REPORTS

Expression of Human Herpesvirus 8-Encoded Cyclin D in Kaposi's Sarcoma Spindle Cells

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Background: **Human herpesvirus 8 (HHV-8) DNA sequences have been detected in Kaposi's sarcoma, in primary effusion lymphoma (an unusual high-grade non-Hodgkin's lymphoma seen primarily in patients with acquired immunodeficiency syndrome [AIDS]), and in Castleman's disease (a rare lymphoproliferative disorder); however, proof that HHV-8 is involved in the pathogenesis of these diseases remains to be established. HHV-8 contains a gene, i.e., v-cyclin D, that is a homologue of the cellular cyclin D2 gene, which encodes a protein that promotes passage through G_1 phase of the cell cycle. Previous studies have identified v-cyclin D messenger RNA (mRNA) in biopsy specimens of Kaposi's sarcoma. In this study, we isolated a full-length v-cyclin D complementary DNA and characterized the pattern of v-cyclin D mRNA expression in Kaposi's sarcoma.** ***Methods:*** **Standard methods were used to construct and to screen HHV-8 genomic and complementary DNA libraries. Reverse transcription–polymerase chain reaction (RT–PCR) methods and *in situ* hybridization with RNA probes were used to examine v-cyclin D mRNA expression.** ***Results:*** **RT–PCR demonstrated the presence of v-cyclin D mRNA in biopsy specimens of AIDS-related Kaposi's sarcoma, in early-passage spindle cells from classical (i.e., not AIDS-related) Kaposi's sarcoma, and in spindle cells isolated from the peripheral blood of patients with AIDS-related Kaposi's sarcoma.** ***In situ*** **hybridization indicated that mRNAs for v-cyclin D and kaposin, an HHV-8 latency-associated gene, were present in approximately 1% of the spindle cells in early patch lesions and approximately 60% of the spindle cells in late nodular lesions of Kaposi's sarcoma.** ***Conclusions:*** **Spindle cells of Kaposi's sarcoma, which have been regarded as the tumor cells of this cancer, contain v-cyclin D mRNA. Expression of v-cyclin D protein may be involved in the pathogenesis of Kaposi's sarcoma by promoting cell proliferation. [J Natl Cancer Inst 1997;89:1868–74]**

Kaposi's sarcoma is a multifocal tumor that occurs predominantly in the skin, visceral organs, and lymph nodes *(1)*. Kaposi's sarcoma lesions evolve over time from patch or plaque-like lesions at early stages to nodular lesions characteristic of later stages. Histologic hallmarks that characterize all stages of Kaposi's sarcoma include a prominent microvasculature, a large inflammatory component, and, especially in later stages of development, bundles of spindle-shaped cells. These spindle-shaped cells have been regarded as the tumor cells of Kaposi's sarcoma lesions *(1)*.

In vitro analyses of Kaposi's sarcoma-derived spindle cells suggest that acquired immunodeficiency syndrome (AIDS)-related Kaposi's sarcoma is a cytokine-mediated disease. Several growth factors and cytokines have been implicated in the stimulation of Kaposi's sarcoma spindle cell proliferation and the recruitment of inflammatory cells and microvessels into Kaposi's sarcoma lesions, including oncostatin M *(2,3)*, platelet-derived growth factor B (PDGF B) *(4,5)*, interleukin (IL) 1β *(5,6)*, IL-6 *(7)*, IL-8 *(8)*, basic fibroblast growth factor (bFGF) *(9)*, scatter factor (SCF) *(10)*, vascular endothelial growth factor (VEGF) *(11)*, macrophage chemotactic protein-1 (MCP-1) *(6)*, and the human immunodeficiency virus-1 (HIV-1) Tat protein *(12)*. Indeed, for most of these factors (IL-1β, PDGF B, bFGF, VEGF, MCP-1, SCF, and HIV-1 Tat), a possible role in the pathogenesis of Kaposi's sarcoma has been further supported by gene expression studies *(5,8,10,11,13)*. From these reports, a model has been put forth that suggests that Kaposi's sarcoma initiation and progression occur within the framework of a cytokine-mediated reactive process *(14,15)*. However, this model does not explain epidemiologic observations suggesting that AIDS-related Kaposi's sarcoma may be linked to a sexually transmitted infectious agent other than HIV-1 *(16)*.

