Journal of Virology, Aug. 1997, p. 5915–5921 Vol. 71, No. 8
0022-538X/97/$04.00+0
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The 222- to 234-Kilodalton Latent Nuclear Protein (LNA) of Kaposi's Sarcoma-Associated Herpesvirus (Human Herpesvirus 8) Is Encoded by orf73 and Is a Component of the Latency-Associated Nuclear Antigen

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Received 17 March 1997/Accepted 6 May 1997

Kaposi's sarcoma (KS)-associated herpesvirus or human herpesvirus 8 (KSHV/HHV8) is the likely cause of KS and primary effusion lymphomas or body cavity-based lymphomas (BCBLs). A latency-associated nuclear immunofluorescence antigen (LANA) (D. H. Kedes, E. Operskalski, M. Busch, R. Kohn, J. Flood, and D. Ganem, Nat. Med. 2:918–924, 1996; S. J. Gao, L. Kingsley, M. Li, W. Zheng, C. Parravicini, J. Ziegler, R. Newton, C. R. Rinaldo, A. Saah, J. Phair, R. Detels, Y. Chang, and P. S. Moore, Nat. Med. 2:925–928, 1996) and a 222- to 234-kDa nuclear protein (LNA) (S. J. Gao, L. Kingsley, D. R. Hoover, T. J. Spira, C. R. Rinaldo, A. Saah, J. Phair, R. Detels, P. Parry, Y. Chang, and P. S. Moore, N. Engl. J. Med. 335:233–241, 1996) have previously been described in BCBL cell lines by immunofluorescence and Western blotting techniques, respectively. To identify the viral gene(s) encoding this antigen(s) we screened a cDNA library from HBL-6 cells, a B-cell lymphoma cell line persistently infected with KSHV/HHV8, with KS patient sera. One set of positive clones contained the 3′ end of orf73, as well as the complete orf72 and orfK13, and another set contained the 5′ end of orf73. Comparison of cDNA sequences with the KSHV/HHV8 genomic sequence revealed a splice event, occurring upstream of orf73. Immunoaffinity purified antibodies to a recombinant carboxy-terminal fragment of the orf73-encoded protein showed the characteristic speckled nuclear immunofluorescence pattern of LANA and reacted with the 222- to 234-kDa LNA on Western blots. Expression of full-length orf73 in bacteria and COS7 cells reproduced the LNA banding pattern. Immunohistochemistry on cases of nodular KS revealed that orf73/LNA is expressed in the nucleus of KS spindle cells. These findings demonstrate that orf73 encodes the 222- to 234-kDa LNA, is a component of LANA, and is expressed in KS tumor cells.

Kaposi's sarcoma (KS)-associated herpesvirus (KSHV), or human herpesvirus 8 (10), is found in all forms of KS (2, 5, 11, 13, 21, 27, 35), in AIDS-associated body cavity-based lymphoma (BCBL) or primary effusion lymphoma (7), and in a proportion of cases of multicentric Castleman's disease (38). Detection of KSHV in peripheral blood of human immunodeficiency virus (HIV)-infected individuals correlates with and predicts the onset of KS (28, 42). Serological studies also indicate that infection with KSHV is common among HIV-infected homosexual men (16, 17, 19, 22, 37), who are known to have a high risk of developing KS (4), but not among other HIV risk groups such as patients with hemophilia and intravenous drug users (16, 17, 19, 37), who develop KS only exceptionally (4, 31). KSHV can be transmitted sexually (19, 22, 37), and its prevalence among homosexual men is increased by the same sexual risk factors (number and geographic origin of partners, receptive anal intercourse) (24) which have previously been found to increase the risk for KS (31). Although the prevalence of KSHV infection in the general United Kingdom and U.S. populations is still controversial, several serological studies indicate that it is higher in some Mediterranean countries and in particular in several parts of Africa (17, 22, 37). The seroepidemiology of KSHV is thus as expected for the infectious cause of KS and strongly suggests that KSHV is indeed the KS agent.

KSHV establishes a latent infection in KS spindle cells as well as BCBL cell lines (6, 14, 32, 43). KSHV genes known to be expressed in KS tissue include that for K12, a putative 60-amino-acid (60-aa) hydrophobic protein and the type D cyclin homolog (v-cyclin) encoded by orf72, the chemokine receptor homolog encoded by orf74, a nuclear RNA (T1.1), and the viral homologs of bcl-2 and MIP-1β (9, 39, 40, 43). Whereas most spindle cells express mRNA for K12 and v-cyclin, only a small proportion of spindle cells, which may undergo lytic replication, express the nuclear RNA (T1.1), v-bcl2, and v-MIP-II (39, 40).

