Equilibrium Association Constants for Oligonucleotide-Directed Triple Helix Formation at Single DNA Sites: Linkage to Cation Valence and Concentration†

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ABSTRACT: The linkage between the energetics of oligonucleotide-directed triple heilx formation and the cationic solution environment has been investigated in mixed-valence salt solutions. Equilibrium constants for formation of the local pyrimidine·purine·pyrimidine structure afforded by binding of the oligonucleotide 5′-d(T*TTTTCTCTCTCTCT)-3′ to a single site within a 339-bp plasmid fragment were measured using quantitative affinity cleavage titrations at pH 7.0 and 22 °C in the presence of various concentrations of KCl, $MgCl_2$, and spermine tetrahydrochloride ($SpmCl_4$). In a solution containing 10 mM NaCl, 140 mM KCl, 1.0 mM $MgCl_2$, and 1.0 mM $SpmCl_4$, the measured binding constant was 3.3 (±1.4) × 10^5 M^{-1}. The equilibrium constant previously reported for the same association reaction in 100 mM NaCl and 1 mM $SpmCl_4$ at the same temperature and pH was 10-fold higher [Singleton, S. F., & Dervan, P. B. (1992) *J. Am. Chem. Soc. 114*, 6957–6965]. Further study demonstrated that varying the potassium ion concentration between 5.0 and 140 mM (in the presence of 10 mM NaCl, 1.0 mM $MgCl_2$, and 1.0 mM $SpmCl_4$) resulted in an overall 100-fold *decrease* in the binding affinity from the lowest to the highest concentration. In contrast, measured binding constants *increased* 500-fold as the spermine concentration was increased from 0.40 to 4.0 mM (in the presence of 10 mM NaCl, 140 mM KCl, and 1.0 mM $MgCl_2$). There was a modest effect on the binding constant (a 3-fold decrease) upon varying the magnesium ion concentration from 0.10 to 10 mM (in the presence of 10 mM NaCl, 140 mM KCl, and 1.0 mM $SpmCl_4$). The results are consistent with a valence-specific cationic stabilization of the local triple-helical complex decreasing in order from the most stabilizing to the least: Spm^{4+} > Mg^{2+} > K^+. The observed trends are in good qualitative agreement with the expected effects of competition among the cations on changes in the thermodynamic binding fraction and the differential phosphate charge screening potential of each cation.

Oligonucleotide-directed triple helix formation is a versatile method for the sequence-specific recognition of double-helical DNA (Moser & Dervan, 1987; LeDoan et al., 1987; Cooney et al., 1988). Pyrimidine oligonucleotides bind parallel to purine-rich strands in the major groove of duplex DNA to form local triple-helical structures (Moser & Dervan, 1987; Praseuth et al., 1988; de los Santos et al., 1989; Rajagopal & Feigon, 1989a,b). Hoogsteen-type hydrogen bonds between T in the third strand and AT base pairs (Felsenfeld et al., 1957; de los Santos et al., 1989; Rajagopal & Feigon, 1989a,b) and between N3-protonated C in the third strand and GC base pairs (Howard et al., 1964; de los Santos et al., 1989; Rajagopal & Feigon, 1989a,b; Live et al., 1991) to afford T·AT and C+GC base triplets, respectively, impart sequence specificity to complex formation (Figure 1). The high stabilities of the local triple-helical complexes (Maher et al., 1990; Plum et al., 1990; Singleton & Dervan, 1992a,b), the sensitivity of triplex stability to single base triplet mismatches (Moser & Dervan, 1987; Mergny et al., 1991; Roberts & Crothers, 1992; Singleton & Dervan, 1992a; Rougée et al., 1992), and the broad range of potential DNA target sequences (Horne & Dervan, 1990; Griffin et al., 1992; Beal & Dervan, 1992; Jayasena & Johnston, 1992; Koh & Dervan, 1992; Miller & Cushman, 1993) suggest an important role for the triple helix as a structural motif for the design of sequence-specific DNA-binding molecules. The utility of this approach has been demonstrated by the use of triplex-forming oligonucleotides as sequence-specific inhibitors of DNA-binding proteins *in vitro* (Maher et al., 1989, 1992; Collier et al., 1991; Strobel et al., 1991; Grigoriev et al., 1992).

In addition to their dependence on length (Moser & Dervan, 1987; Singleton & Dervan, 1992a), sequence composition (Kiessling et al., 1992), base triplet mismatches, and functional groups on the heterocycle (Povsic & Dervan, 1989; Plum et al., 1990; Singleton & Dervan, 1992b; Froehler et al., 1992), the stabilities of local triple-helical complexes are sensitive to solution conditions, including temperature, pH, and the identities and concentrations of counterions (Moser & Dervan, 1987; Maher et al., 1990; Pilch et al., 1990; Plum et al., 1990; Hanvey et al., 1991; Rougée et al., 1992; Singleton & Dervan, 1992b). The experimental determination of oligonucleotide association constants as a function of these solution conditions is necessary to characterize the noncovalent forces which contribute to the affinity and specificity of binding. Moreover, any application of oligonucleotide-directed triple helix formation to control specific gene expression *in vivo*, where the solution composition is tightly regulated, will require an understanding of the functional linkage between binding free energy and solution conditions.

Previous reports of the effects of cation concentrations on the stability of polymeric (Krakauer & Sturtevant, 1968; Krakauer, 1974; Lee et al., 1984; Latimer et al., 1989) and oligomeric (Pilch et al., 1990; Plum et al., 1990; Shea et al., 1990; Durand et al., 1992; Rougée et al., 1992) triple-helical DNAs, as well as on the kinetics of triple helix formation (Maher et al., 1990; Hampel et al., 1991; Rougée et al., 1992), stimulated us to examine the influence of cations and cation concentrations on the equilibrium constants for oligonucle-

