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# Marek's Disease Virus VP22: Subcellular Localization and Characterization of Carboxyl Terminal Deletion Mutations

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Marek's disease virus (MDV) is an alphaherpesvirus that causes T cell lymphoma and severe immunosuppression in chickens. The MDV UL49 gene, which encodes the tegument viral protein 22 (VP22), has been expressed as a green fluorescent protein (GFP) fusion protein in chicken embryonic fibroblasts to examine its subcellular localization. As with both human herpesvirus 1 and bovine herpesvirus 1VP22-GFP fusion proteins, the MDV VP22-GFP product binds to microtubules and heterochromatin. In addition, the MDV protein also binds to the centrosomes. During mitosis, VP22-GFP binds to sister chromatids, but dissociates from the centrosomes and the microtubules of the mitotic spindle. A series of VP22 carboxy terminal truncation mutants were constructed to define regions responsible for these binding properties. These mutants identified separable domains or motifs responsible for binding microtubules and heterochromatin. 

*Key Words:* herpesvirus; Marek's disease virus; viral protein; VP22; GFP; localization.

## INTRODUCTION

Marek's disease virus (MDV) is a highly virulent oncogenic herpesvirus of chicken that causes severe immunosuppression and T cell lymphoma (Venugopal, 2000). Based upon its genomic sequence, MDV is a member of the alphaherpesvirus family which includes the human herpesviruses (HHV1, 2, and 3), bovine herpesvirus 1 (BHV1), and several other herpesviruses (Roizman and Sears, 1996). Members of this family show extensive conservation of their genomic organization and more than 50 homologous genes are readily identified in the comparison of MDV with HHV1 (Lee *et al.,* 2000; Tulman *et al.,* 2000). The structure of the herpesvirus particle is also conserved within this family. The virions are composed of three layers: a capsid containing the genome, an amorphous tegument layer surrounding the capsid, and a membranous envelope (Cho *et al.,* 1999). Most of the structural proteins that comprise these layers also exhibit significant structural and functional homology between the different members of alphaherpesvirus family.

One of the tegument structural proteins that is conserved among the alphaherpesviruses is Virus Protein 22 (VP22). The HHV1 and BHV1 VP22 proteins exhibit several properties when expressed in cells, including association with microtubules, heterchromatin, and chromatids. HHV1, BHV1, and Marek's disease virus VP22 are capable of intercellular transport (Dorange *et al.,* 2000; Elliott and O'Hare, 1997; Harms *et al.,* 2000) and MDV VP22 has been shown to bind DNA (Dorange *et al.,* 2000). Analysis of the sequence of VP22 reveals little similarity to any known cellular protein suggesting it uses unique means to interact with cellular structures and for intercellular transport (Dorange *et al.,* 2000).

To study the specific subcellular localization of MDV VP22 and provide tools to study its relationship to MDV infection, we have constructed green fluorescent protein (GFP) chimeras with VP22. GFP has proven to be an excellent and accurate reporter of subcellular localization when fused to a variety of herpesvirus proteins (Bello *et al.,* 1999; Brideau *et al.,* 1998; Degreve *et al.,* 1999; Desai and Person, 1998; Loimas, *et al.,* 1998; Lomonte and Everett, 1999; Soliman and Silverstein, 2000; Yamada *et al.,* 1999; Zhu *et al.,* 1999) including VP22 (Dorange *et al.,* 2000; Elliott and O'Hare, 1997, 1999a,b, 2000; Harms *et al.,* 2000). All of the properties observed with HHV1 VP22 have also been observed with GFP-VP22 fusion proteins (Dorange *et al.,* 2000; Elliott and O'Hare, 1997, 1999a,b, 2000; Harms *et al.,* 2000). Consequently, we have used fusion proteins between MDV-VP22 and GFP to determine the subcellular localization of MDV-VP22. As with HHV1 and BHV1 VP22, MDV VP22 is localized to microtubules and the nucleus. Within the nucleus, MDV VP22 appeared bound to heterochromatin. During mitosis, MDV VP22 associates with chromatids, but is absent from the microtubules of the spindle. In addition, MDV VP22 binds to centrosomes and also appears to bind the midbody that connects daughter cells following mitosis. Finally, we have constructed a series of carboxy terminal truncation mutations that demonstrate dissociation of microtubule, nuclear, and centrosome localization.

