# Effects of Chain Length Modification and Bis(ethyl) Substitution of Spermine Analogs on Purine−Purine−Pyrimidine Triplex DNA Stabilization, Aggregation, and Conformational Transitions†

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ABSTRACT: The natural polyamines—putrescine, spermidine, and spermine—are known to stabilize pyrimidine−purine−pyrimidine and purine−purine−pyrimidine triplex DNA formation. We studied the ability of two tetramine and two pentamine analogs of spermine and their bis(ethyl) derivatives to stabilize triplex DNA formation between 5′-$TG_3TG_4TG_4TG_3T$-3′ and its target duplex probe, consisting of the oligonucleotides 5′-$TCGAAG_3AG_4AG_4AG_3A$-3′ and 5′-$TCGATC_3TC_4TC_4TC_3T$-3′. We used electrophoretic mobility shift assay (EMSA), melting temperature ($T_m$) measurements, and circular dichroism (CD) spectroscopy to evaluate the effects of these novel polyamine analogs on triplex DNA stability, dissociation constants, aggregation, and conformation. In general, pentamines were more efficacious than tetramines in stabilizing triplex DNA, although most of the polyamines with pendant free amino groups caused DNA aggregation below 50% conversion to triplex DNA. Ethyl substitution of these pendant amino groups lowered their efficacy approximately 2-fold in stabilizing triplex DNA; however, this effect was more than compensated for by the lack of DNA aggregation in the presence of bis(ethyl)polyamines. A concentration-dependent increase in the $T_m$ of triplex DNA was observed in the presence of polyamines. CD spectral measurements showed distinct differences in the conformation of triplex DNA stabilized in the presence of polyamines compared to the CD spectra of the oligonucleotides alone. Temperature-dependent CD spectra of triplex DNA showed monophasic melting in the absence and presence of polyamines, suggesting duplex/triplex → single-stranded DNA transition. These results indicate that structural modifications of polyamines is an effective strategy to develop triplex DNA-stabilizing ligands, with potential applications in antigene therapeutics.

Triple-helical nucleic acid structures were first discovered approximately 40 years ago in synthetic polyribonucleotides consisting of purine and pyrimidine strands (Felsenfeld et al., 1957). In recent years, triplex DNA formation by the binding of oligonucleotides to duplex DNA has attracted much attention as an approach to develop sequence-specific DNA cleavage agents and to suppress the transcription of disease-related genes (Beal & Dervan, 1991; Cooney et al., 1988; Crooke, 1992; Duval-Valentin et al., 1992; Francois et al., 1988; Frank-Kamenetskii & Mirkin, 1995; Hélène, 1991; Plum et al., 1995; Rajagopal & Feigon, 1989; Wells et al., 1988). Recognition of a duplex of purine (Pu)·pyrimidine (Py) tract by an oligopyrimidine or oligopurine single strand leads to the formation of Hoogsteen or reverse Hoogsteen hydrogen bonding and the occupation of the major groove of the duplex by the oligonucleotide. Base triplets that could be formed under different conditions include Py·PuPy form, as in T·AT and $C^+$·GC, or PyPu·Pu form, as in CG·G and TA·A. Because the $C^+$·GC triplet requires protonated cytosines, triplex formation, in this case, requires low pH. On the other hand, Py·Pu·Pu triplex does not have this requirement and is stabilized by metal ions and/or polyamines under neutral pH (Frank-Kamenetskii & Mirkin, 1995; Hampel et al., 1991; Hélène, 1991; Plum et al., 1995; Singleton & Dervan, 1993; Thomas & Thomas, 1993).

Natural polyamines—putrescine [$H_2N(CH_2)_4NH_2$], spermidine [$H_2N(CH_2)_4NH(CH_2)_3NH_2$], and spermine [$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$]—are ubiquitous cellular components and are known to play an important role in cell proliferation and differentiation (Tabor & Tabor, 1984).

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Under physiological ionic and pH conditions, polyamines are positively charged and hence the negative phosphate groups of DNA are their prime target of action (Thomas & Bloomfield, 1983; Wilson & Bloomfield, 1979). Thus polyamines come under the category of ligands that stabilize triplex DNA at least in part by their ability to neutralize the high negative charge density imposed by the association of the three DNA strands. Stabilization of duplex DNA by polyamines has long been recognized (Basu et al., 1990; Basu & Marton, 1987; Tabor, 1962; Thomas & Bloomfield, 1984). Molecular modeling, X-ray crystallography, and solution structural studies have provided evidence for polyamine−DNA interactions (Egli et al., 1991; Feuerstein et al., 1986; Liquori et al., 1967; Thomas & Bloomfield, 1983; Wilson & Bloomfield, 1979). Polyamine structural effects are not observed in the stabilization of duplex DNA, whereas the number of methylene groups separating the charged amino and imino groups is an important factor governing the efficacy of polyamines in the stabilization of Z-DNA and triplex DNA ((Basu & Marton, 1987; Tabor, 1962; Thomas & Bloomfield, 1984; Thomas & Messner, 1988; Thomas & Thomas, 1993).

Triplex-forming sequences consisting of Pu·Py tracts are present in the regulatory regions of several genes and represent up to 1% of eukaryotic genomes studied (Manor et al., 1988; Tripathi & Brahmachari, 1991). Triplex formation at the promoter region of several genes is being investigated as a strategy to develop an antigene therapy for cancer, inflammation and acquired immunodeficiency syndrome (AIDS) (Cooney et al., 1988; Ebbinghaus et al., 1993; Gee et al., 1992; Grigoriev et al., 1992; McShan et al., 1992; Orson et al., 1991; Postel et al., 1991). For example, Porumb et al. (1996) reported that the expression of human oncogene *HER2* was inhibited by a 28-base-pair triplex-forming oligonucleotide when administered in cells as a complex with lipofectin. Thomas et al. (1995a) found that 1,6-diaminopropane could be utilized to stabilize triplex DNA in the promoter region of the c-*myc* gene in cultured cells and thereby downregulate the expression of c-*myc* mRNA. This putrescine homologue was selected on the basis of physical−chemical studies showing the selectivity of di- and trivalent polyamine analogues to stabilize triplex DNA *in vitro* (Thomas & Thomas, 1993).

