

Investigation of a Z α -like Peptide Motif in Koi
Herpesvirus

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Abstract

Koi herpesvirus (KHV) is responsible for high mortality in the common carp, a global food staple of which over 1.5 million metric tons is harvested annually. The objective of this study is to investigate B-DNA interactions with a hypothesized $Z\alpha$, or Z-DNA binding, domain in the KHV open reading frame 112 (ORF112) peptide motif. Mutations analogous to the Y84A and N62D mutations have been shown to be critical for Z-DNA binding in proteins with $Z\alpha$ domains. Therefore, circular dichroism (CD) spectra were collected of the wild type (WT), Y84A mutant, and N62D mutant ORF112 proteins titrated into poly(dGdC) and calf thymus B-DNA. The WT and Y84A mutant proteins bound to two distinct DNA conformations that did not indicate a B- to Z-DNA transition, and the N62D mutation prevented any conformational change. The results indicate that B-DNA interacts with the $Z\alpha$ -like peptide motif in a non-sequence specific manner to form a unique structure that requires the Asn 62 residue and is affected structurally by the Tyr 84 residue.

Summary

Koi herpesvirus (KHV) causes high mortality in the common carp and koi. A KHV protein was hypothesized to bind to DNA in a reversed orientation. However, it was determined that the protein binds to DNA in a unique conformation unlike the three traditional forms of DNA. Furthermore, two changes made in the amino acid sequence of the protein caused important structural changes in the DNA. The insights into DNA-protein interaction gained in this work could aid in understanding the mechanism of KHV's high lethality.

1 Introduction

1.1 Z-DNA binding to $Z\alpha$ domains

Three structural forms of DNA are currently known: A-DNA, B-DNA, and Z-DNA. Among the three, Z-DNA is structurally unique because it is left- rather than right-handed. It is both thinner and more elongated than B-DNA and A-DNA and contains base pairs that are displaced off its axis [1]. The phosphate groups on the two strands of Z-DNA are closer than those in A-DNA and B-DNA, which explains the stabilization of Z-DNA in high salt conditions [2]. Recent *in vivo* studies provide evidence that Z-DNA is formed and stabilized by negative supercoiling [3], which is involved in processes including transcription, DNA recombination [4], and modulation of DNA binding protein interactions in gene regulation [5]. Because of its genetic importance, Z-DNA has been linked to numerous other biological phenomena, including viral pathogenesis, the immune response, and the host response to tumors [6].

The first $Z\alpha$ domain was identified in dsRNA adenosine deaminase (ADAR1), an RNA-editing enzyme containing a $Z\alpha$ and a $Z\beta$ domain that interact together to bind to Z-DNA. ADAR1 deaminates adenosine to produce inosine, an editing process that has powerful biological implications ranging from the hypermutation of viruses *in vivo* [7] to the cGMP response of the serotonin HT2C receptor [8]. $Z\alpha$ domains in other proteins have been identified based on sequence similarities to the ADAR1 $Z\alpha$ domain. For instance, in the *vaccinia* virus, binding of Z-DNA to the E3L $Z\alpha$ domain is essential for virulence. This domain conserves several key structural and Z-DNA binding residues of the $Z\alpha$ domain of ADAR1 [9]. Amino acid mutations introduced in a study by Rich, *et al.* indicated that Z-DNA binding to the $Z\alpha$ domain of *vaccinia* is essential for pathogenicity of the virus. Z-DNA binding in E3L acts to block the interferon response by the immune system, which could be a mechanism for *vaccinia*'s pathogenicity [10].

1.2 A possible $Z\alpha$ domain in KHV

KHV is a dsDNA virus that devastates populations of koi (*Cyprinus carpio koi*) and common carp (*Cyprinus carpio carpio*) throughout the world. KHV is particularly destructive in Europe, Asia, and the Middle East, where the common carp is an essential food staple, with over 1.5 million metric tons harvested per year for consumption [11]. Likewise, KHV causes high mortality in koi raised for the aquaculture industry. However, because KHV was first isolated in 1998, little is known about the mechanism by which KHV is virulent [12].

