Kaposi’s Sarcoma-associated Herpesvirus K8 Exon 3 Contains Three 5’-Splice Sites and Harbors a K8.1 Transcription Start Site*  

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Shuang Tang and Zhi-Ming Zheng‡  
From the HIV and AIDS Malignancy Branch, Center for Cancer Research, NCI, National Institutes of Health, Bethesda, Maryland 20892

Kaposi’s sarcoma-associated herpesvirus (KSHV) K8 and K8.1 open reading frames are juxtaposed and span from nucleotide (nt) 74850 to 76695 of the virus genome. A K8 pre-mRNA overlaps the entire K8.1 coding region, and alternative splicing of KSHV K8 and K8.1 pre-mRNAs each produces three isoforms (α, β, and γ) of the mRNAs. We have mapped the 5’ end of the K8.1 RNA in butyrate-induced KSHV-positive JSC-1 cells to nt 75901 in the KSHV genome and have shown that exon 3 of the K8 pre-mRNA in JSC-1 cells covers most part of the intron 3 defined previously and has three 5’-splice sites (ss), respectively, at nt 75838, 76155, and 76338. Selection of the nt 75838 5’-ss dictates the K8 RNA production and overrules the RNA processing. Alternative selection of other two 5’-ss is feasible and leads to production of two additional bicistronic mRNAs, K5/K8.1α and -β. However, the novel bicistronic K8/K8.1 mRNAs translated a little K8 and no detectable K8.1 proteins in 293 cells. Data suggest that production of the K8/K8.1 mRNAs may be an essential way to control K8 mRNAs, especially K8α, to a threshold at RNA processing level.

Kaposi’s sarcoma-associated herpesvirus (KSHV)1 or human herpesvirus 8 is a newly identified human gamma herpesvirus strongly associated with development of Kaposi’s sarcoma, body cavity-based B cell lymphoma or primary effusion lymphoma (PEL), and Castleman’s disease (1–4). KSHV has a genome size of 145–157 kb, which encodes up to 90 complete open reading frames (ORFs) (5, 6). The majority of the KSHV ORFs were assigned as ORFs 1–75 by their homology to herpesvirus genome side by side, they, respectively, belong to three different categories of genes involved in different stages of viral lytic infection that are just beginning to be understood, considered evidence indicates that expression of each category gene is coordinately regulated and sequentially ordered in a cascade fashion in a viral lytic life cycle.  

A KSHV locus encompassing ORF50 (Rta) that is expressed at 4 h of butyrate induction and is resistant to cycloheximide inhibition. An early gene is defined by its expression after 8–20 h of chemical induction and its being sensitive to cycloheximide but resistant to antiviral drugs such as phosphonoacetic acid (PAA). KSHV genes encoding K3, K5, K8, vIL6, vmiP, vGCR, vDHFR, and viral thymidylate synthase belong to this group. Late genes differ from early genes by their absence prior to 24 h of chemical induction and by their sensitivity to PAA. These include KSHV ORF65 and several viral envelope glycoproteins such as K8.1 (21). Although the molecular events in correlation with each category gene during KSHV lytic infection are just beginning to be understood, considerable evidence indicates that expression of each category gene is coordinately regulated and sequentially ordered in a cascade fashion in a viral lytic life cycle.

Like other gamma herpesviruses, KSHV characteristically establishes latent infections with only a few genes being expressed in PEL-derived B cells. Five genes have been identified as latent: ORF K13 (ORF71, vFLIP), ORF72 (v-cyclin), ORF73 (LANA 1), ORF K10.5 (LANA 2), and ORF K12 (kaposin A) (8, 10–15). Other genes are the inducible genes that can be expressed by induction of various chemicals such as tetradeacetylphorbol acetate (TPA) and n-butyrate (16–19). Induction by the chemicals initiates the viral lytic life cycle of KSHV in PEL-derived B cells, with the accumulation of progeny virus in culture medium (18, 20). Although KSHV gene expression studies remain controversial, a number of studies, based on transcription kinetics, have classified KSHV genes into three categories during lytic infection induced by chemicals. These include immediate-early genes, early genes, and late genes (21, 22). More recently, Jenner et al. (7) clustered the lytic genes as primary (0–10 h), secondary (10–24 h), and tertiary (48–72 h) lytic genes according to its expression pattern by DNA arrays. A typical immediate-early gene is the KSHV ORF50 (Rta) that is expressed at 4 h of butyrate induction and is resistant to cycloheximide inhibition. An early gene is defined by its expression after 8–20 h of chemical induction and its being sensitive to cycloheximide but resistant to antiviral drugs such as phosphonoacetic acid (PAA). KSHV genes encoding K3, K5, K8, vIL6, vmiP, vGCR, vDHFR, and viral thymidylate synthase belong to this group. Late genes differ from early genes by their absence prior to 24 h of chemical induction and by their sensitivity to PAA. These include KSHV ORF65 and several viral envelope glycoproteins such as K8.1 (21). Although the molecular events in correlation with each category gene during KSHV lytic infection are just beginning to be understood, considerable evidence indicates that expression of each category gene is coordinately regulated and sequentially ordered in a cascade fashion in a viral lytic life cycle.

