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Restricted expression of Kaposi sarcoma-associated herpesvirus (human herpesvirus 8) genes in Kaposi sarcoma

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ABSTRACT **Kaposi sarcoma (KS) is the leading neoplasm of HIV-infected patients and is also found in several HIV-negative populations. Recently, DNA sequences from a novel herpesvirus, termed KS-associated herpesvirus (KSHV), or human herpesvirus 8 (HHV-8) have been identified within KS tissue from both HIV-positive and HIV-negative cases; infection with this agent has been proposed as a possible factor in the etiology or pathogenesis of the tumor. Here we have examined the pattern of KSHV/HHV-8 gene expression in KS and find it to be highly restricted. We identify and characterize two small transcripts that represent the bulk of the virus-specific RNA transcribed from over 120 kb of the KSHV genome in infected cells. One transcript is predicted to encode a small membrane protein; the other is an unusual polyadenylylated RNA that accumulates in the nucleus to high copy number. This pattern of viral gene expression suggests that most infected cells in KS are latently infected, with lytic viral replication likely restricted to a much smaller subpopulation of cells. These findings have implications for the therapeutic utility of currently available antiviral drugs targeted against the lytic replication cycle.**

Kaposi sarcoma (KS) is the most common neoplasm occurring in patients with AIDS (1). The lesion is histologically complex, containing proliferating spindle-shaped cells thought to be of endothelial origin, as well as infiltrating mononuclear cells, plasma cells, and abundant slit-like neovascular spaces. Although HIV infection is a major risk factor for KS development, epidemiologic studies suggest that it cannot be the sole determinant of KS risk and that other cofactors, possibly sexually transmitted, play an important role in the etiology of the tumor (2). For example, HIV-positive male homosexuals are at least 20-fold more likely to develop KS than HIV-infected children or hemophiliacs. In addition, KS occurs in several clinical settings in the absence of HIV infection (1). These and other observations have prompted a search for other viral agents that might be implicated in KS pathogenesis. Recently, DNA sequences of a novel member of the herpesvirus family, termed KS-associated herpesvirus (KSHV) or human herpesvirus 8 (HHV-8), have been regularly identified in KS tumors from both HIV-positive (3–5) and HIV-negative (6–9) patients. This agent has been proposed as a candidate for the presumed etiologic cofactor in KS (3, 10), but definitive evidence for a causal role in KS is lacking. As a first step toward understanding the molecular nature of the association between KS and KSHV/HHV-8, we have examined the nature of viral gene expression in KS tissue. Our studies reveal that viral gene expression is dramatically restricted in the tumor. Detailed characterization of the major viral transcripts produced in infection reveals them to be highly unusual RNAs quite distinct from those found in infection by related herpesviruses. These observations suggest that most infected cells in a KS lesion are latently infected, with lytic growth restricted to no more than a small subpopulation of cells, and bear importantly on potential roles for antiviral chemotherapy.

MATERIALS AND METHODS

Viral Gene Expression Screen. Genomic DNA was extracted from an AIDS-associated pulmonary KS lesion using previously described methods (11) and was partially digested with *Sau*IIIA. Restriction fragments from 10 to 20 kb were gel-purified and cloned into the bacteriophage vector Lambda FIX II (Stratagene). The initial probes used to screen the library were derived from two published sequences, one of which (about 900 bp) corresponds to the KSHV minor capsid gene and the other (about 630 bp) to a KSHV tegument gene (3). Each probe was prepared from the same pulmonary KS tumor by PCR amplification followed by plasmid subcloning. Chromosome walking was performed by isolating additional 1–3 kb subclones from the termini of each newly isolated phage insert, labeling them by random priming using synthetic hexanucleotide primers (RediPrime DNA Labeling System, Amersham) and [^{32}P]dCTP, and applying each labeled probe so prepared to the original KS genomic library. To prepare the cDNA probe used in Fig. 1*C*, about 100 ng of poly(A)$^+$ RNA extracted from the same KS tumor was reverse transcribed in a standard reaction containing 0.5 mM each of dATP, dGTP, and dTTP, 0.1 mM dCTP, 50 μCi [α-^{32}P]dCTP, 50 units of RNasin ribonuclease inhibitor (Promega), 1× concentration of hexanucleotide mixture (Boehringer Mannheim), and 200 units of Moloney murine leukemia virus reverse transcriptase in a total volume of 50 μl. The reaction was carried out at 37°C for 1 hr and stopped by heating at 95°C for 5 min The labeled cDNA probe was added to the KSHV filter prehybridized in Church hybridization solution (12) and incubated overnight at 65°C; the filter was then washed in 40 mM $NaHPO_4$, 0.1% SDS, 1 mM EDTA (pH 8.0) and subjected to autoradiography.

