Spectroscopic Characterization of a DNA-Binding Domain, Zα, from the Editing Enzyme, dsRNA Adenosine Deaminase: Evidence for Left-Handed Z-DNA in the Zα−DNA Complex†

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ABSTRACT: Double-stranded RNA adenosine deaminase (ADAR1) is an ubiquitous enzyme in metazoa that edits pre-mRNA changing adenosine to inosine in regions of double-stranded RNA. Zα, an N-terminal domain of human ADAR1 encompassing 76 amino acid residues, shows apparent specificity for the left-handed Z-DNA conformation adopted by alternating (dGdC) polymers modified by bromination or methylation, as well as for $(dGdC)_{13}$ inserts present in supercoiled plasmids. Here, a combination of circular dichroism, fluorescence, and gel-retardation studies is utilized to characterize recombinant Zα peptide and to examine its interaction with DNA. Results from laser-Raman spectroscopy experiments provide direct evidence for the existence of Z-DNA in peptide−DNA complexes.

Z-DNA was first described in structural detail in 1979 (*1*). Since then, considerable knowledge of this unusual DNA motif has been accumulated using biochemical and structural techniques (*2*−*5*). In Z-DNA the glycosidic bond between base and sugar alternates between *anti* and *syn* conformations causing the phosphate backbone to zigzag. In contrast, the phosphate groups of B-DNA follow a smooth right-handed arc, with all glycosidic bonds in the *anti* conformation. Z-DNA is preferentially formed by alternating purine−pyrimidine sequences and best formed by alternating deoxyguanosine−deoxycytidine (*4*). Z-DNA has been proposed to play a role in a number of important biological processes such as positive and negative transcriptional regulation (*6, 7*) and general DNA recombination (*8*−*11*). While the existence of Z-DNA in transcriptionally active encapsulated nuclei has been demonstrated for a number of genes (*12*−*17*), an understanding of the physiological consequences of the occurrence of this left-handed conformation in vivo has been elusive to date.

Recently, this laboratory reported the isolation of a protein with apparent specificity in vitro for the left-handed Z-DNA conformation (*18, 19*). The activity was isolated from chicken lung tissue (*20*) based on a gel-retardation assay (*21*) and identified as the enzyme double-stranded RNA adenosine deaminase (ADAR1). A homologous protein exists in humans (hADAR1), which is ubiquitously expressed in all tissues that have been tested. Subsequently, another mammalian enzyme, ADAR2, has been found that has a similar activity (*22*). ADAR1 and ADAR2 catalyze hydrolytic deamination of adenosine at the C6 position in regions of double-stranded RNA (*23, 24*). This reaction gives rise to inosine, which is subsequently translated as guanosine. Such an editing process involving adenosine deamination has been found to be of importance in mammals during the processing of the glutamate receptor, GluR-B, pre-mRNA. Here a CAG triplet (glutamine) is changed to CGG (arginine), drastically altering the electrophysiological properties of the GluR-B transmembrane receptor by reducing channel permeability for Ca^{2+} ions (*25*).

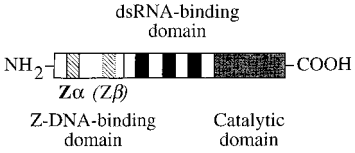

FIGURE 1: Domain structure of ADAR1 (adapted from Herbert, 1996). Human double-stranded RNA adenosine deaminase (ADAR1) enzyme has three functional parts: a C-terminal catalytic domain which is responsible for the conversion of adenosine into inosine, three adjacent segments which have homology with known double-stranded RNA binding domains, and an N-terminal domain which contains Zα, a 78 amino acid domain with apparent specificity for Z-DNA in vitro (Herbert 1996, Herbert et al., 1997). Based on sequence homology, a second Z-DNA-binding motif (*Zβ*) has been identified (Herbert, 1996, Herbert et al., 1997).

The Z-DNA binding region of hADAR1 enzyme was mapped to the N-terminus (Figure 1) and encompasses 78 amino acid residues (*26*). Its affinity for a brominated (dGdC) polymer was determined by BIAcore (Pharmacia, NJ) to be in the low nanomolar range (*26*). In this paper, circular dichroism experiments were carried out using a methylated ($dGdm^5C$) polymer to test for the ability of Zα

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to stabilize Z-DNA at low salt (*26*). Modified polymer was chosen instead of unmodified poly(dGdC), because methylation of cytosine at the C5 position largely reduces the energy needed to reach the midpoint of the transition from B to Z (*27*). The CD experiment produced an inversion of the spectrum that was in reasonable agreement with the original studies carried out by Pohl and Jovin on poly(dGdC) at low-salt (B-form) and high-salt (Z-form) concentrations (*28*). The results were interpreted as supporting data for the specificity of this peptide domain for the left-handed Z-DNA conformation (*26*).

This report confirms that Zα interacts with Z-DNA. By using a variety of biophysical techniques, we examine whether Zα is able to stabilize the left-handed Z conformation adopted by unmodified, linear alternating (dGdC) polymer as well as the methylated form. We also examine the effect of Zα on short, methylated and unmethylated deoxycytidine−deoxyguanosine oligonucleotides. Zα recognizes and binds to unmodified poly(dGdC) and also short $d(CG)_n$ oligonucleotide sequences. In addition, we demonstrate using laser-Raman spectroscopy that the Zα peptide does indeed stabilize unmodified poly(dGdC) in the Z-DNA conformation.

EXPERIMENTAL PROCEDURES

Peptide Preparation. The Zα domain of human ADAR1 protein (*26*) was expressed as a six histidine tag fusion peptide cloned into a pET28a plasmid containing a thrombin cleavage site. Novablue DE3 cells were induced at 0.8−1.0 OD (after 5−6 h) with 1 mM IPTG. Cells were harvested after 3 h and centrifuged at 6000 rpm for 15 min in a Sorvall GS-3 rotor. Pellets were resuspended in 20 mL of ice cold binding buffer from the His−bind buffer kit (Novagen Inc., WI). Cells were lysed with a French press at 1000 psi over ice and centrifuged for 1 h at 17 000 rpm at 4 °C. The supernatant was loaded on a 10 mL column containing a 2 mL volume of Zn-charged His−bind resin (Novagen Inc., WI), except in the case of the protein used in Figure 3C, where Ni-NTA (Qiagen, CA) was used. Purification followed according to the protocol supplied by Novagen except that an additional wash step (wash buffer from His−bind buffer kit containing 1.5 M NaCl) was added to remove bound nucleic acids (monitored by UV absorbance at 260 nm). Eluted peptide was collected in 500 μL aliquots in Eppendorf tubes containing 2 μL of 0.5 M EDTA. The N-terminal histidine tag was removed by incubation with 10 units thrombin/mg of peptide at room temperature. Cleavage was terminated after 4 h with 5−10 μL of PMSF (250 mM in EtOH stock). The peptide solution was then dialyzed into 50 mM Hepes buffer (pH 7.4) containing 25 mM NaCl, 1 mM EDTA, and 1 mM DTT. Any precipitate formed was removed by centrifugation at 17 000 rpm. The supernatant was filtered and purified using a MonoS column. The peptide eluted consistently in a series of three major peaks at 150 mM (pool A), 175 mM (pool B), and 275 mM NaCl (pool C) with pools A and C containing a single peptide (Zα) and pool B containing a contaminant with lower molecular weight in addition to Zα peptide. After several days, peptide sample from pool A eluted as a mixture of pool A and pool C when rechromatographed over the MonoS column, suggesting a possible equilibrium of Zα conformers. Peptide collected as pool C, on the other hand, rechromatographed consistently at 275 mM NaCl. The experiments in this study were all carried out with Zα peptide eluting as pool C. For the individual experiments, Zα was dialyzed extensively into the appropriate buffers (see below). The concentration of pure Zα was monitored by UV absorption at 280 nm assuming an extinction coefficient of $A_{280} = 8250$ M^{-1} cm^{-1} (*29*). In addition to the 76 amino acid domain from hADAR1 (*26*), recombinant Zα peptide contained 5 vector-encoded residues at the N-terminus and 11 vector encoded amino acid residues at the C-terminus resulting in a molecular weight of 10.3 kD. The amino acid sequence of recombinant Zα peptide used throughout this study is as follows:

