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# Structural determination of importin alpha in complex with beak and feather disease virus capsid nuclear localization signal

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ABSTRACT

Circoviruses represent a rapidly increasing genus of viruses that infect a variety of vertebrates. Replication requires shuttling viral molecules into the host cell nucleus, a process facilitated by capsid-associated protein (Cap). Whilst a nuclear localization signal (NLS) has been shown to mediate nuclear translocation, the mode of nuclear transport remains to be elucidated. To better understand this process, *beak and feather disease virus* (BFDV) Cap NLS was crystallized with nuclear import receptor importin-α (Impα). Diffraction yielded structural data to 2.9 Å resolution, and the binding site on both Impα and BFDV Cap NLS were well resolved. The binding mechanism for the major site is likely conserved across circoviruses as supported by the similarity of NLSs in circovirus Caps. This finding illuminates a crucial step for infection of host cells by this viral family, and provides a platform for rational drug design against the binding interface.



## 1. Introduction

*Circoviridae* is a growing family of animal viruses, with members responsible for diseases of both veterinary and economic importance, notably chicken anaemia virus (CAV), the cause of chicken infectious anaemia, porcine circovirus 2 (PCV2), the cause of post-weaning multisystemic wasting syndrome, as well as Anellovirus isolates prevalent in humans. Environmental load of these viruses are likely very high, along with the hypothesis of vertical transmission [1–4] and documented infections of endangered species [5–7], necessitates the requirement for significant research into both the biology of viral replication, and vaccines and therapeutic development. Since the family of viruses is characterized by small circular genomes, encoding a limited number of proteins [8], proteins throughout this family are governed by similar mechanisms and properties, enabling research to be highly focused on these critical proteins, and translatable across related virus isolates.

*Beak and feather disease virus* (BFDV) has a worldwide distribution in wild and captive psittacine birds [9]. The virus is one of the smallest viruses to cause disease, manifesting itself as an acute disease in nestlings and specific parrot species [10,11], chronically with symmetrical feather loss and beak and claw deformations [12], and is a threat to several endangered species. A member of the genus *Circovirus*, BFDV is related to the other avian circoviruses, such as raven circovirus, duck circovirus (DuCV) and columbid circovirus (CoCV), newly discovered fish circoviruses, and the economically important porcine circoviruses. Similar to other circoviruses, BFDV has a circular single-stranded DNA genome of less than 2000 base pairs encoding two major proteins [8], requiring BFDV proteins to be multifunctional, and interact with several host proteins.

The two major proteins of circoviruses are the capsid-associated protein (Cap), which comprises the entirety of the viral capsid, and the replication-associated protein (Rep). In BFDV, Cap must interact with several host cellular proteins to accomplish infection, replication and formation of new viral particles. It must also self-associate to form the viral capsid, as well as interact with a host cell surface receptor in the extracellular environment. BFDV Cap is known to bind to Rep to facilitate nuclear localization, and binds DNA [13], likely localizing its own genome into the host cell nucleus. The nuclear localization signals (NLSs) from several circoviruses have been shown to localize Cap to the nucleus [13–16], however, no mechanism of nuclear transport has been evaluated or suggested. The presence of an extensive NLS in BFDV Cap has been identified, and a large number of positively charged residues at the N-terminal were classified as being a bipartite

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NLS [13]. This sequence is also consistent with other classical NLSs determined to bind with nuclear importin-α [17,18].

Determination of the structural interface between two interacting proteins provides important information of the binding determinants that mediate association, as well as a basis for rational drug design to perturb these interactions. Crystallization of the capsid protein of PCV2 without the NLS has enabled high resolution structural determination of the protein and, in turn, the entire viral capsid [19]. This has allowed further insight to the amino acids thought to be responsible for binding heparin sulfate and the positioning of the NLS inside the viral capsid. Similarities of approximately 43% between the amino acid sequences of BFDV Cap and the solved PCV2 capsid have also allowed for models of BFDV Cap and related viral capsid structures [20]. Structural methods can be applied to determine the pathway of nuclear entry of BFDV Cap, although difficulties arise with expression of the full-length protein [13,21], and, potentially, with subsequent crystallization due to the flexibility of the NLS region [20]. To determine the binding site of BFDV Cap NLS with importin-α (Impα), a peptide, incorporating the NLS region of BFDV Cap, was complexed with Impα. Crystallization of the complex allowed the high−resolution X-ray crystallographic structural determination of BFDV Cap NLS in complex with Impα, providing further insight to the nuclear localization of BFDV Cap and likely the nuclear localization of all related circovirus Caps.