RNA and cDNA Probe Preparation. Total RNA was extracted by the RNAzol method (Tel-Test, Friendswood, TX) according to the manufacturer's directions; hybridization probes were prepared by randomly primed DNA synthesis using synthetic hexanucleotides (Amersham) as described by the manufacturer.

cDNA Library Screening. An oligo(dT) primed cDNA library from poly(A)$^+$ RNA of a cutaneous KS lesion was constructed in this laboratory using the Lambda ZAP-cDNA Synthesis Kit (Stratagene) according to the manufacturer's protocol (S. Chuck, unpublished data). Two probes, 21–0.8 K and 4–1.6 K (see Fig. 2), were used to screen the cDNA library and six positive isolates from each probe were sequenced. The sequences shown in Fig. 3 *A* and *B* represent the longest cDNA isolates. The DNA sequences of these two cDNA clones are identical to the sequences of corresponding genomic regions of KSHV that were cloned from the pulmonary KS specimen (see Fig. 1).

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Abbreviations: KS, Kaposi sarcoma, KSHV, KS-associated herpesvirus; HHV-8, human herpesvirus 8; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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Primer Extension. Primer extension reactions were performed as follows: 5 μg of total KS RNA and 1 pmol of end-labeled oligonucleotide primer were mixed in 10 μl of annealing buffer (100 mM NaCl/20 mM Tris, pH 8.3/0.1 mM EDTA/5 units/μl RNasin), heated at 90°C for 3 min and 56°C for 20 min, and then slowly cooled to room temperature. Extension mixture (40 μl) containing 62.5 mM Tris (pH 8.3), 94 mM KCl, 3.75 mM MgCl2, 12.5 mM DTT, 1.25 mM each of dATP, dCTP, dGTP, and dTTP, 0.6 μg actinomycin D, 30 units of RNasin, and 400 units of SuperScript II reverse transcriptase (GIBCO) was added to the RNA mixture. After incubation for 60 min at 42°C, the reaction was stopped by extraction with phenol and chloroform. Extension products were precipitated with ethanol, resuspended in sample buffer, and then resolved on a 6% acrylamide sequencing gel and autoradiographed.

293T Cell Transfection and Subcellular Fractionation. 293T cells were grown in HME16 medium supplemented with 10% fetal bovine serum. DNA transfection was performed by the calcium phosphate coprecipitation method (13). Three days after transfection, cells (in a 10-cm dish) were rinsed twice with phosphate-buffered saline and collected by low-speed centrifugation. The cell pellet was resuspended in buffer containing 10 mM Hepes (pH 7.9), 3 mM MgCl2, 10 mM KCl, 0.5 mM DTT, and 4 units per ml of RNasin inhibitor (Promega), kept on ice for 10 min, and then homogenized using a Dounce homogenizer. After confirming cell lysis microscopically, cytoplasmic and nuclear fractions were separated by centrifugation at 600 g for 15 min at 4°C. RNA was extracted directly from the cytoplasmic supernatant using RNAzol method (Tel-Test). For nuclear RNA, the nuclear pellet was washed twice with the above buffer and then subjected to RNAzol extraction.

RESULTS AND DISCUSSION

Identification of Expressed Viral Genes. KSHV DNA sequences were first identified by representational difference analysis as DNA present in KS but not uninvolved tissue samples from an AIDS-related KS case. This PCR-based procedure yielded two short (330 and 631 bp) DNA fragments with homology to herpesviral capsid and tegument components, respectively (3). From the published sequence of these clones (3), we designed oligonucleotide primers that allowed us to amplify and clone the corresponding regions from genomic DNA extracted from a fatal case of AIDS-associated pulmonary KS (data not shown). To obtain more extensive clones of KSHV DNA, a library of KS genomic DNA (from the same pulmonary KS tumor) was made in bacteriophage lambda. The library was initially screened with the capsid- and tegument-specific probes described above, and a series of overlapping clones was then isolated that spanned *ca.* 120 kb of the genome (cf. Fig. 1*A*). This large region of the viral genome was then scanned for expression in KS in the following way. First, DNA from each of these clones was digested with *Sal*I or *Sac*I, electrophoresed through 0.8% agarose, and stained with ethidium bromide (Fig. 1*B*). These DNA fragments were transferred to a nylon filter, and then hybridized with a radiolabeled cDNA probe representative of transcripts present in KS. This probe was prepared by reverse transcribing poly(A)$^+$ RNA from the same KS tumor, using random

