Expression of K13/v-FLIP Gene of Human Herpesvirus 8 and Apoptosis in Kaposi's Sarcoma Spindle Cells

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Background: **Human herpesvirus 8 (HHV8) infection is associated with all forms of Kaposi's sarcoma (KS). The HHV8 genome locus ORFK13-72-73 (ORF = open reading frame) encodes proteins that may be important in HHV8-mediated pathogenesis, i.e., the latency-associated nuclear antigen (encoded by ORF73), viral-cyc-D (v-cyc-D), a viral homologue of cellular cyclin D (encoded by ORF72), and viral-FLIP (v-FLIP), a homologue of the cellular FLICE (Fas-associated death domain-like interleukin 1 beta-converting enzyme) inhibitory protein (encoded by ORFK13; is an inhibitor of apoptosis [programmed cell death]). Through differential splicing events, this locus expresses individual RNA transcripts that encode all three proteins (tricistronic transcripts) or just two of them (v-FLIP and v-cyc-D; bicistronic transcripts). We examined expression of these transcripts in KS tissues.** ***Methods:*** **We collected tissues from patients with KS of different stages. By use of an optimized *in situ* hybridization procedure, we examined different ORFK13-72-73 locus transcripts in HHV8-infected cells in skin lesions and in one adjacent lymph node. Apoptosis in KS lesions was determined by use of an *in situ* assay.** ***Results and Conclusions:*** **Our results indicate the following: 1) Transcripts from the ORFK13-72-73 locus appear to be spliced differentially in latently infected KS cells in skin lesions and in HHV8-infected cells in lymph nodes; specifically, ORFK13-ORF72 bicistronic transcripts were expressed abundantly in KS cells, whereas ORFK13-ORF72-ORF73 tricistronic transcripts were detected only in lymph node cells. 2) Sequences encoding the antiapoptotic protein v-FLIP are expressed at very low levels in early KS lesions, but expression increases dramatically in late-stage lesions. 3) The increase in expression of v-FLIP-encoding transcripts is associated with a reduction in apoptosis in KS lesions.** ***Implications:*** **These data suggest that functional v-FLIP is produced *in vivo* and that antiapoptotic mechanisms may be involved in the rapid growth of KS lesions, where only a few cells undergoing mitosis are generally observed. [J Natl Cancer Inst 1999;91: 1725–33]**

Kaposi's sarcoma (KS) is a multifocal tumor that occurs predominantly in the skin, visceral organs, and lymph nodes. KS evolves over time from patch- or plaque-like early-stage lesions to nodular late-stage lesions. Histologic hallmarks of KS are prominent, often abnormal, microvasculature, infiltrating inflammatory cells and spindle-shaped cells that are rare or absent in early lesions but become the predominant cell type in nodular lesions [reviewed in *(1)*]. These so-called KS spindle cells are regarded as the tumor cells of KS.

DNA sequences of a novel human herpesvirus, termed human herpesvirus 8 (HHV8), have been identified in skin biopsies of KS lesions *(2)*. HHV8 has been implicated in KS pathogenesis on the basis of seroepidemiologic and polymerase chain reaction (PCR) studies that have detected viral infection in patients affected with all of the different epidemiologic forms of KS (classical KS, iatrogenic KS, African KS, and acquired immunodeficiency syndrome [AIDS]-associated KS [AIDS–KS]) *(3–7)*. Furthermore, HHV8 infection appears to precede the onset of KS *(8)*.

The mechanisms by which HHV8 is involved in KS pathogenesis are still unclear. Several genes of HHV8 with potentially tumorigenic activities have been identified *(9,10)*. In particular, viral homologues of cellular genes that encode proteins (and corresponding messenger RNA [mRNA] transcripts or open reading frames [ORFs]) with potential transforming, chemoattractive, growth-promoting, and survival properties have been suggested to play a role in KS development. These include the G-protein-coupled receptor homologous to the interleukin 8 receptor B/ORF74 *(11,12)*, the interferon regulatory factor-1 (viral-IRF-1/ORFK9) *(13,14)*, two factors homologous to the β-chemokine macrophage inflammatory protein-1 (viral-MIP-1/ORFK6 and viral-MIP-2/ORFK4) *(9,10,15)*, viral-IL-6/ORFK2 *(15–17)*, and viral-Bcl-2/ORF16 *(18,19)*. These viral proteins have all been shown to be biologically active *in vitro (9–19)*. However, little or no expression of the corresponding mRNA has been detected in KS tissues by reverse transcription (RT)–PCR. In addition, studies of transcription mapping in a primary effusion lymphoma-derived cell line (BC-1 cells) have indicated

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See "Notes" following "References."

that these genes are expressed only in the lytic/productive phase of the viral life cycle *(20)*, whereas most of the cells in KS are latently infected *(21,22)*. In fact, the investigation of the expression of viral-IL-6, viral-Bcl-2, and viral-MIP-1 in KS tissue sections at the single-cell level by *in situ* hybridization has proven that these genes are expressed only by a few productively infected cells, most likely of monocytic or lymphocytic origin *(23–26)*. These results indicate that these genes that are expressed only in the lytic phase of the HHV8 life cycle may have only a limited impact on the development of KS lesions and, more likely, have paracrine function.

In contrast, a high expression of v-IL-6 has been found in lymph nodes *(23)*, suggesting that different microenvironments may influence the HHV8 life cycle. In particular, v-IL-6 may be important in the pathogenesis of HHV8-associated diseases of lymphatic origin, such as primary effusion lymphomas and multicentric Castleman's disease, which are also associated with HHV8 infection *(27,28)*, whereas genes that are expressed also during latent infection are more likely to contribute to KS pathogenesis *(20)*.