## 2. Materials and methods

### 2.1. Expression and purification of Impα

Expression made use of an isolate of mouse Impα lacking the importin β binding domain (mImpαΔIBB; variant α2, residues 70–529) that has previously been crystallized [22]. Recombinant protein was expressed using *Escherichia coli* pLysS cells. *E. coli* cells were grown in 500 mL of autoinduction media (1% tryptone, 0.5% yeast extract, 1X NPS, 1X 5052, 1 mM $MgSO_4$) containing 25 μg/mL kanamycin at 25°C for 27 h. Cells were collected by centrifugation at 4000x*g* for 30 min, resuspended in Buffer A (50 mM phosphate buffer, 300 mM NaCl, 20 mM imidazole, pH 8) and stored at −20°C. Lysis included two freeze/thaw cycles, 20 mg of lysozyme (Sigma–Aldrich), and 0.5 mg of DNaseI (Invitrogen). Debris was pelleted by centrifugation at 40,000*xg* for 30 min followed by filtration of the supernatant through a 0.45 μm low protein-binding filter (Millipore). Supernatant was injected onto a 5 mL HisTrap HP column (GE Healthcare) on an AKTApurifier FPLC (GE Healthcare) and eluted with Buffer B (50 mM phosphate buffer, 300 mM NaCl, 500 mM imidazole, pH 8). Peak fractions were pooled and loaded onto a HiLoad 26/60 Superdex 200 column (GE Healthcare). Peak fractions eluted in GST Buffer (50 mM Tris, 125 mM NaCl, pH 8), collected and concentrated to 33.65 mg/mL.

### 2.2. Crystallization of BFDV Cap NLS:Impα complex

The BFDV Cap NLS from an isolate (GenBank: AAS16925), similar to a sequence capable of localizing the associated Cap to the nucleus [13], was selected and a peptide containing 29 amino acids was produced (Mimotopes). The peptide contained the sequence N- RRRRYARPYYRRRHNRRYRRRRRYFRRRR –C. The Cap NLS peptide was resuspended in GST Buffer at a concentration of 10 mg/mL, and mixed with mImpαΔIBB at a concentration of 33.65 mg/mL, in a 2:1 ratio for crystallization. The crystallization conditions contained 0.675 M trisodium citrate, 0.1 M HEPES (pH 6.5), and 20 mM DTT. The protein complex was mixed in a 1:1 ratio with the crystallization conditions, and crystallized using hanging drop diffusion. Crystals formed after one week.

Crystals of the protein complex were placed in cryoprotectant consisting of the crystallization conditions with 20% glycerol. Diffraction data, collected at the Australian Synchrotron, was scaled and merged using iMosflm [23]. Molecular replacement was performed using the PDB: 1IAL as a search model in Phaser [24] and used to generate an initial electron density map, from which a final model could be built and refined using REFMAC within the CCP4 suite [25], and WinCoot [26] (Table 1). The structure of the complex was deposited in the RCSB Protein Data Bank (PDB: 4HTV). Bond analysis was determined using the server Protein interfaces, surfaces and assemblies (PISA) at European Bioinformatics Institute [27].

## 3. Results and discussion

Nuclear localization signals of several circovirus Caps have been defined to a stretch of amino acids that reside at the N-terminus of the protein [13,14,16]. These sequences possess a large contiguous stretch of positively charged residues, consistent with classical NLSs, and, in the case of PCV, the responsible sequence has been narrowed to a small number of residues [14]. Despite this knowledge, no connection has been made regarding the mechanism of nuclear transport, an essential step for viral replication and infection. Elucidation of this mechanism will enhance both understanding of how these Caps bind nuclear import receptors compared to other previously characterized NLS sequences, and also have wider implications for all circoviruses and circovirus-like viruses. For instance, the N-termini of all known circoviruses isolated from animal tissue, currently spanning several avian species, pigs, fishes, dogs, and bats, as well as Anelloviruses isolated from humans, pigs, and similar viruses from turtles, and dragonfly include conserved clusters of positively charged residues [28–34], suggesting that they all use the same mechanism for nuclear transport (Fig. 1). Although not all circoviruses are associated with disease, their presence in such a variety of species and its ancient origins [35] leads to the hypothesis that this group of viruses will eventually be found in all vertebrates and possibly even further, with varying effects on their hosts.

