Accelerated Publications

Intramolecular Triple-Helix Formation at $(Pu_nPy_n)\cdot(Pu_nPy_n)$ Tracts: Recognition of Alternate Strands via Pu·PuPy and Py·PuPy Base Triplets†

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ABSTRACT: Triple-helical DNA shows increasing potential for applications in the control of gene expression (including therapeutics) and the development of sequence-specific DNA-cleaving agents. The major limitation in this technology has been the requirement of homopurine sequences for triplex formation. We describe a simple approach that relaxes this requirement, by utilizing both Pu·PuPy and Py·PuPy base triplets to form a continuous DNA triple helix at tandem oligopurine and oligopyrimidine tracts. [Triplex formation at such a sequence has been previously demonstrated only with the use of a special 3′–3′ linkage in the third strand [Horne, D. A., & Dervan, P. B. (1990) *J. Am. Chem. Soc. 112*, 2435–2437].] Supporting evidence is from chemical probing experiments performed on several oligonucleotides designed to form 3-stranded fold-back structures. The third strand, consisting of both purine and pyrimidine blocks, pairs with purines in the Watson–Crick duplex, switching strands at the junction between the oligopurine and oligopyrimidine blocks but maintaining the required strand polarity without any special linkage. Although Mg^{2+} ions are not required for the formation of Pu·PuPy base triplets, they show enhanced stability in the presence of Mg^{2+}. In the sequences observed, A·AT triplets appear to be more stable than G·GC triplets. As expected, triplex formation is largely independent of pH unless C^+·GC base triplets are required.

The sequence-specific recognition of nucleic acids is an essential step in almost all cellular processes occurring at the nucleic acid level, and the designing of sequence-specific nucleic acid binding molecules offers an attractive approach for controlling such processes. Recently, intense research has been focused on the nucleic acid triple helix, a structure that was discovered more than 30 years ago in synthetic polyribonucleotides consisting exclusively of purine and pyrimidine strands (Felsenfeld et al., 1957). Triplex DNA has been shown to form in oligopurine–oligopyrimidine mirror repeats under superhelical stress (H-DNA) (Lyamichev et al., 1986; Mirkin et al., 1987; Voloshin et al., 1988; Htun & Dahlberg, 1988; Kohwi & Kohwi-Shigematsu, 1988; Hanvey et al., 1988; Wells et al., 1988; Johnston, 1988; Glover & Pulleyblank, 1990), between linear single strands and native duplex DNA (intermolecular triplexes) (Moser & Dervan, 1987; François et al., 1988; Pilch et al., 1990), and within linear single strands (intramolecular triplexes) (Häner & Dervan, 1990; Chen, 1991) under suitable conditions. Although a detailed X-ray crystal structure of a triple helix is still lacking, a substantial body of chemical and physical evidence indicates that recognition of a duplex oligopurine–oligopyrimidine tract by an oligopyrimidine single strand (Figure 1a, part i) involves the occupation of the major groove of the duplex by the oligopyrimidine strand and the formation of the Py·PuPy base triplets T·AT and C^+·GC via Hoogsteen hydrogen bonding (Felsenfeld et al., 1957; Morgan & Wells, 1968; Lee et al., 1979; Praseuth et al., 1988; Johnston, 1988; Rajagopal & Feigon, 1989; de los Santos et al., 1989). The oligopyrimidine strand lies in parallel orientation to the oligopurine tract of the duplex (Moser & Dervan 1987; Praseuth et al., 1988), and the pairing of T with AT and C^+ with GC makes the recognition sequence-specific. Because the C^+·GC triplet requires protonated cytosines on the Hoogsteen (third) strand, triplex formation is facilitated by low pH (Mirkin et al., 1987).

In addition to Py·PuPy triplets, another general class of base triplets, Pu·PuPy (G·GC and A·AT), has also been proposed to explain the sequence-specific recognition of oligopurine–oligopyrimidine tracts in duplex DNA by oligopurine single strands (Figure 1a, part ii). In this case, the oligopurine single strand, which again lies in the major groove, is antiparallel to the oligopurine tract in duplex DNA (Beal & Dervan, 1991). Triplexes of the Pu·PuPy type can form independently of pH (Kohwi & Kohwi-Shigematsu, 1988; Kohwi-Shigematsu & Kohwi, 1991) and thus are potentially more useful in many applications than triplexes of the Py·PuPy type. In addition to the above base triplets, unusual triplets such as G·TA (Griffin & Dervan, 1989; Radhakrishnan et al., 1991), C^+ (or C)·AT, and U·GC (Pei et al., 1991) and unnatural triplets such as I·AT and I·GC (Letai et al., 1988) have also been documented in triple-helical nucleic acids.

Triple-helix formation has been used as a strategy to (1) block DNA-binding proteins (Maher et al., 1989), (2) afford sequence-specific DNA cleavage (Moser & Dervan 1987; Perouault et al., 1990; Strobel & Dervan, 1990), and (3) repress transcription (Cooney et al., 1988; Orson et al., 1991). The recognition of any base sequence (i.e., any combination of purines and pyrimidines) would provide maximal utility for techniques based on triplex formation. So far, however, except for a few studies (Cooney et al., 1988; Beal & Dervan, 1991; Orson et al., 1991), DNA recognition via triplex formation has been restricted to oligopurine–oligopyrimidine tracts using single strands consisting exclusively of either purines or pyrimidines. Therefore, approaches are indeed needed for recognition of all four bases in triplex formation. Approaches that have been proposed (Horne & Dervan, 1990) include (1)

