Articles

^{1}H NMR Studies of Tris(phenanthroline) Metal Complexes Bound to Oligonucleotides: Characterization of Binding Modes†

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ABSTRACT: The binding of $Ru(phen)_3^{2+}$, $Rh(phen)_3^{3+}$, and $Co(phen)_3^{3+}$ to the oligonucleotides $d(GTGCAC)_2$ and $5'$-$pd(CGCGCG)_2$ has been examined by ^{1}H NMR spectroscopy as a function of temperature, concentration, and chirality of the metal complex. The duplex oligonucleotides act as chiral shift reagents for the metal complexes; phenanthroline protons associated with each enantiomer are resolved upon binding to the oligomer. The spectral titrations, consistent with photophysical studies, indicate that the complexes bind to the oligomer through two modes: one assigned as intercalation favoring the Δ-isomer, and the other assigned as the surface-bound interaction favoring the Λ-isomer. The ligand protons are perturbed in a manner that implies sensitivity of particular protons to binding mode; specifically, the H4,7 protons appear to be altered most for the Λ-enantiomer while the H5,6 protons are perturbed more for the Δ-enantiomer. The NMR chemical shift variations appear particularly sensitive to this surface-bound interaction, which, on the basis of a comparison of binding and photophysical parameters for $Ru(phen)_3^{2+}$, appears more prominant in binding to oligonucleotides than that to polynucleotides. With respect to oligonucleotide proton shifts, the adenine H2 proton, positioned in the minor groove of the helix, shows the largest upfield shifts with metal binding, and more dramatically with Λ-isomers. The major groove thymine methyl protons (TMe) shift downfield to a lesser extent, and more so for Δ-isomers. The different binding modes also differ with respect to their dynamics of association; the longitudinal relaxation rates of Δ- and Λ-4,7 phenanthroline protons of $Rh(phen)_3^{3+}$ are 0.88 and 1.14 s, respectively, in the presence of $d(GTGCAC)_2$. In contrast to studies with the substitutionally inert metal complexes, addition of racemic $Co(phen)_3^{3+}$ to the oligonucleotide solution yields unequal populations of enantiomers, owing to the rapid racemization of the cobalt complex in the presence of oligomer and reequilibration to that form which favors binding. Duplex melting has also been monitored by ^{1}H NMR spectroscopy; the complexes increase the duplex melting temperature by ~5 °C. In the case of $Co(phen)_3^{3+}$, with increasing temperature, as the helix melts, a reequlibration of the enantiomers occurs, indicating that the chiral discrimination arises from enantioselective interactions with the helix rather than with the single-stranded oligonucleotides.

There has been considerable attention focused on the understanding of how small natural products bind DNA with sequence specificity and on the rational design of new synthetic molecules that may also be targeted to specific DNA sites (Dervan, 1988; Hecht, 1986; Zein et al., 1988). In our laboratory we have concentrated on the application of transition metal chemistry to the design of molecules that bind and cleave DNA with specificity based upon *shape selection* (Barton, 1986; Fleisher et al., 1988; Pyle et al., 1989a). In particular, we have focused our efforts on derivatives of tris(phenanthroline) metal complexes (Mei & Barton, 1988; Kirshenbaum et al., 1988).

The tris(phenanthroline) metal complexes and their derivatives share several features that make them amenable to a systematic exploration of DNA site recognition. The complexes are coordinatively saturated and inert to substitution; hence, binding to DNA is determined by a sum of noncovalent interactions rather than through direct metal–nucleotide coordination. The complexes are rigid in structure; once some information regarding the orientation of the molecule with respect to the helix is available, information also becomes known with respect to the relative orientation of all the atoms of the molecule and the DNA helix. Tris(phenanthroline) metal complexes may be prepared with a range of central metal ions; the metal center may therefore be varied to obtain a host of spectroscopic probes of the DNA–complex interactions, and phenanthroline derivatives may be substituted while still preserving synthetic and spectroscopic parameters. The complexes are chiral; enantioselective interactions with DNA aid in the structural characterization of binding modes and in site-specific targeting.

As a foundation for new design, it becomes important, therefore, to characterize in detail the interactions of the parent tris(phenanthroline) metal complexes with double-helical DNA. Photophysical studies of 1,10-phenanthroline (phen) complexes of ruthenium(II) and derivatives thereof have been exceedingly useful in characterizing modes of binding (Kumar et al., 1985; Barton et al., 1986; Pyle et al., 1989b). $Ru(phen)_3^{2+}$ possesses an intense metal to ligand charge-transfer band, and this band is perturbed as a result of DNA binding. On the basis of these studies, two weak, noncovalent binding modes for $Ru(phen)_3^{2+}$ with DNA have been identified: intercalation and surface or groove binding. These

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binding modes are seen commonly in the noncovalent interactions of natural products with DNA. Intercalation leads to an increase in excited-state lifetime of the complex (2 μs compared to 0.6 μs for the free species in aerated solution). Intercalation into a right-handed helix favors the Δ-isomer; competitive binding studies and subsequent DNA cleavage experiments are consistent with intercalation proceeding from the major groove (Kumar et al., 1985; Barton et al., 1986; Pyle et al., 1989a). Surface binding, though identified in photophysical studies, has in contrast been characterized in less detail owing to the fact that the excited-state lifetime for this binding mode is indistinguishable from that of the free form (Kumar et al., 1985; Barton et al., 1986; Pyle et al., 1989b). Nonetheless, on the basis of the differential quenching experiments and studies of $Ru(phen)_3^{2+}$ and its derivatives with a variety of nucleic acids, it was apparent that the surface-bound species is held less rigidly on the DNA than is the intercalatively bound form and that the surface interaction favors the Λ-isomer. It is noteworthy that nonspecific electrostatic interactions contribute some stability but no selectivity under the conditions of our experiments; we find, for example, no detectable spectroscopic changes on addition of $Ru(bpy)_3^{2+}$ to DNA in a buffer of physiological salt.

^1H NMR spectroscopy provides another tool with which to characterize binding interactions of small molecules to oligonucleotides, both dynamically and structurally. Given that the tris(phenanthroline) metal complexes and their interactions with DNA have provided the basis for the design of a host of derivatives that recognize different local DNA conformations based upon structural considerations (Barton, 1986; Mei & Barton, 1988; Kirshenbaum et al., 1988; Pyle et al., 1989a), it appeared important to explore these interactions with NMR spectroscopy as a structural complement to the photophysical studies already performed, despite the low binding affinity of the parent complexes for DNA and the complexity of binding modes. Furthermore, this system has represented the single extensive examination of interactions of molecules with DNA on the basis of chirality, and hence, it appeared important to explore the different consequences of such enantioselective associations on NMR parameters.

We therefore report here the ^1H NMR results of $Ru(phen)_3^{2+}$, $Co(phen)_3^{3+}$, and $Rh(phen)_3^{3+}$ binding to two double-stranded hexamers, $d(GTGCAC)_2$ and 5′-pd-$(CGCGCG)_2$. Enantiomeric discrimination is evident also by NMR spectroscopy. Two distinct binding modes, which differ in terms of their dynamics of association, enantioselectivities, and orientations on the helix, are apparent. In these studies we explore the effects of chirality and charge on DNA binding modes, and furthermore, we examine in much greater detail the surface-bound interaction, pointing to this binding mode as favoring the minor groove of the DNA.

