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Original Article

The Zα domain of fish PKZ facilitates the B–Z conformational transition of oligonucleotide DNAs with d(GC)$_n$ inserts

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PKZ (PKR-like) was discovered as a member of eIF2α kinase family in fish, which possesses a conserved catalytic domain of an eIF2α kinase in C-terminal and also two Z-DNA-binding domains (Zα1 and Zα2) in N-terminal. PKZ can be activated through binding of Zα to Z-DNA. However, the regulatory function of PKZ Zα still remains unclear. To investigate a molecular mechanism of how PKZ Zα interacts with Z-DNA, we expressed Zα polypeptide Zα1α2 in *Escherichia coli* Rosetta strain and purified by affinity chromatography on Ni-NTA resin. Different lengths of oligonucleotide DNAs with various inserts, namely d(GC)$_n$ (n = 6, 8, 10, 13), d(TA)$_n$ (n = 6, 10), non-d(GC), and non-d(TA), were designed and synthesized. Circular dichroism spectrum and gel mobility shift assays were used to investigate the effects of Zα1α2 on the conformational transition of different oligonucleotide DNAs. Results showed that oligonucleotide DNAs retained a conventional B-DNA conformation in the absence of Zα1α2. With the increasing amount of Zα1α2 titration, d(GC)$_n$ were recognized and converted to Z-DNA conformation to some degree. With increasing the repeat number (from n = 6 to n = 13), the tendency of conformational transition became more obvious. However, the conformation of oligonucleotides with d(TA)$_n$ inserts changed a little in the presence of Zα1α2, and Zα1α2 had no effect on conformational transition of oligonucleotides with non-d(GC) or non-d(TA) inserts. Gel mobility shift assays further showed that Zα1α2 could bind to oligonucleotide with d(GC)$_{10}$. In other words, Zα1α2 can turn oligonucleotides with d(GC)$_n$ inserts into Z-DNA conformation and bind to it with high affinity.

Keywords PKZ; Zα domain; Z-DNA; conformation; d(GC)$_n$

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Introduction

Zα is a protein domain that can recognize specifically Z-DNA and stabilize its conformation [1]. In recent years, proteins containing Zα domain have been found, including ADAR1 (Adenosine Deaminases Acting on RNA) [2], DLM-1 (Z-DNA-Binding Protein, ZBP-1) [3], E3L [4], and PKZ (PKR-like) [5–8]. The secondary structure of Zα consists of three α-helices and three β-sheets that arranges as a helix–corner–helix (α + β HTH) and forms a topological structure $\alpha_1\beta_1\alpha_2\alpha_3\beta_2\beta_3$ that is beneficial for Z-DNA binding [9]. Three-dimensional structures of $Z\alpha_{ADAR1}$ [10], $Z\alpha_{E3L}$, and $Z\alpha_{DLM-1}$ [11] have been reported. They share a homologous domain, indicating that the structure and function of Zα are evolutionally conserved [12].

There were many reports on the affinity between Zα and Z-DNA during the past few years. $Z\alpha_{ADAR1}$-fused polypeptides bound to Z-DNA and activated the downstream reporter gene [13]. Schade *et al.* [14] analyzed the binding constant and stoichiometry of Zα/Z-DNA in solution. Wu *et al.* [15] showed that $Z\alpha_{PKZ}$ polypeptides could bind to recombinant plasmids with d(GC)$_n$ (n = 6, 8, 10, 13) inserts. Schwartz *et al*. [16] reported the structure of Zα/Z-DNA complex. Lushnikov *et al.* [17] observed $Z\alpha_{ADAR1}$ interacted with Z-DNA by AFM. Circular dichroism (CD) spectroscopy is one of the most widely used methods to characterize the conformation of protein or DNA because of its accuracy and stability [18]. For example, Herbert *et al.* [1] discovered that $Z\alpha_{ADAR1}$ bound to Z-DNA conformer of many different sequences using CD spectroscopy.