Sera from KSHV-infected individuals react in immunofluorescence with a nuclear antigen in latently infected BCBL cell lines which is characterized by a speckled immunofluorescence pattern (17, 19, 29, 37) and has been termed latency-associated nuclear antigen (LANA) (19). The antigen was first discovered by using the Epstein-Barr virus (EBV)-KSHV coinfected cell line HBL-6 (29) but has since been used as a highly specific antigen for preparations from EBV^{-}-$KSHV^{+}$ BCBL cell lines

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(17, 19). On Western blots of nuclear extracts from the BC-1 cell line, patient sera also react with a 222- to 234-kDa doublet band, termed latent nuclear antigen (LNA) (16). Detection of Western blot-defined LNA correlates closely with LANA reactivity (17). However, whether LNA is identical with LANA has so far not been established. Both the latent immunofluorescence assay (IFA) and the LNA Western blot assay are very specific assays for the detection of KSHV antibodies (16, 17, 37).

In view of the important role that nuclear antigens (EBNAs) of EBV (human herpesvirus 4) a gamma-1 herpesvirus related to KSHV, play in B-lymphocyte transformation, we attempted to identify the viral gene(s) encoding LANA and/or LNA. We show here that LNA is encoded by orf73 of KSHV and that antibodies to this protein produce the speckled nuclear immunofluorescence pattern typical of LANA. This suggests that orf73/LNA is at least a component of, if not identical with, LANA. We also show that orf73/LNA is expressed in the majority of spindle cells of nodular KS lesions and thus is likely to play a role in the pathogenesis of KS.

MATERIALS AND METHODS

Construction and screening of HBL-6 cDNA library. A cDNA phage library of the unstimulated HBL-6 cell line (8, 15) was constructed in the λZapII vector according to the manufacturer's (Stratagene's) instructions and amplified. We screened approximately 10^6 phage on nitrocellulose filters, using a conventional protocol, with a pool of five sera from endemic KS (HIV-negative) patients. Patient sera were diluted 1:500 (each serum was diluted 1:2,500) in phosphate-buffered saline (PBS)–3% dried skim milk (Marvel)–0.1% Triton X-100–0.05% sodium azide–10% fetal calf serum and incubated with filters for 1 h at room temperature. Filters were washed in PBS–0.1% Tween 20, followed by an alkaline phosphatase-conjugated goat-anti human immunoglobulin G (IgG) (1:7,500; Seralab) and nitroblue tetrazolium with 5-bromo-1-chloro-3-indolylphosphate (BCIP) (Promega) as substrate. Positive phage were rescreened twice by the same protocol and then examined for their reactivities with patient sera and normal sera. After conversion of positive phage into a phagemid by the rapid excision system (Stratagene) and 704 helper virus according to the manufacturer's instructions, the inserts were sequenced by automated DNA sequencing (ABI 377 sequencer) and use of internal sequencing primers.

Expression of a carboxy-terminal fragment of orf73 and affinity purification of orf73-specific antibodies. A fragment corresponding to aa 951 to 1163 of the published orf73 sequence (33) was cloned into the *Bgl*II and *Sph*I sites of the bacterial expression vector pQE40 (Qiagen) by using the PCR primers GAG AAG ATC TCC GAT TAC CCT GTT GTT AGC ACA and CTC AAG CTT TTA TGT CAT TTC CTG TGG AGA (the cloning sites are underlined). The resulting expression construct produces a fusion protein with dihydrofolate reductase and the last 212 aa of orf73 which contains a histidine tag and can be purified on a Ni resin. After purification, this protein was coupled to cyanogen bromide activated sepharose, as instructed by the manufacturer (Pharmacia). Serum (500 μl) from an AIDS KS patient was diluted 1:10 in PBS and incubated for 1 h at room temperature with the orf73 Sepharose. Sepharose beads were then transferred into a disposable column and washed extensively (10 column volumes) with PBS, and orf73-specific antibodies were eluted with 50 mM glycine (pH 2.5), neutralized immediately with 1 M Tris (pH 11.0), and stabilized by the addition of 1% bovine serum albumin (BSA) and 0.1% sodium azide. As a control, a similar affinity purification of patient antibodies was carried out on recombinant orf65 and orf52 proteins, two structural KSHV proteins (37). The specificities of the affinity-purified antibodies for their respective antigens were verified by Western blotting on recombinant proteins.

IFA for LANA and Western blotting for LNA. The IFA for the latent nuclear KSHV antigen was carried out as described previously (17, 37) on the BCP-1 and HBL-6 cell lines (15, 17). Patient sera were diluted 1:200 in PBS–2% fetal calf serum, and affinity-purified antibodies were diluted 1:10 in PBS. A rabbit anti-human IgG fluorescein isothiocyanate conjugate (DAKO) was used as the second antibody. The Western blot assays on nuclear extracts of HBL-6 and BCP-1 cells and on transfected COS7 cells and orf73-expressing bacteria were carried out as described previously, by using the serum of an HIV-negative homosexual man with KS or from an HIV-negative U.S. blood donor previously shown to be negative for antibodies to latent KSHV antigens (16, 17).