$hZ\alpha_{ADAR1}$	LSGKLGTP KKE INRVLYSLAKKGK LQ KEA--GTP PLWK
$hZ\alpha_{DLM1}$	LVKECQAP KRE LNQVLYRM-KKEL KV SL---TSPAT WC
$mZ\alpha_{DLM1}$	LVKKCQVP KKT LNQVLYRL-KKED RV SS---PEPAT WS
$vvZ\alpha_{E3L}$	LTRQLNME KRE VNKALYDLQRSAM VY SS--DDI PP RF
$caZ\alpha_{PKZ}$	IAKEIGLD KST VNRHLYNLQRSN QV FNS--NE KPP VWD
$drZ\alpha_{PKZ}$	IFKELKLE KST VNRHLYSLQASK QV FKTVEDN KRP VWD
$khvZ\alpha_{WT}$	IAQSLGAQRSE VNR HLYRMSE DGR VRKHPQHPV WY LPA
$khvZ\alpha_{ND}$	IAQSLGAQRSE VDR HLYRMSE DGR VRKHPQHPV WY LPA
$khvZ\alpha_{YA}$	IAQSLGAQRSE VNR HLYRMSE DGR VRKHPQHPV WAL PA

Figure 1: **Amino acid sequence comparison of identified $Z\alpha$ domains and a hypothesized KHV $Z\alpha$ domain**

The residues highlighted in red are found in the hydrophobic core, indicating a function in protein folding. Those in blue contact nucleic acid, and are essential for Z-DNA recognition. Residues colored purple are important in both nucleic acid interaction and protein folding. The amino acid letters bolded are the sites of mutations induced in the ORF112 peptide motif. The sequences shown are from human $Z\alpha_{ADAR1}$, human $Z\alpha_{DLM1}$, mouse $Z\alpha_{DLM1}$, *vaccinia* virus $Z\alpha_{E3L}$, *D. rerio* $Z\alpha_{PKZ}$, *C. auratus* $Z\alpha_{PKZ}$, KHV WT $Z\alpha$, KHV ND $Z\alpha$, and KHV YA $Z\alpha$, respectively. [13]

NCBI/BLAST sequence analysis has suggested that KHV contains a helix-turn-helix motif in the open reading frame 112 (ORF112) peptide motif, which may bind to double-

stranded nucleic acids [14]. Significant amino acid sequence similarities are illustrated in Figure 1. This study suggests that ORF112 may contain an N-terminal $Z\alpha$ domain. It has been hypothesized that Z-DNA forms *in vivo* during transcription in alternating purine-pyrimidine sequences. If the KHV protein binds to Z-DNA, it is possible that the binding process is a key factor in the pathogenicity of KHV, as it is in *vaccinia* with E3L.

Investigating a possible Z-DNA binding site may be important to understanding the mechanism of KHV pathogenesis. However, it is also possible that the protein domain does not bind to Z-DNA, but rather A-DNA, which forms in dehydrated samples of DNA, or B-DNA, the traditional right-handed, double-helical structure. Thus, identifying this DNA-binding domain is an important step toward understanding the pathogenesis of KHV.

This study uses circular dichroism (CD) spectroscopy to examine the structural interaction of poly(dGdC) B-DNA with the hypothesized $Z\alpha$ domain of ORF112. Wild type, Y84A mutant, and N62D mutant proteins are examined to ascertain the function of the Tyr 84 and Asn 62 residues, which correspond structurally to important residues of previously identified $Z\alpha$ domains.

2 Materials and Methods

2.1 ORF112 purification

$Z\alpha$ was expressed and purified as previously described [15]. A tyrosine-alanine (YA) mutation in amino acid 84 and an asparagine-aspartic acid (ND) mutation in amino acid 62 were induced with QuikChange from Agilent Technologies (Santa Clara, California). The YA mutation was chosen because in $Z\alpha_{\text{ADAR1}}$, a mutation of Tyr 177 to Ala decreases Z-DNA binding [16]. As shown in Figure 1, the $Z\alpha$ domains of ADAR1, DLM1, E3L, and PKZ all share an analogous asparagine residue, suggesting that it could be critical for Z-DNA binding.

