Triplex Formation at the Rat *neu* Gene Utilizing Imidazole and 2′-Deoxy-6-thioguanosine Base Substitutions†

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ABSTRACT: Triplex-forming oligodeoxyribonucleotides (TFOs) can be designed so as to form antiparallel triple helices with duplex DNA by means of GGC and TAT or AAT base triplets, and these have been shown to be useful as sequence-specific DNA binding agents. Using TFOs targeted to the promoter region of the rat *neu* oncogene, it is shown here that substitution of an imidazole-nucleoside chimera at a single site in a *neu* specific TFO results in an increase in TFO binding affinity and specificity. This effect is discussed in terms of the stabilizing effect of local imidazole-TA triplet formation. It is also found that site-selective substitution of 2′-deoxy-6-thioguanosine for guanosine (S6-dG) in the TFO results in an increase in triplex formation in the presence of physiological levels of potassium ion. The utility and positioning of S6-dG base substitutions is discussed in the context of an intramolecular tetrad model.

Homopurine−homopyrimidine-rich tracts of DNA such as the ones found in the rat *neu* promoter have been shown to be good targets for binding by triplex-forming oligonucleotides (TFOs)[1] (Cooney et al., 1988; Durland et al., 1990, 1991; Hélène, 1991; Maher et al., 1992; Miller et al., 1992; Gee & Miller, 1992). A G-rich tract in the human *c-myc* promoter was shown to form a triplex readily with G-rich TFOs targeted to it (Cooney et al., 1988; Durland et al., 1991). TFOs have also been shown to bind to sites in the EGFR, mouse insulin receptor (Durland et al., 1991), and HER2 (Milligan et al., 1993) promoters. The GC box used as a binding site for the Sp1 transcription factor has also been successfully targeted in an artificial construct (Maher et al., 1992), the human dihydrofolate reductase promoter (Gee et al., 1992), and in the human immunodeficiency virus long terminal repeat (McShan et al., 1992).

Binding of synthetic oligonucleotides so as to form a triple helix with duplex DNA has been shown to repress transcription, presumably by preventing the binding of transcription factors to gene promoters (Cooney et al., 1988; Hélène, 1991; Maher et al., 1992) or by distorting the DNA duplex to the point that a transcription initiation complex is destabilized (Maher et al., 1992). Transcription from the *c-myc* promoter has been shown to be repressed by triplex formation (Cooney et al., 1988). Recent work has also indicated triplex-mediated transcriptional inhibition of the HER2 promoter (Ebbinghaus et al., 1993).

The rat *neu* promoter contains many sites for protein binding (Suen & Hung, 1990; Yu et al., 1992; Zhao & Hung, 1992). The sequences from −312 to −174 relative to the ATG initiator codon contain specific elements which mediate *neu* transcription (Suen & Hung, 1990). This segment contains the sequence GGTGGGGGGG known as the GTG enhancer element. The mechanism by which this element exerts its influence is unknown; however, it has been implicated in the negative autoregulation of the *neu* gene (Zhao & Hung, 1992). The −312 to −174 segment also contains the GGAGGA enhancer element which is complementary to the TCCTCC element found in the EGFR gene promoter (Johnson et al., 1988). Although the GGAGGA element does not appear to bind the Sp1 transcription factor as the TCCTCC element does, they may have other regulatory factors in common (Suen & Hung, 1990).

Intermolecular triplex formation occurs when an exogenous third strand of DNA binds specifically to the purine-rich strand of a targeted duplex (Beal & Dervan, 1991; Durland et al., 1991; Hélène, 1991; Gee & Miller, 1992). TFOs can be classified as either pyrimidine-rich (resulting in a YRY triple helix) or purine-rich (resulting in a RRY triple helix). Pyrimidine-rich TFOs typically bind in an orientation parallel to the purine-rich tract of the underlying target duplex. TFOs which are purine-rich typically bind at a physiological pH and lie along the major groove of the target duplex in an antiparallel orientation relative to the purine-rich strand of the target duplex. Guanine and thymine or adenosine in the third strand form reverse-Hoogsteen type bonds with the guanines in the underlying duplex (Beal & Dervan, 1991; Durland et al., 1991; Gee & Miller, 1992; Hélène, 1991). Homopurine/homopyrimidine duplex tracts make the best targets for intermolecular triplex formation of both the YRY and RRY type. Inversions in which a pyrimidine interrupts the homopurine motif can be tolerated although there is a loss in binding affinity to the TFO (Hélène 1991; Durland et al., 1990). The bulk of work to determine the best composition of TFOs has been with the pyrimidine-rich YRY TFO motif (Beal & Dervan, 1991; Durland et al., 1991; Gee & Miller, 1992; Hélène, 1991). To alleviate the need for acidic conditions, the placement of 5-methylcy-

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[1] Abbreviations: S6-dG, 2′-deoxy-6-thioguanosine; TFO, triplex-forming oligodeoxyribonucleotide.

