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Intracellular Localization Map of Human Herpesvirus 8 Proteins▽

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Human herpesvirus 8 (HHV-8) is the etiological agent of Kaposi's sarcoma. We present a localization map of 85 HHV-8-encoded proteins in mammalian cells. Viral open reading frames were cloned with a Myc tag in expression plasmids, confirmed by full-length sequencing, and expressed in HeLa cells. Protein localizations were analyzed by immunofluorescence microscopy. Fifty-one percent of all proteins were localized in the cytoplasm, 22% were in the nucleus, and 27% were found in both compartments. Surprisingly, we detected viral FLIP (v-FLIP) in the nucleus and in the cytoplasm, whereas cellular FLIPs are generally localized exclusively in the cytoplasm. This suggested that v-FLIP may exert additional or alternative functions compared to cellular FLIPs. In addition, it has been shown recently that the K10 protein can bind to at least 15 different HHV-8 proteins. We noticed that K10 and only five of its 15 putative binding factors were localized in the nucleus when the proteins were expressed in HeLa cells individually. Interestingly, in coexpression experiments K10 colocalized with 87% (13 of 15) of its putative binding partners. Colocalization was induced by translocation of either K10 alone or both proteins. These results indicate active intracellular translocation processes in virus-infected cells. Specifically in this framework, the localization map may provide a useful reference to further elucidate the function of HHV-8-encoded genes in human diseases.

Human herpesvirus 8 (HHV-8) belongs to the family of gammaherpesviruses. HHV-8 infection is associated with several severe human diseases such as multicentric Castleman's disease, primary effusion lymphoma, and Kaposi's sarcoma (7, 9, 18, 45, 81).

The HHV-8 genome consists of 165 kbp. To date, 86 different open reading frames (ORFs) have been identified (68). The absolute number of HHV-8-encoded genes is still under investigation due to the detection of differentially spliced gene products in different types of infected cells (68, 80).

Previously, the pathogenic activity of HHV-8 was preferentially analyzed in studies with single genes. More comprehensive analyses may be required to understand the complexity of the HHV-8 pathogenic repertoire. Systems biology approaches are a new powerful tool for the analysis of complex biological processes. However, these methods have been preferentially applied to study the cell biology of yeast (30, 53, 70) and only in a very limited way to study pathogenic activities of infectious agents. Only recently, the first proteome-wide protein interaction study of HHV-8 and varicella-zoster virus was published (82). In this study the K10 protein of HHV-8 was identified as a key interacting protein, binding to at least 15 different HHV-8-encoded proteins (82).

In addition to protein interactions, subcellular localization of proteins is closely associated with protein function. This is generally appreciated, and it is underscored by the rapid growth of localization databases, such as Organelle DB (85). The subcellular localization of most HHV-8-encoded proteins is not known yet. Therefore, we generated a complete localization map of all known HHV-8-encoded genes in mammalian cells. Several unexpected findings were obtained clearly documenting the usefulness of systems biology approaches to study HHV-8.

MATERIALS AND METHODS

Cloning of HHV-8 genes. Specific primers with suitable overhanging restriction enzyme motifs were used to amplify the ORFs of interest via PCR from DNA derived from BCBL-1 cells (67) or from phages containing large fragments of HHV-8 DNA (52). A mixture of Platinum *Taq* (Invitrogen, Karlsruhe, Germany) and *Pfu* Ultra (Stratagene, La Jolla, CA) DNA polymerase was used (16:1 U) for PCR. By using this combination, the constructs of the spliced K8, K10, ORF40/41, and ORF57 genes contained the intron sequences. In addition, the spliced K8.1, K10.5, K11, K15, ORF29, and ORF50 genes were cloned from cDNA isolated from HHV-8-infected cells (83). After digestion with the appropriate restriction enzymes and purification via agarose gel extraction (QIAquick gel extraction kit; Qiagen, Hilden, Germany), the PCR products were cloned in the expression plasmids

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pcDNA3.1 and pcDNA4-Myc/His in frame with a Myc/His tag at the 3′ end. The plasmids containing K15 and LANA-1 were provided by T. Schulz (6, 66). LANA-1 was cloned in pcDNA3 with a His tag at its 5′ end. K10 was also cloned with a Flag tag at its 3′ end in order to allow simultaneous detection of K10 and different HHV-8 proteins in the same cell using anti-Flag and anti-Myc antibodies.

All cloned constructs were confirmed by full-length sequencing. The sequences were aligned with the U93872 (52), U75698 (71), U86667 (38), or AF148805 (25, 68) sequences. When isolated DNA sequences varied from those of the published sequences, the respective reading frames were analyzed to ensure that they were open in full length, and the sequences of three independent clones were determined. When identical sequences were obtained, the isolated sequence was considered as a natural variant of the respective gene. This was the case for the genes of the following proteins: K10, K12, K14, K15, ORF9, ORF16, ORF19, ORF22, ORF40/41, ORF45, ORF48, ORF49, ORF50, ORF52, ORF64, ORF65, ORF66, ORF72, ORF73, and ORF75.

For construction of K13-green fluorescent protein (GFP), the Myc tag was replaced by insertion of a GFP-coding sequence in frame with the K13 sequence. To generate the untagged K2, K8.1, K10.5, and K13, the respective ORFs were amplified via PCR from cDNA derived from BCBL-1 cells with specific primers containing suitable overhanging restriction enzyme motifs (67). After digestion with restriction enzymes and purification via agarose gel extraction (QIAquick gel extraction kit; Qiagen), the PCR products were cloned in the pcDNA4 expression plasmids without a Myc/His tag at the 3′ end.

Cloning of other plasmids. The luciferase reporter plasmid NF-κB-Luc was constructed by inserting a promoter with four tandem repeats of the consensus NF-κB binding site and the thymidine kinase minimal promoter (51) in the luciferase reporter plasmid pGL3-Basic (Promega, Mannheim, Germany).

Cell culture. HeLa cells were grown in Dulbecco's modified Eagle's medium (PAA, Cölbe, Germany) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany), 2 mM L-glutamine (PAA), and 50 U/ml penicillin G and 50 μg/ml streptomycin (PAA).

Antibodies and blocking solution. Fluorescence-labeled secondary antibodies were purchased from Invitrogen. The following primary antibodies were used: polyclonal rabbit antibodies against Flag tag (working dilution, 1:500; Affinity-BioReagents, Golden, CO), Myc tag (1:500; CellSignaling, Danvers, MA), and calnexin (1:100; Abcam, Cambridge, United Kingdom). Mouse monoclonal antibodies against Myc tag (clone 9B11; 1:5,000) (CellSignaling), GFP (clones 7.1 and 13.1; 1:1,000) (Roche, Penzberg, Germany), and the Golgi marker GM130 (1:1,000; BD Bioscience, Erembodegem, Belgium) were also used along with rat monoclonal antibodies against LANA-1 (1:500; Tebu-Bio, Columbia, MD) and K8.1 (1:5,000; Tebu-Bio). The K13 (clone 4C1) and the K10.5 (clone 3G7) monoclonal antibodies were produced by immunization of LOU/C rats with His-tagged purified recombinant K13 (full-length) protein or K10.5 (N-terminal 298-amino-acid fragment) protein (50 μg each) according to a previously described procedure (41). Goat normal serum was purchased from Dianova (Hamburg, Germany).

Indirect immunofluorescence microscopy. HeLa cells were plated on chamber slides (Nunc, Roskilde, Denmark) the day before transfection. Transfection was performed using the calcium phosphate precipitation procedure. At 48 h posttransfection, chamber slides were washed once with phosphate-buffered saline and fixed for 20 min with 100% ethanol at 4°C. For rehydration, cells were incubated in graded ethanol solutions (100%, 96%, 85%, and 70%) two times for 2 min at room temperature. Cells were then washed in Tris-buffered saline (TBS) for 5 min. Permeabilization was carried out by incubating the cells for 20 min in 0.1% saponin (Sigma-Aldrich, Hamburg, Germany) in TBS or 0.1% Triton X-100 (Sigma-Aldrich) in TBS. After permeabilization, the cells were blocked with 10% goat normal serum for 10 min and incubated with anti-Myc tag mouse monoclonal antibody diluted 1:5,000 in 5% goat normal serum for 2.5 h. After two washes in TBS, cells were incubated for 45 min at room temperature with the secondary antibody (goat anti-mouse immunoglobulin G [IgG]-Alexa Fluor 488-conjugated antibody), diluted 1:500 in 5% goat normal serum. Nuclei were counterstained with DAPI (4′,6′-diamidino-2-phenylindole). Finally, cells were washed two times with TBS, and then the slides were mounted with fluorescence mounting medium (DAKO, Glostrup, Denmark) and analyzed by using an immunofluorescence microscope at a magnification of ×1,000 (Leica DMRBE; Bensheim, Germany). Classification of subcellular localization of the proteins was determined by four researchers independently, and categorization was discussed until consensus was reached.