We have, therefore, focused our *in situ* hybridization studies on an HHV8 DNA locus with latency-associated gene expression, which includes three different adjacent ORFs termed ORFK13, ORF72, and ORF73 (ORFK13-72-73 locus) *(29)*. Two transcripts are expressed via differential splicing from this locus, a 6.0-kilobase (kb) RNA encoding all three ORFs and a shorter 1.7-kb bicistronic transcript that encodes only the ORF72-K13 sequences (Fig. 1) *(29–31)*.

ORF73 encodes a high-molecular-weight latent nuclear protein (LNA, LNA1, or LANA), which is a component of the latency-associated nuclear antigen *(29)*. Recently, a latency-associated nuclear antigen (LNA) protein has been detected by immunohistochemical stainings in the spindle cells of KS lesions *(32)*, and it has been suggested that this protein may regulate the segregation of HHV8 episomes to progeny cells *(33)*.

ORF72 encodes a protein homologous to the cellular cyclin D. HHV8 viral-cyc-D (v-cyc-D) is capable of inducing pRb phosphorylation through Cdk6 activation and can release cells from the growth-proliferation blockade induced by pRb expression *(34–37)*.

ORFK13 encodes a protein homologous to cellular FLICE (FADD [Fas-associated death domain]-like interleukin 1 beta-converting enzyme) inhibitory protein (viral-FLIP [v-FLIP]), which has been recently identified in muscle and lymphoid tissues. Virally encoded FLIPs have also been detected in several γ-herpesviruses as well as in the tumorigenic human molluscipoxvirus. FLIPs block early signaling events of the death receptors Fas, TRAMP (wsl/DR-3/apo-3), TRAIL-R1 (DR-4), TRAIL-R2 (DR-5), and tumor necrosis factor receptor 1 (TNF-R1) *(38–43)*. HHV8 v-FLIP contains two death-effector domains *(43)*. One of these may bind to death-effector domains of FADD (MORT-1) or FLICE (Caspase 8/MACH/Mch-5) and interfere with the FADD–FLICE interaction, thereby inhibiting the recruitment and activation of FLICE by Fas *(43)*.

Given these roles, the expression of each of the genes encoded by the ORFK13-72-73 locus in KS lesions may be highly relevant for tumor growth. Thus, we investigated the expression of these genes in KS and lymph node tissues by *in situ* hybridization by use of probes specific for each of the three ORFs.

Materials and Methods

Patients

Fourteen KS biopsy specimens (12 AIDS–KS lesions and two classical KS lesions) in different stages of development (four with early-patch lesions [all from AIDS–KS] and 10 with late nodular KS lesions [eight AIDS–KS lesions and two classical KS lesions]) were studied by *in situ* hybridization. One nodular KS biopsy specimen included an adjacent lymph node. All other biopsy specimens were obtained from skin lesions. In addition, three skin biopsy specimens from a healthy region of the skin of three different patients with AIDS–KS were included as control specimens. Terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) analysis was performed on the three biopsy specimens of nonlesional (healthy) skin and on nine early-patch and 12 late nodular stage AIDS–KS lesions, including all of the four early-stage lesions and five of the late-stage lesions that were used for *in situ* hybridization studies.

All human immunodeficiency virus (HIV)-1-infected donors were homosexual male patients classified as Centers for Disease Control and Prevention group C *(44)*. None of the patients received anti-KS therapy when the biopsy specimens were taken. Control biopsy specimens were obtained from an uninvolved area of the skin of one of the patients with AIDS–KS. All biopsy specimens were taken for diagnostic purposes with written informed consent from the patients.

Synthesis of Plasmids and Probes

For probe synthesis, DNA fragments of the respective HHV8 genomic region (K13 probe [nucleotides 122710–122294], v-cyc-D probe [nucleotides 123566–122796], C14 probe [nucleotides 124897–123959], and ORF73 probe [nucleotides 127293–126337]) (Fig. 1) were inserted into transcription plasmids and were verified by nucleotide sequence analysis. Plasmids for synthesis of T0.7, VP23, and actin hybridization probes have been described earlier *(22,45,46)*.

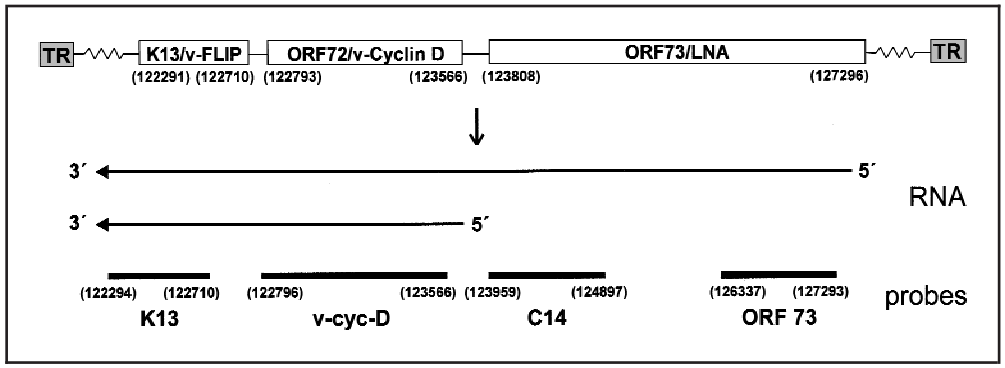

Fig. 1. Structure and expression of the ORFK13-72-73 locus of human herpesvirus-8. The locus is expressed via a 6.0-kilobase (kb) RNA transcript encoding all three open reading frames (ORFs) and a 1.7-kb bicistronic transcript that encodes the viral-cyc-D (v-cyc-D)/viral-FLIP (v-FLIP) sequences *(31)* [nucleotide numbers are according to Russo et al. *(10)*]. **Black bars** indicate the respective nonoverlapping RNA hybridization probes constituting almost all of the sequences encoding K13/v-FLIP (K13 probe, 122710–122294), ORF72/v-cyc-D (v-cyc-D probe, 123566–122796), and sequences encoding the aminoterminal part (ORF73 probe, 127293–126337) and the carboxyterminal part (C14 probe, 124897–123959) of the ORF73/LNA protein. Sense and antisense probes had the same length complementary sequence and polarity. v-FLIP = viral FLICE (Fas-associated death domain-like interleukin 1 beta-converting enzyme) inhibitory protein; LNA = viral latency-associated nuclear antigen. TR = terminal repeat.