A KSHV locus encompassing ORF50 (Rta), K8, and K8.1 extends for about 5,400 nts at map position ~0.43–0.46 of the virus genome. Although the three genes are positioned in the virus genome side by side, they, respectively, belong to three different categories of genes involved in different stages of viral lytic cycle. KSHV Rta gene is an immediate-early gene and expresses a homolog to EBV Rta that mediates transcription of several early genes including the K8, ORF57, K2 (vIL6), and PAN RNA (23, 24). A recent report (25) indicates that interaction of the N terminus of KSHV Rta with a 12-bp binding site, AACAATAATGTT, in both K8 and ORF57 promoters is necessary for transcriptional activation. Thus, expression of KSHV Rta is essential for lytic viral reactivation. The K8 gene is an early gene encoding a basic-leucine zipper (bZIP) protein called K-bZIP (22, 26, 27). The K-bZIP protein is also a homolog to EBV Zta, a transcriptional activator that plays a crucial role in the initiation of the EBV lytic cascade. Accumulated data show that KSHV K-bZIP is a nuclear protein probably related to...
viral DNA replication (27–29). Finding that KSHV K-hZIP forms a dimer in vivo (22) and undergoes extensive phosphorylation by cyclin-dependent kinase (30) as well as co-localizes with cellular promyelocytic leukemia protein (PML) in PML oncopgenic domains along with KSHV core replication proteins (29, 31) implies its roles in initiation of viral transcription and DNA replication. Evidence of the K-hZIP repressing transcription through interaction with p53 and CREB-binding protein further supports such a notion (31–33). KSHV K8.1 gene is a late gene encoding two related glycoproteins, K8.1A and K8.1B (34–36). The K8.1A and -B have identical amino acid sequences in both N terminus and C terminus but differ at the central domain because of alternative RNA splicing. The K8.1A (gp35–37) is a viral envelop protein interacting with cellular receptors heparan sulfate-like moieties (37). However, function of the K8.1B remains unknown.

Structural, functional, and expression analysis of individual viral genes have demonstrated that KSHV Rta, K8, and K8.1 genes share a common polyadenylation signal at nt 76714 downstream of the K8.1 coding region. KSHV Rta and K8 transcribe their RNAs from two separate promoters (22, 24, 30), whereas the K8.1 promoter and its transcription start site remain unknown. As a result, a pre-mRNA transcribed from the Rta promoter overlaps the K8 and K8.1 coding regions and thus is tricistronic with 5 exons and 4 introns. However, a pre-mRNA transcribed from the K8 promoter only overlaps the K8.1 coding region and thus is bicistronic with 4 exons and 3 introns. Chen et al. (38) recently showed that the Rta promoter p71560 is heavily methylated in latently infected cells, and demethylation of the Rta promoter by TPA treatment induces the KSHV lytic life cycle. KSHV K8 promoter p74845 identified by Lin et al. (22) has an Rta-binding site and can be directly activated by Rta (25). It has been documented that transcription of both Rta and K8 mRNAs undergoes extensive alternative splicing which leads to the generation of three spliced forms of the mRNAs (α, β, and γ) (22, 27, 30). In each case, a 3′-terminal intron is excluded from all isoforms of both Rta and K8. The KSHV K8.1 gene shares the 3′-terminal exon with Rta and K8 but utilizes a 3′-terminal intron of the Rta and K8 as its own coding region, which is also alternatively spliced to the same 3′-terminal exon of the Rta and K8 by using two alternative 5′-splice sites (ss) and subsequently produces the following two spliced mRNAs: K8.1α and K8.1β (34, 35). RNA K8.1y is the one left unspliced.

Altogether, these findings suggest that alternative promoter usage and alternative splicing of the three primary transcripts are cascaded and play important roles in KSHV lytic reactivation. We are interested in regulation of KSHV RNA splicing in the virus life cycle. The structures of Rta, K8, and K8.1 pre-mRNAs and their sophisticated splicing provide an ideal model for understanding how a viral pre-mRNA recruits cellular splicing machinery to define its splice sites for removal of an unwanted intron. Our initial approach focuses on a 3′-terminal intron because it involves the processing of all three transcripts Rta, K8, and K8.1. Because of complexity of their RNA structures and splicing, however, we simplify our focus on description only on K8 and K8.1 RNAs for better understanding in this report. Here we provide evidence that 5′ part of the K8 terminal intron is part of the K8 exon 3 (Rta exon 4) which extends to include two additional 5′-ss being used for splicing of the K8.1 pre-mRNA. Alternative selection of three 5′-ss in the K8 exon 3 results in several novel forms of bicistronic K8/K8.1 mRNAs. In addition, we also demonstrate that transcription of late K8.1 RNA is initiated at nt 75901, 14 nts upstream of the first AUG at nt 75915 in KSHV genome.

Experimental Procedures

Cells—Human 293 cell was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% inactivated fetal bovine serum (HyClone), 2 mM L-glutamine, 100 units penicillin/ml, and 100 μg streptomycin/ml at 37 °C under a 5% CO2. The KSHV-positive B cells JSC-1 cells (TEL-derived, coinfected with Epstein-Barr virus (EBV)) (21) was a generous gift from Dr. Richard Anderson (The Johns Hopkins University), and the BCP-1 cell line (KSHV+, EBV+) (CRL-294) was purchased from ATCC. Both cell lines were maintained at 37 °C in RPMI 1640 medium (Invitrogen) containing 2 mM L-glutamine, 100 units of penicillin/ml, 100 μg of streptomycin/ml, and 10 (JSC-1 cells) or 20% (BCP-1 cells) fetal bovine serum in the presence of 5% CO2. For chemical activation of cells, sodium butyrate (Alr) was added to a final concentration of 3 mM and incubated for 24 h. For anti-IgM activation, JSC-1 cells at 1 × 105, after three washes with serum-free RPMI 1640, were resuspended in 1 ml of serum-free RPMI 1640, incubated with 10 μg of anti-human IgM at 37 °C for 30 min, and then fed with 9 ml of RPMI 1640 with 10% fetal bovine serum at 37 °C in the presence of 5% CO2 for another 30 min.

