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The Human Herpesvirus 8 Homolog of Epstein-Barr Virus SM Protein (KS-SM) Is a Posttranscriptional Activator of Gene Expression

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Homologs of the Epstein-Barr virus (EBV) SM protein exist in several human and nonhuman herpesviruses. Structure and function differ significantly among these proteins. We have cloned and characterized the human herpesvirus 8 (HHV8) gene, KS-SM, which is homologous to the EBV SM and herpes simplex virus ICP27 genes, from an HHV8-infected primary effusion lymphoma. KS-SM is shown to be a posttranscriptional activator of gene expression in cotransfection studies. KS-SM activated gene expression in a gene-specific, promoter-independent manner. In particular, KS-SM enhanced the expression of KDR/flk-1, a receptor for vascular endothelial growth factor (VEGF), in cotransfection studies. Since expression of KDR/flk-1 is increased in Kaposi's sarcoma and HHV8-infected cell cultures and VEGF enhances the proliferation of HHV8-infected cells, KS-SM may play a pathogenic role in Kaposi's sarcoma.

The newly discovered human herpesvirus, Kaposi's sarcoma (KS)-associated herpesvirus or human herpesvirus 8 (HHV8), is causally associated with KS, primary effusion lymphoma, and multicentric Castleman's disease (for a review, see reference 1 and references therein). The role of HHV8 infection in the development of KS has not been fully characterized. However, several aspects of HHV8 gene expression are likely to be central to the pathogenesis of KS. First, many HHV8 gene products expressed during the lytic cycle of replication have angiogenic and antiapoptotic properties (1). Second, many of these proteins are secreted viral homologs of cellular cytokines (53). Expression of lytic cycle proteins has been demonstrated both in KS tumor biopsy specimens and in HHV8-infected human umbilical vein endothelial cells (14, 52). Thus, it is likely that formation of KS tumors does not conform to the paradigm of an abnormally proliferating clone of virus-transformed cells. Rather, a subset of infected cells that are permissive of lytic HHV8 replication may secrete factors that enhance proliferation of neighboring uninfected and latently infected cells by a paracrine mechanism (12, 14). Several lines of evidence also indicate that dysregulation of the vascular endothelial growth factor (VEGF)-VEGF receptor axis plays a critical role in the development of KS. KS cells express high levels of VEGF; VEGF receptors are upregulated in KS cell cultures, HHV8-infected human umbilical vein endothelial cells, and tumor tissues (3, 14, 33); downregulation of VEGF expression inhibits its growth of KS cells in vitro and in nude mice (30).

Epstein Barr virus (EBV) encodes a protein (SM, also known as BMLF1, Mta, and EB2) that is a posttranscriptional regulator of gene expression (5, 8, 9, 45, 57). Homologous genes have been described in human alpha-, beta-, and gammaherpesviruses. Examples include herpes simplex virus (HSV) ICP27/IE63, human cytomegalovirus (CMV) UL69, varicella-zoster virus open reading frame (ORF) 4, and herpesvirus saimiri (HVS) IE52/ORF57 (7, 11, 20, 31, 34, 41). Despite similarity among these various proteins, they exhibit considerable functional and structural diversity, which is likely to be related to differences in the biologic behavior and host cell tropism of their parent viruses (32, 37, 46, 47, 55, 56). Because of the potential importance of an HHV8 lytic cycle protein that could activate other HHV8 lytic genes as well as host cell genes, we sought to identify and characterize the function of the HHV8 member of this family of proteins. Sequence analysis of the HHV8 genome had revealed the presence of an ORF (ORF57) beginning with a methionine that is homologous to the carboxy-terminal portions of the EBV SM and HVS IE52 genes (13, 43). However, the size of this ORF is only 218 amino acids, compared to more than 400 amino acids in the saimiri and EBV homologs, suggesting the existence of one or more upstream exons. We have cloned and expressed the complete cDNA for the HHV8 gene, termed KS-SM, and characterized its functional properties in transfection assays. Several aspects of KS-SM activity that are potentially important for activation of HHV8 genes and host cell genes that are critical for angiogenesis and KS tumor growth are described.