GSHMGGVDCL SSHFQELSIY QDQEQRILKF LEELGEGKAT TAHDLSGKLG

TPKKEINRVL YSLAKKGKLQ KEAGTPPLWK IEFPGRLERL TD

Circular Dichroism (CD) Spectroscopy. The CD spectra were recorded at 20 °C, unless otherwise indicated using an AVIV 62DS spectropolarimeter (Lakewood, NJ) equipped with a thermoelectric temperature controller and interfaced to a personal computer. Stock solutions of peptide samples (4 mg/mL) were prepared in a 20 mM sodium phosphate buffer, pH 7.0, containing 20 mM NaF and 0.5 mM DTT (buffer A). DNA was purchased from Pharmacia, NJ (polymers) or DNAgency, PA (oligonucleotides). Prior to mixing with Zα peptide, DNA stocks were dialyzed extensively against buffer A and adjusted to a concentration of 40 μM/basepair. Zα peptide was then added in aliquots not exceeding 1% of the total sample volume in the cuvette. Peptide concentrations of 10 μM or less were used to obtain the spectra. The CD spectra of Zα peptide and peptide−DNA complexes were measured with a step size of 0.5 nm with 5 s averaging time/point in a 0.2 cm cuvette. Spectra were signal averaged by adding three scans, baseline corrected, and smoothed using the software provided by AVIV. Experiments were reproduced at least three times. On the basis of the CD spectra, the percent helicity of Zα peptide was estimated from the equation:

$$\% \ \alpha\text{-helix} = [([\Theta]_{222} + 3000)/(36\,000 + 3000)]100 \quad (1)$$

where $[\Theta]_{222}$ is the mean residue ellipticity at 222 nm. In addition, the α-helical content was approximated by the algorithm k2d (*30, 31*).

For thermal denaturation, scans were performed at $[\Theta]_{222}$ from 283 to 363 K at $\Delta T = 1$ K/min. Spectral data were averaged for 25 s every 30 s and stored along with temperature data. The reversibility of the thermal denaturation was assessed by measuring the signal at native conditions after refolding with the percent renaturation at 293 K (20 °C) calculated as follows:

$$\% \ \text{renaturation} = \frac{\Theta_d - \Theta_r}{\Theta_d - \Theta_i}(100) \quad (2)$$

with Θ_d being the signal of the denatured peptide extrapolated to 293 K, Θ_i the initial signal at 293 K, and Θ_r the signal of the renatured peptide at 293 K.

Gel-Retardation Assay. Oligonucleotides were radioactively end labeled using [32P]γ-ATP and T4 polynucleotide

kinase. Following labeling, samples were heated to 90 °C and allowed to slowly cool overnight. Labeled oligonucleotides were purified by electrophoresis through a 20% nondenaturing polyacrylamide gel. For gel-retardation assays, 500 ng of Zα peptide was preincubated in incubation buffer (45 mM Tris base, 45 mM boric acid (pH 8.3), 5 mM $MgCl_2$, and 0.05 mg/mL γ-globulin) with 0.5 μg of λ-DNA (Life Technologies, NY) as nonspecific competitor, representing a 2000-fold excess on a mass basis. After 15 min, between 10 000 and 20 000 cpm of labeled oligonucleotide was added and incubation continued for 1 h at room temperature. Samples were then loaded on a 5% nondenaturing polyacrylamide gel (200 V). Gels were dried, and the radioactivity was measured using a phosphorimager (Molecular Dynamics, CA).

Raman Spectroscopy. Raman spectra of poly(dGdC) in 20 mM sodium phosphate buffered solution (pH 7) were obtained at low-salt (20 mM NaCl) and high-salt (4 M NaCl) conditions. To measure the Raman spectra of the peptide alone, 25 μL of a solution containing approximately 10 mg/mL Zα peptide (1 mM) was prepared using Centricons (Amicon) to concentrate complexes of Zα and poly(dGdC). Peptide concentration was verified by A_{280} absorption and Bradford assay (Bio-RAD Laboratories, CA). The Zα peptide−poly(dGdC) complex was prepared at a peptide concentration of approximately 1 mM and a basepair-to-peptide ratio of 4:1.

Samples (25 μL) of poly(dGdC) and the peptide−poly(dGdC) complex contained in a capillary were excited with 407 nm light (prefiltered with a Photophysics Laser Filter monochromator) from a Krypton laser (model Innova 90, coherent). Raman signals were collected in a 180° backscattering geometry, Rayleigh light rejected using a holographic notch filter (Kaiser Optical Systems Inc., MI), dispersed in a 0.6 m spectrograph equipped with a 600 lines/mm grating (model 1877 without filter stages, SPEX Industries, NJ), and detected by a liquid nitrogen cooled CCD detector (model LN-CCD-512TK, Princeton Instruments Inc., NJ). Spectra were obtained in 50 s accumulation time using a laser power of 80 mW, with a spectral resolution of 4 cm^{-1}. Spectra of buffer and Zα peptide in buffer (without DNA polymer) were obtained under identical conditions and used as references. The reference spectra were subtracted from the solution spectra to obtain the signal contributed by poly(dGdC) at low salt, at high salt, and in the stoichiometric mixture with Zα peptide, respectively. Raman spectra were corrected for the spectral response of the system (CCD, spectrograph, and notch filter) using a NIST traceable tungsten light source and wavenumber calibrated using indene Raman lines as the Raman frequency standard.

Fluorescence Spectroscopy. The fluorescence experiments were performed on a Perkin-Elmer spectrofluorimeter. To monitor essentially pure tryptophan fluorescence, the excitation wavelength was set to 293 nm. The concentration of peptide in the experiments was 1 μM. DNA was added from concentrated stock solutions in aliquots not to exceed altogether 1% of the sample volume. Fluorescence experiments were carried out in 20 mM sodium phosphate buffer, pH 7.0, containing 20 mM NaCl and 0.5 mM DTT. The UV absorbance of the samples at any time was checked to be below 0.05 OD to prevent inner filter effects.