The expression of fish PKZ can be upregulated by various stresses, so it may act as a kind of intracellular adaptor, which is similar to mammalian PKR [19–21]. Previous reports revealed that PKZ responded to heat shock, phosphorylated the substrate eIF2α, and finally inhibited protein synthesis [9,22]. To better understand the

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relationship between Zα$_{PKZ}$ and DNA conformation, Zα polypeptide Zα1α2 from goldfish (*Carassius auratus*) PKZ (GenBank accession number AY293929) was expressed in *Escherichia coli* Rosetta strain and purified by affinity chromatography using the Ni-NTA His·Bind® resin column. Meanwhile, different lengths of oligonucleotide DNAs containing d(GC)$_n$ or d(TA)$_n$ inserts and some non-d(GC)$_n$ or non-d(TA)$_n$ inserts were designed and synthesized. CD was used to detect the influence of Zα1α2 on DNA conformation. Results showed that Zα1α2 converted oligonucleotide DNAs with d(GC)$_n$ inserts from B-DNA to Z-DNA to some degree. In addition, when the GC repeat number was increased from $n = 6$ to $n = 13$, the tendency of conformational transition became obvious. Furthermore, the efficiency of conformational conversion for d(GC)$_n$ was far more than d(TA)$_n$ in the presence of Zα1α2. However, Zα1α2 did not change the conformation of oligonucleotides with non-d(GC)$_n$ or non-d(TA)$_n$ inserts, such as primers 3, 4, and 5 used in this paper. These results were further confirmed by the gel mobility shift assays.

Materials and Methods

Oligonucleotide DNAs

Oligonucleotide DNAs (**Table 1**) were designed according to previous studies [15,23,24] and synthesized by Shanghai Sangon Biotechnology Co. Ltd (Shanghai, China) and Beijing Genomics Institute (Beijing, China). Except for the core segment, the 5′ and 3′ flanking regions of oligonucleotide DNAs used in this paper are identical. The anti-Z-DNA antibody was purchased from Abcam (Cambridge, UK).

Protein preparation

CaPKZ Zα cDNA was constructed into pET-32a(+) (Novagen, Gibbstown, USA) expression vector, and then resulting pET-32a(+)/Zα cDNA recombinant plasmid was transformed into *E. coli* BL21 (DE3). Zα1α2 fused polypeptide was expressed and purified as described previously [15].

CD spectroscopy

CD spectra and thermal CD melting curves were recorded on a JASCO J-715 spectropolarimeter (Easton, USA) equipped with a programmable, thermoelectrically controlled cell holder. The light path was 1 cm, and the sample volume was 0.2 ml. All solutions contained 50 mM Tris, 25 mM NaCl, and 0.1 mM ethylenediaminetetraacetic acid. Every spectrum was scanned three times between 220 and 320 nm with a scan rate of 200 nm/min at 25°C. Oligonucleotide DNAs and Zα1α2 were mixed at the following molar ratios 24 : 1, 12 : 1, 6 : 1, and 3 : 1. Each mixture was incubated for 30 min at 25°C before measurement. All spectra were corrected by subtracting the buffer baseline. The data were converted to JWS (Java Web Service) and txt file formats were used for final analysis.

Gel mobility shift assay

Gel mobility shift assays were carried out as described previously [25] with some minor modifications to measure the binding of (GC)$_6$, (GC)$_{10}$, (TA)$_{10,}$ or primer 5 oligonucleotide DNA, respectively, to Zα1α2 *in vitro*. Briefly, each oligonucleotide DNA (290 ng) was mixed with Zα1α2 (4 μg) in a final volume of 10 μl. In addition, (GC)$_6$ or (GC)$_{10}$ oligonucleotide DNA was mixed with the same amount of Zα1α2 and 4 μg anti-Z-DNA antibody for the competitive experiment. After incubation for 30 min at 30°C, the reaction products were resolved on a 2% agarose gel. After electrophoresis, the gels were stained with ethidium bromide and photographed.