Construction of orf73 expression vector. A *Sma*I-*Hin*dIII fragment of KSHV, corresponding to nucleotides 122,789 to 127,285 of the published KSHV sequence (33), was cloned into the *Hin*cII and *Hin*dIII sites of pBluescript-KS (Stratagene). An *Xho*I/*Xba*I fragment was obtained from this construct and inserted into the *Xho*I and *Xba*I sites of pcDNA3.1/His B (Invitrogen) to obtain a mammalian expression vector. This construct encodes the complete orf73 sequence except for the first 4 aa, fused with 6 His residues, followed by an enterokinase cleavage site in the amino terminus. An *Xho*I/*Hin*dIII fragment was obtained from this construct and inserted into *Xho*I and *Hin*dIII sites of pTrcHisB (Invitrogen) to obtain a bacterial expression vector. The pcDNA3.1/His-orf73 plasmid was transfected into COS7 cells by the calcium phosphate method employing the CellPhect transfection kit (Pharmacia Biotech). Cells were harvested 72 h following transfection. Expression of orf73 in pH5α was induced by IPTG (isopropyl-β-D-thiogalactopyranoside) according to the manufacturer's instructions.

Immunohistology on KS lesions. To investigate the expression of orf73/LNA in KS tissue, we used tissues from eight nodular- and two early (patch-plaque)-stage KS biopsies, embedded in paraffin. Sections of 5 μm were cut, and the paraffin was removed by sequential immersion in xylene and ethanol. Sections were then treated for 15 min in a microwave oven (high power setting) and endogenous peroxidase was blocked by treatment with methanol–3% hydrogen peroxide. Affinity-purified antibodies to orf73 and orf65, diluted 1:5 in Tris-buffered saline (TBS)–5% BSA, were added for 1 h at room temperature. Following two washes in TBS, a biotin-conjugated anti-human IgG antibody (DAKO), diluted 1:200 in TBS–5% BSA, was added for 30 min. This was followed by treatment with streptavidin-conjugated horseradish peroxidase (DAKO), diluted according to the manufacturer's instructions, and diaminobenzidine (0.5 mg/ml)–0.1% H_2O_2 for 7 min. To identify endothelial tumor (spindle) cells, adjacent sections were stained with a monoclonal antibody to CD34 (QB-END 10; Seratech, Oxford, United Kingdom) by the same procedure, except that biotin-conjugated rabbit anti-mouse antibody (DAKO) was used as the second antibody.

RESULTS

Immunoscreening and mapping of cDNA clones. HBL-6 is a BCBL-derived B-cell line which is dually infected with KSHV and EBV (15, 33), isolated from the same tumor as BC-1 (8) and previously shown to be serologically indistinguishable from BC-1 (29). KSHV is tightly latent in this cell line, and no expression of the orf26 minor capsid gene is detectable by Northern blot, but its expression can be induced by treatment with sodium butyrate or dibutyrate phorbol ester (25, 26, 36). We screened approximately 10^6 clones of a cDNA library, constructed from uninduced HBL-6 cells, using a pool of sera from patients with endemic KS, and obtained five clones which could be grouped into two sets (Fig. 1). Sequence analysis of the inserts revealed that two clones (clones 5 and 7) (Fig. 1), representing the first set, started at different points within orf73, extended to the end of orf73 and beyond through orf72 (v-cyclin) and another downstream open reading frame (orfK13), and terminated at nucleotide 122,066 of the published KSHV sequence (33), 28 bp downstream of an AAT AAA polyadenylation site at nucleotide 122,094. These two clones included a poly(A) tail (Fig. 1). At its 5′ end, located within the internal repeat of orf73, the sequence of clone 5 was identical with the genomic BC-1 sequence, whereas clone 7 lacked nucleotides 124,578 to 124,595, which correspond to a repeat subunit (AGGAGTTAGAGGAGGTGG on the coding strand, translated as ELEEVE). Clone 7 may therefore be derived from a viral episome with a different repeat structure. Another clone (clone 15), representing the second set, started at nucleotide 127,866 of the published sequence (33) upstream of orf73 and ended at nucleotide 125,094 within the internal repeat region of orf73. In addition to containing the sequence matching that of KSHV, this clone contained a stretch of cellular sequence which had provided its poly(A) tail, suggesting that this clone arose from a recombination event or from a KSHV genome which had integrated into cellular DNA. This clone did not contain nucleotides 127,313 to 127,811 of the genomic KSHV sequence. This segment of genomic sequence has typical intron boundaries (GTGAGT. . .TGTCAG on the coding strand) and appears therefore to have been spliced out of the mRNA encoding orf73 (Fig. 1).