Cloning and structure of the KS-SM gene. The amino terminus of the KS-SM gene was obtained by 5′ rapid amplification of cDNA ends (RACE) of unfractionated RNA from BCBL1 cells that were treated with 12-tetradecanoyl phorbol acetate (TPA) to induce HHV8 replication as previously described (39, 44). Reverse transcription was performed with a primer in ORF57 consisting of nucleotides (nt) 82883 to 82864 of the HHV8 genome (43), and nested primers complementary to nt 82416 to 82397 and 82428 to 82447 were used to amplify the amino terminus of the KS-SM gene. A full-length cDNA was independently isolated from an oligo(dT) primed BCBL1 library that has been previously described (6). Sequencing of both RACE products and cDNA library clones demonstrated that the KS-SM gene consists of a 49-bp exon spliced to a 1,316-bp exon at canonical splice donor and acceptor sites (Fig. 1A). The gene is predicted to encode a 455-amino-acid protein. Analysis of 700 bp flanking the putative transcriptional

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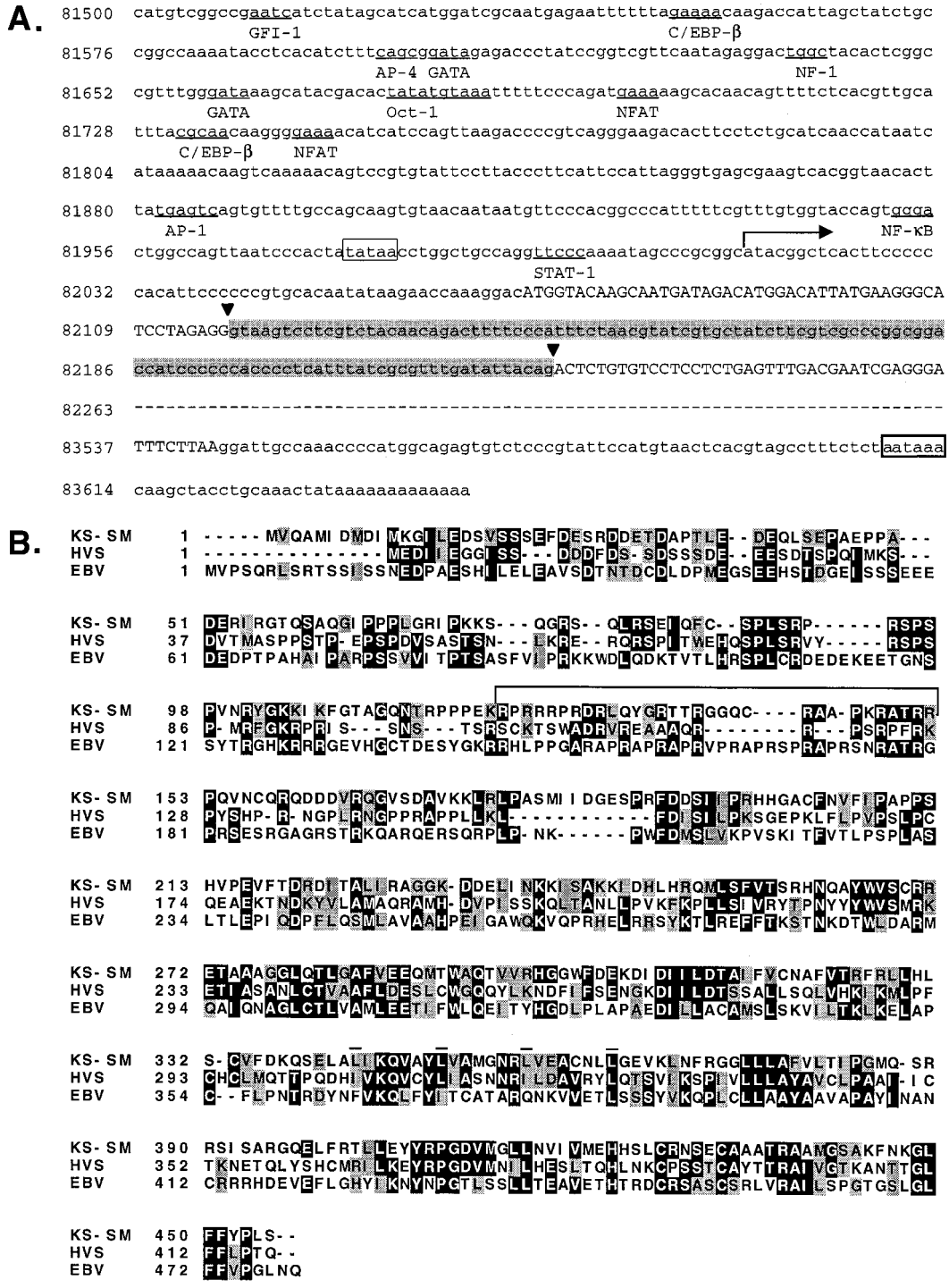