RESULTS

Zα Peptide and Poly(dGdC). The B−Z transition of alternating purine-pyrimidine deoxynucleic acids can be monitored conveniently by CD spectroscopy. The conformational change of poly(dGdC) from dilute to concentrated salt solutions is characterized by inversion of the CD signal between 240 and 310 nm (*28*). The spectra of poly(dGdC) determined at low salt (20 mM NaCl, dashed line) and high salt (4 M, solid line) are shown in Figure 2A to demonstrate the features characteristic of B- and Z-DNA. Superimposed is the spectrum obtained by adding Zα to a 40 μM (per basepair) solution of poly(dGdC) at low salt (20 mM NaCl) until the positive peak at 273 nm reached its maximum (Figure 2B). No baseline correction was applied because Zα alone does not show any significant CD signal above 250 nm (Figure 2D). Although below 250 nm, the signal from the peptide dominates and the spectrum is negative, the spectrum, between 250 and 305 nm, of poly(dGdC) mixed with Zα is similar to that of poly(dGdC) in high salt (Figure 2B). There are, however, several significant differences in the long wavelength region. The maximum is shifted from 258 nm (high-salt form) to 273 nm in the mixture. The negative peak at 285 nm (high-salt form) is less pronounced and shifted to 295 nm for the mixture of Zα and the polymer. Similar observations were made with the ($dGdm^5C$) polymer containing deoxycytidine methylated at the C5 position (*26*), which has been shown previously to form Z-DNA readily under physiological conditions upon the addition of metal ions, synthetic peptides, and polyamines (*27*). The alteration of the CD signal of poly(dGdC) upon addition of the peptide can be interpreted to be indicative of the formation of Z-DNA, stabilized by Zα. The differences in the shape of the curves for the high-salt form of the polymer and in the mixture with Zα could indicate a slightly different conformation of the left-handed polymer in the complex with the peptide, as reasoned before for the ($dGdm^5C$) polymer (*26*). The different isodichroic points for NaCl-induced and Zα-induced changes are consistent with this view. Alternatively, residual B (or any other non-Z) conformation in the mixture of the (dGdC) polymer and the peptide may contribute to the CD spectrum observed at our conditions.

The conformation of Zα, both in the absence and in the presence of DNA, was also studied by CD to determine whether changes occurred in peptide structure following interaction with DNA. The CD spectrum of free Zα peptide is shown in Figure 2C. It is indicative of a significant amount of α-helical content which was determined to be 35% based on formula (*1*) and 32% based on the algorithm k2d. This value rises to more than 50% upon addition of TFE (2,2,2-Trifluoro-ethanol), a chemical that has been shown to stabilize helical structures in shorter peptides (*32*−*34*) and proteins (*35*). It is assumed that TFE acts by disrupting hydrogen bonds between amino acids and solvent, thereby promoting intramolecular hydrogen bonding and helix formation (*32*, *36*). In the case of Zα, addition of TFE causes the spectrum to become more negative, indicating that the molecule is more structured. The minimum at 208 nm as well as the ratio between the signal amplitudes at 208 and 222 nm remains unchanged (Figure 2C). This result suggests that adding TFE stabilizes the native conformation of Zα

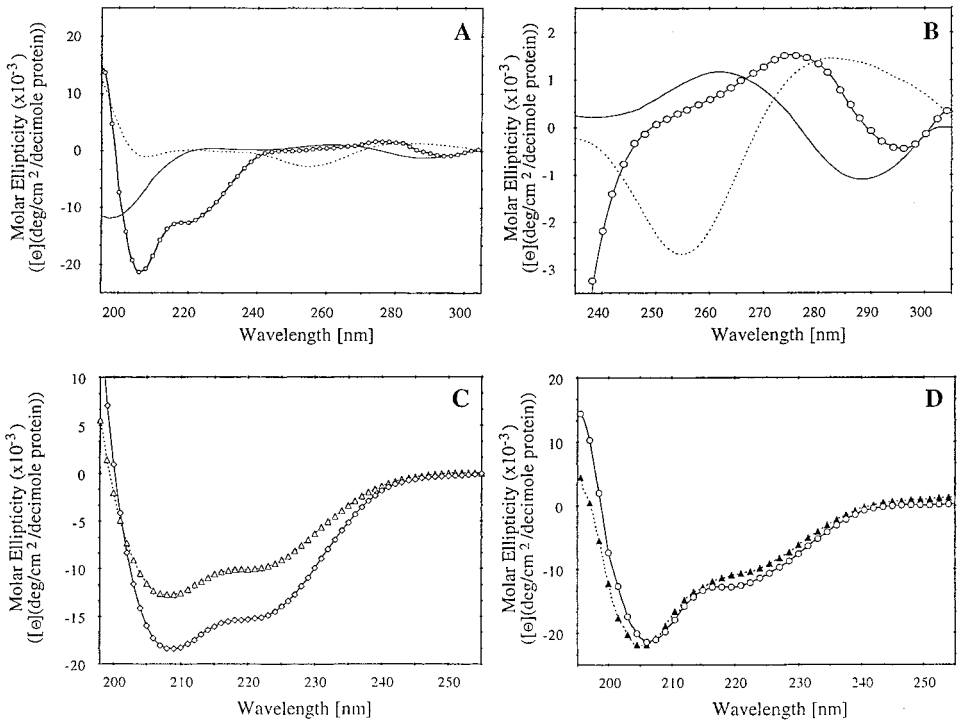

FIGURE 2: Circular dichroism of poly(dGdC) and Zα peptide. (A) CD spectra of poly(dGdC) in low salt (20 mM NaCl, - - -), in high salt (4 M NaCl, —), and at low salt in the presence of Zα peptide at a basepair-to-peptide ratio of 4:1 (-○-). The inversion of the CD signal of the polymer with high salt (Z-form) is also seen in the presence of the peptide. The spectrum of the Zα peptide−DNA mixture (-○-) below 250 nm is dominated by the peptide leading to a large negative peak (see text). Above 250 nm, the spectrum of Zα alone is baseline; no correction has been applied to the spectrum of the mixture. (B) Enlarged view of the CD spectra of poly(dGdC) in the region between 250 and 300 nm. It can be seen that the peptide−DNA spectrum (-○-) differs from the spectrum of poly(dGdC) at 4 M NaCl (Z-form, —). The positive peak at 260 nm (high-salt form) is shifted to 273 nm for the mixture. Correspondingly, the negative peak at 285 nm (high-salt form) is shifted to 295 nm and is less pronounced for the Zα peptide−poly(dGdC) mixture. (C) CD spectra of free Zα peptide in the absence (-△-) and presence (-□-) of 50% TFE. The spectra indicate a large α-helical content of the free peptide under the conditions examined. Addition of TFE causes an increase in signal amplitude; however the relative ratio between the signals at 208 and 222 nm is maintained, indicating that a significant amount of peptide folds into the native conformation under these conditions. (D) Superimposed spectra of the Zα peptide−DNA mixture (-○-) and a calculated sum spectrum of poly(dGdC) at high salt (Z-form) and free peptide (-▲-). The spectra are in reasonable agreement in the region between 200 and 250 nm, indicating that any changes in the conformation of the peptide upon binding to DNA are not detectable within the resolution of CD spectroscopy.

and promotes proper folding of the peptide. The effect of DNA on the Zα conformation was also tested. Figure 2D shows the spectrum between 190 and 255 nm of a mixture of Zα peptide and (dGdC) polymer. A calculated sum spectrum obtained by adding the CD signatures of free Zα peptide and the high-salt form of poly(dGdC) is also shown. Both of these spectra are very similar, suggesting that the conformation of Zα does not change upon binding to DNA. This finding is further supported by our observation that the molar ellipticity at 222 nm of a solution containing Zα peptide remains constant upon addition of stoichiometric amounts of (dGdC) polymer (data not shown). Therefore, poorly folded regions of Zα affected by TFE are unlikely to be involved in the recognition of DNA.

