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Sequences within the VP6 Molecule of Bluetongue Virus That Determine Cytoplasmic and Nuclear Targeting of the Protein

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Genome segment 9 of bluetongue virus serotype 10 encodes the minor protein VP6. The protein is abundant with basic residues particularly in two regions of the carboxy half of the molecule. A series of amino- and carboxy-terminal deletion mutants was expressed in mammalian cells by using a vaccinia virus T7 polymerase-driven transient expression system, and the intracellular fate of the products was monitored by both immunofluorescence staining and cell fractionation techniques. Data obtained indicated clearly that VP6 has nuclear transportation signals which may be correlated with positively charged domains of the molecule. In the intact molecule, though, these signals are masked and the protein is retained in the cytoplasm. The biochemical and immunofluorescence data obtained indicate that sequences in the region of residues 33 to 80 of the 328-amino-acid protein are required for the retention of VP6 within the cell cytoplasm while amino acids 303 to 308 in the carboxy-terminal half of the molecule appear to possess nuclear localization capabilities.

Nuclear targeting sequences are essential for the transport of proteins into the cell nucleus. Generally such sequences are short and contain a high proportion of positively charged amino acids (lysine [K] and arginine [R]). In recent years sequences that are necessary for transportation and accumulation of viral antigens in the nucleus have been identified. Sequences of proteins that are not normally identified in the nucleus may also possess runs of K and R. Why such proteins are not transported to the nucleus is unknown. One possibility is that their transport is inhibited by other aspects of their structure. The minor core protein VP6 (36 kDa) of bluetongue virus (BTV), a member of the *Orbivirus* genus (*Reoviridae* family), is rich in basic amino acids (68 of 328 amino acids [aa]) and has a calculated total net charge of +11 at a neutral pH. The sequence has a number of contiguous R and K residues. Although the function of VP6 is not known, preliminary studies indicate that the protein is likely to be associated with the viral RNA within the cores of virions and may constitute a part of the viral polymerase complex (10). VP6 is not found in the nuclei of BTV-infected mammalian cells.

The BTV genome comprises 10 double-stranded RNA species that are located within the core of the virus particle together with three minor proteins, VP1, VP4, and VP6. Two virus-encoded proteins VP3 and VP7 are the major components of the core which is organized in a bilayered capsid structure. The core is surrounded by an outer capsid consisting of two other major proteins, VP2 and VP5. Under appropriate conditions, the core particle derived from virions exhibits an RNA-dependent RNA polymerase activity which catalyzes the synthesis of mRNA from the double-stranded RNA genome. This activity may involve all three minor proteins. Preliminary studies indicate that VP1 is likely to be the polymerase enzyme (10) and that VP4 is likely to be the guanylyl transferase enzyme (5, 7). The activity of VP6 is not known, although it has been demonstrated that VP6 has a strong affinity for both single- and double-stranded RNA (or DNA) species, even in the presence of denaturing reagents (3, 8). Although many of the basic amino acids of VP6 are scattered throughout the molecule, at least two prominent clusters are located in the carboxy half of the molecule. One comprises 10 residues, RLRDLRRKEK (positions 186 to 195); the other comprises 9 residues, RGGRKQRKK (positions 205 to 213). In addition to these motifs, five consecutive glycine residues are located in the amino-terminal half of the molecule (residues 112 to 116). To investigate the importance of these regions for the intracellular location and structure-function relationships of the VP6 protein, we have generated a series of deletion and substitution mutants and expressed each mutant in mammalian cells using transient assays involving a vaccinia virus T7 polymerase vector system (2). We have found that VP6 possesses nuclear targeting signals, although the intact protein is located in the cytoplasm. Further, we have identified regions of the protein in the amino-terminal half that modulate VP6 nuclear localization.

