Mercury(II) Site-Selective Binding to a DNA Hairpin. Relationship of Sequence-Dependent Intra- and Interstrand Cross-Linking to the Hairpin−Duplex Conformational Transition

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Hg(II) interacted site selectively with only one of three deoxyribooligonucleotides examined; these "oligos" each had a different number of unmatched T residues. Thus, Hg(II) formed an intrastrand T−Hg−T cross-link between the first and fourth T residues of the hairpin, d(GCGCTTTTGCGC) (**T4**). The DNA strand formed a loop around the Hg, as if the Hg atom had been lassoed. The interactions of Hg(II) with two other oligos, d(ATGGGTTCCCAT) (**T2**) and d(GCGCTTTGCGC) (**T3**), were less specific. Previously, we found that at high DNA and salt concentrations, **T2** was a mixture of hairpin and duplex forms while **T3** and **T4** had the hairpin form; modeling studies showed that in the free **T4** hairpin the two T's at the ends of the $(T)_4$ loop form a T·T wobble base pair. Only in **T4** are the T residues positioned to form an intrastrand cross-link readily. The Hg(II)−oligo adducts formed as a function of added Hg(II) were investigated by titrations monitored by UV, CD, and ^{1}H NMR spectroscopy. The appearance of a new set of ^{1}H signals with the concomitant decay of the free oligo ^{1}H signals indicated that 1:1 Hg(II):**T2**, 1.5:1 Hg(II):**T3**, and 1:1 Hg(II):**T4** adducts were formed with $Hg(NO_3)_2$. In H_2O, these adducts all had spectra with very downfield signals for the exchangeable TN(3)H and GN(1)H groups, a characteristic of base-paired regions. All upfield N(3)H signals from the $(T)_2$ and $(T)_3$ sequences of the free oligo disappeared in the spectra of the 1:1 Hg(II):**T2** and 1.5:1 Hg(II):**T3** adducts. The disappearance of the NH signals, the UV spectral changes, and the stoichiometries (1:1 Hg(II):**T2** and 1.5:1 Hg(II):**T3**) indicate that these adducts are duplexes containing two and three T−Hg−T interstrand cross-links for **T2** and **T3**, respectively. The ^{1}H and ^{13}C signals of the 1:1 Hg(II):**T4** adduct in D_2O were nearly completely assigned by 2D NMR spectroscopy. The spectrum of the adduct in H_2O had only two of the four original TN(3)H signals from the $(T)_4$ sequence present in the spectrum of **T4**; this result is consistent with the presence of a TN3−Hg−TN3 cross-link. The ^{13}C chemical shift changes upon Hg(II) binding indicated that the TN3−Hg−TN3 cross-link was between the T's at each end of the $(T)_4$ loop. The NOESY, CD, and UV spectra were all consistent with a hairpin conformation for the 1:1 Hg(II):**T4** adduct. A hairpin conformation also appeared reasonable from molecular modeling calculations. In conclusion, the length of the central $(T)_n$ sequence influenced the type of T−Hg−T cross-link formed and, in turn, the conformation of the adducts. For $(T)_2$ and $(T)_3$, interstrand T−Hg−T cross-linking favored the duplex form. In contrast, for $(T)_4$, intrastrand T−Hg−T cross-linking stabilized the hairpin form.

Introduction

Adducts of Hg(II) compounds with natural and synthetic DNA polymers exhibit interesting behavior, different from that observed with adducts of most other metals. In 1952, Katz, finding that addition of $HgCl_2$ caused a substantial decrease in the viscosity of natural DNAs, attributed this change to a decrease in the overall size of the molecule.[1] It is generally accepted that mercury binds to the bases of DNA. Binding studies[2,3] with natural DNAs showed that the strength of binding increased with increasing A·T content. At small (<0.5) Hg(II):base ratios, 1.5−2 protons were released with the binding of each equivalent of Hg(II).[2] More recent experiments show that Hg(II) induces a large negative band in the CD spectra of natural and synthetic DNA polymers.[4−9] Addition of good complexing ligands for Hg(II) (CN^- or Cl^-) fully restored the original properties of DNA,[1,2,5,6,10] including its biological activity.[5,11] Thus, despite the great difference in many properties of Hg(II)−DNA adducts, the DNA conformation in the adducts may differ little from that of the parent DNA.

The above intriguing observations with DNA polymers have stimulated numerous studies with nucleic acid bases and mononucleosides. These studies were aimed at determining the preferred Hg(II)-binding sites on the nucleobases. Binding constants were found to be highest for N3 of U and T and N1 of G, while weaker binding occurred at various binding sites of C and A.[12] In aqueous solution, $HgCl_2$ probably adds to the amino group in cytidine and adenosine,[13] but in DMSO, it adds to the cytidine N3 and adenosine N1.[14] In DMSO, ^{13}C and ^{15}N chemical shift data suggested that Hg(II) reacts with guanosine at the N7 position,[14−17] but in the presence of base,

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the N1 position is preferred.[15] The structure of a 1:2 Hg(II):1-methylthymine complex determined by X-ray crystallography showed that Hg(II) was bound to the deprotonated N3's.[18] The related 1:2 Hg(II):thymidine and 1:2 Hg(II):guanosine complexes, synthesized by reaction with HgO in aqueous solution at pH 6,[17] did not give N(3)H and N(1)H NMR signals, respectively, in DMSO. These results suggest that Hg(II) binds at these sites in solution. Analysis of the spectra of mixtures in DMSO demonstrated that the Hg(II) thymidine complex was more stable than the guanosine complex.[17]

Binding of Hg(II) to DNA polymers is still poorly understood at a detailed molecular level. Two kinds of binding models have been proposed. For one hypothesis, TN3−Hg−TN3 interstrand cross-links formed by chain slippage[19] have a higher stability than other types of cross-links. The model explains why $[poly(dAdT)]_2$ released 2 H^+/Hg(II) added at ∼0.25 Hg(II):base ratio and why the TNH proton resonance of $[poly(dAdT)]_2$ disappeared at the same ratio,[20] since two deprotonated N3's are formed. The model also explains the reversibility of the Hg(II)−DNA reactions, since a one-base chain shift is the simplest possible rearrangement which can easily be reversed by removal of Hg(II). In another hypothesis, which also explains the reversibility of the Hg(II) binding, Hg(II) is inserted between the original complementary Watson−Crick base pairs to form A−Hg−T and C−Hg−G cross-links. Evidence for an A−Hg−T cross-link was obtained in a 2D NMR study of the d(CGCGAATTCGCG) oligonucleotide (oligo).[21] In this case, formation of T−Hg−T cross-links requires that several Watson-Crick base pairs be disrupted, a process which is unfavorable. We wanted to explore whether the higher stability of the T−Hg−T cross-link can result in DNA conformational changes such as hairpin−duplex transitions.