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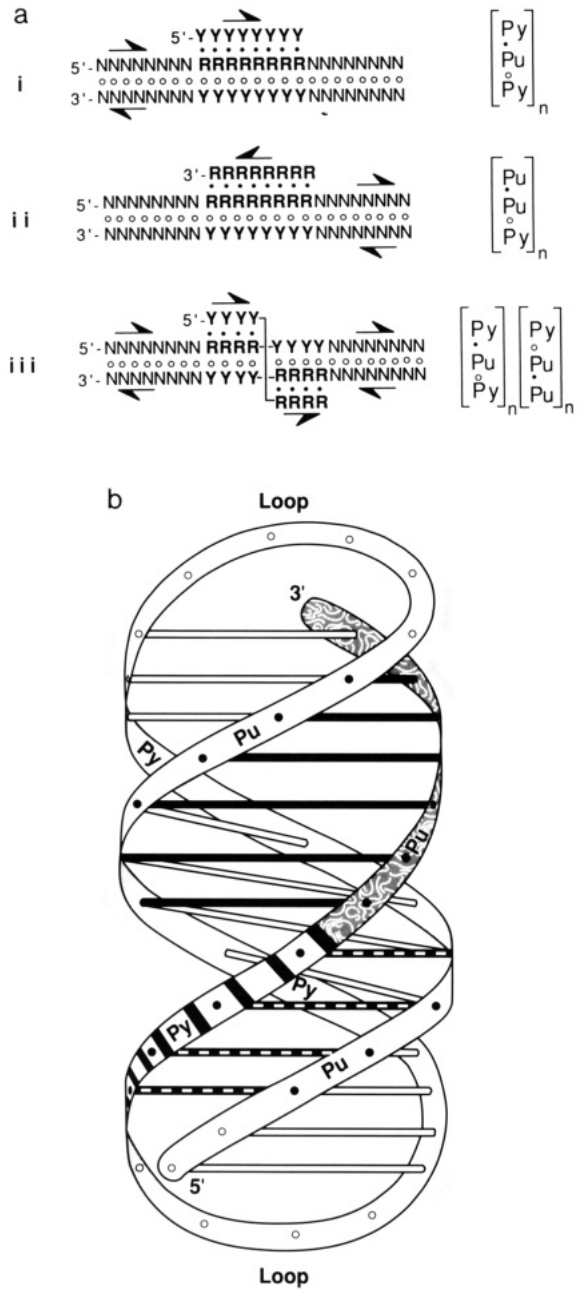

FIGURE 1: (a) Schematic illustration of different triplex motifs. (i) A Py·PuPy triple helix in which the third, oligopyrimidine strand is parallel to the purine strand of the duplex. (ii) A Pu·PuPy triple helix in which the third, oligopurine strand is antiparallel to the purine strand of the duplex. (iii) A triplex spanning tandem oligopurine and oligopyrimidine tracts in which the purine block of the third strand forms hydrogen bonds with purines of one strand of the duplex (via Pu·PuPy base triplets), whereas the pyrimidine block of the third strand hydrogen bonds to the purine tract of the other strand of the duplex via Py·PuPy base triplets. Within this hybrid triplex, the required polarity of the third strand is maintained. The polarity of each strand is shown by a half-arrow. Open circles (○) represent Watson–Crick hydrogen bonding, and closed circles (●) represent Hoogsteen or Pu·Pu-type hydrogen bonding. (b) A ribbon model for an intramolecular triplex of type iii, in which the third strand recognizes alternate strands of a hairpin duplex. Here, the third-strand region (shown in the middle in stippled gray and black-and-white stripes) folds back on the hairpin duplex (white ribbon), forming two loops (top and bottom). The purine tract of the third strand (stippled gray) forms base pairs (black bars) with the purine tract of one strand of the Watson–Crick hairpin (making Pu·PuPy base triplets), whereas the pyrimidine tract of the third strand (black-and-white) forms Hoogsteen base pairs (banded bars) with the purine tract of the other Watson–Crick strand making Py·PuPy base triplets. White bars represent Watson–Crick hydrogen bonding, and arrows indicate the polarity (5′→3′) of the strand.

synthesizing novel, unnatural bases to complete the triplet code; (2) excluding the recognition of certain base pairs in a triplex motif by incorporating abasic sites in the single strand, which may decrease the specificity of triplex formation; and (3) designing homopyrimidine single strands capable of binding to alternate strands of duplex DNA. In the last approach, polarity considerations required the design of an oligonucleotide with an unnatural 3′–3′ linkage of an appropriate length between two oligopyrimidine strings (Horne & Dervan, 1990).

In this study, we explore the feasibility of another approach which does not require the synthesis of unusual bases or linkages. Because two types of base triplets, Pu·PuPy and Py·PuPy, have been documented, a single strand consisting of oligopurine and oligopyrimidine blocks is expected to bind simultaneously to a duplex DNA containing adjacent tracts of oligopurines and oligopyrimidines via both Pu·PuPy and Py·PuPy base triplets, as shown in part iii of Figure 1a. Here, the oligopyrimidine block of the single strand is parallel to one purine tract of the duplex DNA and its oligopurine block is antiparallel to the other purine tract on the alternate strand of the duplex. This polarity of binding requires no special junction between the oligopurine and oligopyrimidine blocks of the single strand occupying the major groove of both oligopurine and oligopyrimidine tracts.

To test whether triple-helix formation can occur in this way at tandem oligopurine and oligopyrimidine tracts, we synthesized oligonucleotides designed to fold into intramolecular triplexes as shown schematically in Figure 1b. Intramolecular triplex formation by oligonucleotides has previously been demonstrated using purely Py·PuPy (Häner & Dervan, 1990) or Pu·PuPy (Chen, 1991) base triplets. Using several different chemical probes, we present evidence indicating that triple-helix formation can indeed occur at tandem oligopurine and oligopyrimidine tracts. This finding suggests that the approach of using both known types of base triplets together with strand switching can significantly relax the requirement of homopurine sequences for triplex formation.

Materials and Methods

Oligonucleotides were synthesized on an Applied Biosystems Model 381 B automated DNA synthesizer using cyanoethyl phosphoramidites. After deprotection, they were purified by electrophoresis on denaturing 20% polyacrylamide gels. Purified oligonucleotides were labeled at 5′ ends using T_4 polynucleotide kinase (United States Biochemicals) and [γ-^{32}P]-ATP (Du Pont–NEN) according to Maxam and Gilbert (1980). Unincorporated ATP was separated from labeled oligonucleotides by passing the labeling reaction mixture through two successive Sephadex G-50 spin columns equilibrated in 10 mM Tris-HCl and 0.1 mM EDTA (pH 7.5).

Potassium Permanganate ($KMnO_4$) Modification (Rubin & Schmid, 1980). A radiolabeled oligonucleotide (~100 ng) was equilibrated in 9 μL of the reaction buffer at 4 °C for 10 min, and then 1 μL of 5 mM $KMnO_4$ (freshly diluted from a 100 mM stock stored at 4 °C) was added. After 10 min of incubation at 4 °C, the reaction was stopped by adding 2 μL of neat allyl alcohol.

Diethyl Pyrocarbonate (DEPC) Modification (Johnston & Rich, 1985; Herr, 1985). An end-labeled oligonucleotide (~100 ng) was equilibrated in 97.5 μL of a reaction buffer for 10 min at 4 °C. Then, 2.5 μL of DEPC (Aldrich) was added, the mixture was vortexed briefly, and the incubation was continued for 30 min.

