Stabilization of Triple-Helical Nucleic Acids by Basic Oligopeptides†

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ABSTRACT: Intermolecular triplex DNA is stabilized by metal cations and polyamines which reduce repulsion between the negatively charged phosphates of the three nucleic acid strands. We use a quantitative chemical-probing assay involving protection of duplex guanines in a homopyrimidine•homopurine (Py•Pu) sequence from dimethyl sulfate modification to study the effects of basic oligopeptides on the stability of triplex DNA. An intermolecular protonated pyrimidine•purine•pyrimidine (Py•Pu*Py*) triplex formed readily between a duplex DNA region and a 14-mer pyrimidine triplex-forming oligonucleotide (TFO) at pH 5. The triplex was stabilized at pH 6 by the addition of magnesium ions. In the presence of spermine and lysine-rich peptides, the intermolecular triplex was stabilized up to pH 6.5−7.0. The effective peptide concentration required for stabilization was 10^{-5}−10^{-2} M. Of the basic peptides studied, pentalysine (Lys-Lys-Lys-Lys-Lys) was the most effective triplex stabilizer. It was effective at concentrations which are lower than those required for Lys-Gly-Lys-Gly-Lys and Lys-Ala-Lys-Ala-Lys and are similar to active concentrations of spermine. Basic peptides were more effective at stabilizing a Py•Pu*Py* triplex than a pyrimidine•purine•purine (Py•Pu*Pu*) triplex. At 1 mM, Lys-Lys-Lys-Lys-Lys stabilized the Py•Pu*Pu* triplex at a level comparable to stabilization by Mn^{2+} and spermine, whereas Lys-Gly-Lys-Gly-Lys and Lys-Ala-Lys-Ala-Lys resulted in weaker TFO binding. The concentrations of TFOs required to form triplex DNA were significantly reduced in the presence of peptides. The difference in triplex stability as a function of amino acid composition suggests the possibility of tuning the triplex-stabilizing effect by varying peptide sequence and the fraction of basic amino acid residues.

Triple-stranded nucleic acid helices (triplexes) were first described in 1957 (Felsenfeld et al., 1957), and their structure and properties have been studied for more than thirty years [see Wells et al. (1988), Thuong and Hélène (1993), Mirkin and Frank-Kamenetskii (1994), Radhakrishnan and Patel (1994), Sinden (1994), and Soyfer and Potaman (1995) for reviews]. Intermolecular triplexes have attracted much attention because of their potential therapeutic application in inhibiting the expression of genes involved in cancer and other human diseases [Chubb and Hogan (1992) and Hélène et al. (1992) for reviews] or for chromosome mapping [Strobel and Dervan (1992) for review]. Intramolecular triplex/single-strand structures (H and H* forms) may play important roles in the processes of replication, genetic recombination, or gene expression [Mirkin and Frank-Kamenetskii (1994), Sinden (1994), and Soyfer and Potaman (1995) for reviews].

The formation of triple-stranded nucleic acids requires a reduction in the repulsion between the negatively charged phosphate groups of the three strands. The repulsion between these phosphates can be screened by submolar concentrations of monovalent cations, including Li^+, Na^+, and K^+ (Felsenfeld & Rich, 1957; Rich, 1960; Krakauer & Sturtevant, 1968). Much lower (millimolar) concentrations of divalent metal cations (e.g., Mg^{2+}), which bind more tightly to phosphates, are effective in the stabilization of triplex structures *in vitro* (Felsenfeld & Rich, 1957). However, the actual concentrations of metal ions in the cell may not be sufficient to stabilize triplex DNA structures. Submillimolar concentrations of polyamines, whose distributed charges allow them to interact simultaneously with different nucleic acid strands, are effective in the stabilization of triple-helical nucleic acids *in vitro* (Glaser & Gabbay, 1968; Raae & Kleppe, 1978; Hampel et al., 1991; Hanvey et al., 1991; Singleton & Dervan, 1993; Thomas & Thomas, 1993). As high as 1 mM concentrations of polyamines can be detected in the cell (Tabor & Tabor, 1976; Sarhan & Seiler, 1989), where they are predominantly bound to macromolecules, including nucleic acids and phospholipids (Davis et al., 1992). Thus, polyamines might be a class of compounds which stabilize triple-helical nucleic acids *in vivo*. However, their effect may be attenuated by other cations which compete for binding to phosphates (Maher et al., 1990; Singleton & Dervan, 1993). The identification of possible triplex stabilizers and an understanding of their combined effects in the cell are important steps in the elucidation of the biological role of triple-stranded nucleic acids and the development of their biotechnological applications.

Basic oligopeptides, another class of biologically relevant polycations, may also stabilize triplex DNA. Cationic amino acid residues of basic peptides might bind to and neutralize phosphate groups of triple-helical nucleic acids. Since the major groove of the duplex target would be occupied by the third strand of the triplex, oligopeptides might bind in the minor groove and/or in the new grooves formed by a third strand and two duplex strands. Here we characterize the stabilization of triplex DNA by lysine-rich peptides.

