecco modified Eagle medium (Sigma) with 10% fetal bovine serum, 2 mM Glutamax I (Invitrogen), penicillin (100 U/ml), and streptomycin (50 μg/ml). All cells were grown at 37°C in 5% carbon dioxide (CO2).

5′ RACE. KSHV-positive BCBL-1 cells were pretreated with either the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) at a final concentration of 25 ng/ml or an equal volume of dimethyl sulfoxide (DMSO) for 40 to 48 h. TPA was prepared in DMSO at 25 μg/ml. Total RNA was isolated from pretreated cells with the RNA STAT-60 kit (Tel-Test, Inc.). Total RNA was further enriched for polyadenylated RNA by using the Oligotex mRNA kit (QIAGEN). Six hundred nanograms of polyadenylated RNA was used for 5′ RACE. First-strand cDNA synthesis and 5′ RACE were performed as specified in the BD SMART RACE kit protocol (Clontech). Reverse transcription was carried out in the presence and absence of Powerscript reverse transcriptase (Clontech). The K15-P-specific primer 5′-AGTAGGGCCCAAAGCATAAATTCCAGA-3′ was used in the appropriate 5′ RACE PCRs. Thermal cycling was run with the following program: (i) 94°C for 5 min, (ii) 94°C for 5 s, (iii) 65°C for 10 s, (iv) 72°C for 3 min, (v) repetition of steps ii to iv for 39 cycles, and (vi) 70°C for 10 min. Amplified cDNA products separated in a 2% low melting-point agarose gel were isolated from the gel and cloned into the pCRII.1 TOPO TA vector (Invitrogen TOPO cloning kit). The TOPO clones were transformed into TOP 10 competent cells (Invitrogen) and plated on Luria-Bertani plates supplemented with ampicillin at 100 μg/ml. Bacterial colonies were picked, and the isolated DNAs were sequenced with the M13 reverse primer by submission to the University of North Carolina at Chapel Hill Genome Analyses Facility.

RPA. Polyadenylated RNA isolated from BCBL-1 cells pretreated with TPA (25 ng/ml) or an equal volume of DMSO was used for RPA. RPA was carried out with the Ambion RPA III kit. To generate the K15 probe, a 500-bp fragment was amplified spanning 150 bp of the K15 first ORF and 350 bp upstream from the ATG translation start site. Genomic DNA from KSHV-positive BCBL-1 cells served as the DNA template. The forward and reverse primers, engineered to contain BamHI sites, used for the amplification reactions were 5′-CGGGCGG GATATCCAGACTCTCCGCCAGAATAGAAG3′ and 5′-CGGGCGGGAGATTCTTA ATAGCCTGACAAACACCA-3′, respectively. The amplified fragment was then cloned into the pSP72 vector at the BamHI site. To make an antisense RNA probe, the plasmid was linearized with HindIII. The Promega Riboprobe kit and [α-32P]CTP were used to transcribe and generate an antisense RNA probe in vitro. The radiolabeled probe was purified with the RNasey Mini kit (QIAGEN). At least 6 × 10^6 cpm of the K15 probe was used for RPA. For the K15 RPA, hybridization of the radiolabeled probe to mRNA was carried out overnight at 55°C. For the positive control, β-actin RPA, the manufacturer’s protocol was followed. Products were separated on a denaturing 6% polyacrylamide-urea gel. The 5′X74 DNA Hindf-digested marker (Promega) was end labeled with [γ-32P]ATP and T4 polynucleotide kinase. The sequencing ladder was prepared from the pSP72 vector and the 32P-end-labeled T7 primer in accordance with the manufacturer’s manual (Epicenter Scoutherm Cycle Sequencing kit).