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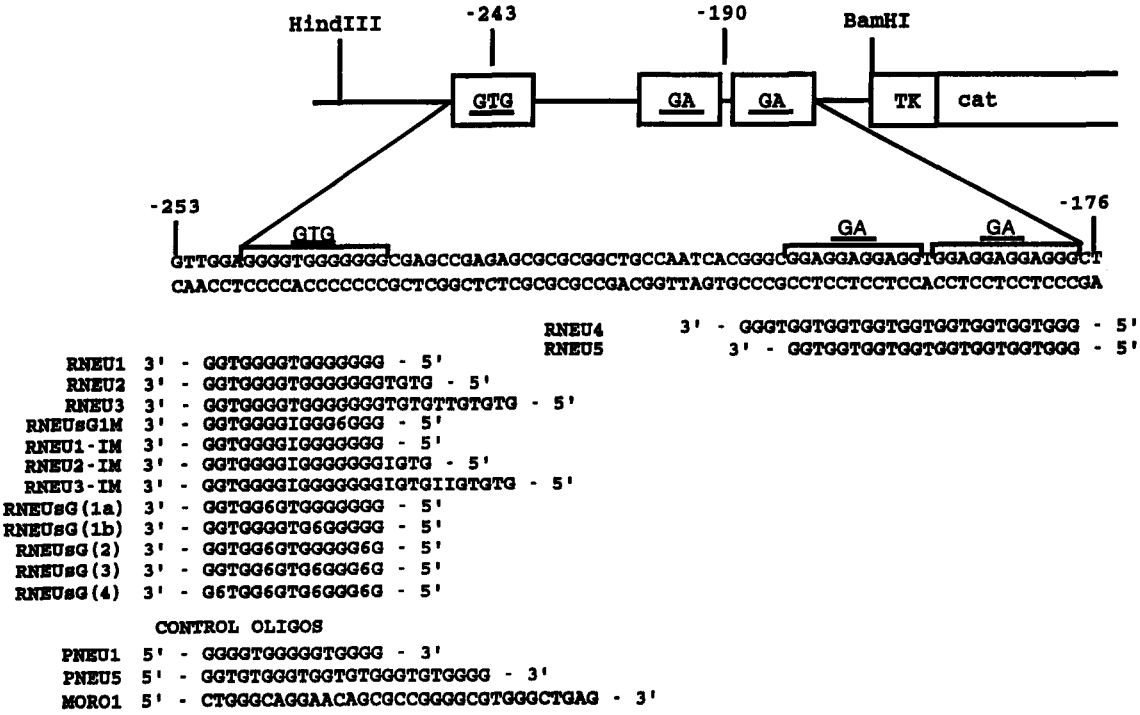

FIGURE 1: Sequences of Rat NEU promoter enhancer region and TFOs. The line drawing at the top indicates positions of the Rat NEU GTG and GA enhancer elements relative to the thymidine kinase (TK) promoter and chloramphenicol acetyltransferase gene in plasmid pNeu(S-X)tkCAT. Numbering shown is base position relative to the translational start site in the wild-type rat NEU gene (Suen & Hung, 1990). TFOs RNEU1, RNEU2, RNEU3, RNEU4, RNEU5, RNEU1-IM, RNEU2-IM, RNEU3-IM, RNEUsG(1a), RNEUsG(1B), RNEUsG-(2), RNEUsG(3), and RNEUsG(4) are shown aligned with their respective binding sites.

tosines (Hélène, 1991) or N^6-methyl-8-oxo-2′-deoxyadenosine (Milligan et al., 1993) in polypyrimidine TFOs was found to be useful. Modified bases such as 8-oxoadenine (Miller et al., 1992), N^4-(3-acetamidopropyl)cytosine (Huang et al., 1993), and 1-(2-deoxy-β-D-ribofuranosyl)-4-(3-benzamidophenyl)imidazole (Griffin et al., 1992) have also been used to increase triplex stability.

Fewer data are available for G-rich TFOs. Recent work has shown that incorporation of 2′-deoxynebularine in a G-rich TFO is useful in stabilizing triplex formation over a CG inversion in a homo-G tract (Stilz & Dervan, 1993). In the case of a T in a run of Gs in a target duplex, the least destabilizing base to use in the TFO is a T over the inversion (Durland et al., 1991; Hélène, 1991). The use of an imidazole-deoxyribose chimera (Figure 3C) to form base triplets at inversion sites has recently been shown to increase the affinity and selectivity of RRY TFO binding in simple test sequences (Durland et al., personal communication). In this paper we report on the utility of this idea within the context of a naturally occurring promoter binding site. Imidazole was chosen because of its synthetic flexibility as a platform for future enhancements.

Although divalent ions are required to drive RRY triplex formation, it has been shown that monovalent cations, especially K^+, can apparently destabilize triplex formation by G-rich TFOs. One explanation for this effect is based upon the possibility that G-rich oligonucleotides will form intramolecular tetrads which coordinate around the K^+ ion (Kim et al., 1991; Blackburn, 1991) thus making them unavailable for intermolecular triplex formation (Figure 3B). Recent work has demonstrated that triplex formation can be facilitated in the presence of K^+ by using 2′-deoxy-6-thioguanosine (Figure 3C) (Rao et al., personal communication) or 7-deaza-2′-deoxyxanthosine (Milligan et al., 1993) which disrupt G-tetrad formation. Here we confirm the utility of a thio-G substitution on a naturally occurring binding site and demonstrate the importance of proper positioning of this base substitution within the context of an intramolecular tetrad folding model.