Dimethyl Sulfate (DMS) Reaction (Maxam & Gilbert, 1980). One microliter of a 1:200 dilution of DMS in water

Table I: Oligonucleotides Used in This Study

I	5′-AAAAAAAATTTTTTTTTTTTTTTTTTTTT-3′
II	5′-TTTTTTTTTTTTAAAAAAAATTTTAAAAAAAA-3′
III	5′-AAATTTTGTTAAAAATTTGGTGGTTTAAAA-3′
IV	5′-AAAACCCCTTTTGGGGTTTTCTTCTTTTGGGG-3′
V	5′-GAGATCTCGTTTGAGATCTCTTTACTCTAGAG-3′

(freshly prepared) was added to an end-labeled oligonucleotide (~100 ng) equilibrated in 9 μL of reaction buffer at 4 °C, and the modification was carried out for 10 min at the same temperature.

For modifications performed at higher temperatures than those noted above, the reaction times were decreased accordingly to control excess modification. The modification reactions were stopped by two ethanol precipitations except in the case of $KMnO_4$. DNA pellets were thoroughly washed with 70% ethanol, dried, and treated with hot piperidine (1 M, 90 °C for 30 min). $KMnO_4$-modified DNA was treated with piperidine directly, without ethanol precipitation. After piperidine was removed by vacuum evaporation, DNAs were resuspended in formamide loading buffer and the cleavage products were resolved on 20% sequencing gels. Sequencing reactions on oligonucleotides were performed according to Williamson and Celander (1990).

Results

Potassium permanganate is sensitive to the secondary structure of nucleic acids, and it specifically reacts with exposed thymines at the 5–6 double bond (Kochetkov & Budovskii, 1972). Thus, thymines in single-stranded DNA are sensitive to $KMnO_4$ modification but become resistant when involved in a duplex (Hayatsu & Ukita, 1967). As recently shown by Häner and Dervan (1991), thymines on a single strand become less reactive toward $KMnO_4$ when the single strand is incorporated into a triple helix by occupying the major groove of a DNA duplex. DMS reacts predominantly at the N-7 position of guanines (Maxam & Gilbert, 1980). In a triple helix, the N-7 position of guanines in the Watson–Crick duplex is involved in hydrogen bonding to the third strand in both C^+·GC and G·GC base triplets, and in the case of C^+·GC triplets, guanines have been shown to become resistant to DMS modification (Johnston, 1988; Hanvey et al., 1988). DEPC, which carbethoxylates purines (A>G) primarily at the N-7 position, is useful for probing adenine residues involved in triplex formation. In T·AT base triplets, adenines in the Watson–Crick strand show protection from DEPC modification because of the Hoogsteen hydrogen bonding involving the N-7 position (Johnston, 1988; Htun & Dahlberg, 1988; Hanvey et al., 1988). Similar protection is expected for the Watson–Crick adenine of the A·AT base triplet.

Oligonucleotide sequences used in the study are listed in Table I. Oligonucleotide I has the general form $(Pu)_nN_4(Py)_nN_4(Py)_n$ and is expected to form an intramolecular triple helix with Py·PuPy base triplets. On the other hand, oligonucleotide II has the general form of $(Py)_nN_4(Pu)_nN_4(Pu)_n$ and thus has a potential of forming an intramolecular triplex with Pu·PuPy base triplets. Oligonucleotides III–V consist of two different triplex-forming motifs fused together—i.e., these oligonucleotides can be considered as hybrids of oligonucleotides I and II. They belong to the general form $(Pu)_n(Py)_nN_4(Pu)_n(Py)_nN_4(Py)_n(Pu)_n$ and can potentially form a triplex consisting of a block of Py·PuPy base triplets connected to a block of Pu·PuPy base triplets. For clarity, each oligonucleotide is described in three segments: 5′, middle, and 3′ segments.

The results of $KMnO_4$ modification on oligonucleotide I performed in 10 mM Tris-HCl (pH 7.0) and 0.1 mM EDTA (TE) buffer are shown in Figure 2. Thymines in the middle segment (T_{12}–T_{17}) are less reactive toward $KMnO_4$ than thymines on either side (lane 2), consistent with the middle segment being base-paired to the 5′ segment, making a Watson–Crick duplex. With increasing concentration of Mg^{2+} ions, thymines in the 3′ segment (T_{23}–T_{28}; bracket) become more and more resistant to $KMnO_4$ modification and the protection of thymines in the middle segment becomes more pronounced (lanes 3–5). In 20 mM $MgCl_2$ (lane 5), the overall reactivity pattern is consistent with an intramolecular triplex having T·AT base triplets, as expected, with only the thymines that occupy the putative loop regions (T_9–T_{11} and T_{18}–T_{22}) being sensitive to $KMnO_4$ modification and thus providing an internal control for the modification. The first two thymines at the 5′ end remain hyperreactive toward $KMnO_4$ throughout all conditions, indicating that these bases are unpaired possibly because of "fraying".

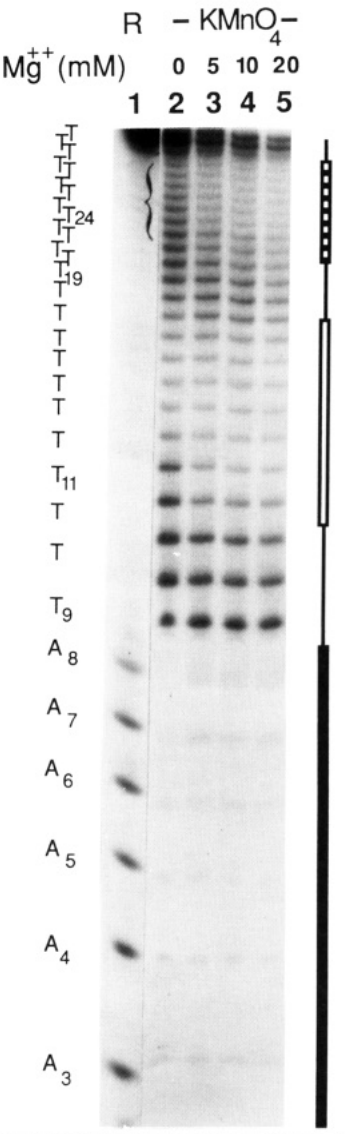

FIGURE 2: $KMnO_4$ reactivity of oligonucleotide I. $KMnO_4$ modification was performed in 10 mM Tris-HCl (pH 7.0) and 0.1 mM EDTA (TE) buffer at 4 °C in the absence (lane 2) and presence of $MgCl_2$ (lanes 3–5, concentrations as indicated). Lane 1 is the A+G sequencing reaction. Modification of thymines within the bracket is sensitive to $MgCl_2$. Open and closed bars represent Watson–Crick duplex region; lines show single-stranded regions and the hatched bar indicates the third "strand" of the triplex.