Additional experiments were performed to further examine the interaction between poly(dGdC) and Zα. Figure 3A shows the result of a titration performed at low salt to determine the amount of Zα peptide needed to obtain complete inversion of the CD spectrum of poly(dGdC). The results indicate that the transition occurs in a single step, with an isodichroic point at 276 nm. The maximal amplitude of the CD signal at 273 nm is reached at a ratio of 4 base pairs/Zα molecule, which is in good agreement with what was observed for the methylated (dGdm^{5}C) polymer (*26*). Next, we investigated the effect of increasing ionic strength on the binding of Zα to poly(dGdC). The result is shown in Figure 3B. Increasing ionic strength causes Zα to release the bound polymer gradually, and at 750 mM NaCl the spectrum in the region between 240 and 305 nm largely converts to that of poly(dGdC) in the regular B-form. The isodichroic point at 276 nm is unchanged, indicating that this process occurs without hysteresis, as is characteristic of the B−Z transition (*27*).

Zα Peptide and Oligonucleotides. We sought to identify alternating deoxycytidine−deoxyguanosine nucleic acid fragments that are shorter in length than the polymers (average length ~100 bp) and hemi-brominated probes (>40 bp) investigated so far (*18*, *26*). We monitored the Zα-induced change in the CD signal of deoxycytidine−deoxyguanosine oligomer duplexes 18 nucleotides and less in length. The experiments were performed in a fashion similar to that described before for the (dGdC) polymer. Results are shown for a hexamer hairpin with the sequence d(CG)$_3$T$_4$(CG)$_3$ (Figure 3C). The CD signal of this hairpin changes detectably upon addition of Zα peptide. The isodichroic point is around 285 nm in the case of the oligonucleotide (Figure 3C) as compared to 276 nm in the case of poly(dGdC). The negative peak between 290 and 300 nm, which is clearly observed in the case of the (dGdC) polymer, is significantly less pronounced in the spectrum of the hairpin. The maximum alteration of the curves is observed at a basepair-to-peptide ratio of approximately 2:1 for the

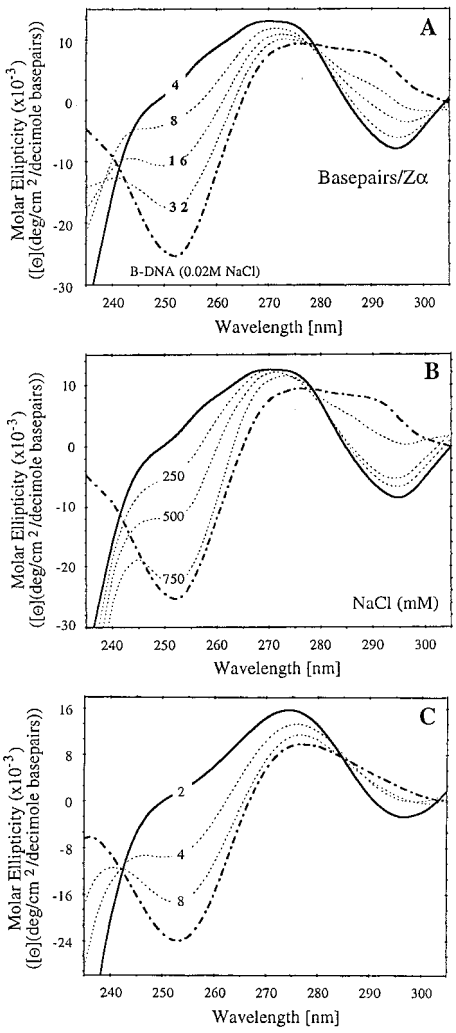

FIGURE 3: Aspects of Zα peptide−DNA interaction. (A) Titration of poly(dGdC) with Zα peptide in 20 mM NaCl, 20 mM Na phosphate, pH 7.0. The positive peak at 273 nm reaches its maximum at a basepair-to-peptide ratio of approximately 4:1 (—), and reversal of the spectrum is observed relative to the B-form (-•-). An isodichroic point for the transition is observed at 276 nm. Spectra at intermediate basepair-to-peptide ratios are drawn with dotted lines. A large negative peak below 250 nm is contributed by the peptide. The CD spectrum of free peptide (Figure 2D) did not show a significant signal above 250 nm, and thus, a corresponding baseline correction was not applied. (B) Influence of ionic strength on DNA binding. Upon addition of increasing amounts of NaCl (mM are indicated), the CD spectrum of a Zα peptide−poly(dGdC) mixture (basepair-to-peptide ratio 4:1) gradually reverts to that of the low-salt conformer and at 750 mM salt concentration is very similar to the spectrum of poly(dGdC) in the B-form (-•-). (C) Titration of a hairpin with the sequence $d(CG)_3T_4(CG)_3$ shows the effect of Zα peptide on these oligonucleotides. The reversal of the spectrum from the B-form (-•-) to Z (—) is clearly observable, albeit less pronounced than in the case of the polymer. The isodichroic point is shifted to 285 nm.

hairpin, whereas a ratio of 4:1 is sufficient for the polymer. Addition of more peptide to the sample does not change either spectrum (data not shown). The higher amount of peptide needed to invert the hairpin spectrum may be due to destabilizing effects stemming from the T_4 turn, and to the lack of cooperativity in the B−Z transition for a short $d(CG)_3$ hexamer stem. With longer oligonucleotides such as $d(m^5CG)_6$, the basepair-to-peptide ratio leading to maximum reversal of the spectrum was also close to 4:1 (data not shown).

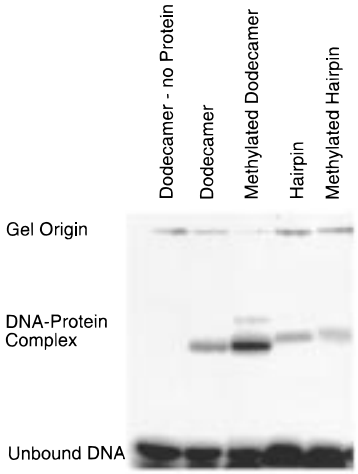

FIGURE 4: Gel-retardation assay of Zα peptide and oligonucleotides. [32P]γ-ATP-labeled oligonucleotides [$d(CG)_6$, $d(CG)_3T_4(CG)_3$ and the m^5C derivatives] in a band shift assay. Both modified and unmodified oligonucleotides show retardation in the presence of Zα. In the case of the methylated $d(m^5CG)_6$ dodecamer, additional bands are observed above the dominant signal, indicating that more than one Zα peptide can bind to an oligonucleotide with this sequence and length.