Double staining procedure. For detection of endoplasmic reticulum (ER) and Golgi localization, HHV-8-encoded proteins were stained as described above; in addition, the ER was stained with a polyclonal rabbit antibody against calnexin, which was detected with a goat anti-rabbit IgG-Alexa Fluor 546-conjugated antibody (1:500; Invitrogen). To determine Golgi-associated localization, HHV-8-encoded proteins were stained with a polyclonal rabbit antibody against the Myc tag and a goat anti-rabbit IgG-Alexa Fluor 488-conjugated secondary antibody. Subsequently, the Golgi was stained with a mouse monoclonal antibody against GM130 (BD Bioscience), and detection was carried out with a goat anti-mouse IgG-Alexa Fluor 546-conjugated secondary antibody (1:500; Invitrogen). Colocalization was analyzed with a Zeiss Axiovert 100 M confocal laser scanning microscope (Oberkochen, Germany).

For detection of Flag-tagged K10 and Myc-tagged interaction partners, K10 interaction partners were stained as described above; in addition Flag-tagged K10 was stained with a polyclonal antibody against Flag (1:250), which was

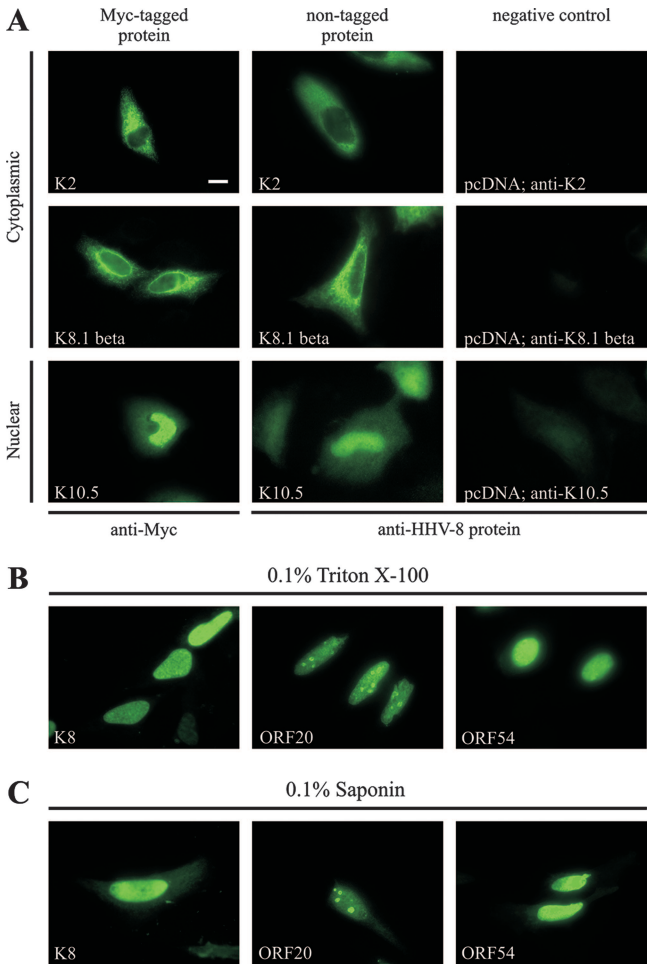

FIG. 1. Influence of Myc tag and cell permeabilization on subcellular localization and immunocytochemical accessibility of HHV-8-encoded proteins. (A) Myc-tagged and untagged HHV-8-encoded proteins were expressed in HeLa cells and detected with either specific antibodies against the different HHV-8 proteins (nontagged proteins) or against the Myc tag. As a negative control, cells were transfected with the vector control (pcDNA4-Myc/His) and stained with antibodies against the viral proteins. Secondary antibodies were conjugated with Alexa Fluor 488. Cells permeabilized by Triton X-100 (B) and saponin (C) were subjected to immunocytochemical analysis of Myc-tagged K8, ORF20, and ORF54. For staining an antibody against the Myc tag was used. Secondary antibodies were conjugated with Alexa Fluor 488. No differences in localization and staining sensitivity were observed under the conditions used. Pictures were obtained using an epifluorescence microscope. The bar in K2 represents 10 μm. The same magnification was used in all panels.

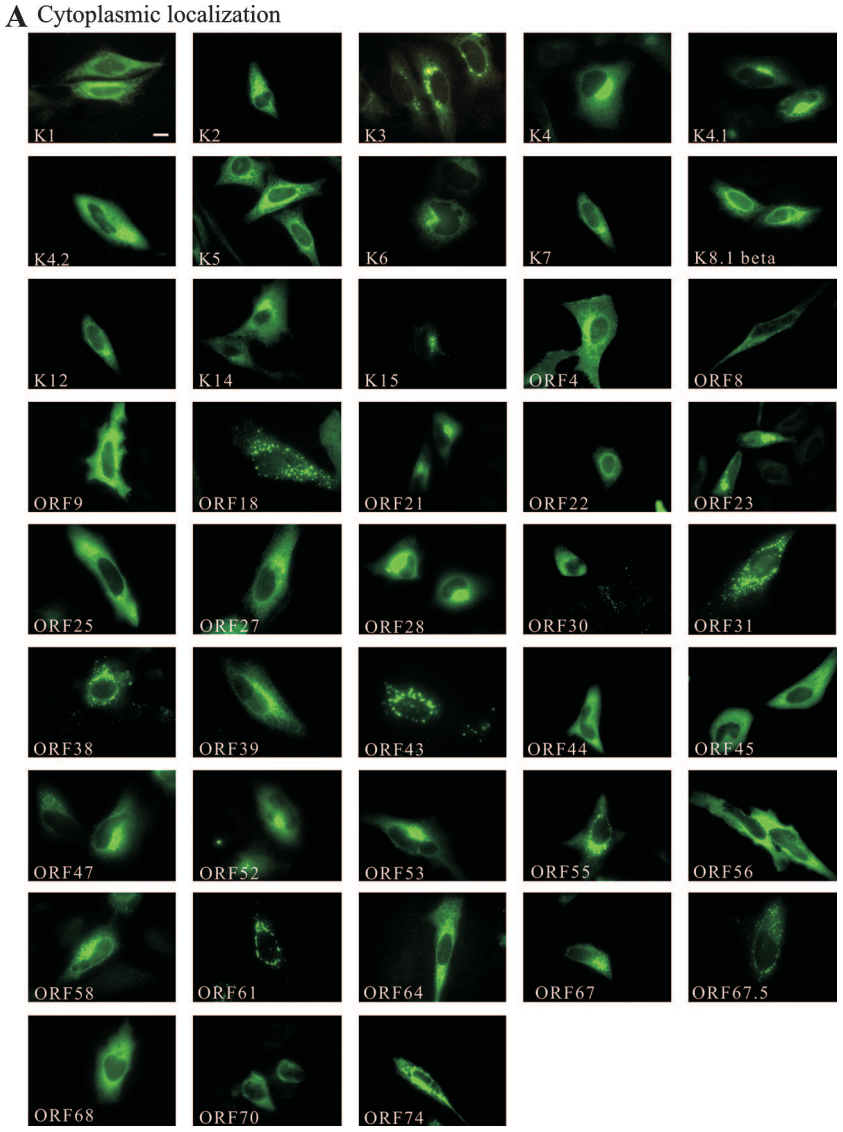

FIG. 2. Subcellular localization of all HHV-8-encoded proteins. Myc-tagged HHV-8 proteins were expressed in HeLa cells, detected with a specific antibody against the Myc tag, and categorized as cytoplasmic (A), nuclear (B), and both nuclear and cytoplasmic (C). Localization of ORF73 (LANA-1) was detected with a specific antibody against LANA-1. As a negative control cells were transfected with the vector control (pcDNA4-Myc/His) and also stained with an antibody against the Myc tag. The secondary antibody was conjugated with Alexa Fluor 488. Pictures were obtained using an epifluorescence microscope. The bar in K1 represents 10 μm. The same magnification was used in all panels.

detected with a goat anti-rabbit IgG-Alexa Fluor 546-conjugated antibody (1:500; Invitrogen).