Results

Effects of Zα1α2 on the conformational transition of oligonucleotides with d(CG)$_n$ or d(TA)$_n$ inserts

To investigate the molecular mechanism responsible for B−Z transition activity of PKZ Zα, we performed the titration analysis of Zα1α2 to oligonucleotides with d(CG)$_n$ ($n = 6, 8, 10, 13$) and d(TA)$_n$ ($n = 6, 10$) inserts. These

Table 1 Oligonucleotide DNAs used in this study

Name	Sequence (5′→ 3′)	bp
Primer 3	CTGATACTACATTGAATTC(CACGTG)$_2$GAATTCAATGTAGTATCAGA	51
Primer 4	CTGATACTACATTGAATTC(CGTACG)$_2$GAATTCAATGTAGTATCAGA	51
Primer 5	CTGATACTACATTGAATTC(CGGCCG)$_2$GAATTCAATGTAGTATCAGA	51
(GC)$_{13}$	CTGATACTACATTGAATTC$\underline{(GC)_{13}}$GAATTCAATGTAGTATCAGA	65
(GC)$_{10}$	CTGATACTACATTGAATTC$\underline{(GC)_{10}}$GAATTCAATGTAGTATCAGA	59
(GC)$_8$	CTGATACTACATTGAATTC$\underline{(GC)_8}$GAATTCAATGTAGTATCAGA	55
(GC)$_6$	CTGATACTACATTGAATTC$\underline{(GC)_6}$ GAATTCAATGTAGTATCAGA	51
(TA)$_{10}$	CTGATACTACATTGAATTC$\underline{(TA)_{10}}$GAATTCAATGTAGTATCAGA	59
(TA)$_6$	CTGATACTACATTGAATTC$\underline{(TA)_6}$GAATTCAATGTAGTATCAGA	51

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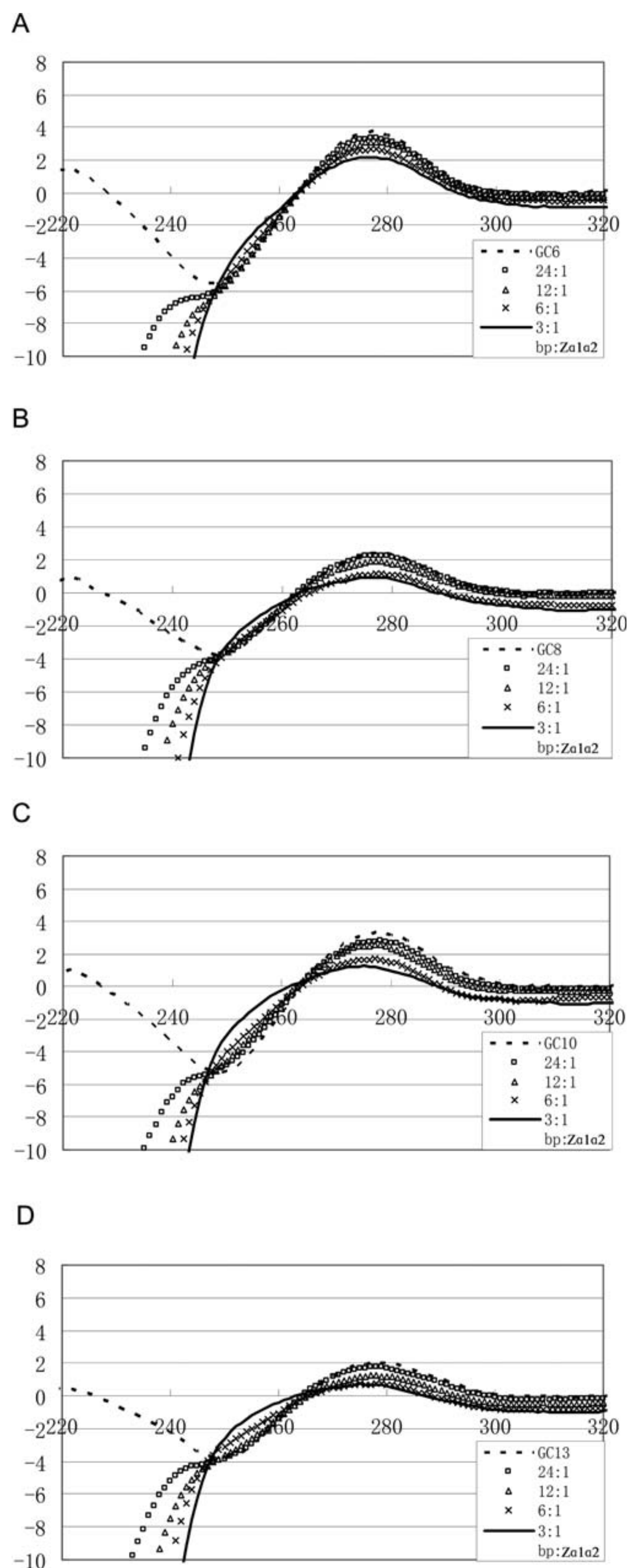