Construction of seven truncation mutants of VP6 and their expression in a transient expression system. To monitor the intracellular fate of the VP6 protein, a mammalian expression system was utilized to generate various mutant forms of the VP6 protein. The system is based on infection of cells with a recombinant vaccinia virus expressing bacteriophage T7 RNA polymerase (VVTF-7) and subsequent transfection with plasmid DNA containing the gene of interest under the control of a copy of the T7 promoter (2). Transcription of the gene is mediated by the T7 polymerase produced by the recombinant vaccinia virus. To use this expression system, a series of transient expression plasmids (pGEM) containing truncated VP6 genes was generated. The deletions were designed to eliminate the N-terminal sequences progressively before and after the VP6 glycine-rich region (aa 112 to 116) as well as from the C-terminal end to remove one (aa 205 to 213) of the two positively charged regions of VP6. For each N-terminal deletion, PCR was used to introduce a *Bam*HI linker and an ATG start codon at the 5′ end of the required coding region; for the C-terminal deletions, a *Bam*HI linker and a stop codon of the BTV-10 S9 gene (pGEM3Z.10BTV-9) which encodes VP6

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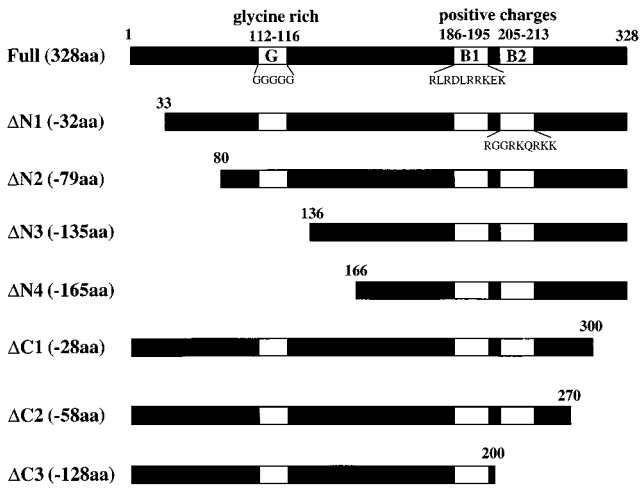


FIG. 1. Schematic maps of VP6 and truncated mutants of VP6. The positions of the glycine-rich region (G) and two positively charged regions (B1 and B2) are indicated.

were introduced (8). Pairs of forward and reverse primers were synthesized for each construction (Table 1). The mutants included four N-terminal deletion mutants (ΔN1, ΔN2, ΔN3, and ΔN4) and three C-terminal deletion mutants (ΔC1, ΔC2, and ΔC3). The N-terminal deletion mutants lacked 32, 79, 135, and 165 residues, respectively, of the 328-aa VP6 (Fig. 1). Similarly, the C-terminal deletion mutants (ΔC1, ΔC2, and ΔC3) lacked 28, 58, or 128 residues (Fig. 1).

HeLa T4$^+$ cells were infected with VVTF-7 and transfected with pGEM3Z plasmid DNAs containing either a full-length VP6 gene or one of the modified vectors, and the synthesis of the derived proteins was analyzed by Western blotting (immunoblotting) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). In each case a band of derived VP6 protein was identified. Each of the modified products exhibited the expected reduction in size.

Intracellular localization of the truncated VP6 proteins. To investigate the fate of each truncated form of VP6 protein synthesized in the transfected cells, immunofluorescence was employed to localize the VP6 derived protein by using an anti-VP6 rabbit serum, together with fluorescein-labeled secondary antibodies. As expected, the full-length VP6 protein (Fig. 3A) was detected within the cell cytoplasm, giving a strong fluorescence signal in contrast to the control, nontrans-