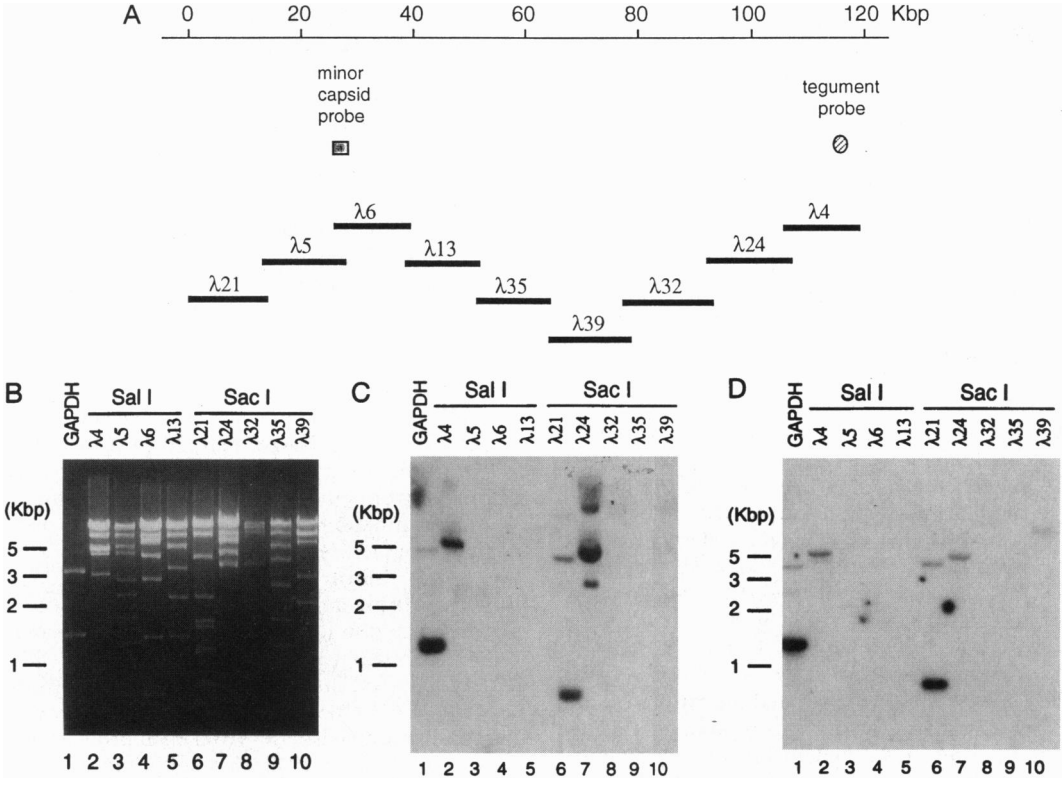

FIG. 1. Cloning of KSHV (HHV8) genomic fragments and identification of regions that are actively transcribed in a KS lesion. (*A*) Summary of overlapping clones of KSHV DNA (≈120 kbp) isolated from a bacteriophage lambda library of KS genomic DNA. (*B*) Ethidium-bromide stained agarose gel (0.8%) of KSHV DNA digested with *Sal*I or *Sac*I (lanes 2 to 10) as indicated. These two restriction enzymes were chosen because they cleave on either side of the insert but not in the phage arms, and generate a suitable number of KSHV-specific fragments. In lane 1, a GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA clone was digested with *Pst*I (to release it from its plasmid vector) and electrophoresed in parallel. (*C*) Hybridization of filter-bound GAPDH DNA (lane 1) or KSHV DNAs (lanes 2–10) with cDNA probe reverse transcribed from poly(A)$^+$ RNA of the pulmonary KS. Hybridization to the GAPDH sequences serves as a control for the integrity and representativeness of the cDNA probe. (*D*) Control hybridization of filter-bound GAPDH (lane 1) or KSHV DNAs (lanes 2–10) to cDNA probe reverse transcribed from poly(A)$^+$ RNA from uninduced BCBL-1 B-lymphoma cells, which are latently infected with KSHV/HHV-8 (14).

oligonucleotide primers and murine leukemia virus reverse transcriptase; the probe thus represents all cellular as well as viral transcripts. As a control for the sensitivity of the method, we showed that transcripts of a single-copy, cellular housekeeping gene, GAPDH, could be readily detected in this fashion (Fig. 1*C*, lane 1).