In parallel to the CD studies, we performed gel-retardation assays with Zα peptide and radiolabeled DNA oligonucleotides, some of which were methylated at the C5 position of deoxycytidine (Figure 4). Both the methylated and the unmethylated dodecamer and hairpin oligonucleotides gave rise to a gel mobility shift in the presence of Zα. The signals obtained with the dodecamer oligonucleotides (both methylated and unmethylated) are stronger than those of the respective hairpins. This is consistent with the higher propensity of the dodecamer oligonucleotide to adopt the Z-DNA conformation, as a result of the cooperativity of the B−Z transition. Methylation led to a stronger band shift signal in the case of the dodecamer. Again, this is consistent with the stabilization of the Z-DNA conformation by methylation. When using the methylated dodecamer, an additional band was observed in the retardation assay, indicating that more than one Zα peptide can bind to the DNA oligomer. Only a single band was observed for the shorter hairpin oligonucleotide, indicating that Zα has only a single DNA binding site.

Melting Studies. Next, we investigated the influence of temperature on both the folding of the Zα peptide as well as the binding of Zα to poly(dGdC). The results are shown in Figure 5. Upon increasing the temperature from 15 to 80 °C, Zα loses its secondary structure and is essentially a random coil at 80 °C (Figure 5A). The thermal denaturation of the peptide is largely reversible (Figure 5B), with a degree of renaturation of more than 90% at 20 °C based on eq *2*. This extreme thermal stability was previously noted for Zα prepared from chicken blood nuclei (*21*). The midpoint of melting occurs at 52 °C, and the existence of an isodichroic point at 203 nm (Figure 5A) is indicative of a two-state transition. The effect of peptide melting upon DNA binding is demonstrated in Figure 5C. A DNA solution titrated to a maximum amplitude of the CD signal at 273 nm by adding aliquots of Zα was heated from 15 to 80 °C. With increasing temperature, the CD spectrum of the DNA in the mixture of Zα peptide and poly(dGdC) gradually reverted to essentially

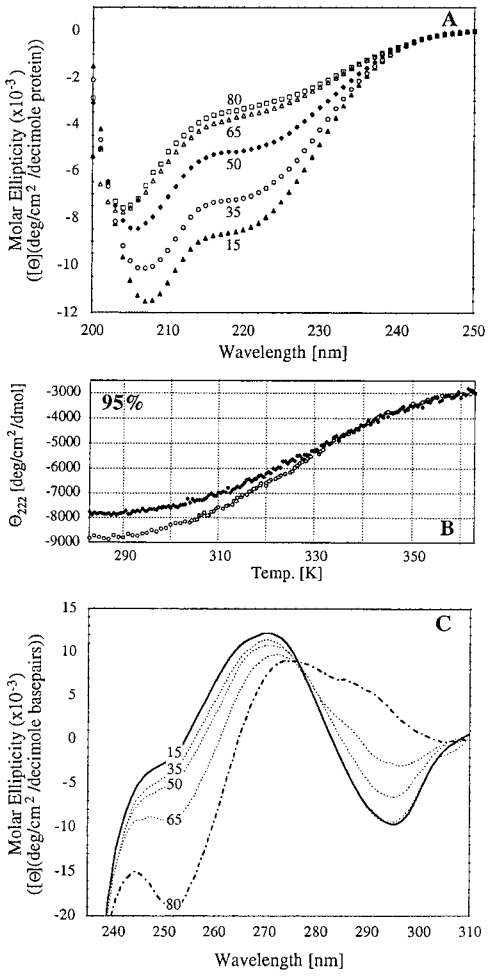

FIGURE 5: Thermal denaturation studies. (A). CD spectra of free Zα peptide recorded at increasing temperatures from 15 ° C (▲) to 80 °C (□). Zα gradually loses its secondary structure components and has the spectrum of a random coil at 80 °C. A two state is suggested by an isodichroic point near 203 nm. (B) Zα peptide melts (○) with a thermal transition midpoint at 52 °C, and thermal denaturation is largely reversible with renaturation (●) leading to >90% signal recovery. Thermal scans were performed monitoring the molar ellipticity at 222 nm. (C) Thermal denaturation of Zα peptide in a mixture with poly(dGdC) at an approximate basepair-to-peptide ratio of 4:1. Upon melting of the peptide, the CD signal of the polymer between 240 and 310 nm gradually reverts to B-type at 80 °C. Numbers denote the temperatures at which the spectra were recorded.

B-form at 80 °C, indicating that correct folding of Zα is necessary for stabilization of Z-DNA.

Laser-Raman Experiments. To gather additional data about the conformation of the (dGdC) polymer in the peptide−DNA complex, we decided to collect and analyze the laser-Raman spectra of poly(dGdC) at low salt, at high salt, and in a complex with Zα. The results of this study are presented in Figure 6.

Figure 6 shows the unmodified spectrum I of poly(dGdC), measured at pH 7 and low salt (20 mM NaCl). The Raman frequencies are characteristic of the B-conformer (*37*). Spectrum III in Figure 6 shows the poly(dGdC) signals measured at high salt (4 M NaCl). Alterations associated with the conformational transition of the polymer from the right-handed B-form at low-salt to the left-handed Z-form at high-salt conditions are evident (*37*). The normal guanine mode at 684 cm^{-1} in the low-salt form is shifted down to

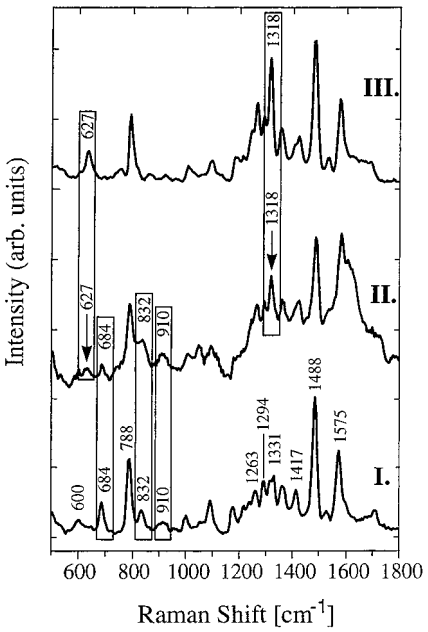

FIGURE 6: Laser-Raman spectra of poly(dGdC). Spectra of poly(dGdC) at low salt (Spectrum I, B-form), at high salt (Spectrum III, Z-form), and in a mixture of poly(dGdC) with Zα peptide at an approximate basepair-to-peptide ratio of 4:1 (Spectrum II). Raman resonance frequencies at 1318 and 627 cm^{-1} (boxed and arrows) indicate the presence of Z-DNA in the mixture. Residual peaks characteristic of the B-DNA conformation (boxed, no arrows) indicate that a portion of the DNA molecules retain the right-handed B-DNA conformation under the conditions studied.