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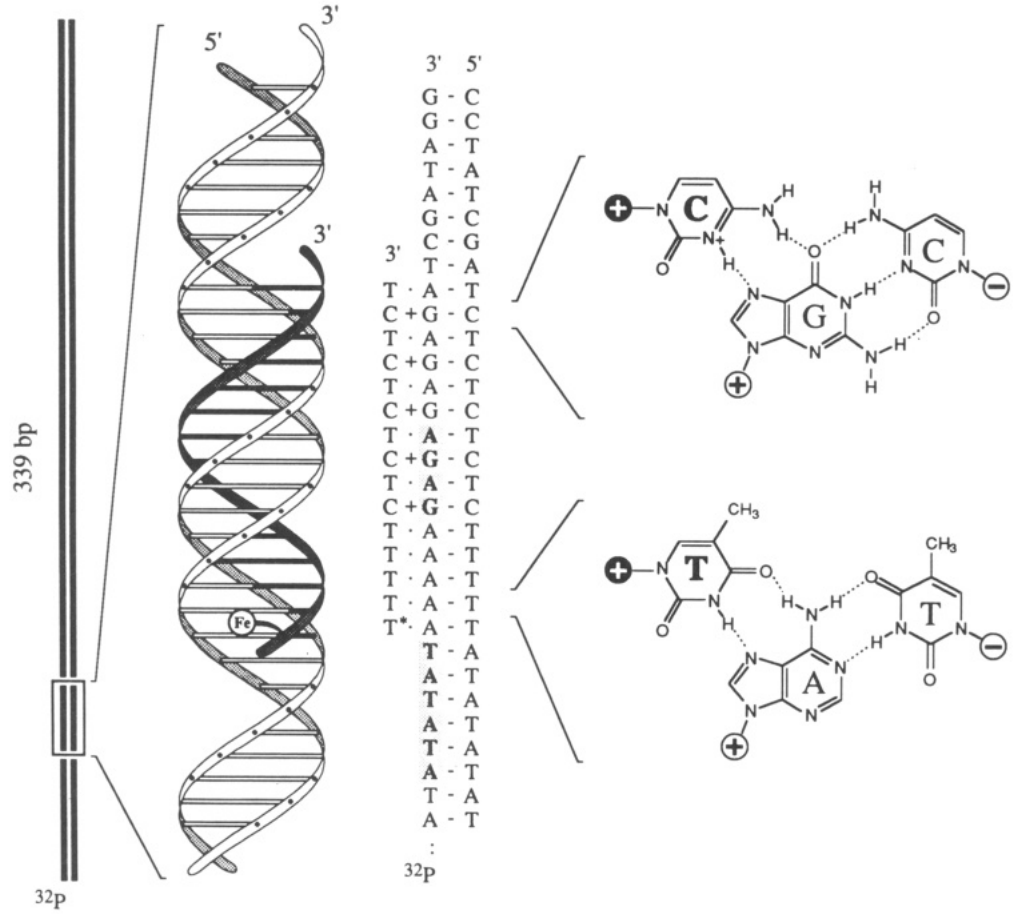

FIGURE 1: (Left) Ribbon model of the local triple-helical structure formed by the binding of the 15mer oligonucleotide to a 15-bp target sequence within a 339-bp end-labeled duplex. The Watson–Crick duplex strands are depicted as white ribbons, while the oligonucleotide–EDTA·Fe is depicted as a dark ribbon. The sequences modeled by the ribbons are shown in the center, where stippled boxes have been drawn around the 10 nucleotide positions cleaved most efficiently by the oligonucleotde–EDTA·Fe conjugate and used to obtain I_{site} values. (Right) Two-dimensional models depicting the C+GC and T·AT base triplets formed by Hoogsteen-type hydrogen bonding of N3-protonated C to a Watson–Crick GC base pair (top) and by Hoogsteen hydrogen bonding of T to a Watson–Crick AT base pair (bottom), respectively. The bases of the third strand are labeled with boldface type, and the bases of the Watson–Crick duplex are labeled with lightface type. The circles attached to N1 of the pyrimidines and to N9 of the purines indicate the positions of attachment to the sugar–phosphate backbone, while the plus and minus signs designate the relative 5′-to-3′ polarity of the strands.

otide-directed triple helix formation. Because potassium, magnesium, and spermine are thought to be the principle mono-, di-, and multivalent cations in eukaryotic cells, respectively, we have chosen to vary each of their concentrations separately in solutions containing all three cations. Using quantitative affinity cleavage titration (Singleton & Dervan, 1992a), we have measured association constants for the binding of the oligonucleotide 5′-d(T*TTTTCTCTCTCTCT)-3′ to a single 15-bp[1] site within a 339-bp plasmid fragment in solutions containing various concentrations of KCl, $MgCl_2$, and $SpmCl_4$ at pH 7.0 and 22 °C. Specifically, we have varied the concentration of potassium ion from 5 to 140 mM, that of magnesium ion from 0.1 to 10 mM, and that of spermine from 0.4 to 4 mM, because the cellular concentrations of these cations have been estimated to be near 140 mM potassium (Darnell et al., 1986), 1 mM magnesium (Darnell et al., 1986), and 1 mM spermine (Tabor & Tabor, 1976; Sarhan & Seiler, 1989).

EXPERIMENTAL PROCEDURES

Oligonucleotide Preparation. The oligonucleotide 5′-d(T*TTTTCTCTCTCTCT)-3′ was synthesized using standard automated solid-phase chemistry on an Applied Biosystems Model 380B DNA synthesizer. Protected thymidine and 2′-deoxycytidine *O*-cyanoethyl *N,N*-diisopropylphosphoramidites were purchased from ABI. The conjugate thymidine-EDTA (T*) phosphoramidite was prepared as described (Dreyer & Dervan, 1985) and incorporated at the 5′ end of the oligonucleotide with the EDTA carboxylates protected as their ethyl esters. Deprotection was carried out in 0.1 N NaOH solution at 55 °C for 36 h. The crude oligonucleotide bearing a dimethoxytrityl protecting group on its 5′-terminal hydroxyl was purified by reverse-phase FPLC using a ProRPC HR10/10 (C_2–C_8) column (Pharmacia LKB) and a gradient of 0–40% acetonitrile in 0.1 M triethylammonium acetate, pH 7.0; detritylated using 80% aqueous acetic acid; and chromatographically fractionated a second time. FPLC-purified oligonucleotide was desalted on a Pharmacia NAP-5 column in water and lyophilized from water twice. The concentration of single-stranded oligonucleotide was determined by UV absorbance at 260 nm. Aliquots of the oligonucleotide solution were lyophilized and stored dry at –20 °C.

End-Labeled DNA Preparation. The 5-^{32}P-labeled duplex DNA was prepared and purified as previously described (Singleton & Dervan, 1992a). The labeled DNA was resuspended in 5 mM Tris-HCl buffer (pH 8.0) at a final activity of 30 000 cpm·μL^{-1} for storage at 4 °C . A typical yield was 2 μg of the desired fragment with a total Cerenkov radioactivity of 6×10^6 cpm.

[1] Abbreviations: bp, base pair; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Spm, spermine; TBE, Tris–borate EDTA.