Despite these exciting developments, triplex DNA-based antigene approach is still in its infancy because of several practical considerations, including the thermodynamic stability of triplex DNA, the slow rate of oligonucleotide uptake within the cell, and the nuclease sensitivity of oligonucleotides (Stein, 1995; Wagner, 1995). Several approaches have been used to maximize the uptake and stability of oligonucleotides and triplex DNA *in vivo*. These include conjugation of the oligonucleotide with cross-linking or intercalating agents (Grigoriev et al., 1993; Tung et al., 1993; Wilson et al., 1993), modification of the oligonucleotides to contain a 3′-propylamine group (Durland et al., 1991) or a cholesterol moiety (Ing et al., 1993), use of cationic lipids or polylysine to enhance uptake (Bennett et al., 1992; Stevenson & Iverson, 1989), and modifications of oligonucleotide backbone by replacement of the phosphate oxygen with sulfur (phosphorothioate oligonucleotides) (Hacia et al., 1994; Uhlmann & Peyman, 1990). Alternatively, optimization of triplex DNA stability might be achieved by variations in oligonucleotide length and sequence (Kandimalla & Agrawal, 1995; Vo et al., 1995). Often oligonucleotide modifications and specific ligands are designed to alter the conformational and configurational flexibility of the oligonucleotide and clamp the oligonucleotide within the major groove of double-helical DNA.

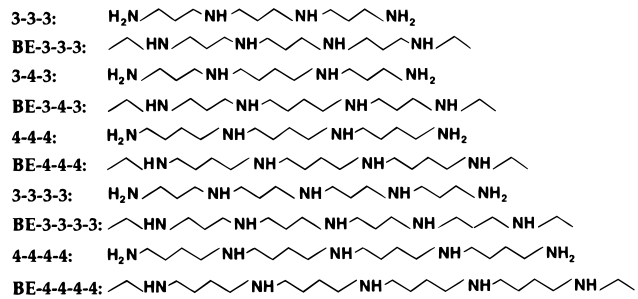


FIGURE 1: Chemical structure of polyamine analogs used in this study.

We used bis(ethyl)-substituted and unmodified tetra- and pentavalent analogs of spermine to stabilize purine−purine−pyrimidine triplex DNA formation. Our results show that bis(ethyl)polyamines can act as efficient ligands to stabilize triplex DNA without the disadvantage of DNA aggregation caused by the free polyamines.

## MATERIALS AND METHODS

*Polyamines.* Spermine tetrahydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO). 1,11-Diamino-4,8-diazaundecane (norspermine, 3-3-3), $N^1$,$N^{11}$-bis(ethyl)-norspermine (BE-3-3-3), $N^1$,$N^{12}$-bis(ethyl)spermine (BE-3-4-3), 1,14-diamino-5,10-diazatetradecane (homospermine, 4-4-4), $N^1$,$N^{14}$-bis(ethyl)homospermine (BE-4-4-4), 1,15-diamino-4,8,12-triazapentadecane (3-3-3-3), 1,15-bis(ethylamino)-4,8,12-triazapentadecane (BE-3-3-3-3), 1,19-diamino-5,10,15-triazanonadecane (4-4-4-4), 1,19-bis(ethylamino)-5,-10,15-triazanonadecane (BE-4-4-4-4) were synthesized according to procedures described earlier (He et al., 1994; Igarashi et al., 1995). The structures and purity of all polyamines were confirmed by elemental analysis, NMR, HPLC, and mass spectrometry. The chemical structures and abbreviations of these compounds are shown in Figure 1. Concentrated solutions of the polyamines were prepared in 10 mM sodium cacodylate buffer, pH was adjusted to 7.2, and small volumes were added to oligonucleotide solutions to make up the necessary concentrations.

*Oligodeoxyribonucleotide and DNA Probe.* The triplex-forming oligonucleotide (ODN1), containing the sequence 5′-TGGGTGGGGTGGGGTGGGT-3′, was synthesized by phosphoramidite chemistry on a Millipore Cyclone DNA synthesizer. Further purification was by *n*-butanol precipitation (Sawadogo & Van Dyke, 1991). Oligonucleotide concentration was determined spectrophotometrically using an average nucleotide molar extinction coefficient at 260 nm of $3.3 \times 10^5$ $M^{-1}$ $cm^{-1}$. The duplex DNA probe (T4) used in assaying triplex formation was composed of the oligonucleotides 5′-TCGAAGGGAGGGGAGGGGAGGGA-3′ and 5′-TCGATCCCTCCCCTCCCCTCCCT-3′ and was $^{32}$P-radiolabeled by Klenow end-filling and purified by ethanol precipitation.

*Electrophoretic Mobility Shift Assays.* For our standard EMSA reaction condition, 1 nM duplex DNA probe and 10 nM ODN1 were incubated for 60 min at 30 °C in a 10-$\mu$L volume containing 40 mM Tris·HCl (pH 7.3, 30 °C), 0.01%

Nonidet P-40, and various polyamine concentrations as indicated in the figure legends. Samples were loaded onto a 6%/0.2% acrylamide/bisacrylamide gel containing 50 mM Tris−borate, 2 mM $MgCl_2$, and 5% glycerol and electrophoresed at 10 V/cm for 3 h at room temperature. DNA species were visualized by autoradiography and quantitated by densitometry using a ScanJet IIcx flatbed scanner (Hewlett-Packard) and NIH Image v1.52 software.