Experimental Procedures

Materials

Metal Complexes. $[Ru(phen)_3]Cl_2$ was synthesized, and the enantiomers were separated as described previously (Barton et al., 1985) as was $[Co(phen)_3]Cl_3$, $\epsilon_{273} = 6.8 \times 10^4$ M^{-1} cm^{-1} (Dollimore & Gillard, 1973). $[Rh(phen)_3]Cl_3$ was prepared by the reaction of rhodium trichloride and phenanthroline in the presence of catalytic hydrazinium monochloride (Gidney et al., 1972). The chloride salt was recovered by successive acetone precipitations, $\epsilon_{273} = 7.62 \times 10^4$ M^{-1} cm^{-1} (McKenzie & Plowman, 1970). FAB mass spectrometry gave a molecular ion at 713 corresponding to $[Rh(phen)_3]Cl_2^+$. ^1H NMR in D_2O: δ (ppm) 9.052 (d, H4,7), 8.656 (s, H5,6), 8.102 (d, H2,9), 7.916 (q, H3,8). Enantiomers of $[Rh(phen)_3]Cl_3$ were resolved by precipitation with $(+)_D$-$K_3[Co(cysu)_3]$ as described previously (Dollimore & Gillard, 1973). The assignment of absolute configuration was first reported in the literature (Mason & Peart, 1973) and is consistent with results obtained by the ^1H NMR chiral shift method (Barton & Nowick, 1984). In the spectrum of enriched Δ-$[Ru(phen)_3]Cl_2$ in CD_3CN, the Λ-H5,6 singlet appears downfield of the Δ singlet. The same proton ordering is observed for enantiomerically enriched $[Rh(phen)_3]Cl_2$ in the presence of the shift reagent. The Λ-enantiomer (100% Λ, $\Delta\epsilon_{280} = +530$ mol^{-1} cm^{-1}) was obtained as the precipitate, and the Δ-enantiomer (85% Δ, $\Delta\epsilon_{280} = -376$ mol^{-1} cm^{-1}) was isolated from the filtrate.

Nucleic Acids. The oligodeoxyribonucleotide $d(GTGCAC)_2$ was synthesized by the phosphoramidite method and purified by reverse-phase HPLC (Atkinson & Smith, 1984). The purified oligonucleotide has a UV maximum at 257 nm and a calculated extinction coefficient at 260 nm of 7500 cm (mol of nucleotide)$^{-1}$ on the basis of the nucleotide composition. 5′-$pd(CGCGCG)_2$ was purchased from Pharmacia P-L Biochemicals, Milwaukee, WI, $\epsilon_{260} = 8400$ M^{-1} cm^{-1}.

Methods

^1H NMR Experiments. Proton Fourier transform NMR spectra and 2-D NOE spectra were obtained at 300 MHz on a Bruker WM-300 NMR spectrometer with an Aspect 2000 computer for data processing or at 400 MHz on a Varian VXR-400 NMR spectrometer or a JEOL GX-400 spectrometer. The full spectral assignments of the oligonucleotides were carried out by 2-D NOESY spectroscopy (Scheek et al., 1984). The pulse sequence utilized for the 2-D experiments has been described previously (Macura et al., 1981).

Samples were prepared by dissolving the oligonucleotide in buffer (pH 7.0) and lyophilizing to dryness. D_2O (99.96% atom purity, Aldrich) was added to the sample, and lyophilization was repeated three times to remove water. Solutions of metal complexes were prepared in a similar manner.

Evaluation of Binding Parameters by NMR. Binding isotherms may be obtained from NMR data by following the chemical shifts of the metal complex protons as a function of the added ratio of metal complex to oligonucleotide (R) (Romer & Anders, 1985). By use of the linear portion of the binding isotherm, the chemical shifts of the bound metal complex (where $R = 0$) may be determined. The proportion of the complex that is free and bound at all R values in the titration may then be calculated on the basis of the chemical shifts obtained. The data are then fit to random-occupancy, anticooperative binding isotherms to obtain the intrinsic site binding constants (McGhee & von Hippel, 1974).

Equilibrium Dialysis. The binding affinity of $Ru(phen)_3^{2+}$ for oligonucleotide 5′-$pd(CGCGCG)_2$ was determined by dialysis of the oligonucleotide (1.1 mM nucleotide, 300 μL total volume) in 20 mM Tris (pH 7.2) and 80 mM NaCl versus $[Ru(phen)_3]Cl_2$ (10–200 μM, 2-mL total volume) in the same buffer. Spectropor-7 from Fisher Scientific (MW cutoff is 1000) was used for the experiment. Each sample was dialyzed at 20 °C for 2–5 days until controls containing no DNA were greater than 93% equilibrated. Concentrations of free and bound $Ru(phen)_3^{2+}$ were determined by absorbance spectroscopy, and enantiomeric selectivity was measured by circular dichroism as described previously (Barton et al., 1985). Evaluation of binding parameters for metal complexes bound to polynucleotides was carried out as described earlier (Barton et al., 1984).

Emission Lifetimes and Steady-State Luminescence Measurements. These experiments were carried out as described

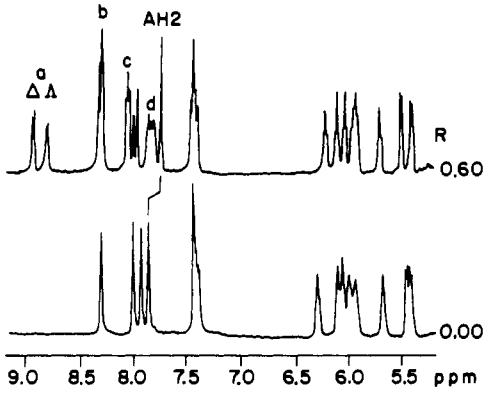

FIGURE 1: 300-MHz ^{1}H NMR spectrum of d(GTGCAC)$_2$ in the absence (bottom) and presence (top) of [Rh(phen)$_3$]Cl$_3$ in D$_2$O (5 mM sodium phosphate, pH 7.0, 15 mM NaCl). *R* is the ratio of metal complex per oligomeric duplex. Proton assignments for the metal complexes are as follows: (a) H4,7; (b) H5,6; (c) H2,9; (d) H3,8. The assignments of the free oligonucleotide were made on the basis of 2-D NOESY spectroscopy (see Experimental Procedures): AH8, 8.31; G1H8, 8.02; G3H8, 7.94; AH2, 7.87; TH6 and C4H6, 7.44; C6H6, 7.40; AH1′, 6.28; C6H1′, 6.10; G1H1′, 6.05; TH1′, 5.98; G3H1′, 5.94; C4H1′, 5.68; CH5, 5.45; TMe, 1.40 (not shown). Note that binding to the oligomer leads to chemical shift differences between bound enantiomers. For the H4,7 resonances (a) this chiral shift difference is easily resolved and the enantiomeric assignments are indicated.

previously (Barton et al., 1986).