K15 promoter cloning. KSHV-positive BCBL-1 genomic DNA was used as the KSHV DNA template in the PCRs to amplify the sequence upstream of the K15 gene. Primers were designed to contain a HindIII restriction site at the 5′ end and a BglII restriction site at the 3′ end of the amplified product. The following reverse primer was used in all of the PCRs: 5′-CGGGGGGAGAAGCTTCTCTAA ATATCCCCAGAACAG-3′. For upstream promoter truncated mutations, the following forward primers were used: (i) 5′-T5K15 (5′-CGGGCGGAGAAGCTTTGTTTITGACGTGTTAAACAA-3′), (ii) 5′-T5K15 (5′-CGGGCGGAGAAGCTTTTCCAGGATACCTTTCTCTCTCTT-3′), (iii) 5′-T5K15 (5′-CGGGCGGAGAAGCAGATGTCCCTCCCGCGGGGGCGGCG-3′), (iv) 5′-T5K15 (5′-CGGGCGGAGAAGCAGATGTCCCTCCCGCGGGGGCGGCG-3′), (v) 5′-T5K15 (5′-CGGGCGGAGAAGCAGATGTCCCTCCCGCGGGGGCGGCG-3′), (vi) 5′-T5K15 (5′-CGGGCGGAGAAGCAGATGTCCCTCCCGCGGGGGCGGCG-3′), (vii) 5′-T5K15 (5′-CGGGCGGAGAAGCAGATGTCCCTCCCGCGGGGGCGGCG-3′).

RESULTS

Identification of the K15 transcription initiation site. In order to identify the promoter element of the full-length K15-P gene, mapping its transcription start site was essential. To date, no literature on the transcription initiation site of K15 has been published. Normal BCBL-1 cells contain ~50 copies of the KSHV genome (40) in the latent state, and when treated with TPA, the viral genome is induced to enter the lytic cycle (19).
K15 is weakly expressed in latently infected B cells but induced in TPA-treated BC3 (15) and BCBL-1 (22) cells. To enrich for K15 transcripts, we sought to map the K15 transcription initiation site with BCBL-1 cells treated with either the vehicle alone (DMSO) or TPA. Briefly, total RNAs from DMSO-treated (latent) and TPA-induced (lytic) BCBL-1 cells were isolated with the Tel-Test RNA Stat-60 kit. RNA was further enriched for polyadenylated [poly(A)]+ RNA isolated from TPA- or DMSO-treated BCBL-1 cells was performed, followed by RNase A-T1 digestion of unprotected RNA fragments. It is important to note that 50 µg of mRNA harvested from BCBL-1 cells pretreated with TPA (lane 8) or 100 µg of DMSO (D, lane 9), i.e., twice as much mRNA, was used in the RNase digestion. The protected probe fragments were separated on a 6% denaturing polyacrylamide-urea gel, and the size of the protected fragment remaining after RNase digestion was determined by comparison to a known RNA sequence ladder. Figure 2 shows the result of the RPA. The K15 RPA yielded a 302-nucleotide protected fragment, as indicated by the symbol ●. The transcript is present in both TPA-treated BCBL-1 cells (lane 8) and DMSO-treated latently infected cells (lane 9). A sequencing ladder (lanes 11 to 14) was also run on the same gel (Fig. 2). As the exact position of the chosen antisense probe was known, the K15 transcription initiation site was confirmed at position 152 upstream from the ATG translation start site of K15. This is the same site obtained in the 5′ RACE experiment described above. Positive controls for the RPA included a β-actin probe which yielded a protected fragment from liver mRNA (Fig. 2, lanes 2 to 4).

K15 promoter basal activity. Elucidation of the cis-acting elements within the K15 promoter will provide important insights into the regulatory mechanisms of K15 gene expression. Figure 3 displays the upstream sequence from the K15 ATG translation start site to the end of the sequenced BCBL genome (GenBank accession no. U85269) (40). The GC-rich terminal repeats start at 270 nucleotides upstream of the ATG translation site, as indicated by an asterisk (GenBank accession no. U85269) (40). The translation start site is marked by a black arrow and the transcription start site by a white arrow (Fig. 3). The sequence was scanned with the Genomatix MatInspector program to identify putative transcription factor binding sites. There appears to be a very weak TATA box (TATTATAT) about 25 nucleotides upstream of the K15 transcription initiation site. Some of the transcription factor binding sites in the K15 promoter include Oct1, E2F, Sp1, NFκB, Stat1, and Smad3.