MATERIALS AND METHODS

A Millipore Milligen 7500 DNA synthesizer using standard phosphoramidite chemistry was used to synthesize all oligonucleotides. The 2′-deoxy-6-thioguanosine and imidazole-2′-deoxyribonucleoside homologue were obtained as the dimethoxytrityl phosphoramidites from Triplex Pharmaceutical Corp. 2′-Deoxy-6-thioguanosine was synthesized as previously described (Rao et al., 1992). Briefly, synthesis of the imidazole nucleoside was achieved by utilizing the stereospecific sodium salt glycosylation procedure (Kazimierczuk et al., 1984). Thus, reaction of the sodium salt of imidazole (generated in situ by the treatment with NaH) with 1-chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-*erythro*-pentofuranose (Hoffer, 1960) in dry acetonitrile (CH_3CN) afforded protected nucleoside in 64.5% yield. Removal of the protecting toluoyl group of the nucleoside was accomplished by treatment with methanolic ammonia at ambient temperature, and the free nucleoside was isolated in a good yield. As is customary, the free nucleoside was converted to the corresponding 5′-*O*-(4,4′-dimethoxytrityl) derivative by treatment with 4,4′-dimethoxytrityl chloride (DMT-Cl) in anhydrous pyridine. Purification of the reaction product by silica gel column chromatography resulted in pure DMT-imidazole nucleoside derivative in 54% yield. Conventional phosphitylation of the DMT with 2-cyano-ethyl-*N*,*N*-diisopropylchlorophosphoramidite in CH_2Cl_2 in the presence of *N*,*N*-diisopropylethylamine gave the corresponding imidazole phosphoramidite in 86% yield.

A.

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5'-TTGGAGGGGTGGGGGGGCGAGCCGAGAG  -3'
3'-  CCTCCCCACCCCCCCGCTCGGCTCTCGC-5'
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B.

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5'-TTGGAGGGGAGGGGGGGGGAGGGGAGAG  -3'
3'-  CCTCCCCTCCCCCCCCCTCCCCTCTCGC-5'
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FIGURE 2: Sequences of the 26 bp duplexes used in band shift analysis. (A) Wild type duplex containing GTG element. (B) Mutant duplex altered to be strictly homopurine/homopyrimidine.

All TFOs (Figure 1) were synthesized as the 3′-propylamine derivative (Glen Research), employing fast deblocking chemistry (Milligen). As previously described, oligonucleotides were purified by ion-exchange HPLC, followed by desalting over Sepharose G-25 (Durland et al., 1991).

Gel mobility shift assays were performed using either a 76 bp oligonucleotide duplex containing both the GTG enhancer element and the GA element (Figure 1A), a 26 bp duplex containing only the GTG enhancer element (Figure 2A), or a 26 bp mutant duplex which was designed to be strictly homopurine/homopyrimidine (Figure 2B). Complementary oligonucleotides were heated to 90 °C for 15 min and allowed to slowly cool for annealing. The probe was end-labeled using T4 polynucleotide kinase (Sambrook et al., 1989). The duplex was run on a 5% (19:1) native gel. The probe was excised from the gel and recovered using a Bio-Rad electroeluter. In band-shift assays the probe was titrated with increasing concentrations of TFOs in a buffer consisting of 20 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, and 10% glycerol. Samples were allowed to incubate for at least 4.5 h at 37 °C to ensure binding equilibrium. Band shifts were run on 12% (19:1) gels containing 90 mM Tris-borate and 10 mM $MgCl_2$ (Cooney et al., 1988; Gee et al., 1992).

DNase I footprinting was performed as described previously (Cooney et al., 1988; Gee et al., 1992) on the plasmid pNeu(S-X)tkCAT (Zhao & Hung, 1992) which contains the *Stu*I−*Xho*I fragment of the rat *neu* promoter (Suen & Hung, 1990). DNAse I footprinting was performed with the *Bam*HI−*Hin*dIII fragment of pNeu(S-X)tkCAT, labeled with [α-^{32}P] dATP (New England Nuclear) and Klenow polymerase at the *Bam*HI terminus. Binding was initiated by incubation with TFO for 4.5 h at 37 °C in a buffer consisting of 20 mM Tris-HCl, pH 7.6, and 10 mM $MgCl_2$. Analytical digestion was performed with 0.01 unit of DNAse I for 1 min. The reaction was quenched using formamide loading dye containing 10 mM EDTA, followed by analysis on a 10% acrylamide 7 M urea sequencing gel (Sambrook et al., 1989).

RESULTS

Rat *neu* specific TFOs (Figure 1) were subjected to band-shift analysis, so as to estimate binding affinity and selectivity. This assay is based upon the observation that the electrophoretic mobility of the bound TFO complex is reduced on native polyacrylamide gels relative to that of the free duplex binding site alone (Cooney et al., 1988). Titration with unmodified TFOs in the presence of Mg^{2+} alone indicated that all are adequately described by a two-state binding equilibrium with a midpoint near 10 nM (Figure 4, A−E) which is indicative of an apparent dissociation constant between 10^{-9} and 10^{-8} M.