In contrast to oligonucleotide I, oligonucleotide II has the potential to form an intramolecular triplex with A·AT base

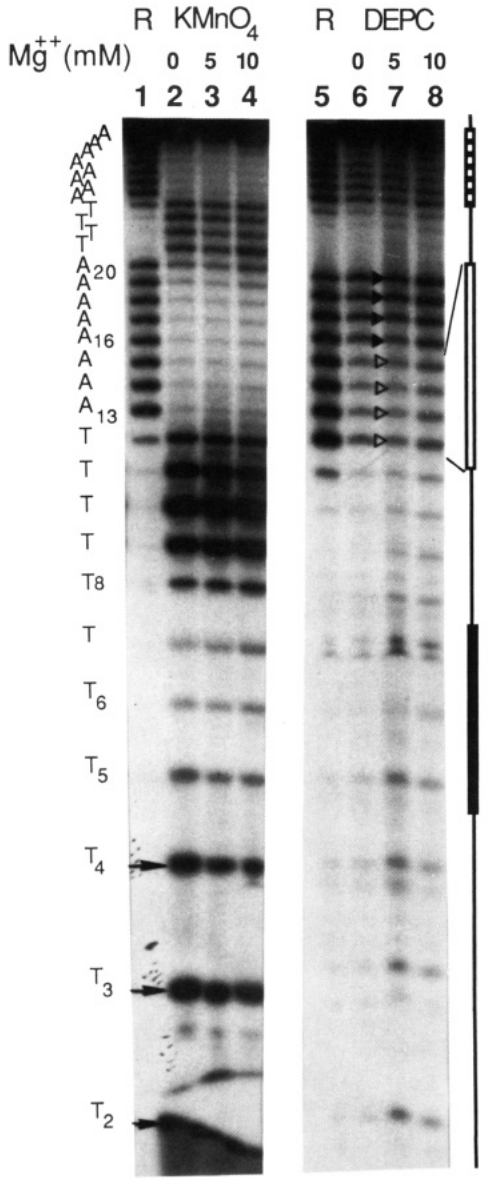

FIGURE 3: $KMnO_4$ and DEPC reactivity of oligonucleotide II. Both modifications were performed at 4 °C in 50 mM sodium cacodylate buffer (pH 7.1) with or without $MgCl_2$. Lanes 1 and 5 are the A+G sequencing reaction. Lanes 2–4 are $KMnO_4$ modification; lanes 6–8 are DEPC modification. $MgCl_2$ concentrations were as shown. Closed arrows show hyperreactive thymines (T_2–T_4) at the 5′ end. Open arrowheads show adenines (A_{13}–A_{16}) that are protected from DEPC modification, whereas closed arrowheads represent reactive adenines (A_{17}–A_{20}). Helical and loop regions are designated as in Figure 2.

triplets. The reactivity patterns of oligonucleotide II in 50 mM sodium cacodylate (pH 7.1) buffer (Figure 3) indeed support a triplex conformation. In addition to the thymines occupying the two potential loops (T_9–T_{12} and T_{21}–T_{24}), the first four thymines (T_1–T_4) at the 5′ end are also reactive toward $KMnO_4$ (lanes 2–4, arrows), suggesting that these bases are at least transiently unpaired or "frayed". The lesser reactivity of internal thymines in the 5′ segment (T_5–T_8) suggests the formation of a Watson–Crick duplex within this region. The reactivity of oligonucleotide II toward DEPC in the same buffer is shown in lanes 6–8 (Figure 3). All adenines of the 3′ segment are equally reactive, whereas those on the middle segment are not; A_{13}–A_{16} are protected from DEPC modification, and A_{17}–A_{20} are reactive. The DEPC protection of A_{13}–A_{16} residues can be attributed to the formation of A·AT base triplets. The reactivity pattern of these adenines does not change with the addition of Mg^{2+} ions, indicating that the formation of A·AT triplets does not require Mg^{2+} ions. This finding is analogous to the behavior of G·GC base triplets, detection of which does not require Mg^{2+} ions in the medium (Chen, 1991). The reactivity of A_{17}–A_{20} indicates that these adenines are not stably involved in the formation of A·AT triplets, apparently because of "fraying" of the homologous Watson–Crick duplex in this region as deduced from $KMnO_4$ reactivity.

Attempts to construct *inter*molecular duplex- or triplex-pairing schemes for either oligonucleotide I or II that were consistent with the observed reactivities were unsuccessful. For example, an intermolecular duplex structure for oligonucleotide I should result in protection of thymines next to the adenine tract (T_9–T_{16}) and not in hyperreactivity as observed.

Oligonucleotide III has two adjacent potential triple-helix-forming motifs, one making T·AT and the other making A·AT triplets. Thymines T_4–T_7 on the 5′ segment are unreactive toward $KMnO_4$ in 50 mM sodium cacodylate (pH 7.1) buffer (Figure 4a, lane 3), suggesting that they are in a Watson–Crick duplex. In contrast to T_4–T_7, T_{24}–T_{26} on the 3′ segment are modified by $KMnO_4$ (Figure 4a, lane 3), indicative of single-stranded character. With the addition of Mg^{2+} ions to the medium, the reactivity of these reactive thymines (T_{24}–T_{26}) is reduced (Figure 4a, lanes 4–6; bracket), suggesting triplex formation with T·AT base triplets. In addition to T_{24}–T_{26}, thymine T_{21} shows Mg^{2+}-dependent resistance toward modification. Because it is flanked by two guanines in the loop, the protection is probably due to stacking with adjacent guanines induced by Mg^{2+}-dependent folding. Such protection from $KMnO_4$ of thymines primarily residing in loops has been observed previously (Häner & Dervan, 1990).