We have shown that Zn^{2+}, Mg^{2+}, and Pt anticancer drugs are capable of controlling the hairpin−duplex equilibrium of oligos.[22−24] The interaction of these metals with d(ATGGGATCCCAT) and d(ATGGGTTCCCAT) was studied by using 2D NMR spectroscopy.[25] The first sequence forms a duplex at 12 °C. However, at low oligo and salt concentrations and at high temperature, a hairpin form was evident. Zn^{2+} eliminated this hairpin form, but Pt anticancer drugs stabilized an irregular hairpin. The second sequence existed as a hairpin at 4 mM concentration. Zn^{2+} and Mg^{2+} were capable of converting the hairpin form into a duplex with a two-base-pair bulge.[25]

Here we show that a DNA molecule can be designed to interact selectively with a metal ion. Such designed species could be useful both as a means to elucidate the metal binding and as a way to create new types of DNA structures. DNA hairpins and other structures with unmatched bases are attractive for initiating such a study since the well-developed background on metal ion binding to isolated residues can be exploited. Here we report our studies on the interaction of Hg(II) with three sequences: $d(A_1T_2G_3G_4G_5T_6T_7C_8C_9C_{10}A_{11}T_{12})$ (**T2**), $d(G_1C_2G_3C_4T_aT_bT_cG_5C_6G_7C_8)$ (**T3**), and $d(G_1C_2G_3C_4T_aT_bT_cT_dG_5C_6G_7C_8)$ (**T4**), by using 1D/2D NMR, UV, and CD spectroscopy. The **T2** sequence was interesting to us because of the earlier studies with Zn^{2+} and Mg^{2+} mentioned above.[25] We chose **T3** and **T4** because they form very stable hairpins even at high salt and oligo concentrations. Addition of Zn^{2+} did not cause a hairpin−duplex transition of these sequences. Molecular modeling studies showed that the loop of the **T4** hairpin contained a T·T wobble base pair formed by the two T residues at the ends of the loop.[26] Formation of T·T base pairs would also be possible in bulged duplex forms of all three oligos. Therefore, we hypothesized that Hg(II) would bind to the $(T)_n$ sequences by insertion into the T·T base pairs of the hairpin or bulged duplex forms. We wanted to know whether the hairpin forms would be converted to duplex forms by Hg(II) and how the length of the $(T)_n$ segment influences the interaction of Hg(II) with these oligos. Such information could be useful in understanding how various features of the sequence and structure influenced the relative amounts of inter- vs intrastrand cross-linking. The extent and type of cross-linking are an important aspect of the mechanism of action of anticancer drugs.

Experimental Section

Sample Preparation. The oligos, **T2**, **T3**, and **T4**, were synthesized as previously described.[23,26] Extinction coefficients of **T2**, **T3**, and **T4** were determined by the nearest neighbor method at 260 nm and used for determining the concentration at 95 °C (ϵ = 7680, 6700, and 6400 M^{-1} cm^{-1} per base, respectively).[27,28] Oligo concentrations are given in strand throughout. In the UV, CD, and 1D NMR experiments four types of buffers were used and abbreviated throughout as follows: 5 mM Na cacodylate, 100 mM $NaNO_3$, pH 7 (cacodylate/$NaNO_3$/pH7); 10 mM Na_2HPO_4, pH 6 (PO_4/pH6); 10 mM Na_2HPO_4, 200 mM $NaNO_3$, pH 6 (PO_4/$NaNO_3$/pH6). Hg^{2+} titrations were performed by adding the correct amount of $Hg(NO_3)_2$ stock solutions to the oligo solutions. The $Hg(NO_3)_2$ stock solutions were prepared by measuring the appropriate amount of $Hg(NO_3)_2$·H_2O (Aldrich) into a 10 mL volumetric flask and adding 2 or 3 drops of concentrated nitric acid, followed by deionized water. The concentration of the $Hg(NO_3)_2$ solution was determined to be 100 mM by atomic absorption spectroscopy. For the UV, CD, and 1D NMR titrations, 10 mM $Hg(NO_3)_2$ solutions were prepared by dilution of the 100 mM solutions. For the 2D NMR experiments, the 1:1 Hg:**T4** samples were prepared by lyophilizing the 2−4 mM solutions of **T4** in 50 mM Na_2HPO_4, pH 7 buffer twice from D_2O, dissolving it in 99.96% D_2O, and adding the calculated amount of a 50 mM $Hg(NO_3)_2$ solution (prepared by lyophilizing $Hg(NO_3)_2$ from H_2O solution, adding 2−3 μL concentrated DNO_3 to it, and dissolving it in 99.9% D_2O). The samples were not lyophilized between the addition of $Hg(NO_3)_2$ and the 2D NMR experiment.

UV and CD Spectroscopy. UV spectra were recorded on a Varian Cary 3 UV−vis spectrophotometer using 0.01 mM samples in cacodylate/$NaNO_3$/pH7 buffer, 0.2 mM oligo in PO_4/$NaNO_3$/pH6 buffer, and 0.1 mM in base $[poly(dGdC)]_2$ in cacodylate/$NaNO_3$/pH7 buffer. CD spectra were recorded on a JASCO 600 spectropolarimeter at ambient temperature using 0.01 mM oligo samples in cacodylate/$NaNO_3$/pH7 buffer. For each CD spectrum, 2 or 3 scans were collected with 0.2 nm/per data step resolution and 100 nm/min scan speed.

NMR Spectroscopy. All NMR data were collected on a GE Ω 600 MHz spectrometer and were processed either with the instrument software or with the FELIX program (Biosym Technologies, Inc.) on an Indigo work station. All 1D ^1H NMR spectral titrations were performed in 10% D_2O/H_2O with 0.4 mM oligo samples in PO_4/$NaNO_3$/pH6 and PO_4/pH6 buffer. The $1\bar{1}$ pulse sequence was used for suppression of the water peak.[29,30]

For the 1:1 Hg(II):**T4** adduct, 2D experiments were performed at 5 °C in 99.96% D_2O. For the NOESY (nuclear Overhauser effect

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Table 1. Summary of Results of Various Spectroscopic Experiments

oligo	R[a]	UV results at high [salt]		CD results at high [salt]	NMR results at high [oligo]		conformational change
		low [oligo]	high [oligo]	low [oligo]	low [salt]	high [salt]	
T2	<1	TT binding		duplex	TT binding		hairpin/duplex→
	1-2	AT binding			AT binding		Hg-duplex
	>2	GC binding					
	~3-6	aggregation		negative CD	aggregation		
T3	<1.5	GC binding	TT binding	CD changed	nonselective binding	TT binding	hairpin→Hg-duplex
	>1.5	GC binding					
	~3-6	aggregation		negative CD	aggregation		
T4	<1	TT binding		no change	TT binding		hairpin→Hg-hairpin
	>1	GC binding					
	~3-6	aggregation		negative CD	aggregation		

[a] [Hg(II)]/[strand]

spectroscopy) data, a 2048 × 256 $t_2 \times t_1$ data point matrix with 128 scans per t_1 increment was collected using a 300 ms mixing time.[31] For the DQF-COSY (double-quantum-filtered correlation spectroscopy) studies, a 2048 × 256 data point matrix with 256 scans per t_1 increment was collected.[32,33] For the HMQC (heteronuclear multiple-quantum coherence spectroscopy) and HMBC (heteronuclear multiple-bond coherence spectroscopy) experiments, the data point matrices were 2048 × 256[34] with 512 and 786 scans per t_1 increment, respectively.