2.2 Gel electrophoresis

SDS PAGE was conducted on each of the three binding domains. A running gel was prepared containing 2.5 mL of lower gel stock (1.5 M Tris-HCl, pH 8.8 and 0.4% SDS), 6 mL of Protogel (Atlanta, Georgia) stock (acrylamide), and 1.5 mL of de-ionized water. After stirring, 50 μ L of ammonium persulfate (APS) and 10 μ L of TEMED were added. Approximately 5 mL of the running gel was used and allowed to polymerize under a layer of water-saturated n-butanol. A stacking gel was prepared containing 0.5 mL of upper gel stock (0.5 mL Tris-HCl, pH 6.8 and 0.4% SDS), 0.3 mL Protogel stock (acrylamide), and 1.2 mL de-ionized water. After stirring, 30 μ L of APS and 4 μ L of TEMED were added. The n-butanol was decanted, and the stacking gel was immediately poured onto the polymerized running gel. The comb was inserted, and the stacking gel was allowed to polymerize.

Loading buffer (2 mL 1 M Tris-HCl, pH 6.8, 0.8 g SDS, 4 mL 100% glycerol, 0.4 mL 14.7 M β -mercaptoethanol, 1 mL 0.5 M EDTA, 8 mg bromophenol blue) was added to the uninduced and induced cells, which were subsequently boiled for 5 minutes. Solutions were prepared by adding loading buffer and then boiling for one minute. The gel was loaded with 5 μ L of uninduced and induced cells, 2 μ L of the supernatant and flow through with 5 μ L of loading buffer, 10 μ L of each of five elution fractions with 3 μ L of loading buffer, and 5 μ L of molecular weight marker in 5 μ L of loading buffer. The gel was run for approximately one hour at 200 V, or until the dye/protein had reached the bottom of the gel. Then, the gel was placed in fixative (25% isopropanol and 10% acetic acid) for 10 minutes, stained with 0.02% Coomassie Blue R250 in 10% acetic acid, soaked with de-ionized water, and scanned with an Epson Perfection 1650 scanner.

2.3 Protein preparation

The proteins from the first three elution fractions, obtained from chromatography during purification, were dialyzed overnight in a solution of 150 mM NaCl solution, 20 mM Tris buffer (pH 8.0), and 0.1 mM EDTA. The proteins were then concentrated with Amicon Ultra-15 Centrifugal Filter Units and centrifuged in a Sorvall RC-5C Plus Superspeed Centrifuge for 15 minutes. The concentration of each sample was determined using a NanoDrop ND-1000 spectrophotometer. The extinction coefficients are $9970 \text{ M}^{-1}\text{cm}^{-1}$ (WT, ND) and $8480 \text{ M}^{-1}\text{cm}^{-1}$ (YA). If the concentration was not at least 1.2 mM, the sample was centrifuged again. The final protein concentrations were 8.47 mg/mL (WT), 14.45 mg/mL (ND), and 18.57 mg/mL (YA).

2.4 Circular dichroism spectroscopy

Spectra were collected using an Aviv Model 410 Circular Dichroism Spectrometer (Lakewood, New Jersey) from 320 to 240 nm in 1 nm steps. The averaging time was 4 seconds with a settling time of 0.330 and 3 scans per sample at 25 °C. A 500 μL cuvette was filled with 295 μL of CD buffer (10 mM HEPES, pH 7.4, 20 mM KF, and 0.1 mM EDTA) as a baseline. Then, 5.1 μL of poly(dGdC) DNA (purchased from GE Healthcare/Amersham Biosciences) was added and run. Protein (KHV WT, Y84A, N62D) was titrated into the cuvette in ratios of 1:10, 1:5, 1:3, 1:2, and 1:1.5 with five minutes between data collection points to allow time for protein-DNA interaction. The procedure was repeated for the wild type using 60 μM calf thymus DNA (purchased from Sigma-Aldrich). CD spectra were also collected for each of the three proteins from 250 nm to 190 nm. The data were adjusted based on the CD buffer baseline and molarity values. The data were graphed after baseline correction, curve smoothing, and normalization at 320 nm using Prism software.