Results

Titrations of Oligonucleotides with Tris(1,10-phenanthroline) Metal Complexes. The oligonucleotide d(GTGCAC)$_2$ was titrated with tris(1,10-phenanthroline)ruthenium(II), -cobalt(III), and -rhodium(III) complexes, and the NMR resonances of the DNA and metal complex protons were monitored as a function of the metal complex concentration. When the enantiomerically pure metal complex is added to the oligonucleotide, one set of ^{1}H resonances is observed, corresponding to the weighted average between bound and free forms of the metal complex. The complex is in fast exchange with the oligonucleotide. This observation is consistent with luminescence lifetime and polarization measurements for the tris(1,10-phenanthroline) metal complex–DNA adducts, which indicate that the residence time of the bound complex is in the microsecond range (Barton et al., 1986).

The spectra of both the metal complex and the oligonucleotide are perturbed in the oligonucleotide–metal complex adduct, and this perturbation varies in degree but not in kind depending upon the metal center or nucleotide sequence utilized. Table I summarizes the changes in phenanthroline proton positions observed from these titrations. Figure 1 displays the 300-MHz ^{1}H NMR spectrum of d(GTGCAC)$_2$ in the absence and presence of racemic [Rh(phen)$_3$]Cl$_3$.

Table I: Change in Chemical Shift (ppm) between Free[a] and Bound[b] Tris(1,10-phenanthroline) Metal Complexes to Specified Oligonucleotide

oligonucleotide	metal	isomer	H4,7		H5,6	H2,9	H3,8
pd(CGCGCG)$_2$	Ru	Λ	0.315	>	0.283	0.175	0.119
		Δ	0.281	<	0.311	0.174	0.164
d(GTGCAC)$_2$	Ru	Λ	0.414	>	0.290	0.171	0.201
		Δ	0.305	<	0.418	0.159	nr[c]
pd(CGCGCG)$_2$	Co	Λ (40%)[d]	0.224	>	0.198	0.103	0.115
		Δ (60%)[d]	0.224	<	0.233	0.103	0.115
d(GTGCAC)$_2$	Co	Λ (65%)[d]	0.274	>	0.202	0.098	nr
		Δ (35%)[d]	0.138	<	0.194	0.064	nr
d(GTGCAC)$_2$	Rh	Λ	0.309	>	0.204	0.104	0.127
		Δ	0.161	<	0.204	0.071	0.068

[a] Chemical shifts for the unbound complexes are reported under Experimental Procedures. [b] Values for bound chemical shifts were obtained graphically by extrapolating the titration curve of chemical shift vs metal complex concentration to zero metal concentration. [c] Not resolved. [d] Co(phen)$_3^{3+}$ racemizes in the presence of oligonucleotides. Tentative assignment of enantiomers was made on the basis of comparison with the rhodium and ruthenium complexes, but owing to the rapid racemization, this could not be verified by circular dichroism.

Most striking is the resolution of enantiomeric protons as a result of DNA binding. The binding orientation differs for the two enantiomers in the diastereomeric association with the DNA helix. The oligonucleotide acts as a chiral shift reagent for the metal complex, and a doubling in many of the phenanthroline proton peaks may be observed. In particular, in Figure 1 it is apparent that the H4,7 resonances corresponding to the Δ- and Λ-enantiomers of the metal complex are clearly separated. On the basis of spectra of enantiomerically enriched material, it was determined that the Λ-H4,7 doublet appears upfield of Δ-H4,7. A similar resolution of some phenanthroline protons is evident for Ru(phen)$_3^{2+}$ (vide infra). The phenanthroline ligand protons of the rhodium complex all experience upfield chemical shifts when bound to the oligonucleotide, and for Rh(phen)$_3^{3+}$, the shift for each Λ-isomer phenanthroline proton equals or exceeds that of the Δ-isomer.

With respect to protons on the oligonucleotide, the chemical shift perturbations of purine base protons and the thymine methyl group as a result of titration with the tris(phenanthroline) complexes of rhodium(III), ruthenium(II), and cobalt(III) are summarized graphically in Figure 2. Protons

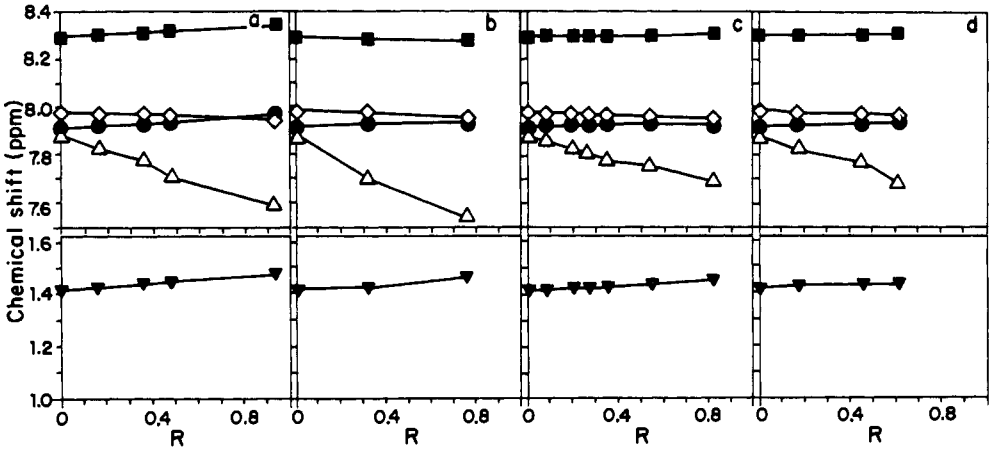

FIGURE 2: Titration of d(GTGCAC)$_2$ with tris(1,10-phenanthroline) metal complexes at 20 °C: Variations in oligonucleotide chemical shifts [(■) AH8; (◇) G1H8; (●) G3H8; (△) AH2; (▼) TMe] with increasing concentrations of (a) Δ-Ru(phen)$_3^{2+}$, (b) Λ-Ru(phen)$_3^{2+}$, (c) Co(phen)$_3^{3+}$, and (d) Rh(phen)$_3^{3+}$. *R* denotes the ratio of metal per duplex oligomer.

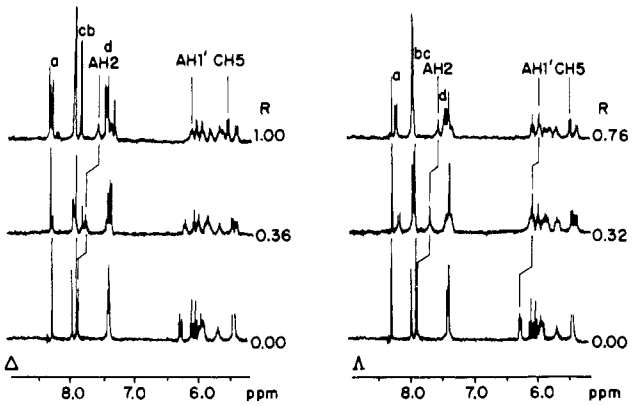

FIGURE 3: 300-MHz ^{1}H NMR spectra of the aromatic and anomeric regions for d(GTGCAC)$_2$ titrated with Δ- (left) and Λ- (right) Ru(phen)$_3^{2+}$ at 20 °C. Ligand proton assignments are as follows: (a) H4,7; (b) H5,6; (C) H2,9; (d) H3,8. Although not shown here, titration data for the TMe protons are given in Figures 2 and 4.