*$T_m$ Measurements.* Absorbance versus temperature heating curves were obtained using a Perkin-Elmer Lambda-2 spectrophotometer (Thomas & Thomas, 1993). The temperature of the five-cell holder was regulated by a thermoelectrically regulated programmer, interfaced to an IBM PS2 computer. The triplex-forming oligonucleotide (ODN1) and duplex target (T4) were dialyzed from 10 mM sodium cacodylate buffer (pH 7.2), mixed in a 1:1 molar ratio, heated to 95 °C for 5 min, cooled to room temperature, and allowed to equilibrate for 16 h. The melting profiles were obtained by increasing the temperature at a rate of 0.5 °C/min with the absorbance and temperature being recorded every 30 s. $T_m$ was taken as the temperature corresponding to half dissociation of the complexes and the reproducibility was within ± 0.5 °C. The first derivative, $dA/dT$ (where $A$ is the absorbance and $T$ is the temperature), of the melting curve was computer-generated and was also used for determining the $T_m$. $T_m$ values obtained from both methods did not differ by >0.5 °C.

*CD Spectral Measurements.* CD measurements were performed on an Aviv Model 62 D circular dichroism spectrophotometer (Aviv Associates, Lakewood, NJ). Rectangular quartz cuvette (Uvonics, New York, NY) with 1-cm optical path lengths were used. Polynucleotide solutions for CD measurements were prepared in a manner similar to that used for UV melting. The CD spectra were recorded from 350 to 200 nm at 25 °C and normalized by subtraction of the background scan with buffer. The molar ellipticity was calculated from the equation $[\theta] = \theta/cl$, where $\theta$ is the relative intensity, $c$ is the molar concentration of oligonucleotides, and $l$ is the path length of the cell in centimeters. To determine changes in CD profiles of triplex DNA as a function of temperature in the absence and presence of polyamines, spectra of oligonucleotides were collected between 0 and 90 °C with 10 °C increments with 5 min of equilibration at each temperature. Spectra were collected between 350 and 210 nm with bandwidths at 1-nm intervals. Spectra at each time point were collected for 1−2 s.

*Deconvolution of the CD Spectra.* The CD spectra of triplex DNA recorded in the absence and presence of polyamines were deconvoluted into two, three, or four basis spectra using a convex constraint analysis program developed by Perczel et al. (1991, 1992). This algorithm allows the deconvolution of sets of spectra into component curves without prior knowledge of the structures that contribute to the chiral elements. In this method, the fractional weights of all components must be positive and the sum of the weights is constrained to equal one. In using the program, one inputs the desired number of basic curves. The program finds the best set of curves which can be combined to generate the entire data set. If the number of requested curves exceeds the number of natural components needed to generate the spectra, one or more of the basis spectra will degenerate. The change in the weights of the component spectra thus generated were plotted to give a curve showing the fraction of individual solution species as a function of temperature. This curve was utilized to determine the $T_m$ from temperature-dependent CD spectral measurements.

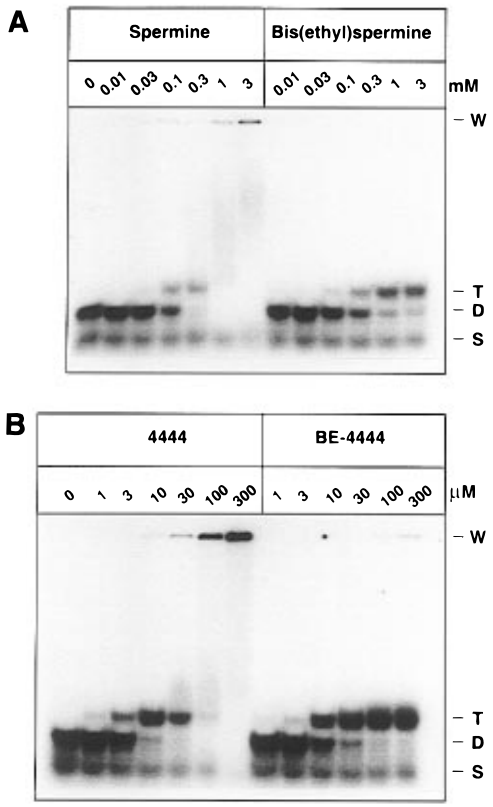


FIGURE 2: Facilitation of purine-motif triplex formation by different polyamines. Shown are electrophoretic mobility shift assays of triplex formation by the oligonucleotide ODN1 in the presence of different polyamine concentrations. D, duplex DNA; S, single-stranded probe; T, triplex; W, probe DNA retained in the polyacrylamide gel well. (A) Spermine and bis(ethyl)spermine. (B) 4444 and BE-4444.

## RESULTS

*Facilitation of Purine-Motif Triplex Formation by Polyamines.* Polyamines are well-recognized for stabilizing both pyrimidine- and purine-motif triplex formation (Musso & Van Dyke, 1995; Thomas & Thomas, 1993). In the present investigation, the capabilities of various polyamines to facilitate purine-motif triplex formation were determined using an electrophoretic mobility shift assay (EMSA). Reactions were performed under our standard conditions [40 mM Tris·HCl (pH 7.3, 30 °C), 0.01% Nonidet P-40, 10 nM ODN1, and 1 nM probe T4] with different concentrations of polyamines as indicated in the figure legends. Triplex formation was visualized by autoradiography and quantitated by densitometry. The results were plotted as a function of polyamine concentration and subjected to nonlinear regression analysis.