Stock Solutions of Buffer, Salts, and Carrier DNA. Spermine was purchased from Sigma as its tetrahydrochloride salt. A 5× stock solution of Tris–acetate (100 mM), NaCl (50 mM), KCl (25 mM), $MgCl_2$ (0.50 mM), and $SpmCl_4$ (2.0 mM) at pH 7.0 in Millipore water was prepared. Separate unbuffered aqueous stock solutions containing KCl (675 mM), $MgCl_2$ (49.5 mM), or $SpmCl_4$ (18.0 mM) were prepared. Sonicated, deproteinized calf thymus DNA (Pharmacia) was dissolved in unbuffered water to a final concentration of 2.0 mM in base pairs. All solutions were stored at 4 °C prior to use.

Quantitative Affinity Cleavage Titrations. The experimental protocol for the titrations has been published (Singleton & Dervan, 1992a,b). A solution containing labeled target DNA, buffer, salts, and carrier DNA was prepared by mixing approximately 200 000 cpm of 5′-end-labeled target DNA, the 5× stock solution containing Tris–acetate and salts, the appropriate volume of each salt solution, and enough water to give the desired final concentrations. This solution was allowed to equilibrate at room temperature for 15 min and was then distributed among sixteen 0.6-mL tubes in 32-μL aliquots. A dried pellet of the oligonucleotide–EDTA was dissolved in a solution of aqueous $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ to produce a solution that was 160 μM in oligonucleotide and 200 μM in Fe(II). The oligonucleotide–EDTA·Fe(II) solution was allowed to equilibrate for 15 min at room temperature and was then diluted serially in 200 μM Fe(II). To each reaction tube was added 4 μL of oligonucleotide–EDTA·Fe(II) at the appropriate concentration. The oligonucleotide–EDTA·Fe(II) and the DNA were allowed to equilibrate for 24 h at 22 °C. The cleavage reactions were initiated by the addition of 4 μL of a 10 mM aqueous DTT solution to each tube. The reactions were incubated for 6 h at 24 °C. Final reaction conditions in a 40-μL total volume were 20 mM Tris–acetate buffer at pH 7.0, 10 mM NaCl, 5–140 mM KCl, 0.1–10 mM $MgCl_2$, 0.4–4 mM $SpmCl_4$, 0.1 mM in base pairs of calf thymus DNA, 1 mM DTT, and approximately 15 000 cpm labeled duplex (the specific activity of the DNA varied slightly from experiment to experiment but was the same for each reaction within a given experiment, and always resulted in a final target site concentration of less than 0.1 nM). Precipitation of the DNA by the addition of glycogen, NaOAc, and $MgCl_2$ to final concentrations of 70 μg/mL, 280 mM, and 10 mM, respectively, followed by the addition of 2.5 vol of ethanol served to quench the cleavage. The precipitate was isolated by centrifugation and dissolved in 20 μL of H_2O. The solutions were frozen, and the water was removed by lyophilization. The DNA in each tube was resuspended in 5 μL of 1× TBE in 80% aqueous formamide. The DNA solutions were assayed for Cerenkov radioactivity by scintillation counting. The DNA was denatured at 90 °C for 4 min, and each sample was loaded onto an 8% denaturing polyacrylamide gel. The DNA cleavage products were electrophoresed in 1× TBE buffer at 50 $V \cdot cm^{-1}$.

Autoradiography and Data Reduction. Cleavage data were obtained using storage phosphor autoradiography as previously described (Singleton & Dervan, 1992a,b). The site-specific cleavage for each oligonucleotide–EDTA concentration was calculated using eq 1, where I_{tot} and I_{ref} are the cleavage intensities in the site and reference blocks, respectively:

$$I_{site} = I_{tot} - \lambda I_{ref} \quad (1)$$

For each experiment, λ was calculated from the average of the minimum I_{tot}/I_{ref} ratios near $\theta = 0$. A theoretical binding curve was fit to the experimental data using the apparent maximum cleavage (I_{sat}) and the K_T as adjustable parameters:

$$I_{fit} = I_{sat}\frac{K_T[O]_{tot}}{1 + K_T[O]_{tot}} \quad (2)$$

The difference between I_{fit} and I_{site} for all data points was minimized using the nonlinear least-squares fitting procedure of KaleidaGraph (version 3.0; Synergy Software) running on a Macintosh IIfx computer. All fits described in this paper were performed without weighting of the data points. All data points were included in the fitting procedure unless visual inspection of the computer image from a storage phosphor screen revealed a flaw at either the target site or the reference blocks, or unless the I_{site} value for a single lane was greater than 2 SE away from both values from the neighboring lanes. Data from experiments for which 80% or fewer of the lanes were usable were discarded. The goodness of fit of the binding curve to the data points was judged by the reduced χ^2 criterion (Bevington, 1969), and fits were judged acceptable for $\chi_\nu^2 \leq 1.5$.

Repeat experiments using a particular set of salt conditions were performed using different serial dilutions of oligonucleotide prepared from different aliquots of the original solution (*vide supra*), different preparations of 5′-end-labeled duplex DNA, and a uniquely prepared buffer solution. All K_T values reported in the text or tables are means of four experimental observations plus or minus the standard error of the mean.

RESULTS

Oligonucleotide–EDTA Equilibrium Association Constants. Previous experiments demonstrated that the equilibrium association constant (K_T) for the binding of an oligonucleotide–EDTA·Fe (O, eq 3) to an individual DNA site (D) to form a local triple-helical structure (T) can be measured using quantitative affinity cleavage titration (Singleton & Dervan, 1992a).

$$O + D \overset{K_T}{\rightleftharpoons} T \overset{k_{cl}}{\rightarrow} P_{cl} \quad (3)$$

Because a comprehensive description and analysis of this method has been presented, only a summary of the rationale and design of the experimental protocol is presented here. We have shown that the apparent fraction of duplex sites bound by oligonucleotide, θ_{app}, can be related to the intensity of bands proximal to the oligonucleotide binding site (I_{site}) on a storage phosphor autoradiogram of a polyacrylamide gel used to separate cleavage products (P_{cl}, eq 3) from intact duplex. Accordingly, for a series of affinity cleavage experiments in which the solution conditions, the concentration of duplex, the reaction volume, and the reaction time are constant, the following relationship holds:

$$\theta_{app} = I_{site}I_{sat}^{-1} \quad (4)$$

where I_{sat} is the intensity of cleavage produced when $\theta \approx 1$. Substitution using the equality expressed in eq 4 into the well-known relation between an equilibrium association constant and θ_{app}, followed by rearrangement, yields the following expression for I_{site} (Singleton & Dervan, 1992a):

$$I_{site} = I_{sat}\frac{K_T[O]_{tot}}{1 + K_T[O]_{tot}} \quad (5)$$

Hence, empirical titration binding isotherms can be constructed by measuring the site-specific affinity cleavage products afforded by the reaction of bound oligonucleotide–EDTA·Fe as a function of $[O]_{tot}$ to determine K_T from nonlinear least-squares analysis of the ($[O]_{tot}$, I_{site}) data points.