Oligonucleotide III was modified with DEPC in the same buffer to detect whether A·AT triplets are formed, in addition to T·AT triplets; the results are shown in lanes 9–12 of Figure 4a. Adenines 13–15 are protected from DEPC (open arrowheads), suggesting the formation of A·AT base triplets. As observed for oligonucleotide II, the reactivity of these less-reactive adenines (A_{13}–A_{15}) is not sensitive to the presence of Mg^{2+} ions. Because it is located in the loop region, the enhanced reactivity of A_{11} (bottom closed arrowhead) is expected, and it serves as an internal control for the modification. However, the high reactivity of A_{12} (a base at the extreme end of the helix) is unexpected and could be due to the distortion caused by the loop making it unavailable for base pairing. Adenines at the 3′ end (A_{27}–A_{30}) are reactive, whereas adenines at the 5′ end (A_1–A_3) are less reactive, as clearly seen in Figure 4b (bottom of the gel, lanes 1 and 2), where DEPC modification was performed at different temperatures. In the absence of Mg^{2+} ions, the less reactive adenines (A_{13}–A_{15}; bracket) become reactive at ~37 °C (Figure 4b, lane 3), suggesting the melting of A·AT base triplets. The addition of Mg^{2+} ions raises the melting temperature, so that maximal reactivity is seen at 55 °C instead of 37 °C (Figure 4b, lane 9). Thus, the results indicate that Mg^{2+} ions are not essential for the formation of A·AT base triplets, but their stability is enhanced by Mg^{2+} ions. Taken together, the results of $KMnO_4$ and DEPC modifications suggest the presence of both Py·PuPy (T·AT) and Pu·PuPy (A·AT) base triplets within oligonucleotide III in the presence of Mg^{2+} ions.

Like oligonucleotide III, oligonucleotide IV also has the potential to form a triplex having juxtaposed Pu·PuPy and Py·PuPy motifs, but with different base triplets (T·AT and G·GC). The results of $KMnO_4$ modification of oligonucleotide IV are shown in Figure 5a. Thymines on the 3′ segment (T_{25}–T_{28}) are reactive toward $KMnO_4$ in TE buffer (lane 3;

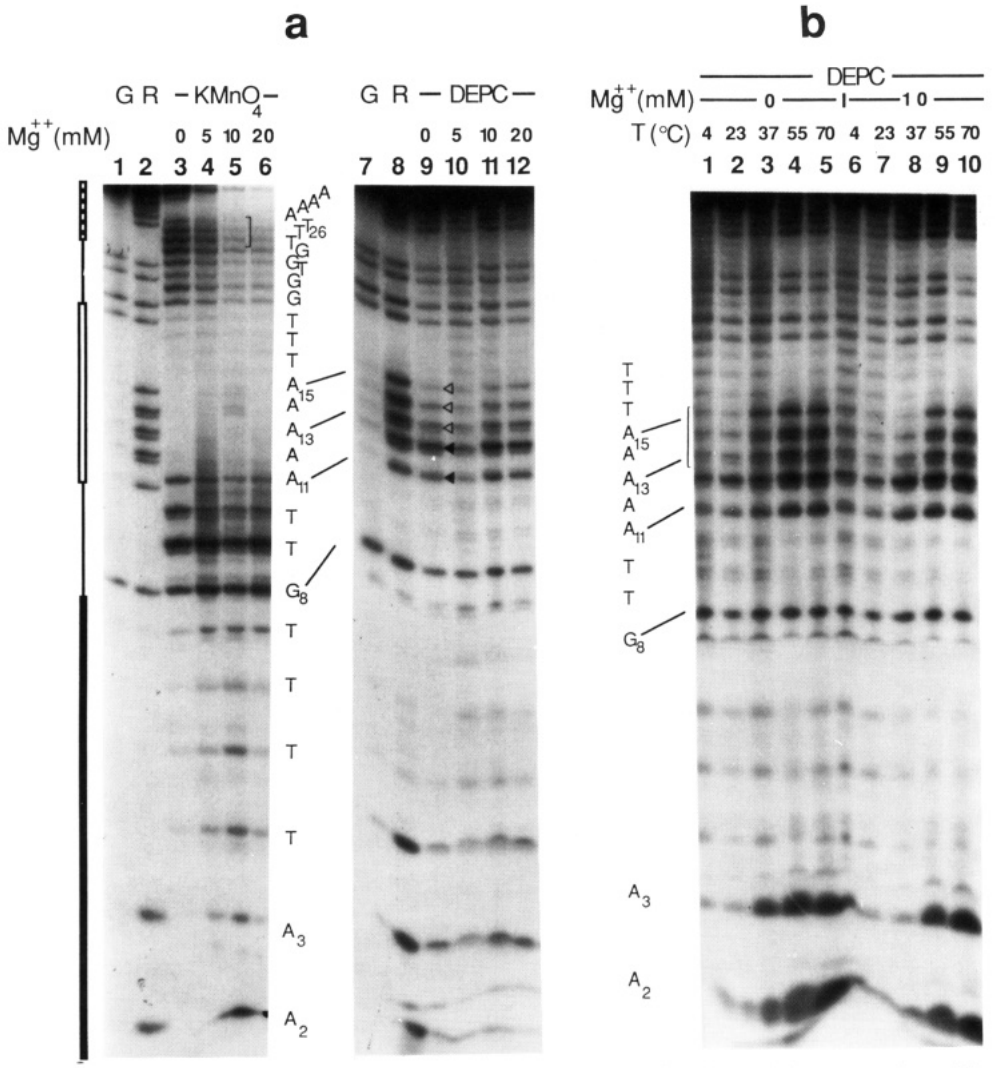

FIGURE 4: $KMnO_4$ and DEPC reactivity of oligonucleotide III. (a) $KMnO_4$ (lanes 3–6) and DEPC (lanes 9–12) modifications performed in 50 mM sodium cacodylate buffer (pH 7.1) at 4 °C. The bracket indicates thymines whose reactivity pattern is sensitive to $MgCl_2$. Lanes 1 and 7 are the G sequencing reaction, and lanes 2 and 8 are the A+G sequencing reaction. $MgCl_2$ concentrations were as indicated. Closed arrowheads indicate adenines that are reactive toward DEPC, whereas those indicated by open arrowheads are protected. (b) DEPC reactivity as a function of temperature in the absence (lanes 1–6) and presence (lanes 7–10) of 10 mM $MgCl_2$. Temperatures were as indicated. Helical and loop regions are designated as in Figure 2.

bracket), indicating that they are in a single-stranded form. However, with the addition of Mg^{2+} ions, T_{25}–T_{28} become less reactive (lanes 4–6), suggesting the formation of T·AT base triplets. On the other hand, at all Mg^{2+} ion concentrations, T_{17}–T_{20} remain less reactive, consistent with their involvement in a duplex. The reactivities of T_9–T_{12} and T_{22}–T_{23} do not change under any condition, indicating their single-stranded nature in the folded (lane 6) as well as unfolded structure (lane 3).