Results of a typical EMSA experiment are shown in Figure 2. As seen in this autoradiogram, increasing concentrations of either spermine, the synthetic polyamine 4-4-4-4, or bis-(ethyl)-substituted analogs of these polyamines all demonstrated the appearance of a slower electrophoretic mobility species corresponding to triplex DNA. Triplex formation occurred at 0.1−0.3 mM spermine, 0.003−0.03 mM 4-4-4-4, 0.3−3 mM BE-spermine, and 0.01−0.3 mM BE-4-4-4-4. Increasing concentrations of either spermine or 4-4-4-4

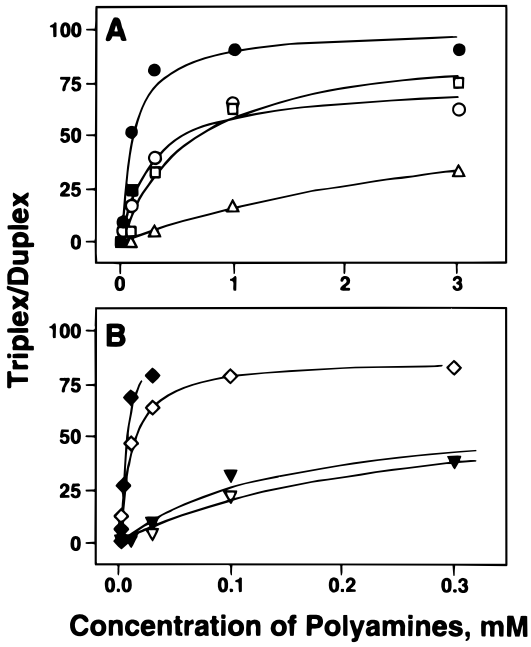


FIGURE 3: Graphical representations of triplex formation by different polyamines. (A) Tetramines: spermine (■), BE-spermine (□), 3-3-3 (▲), BE-3-3-3 (△), 4-4-4 (●), and BE-4-4-4 (○). (B) Pentamines: 3-3-3-3 (▼), BE-3-3-3-3 (▽), 4-4-4-4 (◆), and BE-4-4-4-4 (◇).

caused a significant fraction of the DNA to either remain in the polyacrylamide gel well or be distributed throughout the gel lane. This streaking phenomenon is thought to result from DNA slowly entering the gel during the course of the electrophoresis and is consistent with a polyamine−DNA aggregate that is slowly resolubilizing. However, this phenomenon was not observed with either of the bis(ethyl)-substituted polyamines, even at concentrations that facilitated complete triplex formation. Thus, while bis(ethyl)polyamines may be less capable than their unsubstituted counterparts to facilitate purine-motif triplex DNA formation, this is more than compensated for by their ability to stabilize triplex DNA over a wider range of concentrations in the absence of polyamine-mediated DNA aggregation.

Graphs of the extent of purine-motif triplex formation as a function of polyamine concentration for the different polyamines investigated are shown in Figure 3. From this data, a table of polyamine concentrations necessary to promote 50% triplex formation or cause significant DNA aggregation was also derived (Table 1). We observed an order of effectiveness: 4-4-4-4 > BE-4-4-4-4 > 4-4-4 > BE-spermine = 3-3-3-3 = BE-3-3-3-3 for those polyamines capable of promoting at least 50% triplex formation under our experimental conditions. The other polyamines were less capable of promoting triplex formation, either as a consequence of facilitating polyamine-mediated DNA aggregation (e.g., 3-3-3) or as an intrinsic property of the polyamine itself (e.g., BE-3-3-3). The concentrations at which these polyamines induce aggregation of the oligonucleotides are also shown in Table 1.

To quantify the ability of different polyamines to stabilize purine-motif triplex DNA formation, we determined the triplex DNA dissociation constant in the presence of various polyamines through a range of target DNA concentrations. Results from a representative experiment are shown in Figure 4. As expected, increasing target concentration demonstrated

Table 1: Polyamine Concentrations Required for Observing 50% Purine-Motif Triplex DNA Formation under Standard Conditions

| polyamine | concentration required for 50% triplex formation[a] ($EC_{50}$, mM) | concentration at which substantial DNA aggregation is observed[b] (mM) |
|---|---|---|
| 3-3-3 | (<5% at 0.03 mM) | 0.1 |
| BE-3-3-3 | (34% at 3 mM) | >3 |
| 3-4-3[c] | (24% at 0.1 mM) | 0.3 |
| BE-3-4-3 | 0.69 | >3 |
| 4-4-4 | 0.11 | >3 |
| BE-4-4-4 | 0.56 | >3 |
| 3-3-3-3 | 0.52 | >3 |
| BE-3-3-3-3 | 0.66 | >3 |
| 4-4-4-4 | 0.0066 | 0.1 |
| BE-4-4-4-4 | 0.015 | >3 |

[a] If less than 50%, maximal triplex formation observed and the polyamine concentration used are indicated in parentheses. [b] Concentration where >50% loss of labeled probe was first observed. [c] 3-4-3 is spermine.

