VP22, a tegument protein of bovine herpesvirus 1, accumulates in the nucleus of infected and transiently transfected cells. Previous studies indicated a possible regulatory function of VP22 within nuclei, but how VP22 enters nuclei is unknown. Despite the abundance of basic residues within this protein, no classic nuclear localization signal (NLS) motif has been identified. To identify the signal directing nuclear accumulation, a series of truncations, internal deletions, and point mutations were constructed. Fluorescence microscopy of cells transfected with VP22 constructs indicated that a sequence of 103 residues is necessary and sufficient for nuclear localization. This NLS sequence is conformation-sensitive in contrast to a classical sequential NLS. Energy depletion assays and co-immunoprecipitation suggested that this NLS sequence also binds histone H4, resulting in nuclear retention of VP22. In addition, a mitochondrial targeting sequence was identified at the C-terminal 49 amino acids, which overlapped the sequence required for nuclear targeting. Our findings demonstrate the diversity of VP22 protein to localize within the cell and provide the opportunity for VP22 to direct cargo specifically to different subcellular compartments.

VP22 is a major component of the herpesvirus tegument, a viral compartment located between the capsid and envelope. As is true for many viral tegument proteins, the function of VP22 in viral replication and infection is yet to be elucidated. Bovine herpesvirus 1 (BHV-1) VP22 is dispensable for viral growth in cell culture, but deletion of VP22 from the virus genome delays virus growth and greatly reduces virus yield (1). A herpes simplex virus-1 mutant expressing a truncated form of VP22 has been characterized (2). Interestingly expression of VP22 in transfected cells up-regulates mRNA to adjacent cells where the mRNA is translated (8). By importing specific mRNA to target cells, VP22 may prepare cells for the arrival of herpesvirus.

Despite a variety of potential functions for VP22 within nuclei, little is known regarding how this protein is transported into the nuclei. BHV-1 VP22 localizes to nuclei in transiently transfected cells in the absence of other viral proteins, suggesting that this process is independent of other viral factors (6, 9). In the present study, we sought to delineate the nuclear localization signal of VP22. We constructed a series of truncations and internal deletions of VP22 as fusion proteins with yellow fluorescent protein (YFP) to visualize their subcellular localization in transfected cells. Our results indicated that nuclear accumulation requires an unusually long sequence comprising 103 amino acid residues. This localization signal is sensitive to disruption of protein conformation, suggesting a non-conventional signal, which depends on its complex protein structure. Energy-depleting assays and co-immunoprecipitation indicated that nuclear retention, resulting from association of the VP22 nuclear retention sequence with histones, is also involved in nuclear accumulation of VP22. Furthermore the C-terminal 49 amino acids of VP22 were identified as a mitochondrial targeting sequence, which functioned only when the conformation of the nuclear localization signal was disrupted. The potential roles of these overlapping signal sequences directing VP22 to different subcellular locations are discussed.
inserting the products in the BamHI and AgeI sites of pEYFP-N1 vector (BD Biosciences). VP22 fragments smaller than 50 amino acids were amplified as a VP22-YFP fusion gene from appropriate VP22-YFP plasmids and digested with BamHI/HpaI and then cloned into the pEYFP-N1 vector.

Mutant DNA lacking the encoding sequence for 8 amino acids was synthesized by PCR using pEYFP-VP22-(118–258)-YFP as a template and appropriate forward and reverse primers. The resulting PCR products, containing the entire sequence of vector pEYFP-VP22-(118–258)-YFP except the targeted 8 amino acids, were blunt end-ligated with T4 ligase. A similar method was used to construct glycine substitution mutants (forward primer, 5′-GGG GAT CC A TG CCG CCG AAG AAG AAG CGA AAG GTC ATG GGG TCC TCC GGC GGC GGC GGG-3′; and reverse primer, 5′-GCC CCC GAG CGC GAC GGC GGC GGC GGG-3′) for NLS VP22-(118–221) and appropriate reverse primers, and the resulting PCR products were cloned into pEYFP-N1 in the BamHI and AgeI sites. All mutations were verified by sequencing, and expression of the fusion proteins was confirmed by Western blot analysis using anti-YFP antibody (JL-8, BD Biosciences) or rabbit anti-VP22 antiserum prepared in our laboratory. All PCRs were performed using Pfu DNA polymerase (Stratagene, La Jolla, CA). Restriction enzymes and T7 DNA ligase were purchased from Promega (Madison, WI).