The scrambled-sequence control oligonucleotides PNEU1 and PNEU5 were also analyzed along with the control oligonucleotide MORO1 (Figure 1) in band-shift assays. MORO1 and PNEU1 gave no indication of binding at any

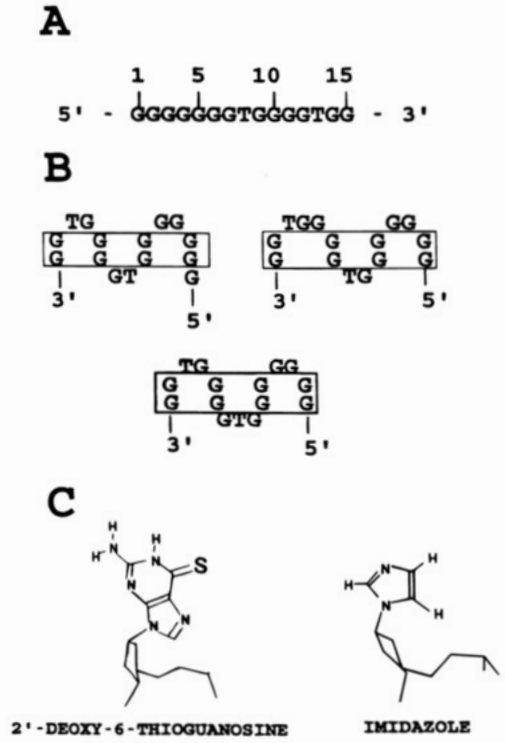

FIGURE 3: (A) RNEU1 TFO with numbering scheme. (B) Models for three equivalent schemes to fold RNEU1 by means of intramolecular G tetrad formation. (C) Structure of imidazole and 2′-deoxy-6-thioguanosine (S6-dG) modified deoxyribonucleotides.

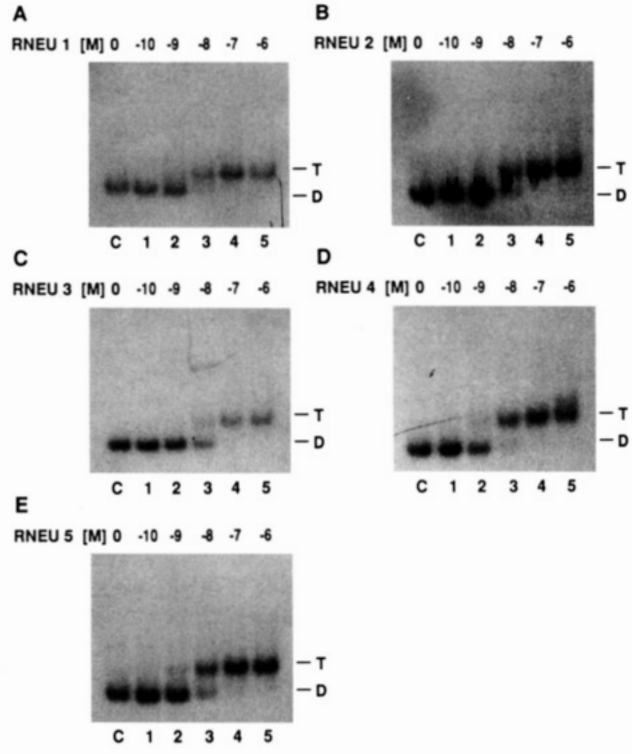

FIGURE 4: Gel mobility shift analysis of unmodified TFOs in 10 mM $MgCl_2$ and 20 mM Tris-HCl, pH 8. ^{32}P-labeled 76 bp duplex (Figure 1) is at 10^{-10} M in all lanes. In each band-shift assay, lane C refers to the control lane which contains the duplex alone, lane 1 contains 10^{-10} M TFO, and lanes 2−5 contain a 10-fold increase of TFO concentration per step. The log of concentration in each of these titration points is repeated at the top of the figure. Markings to the right of each assay indicate positions of duplex (D) band and triplex (T) band.

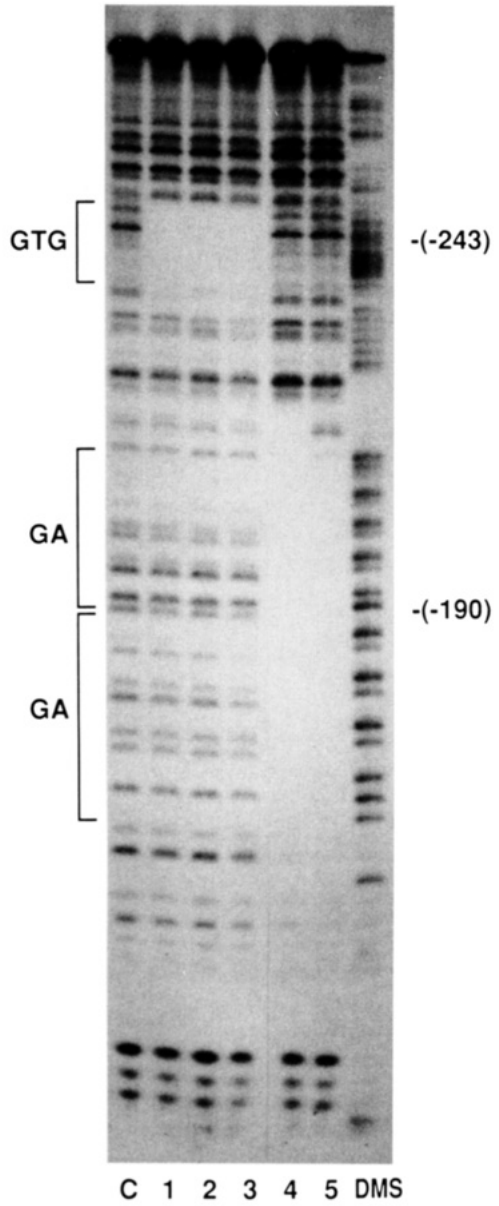

FIGURE 5: DNAse I footprinting of unmodified TFOs in 10 mM $MgCl_2$ and 20 mM Tris-HCl, pH 8. The ^{32}P-labeled *Hin*dIII−*Bam*HI fragment of the rat *neu* promoter enhancer region is held at a concentration of 10^{-9} M in all lanes. TFO was added to a final concentration of 10^{-6} M for all lanes. Lane C contains the control oligonucleotide MORO1. Lanes 1−5 contain RNEU1, RNEU2, RNEU3, RNEU4, and RNEU5 respectively. Brackets to the left indicate the position of the GTG and GA enhancer elements, relative to the rat *neu* transcription initiation site.