Linearized forms of these plasmids were used for synthesis of ^{35}S-radiolabeled sense and antisense RNA hybridization probes (specific activity $\geq 1 \times 10^9$ cpm/μg RNA) with RNA polymerases T3 and T7 as described *(22,45,46)*.

In Situ Hybridization

All biopsy specimens used in our study were processed as previously described with an optimized, highly sensitive *in situ* hybridization protocol that allows detection of RNA encoding cytokines, growth factors, chemokines, and HHV8 transcripts in KS *(22,24,45,47,48)*.

In brief, immediately after removal, biopsy specimens were fixed in a cold (4 °C) solution of freshly prepared 4% paraformaldehyde in phosphate-buffered saline (0.13 *M* NaCl, 7 m*M* Na_2PO_4, and 3 m*M* $NaH_2PO_4 \times 2H_2O$ [pH 7.4]). Dehydration and paraffin embedding were performed as described *(45,48)*. Thin sections (5–10 μm) on silanized slides were deparaffinized and were subjected to *in situ* hybridization as follows:

The RNA probe solution (10–15 μL) was applied directly to the tissue sections at a final concentration of 50 000 cpm/μL in hybridization buffer (50% deionized formamide, 0.3 *M* NaCl, 20 m*M* Tris–HCl [pH 7.4], 5 m*M* EDTA, 10 m*M* $NaPO_4$ [pH 8.0], 10% dextran sulfate, 1× Denhardt's reagent, and 50 μg/mL total-yeast RNA). Hybridization was carried out under coverslips at 50°C for 16 hours in a humid chamber. The coverslips were gently floated off in 5× standard saline citrate (SSC) (1× SSC = 0.15 *M* NaCl and 0.015 *M* sodium citrate) and 10 m*M* dithiothreitol (DTT) at 50 °C, and the sections were subjected to a stringent washing at 60 °C in 50% formamide, 2× SSC, and 10 m*M* DTT covered with film emulsion and then exposed for 14 days. After photographic development, slides were stained with hematoxylin–eosin.

Controls for hybridization specificity were as follows: Negative controls included 1) the predigestion of adjacent sections with 100 μg/mL ribonuclease A for 1 hour at 37 °C prior to hybridization, 2) the use of the sense-strand riboprobe, 3) the use of an unrelated riboprobe, and 4) hybridization of the antisense riboprobe to sections from three different nonlesional healthy skin biopsy specimens. In no case were signals observed with these control sections. A positive control experiment was carried out by hybridization with a probe for the detection of cellular actin mRNA, which is highly expressed in every cell. Hybridizations with up to six different probes were carried out on consecutive sections. Similar signal patterns were obtained with probes specific for latent and lytic genes, respectively, providing a clear evidence for hybridization specificity.

Immunohistochemistry

The BCL-2 protein was detected in KS tissue sections with an immunochemical procedure. Paraffin-embedded tissue sections were deparaffinized and gradually rehydrated. Subsequently, sections were immersed in target retrieval solution (Dako, Hamburg, Germany) and boiled in a microwave oven (800 W) three times for 10 minutes each. Tissue peroxidases were blocked by incubation of the sections for 10 minutes in H_2O_2 (7.5%), and unspecific antibody-binding sites were blocked by incubation for 1 hour in white medium (Dako). The tissue sections were then incubated with an anti-BCL-2 antibody (Dako) at a 4-μg/mL final concentration for 2 hours. Bound primary antibody was detected with a commercially available peroxidase-coupled streptavidin–biotin kit (LSAB-2-Kit; Dako) by use of 3-amino-9-ethylcarbazole (Sigma, Deisenhofen, Germany) as a substrate.

TUNEL Analysis

TUNEL assay for the detection of apoptosis (programmed cell death) in cells in KS tissue sections was carried out with an *in situ* cell death detection kit from Boehringer Mannheim GmbH (Mannheim, Germany) and with the TdT-FragEL™ DNA fragmentation kit from Oncogene Research (Cambridge, MA), according to the manufacturer's instructions. Incorporated fluorescein-dUTP (f-dUTP) and biotinylated-deoxynucleoside triphosphate (b-dNTP) were detected with an antifluorescein antibody (for f-dUTP) or streptavidin (for b-dNTP), both conjugated with peroxidase and appropriate substrate reaction.

RESULTS

Structure and Expression of the HHV8 ORFK13-72-73 Locus

Expression of the ORFK13-72-73 locus was investigated in biopsy specimens from lesions from 12 patients with AIDS–KS and two patients with classic KS at different stages of development (four early-patch lesions [all AIDS–KS lesions], 10 late nodular KS lesions [eight AIDS–KS lesions and two classic KS lesions]). One of the nodular lesions developed adjacent to a lymph node, which allowed comparison of ORFK13-72-73 expression in both tissues. ORFK13-72-73 gene expression was analyzed by *in situ* hybridization with strand-specific radiolabeled nonoverlapping RNA probes that were complementary to almost all of the sequences encoding K13/v-FLIP (K13 probe, 122710–122294) and ORF72/v-cyc-D (v-cyc-D probe, 123566–122796) and to the sequences encoding the aminoterminal part (ORF73 probe, 127293–126337) and the carboxyterminal part (C14 probe, 124897–123959) of the ORF73/LNA protein (Fig. 1).