**Transfection, ATP Depletion, and Fluorescence Microscopy**—Cells were grown in 4-well Lab-Tek chambered coverglasses (Nalge Nunc International, Naperville, IL) to semiconfluence and transfected with 0.3 μg of DNA/well using Lipofectamine (Invitrogen). Thirty hours after transfection, cells were washed and examined with an Axiovert S100 microscope.

**Fig. 1.** A core sequence of 103 amino acids supports VP22-YFP fusion protein nuclear accumulation. A, schematic representation of VP22 truncations generated as in-frame fusions to YFP; the subcellular localization of these fusions in the transfected cells is present on the right. B, fluorescence microscopy of VP22-YFP fusion proteins in transfected D17 cells. Note that VP22-(130–232) is essential for nuclear localization; trimming from either end of the fragment will compromise VP22 nuclear accumulation. Scale bar, 10 μm. C, subcellular fractionation and Western blot analysis of D17 cells transfected with plasmids expressing YFP (panel A), VP22-(1–258)-YFP (panel B), VP22-(1–123)-YFP (panel C), VP22-(118–258)-YFP (panel D), VP22-(130–258)-14YFP (panel E), VP22-(137–258)-YFP (panel F), VP22-(159–258)-YFP (panel G), VP22-(118–249)-YFP (panel H), VP22-(118–238)-YFP (panel I), VP22-(118–232)-YFP (panel J), VP22-(118–226)-YFP (panel K), and VP22-(130–232)-YFP (panel L). Thirty-six hours after transfection, cells were harvested, and nucleus and cytoplasm were separated. The resulting extract from cytoplasm (P) or nucleus (N) was subjected to SDS-PAGE and followed by Western blot analysis with YFP-specific antibody. The protein bands less than 35 kDa are degraded fusion proteins. Note that the subcellular localization of each fusion correlates with the result from fluorescent microscopy.
microscopy (Carl Zeiss, Inc., Thornwood, NY). To visualize mitochondria, cells were incubated with 500 nm Mitofluor Red 589 dye (Molecular Probes, Eugene, OR) for 40 min at 37 °C, then washed, and examined for YFP or mitochondrial staining. Photographs were taken using a Zeiss Axiocam HRm cooled charge-coupled device camera, and images were edited or overlaid using Zeiss Axiovision software (Carl Zeiss). To block active nuclear import, ATP was depleted by incubating D17 cells with 50 mM sodium azide (Sigma) 30 h after transfection (10, 11). Cells were then examined by fluorescence microscopy 30 min, 1 h, or 2 h after adding sodium azide.

Subcellular Fractionation and Western Blot Analysis—Thirty hours after transfection with VP22 constructs, D17 cells were harvested, and nuclei were separated from cytoplasm using Nuclei EZ Prep (Sigma). Nuclei were collected by centrifugation at 500 × g for 5 min at 4 °C, and supernatants containing the cytoplasmic components were subjected to Western blot analysis. Briefly, nuclei were washed twice with lysis buffer and dissolved in sample buffer. The nuclear or cytoplasmic extract was separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). Mitochondria were isolated using a mitochondria/ cytosol fractionation kit (BioVision, Mountain View, CA) and detected by Western blot using an anti-mitochondria specific antibody (MTC02, Abcam, Cambridge, MA). VP22-YFP fusion protein was detected with anti-YFP antibody (JL-8, BD Biosciences) followed by horseradish peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (Pierce) and visualized by enhanced chemiluminescence (Pierce). Expression of VP22 containing an internal deletion was confirmed by Western blot analysis using anti-VP22 antiserum.