Figure 1 Effects of Zα1α2 on the conformation of oligonucleotides with d(GC) inserts by CD *X*-axis is wavelength range from 220 to 320 nm; *Y*-axis is ellipticity in millidegrees in CD spectrometry (MDEG). Four different double-stranded oligonucleotides $(GC)_6$ (A), $(GC)_8$ (B), $(GC)_{10}$ (C), or $(GC)_{13}$ (D) were incubated with increasing amounts of Zα1α2, respectively, and DNA-CD spectra were measured. Ratios were 0 (dash), 1 : 24 (open square), 1 : 12 (open triangle), 1 : 6 (multiplication symbol), 1 : 3 (continuous line) as measured in mole of protein:mol of d(GC) base pairs within Z-forming core of oligonucleotide. Zα1α2 alone had no observable effect on spectra in the region of 250–320 nm. CD spectrum of oligonucleotide DNA alone (dash) was characteristic of B-DNA. With titration of Zα1α2, all spectra contained a higher ellipticity at 255 nm and a lower ellipticity at 278 nm.

oligonucleotides possessed Zα binding sites, d(CG)$_n$ or d(TA)$_n$, were flanked by sequences in B-form. The oligonucleotides were incubated in a titration series with increasing Zα1α2 concentrations, and CD spectra were employed to detect the conformational transition in the DNAs.

The molar ellipticities from 253 to 296.5 nm were altered dramatically upon addition of increasing amounts of Zα1α2. In the absence of Zα1α2, DNAs adopted a conventional B-DNA conformation (**Fig. 1**). When Zα1α2 concentration was increased from 1 : 24 to 1 : 3, CD spectrum changed from a pure B-DNA spectrum to a spectrum that combined a B- and a Z-DNA spectrum, because there were two B–Z junctions, approximately at 247 and 265 nm (**Fig. 1**). The CD curves at stoichiometries of 24, 12, 6, and 3 bp mol to 1 Zα1α2 mol showed a plateau when the ratio was 3 bp mol to 1 Zα1α2 mol, indicating that a part of B-DNA conformation remained unchanged upon overtitration of Zα1α2.

When Zα1α2 was added to oligonucleotides with d(TA)$_n$ inserts, the molar ellipticities of Zα1α2/DNA complexes were about the same $<$260 nm. CD plots of $d(TA)_{10}$ and $d(TA)_6$ oligonucleotide DNA were almost the same. Compared with d(GC)$_n$ inserts, the molar ellipticities of Zα1α2/d(TA)$_n$ were obviously lower $>$260 nm. CD plots also showed ellipticities at stoichiometric ratios of 24, 12, 6, and 3 bp mol to 1 Zα1α2 mol. When the stoichiometry ratio was 3 bp mol to 1 Zα1α2 mol, the molar ellipticities $>$260 nm changed a little (**Fig. 2**).

