Alternate-Strand Triplex Formation: Modulation of Binding to Matched and Mismatched Duplexes by Sequence Choice in the Pu-Pu-Py Block†

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ABSTRACT: In double-stranded DNA, tandem blocks of purines (Pu) and pyrimidines (Py) can form triplexes by pairing with oligonucleotides which also consist of blocks of purines and pyrimidines, using both Py·Pu·Py (Y-type) and Pu·Pu·Py (R-type) pairing motifs in a scheme called "alternate-strand recognition," or ASR [Jayasena, S. D., & Johnston, B. H. (1992) *Biochemistry 31*, 320−327; Beal, P. A., & Dervan, P. B. (1992) *J. Am. Chem. Soc. 114*, 1470−1478]. We investigated the relative contributions of the Py·Pu·Py and Pu·Pu·Py blocks in the 16-bp duplex sequence 5′-AAGGAGAATTCCCTCT-3′ paired with the third-strand oligonucleotides 5′-TTCCTCTTXXGGGZGZ-3′ (XZ-16), where X and Z are either T or A and C is 5-methylcytosine, using chemical footprinting and gel electrophoretic mobility shift measurements. We found that the left-hand, pyrimidine half (Y-block) of the third strand (TTCCTCTT, Y-8) forms a Py·Pu·Py triplex as detected by both dimethyl sulfate (DMS) probing and a gel-shift assay; in contrast, the triplex formed by the right-hand half alone (R-block) with X = T (TTGGGTGT, R-8) is not detectable under the conditions tested. However, when tethered to the Y-block (i.e., as XZ-16), the R-block contributes greatly increased specificity of target recognition and confers protection from DMS onto the duplex even under conditions unfavorable for Pu-Pu-Py triplexes (lack of divalent cations). In general, the 16-mer (XZ-16) can bind with apparent strength either greater or lesser than Y-8, depending on whether X and Z are A or T. The order of apparent binding strength, as measured by the target duplex concentration necessary to cause retardation of the third strand during gel electrophoresis, is TT-16 ∼ AT-16 > Y-8 > AA-16 > TA-16. Chemical probing experiments showed that both halves of the triplex form even for AA-16, which binds with less apparent binding strength than the pyrimidine block alone (Y-8). The presence of the right half of the 16-mers, although detracting from affinity in cases of AA-16 and TA-16, provides strong specificity for the correct target compared to a target incapable of forming the Pu·Pu·Py part of the triplex. We discuss possible explanations for these observations in terms of alternate oligonucleotide conformations and suggest practical applications of affinity modulation by A-to-T replacements.

Binding of single-stranded oligonucleotides to double-stranded DNA through triplex formation may be a useful approach for artificial gene regulation as well as for mapping and isolating fragments from genomic DNA. It is therefore important to know which sequences in double-stranded DNA are capable of triplex formation and what rules or "codes" may exist for their sequence-specific recognition by an oligonucleotide. For homopurine−homopyrimidine target duplexes, recognition is afforded by the following interactions and associated third-strand polarities [for review, see Frank-Kamenetskii and Mirkin (1995)]: Thymines of the third strand bind to adenines of the duplex (T·AT; parallel orientation in relation to adenine is preferred, although antiparallel orientation is also possible); protonated cytosines of the third strand bind to guanines of the duplex in parallel orientation (C+GC); adenines of the third strand bind to adenines of the duplex in antiparallel orientation (A·AT); and guanines of the third strand bind to guanines of the duplex (G·GC; antiparallel orientation preferred). Parallel orientations contain Hoogsteen-type hydrogen bonding (pyrimidine motif), and antiparallel orientations contain reverse-Hoogsteen-type pairings involving G, A, or T on the third strand ("purine motif"). These interactions we designate as "canonical." Other, "noncanonical" interactions are also possible; for example, protonated adenines of the third strand also can bind to guanines in the duplex in antiparallel orientation (A+GC) (Malkov et al., 1993). In all these cases, the bases of the third strand bind purines of the duplex, but not pyrimidines. Some specific interaction between guanine in the third strand and thymine in the duplex has been shown (G·TA) (Griffin & Dervan, 1989), as well as between thymine in the third strand and cytosine in the duplex (T·CG) (Yoon et al., 1992), but no triplex stabilized mainly by interactions with pyrimidines of the duplex has yet been described. Structures have been proposed, however, for recombinase-induced triplexes that involve hydrogen bonds

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to both bases of a Watson−Crick base pair (Zhurkin et al., 1994; Kim et al., 1995; Rao et al., 1993). In this study we restrict ourselves to those triplexes which form spontaneously in the absence of proteins. Evidence for formation of a triplex structure in a self-folding oligonucleotide of mixed sequence, proposed as similar to a recombinase-induced triplex, has been reported (Shchyolkina et al., 1994; Dagneaux et al., 1995), but the sequence requirements and the specificity of this structure have not yet been carefully investigated.

For each purine in a duplex, the "canonical rules" of triplex formation specify a particular nucleotide residue which will bind that purine for either strand polarity (except that, in the case of AT pairs and antiparallel orientation, two residues [T or A] will bind). It was therefore proposed that a duplex containing mixed blocks of purines and pyrimidines could be targeted by a third strand that, through "canonical" interactions, could pair with purines on one strand of the duplex, switching to pair with the other strand when the sequence of the first strand changes from purine to pyrimidine. This scheme was termed alternate-strand recognition (ASR). Because the strands within the duplex are antiparallel, the third strand should bind to one strand of the duplex in parallel orientation, and to the other in antiparallel (considering only third strands that contain natural 5′−3′ linkages). The simplest way of accommodating the required alternation in polarity is to alternate pyrimidine and purine motifs. That approach was demonstrated for both intramolecular (Jayasena & Johnston, 1992a) and intermolecular (Beal & Dervan, 1992; Jayasena & Johnston, 1992b) triplex formation. A variant of this approach, in which the third strand contains only G and T residues and the triplex is stabilized by an intercalator, was recently reported (de Bizemont et al., 1996; Bouziane et al., 1996).

The strand switch can be easily accommodated if the sequence of the duplex is 5′-$(R)_n(Y)_m$-3′ (Jayasena & Johnston, 1992a,b; Beal & Dervan, 1992). In contrast, for duplex sequences of the type 5′-$(Y)_n(R)_m$-3′, triplex formation by ASR is less favorable (Beal & Dervan, 1992; Jayasena & Johnston, 1992b); molecular modeling results suggest that this may be due to strain in the backbone in this type of strand switch (A. Liu and B. H. Johnston, unpublished results). Insertion of additional bases in the third strand at the site of the strand switch can provide further stabilization for some sequences (Beal & Dervan, 1992), but in other cases it is either unnecessary or ineffective (Jayasena & Johnston, 1993).

While a careful study to determine the minimum length of the homopurine blocks for pairing by alternate-strand recognition has not been carried out, published data establish some limits. Pairing has been established by protection assays for blocks as short as four nucleotides for intramolecular triplexes (Jayasena & Johnston, 1992a) and eight for intermolecular triplexes (Jayasena & Johnston, 1992b). The minimum length is almost certainly greater than one nucleotide, since the bases nearest a strand switch do not appear to participate in base pairing as evidenced by one case where removal of two bases from the center of the third strand did not affect its binding at a 5′-$(R)_n(Y)_m$-3′ duplex sequence (Beal & Dervan, 1992); and in another case, the nucleotides of the duplex at the strand switch were hyperreactive to chemical probes (Jayasena & Johnston, 1993).