Immunoprecipitation Analysis—D17 cells were transfected with constructs encoding either NLS-VP22-(1–123) or VP22-(130–232). Cells were collected 40 h after transfection and lysed in 1 ml of M-PER lysis buffer (Pierce) containing protease inhibitor mixture (Sigma). The cell extract was precleared with protein A/G-agarose beads (Pierce) and then incubated with anti-histone H4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 4 h at 4 °C. Protein A/G-agarose beads were then added to the cell lysate for a 2-h incubation at room temperature. Agarose beads were washed once with lysis buffer and three times with phosphate-buffered saline, and the precipitate was recovered by boiling in SDS-PAGE sample buffer and analyzed by Western blotting.

RESULTS

Nuclear Accumulation of VP22 Requires a Minimal 103-amino Acid Sequence—Previously we showed that only the C-terminal residues (118–258) were involved in nuclear accumulation (6). To more precisely define and characterize the NLS within this fragment, successive truncations from either the C or N terminus of VP22-(118–258) were performed. Each truncated VP22 fragment was fused at its C terminus to YFP (Fig. 1A). We investigated the subcellular localization of the VP22 truncations by transfecting plasmids into D17 cells and examining the cells 30 h after transfection by fluorescence microscopy (Fig. 1B).

Removal of 12 additional residues from the N terminus or 26 residues from the C terminus of VP22-(118–258) did not alter its nuclear localization pattern (Fig. 1B, panel E). In contrast, further removal of residues from the N or C terminus abolished nuclear accumulation of the fusion protein (Fig. 1B, panels F and G). Thus, the core NLS is comprised of amino acid 130 to a residue between 226 and 232 (Fig. 1B, panels F, I, J, and K). To test whether fragment 130–226 was sufficient for nuclear translocation, VP22-(130–232) was fused to YFP and expressed in D17 cells. As expected, VP22-(130–232)-YFP fusion protein accumulated in nuclei (Fig. 1, B and C, panel L).

To confirm the fluorescence microscopy results, nuclear and cytoplasmic fractions of D17 transfected cells were analyzed by Western blot analysis using anti-YFP antibody (Fig. 1C). The presence of fusion protein in the nuclear or cytoplasmic extracts was generally in agreement with the subcellular localization observed by fluorescence microscopy except for cells expressing VP22-(1–123)-YFP and YFP alone. In those cells, VP22-(1–123)-YFP or YFP was not detected in nuclei by Western blot, whereas the fluorescence signal appeared evenly distributed throughout the cell. We suspect that the diffuse distribution in the cytoplasm made it appear that these proteins were distributed in the nucleus by fluorescence microscopy.

Disruption of a Potential Helix within VP22-(130–232) Severe Impaired Nuclear Transport—Conventional nuclear localization signals involve a short stretch of amino acids (12). The relatively long amino acid sequence required for VP22 nuclear localization suggests that secondary or higher structure may be critical for nuclear accumulation. Secondary structure prediction software (3D-PSSM, www.sbg.bio.ic.ac.uk/~3dpsmm (13, 14)) predicted three helices within this region that had 22% similarity to the histone fold motif. To test the importance of the helices, amino acids 180–183 (VAAE) within the long helix of VP22-(130–232) were substituted with four glycine residues. Since glycine disrupts helix formation (15–17), this substitution was predicted to perturb nuclear accumulation of VP22. As expected, when the glycine substitutions were introduced in VP22-(130–232) (Fig. 2A), the resulting mutant lost nuclear accumulation with only diffuse distribution in cells (Fig. 2B, panel B). This result supports the concept that residues within a putative helix in the histone foldlike structure may be critical for nuclear transport or retention.

Nuclear Accumulation Correlates with the Ability of VP22 Fragments to Associate with Histones—We previously reported the interaction of full-length VP22 and histones (6). Association with histones might contribute to VP22 nuclear localization. To address this question, the association between VP22 fragments and histones was examined by an immunoprecipitation assay. An NLS peptide from SV40 large T antigen comprised of 8 amino acids was fused to the N terminus of VP22-(1–123)-YFP, a VP22 truncation that fails to accumulate in the nucleus. The resulting fusion protein localized to the nucleus, most likely through the classic nuclear import pathway, with an efficiency similar to that of VP22-(130–232). However, when cell lysate containing either NLS-VP22-(1–123)-YFP or VP22-(130–232)-
YFP was incubated with anti-histone H4 antibody and protein A/G beads, only VP22-(130–232)-YFP co-immunoprecipitated with histone H4 (Fig. 3). Thus, VP22-(130–232) retained nuclear localization and possessed the region that binds histones, whereas NLS-VP22-(1–123) failed to interact with histones.