When the same probe was hybridized to the filter-bound KSHV cloned DNAs, a strikingly simple pattern was observed (Fig. 1*C*, lanes 2–10). The majority of the cloned KSHV fragments annealed poorly or not at all to the probe, with detectable hybridization limited primarily to clones 4, 24, and 21. Subsequent experiments using plasmid subclones of these phage (not shown) revealed that (*i*) the hybridization to clones 4 and 24 mapped to a 1.6-kb restriction fragment in their overlapping region and that (*ii*) the hybridization to clone 21 was primarily to a 0.8-kb subfragment. To more directly visualize the transcripts corresponding to these genomic regions, radiolabeled probes were prepared from these 1.6 and 0.8 kb KSHV-specific subclones and annealed to Northern blots of total RNA from the aforementioned pulmonary KS tumor (Fig. 2). This showed that the RNA annealing to clone 21 corresponded to a 1.1-kb transcript (Fig. 1*A*, lane 1), whereas that corresponding to clones 4 and 24 was a 0.7-kb RNA (Fig. 1*B*, lane 1). (We shall refer to these transcripts as T1.1 and T0.7, respectively.) In other experiments (data not shown), both species were shown to be enriched by selection on oligo(dT)-cellulose, indicating that they correspond to polyadenylylated transcripts; this inference has been confirmed by analysis of cDNA clones (see below). Neither transcript was detectable in normal lung tissue from the same patient (Fig. 2 *A* and *B*, lanes 2), and hybridization to GAPDH probe (Fig. 2*C*) confirmed that equal amounts of KS and non-KS RNA were loaded in each lane. The same transcriptional pattern seen here was also seen in a second KS tumor by Northern blot analysis; in addition, cDNA cloning from a third KS tumor also revealed the expression of these same two transcripts in that tumor. This indicates that this pattern is highly representative of gene expression in AIDS-associated KS.

Recently, we have identified a B cell line (BCBL-1) that is latently infected with KSHV/HHV8 (ref. 14; K. Komanduri, J. Luce, M. McGrath, and V. Ng, unpublished work). These cells were derived from a patient with a body cavity-based B cell lymphoma, a rare tumor in which KSHV/HHV8 DNA sequences are uniformly found (ref. 16; K. Komanduri, J. Luce, M. McGrath, and V. Ng, unpublished work). Under normal growth conditions, these cells display a highly restricted pattern of gene expression (14). However, on treatment with the phorbol ester TPA (phorbol 12-tetradecanoate 13-acetate), lytic viral replication is induced, as judged by the induction of viral DNA synthesis, transcription of extensive regions of the viral genome, and the accumulation of enveloped virions in the cells and medium (14). This indicates that the restricted pattern of gene expression seen in the absence of TPA corresponds to an authentic latent infection. To ask how the pattern of expression we observed in KS compares with this bona fide latency pattern, a control experiment was performed in which RNA from untreated BCBL1 cells was used to generate labeled cDNA that was then applied to filter-bound cloned KSHV DNAs. As previously reported (14), only a few regions of viral DNA are transcriptionally active before TPA induction (Fig. 1*D*); comparison with Fig. 1*C* shows that the patterns of expression in KS tumors and in uninduced BCBL-1 cells are strikingly similar, with the principal signals in both cases emanating from lambda clones 4, 21, and 24. These findings strongly support the notion that most infected cells in KS are latently infected.

We consider it unlikely that the two RNAs identified thus far are the sole viral transcripts present in KS for several reasons. First, the library of cloned fragments screened in Fig. 1 does not include the complete viral genome (whose size we estimate at ≈165 kb), raising the possibility that additional transcripts may be encoded by regions we have not yet cloned. Second, our screening method (Fig. 1) is likely biased in favor of abundantly expressed RNAs, so that low-level transcripts may have passed undetected. (In fact, long autoradiographic exposures of the filter of Fig. 1*C* do reveal a small number of very faint additional bands whose origin is curently under study.) Nonetheless, the data clearly establish that KSHV/HHV-8 infection