In previous work, we demonstrated that quantitative affinity cleavage titration affords equilibrium association constants

that are identical, within experimental uncertainty, to those obtained from the established DNase I footprint titration method of Brenowitz et al. (1986) (Singleton & Dervan, 1992a). Moreover, covalent attachment of EDTA·Fe to thymine at the 5′-terminus of an oligonucleotide does not have a measurable effect on the binding constant (Singleton & Dervan, 1992a). Recent work has demonstrated the utility of this technique for measuring oligonucleotide-directed triple helix formation constants as a function of pH and base substitution (Singleton & Dervan, 1992b), in systems of cooperatively binding oligonucleotides (Distefano & Dervan, 1993; Colocci et al., 1993), as a function of the nucleoside sugar moiety in each of the three strands (Han & Dervan, 1993), and in a purine·purine·pyrimidine triple helix (Stilz & Dervan, 1993).

Association Constants as a Function of Counterion Concentration. It is well established that the association of single-stranded nucleic acids in solutions of monovalent cations is strongly enhanced by increasing cation concentrations (Manning, 1978; Record et al., 1978, 1981). There is similar evidence demonstrating the stabilizing influence of monovalent cations on polymeric (Krakauer & Sturtevant, 1968; Lee et al., 1984; Latimer et al., 1989) and oligomeric triple-helical complexes (Plum et al., 1990; Shea et al., 1990; Durand et al., 1992; Rougée et al., 1992). To assess the influence of monovalent counterion concentration on the energetics of oligonucleotide-directed triple helix formation in mixed-salt solutions, the equilibrium association constant for an oligonucleotide–EDTA conjugate binding to a 339-bp DNA duplex containing a 15-bp target sequence (Figure 1) was measured in buffers containing different concentrations of KCl. At a given KCl concentration, ^{32}P-5′-end-labeled DNA (<5 pM) and various concentrations of oligonucleotide–EDTA·Fe (1 nM–16 μM) were mixed in buffer (5.0–140 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM $SpmCl_4$, 10 mM NaCl, and 20 mM Tris–acetate at pH 7.0) at 22 °C. Only after the association reactions had been allowed to reach equilibrium over 24 h was DTT (1 mM final concentration) added to initiate the EDTA·Fe-mediated cleavage chemistry. The reactions were allowed to proceed for 6 h at 22 °C, allowing a maximum site-specific cleavage yield of about 15%, and the products were separated by PAGE under strand-denaturing conditions. The amounts of radiolabeled DNA in the bands at the target cleavage site (Figure 1) and at a reference site were measured from storage phosphor autoradiograms, and I_{site} for each $[O]_{tot}$ was calculated using eq 1. The ($[O]_{tot}$, I_{site}) data points were fitted using a nonlinear least-squares method and eq 2, with K_T and I_{sat} as adjustable parameters.

The data points obtained for the 15mer oligonucleotide at KCl concentrations of 5, 25, 90, and 140 mM (in the presence of 20 mM Tris–acetate, 10 mM NaCl, 1.0 mM $MgCl_2$, and 1.0 mM $SpmCl_4$) were averaged from four experiments and are plotted along with average best-fit titration binding isotherms in Figure 2A. The mean K_T values obtained from the analyses of these data are contained in Table I. The value of K_T measured in the presence of 90 mM KCl (100 mM total monovalent cation concentration), 1 mM $MgCl_2$, and 1 mM $SpmCl_4$ is similar to that reported previously for the same triple helix at the same pH and temperature in the presence of 100 mM NaCl and 1 mM $SpmCl_4$ (Singleton & Dervan, 1992a). Remarkably, this value is 20-fold higher than that measured in the presence of 140 mM KCl. Overall, the measured K_T values demonstrate that increasing the concentration of KCl 28-fold, from 5.0 to 140 mM, causes a 100-fold *decrease* in the association constant. This trend of decreasing triple helix stability with increasing concentration of KCl is