AH8, G3H8, and TMe experience downfield shifts, while protons G1H8 and AH2 shift upfield with binding of the metals. The AH2 resonance is dramatically affected by metal binding; this proton, positioned in the minor groove of the helix, experiences the largest chemical shift changes when titrated with the complex. Again, results with the different metal complexes are seen to be qualitatively similar.

Since the two isomers display differing relative shifts on binding to the oligonucleotide for different phenanthroline protons, it is apparent that more than a single binding mode for the different complexes exists. The different binding modes may be more clearly compared with respect to NMR parameters by examination of the enantiomerically pure material in the presence of the oligonucleotide. The effects of binding Λ- and Δ-Ru(phen)$_3^{2+}$ on the aromatic and anomeric regions of the ^{1}H NMR spectrum of d(GTGCAC)$_2$ are shown in Figure 3. Again, for both isomers, a large chemical shift change is seen for the AH2 protons in addition to shift perturbations in the anomeric region. Interestingly, the Λ-enantiomer of Ru(phen)$_3^{2+}$ appears to influence the upfield chemical shift of the AH2 and AH1′ protons more than does the Δ-enantiomer. In contrast, the major groove thymine methyl group resonances, which experience downfield shifts in the presence of the complex, are perturbed more by the Δ-enantiomer than by the Λ-enantiomer. This difference in the effects on major and minor groove proton shifts with the two enantiomers is illustrated graphically in Figure 4. The observation again points to the presence of different binding modes, with different structural orientations for the different enantiomers being favored. Since, however, both Λ- and Δ-isomers show both a downfield shift for the TMe resonance and an upfield shift for AH2 but to differing extents, it is likely that neither binding mode is enantiospecific. Instead, as was found in the examination of photophysical properties for Ru(phen)$_3^{2+}$, both isomers likely bind through both binding modes, but the two binding modes differ with respect to their enantioselectivities.

The ligand resonances of both Λ- and Δ-Ru(phen)$_3^{2+}$ either overlap with or fall very close to the DNA proton resonances of the oligonucleotide d(GTGCAC)$_2$, limiting the information that can be obtained about the metal complex geometry by following the titration. The H4,7 doublet, however, is clearly resolved in the case of the Λ-enantiomer and is partially resolved for the Δ-enantiomer. As in the case of the rhodium complex, these protons, as well as the other resonances in the metal complex, are shifted upfield upon complexation with the oligonucleotide, and the H4,7 resonances of the Λ-enantiomer

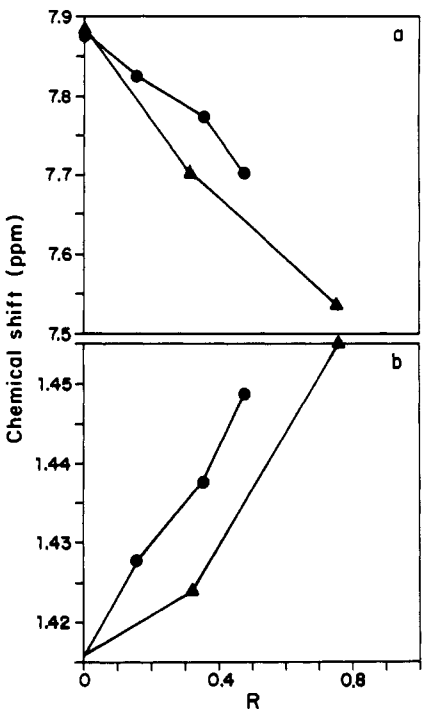

FIGURE 4: Chemical shift variation in AH2 (a) and TMe (b) resonances, upon titration with Λ- (▲) and Δ- (●) Ru(phen)$_3^{2+}$. See Figure 1 for experimental conditions. The minor groove proton, AH2, is perturbed more by the Λ-isomer, while the TMe protons, which lie in the major groove, are affected more by the Δ-isomer.

undergo larger upfield shifts than the Δ-H4,7 resonances. This observation is apparent through a comparison of the spectra of bound Λ- and Δ-isomers at *R* values of 0.32 and 0.36, respectively, in Figure 3. The H5,6 singlet of the Δ-enantiomer, in contrast, undergoes a larger chemical shift than the Λ-H5,6 protons. The H5,6 resonances appear upfield of the H2,9 doublet in the NMR spectrum of the Δ-Ru(phen)$_3^{2+}$–oligonucleotide complex, while the two metal complex resonances overlap with the oligonucleotide guanine H8 protons in the spectrum containing the Λ-isomer (Table I). These differing shift changes for enantiomers may indicate the differing geometric sensitivities of the different proton resonances to binding mode; the 5,6 resonances may be more sensitive indicators to binding through that mode which favors the Δ-isomer, whereas the 4,7 resonances are perturbed most strongly in that mode favoring the Λ-isomer.

Figure 5 shows the NMR spectrum of racemic [Co(phen)$_3$]Cl$_3$ bound to d(GTGCAC)$_2$. The chemical shifts of the oligonucleotide and metal complex protons follow the same general trends as seen previously for Ru(phen)$_3^{2+}$ and Rh(phen)$_3^{3+}$. Interestingly, however, addition of racemic Co(phen)$_3^{3+}$ results in unequal populations of enantiomers in the presence of the oligonucleotide (35%:65% for R = 0.21; see inset in Figure 5). This reequilibration in the presence of the oligonucleotide contrasts the results seen with the substitutionally inert ruthenium and rhodium complexes, where equal integrated intensities (but not line widths, vide infra) of enantiomeric resonances are observed. An attempt was made to assign the enantiomers by titrating the oligonucleotide with Λ-Co(phen)$_3^{3+}$, but the identical enantiomeric product mixture was observed in the NMR spectrum. Clearly, Co(phen)$_3^{3+}$ racemized rapidly in the presence of d(GTGCAC)$_2$ under these conditions to generate a stable mixture of enantiomers enriched in the isomer that interacts most favorably with the oligonucleotide. Rapid racemization of Co(phen)$_3^{3+}$ isomers through electron transfer is catalyzed

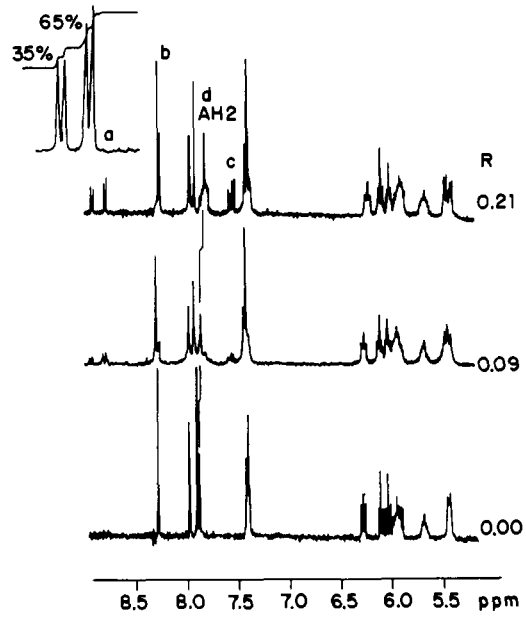

FIGURE 5: 300-MHz ^{1}H NMR spectra of d(GTGCAC)$_2$ in the absence and presence of increasing concentrations of [Co(phen)$_3$]Cl$_3$. Proton assignments for the metal complexes are as follows: (a) H4,7; (b) H5,6; (c) H2,9; (d) H3,8. The metal complex, which racemizes rapidly in the presence of the oligonucleotide, gives an unequal distribution of enantiomers upon equilibration with the duplex. The inset shows the resolution of H4,7 resonances and their relative distributions. The upfield resonance may be assigned tentatively to the Λ-isomer, on the basis of comparisons with oligonucleotide spectra in the presence of ruthenium and rhodium complexes.

