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Right- and Left-Handed (Z) Helical Conformations of the Hairpin $d(C\text{-}G)_5T_4(C\text{-}G)_5$ Monomer and Dimer†

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ABSTRACT: The partial self-complementary 24-mer oligodeoxynucleotide $d(C\text{-}G)_5T_4(C\text{-}G)_5$ forms a hairpin which can be enzymatically dimerized to a dumbbell structure. The blunt-ended nature of the hairpin is indicated by its ability to inhibit the T_4 DNA ligase catalyzed joining of ϕX174 *Hae*III fragments. The hairpin monomer and dimer (dumbbell) undergo a reversible B to Z transition as shown by ultraviolet, circular dichroism, and ^{31}P NMR spectroscopy. The Z form of the hairpin monomer and dimer is supported by monovalent ions (Na^+), divalent ions (Mg^{2+} but *not* Mn^{2+}), and dehydrating (ethanol) conditions. The conformational transition of $d(C\text{-}G)_5T_4(C\text{-}G)_5$ monomer requires higher ionic or dehydrating conditions than those necessary for the corresponding linear oligomer $d(C\text{-}G)_5$. The contribution of the loop ($-T_4-$) of the hairpin to the apparent free energy change for the B to Z conformational transition at the midpoint was calculated to be 3.8 kJ mol^{-1}.

Alternating purine/pyrimidine sequences in linear DNA (Pohl & Jovin, 1972; Wang et al., 1979; Jovin et al., 1983) and RNA (Hall et al., 1984) or plasmids (Singleton et al., 1982; Nordheim et al., 1982; Haniford & Pulleyblank, 1983) can, under appropriate conditions, adopt a left-handed conformation. Supercoiled plasmids containing inverted repeats can also extrude into a cruciform structure (Panayotatos & Wells, 1981; Mizuuchi et al., 1982; Courey & Wang, 1983; Lilley, 1981). Self-complementary alternating purine/pyrimidine sequences, such as $d(G\text{-}C)_n$ present in supercoiled DNA, have the inherent ability to either adopt a Z conformation or extrude into a cruciform. A minimal insert length is required for the latter mechanism (Frank-Kamenetskij & Vologodski, 1984). The conformational freedom of DNA hairpin structures, parts of cruciforms, and the possible structural restrictions in hairpin loops have not been studied in detail. In particular, can hairpin structures, which contain potential Z-DNA sequences, undergo a B to Z transition, or does the loop provide a barrier to this transition? Superhelical free energy is not expected to promote a conformational change in cruciforms, since the hairpin arms are topological independent domains from the rest of the superhelical DNA molecule. However, Z-binding proteins or Z-specific ligands may provide the necessary free energy for a conformational transition in the constituent hairpin arms of a cruciform.

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To answer these questions, the conformational flexibility of DNA hairpins has been studied by using $d(C\text{-}G)_5T_4(C\text{-}G)_5$ as a model substrate. Here we report on a B to Z conformational transition of the monomer hairpin and dimer dumbbell of $d(C\text{-}G)_5T_4(C\text{-}G)_5$.

Experimental Procedures

Synthesis, Purification, and Characterization of $d(C\text{-}G)_5T_4(C\text{-}G)_5$. Deoxyoligonucleotide $d(C\text{-}G)_5T_4(C\text{-}G)_5$ was synthesized on an Applied BioSystems Model 380A DNA synthesizer using phosphoramidate chemistry (Beaucage & Caruthers, 1981). Step yields greater than 99% were obtained. After deblocking and detritylation, the synthesis product was purified by anion-exchange chromatography at pH 13.0 on NACS-20 (Bethesda Research Laboratories).

The purified oligonucleotide was 5′-end labeled with [γ-^{32}P]ATP and T_4 polynucleotide kinase (Chaconas & van de Sande, 1980) and then analyzed by digestion to 5′-mononucleotides (Kleppe et al., 1970). Thermal denaturation profiles were determined in 0.5 mM sodium phosphate and 10^{-5} M ethylenediaminetetraacetic acid (EDTA), pH 7.0.