Nuclear/cytosol fractionation. Fractionation was done with a nuclear/cytosol fractionation kit from BioVision (Wiesbaden, Germany). The protein concentration was determined with a Bio-Rad detergent-compatible (DC) protein assay kit in a microplate reader (München, Germany) at 750 nm.

Luciferase reporter gene assay. Cells were harvested with 200 μl of 1× passive lysis buffer (luciferase reporter assay system; Promega) according to the manufacturer's instructions. Expression of firefly luciferase was determined quantitatively using a luminometer (Luminoskan Ascent; ThermoFisher, Langenselbold, Germany) employing the luciferase assay reagent (Promega) as a substrate. Obtained values were normalized according to their total protein content as determined by the DC protein assay (Bio-Rad).

Computer-assisted nuclear localization signal (NLS) prediction. For each HHV-8 protein, the presence of a possible nuclear localization was predicted using the PredictNLS server (15).

RESULTS

Cellular localization map of all HHV-8 proteins. The coding sequences of all HHV-8-encoded genes except the ORF17.5 gene, which is a splice-variant of ORF17, were isolated, and an immunological tag (Myc tag)-encoding sequence was fused in frame at the 3′ end of the coding sequences to allow immunochemical detection of the respective proteins. The amplified sequences were cloned into expression plasmids, confirmed by full-length sequencing, and expressed in HeLa cells. HeLa cells were used as a cell system for these studies because they exhibit a larger cytoplasm than HEK 293 cells and are easier to transfect than endothelial cells, which are the more commonly used cell types of HHV-8 research.

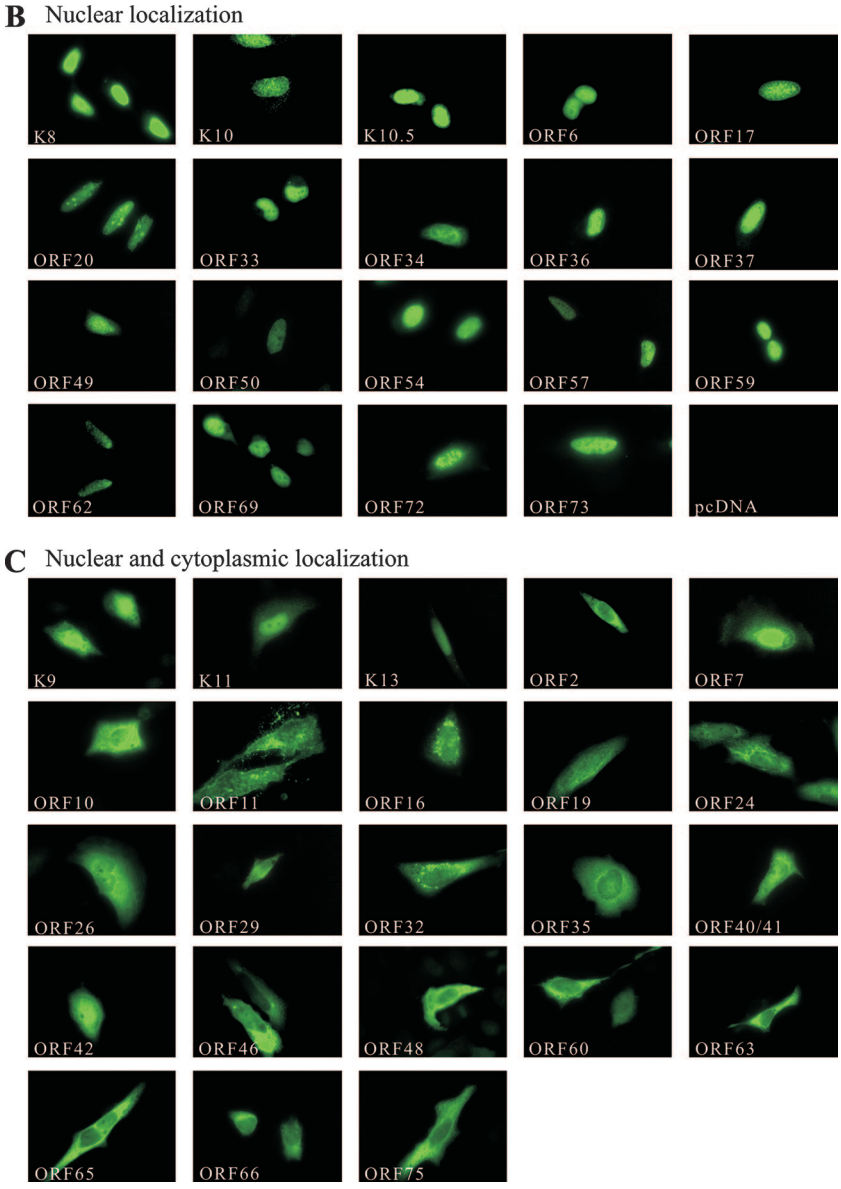

FIG. 2—*Continued*.

In order to determine whether the Myc tag affected cellular localization, two cytoplasmic (K2 and K8.1) and one nuclear (K10.5) HHV-8 proteins against which specific antibodies were available were expressed with and without a Myc tag (Fig. 1A). Subsequently, the different proteins were detected immunocytochemically either with specific antibodies directed against the different HHV-8 proteins or with an antibody against the Myc tag. In all three cases untagged and Myc-tagged proteins showed identical localizations, suggesting that the Myc tag does not have significant effects on cellular localization of HHV-8 proteins.

In addition, it has been reported that immunocytochemical detection of nuclear proteins is critically dependent on the method used for cell permeabilization (22, 59). For this reason we compared the performance of two different permeabilization procedures (Triton X-100 and saponin) for the detection of three different nuclear HHV-8 proteins (Fig. 1B and C). Under both conditions each protein could be detected with identical sensitivity regardless of which method was used for permeabilization (Fig. 1B and C). In all further immunocytochemical stainings, saponin treatment was used for permeabilization.

To investigate subcellular localization, expression plasmids encoding all 85 HHV-8 genes were transfected into HeLa cells, and the proteins were detected by immunocytochemical staining (Fig. 2). In order to obtain a general overview of the subcellular localization of each protein, epifluorescence microscopy was used. With this approach all HHV-8 proteins could be clearly detected in numerous transfected cells.

TABLE 1. Subcellular localization of HHV8 encoded protein[a]