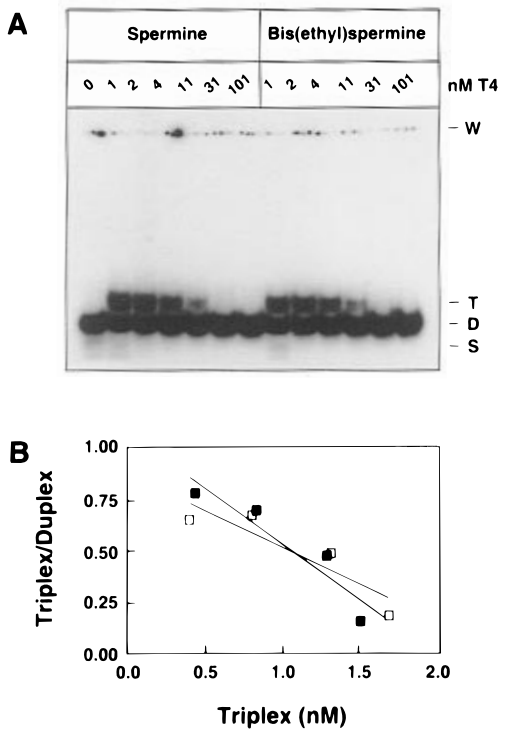


FIGURE 4: Triplex formation constant determination in the presence of spermine or bis(ethyl)spermine. Reactions were performed in a buffer containing 40 mM Tris HCl (pH 7.3, 30 °C), 0.01% Nonidet P-40, 10 nM ODN1, and the concentration of probe T4 as indicated. (A) EMSA of triplex formation. The locations of single-stranded probe (S), duplex probe (D), triplex (T), and material retained in gel well (W) are indicated at right. C, duplex DNA control. (B) Scatchard analysis of triplex formation data for spermine (■) and BE-spermine (□).

a decreased percentage of duplex target conversion into a triplex species. Using a Scatchard analysis, we were able to determine apparent triplex dissociation constants of 1.8 and 2.7 nM for spermine and bis(ethyl)spermine, respectively. Apparent dissociation constants for several pentamines are shown in Table 2. From these data it would appear that polyamines in the order 3-3-3-3 > BE-3-3-3-3 > 4-4-4-4, spermine > BE-4-4-4-4, BE-spermine increased the binding between oligonucleotide and duplex target.

*Differential Effects of Unsubstituted and Bis(ethyl)-Substituted Polyamines on the $T_m$ of Triplex DNA.* Polyamines are known to stabilize duplex and triplex DNA by dramati-

Table 2: Apparent Purine-Motif Triplex Dissociation Constants in the Presence of Different Polyamines

| polyamine | dissociation constant[a] (nM) |
|---|---|
| 3-4-3 (spermine) | 1.8 |
| BE-3-4-3 | 2.7 |
| 3-3-3-3 | 0.89 |
| BE-3-3-3-3 | 1.3 |
| 4-4-4-4 | 1.8 |
| BE-4-4-4-4 | 2.4 |

[a] Dissociation constants were calculated by a Scatchard analysis, as shown in Figure 4.

cally increasing their melting temperature (Thomas & Bloomfield, 1984; Thomas & Thomas, 1993). We therefore examined the effects of the polyamines on the $T_m$ of triplex DNA formed by mixing the oligonucleotides, duplex T4, and ODN1. These experiments were conducted in a buffer of 10 mM sodium cacodylate (pH 7.2) and 1 mM EDTA, as used in our previous studies (Thomas & Thomas, 1993). The $T_m$ was determined from measurements of absorbance (at 260 nm) as a function of temperature from 25 to 98 °C. There was only a single melting transition (45 °C) of the oligonucleotide mixture in the absence of polyamines and this melting transition corresponded to the $T_m$ of the duplex target, T4. When this mixture was treated with as little as 5 $\mu$M 4-4-4-4, the $T_m$ was raised to 73.9 °C. From our EMSA results and CD data (see below), we conclude that this temperature corresponds to the $T_m$ of triplex DNA. It should be noted here that only a single melting transition was observed, indicating the melting of triplex DNA to single strands, as previously reported for the melting of poly(dA)·2poly(dT) in the presence of spermine (Thomas & Thomas, 1993).

There was a concentration-dependent increase in the $T_m$ of triplex DNA in the presence of various polyamines up to 25 $\mu$M of polyamines (Table 3). The bis(ethyl)polyamines were less efficient in stabilizing triplex DNA compared to their unsubstituted analogs. This result is consistent with the data obtained from EMSA experiments. From $T_m$ measurements, an order of efficacy of polyamines in stabilizing triplex DNA could be deduced: 4-4-4-4 > 3-3-3-3 > BE-3-3-3-3 ∼ BE-4-4-4-4 > 4-4-4 ∼ 3-4-3 > BE-3-4-3 > 3-3-3 ∼ BE-4-4-4 > BE-3-3-3.

*Effects of 4-4-4-4 and BE-4-4-4-4 on the Conformation of Triplex DNA.* Polyamines have been shown to induce and stabilize conformational alterations in DNA, including the left-handed Z-DNA, A-DNA, and Ψ-DNA (Behe & Felsenfeld, 1981; Minyat et al., 1978; Thomas & Bloomfield, 1985; Thomas & Messner, 1988). In a recent investigation of the conformation of triplex DNA, Thomas et al. (1996) found that a series of trivalent analogs of spermidine induced the formation of Ψ-DNA, a twisted liquid crystalline form of DNA, in poly(dA)·2poly(dT). We therefore examined

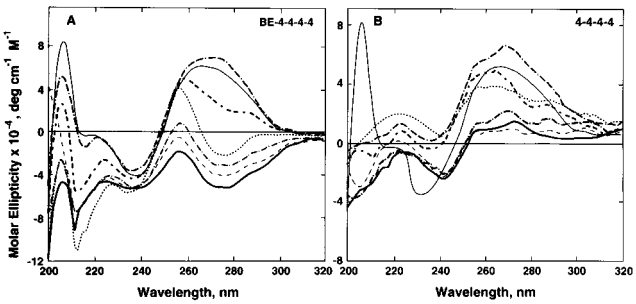


FIGURE 5: CD spectra of the effect of BE-4-4-4-4 (A) and 4-4-4-4 (B) on purine-motif triplex DNA. T4 and ODN1 were mixed in a 1:1 molar ratio in 10 mM sodium cacodylate buffer in the presence of different concentrations of polyamines, heated to 90 °C for 10 min, and cooled to room temperature, and the CD spectra were recorded with an Aviv Model 62D circular dichroism spectrophotometer. Polyamine concentrations were as follows: 0 (—, thin), 2.5 (· — — ·), 5 (- — — -), 10 (···), 25 (— · —), 50 (- - -), and 100 (—, thick) $\mu$M.