T_4 DNA Ligase Reactions. Ligation of ϕX174 *Hae*III restriction fragments (3.6 μg) in 10 μL of ligase buffer [50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), and 1 mM ATP] and T_4 DNA ligase (6 units) was carried out at ambient temperatures for 18 h in the presence or absence of a 70-fold excess of $d(C\text{-}G)_5T_4(C\text{-}G)_5$. Dimerization

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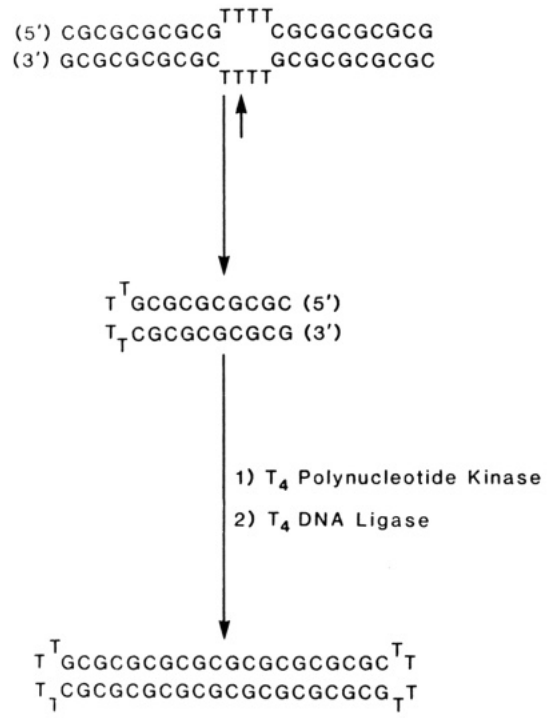

FIGURE 1: Monomer–dimer equilibrium of $d(C\text{-}G)_5T_4(C\text{-}G)_5$ intra- and intermolecular forms. T_4 DNA ligase catalyzed joining yields the dumbbell structure $[d(C\text{-}G)_5T_4(C\text{-}G)_5]_2$.

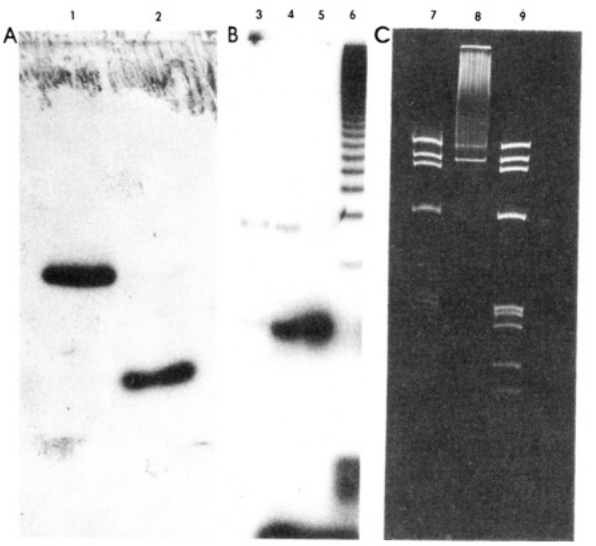

FIGURE 2: (A) Autoradiogram of denaturing gel electrophoretic analysis of ^{32}P-labeled $d(C\text{-}G)_5T_4(C\text{-}G)_5$ (lane 1) and $d(CATG)_2$ marker (lane 2). (B) Autoradiogram of a polyacrylamide gel under renaturing conditions of T_4 DNA ligase reaction of 5′-end-labeled $d(C\text{-}G)_5T_4(C\text{-}G)_5$ before (lane 4) and after (lane 3) phosphomonoesterase treatment. Lane 5, ^{32}P-labeled $d(C\text{-}G)_5T_4(C\text{-}G)_5$; lane 6, ligated ^{32}P-labeled $(CATG)_2$. (C) Ethidium bromide stained polyacrylamide gel of ϕX174 RF *Hae*III fragments treated with T_4 DNA ligase in the absence (lane 8) or presence of $d(C\text{-}G)_5T_4(C\text{-}G)_5$ (lane 7). Lane 9, *Hae*III restriction fragments of ϕX174 RF.

of $d(C\text{-}G)_5T_4(C\text{-}G)_5$ was carried out at oligonucleotide concentrations from 4 to 130 μM in ligase buffer in the presence of 6 units of T_4 DNA ligase per 10-μL reaction. Phosphomonoesterase sensitivity was determined by incubating aliquots of the ligase reaction mixture with bacterial alkaline phosphatase (Sigma, 2 μg/10 μL) followed by diethylaminoethyl (DEAE) paper chromatography analysis. Preparative dimerization reactions were analyzed by gel electrophoresis, and the appropriate product was isolated from the gel by electroelution.