The results of DMS modification of oligonucleotide IV performed in TE buffer at different temperatures are shown in Figure 5b. In the absence of Mg^{2+} ions, G_{13}–G_{16} become more reactive at temperatures above 23 °C (lanes 1–6), suggesting the presence of G·GC base triplets at 23 °C or below. In the presence of Mg^{2+} ions, these guanines are less reactive at 23 °C than they are in the absence of Mg^{2+}, indicating that, analogous to A·AT base triplets, the stability of G·GC base triplets is also enhanced in the presence of Mg^{2+} even though Mg^{2+} is not essential for their formation. Thus, the results of $KMnO_4$ and DMS modifications indicate the formation of a triple helix containing both T·AT and G·GC base triplets in oligonucleotide IV. Comparison of the melting temperatures for oligonucleotides III and IV suggests that A·AT base triplets are more stable than G·GC base triplets.

The potential triple-helix-forming regions of oligonucleotide V consist of mixed purines and mixed pyrimidines. Unlike the case for other oligonucleotides, the formation of an intramolecular triplex by oligonucleotide V is expected to be pH-dependent because of the involvement of C^+·GC base triplets. The results of $KMnO_4$ modification of oligonucleotide V at different pH values are shown in Figure 6a. At pH 4.5, both T_{26} and T_{28} on the 3′ segment are protected from $KMnO_4$ modification (lane 3, arrowheads), whereas at pH 6 and 7 these two thymines are modified. This pH-dependent reactivity is consistent with the formation of T·AT base triplets in the 3′ segment at a pH permitting C^+·GC triplets to form. In 10 mM Mg^{2+}, T_{26} and T_{28} remain unreactive at both pH 6 and 7 (data not shown).

The formation of A·AT and G·GC base triplets in oligonucleotide V is detected by reacting it with DEPC and DMS, respectively. As seen in lanes 3–7 of Figure 6b, A_{14} and A_{16} are less reactive toward DEPC (open arrowheads) than A_{24} (closed arrowhead), consistent with A_{14} and A_{16} being involved in A·AT base triplets and A_{24} residing in a loop. Both A_2 and A_4 are less reactive, consistent with their involvement in T·AT base triplets. With DMS modification (lanes 8–12), G_{13} and

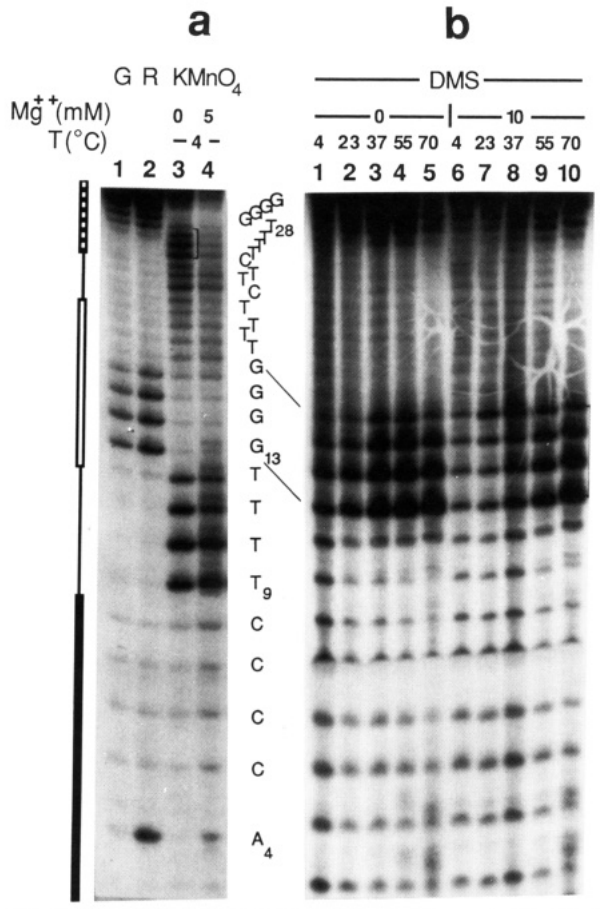

FIGURE 5: $KMnO_4$ and DMS reactivity of oligonucleotide IV. (a) $KMnO_4$ modification performed in TE buffer at 4 °C in the absence (lane 3) and presence (lane 4) of 5 mM $MgCl_2$. Lane 1 is the G sequencing reaction; lane 2 is the A+G sequencing reaction. (b) DMS reactivity as a function of temperature in the absence (lanes 1–5) and presence (lanes 6–10) of 10 mM $MgCl_2$. Temperatures were as indicated. Bracketed bases are affected by $MgCl_2$ concentration (a) or temperature (b). Helical and loop regions are designated as in Figure 2.

G_{15} remain less reactive than the internal control G_9 (in a loop; closed arrowhead), suggesting the involvement of G_{13} and G_{15} in G·GC base triplets. Here again, the formation of A·AT and G·GC base triplets is not dependent on Mg^{2+} ions. Furthermore, as expected, their formation is independent of pH as shown by the identical reactivity patterns observed for both DEPC and DMS modifications at pH 4.5 and 7.0 (compare lanes 4 and 5 with 6 and 7 and lanes 9 and 10 with 11 and 12). The reason for the unexpected reactivity of guanine G_3 is not clear.

Figure 6c shows the melting of oligonucleotide V as monitored by DEPC reactivity. As was the case with Pu·PuPy base triplets in oligonucleotides III and IV, A·AT triplets (at A_{14} and A_{16}) melt at a higher temperature in the presence of Mg^{2+} ions [>70 °C at 10 mM Mg^{2+} compared to <55 °C without Mg^{2+}; compare lanes 3–6 with lanes 7–10 (arrow heads)]. On the other hand, G_3 and A_4 show a transition to increased reactivity well below 70 °C even in the presence of Mg^{2+} ions, indicating the melting of C^+·GC and T·AT base triplets. Therefore, in the presence of Mg^{2+} ions, A·AT base triplets in this mixed purine tract appear to be more stable than T·AT base triplets. The different melting temperatures observed for A·AT base triplets generated within oligonucleotides V and III could be due to a sequence effect (oligonucleotide V contains 50% GC). In summary, the results of chemical modifications of oligonucleotide V show the formation of intramolecular triplexes utilizing alternate strands of duplex DNA containing all four bases.