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MATERIALS AND METHODS

Materials. Oligonucleotides were purchased from Genosys Biotechnologies (The Woodlands, TX). Their concentrations in stock solutions were determined using molar extinctions at 260 nm of 15400 (A), 7300 (C), 11700 (G), and 8800 (T) for monomers and the UV absorbance spectra at 90 °C where the oligomers are denatured. Interactions between monomers are minimal, and to a good approximation, the oligomer absorbance is the sum of absorbances of constituent monomers. To construct plasmid pRDH7, the 41 bp[1] insert shown in lowercase letters in Figure 1A was cloned into the *Eco*RI site of plasmid pUC8. A 151 bp fragment of a promoter region of the human Na,K-ATPase α2 gene (Shull et al., 1989) was cloned into the *Bam*HI site of plasmid pUC8, resulting in plasmid pBB151-8. This promoter region contains a singly interrupted 35 bp Py·Pu tract, in which the 26 bp sequence constitutes a quasi-mirror repeat (Figure 1B).

Oligopeptides Lys-Gly-Lys-Gly-Lys, Lys-Ala-Lys-Ala-Lys, and Lys-Lys-Lys-Lys-Lys were synthesized by Dr. S. Gurusiddappa (Institute of Biosciences and Technology, Texas A&M University) and were >90% homogeneous according to HPLC analysis. *Pvu*II (New England Biolabs), *Taq* DNA polymerase (Gibco BRL), and the Stoffel fragment of *AmpliTaq* DNA polymerase (Perkin-Elmer) were used according to the manufacturer's specifications.

*Intermolecular Triplx Formation and Probing. Pvu*II digestion of the plasmids gave Py·Pu tract-containing fragments of 375 bp from pRDH7 and 446 bp from pBB151-8. DNA target (0.5 pmol) (about 1 μg of restricted plasmid) was mixed with the triplex-forming oligonucleotide (TFO) (at molar ratios indicated in the figure legends) in 20 μL of appropriate buffer containing triplex stabilizers (specified in the figure legends). The resulting mixture was incubated at room temperature overnight to allow the triplex to reach equilibrium (Maher et al., 1990). Dimethyl sulfate (DMS) (2%, 1 μL) was added to the reaction mixture, vortexed, and incubated at room temperature for 10 min, after which 4 μL of 2.5 M β-mercaptoethanol was added to stop the modification reaction. Following two ethanol precipitations, samples were resuspended in 20 μL of TE (10 mM Tris-HCl (pH 7.5) and 1 mM EDTA). Ten microliters was removed for quantitative analysis by a linear amplified primer extension reaction as described by Ussery et al. (1992). The primer extension reaction contained 5 units of a Stoffel fragment of *AmpliTaq* DNA polymerase, 0.25 pmol of modified DNA, 2 pmol of a 23 nt ^{32}P-labeled primer complementary to the strand containing the purine tract of the Py·Pu sequence, and 0.5 mM deoxynucleotide triphosphates in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 5 mM $MgCl_2$. Each of ten reaction cycles consisted of a melting step (96 °C, 2 min), a primer-annealing step (60 °C, 30 s), and a primer extension step (80 °C, 5 min) with a 20 s autoextension. After ethanol precipitation, the products of primer extension were resolved on a 5% denaturing sequencing polyacrylamide gel in TBE buffer (90 mM Tris-borate (pH 8.3) and 2.5 mM EDTA), with sequence markers obtained by dideoxy sequencing of the plasmids using *Taq* DNA polymerase. The gel was dried and exposed to a PhosphorImager plate overnight for analysis on a Molecular Dynamics PhosphoImager using ImageQuant software.

A. Py·Pu sequence in plasmid pRDH7 and complementary triplex-forming oligonucleotides (TFO)

```
3'CTTAAggattccttccttccttatattccttccttccttaacttaaG
5'GaattcctaaggaaggaaggaatataaggaaggaaggaattgAATTC
          oo++oo++oo++oo    oo++ o++oo++oo
      5'-TTCCTTCCTTCCTT    TTCCGTCCTTCCTT-3'
            TFO I               TFO II
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B. Py·Pu sequence in plasmid pBB151-8 and complementary TFO

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3'AAAGCCTCCTCCCCTTCCGCCCCCTCTCCCCCTCTTCCTGGATAAATTT
5'TTTCGGAGGAGGGGAAGGCGGGGGAGAGGGGGAGAAGGACCTATTTAAA
      oooooooooooooo          oooo oo ooooo
   3'GGAGGAGGGGAAGG      3'GGAGGAGGGGAAGG
        TFO III               TFO III
```

FIGURE 1: DNA sequences of the Py·Pu*Py and Py·Pu*Pu* intermolecular triplexes. Sequences of Py·Pu tracts in plasmids pRDH7 and pBB151-8 are shown in parts A and B, respectively. (A) Sequences of triplex-forming oligonucleotides TFO I and TFO II capable of forming a perfect Py·Pu*Py triplex and a triplex with one mismatch, respectively, are shown below the Py·Pu tract in pRDH7. (B) The sequence of TFO III is shown forming a perfect and imperfect Py·Pu*Pu triplex at the 5′ and 3′ ends of the Py·Pu tract, respectively. Hoogsteen bonds are indicated by O and protonated Hoogsteen bonds are denoted by +.

Quantitation of Triplex Stabilization by Basic Peptides. The N7 position of quanines in DNA is susceptible to modification by DMS. However, when involved in Hoogsteen hydrogen bonding with a TFO, they are protected from modification by DMS. This assay has been widely used to detect triplex formation (Soyfer & Potaman, 1995). We have determined the extent of guanine protection from modification as a quantitative measure of the level of triplex DNA formation. The primer extension protocol of Cartwright and Kelly (1991) allows linear amplification of the primer extension products, thereby providing the basis for quantitative analysis (Ussery et al., 1992). The primer extension products in the Py·Pu tract, which are triplex formation-dependent, are quantitated and normalized to the sum of specific termination products in the 5′ and 3′ flanking regions (indicated by arrows in Figures 2 and 6) which are independent of triplex formation. The level of DMS modification in the absence of the TFO is taken as 100%, and the percent of DNA modification upon triplex formation is calculated relative to that maximum value.