Even where individual sequence blocks are long enough, some blocks within an ASR triplex—especially certain purine-motif blocks—are so weak that they contribute little or nothing to the overall stabilization of the triplex (Jayasena & Johnston, 1993; Olivas & Maher, 1994). In such cases, the question arises as to whether those blocks contribute at all to the ability of the third strand to recognize a unique target sequence and to reject similar but mismatched sequences. Such questions are critical for avoiding side effects in therapeutic applications of triplex formation.

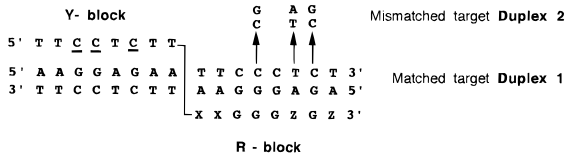
In a quantitative study of such a system, Olivas and Maher (1994) observed only a very modest increase in affinity upon the addition of a more weakly binding purine block to a pyrimidine block that bound strongly enough to be stable by itself. This example shows that the addition of the R-block to the Y-block has a very different effect from simply lengthening an ordinary triplex (one lacking a strand switch) by the same number of nucleotides. For ordinary triplexes of 10−20 base triads without mismatches, detachment of an entire section of the third strand is expected to be a rare event due to the stacking interactions between the bases of third strand within the triplex. In the case of strand-switching triplexes, however, the strand-switch junction may be energetically unfavorable due to imperfect stacking and conformational distortions, and part of the binding energy is needed to compensate for this unfavorable junction. This is why in some cases the stabilizing effect of the R-block is very weak, and complete detachment of the weaker half of the triplex is much more likely than for ordinary triplexes.

We were interested to know whether R-blocks can contribute to the specificity of binding even if they contribute only weakly to affinity compared to the Y-block alone. We found that extending a Y-type triplex by adding a strand switch and a second block of sequence to the third strand greatly increases specificity but, remarkably, may either increase or decrease the apparent affinity. By careful choice between thymine or adenine for recognizing AT base pairs in the second (reverse Hoogsteen) block, the level of stringency (i.e., the degree to which potential specificity is realized) may be adjusted. This approach is similar to the method of "stringency clamping" (Roberts & Crothers, 1991), but in the case of alternate-strand recognition the same sequences can participate in both "stringency clamping" and target recognition.

MATERIALS AND METHODS

Oligonucleotides. The sequences of all oligonucleotides are shown on Figure 1. Oligonucleotides TT-16, Y-8, R-8, and MR-8 were synthesized on an Applied Biosystems Model 380 B automated DNA synthesizer using standard phosphoramidite chemistry. After deprotection, the oligonucleotides were purified by electrophoresis on denaturing 20% polyacrylamide gels. Oligonucleotides AT-16, TA-16, and YS-16 and the two complementary strands for duplex 2 were purchased from Midland Certified Reagent Company. Oligonucleotides AA-16 and the two complementary strands for duplex 1 were purchased from Oligos, Etc. Purchased oligonucleotides were also purified by electrophoresis on denaturing 20% polyacrylamide gels before use.

Plasmid Construction. To obtain the plasmid pD1, duplex 1 was ligated to pBluescript II SK^+ (Stratagene) DNA which had been previously digested with *Sma*I. *Escherichia coli*



The third strand	Y-block	X	Z	R-block
Y-8	5' T T C C T C T T 3'	-	-	-
TT-16	5' T T C C T C T T 3'	T	T	5' T T G G G T G T 3'
TA-16	5' T T C C T C T T 3'	T	A	5' T T G G G A G A 3'
AT-16	5' T T C C T C T T 3'	A	T	5' A A G G G T G T 3'
AA-16	5' T T C C T C T T 3'	A	A	5' A A G G G A G A 3'
YS-16	5' T T C C T C T T 3'	-	-	5' G G T T T G T G 3'
R-8	-	T	T	5' T T G G G T G T 3'
MR-8	-	-	-	5' T G T G G G T T 3'

FIGURE 1: General scheme of alternate-strand triplex formation and DNA sequences used. Underlined C residues represent 5-methylcytosine. The duplex shown is matched duplex 1; arrows show the base pair replacements which convert duplex 1 to mismatched duplex 2, which cannot form a triplex in its right half.

JM101 cells were transformed with ligated products and grown on X-gal- and IPTG-coated, ampicillin-containing LB plates. Transformants were identified by blue-white colony screening and further characterized by restriction and sequence analysis. Recombinant plasmid was purified from large-scale cultures by alkaline lysis (Sambrook et al., 1989).

Dimethyl Sulfate (DMS) Footprinting. DMS footprinting was performed on *Pvu*II/*Hind*III and *Pvu*II/*Xba* I fragments of plasmid pD1. Each fragment was 3′-end-labeled using the Klenow enzyme (Promega) and [α-^{32}P]dCTP (Dupont-NEN) and purified from a native 8% polyacrylamide gel. The labeled strand of the *Pvu*II/*Hind*III fragment contains the guanines that participate in Y-type triplex formation, and the labeled strand of the *Pvu*II/*Xba* I fragment contains the guanines that participate in R-type triplex formation. For triplex formation, the labeled fragments (concentration <0.02 μM) were preincubated with an excess (8 μM) of various triplex-forming oligonucleotides under varying buffer conditions. The samples were incubated at 20 °C with agitation for 30 min and then further incubated overnight at 4−6 °C. After incubation, 1 μL of a freshly-prepared 5% (v/v) solution of DMS in ethanol was added to 10 μL of the preincubated mixture, allowed to react for 3 min at 4−6 °C, and stopped by the addition of 4 μL of DMS-stop solution (Maxam & Gilbert, 1980). Samples were then twice precipitated with ethanol, rinsed with 70% ethanol, treated with 10% piperidine at 90 °C for 30 min, dried, dissolved in 80% formamide loading solution, and electrophoresed on 8% polyacrylamide sequencing gels.

Gel-Shift Assay. Gel electrophoretic mobility shifts were the primary means of assaying for triplex formation. In most of the gel-shift experiments, the triplex contained ^{32}P-labeled third strand and unlabeled duplex. [We used this approach instead of the more common labeled duplex with unlabeled third strand, because, under our conditions, the target duplexes had similar electrophoretic mobility to one of the triplex bands observed (T1; see below). By contrast, the triplex and the third strand were always well-resolved on gels.] Standard conditions for triplex formation consisted of incubating equimolar amounts of the two duplex-forming strands plus labeled third strands in 10 mM $Mg(OAc)_2$ and 40 mM NaOAc (pH 5.1) at 4−6 °C for 16−18 h. For each experiment, samples were prepared containing a constant amount of third strand, with increasing amounts of duplex strands. Even for the lowest level of duplex, the duplex strands were in significant molar excess over the third strands. After incubation, the samples were electrophoresed for 6−7 h at 6 V/cm on native 10% acrylamide gels using the same buffer and temperature for electrophoresis as for incubation. Any modifications of these standard conditions are described in the figure and table legends. For triplexes formed by duplex 1 and the third strands Y-8, TT-16, AA-16, AT-16, and TA-16, radioactive bands corresponding to the single-stranded oligonucleotide and the triplex were excised from the dried gel, and the radioactivity was measured by scintillation counting. This additional analysis was performed to obtain the total yield of triplex in cases where it was distributed across more than one band (see Results).