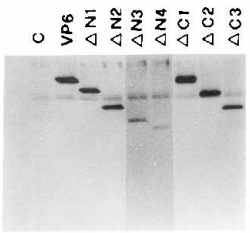
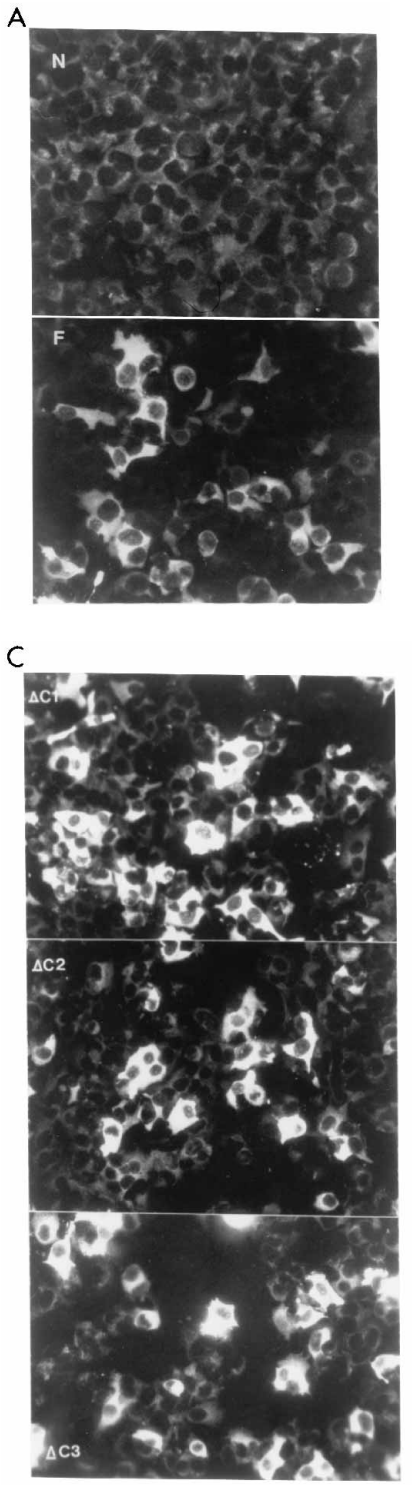


FIG. 2. Expression of truncated VP6 mutants in HeLa T4$^+$ cells. HeLa T4$^+$ cells were infected with VVTF-7 at a multiplicity of infection of 10 to 20 for 1 h at 37°C. Ten to 20 μg of pGEM3Z plasmid DNA containing either a full-length VP6 gene or one of the truncated genes (ΔN1 to ΔN4 or ΔC1 to ΔC3) was mixed gently with an equal volume of Lipofectin, incubated at room temperature for 15 min, and added to the infected cells. After 14 h of incubation, cell extracts were prepared and resolved by SDS–15% PAGE before Western blot analysis using an anti-VP6 antiserum. As a control (lane C), cells were infected with vaccinia virus alone.

TABLE 1. Oligonucleotides used for construction of truncation mutants and for internal-deletion and substitution mutants

Mutant (aa)	Forward primer(s)	Reverse primer(s)
ΔN1 (−32)	GAGGGATCC**ATG**GAGAGTGAGGGC (P1)	GCGGGATCC**TTA**GAGGTGATCGATCAAATGTA (P2)
ΔN2 (−79)	GCAGGATCC**ATG**GATAGACGCATACATACTG (P3)	P2
ΔN3 (−135)	GCGGGATCC**ATG**ACGGAAGAGATTGC (P4)	P2
ΔN4 (−165)	GCAGGATCC**ATG**GAACGCAGTCTC (P5)	P2
ΔC1 (−28)	GGAGGATCC**ATG**TCAGCTGCGATACTTCTT (P6)	GCGGGATCC**TTA**GCGAGCCACTTCTTTC (P7)
ΔC2 (−58)	P6	GCAGGATCC**TTA**CATAACCGCGCTCTC (P8)
ΔC3 (−128)	P6	GCGGGATCC**TTA**AGCATGCGTCCCATTCT (P9)
ΔG (112–116)	P6, TGGAAAAGGTTGATGCTGATGCGGGAGTTGG (P11)	CATCAGCATCAACCTTTCCATCTCCTCTGT (P10), P2
ΔB1 (186–195)	P6, GCAGACCGAGAATGGGACGCATGCTAAAGCC (P13)	GCGTCCCATTCTCGGTCTGCTCGGCCACTC (P12), P2
ΔB2 (205–213)	P6, AGCCGTTGAGGCCCATGGTGACGCGCAGAG (P15)	CACCATGGGCCTCAACGGCTTTAGCATGCGT (P14), P2
GS (112–116[a])	P6, GCTAGTGCTAGTGCTGATGCTGATGCGGGAGTT (P17)	AGCACTAGCACTAGCAACCTTTCCATCTCCTCTGTT (P16), P2
B1S (186–195[b])	P6, CCAAAAAAGAAGAAAGGTCAATGGGACGCATGCTAAAGCC (P19)	GACCTTTCTCTTCTTTTTTGGCTCGGTCTGCTCGGCAACTCC (P18), P2
B2S (207–213[c])	P6, CCAAAAAAGAAGAAAGGTCGCCCATGGTGACGCTCAGAGA (21)	GACCTTTCTCTTCTTTTTTGGTCCTCTCTCAACGGCTTTAGC (P20), P2