**Table 1**
Crystallographic data for structure of mImpαIBB in complex with BFDV Cap NLS.

| Space group | Overall (outer shell)<br>$P2_12_12_1$ |
|---|---|
| Unit cell | |
| Length (Å) | $a$ = 78.2, $b$ = 89.9, $c$ = 99.16 |
| Data collection | |
| Resolution range (Å) | 29.99–2.90 (3.06–2.90) |
| Completeness (%) | 99.9 (100.0) |
| Unique reflections | 16074 (2295) |
| $R_{merge}$ | 0.153 (0.583) |
| Avg $I/\sigma$ | 9.9 (3.3) |
| Redundancy | 7.1 (7.2) |
| Refinement statistics | |
| $R_{free}/R_{work}$ | 0.22804/0.17293 |
| Avg B-factor | 33.135 |
| Number of non-hydrogen atoms | 3332 |
| R.m.s. deviations from ideality | |
| Bond lengths (Å) | 0.014 |
| Bond angles (°) | 1.848 |
| Ramachandaran plot (%) | |
| Favored | 95.3 |
| Allowed | 4.5 |
| Outliers | 0.3 |

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BFDV AAS16925...RRRRYARPYYRRRHNRRYRRRRRYFRRRR
PCV2 AAF87231...RRRYRRRRHRPRSHLGQILRRRPWLVHPRHRYRWRRK
DuCV ABN13870...RRSTYRRAYRARRRRRGLRRRLRRRRLRIGRPRRR
CAV ACT31558....RRARRPRGRFYAFRRGRWHNLKRLRRRYKFRHRRRQRYRRR
TTV BAA93589....RRRWRPRYRKRTWRLRRRRPRRTFRRRRRRQYVSRRRRRRYYRRRLRRGRRRGRRKRHR
GoCV ABA39154...RARPRSLYYRRRRAANRRRRYRRRRLHIGRIR
BatCV AEL87793..RFRRRVARRRPVRSIRRIRRRRRYGRRR
CoCV AEC03084...RRRRFYRRRRAPIRRRRIRRRRTRLSRMRRGHR
DrCV ADY18001...RYRRAARRPVRRARRSRVRKLRFRRRRRYHRR
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**Fig. 1.** The NLS sequences of several circoviruses or circovirus-like viruses. Virus abbreviations are followed by their GenBank accession number. Underlined residues are those found to bind to the major site of Impα (as in BFDV), or are predicted to be able to bind the major site of Impα. Predicted sequences contain a minimum of four arginine residues, which were found to provide the most contacts in BFDV Cap NLS. The highlighted residues for PCV2 were those found to affect nuclear localization in mutation experiments by Liu et al. [14]. BFDV = beak and feather disease virus; PCV2 = porcine circovirus 2; DuCV = duck circovirus; CAV = chicken anaemia virus; TTV = Torque teno virus; GoCV = goose circovirus; BatCV = bat circovirus; CoCV = columbid circovirus; DrCV = dragonfly cyclovirus.

The current state of BFDV research has placed restrictions on experimental methods that can be used to study its viral biology. No cell culture method has been devised to maintain BFDV infection, therefore to date, recombinant protein expression technologies have been utilized to characterize the function, and in this study, the structural and functional relationships of proteins critical for viral infection and replication. The field is further hampered due to expression of recombinant, full-length BFDV Cap that includes the NLS region resulting in reduced expression of the protein [13,21], and different isolates of BFDV Cap exhibiting reduced solubility based on amino acid sequence [36]. Since the aim of this study was to characterize the structural determinants that mediate binding to the classical nuclear import receptor Impα, a peptide incorporating the N-terminal NLS region of BFDV Cap was co-crystallized with recombinantly expressed Impα.