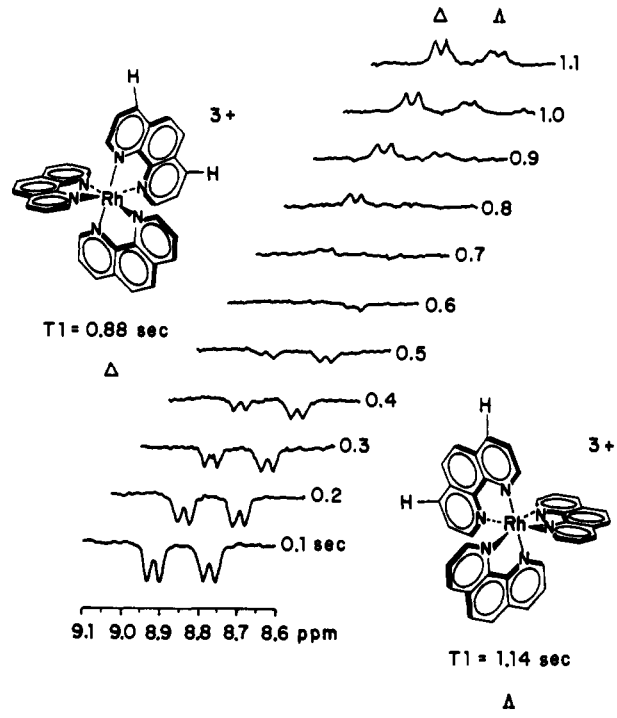

FIGURE 6: Determination of the different longitudinal relaxation rates for the resolved Λ- and Δ-H4.7 phenanthroline protons of Rh(phen)$_3^{3+}$ in the presence of d(GTGCAC)$_2$. The proton relaxation rates were measured by the inversion recovery method (180–τ–90 pulse sequence) and calculated on the basis of a nonlinear least-squares fit of the measured peak height. Variations in spectra as a function of τ are shown. Schematic illustrations of the isomer structures are also given and the 4,7 proton positions denoted.

by trace cobaltous ion (Basolo & Pearson, 1968). On the basis of the chemical shift order of the H4,7 protons observed in the Ru(phen)$_3^{2+}$ and Rh(phen)$_3^{3+}$ titrations, this enantiomer is suggested to be in the Λ-configuration. For the tris(phenanthroline) metal dications and for the cobalt complex with pd(CGCGCG)$_2$ (vide infra), in contrast, binding favors the Δ-isomer.

The relative quantities of each Co(phen)$_3^{3+}$ enantiomer also vary as a function of ionic strength and temperature, and thus this reequilibration permits a facile measure of relative binding association for isomers as a function of environmental perturbation. For the Co(phen)$_3^{3+}$–d(GTGCAC)$_2$ mixture, as the ionic strength increases, the enantiomerically enriched solution gradually becomes racemic. The tendency of the metal complex to reequilibrate to a racemic distribution reflects the decrease in overall binding to the helix with increasing sodium ion. Similar effects are obtained with increasing temperature, as the helix melts (vide infra).

Measurements of Relaxation Parameters. An indication of the differing dynamics associated with the two binding modes may be obtained through the examination of relaxation properties for molecules in the different bound forms. Inspection of the enantiomerically resolved H4,7 protons for racemic Rh(phen)$_3^{3+}$ bound to the oligonucleotide (Figure 1) reveals not only the resolution of protons based upon metal chirality but also differences in the line widths for the enantiomers. The Λ-H4,7 doublet is broadened more than that of the Δ-isomer, indicating different relaxation rates for the bound Δ- and Λ-isomers. As shown in Figure 6, the longitudinal relaxation rates ($1/T_1$) of the Δ- and Λ-H4,7 protons may be differentiated in the presence of the oligonucleotide. In the presence of oligomer, T_1(Δ-H4,7) is 0.88 s while T_1(Λ-H4,7) is 1.14 s. These results also support the notion that the different binding modes, favored for the different enantiomers, differ in their dynamics of association with the helix.

Titrations of 5′-pd(CGCGCG)$_2$ with Tris(1,10-phenanthroline) Metal Complexes. Titrations of Ru(phen)$_3^{2+}$ and Co(phen)$_3^{3+}$ with the oligonucleotide 5′-pd(CGCGCG)$_2$ were also conducted so as to examine any sequence dependencies in the interactions. The ^{1}H chemical shifts for the oligonucleotide and metal complexes were altered with binding, and shift differences between enantiomers were also evident. In contrast to d(GTGCAC)$_2$, however, very little perturbation of the oligonucleotide chemical shifts is apparent. There were, nonetheless, small chemical shift changes in the anomeric region of the spectrum when the oligonucleotide was titrated with Λ-Ru(phen)$_3^{2+}$.

Chemical shift changes experienced by the ligand protons are summarized in Table I. As in the case of d(GTGCAC)$_2$, the phenanthroline protons experience upfield shifts when bound to the oligonucleotide. The exterior H5,6 and H4,7 protons are perturbed more than the H3,8 and H2,9 protons, which are closer to the metal center. Interestingly, the same enantiomeric selectivity is observed in the magnitude of the phenanthroline ligand proton shifts for both oligonucleotides; the H5,6 proton shows a greater shift for the Δ-isomer compared to Λ-isomer for both oligomers while the H4,7 protons show a greater change for the Λ-isomer. Structural features of binding are therefore likely to be similar with these two oligonucleotides. The Co(phen)$_3^{3+}$ enantiomers also experience rapid racemization in the presence of 5′-pd(CGCGCG)$_2$, but interestingly, here the equilibration occurs so as to favor the Δ-isomer. This observation suggests that the relative proportion of binding by the two modes may differ between these different oligonucleotides. Differing levels of binding for analogous synthetic polymers through the surface-bound interaction and by intercalation was seen earlier with photophysical techniques (Barton et al., 1986), with intercalative binding by Δ-Ru(phen)$_3^{2+}$ being favored, almost to the ex-