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## RESULTS

### VP22 interacts with components of the microtubule network

Marek's disease virus VP22 was cloned in frame with the coding sequence of the green fluorescent protein (GFP) and the subcellular localization of the resulting fusion protein was determined by confocal microscopy. The VP22-GFP encoding plasmid was transfected into secondary chicken embryonic fibroblasts. VP22-GFP expressed protein was found in both the cytoplasmic and nuclear compartments. Within the cytoplasm, VP22-GFP exhibited at least two distinct, specific patterns (Fig. 1). Typically, it formed a filamentous network reminiscent of microtubules. It also exhibited a punctuate pattern with multiple spots of different sizes. This differs dramatically from the cytoplasmic distribution of GFP alone, which is found diffusely throughout the cytoplasm, with no evidence of filaments or punctuate distribution.

To characterize further the subcellular localization of VP22-GFP, transfected cells were fixed, incubated with anti-alpha-tubulin antibodies, and then visualized with Texas red conjugated anti-mouse IgG. As seen in Fig. 1a–c, filamentous VP22 green fluorescence was found to localize with the microtubules. Typically, much of the cytoplasmic VP22-GFP formed a continuous network with the microtubules. The VP22-GFP giving rise to punctuate fluorescence is presumably aggregated fusion protein. These aggregates were typically in close approximation to the microtubule network. In some cells, VP22-GFP protein was also found in a perinuclear spot that appeared as part of the centrosome (Fig. 1d–f). Staining transfected cells using anti-gamma tubulin, which is found in centrosomes (Dictenberg *et al.,* 1998; Moritz *et al.,* 1995; Oakley *et al.,* 1990), supported this conclusion (Fig. 1e). In some instances, this VP22 appeared to be composed of two distinct spots, suggesting association with individual centrosomes (Fig. 1f). VP22-GFP also appeared on some, but not all microtubules, radiating from these centrosomes. In several cases, VP22-GFP associated with the nuclear envelope appeared more concentrated in a region opposite the centrosome and then decreased in a gradient radiating from this location. (Fig. 1d).

### MDV VP22 interacts with heterochromatin and chromatids

In addition to the cytoplasm, MDV VP22-GFP was also found within the nucleus (Fig. 1). Staining experiments with heterochromatin binding Hoechst 33342 revealed localization of nuclear MDV VP22-GFP to the heterochromatin (Fig. 1g–i). This contrasts with the nuclear distribution of GFP alone, which is distributed diffusely in the nucleus, although it is excluded from the nucleoli.

While the MDV VP22-GFP fusion protein was found both within the nucleus and associated with microtubules in interphase cells, in mitotic cells the fusion protein was only found associated with the chromatids (Fig. 1j). No VP22-GFP was found associated with the mitotic spindle. In postmitotic cells, VP22-GFP appeared bound to a microtubular structure, possibly the midbody, connecting the two daughter cells (Fig. 1k).

TABLE 1

Effect of Deletion Mutations on VP22 Binding Properties

| Deletion | Microtubules | Heterochromatin | Centrosome |
|---|---|---|---|
| D214 and D183 | + | + | + |
| D171 | − | + | + |
| D144 | − | − | + |

### Mutations dissociate nuclear and microtubule binding

A series of carboxy terminal mutations were constructed to begin defining regions responsible for the VP22-properties (Table 1, Fig. 2). These truncations indicate that the microtubule, heterochromatin, and centrosome binding domains are separable. The truncation mutants D214 and D183 exhibit the properties of wild-type VP22 and bind microtubules, heterochromatin, and centrosomes (Fig. 2a–b). The D171 carboxy terminal truncation binds heterochromatin and centrosomes (Fig. 2c). However, it does not form filamentous structures and appears to have lost its ability to interact with microtubules. Another truncation, D144, binds centrosomes, but not longer interacts with either heterochromatin or microtubules (Fig. 2d). The centrosomal localization in all of these mutants indicates that the expressed VP22 protein is not simply denatured, but retains some folded structure.

### Sequence alignment

Given that MDV, HHV1, and BHV1 share similar properties, comparison of the amino acid sequences and predicted secondary structures, combined with results from the mutation studies, may help define motifs responsible for these properties. Figure 3 shows a plot of similarity for these three proteins as a function of amino acid position. There is a notable lack of similarity throughout the amino and carboxy terminal regions of the VP22 proteins. A core region, corresponding to MDV VP22 amino acids 94–180, exhibits significant conservation of both primary amino acid sequence and predicted secondary structure (Fig. 3). Analysis with the program MAST, which identifies potentially unique motifs in multiple sequence alignments, identified three potential motifs within the aligned VP22 proteins. All three predicted motifs fall within the core sequence, and two are located within a region apparently important for binding heterochromatin.