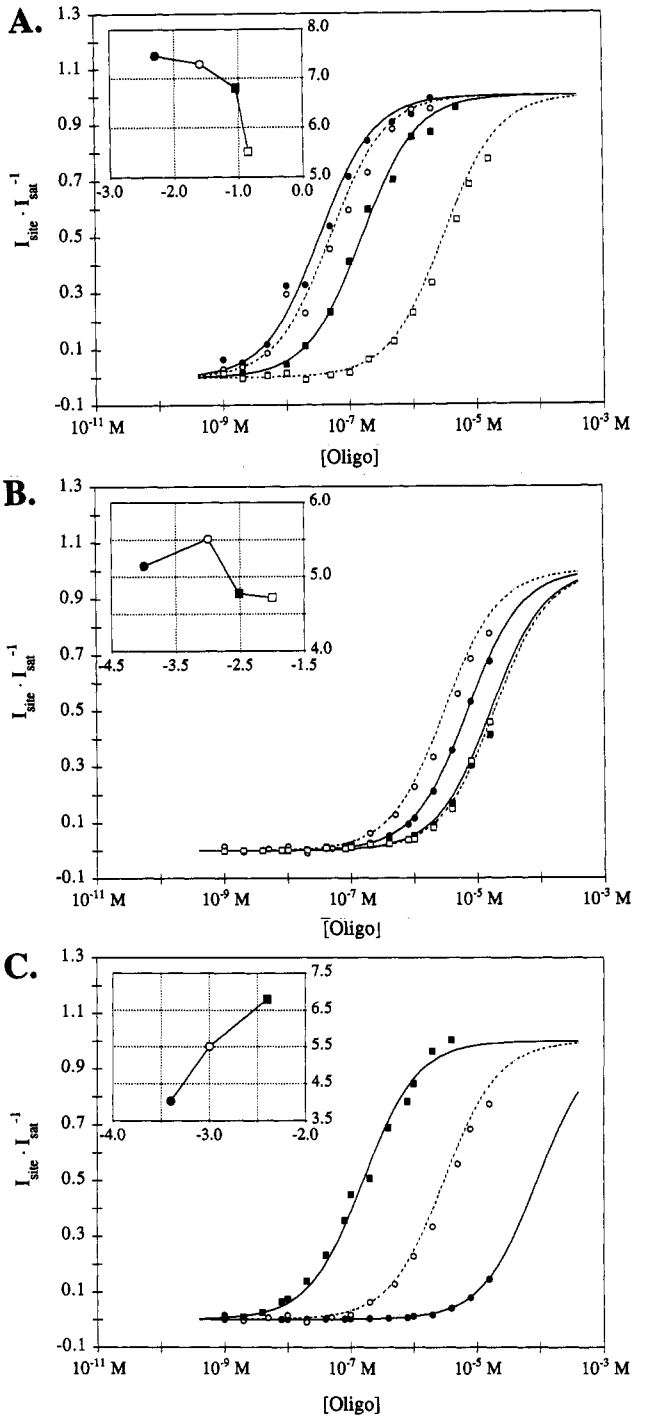

FIGURE 2: Data for quantitative affinity cleavage titrations performed in solutions containing 20 mM Tris–acetate (pH 7.0), 10 mM NaCl, 5–140 mM KCl, 0.1–10 mM $MgCl_2$, and 0.4–4 mM $SpmCl_4$ at 22 °C. The data points represent the average site-specific cleavage signal intensities from four experiments. The sigmoidal curves show the titration binding isotherms plotted using the mean values of K_T (Table I) and eq 2. The data points were normalized using I_{sat} from each experiment, and the binding curves were subsequently normalized using I_{sat} = 1 for eq 2. (A) The experimental binding isotherms were measured in solution containing 1.0 mM $MgCl_2$, 1.0 mM $SpmCl_4$, and either 5.0 (●), 25 (○), 90 (■), or 140 mM KCl (□). (A, inset) The apparent equilibrium association constant is plotted as a function of KCl concentration on a log–log scale. (B) The experimental binding isotherms were measured in solution containing 140 mM KCl, 1.0 mM $SpmCl_4$, and either 0.1 (●), 1.0 (○), 3.0 (■), or 10 mM $MgCl_2$ (□). (B, inset) The apparent equilibrium association constant is plotted as a function of $MgCl_2$ concentration on a log–log scale. (C) The experimental binding isotherms were measured in solution containing 140 mM KCl, 1.0 mM $MgCl_2$, and either 0.40 (●), 1.0 (○), or 4.0 mM $SpmCl_4$ (■). (C, inset) The apparent equilibrium association constant is plotted as a function of $SpmCl_4$ concentration on a log–log scale.

Table I: Counterion Concentration Dependence of the Equilibrium Association Constant for Oligonucleotide-Directed Triple Helix Formation at pH 7.0 and 22 °C[a]

variation of [KCl][b]		variation of [$MgCl_2$][c]		variation of [$SpmCl_4$][d]	
[KCl]	K_T	[$MgCl_2$]	K_T	[$SpmCl_4$]	K_T
5.0	$3.0 (\pm 0.8) \times 10^7$	0.10	$1.4 (\pm 0.3) \times 10^5$	0.40	1.1×10^4 [e]
25	$1.9 (\pm 0.8) \times 10^7$	1.0	$3.3 (\pm 1.4) \times 10^5$	1.0	$3.3 (\pm 1.4) \times 10^5$
90	$6.2 (\pm 1.8) \times 10^6$	3.0	$6.0 (\pm 2.1) \times 10^4$	4.0	$6.2 (\pm 1.0) \times 10^6$
140	$3.3 (\pm 1.4) \times 10^5$	10	$5.3 (\pm 0.3) \times 10^4$		

[a] The K_T values in the table are mean values (±SEM) of four independent measurements. The K_T values and salt concentrations are reported in M^{-1} and mmol·L^{-1} units, respectively. [b] The concentration of KCl was varied in aqueous buffer containing 20 mM Tris–acetate, 10 mM NaCl, 1.0 mM $MgCl_2$, and 1.0 mM $SpmCl_4$. [c] The concentration of $MgCl_2$ was varied in aqueous buffer containing 20 mM Tris–acetate, 10 mM NaCl, 140 mM KCl, and 1.0 mM $SpmCl_4$. [d] The concentration of $SpmCl_4$ was varied in aqueous buffer containing 20 mM Tris–acetate, 10 mM NaCl, 140 mM KCl, and 1.0 mM $MgCl_2$. [e] Of the four titration experiments performed to measure this association constant, three resulted in apparent association constants which were too small to be accurately measured ($\leq 10^4$ M^{-1}) and one resulted in an apparent association constant of 4.0×10^4 M^{-1}.

more clearly indicated by the plots of log K_T *versus* log[KCl] displayed in the inset of Figure 2A. Previous investigations have shown that θ_{app}, extracted from the extent of restriction endonuclease cleavage protection, decreases with increasing concentration of NaCl in the presence of $MgCl_2$ and $SpmCl_4$ at 37 °C (Maher et al., 1990; Hanvey et al., 1991).

To clarify the origin of this result, the concentrations of $MgCl_2$ and $SpmCl_4$ were independently varied between 0.10 and 10 mM and between 0.40 and 4.0 mM, respectively, in solutions containing 10 mM NaCl, 140 mM KCl (the least stabilizing concentration), and either 1.0 mM $SpmCl_4$ (for the magnesium experiments) or 1.0 mM $MgCl_2$ (for the spermine experiments) at pH 7.0 and 22 °C. The results of the experiments in which the magnesium ion concentration was varied are shown in Figure 2B. The observed equilibrium association constant (Table I) decreased whether the concentration of $MgCl_2$ was raised or lowered from 1.0 mM. Overall, as the concentration of $MgCl_2$ is increased 100-fold, from 0.10 to 10 mM, the association constant decreases 3-fold (Figure 2B, inset). In contrast, the result of increasing the spermine concentration 10-fold, from 0.40 to 4.0 mM (Figure 2C), is a greater than 500-fold *increase* in the observed association constant. Thus, in a solution containing potassium, magnesium, and spermine cations at or above millimolar concentrations, potassium ions are strongly inhibitory to triple helix formation, magnesium ions are slightly inhibitory, and the tetravalent spermine ion strongly enhances the stability of a local triple-helical structure.