[a] GGGGG to ASASA.
[b] RLRDLRRKEK to PKKKRKV.
[c] GRKQRKK to PKKKRKV.



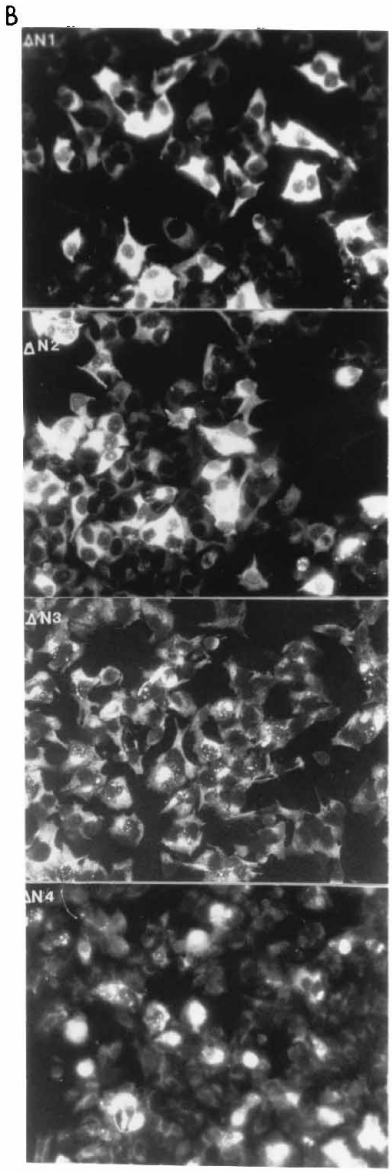


FIG. 3. Intracellular localization of the expressed VP6 derived proteins in transfected cells. HeLa $T4^+$ cells grown on coverslips were infected with VVTF-7, transfected with various pGEM-VP6 plasmid DNAs as described for Fig. 2, and incubated for 8 to 16 h before fixation for immunofluorescence analysis. The fixed cells were reacted with an anti-VP6 rabbit serum at 37°C for 1 h and incubated at 37°C for 1 h with fluorescein isothiocyanin-conjugated goat anti-rabbit immunoglobulin G. (A) VVTF-7-infected control cells (N) and cells transfected with pGEM-VP6, showing the presence of VP6 in the cytoplasm (F); (B) N-terminal deletion mutants ΔN1 to ΔN4; (C) C-terminal deletion mutants ΔC1 to ΔC3.

fected cells. Similarly, all the C-terminal deletion mutants, including the mutant ΔC3, which lacked 128 residues and the aa 205-to-213 motif, remained in the cytoplasm (Fig. 3C). In contrast, although deletion of the first 32 residues from the N terminus did not seem to have any effect on the localization of the VP6 molecule (ΔN1), larger deletions (e.g., ΔN3 and ΔN4) gave clear evidence of punctate fluorescence associated with the nuclei (Fig. 3B). Some nuclear fluorescence was also observed with the ΔN2 mutant. Transfected cells analyzed after a longer period (e.g., 16 h) gave similar results. The data indicate that the N-terminal half of VP6 is responsible for the cytoplasmic retention of the molecule and that in the absence of this region some of the protein associates with the nucleus.