Table II: Comparisons in Binding Parameters and Photophysical Properties of Tris(phenanthroline) Metal Complexes with Oligo- and Polynucleotides

metal complex	DNA	K_i (M^{-1})[a]	luminescence lifetimes (ns)[b]
$Ru(phen)_3^{2+}$	$d(GTGCAC)_2$	2200 ± 700	Δ, 890 (52%), 3370 (48%)
		1700 ± 900[c]	Λ, 910 (66%), 2760 (34%)
	$pd(CGCGCG)_2$	3050 ± 600	
	calf thymus DNA	3100[c,d]	Δ, 760 (29%), 2180 (71%)
			Λ, 750 (39%), 2050 (61%)
$Rh(phen)_3^{3+}$	$d(GTGCAC)_2$	1750 ± 600	
	calf thymus DNA	5000 ± 2000[c]	
$Co(phen)_3^{3+}$	$d(GTGCAC)_2$	1950 ± 700	
	$pd(CGCGCG)_2$	2100 ± 700	

[a] K_i corresponds to the intrinsic binding constant per nucleotide. All binding parameters were obtained by NMR except where indicated. Binding constants, based on changes in chemical shift as a function of metal complex concentration, were calculated for each ligand proton. The reported values represent an average over all of the ligand protons. The uncertainties reflect the standard deviations in these averages. [b] ±10% uncertainty. Shown in parentheses are values that correspond to the integrated areas of both the short- and long-lived luminescent components obtained from the deconvoluted biexponential decay curves. [c] This value was obtained by equilibrium dialysis. [d] Barton et al., 1986.

clusion of surface binding, in the case of poly[d(GC)].

Evaluation of Binding Parameters for Tris(1,10-phenanthroline) Metal Complexes Bound to Oligonucleotides. Binding constants were measured for tris(1,10-phenanthroline) metal complexes associated with $d(GTGCAC)_2$ and 5′-pd-$(CGCGCG)_2$ by titration of the chemical shifts of the metal complex with binding (see Experimental Procedures) and, in a single case, by equilibrium dialysis. Table II summarizes the results of these experiments and, for comparison, provides similar binding data for these complexes associated with a native polynucleotide, calf thymus DNA. The similarity between the binding constants obtained by equilibrium dialysis and the NMR method verifies the validity of the latter approach. Intrinsic binding constants for the metal complexes with the oligonucleotides are seen overall to be comparable to those found with polynucleotides, although the values suggest that the overall binding may be somewhat reduced with the oligonucleotides. (This comparison is, however, on the order of the certainty of the measurement.) Certainly these results indicate that all of the metal complexes have affinity for the oligonucleotides, and the strength of the binding is on the same order of magnitude as binding to polynucleotides.

In comparing binding of the metal complexes to the two oligonucleotides, it is noteworthy that the intrinsic binding to 5′-$pd(CGCGCG)_2$ appears higher than to $d(GTGCAC)_2$ despite the fact that overall shift changes with binding were smaller with this oligomer. Since the relative extent of binding by the two binding modes seems to be different with these two oligonucleotides, intercalation being more favored for 5′-pd-$(CGCGCG)_2$, these data indicate that the NMR chemical shift changes are weighting the two modes to differing extents; the chemical shift changes appear more sensitive to surface binding than to intercalation.

Emission Lifetimes of Δ- and Λ-$[Ru(phen)_3]Cl_2$ Bound to $d(GTGCAC)_2$. A comparison in binding of the complexes between the polymers and oligomers may also be drawn through an examination of luminescent properties of the metal complexes with the oligonucleotide. The binding of Λ- and Δ-$Ru(phen)_3^{2+}$ associated with $d(GTGCAC)_2$ was characterized through luminescence measurements as conducted earlier with polynucleotides (Barton et al., 1986), and these results are also summarized in Table II. As was found with polynucleotides, the luminescence of the ruthenium complex in the presence of oligonucleotide may be fit to a biexponential decay, with a long-lived component approximately a factor of 3 longer in lifetime than the short-lived component, which is experimentally indistinguishable from the free form. The long-lived component, established for polynucleotides to correspond to decay from the intercalated form, displays a characteristic lifetime of 3.4 μs for the Δ-isomer and 2.8 μs for the Λ-isomer, when the metal complex is bound to d-$(GTGCAC)_2$. Emission lifetimes obtained in the presence of oligomer were longer than found earlier with calf thymus DNA. Importantly, as for polynucleotides, the percentage of emission from the long-lived component compared to the short-lived components is greater for the Δ-isomer than for the Λ-isomer, consistent with the enantioselective preference for intercalation which favors the Δ-isomer. The proportion of the long-lived component that is found in the case of the oligonucleotide–metal complex adduct, however, is significantly less than that of the short-lived component and is reduced from that which occurs when calf thymus DNA is present (Barton et al., 1986). The short-lived mode is instead predominant when the metal complex is bound to the oligonucleotide. This finding may indicate a decreased extent of binding by the metal complex to this oligonucleotide through the intercalative mode.

Table III: Midpoint of the Helix–Coil Transition (T_m) for Oligonucleotide $d(GTGCAC)_2$ both Free and Bound to $Rh(phen)_3^{3+}$ and $Ru(phen)_3^{2+}$ Based upon Chemical Shift Changes for Selected Oligonucleotide Protons

	T_m (°C)			
proton	$d(GTGCAC)_2$[a]	$Rh(phen)_3^{3+}$	$Ru(phen)_3^{2+}$	ΔT_m (°C)
AH2	39.5	52	53.5	+12–14
C4H1′	38	42	42	+4
AH1′	50	56	55	+5–6
TMe	38.5	44	41	+2.5–5.5
C4H5	42	47	47	+5
C6H5	39	44	44	+5

[a] Duplex hexamer concentration is 1.9 mM for all samples. $R = 0.6$.

Helix to Coil Transition. The midpoints of the helix to coil transition for the oligonucleotide $d(GTGCAC)_2$ in the presence and absence of bound tris(1,10-phenanthroline)ruthenium and -rhodium complexes are given in Table III on the basis of measurements of chemical shift changes for protons associated with the oligonucleotide and metal complex. The melting temperature of the duplex increases when either metal complex binds, irrespective of the proton being used to follow the transition. The metal complexes therefore are seen clearly to stabilize the helical form preferentially over the separated strands of the oligomers. No substantial difference between the ruthenium and rhodium complexes in their ability to promote stabilization of the duplex is evident.

As may be seen in Figure 7, the shapes of the melting profiles for the minor groove AH2 and anomeric protons differ from the curves observed for the major groove protons and for the free oligonucleotide. The minor groove AH2 protons and all but the terminal H1′ protons experience an initial upfield shift as a function of increasing temperature. This shift may