A very likely candidate for such an infectious agent is a novel human herpesvirus, human herpesvirus 8 (HHV-8) *(17)*. HHV-8 was initially detected in biopsy specimens of AIDS-related Kaposi's sarcoma *(17)*. Subsequently, HHV-8 DNA sequences have been identified in all clinical forms of Kaposi's sarcoma (classical, endemic, iatrogenic, and AIDS-related), suggesting that HHV-8 may be the common pathogenic mediator functioning in Kaposi's sarcoma *(17–21)*. In addition, HHV-8 DNA sequences have been detected in primary effusion lymphoma *(18,22,23)*, an unusual high-grade non-Hodgkin's lymphoma primarily seen in AIDS patients, and in multicentric Castleman's disease *(24–26)*, a rare lymphoproliferative disorder often associated with an increased incidence of Kaposi's sarcoma *(27–30)*.

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See ''Notes'' following ''References.''

The mechanisms by which HHV-8 infection may contribute to the development of Kaposi's sarcoma, primary effusion lymphoma, and Castleman's disease are not known. However, the identification and characterization of viral genes expressed in these HHV-8-associated proliferative diseases may provide new clues toward understanding the pathogenic potential of HHV-8. It is interesting that the HHV-8 genome contains several viral homologues of cellular genes that encode proteins with potential growth-promoting and survival properties *(31–35)*, including two beta chemokines that are homologous to macrophage inflammatory protein-1 *(33,36)*, IL-6 *(33,36)*, interferon regulatory factor-1 *(33,36)*, Bcl-2 *(33,36)*, a G-protein-coupled receptor homologous to the IL-8 receptor B *(32,34)*, and cyclin D2 *(34)*. Recently, the viral cyclin D gene, i.e., v-cyclin D, has been shown to encode a functional protein that is able to activate cell cycle progression through G_1 phase *(31,37)*. In addition, reverse transcription–polymerase chain reaction (RT–PCR) studies on a limited number of clinical samples suggest that at least two of the genes, i.e., the ones encoding the G-protein-coupled receptor and v-cyclin D, are expressed in Kaposi's sarcoma tissues *(32,34)*. However, which cells within the Kaposi's sarcoma lesions express these genes remains to be determined.

Herein, we report the isolation of an HHV-8 complementary DNA clone encoding the viral homologue of cellular cyclin D2 (v-cyclin D), and we describe the stage-specific expression of v-cyclin D messenger RNA (mRNA) in Kaposi's sarcoma.

Patients, Materials, and Methods

Patients. Clinical specimens were obtained and studies performed as defined in Table 1. All patients gave written informed consent for the analysis of nondiagnostic biopsy specimens. Moreover, the collection of peripheral blood and biopsy materials used in this study was approved by the Institutional Review Board at Vanderbilt University. Kaposi's sarcoma specimens used for *in situ* hybridization were used in several preceding studies *(4,5,38,39)* and have been shown to give optimal hybridization results. At the time of biopsy, individuals included in this study were not receiving specific therapy for Kaposi's sarcoma.

Cell culture. Single-cell suspensions were prepared from skin biopsy specimens of classical Kaposi's sarcoma by overnight treatment with 100 U/mL collagenase (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD) contained in RPMI-1640 medium supplemented with 10% fetal calf serum (Life Technologies, Inc.), 100 U/mL penicillin G, and 100 μg/mL streptomycin (Life Technologies, Inc.). Nucleated pleural fluid cells and cells obtained from the classical Kaposi's sarcoma tumor specimens were propagated in fibronectin (Boehringer Mannheim, Indianapolis, IN)-coated 24-well tissue culture plates (Falcon, Becton Dickinson and Company, Lincoln Park, NJ) either in RPMI-1640 medium supplemented with 10% fetal calf serum, 10 ng/mL endothelial cell growth factor (Life Technologies, Inc.), and 5 U/mL heparin (Sigma Chemical Co., St. Louis, MO) or as described previously *(40)* in conditioned medium from activated lymphocytes. Peripheral blood-derived spindle cells from patients with AIDS-related Kaposi's sarcoma were isolated and cultured as described previously *(40)*. The Epstein-Barr virus-infected cell line P3HR1 was obtained from the American Type Culture Collection (Rockville, MD) and grown in RPMI-1640 medium supplemented with 10% fetal calf serum. The lymphoblastoid cell line Ram (Epstein-Barr virus-infected) was established in our laboratory from the peripheral blood of a patient with AIDS-related Kaposi's sarcoma and cultured by use of the same conditions as described for P3HR1.