To determine whether VP22 nuclear accumulation correlated with nuclear retention of VP22 through histone binding, an energy-depleting assay was performed. Sodium azide, an ATP-depleting agent, can inhibit active nuclear import and lead to the redistribution of classic NLS-containing proteins throughout the nucleus and cytoplasm (18–20). However, when protein is retained in the nucleus through association with nuclear components, sodium azide will not affect the distribution of this protein. As expected, nuclear accumulation of VP22-(130–232)-YFP was not altered following a 2-h incubation with sodium azide (Fig. 4A). However, when the NLS-VP22-(1–123)-YFP-transfected cells were incubated with sodium azide, redistribution of NLS-VP22-(1–123)-YFP from nucleus to cytoplasm was observed within 30 min as demonstrated by increased cytoplasmic staining coupled with decreased nuclear staining (Fig. 4B, lower panels).

An 8-amino Acid Internal Deletion within the VP22 C-termina l Region Relocates the Fusion Protein to a Different Subcellular Compartment—To further analyze the VP22 nuclear targeting sequence, VP22-(118–258)-YFP was used as a template to construct a series of successive internal deletions, each lacking 8 amino acids (Fig. 5A). Interestingly all internal deletions no longer demonstrated nuclear localization (Fig. 5B). Of the 12 deletions, 10 fragments with internal deletions covering amino acids 130–209 displayed a cytoplasmic filamentous network in the transfected cells, whereas deletions of amino acids 210–217 or 225–233 no longer bound to this network (Fig. 5B, panels J and L). The internal deletions did not possess nuclear trafficking ability and did not interact with histones by Western blot analysis (data not shown).

The C-terminal 49 Amino Acids of VP22 Are a Mitochondrial Targeting Sequence—Closer microscopic examination revealed that the abundance and polyphenotype of this cytoplasmic network resembled mitochondria. To confirm whether network-targeting VP22-YFP fusions localized to mitochondria, cells were treated with the mitochondria-specific dye MitoFluor Red prior to microscopy. An overlay of the fluorescent signals from VP22-YFP and MitoFluor Red revealed co-localization of mitochondria and the VP22 fusion-associated cytoplasmic network (Fig. 6, panels A, C, and E). No co-localized image was observed in cells expressing VP22 constructs that did not target mitochondria (Fig. 6, panels B, D, and F). Thus, the 49-amino acid sequence at the C terminus of VP22 is a mitochondria-targeting sequence.

Mitochondrial Targeting by VP22-(210–258) Occurs in Other Cells—To determine that mitochondrial targeting by VP22-(210–258)-YFP was used as a template to construct a series of successive internal deletions, each lacking 8 amino acids (Fig. 5A). Interestingly all internal deletions no longer demonstrated nuclear localization (Fig. 5B). Of the 12 deletions, 10 fragments with internal deletions covering amino acids 130–209 displayed a cytoplasmic filamentous network in the transfected cells, whereas deletions of amino acids 210–217 or 225–233 no longer bound to this network (Fig. 5B, panels J and L). The internal deletions did not possess nuclear trafficking ability and did not interact with histones by Western blot analysis (data not shown).

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258) was not cell type-specific, the fusion protein was expressed in cells other than D17. The VP22 construct was transfected into murine NXS2 neuroblastoma cells followed by fluorescence microscopy. Again the fusion protein localized to a filamentous cytoplasmic network that co-localized with mitochondria stained by MitoFluor Red (Fig. 7, upper panels). In addition, the mitochondrial association was observed in all transfected NXS2 cells, whereas in transfected D17 cells, a diffuse pattern with bright fluorescence intensity was observed in a subpopulation. The diffuse staining pattern likely resulted from extraordinarily high expression of VP22 in these D17 cells, and the subcellular structures were obscured by the intense fluorescence emission from VP22 fusion protein produced in the cytoplasm. The lower expression of VP22 fusion protein did not obscure mitochondrial staining in NXS2 cells. Mitochondrial targeting of VP22-(210–258)-YFP was also observed in Madin-Darby bovine kidney cells, which are derived from the natural host for bovine herpesvirus (Fig. 7, lower panels). Thus, VP22-(210–258) targeting to mitochondria is not a cell-specific phenomenon.