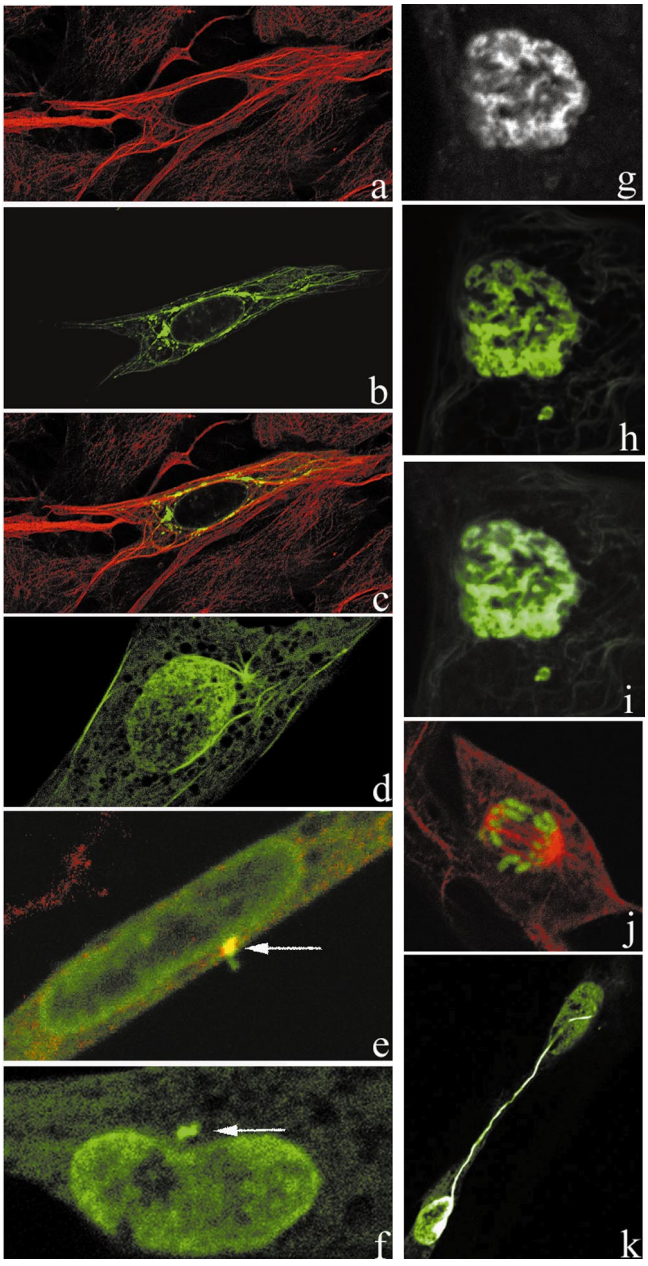



FIG. 1. Distribution of VP22-GFP. (a–c) CEF cells were transfected with VP22-GFP and replated onto microscope slides. Cells were incubated with anti-alpha-tubulin antibody and then stained with Texas red anti-mouse IgG. (a) Bundled microtubules stained red in a cell expressing VP22-GFP. Contrast with thickness of microtubules in surrounding cells. (b) VP22-GFP fluorescence showing similarity to the distribution of tubulin. (c) Composite image of panels a and b. (d) Nuclear and centrosomal localization of VP22-GFP. VP22-GFP fluorescence showing association with the centrosome, filaments, and the nucleus. (e) Centrosomal localization of VP22-GFP (arrow). Anti-gamma tubulin combined with Texas red conjugated anti-mouse IgG was used to label centrosomes of CEF cells transfected with VP22-GFP. (f) Centrosomal GFP-VP22 appearing as two spots (arrow). (g–i) VP22-GFP associates with heterochromatin. CEF cells transfected with VP22-GFP were stained with Hoechst 33342 to reveal heterochromatin. (g) Hoechst 33342 staining. (h) VP22-GFP. (i) Composite of panels g and h. Note extensive colocalization of nuclear VP22-GFP and the heterochromatin stain. (j) VP22-GFP associates with mitotic chromatids. Composite image of a CEF cell expressing VP22-GFP and stained for tubulin. Note association of VP22-GFP with chromatids, but its absence from the mitotic spindle. (k) VP22-GFP links the nuclei of two cells by apparent association with the midbody.