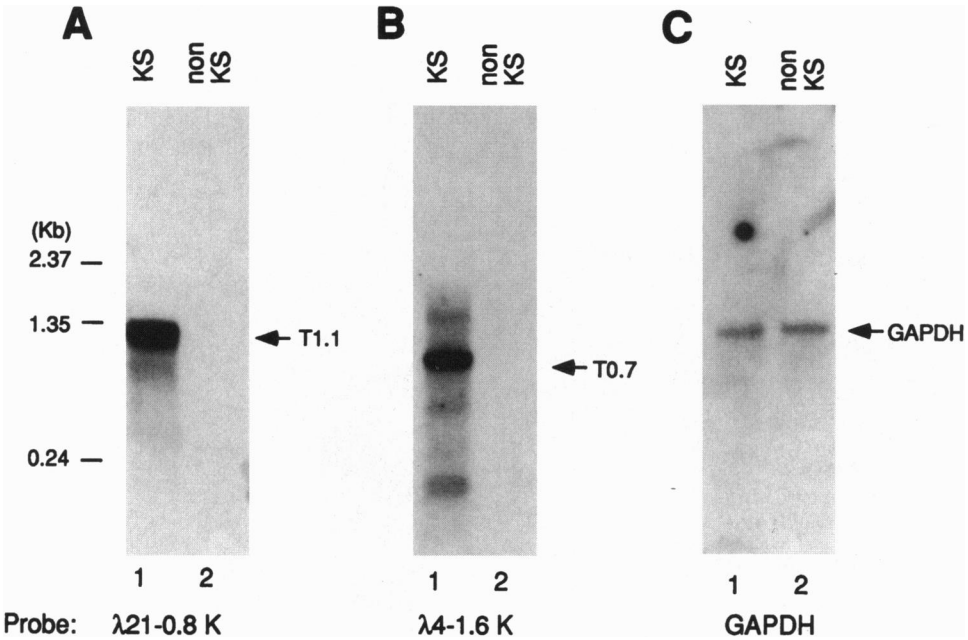

FIG. 2. Northern blot hybridization of total RNA from KS and normal pulmonary tissue of the same patient. Total RNA (≈10 μg per lane) extracted from KS-involved lung (lanes 1) and normal lung (lanes 2) was separated on a 1.2% agarose-formaldehyde gel and transferred to nylon filters. The filters were probed with probes 21–0.8 K (a 0.8-kbp fragment from lambda clone 21; *A*), 4–1.6 K (a 1.6-kbp fragment from lambda clone 4; *B*), and GAPDH (*C*). GAPDH control was used to ensure the integrity of the RNA samples and that equal amounts of RNA were present in each lane.

of cells in KS involves a dramatic restriction of viral gene expression, indicating that most infected cells in KS are not productively infected (see below).

Characterization of the Viral Transcripts. To characterize in more detail the structure of these RNAs, we used the probes described in Fig. 2 to screen an oligo(dT)-primed cDNA library prepared from a cutaneous KS lesion of a different AIDS patient. Six positive cDNA clones were identified for each of the two viral transcripts; the DNA sequences of the longest of each of the clones is presented in Fig. 3. Both sequences contained canonical polyadenylylation signals (AAUAAA) 12–24 nt upstream of a homopolymeric run of adenosine. The sizes of the clones (1096 and 717 nt) conformed closely to the approximate sizes of the transcripts estimated by Northern blot analysis, suggesting that the clones were full-length. To verify this, we mapped the 5′ end of each RNA by