FIG. 1. Structure of the KS-SM gene. (A) The KS-SM gene is predicted to encode a 455-amino-acid protein. The first exon (nt 82069 to 82117) and the coding region of the second exon (nt 82226 to 83541) are separated by a 108-bp intron (shaded box). Intron-exon boundaries are shown with vertical arrows. A horizontal arrow shows the approximate transcriptional start site at nt 82014 of the HHV8 genome. A TATA box at −38 bp relative to the transcriptional start site and a canonical hexanucleotide polyadenylation signal 63 bp after the stop codon are enclosed in open boxes. Potential transcription factor-binding sites are underlined, and the corresponding transcription factors are shown below the nucleotide sequence. The nt 82263 to 83534 (represented by a dashed line) are identical to the published BC-1 HHV8 sequence (43) except for the presence of a C instead of a T at position 82959 that does not change the predicted amino acid sequence. (B) Comparison of the amino acid sequence of KS-SM and homologs in HVS and EBV. Amino acids that are identical or similar to KS-SM are shaded black or gray, respectively. A potential leucine zipper in KS-SM is shown with the four component leucines (bars), each separated by six amino acids. The arginine-rich region is bracketed.

start site with the TSSW and MatInspector promoter analysis programs (38, 51) identified a TATA box 88 bp 5′ to the first ATG in the spliced gene and numerous transcription factor binding sites, as shown in Fig. 1A. It should be noted that four potential initiator methionines are present in the first 11 amino acids of the predicted protein (Fig. 1B). Although it is not possible to predict a priori which are used to initiate translation in vivo, all four have a purine in the −3 position and thus

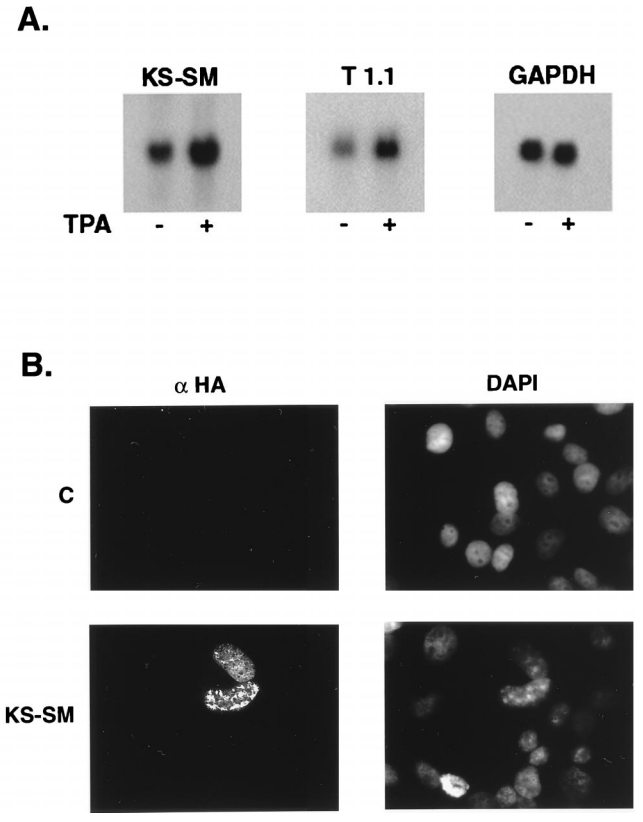

FIG. 2. KS-SM expression in HHV8-infected and KS-SM-transfected cells. (A) KS-SM is expressed during lytic replication of HHV8. BCBL1 cells were induced to permit lytic replication of HHV8 with 20 ng of TPA per ml. RNA was harvested after 48 h from induced (+) or uninduced cells (−). Each RNA (10 μg) was electrophoresed, blotted, and probed with ^{32}P-labeled KS-SM cDNA. The blot was stripped and reprobed for T1.1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. (B) Nuclear localization of KS-SM in transfected Cos-7 cells. KS-SM tagged with influenza virus HA was transfected into Cos-7 cells. Cells were stained with anti-HA monoclonal antibody, 16B12 (Babco-Covance). Nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI) stain. Diffuse speckled intranuclear staining was seen in HA–KS-SM-transfected Cos-7 cells (lower left panel) but not in control-transfected cells (upper left panel). Corresponding DAPI-stained nuclei are shown in the upper right (control) and lower right (KS-SM) panels.

may be considered to be in a strong context for initiation (25). The first and third methionines also contain a guanine in the +4 position and may therefore be in a particularly favorable context for initiation.