RNA Preparation and RT-PCR—Total cell RNA was extracted by TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The poly(A)− mRNAs was isolated with Oligotex mRNA isolation mini kit (Qiagen). Following DNase I digestion, 1 μg of total RNA or 0.2 μg poly(A)− mRNAs was reverse-transcribed at 42 °C for 1 h using random primers and the cDNA amplified by PCR with primers of KSHV polymerase (PerkinElmer Life Sciences) using different pairs of primers as described in each figure. The followings are the primer sequences named by the locations of their 5′ ends: Pr75183 (oST1, 5′-TAATACGACTCACTATAGGGGACACACCAGAACCAATCTATATTCCC-3′), Pr75864 (oST2, 5′-GCTGGCCAGGATATATTCTCTTTC-3′), Pr75838 (oST10, 5′-GATCCTACCACTTTTCACCCACACCAATCTATATCTTC-3′), Pr75171 (oST11, 5′-TTAGTCGAGTGTTAGTTAGTTCTTTC-3′). P75769 (oST3, 5′-TACGCCGGGATATATTCTCTTTC-3′), Pr75605 (oST4, 5′-ATGCATTAGTGTTAGTTAGTTTCTTGT-3′) was linearized plasmid pST22 DNA templates or treated RNA fragments were separated by using a 6% polyacrylamide gel in TBE buffer and then followed by RNase digestion with RPA (Research Genetics, Huntsville, AL). The radioactive antisense RNA probe was detected by autoradiography with exposure to PhosphorImager film (Fuji Film). The radioactive antisense RNA probe was detected by autoradiography with exposure to PhosphorImager film (Fuji Film). The radioactive antisense RNA probe was detected by autoradiography with exposure to PhosphorImager film (Fuji Film). The radioactive antisense RNA probe was detected by autoradiography with exposure to PhosphorImager film (Fuji Film).
with an antisense primer Pr76687 (oST47, 5'-TCGAGGGCCCAAGGGACATTTGGATTGTCATTCGACGGAGAT-3'), an amplified DNA fragment was inserted into an expression vector pCDNA3.1/D/V-His-TOPO (Invitrogen) at a polylinker region downstream of a CMV promoter. Plasmid pST2 containing an inverted KSHV fragment (nt 76136 to 75569) for the RPA assay in Fig. 1C was constructed by inserting into the same expression vector a fragment amplified by PCR using a primer pair of Pr75679 and Pr76136 (oST50, 5'-CCAGGTATGCTGCTCCCATGACTACTCATGGTCACG-3'). To construct the plasmid (pST2) with a U to G mutation at 75583 5'-ss, a high fidelity PCR Taq enzyme (Roche Molecular Biochemicals) was used for overlapping PCR (3b). Two primer sets, Pr74850 and Pr75857 (oST49, 5'-GACCTAATTCCTCCTGCAACAAAGTCTGGC-3') and Pr75824 (oST48, 5'-GGAGACATTTGGATTGTCATTCGACGGAGAT-3') were mixed, re-amplified using a primer set of Pr74850 and Pr76687, respectively. The amplified PCR products from each set of the primers were mixed and re-amplified using a primer set of Pr74850 and Pr76687. Following gel purification, the PCR fragment with a U to G mutation at 75583 5'-ss was inserted into the expression vector pCDNA3.1/D/V-His-TOPO as described above. The same strategy was used to create plasmid pST5 derived from plasmid pST2 by overlapping PCR using two other primer sets, Pr74850 and Pr76180 (oST55, 5'-GATACGGAGACATTTGGATTGTCATTCGACGGAGAT-3') and Pr76145 (oST56, 5'-ACTCCGTCGAATGACAAATCAATGCTCCTGATC-3') and Pr76687. The individual PCR products were then mixed, re-amplified by using a primer set of Pr74850 and Pr76687, and cloned into the same expression vector. Resulting plasmid pST5 had mutations in 5'-ss, respectively, at nt 75583 (GU to GG) and at nt 76155 (GUGAGU to AUGCA). All mutations described in plasmids pST2 and pST5 were verified by sequencing. To construct a K8.1 cDNA plasmid (pST3), the RT-PCR fragment corresponding to K8.1 amplified from the JSC-1 total RNA using the primer set Pr74850 and Pr76687 was cloned. Plasmid pST6 was derived from plasmid pST2 by deletion of the CMV promoter with BamHI and SphI. LipofectAMINE (Invitrogen) was used to transfect 2 μg of plasmid DNA into 293 cells according to the manufacturer's instructions. Total cellular RNA was extracted from the transfected cells after 24 h.

Western Blot Analysis—JSC-1 cells and 293 cells with or without transfection were washed with PBS buffer and lysed in 2 x SDS sample buffer (4% SDS, 10% β-mercaptoethanol, 20% glycerol, 2 mM EDTA, 120 mM Tris-HCl (pH 6.8)). Protein samples were resolved on a 12% SDS-PAGE gel, blotted overnight onto a nitrocellulose membrane, and then probed with polyclonal anti-K-bZIP antibody (30) and anti-K8.1 A/B monoclonal antibody 4A4 (40).

