Inhibition of Transcription Elongation in the HER-2/neu Coding Sequence by Triplex-Directed Covalent Modification of the Template Strand†

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ABSTRACT: Triplex formation may be of potential utility to inhibit the expression of individual genes. We describe the formation of a triple helix in the coding sequence of the HER-2/neu gene. In vitro transcription analysis in the presence and absence of triplex formation demonstrates that an unmodified DNA triplex-forming oligonucleotide is incapable of inhibiting RNA polymerase elongation. Triplex formation by an oligonucleotide−psoralen conjugate was used to form a covalent photoadduct with a thymine on the nontemplate strand of the HER-2/neu gene. In the native HER-2/neu gene, covalent attachment of the triplex-forming oligonucleotide to the nontemplate strand did not prevent RNA polymerase elongation. Using HER-2/neu point mutants that would place the target thymine on the template strand, we demonstrated that covalent modification of the template strand was necessary to inhibit RNA polymerase elongation. Based on these data, we synthesized oligonucleotide−alkylator conjugates that would react with a specific guanine residue on the template strand of the HER-2/neu coding sequence. The oligonucleotide−alkylator conjugates inhibited transcription elongation by T7 RNA polymerase and eukaryotic RNA polymerase II from a HeLa nuclear extract. These studies demonstrate the successful application of triplex-forming oligonucleotide−alkylator conjugates to inhibit transcription elongation in the HER-2/neu gene, and show that covalent modification of the DNA strand used as the transcription template is necessary to prevent RNA polymerase elongation.

Triplex-forming oligonucleotides (TFOs) have the ability to bind to double-stranded DNA with exquisite specificity. They are capable of recognizing a specific target sequence in a gene of interest in the context of mega- and giga-base chromosomes (*1*). Triplex formation has been demonstrated intracellularly in a plasmid target (*2*−*4*) and in chromatin (*5*, *6*). This specificity derives from the hydrogen bonding scheme responsible for triplex formation, and three structural motifs for triple helix formation have been described, based on the Hoogsteen (or reverse Hoogsteen) hydrogen bonding of the third-strand oligonucleotide [reviewed in (*7*)]. Triplex formation has been limited to target sequences which are nearly homopurine−homopyrimidine in nature, but several important advances to the three basic structural motifs have been made by introducing modified bases, linkers, or changes in the backbone of TFOs [reviewed in (*7*, *8*)].

Many investigators have attempted to take advantage of this high degree of binding specificity in the development of "anti-gene" TFOs to inhibit the expression of clinically important genes whose pathological expression may be modulated to therapeutic advantage. In this regard, a TFO can be designed to bind to a polypurine−polypyrimidine tract in a gene promoter to prevent the formation of the transcription initiation complex (*9*−*14*). A second potential mechanism by which TFOs may lead to a reduction in gene expression is by inhibiting transcription elongation. The oligonucleotide is designed to form a triple helix in the coding sequence of the gene of interest in order to prevent the progression of the RNA polymerase in its synthesis of the nascent chain of messenger RNA. One advantage of this approach is to increase the number of potential triplex target sequences since the transcribed region of a gene is generally much larger than the cis-regulatory elements of the promoter. Another possible advantage of a target in the coding region of a gene is a somewhat greater accessibility of the coding sequence to triplex formation, since the TFO will not be required to displace a previously bound transcription factor. Indeed, TFOs have not been shown to displace previously bound transcription factors or histone proteins, and triple helix formation is excluded in the histone−DNA contacts of a preformed nucleosome (*15*).

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[1] Abbreviations: CMV I/E, cytomegalovirus immediate/early; RNAP II, (eukaryotic) RNA polymerase II; TFO, triplex-forming oligonucleotide; TFO−pso, triplex-forming oligonucleotide−psoralen conjugate; TFO−chl, triplex-forming oligonucleotide−chlorambucil conjugate; TFO−pam, triplex-forming oligonucleotide−phenylacetate mustard conjugate; bp, base pairs; nt, nucleotides; PAGE, polyacrylamide gel electrophoresis; ppG, pyrazolopyrimidine analogue of guanine; UV, ultraviolet.