The data obtained from immunofluorescence studies were confirmed by biochemical studies. HeLa $T4^+$ cells were infected with VVTF-7, transfected with the recombinant plasmids and pulse-labeled with ^{35}S-labeled methionine. The infected cells were homogenized, and nuclei and cytoplasmic

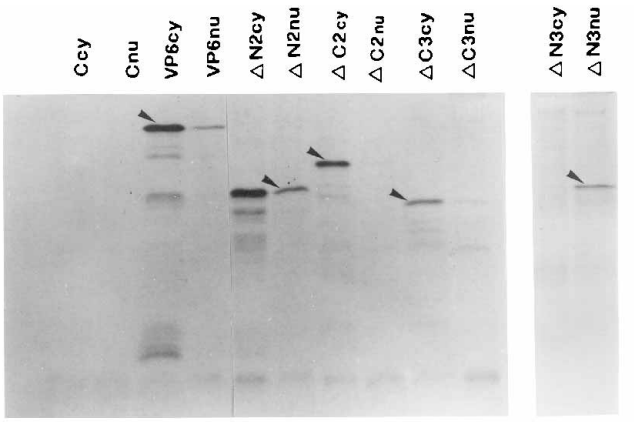


FIG. 4. Subcellular localization of truncated VP6 mutants analyzed by immunoprecipitation. HeLa $T4^+$ cells in six-well plates were infected with VVTF-7 and then transfected by various VP6 deletion mutant pGEM constructs described for Fig. 2. At 14 h posttransfection the medium was replaced with 0.5 ml of serum-free minimal essential medium supplemented with 40 μCi of [^{35}S]methionine, and the cells were harvested after 3 h of incubation. The posttransfected cells were processed, and nuclei were separated from their cytoplasmic fractions. The presence of VP6 protein in each fraction was analyzed by immunoprecipitation using VP6 antisera and SDS–15% PAGE followed by autoradiography.

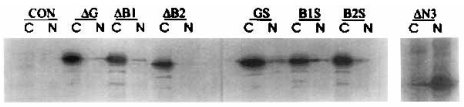


FIG. 5. Subcellular localization of internal-deletion and substitution mutants of VP6 by immunoprecipitation. HeLa $T4^+$ cells were infected with VVTF-7, transfected with the recombinant vectors, and pulse-labeled with [^{35}S]methionine as described for Fig. 2 and 4. Nuclear (N) and cytoplasmic (C) fractions were prepared, and the presence of VP6 protein in each fraction was analyzed by immunoprecipitation using anti-VP6 antisera as described for Fig. 4. Cells were also infected with VVTF-7 alone as a control (CON).

fractions were separated by centrifugation. The absence of intact cells and presence of nuclei were checked by hematoxylin staining. The presence of VP6 protein in each fraction was analyzed by immunoprecipitation using VP6 antisera and SDS-PAGE. As shown in Fig. 4, truncation of 135 residues from the N-terminal region, including deletion of the glycine-rich region, clearly altered the pattern of localization of the protein in the cells. The truncated protein was recovered in the nuclear fraction, while the native VP6 or the VP6 mutants with deletions of the C-terminal regions remained predominantly in the cytoplasm.

Specific internal deletion and substitution mutants to identify cytoplasmic and nuclear localization signals of VP6. A peculiar feature of the VP6 sequence between aa 33 and 135 is a stretch of five glycines. To determine whether this glycine-rich region plays any role in preventing transportation of VP6 to the nucleus, two mutants were prepared: one (ΔG) lacked all five glycine residues (aa 112 to 116) (Fig. 1), and the other (GS) contained alternate alanine and serine substitutions of the five glycines (ASASA [aa 112 to 116]) (Table 1). In addition, to investigate the importance of the two basic regions (B1 [aa 186 to 195] and B2 [aa 205 to 213]), four other VP6 mutants (two deletion mutants [ΔB1 and ΔB2] and two substitution mutants [B1S and B2S]) were constructed. For the internal-deletion and substitution mutants PCR was used to introduce the required site-directed mutations into the VP6 gene. For example, for the deletion construct ΔG, which lacked sequences corresponding to aa 112 to 116, a mutant DNA lacking the target sequence was synthesized by PCR using pGEM3Z.10BTV9 and the appropriate forward- and reverse-sense primers (Table 1). The protocol to make ΔG involved synthesizing a PCR product that included the protein sequence immediately upstream of the deletion site (primers 6 and 10) and a second PCR product that included the protein sequence immediately downstream (primers 11 and 2). Since the products were designed to have overlapping sequences, they were recovered, pooled, melted, and used with primers 6 and 2 to obtain a single PCR product lacking the required sequences. The same approach was taken to construct the two other deletion mutants (ΔB1 and ΔB2) and the three substitution mutants (GS, B1S, and B2S) involving the appropriate forward- and reverse-sense primers. The sequences of these primers are shown in Table 1. By these procedures ΔB1 lacked aa 186 to 195 (RLRDLRRKEK) and ΔB2 lacked aa 205 to 213 (RG GRKQRKK). The two substitution mutants contained the sequence PKKKRKV (representing the well-defined nuclear localization signal [NLS] of simian virus 40 large-T antigen [4]) in lieu of the natural B1 or B2 sequences and were designated B1S and B2S, respectively.