Increased Expression of K13/v-FLIP in KS Lesions With Disease Stage

Hybridization with the K13 probe showed only a very few (one lesion) or no (three lesions) hybridization signals in the four early-patch KS lesions examined (Fig. 2, A, bright field; Fig. 2, B, corresponding dark field). By contrast, all the 10 nodular lesions showed strong hybridization signals with the K13 probe in almost 70% of the spindle cells (Fig. 2, C and D [KS]). In adjacent noninvolved lymph nodes (Fig. 2, C and D, (LN)] and other peritumoral tissues (Fig. 2, C and D, left part), no hybridization signals were found. At a higher magnification, it was clear that K13 was predominantly expressed by the spindle cells with typical plump, elongated nuclei (Fig. 2, E and F; arrow). In addition, signals were observed in endothelial cells lining morphologically recognizable vessels in the lesions (Fig. 2, E and F; arrowhead).

To control reliability of the technique, the tissue was hybridized with an actin-specific hybridization probe. Signals occurred over all cells of the sections, demonstrating that all parts of the tissue were similarly accessible for hybridization (Fig. 2, G and H). Tissue sections of healthy skin regions of patients with AIDS–KS did not show signals with the K13 probe (Fig. 2, I and J), indicating a specific association with KS tissues. In addition, in no case were signals observed by hybridization with the K13 sense control probe, excluding hybridization to viral DNA as the reason for the signals obtained with the K13 antisense probe (data not shown). Additional control experiments were also performed to demonstrate hybridization specificity (*see* "Materials and Methods" section).

Bicistronic ORF72-K13 mRNA: the Predominant Transcript of the ORFK13-72-73 Locus in KS Lesions

To investigate whether the bicistronic ORF72-K13 mRNA and the tricistronic RNA, encoding also LNA, were both detected by the K13 antisense probe, serial sections of KS biopsy specimens were hybridized with four different probes complementary to the mRNA encoding K13/v-FLIP (K13 probe), ORF72/v-cyc-D (v-cyc-D probe), the aminoterminus (ORF73 probe), and the carboxyterminus (C14 probe) of ORF73/LNA (*see* Fig. 1). As shown in Fig. 3, prominent signals were obtained in KS lesions with the K13 probe (Fig. 3, A and B) and the v-cyc-D probe (Fig. 3, C and D), whereas no signals were observed with the ORF73 probe (Fig. 3, E and F) and the C14 probe (data not shown). These results demonstrated that signals obtained with the K13 and the v-cyc-D probe represent hybrid-

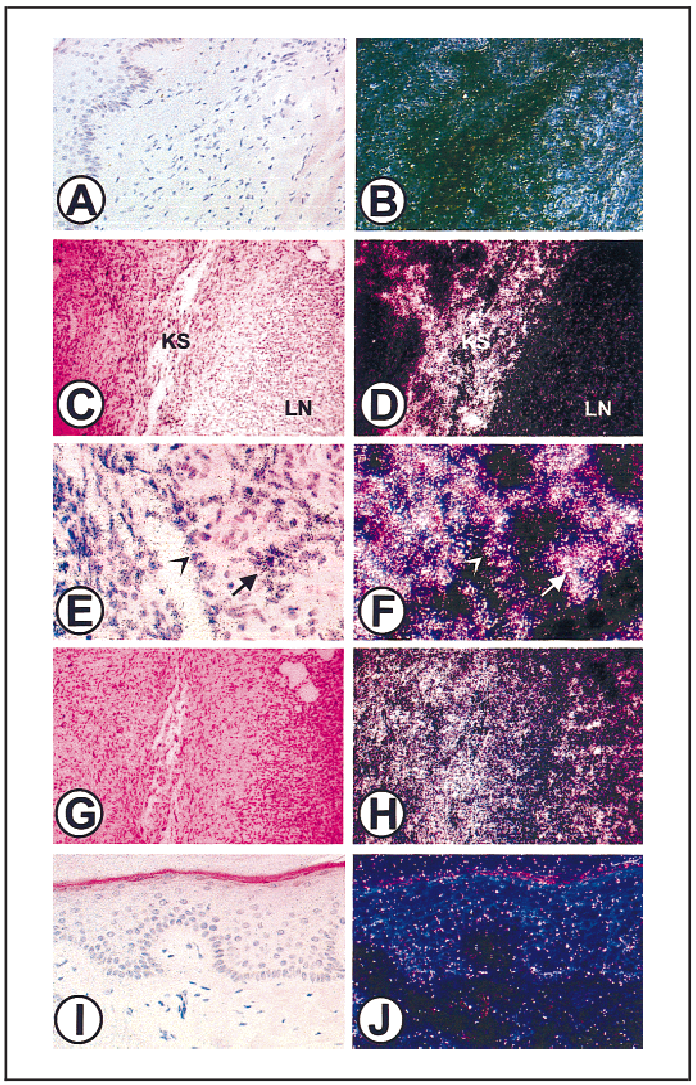

Fig. 2. Detection of RNA transcripts of the human herpesvirus-8–K13 gene in Kaposi's sarcoma (KS) lesions. Hybridization with the K13 antisense probe showed no signals in early-patch KS lesions (**A** = bright field; **B** = corresponding dark field). Strong signals were obtained in almost 70% of the spindle cells present in a late-stage KS lesion (**C** and **D** [KS]), whereas the adjacent lymph node tissue (**C** and **D** [LN]) and the peritumoral tissue outside the lymph node (**C** and **D;** left part) did not reveal any hybridization signal. Higher magnification demonstrated hybridization signals overlaying the spindle cells of KS with typical plump, elongated nuclei (**E** and **F; arrow**) and endothelial cells lining morphologically recognizable vessels in the lesion (**E** and **F; arrowhead**). Hybridization with an actin-specific probe revealed signals over all cells of the section (**G** and **H**). No signals were observed when the K13 antisense probe was hybridized to a healthy skin region of a patient with acquired immunodeficiency syndrome-associated KS (**I** and **J**).