the effects of 4-4-4-4 and BE-4-4-4-4 on the conformation of the purine-motif triplex DNA formed from ODN1 and T4. We used CD spectroscopy as a convenient method to investigate the conformational changes of triplex DNA. Figure 5 shows the CD spectra of triplex DNA in the presence of different concentrations of BE-4-4-4-4 (A) and 4-4-4-4 (B). In the absence of polyamines, the CD spectrum of the oligonucleotide mixture consisted of two positive peaks, 205 and 265 nm, and a negative peak at 238 nm. This is predominantly the CD spectrum of duplex B-DNA (Gray et al., 1992; Park & Breslauer, 1992; Scaria and Shafer, 1991). In the presence of 2.5 $\mu$M BE-4-4-4-4, the CD spectrum of the oligonucleotide mixture underwent significant changes, including a broadening and increase in the intensity of the positive peak centered at 265 nm. There was also a red shift in the peak to 275 nm. The low-wavelength peak centered at 208 nm decreased in intensity, and the small dip at 217 nm was converted to a small negative peak centered at 215 nm. This negative peak between 210 and 220 nm is considered a hallmark of triplex DNA formation. Therefore, triplex DNA stabilization occurred at BE-4-4-4-4 concentrations as low as 2.5 $\mu$M.

Progressive addition of BE-4-4-4-4 produced marked changes in the broad positive band centered at 275 nm, converting it into a broad negative band centered at 275 nm in the presence of 100 $\mu$M BE-4-4-4-4. The intensity of the negative band between 210 and 220 nm also increased and leveled off at about 50 $\mu$M BE-4-4-4-4. There was no major change in the CD spectrum of the triplex DNA above this concentration of polyamine. Control experiments with BE-4-4-4-4 showed no spectral contribution from the polyamine.

The unethylated polyamine, 4-4-4-4, also produced marked changes in the CD spectrum of the oligonucleotides mixed

Table 3: Effect of Polyamines on the Melting Temperature of Purine-Motif Triplex DNA

| concn ($\mu$M) | melting temperature[a] (°C) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 3-3-3 | BE-3-3-3 | 3-4-3 | BE-3-4-3 | 4-4-4 | BE-4-4-4 | 3-3-3-3 | BE-3-3-3-3 | 4-4-4-4 | BE-4-4-4-4 |
| 0 | 45.3 | 45.4 | 45.2 | 46.3 | 44.8 | 45.1 | 44.8 | 45.0 | 45.3 | 44.9 |
| 5.0 | 64.6 | 62.4 | 65.9 | 63.2 | 63.5 | 62.6 | 75.6 | 74.1 | 73.9 | 70.6 |
| 10.0 | 73.2 | 70.8 | 69.3 | 67.5 | 65.2 | 64.2 | 84.2 | 79.2 | 82.7 | 79.2 |
| 25 | 74.0 | 73.2 | 75.8 | 73.7 | 73.2 | 73.1 | 84.2 | 82.8 | 92.2 | 84.2 |
| 100 | 74.1 | 73.8 | 78.2 | 75.9 | 76.4 | 75.2 | 85.4 | 85.0 | 92.8 | 84.5 |

[a] Melting temperature was determined by monitoring changes in UV absorption as a function of temperature at a heating rate of 0.5 °C/min.

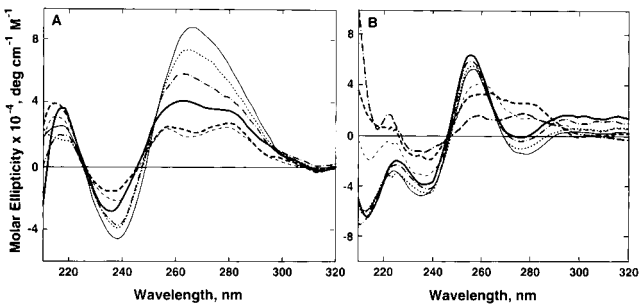


FIGURE 6: Temperature-dependent CD spectra of purine-motif triplex DNA in the absence (A) and presence (B) of 5 $\mu$M of BE-4-4-4-4. CD spectra were recorded at 10 °C intervals from 0 to 90 °C. Spectra are shown at selected temperatures for clarity: 10 °C (—, thin); 30 °C (···); 40 °C (— · —); 50 °C (—, thick); 60 °C (- - -, thick); 70 °C (- - -, thin); and 90 °C (· — — ·).

to form the triplex DNA; however, the spectral changes were not as confirmatory for triplex DNA formation as those found with BE-4-4-4-4. Although the positive peak centered at 265 nm underwent changes with a significant reduction of its positive amplitude, this peak remained in the positive range up to 100 $\mu$M of 4-4-4-4. There was no further change up to 300 $\mu$M (results not shown). The spectral changes at the low-wavelength region were also different from those observed with BE-4-4-4-4 and may signify the interaction of circularly polarized light with triplex DNA aggregates formed in the presence of 4-4-4-4. There was also a positive ellipticity at >300-nm wavelength, further indicating scattering of circularly polarized light by DNA aggregates (Tinoco et al., 1980).

We also examined the effects of BE-4-4-4-4 and 4-4-4-4 on the CD spectra of duplex T4 and single-stranded ODN1. There were significant changes in the CD spectra of these oligonucleotides in the presence of both polyamines; however, these changes were distinct from those we observed for the triplex DNA (results not shown).