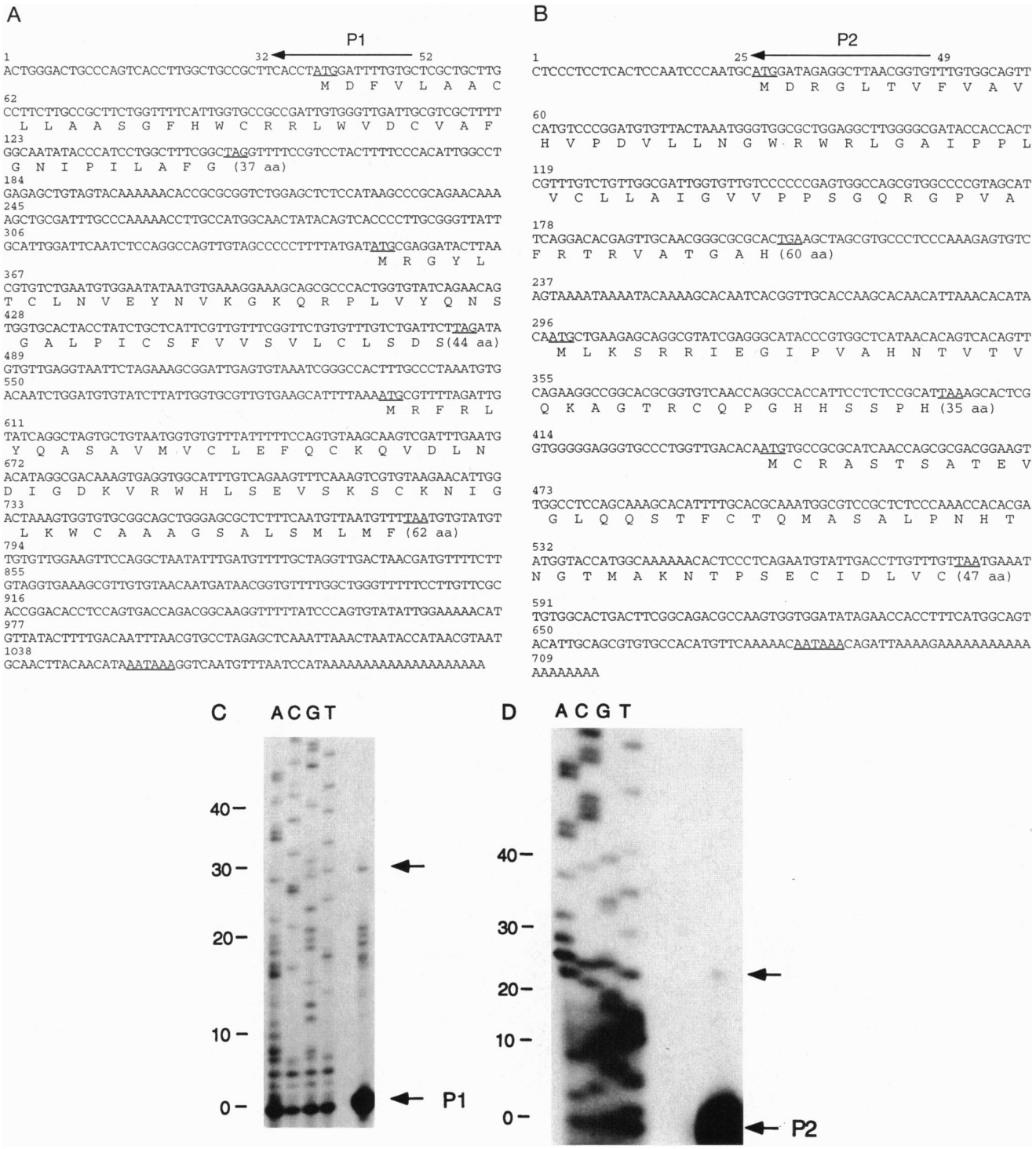

FIG. 3. Fine structure of the cDNAs corresponding to the two KSHV transcripts identified in Figs. 1 and 2. (*A*) T1.1 and its putative ORFs. (*B*) T0.7 and its putative ORFs. The start and stop codons of each putative ORF and the polyadenylylation signal are underlined; the region corresponding to the primer used for primer extension experiments is overlined. (*C*) Primer extension reaction to map the 5′ end of T1.1. A synthetic oligonucleotide spanning nt 32–52 (depicted as P1 in *A*) was end-labeled, annealed to total RNA from a KS tumor, and extended with SuperScript II reverse transcriptase, and the products were resolved on a sequencing gel. (*D*) Primer extension mapping of the 5′ end of T0.7, using the primer P2 depicted in *B*; this primer spans nt 25–49. Primer extension was carried out as described for *C*.

primer extension, using the synthetic oligonucleotide primers indicated in the legend to Fig. 3. As shown in Fig. 3*C* for T1.1 and Fig. 3*D* for T0.7, the 5′ ends of each RNA corresponded closely to those defined by the 5′ most nucleotide of the longest isolated cDNA clones. In addition, the position of the 5′ end of T1.1 was independently confirmed by RNase protection experiments using RNA probes spanning the start site depicted in Fig. 3*A* (data not shown).

We next determined the nucleotide sequences of the genomic loci corresponding to these RNAs and compared them with their respective cDNA sequences. This revealed complete colinearity in the body of each transcript, indicating that (*i*) neither RNA is derived by splicing or RNA editing and that (*ii*) the sequences encoding both transcripts are entirely conserved in two epidemiologically unrelated isolates of KSHV. The KSHV genomic DNA sequence 5′ to each start site is shown in Fig. 4*A* (for T1.1) and Fig. 4*B* for (T0.7). The putative regulatory sequences for T1.1 appear to conform to expectation for a typical polymerase II promoter: they include an appropriately positioned TATA element, a potential CAAT box, and several potential AP1 sites. In addition, the sequence displays two so-called CArG (*C A-rich G*-containing) boxes, sequences that have been previously identified to control expression in muscle, including vascular smooth muscle (17–19). By contrast, the T0.7 upstream regulatory sequences are highly distinctive. No TATA box is present, and the start site is embedded within the distal region of the sequence CGGGACCTGGTGCCCTCCTCCC, which is directly repeated over 12 times 5′ to the start site. Because we have found no evidence for RNA splicing in this region, we presume that these sequences must indeed supply promoter function, and experiments are underway to test this hypothesis.