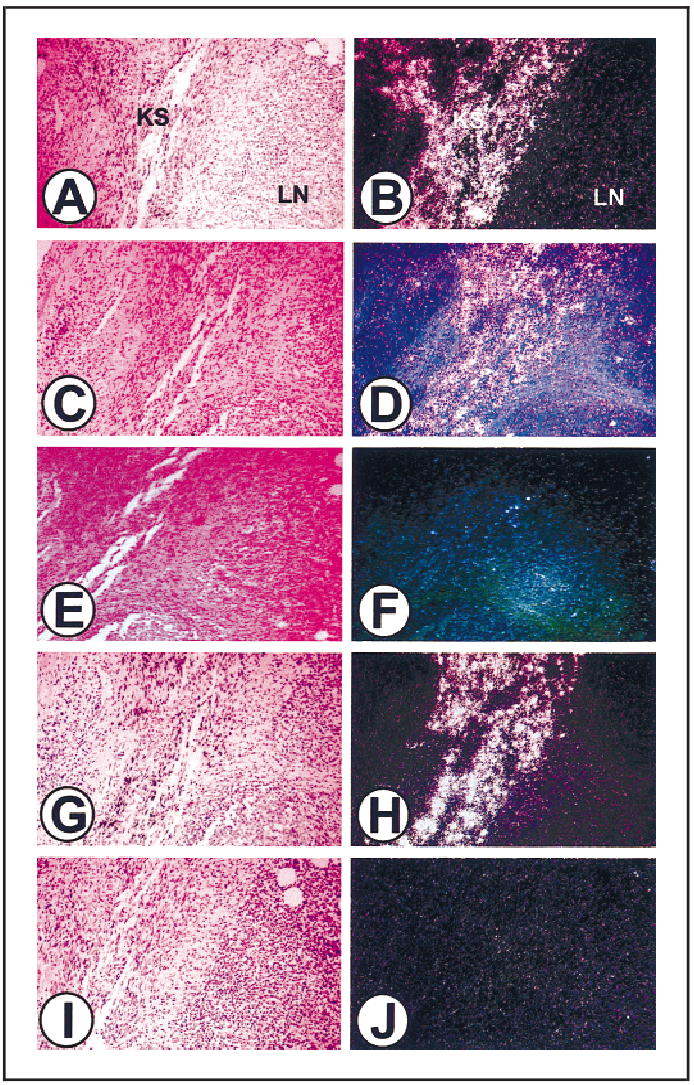

Fig. 3. Identification of various RNA transcripts arising from the ORFK13-72-73 locus of human herpesvirus 8 by hybridization of consecutive Kaposi's sarcoma (KS) tissue sections. Serial sections of late-stage KS lesion biopsy specimens were hybridized with the K13 probe (**A** = bright field; **B** = dark field), viral-cyc-D probe (**C** and **D**), ORF73 probe (**E** and **F**), T0.7 probe (**G** and **H**), and VP23 probe (**I** and **J**). Prominent signals were obtained in KS lesions with the K13 probe (**A** and **B** [KS]) and the v-cyc-D probe (**C** and **D**). No signals were observed with the ORF73 probe (**E** and **F**). Robust signals were obtained with the T0.7-specific probe (**G** and **H**) and a similar distribution on the tissue as those obtained with the K13 and v-cyc-D probes (compare **A–D**), respectively. The VP23 gene was not expressed in this specimen (**I** and **J**), indicating a predominantly latent infection. Panels **A** and **B** have been repeated from Fig. 2 (**C** and **D**) to demonstrate the hybridization pattern of the different probes on consecutive sections.

ization with the bicistronic v-cyc-D-K13 mRNA and not hybridization with the tricistronic RNA, which also contains the ORF73 sequences.

To investigate whether the bicistronic ORF72-K13 mRNA is expressed in KS lesions during the productive/lytic or the latent phase of the viral life cycle, expressions of the capsid gene VP23 (lytic cycle associated) and of the latency-associated T0.7/kaposin gene were investigated in two further sections that were consecutive to those hybridized with the K13 probe (Fig. 3, A–F). Strong hybridization signals were obtained with a T0.7-specific probe (Fig. 3, G and H), with a distribution in the tissue section similar to that obtained with the K13 and the v-cyc-D probes (compare Fig. 3, A–D). By contrast, VP23 mRNA was either undetectable (Fig. 3, I and J) or expressed only in very few cells of the lesions (data not shown), which is consistent with earlier reports *(22,26,46).* These findings indicate that the bicistronic ORF72-K13 mRNA is expressed in latently infected KS spindle cells of late-stage nodular KS lesions.

Full-Length ORF73-72-K13 mRNA and an Abundant Expression in Lymph Node Tissue

Hybridization analysis of the tissue (including a lymph node and the adjacent nodular KS lesion) from a patient (Figs. 2 and 3) showed HHV8 infection in three follicles distant from the KS

lesion and hybridization signals with the K13 probe (Fig. 4, A [bright field], B [dark field], and C [increased magnification of positive cells]) and the v-cyc-D probe (Fig. 4, D–F) but also with the ORF73 (Fig. 4, G–I) and the C14 probe (data not shown). These results indicate that the ORFK13-72-73 locus is differentially expressed in KS and lymph node tissues. Moreover, T0.7 mRNA was also detected in lymph node cells (Fig. 4, J–L). In contrast to KS tissues, VP23 mRNA was expressed in more than 30% of the HHV8-infected lymph node cells (Fig. 4, M–O), demonstrating that lytic/productive infection is more prominent in the lymph node cells compared with KS tissues where the latent infection is predominant.