The KS-SM gene is similar to its homologs in other human herpesviruses, particularly HVS IE52 (Fig. 1B). Overall, the identity at the amino acid level is approximately 30% among KS-SM, EBV SM, and HVS IE52. As in ICP27 (19), an arginine-rich region and arginine-serine dipeptides characteristic of many RNA-binding proteins (28) are present in KS-SM. Several differences are also present that may be functionally important. First, KS-SM does not contain a leucine-rich nuclear export signal motif potentially capable of binding CRM1 (exportin 1) (15, 16, 35, 54) as found in EBV SM, ICP27, and HVS IE52 (2, 34, 46). Unlike its homologs in other herpesviruses, KS-SM contains a leucine zipper motif between amino acids 343 and 364, suggesting a possible role in dimerization and DNA binding (24, 26) that has not been described for its homologs.

Expression and localization of KS-SM in HHV8-infected and KS-SM-transfected cells. Other herpesvirus genes homologous to KS-SM are expressed during the lytic phase of viral replication. In order to determine whether KS-SM is also expressed in a similar manner, we examined the expression of the KS-SM gene in BCBL1 cells, which are derived from an EBV-negative primary effusion B-cell lymphoma (39). RNA was isolated from untreated BCBL1 cells and from cells treated with TPA to induce lytic replication as previously described (44). Northern blotting demonstrated the presence of a single transcript of 1.8 kb, which correlates well with the predicted size of the cDNA described above (Fig. 2A). Zhu et al. have described a butyrate-inducible, cycloheximide-sensitive 1.5-kb transcript in BC-3 cells that hybridizes to DNA containing a portion of the second exon of KS-SM (59). This transcript is likely to be the mRNA for KS-SM in BC-3 cells. The level of KS-SM induction in BCBL1 cells is similar to that seen with the T1.1 lytic RNA transcript (Fig. 2A). The level of KS-SM expression seen in uninduced cells is likely due to the small percentage of BCBL1 cells that is spontaneously permissive of lytic replication (39).

In order to study the intracellular location of KS-SM, we performed immunofluorescence microscopy of cells transfected with a plasmid in which epitope-tagged KS-SM expression was driven by the CMV IE promoter (pCDNA3; Invitrogen Corp.). Cos-7 cells were transfected with influenza hemagglutinin epitope (HA)-tagged KS-SM and examined 48 h after transfection as previously described (2). As shown in Fig. 2B, KS-SM localizes to the nucleus in a speckled pattern similar to that seen with EBV SM and ICP27. We have previously shown that EBV SM translocates from nucleus to cytoplasm upon overexpression of the cellular exportin CRM1 (2). EBV SM has also been shown to shuttle from nucleus to cytoplasm in heterokaryon assays (49). Similarly, ICP27-mediated RNA export is nuclear export signal dependent and is blocked by an inhibitor of CRM1 complex formation (46; S. Silverstein, personal communication). In order to determine whether KS-SM interacts with CRM1 in a similar manner, we overexpressed CRM1 in Cos-7 cells by cotransfection with HA–KS-SM. Unlike the results with EBV SM, no cytoplasmic translocation was observed (data not shown). Thus, although we cannot rule out nucleocytoplasmic shuttling by KS-SM, its interaction with cellular export factors is likely to differ from that of homologs in other herpesviruses.