Pre-mRNA Substrates and in Vitro Splicing—DNA templates used for in vitro transcription were obtained from plasmids pST1 and pST2 using a primer set of chimeric SP6/P7/Pr75860 (oST47, 5'-ATTAGGTTGCACATTTAGAGCCGGCAAGGTGAC-3') combined with Pr76576 or Pr76576/U (oST50, 5'-GATACCCGGCCGGTTCCTGATC-3'). In vitro transcription using SP6 RNA polymerase was performed in the presence of the cap analog (m7GpppG) and [α-32P]GTP. In vitro splicing of the transcribed pre-mRNAs and analysis of the spliced products were carried out as described in our previous publication (41).

RESULTS

Mapping of KSHV K8.1 RNA Transcription Start Site—Several laboratories (22, 26, 42) have reported that the size of KSHV K8 mRNA isolated from chemically stimulated PEL-derived B cells is ~0.9 kb by Northern blot analysis. However, the exact 5' end of the transcript remains unknown. If the K8.1 does have its own promoter, the nascent transcript should have a 5' end distinguishable from that of the K8 and Rta transcripts. Otherwise, the K8.1 protein must be translated from tricistronic Rta RNA or bicistronic K8 RNA by one of the following mechanisms: translation re-initiation, leaky scanning, or internal ribosome entry site. This seems unlikely because the published Northern blot results on the K8.1 mRNA do not support the latter scenario. Thus, locating the 5' end of the nascent K8.1 transcript will be very useful for our understanding on the K8.1 gene expression. To address this question, three K8.1 antisense RNA probes with different lengths of their 3' ends, but starting with the same 5' end at nt 76136 downstream of the K8.1 initiation codon AUG at nt 75915, were designed to cover K8.1 exon 1 (probe A in Fig. 1A) or both K8 exon 3 and K8.1 exon 1 as well as a putative junction region (probes B and C in Fig. 1A). RNase protection assays by using these probes were then performed to map the K8.1 transcription start site with total cell RNA isolated at different time points from JSC-1 cells induced by sodium butyrate. Results in Fig. 1, B and C, show that an RNA fragment with ~240 nts was protected from RNase digestion by all three probes. The protected RNA product appeared at 24 h of butyrate induction (lanes 3 and 9 in Fig. 1B and lane 2 in Fig. 1C) and reached a maximum at 48 h (lanes 4 and 10 in Fig. 1B and lane 3 in Fig. 1C), the latest time point of butyrate induction in this study. More importantly, the appearance of the protected 240-nt product induced by butyrate was sensitive to PAA inhibition (Fig. 1C, lane 4), suggesting that it was a transcript from a late gene and should represent a K8.1 transcript. Transcription of this product should be initiated within intron 3 of the K8 but upstream of the first K8.1 initiation codon AUG at nt 75915. By comparison with a sequencing ladder generated from pUC19, the protected RNA product was determined with a size of ~234 nts (data not shown). To map precisely the 5' end of the K8.1, primer extension analysis was further performed on those JSC-1 RNAs used for RPA with the 32P-labeled antisense primer Pr75986. The same primer was also used in deoxy DNA sequencing reactions on pST1 DNA, a KSHV K8 and K8.1 expression vector. Thus, the K8.1 transcription start site could be mapped by comparing the extended products with the DNA sequencing ladder. The data in Fig. 1D show that a product from butyrate-induced JSC-1 RNA (lanes 1 and 6) was extended and stopped at an A residue on the antisense strand at nt 75901. These results indicate that the K8.1 transcription is initiated at nt 75901, 14 nts upstream of the first AUG at nt 75915.

It was notable that expression of the K8 exon 3 (160 nts) protected by probes B and C also increased with butyrate induction (Fig. 1B, lanes 7–10, and Fig. 1C, lanes 2–4). However, its time course (Fig. 1B, lanes 7–10) was obviously different from that of the K8.1 mRNA. Importantly, expression of the K8 exon 3 was not sensitive to PAA inhibition (compare lane 4 to lane 3 in Fig. 1C), further confirming that the K8 is an early gene and the K8.1 is a late gene. In addition, we also found there were other protected fragments in the sizes of the full-length probes A and B, suggesting that these RNAs retained intron 3 of the K8 (Fig. 1B, lanes 3 and 4 and 8–10). The presence of this RNA species with the same sizes of probes A and B was further confirmed by using probe C (Fig. 1C, lanes 2 and 3). This was surprising because previous reports from several laboratories (22, 24, 27) have indicated that intron 3 of K8 is spliced out in all isoforms of Rta and K8 mRNAs. The expression profile of these RNAs with intron 3 retention in butyrate-induced JSC-1 cells (Fig. 1B, lanes 8–10) was also different from that of K8.1 transcripts, but similar to the pattern of K8 exon 3; therefore, they might be a part of the K8 transcription products.
treatment of JSC-1 cells not only increased K8 expression (Fig. 2C, lane 3) but also induced expression of the putative K8.1 mRNA with retention of both intron 2 and 3 (Fig. 2C, lane 5), which was designated as a putative K8/K8.1 mRNA. Similar results were also observed in BCP-1 cells. The results were further verified by sequencing of the RT-PCR products amplified by using a primer pair spanning from nt 75605 to 75935 of the virus pre-mRNAs (data not shown) and by RPA using a probe spanning over part of K8 intron 2, the entire exon 3, and part of the intron 3 (Fig. 2, A and D). Quantitative analysis of the protected products by RPA showed a molar ratio of K8α, -β, and the putative K8/K8.1 to be 10:2:1 in uninduced JSC-1 cells (Fig. 2D, lane 1). Butyrate treatment of JSC-1 cells significantly increased expression of all three forms of the RNAs (16-fold for the α) with a relative constant molar ratio changed to approximately 5:1:1. More-
over, a 20-fold increase of the K8β was also accompanied by about a 32-fold induction of the putative K8/K8.1 mRNA (Fig. 2, D and E, lanes 2). The expression profile of all three RNAs in JSC-1 cells following anti-IgM (a B cell activator) stimulation was similar to the results obtained from butyrate treatment. Compared with butyrate, anti-IgM was a much weaker inducer (Fig. 2D, lane 3) that led to an increased expression of all three species of the RNAs by close to 5-fold with a molar ratio of 10:2:1.