The coding organization of these transcripts revealed several surprises (cf. Fig. 3). Although T1.1 is translationally open from its start site, the first in-frame AUG is not reached until nt 39, and this start codon is in a suboptimal sequence context for initiation (20). As a result, the 5′ most open reading frame (ORF) is only 37 aa long. Two other short ORFs are also present on the RNA, though their internal position makes their translation quite problematic. These peculiarities suggested that this transcript might not serve as a mRNA *in vivo*. This inference is further supported by analysis of the subcellular localization of this RNA. During a separate *in situ* hybridization study of infected cell types in KS (K. Staskus, W.Z., K. Gebhardt, H.W., B.H., D.G., and A Haase, unpublished work), we noticed that cells expressing T1.1 appeared to have the transcript localized primarily to the nucleus (data not shown).

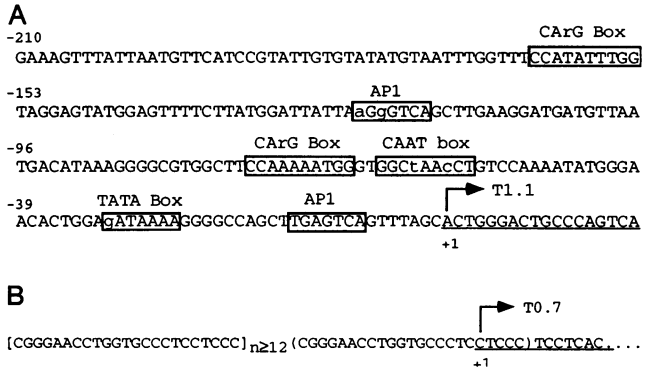

FIG. 4. (*A*) Genomic sequence upstream of T1.1 RNA. Boxes denote sequences homologous to binding sites for known transcription factors; lowercase letters indicate nucleotides within these sequences that are different from the consensus recognition sequence. Arrow indicates position of the 5′ end of the transcript; body of transcript is underlined. (*B*) Genomic sequence upstream of T0.7 RNA. Multiple copies of a 23-bp G+C-rich repeat are present in tandem; transcription initiates within the last repeat (at nucleotide denoted by arrow).

To affirm this result biochemically and to determine whether other viral gene products are important in the nuclear localization of this RNA, we examined the subcellular distribution of T1.1 RNA in transfected cells expressing only this viral gene product. 293T cells were transiently transfected with a genomic clone spanning the T1.1 locus (including all of the regulatory signals shown in Fig. 4*A*) and poly(A)$^+$ RNA from nuclear and cytoplasmic fractions was examined by Northern blot analysis. As shown in Fig. 5, T1.1 RNAs are localized primarily to the nucleus, under conditions in which GAPDH mRNA is largely cytoplasmic. This indicates that information for nuclear localization is intrinsic to the transcript and that no other viral functions are required to assure its nuclear delivery or retention. Most likely, interactions with cellular factor(s) are required for nuclear localization, and a search for such factors is currently underway. By comparison of the signal intensity of T1.1 RNA on Northern blots with that of U12 RNA and normalizing for the number of cells expressing this transcript (K. Staskus, W.Z., K. Gebhardt, H.W., B.H., D.G., and A. Haase, unpublished work; see below), we estimate that each T1.1-positive nucleus harbors ≈25,000 copies of the RNA (data not shown).

In this connection, it is noteworthy that several lymphotropic herpesviruses are known to produce abundant nuclear RNAs. Cells latently infected by Epstein–Barr virus produce the so-called EBER RNAs, small, structured RNAs that associate in the nucleus with several host proteins, including the autoantigen La (21). EBERs, however, are nonpolyadenylylated and are transcribed by RNA polymerase III (22). Herpesvirus saimiri, a simian virus related to HHV8, encodes several abundant U-like nuclear RNAs during latent infection. Like host U RNAs, they are complexed with cellular Sm proteins in ribonucleoprotein particles; although transcribed by RNA polymerase II, they are also nonpolyadenylylated (23). The accumulation of stable, nuclear poly(A)$^+$ transcripts in KSHV/HHV-8 infection is unique among herpesviruses. Perhaps they too are organized into a small nuclear ribonucleoprotein-like particle with actions on RNA processing or transport. Alternatively, the naked RNA might play a structural role in the nucleus or have catalytic function; similar speculations have been proffered for the cellular RNA Xist, a large, noncoding, polyadenylylated nuclear transcript implicated in X chromosome inactivation (24, 25). Another possibility is that these RNAs might be stored in the nucleus, awaiting transport to the cytosol under the influence of another viral gene product, e.g., one analogous to HIV rev. If so, however, such a product is not being expressed during latent infection of target cells in KS.