Additional support for VP22 interaction with mitochondria was observed when mitochondria were isolated from cytoplasmic extract, subjected to SDS-PAGE, and transferred to nitrocellulose. The membrane was incubated with full-length VP22 followed by anti-VP22 antibody. VP22 bound two proteins (60 and 85 kDa) present only in the mitochondrial fraction (Fig. 8), suggesting specificity of VP22 for mitochondria. To determine whether truncated VP22-(210–258)-YFP could also associate with mitochondria, mitochondria were isolated from transfected cells and subjected to Western blot analysis using a mitochondria-specific or anti-YFP specific antibody (Fig. 9). The data support the trafficking of VP22-(210–258)-YFP to the mitochondria.

**DISCUSSION**

VP22, a tegument protein of bovine herpesvirus 1, accumulates in nuclei of infected and transiently transfected cells, but the mechanism of nuclear entry is unknown. Although VP22 contains an abundance of basic residues, no classic NLS has been identified.

A prototypic or classic NLS, such as the SV40 large T antigen, consists of short stretches of basic amino acids (21, 22), whereas a bipartite NLS consists of two clusters of basic residues separated by 10–12 amino acids, often including proline residues (23). Previously a C-terminal VP22 fragment (amino acids 118–258) was shown to possess nuclear accumulation similar to that of full-length VP22 (6), and in the present study,
we searched for an NLS within 118–258. A 103-amino acid sequence from residues 130 to 232 was sufficient for nuclear localization, and deletion or insertion of 8 amino acids within this 103-amino acid sequence prevented VP22 accumulation in the nucleus. We reasoned that the 8-amino acid internal deletion may disrupt the secondary or higher structure of the C-terminal domain, a structure that may be required for nuclear localization. The relatively long amino acid sequence required for nuclear localization compared with the classic NLS and the unusual sensitivity to truncations and deletions suggests VP22 does not possess a sequential NLS but rather a conformation-dependent NLS. Recently an increasing number of cellular or viral proteins have been found to utilize nonclassical NLSs for nuclear localization (24–29).

The three-dimensional structure of VP22 has yet to be elucidated, but the minimal 103-amino acid sequence required for VP22 nuclear localization is predicted to be highly structured, containing several potential α-helices including a histone fold-like motif as well as β-turns (3D-PSSM program, www.sbg.bio.ic.ac.uk/~3dpssm). A histone fold motif containing three helices is common to all four core histones as well as a variety of DNA-binding and multimeric proteins (30, 31). Interestingly recent studies suggest that a histone fold motif may also be critical for the non-conventional conformation-dependent NLS within the globular domain of core histones (18). We reasoned that if nuclear localization of VP22 was sensitive to conformational changes, then disrupting the helix structure within the histone fold-like motif might similarly impair nuclear accumulation. Interestingly when the integrity of the longest putative helix was disrupted by substituting four adjacent residues with glycine, the efficiency of nuclear localization by full-length VP22 was greatly reduced, whereas the same point mutations in VP22-(130–232) eliminated nuclear localization (Fig. 2).
These findings further support that the non-conventional nuclear localization sequence of VP22 may be sensitive to conformational change.