The expression of the six mutants (ΔG, GS, ΔB1, ΔB2, B1S, and B2S) in HeLa cells was analyzed by SDS-PAGE followed by Western analysis. The derived products exhibited the expected molecular sizes (data not shown). To monitor the subcellular distribution of ΔG and GS, immunofluorescence and cell fractionation were employed as described above. Both mutants were recovered predominantly in the cytoplasm. The gel profiles demonstrating the accumulation of proteins in the cytoplasm are shown in Fig. 5. Likewise, the B1 and B2 mutants were also obtained in the cytoplasm, in contrast to the results obtained with ΔN3 (Fig. 5). These results were also confirmed by the immunofluorescence experiments (data not shown).

The C-terminal half of VP6 has nuclear targeting activity. The carboxy-terminal region (aa 303 to 308, RKRRNI) of VP6 has high homology with the NLS sequence of the simian virus 40 T antigen (up to 83% similarity [1]). To determine whether this region can act as an NLS for VP6, an additional mutant VP6 was prepared. Since ΔN3 is readily transported to the nucleus, we deleted aa 303 to 308 from ΔN3 (N3-NLS) and examined the fate of the products using the transient expression system. Transfected cells were fractionated into cytoplasmic and nuclear fractions, and the presence of the mutant protein was identified by immunoprecipitation followed by SDS-PAGE. As shown in Fig. 6A, the truncated proteins under the control of the T7 promoters exhibited the expected molecular sizes. However, the pattern of the N3-NLS accumulation differed significantly from that of the ΔN3 mutant. The N3-NLS protein segregated predominantly with the cytoplasmic fraction of the transfected cells. To confirm that deletion of aa 303 to 308 of the ΔN3 deletion altered the NLS of the protein, we also examined transfected cells by immunofluorescence, as described above. As shown in Fig. 6B, deletion of aa 303 to 308 altered the localization of the ΔN3 protein. The signals were predominantly associated with the cytosol and not with the nucleus. Thus, it seems that aa 303 to 308 have the potential to function as an NLS for the VP6 protein, although the complete VP6 protein is located in the infected cell cytoplasm.

The replication cycle of orbiviruses is confined to the cytoplasm of infected cells, as for reo- and rotaviruses. Recombinant BTV capsid proteins, including the VP6 protein, when expressed either in insect cells or in mammalian cells, are also found only in the cell cytoplasm. In the present study, it was