Table 1. Clinical samples and studies performed*

Sample	Total No.	No. tested		
		RT–PCR	Culture	*In stiu* hybridization
Classical Kaposi's sarcoma, late nodular stage	1	ND	ND	1
Classical Kaposi's sarcoma, late nodular stage	2	2	2	ND
AIDS-related Kaposi's sarcoma, late nodular stage	4	ND	ND	4
AIDS-related Kaposi's sarcoma, early patch stage	3	1	ND	2
AIDS-related Kaposi's sarcoma, pleural effusion†	1	1	ND	ND
AIDS-related Kaposi's sarcoma, uninvolved skin	1	ND	ND	1
AIDS-related Kaposi's sarcoma, peripheral blood‡	6	6	6	ND
Classical Kaposi's sarcoma, peripheral blood§	1	1	ND	ND

*RT–PCR = reverse transcription–polymerase chain reaction; ND = not done.
†Patient had bilateral pulmonary lesions and pleural effusions.
‡Peripheral blood-derived spindle cells from patients with nodular stage AIDS-related Kaposi's sarcoma.
§Peripheral blood mononuclear cells from a patient with nodular stage Kaposi's sarcoma.

RNA isolation and polyadenylated RNA purification. Total RNA was isolated from cultured cells and biopsy specimens by use of TriReagent (Molecular Research Center, Inc., Cincinnati, OH) in accordance with the manufacturer's recommendations. Polyadenylated RNA was isolated with the aid of oligo dT-conjugated magnetic beads (Dynal, Lake Success, NY), following the manufacturer's protocol.

Analyses of HHV-8 gene expression. Polyadenylated RNA was isolated from the clinical samples (Table 1) as described above. Complementary DNA was synthesized as follows: 100 ng polyadenylated RNA was reverse transcribed (1 hour at 37 °C) in a reaction containing 50 m*M* Tris–HCl (pH 8.3), 75 m*M* KCl, 3 m*M* $MgCl_2$, 10 m*M* dithiothreitol, 500 μ*M* each of deoxyadenosine triphosphate, deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate, and deoxythymidine triphosphate (Boehringer Mannheim), 10 ng random hexamer primers (Boehringer Mannheim), and 10 U/mL SuperScript II reverse transcriptase (Life Technologies, Inc.). Complementary DNAs were diluted to a final concentration of 1 ng/μL based on the total amount of RNA used to prepare them, and 1 ng was used per PCR reaction. The primers used for PCR amplification and the detection of PCR products by means of Southern blot hybridization were as follows: β-actin PCR (sense, 5′-GAAACTACCTTCAACTCCATC-3′; antisense, 5′-CTAGAAGCATTTGCGGTG-GACGATGGAGGGGCC-3′), v-cyclin D PCR (sense, 5′-ATGGCAACTGCCAATAACC-3′; antisense, 5′-ATAGCTGTCCAGAATGCG-3′), and v-cyclin D probe (5′-CTCTTACTTCGCATATGC-3′). The amplification conditions were 40 cycles at 95 °C for 1 minute, 54 °C for 1 minute, and 72 °C for 1 minute for v-cyclin D and 40 cycles at 95 °C for 1 minute, 58 °C for 1 minute, and 72 °C for 1 minute for β-actin. PCR products were resolved by means of agarose gel (1.5%) electrophoresis, and the gels were stained with ethidium bromide, followed by capillary transfer to Nytran membranes (Schleicher and Schuell, Inc., Keene, NH). The membranes were hybridized with the defined v-cyclin D oligonucleotide probe after it was labeled at its 5′ end with $^{32}PO_4$. The predicted sizes of the PCR products were as follows: β-actin, 303 base pairs; v-cyclin D, 771 base pairs. To control for possible DNA contamination of mRNA, we performed PCR reactions with 10 ng mRNA in the absence of reverse transcription. In no case did observable specific amplification occur in these control reactions.

Genomic library construction and screening. DNA from tumor cells in the pleural fluid of a patient with HHV-8-related primary effusion lymphoma was used to construct a genomic library as described *(32)*. The HHV-8 tegument probe that was used to screen the genomic library was homologous to open reading frame 75 of *Herpesvirus saimiri (32)*. The HHV-8 tegument probe was labeled with [α-^{32}P]dCTP (Amersham Life Sciences, Arlington Heights, IL), using the RediPrime Random Primer Labeling kit (Amersham Life Sciences), and was used to isolate HHV-8 genomic clones, following specifications of the manufacturer of the lambda cloning vector (Stratagene, La Jolla, CA). Sequencing of positive genomic clones was performed by use of primer walking and nested deletions of both DNA strands. Sequence analyses were performed by use of the Beauty and Blast programs (from Baylor University College of Medicine, Houston, TX) and

the DNAstar analysis program (DNAstar, Madison, WI). A 20-kilobase HHV-8 fragment, clone 7-2, was isolated from the genomic library. Several HHV-8 genes were contained within clone 7-2, including genes homologous to cellular cyclin D2 (v-cyclin D) and IL-8 receptor B as well as a homologue of the major tegument protein of *H. saimiri (41).* This HHV-8 genomic clone was used as a probe to screen the complementary DNA library described below.