Antibodies to orf73 react with LANA in immunofluorescence and the 222- to 234-kDa LNA in Western blots. The first open reading frame within cDNA clones 5 and 7 corresponded to that of orf73 and was contiguous with the β-galactosidase-

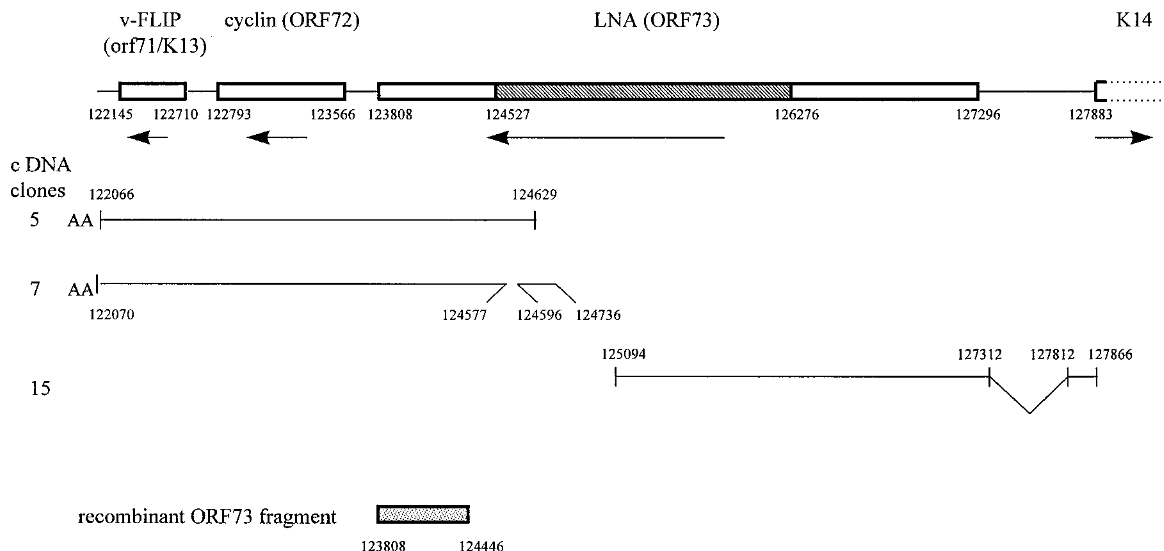

FIG. 1. Mapping of cDNA clones obtained by immunoscreening with respect to the genomic KSHV sequence. The positions of open reading frames in this nonconserved genomic region are indicated by open boxes and the nucleotide numbers marking their beginning and end listed in the published orientation of the genome (33). The coordinates of the corrected v-FLIP/orf71/K13 sequence are shown (41a). The internal repeat region of orf73 is indicated by hatched shading. Arrows indicate direction of transcription. The position of the recombinant orf73 fragment used to affinity purify orf73-specific antibodies is also shown. Lines indicate the locations of three distinct cDNA clones (clones 5, 7, and 15), representative of those obtained by immunoscreening, and their splicing patterns.

encoding gene in the λZap vector. This suggested that the carboxy-terminal 256 aa of orf73, which were included in both clones 5 and 7, contained an epitope(s) reactive with patient sera and that the protein encoded by orf73 was expressed in latently infected BCBL cell lines. We expressed the last 212 aa of orf73 as a fusion protein with dihydrofolate reductase in *Escherichia coli*. This region of orf73 was chosen because it does not extend into the internal repeat region within orf73 (Fig. 1), which is predicted to encode a highly negatively charged protein sequence. When used as an antigen in an enzyme-linked immunosorbent assay, the purified recombinant orf73 fragment reacted with 12 of 18 serum samples (66%) from endemic KS (HIV-negative) cases and 1 of 25 sera (4%) from matched controls. Therefore, not all latent immunofluorescence-reactive patient sera react with this recombinant protein, most likely because it represents only a small segment of the protein encoded by orf73.

To establish the relationship between the orf73-encoded protein, the immunofluorescence-defined LANA, and the Western blot-defined 222- to 234-kDa LNA (16, 17, 37), we affinity purified orf73-specific human antibodies from patient sera on the 212-aa recombinant orf73 fragment. These orf73-specific antibodies produced the speckled nuclear staining pattern characteristic of LANA also seen with KS patient sera on BCP-1 cells (Fig. 2), which are only infected with KSHV (17), and on HBL-6 cells (not shown), which are latently infected with both KSHV and EBV (15). Antibodies affinity-purified on recombinant orf52 and orf65 proteins did not stain these two (uninduced) cell lines (Fig. 2).