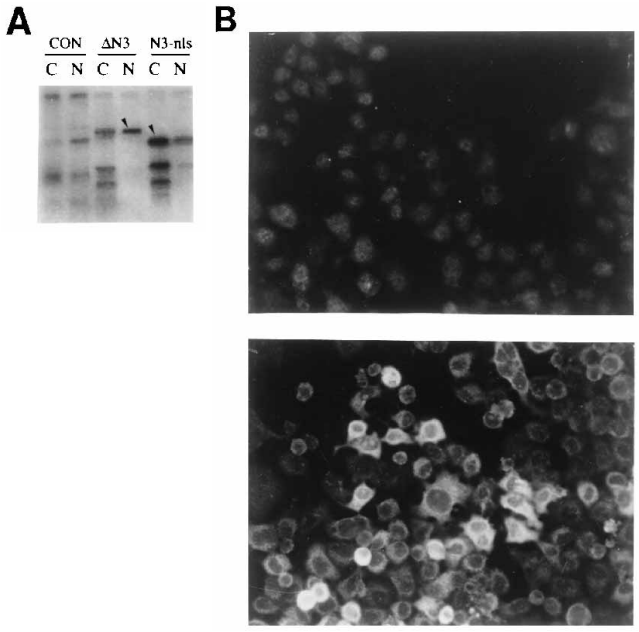


FIG. 6. Expression and localization of VP6 mutants with or without the putative NLS sequence. (A) HeLa T4$^+$ cells were infected with VVTF-7, transfected with recombinant vectors (ΔN3 or N3-NLS), and pulse-labeled with [^{35}S]methionine as described for Fig. 4. Cells were also infected with VVTF-7 alone as a control (CON). Nuclear (N) and cytoplasmic (C) fractions were prepared, and the presence of VP6 protein in each fraction was analyzed by immunoprecipitation using anti-VP6 antisera and SDS–15% PAGE. The positions of the modified VP6 proteins (arrowheads) are indicated. (B) Indirect immunofluorescence was employed to localize the VP6 derived proteins as described for Fig. 3. Upper panel, VVTF-7-infected control cells; lower panel, cells transfected with the recombinant vector containing the deletion of aa 303 to 308 from ΔN3 (N3-NLS), showing the presence of the mutant VP6 in the cytoplasm.

observed that when certain amino-terminal sequences of VP6 were deleted (e.g., in ΔN3) the protein was found in association with the nucleus whereas intact VP6 was located in the cytoplasm. Analysis of several carboxy-terminal deletions, including a deletion of at least one of two regions rich in arginines and lysines (aa 205 to 213), showed that VP6 remained within the cytoplasm. Taken together, these data indicate that N-terminal regions are likely to be involved in preventing the protein from being transported to the nucleus. It seems that in some manner the amino-terminal half of VP6 masks the nuclear localization signal(s) present in the remainder of the protein. We generated site-directed mutants either lacking the contiguous five glycines or involving their substitution by alternate alanines and serines to determine whether this region of the amino half of the protein was involved in preventing nuclear transportation. Both mutant proteins were retained in the cell cytoplasm, indicating that by itself this sequence is not responsible.

Recently, it was reported by Suzuki et al. (9) that the core protein of hepatitis C virus, which is normally localized within the infected cell cytoplasm, is translocated into the nucleus when the C-terminal hydrophobic domain is deleted. Further studies indicated that a cluster of basic amino acids plays an important role in translocation of the truncated hepatitis C virus core protein to the cell nucleus. In the analyses reported here, removal of one or the other of the basic regions encompassed by VP6 aa 186 to 195 or aa 205 to 213 did not alter the cytoplasmic location of VP6. Likewise, exchange of the natural basic sequences with the simian virus 40 NLS did not affect the cytoplasmic location of VP6.

Since truncation of the N terminus targeted the protein into the nucleus, it is reasonable to consider that the C-terminal region contains signals for nuclear transportation. Interestingly, the RKRRNI sequence at aa 303 to 308 has high homology with the NLS of simian virus 40 T antigen. When these sequences were deleted from the ΔN3, the derived protein (N3-NLS) remained in the cytoplasm in contrast to ΔN3, suggesting that this C-terminal sequence contains the signal for nuclear transportation of the protein.

The amino-terminal region is responsible for the cytoplasmic retention of the protein. Since aa residues 30 to 140 (including the glycine rich region, aa 112 to 116) have strong homology (up to 62%) with yeast NLS-binding protein (6), this region is likely to bind and mask the C-terminal NLS domain. Indeed, removal of this domain from the N terminus restored the nuclear targeting activity of the protein. Thus, in addition to the nuclear transportation signal, the NLS binding domain also resides on the VP6 molecule.

The evidence presented here clearly demonstrates the possibility that a restricted region of the molecule (around aa 303 to 308) has the potential to target the protein to the nucleus. Further experiments are in progress to determine more precisely how this region interacts with the rest of the molecule.

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