RESULTS

Experimental Rationale. To test the possible triplex-stabilizing effect of basic peptides, we have analyzed triplex formation at two duplex DNA targets which contain Py·Pu tracts appropriate for the formation of either Py·Pu*Py or Py·Pu*Pu* intermolecular triplex structures (Figure 1). Triplex-forming oligonucleotide I (TFO I, 14-mer) forms a perfect Py·Pu*Py triplex at the Py·Pu sequence in pRDH7 at moderately acidic pH, necessary for protonation of cytosines in the third strand. TFO II (14-mer) forms a triplex with one mismatched T·A*G triad at the same target. TFO III (14-mer) forms a perfect Py·Pu*Pu triplex at the 5′ end of the Py·Pu tract in pBB151-8 at neutral pH. This oligonucle-

[1] Abbreviations: bp, base pair; nt, nucleotide; Pu, homopurine; Py, homopyrimidine; DMS, dimethyl sulfate; TFO, triplex-forming oligonucleotide; *R*, molar TFO/DNA ratio; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid. Pu·Pu*Py and Py·Pu*Pu* denote a Py·Pu Watson–Crick duplex with either a *Py* or *Pu* third Hoogsteen base-paired strand.

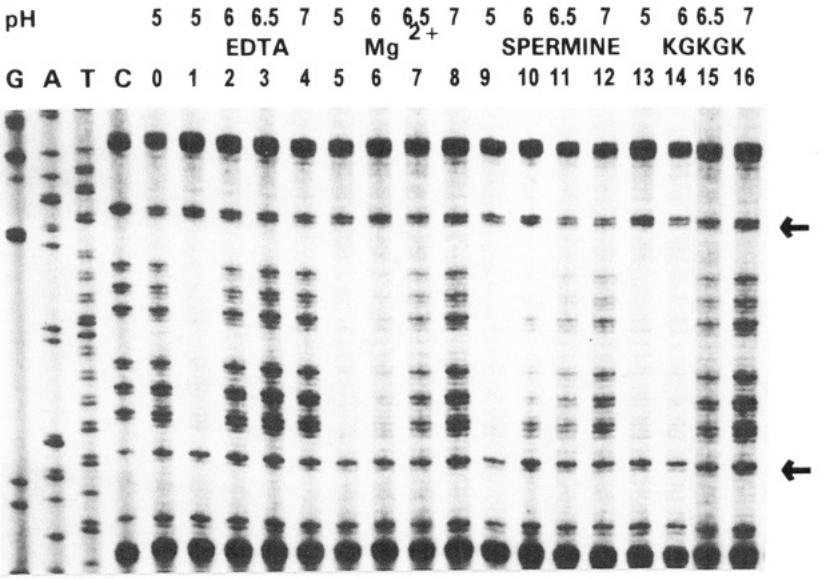

FIGURE 2: Comparison of effects of Mg^{2+}, spermine, and Lys-Gly-Lys-Gly-Lys on the protection of guanines from DMS modification by TFO I. A 375 bp fragment of plasmid pRDH7 was incubated with the 100-fold excess of TFO I overnight in the following buffers containing 50 mM Na^+ with or without stabilizers: pH 5, 50 mM sodium acetate; pH 6, 20 mM MES and 42 mM NaCl; pH 6.5, 20 mM MES and 36.5 mM NaCl; and pH 7, 20 mM HEPES and 46 mM NaCl. Samples without stabilizing additives contained 1 mM EDTA (lanes 1−4). Stabilizers included 10 mM $MgCl_2$ (lanes 5−8), 0.3 mM spermine (lanes 9−12), and 1 mM Lys-Gly-Lys-Gly-Lys (lanes 13−16). Following incubation, samples were treated with DMS and analyzed by primer extension as described in Materials and Methods. Lane 0 shows the DMS modification pattern at pH 5 in the absence of any TFO. Lanes denoted G, A, T, and C are the dideoxy sequencing lanes of the complementary strand. The reference, triplex formation-independent bands used for quantitation of guanine protection in the triplex region are indicated by arrows.

otide can also potentially form an imperfect Py·Pu**Pu* triplex at the 3′ end of the Py·Pu tract.

As an initial test system, we have analyzed stabilization of Py·Pu**Py* and Py·Pu**Pu* triplexes by five-amino acid oligopeptides which are close in size to that which stabilizes Z-DNA (Takeuchi et al., 1991, 1994). To test the importance of positive charge density in oligopeptides, we designed peptide sequences containing cationic amino acid residues at every position in Lys-Lys-Lys-Lys-Lys and containing alternating cationic and neutral residues in Lys-Gly-Lys-Gly-Lys and Lys-Ala-Lys-Ala-Lys. The latter two peptides may have unequal stabilizing effects because glycine and alanine may provide different conformational flexibilities to peptides. Therefore, they may adjust differently to the geometry of a binding site, and as a result, they may have different affinities for triplex DNA.