Affinity-purified antibodies to the recombinant carboxy-terminal orf73 fragment, but not to orf52 protein, also reacted with the high-molecular-weight doublet band (LNA) in nuclear extracts of BCP-1 and BC-1 cells (Fig. 3A and B) which is recognized by most sera from KS patients (Fig. 3C and reference 16). The same antibody preparation did not recognize any bands in nuclear extracts from P3HR-1, an EBV-infected B-cell line (Fig. 3A). Interestingly, the molecular weight of the LNA doublet was slightly higher in BC-1 cells than in BCP-1 cells (Fig. 3). PCR amplification with primers flanking the internal repeat region within orf73 revealed that this region is approximately 300 bp longer in BC-1 cells than in BCP-1 cells (not shown), providing a possible explanation for the size difference between the LNA doublets in these cell lines. These findings show that the Western blot-defined 222- to 234-kDa LNA protein is encoded by orf73, and that it is either identical with, or at least a component of, immunofluorescence-defined LANA.

Expression of orf73 in *E. coli* and COS7 cells gives rise to LNA. We cloned orf73, derived from BC-1 cells (8, 33), into the mammalian expression plasmid pcDNA3.1/His B and the bacterial expression plasmid pTrcHis (Invitrogen), such that aa 5 to 1162 of orf73 were preceded by six His residues and an enterokinase cleavage site. Western blotting of both bacterial and transfected COS7 lysates with KS patient sera, but not control patient sera, reproduced the high-molecular-weight doublet pattern of the LNA (Fig. 4). Affinity purification of the His-tagged orf73 protein from bacterial extracts (Fig. 4) provides final confirmation that orf73 encodes the LNA and indicates that the much-larger-than-expected apparent molecular mass of the orf73 protein (expected molecular mass, approximately 135 kDa) is likely due to its primary structure rather than posttranslational modification. However, the size difference between the orf73 expressed in mammalian cells and that in *E. coli* (Fig. 4) indicates that posttranslational modification does occur.

orf73/LNA is expressed in KS endothelial tumor (spindle) cells. Using the affinity-purified human antibodies to orf73 and orf65, we investigated the expression of these two viral proteins in KS tissue. As shown in Fig. 5A and B, orf73/LNA was expressed in KS nodules in cells with a spindle-shaped morphology, which on parallel sections stained strongly with the endothelial marker CD34 (Fig. 5D) and therefore meet accepted criteria for KS spindle cells, the endothelial tumor cells of KS lesions. Expression of orf73/LNA was confined to the nucleus, in keeping with its expression in lymphoma cell lines (Fig. 2 and 5). Antibodies to orf65 never produced any nuclear

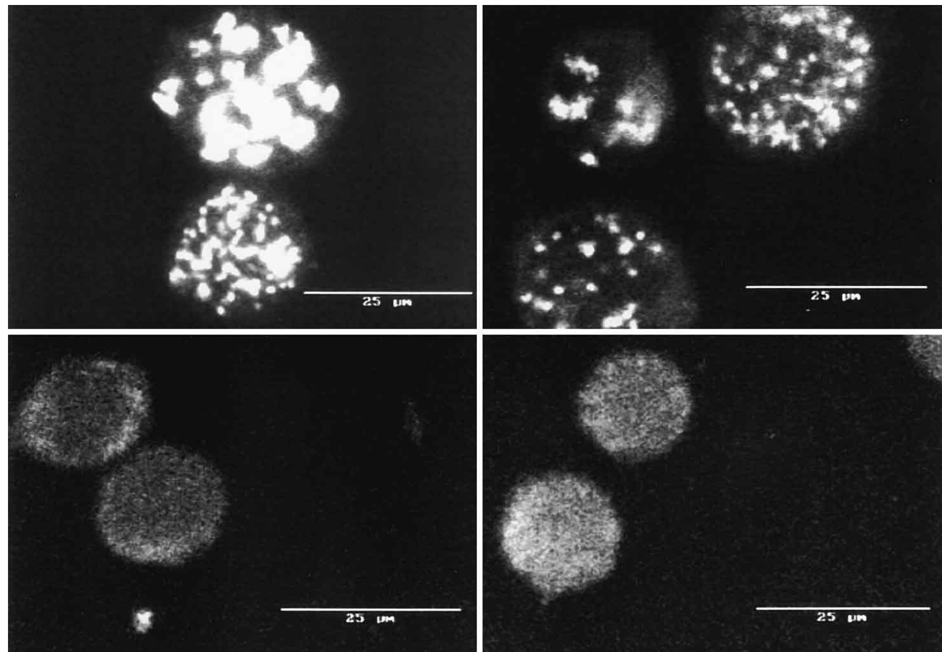

FIG. 2. Antibodies to orf73 protein react with LANA. IFA was carried out on BCP-1 cells by using serum from a KS patient (top left), human antibodies affinity purified on a recombinant carboxy-terminal fragment of orf73 (top right), and human antibodies affinity-purified on two structural KSHV proteins, encoded by orf65 (bottom left) and orf52 (bottom right).