To examine the cis-acting elements necessary to activate the K15 gene, a series of 5′-truncated promoter deletion constructs...
were synthesized. Fragments containing sequences from the K15 transcription initiation site to 75, 100, 125, 150, 225, and 271 bp upstream were generated by PCR with genomic DNA from KSHV-positive BCBL-1 cells as the DNA template. These PCR-generated fragments were subcloned in front of the luciferase reporter gene in the pGL2Basic vector. Figure 4 illustrates the six deletion constructs. The nomenclature for each plasmid is listed on the right. The −125K15p, −150K15p, −225K15p, and −271K15p promoters contain sequences within the terminal repeats.

To investigate the ability of each of the K15 deletion promoter elements to drive luciferase expression, reporter assays were carried out with various cell lines. The K15 promoter-reporter plasmids were each transiently transfected into 293 human kidney epithelial cells with Lipofectamine (Invitrogen) and into BJAB and BCBL-1 cells by electroporation. Cotransfection with constant amounts of a β-galactosidase reporter plasmid was used to normalize for transfection efficiency. The transfections were performed in duplicate and repeated numerous times. Figure 5 shows the results of these reporter assays. Transfection assays with KSHV-negative 293 cells (Fig. 5A) and BJAB cells (Fig. 5B) demonstrate 15- to 35-fold activation of the K15 promoter constructs over that of the empty vector (pGL2basic) in both cell lines. Only the −75K15p promoter containing the first 75-bp sequence from the transcription initiation codon, and the white arrow represents the transcriptional start site.
tion site showed minimal activity in both 293 and BJAB cells. A similar profile was seen in KSHV-positive BCBL-1 cells (Fig. 5C). The \(100\)K15p, \(125\)K15p, \(150\)K15p, \(225\)K15p, and \(271\)K15p promoter elements displayed basal activity 30- to 60-fold higher than that of the empty vector. These results suggest that the minimum sequence that can function as a promoter element for K15 gene expression contains the first 100 nucleotides upstream of the K15 transcription start site.

TPA-induced lytic infection activates the K15 promoter elements. The K15 gene is weakly expressed in latent BCBL-1 cells, but expression increases after chemical induction of viral lytic replication (8, 13, 23), thus implying differential transcriptional regulation at variable stages of the viral cycle and/or the host environment. We investigated whether lytic induction with TPA could activate K15 gene expression. The two minimal promoter element \(-75\)K15p and \(-100\)K15p constructs were transfected into BJAB and BCBL-1 cells by electroporation. Four to 6 h after electroporation, cells were treated with TPA at 25 ng/ml (hatched bars) or an equal volume of DMSO (black bars). Cells were harvested 48 h posttransfection and assayed for luciferase activity (RLU). Promoter activity (RLU) was calculated as normalized luciferase activity (RLU/β-galactosidase activity) minus the background activity of cells transfected with the pGL2Basic reporter construct alone. Error bars represent the standard deviation of duplicate samples.