Decrease in Apoptosis in KS Lesions With Disease Stage

Since the K13/v-FLIP gene product may inhibit receptor-mediated apoptosis in latently infected spindle cells, we examined whether its expression is associated with a reduced apoptosis of these cells. For this purpose, nine early-patch KS lesions, 12 late nodular-stage KS lesions, and, as a control, three biopsy specimens from nonlesional healthy skin of patients with AIDS–KS were analyzed by the TUNEL assay. In the three biopsy specimens from the healthy skin, the apoptosis was detected only in the upper cell layers of the epidermis but not in the dermis (Fig. 5, A; arrow). *In situ* hybridization of consecutive sections with the K13 probe did not show any signal (Fig. 5, B [bright field] and C [dark field]). In eight of the nine early KS lesions, numerous cells appeared to be apoptotic (Fig. 5, D; arrows). Some of these cells were lining vascular spaces apparently representing endothelial cells (Fig. 5, D; lower arrow). *In situ* hybridization of a consecutive section with the K13 probe revealed no signals (Fig. 5, E and F). By contrast, apoptosis was either absent (10 lesions) or very rare (two lesions) in the late-stage KS lesions examined (Fig. 5, G) and high expression of K13 was detected by *in situ* hybridization on consecutive sections (Fig. 5, H and I).

It has been reported that BCL-2, a cell-encoded antiapoptotic factor, is expressed in the spindle cells of KS and that its expression increases in a stage-related manner during KS lesion development *(49)*. By immunohistochemistry on the specimen with the late-stage lymph nodal KS, prominent staining of the BCL-2 protein was observed in the mantle zone cells of the lymph node, whereas the cells in the germinal centers were negative (Fig. 5, J [LN]). In comparison to the lymph node, BCL-2 staining intensity in the KS lesion was lower (Fig. 5, J [KS]). However, higher magnification of the KS area revealed that BCL-2 was expressed in almost all of the cells of the lesion (Fig. 5, K), predominantly in the spindle cells (Fig. 5, L, arrows).

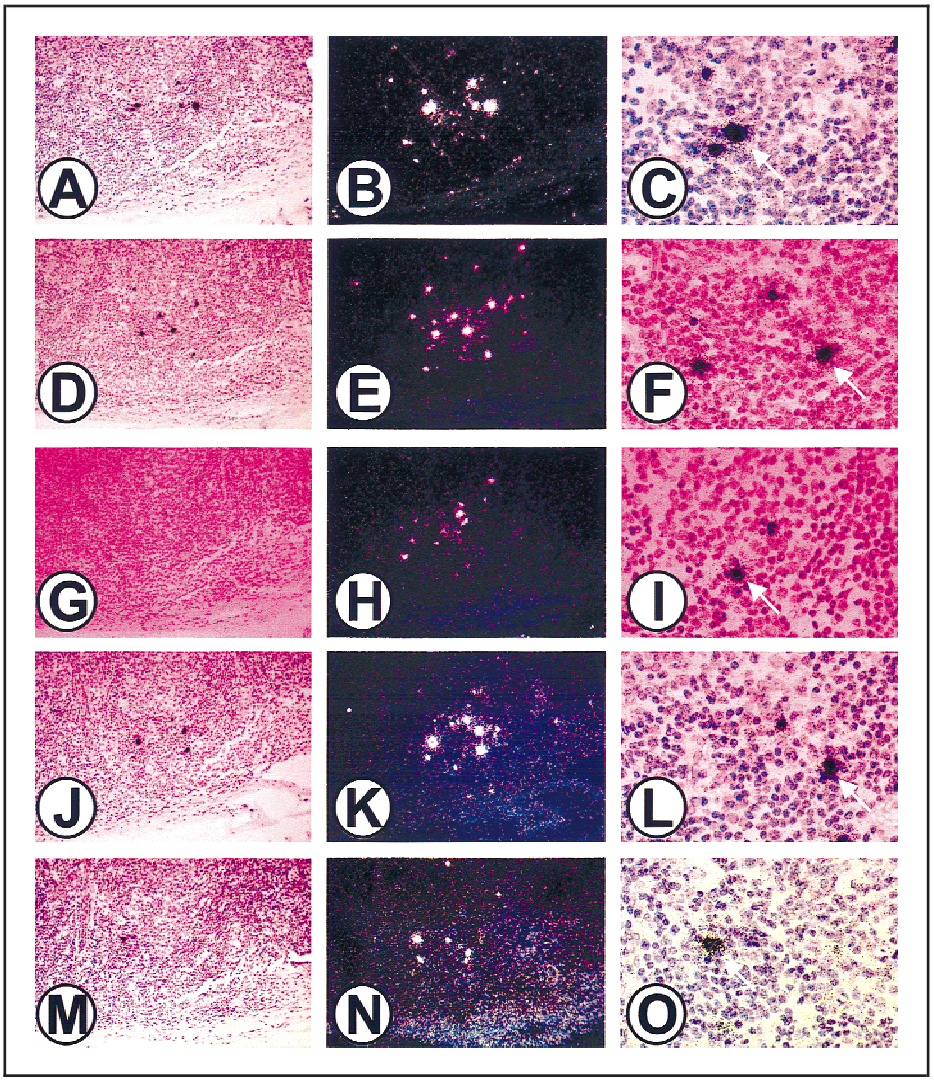

Fig. 4. Determination of the expression of the human herpesvirus 8 (HHV8) ORFK13-72-73 locus in lymph node tissue adjacent to Kaposi's sarcoma (KS) lesion. HHV8 infection was detected in three follicles of a lymph node adjacent to the nodular KS lesion shown in Figs. 2 and 3. Various cells of these follicles hybridized with the K13 probe (**A** = bright field; **B** = dark field; and **C** = increased magnification of positive cells), the viral-cyc-D probe (**D, E,** and **F**), the ORF73 probe (**G, H,** and **I**), the T0.7 probe (**J, K,** and **L**), and the VP23 probe (**M, N,** and **O**). Panels **J–O** indicate that a high fraction (≥30%) of HHV8-infected lymph node cells is lytically/productively infected (compare ratio of T0.7-positive [**J, K,** and **L**] and VP23-positive [**M, N,** and **O**] cells).