Nevertheless, our data demonstrate that a K8 transcript containing both introns 2 and 3 could be a novel K8/K8.1 mRNA that was not reported before. Interestingly, a predicted 291-nt band corresponding to a protected fragment with intron 3 retention but no intron 2 was out of a detectable level in those RPA experiments (Fig. 2, D and E), indicating that an RNA with intron 3 retention most likely had an intron 2. This was consistent with our RT-PCR results (lane 5 in Fig. 2B and lanes 4 and 5 in Fig. 2C). Moreover, K8γ RNA with both introns 1 and 2 retention but with no intron 3 previously described in other cell lines (22, 27) was hard to detect by RT-PCR in JSC-1 cells (Fig. 2B, lane 4; Fig. 2C, lanes 2 and 3), indicating that K8 intron 1 removal was very efficient during RNA splicing. To confirm this assumption, several sets of the primers from exon 1 or intron 1 and intron 2 were designed to amplify this region by RT-PCR. Results from those studies showed that an RNA with intron 1 retention always had an intron 2, but an RNA containing an intron 2 generally had no intron 1 (data not shown). The data conclude that removal of intron 1 preceded that of intron 2.

K8 Exon 3 Has Three 5′-ss—Because the K8 intron 3 is a coding region for K8.1 exon 1 (Fig. 1), an intron 3 retention in the putative K8/K8.1 mRNAs probably extends the K8 exon 3 to include two additional 5′-ss downstream being used for K8.1 RNA splicing. Thus, the exon 3 in this circumstance would have three 5′-ss, respectively, at nt 75838, 76155, and 76338 (Fig. 3A) which then could be alternatively selected for RNA splicing. If so, an antisense K8.1c- or β-specific primer covering the splicing junction of each K8.1 mRNA, when combined with a sense primer positioned in the K8 intron 2 or exon 3, would generate by RT-PCR a corresponding product α or β. As predicted, both α (Fig. 3B, lanes 3 and 7) and β forms (Fig. 3B, lanes 1 and 5) of the K8/K8.1 RNAs were specifically amplified from JSC-1 cell RNA by using those specially designed primers. Butyrate that stimulates KSHV K8 and K8.1 expression also increased production of both K8/K8.1α and β forms (Fig. 3C, below the pre-mRNA and are named by the locations of their 5′ ends. Tailed primers Pr75183 and Pr75838 had, respectively, 21 and 11 nts (nonspecific) attached to each 5′ end as indicated by a dashed line. Shown below the predicted from the RT-PCR products predicted by the two sets of primers used for amplification in B and C. See other descriptions in Fig. 1. B, RT-PCR analysis of poly(A)+ mRNAs. Total cell RNA isolated from JSC-1 cells was subjected to RNase-free DNase I digestion before poly(A) selection. The poly(A)+ mRNAs with or without reverse transcription were then amplified by PCR using a 5′ primer combined with a 3′ primer as indicated. C, RT-PCR of total cell RNA isolated from JSC-1 cells with or without butyrate treatment. DNase I digestion of the RNA and amplification strategy were the same as in B. D and E, RNase protection assays of total JSC-1 cell RNA. Total cell RNA isolated from JSC-1 cells with or without (control) butyrate or anti-IgM treatment was hybridized with 1 × 10⁵ CPM of [32P]-labeled antisense viral probe mixed with a human cyclophilin antisense RNA probe (165 nts). The latter generated a protected product with size of 103 nts and was used as an internal control for normalization of sampling. The cyclophilin probe was also used as a reference for efficiency of RNase digestion. Yeast RNA was used as a negative control. The protected fragments from RNase A and T1 digestion were resolved on 6% polyacrylamide/8 M urea gel. The identities of corresponding products protected are shown between D and E. The numbers to the left of the D and to the right of the E are the size markers of a 100-bp ladder.
Alternative Splicing of KSHV K8 and K8.1 Pre-mRNAs

Fig. 3. Detection of K8/K8.1 mRNAs in JSC-1 cells. A. diagram of a hypothetical K8 exon 3 containing three 5'-ss. Splicing using nt 75838 5'-ss produces a K8 mRNA. Utilization of nt 76155 and 76338 5'-ss is assumed to be responsible, respectively, for production of K8/K8.1α and β mRNAs as indicated in the diagram. Arrows are PCR primers used in B and C. B, C: RT-PCR analysis of K8/K8.1α- and β-specific mRNAs in JSC-1 cells. Total cell RNA extracted from JSC-1 cells with or without (B, controls in C) butyrate treatment was digested with RNase-free DNase I. The digested RNAs with or without reverse transcription were amplified by PCR using a primer pair as indicated at the top of each panel. The identities of corresponding products are shown on the right of each panel, and the size markers of a 100-bp ladder are indicated on the left.

lanes 5 and 7). The primary substrate RNAs used to generate α and β forms of the K8/K8.1 RNAs must be the K8 pre-mRNAs because the K8.1 transcription initiates at the 5' end of intron 3, and the sense primers upstream of the intron were used in those experiments. However, we cannot rule out a tricistronic Rta pre-mRNA in butyrate-induced JSC-1 cells also being used as a substrate for the splicing because a tricistronic Rta pre-mRNA transverses both K8 and K8.1 coding regions.