*Stabilization of a Py·Pu*Py Triplex by Basic Oligopeptides.* Figure 2 shows a representative comparison of the effects of triplex stabilizers on the DMS modification patterns for the Py·Pu tract in pRDH7 in the presence of TFO I. In the absence of TFO I, a strong DMS modification at pH 5 was observed throughout the 31 bp Py·Pu sequence (lane 0). Irrespective of the presence of stabilizers, TFO I strongly bound to the purine strand at pH 5 to form a Py·Pu**Py* triplex and effectively protected only the Py·Pu tract guanines from modification (lanes 1, 5, 9, and 13) as shown by the minimal signal from primer extension. Triplex formation and concomitant guanine protection in an EDTA-containing buffer was significantly less efficient at pH 6 (lane 2), and virtually no protection was observed at pH 6.5 (lane 3) and 7.0 (lane 4). Mg^{2+} ions at 10 mM afforded significant protection at pH 6 (lane 6) and pH 6.5 (lane 7), where 80 and 52% of maximum DMS protection were observed. Relatively weak triplex formation occurred at pH 7 in the presence of Mg^{2+} (lane 8, 24% DMS protection). Spermine at 0.3 mM resulted in a strong binding of TFO I to duplex up to pH 6.5, where protection of guanines from DMS modification was 75% (lane 11). At pH 7.0, weaker triplex stabilization by spermine was observed (lane 12, 27% DMS protection), where modification was comparable to that of magnesium ions at pH 7. Lys-Gly-Lys-Gly-Lys at 1 mM had a triplex-stabilizing effect similar to that of 0.3 mM spermine at all pH values studied and had a stronger effect than that of 10 mM Mg^{2+}.

Figure 3 shows a comparison of the triplex-stabilizing effects of 1 mM Lys-Gly-Lys-Gly-Lys, Lys-Ala-Lys-Ala-Lys, and Lys-Lys-Lys-Lys-Lys as a function of pH. Of the three peptides studied, Lys-Lys-Lys-Lys-Lys had the most pronounced effect. At pH values up to 6.5, almost 100% of the Py·Pu was bound by the third strand TFO I. Even at pH 7, TFO I bound to its duplex target, reducing DMS modification to 60%. Lys-Ala-Lys-Ala-Lys had the least stabilizing effect of the three oligopeptides, an effect comparable to that of 10 mM Mg^{2+} throughout the pH range studied.

The peptide concentration dependence for Py·Pu**Py* triplex stabilization at a molar TFO/DNA ratio (R) of 100 at pH 6.5 is shown in Figure 4. Consistent with the pH dependence, Lys-Lys-Lys-Lys-Lys had the greatest stabilizing effect of the three peptides. Lys-Lys-Lys-Lys-Lys reduced DMS modification to ~70% even at the lowest concentration studied (30 μM). At 1 mM, DMS modification was reduced to a background level indicative of the formation of triplex DNA by 100% of the Py·Pu target sequence. Higher concentrations of Lys-Gly-Lys-Gly-Lys and Lys-Ala-Lys-Ala-Lys were required for similar levels of triplex stabilization compared to that of Lys-Lys-Lys-Lys-Lys, and they were maximum at 3 mM. Thus, the effective peptide concentrations for triplex stabilization are in the range of 10^{-5} to 10^{-2} M. Analysis of other experiments showed that

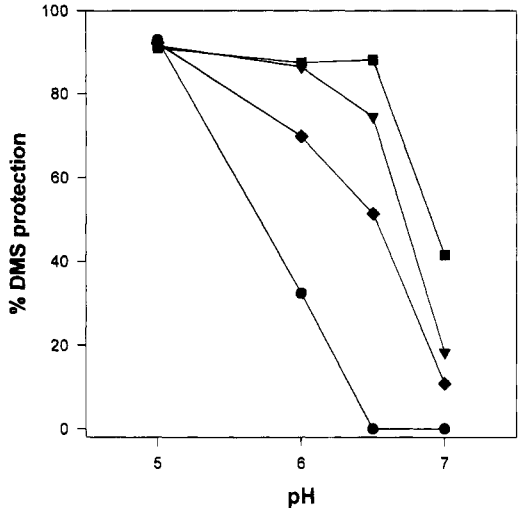

FIGURE 3: Comparison of the triplex-stabilizing effects of Lys-Ala-Lys-Ala-Lys, Lys-Gly-Lys-Gly-Lys, and Lys-Lys-Lys-Lys-Lys. The triplex between the Py·Pu tract in pRDH7 and TFO I was formed at a molar TFO I/DNA ratio of 100 as described in the legend to Figure 2, except that the reaction mixtures contained 1 mM EDTA (●), Lys-Ala-Lys-Ala-Lys (◆), Lys-Gly-Lys-Gly-Lys (▼), or Lys-Lys-Lys-Lys-Lys (■). The percent of guanine protection from DMS modification (% DMS protection), which represents a measure of triplex formation, was calculated as described in Materials and Methods.

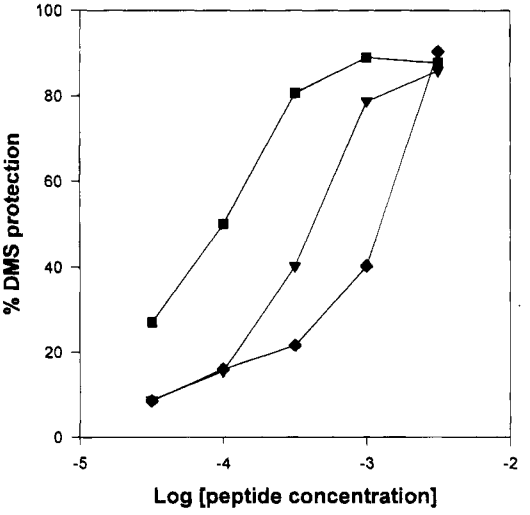

FIGURE 4: Peptide concentration dependence for the stabilization of the Py·Pu*Py triplex. The triplex between the Py·Pu region of pRDH7 and TFO I was formed at a molar TFO I/DNA ratio of 100 in 20 mM MES and 36.5 mM NaCl (pH 6.5). The reaction mixtures contained 30 μM, 100 μM, 300 μM, 1 mM, and 3 mM Lys-Ala-Lys-Ala-Lys (◆), Lys-Gly-Lys-Gly-Lys (▼), or Lys-Lys-Lys-Lys-Lys (■).

equal concentrations of spermine and Lys-Lys-Lys-Lys-Lys (0.3 mM) result in the same extent of DMS protection at pH 6.5 (75% for spermine and 73% for Lys-Lys-Lys-Lys-Lys).