Discussion

Oligonucleotide I has the general form $(Pu)_nN_4(Py)_nN_4(Py)_n$ and is expected to form an intramolecular triple helix with Py·PuPy base triplets, whereas oligonucleotide II has the general form $(Py)_nN_4(Pu)_nN_4(Pu)_n$ and thus has the potential to form an intramolecular triplex with Pu·PuPy base triplets. Oligonucleotides III–V are designed to have the Py·PuPy and Pu·PuPy motifs juxtaposed in the same helix. The reactivity patterns of all these oligonucleotides, as well as the deduced structures for each, are summarized in Figure 7. On the basis of $KMnO_4$ reactivity patterns, we conclude that oligonucleotide I forms an intramolecular triplex containing T·AT base triplets only in the presence of Mg^{2+} ions. In contrast, oligonucleotide II forms a triplex helix consisting of A·AT base triplets regardless of the presence of Mg^{2+} ions. Analogous results have been observed for G·GC base triplets formed in intramolecular triplexes of oligonucleotides using spectroscopic techniques (Chen, 1991). However, Mg^{2+} ions were reported to be required for intramolecular triple-helix formation involving identical base triplets but within longer tracts of guanines and driven by negative supercoiling (H-DNA formation, which requires the disruption of Watson–Crick base-pairing and restructuring; Kohwi & Kohwi-Shigematsu, 1988; Kohwi-Shigematsu & Kohwi, 1991), as well as the formation of an intermolecular triplex (a bimolecular process; Beal & Dervan, 1991).

Two different hydrogen-bonding schemes have been proposed for the A·AT base triplet (Beal & Dervan, 1991), one with two hydrogen bonds involving N-7 and N-6 of the Watson–Crick adenine, and the other with only one hydrogen bond forming at the N-6 of the same adenine. Because the Watson–Crick adenine is protected from DEPC, which reacts primarily at the N-7 of purines, we conclude that the A·AT base triplet has two hydrogen bonds involving both N-7 and N-6 of the Watson–Crick adenine.

Oligonucleotides III–V, containing tandem tracts of oligopurines and oligopyrimidines in different base combinations, exhibit chemical reactivity patterns that are in good agreement with an intramolecular triplex structure forming both Pu·PuPy and Py·PuPy base triplets simultaneously. In each case, the reactivity patterns are consistent with the bases of a single-stranded region occupying the major groove of the duplex hairpin, base-pairing to the purine tract in each Watson–Crick strand, as depicted in Figure 1b. The results of chemical modification performed at different temperatures suggest that Mg^{2+} ions enhance the stability of Pu·PuPy base triplets, although their formation does not require Mg^{2+}. Surprisingly, the stability of the G·GC base triplet seems to be lower than that of the A·AT base triplet although they are expected to have similar stability, each having two hydrogen bonds to the third strand (see above). The difference in stability of these two base triplets could be due to steric or electronic effects or both, including stacking interactions. The presence of the single-stranded loops might also contribute subtly to the energetics; thus, some caution in generalizing relative stabilities to intermolecular triplexes may be advisable.

The formation of a triple helix using a single strand consisting of both purines and pyrimidines was initially reported by Cooney et al. (1988) and was further investigated by Beal and Dervan (1991). In both studies, purine-rich oligonucleotides were designed to recognize purine-rich strands of the duplex. Because the favored polarity of the third strand is different for Pu·PuPy than for Py·PuPy triplets (antiparallel