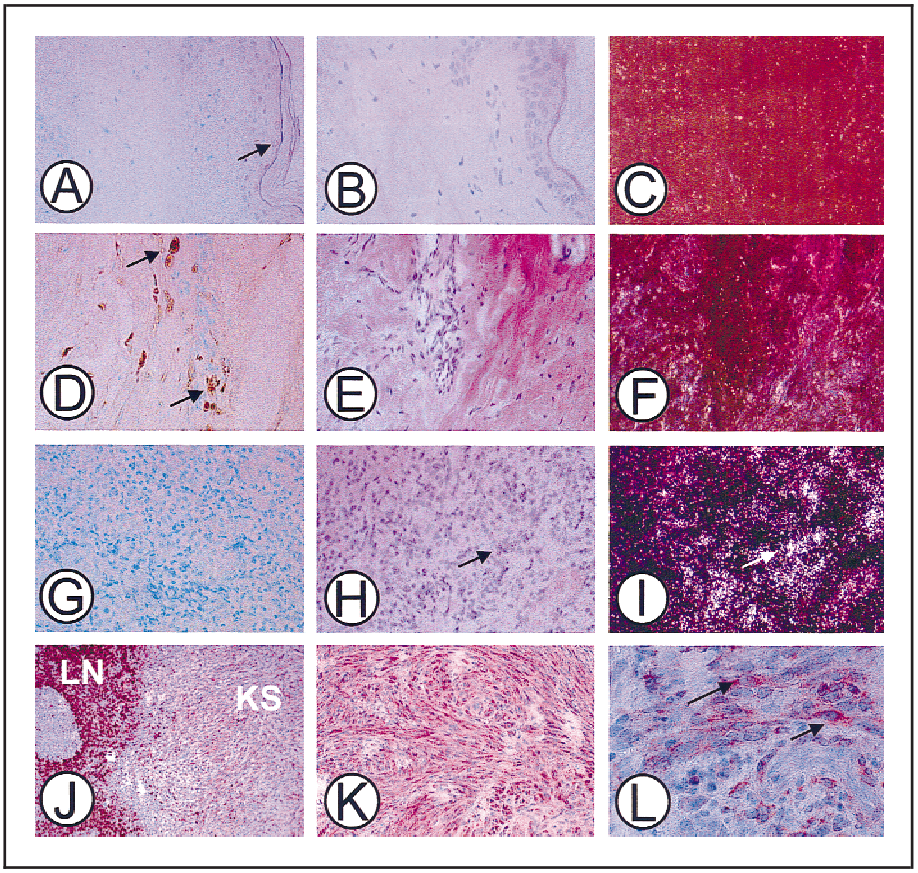

Fig. 5. Detection of apoptosis (programmed cell death) and expression of antiapoptotic factors (human herpesvirus 8–K13 gene encoded viral-FLIP and cell encoded BCL-2) in early-stage and late-stage Kaposi's sarcoma (KS) lesions. Terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick-end labeling analysis of nonlesional healthy skin of patients with acquired immunodeficiency syndrome-associated KS indicated that apoptosis is detected only in the upper cell layers of the epidermis but not in the dermis (**A; arrow**). *In situ* hybridization with the K13 probe of consecutive sections did not show any signal (**B** = bright field; **C** = dark field). In early KS lesions, numerous cells appeared apoptotic (**D: brown staining; arrows**). Some of these cells were lining vascular spaces apparently representing endothelial cells (**D; lower arrow**). *In situ* hybridization with the K13 probe of a consecutive section revealed no signals (**E** and **F**). No apoptosis was detected in late-stage KS lesions (**G**), and K13 was highly expressed (**H** and **I**). In a section with a late-stage KS overlying a lymph node (LN), BCL-2 protein was found to be highly expressed in the mantle zone cells of lymph follicles (**red staining**). In KS, anti-BCL-2 staining intensity was lower and the cells in the germinal centers did not express this cell-encoded antiapoptotic factor (**J**). Higher magnification of the KS tissue revealed that BCL-2 is expressed in almost all cells of the lesion (**K**) and predominantly in the spindle cells (**L; arrows**). viral-FLIP = viral-FLICE (Fas-associated death domain-like interleukin 1 beta-converting enzyme) inhibitory protein.

DISCUSSION

Most previous studies on the expression of HHV8 genes in KS lesions have been performed by the RT–PCR method, which does not allow determination of the gene expression at the cellular level and of the level of expression and whether lytic or latent infection is predominant.

During lytic infection, almost every viral gene is expressed, whereas only a few genes are expressed in latently infected cells *(50).* Lytic infection may be required to maintain the virus in a tissue, but lytically infected cells will die. Therefore, only latently infected cells, which survive infection, may contribute to lesion growth. This suggests that, for HHV8 to perpetuate growth of KS lesions, the presence of both lytically and latently infected cells may be required.