3 Results and Discussion

3.1 Structural characteristics of the ORF112 WT and mutated peptide motifs

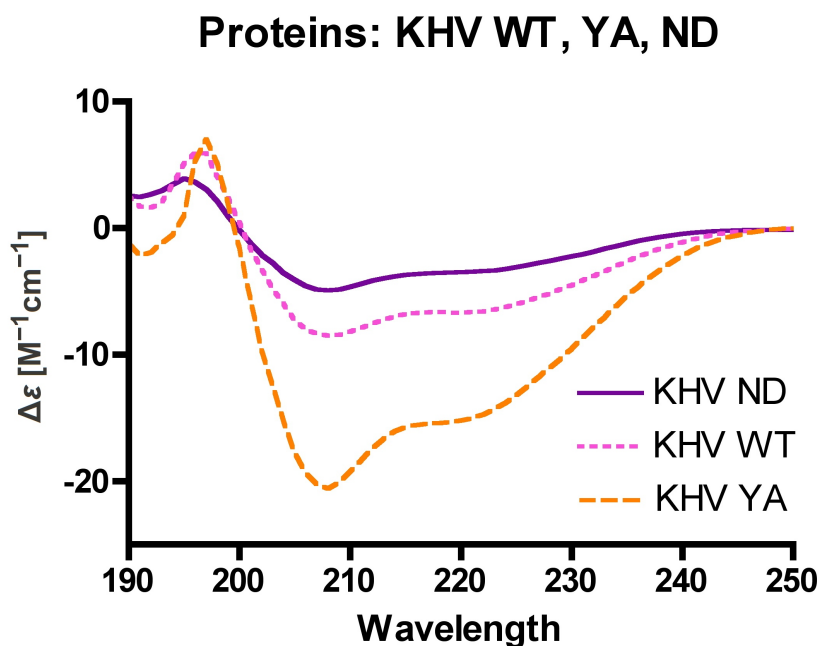


Figure 2: Spectra of the KHV WT, Y84A, and N62D $Z\alpha$ -like peptide motifs. The proteins are unstable and have a tendency to precipitate, and therefore it is likely that the significant differences in intensity of the graphs are due to error in concentration. The major structural features appear in analogous locations, indicating a relatively similar structure of the proteins.

The spectra of the three proteins are illustrated in Figure 2. It is possible that the calculated concentrations were incorrect because all three proteins have a tendency to precipitate. When protein concentrations were checked after collecting the CD data, they had decreased 4.31 mg/mL (WT), 5.41 mg/mL (ND), and 9.57 mg/mL (YA), as determined by NanoDrop spectroscopy. The spectra share similar features, such as peaks at identical wavelengths and

the same overall shape, and differ only in intensity because of error in calculated concentrations. However, the fact that the spectra are essentially identical in shape indicates that the conformations of the proteins, even with the two mutations, are similar. This demonstrates that mutagenesis did not cause destabilization of the proteins, and that any lack of interaction of DNA (in the ND mutation) was not due to degraded protein structure.

3.2 Interaction of ORF112 WT with poly(dGdC) B-DNA

The expression of ORF112 was verified by SDS PAGE.

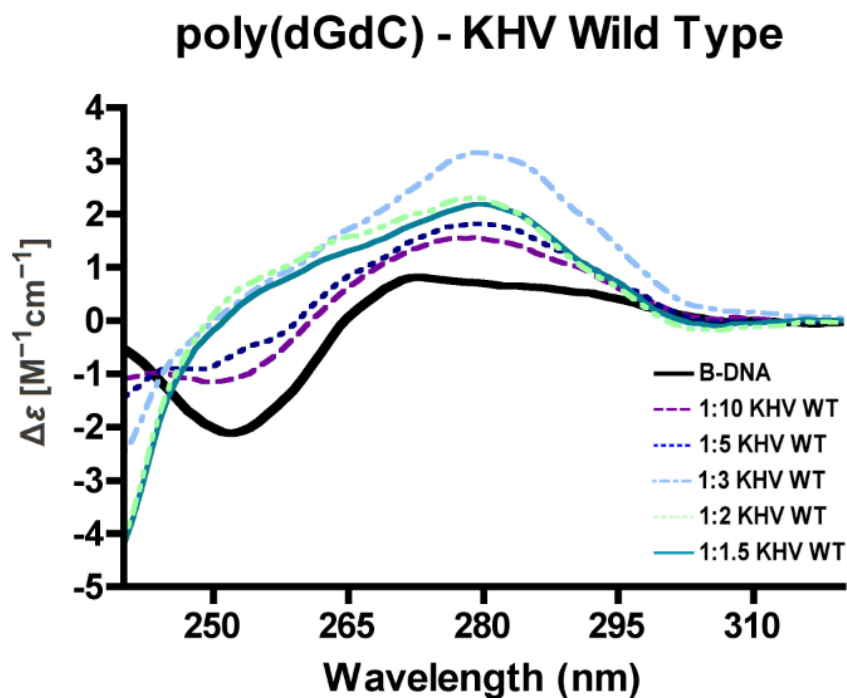


Figure 3: Titration of WT ORF112 into poly(dGdC) B-DNA

The initial B-DNA structure, with a trough at 254 nm and a peak at 272 nm, showed an inversion of bands below 265 nm starting from titration at the 1:5 ratio. The peak consistently shifted as protein was added until it reached 280 nm at the 1:1.5 ratio.