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The formation of a highly stable triple helix with modified or chemically reactive oligonucleotides has enhanced the ability of TFOs to inhibit transcription elongation. Young and colleagues demonstrated that triplex formation in the transcribed sequence of a G-free cassette induced a transient block in transcription elongation, but all of the initiated transcripts were elongated with further incubation (*16*). By conjugating an alkylating group to the TFO, thereby covalently binding to a guanine base in the template strand, these investigators demonstrated a persistent block in RNA polymerase elongation. Interestingly, they commented that placing the alkylating moiety at either the 5′ or the 3′ end of the TFO gave rise to the same size truncated transcript, implying that the stabilization of triplex formation rather than the formation of a covalent guanine adduct was responsible for the transcription block. The findings of Duval−Valentin and colleagues corroborated and expanded these observations, demonstrating a transient and partial block of *E. coli* RNA polymerase elongation by a triplex downstream of the promoter (*17*). Transcription inhibition was dependent on both the temperature and the time of the in vitro transcription reaction, demonstrating that the stability of the triple helix is the critical factor in blocking transcription elongation. By using a psoralen conjugate, these investigators were able to permanently block the elongation of RNA polymerase. Recent data have also suggested that the formation of a highly stable triple helix may be capable of preventing transcription elongation independent of the formation of a covalent bond with the triplex target. A triple helix formed by an oligonucleotide containing N3′→P5′ phosphoramidate linkages (*18*) or an acridine intercalator (*19*) was capable of inhibiting transcription elongation in vitro without covalent modification of the triplex target.

The purpose of the present work was to develop a TFO strategy to inhibit the expression of the HER-2/neu gene by preventing RNA polymerase elongation through the coding sequence of the HER-2/neu gene. While triplex formation has been previously demonstrated in the HER-2/neu promoter (*13*, *14*, *20*, *21*), the only potential triplex target site in the HER-2/neu coding sequence is a nearly perfect homopurine−homopyrimidine tract at +205 − +228 (map numbers are according to the cDNA sequence in GenBank, accession number X03363)(Figure 1A). This tract begins 30 base pairs (bp) downstream from the initiation codon, so that inhibition of transcription elongation at this point would lead to a truncated transcript that would not be translated into a meaningful protein product. This sequence contains two C:G interruptions in the homopurine strand, and it is not flanked by a TpA step for psoralen-directed photo-cross-linking. Based on recent data suggesting that a highly stable triple helix was capable of inhibiting transcription elongation in vitro without covalent modification of the triplex target (*18*, *19*), as well as the previous observation that stabilization of the triple helix rather than covalent modification of the triplex target was responsible for arresting RNA polymerase elongation (*16*), we designed TFO−psoralen conjugates (TFO−pso) that would be capable of inducing photoadduct formation only with the nontemplate strand. To further delineate the conditions necessary for triplex-mediated inhibition of transcription elongation, we used the same oligonucleotide in mutated triplex target sequences in which the target thymine was placed only on the template strand or on both the nontemplate and template strands. Finally, we designed TFOs conjugated to chlorambucil or phenylacetate mustard that would covalently bind to a specific guanine in the template strand of the native HER-2/neu gene. We used in vitro transcription analysis to predict the ability of these oligonucleotides to effectively inhibit HER-2/neu gene expression. In this report, we describe a novel triplex target in the HER-2/neu gene, and our findings demonstrate the need for covalent modification of the template strand to inhibit both phage and eukaryotic RNA polymerase elongation.

MATERIALS AND METHODS

Oligonucleotide Synthesis and Purification. Unmodified oligonucleotides were purchased from commercial sources. Oligonucleotides were analyzed for integrity and quality by denaturing polyacrylamide gel electrophoresis (PAGE), and if failure sequences were detected, oligonucleotides were purified by PAGE. Oligonucleotides were conjugated via a 5′-terminal phosphate to the 4′ position of 4′-hydroxymethyl-4,5′,8-trimethylpsoralen by a two-carbon linker arm (psoralen 5′ C-2 conjugates were purchased from Oligos, Etc., Wilsonville, OR). Oligonucleotide−alkylator conjugates were prepared by Epoch Pharmaceuticals (Bothell, WA) according to methods published elsewhere (*22*). Oligonucleotides linked to nitrogen mustards were stored in aqueous solution at −80 °C, and stock solutions were thawed and kept on ice prior to use.

Plasmids and Cloning. The triplex target sequence was cloned into several plasmid vectors for in vitro transcription analysis (Figure 1B). Oligonucleotides corresponding to the triplex target site were ligated into the pGEM-7f+ plasmid vector (Promega, Madison, WI) to yield the recombinant vector pGEM7/HNC. This vector contains T7 and Sp6 promoters on either side of the multiple cloning site so that in vitro transcription from the T7 promoter will use the bottom strand as a template and in vitro transcription from the Sp6 promoter will use the top strand as a template. The oligonucleotides used for the cloning contain the 24 base pair triplex target sequence, 8 bp of flanking sequence, and *Eco*RI and *Hin*dIII compatible ends for cloning. Native and mutant HER-2/neu triplex target sites were cloned into the pcDNA1.1 vector (Invitrogen, Carlsbad, CA) by ligating double-stranded oligonucleotides as described for the pGEM7/HNC plasmid. The pcDNA1.1 plasmid contains both T7 bacteriophage and cytomegalovirus immediate/early (CMV I/E) promoters upstream of the multiple cloning site for use in in vitro transcription analysis. Finally, the HER-2/neu cDNA sequence under the control of the CMV I/E promoter was contained in the pcDNA3/erbB2 vector (the kind gift of Teresa Strong, University of Alabama at Birmingham).