KS-SM activates gene expression by a posttranscriptional mechanism. The EBV SM gene, HSV ICP27, and ORF57/IE52 genes all activate expression of other genes via posttranscriptional mechanisms (5, 10, 21, 44, 47, 55). In order to determine whether KS-SM has similar properties, we examined the effect of KS-SM in chloramphenicol acetyltransferase (CAT) reporter assays. BJAB cells, which are derived from an EBV-negative Burkitt lymphoma, were transfected by electroporation with a series of CAT reporter plasmids and either KS-SM expression vector (KS-SM cDNA cloned in pCDNA3) or control vector (Fig. 3A). Cotransfection of KS-SM led to a 4- to 10-fold increase in CAT activity, compared to the control. As has been demonstrated previously for EBV SM, KS-SM was capable of activating CAT transcribed from a variety of promoters (5, 22, 27, 57).

To determine if KS-SM-mediated activation occurs at the RNA level, steady-state CAT mRNA levels in KS-SM or control-transfected cells were measured by Northern blotting. RNA was isolated from transfected BJAB cells 48 h after transfection with intronless CMV-CAT and either KS-SM or control plasmids and processed exactly as previously described (44). As shown in Fig. 3B, both nuclear and cytoplasmic levels of the target CAT mRNA were increased in KS-SM-transfected cells. The fact that nuclear levels of target CAT mRNA are also increased suggests that KS-SM-mediated activation of

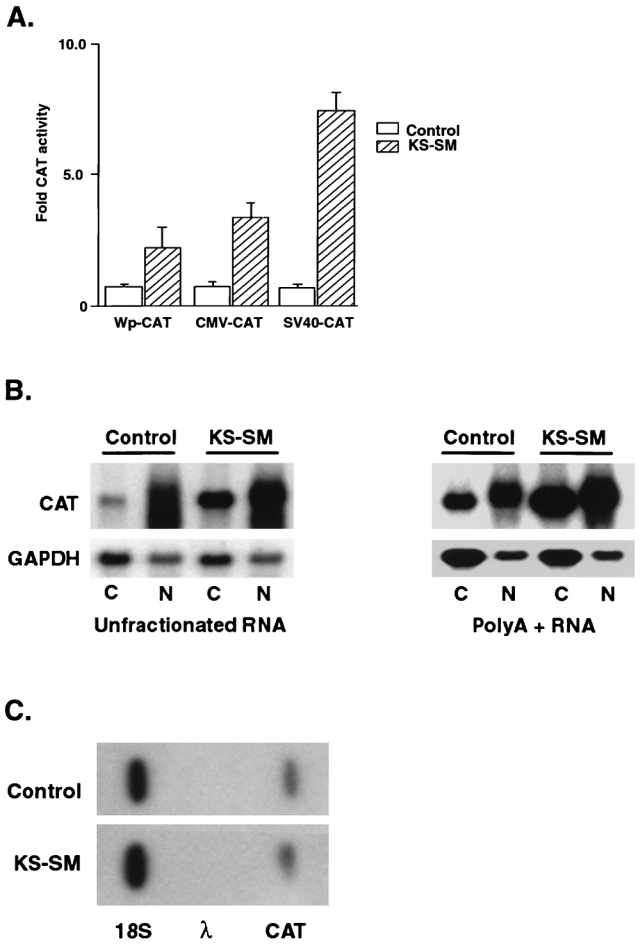

FIG. 3. Posttranscriptional regulation of gene expression by KS-SM. (A) BJAB cells (from an EBV-negative Burkitt lymphoma) were cotransfected with 10 μg of either KS-SM expression plasmid or control plasmid and intronless CAT reporter constructs driven by either the EBV latent promoter Wp, the CMV IE promoter, or the SV40 late promoter. CAT assays were performed on lysates from cells harvested 18 h after transfection. CAT activity was calculated as percent CAT conversion per microgram of protein, and the results are expressed as fold activation relative to control. Data represent the means ± the standard errors of the means of three independent experiments. (B) KS-SM increases steady-state RNA levels of the target gene. BJAB cells were cotransfected by electroporation with 10 μg of CMV-CAT and either KS-SM or control plasmid. Unfractionated RNA or poly(A) RNA from cytoplasmic and nuclear fractions was harvested 48 h after transfection. Each RNA (10 μg) was electrophoresed, blotted, and probed for CAT expression. (C) KS-SM has no effect on the transcript initiation rate of CMV-CAT. BJAB cells were transfected with either KS-SM or control plasmid and CMV-CAT. Nuclei were harvested 48 h after transfection. In vitro-labeled nuclear transcripts were hybridized to immobilized cDNA corresponding to 18S RNA, λ phage (negative control), or CAT.