Complementary DNA library construction and characterization. Polyadenylated RNA prepared from uncultured primary effusion lymphoma cells (1×10^8 cells) was used to generate a size-selected complementary DNA library (average insert size >1.5 kilobases) by use of the SuperScript Lambda system (Life Technologies, Inc.). The 20-kilobase HHV-8 clone 7-2, noted above, was radiolabeled with [α-^{32}P]dCTP and used to screen the primary effusion lymphoma complementary DNA library. Inserts derived from positive complementary DNA phage clones were subcloned into the pBKCMV eukaryotic expression vector (Stratagene) and sequenced as described above.

Southern blot hybridization. To determine DNA sequence specificity, we used complementary DNA isolates in Southern blot hybridization. Genomic DNA was isolated from primary effusion lymphoma cells, P3HR1 cells, Ram cells, and cells from the pleural effusion of a patient with AIDS-related Kaposi's sarcoma as described above in the methods for genomic library construction. DNA samples (10 μg each) were digested with the restriction enzyme *Bam*HI, resolved by means of agarose gel (0.9%) electrophoresis, and transferred to Nytran membranes as described previously. The isolated HHV-8 complementary DNA clones or the Epstein-Barr virus terminal repeat DNA fragments *(17)* were labeled with [α-^{32}P]dCTP (Amersham Life Sciences) and used to probe prepared membranes containing HHV-8-positive and HHV-8-negative DNAs. After two stringent washes at 65 °C with 0.1× SSC (15 m*M* NaCl and 15 m*M* sodium citrate) that contained 1% sodium dodecyl sulfate for 30 minutes, the hybridized membranes were exposed to x-ray film for 16 hours at −70 °C.

***In situ* hybridization.** Immediately after biopsy, Kaposi's sarcoma specimens were transferred to freshly prepared 4% paraformaldeyde in phosphate-buffered saline. After dehydration and embedment in paraffin, thin sections (5–10 μm) were prepared and subjected to *in situ* hybridization *(4,5).* The coding region for v-cyclin D and the HHV-8 latency-associated gene, kaposin *(42),* were amplified from HHV-8 clone 7-2 or Kaposi's sarcoma genomic DNA (kaposin) by use of the following PCR primers: cyclin (sense, 5′-ATGGCAACTGC-CAATAACC-3′; antisense, 5′-ATAGCTGTC-CAGAATGCG-3′) and kaposin (sense, 5′-CTCCTCACTCCAATCCCAATGC-3′; antisense, 5′-TTATTGTTTTTGAACATGTGGC-3′). The amplified DNA was then subcloned into the plasmid vector Bluescript SK+ (Stratagene). The orientation and sequence of the 775-base-pair v-cyclin D and 680-base-pair kaposin PCR products were confirmed by means of automated DNA sequencing as previously described. Both plasmids were linearized to generate sense and antisense RNA probes. Synthesis of ^{35}S-labeled complementary RNA probes and *in situ* hybridization were carried out essentially as described *(4,5).* After photographic development of probed sections for 10 days, the specimens were fixed, stained with hematoxylin, and counterstained with eosin.

Results

v-Cyclin D transcripts detected by means of RT–PCR. RT–PCR was used to examine v-cyclin D expression in clinical samples obtained from patients with Kaposi's sarcoma and in HHV-8 PCR-positive cell cultures (Fig. 1). Transcripts for v-cyclin D were clearly detected in RNA isolated from peripheral blood mononuclear cells obtained from a patient with nodular stage classical Kaposi's sarcoma (Fig. 1, lane 1), from pleural effusion cells from a patient with AIDS-related pulmonary Kaposi's sarcoma (Fig. 1, lane 3), and from a skin biopsy from a patient with early stage AIDS-related Kaposi's sarcoma (Fig. 1, lane 5). Moreover, transcripts for v-cyclin D were identified in cultures of spindle cells derived from the peripheral blood of patients with AIDS-related Kaposi's sarcoma (both early patch and late nodular lesions) (five of six examined) (Fig. 1, lanes 10–14) and in RNA isolated from a primary effusion lymphoma (Fig. 1, lane 4). Spindle cells derived from nodular stage lesions of classical Kaposi's sarcoma contained v-cyclin D transcripts only at early passages (Fig. 1, lanes 7 and 8), whereas no v-cyclin D transcripts were observed in RNA isolated from the same cultures at later passages (>4) (one of two is shown) (Fig. 1, lane 2). These findings were in agreement with our inability to amplify HHV-8 genomic sequences from spindle cells of classical Kaposi's sarcoma at later passages (data not shown).