627 cm^{-1} at high salt. This change is due to the different chemical environment of the guanine base in Z-DNA which is in the syn conformation, whereas all glycosidic bond angles in B-DNA are in the anti conformation. Major differences in the phosphodiester vibrational modes (780−1100 cm^{-1}) are apparent, associated with loss of B-DNA specific bands at 832 and 910 cm^{-1}. Differences in the guanine modes between 1300 and 1600 cm^{-1} also exist. The guanine doublets at 1315 cm^{-1} (not labeled) and 1331 cm^{-1} (labeled) in the low-salt form (B-DNA), for example, become a strong single band at 1318 cm^{-1} in the high-salt Z-form. These spectral features, particularly at 627 and 1318 cm^{-1} in the high-salt form (Figure 6, Spectrum III), are also found in crystals of Z-DNA and can be used as fingerprints for the left-handed Z conformation (*37*). Spectrum II was collected using poly(dGdC) complexed to Zα at a stoichiometric ratio of 4 basepairs/peptide. Data were baseline corrected by subtracting the spectrum of a solution containing Zα peptide at the same concentration (approximately 1 mM). Spectrum II exhibits striking similarities to that observed for poly(dGdC) at high salt. The Z-conformer specific modes at 627 and 1318 cm^{-1} are clearly observed, indicating the presence of Z-DNA in the peptide−DNA mixture. Spectrum II also shows several bands at 684, 832, and 910 cm^{-1} (boxed in Figure 6) which are characteristic for the right-handed B conformation, indicating that some residues remain as B-DNA under the conditions studied. In the spectrum of the Zα−poly(dGdC) mixture, additional bands are present at 1060 cm^{-1} and between 1575 and 1650 cm^{-1} which are not observed in the spectra of either the B-form or the Z-form, and these currently cannot be assigned. The guanosine peak at 1488 cm^{-1} appears to be reduced in magnitude relative to that at 1595 cm^{-1} in Spectrum II. The

significance of this is currently unclear, although it may suggest an interaction between the peptide and some guanosine residues leading to changes in these particular vibrational modes.

Fluorescence Experiments. The amino acid sequence of Zα contains a single tryptophan residue in the C-terminal region that is part of a conserved motif in ADAR1 enzymes from various species (*26*). Tryptophan fluorescence is a useful spectroscopic marker for major state transitions in proteins in many different systems (*38*). With this in mind, we performed steady-state experiments to examine the interaction of Zα peptide with DNA. The fluorescence emission maxima and quantum yields of tryptophan are very sensitive to the microenvironment of this amino acid residue. We sought to establish whether we could probe the microenvironment of this tryptophan for changes in the fluorescence emission upon binding to different DNA sequences. The changes in the steady-state emission spectrum of tryptophan as a function of DNA concentration are shown in Figure 7. Zα peptide showed an intrinsic fluorescence spectrum with a maximum emission wavelength centered around 329 nm when excited at 293 nm (Figure 7A), which is significantly blue shifted as compared to the maximum emission wavelength of L-tryptophan in solution (around 355 nm). This indicates that, on average, the tryptophan residue in unbound Zα is in a nonpolar (hydrophobic) environment. Upon addition of aliquots of poly(dGdC), the fluorescence signal was quenched considerably (Figure 7A) reaching a plateau at a basepair-to-peptide ratio of 5−6. An essentially identical behavior was observed for the methylated (dGdm^{5}C) polymer (Figure 7B). To verify that signal quenching was actually due to specific binding of Zα to DNA, calf thymus DNA was used as a negative control. No quenching was found showing that this effect is specific for poly(dGdC) polymers. Interestingly, the alternating polymer (dCdA)−(dTdG) also caused a significant, if less pronounced, change of the fluorescence signal (Figure 7B). This asymmetric polymer can also adopt a left-handed conformation; however it requires more stringent conditions than required for alternating (dGdC) sequences (*39*−*42*). This property may account for the reduced ability of this polymer to quench Zα fluorescence. No significant shift of the maximal wavelength of the fluorescence spectrum was observed in any of these experiments, indicating that the microenvironment of the tryptophan chromophore did not change dramatically upon DNA binding.

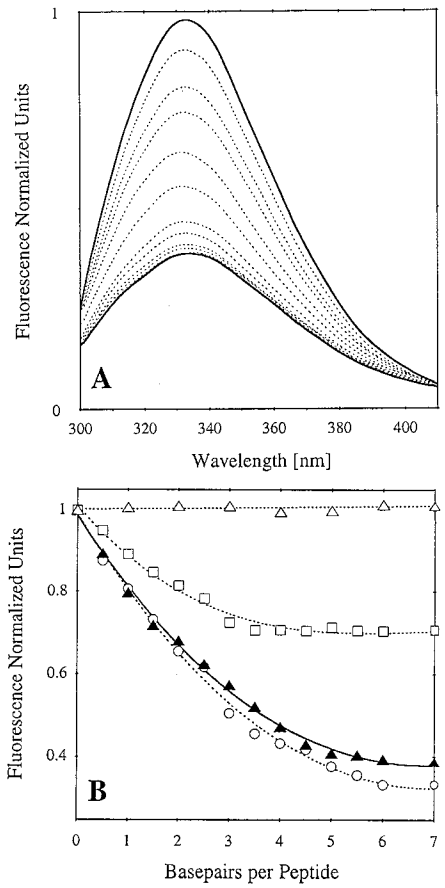

FIGURE 7: Steady-state fluorescence of Zα peptide. (A) Fluorescence emission signal of the single tryptophan residue in the Zα peptide in the region from 300 to 400 nm. The fluorescence signal of the tryptophan of unbound Zα peptide is centered around a maximum at 329 nm. Upon adding aliquots of (dGdC) polymer (dotted lines) to a solution containing 1 μM Zα peptide, the fluorescence intensity is significantly quenched. No blue or red shifting of the fluorescence signal is observed. (B) Fluorescence signal at 330 nm plotted against the relative basepair-to-peptide ratio upon addition of aliquots of different DNA sequences to a solution containing 1 μM Zα peptide. The (dGdC) polymer (▲) and methylated poly(dGdm^{5}C) (○) give rise to a comparable reduction of the fluorescence intensity and reach a plateau at a ratio of between 5 and 7 basepairs/peptide. The asymmetric polymer, (dCdA)−(dTdG), also quenches the fluorescence emission signal of Zα peptide (□), but not to the extent of poly (dGdC). Signal quenching was not observed when calf thymus DNA was added (△).