CAT is not simply due to enhanced nucleocytoplasmic transport of CAT mRNA. However, in the presence of KS-SM, the amounts of cytoplasmic CAT poly(A) RNA were increased more than nuclear CAT poly(A) RNA. Direct radiometric quantitation with an Instant Imager (Packard Instruments, Meriden, Conn.) revealed that KS-SM led to a 6-fold increase in cytoplasmic poly(A)$^+$ CAT mRNA versus a 2.6-fold increase in nuclear CAT poly(A)$^+$ mRNA. This finding suggests that KS-SM may facilitate nucleocytoplasmic transport of target mRNAs. Nuclear run-on transcription assays were performed to determine whether KS-SM directly activated transcription (44). As shown in Fig. 3C, no difference in CAT transcript initiation rate was observed in BJAB cells cotransfected with CMV-CAT and KS-SM, compared to control transfected cells. Although it is not possible to rule out direct activation of other promoters by KS-SM, these data indicate that the activating effect seen for CAT expressed from the CMV promoter is posttranscriptional.

Activation by KS-SM is gene-dependent. It has previously been shown that the activating effect of EBV SM and ICP27 is gene dependent. For example, EBV SM does not activate β-galactosidase, luciferase, or certain lytic EBV genes in cotransfection assays (21, 29, 49; V. Ruvolo and S. Swaminathan, unpublished observations). Similarly, ICP27 does not activate all HSV genes equally (40, 50). The difference in the sensitivity of various genes to activation by EBV SM and ICP27 remains to be completely explained. However, these differences may be due to multiple factors, including the sequence of the gene itself, as well as the sequence of the 3′ untranslated region (4,

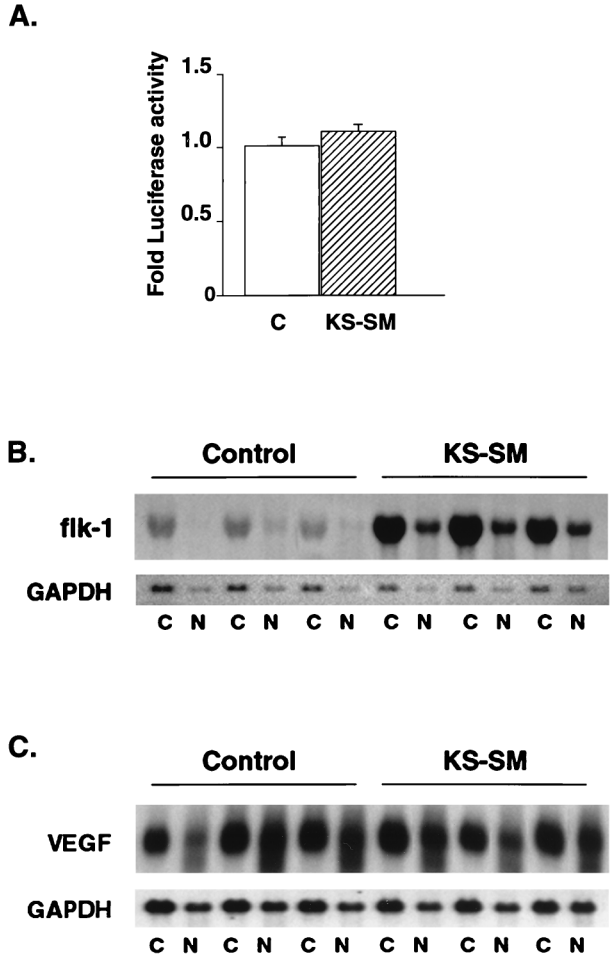

FIG. 4. KS-SM-mediated activation is gene dependent. (A) BJAB cells were cotransfected by electroporation with either KS-SM or control plasmid and an SV40-luciferase reporter plasmid (pGL3 promoter; Promega). Cells were harvested 48 h after transfection, and the luciferase assay was performed per the manufacturer's protocol. The results are represented as fold luciferase activity relative to control. Data represent the means ± the standard errors of the means of three independent experiments. (B) KS-SM upregulates KDR/flk-1 expression. BJAB cells were cotransfected with KS-SM or control and KDR/flk-1 cDNA expression plasmid. RNA from cytoplasmic or nuclear fractions was isolated 18 h after transfection. Each RNA (10 μg) was electrophoresed, blotted, and probed with ^{32}P-labeled flk-1 cDNA. The blot was stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (C) KS-SM does not affect VEGF expression. BJAB cells were cotransfected with CMV-VEGF and either KS-SM or control. RNA from cytoplasmic or nuclear fractions was isolated and probed with radiolabeled VEGF cDNA as described for panel B.