staining (Fig. 5C). We found expression of orf73/LNA in eight of eight cases of nodular KS, but not in two early-stage (patch or plaque) KS lesions. Expression in spindle cells of nodular KS lesions was variable, with some nodules showing orf73/LNA expression in the majority of spindle cells (Fig. 5A) and others showing only a few positive cells (Fig. 5B). We cannot exclude the possibility that our affinity-purified human antibodies may have failed to detect weak expression of orf73/LNA in some cells or that different fixation conditions may have been responsible for these differences. Whether expression of this protein is limited to fully developed KS lesions therefore remains to be investigated further. We also noted that within the same biopsy sample, some spindle cells showed a diffuse nuclear staining (Fig. 5A) whereas the staining in others was confined in a dot-like pattern to subnuclear domains (Fig. 5B), reminiscent of the coarse staining seen by immunofluorescence

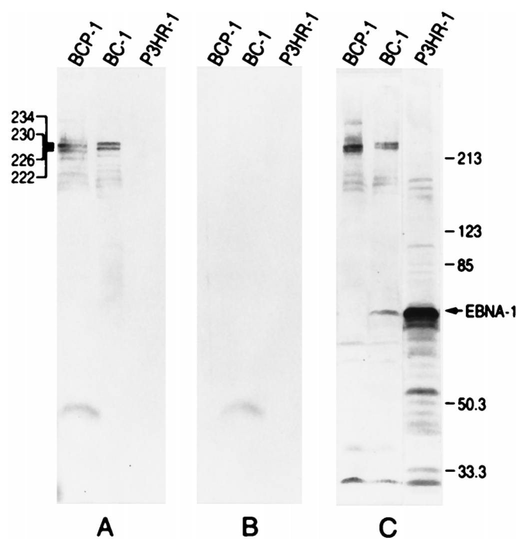

FIG. 3. Antibodies to orf73 protein recognize the Western blot-defined 222- to 234-kDa LNA. Western blots of nuclear extracts from two KSHV-infected BCBL cell lines (BCP-1 and BC-1) and an EBV cell line (P3HR-1) were stained with affinity-purified human antibodies to the carboxy-terminal end of orf73 (A) or to orf52 (B) and unabsorbed serum from a KS patient (C). Numbers to the left and right of the gels are molecular masses (in kilodaltons).

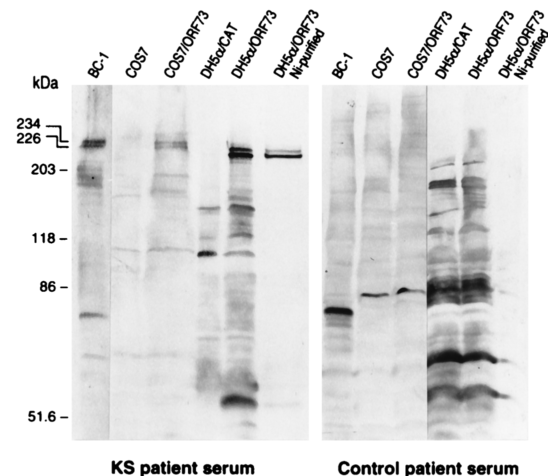

FIG. 4. Transfection of an orf73 expression plasmid gives rise to LNA. COS7 cells and *E. coli* DH5α were transfected or transformed with plasmids directing expression of aa 5 to 1162 of orf73 with a polyhistidine tag. Nclear extracts of BC-1, untransfected COS7, COS7 transfected with the orf73 expression construct, and whole-cell lysates of DH5α transformed with either a chloramphenicol acetyl transferase (CAT) construct or an orf73 construct were immunoblotted against serum from a KS patient or a control patient. Additionally, polyhistidine-tagged orf73 protein was purified from DH5α/orf73 lysates on a nickel column before loading.