The efficiency of conformational transition of oligonucleotides with d(GC)$_n$ inserts was far higher than that with d(TA)$_n$ in the presence of Zα1α2. When the number of repeats in d(GC)$_n$ was increased, this tendency became more obvious. The hierarchy for base-pairs that can form Z-DNA was $d(GC)_{13} > d(GC)_{10} > d(GC)_8 > d(GC)_6$.

Effects of Zα1α2 on the conformation of oligonucleotides with non-d(GC) or non-d(TA) inserts

Primers 3, 4, and 5 were oligonucleotides with non-d(GC) or non-d(TA) repeat (**Table 1**). To understand the influence of Zα1α2 on conformation of these oligonucleotides, we analyzed CD spectra of the mixture of Zα1α2 with each oligonucleotide. In each case, the conformation of oligonucleotides remained unchanged (**Fig. 3**).

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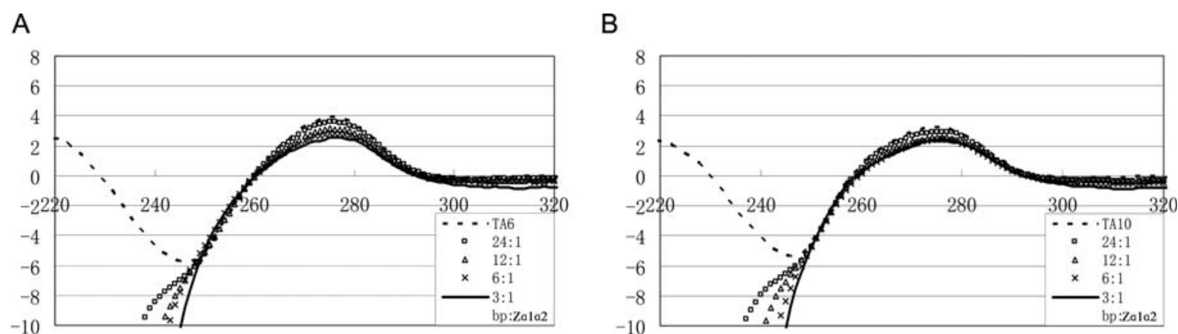

Figure 2 Effects of Zα1α2 on the conformation oligonucleotides with d(TA) inserts by CD *X*-axis is wavelength range from 220 to 320 nm; *Y*-axis is ellipticity in MDEG. Two different double-stranded DNA oligonucleotides $(TA)_6$ (A) or $(TA)_{10}$ (B) were incubated with increasing amounts of Zα1α2 respectively and DNA-CD spectra were measured. Ratios were 0 (dash), 1 : 24 (open square), 1 : 12 (open triangle), 1 : 6 (multiplication symbol), 1 : 3 (continuous line) as measured in mole of protein:mol of d(TA) base pairs within Z-forming core of oligonucleotide. The Zα1α2 alone (dash) had no observable effect on spectra in region of 250–320 nm. CD spectrum of d(TA) DNA alone (dash) was characteristic of B-DNA. Compared with d(TA) DNA alone (dash), CD spectrum of titration of Zα1α2 had obvious lower ellipticity at 278 nm for $d(TA)_6$ or $d(TA)_{10}$.

Binding of Zα1α2 to oligonucleotides detected by gel mobility shift assays

Although there are some specific bands lagging behind by gel mobility shift assays, the lag is incomplete [**Fig. 4(A)**, lanes 2 and 5]. This reveals that Zα1α2 can convert only a part of oligonucleotide DNA with $d(GC)_6$ or $d(GC)_{10}$ insert to Z-DNA, then bind to them. In the competition assays, when Z-DNA antibodies (4 μg) were added to Zα1α2 (4 μg)/$d(GC)_6$ or /$d(GC)_{10}$ oligonucleotide (290 ng) complexes, the bands displayed sharp broad smear [**Fig. 4(A)**, lanes 3 and 6]. Results showed that the antibodies could bind to Z-DNA induced by Zα1α2, and $d(GC)_{10}$ had a higher affinity with Zα1α2 than $d(GC)_6$. Compared with $d(GC)_n$, the band shift pattern of primer 5 (290 ng) or $(TA)_{10}$ (290 ng) oligonucleotides was very weak [**Fig. 4(B)**, lanes 2 and 4].