DISCUSSION

The RNA editing enzyme ADAR1 has been identified as the first naturally occurring protein with apparent in vitro specificity for hemi-brominated and methylated DNA probes with alternating deoxyguanosine−deoxycytidine sequences (*19*, *26*). Further, it has been demonstrated that supercoiled plasmids containing alternating deoxyguanosine−deoxycytidine inserts competed with the hemi-brominated probe (*20*). The same plasmids failed to compete when relaxed with topoisomerase I. An extensive panel of other nucleic acids, including r(GC) polymer, also failed to compete. An N-terminal 76 amino acid DNA binding domain of ADAR1, Zα, was demonstrated to bind to chemically brominated (dGdC) polymer with a K_D of 4 nM (*26*). Studies carried out with an artificial restriction endonuclease, made by fusing Zα-peptide and the catalytic domain of *Fok*I restriction enzyme, show that this chimera can selectively cleave supercoiled plasmids that contain Z-forming inserts, such as $(dGdC)_{13}$ (*43*). These data lead to the interpretation that Zα recognizes and binds to the left-handed Z-DNA conformation. In particular the observation that Zα peptide causes a reversal of the circular dichroism spectrum between 250 and 310 nm of methylated (dGdm^{5}C) polymer (*26*), as well as unmodified poly(dGdC) and (dGdm^{5}C) oligonucleotides (this study) is in reasonable agreement with original studies of the B to Z transition (*28*). Although no biological function for ADAR1 binding to Z-DNA has been established, these results provide strong circumstantial evidence that Zα peptide recognizes and binds to left-handed Z-DNA.

Circular dichroism spectroscopy is a powerful tool to monitor conformational changes in biological macromolecules and is particularly well suited to examine the B−Z

transition of DNA polymers. However, the underlying quantitative biophysical principles of this method are not known in great detail. Thus, changes in the CD spectrum must be interpreted in the context of other evidence. For instance, titration of a solution of poly(dGdC) with Mitomycin C, an antibiotic which covalently binds to and cross-links DNA at d(GC) dinucleotide steps, leads to a reversal of the CD spectrum with increasing drug concentrations, which is strikingly similar to that observed for the transition of poly(dGdC) from the right-handed to the left-handed conformation (*44*). The CD spectrum of a Mitomycin C−poly(dGdC) complex exhibits a positive peak around 260 nm and a negative peak around 295 nm (*44*), in excellent agreement with the spectrum reported for the high-salt form of the polymer (*28*). Yet, as high-resolution NMR solution studies revealed recently, the complex of Mitomycin C and DNA contains no left-handed Z-DNA (*45*).

We therefore recognized the need to augment the existing body of evidence for the existence of left-handed Z-DNA in the complex between Zα and poly(dGdC), using a different approach. The question of whether the conformation of poly(dGdC) observed at high salt (*28*) was identical with the left-handed Z-DNA conformation observed in the crystal of the $d(CG)_3$ hexamer was answered unambiguously by a Raman spectroscopic investigation, which compared these two samples (*37*). Distinct bands in the Raman spectra of crystalline $d(CG)_3$ and poly(dGdC) at high salt were identified as characteristic for the left-handed Z conformer. Those at 1318 and 627 cm^{-1} are present only in Z-DNA.

In this study, we provide evidence for the existence of left-handed Z-DNA in the complex formed between Zα and unmodified poly(dGdC). The laser-Raman spectrum of a mixture of poly(dGdC) and Zα at a basepair-to-peptide ratio of 4:1 at low salt allows us to clearly identify characteristic signals at 1318 and 627 cm^{-1}. The conversion to Z-DNA is not complete, as demonstrated by the presence of several bands which are diagnostic of residual right-handed B-DNA. At the same basepair−peptide ratio, the CD spectrum shows maximal inversion between 240 and 305 nm.

The peptide used here is not able to induce and stabilize Z-DNA formation in poly(dGdC) as efficiently as mono- or divalent metal ions, short peptides, polyamines, or alcohols (*27*). This may be explained by the fact that the smaller molecules, and, in particular individual metal ions, can contact the DNA polymer at any position. Interaction involving the Zα peptide may be limited by its significantly larger size. This interpretation is further supported by the CD of short oligonucleotides in the presence of Zα. Unmodified hairpins with a stem built by merely three d(CG) dinucleotide steps can be completely flipped from B to Z at room temperature in 4 M $NaClO_4$ (*46*). In contrast, the Zα peptide used in these studies shows a less pronounced effect. Other constructs may be more effective in altering the B−Z equilibrium of shorter oligonucleotides (Schwartz et al., manuscript in preparation). In addition, the data presented here cannot rule out the possibility that Zα binds to B−Z junctions (*47, 48*).

These results suggest that recombinant Zα peptide adopts a structure with a large α-helical content. CD spectra indicate that about 30% of the residues are in α-helices, and this number increases to 50% on addition of TFE. Predictions based on sequence analysis of Zα family members (*26*) predict a helix-turn-helix motif. Molecular modeling studies consistently indicate a helix-loop-helix or helix-turn-helix tertiary structure for a core segment of the Zα peptide encompassing approximately 50 amino acid residues (Ortiz & Ribas de Pouplana, personal communication to I.B.). The peptide does not alter its conformation detectably over a broad concentration range as indicated by the molar ellipticity of the peptide remaining constant at concentrations from 2 μM to 0.5 mM (data not shown). Proper folding of Zα is required for the transition of poly(dGdC) from the B-conformation to left-handed Z-DNA. Unfolding of the peptide occurs with a midpoint of melting at 52 °C and leads to a gradual release of bound DNA such that there is no significant amount of Z-DNA detectable by CD at 80 °C. Zα does not become further structured upon binding to DNA, as suggested by the constant molar ellipticity at 222 nm observed when adding (dGdC) polymer to a peptide solution.

CD experiments involving both poly(dGdC) and short oligonucleotide fragments show a pronounced effect at a basepair-to-peptide molar ratio of approximately 4:1. In addition, the fluorescence intensity of the single tryptophan residue at 330 nm is fully quenched at a basepair-to-peptide ratio of between 5:1 and 6:1. This result suggests that a molecule of Zα may interact with a stretch of 4−6 basepairs of alternating deoxyguanosine−deoxycytidine under the conditions studied. This interpretation is further supported by the occurrence of higher bands in the gel shift assay when a methylated $d(m^5CG)_6$ dodecamer is used as a probe and by our finding that a hairpin containing only a short $d(CG)_3$ hexamer stem can be shifted by Zα peptide in a gel-retardation assay. Short repeats of alternating deoxyguanosine and deoxycytidine appear frequently in the 5′ region of eukaryotic genes (*49*) where they could possibly form Z-DNA stabilized by negative supercoiling generated in the wake of a transcribing RNA polymerase (*50*). These stretches might provide potential binding sites for the Zα domain of ADAR1, if they are present in the left-handed Z conformation (*5*). The fluorescence studies indicate that Zα also binds to poly(dCdA)−poly(dTdG), but not as well as to poly(dGdC). DNA containing (dCdA)−(dTdG) dinucleotide repeats is able to flip into the Z conformation, albeit with significantly reduced propensity than (dGdC) repeats. Zα binding in vivo may also involve more diverse DNA sequences and thus affect a large number of genes in the genome.