Gel Electrophoresis. Analysis of oligonucleotides was carried out on either denaturing 20% polyacrylamide gels run in 8 M urea in TBE buffer (90 mM Tris–borate and 5 mM EDTA, pH 8.3) or 20% polyacrylamide gels under renaturing conditions in TBM buffer (90 mM Tris–borate, pH 8.3, and 5 mM $MgCl_2$). Kodak X-Omat 5 film was used for autoradiography. Restriction fragments were analyzed on 8% polyacrylamide gels containing TBE buffer, and bands were visualized by staining with ethidium bromide (0.5 μg/mL).

Spectroscopy. Ultraviolet (UV) absorption spectra and thermal denaturation profiles were recorded with a Varian 2280 spectrophotometer equipped with temperature-regulated cuvette holders. A molar extinction coefficient of $\epsilon_p^{257} = 7000$ M^{-1} cm^{-1} (in 1 M NaCl and 1 mM sodium phosphate, pH 7.0) was used for $d(C\text{-}G)_5T_4(C\text{-}G)_5$. A Jasco J-500C spectropolarimeter, calibrated with androsterone, was used for circular dichroism (CD) measurements which are expressed as $\Delta\epsilon = \epsilon_l - \epsilon_r$. A Varian 200 spectrometer operating at 81.98 MHz was used to obtain ^{31}P NMR spectra. Chemical shifts are referenced to an external trimethyl phosphate standard. Negative values indicate an upfield shift relative to this standard.

Results and Discussion

The structure of the 24-mer substrate containing both an inverted repeat and a purine/pyrimidine alternation, $d(G\text{-}C)_5T_4(C\text{-}G)_5$, is shown in Figure 1. The oligonucleotide was synthesized by using automated phosphoramidate chemistry, and after complete deprotection, the 24-mer was isolated by NACS-20 anion-exchange chromatography at pH 13.0. This extreme condition was necessary to denature the oligonucleotide for the purification. The $d(C\text{-}G)_5T_4(C\text{-}G)_5$ showed a single band with an anomalous mobility (Figure 2A, lane 1) on denaturing polyacrylamide gel electrophoresis which has also been reported for other potential hairpin structures (Betz & Sadler, 1981; B. W. Kalisch, unpublished results). Digestion of 5′-end-labeled $d(C\text{-}G)_5T_4(C\text{-}G)_5$ to mononucleotides showed >95% of the radioactivity in dpC. This oligonucleotide can form either an intramolecular monomer (hairpin) or an intermolecular dimer. Such a monomer–dimer equilibrium is shifted to the intramolecular hairpin conformation at low DNA concentration and at high temperature, whereas the addition of salt favors the formation of the intermolecular dimer. These generalizations were recently confirmed by studies on the dodecanucleotide d(CGCGAATTCGCG) (Marky et al., 1983; Patel et al., 1982). However, in the study presented here, the hairpin monomer is more stable than the dimer conformation because the latter contains internal loops which are unable to base pair, preventing a lowering of the free energy of the dimer structure. Studies on $d[ATCCTA(T)_nTAGGAT]$ ($n = 2$) showed that even at a strand concentration of 3 mM, hairpin conformation is present (Haasnoot et al., 1980). A hairpin loop with only two nucleotides is postulated to be sterically impossible (Tinoco et al., 1971), and consequently, an adjacent A-T base pair opens to form a hairpin with a four-membered loop and 5 base pairs in the stem of the 14-mer (Haasnoot et al., 1980). Oligonucleotides in this series with $n > 2$ exclusively form hairpin conformations, with a loop size of 4 or more noncomplementary bases being highly favorable for hairpin formation (Haasnoot et al., 1983).