A strong triplex-stabilizing effect of peptides means longer residence times for TFOs on their duplex targets. We asked whether the presence of basic peptides would reduce the concentration of a TFO needed to obtain a certain level of triplex DNA formation. Figure 5 shows that Lys-Gly-Lys-Gly-Lys at 1 mM effectively reduced the amount of TFO I necessary for duplex protection from DMS modification. In the absence of stabilizers, an about 20-fold molar excess of TFO I was required for 50% protection at pH 5. In the

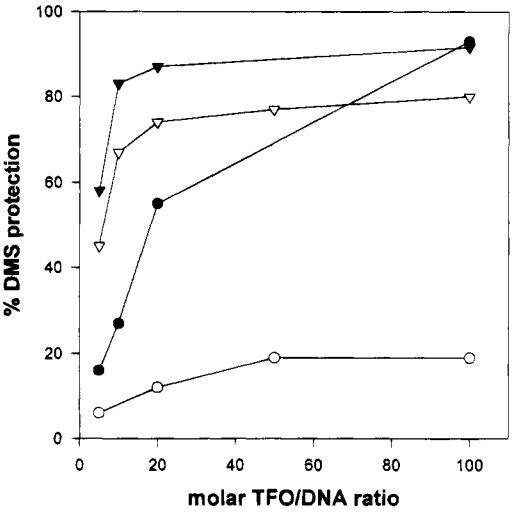

FIGURE 5: TFO concentration dependence of the Py·Pu*Py triplex. Triplexes were formed between the Py·Pu region of pRDH7 and a perfectly matched TFO I or TFO II that will form a Py·Pu*Py triplex containing a single mismatch. The TFO/DNA complexes were formed overnight in 50 mM sodium acetate (pH 5) containing either 1 mM EDTA or 1 mM Lys-Gly-Lys-Gly-Lys before analysis using the DMS protection assay. Experimental points are as follows: pRDH7 plus TFO I in the presence of EDTA (●) or Lys-Gly-Lys-Gly-Lys (▼); pRDH7 plus TFO II in the presence of EDTA (○) or Lys-Gly-Lys-Gly-Lys (▽).

presence of 1 mM Lys-Gly-Lys-Gly-Lys, only a 3−4-fold TFO I excess was necessary to elicit a similar level of protection.

Third strand mismatches within a triplex DNA region significantly weaken the interactions between the duplex target region and a TFO (Moser & Dervan, 1987; Roberts & Crothers, 1991). The effect of basic peptides on the stabilization of a Py·Pu*Py triplex containing a single mismatch is shown in Figure 5. In the case of TFO II, which is capable of forming an imperfect triplex with one mismatch at the same target site as that for TFO I, very little triplex DNA formed in the absence of stabilizers at pH 5.0. At a TFO/DNA ratio (R) of 100, the level of DMS protection was about 20%. A further increase in the TFO II concentration resulted in a slow increase in DMS protection to 50% at R = 1000 (data not shown). When 1 mM Lys-Gly-Lys-Gly-Lys was added with TFO II, 50% protection from DMS modification was observed at $R < 10$ (at a 100-fold lower TFO II concentration than without the peptide). The level of DMS protection was further increased with increasing concentrations of TFO II, with about 80% protection at R = 100. A similar nonspecific triplex stabilization by spermine was also observed (data not shown).

*Stabilization of a Py·Pu*Pu Triplex by Basic Oligopeptides.* Figure 6 shows the DMS modification pattern of the Py·Pu*Pu triplex formed at neutral pH in the presence of either 1 mM EDTA, 1 mM Mn^{2+}, 0.1 mM spermine, 1 mM Lys-Gly-Lys-Gly-Lys, 1 mM Lys-Ala-Lys-Ala-Lys, or 1 mM Lys-Lys-Lys-Lys-Lys. Lane 0 shows the maximal level of DMS modification for duplex DNA in the absence of TFO III. In the presence of TFO III, no triplex formation and concomitant protection of the duplex Py·Pu tract was observed without multivalent cations (lane 1). The addition of 1 mM Mn^{2+} or 0.1 mM spermine resulted in protection from DMS, indicative of triplex formation between the target region of the Py·Pu tract and TFO III (lanes 2 and 3). The observed stabilization of the Py·Pu*Pu triplex by Mn^{2+} or