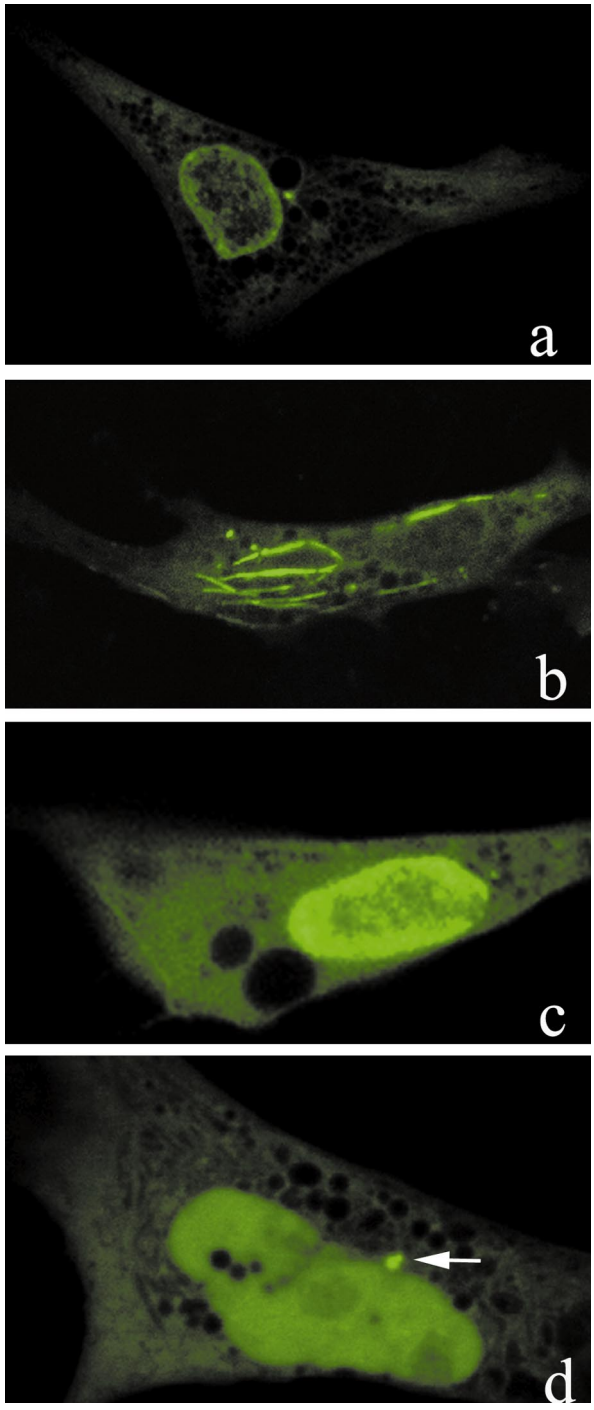
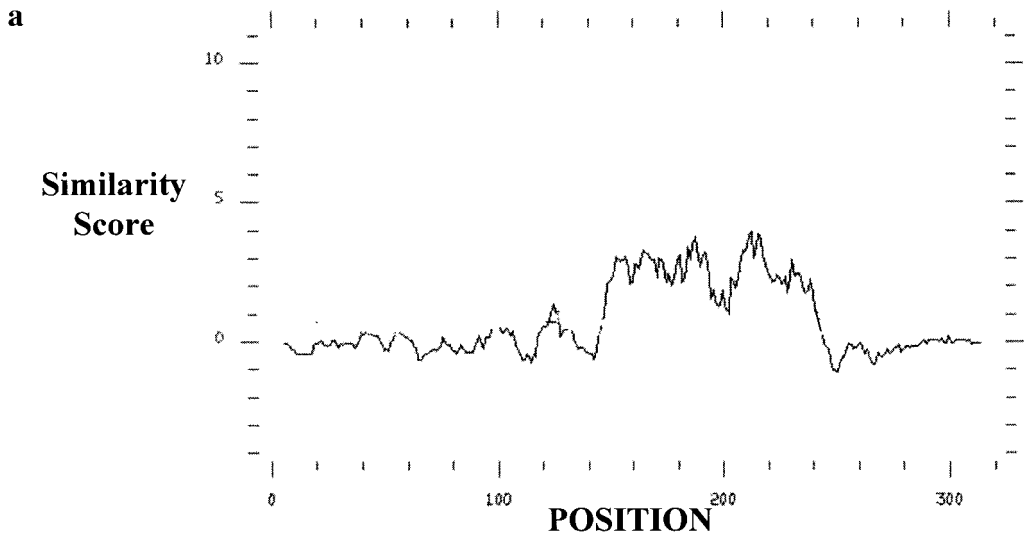



FIG. 2. Distribution of carboxy terminal deletion mutants of VP22-GFP. (a) Carboxy terminal deletion mutant D214, showing both heterochromatin and centriole binding. (b) Mutant D183VP22-GFP showing assoication with microtubule structures. (c) D171VP22-GFP showing heterochromatin association in nucleus. (d) D144VP22-GFP showing association with the centriole.