Circular dichroism spectroscopy was used to monitor the change in conformation of poly(dGdC) B-DNA during interaction with the KHV ORF112 Z α -like peptide motif. Fig-

Figure 3 shows the titration of 60 μM poly(dGdC) B-DNA with ratios of protein:DNA from 1:10 to 1:1.5 (increasing protein concentration). The CD buffer baseline was subtracted from all data, which was smoothed by averaging nine adjacent data points at a time. After the addition of 1:10 protein:DNA, a peak appeared at 278 nm, which grew sharper up to the 1:3 ratio and subsequently dropped. Inversion of the CD bands from approximately 250-265 nm also occurred at the 1:3 ratio. While the spectra do not intersect at one point around 300 nm, they are similar enough that it is possible that any deviation is the result of experimental error. There is a consistent trend of decreased data values with increasing protein concentration at wavelengths below 245 nm. This can be attributed to addition of the protein itself rather than interaction of protein with DNA because protein secondary structure is shown in CD spectra at wavelengths of approximately 190-240 nm.

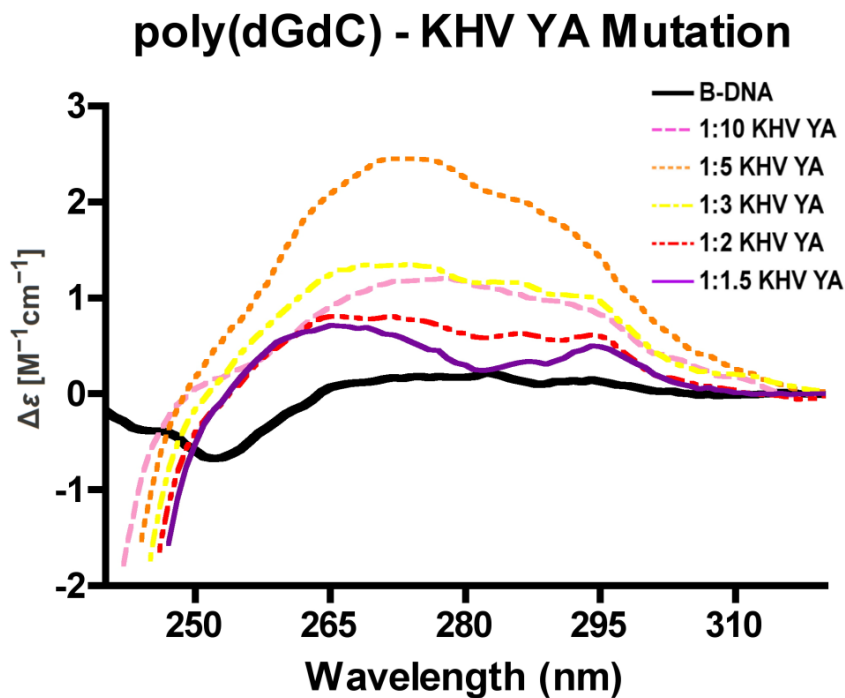


Figure 4: **Titration of YA ORF112 into poly(dGdC) B-DNA**

The initial B-DNA peak at 252 nm shifted to 267 nm as more protein was added, and peaks appeared in the range of 272-282 nm for all of the titration ratios. A third set of peaks appeared at the 1:3 ratio at 293-294 nm.