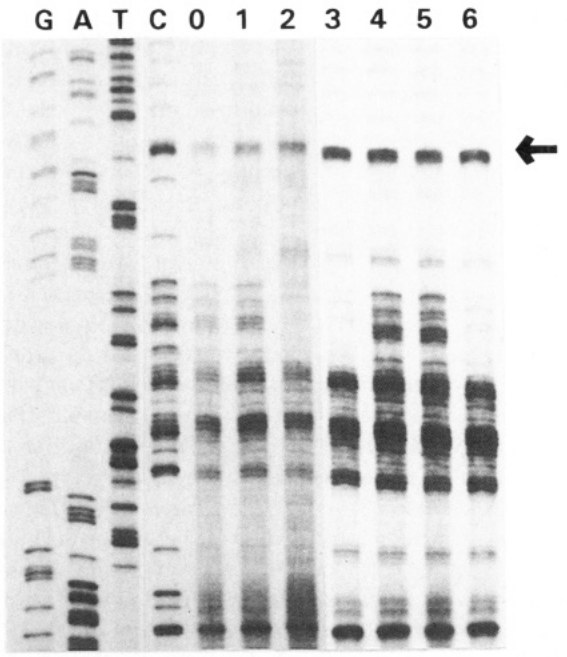

FIGURE 6: Comparison of effects of Mn^{2+}, spermine, Lys-Gly-Lys-Gly-Lys, Lys-Ala-Lys-Ala-Lys, and Lys-Lys-Lys-Lys-Lys on the protection of guanines from DMS modification by TFO III. Before treatment with DMS, a 446 bp fragment of plasmid pBB151-8 was incubated with a 100-fold excess of TFO III overnight in 25 mM Tris-HCl (pH 7.6), 20 mM NaCl, and one of the following: 1 mM EDTA (lane 1), 1 mM $MnCl_2$ (lane 2), 0.1 mM spermine (lane 3), 1 mM Lys-Gly-Lys-Gly-Lys (lane 4), 1 mM Lys-Ala-Lys-Ala-Lys (lane 5), or 1 mM Lys-Lys-Lys-Lys-Lys (lane 6). Lane 0 shows the DMS modification pattern in the absence of a TFO. Lanes denoted G, A, T, and C are the dideoxy sequencing lanes of the complementary strand. The reference band used for quantitation is indicated by an arrow.

spermine was consistent with the previous results of Beal and Dervan (1991) and Malkov et al. (1993). Lys-Lys-Lys-Lys-Lys (1 mM) (lane 6) stabilized the Py·Pu*Pu* triplex as effectively as Mn^{2+} or spermine (only background levels of primer extension pausing were observed, indicative of stable triplex formation). Lys-Gly-Lys-Gly-Lys (1 mM) (lane 4) or Lys-Ala-Lys-Ala-Lys (1 mM) (lane 5) reduced DMS modification by only 20%, indicative of relatively insignificant triplex stabilization. No reduction in the extent of DMS modification was detected under any conditions at the adjacent Py·Pu sequence, where TFO III could potentially form a triplex with two mismatches and an unpaired terminal base.

DISCUSSION

Stabilization of Intermolecular Triplex DNA by Basic Oligopeptides. Our data show that lysine-rich oligopeptides can substitute for other triplex-stabilizing factors including low pH, divalent metal cations, or polyamines. A protonated intermolecular triplex between a Py·Pu tract and a pyrimidine TFO that requires pH 5 for formation was stabilized up to pH 6.5 in the presence of lysine-rich peptides. The level of stabilization by 1 mM peptide was generally greater than stabilization by 10 mM Mg^{2+} and equivalent to stabilization by 0.3 mM spermine. Effective peptide concentrations for stabilization were in the range of 10^{-5} to 10^{-2} M. Pentalysine was the most effective triplex stabilizer of the three basic peptides studied. It is effective at concentrations lower than those required for stabilization by other peptides and similar to the effective concentrations of spermine which is a well-known triplex stabilizer (Glaser & Gabbay, 1968; Raae & Kleppe, 1978; Hampel et al., 1991; Hanvey et al., 1991; Singleton & Dervan, 1993; Thomas & Thomas, 1993). Significant triplex formation was observed at pH 7 in the presence of pentalysine. The differences in triplex-stabilizing effects of the three peptides as well as spermine might be explained in terms of the positive charge densities. For example, a strong stabilizing effect of Lys-Lys-Lys-Lys-Lys might result from its high total positive charge (five positive charges in side chains and one positive charge at the amino terminus). The two other peptides studied and spermine have only four charges each. The higher charge density in the smaller spermine molecule might result in triplex stabilization at lower concentrations compared to stabilization by Lys-Gly-Lys-Gly-Lys with a lower charge density. However, the difference in stabilization by Lys-Gly-Lys-Gly-Lys and Lys-Ala-Lys-Ala-Lys with the same charge density suggests the importance of other contributions similar to the polyamine stabilization effects. For example, triplex stabilization by polyamine analogs is sensitive to the distance between the consecutive charges in polyamine chains with the same total charge (Thomas & Thomas, 1993). The increasing size of hydrophobic substituents in polyamine analogs decreases the degree of stabilization of triple helices (Glaser & Gabbay, 1968). The latter effect might be correlated with a reduced triplex stabilization by Lys-Ala-Lys-Ala-Lys in which a methyl side group makes an Ala residue more hydrophobic than Gly. The relative contributions of stabilizing/destabilizing effects of different types of interaction between basic peptides and triple-stranded nucleic acid remain to be clarified. However, in general, the basic peptide stabilization of the Py·Pu*Py* triplex is comparable to that of polyamines and is stronger than the effect of magnesium ions.