Results and Discussion

In this section, the results are presented and discussed in the following order: First, we describe and analyze the titrations monitored by spectral methods. Second, we describe the 2D NMR characterization of the selectively formed 1:1 Hg(II):**T4** adduct. Third, we discuss the effect of the length of the $(T)_n$ sequence on the hairpin−duplex conformational transition induced by Hg(II) binding. The results are summarized in Table 1.

Titrations: UV Experiments. UV spectroscopy permits evaluation of many ratios and has the additional advantage that polymers can be studied and compared to the oligos. The titration of poly(dT) and $[poly(dAdT)]_2$ with Hg(II) has been monitored by UV spectroscopy in the 260 nm region.[8,9] The binding is characterized by absorption decreases and small red shifts for poly(dT) at <0.5 and $[poly(dAdT)]_2$ at <0.25 Hg(II):base ratios.[8,9] However, for $[poly(dAdT)]_2$ at > 0.25 Hg(II):base ratios, an absorbance increase was seen at ~290 nm, consistent with T binding at <0.25 and A binding at >0.25 Hg(II):base ratios.[9] We could not find a similar study for $[poly(dGdC)]_2$. Hg(II) binding to $[poly(dGdC)]_2$ had two distinct spectral phases (Figure 1). In the first phase at ≤0.4 Hg(II):base ratios, the absorbance at 260 nm decreased, while a new more intense band appeared at 270 nm and an isosbestic point was maintained at 258 nm. Isosbestic points were also found near this wavelength for natural DNAs (calf thymus, *Micrococcus lysodeikticus*, *Escherichia coli*) at 0−0.5 Hg(II):base ratios.[2,10] Gruenwedel also observed isoelliptic points for $[poly(dGdC)]_2$ at 0−0.5 Hg(II):base ratios.[4] In the second phase at >0.4 Hg(II):base ratios, the 270 nm band shifted to longer wavelengths and increased further in intensity; the 258 nm isosbestic point was lost. The changes at Hg(II):base ratios greater than 1 were small. These very distinctive spectral changes on Hg(II) binding to T, AT, and GC residues were used to interpret the UV spectral titration data on oligos.

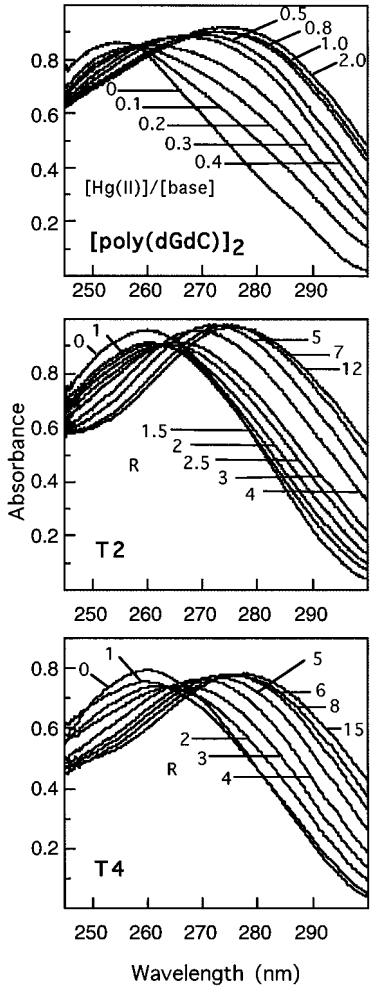

Figure 1. Representative absorption spectra showing the effect of Hg(II) on DNAs in 5 mM Na cacodylate/100 mM $NaNO_3$, pH 7 buffer: top, $[poly(dGdC)]_2$ (0.1 mM in base); middle, **T2** (0.01 mM in strand); bottom, **T4** (0.01 mM in strand). Values indicated in the panels are [Hg(II) added]/[base] for polymer, and R = [Hg(II) added]/[strand] for oligos.

UV spectral titrations were performed at low (0.01 mM) and high (0.2 mM) oligo concentrations for all three oligos. These different concentrations were useful since single-stranded species are favored at low concentrations and duplexes or higher aggregates are favored at high concentrations.

UV Spectral Titration of T2. At low **T2** concentration, the decrease of the absorbance at 260 nm and a very small hyperchromic effect around 290 nm found at R values 0.25−1.0 (R = [Hg(II) added]/[strand]) (Figure 1) suggest Hg(II) binding to the T residues .[8,9] At R = 1.5 and 2, an increased hyperchromic effect was observed at longer wavelengths, consistent with an interaction with A·T base pairs.[8,9] At R values 2.5−4, a new maximum appeared at 270 nm and increased in intensity while an isosbestic point was maintained at 262 nm, suggesting the formation of 4:1 adducts. At R > 4, the absorbance maximum shifted to longer wavelength, but there was no shift at R = 7−12. Since these spectral changes at R > 2.5 were very similar to those found for $[poly(dGdC)]_2$, we attribute the changes to Hg(II) interaction with the G·C base pairs.

At high **T2** concentration, the UV spectral changes (not shown) over all R ranges were quite similar to those observed with the low-concentration sample. Thus, at both high and low

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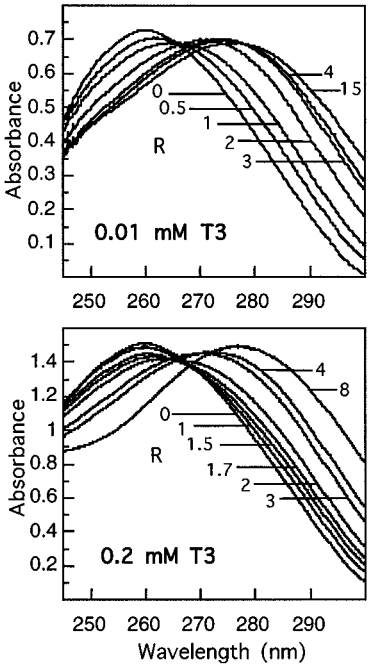

Figure 2. Representative absorption spectra of **T3** at various *R* values: top, 0.01 mM in 5 mM Na cacodylate/200 mM $NaNO_3$ at pH 7; bottom, 0.2 mM in 10 mM Na_2HPO_4 and 200 mM $NaNO_3$, pH 6.

concentrations of **T2**, Hg(II) binds to T at low *R* and then also to GC at higher R.

UV Spectral Titration of T3. In contrast to the **T2** results, different UV spectral changes were observed at low and high **T3** concentrations upon Hg(II) binding. At low concentration, initial addition of Hg(II) resulted in an absorbance decrease at 260 nm and a substantial increase at ~270 nm (Figure 2). A clear isosbestic point was seen for *R* values ≤2. At $R > 2$, the absorption maximum shifted to longer wavelengths. At $R > 10$, very little spectral change occurred relative to the amount of Hg(II) added. The increase of the 270 nm band and red shift at higher Hg(II) concentration were observed for $[poly(dGdC)]_2$ as well. Therefore, at low **T3** concentration, Hg(II) was bound to the d(GCGC)·d(GCGC) sequence, and the UV data gave no sign of binding to the $(T)_3$ sequence.