3.3 Effects of Y84A and N62D mutations on DNA conformational changes

A titration with poly(dGdC) B-DNA was conducted for both the ORF112 Y84A and N62D mutants using identical concentrations and the same procedure. In Figure 4, the conformational change in B-DNA with titration of ORF112 Y84A can be seen. Initial addition of protein causes an immediate inversion of CD bands from 250-265 nm, whereas in the wild type, inversion did not occur until titration to the ratio of 1:3. At a ratio of 1:5, the highest peak is observed at about 275 nm, whereas in the wild type, the greatest peak was consistently around 280 nm and merely grew higher up to the 1:3 ratio. The Y84A mutation does not exhibit a consistent peak; rather, the peak at 275 nm appears to shift toward a smaller wavelength as higher protein concentrations are added, reaching about 260 nm at a concentration of 1:1.5. It appears that beginning at the ratio of 1:2, two peaks appear in the spectrum: one around 260 nm and one around 295 nm. These peaks remain in the same location for the 1:1.5 ratio. The increase of spectral intensity up to the ratio of 1:3 (WT) and 1:5 (Y84A) suggests that the domain may bind as a unit, such as a trimer or pentamer. It has been shown that the $Z\alpha$ domain of ADAR1 must dimerize to bind properly to Z-DNA [8], so it is possible that the hypothesized $Z\alpha$ domain of ORF112 may also require multiple protein domain interactions for binding.

By contrast, Figure 5 clearly shows that there is no significant conformational change in the ORF112 N62D mutant. The six spectra intersect with the exception of wavelengths below 250 nm, which shifted downward because of increasing protein concentration rather than interaction with B-DNA [17]. The N62D mutation prevented any conformational change from occurring in the B-DNA, implying that the asparagine residue is critical for the interaction of the $Z\alpha$ -like peptide motif of ORF112 with poly(dGdC). As previously noted, this asparagine residue is present in an analogous location in many proteins that bind to Z-DNA selectively.

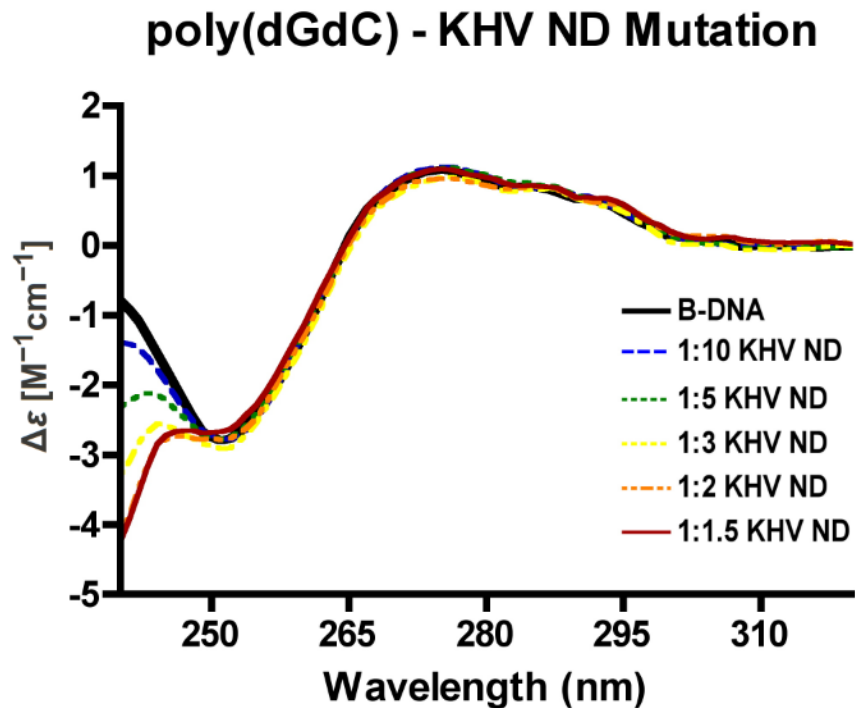


Figure 5: **Titration of ND ORF112 into poly(dGdC) B-DNA**

Addition of protein had minimal impact on the trough and peak, which consistently appeared from 250-254 nm and 274-276 nm, respectively.

This could imply that a conformational change similar to the B- to Z-DNA transition is being prevented by the N62D mutation, and moreover that perhaps a conformational change similar to the B- to Z-DNA transition is occurring in the ORF112 WT peptide motif.