CONCLUSION

In the present study, we have provided laser-Raman spectroscopic evidence for the existence of the left-handed Z-DNA conformation in a complex of poly(dGdC) with Zα, a novel DNA binding domain in the N-terminal region of human double-stranded RNA adenosine deaminase (ADAR1) enzyme. These studies represent the first such data on a naturally occurring peptide domain with apparent specificity for left-handed Z-DNA, and are direct evidence to date for the interaction of Zα with the left-handed DNA conformer. Our analyses of the Zα peptide itself and its interaction with DNA constitute a reference point for additional biophysical studies.

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REFERENCES

1. Wang, A. H.-J., Quigley, G. J., Kolpak, F. J., Crawford, J. L., van Boom, J. H., van der Marel, G., and Rich, A. (1979) *Nature 282*, 680−686.
2. Rich, A. (1983) *Cold Spring Harbor Symp. Quant. Biol. 47*, 1−12.
3. Rich, A., Nordheim, A., Wang, A. H.-J. (1984) *Annu. Rev. Biochem. 53*, 791−846.
4. Rich, A. (1993) *Gene 135*, 99−109.
5. Herbert, A. G., and Rich, A. (1996) *J. Biol. Chem. 271*, 11595−11598.
6. Nordheim, A., and Rich, A. (1983) *Nature 303*, 1661−1677.
7. Naylor, L. H., and Clark, E. M. (1990) *Nucleic Acids Res. 18*, 1595−1601.
8. Bullock, P., Miller, J., and Botchan, M. (1986) *Mol. Cell. Biol. 6*, 3948−3953.
9. Treco, D., and Arnheim, N. (1986) *Mol. Cell. Biol. 6*, 3934−3947.
10. Blaho, J. A., and Wells, R. D. (1987) *J. Biol. Chem. 262*, 6082.
11. Wahls, W. P., Wallace, L. J., and Moore, P. D. (1990) *Mol. Cell. Biol. 10*, 785−793.
12. Wittig, B., Dorbic, T., and Rich, A. (1989) *J. Cell Biol. 108*, 755−764.
13. Wittig, B., Dorbic, T., and Rich, A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2259−2263.
14. Wittig, B., Wölfl, S., Dorbic, T., Vahrson, W., and Rich, A. (1992) *EMBO J. 11*, 4653−4663.
15. Wölfl, S., Wittig, B., and Rich, A. (1995) *Biochim. Biophys. Acta 1264*, 294−302.
16. Müller, V., Takeya, M., Brendel, S., Wittig, B., and Rich A. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 780−784.
17. Wölfl, S., Martinez, C., Rich, A., and Majzoub, J. A. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 3664−3668.
18. Herbert, A. G., Lowenhaupt, K., Spitzner, J., and Rich, A. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 7550−7554.
19. Herbert, A. G. (1996) *Trends Genet. 12*, 6−9.
20. Herbert, A. G., Lowenhaupt, K., Spitzner, J., Berger, I., and Rich, A. (1996) in *Biological Structure and Dynamics* (Sarma, R. H., and Sarma, M. H. Eds.) Vol. 2, pp 189−198, Adenine Press, New York.
21. Herbert, A. G., and Rich, A. (1993) *Nucleic Acids Res. 21*, 2669−2672.
22. Melcher, T., Maas, S., Herb, A., Sprengel, R., Seeburg, P. H., and Higuchi, M. (1996) *Nature 379*, 460−464.
23. Bass, B. L. (1993) in *The RNA World* (Getseland R. F., and Atkins, J. F. Eds.) pp 383−418, Cold Spring Harbor Laboratory Press, Plainview, NY.
24. Maas, S., Melcher, T., Herb, A., Seeburg, P. H., Keller, W., Krause, S., Higuchi, M., and O′Connell, M. A. (1996) *J. Biol. Chem. 271*, 12221−1226.
25. Sommer, B., Kohler, M., Sprengel, R., and Seeburg, P. H. (1991) *Cell 67*, 11−19.
26. Herbert, A., Alfken, J., Kim, Y.-G., Mian, I. S., Nishikura, K., and Rich, A. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 8421−8426.
27. Behe, M., and Felsenfeld, G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1619−1623.
28. Pohl, F. M., and Jovin, T. M. (1972) *J. Mol. Biol. 67*, 375−396.
29. Gill, S. C., and von Hippel, P. H. (1989) *Anal. Biochem. 182*, 319−126.
30. Andrade, M. A., Chacón, P., Merelo, J. J., and Morán, F. (1993) *Protein Eng. 6*, 383−390.
31. Merelo, J. J., Andrade, M. A., Prieto, A., and Morán, F. (1994) *Neurocomputing 6*, 443−454.
32. Sönnichsen, F. D., Van Eyk, J. E., Hodges, R. S., and Sykes, B. D. (1992) *Biochemistry 31*, 8790−8798.
33. Jasanoff, A., and Fersht, A. R. (1994) *Biochemistry* 33, 2129−2135.
34. Waterhous, D. V., and Johnson, W. C., Jr. (1994) *Biochemistry 33*, 2121−2128.
35. Shiraki, K., Nishikawa, K., and Goto, Y. (1995) *J. Mol. Biol. 245*, 180−194.
36. Zhou, N. E., Kay, C. M., Sykes, B. D., and Hodges, R. S. (1993) *Biochemistry 32*, 6190−6197.
37. Thamann, T. J., Lord, R. C., Wang, A. H.-J., and Rich, A. (1981) *Nucleic Acids Res. 9*, 5443−5457.
38. Beechem, J., and Brand, L. (1985) *Annu. Rev. Biochem. 54*, 43−71.
39. Vorlickova, M., Kypr, J., Stokrova, S., and Sponar, J. (1982) *Nucleic Acids Res. 10*, 1071−1080.
40. Zimmer, C., Tymen, S., Marck, C., and Guschlbauer, W. (1982) *Nucleic Acids Res. 10*, 1081−1091.
41. Wells, R. D., Miglietta, J. J., Klysik, J., Larson, J. E., Stirdivant, S. M., and Zacharias, W. (1982) *J. Biol. Chem. 257*, 10166−10171.
42. Taboury, J. A., and Taillandier, E. (1985) *Nucleic Acids Res. 13*, 4469−4483.
43. Kim, Y. G., Kim, P. S., Herbert, A., and Rich, A. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 12875−12879.
44. Mercado, C. M., and Tomasz, M. (1977) *Biochemistry 16*, 2040−2046.
45. Sastry, M., Fiala, R., Lipman, R., Tomasz, M., and Patel, D. J. (1995) *J. Mol. Biol. 247*, 338−359.
46. Amaratunga, M., Pancoska, P. O., Paner, T. M., and Benight, A. S. (1990) *Nucleic Acids Res. 18*, 577−587.
47. Peticolas, W. (1995) in *Methods in Enzymology: Biochemical Spectroscopy* (Sauer, K., Abelson, J. N., and Simon, M. I. Eds.) Vol. 246. pp 389−417, Academic Press, New York.
48. Dai, Z. Y., Thomas, G. A., Evertsz, E., and Peticolas, W. L. (1989) *Biochemistry 28*, 6991−6996.
49. Schroth, G. P., Chou, P. J., and Ho, P. S. (1992) *J. Biol. Chem. 267*, 11846−11855.
50. Liu, L. F., and Wang, J. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7024−7027.

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