Recombinant expression of Impα that lacked the N-terminal Importin-β binding (IBB) domain was over-expressed and soluble to greater than 10 mg/L. Truncation of 69 N-terminal residues on mImpαΔIBB prevents autoinhibition of the protein by the IBB domain [37]. The remaining features of Impα, 10 armadillo (ARM) motifs each comprised of α-helices H1, H2 and H3, and major and minor binding sites on the H3 helices of ARM 1–4 and 6–8, respectively, are present in mImpαΔIBB [22,37–39]. Crystallization of ImpαΔIBB in complex with BFDV Cap NLS was performed by co-crystallization of the peptide in a 2-fold molar excess to the concentrated Impα molecule, as in Takeda et al. [39]. Diffraction

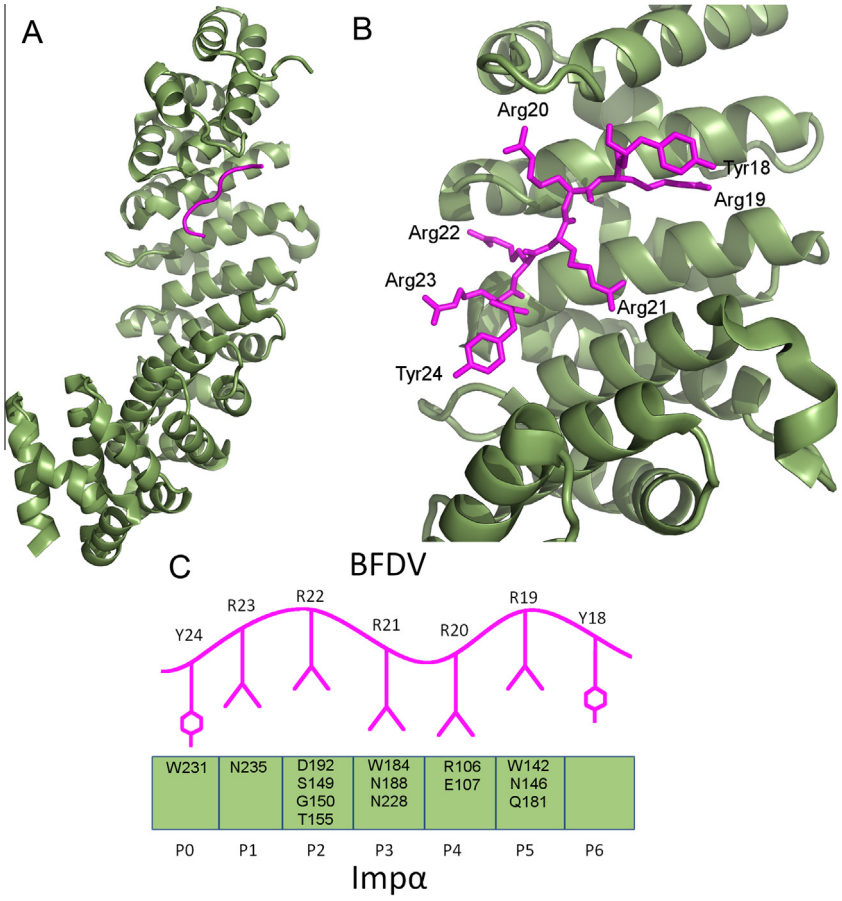


**Fig. 2.** BFDV Cap NLS (magenta) binding the major site of mImpαΔIBB (green). (A) Shows the entire mImpαΔIBB molecule with BFDV Cap NLS in the major site. (B) Shows labeled side chains of the NLS amino acids bound to the major site. (C) Is a schematic representation of the NLS residues occupying each pocket of the mImpαΔIBB major binding site. Residues listed in the Impα pocket form bonds with corresponding BFDV Cap NLS residues; P6 does not form a bond with Y18. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

quality crystals were obtained after 2 weeks in crystallization conditions.