To determine if this is the case, the 1:1.5 ratio spectra for ORF112 WT and Y84A are compared to standard spectra of Z-DNA and B-DNA in Figure 6. From this comparison, it is clear that the conformational change in both the WT and Y84A mutation interactions with B-DNA is not a B- to Z-DNA transition. However, the spectra for the wild type and mutant are in fact different, particularly in that the wild type exhibits a peak at approximately 280 nm, while the Y84A mutation exhibits an inversion around 280 nm and smaller peaks at around 260 and 295 nm. Although this provides evidence that the identified ORF112 peptide motif may not bind Z-DNA under the experimental conditions, the fact that the WT and

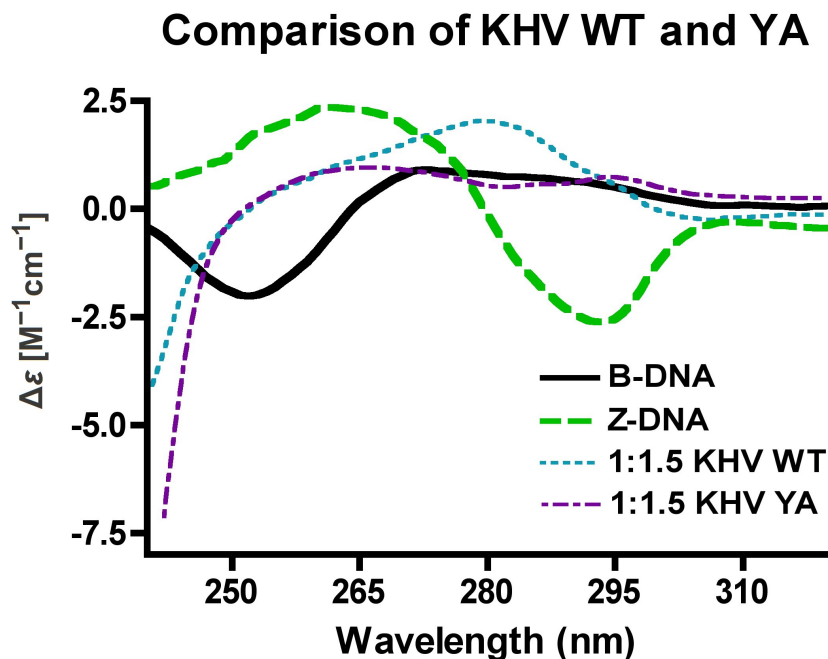


Figure 6: **Comparison with B- and Z-DNA of structures formed by WT and YA**. The figure illustrates that the DNA conformations formed upon binding with the WT and YA $Z\alpha$ -like peptide motifs clearly do not match the spectrum of either B- or Z-DNA.

Y84A spectra differ implies that the mutation changes the way in which the protein interacts with B-DNA. In fact, it is possible that the Y84A mutation forms a different conformation of DNA than that formed by the wild type.

3.4 Sequence specificity determination by titration of calf thymus DNA with ORF112 WT

To test sequence specificity of the ORF112 WT $Z\alpha$ -like peptide motif, a titration with concentrations and a procedure identical to that of the poly(dGdC) experiments was conducted with sheared calf thymus DNA. Figure 7 shows an inversion in the CD bands from approximately 245-260 nm starting with the 1:3 ratio. The 1:3 ratio also exhibited the highest peak at about 280 nm. These characteristics are extremely similar to those exhibited by the

ORF112 WT when titrated with poly(dGdC) B-DNA.