Discussion

It is generally accepted that two or more conformations may exist within the same DNA molecule, thereby generating conformational junctions due to stereochemical considerations. Z-DNA is thought to be one of three biologically active double helical structures along with A- and B-DNA. Because of the possible involvement of Z-DNA in gene expression, both Z-DNA and B-Z transition were the subjects of intense studies. The Z-DNA-binding proteins play an essential role in the process of the conformational transition. Some phosphatides binding proteins such as yolk proteins from nematodes, chickens, and frogs bound specifically to Z-DNA by interacting with the phosphoric acid backbone and *cis–trans* alternant bases [26]. These proteins bound to Z-DNA based on the conformation specifically. However, in most cases, the identification and binding to Z-DNAs are still dependent on Zα domain of these proteins. Human $Zα_{ADAR1}$ possessed a high affinity with BZB666 and BZB888 oligonucleotide DNAs, and formed complexes with the DNAs such as Z-DNA, B-DNA, and two B–Z junctions [23]. So $Zα_{ADAR1}$ recognized and interacted with poly(dC-dG), no matter what conformation it was. Similarly, Lushnikov *et al*. [17] found that site-specific Zα of human ADAR1 bound to $d(GC)_{13}$ or $d(GC)_2C(GC)_{10}$ inserts when DNA supercoiling was insufficient to induce Z-DNA conformation. Therefore alternating purine–pyrimidine sequences, especially poly(dC–dG), may be the favorable and potential Z-DNA sequence for Zα.

In the past few years, significant progress has been made in the understanding of molecular mechanism of B-Z transition in [d(G–C)·d(G–C)] polymers. The conversion of B-DNA to Z-DNA is associated with a 'flipping over' of the base pairs so that they are upside down in their orientation relative to what is found in B-DNA [27]. Oligonucleotide DNAs used in the paper contained about a 19-bp flanking sequence on each end. In the middle of DNAs was the core segment with $d(GC)_n$ or $d(TA)_n$ alternant purine and pyrimidine bases. CD spectrum of Z-DNA at around 250–260 nm had a higher molar ellipticity than that of B-DNA. In addition, there were two B–Z junctions when Z-DNA was within a B-DNA environment and junctions were characteristic of the beginning and transition from B- to Z-DNA [23,27]. Our results are consistent with these previous studies [23,27,28]. In our experiments, Zα1α2 can identify and partly turn on the conformational transition of DNAs with $d(CG)_6$ or $d(CG)_{10}$ insert embedded in B-DNA (**Fig. 1**). When the value for '*n*' increased, the overturn became more obvious.

$Zα_{PKZ}$ has the functions similar to $Zα_{ADAR1}$. Ho *et al.* [29] found that $d(GC)_{12}$ had a predicted Z-forming potential almost as great as those of $d(CA)_{12}$ or $d(AT)_{12}$ and that