Mutation of the nt 75838 5'-ss in K8 Exon 3 Leads to Production of a K8/K8.1β mRNA in Vitro—To simplify the data interpretation complicated by the presence of tricistronic Rta pre-mRNAs in JSC-1 cells, we constructed several K8 minigene expression vectors with different 5'-ss mutation to verify if the three 5'-ss within exon 3 of a K8 pre-mRNA are alternatively selected. A single nucleotide mutation (U to G) was introduced into the nt 75838 5'-ss of a K8 minigene expression vector to convert the wt 5'-ss GU (plasmid pST1) to a mt 5'-ss GG (plasmid pST2). In vitro splicing of the pre-mRNAs transcribed from those templates showed that the RNAs containing a wt nt 75838 5'-ss efficiently spliced and generated a K8 mRNA (fully spliced at nt 75838 to 76433) with a size of 301 (pre-mRNA 1 in Fig. 4) or 312 nts (pre-mRNA 2 in Fig. 4) regardless of whether or not a U1-binding site was attached to their terminal exons (Fig. 4, compare pre-mRNAs 1 and 2). A U1-binding site attached to a pre-mRNA terminal exon was thought to function as a splicing enhancer (39, 43). The pre-mRNAs with a mt nt 75838 5'-ss spliced poorly, and no K8 mRNAs were detectable from the splicing, indicating that the nt 75838 5'-ss was the most favorable 5'-ss being used for the splicing. However, another alternative 5'-ss, obviously a nt 76338 5'-ss downstream of the nt 75838 5'-ss in the mt pre-mRNAs, was selected for the splicing, leading to production of more K8/K8.1β mRNAs (fully spliced at nt 76338 to 76433) with a size of 801 (pre-mRNA 3 in Fig. 4) or 812 nts (pre-mRNA 4 in Fig. 4). Thus, our data demonstrated that a K8 pre-mRNA was indeed a substrate RNA responsible for generation of the novel K8/K8.1 mRNA.

Novel K8/K8.1α and β mRNAs Are Spliced Products of K8 Pre-mRNAs by Alternative Selection of Three 5'-ss in K8 Exon 3 in Transient Transfected 293 Cells—Transfection of 293 cells with plasmid pST1 (wt K8 minigene) (Fig. 5A) showed that splicing patterns of the K8 pre-mRNAs were exactly the same as seen in JSC-1 cells. Selection of the nt 75838 5'-ss in K8 exon 3 also overwhelmed other two alternative 5'-ss downstream (Fig. 5B, compare lanes 2 with 5). However, the RNAs with a mt nt 75838 5'-ss obtained from plasmid pST2 transfection, when compared with their wt counterparts, were unable to use this 5'-ss (Fig. 5B, compare lanes 4 with 8). Instead, these RNAs switched to select the nt 76155 and 76338 5'-ss for the splicing (Fig. 5B, compare lanes 4 and 5 with 8 and 9). Transfection of 293 cells with plasmid pST5 that had mutations in both nt 75838 5'-ss and 76155 5'-ss showed a similar splicing profile as seen in pST2-transfected 293 cells (compare lanes 11 and 12 with lanes 6 and 7 in Fig. 5B). Because plasmid pST5 had only one 5'-ss (nt 76338 5'-ss) left after double 5'-ss mutations, only two RT-PCR products, one from unsliced and another from spliced (lanes 13 and 14 in Fig. 5B), could be
amplified from pST5-transfected 293 cell RNA. In contrast, there were three RT-PCR products (lanes 8 and 9 in Fig. 5B) detectable from pST2-transfected cell RNA due to a single 5'–ss mutation made at nt 75838. These data provided strong evidence that a bicistronic K8 pre-mRNA could be spliced through alternative selection of three 5'–ss within its exon 3. Among the three 5'–ss, selection of the nt 75838 5'–ss overwhelms the other two, leading to production of a K8 mRNA. Selection of the nt 76155 5'–ss is favored if a blockade of the nt 75838 5'–ss occurs, resulting in production of a K8/K8.1 mRNA. The nt 76338 5'–ss is the least favorable one and its selection generates a K8/K8.1 mRNA.

Furthermore, our results also showed that blockade of the nt 75838 5'–ss or together with the nt 76155 5'–ss not only promoted the K8 pre-mRNAs to use other 5'–ss downstream but also affected efficiency of intron 2 removal (Fig. 5B, compare lanes 2 with 6 and 11), consequently leading to accumulation of the K8 RNA retaining intron 2. Data suggest that selection of the nt 75838 5'–ss might play an important role in removal of the K8 intron 2.