Isolation of v-cyclin D complementary DNA. Complementary DNA cloning was used to isolate and characterize HHV-8 transcripts containing v-cyclin D sequences. The 20-kilobase HHV-8 clone 7-2, known to contain several HHV-8-encoded genes [including v-cyclin D, a G-protein-coupled receptor homologue, and the major tegument protein *(43)*], was used to screen a primary effusion lymphoma complementary DNA library generated from uncultured tumor cells. Several complementary DNA clones, with inserts ranging from 1.5 to 2.9 kilobases, that cross-hybridized with each other were isolated (data not shown). A representative 2.9-kilobase clone, 1A, was used in further analyses. To evaluate whether clone 1A originated from HHV-8-encoded sequences, Southern blot hybridization was performed (Fig. 2). Radiolabeled clone 1A hybridized with genomic DNA derived from cells of an AIDS-related Kaposi's sarcoma pleural effusion and from cells of a primary effusion lymphoma (Fig. 2, A, lanes 3 and 4, respectively), whereas there was no apparent hybridization in lanes containing DNA from P3HR1 cells or cells of the Epstein-Barr virus-infected lymphoblastoid cell line Ram (Fig. 2, A, lanes 1 and 2, respectively). Hybridization of the same blot with an Epstein-Barr virus terminal repeat probe demonstrated the pres-

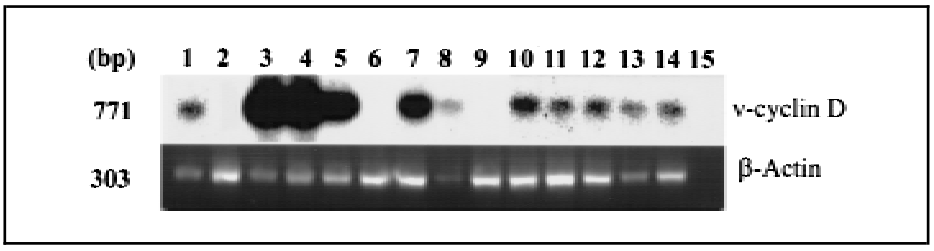

Fig. 1. Reverse transcriptase–polymerase chain reaction (RT–PCR) analysis demonstrating human herpesvirus-8 gene expression in specimens from patients with Kaposi's sarcoma. Lane 1, peripheral blood mononuclear cells from a patient with classical Kaposi's sarcoma; lane 2, spindle cell culture from a patient with classical Kaposi's sarcoma, passage 6; lane 3, pleural effusion cells from a patient with acquired immunodeficiency syndrome (AIDS)-related Kaposi's sarcoma; lane 4, primary effusion lymphoma cells, uncultured; lane 5, AIDS-related early stage Kaposi's sarcoma skin biopsy; lane 6, AIDS-related B-cell lymphoma; lanes 7 and 8, spindle cell cultures from patients with classical Kaposi's sarcoma, passages 1 and 2, respectively; lanes 9 through 14, peripheral blood-derived spindle cell cultures from patients with AIDS-related Kaposi's sarcoma; and lane 15, water only control (i.e., no template RNA). Top panel, Southern blot autoradiograph for the detection of v-cyclin D transcripts; bottom panel, ethidium bromide-stained gel of the β-actin control for each RNA sample. The RT–PCR conditions and the primers used are described in the ''Patients, Materials, and Methods'' section. bp = base pair.