Structural data from X-ray crystallography of mImpαΔIBB in complex with the 29 amino acid peptide of the BFDV Cap NLS was analyzed and refined to 2.9 Å in the $P2_12_12_1$ space group (Table 1), and showed the Cap NLS in the major binding site of the mImpαΔIBB molecule (Figs. 2 and 3). Electron density for seven amino acids of the Cap NLS was clearly discernible, corresponding to $^{NLS}$YRRRRRY$^{18-24}$. Dissection of the binding profile of the BFDV Cap NLS reveals a binding arrangement with six amino acids interacting to residues in the major site of mImpαΔIBB. At the P1 major binding site of Impα, $^{NLS}$Arg23 interacts with $^{mImp\alpha\Delta IBB}$Asn235 through hydrogen bonding. This is consistent with other P1 residue motifs at this position that have shown to be able to accept several amino acids [40]. At P2, $^{NLS}$Arg22 formed multiple interactions with Impα; a salt bridge and hydrogen bond to $^{mImp\alpha\Delta IBB}$Asp192, and hydrogen bonds to $^{mImp\alpha\Delta IBB}$Ser149, $^{mImp\alpha\Delta IBB}$Gly150 and $^{mImp\alpha\Delta IBB}$Thr155. P2 strongly prefers a Lys, which is shown to be conserved among classical NLS sequences [17,18,22], therefore, $^{NLS}$Arg22 is not an ideal binding partner, however, atypical NLS sequences that do not possess a Lys but a conserved Arg have been shown to bind the major site of Impα [41]. The $^{NLS}$Arg21 residue in the P3 site interacts with several residues on Impα including $^{mImp\alpha\Delta IBB}$Trp184, $^{mImp\alpha\Delta IBB}$Asn188, and $^{mImp\alpha\Delta IBB}$Asn228. The P3 site prefers an Arg or Lys but contributes less to the binding of an NLS than P2 [18,42], which may explain the range of binding residues among known NLSs [17]. The $^{NLS}$Arg20 bound in position P4 provides the most stability and binds through hydrogen bonds to $^{mImp\alpha\Delta IBB}$Arg106 and $^{mImp\alpha\Delta IBB}$Glu107, although this residue need not be specific and would be accepting of even a small hydrophobic residue [17]. Occupying P5 was $^{NLS}$Arg19 through hydrogen bonds with $^{mImp\alpha\Delta IBB}$Trp142, $^{mImp\alpha\Delta IBB}$Asn146, and $^{mImp\alpha IBB}$Gln181. P5 prefers an Arg or Lys, matching that seen in BFDV Cap NLS, and, along with P3 contributes the second highest energy for binding following P2 [17,18]. The $^{NLS}$Tyr18 residue occupies position P6, although no bonding with mImpαIBB was observed, consistent with previous observations that P6 will accommodate a range of residues and is less important for binding [40]. In addition to contacts within the Impα major binding site, $^{NLS}$Tyr24 formed a hydrogen bond with $^{mImp\alpha IBB}$Trp231 in a potential linker region. When viewing the sequence of the entire BFDV Cap NLS, the interaction of a region not containing a Lys residue may not be surprising, as molecules with extremely high affinity to Impα will act as an inhibitor, and the of Arg residues present provide ideal binding partners for most of the sites [43,44].

The structural determination of BFDV Cap NLS in complex with mImpαIBB illuminated a pathway of nuclear localization for the viral Cap. The BFDV Cap NLS was shown to bind to the major site on the importin molecule. Additional residues outside of the NLS region were not discernible from the electron density, likely because of a combination of flexibility in the region and the Impα residues in the linker region being positively charged [22], repelling the positively charged regions of the Cap NLS.

Several examples of NLSs binding to Impα have been investigated and may be compared with the BFDV Cap NLS. Most notably, characterization of simian virus 40 (SV40) large-T NLS with a wild-type sequence of PKKKRKV binding to Impα major sites P0–P6, respectively, showed that mutation of the Lys residue bound to P2 to an Arg still allowed for nuclear localization and viral replication [18,45]. Additionally, mutation of the P3 bound Lys to Arg also showed nuclear localization and viral replication. The mutated and viable SV40 large-T NLSs, PKRKRKV and PKKRRKV, have very similar sequences to the BFDV Cap NLS seen bound to the Impα major site, as both have a bulky residue in P0, followed by basic residues bound to P1–P5. This is in contrast to bipartite sequences Bimax1, Bimax2, mCBP80, and yCBP80, which bind the Impα major and minor sites, as well as the linker region [22]. These structures solved by Marfori et al. [22] have similar sequences bound to the