## DISCUSSION

As with human herpesvirus 1 (HHV1) and bovine herpesvirus (BHV), VP22, the MDV VP22 protein exhibits multiple distinct patterns of subcellular localization (Dorange *et al.,* 2000; Elliott and O'Hare, 1997, 1998; Elliott *et*




b

```
MDV1     94   FAFSTAPSSA SSTWRSNTVA FNQRMFCGAV ATVAQYHAYQ GALALWRQDP
EHV1    177   LAFSGTPKTP KSQWYGATHL FNKNVFCAAV SRVAAAHASD AASALWDLNP
HHV3    156   ISFSTAPKTA TSSWCGPTPS YNKRVFCEAV RRVAAMQAQK AAEAAWNSNP
BHV1    148   LAFSAAPKTP KAPWCGPTHA YNRTIFCEAV ALVAAEYARQ AAASVWDSDP
HHV1    176   LHFSTAPPNP DAPWTPRVAG FNKRVFCAAV GRLAAMHARM AAVQLWDMSR
HHV2    176   LHFSTAPPSP TAPWTPRVAG FNKRVFCAAV GRLAATHARL AAVQLWDMSR
consensus       FS  P       W        N   FC AV    A   A    A   W
ssp             ---------- ---------- -----HHHHH HHHHHHHHHH HHHHH-----
motifs                               {FNKRVFCAAV}                {WDMDP
```

D144 D171 D183

Heterochromatin Microtubule

```
MDV1    144   PRTNEELDAF LSRAVIKITI QEGPNLMGEA ETCARKLLEE SGLSQGNENV
EHV1    227   PKTNEDLDRF LKAAAIRILV CEGAQLLEVA NSTMESTPDG YAAAGPNGYD
HHV3    206   PRNNAELDRL LTGAVIRITV HEGLNLIQAA NEADLGEGAS VSKRGHNRKT
BHV1    198   PKSNERLDRM LKSAAIRILV CEGSGLLAAA NDILAARAQR PAARGSTSGG
HHV1    226   PRTDEDLNEL LGITTIRVTV CEGKNLLQRA NELVNPDVVQ DVDAATATRG
HHV2    226   PHTDEDLNEL LDLTTIRVTV CEGKNLLQRA NELVNPDAAQ DVDATAAARG
consensus     P   E L    L    I      EG  L   A
ssp           -----HHHHH HHHH EEEEE E   HHHHH----------------------
motifs        PRTNEDLDRL L}   {IRITV CEG}
```

FIG. 3. Conservation of VP22. (a) A plot of the similarity of herpesvirus VP22 protein. Sequences aligned were derived from MDV1, equine herpesvirus 1 (EHV1), human herpesvirus 3 (HHV3), bovine herpesvirus 1 (BHV1), human herpesvirus 2 (HHV2), and human herpesvirus 1 (HHV1). (b) Alignment of VP22 protein sequences from the core region (see text). Positions of the three truncation mutations are indicated. Heterochromatin and microtubule indicate that loss of the region bounded by the mutations confers loss of binding to the respective structure.

*al.,* 1999; Harms *et al.,* 2000). The work presented here demonstrates that MDV VP22-GFP expressed in chicken embryonic fibroblasts by transfection localizes to microtubules, nuclei, mitotic chromatids, and the centrosome. Furthermore, the carboxy terminal deletion series demonstrates that the microtubular, nuclear, and centrosome localization are separable, arguing these properties are attributes of independent VP22 domains or motifs.

The interaction of MDV VP22 with microtubules is similar to that reported for HHV1 and BPV proteins. Microtubules with bound MDV VP22-GFP appeared both rearranged and thicker relative to surrounding cells not expressing the fusion protein (Fig. 1). This is consistent with reports that human herpesvirus VP22 causes acetylation, bundling, and stabilization of cellular microtubules (Elliott and O'Hare, 1998). Cells containing these thick microtubules decorated with MDV VP22-GFP have frequently lost their centrosome as has been seen with VP22 encoded by other herpesviruses. The role of this VP22 activity during viral infection is uncertain and the BHV VP22 gene is dispensible for *in vitro* passage of the virus (Liang *et al.,* 1995). During early stages of infection, herpesvirus capsids bind microtubules for transport to the nucleus. (Granzow *et al.,* 1997; Sodeik *et al.,* 1997). Perhaps the ability of VP22 to bind and bundle microtubules plays an important role in this step of the herpesvirus replicative cycle.