Gene[b]	Alternative description or product (designation)[c]	Position in the genome[d]	Reference	Subcellular localization	Golgi or ER localization[e]
K1[f]	Signaling molecule K ITAM-signaling (KIS)	105–944	39, 72	Cytoplasmic heterogeneously	
ORF4	Complement control protein (KCP)	1112–2764	78	Cytoplasmic heterogeneously	
ORF6	Major single-stranded DNA binding protein	3179–6577	86	Nuclear	
ORF7	Processing and transport protein (ICP18.5)	6594–8681		Nuclear and cytoplasmic	
ORF8	Glycoprotein B (gB)	8665–11202	4, 63	Cytoplasmic heterogeneously	ER
ORF9	DNA polymerase	111329–14367	86	Cytoplasmic diffuse	
ORF10	HVS homologue	14485–15741		Nuclear and cytoplasmic	
ORF11	HVS homologue	15756–16979		Nuclear and cytoplasmic	
*K2**	Viral interleukin-6 (vIL-6)	c17227–17841	3, 62	Cytoplasmic heterogeneously	
ORF2	Dihydrofolate reductase (DHFR)	c17887–18159		Nuclear and cytoplasmic	
K3[f]	Modulator of immune recognition 1 (vMIR1)	c18574–19542	16, 31, 73	Cytoplasmic granular	
ORF70	Thymidylate synthase (TS)	c20023–21036		Cytoplasmic heterogeneously	
K4	Macrophage inflammatory protein-II (vMIP-II; vCCL-2)	c21480–21764	49	Cytoplasmic perinuclear, focally enriched	Golgi
K4.1	vMIP-III; vCCL-3	c22117–22461		Cytoplasmic perinuclear, focally enriched	Golgi
K4.2[f]		c22530–23078	31	Cytoplasmic diffuse	
K5	vMIR2	c25865–26635	29, 31	Cytoplasmic heterogeneously	ER
K6	vMIP-I; vCCL-1	c27289–27576	49	Cytoplasmic perinuclear, focally enriched	
K7	Viral inhibitor of apoptosis (vIAP)	28774–29154	20, 84	Cytoplasmic heterogeneously	
ORF16	Viral B-cell-lymphoma 2 (v-Bcl-2)	30242–30769	56	Nuclear and cytoplasmic	
ORF17	Capsid assembly protein, protease	30857–32524		Nuclear	
ORF18	HVS homologue	32523–33296		Cytoplasmic granular	
ORF19	Virion/tegument protein	c33293–34942		Nuclear and cytoplasmic	
ORF20	HVS homologous, fusion protein	c34710–35672		Nuclear	
ORF21	Thymidine kinase (TK)	35482–37224	23	Cytoplasmic perinuclear focally enriched	Golgi
ORF22	Glycoprotein H (gH)	37212–39404	50	Cytoplasmic heterogeneously	ER
ORF23	HVS homologue	c39401–40615		Cytoplasmic perinuclear, focally enriched	
ORF24	HVS homologue	c40619–42877		Nuclear and cytoplasmic	
ORF25	Major capsid protein	42876–47006		Cytoplasmic diffuse	
ORF26[f]	Minor capsid protein	47032–47949	34, 58	Nuclear and cytoplasmic	
ORF27	HVS homologue	47973–48845		Cytoplasmic heterogeneously	
ORF28	HVS homologous glycoprotein	49091–49399		Cytoplasmic perinuclear, focally enriched	Golgi
ORF29[g]	DNA packaging protein, terminase	c49462–50604 + 53855–54775		Nuclear and cytoplasmic	
ORF30	HVS homologue	50723–50956		Cytoplasmic diffuse	
ORF31	HVS homologue	50863–51537		Cytoplasmic granular	
ORF32	HVS homologue	51504–52868		Nuclear and cytoplasmic	
ORF33	HVS homologue	52861–53865		Nuclear	
ORF34	HVS homologue	54774–55757		Nuclear	
ORF35	HVS homologue	55738–56190		Nuclear and cytoplasmic	
ORF36	Serine protein kinase, phosphotransferase	56075–57409	60	Nuclear	
ORF37	Alkaline DNA-exonuclease shutoff and exonuclease (SOX)	57372–58832	24	Nuclear	
ORF38	Myristylated tegument protein EHV-2 homologue	58787–58972		Cytoplasmic granular	
ORF39	Glycoprotein M (gM), integral membrane protein	59072–60274		Cytoplasmic perinuclear, focally enriched	ER
ORF40/41[h]	DNA helicase-primase complex component	60407–61756 + 61884–62543	86	Cuclear and cytoplasmic	
ORF42	HVS homologue	c62535–63371		Nuclear and cytoplasmic	
ORF43	Minor capsid protein	c63235–65052		Cytoplasmic granular	Golgi
ORF44	DNA replication protein (helicase/primase subunit)	64991–67357	86	Cytoplasmic diffuse	
ORF45	KSHV-immediate-early-2 (KIE-2)	c67452–68675	89, 90	Cytoplasmic diffuse	
ORF46	Uracil DNA glucosidase	c68736–69503		Nuclear and cytoplasmic	
ORF47	Glycoprotein L (gL)	c69511–70014		Cytoplasmic heterogeneously	ER
ORF48	HVS homologue	c70272–71480		Nuclear and cytoplasmic	
ORF49	HVS homologue	c71728–72637	27	Nuclear	
ORF50[g]	Replication and transcription activator (RTA)	71695–71712 + 72671–74728	11, 28, 42, 57	Nuclear	
K8[h]	K-basic leucin zipper/replication-associated protein (K-bZIP/RAP)	74949–75662 + 75744–75890	14, 34, 65, 86	Nuclear	

Continued on following page

TABLE 1—*Continued*

Gene[b]	Alternative description or product (designation)[c]	Position in the genome[d]	Reference	Subcellular localization	Golgi or ER localization[e]
K8.1 beta[g]*	Glycoprotein (gp35-37)	76014–76437 + 76532–76794	34, 40, 42, 46, 87, 91, 92	Cytoplasmic heterogeneously	
ORF52	HVS homologue	c76901–77296		Cytoplasmic perinuclear, focally enriched	
ORF53	HVS homologue	c77432–77764		Cytoplasmic perinuclear, focally enriched	Golgi
ORF54	dUTPase homologue	77835–78722		Nuclear	
ORF55	HVS homologue	c78864–79547		Cytoplasmic perinuclear, focally enriched	Golgi
ORF56	DNA replication protein (helicase/primase subunit)	79535–82066	86	Cytoplasmic diffuse	
ORF57[h]	Immediate-early protein (MTA)	82169–82217 + 82326–83644	5, 36, 43, 55, 86	Nuclear	
K9	vIRF-1	c83960–85309	61, 75	Cuclear and cytoplasmic	
K10[h]	vIRF-4	c86174–88442, 88544–89010	32, 34	Nuclear	
K10.5[g]*	vIRF-3; latency-associated nuclear antigen-2 (LANA-2)	c89700–90945, 91042–91496	1, 47, 69	Nuclear	
K11[f,g]	vIRF-2	c92066–93620, 93742–94229	34	Nuclear and cytoplasmic	
ORF58	HVS homologue	c94577–95650		Cytoplasmic perinuclear, focally enriched	Golgi
ORF59	DNA polymerase processivity factor (PF-8)	c95655–96845	8, 21, 33, 34, 46, 86, 92	Nuclear	
ORF60	Ribonucleotide reductase small subunit homologue	c96976–97893		Nuclear and cytoplasmic	
ORF61	Ribonucleotide reductase large subunit homologue	c97922–100300		Cytoplasmic granular	
ORF62	Capsid assembly and DNA maturation protein	c100305–101300		Nuclear	
ORF63	Tegument protein	101314–104100		Nuclear and cytoplasmic	
ORF64	Large tegument protein	104106–112013		Cytoplasmic heterogeneously	
ORF65[f]	Capsid protein	c112037–112549	34	Nuclear and cytoplasmic	
ORF66	HVS homologue	c112576–113865		Nuclear and cytoplasmic	
ORF67	Tegument protein	c113799–114614		Cytoplasmic perinucelar, focally enriched	Golgi
ORF67.5	EHV-2 ORF67A homologue	c114669–114911		Cytoplasmic granular	
ORF68	Major envelope glycoprotein	114874–116511		Cytoplasmic diffuse	
ORF69	HVS homologue	116544–117452		Nuclear	
K12	Kaposin (virus structure protein, T0.7)	c118025–118207	48	Cytoplasmic heterogeneously	
K13	FLICE-inhibitory protein cellular homologue (vFLIP)	c122393–122959		Nuclear and cytoplasmic	
ORF72	Viral cyclin (v-cyc)	c123042–123815	26	Nuclear	
*ORF73***	Latency associated nuclear antigen 1 (LANA-1)	c124057–127446	17, 34, 35, 64, 66	Nuclear	
K14	OX-2 membrane-glycoprotein homologue (vOX-2)	128264–129079		Cytoplasmic diffuse	
ORF74	Viral G protein coupled receptor (vGPCR)	129520–130548	12	Cytoplasmic heterogeneously	Golgi
ORF75	Tegument protein, phosphoribosylformylglycineamide amidotransferase homologue (FGARAT)	c130699–134589		Nuclear and cytoplasmic	
K15[f,g]	Latency-associated membrane protein (LAMP)	c134824–135287, 135373–135474, 135557–135664, 135747–135889, 135977–136066, 136155–136397, 136481–136573, 136683–136899	13, 25, 76	Cytoplasmic perinuclear, focally enriched	

[a] Subcellular localization of all proteins was determined with an antibody against the Myc tag except where indicated.
[b] Order according to the 5′–3′ appearance in the HHV-8 genome. *, subcellular localization of proteins was detected with an antibody against the Myc tag and in addition with a specific antibody directed against the protein; **, subcellular localization of LANA-1 was determined with a specific antibody against LANA-1.
[c] HVS, herpesvirus samiri; EHV-2, equine herpesvirus 2; vIRF, viral interferon regulatory factor.
[d] Position numbers are according to GenBank accession number AF148805 (25, 68). c, coding sequence complementary.
[e] Colocalization with the marker GM130 or calnexin for the Golgi or ER, respectively.
[f] There were slight differences between our findings and previous reports. ORF26 was detected only in the cytoplasm, and K11 and ORF65 were detected only in the nucleus by others. K1, K3, and K4.2 were found in the ER or Golgi by other investigators.
[g] Spliced genes isolated from cDNA.
[h] Spliced genes expressed via isolated genomic DNA.