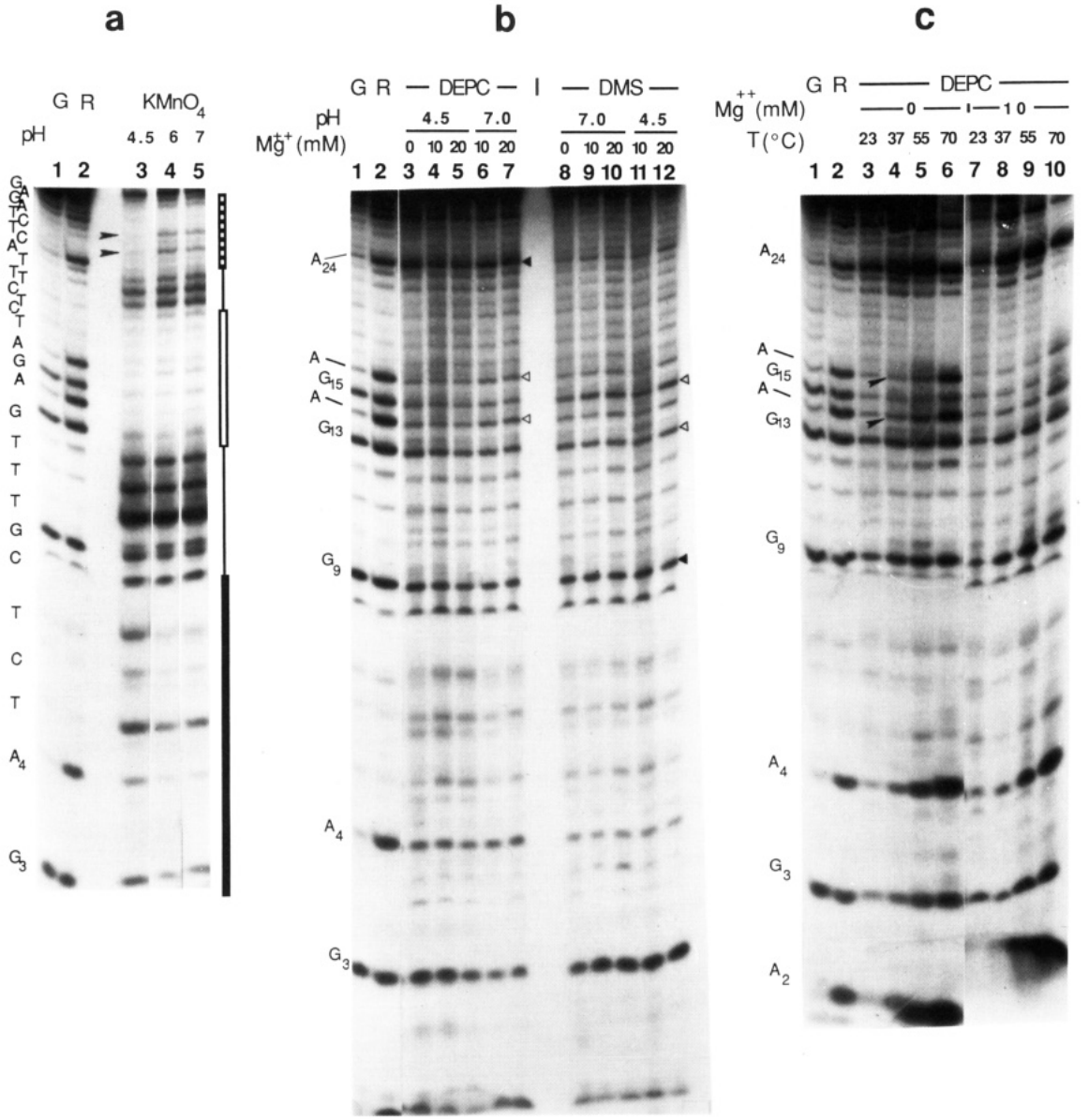

FIGURE 6: Reactivity of oligonucleotide V toward $KMnO_4$, DEPC, and DMS. (a) $KMnO_4$ reactivity in a buffer containing 25 mM NaOAc and 25 mM Tris-HCl at pH 4.5 (lane 3), pH 6.0 (lane 4), and 7.0 (lane 5) at 4 °C. (b) DEPC (lanes 3–7) and DMS (lanes 8–12) reactivity at 4 °C in the same buffer. The pH and $MgCl_2$ concentrations were as indicated. Bases indicated by closed arrowheads are reactive toward the chemical probes, whereas those indicated by open arrowheads are protected. (c) DEPC reactivity in pH 7.0 buffer as a function of temperature in the absence (lanes 3–6) and presence (lanes 7–10) of 10 mM $MgCl_2$. Temperatures were as indicated. Arrowheads designate adenines whose reactivities are temperature-dependent. In each panel, lane 1 shows the G sequencing reaction and lane 2 shows the A+G sequencing reaction. Helical and loop regions are designated as in Figure 2.

vs parallel, relative to the Watson–Crick purine strand), the formation of a triplex using a mixed (both purines and pyrimidines) single-stranded oligonucleotide is expected to be unfavorable. On the other hand, it could be argued that the presence of one or two pyrimidine bases buried in a purine-rich oligonucleotide [as in Cooney et al. (1988) and Beal and Dervan (1991)] does not significantly alter the energetics of triplex formation. However, the polarity conflict becomes problematic when the target sequence has distinct tracts of purines and pyrimidines. One solution to this problem is to design oligonucleotides capable of recognizing purine tracts in alternate strands. Due to polarity requirements in the triplex, the recognition of purine tracts in alternate strands by a third strand consisting of two pyrimidine blocks (forming only Py·PuPy base triplets) necessitates the placement of an unusual 3′–3′ linkage between these blocks (Horne & Dervan, 1990). Similar constraints would be expected if the third strand consists of two blocks of purines as well. However, as demonstrated in this paper, no such linkage is required if both Py·PuPy and Pu·PuPy base triplets are incorporated into the triplex by using a third strand consisting of oligopurine and oligopyrimidine blocks. This approach thus allows triplex formation at sequences consisting of tandem tracts of oligopurines and oligopyrimidines. While the experiments described here were in progress, Orson et al. (1991) reported the inhibition of in vivo transcription via triplex formation using T·AT and G·GC triplets.

Designing oligonucleotides to bind short purine tracts on alternate strands appears to be a viable approach toward triplex formation involving any sequence of DNA, relaxing the restriction that target sequences be long (>10 nucleotides) oligopurine tracts. To assess the full potential of this approach, two questions must be answered: how many alternating purine tracts can be tolerated within a single triple helix, and how short can the individual tracts be? If the shortest length turns out to be one base, then the triplex structure can be accom-

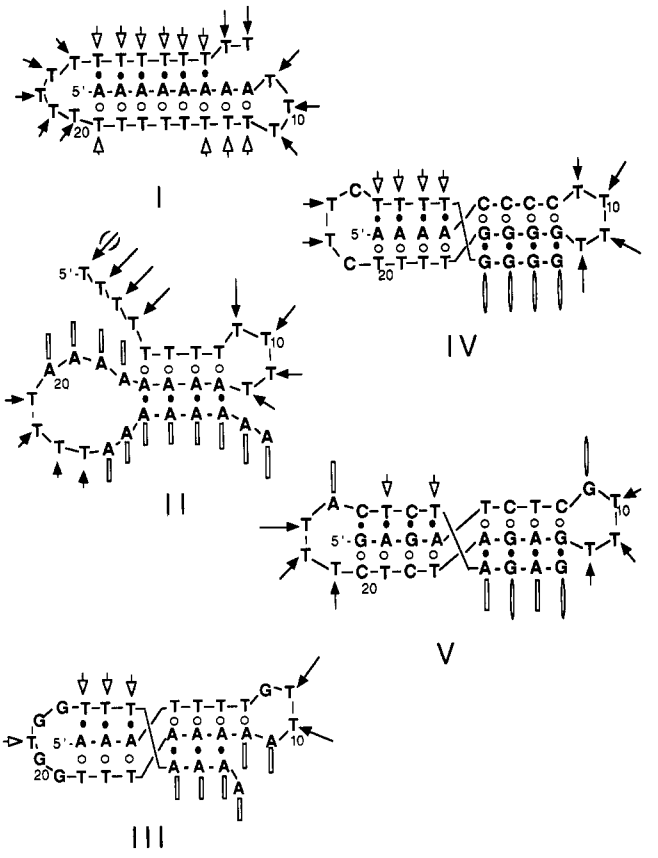

FIGURE 7: Summary of reactivity patterns and deduced secondary structures for each oligonucleotide. Closed arrows indicate $KMnO_4$ reactivity that is not sensitive to Mg^{2+} ions; the length of the arrow roughly correlates with the extent of base reactivity. (The arrow shown in parentheses is a presumably reactive thymine that was run off the gel.) Open arrows indicate $KMnO_4$ reactivity of thymines sensitive to the presence of Mg^{2+} ions; open rectangles indicate DEPC reactivity of adenines, with the symbol size roughly correlating with the extent of reactivity; open cigar-shaped symbols indicate DMS reactivity of guanines. Lines connecting bases show the continuity of the backbone. Open circles indicate Watson–Crick hydrogen bonding; closed circles indicate Hoogsteen or Pu·Pu-type hydrogen bonding.

modated by virtually any given sequence, an ideal situation for sequence-specific DNA targeting. Experiments are currently underway to answer these questions.

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