**Novel K8/K8.1 mRNAs Are Poorly Translatable—Demonstration of the K8/K8.1 mRNAs derived from K8 pre-mRNAs through alternative selection of two 5'–ss previously identified for K8.1 mRNA processing also implies that the novel K8/K8.1 mRNAs should have coding potential for K8 and K8.1 and are bicistronic. It is predicted that a K8.1 protein will be expected to be translated in addition to K8 proteins in our transient transfection assay. Thus, Western blot analysis was further performed to look for both K8 and K8.1 proteins translated from the novel K8/K8.1 bicistronic mRNAs in several K5 mimic-transfected 293 cells by using anti-K8 or anti-K8.1 antibodies. Data in Fig. 5C showed that K8a (K-bZIP) but not K8β protein was efficiently translated in pST1-transfected 293 cells (lane 2). The K8a protein expression in 293 cells transfected with pST2 containing a mt nt 75838 5'–ss or with pST5 containing a mt nt 75838 5'–ss plus a mt nt 76155 5'–ss was greatly reduced along with appearance of a very small amount of K8β protein (lanes 3 and 5), even though a greater number of the bicistronic K8/K8.1 mRNAs existed in the cells (Fig. 5B, lanes 7–9 and 12–14). Moreover, several attempts were tried to detect K8.1 proteins and failed with negative results when the protein samples described above were probed with an anti-K8.1 antibody (Fig. 5D, lanes 2–6). Data suggest that the novel K8/K8.1 mRNAs are poorly translatable. This is very different from a bicistronic v-cyclin/vFLIP mRNA in which vFLIP is translated by using an internal ribosome entry site residing in the v-cyclin coding region (44–46).

**DISCUSSION**

In this report, we mapped transcription start site for initiation of K8.1 transcription and found that K8.1 transcripts start at nt 75901, 14nts upstream of the first AUG at nt 75915 in KSHV genome. By scanning the region 5' to the K8.1 transcription start site, we found no consensus TATA box but instead the presence of several potential transcription factor binding sites, including a TGACCTCA-like site (AP-1) (47) at nt 75602, a CANNTG site, upstream stimulatory factor (48) at nt 75607, a CAAT (CP1, CP2, C/EBP, and ACF) site (49, 50) at nt 75617, and a GC-like box (SP) (51) at nt 75766. This indicates that a potential K8.1 promoter is TATA-independent and lies in the body of K8 ORF. Nevertheless, implication of an existing K8.1 promoter upstream of the K8.1 transcription site mapped in this report suggests that expression of Rta, K8, and K8.1 genes is through alternative promoter usage in three stages (immediate-early, early, and late) of a lytic viral cycle. There are a number of reports suggesting that viral late promoters are generally TATA-independent promoters including KSHV TK promoter (52) and assembly protein promoter (53) as well as EBV late promoters (54), and their activities are associated mainly with viral DNA replication. In KSHV-positive JSC-1 cells, K8.1 mRNAs was not detected until 24 h of butyrate stimulation (Fig. 1B) and was inhibited by PAA, a viral DNA polymerase inhibitor. Moreover, the transcriptional activity of the potential K8.1 promoter was examined in 293 cells with or
viruses is an important mechanism for regulating diversity of gene expression. The mechanisms that control alternative RNA splicing remain understood. ORF50, K8, and K8.1 are three split genes positioned at the same locus side by side but represent three completely different categories of genes (immediate-early, early, and late) in the virus life cycle. Three genes share a common poly(A) signal downstream of the K8.1 coding region. With such an arrangement, expression of each gene takes place based upon what we have learned. In this model, alternative pathways involved in processing of K8 and K8.1 pre-mRNAs provide a strong evidence of an even more complex splicing regulation in expression of these genes. For better understanding, a model in Fig. 6 is proposed to lay out different splicing pathways involved in processing of K8 and K8.1 pre-mRNAs based upon what we have learned. In this model, alternative selection of three 5' ss within exon 3 of a bicistronic K8 pre-mRNA at early stage of virus life cycle determines a different splicing pathway, dictating production of the K8 mRNAs. Splicing of intron 3 in the K8 pre-mRNA apparently activates the splicing of the intron 4, leading to production of K8A/B proteins expressed from bicistronic K8/K8.1 mRNAs in 293 cells transfected by plasmids pST1, pST2, pST3, pST5, or pST6 (pST1 without a CMV IE promoter). Protein samples prepared from Raji cells and 293 cells without transfection were used as positive controls.

This report also provides some evidence that anti-IgM stimulation of KSHV K8 and K8.1 gene expression in JSC-1 cells. Although expression induced by anti-IgM of KSHV K8 and K8.1 was less efficient compared with butyrate, this was unexpected because many PEL-derived B cells lack a B cell receptor, an IgM-type antigen on the B cell surface. Lack of B cell receptors may be due to Ig gene rearrangement or mutation (55–57) or probably due to blockade by KSHV K1 of BCR transport to cell surface (58). Immunoglobulin heavy chain rearrangements in JSC-1 cells may be due to Ig gene rearrangement or mutation (55–57) or probably due to blockade by KSHV K1 of BCR transport to cell surface (58). Immunoglobulin heavy chain rearrangements in JSC-1 cells have been demonstrated by Southern blot analyses (20). Attempts to detect such B cell receptors on JSC-1 cells were unsuccessful by Western blot (data not shown). However, in our study we cannot rule out the presence of any trace amount of B cell receptors on JSC-1 cells, which may be enough for the unexpected stimulation in our experiment.