The 24-mer CG hairpin was found to be a substrate for T_4 DNA ligase to yield a single end to end dimer (dumbbell) product, confirming the hairpin conformation for the oligonucleotide. This dimer was found to be resistant to phosphomonoesterase (lane 3, Figure 2B), and gel analysis of the starting material and ligase reaction products under renaturing

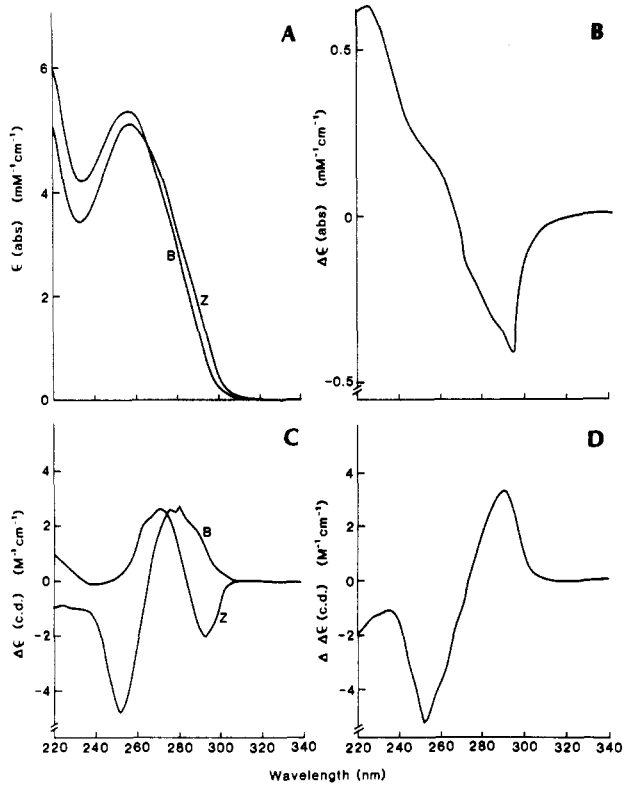

FIGURE 3: Absorbance (A) and circular dichroism (C) spectra of $d(C\text{-}G)_5T_4(C\text{-}G)_5$ in 1 mM sodium phosphate, pH 7.0 (B form), and 1 mM sodium phosphate and 5 M NaCl, pH 7.0 (Z form). As shown in the UV difference spectrum (B), the B–Z transition is characterized by a slight red shift at the λ_{max} and by hyperchromicity at 295 nm. Additionally, an inversion of the circular dichroism accompanies the transition, yielding the B–Z difference spectrum shown in (D).

conditions produced no evidence relating any observed bands to the corresponding intermolecular dimer with internal loops. This indicates that the monomer–dimer equilibrium of the synthetic CG 24-mer lies exclusively on the monomer (hairpin) side (Figure 1). In addition, $d(C\text{-}G)_5T_4(C\text{-}G)_5$ inhibits the intermolecular ligation of blunt-ended ϕX174 RF *Hae*III fragments to large molecular weight products. Mobility shifts observed in the latter reaction (lane 7, Figure 2C) are due to the sequential addition of the CG hairpin to the blunt-ended DNA restriction fragments.

The effect of counterions and dehydrating solvents on the conformation of the hairpin $d(C\text{-}G)_5T_4(C\text{-}G)_5$ was determined. The absolute and difference ultraviolet (UV) absorption and circular dichroism (CD) spectra of $d(C\text{-}G)_5T_4(C\text{-}G)_5$ under different salt conditions are shown in Figure 3. Increasing the salt concentration from 0 to 5 M NaCl results in a cooperative fully reversible conformational transition, characterized by a dramatic inversion of the CD spectrum (isoelliptic point at 275 nm, Figure 3C,D) and a red shift in the UV absorption spectrum, with hypochromicity at 258 nm and an isosbestic point at 254 nm (Figure 3A,B). The A_{295}/A_{260} ratio was 0.13 for the low-salt form and 0.25 for the high-salt form. The latter ratio is slightly lower than that reported for $(C\text{-}G)_n$ (n approximately 13) as 0.31 (Pohl & Jovin, 1972). These transitional properties are similar to those reported for the B to Z isomerization of poly[d(G-C)] (Pohl & Jovin, 1972) and oligo[d(C-G)] (Pohl, 1982; Quadrifoglio et al., 1981). The helical form in high salt is designated as the Z form and that in low salt as the B form.