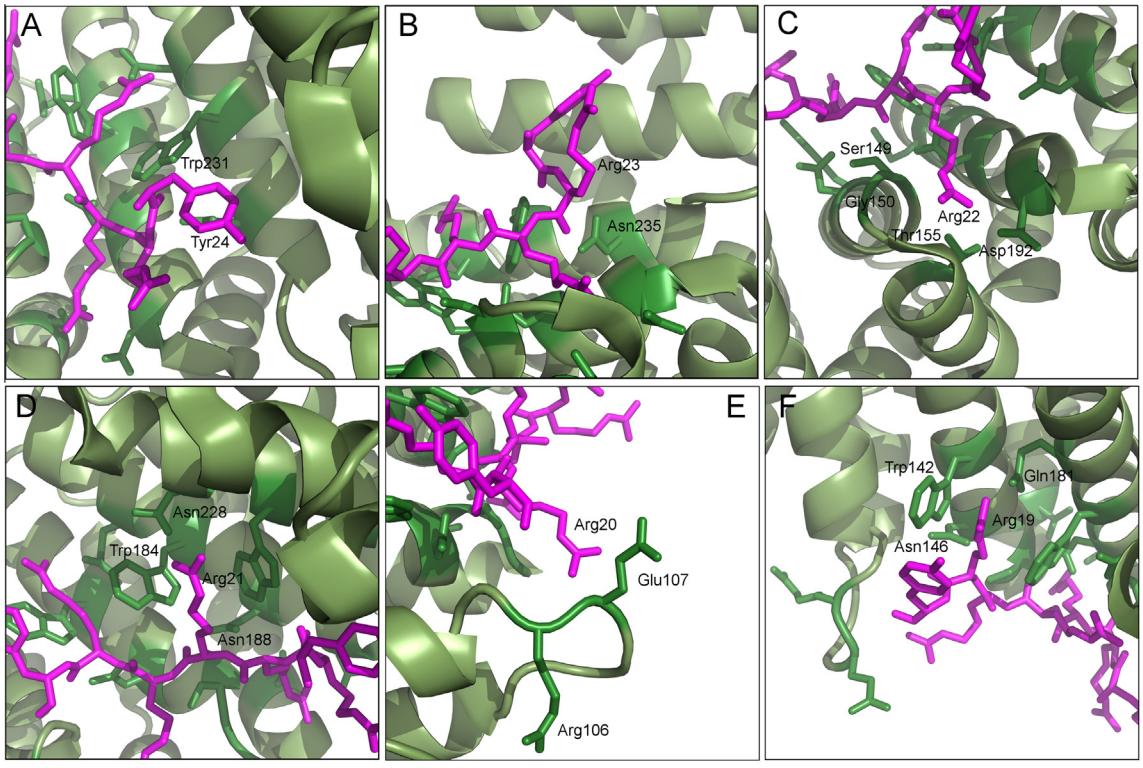


**Fig. 3.** BFDV Cap NLS (magenta) binding the major site of mImpαΔIBB (green). (A–F) show each BFDV Cap NLS residue involved in binding mImpαΔIBB in sites P0–P5, respectively. Impα residues involved in binding are in green highlight and labeled. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

major site, however, the residues separating the two NLS regions have a large concentration of negatively charged amino acids, allowing them to bind to the linker region. This negatively charged region is not present in the BFDV Cap NLS or any other circovirus NLS sequence. Of even greater contrast is the TPX2 NLS, which is bipartite but preferentially binds the minor site [46]. The TPX2 NLS sequence contains only two basic residues, both Lys, bound to the major site of Impα in positions P2 and P5.

Experiments by Heath et al. [13] were the first to show nuclear localization of BFDV Cap, however, there has been no further elucidation of the pathway, or any critical binding regions. In PCV2 an NLS was identified by sequentially mutating groups of positively charged amino acids in the NLS region [14]. Mutations in regions that contained clusters of positively charged residues resulted in decreased nuclear and increased cytosolic localization. Experimentation to isolate the NLS in DuCV was similar to that seen in previous experiments with BFDV, using truncations of several amino acids to determine the regions responsible for nuclear localization [16]. Additional regions containing positively charged amino acids likely play a role in DNA binding, thus, requiring large stretches of positive residues to congregate in the N-terminal to ensure binding to two molecules necessary for infection. Using this information to guide drug or vaccine development may provide new classes of therapeutics to limit efficient binding of the viral Cap to host molecules and control, specifically, BFDV, but also the entire range of related viruses with significant veterinary and potential medical impact.

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