23, 44, 49). In order to determine whether KS-SM function is also gene dependent, we performed cotransfection assays with KS-SM and intronless luciferase expression constructs. KS-SM did not activate luciferase expression (Fig. 4A).

Since increased expression of the VEGF receptor KDR/flk-1 may be a necessary step in proliferation and transformation of HHV8-infected cells (14), we examined the effect of KS-SM on the expression of VEGF and KDR/flk-1. Cotransfection assays were performed with CMV-driven expression vectors that contained either VEGF or KDR/flk-1 cDNAs (42, 58) and either KS-SM or control plasmid. Northern blots of RNA from transfected BJAB cells revealed that KS-SM did not activate VEGF expression. Quantitative measurements indicated that the ratio of VEGF mRNAs in the presence or absence of KS-SM was 1.1 (Fig. 4C). However, KS-SM expression led to an approximately sixfold increase in KDR/flk-1 expression (Fig. 4B). These data confirm that KS-SM-mediated activation is gene dependent. The fact that KDR/flk-1 expression is enhanced by KS-SM suggests that KS-SM may play a role in mediating changes in endothelial cell gene expression associated with the HHV8-transformed phenotype. However, it should be noted that these results were obtained in transient transfection experiments with KDR/flk-1 cDNA. The KDR/flk-1 gene is multiply spliced, and its expression is highly restricted to cells of endothelial lineage (36, 58). Therefore, the effect of KS-SM on KDR expression in endothelial cells and its physiological role in endothelial cell growth and VEGF responsiveness remain to be directly determined.

Effect of introns on activation by KS-SM. Several members of the SM family, including ICP27, HVS IE52, and EBV SM, appear to inhibit the expression of genes containing introns. Such inhibition may be due to interference with the normal processing of intron-containing pre-mRNAs (4, 17, 18). In order to determine whether the presence of introns similarly interferes with KS-SM-mediated activation, we performed CAT activation assays with several intron-containing reporter constructs. Cotransfection experiments were performed with intron-containing CMV-CAT, Wp-CAT, or simian virus 40 (SV40) CAT and KS-SM or control plasmid. In each case, a slight decrease in reporter activity was found in the presence of KS-SM (Fig. 5A). The inhibition was not as marked as seen in similar experiments with EBV SM (44). However, in no instance was there an increase in CAT activity as seen with reporter plasmids that were identical except for the absence of the intron (Fig. 3A).

We also examined the effect of KS-SM on an expression construct that encodes a genomic copy of the human growth hormone (hGH) gene containing four introns (48). Expression of the hGH gene has previously been shown to be markedly inhibited by EBV SM at the posttranscriptional level (44). Reporter assays were performed with BJAB cells transfected with intron-containing hGH reporter plasmid and KS-SM or control plasmid. hGH secreted by the transfected cells was then measured by radioimmunoassay of the growth medium (Nichols Institute, San Juan Capistrano, Calif.). In addition, hGH mRNA levels were measured by Northern blotting. In both cases, there was a moderate inhibition of hGH expression by KS-SM. Secreted hGH levels in the presence of KS-SM were 51% of those of the control (Fig. 5B), and hGH RNA levels in KS-SM-transfected cells were 82% of those in control transfected cells (Fig. 5C). These results indicate that although the presence of introns may interfere with activation by KS-SM, KS-SM may not possess a strong intrinsic inhibitory function compared with EBV SM or ICP27.