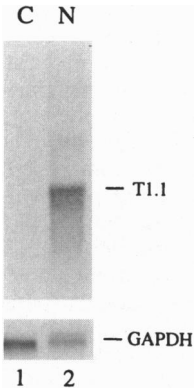

FIG. 5. Subcellular localization of T1.1 RNA in transfected 293T cells. 293T cells were transfected with a T1.1 genomic DNA clone (including sequences flanking T1.1), and then fractionated into nuclear and cytoplasmic fractions as described. Cytoplasmic (C) and nuclear (N) RNA were extracted with RNAzol and probed for T1.1 RNA sequences (*Top*) or GAPDH (*Bottom*).

Like that of T1.1, the sequence of T0.7 likewise displays only short ORFs (Fig. 3). Its longest coding region, only 60 aa long, is positioned at the 5′ end of the RNA, a position compatible with its translation by scanning ribosomes. *In situ* hybridization studies reveal that T0.7 RNAs do access the cytoplasmic compartment (K. Staskus, W.Z., K. Gebhardt, H.W., B.H., D.G., and A. Haase, unpublished work), consistent with their likely messenger function. The sequence of the 5′ ORF has no homologs in the GenBank data base; however, it displays a strikingly hydrophobic nature, strongly suggesting that its product is a membrane protein, most likely with at least two transmembrane domains. The small size and extreme hydrophobicity of this polypeptide recall similar attributes of the 44 aa long E5 protein of bovine papilloma virus, a major transforming protein that acts through interaction with the platelet-derived growth factor receptor in cell membranes (26, 27); thus, its small size does not exclude important potential biological roles. Its expression during latency, its likely membrane association, and its location downstream of genomic repeats also recall like features of the major transforming protein of Epstein–Barr virus, latent membrane protein 1 (LMP-1) (28). However, the product of T0.7 has no homology to latent membrane protein 1 or to E5 at the primary sequence level, and we emphasize that we have not yet determined whether this product has transforming properties *in vivo*.

Implications. These results have several implications for biology of infection by KSHV/HHV-8 in KS tumors. Herpesviruses generally can produce one of two modes of infection, latent or productive, depending on the cell type infected and other physiologic parameters (29). The productive (or lytic) infection is characterized by vegetative viral replication and the release of progeny virions. Latent infection is noncytolytic and is generally characterized by (*i*) the maintenance of the viral genome in the nucleus at low copy number; (*ii*) a dramatic restriction of viral gene expression; and (*iii*) the lack of progeny virion production. Often, latent and lytic infections involve different cell types. However, because latently infected cells harbor the entire viral genome, they retain the capacity to produce lytic viral growth upon receipt of the appropriate signal; in latently infected B cells, for example, productive Epstein–Barr virus infection can be evoked by phorbol ester exposure (30, 31). Our data are most consistent with latent infection of one or more cell types as the predominant mode of infection in the KS lesion.

The predominantly nonproductive nature of KSHV infection in KS implies that antiviral drugs like acyclovir, which target the lytic viral replicative cycle (32), will have little effect on the infection at the level of most individual tumor cells. However, it is possible to envision that such drugs could alter the natural history of KS in the whole organism if (*i*) latent viral infection drives proliferation of target spindle cells, and (*ii*) this proliferative stimulus is not sustained (or is limited by apoptosis). If so, then the growth of the lesion as a whole would require the continuing recruitment of new latently infected cells by virus released from productively infected cells.

Recently, we have examined a larger number of KS tumors by *in situ* hybridization using probes for T1.1, T0.7, and the gene corresponding to the major viral capsid protein (K. Staskus, W.Z., K. Gebhardt, H.W., B.H., D.G., and A. Haase, unpublished work). These studies show that viral infection of KS is targeted to the endothelial (spindle) cell component, thought to be the central cell in the pathogenesis of the lesion; this result agrees well with independent *in situ* PCR analyses of the distribution of viral DNA (15). The vast majority of these cells express only T0.7 and are presumably latently infected. Interestingly, only ≈0.5–1% of these cells express T1.1 and many of these also express detectable quantities of the major capsid mRNA (K. Staskus, W.Z., K. Gebhardt, H.W., B.H., D.G., and A. Haase, unpublished work). Because the latter is expected to be expressed primarily in lytic growth, it is conceivable that T1.1 accumulation actually is a marker for the subpopulation involved in productive infection or in abortive versions of the lytic cycle. If such cells are indeed producing virions, they could be one source of virus to sustain ongoing cycles of infection in the lesion.

This manuscript is dedicated to the memory of Dr. Bernard Fields, whose innumerable contributions to the study of viral pathogenesis inspired a generation of American virologists. We gratefully acknowledge the support of the Howard Hughes Medical Institute, without which this work would not have been possible.

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