of the concentrations tested. PNEU5 gave some evidence for metastable complex formation, but only at 10^{-6} M (data not shown). Consistent with these observations DNase I footprinting provided no evidence for binding of either PNEU1, PNEU5 (data not shown), or MORO1 (Figure 5, lane C).

The DNase I footprints for RNEU1, RNEU2, and RNEU3 (Figure 5, lanes 1−3) were virtually identical indicating that only the 15 bp core binding site, defined by the binding of RNEU1, was being occupied in triplex formation (Figure 5, lane 1). RNEU4 and RNEU5 (Figure 4, lanes 4−5) gave full footprints at their respective target sequences indicating that both formed a full-length triplex.

As expected, the imidazole modified TFO RNEU1-IM (Figure 1) gave a DNAse I footprint pattern identical to the

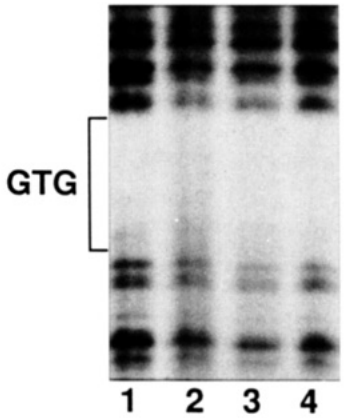

FIGURE 6: DNAse I footprinting of imidazole substituted TFOs. Footprinting was performed as in Figure 5, at 10^{-9} M in duplex and 10^{-6} M in TFO. Lane 1 contains RNEU1. Lane 2 contains RNEU1-IM. Lane 3 contains RNEU2-IM. Lane 4 contains RNEU3-IM.

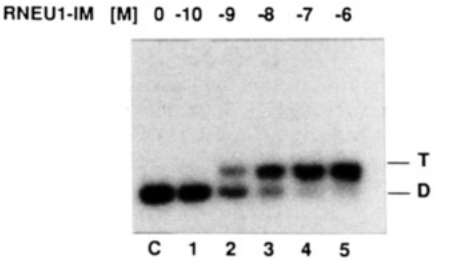

FIGURE 7: Gel mobility shift titration of RNEU1-IM in 10 mM $MgCl_2$ and 20 mM Tris-HCl, pH 8. ^{32}P-labeled 26 bp duplex (Figure 2A) is at a concentration of 10^{-10} M in all lanes. Lane C refers to the control lane which contains the duplex alone. Lane 1 contains 10^{-10} M TFO. The concentration of TFO is increased 10-fold per lane going left to right. The log of added TFO concentration is listed at the top of the figure. Markings to the right of each assay indicate positions of duplex (D) band and triplex (T) band.

unmodified TFO RNEU1 (Figure 6, lane 2). The longer homologues RNEU2-IM and RNEU3-IM which contain imidazole substitutions within the region flanking the core site defined by RNEU1 (Figure 1) continued to give footprints only within the core 15 bp binding site (Figure 6, lanes 3−4), suggesting that imidazole substitution had not induced stable triple formation within the flanking domain.

In gel mobility shift assays, RNEU1-IM appeared to induce binding at approximately 5−10 times lower added TFO concentration (Figure 7) which suggests that a single imidazole substitution has improved binding affinity approximately 5−10 times, relative to the unmodified TFO RNEU1.

To test the selectivity of the imidazole-modified TFO binding, a homologue of the natural promoter target duplex was employed in which the TA inversion at −273 is reversed, so as to generate a simple homopurine/homopyrimidine duplex element (Figure 2B). Gel mobility analysis with RNEU1 showed that, as expected, the unmodified TFO binds more tightly to the homopurine duplex homologue. This is almost certainly a result of the greater stability of a TAT triplet which forms in the homologue as compared to a T binding to form a TTA triplet (Figure 8A, lane 2).

In contrast, the imidazole-containing homologue RNEU1-IM even at concentrations as high as 10^{-6} M is not capable of forming a stable complex with this homopurine binding site (Figure 8B). As discussed below, this observation