At high **T3** concentration, initial addition of Hg(II) caused an absorbance decrease at 260 nm but only a small hyperchromic effect at ~275 nm (Figure 2), features characteristic of binding to T residues.[8,9] At $R = 1.5-3$, the spectral changes (absorbance decrease and increase at 260 and 275 nm, respectively, with an isosbestic point at 264 nm; and at $R > 4$, red shift of the 275 nm absorbance maximum) were similar to those found for $[poly(dGdC)]_2$. Thus, at high **T3** concentration, Hg(II) binds to the T residues at low *R*; at higher *R*, it also binds to the d(GCGC)·d(GCGC) sequence.

UV Spectral Titration of T4. At low **T4** concentration, at $R = 1$ the absorbance at 260 nm decreased but increased very little at longer wavelengths (Figure 1). Therefore, at $R = 1$, Hg(II) binds to the T residues. At $R > 1$, the UV spectral changes (at $R = 1-4$, hypochromic effect at 260 nm, hyperchromic effect at 270 nm, and isosbestic point at 263 nm; at $R > 4$, red shift of the 270 nm absorbance maximum) were interpreted as binding to the d(GCGC)·d(GCGC) sequence.

At high **T4** concentration, the UV spectral changes (at $R < 1$, hypochromic effect at 260 nm; at $R > 1$ hyperchromic effect at 270 nm) were similar to those at low **T4** concentration, suggesting similar binding modes. Thus, of the three oligos, only **T3** formed different adducts at high and low oligo concentrations.

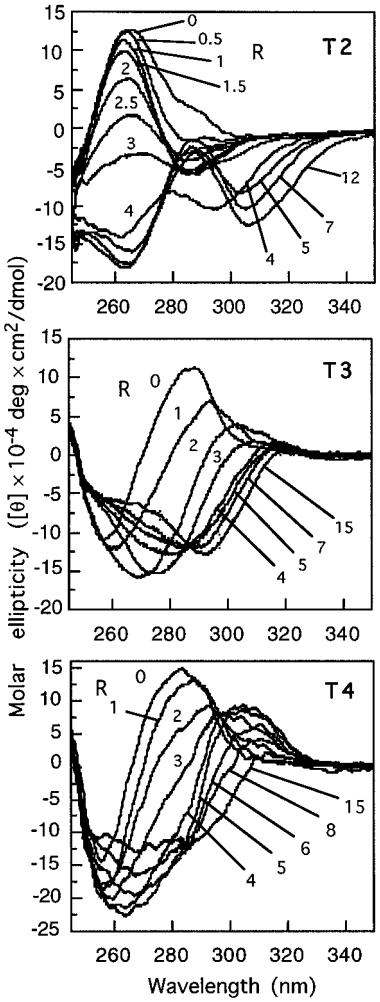

Figure 3. CD spectra of 0.01 mM solutions of **T2** (top), **T3** (middle), and **T4** (bottom) at various *R* values in 5 mM Na cacodylate/200 mM $NaNO_3$, pH 7.

CD Experiments. Hg(II) titrations of poly(dT), $[poly(dAdT)]_2$, and $[poly(dGdC)]_2$ have been monitored by CD spectroscopy.[4,9] At ~1 Hg(II):base, the CD spectra of all three polymers contain a negative band and no positive bands. The shapes of the CD spectrum of $[poly(dGdC)]_2$ were similar at 1 Hg(II):base and in 3 M salt solution with no Hg(II);[4] $[poly(dGdC)]_2$ is known to have the *Z* conformation at 3 M salt. On the basis of this similarity of the CD spectra, Gruenwedel et al. proposed that Hg(II) induces a left-handed *Z*-like DNA conformation. Hg(II)-induced negative CD spectra were observed for several natural and other synthetic polynucleotides[4-9] and even for filamentous viruses.[35] A CD spectrum with negative bands at 260 and 285 nm was also found for an oligo, d(CGCGATATCGCG).[7] We wanted to know if **T2**, **T3**, and **T4** would exhibit similar negative CD bands, so we studied all three oligos at low concentration (0.01 mM).

CD Spectral Titration of T2. In the absence of Hg(II), **T2** exhibited a conservative CD spectrum with a negative and a positive ellipticity at 240 and 265 nm, respectively (Figure 3). The negative ellipticity was partially obscured because of the absorbance of $NaNO_3$. Since there were only small changes in the CD spectra at $R < 2.5$ (a slight decrease in the positive ellipticity at 265 nm and the appearance of only a small negative band at 285 nm), **T2** remains in a more or less right-handed B-DNA-like conformation. These moderate spectral changes

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correspond with the UV spectral changes that suggested Hg(II) binding to the T residues and the A·T base pairs. Much more dramatic CD spectral changes occurred for **T2** at higher *R* values. The CD feature at ~260 nm became negative, and a second negative feature emerged near 310 nm. These changes appear to be associated with binding to the G·C base pairs and suggest a large conformational change away from B-form DNA.

CD Spectral Titration of T3. In the absence of Hg(II), **T3** had a conservative CD spectrum indicative of a right-handed DNA helix, with negative and positive bands at 256 and 287 nm, respectively (Figure 3). Major spectral changes started to occur even at low *R* values. Up to $R = 3$, the negative band at ~260 nm became broad and red-shifted; with further addition of Hg(II), this band began to decrease with the concomitant increase of the negative ellipticity at 295 nm. The UV spectral changes for these solutions indicated Hg(II) binding to the GCGC·GCGC sequence. Therefore, as for **T2**, the appearance of negative ellipticity can be correlated with GC binding.

CD Spectral Titration of T4. **T4** exhibited negative and positive Cotton effects around 245 and 280 nm, respectively (Figure 3). At $R = 1.0$, the conservative CD spectrum of the free **T4** was almost unaffected, indicating essentially no difference in the conformations of free **T4** and the 1:1 Hg(II):**T4** adduct. At increasing *R* values, first a broad negative band appeared at 260 nm, and this band then decreased as another negative band appeared at 290 nm.

Thus, at $R \leq 1$ the smallest CD spectral changes occurred for **T4**; at these *R* values the UV data suggested Hg(II) binding to the T residues. At $R > 4$, all three oligos exhibited negative CD spectra similar to those found for native or other synthetic DNAs.[4−9] Again, these negative CD bands appeared when the UV spectral changes indicated that Hg(II) was binding to the G·C base pairs.

1D NMR Experiments. For all three oligos, two 1D NMR spectral titrations were performed: one at high salt, 200 mM $NaNO_3$ ($NaNO_3$/PO_4/pH6 buffer) and the other at low salt with no added $NaNO_3$ (PO_4/pH6 buffer).

1D ^{1}H NMR Spectral Titration. The ^{1}H signals of the free **T2** were assigned previously.[25] In high salt, the eight CH_3 peaks observed in the ^{1}H spectrum at $R = 0$ (Figure 4) are consistent with the simultaneous presence of hairpin and duplex forms.[25] In the GN(1)H/TN(3)H region, there are two signals at ~11 ppm and at least eight signals downfield of 12 ppm. The downfield signals are characteristic of base pairs and are attributed to the d(ATGGG)·d(CCCAT) segments of the hairpin and the duplex forms. The very broad signal at 12.15 ppm is from N(1)H of G_5 of the hairpin loop. Previous experiments[25] showed that the N(3)H signals of T_6 and T_7 from the free hairpin and the duplex forms both were found at ~11 ppm. Consequently, the signals at ~11 ppm are probably overlapped N(3)H signals from T_6 and T_7 of the hairpin and duplex forms.