As a measure of affinity between the duplex and the third strand, we used $D_{1/2}$, defined as the duplex concentration at which half of the third strand radioactivity is retarded from the position of free third strand. $D_{1/2}$ was calculated as the interpolated average between the largest duplex concentration at which less than half of the third strands are bound to the duplex and the smallest duplex concentration at which more than half are bound to the duplex. The error of such an estimation cannot exceed one-half the interval between two consecutive duplex concentrations, which for estimation of $D_{1/2}$ usually differed by a factor of 2−3. The estimated errors are given in Tables 1 and 2.

In all experiments, the duplex was in excess relative to the third strand, such that the upper limit of the third strand concentration (calculated by assuming no losses during the preparation of the labeled third strand) was significantly smaller than the lowest concentration of duplex tested; thus duplex concentration always remained largely unaffected by addition of third strand.

The minimal molar concentration of the duplex-forming strands in these experiments was 3 nM, which is significantly higher than the expected dissociation constant of these duplexes under our experimental conditions (Wetmur, 1991). Even at this concentration of the duplex-forming strand, it is estimated that duplex formation should not require more than 1 h (Wetmur, 1991), whereas the incubation period in our experiments was at least 16 h. It was also established that no complexes which can interfere with duplex formation are formed between any of the third strands and any of the duplex-forming strands used (results not shown). Thus, we consider the formation of duplex a relatively fast and practically irreversible process under our conditions, and hence we may characterize the mixtures in terms of duplex concentrations even though the duplex was not preformed before being incubated with the third strand (except for the kinetic measurements for AT-16).

RESULTS

Effect of Purine Moiety on Binding to Matched Target. Figure 1 shows the alternate-strand triplex sequence that we investigated. It involves a single strand switch in a target sequence of the type 5′-Pu_nPy_n-3′ and a variety of third strand sequences. To test the contribution of each half toward

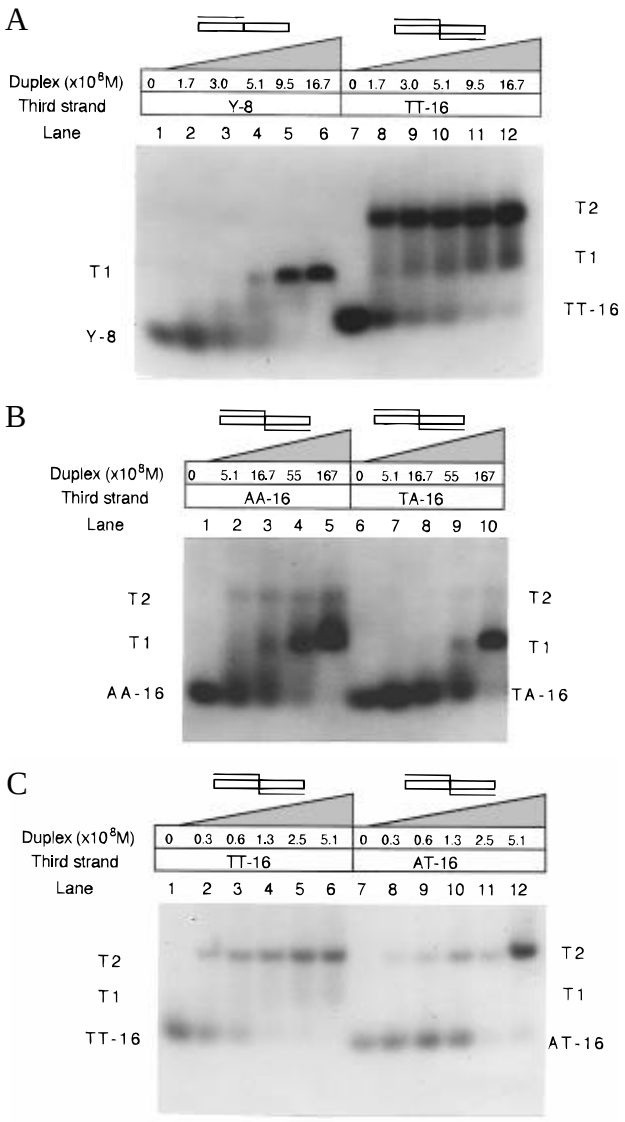


FIGURE 2: Affinity of the various third strands for triplex binding to duplex 1 as determined by gel-shift experiments. Labeled third strands Y-8 and TT-16 (A), AA-16 and TA-16 (B), TT-16 and AT-16 (C) were incubated with various concentrations (but always an excess) of duplex 1 under standard conditions favoring triplex formation and then electrophoresed on polyacrylamide gels (for conditions, see Materials and Methods). For each third strand, the corresponding triplex is shown schematically above the lane headings; the left and right parts of the white rectangles symbolize the recognition sites on the duplex for the Y- and R-blocks of the third strands, respectively. Upper limits for initial concentrations of the third strands were as follows: for A, 8×10^{-9} M; for B, 4×10^{-9} M; for C, 8×10^{-10} M.

triplex stability, we end-labeled each third-strand oligonucleotide, incubated it for ~17 h with increasing concentrations of unlabeled duplex, and then analyzed the products on polyacrylamide gels, as described in Materials and Methods. Typical results of these gel-shift experiments are shown in Figure 2. The lower band corresponds to free, labeled third strand. In the case of Y-8 (which contained only pyrimidines), only one clear-cut, slowly migrating complex (T1) appeared when duplex was added (Figure 2A). In the case of TT-16, most of the complex was in the even more slowly migrating fraction T2, although a significant amount of material was distributed between T1 and T2 (Figure 2A). Fraction T1 has an electrophoretic mobility very close to that of pure target duplex (data not shown). No retarded bands were detected when only one of the duplex-forming strands was added to any of the third strands used in our experiments (data not shown). Thus, we conclude that the retarded bands correspond to triplex. In the case of AA-16 (Figure 2B), T2 was predominant at lower concentrations of duplex, but T1 predominated at high concentrations. T1 was predominant for TA-16 and T2 for AT-16 (Figure 2B,C). The identity of T1 and T2 has been established from experiments reported elsewhere (Belotserkovskii & Johnston, 1996); a summary is given under Discussion.

Table 1: Relative Affinities of the Various Third Strands to the Matched Target Duplex 1[a]

TT-16		A = 8
AT-16		A = 2 (6)
Y-8		A ≡ 1
YS-16		A = 0.5
AA-16		A = 0.5
TA-16		A = 0.1

[a] Relative affinity A is the ratio of $D_{1/2}$ (the half-transition duplex concentration) for the triplex formed by duplex 1 and Y-8 to $D_{1/2}$ for the triplex formed by duplex 1 and the given third strand. $D_{1/2}$ for Y-8 is 7×10^{-8} M. All data were obtained after the standard incubation time (17−18 h). For AT-16, the extrapolated value for infinitely long incubation is given in parentheses (see *Estimation of the Kinetic Parameters for Triplex Formation* under Results). Errors are estimated to be 60% for relative affinities of AA-16 and TA-16 and 50% in all other cases.