DISCUSSION

The influence of the cationic environment on triple helix stability in solutions containing several cations of varying valence, conditions expected to be found within the cellular matrix (Darnell et al., 1986), is of considerable interest. In this series of experiments, we have used quantitative affinity cleavage titration to measure the effects of various cations on the stability of a local triple-helical complex in mixed-valence salt solutions of varying composition at pH 7.0 and 22 °C. Binding isotherms were measured in solutions containing sodium, potassium, magnesium, and spermine ions as the concentrations of K^+, Mg^{2+}, and Spm^{4+} were individually varied at constant concentrations of the other three cations. Analysis of the trends in the apparent equilibrium association constant allows us to elucidate the influence of each cation on triplex formation in the context of basic polyelectrolyte theory. The results indicate that the effect of a given counterion's concentration on the stability of a local triple-helical complex in solutions containing more than one type of counterion depends on the valence of that counterion. Specifically, increasing the concentration of the monovalent potassium ion from 5.0 to 140 mM in a solution containing 10 mM NaCl, 1.0 mM $MgCl_2$, and 1.0 mM $SpmCl_4$ at pH 7.0 and 22 °C results in a 100-fold decrease in the apparent equilibrium association constant for a 15mer binding to a single 15-bp homopurine·homopyrimidine site in a 339-bp plasmid fragment. Increasing the concentration of divalent magnesium ions from 0.1 to 10 mM in a solution containing 10 mM NaCl, 140 mM KCl, and 1.0 mM $SpmCl_4$ results in a 3-fold decrease in the association constant for the same system. The equilibrium constant for this association reaction is increased nearly 500-fold when the concentration of the spermine tetracation is raised from 0.40 to 4.0 mM in the presence of 10 mM NaCl, 140 mM KCl, and 1.0 mM $MgCl_2$.

It is important to note that related trends have been observed in the thermal denaturation behavior of duplex DNA in solutions containing both Na^+ and either Mg^{2+} (Dove & Davidson, 1962; Manning, 1972; De Marky & Manning, 1975; Record, 1975) or a polyamine (Thomas & Bloomfield, 1984). In these solutions, the melting temperature of the DNA decreases with increasing [Na^+] until the melting temperature reaches a minimum at a critical sodium ion concentration (near 10 and 100 mM in the presence of Mg^{2+} and Spm^{4+}, respectively). For increasing [Na^+] above the critical concentration, the DNA melting behavior is similar to that of the DNA in solution free of oligovalent cations. The results for both oligonucleotide-directed triple helix formation and duplex DNA melting are consistent with competition among the different cations for nucleic acid phosphate binding sites and valence-specific abilities of the cations to stabilize the triple helix.

The Counterion Condensation Model. The molecular counterion condensation model developed by Manning (1978) and elaborated by Record and co-workers (Record et al., 1978), although an approximation to the real behavior of linear polyelectrolytes in solution, is a theoretical framework that provides reasonable descriptions of the interactions of small cations with nucleic acids and their thermodynamic consequences for nucleic acid conformational transitions (Record et al., 1981; Anderson & Record, 1982; Lohman, 1985). In the presence of a single type of counterion, the association of a single-stranded nucleic acid molecule with a duplex to form a triple-helical complex results in an overall increase in the linear charge density of the nucleic acid species in solution. Hence, triple helix formation is accompanied by the condensation of free cations (decreased entropy) and a decrease in the repulsive electrostatic free energy. At monovalent cation concentrations below 1 M, the first term is dominant, and although it destabilizes the triple helix, it decreases in magnitude with increasing cation concentration. Therefore, the triplex is stabilized relative to its unbound components when the bulk concentration of the counterion is increased. In accord with these expectations, the stabilities of polymeric

(Krakauer & Sturtevant, 1968; Krakauer, 1974; Lee et al., 1984; Latimer et al., 1989) and oligomeric triplexes (Pilch et al., 1990; Plum et al., 1990; Shea et al., 1990; Durand et al., 1992; Rougée et al., 1992) in buffers containing single alkali or alkaline earth metal ions are found to increase with increasing concentrations of the metal ions.

In mixed-valence salt solutions, the situation is more complicated than that found in solutions containing only nucleic acid and a single metal cation. The equilibrium between oligonucleotide, duplex, and triplex in the presence of three counterions of different valence can be written

$$O + D + iK^+ + jMg^{2+} + kSpm^{4+} \overset{K_T^\circ}{\rightleftharpoons} T \qquad (6)$$

where i, j, and k represent the numbers of K^+, Mg^{2+}, and Spm^{4+} ions, respectively, thermodynamically bound per phosphate during the association reaction; and n is the length of the oligonucleotide. The thermodynamic equilibrium constant for this reaction can be written

$$K_T^\circ = \frac{a_T}{a_D a_O} a_{K^+}^{-ni} a_{Mg^{2+}}^{-nj} a_{Spm^{4+}}^{-nk} \qquad (7)$$

were a values are activities. The apparent equilibrium constant for eq 6 is simply

$$K_T = [T]/[D][O] \qquad (8)$$

It follows from eqs 7 and 8 that

$$\ln K_T = \ln K_T^\circ + n\{i \ln[K^+] + j \ln[Mg^{2+}] + k \ln[Spm^{4+}]\} - \ln(\gamma_T/\gamma_D\gamma_O) \qquad (9)$$

where γ values are activity coefficients. Record (1975) has shown that, when the magnitudes of the activity coefficients originate from the purely electrostatic interactions between the nucleic acid species and its counterions, eq 9 can be rewritten

$$\ln K_T = \ln K_T^\circ + n\{i \ln[K^+] + j \ln[Mg^{2+}] + k \ln[Spm^{4+}] - \eta' \ln \kappa\} \qquad (10)$$

where κ is the Debye–Hückel screening parameter (proportional to the square root of the ionic strength), and the factor η is defined by

$$\eta' \equiv \left[\frac{1}{3}\left(\frac{\xi_{net}^2}{\xi}\right)_O + \frac{2}{3}\left(\frac{\xi_{net}^2}{\xi}\right)_D\right] - \left(\frac{\xi_{net}^2}{\xi}\right)_T \qquad (11)$$

where ξ is the dimensionless structural charge density parameter for the nucleic acid molecule, and ξ_{net} is the effective value of ξ after counterion condensation has been considered. For idealized single-, double-, and triple-stranded DNA, the linear charge spacings are 4.3, 1.7, and 1.1 Å, respectively. Hence, the values of ξ were taken to be 1.7, 4.2, and 6.6, respectively, for each of the conformations. In the mixtures of mono-, di-, and tetravalent cations, the value of ξ_{net} should be in the range $1 \geq \xi_{net} \geq 0.25$ and depends on the exact counterion composition (Manning, 1972; De Marky & Manning, 1975). The factor η indicates differential screening of the phosphate charges between the free and bound states of the nucleic acid system, and larger positive values of η represent better relative screening of the charge repulsions in the triple helix.