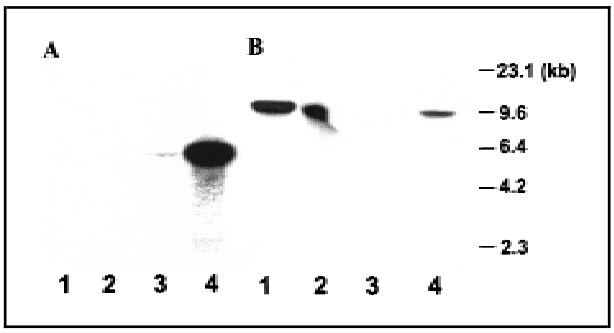

Fig. 2. Southern blot analysis. DNA was extracted from the following: 1) P3HR1, an Epstein-Barr virus-infected cell line; 2) Ram, an Epstein-Barr virus-infected lymphoblastoid cell line; 3) pleural effusion cells from a patient with acquired immunodeficiency syndrome-related Kaposi's sarcoma who had pulmonary lesions; and 4) primary effusion lymphoma cells. Ten micrograms of genomic DNA each was digested with the restriction enzyme *Bam*HI. The digested DNA was resolved electrophoretically in a 0.9% agarose gel, transferred to a Nytran membrane, and hybridized with a ^{32}P-labeled DNA probe from clone 1A **(A).** The same filter was stripped and rehybridized with an Epstein-Barr virus terminal repeat probe **(B).** The filters were exposed to Kodak X-Omat film for 16 hours at −70 °C. The lane numbers in **A** and **B** correspond to the specimen numbers given above, and the numbers to the right of the figure indicate the position of DNA size markers in the gel. kb = kilobase. *See* the text for additional details.

ence of Epstein-Barr viral sequences only in those lanes containing DNA from P3HR1 cells, Ram cells, and cells of the primary effusion lymphoma (Fig. 2, B, lanes 1, 2, and 4, respectively). There was no apparent hybridization observed in the lane containing DNA from the AIDS-related Kaposi's sarcoma pleural effusion cells (Fig. 2, B, lane 3), even after prolonged exposure. These findings suggested that clone 1A encoded an HHV-8-specific gene product. *In vitro* transcription and translation were performed with clone 1A to determine the apparent mass of the encoded protein. A protein of approximately 29 kd was observed when the translation products were resolved by means of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (data not shown). DNA sequencing and analysis of clone 1A identified an open reading frame that encoded a protein with homology to open reading frame 72 of *H. saimiri* (33.6% homology) *(44)*, several proteins of the human D-type cyclin family *(45)*, and the recently reported cyclin homologue encoded by HHV-8 *(34)*. The size of the cyclin protein encoded by clone 1A is in agreement with the predicted size of v-cyclin D determined by sequencing of previously isolated HHV-8 genomic clones *(34)*.

Detection of v-cyclin D transcripts by means of *in situ* hybridization. Transcripts for v-cyclin D are found in Kaposi's sarcoma lesions; however, the cell type-specific expression of v-cyclin D in Kaposi's sarcoma is not known. To determine whether Kaposi's sarcoma spindle cells contain v-cyclin D transcripts, *in situ* hybridization with specific RNA probes was performed (Fig. 3). In all tissue sections of late nodular Kaposi's sarcoma examined (four AIDS-related Kaposi's sarcomas and one classical Kaposi's sarcoma), approximately 60% of the cells were positive (Fig. 3, A and B). Cells containing v-cyclin D transcripts were arranged in bundles and revealed elongated nuclei, which are histologic hallmarks of Kaposi's sarcoma spindle cells. In some cases, endothelial cells lining blood vessels in Kaposi's sarcoma lesions were positive, whereas, in the overlying epidermal layer, no apparent signal for v-cyclin D was observed (data not shown). The staining pattern for v-cyclin D mRNA was similar to that found for transcripts of the HHV-8-encoded latency-associated gene, kaposin *(39,46)* (Fig. 3, G and H), which has been shown to be expressed in most spindle cells and endothelial cells lining blood vessels in Kaposi's sarcoma lesions *(39,46)*. As a result of the consequential altered morphology produced in the tissue sections by the *in situ* hybridization procedure, it was not possible to determine whether a subpopulation of the v-cyclin D-expressing cells could have been lymphocytes or monocytes, which have been recently determined to be permissive for HHV-8 infection *(38,47)*. It is interesting that, in Kaposi's sarcoma lesions at earlier stages of development (patch stage), very few cells (approximately 1%) were positive for v-cyclin D (Fig. 3, C and D). To control for hybridization specificity, a biopsy specimen from an uninvolved skin area of a patient with AIDS-related Kaposi's sarcoma was hybridized with the antisense v-cyclin D probe, and sections of nodular Kaposi's sarcoma lesions were hybridized with the v-cyclin D sense probe. There were no apparent hybridization signals observed in either of these two control experiments (Fig. 3, E and F, respectively).