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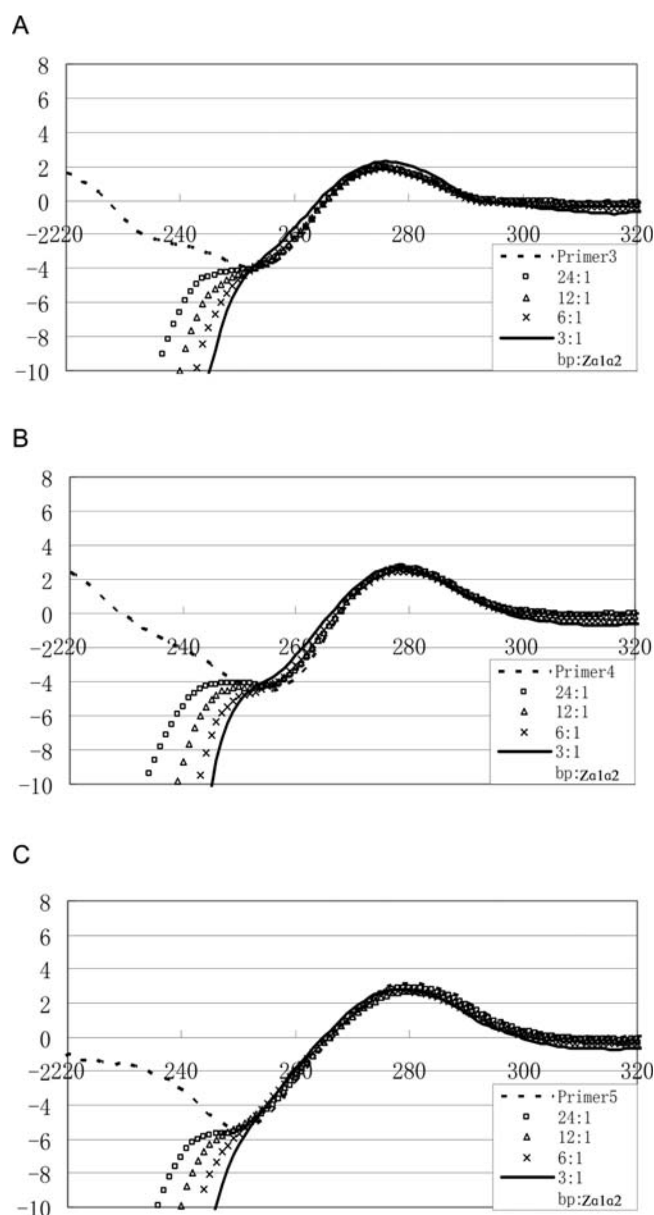

Figure 3 Effects of Zα1α2 on the conformation of oligonucleotides with non-d(GC) or non-d(TA) inserts by CD *X*-axis is wavelength range from 220 to 320 nm; *Y*-axis is ellipticity in MDEG. Three double-stranded DNA oligonucleotides primer 3 (A), primer 4 (B), or primer 5 (C) were incubated with increasing amounts of Zα1α2, respectively, and DNA-CD spectra were measured. Ratios were 0 (dash), 1 : 24 (open square), 1 : 12 (open triangle), 1 : 6 (multiplication symbol), 1 : 3 (continuous line) as measured in mole of protein:mol of primer base pairs within Z-forming core of oligonucleotides. Zα1α2 alone (dash) had no observable effect on spectra in the region of 250–320 nm. CD spectrum of primer DNA alone (dash) was characteristic of B-DNA. With the titration of Zα1α2, nothing had been changed at approximately 255 nm, 278 nm, and 285 nm for primers 3, 4, and 5, respectively.