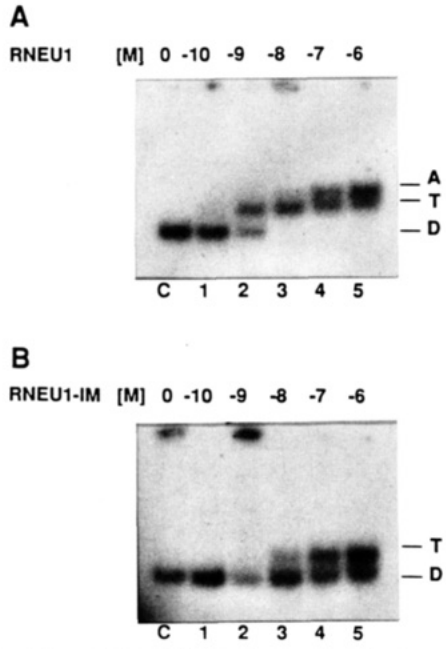

FIGURE 8: Gel mobility shift titrations using the homopurine 26 bp duplex isomer (Figure 2B) in 10 mM $MgCl_2$ and 20 mM Tris-HCl, pH 8. (A) Binding of RNEU1. (B) Binding of RNEU1-IM, the singly-modified imidazole homologue. ^{32}P-labeled mutant duplex is at a concentration of 10^{-10} M in all lanes. In each band-shift assay lane C refers to the control lane which contains the duplex alone. For each assay, lane 1 contains 10^{-10} M TFO. The concentration of TFO is increased 10-fold per lane going left to right and is repeated to the top of the figures. Markings to the right of each assay indicate positions of duplex (D) band and triplex (T) band.

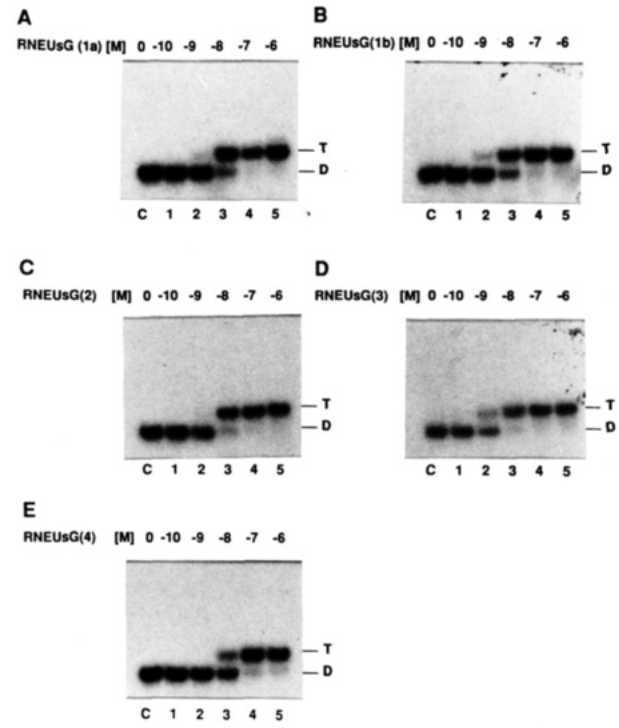

FIGURE 9: Gel mobility shift titrations of S6-dG modified TFOs in 10 mM $MgCl_2$ and 20 mM Tris-HCl, pH 8. The ^{32}P-labeled 26 bp duplex (Figure 2A) is at a concentration of 10^{-10} M in all lanes. In each band-shift assay, lane C refers to the control lane which contains the duplex alone. For each assay lane 1 contains 10^{-10} M TFO. The concentration of TFO is increased 10-fold per lane going left to right, as indicated to the top of the figure. Markings to the right of each assay indicate positions of duplex (D) band and triplex (T) band.

confirms a recent study (Durland et al., personal communication) and suggests that the imidazole homologue has begun to diminish the requirement for simple homopurine binding site symmetry.

TFOs of the RNEU class were synthesized with S6-dG modification, based upon the possibility that the free TFO might form an intramolecular tetrad at a physiological concentration of K^+ (Figure 1A). For RNEU1 there are three possible intramolecular tetrad conformations (Figure 2B). RNEUsG(1a), RNEUsG(1b), RNEUsG(2), RNEUsG(3), and RNEUsG(4) contain S6-dG substitutions at different sites in the presumed tetrad forming region (Figure 2B). RNEUsG1M contains an imidazole in place of T8 and an S6-dG in place of G4 (numbering as shown in Figure 3A) which is in the loop region of all three putative intramolecular tetrad structures.

Gel mobility shift assays, done in the absence of KCl (20 mM Tris-HCl, pH 8, and 10 mM $MgCl_2$), indicate that RNEUsG(1a), RNEUsG(1b), RNEUsG(2), RNEUsG(3), and RNEUsG(4) bind between 10^{-9} and 10^{-8} M (Figure 9A–E), as does RNEUsG1M (data not shown).

In the presence of 25 mM KCl (20 mM Tris-HCl, pH 8, 25 mM KCl, and 10 mM $MgCl_2$) RNEU1 did not shift the duplex at any concentration tested while RNEUsG1M gave a shift with a midpoint near 10^{-6} M. The binding of RNEUsG(1a), RNEUsG(1b), RNEUsG(2), RNEUsG(3), and RNEUsG(4) were only modestly affected. Observed ranges of binding are summarized in Table 1. In the presence of 50 mM KCl, 20 mM Tris-HCl, pH 8, and 10 mM $MgCl_2$, RNEU1 and RNEUsG1M gave no detectable band shifts (data not shown).

At high KCl (20 mM Tris-HCl, pH 8, 100 mM KCl, and 10 mM $MgCl_2$) RNEUsG(1a) and RNEUsG(1b) begin to show a measurable decrease in binding in the band-shift assay (Figure 10A–B). However, the homologues with two or greater 6-thio G substitutions are much less effected. These data are also summarized in Table 1.