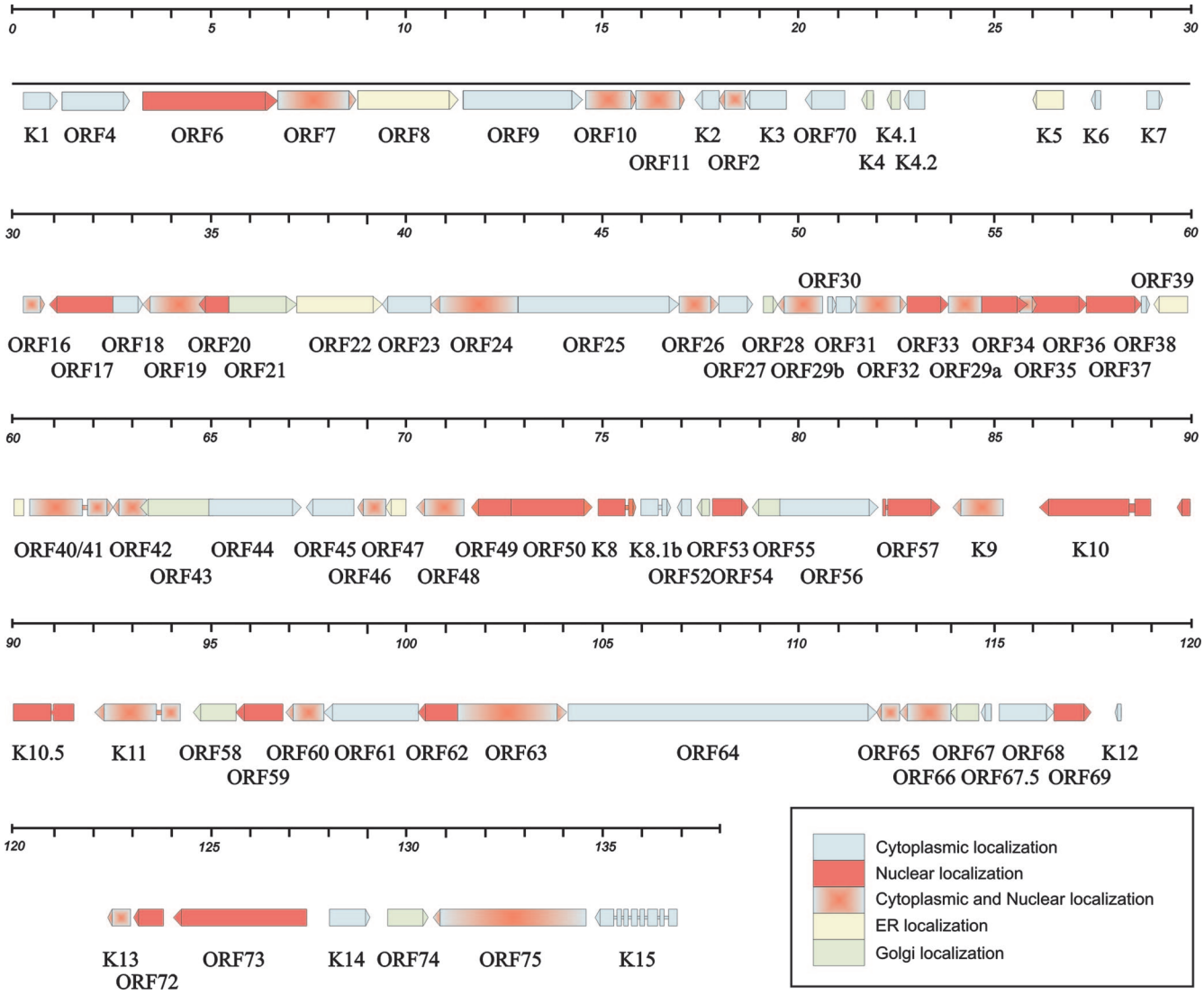

FIG. 3. Gene map and intracellular localization of HHV-8 proteins. Protein coding regions are indicated by colored arrows, and gene names are given. Orientations of the arrows indicate the transcriptional orientations. Genes are color coded as shown on the figure.

TABLE 2. Nuclear localization sequences of HHV-8-encoded proteins

Protein	NLS(s)[a]	Amino acid position	Reference
K10.5	RRHERPTTRRIRHRKLRS	367–384	47
K11	KHREKALRRSLRKK	146–159	PredictNLS
ORF37	PRKKRKL	315–320	24
ORF50	KRKQRSKERSSKKRK	515–529	11
ORF57	RYGKKIK	101–107	43
	KRPRRRPRDR	121–130	
	RAAPKRATRR	143–152	
ORF73	RKRNRSP	24–30	64

[a] Amino acids are given in the single-letter code; ORF57 encodes three different NLSs.

According to their localization, the proteins could be classified into three groups: those with cytoplasmic localization (51%) (Fig. 2A) or nuclear localization (22%) (Fig. 2B) and those which were localized in both the cytoplasm and the nucleus (27%) (Fig. 2C). Proteins with purely cytoplasmic localization were further subcategorized into four groups: cytoplasmic granular (16%); cytoplasmic perinuclear, focally enriched (30%); cytoplasmic diffuse (21%); and cytoplasmic heterogeneous (33%). A summary of all results is presented in Table 1 and Fig. 3. Graphical depiction showed that nuclear proteins are mainly encoded by genes in the second half of the viral genome (Fig. 3, red).

We further analyzed whether nuclear proteins (Fig. 2B and C) exhibit an NLS using the prediction algorithm PredictNLS (15). In this study an NLS was detected in K11 in addition to the proteins K10.5, ORF37, ORF50, ORF57, and ORF73, in which an NLS has been detected previously by other investigators (Table 2). All of these proteins were detected in the nucleus in our study.

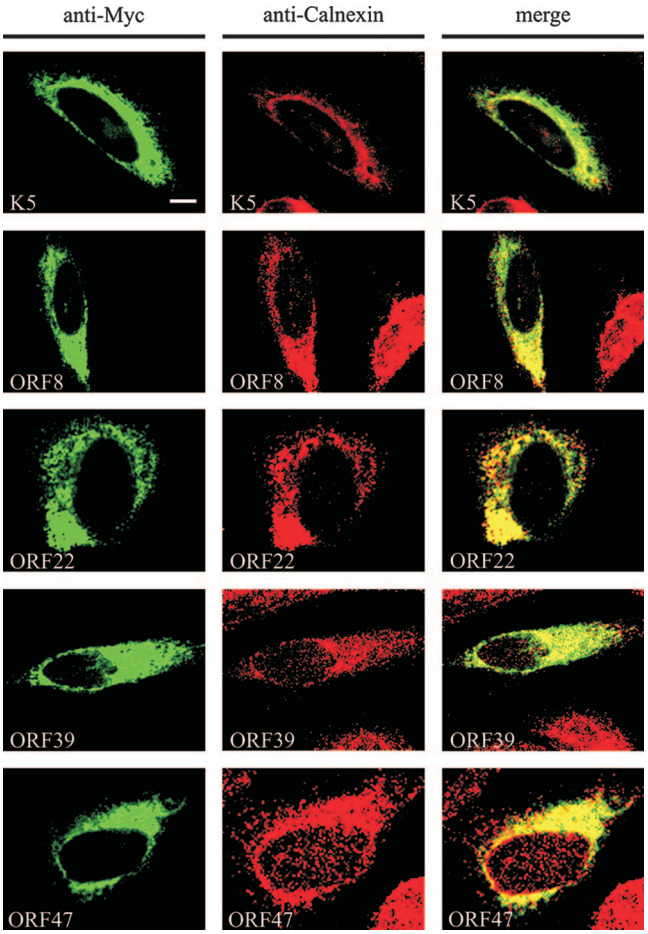

FIG. 4. ER localization of HHV-8 proteins. ER localization of HHV-8 proteins was determined by costaining with an antibody against calnexin. Colocalization was analyzed by confocal laser scanning microscopy. The bar in K5 represents 10 μm. The same magnification was used in all panels.