Figure 4A shows the NaCl transition profile with a midpoint at 3.35 M NaCl. The conformational transition was temperature independent (20–60 °C, $\Delta H \sim 0$ kJ mol^{-1}) in contrast to the observed preference of the B form of $d(CG)_n$ (n = 3 or 4) at higher temperatures. However, the Z form becomes more thermostable in (CG) oligonucleotides of increasing chain length (enthalpy approaches zero) (Holak et al., 1984). The B to Z conformational transition in the CG hairpin is also observed in the presence of $MgCl_2$ ($\theta_{1/2}$ is 0.94 M), where a slight preference for the Z form is observed at elevated temperatures. These findings are similar to those reported for the B to Z transition for the prototypic polynucleotide poly[d(G-C)] (Pohl & Jovin, 1972; van de Sande et al., 1982). The transition-metal ions Ni^{2+} and Mn^{2+} induce a B to Z conformational transition in poly[d(G-C)] at sub-

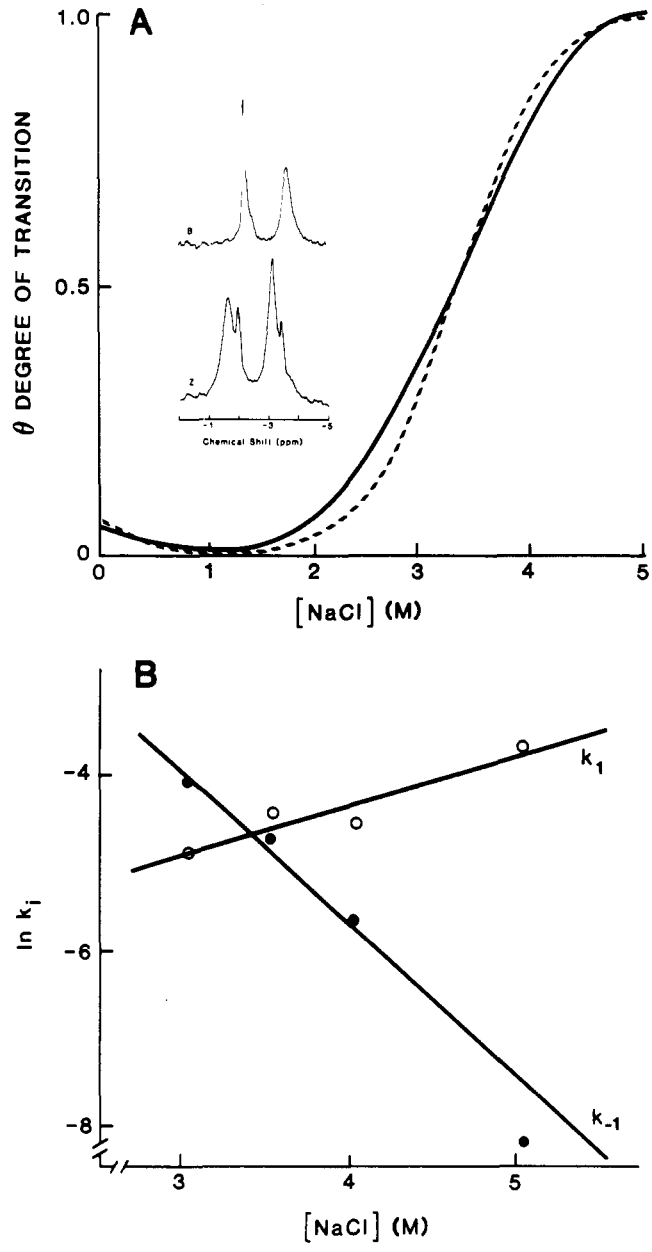

FIGURE 4: (A) Transition profiles for the B to Z transition of $d(C\text{-}G)_5T_4(C\text{-}G)_5$ monitored by the absorbance A_{295}/A_{260} ratio (—) or by circular dichroism at 252 nm (---). The degree of transition, $\theta = [Z]/([B] + [Z])$, is plotted vs. the sodium chloride concentration. Transition profiles were obtained in 1 mM sodium phosphate, pH 7.0, at a strand concentration of 2–4 μM. Insert: ^{31}P NMR spectra of 0.4 mM $d(C\text{-}G)_5T_4(C\text{-}G)_5$ in 30% D_2O (v/v) and 10 mM Tris, pH 7.6, in the absence (B form) or presence of 4.5 M NaCl (Z form) at 24 °C. Line broadening of 2 and 5 Hz, respectively, was employed. (B) Dependence of the overall rate constants for the B–Z equilibrium on the sodium chloride concentration, after a rapid change of the initial salt concentration.