DISCUSSION

The use of TFOs in the so called antigene (Hélène, 1991) or triple helix strategy has been recognized a basis for the design of sequence-specific DNA binding drugs (Hélène, 1991; Durland et al., 1991; Maher et al., 1989; Gee & Miller, 1993). Although homopurine/homopyrimidine tracts occur throughout the human genome, the ability to target more random targets would greatly expand the therapeutic usefulness of this approach (Durland et al., 1990; Milligan et al., 1993; Hélène, 1991). Strategies such as substituting non-natural oligonucleotide bases to enhance triplex formation, especially with target duplexes containing inversions in the homopurine/homopyrimidine tract, are currently under investigation (Stilz & Dervan, 1993; Griffin et al., 1992; Mohan et al., 1993).

In this study, a series of triplex forming oligonucleotides (TFOs) was synthesized so as to bind to duplex targets within the rat *neu* promoter (Figure 1). RNEU1, RNEU2, and RNEU3 were targeted to the GTG element. RNEU4 and RNEU5 were targeted to the GGAGGA element. PNEU1 and PNEU5 have the same composition as RNEU1 and RNEU5, respectively, but with a scrambled sequence.

DNase I footprinting analysis of RNEU1, RNEU2, and RNEU3 gave nearly identical results (Figure 5). This

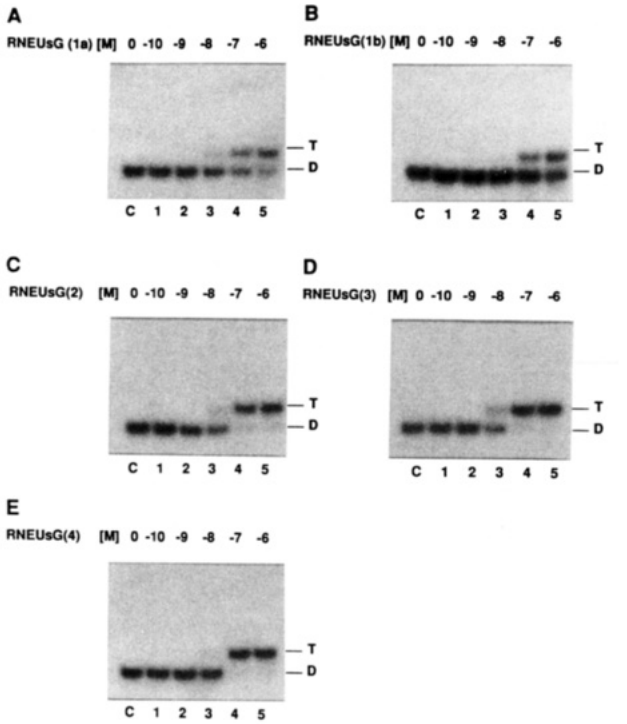

FIGURE 10: Gel mobility shift titrations of S6-dG modified TFOs in 100 mM KCl, 10 mM $MgCl_2$, and 20 mM Tris-HCl, pH 8. ^{32}P-labeled 26 bp duplex (Figure 2A) is at a concentration of 10^{-10} M in all lanes. In each band-shift assay, lane C refers to the control lane which contains the duplex alone. For each assay, lane 1 contains 10^{-10} M TFO. The concentration of TFO is increased 10-fold per lane going left to right, as indicated to the top of each figure. Markings to the right of each assay indicate positions of duplex (D) band and triplex (T) band.

Table 1: Comparison of Triplex-Forming Ability of TFOs in 10 mM $MgCl_2$[a]

TFO	0 mM KCl	25 mM KCl	100 mM KCl
RNEU1	10^{-9}↔10^{-8}	$>10^{-6}$	N/A
RNEUsG1M	10^{-9}↔10^{-8}	$\sim10^{-6}$	N/A
RNEUsG(1a)	10^{-9}↔10^{-8}	$\sim10^{-8}$	$\sim10^{-7}$
RNEUsG(1b)	10^{-9}↔10^{-8}	10^{-8}↔10^{-7}	$\sim10^{-6}$
RNEUsG(2)	10^{-9}↔10^{-8}	10^{-8}↔10^{-7}	10^{-8}↔10^{-7}
RNEUsG(3)	10^{-9}↔10^{-8}	10^{-8}↔10^{-7}	10^{-8}↔10^{-7}
RNEUsG(4)	10^{-8}↔10^{-7}	10^{-8}↔10^{-7}	10^{-8}↔10^{-7}

[a] Numbers refer to an estimation of the titration midpoint for each TFO, interpolated from band-shift data.

similarity is reinforced by band-shift analysis (Figure 4). Thus, both sets of measurements suggest that the oligonucleotide sequence elements of RNEU2 and RNEU3, which extend beyond the 15 bp binding site defined by RNEU1, do not form stable associations with the flanking duplex. Thus, the multiple CG inversions which disrupt the homopurine/homopyrimidine nature of this flanking sequence (Figure 1) appear to disrupt triplex formation by their close proximity to each other. As expected from the more nearly uniform structure of their duplex target, RNEU4 and RNEU5 bind tightly throughout their respective target sites (Figure 5).

RNEU1 TFOs containing an imidazole at the TA inversion site within the core binding domain, bound more tightly than the unmodified parent molecules, as indicated by the gel mobility shift assay (Figure 7). The increased affinity of such imidazole-TA triplet associations relative to other bases at a TA inversion could be due to a reduction of unfavorable steric interactions and the induction of favorable hydrogen bonding (Durland et al., personal communication). To resolve those possibilities, work is in progress to deduce the structure of the imidazole-TA triplet association, employing modeling and NMR methods.