It is apparent from Figure 2A that the 8-nt pyrimidine block alone (Y-8) forms detectable triplex at duplex concentrations of about 5×10^{-8} M and higher. In contrast, oligonucleotide R-8, which corresponds to the R-block of TT-16, does not form detectable triplex at duplex concentrations of up to at least 7×10^{-6} M (data not shown). (For simplicity, we refer to the R-block as the purine-containing part of a third strand, although it may also contain thymines.) Oligonucleotide MR-8, which contains only the R-block but in reverse orientation (see Figure 1), also fails to form a detectable triplex (not shown). Joined with the Y-block, however, the R-block can significantly affect the affinity of the third strand to the duplex. We calculated the relative affinity (A) of an oligonucleotide (O) for the target as

$$A = D_{1/2}(\text{Y-8})/D_{1/2}(\text{O})$$

where $D_{1/2}$(O) is the interpolated duplex concentration at which half of the oligonucleotide O is in one or more electrophoretically retarded bands. The larger the value of A, the more favorable the triplex formation. Thus, the parameter A characterizes the contribution of the R-blocks to triplex formation. Affinity data for the third strands listed in Figure 1 are summarized in Table 1.

Table 1 shows that A is maximal in the case of oligonucleotide TT-16 and AT-16. It is seen that thymine-to-adenine replacements in the vicinity of the strand-switch cause little if any change in A. However, the same

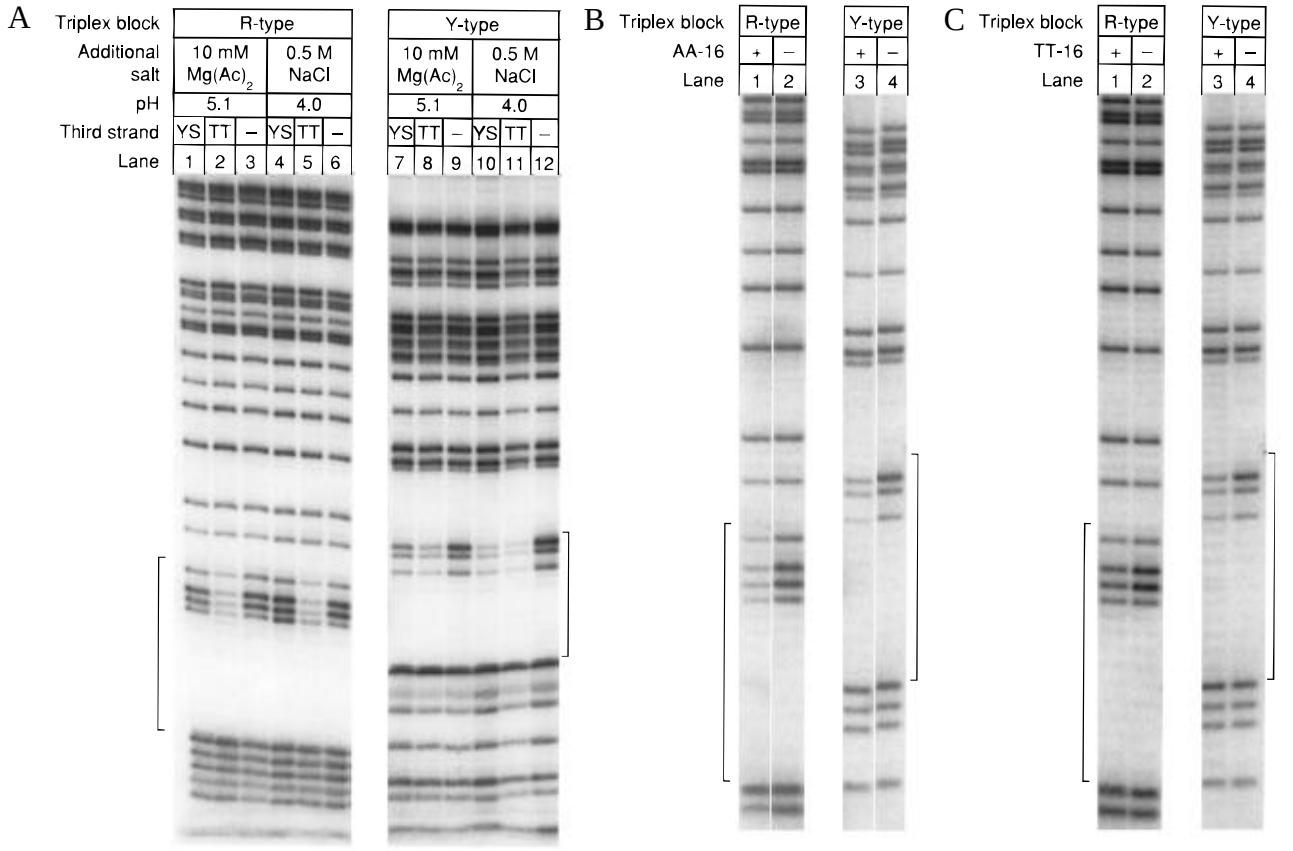


FIGURE 3: Protection from dimethylsulfate of both halves of the matched target sequence by third-strand oligos. For footprinting purposes, duplex 1 was inserted into a plasmid; the position of the insert is indicated by brackets, with the 5′-to-3′ direction oriented top-to-bottom. The sequence spanned by the brackets for lanes labeled "R-type" corresponds to the bottom strand of duplex 1 as shown on Figure 1; the 5′-half of this strand serves as the recognition site for the R-block of the third strands. The sequence spanned by the brackets for lanes labeled "Y-type" corresponds to the top strand of duplex 1; the 5′-half of this strand serves as the recognition site for the Y-block of the third strands. In each panel, DMS reaction was performed in the salt and buffer conditions indicated, at 4−6 °C (for other conditions, see Materials and Methods), and all lanes were taken from the same X-ray films of a single gel. (**A**) 40 mM NaOAc, additional salt and pH as indicated above the lanes. Third strands are as indicated above the lanes (TT is TT-16, and YS is YS-16). (**B**) 40 mM NaOAc, 10 mM $Mg(OAc)_2$, pH 5.1. (**C**) 40 mM NaOAc (pH 5.1), 0.5 M NaCl.

replacements made farther from the strand-switch (TA-16) cause a much greater loss of affinity (Figure 2B). In this case, the presence of the purine block increases $D_{1/2}$ to a value below even that of Y-8 alone (i.e., $A < 1$). Replacement of all the thymines by adenines (AA-16) results in an affinity intermediate between those of TA-16 and AT-16 and similar to that of the scrambled-R-block oligonucleotide YS-16.