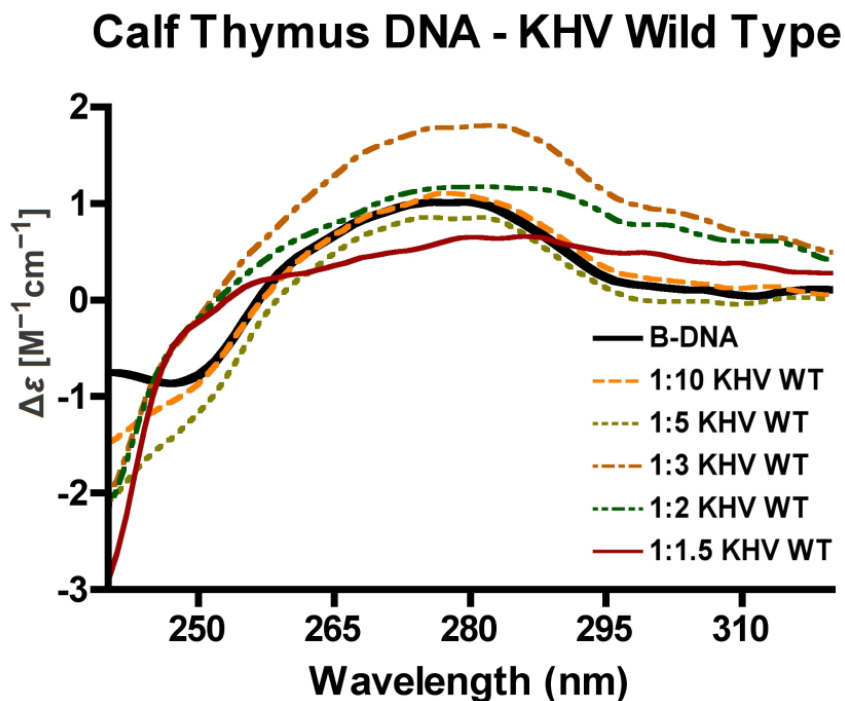


Figure 7: Testing for sequence specificity: titration of ORF112 WT with calf thymus B-DNA

The figure shows an inversion of bands below beginning with the 1:5 ratio similar to that seen in the poly(dGdC) WT experiment. There is also a similar shifting of peaks to the right.

Since the protein causes a nearly identical conformational change in the B-DNA when titrated with both random sequence and alternating C-G DNA, it can be inferred that the protein does not exhibit sequence-specific B-DNA binding. $Z\alpha$ in ADAR1 has similarly been shown to be dependent on energetic conditions rather than sequence specificity [8]. Although both the $Z\alpha$ -like domain identified in this study and that of ADAR1 are not sequence specific, there is still not sufficient evidence to show that the domain binds Z-DNA. In addition, winged helix proteins that bind specifically to B-DNA are characterized by sequence-specific binding [18]. Thus, it is unlikely that the $Z\alpha$ -like peptide motif of ORF112 is a B-DNA-specific binding domain.

4 Conclusion

The CD spectra indicate that the $Z\alpha$ -like peptide motif of ORF112 does not exhibit sequence specificity or a B- to Z-DNA transition, like most $Z\alpha$ domains. However, it is affected by amino acid residue mutations that are significant in most $Z\alpha$ domains. The wild type protein showed inversion of bands from 250-265 nm and a peak at 280 nm. The Y84A mutation produced a different structure, with an inversion around 280 nm and two smaller peaks at 260 and 295 nm. While an interaction still occurred with the Y84A mutation, it is noteworthy that the mutation was significant enough to change the DNA conformation. The N62D mutation prevented any conformational change of B-DNA, suggesting that the asparagine 62 residue is essential for the the protein-DNA interaction observed in this study.

In many Z-DNA binding $Z\alpha$ domains, the corresponding tyrosine and asparagine residues mutated in this study are essential for effective Z-DNA binding. While this study has not determined in which conditions Z-DNA may bind to the $Z\alpha$ -like peptide motif of ORF112, there is a striking impact of the Y84A and N62D mutations on the interaction of B-DNA with the peptide motif.

5 Future Work

Since the peptide motif does not readily bind Z-DNA under the tested conditions, a B-Z midpoint assay can be conducted using cobalt hexamine to assess Z-DNA binding of the peptide motif in a solution of high Z-DNA concentration [19]. In addition, titrating the Y84A and N62D mutations with calf thymus DNA could determine whether sequence specificity is dependent on the amino acid mutations. Inducing different mutations that are shown to be important either structurally or for Z-DNA binding would reveal the importance of various residues of the protein.

Elucidating the structure of ORF112, ORF112 bound to DNA, and the DNA conforma-

tion itself would provide valuable information about the mechanism of nucleic acid binding to ORF112. This could be achieved by structural studies, including nuclear magnetic resonance (NMR) [20] spectroscopy, Raman spectroscopy, x-ray crystallography, and cocrystallization. Secondary structure information can be derived from K2D2, which estimates percentages of α helices and β sheets based on CD spectra [21].

If the $Z\alpha$ -like peptide motif is found to bind to Z-DNA, its role in KHV pathogenesis can be tested in an experiment similar to that of Rich, *et al.* [10] Chimeric viruses can be constructed containing point mutations that decrease Z-DNA binding capabilities. The change in mortality can be assessed for each point mutation to determine its importance in Z-DNA binding. A comparison of lethality of viruses that express domains that bind to Z-DNA and those that do not can demonstrate whether Z-DNA binding is essential for KHV pathogenesis. If it is, treatments that prevent Z-DNA binding in KHV could prove to be an effective treatment for a thus far uncured and highly lethal disease.

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