The nuclear VP22-GFP appears localized predominantly to the heterochromatin. This is consistent with the reports of Dorange *et al.* (2000) that VP22 is capable of directly binding DNA. In mitotic cells, MDV VP22-GFP associates with the chromatids, possibly by direct inter-

action with the condensed DNA. MDV VP22 contains no canonical nuclear import signal, suggesting that it may enter the nucleus either attached to some other nuclear protein or by passive diffusion. The function of the nuclear VP22 is unknown. A conserved characteristic of all VP22 proteins examined to date is that they are capable of intercellular transport, and the transported VP22 is concentrated in the nucleus of the target cell. These properties suggest that the nuclear VP22 may modulate transcription or replication of host cells, perhaps preparing them for infection (Elliott and O'Hare, 1997, 2000).

MDV VP22 also appears capable of localizing to the centrosome. VP22 associated with the centrosomes was typically visible in cells that also exhibited nuclear VP22-GFP fluorescence. In many cases, the nuclear fluorescence appeared concentrated at a position of the nuclear envelope opposite the labeled centrosomes. The nuclear fluorescence then diminished in a gradient fashion as a function of distance from the centrosome. This may reflect preferential localization of heterochromatin to regions of the nuclear envelope opposing the centrosomes as cells proceed through early mitosis.

Despite the ability of VP22-GFP to bind microtubules, during mitosis, only the chromatids have bound fusion protein. VP22-GFP is absent from the microtubular mitotic spindle associated with mitosis. Undoubtedly, this reflects some modification of the ability of VP22 to interact with microtubules. HHV1 VP22 can be phosphorylated at multiple sites, and phosphorylation has been implicated in nuclear localization (Elliott *et al.,* 1996; Pomeranz and Blaho, 1999). Perhaps phosphorylation augments chromatin binding and inhibits interaction with microtubules. Also possible are alterations in the microtubules that prevent interactions with VP22. Such alterations could include modifications, such as phosphorylation, or mitosis-specific degradation of microtubular-associated proteins that are responsible for binding VP22.

The carboxyl terminal truncation series demonstrates that the microtubule, nuclear, and centrosome binding properties of VP22 are separable. In addition, others have shown that the region of HHV1 VP22 responsible for intracellular transport can be distinguished from microtubule binding. Taken together, these observations suggest that VP22 may consist of as many as four distinct domains or motifs. Despite interactions with disparate cellular compartments and structures, comparison of MDV VP22 with the protein database by both Blast and Psi-Blast (Altschul *et al.,* 1997), does not reveal any significant similarities beside other viral VP22 proteins. This suggests that these properties of VP22 were not pirated from cellular genes but arose during the evolution of herpesviruses.

Alignment of VP22 proteins encoded by different herpesviruses indicates a core region of conservation, spanning from MDV VP22 amino acid residues 94–180, with significant conservation of both primary amino acid sequence (Dorange *et al.,* 2000) and predicted secondary structure. Outside of this region, the secondary structure is predicted to be predominantly disordered, which is consistent with the direct measurements of VP22 secondary structure (Kueltzo *et al.,* 2000). The deletion mutations have mapped a regions of MDV VP22 between residues 144 and 171 and between 171 and 183 as important to heterochromatin and microtubule binding, respectively. With this information, combined with the predicted motifs (Fig. 3), it may be possible to design point mutations that affect only one of these binding properties and resolve their relative contributions to the virus replicative cycle. Also, because at least part of the primary sequence responsible for MDV-VP22 recognition of heterochromatin and microtubules resides in the conserved core region, these sites may provide useful targets for the design of future anti-herpesvirus drug therapies.

TABLE 2

VP22 Primers

| | |
|---|---|
| 5′ VP22 primer | TCTAAGCTTGTTTAATATTATATCTTAGTTAC |
| D214 primer | GATCGGATCCGATTTCAATTTCACCGCCGCGTCTT |
| D183 primer | GATCGGATCCCTCTTCCAATAGTTTGCGGGCACAG |
| D171 primer | GATCGGATCCCATCAAATTTGGACCCTCTTGAATG |
| D144 primer | GATCGGATCCCGGAGGATCTTGACGCCAAAGGGCG |

## MATERIALS AND METHODS

### Plasmid construction

The VP22 gene and the deletion mutants were amplified from the RB1B strain of Marek's disease virus by the polymerase chain reaction and the primers indicated in Table 2. The product was inserted into Bam HI-EcoRI digested pEGFP plasmid (Clontech, Palo Alto, CA) and the sequence of the insert determined to verify that no errors were introduced by PCR. DNA sequencing was done using a Perkin Elmer ABI-377 Sequencer and dye terminators.