The apparent equilibrium constant, K_T, will be a function of all three cation concentrations:

$$\frac{\partial \ln K_T}{\partial \ln[K^+]} = i + \frac{\partial i}{\partial \ln[K^+]} \ln[M^+] + \frac{\partial j}{\partial \ln[K^+]} \ln[Mg^{2+}] + \frac{\partial k}{\partial \ln[K^+]} \ln[Spm^{4+}] - \frac{\partial \ln \kappa}{\partial \ln[K^+]} \eta' - \frac{\partial \eta'}{\partial \ln[K^+]} \ln \kappa \qquad (12a)$$

$$\frac{\partial \ln K_T}{\partial \ln[Mg^{2+}]} = j + \frac{\partial i}{\partial \ln[Mg^{2+}]} \ln[K^+] + \frac{\partial j}{\partial \ln[Mg^{2+}]} \ln[Mg^{2+}] + \frac{\partial k}{\partial \ln[Mg^{2+}]} \ln[Spm^{4+}] - \frac{\partial \ln \kappa}{\partial \ln[Mg^{2+}]} \eta' - \frac{\partial \eta'}{\partial \ln[Mg^{2+}]} \ln \kappa \qquad (12b)$$

$$\frac{\partial \ln K_T}{\partial \ln[Spm^{4+}]} = k + \frac{\partial i}{\partial \ln[Spm^{4+}]} \ln[K^+] + \frac{\partial j}{\partial \ln[Spm^{4+}]} \ln[Mg^{2+}] + \frac{\partial k}{\partial \ln[Spm^{4+}]} \ln[Spm^{4+}] - \frac{\partial \ln \kappa}{\partial \ln[Spm^{4+}]} \eta' - \frac{\partial \eta'}{\partial \ln[Spm^{4+}]} \ln \kappa \qquad (12c)$$

The effect of a particular cation's concentration on K_T originates from a combination of the negative entropy of condensation of cations during triple helix formation and the electrostatic free energy change which results from phosphate charge neutralization during the transition (*vide infra*). The former effect is represented in the first four terms in eqs 12a–c, while the latter effect is represented by the last two terms.

In order to examine the observed counterion effects semiquantitatively using eqs 12a–c, we have estimated binding densities for each of the cations on idealized triple-helical, double-helical, and single-stranded DNA under the experimental salt conditions. By analyzing the changes in the estimated cation binding densities at different cation concentrations, it is possible to compare the signs and the relative magnitudes of the terms in eqs 12a–c, as well as the overall signs of the derivatives of $\ln K_T$. The values of the association constants for Mg^{2+} and Spm^{4+} binding to each nucleic acid species were calculated from the following equations:

$$\log K_{Mg} = 2\psi(1 - 4\theta_{Spm}) \log[K^+] + \log K_{Mg}^\circ \qquad (13a)$$

$$\log K_{Spm} = 4\psi(1 - 2\theta_{Mg}) \log[K^+] + \log K_{Spm}^\circ \qquad (13b)$$

where ψ is the thermodynamic monovalent cation binding parameter for a particular nucleic acid conformation, θ-values are oligovalent cation binding densities, and values of K° represent oligovalent cation binding constants at 1 M monovalent cation concentration (Record et al., 1978). From eqs 13a,b, it is clear that cation binding is competitive, *i.e.*, binding of one oligovalent cation reduces the apparent binding constants of other cations. Initial estimates of K_{Mg}° and K_{Spm}° were made using eqs 13a,b with $\theta_{Mg} = \theta_{Spm} = 0$ and $\log K_{Mg} = \log K_{Spm}^\circ = 0$. On the basis of the computed values of the association constants and the assumed binding site sizes of $n_{Mg} = 2$ and $n_{Spm} = 4$, binding densities for Mg^{2+} and Spm^{4+} were calculated using the model of McGhee and von Hippel (1974) for ligands binding to overlapping sites on a uniform linear lattice of phosphates. This model has been shown to fit experimental data for the binding of Mg^{2+} and Spm^{4+} to duplex DNA adequately (Braunlin et al., 1982). The values of K_{Mg} and K_{Spm} were then adjusted to account for the binding densities of Spm^{4+} and Mg^{2+}, respectively. This process was repeated until values of K_{Mg} and K_{Spm} from successive iterations differed by less than 1%. Equilibrium constants for Spm^{4+}

binding to duplex DNA calculated in this manner were in good agreement with those predicted using an empirically derived relation (Braunlin et al., 1982). The spermine–single strand binding constants were about 10-fold lower than those for duplex binding, in agreement with experiment (Morgan et al., 1986), while spermine–triplex binding was about 1.5-fold stronger than spermine–duplex binding, in accord with the difference in the charge densities of the DNA complexes (Murray & Morgan, 1973; Record et al., 1978). As a result of competition with Spm^{4+}, the estimated binding constants for Mg^{2+} interacting with all three DNA species were about 10-fold lower than predicted (Braunlin et al., 1982; Morgan et al., 1986). Following calculation of the binding densities of Spm^{4+} and Mg^{2+}, the thermodynamic binding density of K^+ was corrected for the number of potassium ions displaced by Mg^{2+} and Spm^{4+} (Record et al., 1978).

Influence of Potassium Ion Concentration on the Association Constant. It is clear that neither Na^+ nor K^+ is intrinsically destabilizing to double- or triple-helical nucleic acids because, in the presence of these ions alone and in the presence of high concentrations of these ions with oligovalent cations, the complex stabilities are enhanced by increasing the monovalent cation concentration. Rather, the observed trend of decreasing triplex stability with increasing $[K^+]$ up to 140 mM in the presence of millimolar magnesium and spermine results from the influence of these oligovalent cations on the triplex formation equilibrium. The ability of a counterion to stabilize a nucleic acid complex of higher charge density increases with the charge on the counterion (Manning, 1972; Morgan et al., 1986). Thus, Spm^{4+} is more stabilizing to the triple helix than is Mg^{2+}, which, in turn, is more stabilizing than K^+. The effect of spermine is great enough that its presence at micromolar concentration effects the complete dismutation of polymeric DNA (Hampel et al., 1991) and RNA duplexes (Glaser & Gabbay, 1968) to the corresponding triplexes plus single strands at neutral pH.