Golgi and ER localization of HHV-8 proteins. Cytoplasmic proteins with heterogeneous distribution, granular staining patterns, or perinuclear enrichment may be associated with the ER or the Golgi. In order to confirm the putative association with these intracellular organelles, double staining experiments of the HHV-8 proteins and the ER marker calnexin (Fig. 4) or the Golgi marker GM130 (Fig. 5) were carried out and analyzed by laser scanning microscopy. Colocalization with calnexin confirmed ER localization of five proteins (K5, ORF8, ORF22, ORF39, and ORF47) (Fig. 4 and Table 1). Enrichment in the Golgi was observed for 10 proteins (K4, K4.1, ORF21, ORF28, ORF43, ORF53, ORF55, ORF58, ORF67, and ORF74) (Fig. 5 and Table 1), all of which colocalized with GM130. Of note, ER-associated proteins were exclusively encoded in the first half of the viral genome (Fig. 3, yellow), whereas the genes of Golgi-associated proteins were randomly distributed (Fig. 3, green).

Nuclear localization of v-FLIP. A surprising observation was obtained with the K13 gene product. K13 encodes a viral Fas-associated death domain-like interleukin-1β-converting enzyme-inhibitory protein (v-FLIP). Cellular FLIPs are exclusively localized in the cytoplasm (44, 54). Unexpectedly, the protein encoded by the K13 gene was localized in the cytoplasm and the nucleus (Fig. 6A, arrow). In order to determine whether nuclear localization was due to the Myc tag, a GFP-tagged K13 protein (K13-GFP) was expressed in HeLa cells and detected by direct fluorescence analysis (Fig. 6B). In addition, a rat monoclonal K13-specific antibody was generated and used to detect the localization of an untagged K13 protein (Fig. 6C). All of these controls showed concordantly that K13 is resident in both the cytoplasm and the nucleus of the cell (Fig. 6A to C, arrows). No signal was observed in a control staining with only the secondary antibody (Fig. 6D). To confirm these results, we isolated nuclear and cytoplasmic fractions of HeLa cells that expressed K13 with a Myc and a GFP tag (Fig. 6E). Western blot analyses of the isolated cell fractions clearly confirmed that both Myc-tagged (Fig. 6E, upper panels) and GFP-tagged (Fig. 6E, lower panels) K13 proteins are clearly present in the cytoplasm and in the nucleus. To exclude the possibility that the Myc tag may affect the function of K13, we compared a Myc-tagged and an untagged K13 in a functional test. A major function of K13 is its capability to activate the NF-κB pathway (10). In an NF-κB reporter test, the Myc-tagged and the untagged K13 activated NF-κB at comparable levels (Fig. 7).

Effects of K10 binding partners on subcellular localization of K10. Recently it has been shown that K10 interacts with at least 15 different HHV-8 proteins (K12, ORF2, ORF9, ORF28, ORF29b, ORF31, ORF37, ORF39, ORF41, ORF47, ORF59, ORF60, ORF61, ORF67.5, and ORF68) (82). According to Rezaee et al. (68) truncated forms of ORF29 and ORF40/41 were used by Uetz et al. (82) in an interaction study of all HHV-8 genes. In order to allow comparison of our results with those of Uetz and colleagues, we also used the truncated forms of ORF29 and ORF40/41 (ORF29b and ORF41, respectively) for this study. We noticed that K10 localized in the nucleus. In contrast, only five (ORF2, ORF37, ORF41, ORF59, and ORF60) of the potential K10 interacting proteins were also detected in the nucleus when they were expressed alone in HeLa cells (Table 1 and Fig. 2). However, coexpression of K10 with the putative interacting proteins resulted in a clear colocalization in 13 cases (Table 3 and Fig. 8) (K12, ORF2, ORF9, ORF29b, ORF31, ORF37, ORF39, ORF41, ORF47, ORF59, ORF60, ORF67.5, and ORF68), as detected in an analysis with the laser scanning microscope. Colocalization was induced either by a change of K10 subcellular localization (K12, ORF9, ORF29b, ORF31, ORF39, ORF47, and ORF67.5) or by a change of the localization of both K10 and the putative binding protein (ORF41, ORF60, and ORF68) (Fig. 8). K10 did not colocalize with ORF28 and ORF61 in our experiments. To demonstrate that the relocalization of K10 is not simply an artifact of overexpression, we tested six different HHV-8 proteins (K3, K4, K5, K8, ORF38, and ORF54) that did not interact with K10 (82). No relocalization of any of the proteins was observed when they were coexpressed with K10 (data not shown).

DISCUSSION

We determined the intracellular localization of all HHV-8-encoded proteins in mammalian cells. At present, antibodies

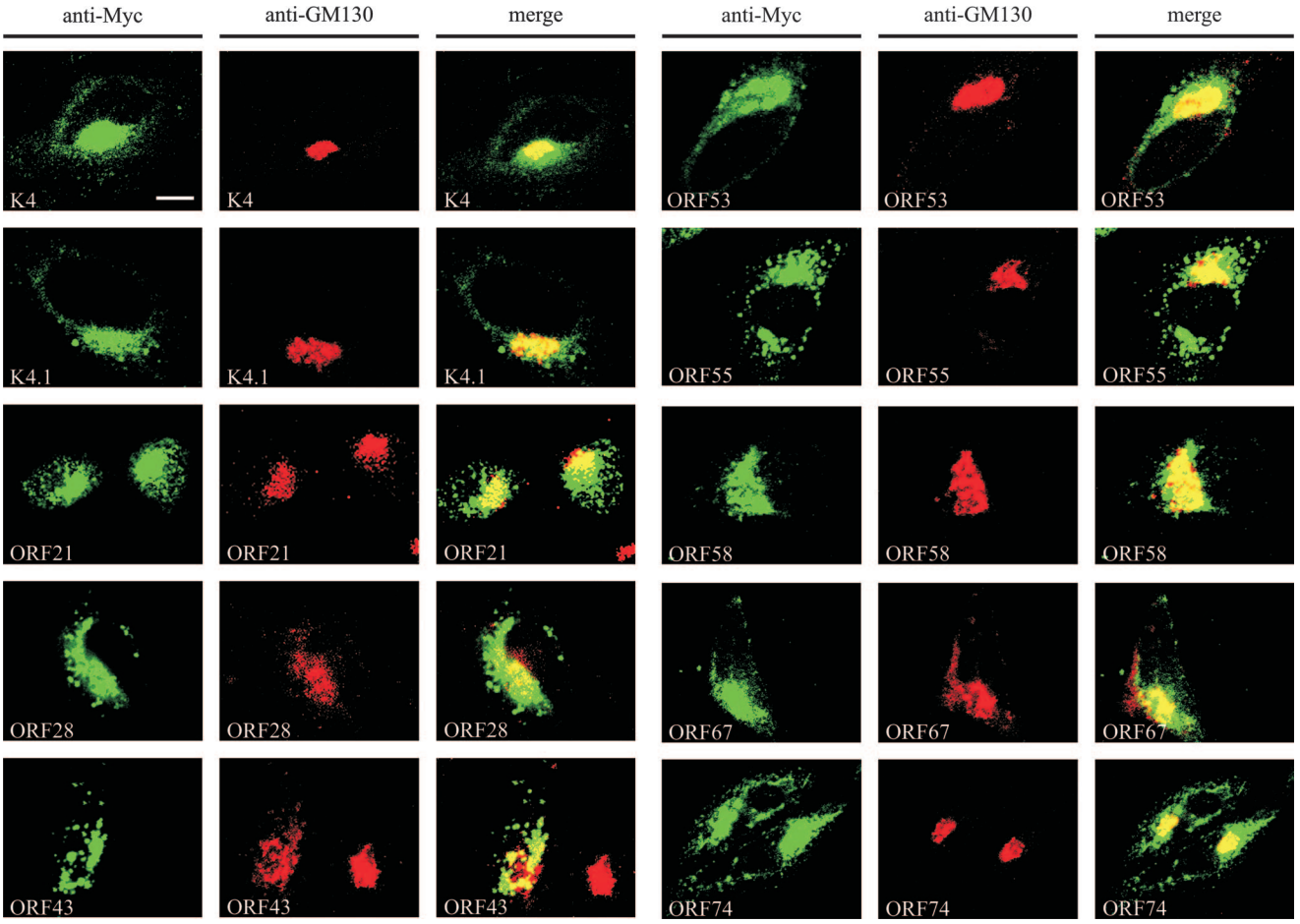

FIG. 5. Golgi localization of HHV-8 proteins. Golgi localization of HHV-8 proteins was determined by costaining with an antibody against GM130. Colocalization was analyzed by confocal laser scanning microscopy. The bar in K4 represents 10 μm. The same magnification was used in all panels.