Alternative RNA splicing in eukaryotes and some animal
Alternative Splicing of KSHV K8 and K8.1 Pre-mRNAs

![Diagram of K8 and K8.1 pre-mRNA splicing]

**Fig. 6. Proposed model for expression of KSHV K8, K8/K8.1, and K8.1 mRNAs.** Genomic locations of ORF K8 (22, 27, 30), K8.1 (34–36), and the 3′ end of ORF50 are depicted at the top of KSHV genome (heavy line). Shown below the heavy line are the K8 and K8.1 pre-mRNA structures with exons (boxes) and introns (lines) as well as splicing directions (dashed lines) and splicing products (α, β, and γ isoforms). The numbers above the ORFs and pre-mRNAs or below the heavy line are nt positions in KSHV (BC-1) genome (GenBank™ accession number U75698) (5). The K8 and K8.1 promoters are named based on the nt position where the transcription is initiated. The 5′-ss and 3′-ss are shown below each pre-mRNA. The K8 ORF terminates at nt 75791 for K-bZIP instead of nt 75569 (5) for K8β because the K-bZIP is a major form predominantly expressed in viral lytic infection. Downstream of the K8 termination codon are 3′-untranslated sequences including part of the exon 3 and the entire exon 4. The K8 shares with the K8.1 a single polyadenylation site (pA) at nt 76714 and a potential cleavage site at nt 76730 (22). Splicing of the K8 pre-mRNAs is first to remove intron 1. The resulting products are then further spliced by using a nt 75838 5′-ss to remove intron 3, leading to generation of the K8β mRNA. Finally, intron 2 is removed, and consequently the K8α mRNA is produced. Lack of intron 1 in all K8 mRNAs in this model is also consistent with the absence of a detectable protein containing a peptide sequence translated from the intron 1 in TPA-induced PEL cells (30). However, alternative splicing by using other 5′-ss in the K8 exon 3 is feasible, and the resulting K8/K8.1 and β mRNAs are bicistronic due to their features of having both K8 and K8.1 coding regions. The K8.1 mRNAs are expressed only at late stage of virus infection because the K8.1 promoter P75901 is a late promoter, and its activity requires viral DNA replication (Fig. 2C). Splicing by selection of two alternative 5′-ss (the same two 5′-ss in the K8 exon 3) in its exon 1 of the pre-mRNA produces either a K8.1α (splicing from the nt 76115 5′-ss to the nt 76433 3′-ss) or a K8.1β (splicing from the nt 76338 5′-ss to the nt 76433 3′-ss) mRNA. The K8.1γ mRNA is the one left unspliced.

K-bZIP protein (22, 27, 30). Removal of intron 2 is not as efficient as intron 1, leading to a minor product of K8β. However, the nt 75838 5′-ss is not chosen sometimes, and consequently, alternative selection of other two 5′-ss downstream produces either a K8/K8.1α or a K8/K8.1β mRNA which often retains an intron 2. The nt 76155 5′-ss appeared preferentially selected over the nt 76338 5′-ss in our transient transfection assay (Fig. 5B). Selection of the nt 76338 5′-ss is problematic due to size of the exon. Because an internal exon is defined by cross-talking of a 3′-ss and a 5′-ss over the exon (59), an oversized exon (>500 nts) makes it more difficult to be defined by cellular splicing machinery (60). When the K8.1 promoter is activated at a late stage of the virus life cycle, the resultant K8.1 transcripts with only two exons and one intron undergo splicing through alternative selection of two 5′-ss in their exon 1 (34, 35). A majority of K8.1β mRNAs (encoding for K8.1A protein) are produced by preferential selection of nt 76338. K8.1α mRNAs (encoding for K8.1B protein) are less prominent and come from selection of the nt 76155 5′-ss. This is consistent with our K8.1 protein data (Fig. 5D) and reports from other laboratories (34, 35, 40).

There are many examples of alternative selection of 5′-ss involved in expression of viral and eukaryotic genes including expression of SV40 large T and small t antigen and adenovirus E1A (61–63). Many studies (64–68) show that the strength of the 5′-ss and the cis elements in the exon and intron and status and level of cellular splicing factors all devote to regulation of alternative 5′-ss selection. In this regard, we know nothing about how three 5′-ss in exon 3 of the K8 are alternatively selected. Considering that exon definition is limited by exon size (59) and recognition of a 5′-ss is scanned 5′ to 3′ by cellular splicing machinery during RNA splicing (69), three 5′-ss, respectively, at nt 75838, 76155, and 76338, should be selected in order by a simple scenario of the first come and first served. However, why the nt 76338 5′-ss is preferentially selected over the nt 76155 5′-ss during KSHV K8.1 expression remains questionable. Looking for viral cis elements in this region and
trans-acting factors involved in this regulation is currently under investigation. It is also unclear why KSHV produces a bicistronic K8/K8.1 or K8/K8.1α mRNA even if it has little protein coding function. Because the bicistronic K8/K8.1α and β mRNAs exist in a relative amount and are inducible in JSC-1 cells by butyrate, one of the possibilities is that these RNAs, in addition to T1.1 or PAN RNAs (70, 71), may represent another class of regulatory RNAs. Through alternative selection of three 5′-ss in the K8 exon 3, the virus is able to keep the production level of the K8α mRNA in check. Thus, production of bicistronic K8/K8.1α and β mRNAs or other derivatives may be an essential mechanism to control K8α mRNAs to a threshold during processing of bicistronic K8 pre-mRNAs.

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