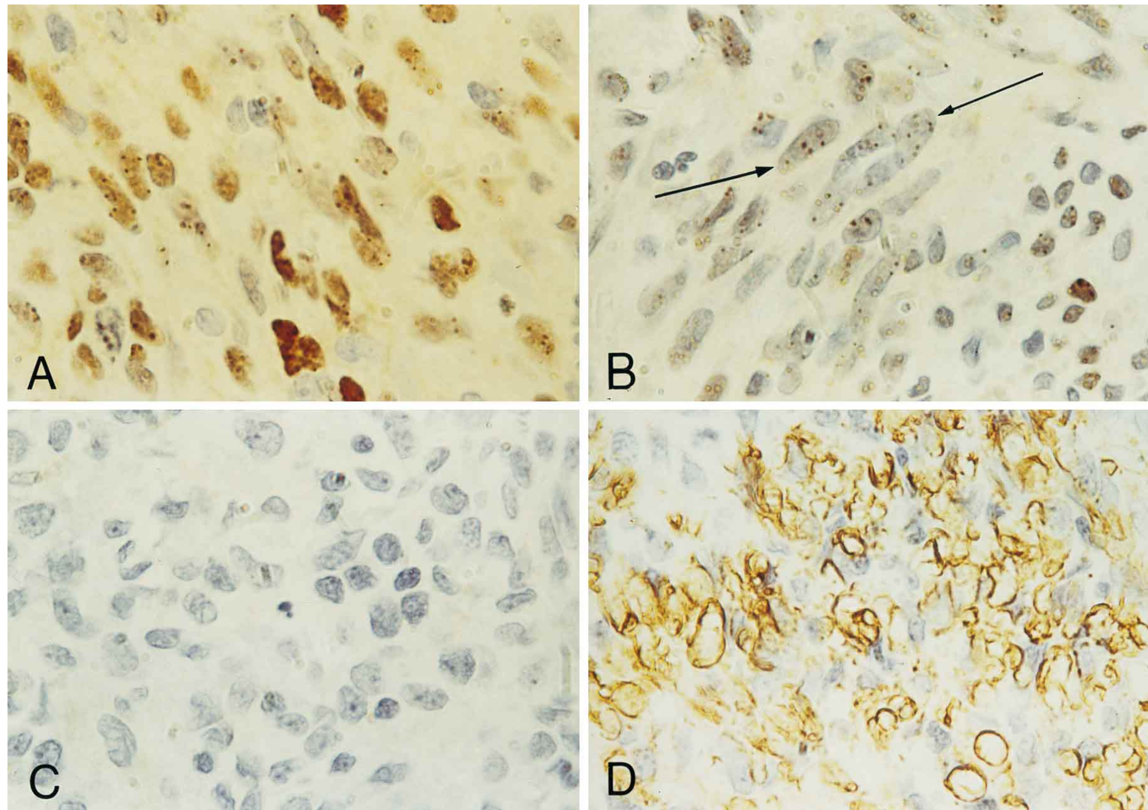

FIG. 5. orf73/LNA is expressed in KS tumor (endothelial spindle) cells. A biopsy sample of nodular KS tissue was stained with affinity-purified human antibodies to orf73 (A and B), to the structural (capsid-related) protein encoded by orf65 (C) and with a monoclonal antibody (QB-END 10) to CD34 (D) to demonstrate the endothelial origin of the KS tumor (spindle) cells shown. Most spindle cells in one region of this biopsy sample (A) and a smaller proportion of them in another region (B) express orf73/LNA in the nucleus. In some cells nuclear staining is confined to a few discrete dots (arrows [B]), whereas in others it is more diffuse. No cytoplasmic staining was ever seen with this antibody.

in some lymphoma cells. Whether these two staining patterns correspond to different stages of infection or reflect an association of orf73/LNA with different nuclear structures at different points in the cell cycle is currently under investigation. With the exception of plasma cells reacting with the anti-human conjugate antibody, no cytoplasmic staining was ever seen, nor did orf73-specific antibodies stain any of a series of control biopsy specimens, including tonsil, colon, pancreas, and adenocarcinoma specimens.

DISCUSSION

We show in this report that KSHV orf73 encodes a previously described high-molecular-weight nuclear protein (LNA) (16) and is a component of the LANA defined by immunofluorescence (17, 19). This conclusion is based on our observations (i) that two sets of cDNA clones extending into orf73 were recognized by patient sera, (ii) that human antibodies affinity purified on a recombinant orf73 fragment give rise to the typical nuclear immunofluorescence pattern of LANA and react with the 222- to 234-kDa LNA, and (iii) that orf73 expression plasmids encoding aa 5 to 1162 of orf73 in COS7 cells and *E. coli* produce the characteristic 222- to 234-kDa Western blot doublet in nuclear extracts. We also show that orf73/LNA is expressed in the nuclei of KS spindle cells. This is in agreement with a recent report (9) that RNA sequences spanning the orf73 region can be detected in KS lesions by reverse transcription-PCR (RT-PCR). However, neither the expression of the corresponding protein nor the nature of the cell expressing it could be established by this technique.