Chemical Probing of Complexes. The unexpected destabilizing effects of adding certain R-block sequences to the Y-8, pyrimidine half of the third strand (as in AA-16 and TA-16) raised the question of whether the R-block was indeed involved in triplex pairing in those oligonucleotides. To address this question, we cloned the matched target duplex into a plasmid and tested how much of the target sequence was protected from modification by dimethyl sulfate (DMS) due to the presence of several third-strand oligonucleotides. [DMS reacts with the N7 position of guanine, which is unavailable when involved in Hoogsteen pairing to a third strand (Johnston, 1988; Voloshin et al., 1988; Jayasena & Johnston, 1992a)]. We observed partial DMS protection under our standard conditions (10 mM Mg^{2+}, 40 mM NaOAc, pH 5.1) in the sequence that binds with the pyrimidine blocks of Y-8 (data not shown), YS-16 (Figure 3A), TT-16 (Figure 3A), and AA-16 (Figure 3B). TT-16 and AA-16, but not YS-16, exhibited partial DMS protection in the sequence that binds with the R-block of the third strand [Figure 3, panels A (lanes 1−3) and B (lanes 1−2)] under the standard conditions. Surprisingly, TT-16 exhibited partial DMS protection in the sequence that binds with the R-block even under conditions usually considered unfavorable for formation of the canonical Pu·Pu·Py triplex (0.5 M NaCl in the absence of Mg^{2+}). This protection is noticeable at pH 5 (Figure 3C, lane 1) and even more evident at pH 4 (Figure 3A, lane 5). To exclude the possibility that this protection is due simply to the proximity of the destructured purine half of the oligonucleotide to the major groove of the duplex, rather than to formation of a Pu·Pu·Py triplex, we performed DMS modification in the presence of YS-16, which contains a scrambled R-block. No protection of the purine-binding block was obtained in this case (Figure 3A, lane 4), so we conclude that the Pu·Pu·Py triplex is formed by the R-blocks of TT-16 even in the absence of Mg^{2+}. Similar results at pH 4 were obtained for AA-16 (data not shown).

As was mentioned above, oligonucleotide R-8, which corresponds to the R-block of TT-16, did not produce any detectable complex with duplex 1 under standard conditions. R-8 also did not produce DMS protection at pH 4 and 0.5 M NaCl. Thus the R-block must be tethered to the Y-block for noticeable interaction with the target.

Effect of the Purine Block on the Specificity of Triplex Formation. We define the specificity (S) of triplex formation as $S = D_{1/2}^{mm}/D_{1/2}^{m}$, where $D_{1/2}^{m}$ and $D_{1/2}^{mm}$ are half-

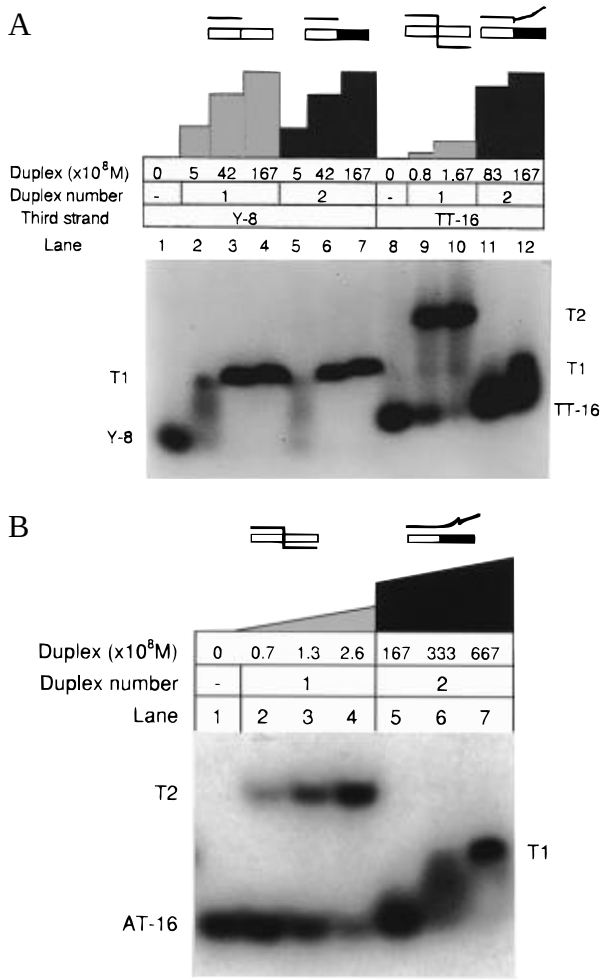
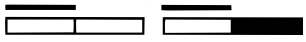
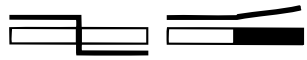
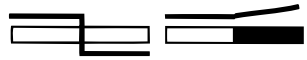


FIGURE 4: Specificity of oligonucleotides XZ-16 for matched target duplex 1 versus mismatched target duplex 2 as determined by gel-shift experiments. (**A**) Binding of Y-8 and TT-16 to duplex 1 and duplex 2. The concentrations are shown by gray and black rectangles, respectively. In the schematic of the expected triplexes shown at the top, the scrambled recognition site for the R-block in duplex 2 is shown in black. The upper limits for the concentrations of labeled third strands were 8×10^{-9} M for Y-8 and 4×10^{-9} M for TT-16. In the case of Y-8, for both duplex 1 and duplex 2 the patterns are indistinguishable. The transition from single strand to triplex occurs at approximately 5×10^{-8} M duplex (lanes 2 and 5); and at 42×10^{-8} M duplex, the transition is very nearly complete (lanes 3 and 6). More detailed experiments have shown that the transition points in the case of Y-8 for duplex 1 and duplex 2 coincide within 40% (data not shown). In contrast, for TT-16 the half-transition from single strand to triplex requires at least a 200-fold higher concentration of duplex 2 than of duplex 1 (compare lanes 9 and 12). Note that although TT-16 binds duplex 1 much better than does Y-8 (compare lanes 2 and 10), Y-8 binds duplex 2 much better than does TT-16 (compare lanes 3 and 12). (**B**) Binding of AT-16 to duplex 1 and duplex 2. The upper limit for the concentrations of labeled third strand was 5×10^{-10} M. In both A and B, standard conditions (see Materials and Methods) were used for incubation and gel electrophoresis.

transition duplex concentrations obtained, respectively, by titrating a third strand by the matched Duplex 1 (see Figure 1), which can pair with all the third-strand oligonucleotides (except the scrambled YS-16) by canonical interactions, and duplex 2, which is mismatched in the right-hand half that normally would bind the R-block.

Figure 4 shows several examples of gel-shift experiments with duplexes 1 and 2 for various oligonucleotides, and the data are summarized in Table 2. As expected, there was no detectable specificity for Y-8, which contains only the Y-block. In contrast, the specificity of TT-16 was between 200 and 400. The R-block thus has at least a 20-fold greater effect on specificity than on affinity (compare Table 1). Interestingly, AA-16 and TA-16 also demonstrate high specificity for the matched target, even though their affinity to this target is less than that of the nonspecific Y-8. We were unable to detect triplexes between those two oligonucleotides and duplex 2 for duplex concentrations of up to 33×10^{-6} M for AA-16, and 40×10^{-6} M for TA-16, from which we calculate the lower limit of specificity for AA-16 to be 160 and for TA-16 to be 50. Surprisingly, YS-16 also demonstrated moderate specificity [$S = 4-7$; data from Belotserkovskii and Johnston (1996)] for the matched over the mismatched target, which might be explained by unintended noncanonical interactions.

Table 2: Specificity of Triplex Formation for Various Third Strands[a]

Y-8		S = 1
YS-16		4 < S < 7
AA-16		S > 160
TA-16		S > 50
TT-16		200 < S < 400
AT-16		130 < S < 260 (390 < S < 780)

[a] Specificity S of a given third strand is determined as the ratio of its value of $D_{1/2}$ with the mismatched target duplex 2 to that with the matched target duplex 1. For Y-8, the apparent binding constants with duplexes 1 and 2 coincide within 40%. For AT-16, the extrapolated value for infinitely long incubation is given in parentheses (see Results).