In the next set of experiments, we recorded the CD spectra of the triplex DNA in the absence and presence of 5 $\mu$M BE-4-4-4-4 at 10 °C intervals between 0 and 90 °C, after allowing equilibration for 5 min at each 10 °C step. Figure 6 shows representative temperature-dependent CD spectra of the oligonucleotide mixture in the absence (A) and presence (B) of BE-4-4-4-4. The CD spectrum of the oligonucleotides underwent significant changes starting at 40 °C in the absence of the polyamine, with reduced intensity of the positive and negative peaks centered at 265 and 240 nm, respectively. At 70 °C these changes were almost complete and the CD spectrum was comparable to that of single-stranded DNA. In the presence of BE-4-4-4-4, spectral changes were not evident up to 60 °C. At higher temperatures, major changes in the CD spectra of the oligonucleotide occurred and the spectrum was similar to that of single-stranded DNA at 80 °C (Gray et al., 1992; Thomas et al., 1995b).

We converted the CD spectra of the oligonucleotide mixture to fractional contributions of chiral species contributing to the overall CD spectra using a deconvolution analysis program developed by Perczel et al. (1991, 1992). There were only two major solution species up to 90 °C, and we consider them as the helix and coil forms of the oligonucleotides. Figure 7 shows plots of fractional helical DNA against temperature. The melting transition is monophasic

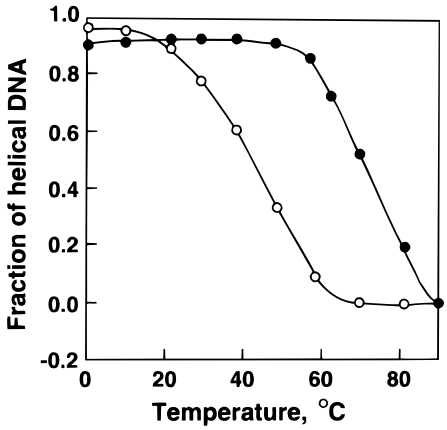


FIGURE 7: Temperature-dependent changes in the fraction of helical DNA in the absence (○) and presence (●) of 5 $\mu$M BE-4-4-4-4. The CD spectra recorded at 10 °C intervals were deconvoluted to obtain the fractions of solution species contributing to the overall spectra. Two predominant species (helix and coil) contributed to the spectra. The melting temperatures determined from these CD spectral measurements were similar to that obtained from UV absorption measurements.

in both cases, and a 34 °C increase in the midpoint of the helix → coil transition temperature is observed in the presence of 5 $\mu$M BE-4-4-4-4.

## DISCUSSION

Results presented in this report show that the tetra- and pentavalent polyamines are excellent promoters of purine-motif triplex DNA. In addition to a structural specificity effect depending on the number of methylene groups separating the amino and imino groups of the polyamines, we discovered a distinct advantage of bis(ethyl) substitution in stabilizing triplex DNA formation in the absence of DNA aggregation. In most cases, aggregation was the predominant effect of unsubstituted polyamines and even 50% triplex DNA formation could not be achieved with 3-3-3 and 3-4-3. Bis(ethyl)polyamines were less effective than the unsubstituted polyamines in stabilizing triplex DNA because of steric constraints imposed by the substituent. However, there was only a 2-fold reduction in $EC_{50}$ (Table 1) due to bis(ethyl) substitution, whereas the tendency of aggregation was reduced by at least 30-fold. Similarly, there was a 46-fold increase in efficacy of BE-4-4-4-4 compared to BE-3-4-3.

The apparent dissociation constants of triplex DNA in the presence of bis(ethyl)polyamines were consistently greater than those determined in the presence of their unmodified counterparts. However, the range in binding constants observed was only 3-fold between the different polyamines. This result is in agreement with the results presented in Table 1, and demonstrates a differential effect of bis(ethyl)-substituted and unsubstituted polyamines on facilitating triplex DNA formation. In this context, it is important to note that Singleton and Dervan (1993) reported a 500-fold increase in the association constant of triplex DNA formation by increasing the concentration of spermine; however, this is the first report showing a steric hinderance effect of polyamines in triplex DNA stabilization.

$T_m$ data presented in Table 3 provide another measure of the ability of these novel polyamine analogs to stabilize the purine-motif triplex DNA. As in previous studies (Thomas & Bloomfield, 1984), electrostatic effects are predominant; the pentamines are much more efficient than the tetramines.