The characteristic Western blot pattern of LNA is a doublet of approximately 226- to 234-kDa in HBL-6 or BC-1 cells and a slightly lower molecular mass (approximately 220- to 230-kDa) in BCP-1 cells. This size difference seen in different cell lines is probably related to the length of the internal repeat region in orf73, which differs in these two cell lines and also in KSHV obtained from different patients (13a). There are several possible explanations for the presence of a doublet band in both cell lines: the observation that expression of a cloned orf73 sequence in COS7 cells and bacteria (Fig. 4) also produces this doublet suggests that it results from differences in posttranslational modifications of the orf73 protein sequence. Differences in posttranslational modification between mammalian cells and *E. coli* may also explain why the molecular weight of the orf73/LNA doublet in *E. coli* is slightly lower than that in COS7 or BC-1 cells (Fig. 4). Alternatively, proteolytic cleavage at a particular site may produce the doublet pattern or explain the weaker bands also seen in Fig. 3A. The sequence difference between two of our cDNA clones (clones 5 and 7) in the internal repeat region of orf73 could suggest that a different arrangement of repeat subunits on individual episomes within a cell line may occur, but is unlikely to be responsible for these two major protein bands. Although it would be an unlikely explanation in view of the doublet nature of orf73 expressed in *E. coli*, we have investigated the possibility of alternative splicing within the internal repeat region, using RT-

PCR and several combinations of primers flanking this domain, but have failed to find any evidence for this (not shown). The apparent molecular mass of all orf73/LNA bands on sodium dodecyl sulfate (SDS)-polyacrylamide gels is much higher than that predicted on the basis of the primary sequence (predicted molecular mass, approximately 135 kDa); this is likely due to the highly negatively charged stretch of amino acid residues encoded by the internal repeat region of orf73, resulting in the aberrant migration of the protein on SDS-polyacrylamide gels. Interestingly, the transforming STP protein of herpesvirus saimiri (HVS), which contains a collagen-like internal repeat domain, also migrates during SDS-polyacrylamide gel electrophoresis as a doublet band with a molecular weight much higher than expected (18).

Our findings suggest that both the carboxy-terminal region of orf73 (which we expressed as a recombinant protein fragment) and its amino-terminal domain (included in clone 15) are recognized by patient sera. Our carboxy-terminal recombinant fragment reacted with only approximately 60% of sera from latent IFA-positive endemic-KS patients, suggesting that individual sera may recognize different epitopes in orf73/LNA. A full-length recombinant protein is at present being evaluated.

KSHV infects endothelial and spindle cells of KS lesions (6), and an abundant latent transcript with the potential to encode a small 60-aa hydrophobic protein (orfK12) is expressed in the majority of KS spindle cells (39, 40). In addition, a nuclear RNA is strongly expressed in a few spindle cells which also express mRNA for a minor capsid protein and may therefore undergo lytic replication (39). The expression of orf73/LNA in the majority of KS spindle cells of nodular KS lesions and uninduced BCP-1 and HBL-6 cells (Fig. 2 and 5) suggests that it is expressed in persistently infected cells. Persistently infected BCBL-derived cell lines also express a viral cyclin homolog (orf72), and a homolog of interleukin-6 (orfK2) (30, 34). The expression of mRNA for orf72 has also been noted in KS lesions (9). Therefore, the role of KSHV in promoting the growth of endothelial cells most likely involves the expression of at least two nuclear proteins (orf73/LNA and orf72/v-cyclin), one of which (v-cyclin) can interact with and phosphorylate the Rb cell cycle checkpoint control protein (12).

orf73 is located in a genomic region which is poorly conserved between the different gamma herpesviruses sequenced so far (1, 3, 23, 41). Among the gamma-2 herpesviruses whose sequences have been published, i.e., KSHV (33), HVS (1), equine herpesvirus 2 (41), and bovine herpesvirus 4 (23), equine herpesvirus 2 lacks an orf73 homolog. The orf73 homolog of bovine herpesvirus 4 does not contain the internal repeat domain present in KSHV and HVS (23). The corresponding genomic region of EBV, a gamma-1 herpesvirus, is much larger than that in gamma-2 herpesviruses and contains the extended *Bam*HI W repeat and adjoining regions encoding EBNA-LP and EBNA-2 (1, 3). EBNA-2 and EBNA-LP mRNAs are extensively spliced in the *Bam*HI W repeat. We however failed to detect any major splicing within the internal repeat of orf73 by RT-PCR in BCBL cell lines. The speckled nuclear staining pattern of orf73/LNA is reminiscent of that seen with EBNA-2 and EBNA-LP and may reflect the association with subnuclear structures. Whether there are functional similarities between orf73/LNA and EBNA-2 or EBNA-LP, which are the earliest proteins to be expressed in newly infected B lymphocytes and are essential for B-lymphocyte transformation (20), is presently under investigation.

ACKNOWLEDGMENTS

This study was supported by the Medical Research Council of Great Britain, the Northwest Cancer Research Fund, the Cancer Research Campaign, the European Concerted Action on the Pathogenesis of Kaposi's Sarcoma (BMH4-97-2302), and the Centers for Disease Control (cooperative agreement U64CCU210852).

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