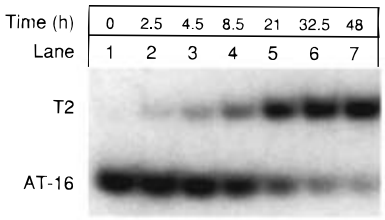


FIGURE 5: Kinetics of triplex formation for AT-16 and duplex 1. The duplex concentration was 19.5×10^{-9} M; the upper limit for the concentrations of labeled third strand was 8×10^{-10} M. The other conditions were standard (see Materials and Methods), except that the time of electrophoresis was 3 h instead of the usual 6−8 h.

Estimation of the Kinetic Parameters for Triplex Formation. The most detailed kinetic measurements were performed for the triplex formed by AT-16 and duplex 1 (Figure 5). In these experiments the unlabeled duplex-forming strands were allowed to hybridize by incubating under standard conditions for 3 h and then aliquoted and frozen. Individual aliquots were thawed at different times, mixed with the labeled third strand, and incubated under the standard buffer conditions. This procedure permitted all incubations to end at the same time, whereupon all samples were loaded on the gel together.

After electrophoresis, bands corresponding to the triplex (which exists effectively only as T2 in the case of AT-16)

and the single strand were excised from the gel. We calculated the relative amount of the triplex

$$\theta = \frac{T}{T+S}$$

where T and S are the amounts of triplex and single strand, respectively, as described in Materials and Methods.

In our case of significant excess of duplex over third strand, the duplex concentration (D) effectively remains constant during triplex formation. Thus triplex formation should obey the first-order rate equation

$$\theta(t) = \theta_{eq}\{1 - \exp[-(k_{on} D + k_{off})t]\} \quad (1)$$

where θ_{eq} is the equilibrium value of θ, k_{on} and k_{off} are rate constants for formation and dissociation of triplex, and t is the time of incubation, respectively.

The same equation can be written in more compact form:

$$\theta(t) = \theta_{eq}[1 - (1/2)^{t/r}] \quad (1')$$

where r is the time at which the yield of triplex reaches half of its equilibrium value:

$$r = \frac{\ln 2}{k_{on}D + k_{off}} = \frac{\ln 2}{k_{off}\left(\frac{D}{K_d} + 1\right)} \quad (2)$$

where K_d is the dissociation constant of the triplex. The dissociation constant can be calculated as

$$K_d = \frac{D(1 - q_{eq})}{q_{eq}} \quad (3)$$

From eq 1′, r can be calculated from θ and t:

$$r = \frac{t \ln 2}{\ln\{q_{eq}/[q_{eq} - q(t)]\}} \quad (4)$$

From Figure 5 one can infer that the reaction is nearly complete after 48 h; i.e., θ(48 h) = 0.74 is close to the equilibrium value θ_{eq}. If this assumption is correct, inserting θ(48 h) in place of θ_{eq} in eq 4 should yield similar values of r (which should be independent of t) for different values of t. Data in Figure 5 show that this is indeed the case: Incubation times of 2.5, 4.5, 8.5, 21, and 32.5 h yielded r values (half-times for equilibration) of 8.4, 10.2, 9.4, 9.9, and 9.2 h, respectively (average = 9.4 h; maximum deviation = 1 h). Thus the dissociation constant for this triplex can be calculated from eq 3 with θ(48 h):

$$K_d = 7 \times 10^{-9}\ \mathrm{M}$$

From eq 2, using r = 9 h,

$$k_{off} = 2 \times 10^{-2}\ \mathrm{h}^{-1}$$

$$k_{on} = 3 \times 10^{6}\ \mathrm{M}^{-1}\ \mathrm{h}^{-1}$$

The lifetime of this triplex $\tau_T = 1/k_{off}$ = 50 h, which is significantly greater than the time of gel electrophoresis. However, the lifetime of the triplex formed by the same third strand and mismatched target duplex 2, estimated assuming that the specificity is mostly due to the difference in off-rates, should be less than (50 h/300) = 10 min, which is much shorter than the time of gel electrophoresis. Thus it seems paradoxical that this triplex can still be detected by the gel-shift assay. Our model of CCD comigration explains this apparent paradox (see Discussion).

The K_d value obtained in this experiment for the triplex formed by AT-16 and duplex 1 is about three times smaller than $D_{1/2}$ obtained for the same system at our standard time of incubation (17 h). Thus for this triplex, the deviation from equilibrium at 17 h is significant, and estimated values correcting for this deviation from equilibrium are given in parentheses in Tables 1 and 2. In contrast, a similar analysis for TT-16 shows no significant deviation from equilibrium after 17 h (data not shown).

Thus increasing the time of incubation from the standard 17 h has a significant effect on results obtained for AT-16 with duplex 1 but not for TT-16 with duplex 1. Note that in the case of these two triplexes the amount of T2 at saturating duplex concentrations is much greater than for all other triplexes used in this study. We have shown elsewhere (Belotserkovskii & Johnston, 1996; see Discussion) that the greater the proportion of T2 at saturating concentrations of the duplex, the greater the lifetime of the triplex. From eq 2 at $D = K_d$, the half-time of equilibration r is equal to ln 2 $\tau_T/2$; thus if 17 h is long enough to approach equilibrium at duplex concentrations around K_d (and greater) in the case of TT-16 with duplex 1, it will be also enough for any triplex having a greater proportion of T1 and hence shorter lifetime τ_T. This applies to all other triplexes used, except AT-16 with the duplex 1, which has been analyzed separately. Consequently, since the extrapolated value of $D_{1/2}$ for infinitely long incubation cannot be less than K_d (see Discussion), the corrections for $D_{1/2}$ to account for insufficient time to achieve equilibrium should be insignificant in all cases other than AT-16 with duplex 1.

DISCUSSION

Both Halves of the Triplex Are Formed even in the Absence of Divalent Cations. Data on DMS modification of the matched target inserted into plasmid pD1 show that guanines in both blocks of the target are partially protected in the presence of AA-16 and TT-16. This result, supported by the gel shift experiments addressing specificity assays performed under the same conditions (10 mM Mg^{2+}, 40 mM Na^+, pH 5.1), means that both the Y- and R-blocks of these oligonucleotides participate in target recognition.

When the experiment was performed in nonstandard conditions without magnesium ions (0.5 M NaCl, 40 mM NaOAc, pH 4.0), the same pattern of protection was seen as with the standard conditions, despite the fact that the formation of canonical Pu·Pu·Py triplexes, which contain only GGC, AAT, and TAT (reverse Hoogsteen) triads, usually requires divalent or polyvalent cations (Cooney et al., 1988; Pilch et al., 1991; Lyamichev et al., 1991; Malkov et al., 1992). Moreover, monovalent cations like Na^+ and K^+ are known to inhibit the formation of such triplexes (Olivas & Maher, 1995; Milligan et al., 1993; Cheng & Van Dyke, 1993). Thus, we did not expect to see DMS protection in the recognition sequence for the R-block in this case. However, it has been shown that inhibition of Pu·Pu·Py triplexes by monovalent cations is at least partially due to formation of four-stranded, "G-quartet" complexes by the

third strand (Olivas & Maher, 1995; Gee et al., 1995); such complexes are dramatically stabilized by Na^+ and K^+ [for review, see Guschlbauer (1990)]. Thus, it seems that di- and polyvalent cations and low concentrations of monovalent cations are not absolute requirements for Pu·Pu·Py triplex formation if the local concentration of the purine moiety of the third strand in the vicinity of the duplex is high due to tethering by a firmly bound pyrimidine moiety. Indeed, previous studies with intramolecular triplexes revealed that, although magnesium ions are stabilizing, they are not required for formation of a Pu·Pu·Py triplex (Jayasena & Johnston, 1992a; Chen, 1991). It is worth noting that, in the sequences examined, the R-block part of the triplex forms despite potential competition with quadruplex structures due to the three adjacent guanines and the rather high concentration (8 μM) of the free oligonucleotide.