### Transfections

Chicken embryonic fibroblasts were prepared from 10-day-old embryos using the method of Silim *et al.* (1982). Cells were transfected by the calcium phosphate technique (Graham and van der Eb, 1973) and transferred to 2-well, 25 mm slides prior to observation by confocal microscopy.

### Antibody staining

Monoclonal anti-alpha tubulin (Amersham, Arlington Heights, IL) and anti-gamma tubulin (Sigma, St. Louis, MO) were used at a 1:500 dilution and visualized with Texas red coupled anti-mouse antibody (Amersham) using standard protocols (Spector *et al.,* 1997). Images were obtained using a Zeiss LSM 510 confocal microscope.

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## REFERENCES

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* **25**(17), 3389–3402.

Bello, L. J., Davison, A. J., Glenn, M. A., Whitehouse, A., Rethmeier, N., Schulz, T. F., and Barklie Clements, J. (1999). The human herpesvirus-8 ORF 57 gene and its properties. *J. Gen. Virol.* **80**(Pt 12), 3207–3215.

Brideau, A. D., Banfield, B. W., and Enquist, L. W. (1998). The Us9 gene product of pseudorabies virus, an alphaherpesvirus, is a phosphorylated, tail-anchored type II membrane protein. *J. Virol.* **72**(6), 4560–4570.

Cho, K. O., Ohashi, K., and Onuma, M. (1999). Electron microscopic and immunohistochemical localization of Marek's disease (MD) herpesvirus particles in MD skin lymphomas. *Vet. Pathol.* **36**(4), 314–320.

Degreve, B., Esnouf, R., De Clercq, E., and Balzarini, J. (1999). Characterization of multiple nuclear localization signals in herpes simplex virus type 1 thymidine kinase. *Biochem. Biophys. Res. Commun.* **264**(2), 338–342.

Desai, P., and Person, S. (1998). Incorporation of the green fluorescent protein into the herpes simplex virus type 1 capsid. *J. Virol.* **72**(9), 7563–7568.

Dictenberg, J. B., Zimmerman, W., Sparks, C. A., Young, A., Vidair, C., Zheng, Y., Carrington, W., Fay, F. S., and Doxsey, S. J. (1998). Pericentrin and gamma-tubulin form a protein complex and are organized into a novel lattice at the centrosome. *J. Cell Biol.* **141**(1), 163–174.

Dorange, F., El Mehdaoui, S., Pichon, C., Coursaget, P., and Vautherot, J. F. (2000). Marek's disease virus (MDV) homologues of herpes simplex virus type 1 UL49 (VP22) and UL48 (VP16) genes: High-level expression and characterization of MDV-1 VP22 and VP16. *J. Gen. Virol.* **81 Pt 9,** 2219–2230.

Elliott, G., and O'Hare, P. (1997). Intercellular trafficking and protein delivery by a herpesvirus structural protein. *Cell* **88**(2), 223–233.

Elliott, G., and O'Hare, P. (1998). Herpes simplex virus type 1 tegument protein VP22 induces the stabilization and hyperacetylation of microtubules. *J. Virol.* **72**(8), 6448–6455.

Elliott, G., and O'Hare, P. (1999a). Intercellular trafficking of VP22-GFP fusion proteins. *Gene Ther.* **6**(1), 149–151.

Elliott, G., and O'Hare, P. (1999b). Live-cell analysis of a green fluorescent protein-tagged herpes simplex virus infection. *J. Virol.* **73**(5), 4110–4119.

Elliott, G., and O'Hare, P. (2000). Cytoplasm-to-nucleus translocation of a herpesvirus tegument protein during cell division. *J. Virol.* **74**(5), 2131–2141.

Elliott, G., O'Reilly, D., and O'Hare, P. (1996). Phosphorylation of the herpes simplex virus type 1 tegument protein VP22. *Virology* **226**(1), 140–145.

Elliott, G., O'Reilly, D., and O'Hare, P. (1999). Identification of phosphorylation sites within the herpes simplex virus tegument protein VP22. *J. Virol.* **73**(7), 6203–6206.

Graham, F., and van der Eb, A. (1973). A new technique for the assay of infectivity of human adenovirus 6 DNA. *Virology* **52,** 456.