Table I: Transition Properties of CG Deoxyoligonucleotides[a]

	d(C-G)$_5$T$_4$(C-G)$_5$	d(C-G)$_4$[b]	d(C-G)$_5$[b]	d(C-G)$_{13}$[c]
	Transition Midpoints (mol L^{-1})			
NaCl	3.35	2.66	2.57	2.56
$MgCl_2$	0.94			0.66
	Hill Coefficients			
NaCl	5.5	4.8	6.0	11.0
$MgCl_2$	3.3			
	Activation Energy (kJ mol^{-1})			
NaCl	141			92 ± 8[d]

[a] Measurements at ambient temperature. [b] From Pohl (1982). [c] From Pohl & Jovin (1972). [d] A fraction with approximately half the base pairs shows an activation energy of 96 ± 12 kJ mol^{-1} (Pohl & Jovin, 1972).

millimolar concentrations (Jovin et al., 1982). These ions failed to induce the conformational change for the CG hairpin under the conditions investigated, indicating that the cooperative length for the transition is not reached in this particular hairpin substrate. The presence of these metal ions resulted in an ionic strength dependent decrease in the CD band at 280 nm, indicating that an interaction of the ions with the CG hairpin occurred. This binding is reversible, and the addition of a stoichiometric amount of EDTA restored the original CD spectra.

Rate constants for the forward, B → Z (k_1), and backward, Z → B (k_{-1}), reactions in the presence of sodium chloride were determined by monitoring the absorption at 295 nm after a rapid change of the initial salt concentration. Rate constants were calculated by using the relaxation time $\tau = 1/(k_{-1} + k_1)$ in conjunction with the equilibrium constant $K = k_1/k_{-1} = \theta/(1 - \theta) = [Z]/[B]$ and are shown in Figure 4B. The maximal values for the time constants are reached at the midpoint of the transition similar to that reported for oligo[d(C-G)], and the kinetic and thermodynamic data are consistent with a two-state mechanism (Pohl & Jovin, 1972). A comparison of the B to Z transitional parameters of the CG hairpin with linear $(CG)_n$ oligomers is presented in Table I. The transition midpoints of the hairpin in NaCl and $MgCl_2$ are both shifted to higher concentrations, compared to those of the linear oligomers. The Hill coefficient for the transition of the CG hairpin in the presence of NaCl is 5.5, close to the coefficient of 6 for the corresponding linear oligonucleotide d(C-G)$_5$ [calculated from data in Pohl (1982)]. Therefore, the presence of the d(-T-)$_4$ loop in the CG hairpin has little influence on the cooperativity of the B–Z transition of the stem region of the hairpin. The activation energy for the transition of this hairpin is 141 kJ mol^{-1}, which is considerably higher than the value reported for a corresponding linear oligomer fraction (Pohl & Jovin, 1972). The effect of the loop on the apparent free energy change for the B to Z transition was calculated to be 3.8 kJ mol^{-1}.

The B and Z forms of poly[d(G-C)] and d(C-G)$_n$ oligonucleotides can easily be diagnosed by ^{31}P NMR (Patel et al., 1979). The high- and low-salt ^{31}P NMR data on d(C-G)$_5$T$_4$(C-G)$_5$ are shown in the inset of Figure 4A. The low-salt spectrum of the hairpin shows two resonances of equal intensity at −2.27 and −3.71 ppm separated by 1.44 ppm. In high salt, a downfield shift of each of these two peaks occurs concomitant to a superimposed splitting of each peak by 0.35 and 0.31 ppm, respectively. These NMR changes occur at the same NaCl concentration range in which the inversion of the CD spectra and the characteristic change in the UV spectra take place. A detailed analysis of the ^{31}P NMR of d(C-G)$_5$T$_4$(C-G)$_5$ in high and low salt awaits the isolation of large quantities of this hairpin.