The nuclear localization and retention of VP22 occurs by a non-classical NLS. This VP22 NLS does not have similarity to other known non-classical NLSs such as in Tat or Antennapedia or bipartite NLs such as in parathyroid hormone-related protein (www.ncbi.nlm.nih.gov/blast). An NLS is not identified in VP22 using a SORT or Prosite search. Indeed in a database search and comparison, the only proteins with sequence similarity to BHV-1 VP22 are VP22 and VP22 homologs encoded by the UL49 gene from other herpesviruses (www.ebi.ac.uk/fasta33). In contrast, a BLAST search for similar sequences to the C terminus (amino acids 210–258) revealed only the full-length BHV-1 VP22 (BLAST, FastA). The C terminus of the VP22 homologs is the region of highest diversity, likely leading to the variation in function observed among members of this family. Although further study is needed, this lack of homology suggests several potential mechanisms for the novel functions of VP22-(210–258). There may be conformational or charge factors, or VP22-(210–258) might bind to another mitochondria-targeted protein and piggyback to the mitochondria.

The molecular mass of VP22-(130–232)-YFP is ~40 kDa, which would permit passive diffusion. Small proteins like VP22-(130–232)-YFP can diffuse across the nuclear envelope, and their accumulation would require either constitutively active import rendered by nuclear transport signals, retention in the nucleus by nuclear retention signals, or both. Efficient nuclear accumulation involving both active import and nuclear retention has been demonstrated for other proteins (29, 30). We previously reported the interaction of full-length VP22 with histones by far-Western blot analysis (6), an observation that makes the retention mechanism attractive. In the present study, retention of VP22-(130–232)-YFP was supported by an energy-depleting assay. When constitutively active import was blocked by sodium azide, VP22-(130–232)-YFP was retained in the nucleus, whereas VP22-(1–123)-YFP redistributed back to the cytoplasm within 30 min. Co-immunoprecipitation assays indicated that VP22-(130–232)-YFP but not VP22-(1–123)-YFP associated with histones, indicating that interaction with histones contribute, at least partially, to nuclear retention of VP22-(130–232). Interestingly two other VP22 truncations, VP22-(210–258) and VP22-(210–232) (data not shown), that did not localize in nuclei still contained information for nuclear retention. Thus, the energy-depleting assay suggested that VP22-(130–232) contains a nuclear retention signal as well as an NLS.

Also by studying BHV VP22 internal deletions and truncations, we identified the unusual property of a 49-amino acid mitochondrial targeting sequence located at the C terminus. Although VP22-(210–258) can target the YFP fusion protein to mitochondria, it is still unclear whether this targeting signal is functional in the context of full-length VP22. We failed to observe mitochondrial association of full-length VP22 in transiently transfected cells. Also conformational change might expose a cryptic linear targeting sequence that directs the deletions to a new subcellular compartment. The target signal requires residues within 210–217 and 225–233. In addition, VP22-(210–258) alone can function as the new targeting sequence (Fig. 5, B and C, panel M), whereas further deletions from the C terminus resulted in loss of cytoplasmic network targeting (Fig. 5B, panel N). Also Fig. 5B, panels A, J, L, and N, shows diffuse staining, whereas other panels show a staining reminiscent of endoplasmic reticulum. Additional studies are required to identify such differences.

Recent studies have indicated that mitochondria play a central role in apoptosis. Mitochondrial membrane permeability can be induced during the onset of apoptosis resulting in leakage of apoptogenic factors into the cytoplasm from the mitochondrial intermembrane space leading to a cascade of activated caspases, nuclear damage, and cell death (18, 29). Virus infection triggers numerous cellular defense responses to limit viral replication; one cellular defense mechanism is apoptosis of the host cells. Some viral proteins, like PB1-F2 of influenza virus, target mitochondria to induce apoptosis and kill host immune cells responding to virus infection (14). Previously we have shown that full-length VP22 can induce apoptosis by up-regulating the expression ratio of Bax/Bcl-2 (7). Whether VP22 can affect apoptosis of host cells through interaction with mitochondria during virus infection is of future interest.

Here we identified a 103-amino acid sequence that is sufficient for VP22 to target nuclei. The NLS sequence is conformation-sensitive and binds histone H4 permitting nuclear retention. Also a mitochondrial targeting sequence was identified comprising the C-terminal 49 amino acids of VP22 that overlaps the nuclear targeting sequence. These findings demonstrate the diversity of intracellular localization for VP22 and provide the opportunity to specifically direct cargo to a given subcellular compartment.

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