Basic peptides were more effective at stabilization of a Py·Pu*Py* triplex than a Py·Pu*Pu* triplex. At 1 mM, only Lys-Lys-Lys-Lys-Lys stabilized the Py·Pu*Pu* triplex at the level characteristic of spermine, whereas Lys-Gly-Lys-Gly-Lys and Lys-Ala-Lys-Ala-Lys resulted in weaker TFO III binding. The difference between the stabilizing effects of Lys-Gly-Lys-Gly-Lys and Lys-Ala-Lys-Ala-Lys on Py·Pu*Py* and Py·Pu*Pu* triplexes may be a result of structural differences between the two types of triplexes. According to molecular mechanics simulations, the conformations of the three strands in both types of triplexes are close to B-DNA (Laughton & Neidle, 1992). However, the third strand in the Py·Pu*Pu* triplex is shifted in the major groove so that the phosphate groups of the third and purine strands are much closer than in the Py·Pu*Py* triplex. Therefore, the requirements for cationic stabilization of the Py·Pu*Py* and Py·Pu*Pu* triplexes may differ significantly. Consistent with this interpretation, the Py·Pu*Py* triplexes can be stabilized by mono- and multivalent cations (Felsenfeld & Rich, 1957; Rich, 1960; Krakauer & Sturtevant, 1968; Hampel et al., 1991; Hanvey et al., 1991; Singleton & Dervan, 1993), whereas the Py·Pu*Pu* triplexes require stabilization by divalent cations and cations of higher valences (Kohwi & Kohwi-Shigematsu, 1988; Beal & Dervan, 1991; Malkov et al., 1993).

The concentrations of TFOs required to protect the duplex target from interactions with chemicals were significantly reduced in the presence of peptides. In a similar way, peptides may reduce the amount of TFO required to protect

DNA from interactions with proteins. This may be relevant to antigene strategies where TFOs are designed to form specific triplex structures that will prevent proteins from interacting with their duplex targets. Since, after introduction of TFOs into cells, they may be digested by intramolecullar nucleases reducing their intracellular concentration, the possibility of formation of a triplex at lower TFO concentrations may be important for gene therapy. However, strong interactions of positive charges in basic peptides with DNA may result in nonspecific stabilization of triplex structures containing mismatches. In addition to peptide concentration, variations in peptide length, the fraction of basic amino acid residues, and the presence of other nonbasic amino acid residues may allow a "tuning" of the triplex-stabilizing effect.

The strong triplex-stabilizing effect of peptides might be important for the stabilization of intramolecular triplex structures, which require mirror-repeated Py•Pu tracts. Py•Pu tracts with perfect mirror symmetry do not frequently occur in natural sequences, and many intramolecular triplex structures that may be biologically relevant could contain some mismatches. Such mismatched triplex structures might be stabilized by polycations such as polyamines or polypeptides.

Triplex stabilization by basic peptides is consistent with the stabilizing effects of several compounds on A-DNA, Z-DNA, and condensed forms of DNA, in which the charge density is comparable or higher than in conventional B-DNA. Divalent metal cations promote the B to A (Xu et al., 1993) and B to Z (Pohl & Jovin, 1972; Behe & Felsenfeld, 1981) transitions in DNA, as well as DNA condensation [see Bloomfield (1991) for review]. Polyamines are known to stabilize nucleic acids against thermal denaturation (Tabor, 1962; Glaser & Gabbay, 1968; Thomas & Bloomfield, 1984), facilitate DNA condensation (Gosule & Schellman, 1978), promote the B to A transition (Minyat et al., 1978), and induce left-handed Z-DNA (Wang et al., 1979; Behe & Felsenfeld, 1981). Both divalent metal cations and polyamines have been shown to stabilize triple-helical nucleic acids (Felsenfeld & Rich, 1957; Glaser & Gabbay, 1968; Raae & Kleppe, 1978; Kohwi & Kohwi-Shigematsu, 1978; Hampel et al., 1991; Hanvey et al., 1991; Malkov et al., 1993; Singleton & Dervan, 1993; Thomas & Thomas, 1993). The interaction of nucleic acids with lysine- and arginine-rich polypeptides as reasonable models for basic proteins has been extensively studied [Hélène and Maurizot (1981) for a review]. The polypeptide side chains may entirely neutralize the phosphate charges of single- and double-stranded nucleic acids and at sufficient concentration lead to the condensation of the DNA−peptide complex formed (Leng & Felsenfeld, 1966; Latt & Sober, 1967). Upon addition of basic oligopeptides or polypeptides, the melting temperatures of RNA and DNA are elevated, indicating an increased stability of the double helix (Tsuboi et al., 1966; Olins et al., 1968; Gabbay et al., 1973; Mandel & Fasman, 1974). Lysine-containing peptides with $(Ala\text{-}Lys)_n$, $(Gly\text{-}Lys)_n$, $(Lys\text{-}Ala\text{-}Ala)_n$, and $(Lys\text{-}Leu\text{-}Ala)_n$ sequences act in a manner similar to that of polyamines in inducing Z-DNA (Takeuchi et al., 1991, 1994; Votavová et al., 1991; Votavová & Šponar, 1993). Our data extend the list of DNA structures known to be stabilized by basic oligopeptides to include intermolecular triplex DNA.