Two Retarded Bands; One Triplex. A gel-shift assay was used to compare the binding of different deoxyribonucleotides to a given DNA target. In our version of this assay, a small amount of labeled third strand is mixed with an excess of unlabeled duplex. In these experiments, one of the two retarded fractions seen (T1) has the same electrophoretic mobility as the duplex; thus it could not be detected by the more commonly used gel-shift assay in which an excess of unlabeled third strand is mixed with a small amount of labeled duplex.

We recently presented evidence (Belotserkovskii & Johnston, 1996) that the T1 band results from a fraction of the original triplex that dissociates during gel electrophoresis. The newly liberated third strand migrates faster (in our case) than the duplex and eventually overtakes the band containing excess duplex, whereupon, with some probability, it is captured by the duplex through triplex formation and again becomes retarded (Figure 6). The average mobility of the third strand molecules participating in this movement [which we have termed "cyclic capture and dissociation" (CCD)] is equivalent to the mobility of the duplex (Belotserkovskii & Johnston, 1996). The triplex fraction that remains undissociated migrates according to its intrinsic mobility, forming the T2 band. If the lifetime of the triplex is longer than the duration of electrophoresis, T2 predominates (normal comigration). When the lifetime of triplex is short compared to the duration of electrophoresis, T1 predominates, and T2 may disappear entirely (pure CCD comigration).

If the preincubation period is long enough to achieve equilibrium, in the case of normal migration, at the midtransition duplex concentration $D_{1/2}$, $K_d = k_{off}/k_{on} = D_{1/2}$, where K_d is the dissociation constant of the triplex and k_{on} and k_{off} are the rate constants for triplex formation and dissociation.

In the case of CCD comigration, the picture is more complex. $D_{1/2}$ depends both on the efficiency of triplex formation in free solution during preincubation (because only those third strands that were initially bound to the duplex can become involved in CCD comigration) and on the efficiency of third strand capture by the duplex in the gel. In free solution, the duplex concentration at which half of the third strands are bound to the duplex corresponds to K_d. Under given conditions in the gel, the duplex concentration at which half of the third strands traversing the duplex band become involved in comigration, is a critical value we term c_{cr}. $D_{1/2}$ should be equal to the greater of K_d and c_{cr}.

The exact dependence of c_{cr} on k_{on}, k_{off}, and the various electrophoretic parameters is unknown, although we have

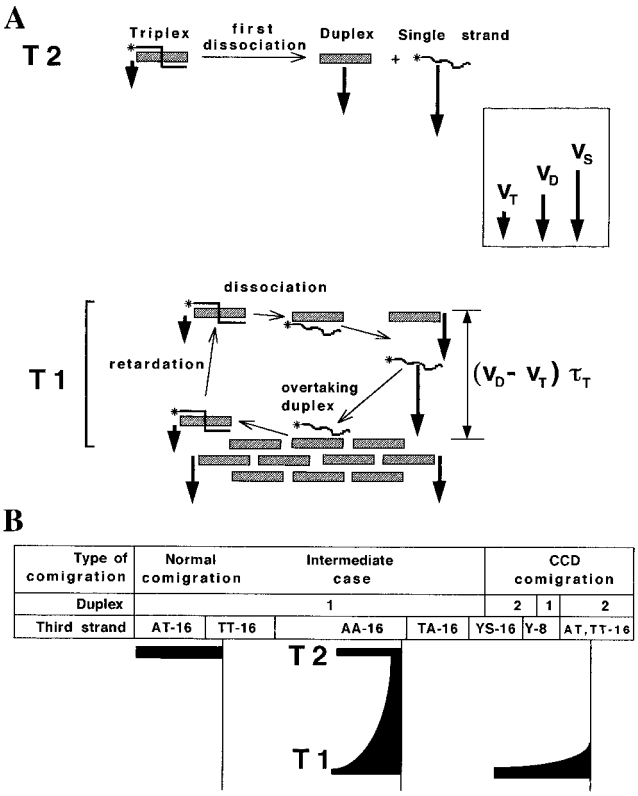


FIGURE 6: Explanation for the existence of dual triplex bands T1 and T2. (A) The general scheme for CCD comigration is as follows: After the first dissociation from the triplex, the free third strands have overtaken the duplex band and been captured; the resulting triplex migrates more slowly once again. Designations: τ_T is the lifetime of the triplex; v_S, v_D, and v_T are electrophoretic mobilities of single strand, duplex, and triplex, respectively. Note that the "width" of the T1 band (more precisely, the characteristic width of the exponential decline in T1 intensity toward the T2 band) decreases with decreasing triplex lifetime; thus the shorter the lifetime, the sharper the T1 band. (**B**) Schematic representation of distribution of the labeled third strand in the gel at saturating duplex concentrations, with the horizontal axis representing intensity of radioactivity, and the vertical axis, the position in the gel. From left to right: Normal comigration—the lifetime of the triplex is significantly longer than the time of electrophoresis (a typical example is AT-16 with duplex 1). Next, an intermediate case—part of the initially presented triplexes have dissociated in the course of electrophoresis; the released third strands become involved in CCD comigration; the "width" of the T1 band is bigger than the width of the true electrophoretic band, thus T1 is noticeably more "smeary" than T2 (a typical example is AA-16 with duplex 1). Pure CCD comigration—effectively all initially formed triplex molecules have dissociated very early in electrophoresis (thus T2 is not seen), and the T1 form has the appearance of a normal electrophoretic band (examples include Y-8 with both targets and all XZ-16 with mismatched target duplex 2) . Boxes designating duplexes and third strands are positioned according to the type of gel pattern seen for the corresponding triplexes at saturating duplex concentrations. The complex of duplex 1 with YS-16 provides a trace amount of T2 (Belotserkovskii & Johnston, 1996).

presented arguments that it may be proportional to K_d with a proportionality constant that depends on electrophoretic mobilities and gel conditions (Belotserkovskii & Johnston, 1996). However, some properties of this dependence can be deduced directly from the model of CCD comigration. Those properties are listed below, with the assumption that all samples being compared are loaded on the same gel, or the gels contain common internal controls.

(i) c_{cr} should decrease with increasing k_{on}, because as k_{on} increases, smaller concentrations of duplex are required to provide a given probability of capture.

(ii) c_{cr} should decrease with increasing triplex lifetime (the reciprocal of k_{off}) and with increasing difference in mobility between the duplex and the triplex, because both of these values increase the distance of retardation (Figure 6).

(iii) c_{cr} should increase with increasing difference between third strand and duplex mobilities, because the faster the free third strand overtakes and transverses the duplex band, the faster its capture must be before it outdistances the duplex and is unavailable for further triplex formation.

Since for given gel conditions and mobilities (which in our experiments were very close for the different third strands tested) c_{cr} decreases with k_{on} and increases with k_{off}, $D_{1/2}$ (being the greater of K_d and c_{cr}) still provides a measure of the binding strength of the third strand for the duplex even in the case of CCD comigration.