Discussion

Our findings demonstrate, by means of *in situ* hybridization, that Kaposi's sarcoma spindle cells and endothelial cells lining blood vessels in Kaposi's sarcoma lesions contain transcripts for a viral homologue of cellular cyclin D2, i.e., v-cyclin D. Moreover, the pattern of detection of v-cyclin D transcripts in spindle cells from the clinical stages of Kaposi's sarcoma (early patch and late nodular) parallels a pattern seen for transcripts of the HHV-8 latency-associated gene, kaposin *(39,46)*. The post-transcriptional processing of herpesvirus mRNA species is complex, frequently involving multiple splicing events *(48)*. The isolation and characterization of a v-cyclin D complementary DNA from an uncultured primary effusion lymphoma library suggest that transcripts encoding v-cyclin D are indeed present *in vivo* and are, therefore, likely to be translated into protein. The significance of these results is not known; however, several hypotheses can be proposed. There are several lines of evidence that suggest HHV-8 latently infects most Kaposi's sarcoma spindle cells *(17,39,42,46,49)*. First, in their initial report, Chang et al. *(17)* observed that Kaposi's sarcoma lesions contain at least one copy of the HHV-8 genome per cell, a characteristic of latent infection by herpesviruses. Likewise, Decker et al. *(49)* showed that HHV-8 genomic DNA in Kaposi's sarcoma lesions was circular, as opposed to the linear form associated with lytic or productive infection. Therefore, this single-copy, circular presentation of the HHV-8 genome in Kaposi's sarcoma lesions suggests a latent mode of infection. Second, viral gene expression is generally restricted during latent herpesvirus infection. In Kaposi's sarcoma lesions, *in situ* hybridization has detected transcripts for the minor capsid protein *(38)*, v-bcl-2, v-G-protein-coupled receptor, and v-macrophage inflammatory protein-1A with similar low-level positivity (1%) (Stürzl M, Blasig C, Opalenik SR, Browning PJ: unpublished observations). These expressing cells may represent the small subset of those that, as demon-