are available against only a few HHV-8-encoded proteins. In order to allow immunocytochemical detection of the different proteins, a tag was fused in frame at the 3′ end of each coding sequence. Several observations indicated that the tag did not exert significant effects on subcellular localization of HHV-8 proteins. First, the cellular localization of three proteins (K2, K8.1, and K10.5) against which specific antibodies were available was not affected by the tag. Second, the great majority (82%) of the available published results on localization of HHV-8 proteins were in clear agreement with our findings. Of the 85 different HHV-8 proteins, 38 have been investigated by others to our knowledge. Only in seven cases were slight differences between previous findings and our study results observed (Table 1). Specifically, ORF26 was detected only in the cytoplasm, and K11 and ORF65 were found only in the nucleus by other investigators, whereas in our study all three proteins were detected in both the cytoplasm and the nucleus (34, 58). In addition, K1, K3, K4.2, and K15 were found in the ER or Golgi by others (16, 31, 39, 76). We also detected each of these four proteins in the cytoplasm but could not confirm colocalization with the respective compartment markers calnexin and GM130 (Table 1). Altogether, a high concordance with the available published results was observed, which clearly supported the validity of the localization map described here.

It is of interest that 22% of the HHV-8-encoded proteins were detected in the nucleus, whereas only 12% of randomly selected cellular proteins showed nuclear localization (77). Nuclear preponderance of HHV-8-encoded proteins is in good agreement with the viral life cycle, which is preferentially associated with the nucleus. Comparing protein localization with the expression state during the viral life cycle, we noticed that all latency-associated proteins showed a nuclear staining pattern, whereas only 47% of primary lytic, 43% of secondary lytic, and 43% of tertiary lytic proteins showed a nuclear staining pattern (37). This apparently is in agreement with the fact that latency is a regulatory state, which may predominantly depend on nuclear proteins to control host cell and viral transcription.

ER and Golgi localization was observed for 17% of the HHV-8 proteins. It is in clear agreement with these results that many of the proteins were suspected to associate with these organelles. The putative Golgi and/or ER proteins were the following: (i) glycoproteins (gB/ORF8, gH/ORF22, gM/ORF39, and gL/ORF47) (4, 50, 63), which are known to be incorporated into the viral envelope, as well as ORF28, which

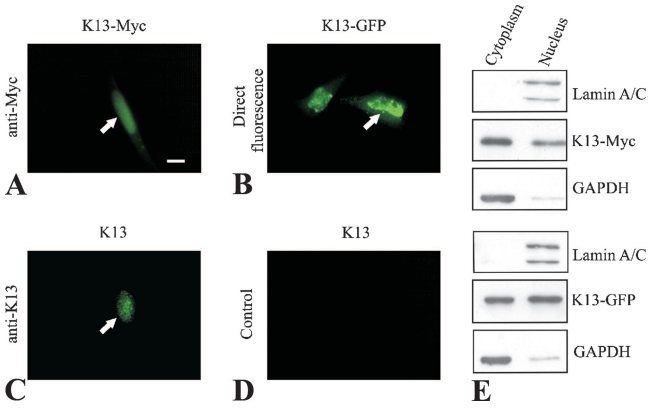

FIG. 6. Nuclear localization of v-FLIP/K13. K13 was expressed in HeLa cells with a Myc tag (A), in fusion with GFP (B), and without a tag (C). Recombinantly expressed K13 was detected with antibodies against the Myc tag or against K13 or by direct epifluorescence. As a control cells were transfected with pcDNA4-Myc/His and subjected to immunocytochemical staining using the anti-K13 antibody (D). Pictures were obtained using an epifluorescence microscope. The bar shown in K13-Myc represents 10 μm. The same magnification was used in all panels. For cell fractionation experiments HeLa cells were transfected with Myc-tagged or GFP-tagged K13, and nuclear and cytoplasmic fractions were isolated and analyzed by Western blotting (E). K13 proteins were detected with antibodies against the Myc or the GFP tag.

reveals similarities to a glycoprotein from saimiriine herpesviruses (19); (ii) membrane-associated proteins such as viral interleukin-8 receptor-like G protein-coupled receptor homolog (vGPCR; ORF74), vMIR2, K5 (29, 31), and the tegument protein ORF67 with homology to the membrane-associated phosphoprotein LF2 from HHV-6; and (iii) chemokine-like proteins (vMIP-II/K4 and vMIPIII/K4.1) which may be secreted

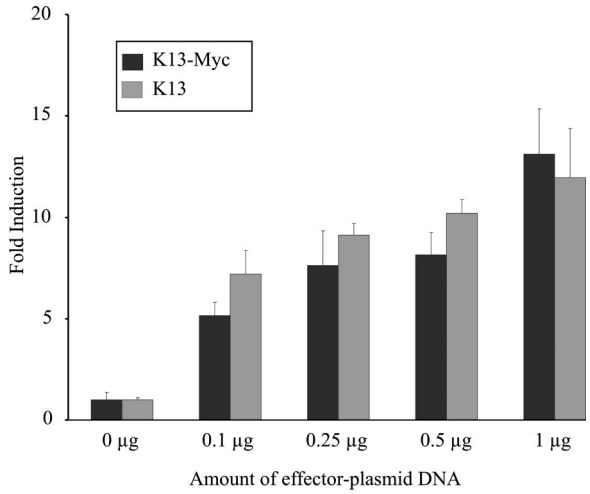

FIG. 7. NF-κB activation with Myc-tagged and untagged K13. HeLa cells were cotransfected with reporter plasmid (pNF-κB-Luc; 1 μg) and increasing concentrations of effector plasmids encoding Myc-tagged or untagged K13. Activation of the NF-κB promoter was analyzed by luciferase measurement 48 h after transfection. Values were adjusted to total protein content, and the results are expressed in terms of the relative increase in induction in comparison with the negative control (0 μg).

TABLE 3. Subcellular localization of K10 and putative K10 interacting proteins

Putative K10 interaction partner	Localization change at coexpression[a]		Colocalization
	Interaction partner	K10	
K12	−	+	Yes
ORF2	−	−	Yes
ORF9	−	+	Yes
ORF28	−	+	No
ORF29b	−	+	Yes
ORF31	−	+	Yes
ORF37	−	−	Yes
ORF39	−	+	Yes
ORF41	+	+	Yes
ORF47	−	+	Yes
ORF59	−	−	Yes
ORF60	+	+	Yes
ORF61	−	−	No
ORF67.5	−	+	Yes
ORF68	+	+	Yes

[a] +, change in localization was observed; −, no change in localization was observed.

(79). Unexpected Golgi localization was observed with thymidine kinase (ORF21) and the minor capsid protein (ORF43). It remains to be determined in future studies whether this may indicate membrane-associated and/or secretory functions of these proteins.

Among the most surprising findings of our study was the partial nuclear localization of v-FLIP/K13. Nuclear localization is not observed for cellular FLIPs, which are resident exclusively in the cytoplasm (44, 54). This indicates that HHV-8-encoded v-FLIP may exert different and/or additional functions compared to cellular FLIPs. It is in line with our finding that nuclear localization of other death effector domain (DED)-containing molecules such as DEDD, DEDD2, partially processed caspase-8, and the N-terminal DED of caspase-8 (DEDa) has been detected recently and associated with their regulatory function in apoptosis (2, 74, 88).

We noticed that the nuclear K10 protein was identified recently as a major interacting protein of HHV-8, which can bind to at least 15 different HHV-8 proteins (82). Interestingly, only 33% (5 of 15) of the potential binding factors of K10 were detected also in the nucleus when the proteins were expressed alone. However, when K10 was coexpressed with its putative binding factors, colocalization was observed in 87%. These findings demonstrate that mutual protein interactions in an infected cell affect subcellular localization. In agreement with this, other investigators have described significant relocations of HHV-8 proteins in latently rather than lytically infected primary effusion lymphoma cells (34, 86). The localization map of individually expressed HHV-8 proteins described here may provide a useful reference to detect positional effects of other HHV-8 proteins in virus-infected cells. In addition, the intracellular localization map may provide a valuable platform to further elucidate the function of HHV-8-encoded genes in human diseases.