KSHV Orf50/Rta activates K15 promoter elements. Since our study showed that activation of the two K15 minimal promoter elements, \(-75\)K15p and \(-100\)K15p, by TPA induction to lytic replication was KSHV dependent, we sought to identify viral lytic gene products involved in K15 transcriptional regulation. KSHV immediate-early protein Orf50/Rta was a primary candidate. Orf50/Rta is a potent transactivator of early and late viral genes, thus triggering the cascade of viral replication. The
KSHV Orf50/Rta protein has been previously shown to activate a number of different downstream KSHV promoters, including nut-1, ORF57, K8, K1, and vIL-6 (2, 11, 17, 29–31, 36). Orf50/Rta recognizes and binds diverse DNA sequence motifs in responsive promoters. A number of Rta-responsive elements (RREs) have been identified (6, 11, 12, 16, 25, 29). The consensus RRE includes a pattern of multiple A/T triplets repeated every 7 or 17 nucleotides with high-GC sequences preferred for the spacer (16) (Fig. 7A). To determine the effects of Orf50/Rta on the K15 promoter, the empty vector pGL2Basic and the K15 promoter deletion construct /H11002\75K15p and /H11002\100K15p promoter-luciferase plasmids were each transfected into 293, BJAB, and BCBL-1 cells with or without the Orf50/Rta expression plasmid (Fig. 7B, C, and D). In 293 cells, Orf50/Rta activated /H11002\75K15 13-fold and /H11002\100K15 3-fold over the activity of cells cotransfected with pCDNA3 (empty vector) (Fig. 7B). Similarly, in BJAB cells, −75K15p and −100K15p were activated by Orf50/Rta 4.5-fold and 5-fold, respectively (Fig. 7C). In BCBL-1 cells, Orf50/Rta increased the activation of −75K15p and −100K15p fivefold and threefold, respectively, over cotransfection with pCDNA3 (Fig. 7D). Thus, the immediate-early gene product Orf50/Rta can activate transcription of the K15 promoter element.

In this study, we have identified, for the first time, the major transcription start site of the full-length K15 KSHV gene. We utilized both 5’ RACE and RPAs to confirm the transcription start site of the K15 gene. Both 5’ RACE and RPA mapped the K15 transcription initiation site to 152 nucleotides upstream from the K15 translation start site (ATG). Most eukaryotic promoters contain a TATA box element located 25 to 30 bp upstream from the transcription start site. We have identified a putative weak TATA box, TATTTAT, located 26 bp upstream of the K15 transcription initiation site. Cellular transcription binding sites in the K15 promoter include Oct-1, E2F, Stat-1, Sp1, and NFκ-B. Interestingly, we compared the K15 promoter sequences of four different isolates of KSHV which encode the predominant, K15-P, form of the protein: the BCBL-R isolate from PEL cells (accession number U85269), the GK18 isolate (accession number AF148805) from KS tumors, and two Ugandan K15-P isolates (accession numbers AY042968 and AY042973). The promoter element we have mapped for K15-P was 100% conserved among all of these viral genomes (Fig. 8), suggesting that K15-P transcription is regulated by factors that are conserved among these different KSHV genomes. However, the
but not in KSHV-negative BJAB cells, implying that KSHV proteins play a role in activating K15 expression.

The KSHV immediate-early gene product, Orf50/Rta, is known to transactivate several downstream KSHV promoters, including nut-1, ORF57, K8, K1, and VIL-6 (2, 10, 17, 29–31), and our data support adding the K15 promoter to this set. Cotransfection experiments showed that both the −75K15p and −100K15p promoter elements were activated by Orf50/Rta compared to the vector alone in both KSHV-negative and KSHV-positive cells. Transcriptional activation by Orf50/Rta was not surprising since both the −75K15p and −100K15p promoter elements contain multiple consensus RREs. There are two (A/T)3N2(A/T)3 and six (A/T)3N2(A/T)3 RRE motifs, some of which overlap, spanning the 75-bp region upstream from the K15 transcription start site. One additional (A/T)3N2(A/T)3 motif and three (A/T)3N2(A/T)3 motifs are present in the −100K15p promoter element. No RRE sequences were identified outside the 100-bp region immediately upstream of the K15 initiation site. Thus, both our reporter assays and identifications of multiple RRE motifs within −75K15p and −100K15p strongly support Orf50/Rta-mediated transcriptional activation of the K15 gene.

Elucidation of the transcription initiation site and the cis-acting elements within the K15-P promoter imparts important insights into the regulatory mechanisms of K15 gene expression. This study will provide the basis for future investigations.

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REFERENCES