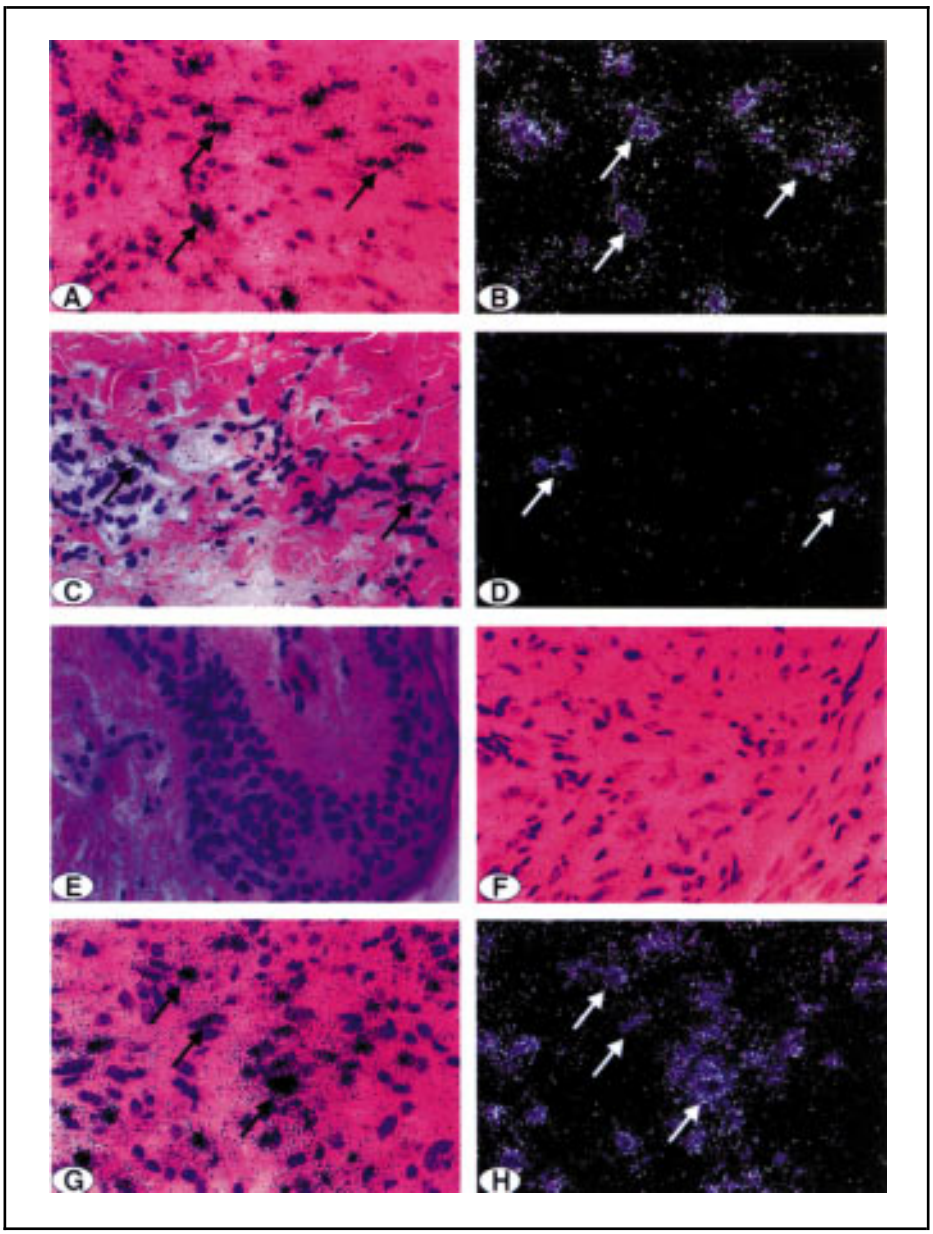

Fig. 3. *In situ* hybridization analyses. Hybridization with ^{35}S-labeled RNA probes was performed on sections of tumor stage Kaposi's sarcoma **(A, B,** and **F** through **H),** early stage Kaposi's sarcoma **(C** and **D),** and uninvolved skin from a patient with acquired immunodeficiency syndrome (AIDS)-related Kaposi's sarcoma **(E).** Panels **A** through **D:** antisense v-cyclin D probe; bright field (**A** and **C**) and dark field (**B** and **D**) microscopy. Transcripts for v-cyclin D are detected in the majority of cells in late nodular Kaposi's sarcoma (**A** and **B**) and in a few cells of early stage Kaposi's sarcoma (**C** and **D**). No apparent hybridization signals were observed in the uninvolved skin area from the patient with AIDS-related Kaposi's sarcoma (**E**) and in the control hybridization sample probed with a sense v-cyclin D probe (**F**). Hybridization with a kaposin antisense probe suggested that, in AIDS-related Kaposi's sarcoma lesions, most of the cells are latently infected with human herpesvirus-8; bright field (**G**) and dark field (**H**) microscopy.

strated by Orenstein et al. *(50)* with transmission electron microscopy, contain herpesvirus-like particles. In contrast, most spindle cells from late nodular Kaposi's sarcoma lesions produced transcripts for v-cyclin D and kaposin *(39,46)*, as well as for latent nuclear antigen *(51)*. Therefore, this pattern of restricted HHV-8 gene expression in the majority of Kaposi's sarcoma spindle cells provides additional support favoring a latent mode of HHV-8 infection in Kaposi's sarcoma lesions.

With regard to Kaposi's sarcoma pathogenesis, the expression of v-cyclin D mRNA in Kaposi's sarcoma spindle cells may have an important impact on tumor progression. Chang et al. *(31)* and Li et al. *(37)* have previously demonstrated that HHV-8 encodes a functional cyclin protein. Overexpression of cellular D-type cyclin proteins has been shown to accelerate cell cycle progression through the G_1 phase, leading to increased numbers of cells in S phase (DNA synthesis) *(52)*. Moreover, several studies *(53–55)* suggest a direct association between the stage of Kaposi's sarcoma and the percentage of cells in S phase. Kaposi's sarcoma lesions at all stages have an S-phase fraction, on the basis of flow cytometry, of 2%–18% *(54)*. Furthermore, the percentage of cells in S phase and G_2M phase is highest in late stage nodular Kaposi's sarcoma lesions *(53,55)*. The data presented in this report demonstrate expression of v-cyclin D in approximately 60% of Kaposi's sarcoma spindle cells. Therefore, expression of v-cyclin D may explain the previously reported increase of late nodular Kaposi's sarcoma spindle cells in S phase and G_2M phase. Moreover, overexpression of v-cyclin D in peripheral blood spindle cells derived from patients with Kaposi's sarcoma may explain, in part, why these HHV-8-infected cells are increased in the circulation of human immunodeficiency virus-1-infected individuals with Kaposi's sarcoma and those individuals at highest risk to develop this disease—homosexual and bisexual men, as well as female sexual partners of bisexual men *(40)*. In conclusion, the results of this study clearly demonstrate that v-cyclin D mRNA is predominant in the tumor cells of Kaposi's sarcoma. Therefore, v-cyclin D protein may be involved in the pathogenesis of Kaposi's sarcoma and, consequently, may represent a target for therapeutic intervention.

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Notes

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