For example, 5 $\mu$M 3-3-3 increased the $T_m$ of the triplex DNA by 19 °C, whereas the same concentration of 3-3-3-3 facilitated $T_m$ by 31 °C. Bisethylation had a slight destabilizing effect, as observed with the results of EMSA and dissociation constant measurements. The structural specificity of polyamines is consistent with previous studies of polyamine effects on the $T_m$ of triplex DNA (Thomas et al., 1996; Thomas & Thomas, 1993) and the effects of these compounds in provoking unusual conformations, including the left-handed Z-DNA (Basu et al., 1990; Basu & Marton, 1987; Thomas et al., 1985; Thomas & Messner, 1988). In contrast, polyamine homologues had only a minor effect in stabilizing duplex DNA (Tabor, 1962; Thomas & Bloomfield, 1984). The reason for this differential effect is not as yet known; however, this effect may be viewed in terms of the proposed mechanisms of polyamine−DNA interactions (Bloomfield, 1991; Feuerstein et al., 1986, 1991; Padbhanabhan et al., 1991; Plum & Bloomfield, 1990: Schellmann & Parthasarathy,1984; Thomas & Bloomfield, 1983). Using $^{13}C$ NMR spectroscopy, Hague and Moreton (1994) showed that polyamine analogues with at least a trimethylene separation between the amino and/or imino groups are fully protonated at pH >7.2, the pH value used in our study. Because of this cationic charge under physiological conditions, the polyamine analogues used in our study are expected to interact with DNA phosphate charges by electrostatic interactions. Thomas and Bloomfield (1983) and Wilson and Bloomfield (1979) utilized the counterion condensation theory developed by Manning (1978) and Record et al. (1978) to calculate the percent charge neutralization in the presence of mono-, di-, tri-, and tetravalent cations at concentrations necessary to collapse DNA into toroidal structures. Molecular modeling (Feuerstein et al., 1986) and X-ray crystallographic studies (Egli et al., 1991) indicate site-specific interactions of polyamines with DNA. The tetramethylene spacing between amino and imino groups of polyamines appears to be amenable to form hydrogen bonding between the amino/imino hydrogens of the DNA bases and phosphates in adjacent strands, with the preferential docking of the polyamine being in the major groove of DNA (Feuerstein et al., 1986). Thus the destabilizing effect of bis(ethyl)polyamines on triplex DNA formation, compared to that of their unsubstituted parent compounds, might be a steric hindrance effect of the bulky substituents on hydrogen-bond formation.

Since the third strand of triplex DNA exists in the major groove of the duplex DNA, it is conceivable that polyamines occupy alternate sites in triplex DNA compared to that on duplex DNA. Recent X-ray crystallographic studies also provide evidence for the binding of spermine across the narrowed minor groove rather than being embedded in it (Tari & Secco, 1995). Low-temperature single-crystal X-ray crystallography has also provided evidence for the binding of polyamines in the minor groove of DNA (Bancroft et al., 1994). It is thus possible that a polyamine such as 4-4-4-4 might wrap around DNA rather than being localized in any of the grooves because of the longer methylene chain length. The presence of a highly flexible tetramethylene bridge between the amino and imino groups in this compound may also aid in establishing hydrophobic contacts between polyamines and the DNA bases (Jain et al., 1994). Reduction in the number of methylene groups in compounds such as 3-3-3 and 3-3-3-3 makes this mode of interaction difficult and hence these molecules may act like metal cations with high charge density. Our results on the effects of various polyamines on increasing the $T_m$ of triplex DNA is consistent with such a hypothesis and is supported by the increased efficacy of $Co(NH_3)_6^{3+}$ compared to its isovalent polyamine analog, spermidine$^{3+}$, in provoking the condensation and conformational transitions of DNA (Thomas & Bloomfield, 1983; Widom & Baldwin, 1983).

Another important finding of our study is the conformational alterations induced by the polyamine analogs on triplex DNA. At low concentrations of these polyamines, there was a red shift in the peak intensity from 265 to 273 nm. This shift is indicative of a B-DNA to A-DNA conformational transition (Gray et al., 1992; Manzini et al., 1990). The conformational status of triplex DNA is not as yet precisely defined; however, it appears that the conservative B-DNA structure is altered on the association of the third strand. CD and NMR spectroscopic measurements indicate that triplex DNA adopts a conformation that is distinct from both canonical A-form and B-form helices (Gray et al., 1992; Radhakrishnan & Patel, 1994a,b). A recent X-ray crystallographic analysis (Betts et al., 1995) also showed an atypical P-DNA conformation for triplex DNA. The negative bands at 215 and 277 nm in the CD spectrum of triplex DNA are not present in duplex or single-stranded DNA and result from the binding of ODN1 to T4 target to form triplex DNA. The negative ellipticity near 277 nm has been previously described in the case of poly(dG)·poly(dG)·poly(dC) triplexes (Marck et al., 1978; Thiele et al., 1978). Scaria et al. (1995) also found a strong negative ellipticity centered at 277 nm in a triplex formed from $d(G_3T_4G_3)$·$d(G_3A_4G_3)$·$d(C_3T_4C_3)$. These results, however, contrast with the ability of polyamine analogs to provoke global structural modifications in poly(dA)·2poly(dT) triplex to a $\psi$-DNA conformation in the presence of polyamines (Thomas et al., 1996; Thomas et al., unpublished results). Thus significant DNA sequence specificity effects are also present in polyamine-mediated triplex DNA formation in addition to polyamine structural specificity effects. Preferential binding of polyamines to certain duplex sequences was previously reported by Marquet et al. (1989) and Xiao et al. (1991).

*Conclusions.* The results presented here indicate the possibility of developing novel polyamine analogs as secondary ligands to stabilize triplex DNA. Our results delineate the problem of polyamine-mediated aggregation and a strategy to eliminate this adverse effect by using substituted polyamines. Several trends can be derived from the present data: (i) Pentamines stabilized triplex formation better than tetramines (e.g., 4-4-4-4 vs 4-4-4); (ii) polyamines containing amine substituents separated by a four-methylene bridging region provided more stabilization than those containing amines separated by three methylenes (e.g., 4-4-4-4 vs 3-3-3-3); (iii) bis(ethyl)-substituted polyamines facilitated triplex formation slightly less well than their unsubstituted counterparts but were significantly less able to cause polyamine−DNA aggregation (e.g., 4-4-4-4 vs BE-4-4-4-4); (iv) $T_m$ data confirm ionic and structural effects in the ability of these polyamines to stabilize triplex DNA and suggest the possibility of different modes of polyamine−triplex DNA interactions depending on the number and spacing of positive charges in the polyamine; and (v) CD results provide evidence for a triplex DNA conformation that is different from typical A-DNA and B-DNA structures.

Further research is necessary to determine which of the spermine analogs will be useful in stabilizing triplex DNA *in vivo* and in advancing triplex DNA strategy as a therapeutic approach.

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