RNEU2-IM and RNEU3-IM were synthesized with imidazoles positioned at the core TA inversion site and also at inversions in the flanking region to the 3′ side of the 15 bp core GTG sequence (Figure 1). However, RNEU2-IM and RNEU3-IM form 15 bp long footprints which are identical to those of the unmodified homologues (Figure 6). Therefore, although imidazole can increase TFO binding affinity when placed at isolated base-pair inversion sites, such as that in the rat *neu* core domain, these footprinting data suggest that imidazole is not able to overcome the destabilizing influence of several closely spaced base-pair inversions which interrupt an extended region within a homopurine/homopyrimidine motif.

In addition to the increase in affinity seen in RNEU1-M as a result of imidazole substitution, it is important to note that the selectivity of triple-helix formation has increased also. Using a homopurine/homopyrimidine binding-site isomer as a reference (Figure 2B), it was observed that the RNEU1 TFO containing standard bases bound more tightly to the homopurine isomer than to the naturally occurring TGT sequence (Figures 4 and 7). This is due to the destabilizing effect of a TTA triplet which is formed at the TA inversion site upon binding of RNEU1 to the wild-type TGT site, as compared to the TAT triplet formed upon binding RNEU1 to the homopurine isomer.

Conversely, the imidazole-containing TFO RNEU1-M binds more tightly to the wild-type duplex than to the homopurine binding site isomer (Figure 8). This observation is consistent with studies of synthetic test sequences (Durland et al., personal communication). Therefore the data presented here constitute the first evidence from a biologically relevant target that the homopurine/homopyrimidine requirement for triple-helix formation can be reversed by site selective base modification in a TFO.

However, the imidazole substitution described here is not a perfect one in that regard. Durland et al. (personal communication) report that the imidazole base cannot distinguish TA and CG inversions, and the data presented here suggest that imidazole containing triplets are not useful in triple helices with closely spaced sites of pyrimidine/purine base-pair inversion in the duplex target. Clearly, additional refinement of the approach is warranted, in order to improve upon those shortcomings.

In the presence of K^+, G-rich oligonucleotides can fold to form tetrads (Kim et al., 1991; Blackburn, 1991). Of particular interest here is the formation of K^+ dependant intramolecular tetrads, such as the thrombin binding aptamer (Shultze et al., 1994). This 15-mer oligodeoxynucleotide has been shown to fold in KCl to form an intramolecular quadraplex comprising two G-quartets connected by one T-G-T and two T-T loops. The rat *neu* specific TFOs described here are all G-rich. In particular, the RNEU1 series has a sequence structure which is very similar to that of the thrombin-binding aptamer (Figure 3) and should be similarly capable of stable intramolecular quadraplex formation. Folding to form such a quadraplex should directly compete with triple-helix formation and would be expected to result in a significant reduction in the availability of such TFOs for duplex DNA binding at physiological KCl concentration.

The incorporation of modified bases such as 7-deazaguanosine (dzaG) has recently been utilized in inhibiting G-quartet formation (Milligan et al., 1993). 2′-Deoxy-6-thioguanosine (S6-dG) has recently been shown to disrupt tetrad formation (Rao et al., personal communication) by making unavailable the O^6 atoms which engage in tetrad specific hydrogen bonding and coordination of monovalent cations (Kim et al., 1991; Blackburn, 1991). Here, we have shown that a single S6-dG substitution can substantially diminish the KCl dependance of TFO binding (Figure 10). Interpretation of the position dependance of those data benefits from reference to models for intramolecular tetrad formation (Figure 3B). Specifically, RNEUsG1M, with an S6-dG substitution at a site which would be placed in a loop, is unavailable to form triple helix at 50 mM KCl. Conversely, RNEUsG(1a) and RNEUsG(1b), which have S6-dG placed within the octamer stem region, showed a significant reduction in the KCl dependance of triple-helix formation (Figure 10).

As evidenced by the data in Figure 10, none of the single S6-dG substitutions have completely eliminated the interference of triple-helix formation by KCl. Therefore RNEU1 homologues with two, three, or four substitutions were synthesized (Figure 1). RNEUsG(2), the homologue with two substitutions, displayed an apparent dissociation constant for triple-helix formation at 100 mM KCl, 10 mM $MgCl_2$ which was within a factor of 10 of that in the absence of KCl (Figure 9). Additional S6-dG substitution to generate RNEUsG(3) and RNEUsG(4) had no additional effect on binding affinity in the presence or absence of KCl.

Together, these data suggest that modification of guanosines at the 6 position constitutes a useful tool for retaining tight TFO binding at physiological potassium ion concentration, without the requirement for extensive chemical modification [e.g., RNEU1 and RNEUsG(2) differ by two atoms]. Although not definitive, the site dependance of the data suggests that tetrad formation may be the origin of the observed KCl interference with triple-helix formation, presumably mediated by direct coupling of the two oligonucleotide dependant equilibria.

The practical ramification of the studies described above is that by substitution of a single imidazole and two S6-dG groups within the RNEU class of TFO, it is possible to obtain a significant increase in the selectivity of rat *neu* specific triple-helix formation and tight binding which is retained at physiological pH, temperature, and potassium concentration. Work is in progress to assess the effect of these physical improvements upon the activity of such TFOs in mammalian cells.

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