At $R = 0.6$, there was a noticeable decrease in the intensity of the signals of the free hairpin and duplex forms (Figure 4). At the same *R* value, several new signals appeared; the overall number of the CH_3 signals was at least 13. Therefore, at least two Hg−**T2** adducts must be present besides the free hairpin and duplex forms. One adduct was transient and was not observed at $R = 1.2$. This transient adduct had signals at ~10.5 ppm, consistent with the N(3)H signals of T_6 and T_7. The other adduct present at $R = 0.6$ was the dominant form at $R = 1.2$. This form did not have TN(3)H signals for T_6 and T_7.

The signals of the free **T2** and the minor adduct disappeared completely at $R = 1.2$; therefore, the Hg(II):**T2** ratio in the remaining adduct is ~1:1. There were four sharp NH signals at ~13 ppm for the four types of nonterminal complementary

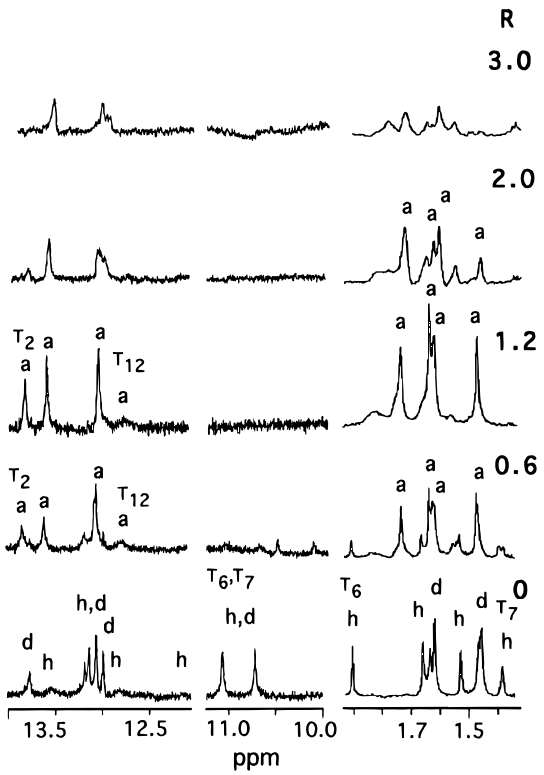
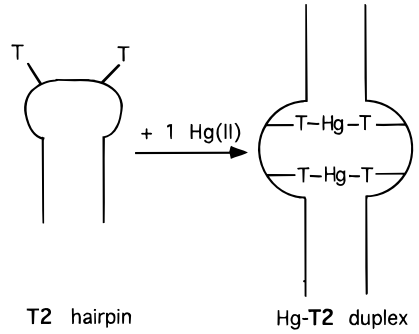


Figure 4. Imino (left) and CH_3 (right) regions of the ^{1}H NMR spectra of **T2** (0.4 mM) at various *R* values in 10 mM Na_2HPO_4/200 mM $NaNO_3$, pH 6 buffer in 90% H_2O/10% D_2O at 20 °C. Peak labels: h, free hairpin form; d: free duplex form; a, 1:1 Hg(II):**T2** adduct.

Chart 1



base pairs but no signals at ~11 ppm for the wobble base pair. Two GN(1)H signals overlapped (supplementary figure) making complete assignment difficult, but we were able to assign unambiguously the sharp TN(3)H signal. The number of signals requires that all nonterminal complementary base pairs are intact. This pattern is evidence that Hg(II) forms two interstrand cross-links between T_6 and T_7 (Chart 1), since Hg(II) cannot link two adjacent T bases in a single strand, and the elimination of the ~11 ppm signals is strong evidence for N(3) binding. One Hg(II):strand is the equivalent of two Hg(II):duplex, consistent with 1 Hg(II) for each T·T base pair. The adduct formation was in the slow exchange regime on the NMR time scale, and only one signal per proton was detected for the 1:1 adduct. These data at high salt are consistent with a self-complementary duplex with C_2 symmetry, the C_2 axis passing between the two equivalent T−Hg−T interstrand cross-links.

Continuation of the titration until $R = 2$ provided evidence that additional Hg(II) added to **T2**. The added Hg(II) caused the appearance of new signals in the CH_3 region (Figure 4). However, all the signals became much broader. The NH region became noisier as well (Figure 4). Nevertheless, it is clear that

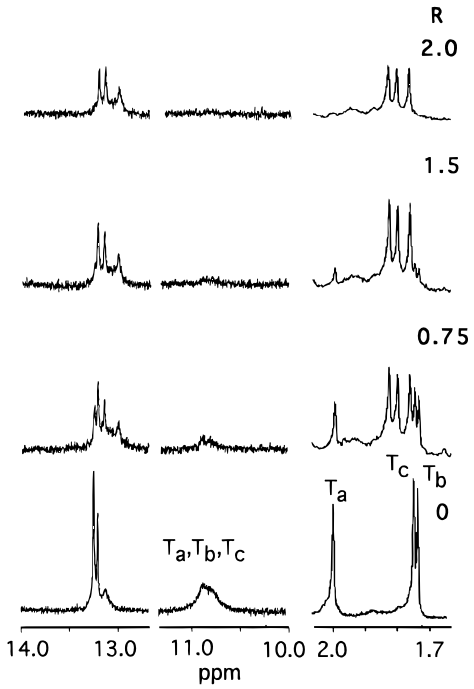


Figure 5. Imino (left) and CH_3 (right) regions of the ^{1}H NMR spectra of **T3** (0.4 mM) at various *R* values in 10 mM Na_2HPO_4/200 mM $NaNO_3$, pH 6 buffer in 90% H_2O/10% D_2O at 20 °C.

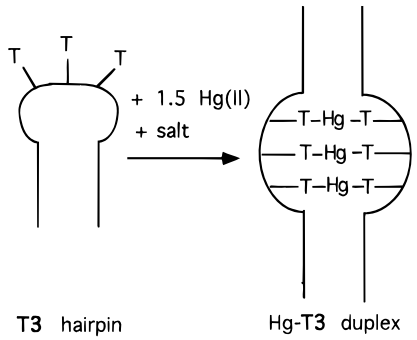
the intensity of the T_2N(3)H signal is much smaller relative to the GN(1)H signals. The decrease of the T_2N(3)H signal indicates that the second equivalent of Hg(II) per strand binds preferentially, but not exclusively, to the T_2·A_{11} base pairs of the Hg−**T2** duplex. Therefore, this titration of **T2** with Hg(II) demonstrated that Hg(II) binds preferentially to the T·T over the A·T base pairs. However, the specificity for addition of Hg(II) to the primary T·T site contrasts with the addition of Hg(II) to the secondary site (A·T), which is only selective.