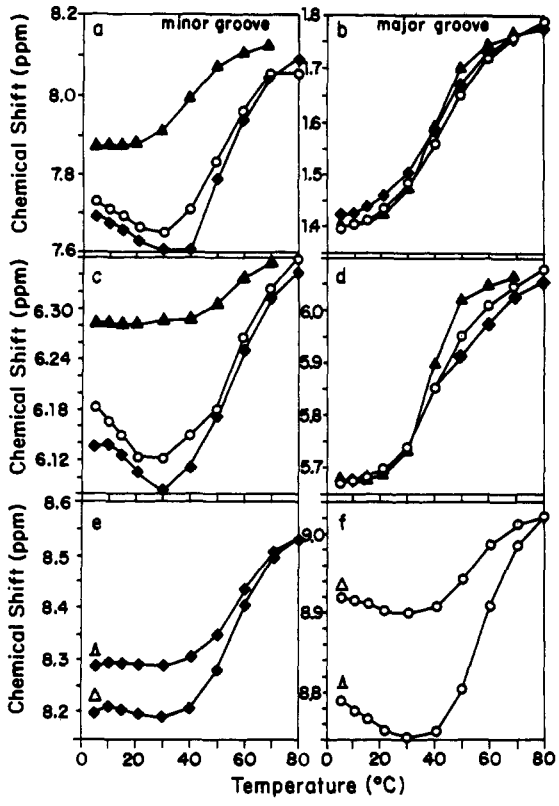

FIGURE 7: Chemical shift variations with temperature in the absence (▲) and presence of $[Rh(phen)_3]Cl_3$ (○) and $[Ru(phen)_3]Cl_3$ (◆) of selected oligonucleotide protons: (a) AH2, (b) TMe, (c) AH1′, (d) C6H5, and ligand H4,7 protons of bound $Ru(phen)_3^{2+}$ (e) or $Rh(phen)_3^{3+}$ (f) isomers. The duplex oligomer concentrations are 1.9 mM, and $R = 0.6$.

reflect an increase in binding of the metal complexes to the minor groove by that mode favored at higher temperature before the duplex begins to melt. At the onset of melting (between 25 and 35 °C), these protons begin to shift downfield, as they do in the absence of metal complex. The phenanthroline ligand protons display this same chemical shift behavior (to differing extents for the different protons) at initial temperatures, reflecting this shift in binding. Interestingly, the TMe and C6H5 protons, located in the major groove of the DNA helix, do not show this behavior, reflecting the insensitivity of these protons to this binding interaction.

Tris(1,10-phenanthroline)cobalt(III) chloride, which racemizes in the presence of oligonucleotides, undergoes a change in enantiomeric composition as a function of temperature, and this provides a measure also of the melting process and of the change in binding interaction before the helix melts. Figure 8 displays the enantiomeric distribution of $Co(phen)_3^{3+}$ in the presence of $d(GTGCAC)_2$ as a function of temperature. As is evident in Figure 8, the effect of temperature on the enantiomeric composition parallels the chemical shift behavior. As the temperature is increased, the proportion of the complex in the Λ configuration first increases from 60:40 Λ:Δ at 5 °C to 65:35 Λ:Δ at 22 °C. In this temperature regime, the percentage of metal complex bound in that mode favored for the Λ-isomer increases; increasing temperature favors the surface-bound mode. Above 22 °C the distribution moves back to a racemic, 50:50 Λ:Δ equilibrium composition as the duplex melts and the complex dissociates from the oligonucleotide.

Discussion

Comparison of Binding Tris(phenanthroline) Metal Complexes to Oligo- and Polynucleotides. Equilibrium binding and photophysical experiments of the metal complexes with

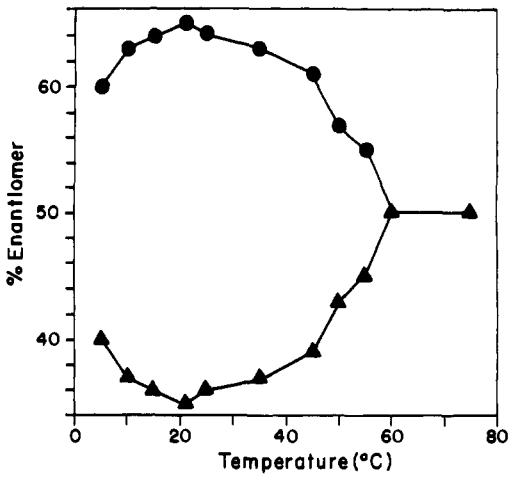

FIGURE 8: Temperature dependence of the enantiomeric composition of $Co(phen)_3^{3+}$ in the presence of $d(GTGCAC)_2$. The duplex oligonucleotide concentration is 1.1 mM, and $R = 0.6$. Tentative enantiomeric assignments for the complex: (●) Λ; (▲) Δ.

oligonucleotides were carried out to characterize the interactions between oligonucleotide and tris(1,10-phenanthroline) metal complexes in comparison to results obtained earlier with longer polymeric DNAs. The binding experiments, summarized in Table II, verify that the metal complexes do associate with oligonucleotides and that their affinity for oligonucleotides is comparable to their affinity for polynucleotides. Emission lifetime experiments furthermore indicate that both Δ- and Λ-$Ru(phen)_3^{2+}$ associated with $d(GTGCAC)_2$ show a biexponential decay in luminescence, as is seen with polynucleotides. This biphasic luminescence decay has been shown to correspond to excited-state emission from complexes in two different binding modes on the helix (Kumar et al., 1985; Barton et al., 1986): intercalation, which favors the Δ-enantiomer, and a surface-bound mode, favoring the Λ-enantiomer. The surface-binding mode, however, may be more favorable for $Ru(phen)_3^{2+}$ enantiomers when bound to the oligonucleotide. Variations in binding mode with sequence are also evident with the oligonucleotides and are comparable to results obtained earlier with polynucleotides. Binding to $d(CGCGCG)_2$ seems to favor the intercalative interaction, whereas a greater proportion of surface binding is evident upon binding to the AT-containing $d(GTGCAC)_2$.

Comparison of NMR Results with Those Found with Other Small Molecules and Oligonucleotides. Some comparisons to results obtained with other small molecules bound to oligonucleotides may be drawn. The phenanthroline ligand protons of the tris(1,10-phenanthroline)ruthenium(II), -cobalt(III), and -rhodium(III) complexes are shifted upfield when bound to $d(GTGCAC)_2$. The direction and magnitude of these shifts are comparable to those observed for known intercalators such as ethidium (Patel, 1980), daunomycin (Patel & Canuel, 1978), actinomycin D (Patel, 1976), 9-aminoacridine (Reuben et al., 1978), and bleomycin (Booth et al., 1983), while the protons of groove binding drugs such as netropsin (Patel & Shapiro, 1985) and steroid diamine dipyrandium (Assa-Munt et al., 1982) demonstrate both upfield and downfield shifts. These shifts cannot however be taken as proof of a particular binding geometry. The protons that experience the greatest shifts on binding to the oligonucleotide lie on the periphery of the phenanthroline ring in the H5,6 and H4,7 positions. This part of the molecule would be expected to interact most strongly with the oligonucleotide.

The AH2 oligonucleotide proton in the minor groove of the helix experiences the greatest change in chemical shift as a

result of complex binding for all of the metal complexes studied. The sensitivity of the AH2 proton to small molecule binding is very common. The sensitivity of a given proton depends on its location with respect to the stacked aromatic rings in the duplex. Alterations in stacking interactions have been invoked to explain upfield shifts of the AH2 proton when the intercalators ethidium (Patel, 1980), binding from the minor groove, and $[Pt(bipy)(en)]^{2+}$ (Yamauchi et al., 1986), bound from the major groove direction, associate with poly(dAdT) and AMP, respectively. The upfield shift has been attributed to intercalation of the complex directly above the adenosine AH2 proton. Downfield shifts of the AH2 proton are observed when the groove binders, such as distamycin (Klevit et al., 1986) and netropsin (Patel & Shapiro, 1985), or the intercalator daunomycin (Phillips & Roberts, 1980; Patel & Canuel, 1978) binds to oligonucleotides. Generally these shifts are rationalized in terms of both disruption of DNA base stacking upon drug binding and close proximity between the DNA binding drug and the AH2 proton.