In summary, we have cloned the KSHV/HHV8 member of a family of herpesvirus gene regulatory proteins that includes

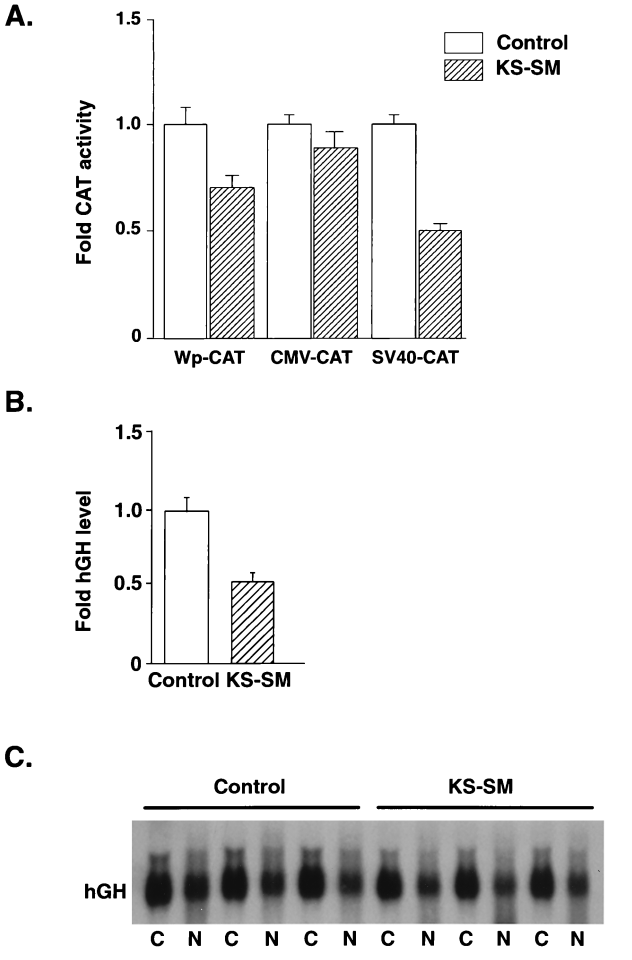

FIG. 5. KS-SM does not activate intron-containing reporter genes. (A) BJAB cells were cotransfected with 10 μg of KS-SM or control plasmid and a CAT reporter plasmid driven by either the EBV latent promoter Wp, the CMV IE promoter, or the SV40 late promoter. CAT assays were performed as described in the legend for Fig. 3. Data represent the means ± the standard errors of the means of three independent experiments. (B) Effect of KS-SM on a gene with multiple introns. BJAB cells were transfected with a plasmid (pXGH5) encoding genomic hGH, which contains four introns (48) and either KS-SM or control plasmid. Radioimmunoassays for hGH were performed by using the medium from cells 48 h after transfection. (C) Effect of KS-SM on hGH RNA levels. BJAB cells were cotransfected with pXGH5 and either KS-SM or control plasmid as above. RNA isolated from cytoplasmic or nuclear fractions was electrophoresed, blotted, and probed with ^{32}P-labeled hGH cDNA.

HSV ICP27 and EBV SM, among others. This HHV8 gene (KS-SM) is shown to be a nuclear protein expressed during lytic replication that posttranscriptionally activates the expression of reporter genes in B lymphocytes. KS-SM leads to increased accumulation of target mRNAs, particularly in the cytoplasm, although we have no direct evidence of a role for KS-SM in nuclear mRNA export. Activation by KS-SM is also gene dependent. In addition, the presence of introns in the target gene appears to interfere with KS-SM-mediated activation. Such an effect may be important in selectively enhancing expression of HHV8 genes, which are predominantly intronless. However, the net effect of KS-SM on the expression of a specific gene is likely to depend on multiple gene-specific factors. These include the presence or absence of introns as well as the 3′ untranslated region and the coding sequence of the gene. These data suggest that KS-SM, like its counterparts in HSV, EBV, and HVS, may be important for the activation of other viral lytic genes, particularly those that are encoded as

unspliced ORFs, and for facilitation of the lytic cascade. Our data also suggest that KS-SM could play a role in enhancing proliferation of HHV8-infected endothelial cells by upregulating host cell genes such as KDR/flk-1. Although the KDR/flk-1 gene is spliced from an extremely long transcript in vivo (36), the strongly stimulatory effect on expression of KDR/flk-1 cDNA leaves open the possibility that KS-SM enhances KDR/flk-1 expression in vivo and thereby increases VEGF-mediated angiogenesis. Physiological expression of cellular KDR/flk-1 expression is highly restricted to cells of endothelial origin (36). Therefore, any potential role for KS-SM in enhancing cellular expression of KDR/flk-1 needs to be validated in endothelial cells. Nevertheless, KS-SM activation of HHV8 and critical host cell genes may play an important role in the pathogenesis of KS and provide potential targets for specific antiviral and antiangiogenic therapy.

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