Because of the broadening of the signals, we were not able to establish the specific binding mode to the A·T pair. Frøystein et al. also observed the disappearance of the TN(3)H signals of the d(CGCGAATTCGCG)$_2$ duplex upon addition of Hg(II).[21] They observed substantial downfield shifts of the AN(1) ^{15}N signals but no change in the splitting pattern of the AH2−AN1 HMQC cross-peaks. These authors proposed that four Hg(II) ions cross-linked all four A·T base pairs in the duplex through AN6 and TO4, and they attributed the disappearance of the TNH signals to signal broadening due to an increase in the rate of exchange of N(3)H with H_2O. An alternative binding mode in which the Hg(II) forms a cross-link between TN(3) and AN(1) is entirely consistent with our results.

Under low-salt conditions, the Hg−**T2** adduct formed had broader signals than the high-salt adduct. However, the CH_3 signals had shifts similar to those of the Hg−**T2** duplex observed at high salt. Therefore, it is essentially the same duplex. Thus, Hg(II) can convert **T2** from the hairpin to the duplex form even under low-salt conditions.

1D ^{1}H NMR Spectral Titration of T3. The ^{1}H signals of the free **T3** hairpin in the absence of any buffer or salt were assigned previously.[26] High-salt conditions did not change the chemical shifts; therefore, **T3** remained in the hairpin form. When Hg(II) ($R = 0.75$) was added, the number of the signals doubled (Figure 5). The new CH_3 signals had higher intensity, but the relative intensity of the signals indicated that a substantial amount of the DNA was still in the free hairpin form. The free hairpin CH_3 signals disappeared only at $R \sim 1.5$, and the N(3)H signals at ∼11 ppm from T_a, T_b, and T_c also disappeared.

Chart 2



Evidence for the formation of a 1.5:1 Hg:**T3** adduct was also found in the UV titration. The simplest explanation of the 1.5:1 Hg:**T3** ratio and the disappearance of the TNH signals is the formation of three T−Hg−T interstrand cross-links (Chart 2). Since all the signals indicate C_2 symmetry and the system is in slow exchange on the NMR time scale, the results are most consistent with the presence of a Hg−**T3** duplex form at high salt. The C_2 axis passes through the Hg of the central T−Hg−T interstrand cross-link.

The effects of the addition of Hg(II) on the **T3** NMR signals in low salt were different from those in high salt. In low salt, two new sets of small CH_3 signals were observed. The chemical shifts of neither set resembled those for the Hg−**T3** duplex found at high salt; thus, both of these adducts were new and different. However, even at $R = 2$, the intensities of these signals were quite small relative to those of the free **T3** signals. The major spectral change observed upon addition of Hg(II) was a decrease of *S*/*N*, which we attribute to aggregation. Thus, there is weak or nonselective binding of Hg(II) under low-salt conditions. This observation and the finding in the UV spectral titration that Hg(II) does not interact well with the T residues at low **T3** concentration may be related. Since both low DNA and low salt concentrations stabilize the hairpin form, both the UV and NMR spectral changes found for these conditions may be the result of Hg(II) binding to the hairpin form of **T3**. This lack of selective binding to the hairpin form is consistent with our modeling studies with the free **T3** hairpin. The conformation of the **T3** hairpin loop (with T_a in the minor groove, T_b in the major groove, and T_c outside the loop)[26] does not place the T residues in an orientation favoring an intrastrand T−Hg−T cross-link.

1D ^{1}H NMR Spectral Titration of T4. The free **T4** hairpin signals were assigned previously by 2D NMR methods in the absence of salt or buffer.[26] In high salt buffer, almost exactly the same chemical shifts were measured at $R = 0$, indicating that the conformation did not change on addition of salt. From $R = 0.5$ to $R = 1.0$, a new set of signals appeared with a simultaneous decay of the free **T4** signals (Figure 6). On the basis of the relative intensity of the signals, more than 95% of a Hg−**T4** adduct seemed to be formed at $R \sim 1.0$. Two of the four CH_3 signals were broad at 20 °C but sharp at 5 °C (Supporting Information), the temperature used for NOESY studies. Although the TNH signals were quite broad even at $R = 0$, the integration made it clear that the ratio of the GNH to the TNH signal is ∼4:4 at $R = 0$, but ∼4:2 at $R = 1.0$. Additionally, in the 5 °C spectrum, four GNH signals and two TNH signals were clearly present. Our 2D NMR studies on this species (see below) indicate that it is a hairpin in which Hg(II) links T_aN3 and T_dN3 (Chart 3), explaining the distribution of NH signals. At low salt and $R = 0-1$, the same spectral changes occurred as at high salt (not shown). Thus, the same species is formed at low *R* and low salt as at high salt.

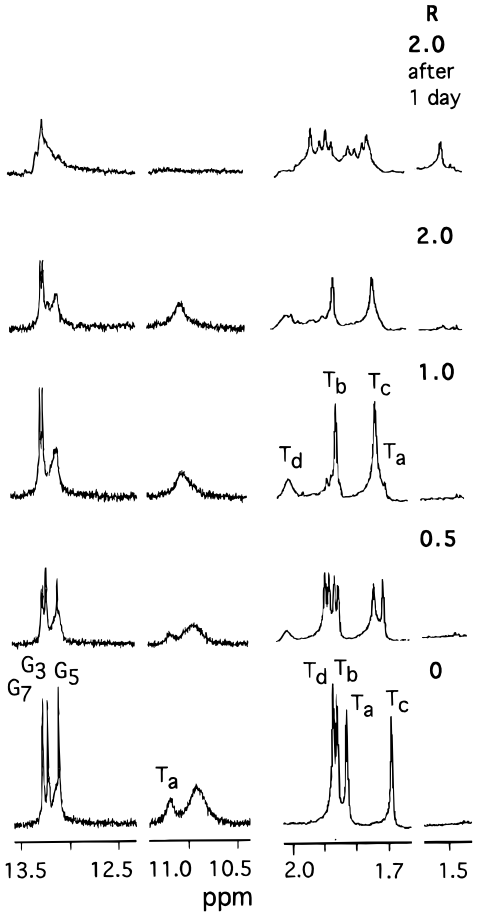
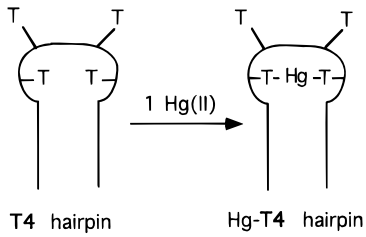


Figure 6. Imino (left) and CH_3 (right) regions of the ^{1}H NMR spectra of **T4** (0.4 mM) at various R values in 10 mM Na_2HPO_4/200 mM $NaNO_3$, pH 6 buffer in 90% H_2O/10% D_2O at 20 °C.

Chart 3



In high salt, immediately after the addition of more Hg(II) ($R = 2.0$), little change was apparent in the spectrum of the 1:1 Hg:**T4** adduct in high salt; the only change was the appearance of some very small signals nearly lost in base line noise (Figure 6). However, after 1 day, the spectrum became complicated and contained evidence for at least two unknown species. The spectrum showed no further change with time. Thus, the 1:1 Hg:**T4** adduct does not readily add a second Hg(II).