Granzow, H., Weiland, F., Jons, A., Klupp, B. G., Karger, A., and Mettenleiter, T. C. (1997). Ultrastructural analysis of the replication cycle of pseudorabies virus in cell culture: A reassessment. *J. Virol.* **71**(3), 2072–2082.

Harms, J. S., Ren, X., Oliveira, S. C., and Splitter, G. A. (2000). Distinctions between bovine herpesvirus 1 and herpes simplex virus type 1 VP22 tegument protein subcellular associations. *J. Virol.* **74**(7), 3301–3312.

Kueltzo, L. A., Normand, N., O'Hare, P., and Middaugh, C. R. (2000). Conformational lability of herpesvirus protein VP22. *J. Biol. Chem.* **275**(43), 33213–33221.

Lee, L. F., Wu, P., Sui, D., Ren, D., Kamil, J., Kung, H. J., and Witter, R. L. (2000). The complete unique long sequence and the overall genomic organization of the GA strain of Marek's disease virus. *Proc. Natl. Acad. Sci. USA* **97**(11), 6091–6096.

Liang, X., Chow, B., Li, Y., Raggo, C., Yoo, D., Attah-Poku, S., and Babiuk, L. A. (1995). Characterization of bovine herpesvirus 1 UL49 homolog gene and product: Bovine herpesvirus 1 UL49 homolog is dispensable for virus growth. *J. Virol.* **69**(6), 3863–3867.

Loimas, S., Wahlfors, J., and Janne, J. (1998). Herpes simplex virus thymidine kinase-green fluorescent protein fusion gene: New tool for gene transfer studies and gene therapy. *Biotechniques* **24**(4), 614–618.

Lomonte, P., and Everett, R. D. (1999). Herpes simplex virus type 1 immediate-early protein Vmw110 inhibits progression of cells through mitosis and from G(1) into S phase of the cell cycle. *J. Virol.* **73**(11), 9456–9467.

Moritz, M., Braunfeld, M. B., Sedat, J. W., Alberts, B., and Agard, D. A. (1995). Microtubule nucleation by gamma-tubulin-containing rings in the centrosome. *Nature* **378**(6557), 638–640.

Oakley, B. R., Oakley, C. E., Yoon, Y., and Jung, M. K. (1990). Gamma-tubulin is a component of the spindle pole body that is essential for microtubule function in Aspergillus nidulans. *Cell* **61**(7), 1289–1301.

Pomeranz, L. E., and Blaho, J. A. (1999). Modified VP22 localizes to the cell nucleus during synchronized herpes simplex virus type 1 infection. *J. Virol.* **73**(8), 6769–6781.

Roizman, B., and Sears, A. E. (1996). "Herpes Simplex Viruses and Their Replication," 3rd Ed. Fundamental Virology (B. N. Fields, D. M. Knipe, and P. M. Howley, Eds.), Lippincott-Raven, Philadelphia.

Silim, A., El Azhary, M. A., and Roy, R. S. (1982). A simple technique for preparation of chicken-embryo-skin cell cultures. *Avian Dis.* **26**(1), 182–185.

Sodeik, B., Ebersold, M. W., and Helenius, A. (1997). Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. *J. Cell Biol.* **136**(5), 1007–1021.

Soliman, T. M., and Silverstein, S. J. (2000). Identification of an export control sequence and a requirement for the KH domains in ICP27 from herpes simplex virus type 1. *J. Virol.* **74**(16), 7600–7609.

Spector, D., Goldman, R., and Leinwand, L. (1997). "Cells — A Laboratory Manual," Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Tulman, E. R., Afonso, C. L., Lu, Z., Zsak, L., Rock, D. L., and Kutish, G. F. (2000). The genome of a very virulent Marek's disease virus. *J. Virol.* **74**(17), 7980–7988.

Venugopal, K. (2000). Marek's disease: An update on oncogenic mechanisms and control. *Res. Vet. Sci.* **69**(1), 17–23.

Yamada, H., Jiang, Y. M., Zhu, H. Y., Inagaki-Ohara, K., and Nishiyama, Y. (1999). Nucleolar localization of the UL3 protein of herpes simplex virus type 2. *J. Gen. Virol.* **80**(Pt 8), 2157–2164.

Zhu, H. Y., Yamada, H., Jiang, Y. M., Yamada, M., and Nishiyama, Y. (1999). Intracellular localization of the UL31 protein of herpes simplex virus type 2. *Arch. Virol.* **144**(10), 1923–1935.