At low ionic strengths, spermine binds with high affinity ($K_{Spm} > 10^5\ M^{-1}$) to all three DNA species and dominates the counterion condensation. At $[Spm^{4+}] = 1.0$ mM and $[M^+] = 15$ mM, all three DNA species are nearly saturated by Spm^{4+} and the triple helix is particularly stable ($K_T \approx 10^8\ M^{-1}$). Because there is little change in Spm^{4+} condensation upon triplex formation, there is little change in the overall degree of counterion condensation or in the electrostatic free energy, and K_T is near a maximum. As the concentration of K^+ increases, the equilibrium constant for Spm^{4+} binding to each of the DNA species decreases; however, because the spermine–triplex binding constant is always higher than the other two, there is a net condensation of Spm^{4+} and the entropy of condensation becomes more negative (fourth term of eq 12a). Moreover, the net difference in counterion binding to the triplex versus the duplex and the oligonucleotide results in a decrease in the electrostatic free energy of association ($\eta > 0$ and increases with increasing $[K^+]$). This effect (fifth and sixth terms of eq 12a) stabilizes the triplex but decreases in magnitude with increasing ionic strength. The result of increasing $[K^+]$ in the presence of magnesium and spermine is therefore a reduction in the apparent equilibrium constant, K_T.

Above 0.5 M potassium ion concentration, Spm^{4+} binds poorly to all three DNA species, K^+ significantly contributes to the net counterion condensation, and K_T should increase with further increases in $[K^+]$. However, K^+ is sufficiently weaker than Spm^{4+} for triple helix stabilization that no site-specific affinity cleavage is detected at a potassium ion concentration of 500 mM (data not shown). In fact, it has been demonstrated that the oligonucleotide used here binds to 15 bp within a 21-bp target duplex with an equilibrium constant of only 10 M^{-1} at 25 °C and pH 6.5 in the presence of 200 mM NaCl without spermine (Plum et al., 1990).

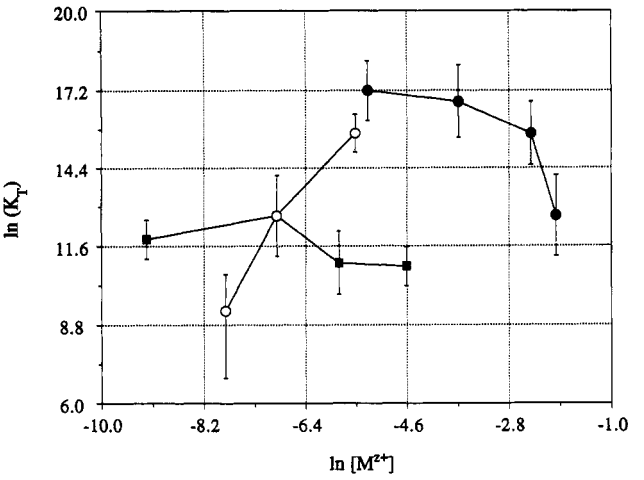

FIGURE 3: Plot of the natural logarithms of the mean association constants as a function of the natural logarithm of the concentration of KCl (●), $MgCl_2$ (■), or $SpmCl_4$ (○) for comparison with eq 12 (see text). The error bars represent estimated confidence limits.

Influence of Spermine Ion Concentration on the Association Constant. At all concentrations of Spm^{4+} investigated, the tetracation is preferentially condensed upon triple helix formation, resulting in higher values of K_T at higher $[Spm^{4+}]$. The extent of net Spm^{4+} condensation decreases from 0.40 to 4.0 mM because the ion begins to saturate all forms of the DNA, and the entropic penalty (fourth term of eq 12c) is reduced. Concurrently, the differential screening parameter (sixth term of eq 12c) becomes smaller. These two effects add together to give the large positive slope for the data plotted in Figure 3. The condensation of Spm^{4+} is predicted to reach a minimum ($k \approx 0$) at a spermine concentration above 10 mM; however, precipitation of the DNA at elevated spermine concentrations prevents measurement of the association constant.

Influence of Magnesium Ion Concentration on the Association Constant. The stabilization of the triple helix by Mg^{2+} is intermediate between the stabilizations afforded by Spm^{4+} and K^+, and the influence of $[Mg^{2+}]$ is likewise intermediate between those of $[Spm^{4+}]$ and $[K^+]$. Over the range of magnesium ion concentrations studied, five of the six terms in eq 12b have the same sign and magnitude as the corresponding terms in eq 12a. The exception is the screening parameter η (the last term), whose magnitude changes less dramatically with changes in $[Mg^{2+}]$ than with changes in $[K^+]$. For $[Mg^{2+}] \leq [Spm^{4+}]$, the condensation is dominated by Spm^{4+} and the effect of $[Mg^{2+}]$ is neglible ($\partial\eta/\partial\ln[Mg^{2+}] \approx 0$). As $[Mg^{2+}]$ increases, it competes effectively with Spm^{4+} and the stability of the triplex is diminished; however, because Mg^{2+} is more stabilizing than K^+, this decrease in triple helix stability is less dramatic than that caused by increasing $[K^+]$. At concentrations of Mg^{2+} above 10 mM, the triplex is expected to be stabilized by increasing the concentration of this cation because the apparent equilibrium fraction of a target duplex bound by a 21mer oligonucleotide was observed to increase from 20 to 160 mM Mg^{2+} in the presence of 600 mM Na^+ and 0.4 mM Spm^{4+} (Maher et al., 1990).

Implications for Oligonucleotide-Directed Triple Helix Formation. The observed influence of changes in the cationic environment on K_T demonstrates the importance of millimolar spermine concentrations or >10 mM magnesium concentrations for achieving equilibrium constants on the order of 10^6

M^{-1} or higher for oligonucleotide-directed triple helix formation near neutral pH in the presence of monovalent cations at a concentration of 150 mM. This dramatic linkage between the oligonucleotide association constant and the concentrations and valences of the counterions in solution is a consequence of the increase in charge density that accompanies oligonucleotide binding. The observations that the dependence of polymer triplex thermal denaturation temperature on $[Na^+]$ decreases as the fraction of C·GC triplets increases (Lee et al., 1984; Latimer et al., 1989) and that protonation of C·GC sites plays a crucial role in triplex stability (Singleton & Dervan, 1992b; Völker et al., 1993) demonstrate the importance of reducing the increase in charge density in order to stabilize a triplex. It is important that protonation of C·GC sites in the triplex accompanies binding of an unprotonated single strand near neutral pH because the protonation specifically stabilizes the triplex relative to the unbound components.

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