^{1}H NMR Spectral Titration of T2, T3, and T4 at $R > 2$. As described above, in the UV titrations of all three oligos at $R > 2$, the appearance of a 270 nm band and the presence of the isosbestic point (for **T2** at 262 nm, for **T3** at 266 nm, and for **T4** at 264 nm) suggested that Hg(II) interacts with the G·C base pairs. At $R > 6$, negative CD spectra were observed. After finding these interesting UV and CD spectral changes, we wanted to study these higher Hg(II)−strand adducts of **T2**, **T3**, and **T4** by a more informative technique like NMR. Therefore, we continued the ^{1}H NMR titration in high-salt buffer. However, at higher R values, the NMR spectra of all three oligos became increasingly noisy and the signals of the adducts for lower R values gradually disappeared; only extremely broad peaks remained. During both the UV and ^{1}H NMR titrations at high oligo concentration, the solutions started to become cloudy at $R \sim 6$. Addition of 10 mM KCN restored the original free oligo ^{1}H NMR signals. Similar uninterpretable ^{1}H NMR spectra were observed at higher Hg(II) concentration for the d(CGCGAATTCGCG) oligo[21] and [poly(dAdT)]$_2$.[20] We attributed the signal broadening to aggregation. In his early light-scattering measurements, Katz also observed aggregation of DNA.[1] However, later, sedimentation studies by Thomas did not show excessive increase in the molecular weight of DNA upon Hg(II) binding.[10] Unfortunately, the interesting left-handed type CD spectra were found only at $R > 4$. Under these conditions, we could not obtain satisfactory NMR data to evaluate the hypothesis that the DNA has a *Z*-like conformation.

2D NMR Studies with the 1:1 Hg(II):T4 Adduct. (a) NOESY Spectroscopy. Assignments of the Hg−**T4** ^{1}H signals were based on the NOESY and DQF-COSY data (Figure 7 and Supporting Information). For the $G_1C_2G_3C_4$·$G_5C_6G_7C_8$ signals, the same type of classical sequential "walk" can be made in the NOESY spectrum of the Hg−**T4** adduct as in that of the free **T4** hairpin.[26]

For the $C_4T_aT_bT_cT_dG_5$ sequence, C_4H5 had an NOE with one of the T CH_3 signals; therefore, this CH_3 signal was assigned to T_a. G_5H8 had NOEs with H1′/H2′/H2″ of a T residue; these signals were assigned to T_d. The H6 signal with NOEs to T_d-H2′/H2″ was assigned to T_cH6. The H2′/H2″ signals with NOEs to T_cH6 were assigned to T_b. On the basis of these assignments, other interresidue NOEs could be assigned for the T residues: T_aH1′/H2′−$T_c$$CH_3$, T_aH4′−$T_b$$CH_3$, and T_bH3′−T_c-CH_3.

The NOESY spectrum of Hg−**T4** at 5 °C contains all the most important NOEs used in the determination of the conformation of the free **T4** hairpin. T_b is in the minor groove (T_a-H4′−$T_b$$CH_3$ NOE) and T_c is in the major groove above T_a (T_aH1′/H2′−$T_c$$CH_3$ and T_bH3′−$T_c$$CH_3$ NOEs). On the basis of the similarity between the NOE cross-peak patterns of the Hg−**T4** adduct and the free **T4** hairpin, we conclude that these are both hairpins with the same conformation at 5 °C; in particular, the Hg−**T4** loop conformation is similar to that found for the free **T4**.

The combined data suggest that Hg(II) inserts between the N3's of T_a and T_d of a haripin with essentially the same conformation as the free **T4** hairpin. To explore this model further, we generated such a Hg−**T4** hairpin structure by unconstrained energy minimization. Superimposition of the free **T4** and the Hg−**T4** structures (Figure 8) shows that insertion of Hg(II) between T_a and T_d did not cause any major conformational change, in agreement with the NOESY spectrum of Hg−**T4** at 5 °C. Thus, the $(T)_4$ loop seems ideal for capturing a Hg(II) ion.

Two of the ^{1}H CH_3 signals were broad at 25 °C. However, these signals, from T_a and T_d in the T−Hg−T cross-link, were sharp at 5 and 40 °C (Supporting Information). The temperature dependence of the signals indicates a temperature-dependent conformational equilibrium. Probably just two different conformers with very similar conformations are involved, since the overall shifts are temperature dependent and the signal broadening is localized to the two residues. The conformational change is probably localized to T_a and T_d. If the whole loop conformation were changing, a temperature-dependent broadening of the T_b and T_c signals would be expected as well.

(b) ^{13}C NMR Spectroscopy. The ^{13}C signals from the ^{1}H-bonded carbons of the free **T4** and the 1:1 Hg(II):**T4** adduct were assigned from the HMQC spectrum (Table 2 and Supporting Information). The ^{13}C CH_3 signals of T_a and T_d shifted 0.9 and 1.1 ppm downfield, while those of T_b and T_c did not

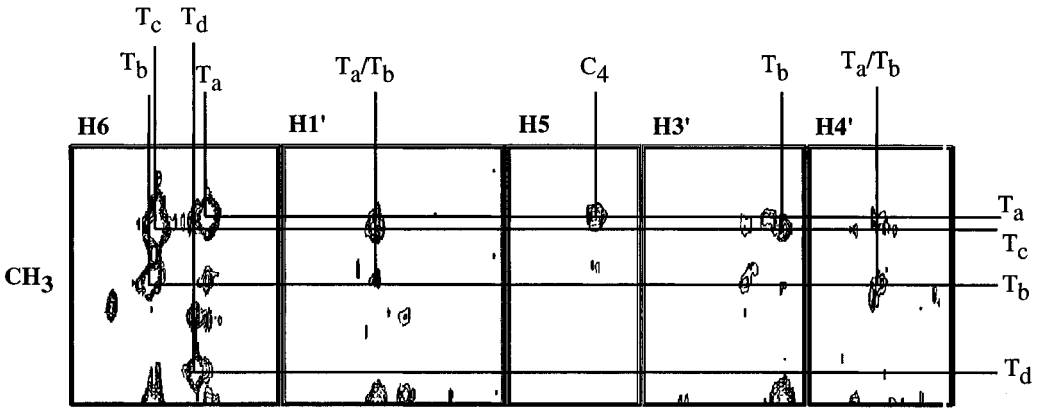


Figure 7. Selected regions of the 300 ms mixing time NOESY spectrum of the 1:1 Hg:**T4** adduct (2 mM) in 50 mM Na_2HPO_4, pH 7 buffer in D_2O at 5 °C.