By use of *in situ* hybridization, we mapped the different transcripts of the HHV8-encoded ORFK13-72-73 locus in lytically and latently infected cells in KS and lymph node tissues. We obtained for the first time detailed insights into the expression of this locus *in vivo* by hybridization of up to six consecutive sections with different probes (K13, v-cyc-D, C14, ORF73, VP23, and T0.7). The results indicate that 1) the ORFK13-72-73 locus is differentially expressed at the RNA level in KS tissues as compared with lymph nodes, 2) the expression of the HHV8-encoded antiapoptotic K13/v-FLIP gene increases dramatically from early- to late-stage KS lesions, and 3) the increase of K13/v-FLIP gene expression parallels the decreased apoptosis in late-stage lesions.

Specifically, in late-stage nodular KS lesions, the bicistronic ORF72-K13 mRNA is conspicuously expressed in almost 70% of the spindle cells, whereas the tricistronic RNA, which also encodes ORF73, is below the *in situ* detection limit (≈50 copies). In contrast, a recent report *(32)* has shown the presence of the LNA protein in KS spindle cells. This finding is similar to previous data with the Epstein-Barr virus-encoded nuclear antigen-1 (EBNA-1), whose RNA is expressed at a very low level *(51),* whereas the EBNA-1 protein is relatively abundant and can be easily detected in infected cells by immunochemical methods *(52,53).* However, in lymph node tissue, the ORF73-encoding RNA sequences are expressed at levels detectable by *in situ* hybridization. Since the lymph node and the KS tissue were both present in the same biopsy sections and, therefore, identically fixed and processed during hybridization, and since an actin probe revealed similar signals in KS and lymph node tissues, the different expression patterns are not due to methodologic variations. In contrast, they prove that the tricistronic ORF73-72-K13 RNA is the predominant transcript in cells of the lymph nodes, whereas the bicistronic ORF72-K13 RNA is expressed mostly in the KS lesions.

In pleural effusion lymphoma-derived cell lines, the bicistronic ORF72-K13 RNA has been shown to be generated by the splicing of a tricistronic ORF73-72-K13 precursor RNA *(30).* Thus, the different expression pattern observed in KS and lymph nodes is likely because of differential splicing events. Differential processing of the tricistronic precursor RNA may be coupled with the viral life cycle. This hypothesis is suggested by our observation that in KS almost all of the infected spindle cells are latently infected (⩾98), whereas in lymph nodes more than 30% of the infected cells show lytic gene expression (as determined by the ratio of VP23- and T0.7-expressing cells). Of interest, induction of lytic infection *in vitro* by 12-*O*-tetradecanoylphorbol-13-acetate treatment of latently infected

BCBL-1 cells has no effect on the splicing of this locus *(30)*. Our data suggest that *in vivo* the ORF73-72-K13 locus is differentially expressed in lytically and latently infected cells. However, hybridization on consecutive sections did not allow us to determine unequivocally whether ORF73 sequences are expressed in VP23-positive cells. Therefore, our results may also indicate that there are two different phases of HHV8 latency: 1) a KS-associated latency with abundant expression of the bicistronic ORF72-K13 RNA and low expression of the tricistronic transcript and 2) a lymph node-associated latency where the ORF73–72-K13 transcript is expressed abundantly.

However, with regard to KS, our study clearly shows that the bicistronic ORF72-K13 RNA, which encodes the antiapoptotic v-FLIP, is highly expressed in late-stage KS lesions but not in early lesions and that this stage-related increase of K13/v-FLIP expression parallels the reduction of apoptosis.

There may be several mechanisms for the high apoptotic rate in early-stage KS. For example, high concentrations of inflammatory cytokines, such as TNF-α and interferon gamma, that are present in the lesions *(54)* may induce endothelial cell apoptosis *(55–59)*. Alternatively, apoptosis could be due to activated T cells expressing the proapoptotic Fas ligand (FasL) *(60,61)*. Activated T cells can be detected in all stages and in all epidemiologic forms of KS *(1,48,54,62,63)*. This fact suggests that potent antiapoptotic mechanisms have to be operative to allow growth of KS lesions under these conditions.

BCL-2, a cell-encoded antiapoptotic factor, was found to be highly expressed in the mantle zone cells of lymph node tissue and also in the spindle cells and endothelial cells of a late-stage KS lesion, confirming previous data on a stage-related increase of BCL-2 expression in KS development *(49)*. The expression of BCL-2 and v-FLIP in almost all cells of KS indicates that both factors are co-expressed in the spindle cells. However, BCL-2 is not an efficient inhibitor of receptor-mediated apoptosis pathways induced by inflammatory cytokines like TNF-α or the FasL *(64,65)*. This, in fact, is a specific task of FLIPs *(38–43)*, suggesting that the HHV8-encoded v-FLIP may inhibit the receptor-mediated apoptosis in late-stage KS lesions.

The cooperative activity of the cellular BCL-2 and v-FLIP may explain how KS lesions can rapidly grow and progress in the presence of potent pro-apoptotic factors and relatively low mitotic activity *(66)*, suggesting that the modulation of apoptosis in spindle cells may play a key role in the rapid growth of KS lesions.

In conclusion, we found that the bicistronic v-cyc-D/v-FLIP-encoding mRNA is highly expressed in KS spindle cells of late-stage nodular lesions but not in early lesions. Furthermore, a stage-related inverse relation is observed between the expression of this bicistronic mRNA and apoptosis. Thus, apoptosis is relatively frequent in early lesions but low or absent in the late-stage nodular lesions, whereas mRNA for v-cyc-D/v-FLIP is expressed in almost every tumor cell. These data suggest that the antiapoptotic v-FLIP protein of HHV8 may contribute to KS progression by modulating receptor-mediated apoptosis in KS spindle cells.

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NOTES

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