It should be noted that chemical shift changes can only be used to infer plausible structural information regarding the oligonucleotide–metal complex adduct, since a change in chemical shift often reflects the combined contribution of multiple structural and conformational effects. In addition, the complex set of ring currents generated by the three orthogonal phenanthroline rings makes chemical shift changes even more difficult to interpret for tris(1,10-phenanthroline) metal complexes bound to oligonucleotides. Nonetheless, it is likely that large changes in chemical shift on metal complex binding are a result of a close interaction between the metal complex and the oligonucleotide proton.

Binding of Tris(phenanthroline) Metal Complex to Oligonucleotides through NMR Experiments. The NMR results are consistent with binding of tris(phenanthroline) metal complexes to duplex DNA via two binding modes and allow some additional characterization of these interactions with the helix. Most simply, as was seen with polynucleotides, chiral discrimination is evident in binding the complexes to duplex oligonucleotides. This notion is illustrated quite clearly in that the oligomer is seen to act as a chiral shift reagent for the metal complex, shifting proton resonances of the metal complex enantiomers to differing extents in the diastereomeric complexes with the helical oligomer. Moreover in experiments conducted with $Co(phen)_3^{3+}$, which can undergo rapid racemization, an unequal concentration of enantiomers is evident in the presence of the helical duplex, reflecting this chiral discrimination in binding, but with increasing temperature, as the helix melts, a reequilibration of the enantiomers occurs, resulting finally in a racemate. Therefore, the chiral discrimination arises necessarily from enantioselective interactions with the helix rather than with the single-stranded oligonucleotides themselves.

The NMR experiments reveal additional features associated with this chiral discrimination. The enantiomers bound to the helix exhibit differing dynamics, on the basis of the different spin-relaxation parameters obtained for the same proton of the two isomers bound in their differing modes. Furthermore, it is clear that both isomers bind to the helix through both modes, but one mode favors the Λ-isomer and the other the Δ-isomer. Photophysical experiments with polynucleotides earlier indicated that intercalation favors the Δ-isomer and surface binding the Λ-isomer. Here, in NMR experiments we see that the H4,7 of the phenanthroline complex shifts upfield to a greater extent in binding the Λ-isomer while the H5,6 of the phenanthroline shifts upfield to a greater degree with the Δ-isomer. This difference surely must indicate differences in orientations associated with binding of the complexes in the different modes. The different modes may also reflect the association of the complexes at substantially different sites on the DNA. The AH2 proton, located in the minor groove of the helix, is shifted appreciably and to a greater extent with Λ-$Ru(phen)_3^{2+}$, while the TMe resonance, associated with protons residing in the major groove, though shifted to a smaller extent on binding by the metal complexes than is AH2, shifts downfield more with Δ-$Ru(phen)_3^{2+}$ than with the Λ-isomer.

Characterization of Intercalative and Surface-Bound Modes. These NMR studies do not reveal sufficient structural detail to establish intercalation or even the surface-bound interaction of the complex with the helix. The complexes bind only weakly to the helix. They are in fast exchange between the bound and free form and likely bind in several orientations along the oligomer rather than at unique sites [see Rehmann and Barton (1990)]. Transfer NOEs could not be detected between the bound complexes and the oligomers (data not shown), likely because of the weak binding and the presence of three nonequivalent ligand resonances which are averaged owing to the fast tumbling of the complex. Nonetheless, these NMR studies have pointed to new features associated with these different binding modes. First and perhaps foremost is the high extent of chemical shift change seen in the AH2 proton as a function of binding. This variation, greatest with the Λ-isomer, may indicate that surface binding occurs in the minor groove of the helix. In contrast, since major groove protons are affected more by the Δ-isomers than by the Λ-isomers, these results may suggest also that intercalation occurs in the major groove. It is certainly clear, however, that these results, all in all, are more sensitive to groove binding than to binding by the intercalative mode. The small effects seen in binding the complexes to $pd(CGCGCG)_2$, where intercalation should be appreciable, compared to $d(GTGCAC)_2$ supports this conclusion.

On the basis of NMR results, it is also apparent that increasing complex charge favors surface binding in comparison to intercalation. This may be a result of the differing electrostatics associated with the two binding modes, or perhaps, it is a structural consequence of the fact that the more highly charged species would move further out of the base stack toward the phosphate backbone, substantially limiting the surface area of the phenanthroline for stacking. Association of $Co(phen)_3^{3+}$ with $pd(CGCGCG)_2$ does, however, still favor the Δ-isomer. Hence, the stacking interaction depends both on complex charge and the sequence of bases involved in the stack. How the metal complexes actually sit in each of the bound modes is not made clear from these experiments. For intercalation, the greatest perturbations occur in the H5,6 positions, as might be expected if these protons were most deeply inserted between the base pairs in an intercalative mode. For surface binding, with phenanthroline ligands lying along the helical groove, the ancillary H4,7 might be most strongly affected, and indeed, the results are consistent with this view.

The temperature dependences in the NMR experiments with cobalt complexes also provide some information to characterize the binding modes. As the temperature is raised from 5 to 22 °C, the proportion of the Λ-enantiomer increases from 60% to 65% of the total bound complex (Figure 8). This increase with increasing temperature by the Λ-enantiomer, binding in a surface-bound fashion, indicates the surface-binding interactin to be an entropy-driven process. This variation with temperature may be understandable for a surface interaction

stabilized by electrostatic attraction of the cationic metal complex to the oligonucleotide and a hydrophobic displacement of bound water from the solvated complex and DNA. The tris(phenanthroline) metal complexes lack hydrogen-bonding donors and acceptors.

Conclusions. These NMR studies show that the duplex oligonucleotide provides a template to discriminate between enantiomers of tris(phenanthroline) metal complexes. This chiral discrimination in binding to the oligonucleotide is easily revealed in the differential shifts and relaxation parameters associated with proton resonances of each enantiomer for $Ru(phen)_3^{2+}$ and $Rh(phen)_3^{3+}$ and in the differing distribution of enantiomers as a function of ionic strength and temperature in the case of the rapidly racemizing $Co(phen)_3^{3+}$. The NMR results, consistent with earlier photophysical studies, indicate that the tris(phenanthroline) metal complexes associate with the helix through two binding modes, intercalation, favoring the Δ-isomer, and a surface-bound interaction, favoring the Λ-isomer. The intercalative interaction could not be more clearly characterized through these studies. However, the sensitivity in chemical shift perturbations to the surface-bound interaction has permitted a greater characterization of this binding mode with respect to ionic strength, temperature, metal complex charge, and enantiomeric preferences. In particular, the substantial variation in the adenine H2 resonance with complex binding may point to the surface-bound mode as favoring the minor groove of the helix. Lastly, the examination of these weak, noncovalent binding interactions of tris(phenanthroline) metal complexes with nucleic acids is made more facile through simple coordination chemistry owing to (i) the ready variation in metal center, with both inert and labile species and the inclusion of di- and trications, and (ii) the chirality of the metal center, with sensitive probes for binding mode and geometry.

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