Several firm conclusions can be made from the gel-shift patterns regarding sequence effects in the R-block, as follows:

(1) For triplexes without mismatches, replacement of R-block thymines by adenine away from the strand switch (in the Z-positions) decreases the triplex lifetime ($1/k_{off}$), whereas replacement of T by A near the strand switch (in the X-positions) increases it. The amount of T2 at saturating concentration of duplex (where there is no radioactivity at the free third strand position) is much less in the case of TA-16 (Figure 2B) than with TT-16 (Figure 2A,C). The absence of radioactivity at the free third strand position means that all third strands were bound to the duplexes at the moment of entering the gel. Thus, the lower proportion of T2 for TA-16 in comparison with TT-16 implies that a T-to-A substitution in the Z-position results in a decrease in triplex lifetime. T-to-A substitution in both the X and Z-positions (AA-16; Figure 2B) produces a pattern intermediate between those of TT-16 (Figure 2A,C) and TA-16 (Figure 2B). The proportion of T1 in the case of AT-16 is noticeably less than for TT-16 (Figure 2C). Thus, A in the X-position increases the lifetime of the triplex.

(2) AA-16 and TA-16 have smaller off-rate and on-rate constants in the reaction with matched target than does Y-8. The half-transition concentration of duplex $D_{1/2}$ is greater in the cases of AA-16 and TA-16 than for Y-8. Since there is no noticible T2 band in the case of Y-8 (pure CCD comigration), whereas for AA-16 and TA-16 some amount of T2 is clearly seen, the off-rate constant for Y-8 must be greater. Thus the larger $D_{1/2}$ for AA-16 and TA-16 can only mean that the on-rate constant for these oligonucleotides is smaller.

(3) The stringency of triplex formation can be adjusted by appropriate sequence choice in the R-block. Two duplexes were used in the titration experiments: the matched target duplex 1, which contains recognition sites for both the Y- and R-blocks of the third strand, and the mismatched target duplex 2, which differs from duplex 1 by three inversions in the recognition site for the R-block. Because these inversions disturb the homopurine−homopyrimidine character of this site, there exists no R-block that can recognize this site via formation of canonical triads. In the cases of AA-16 and TA-16, the presence of the R-block decreases the on-rate for triplex formation over that of the Y-block alone (see above) but at the same time confers a high degree of specificity for the matched duplex. This observation suggests that these R-blocks function simultaneously as recognition elements and as "stringency clamps".

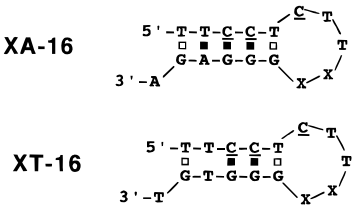


FIGURE 7: Possible hairpins formed by the oligonucleotides XA-16 and XT-16 (X = A or T). It is clear that hairpins formed by XA-16 should be more stable. The effect of replacements in the X-positions on hairpin stability is not obvious.

This term was introduced by Roberts and Crothers (1991) to refer to sequences appended to a triplex-forming oligonucleotide that do not recognize target sequences (unlike our R-blocks) but form secondary structures with parts of the oligonucleotide that do bind to the target, thereby increasing the stringency and permitting discrimination between matched and mismatched targets. In that case, stringency could be arbitrarily adjusted by choice of the sequence (and hence stability) of the clamp. Since in our system the clamp sequence is also part of the duplex recognition element, stringency can only be adjusted (without changing the environmental conditions) by virtue of our ability to choose between A and T in recognizing R-block AT base pairs. The secondary structures responsible for this stringency clamping effect are most likely hairpins; possible hairpins whose expected stabilities correlate inversely with the stability of the corresponding triplexes are shown in Figure 7.

Unlike the cases of AA-16 and TA-16, a complex formed between TT-16 and mismatched target duplex 2 was detected. Even so, the R-block in TT-16 contributes negatively to affinity in the case of duplex 2, since the concentration of duplex 2 needed to provide comigration was nearly 40-fold greater for TT-16 than for Y-8 (Figure 4A). This negative affinity contribution can also be explained by hairpin formation by TT-16, although this hairpin is expected to be less stable than for AA-16 and TA-16 (Figure 7).

The decrease in triplex lifetime resulting from T-to-A replacements in the Z-positions might be explained by a lower stability of AAT triads in comparison with TAT triads. Depending on the sequence context, such replacements may stabilize (Scaria et al., 1995), destabilize (Beal & Dervan, 1991), or have no effect (Mayfield et al., 1994) on triplex stability. In our case, the stabilizing effect of A in the X-position might be due to a better ability of purines to stack at the strand switch, where the helix appears to be distorted (Jayasena & Johnston, 1992b; A. Liu and B. H. Johnston, unpublished molecular modeling studies).

However, another explanation is also possible. Although the dissociation of short duplexes or triplexes is generally described as an all-or-none transition, at the microscopic level it involves "fraying", a progressive series of base pair openings starting from each end of the complex, each of them reversible (Anshelevich et al., 1984). If the R-block is unbound, either transiently due to thermal fluctuations in the case of a matched target, or permanently if its target sequence contains mismatches, it can interact with a transiently frayed end of the Y-block, stabilizing the frayed state and facilitating decay of the triplex. This scheme is equivalent to displacement of the duplex from the triplex by hairpin formation in the third strand; hence the effect should be stronger for sequences that can form more stable hairpins, such as those

having adenine residues in the Z-positions (see Figure 7). In this view, we need not assume that the relative stabilities of AAT and TAT triads in the middle of the triplex differ from those near the strand switch. Instead, we can suggest that AAT is generally more stable than TAT, but in the case of the Z-positions this effect is overwhelmed by the contribution of adenines to the hairpin formation, while in the case of the X-positions that contribution is less. This hypothesis also explains why the lower limit of specificity *S* is not much smaller for AA-16 than for TT-16 despite their very different affinities, since *S* should depend only on interactions of the R-block with the duplex and not on any interactions among different parts of the third strand.

It is unlikely that the effect of T-to-A replacements in our case is due to intermolecular intereactions between the purine blocks of the third strand, which were shown to interfere with triplex formation in the case of the GA motif, but not the GT motif (Noonberg et al., 1995). In fact, the possible interactions between the third strand molecules cannot affect the lifetime of the complex in the gel, and the effects in solution also should be small due to low concentration of the strands in our experiments.

Concluding Remarks. In this study we have investigated the effect of different R-block sequences on the formation and stability of a strand-switching triplex. We detected participation of the R-block in target recognition under conditions which are not strongly stabilizing for R-type triplexes, i.e., monovalent cations alone. Even Mg^{2+} is known to be a relatively weak stabilizer of R-type triplexes compared to Mn^{2+}, Zn^{2+}, polyamines, or intercalators. Thus we think that the range of conditions for applicability of R-type triplexes can be extended if they are bound to a Y-block.

The ability to decrease the lifetime of a complex without significant loss in specificity by enabling alternate third-strand conformations (which we observed in the case of T-to-A replacements in the Z-position) could be useful in cases where triplex formation is followed by some irreversible reaction such as covalent bond formation or cleavage (Herschlag, 1991). In such cases, the A-T replacements might be used to adjust the selectivity for a matched target, even if specificity (defined as the difference in energy between matched and unmatched triplexes) is unchanged.

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