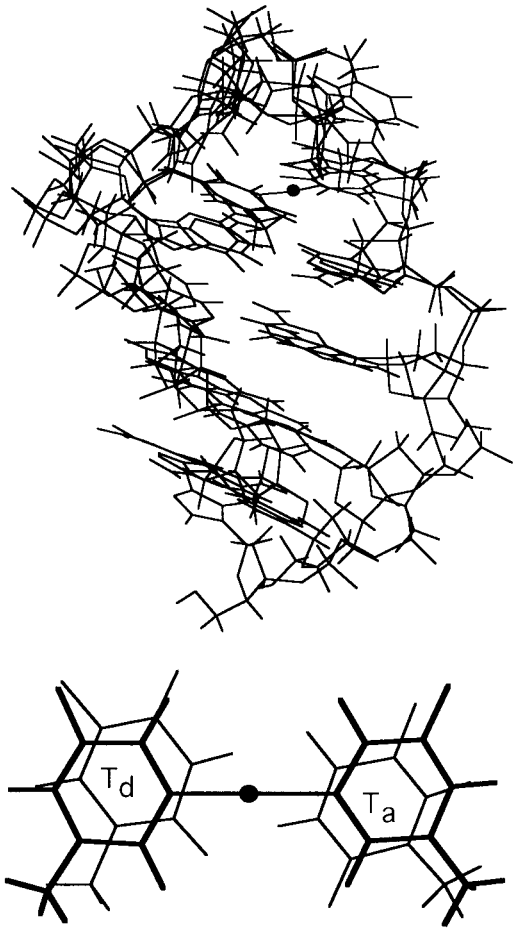


Figure 8. Superimposition of the Hg−**T4** and the free **T4** hairpin structures. The structure of the Hg−**T4** hairpin was generated by unconstrained energy minimization starting with the model for free **T4** and T_a−Hg−T_d bond lengths and bond angles from the 1:2 Hg(II):1-methylthymine complex.[18] The bottom inset shows a magnification of the T_aT_d region.

show such downfield shifts, suggesting Hg(II) binding to T_a and T_d. For comparison, we collected ^{13}C chemical shift data for thymidine 5′-monophosphate (5′-dTMP, Supporting Information). ^1H NMR spectroscopy indicated that the expected 1:2 Hg:5′-dTMP adduct formed (Supporting Information). The ^{13}C CH_3 signal of Hg−5′-dTMP was also shifted downfield, in this case by 0.7 ppm. Thus, the results are consistent with a T_a−Hg−T_d cross-link.

The C4 and C2 signals of Hg−5′-dTMP showed a much greater downfield shift, ∼3 ppm. Therefore, better evidence for Hg(II) binding to T_a and T_d would be the detection of highly

Table 2. ^{13}C Chemical Shifts for the T Bases of **T4** and Hg−**T4**

	chemical shift (ppm				
residue	C6	C5	C4	C2	CH_3
T4−Ta	139.0	114.1	167.7	153.8	14.3
Hg**T4**−Ta	139.4	114.2			15.2
T4−Tb	139.7	114.2	168.9	153.7	14.2
Hg**T4**−Tb	139.8	114.5	168.8	154.2	13.9
T4−Tc	139.6	114.2	168.1	153.8	14.5
Hg**T4**−Tc	139.8	113.9	168.7	154.3	14.2
T4−Td	139.3	114.1	168.8	153.7	14.2
Hg**T4**−Td	139.6	115.0			15.3

downfield-shifted C4 and C2 signals. In the HMBC spectrum of the Hg−**T4** adduct at 5 °C (Supporting Information), the CH_3−C4 and H6−C5/C2 cross-peaks could be detected only for T_b and T_c. The CH_3−C6/C5 cross-peaks could be found for all T residues but were very weak. Nevertheless, the signals detected for T_b and T_c did not shift upon Hg(II) binding, confirming that there is no Hg(II) binding at T_b and T_c. The ^{13}C signal shifts for the stem of the **T4** hairpin were unaffected by mercuration as well. Therefore, the HMBC data for Hg−**T4** support, albeit indirectly, Hg(II) binding to T_a and T_d.

Effect of the Length of the $(T)_n$ Sequence on the Hg(II)-Induced Hairpin−Duplex Transition. The shapes and the similar ellipticities of the CD spectra at $R = 0$ and $R = 1.0$ at low **T4** concentration are consistent with similar hairpin conformations for the free **T4** and the 1:1 Hg(II):**T4** adduct. At this low **T4** concentration the UV data also suggested Hg(II) binding to the T residues. Therefore, the UV, CD, and NMR data all suggest that the 1:1 Hg:**T4** adduct has a hairpin structure with Hg(II) bound to the deprotonated N(3)'s of T_a and T_d in a T_a−Hg−T_d cross-link in the loop.

Previous modeling studies of **T2**, **T3**, and **T4** showed that only the **T4** hairpin contains a T·T base pair in the loop; **T2** and **T3** contain T·T base pairs only in the duplex form.[25,26] Indeed, the hairpin form was not stable for **T2**.

The Hg−duplex form is most readily formed by **T2**. **T3** formed a Hg−duplex only at high salt concentration. In the absence of salt or at low **T3** concentration, Hg(II) was found to interact with the G·C base pairs and there was very little or no binding to the T residues. These findings are consistent with the higher stability of the hairpin form for **T3** over **T2** and with the lack of T·T base pairing in the $(T)_3$ loop. Under all the conditions used, **T4** was in a hairpin form at $R = 1$. The finding that **T4** formed a stable 1:1 Hg:**T4** hairpin but that **T2** and **T3** did not is consistent with the preexisting T·T base pair in the **T4** hairpin.

Summary. This study has confirmed and extended our understanding of the nature of Hg(II) DNA adducts. Upon Hg-

(II) binding, the oligos studied exhibited UV and CD spectral changes similar to those observed for many natural and other synthetic DNAs. At <2 Hg(II):strand ratios, the UV and CD spectral changes correlate with the NMR data. We demonstrated that Hg(II) is able to stabilize the duplex form of oligos with $(T)_2$ and $(T)_3$ sequences flanked by self-complementary sequences. Our results with the Hg−**T2** duplex showed the preference of Hg(II) for forming T−Hg−T over A−Hg−T cross-links. Our results for **T3** indicated that, under conditions that stabilize the hairpin form (low DNA and low salt concentrations), Hg(II) binds primarily to the G·C base pairs, most likely because a T−Hg−T intrastrand cross-link cannot form in the **T3** hairpin. All the data obtained for the 1:1 Hg−**T4** adduct are consistent with a hairpin form in which Hg(II) is bound to the deprotonated N(3)'s of T_a and T_d in a T_a−Hg−T_d interstrand cross-link in the loop. Because the T residues are correctly positioned for the intrastrand cross-link, there was no significant change in the DNA hairpin conformation on Hg binding.

Acknowledgment. We thank the NIH for support through Grant GM 29222. Purchase of instrumentation used in this study was funded in part by grants from the NIH and NSF.

Supporting Information Available: Figures showing 1D NOE spectra of Hg−**T2**, temperature dependence of ^{1}H NMR signals of Hg−**T4**, selected regions of the DQF-COSY, HMQC, and HMBC spectra of Hg−**T4**, and imino and methyl regions of the ^{1}H NMR spectra as a function of added Hg for **T3** and for 5′-dTMP; tables giving ^{1}H and ^{13}C chemical shifts for **T4**, Hg−**T4**, 5′-dTMP, and Hg-5′-dTMP (9 pages). Ordering information is given on any current masthead page.

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