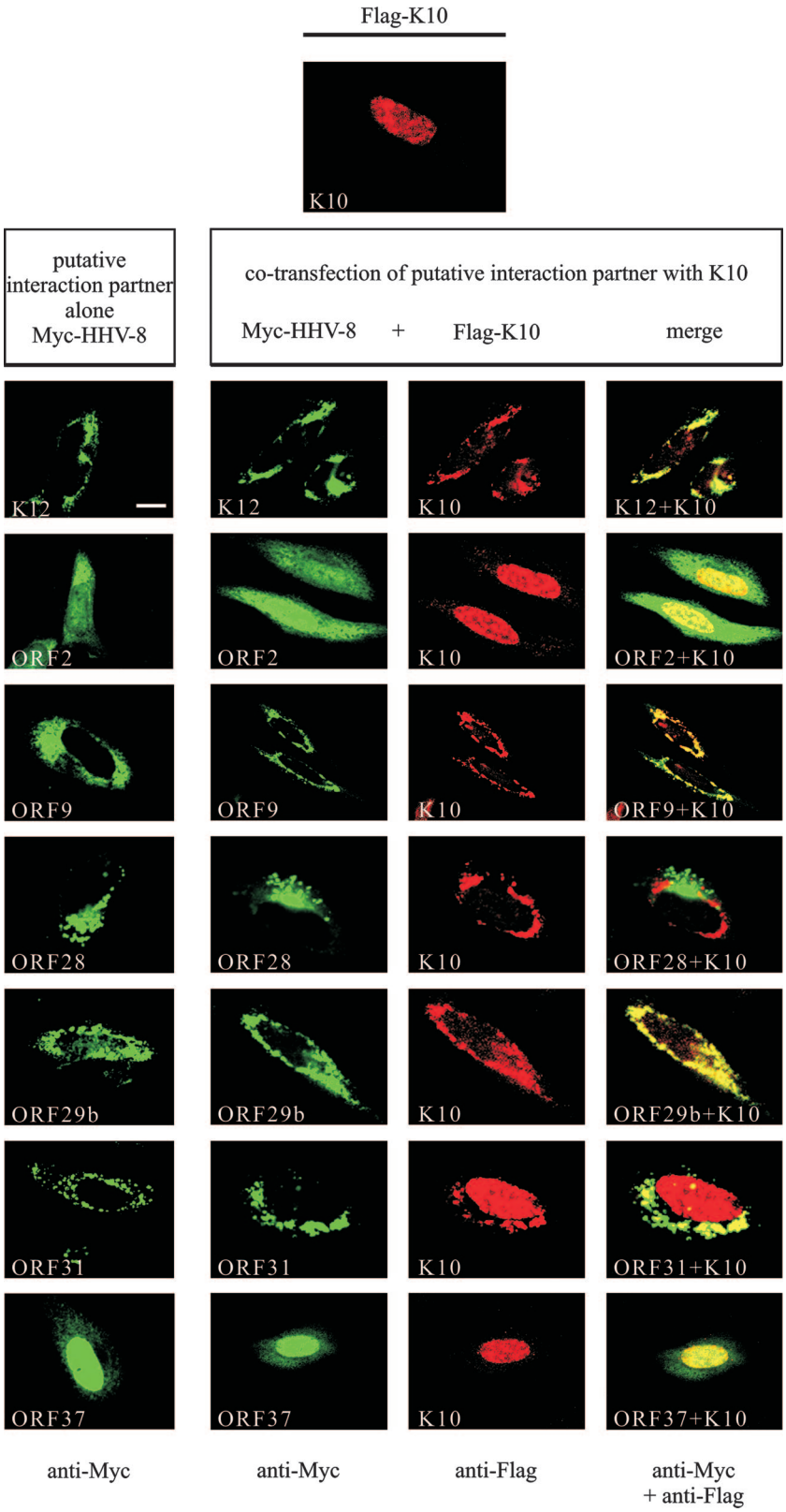

FIG. 8. Colocalization of K10 and its putative interaction partners. A Flag-tagged K10 protein (Flag-K10) and different Myc-tagged HHV-8 proteins (Myc-HHV-8) were expressed in HeLa cells either alone or in combination (Myc-HHV-8 + Flag-K10). The different proteins were detected with antibodies directed against either the Myc tag or the Flag tag or against both epitopes simultaneously (bottom line). Colocalization was analyzed by confocal laser scanning microscopy. The bar shown in K12 represents 10 μm. The same magnification was used in all panels.

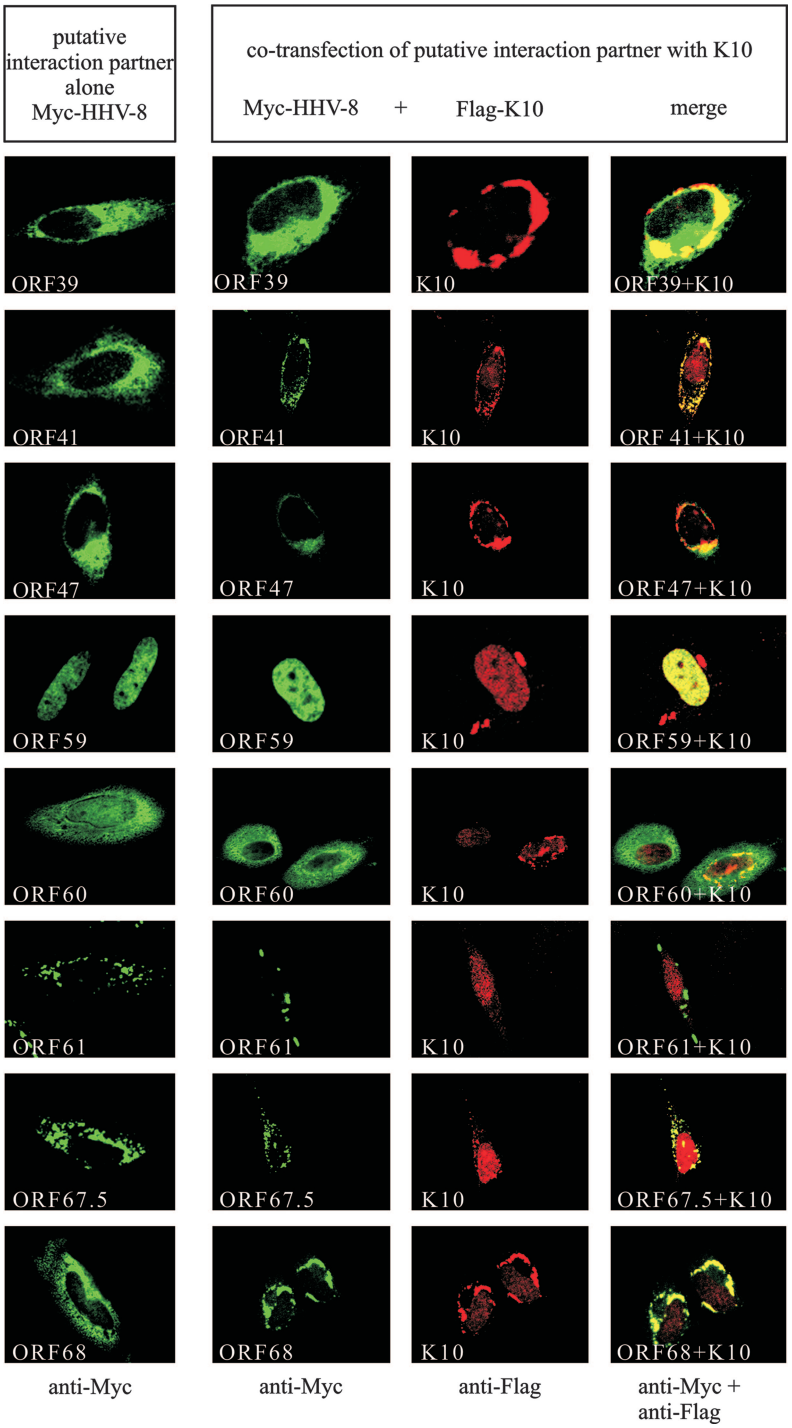

FIG. 8—*Continued*.

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