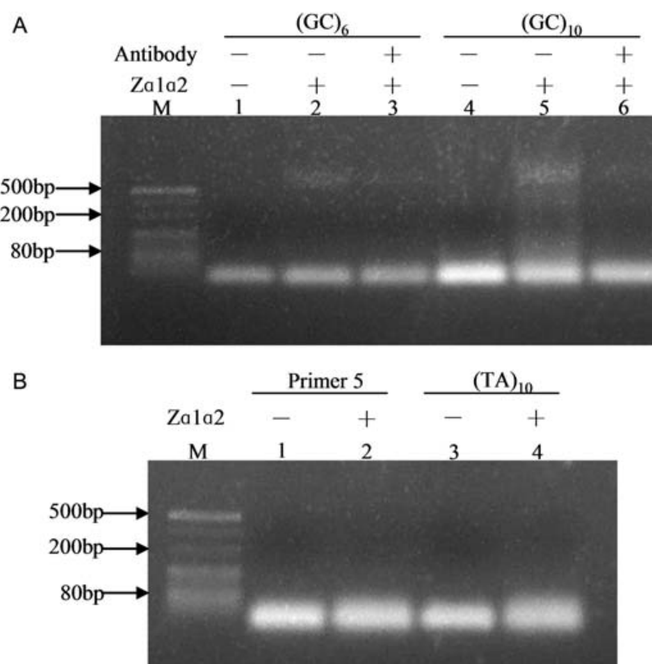

Figure 4 Gel mobility shift assays of the binding of Zα1α2 to oligonucleotides Effect and affinity of Zα1α2 on oligonucleotides were detected by gel mobility shift assays. (A) $d(GC)_6$ (lane 2) and $d(GC)_{10}$ (lane 5) were subjected to gel mobility shift assays in the presence of Zα1α2. Lanes 1 and 4 lack Zα1α2, used as controls. The competitive experiment was performed with adding Z-DNA antibody to the mixture of Zα1α2 with d(GC)6 (lane 3), or with $d(GC)_{10}$ (lane 6). (B) The interaction of $d(TA)_{10}$ or primer 5 with Zα1α2 were performed by gel mobility shift assays in the presence (plus sign) of Zα1α2 (lanes 2 and 4) or in the absence (minus sign) of Zα1α2 (lanes 1 and 3).

the hierarchy for base pairs that can form Z-DNA was $d(GC)_n > d(CA)_n > d(AT)_n$. The effects were thought to be due to differences in hydration of $d(TA)_n$ [30]. Our results also showed that the degree of transition of d(TA) was tremendously lower than that of d(GC) (**Fig. 2**).

The hairpin $d(GC)_3T_4d(GC)_3$ was overturned completely in the presence of Zα [14,30]. It was possible that DNAs used in this paper needed further energy for overturning, so the trend of forming Z-DNA for $d(CG)_n$ embedded in B-DNA was weaker than hairpin DNA. Compared with hairpin $d(GC)_nT_4d(GC)_n$ [14] and recombinant plasmid with $d(GC)_n$ inserts [15], oligonucleotide DNAs with $d(CG)_n$ embedded in B-DNA may be more related to B-Z transition and gene expression.

$(CACGTG)_2$, $(CGTACG)_2$, or $(CGGCCG)_2$ was the core sequence of primers. The sequences were the substitute for $d(CG)_6$ and often used for discussion on the manner of interaction between Zα and Z-DNA [31,32]. The sequence $(CACGTG)_2$, $(CGTACG)_2$, or $(CGGCCG)_2$ can co-crystallize with human $Z\alpha_{ADAR1}$, respectively [31], so Ha *et al*. [31] considered $hZ\alpha_{ADAR1}$ recognized Z-DNA in a conformation-specific manner, but not in a sequence-specific manner. In our paper, we used primers to prove whether PKZ Zα could convert and bind to non-CG or TA repeat DNA duplexes. The molar elipticities of these Zα1α2/primers complexes were very similar at the ratio of 24 : 1, 12 : 1, 6 : 1, 3 : 1 (bp : Zα) (**Fig. 3**). We did not observe the obvious affinity between Zα1α2 and primers.

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Of course, the transition of DNA conformation did not happen in Zα1α2 titration experiments. This implies that $Z\alpha_{PKZ}$ cannot recognize, convert, or bind to non-d(GC) or non-d(TA) repeat short segment of DNAs in solution. Primers could not be turned to Z-DNA by PKZ Zα (**Figs. 3** and **4**). It illustrated that the forming of Z-DNA might acquire intense sequence discrimination. The non-(GC) repeat sequences were more difficult to form Z-DNA than d(CG) repeat sequences. Because non-(GC) repeat sequences had very low-binding affinity for $Z\alpha_{ADAR1}$ than alternating d(CG) DNA, Seo *et al.* [32] considered the possibility of flipping B-DNA into Z-DNA for non-(GC) repeat sequences was far below than alternating d(CG) DNA duplex.

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