Interaction of Basic Peptides with Triplex DNA. Peptides may be localized in the minor groove, since the major groove is occupied by the third polynucleotide strand. Alternatively, basic peptides may bind in the new grooves which are formed upon third strand binding, or elsewhere on the triplex helix surface. Most likely, oligopeptides bind to triplex DNA through interactions in the minor groove. The affinities of oligolysine binding to poly(U)•poly(A) and poly(U)•poly(A)•poly(U) were similar, implying that the same binding site is present in each of these two polynucleotide complexes (Latt & Sober, 1967). Only the minor grooves of double- and triple-stranded complexes would be expected to be similar, since the major groove of duplex DNA becomes inaccessible upon third strand binding. For double-stranded DNA, basic polypeptides have been suggested to bind in the minor groove in an extended form with the side chains alternatively pointed at one or another polynucleotide strands to neutralize charges on phosphates (Feughelman et al., 1955; Tsuboi, 1967). Moreover, peptide-related antibiotics such as netropsin and distamycin A, which bind to the minor groove of double-stranded DNA [Zimmer and Wähnert (1986) for review], have also been shown to bind to triplex nucleic acids (Umemoto et al., 1990; Durand et al., 1992; Howard et al., 1992; Park & Breslauer, 1992; Pilch & Breslauer, 1994). In addition, because of the interaction of the third strand in the major groove and the peptide in the minor groove, they should not interfere with binding of each other to different sites on the duplex target. Evidence that two ligands can bind to the same DNA sequence in the major and minor grooves was presented by Oakley et al. (1992), who showed that a major groove-bound protein did not noticeably change the minor groove binding of a netropsin analog. Thus, basic peptides may be localized in the minor groove of triplex DNA.

Some degree of oligopeptide sequence specificity may exist for the formation of triplex DNA. Studies of oligopeptide−duplex DNA interactions have shown that polylysines preferentially bind to the A+T-rich DNA and that polyarginines show a marked preference for the G+C-rich DNA (Leng & Felsenfeld, 1966; Wehling et al., 1975). Sequence specificity results from the contribution of specific amino acid−nucleobase hydrogen bonds in the basic peptide−DNA interaction [see Hélène and Maurizot (1981) and Saenger (1984) for discussion of possible hydrogen-bonding schemes between the ϵ-amino group of lysine and either adenine or thymine and between the guanidino group of arginine and guanine].

Although basic peptides may be localized in the minor groove of DNA where peptide-related antibiotics can bind, their modes of interaction with DNA are different. For example, the major contribution to netropsin-binding affinity to A+T-rich DNA sequences comes from the hydrogen bonding between the peptide backbone imino groups and the O2 of thymine and N3 of adenine (Zimmer & Wähnert, 1986). The resulting effect of netropsin on non B structures is a destabilization of the Z form (Zimmer et al., 1983) or the $(dT)_n$•$(dA)_n$•$(dT)_n$ triplex (Durand et al., 1992; Park & Breslauer, 1992). The largely electrostatic interactions between the numerous positive charges of basic peptides (e.g., polylysine) and the negative charges of DNA effectively stabilize the Z form (Takeuchi et al., 1991, 1994; Votavová et al., 1991; Votavová & Šponar, 1993) and, according to our results, triple-stranded DNA.

Biological Source of Basic Polypeptides. Many eukaryotic and prokaryotic proteins contain clustered basic amino acid

residues, and basic oligopeptides may provide a model for surface-localized protein domains which might interact with triple-helical nucleic acids. The histones, which are used to package the entire genome into chromatin, are abundant in lysine. In histone H1, almost one-third of the total amino acid residues are lysine (Mirzabekov, 1980). However, histones might not be likely candidates for binding to triplex DNA because their interactions with DNA primarily involve the major groove (Mirzabekov et al., 1978). Protamines are small proteins (molecular mass of about 4.2 kDa), which condense the DNA in sperm. They are highly basic and contain regions consisting of four to six arginines (Saenger, 1984). Models of protamine−DNA complexes suggest association of protamine α-helices with the major groove (Warrant & Kim, 1978) or binding of protamines in the extended form of the minor groove (Feughelman et al., 1955). Prokaryotic cells produce small basic histone-like proteins that bind in the minor groove of DNA through hydrogen bonding of clustered arginines and lysines with the phosphate backbone [Drlica and Rouviere-Yaniv (1987) for review]. A DNA (cytosine-5-)-methyltransferase in mouse cells contains a run of 13 alternating lysine and glycine residues (Bestor et al., 1988). A human DNA-binding factor that represses transcription (Kageyama & Pastan, 1989) has a sequence of seven lysines in its basic N-terminus. The basic peptides Tyr-Pro-Lys-Arg-Pro-Arg-Gly-Arg-Pro-Lys-Lys, Pro-Arg-Gly-Arg-Pro-Lys-Lys, and Pro-Arg-Gly-Arg-Pro derived from the non-histone chromosomal protein HMG-I/Y have been shown to bind in the minor groove of some model DNA duplexes (Geierstanger et al., 1994). It is possible that basic oligopeptides exist in the cell. A family of nuclear oligopeptides of molecular masses 1.5−6 kDa, which bind single-stranded oligonucleotides, RNA, and double-stranded DNA, has been found in nuclear extracts (Svinarchuk et al., 1993). Because they bind to DNA, these polypeptides may be basic and their nonspecific binding to nucleic acids may result from electrostatic interactions between negatively charged phosphates and positively charged basic amino acid residues. A single report has described the partial characterization of a protein which bound to a poly(dT)·poly(dA)·poly(dT) triplex with a 2−5-fold preference over a poly(dT)·poly(dA) duplex (Kiyama & Camerini-Otero, 1991).

Cellular proteins containing clustered basic amino acid residues clearly exist and may stabilize the formation of inter- and intramolecular triplex DNA *in vivo*. Understanding the potential biology of triplex DNA *in vivo*, including natural intramolecular triplexes, as well as the therapeutic applications of TFOs, will require an understanding of the interaction of triplex DNA with protein motifs.

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