The CG hairpin is found to also undergo an ethanol-induced transition with a midpoint at 58% v/v ethanol as compared to a midpoint of 48% v/v reported for poly[d(C-G)] (Pohl, 1976). A second cooperative conformational transition is observed at higher ethanol (67% v/v) concentration, reminiscent of a Z–A transition (Pohl, 1976; Ivanov & Minyat, 1981). The Z form of the hairpin generated in 64% v/v ethanol remains stable upon dilution to a concentration of 25% ethanol following the addition of 16 mM $MgCl_2$. This is analogous to the synergism of this binary solvent previously observed for the polynucleotide poly[d(G-C)] (van de Sande & Jovin, 1982). In contrast to the polynucleotide which forms an aggregated (Z*) form under these conditions, no aggregation of the hairpin is observed. The transition-metal Mn^{2+} could not maintain Z DNA upon dilution from 64% ethanol.

The dimeric (dumbbell) structure [d(G-C)$_5$T$_4$(C-G)$_5$]$_2$ isolated after T$_4$ DNA ligase catalyzed joining of the hairpin was also studied under different ionic conditions. Spectroscopic analysis (UV and CD) showed that the dumbbell structure did undergo a B to Z transition in the presence of NaCl ($\theta_{1/2}$ = 2.7 M) and $MgCl_2$ ($\theta_{1/2}$ = 0.81 M) but again not with $MnCl_2$.

The demonstration that a CG hairpin can adopt a Z conformation is the first report of a major conformational change in this type of structure. The cooperativity of the B to Z transition in the stem of the hairpin is only marginally affected by the presence of the -T$_4$- loop. However, the contribution of the loop to the apparent free energy change of the conformational transition is reflected in the higher ionic requirements to attain the Z form. The finding that the dumbbell structure too can undergo a B to Z transition clearly shows that the transition in oligonucleotides does not require a free end and possibly can start at hairpin loops.

The potential of inverted repeat sequences to extrude into a cruciform, containing hairpin components, under torsional stress has been implicated as models for transcriptional regulation of gene activity (Gierer, 1966). A B to Z transition of the hairpin arms of a cruciform might serve as an additional recognition or possibly protein binding site which could be involved in regulation processes.

Registry No. d(C-G)$_5$T$_4$(C-G)$_5$, 97950-79-3; d(C-G)$_5$T$_4$(C-G)$_5$ dimer, 97950-80-6.

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Articles

Mapping Labeled Sites in *Escherichia coli* Ribosomal RNA: Distribution of Methyl Groups and Identification of a Photoaffinity-Labeled RNA Region Putatively at the Peptidyltransferase Center†

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ABSTRACT: We have developed a method for the rapid localization of sites of ribosomal RNA labeling to limited regions (~200 bases). The method is based on the formation and polyacrylamide gel electrophoretic separation of hybrids between restriction fragments of *rrn*B DNA and isotopically labeled rRNA and the subsequent determination of radioactivity across the gel. Using [^{3}H]adenine-labeled rRNA as a control sample, we optimized experimental conditions with respect to a number of variables, including rRNA:DNA stoichiometric ratio, temperature of the annealing step, and levels of nucleases. An important result is that different rRNA·DNA hybrid fragments are obtained in different yields. The method was then applied to analyses of C^3H_3-labeled rRNA, giving results in good accord with known and proposed sites of rRNA methylation, and of rRNA that has been photoaffinity-labeled with 5-azido-2-nitrobenzoyl-[^{3}H]Phe-tRNA^{Phe}, a probe directed toward the peptidyltransferase center. The latter study showed a single major site of RNA labeling, falling within bases 2445-2668 of 23S rRNA. The extent of labeling was shown to be dependent on light-induced formation of a reactive intermediate and to be decreased in the absence of poly(uridylic acid) or in the presence of puromycin. The location of this major site of labeling is consistent with recent results obtained with an analogous tRNA photoaffinity label [Barta, A., Steiner, G., Brosius, J., Noller, H. F., & Kuechler, E. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3607-3611] and with related genetic and biochemical studies of antibiotic interaction with ribosomes suggesting that the peptidyltransferase center falls within region V (bases 2043-2625) of 23S rRNA.

In recent years increasing emphasis has been placed on the possible functional roles of rRNA in overall ribosomal function (Noller, 1984). An important step for the development of appropriate models is the placement of specific functional sites within the rRNA structure. Although affinity labeling is a potentially powerful way of identifying such sites and several affinity labels of ribosomal ligands have been known for some time to incorporate covalently into rRNA (Cooperman, 1980), localization of such incorporation to either specific bases or limited rRNA regions has proven to be quite difficult. As a

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