Triplex Reactions and Native Gel Mobility Shift Analysis. Triplex formation was demonstrated by nondenaturing gel mobility shift analysis as previously described (*13*). Triplex reactions were incubated at 37 °C for at least 1 h in TBM buffer consisting of 90 mM Tris-HCl, 90 mM boric acid, 10 mM $MgCl_2$, pH 7.4. Electrophoresis was carried out in TBM, pH 8.0.

Computer Image Densitometry and Calculation of the Apparent Dissociation Constant. Autoradiographs of mobility shift gels were scanned with a Hewlett-Packard ScanJet 4c.

Band intensity of the scanned image was analyzed with SigmaGel 1.0 (Jandel Scientific software). The equilibrium dissociation constant for triplex formation was estimated by the formula $K_d = (D/T)[TFO]$ where D/T is the ratio of duplex DNA to triplex DNA formed at TFO concentration [TFO] as described (*9*, *12*).

Irradiation of Psoralen-Containing Oligonucleotides and Denaturing Gel Mobility Shift Analysis. After triplex formation with TFO−pso conjugates, samples were irradiated on ice at 366 nm for 10 min (unless otherwise indicated) essentially as described (*23*). After irradiation, the reactions were denatured and resolved by denaturing PAGE.

Piperidine Cleavage Reactions. The sites of DNA alkylation may be mapped by converting them to single-strand breaks with mild alkali and heat. For this work, either a 248 base pair *Hin*dIII/*Eco*RV restriction fragment of the pcDNA3/erbB2 vector or a 57 base pair *Hin*dIII/*Eco*RI restriction fragment of the pcDNA1.1/HNC1 vector was labeled on the template strand by the Klenow reaction. Triplex formation with the oligonucleotide−alkylator conjugates took place in TBM buffer for 2−4 h at 37 °C. After triplex formation, piperidine was added to a final concentration of 1 M, and the reactions were heated to 80 °C for 30 min. The cleavage products were resolved by denaturing PAGE. For identification of the precise sites of alkylation, a Maxam−Gilbert G-reaction was performed by standard methods and loaded in an adjacent lane on the same gel. Bands were visualized by autoradiography.

Runoff in Vitro Transcription Analysis. For phage RNA polymerase transcription, a plasmid containing the triplex target site was linearized by restriction digestion downstream of the triplex target site. One microgram of plasmid was used as the template for T7 or Sp6 transcription, using an Ambion (Austin, TX) Maxiscript In vitro Transcription Kit according to the manufacturer's procedures. Triplex formation and UV irradiation were conducted in the transcription buffer (T7 system: 40 mM Tris-HCl, 8 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl, pH 8.0; Sp6 system: 40 mM Tris-HCl, 6 mM $MgCl_2$, 2 mM spermidine), and transcription was initiated by the addition of NTPs including [α-^{32}P]GTP and T7 or Sp6 RNA polymerase. Transcription reactions were incubated at 37 °C for 15 min, and the transcripts were resolved on denaturing PAGE gels followed by autoradiography.

For eukaryotic RNAP II runoff transcription analysis, an 851 base pair DraI fragment containing the CMV I/E promoter and 228 bp of coding sequence was purified from the pcDNA1.1/HNCx series of plasmids by agarose gel electrophoresis. Triplex reactions, including 100 ng of purified template and TFO (at the concentrations specified in the individual experiments), were incubated in HEPES−Mg buffer (25 mM HEPES, 7.5 mM $MgCl_2$, pH 7.9) in a 10 μL volume at 37 °C for 1 h (GA24tfo and GA23pso oligonucleotides) or for 4 h (oligonucleotide−alkylator conjugates). Triplex formation with GA23pso was followed by UV irradiation. The transcription reaction took place in a 25 μL volume in 10 mM HEPES, pH 7.9, 3 mM $MgCl_2$, 50 mM KCl, 0.1 mM EDTA, 10% glycerol, and 0.2 mM DTT. The reaction was initiated by adding NTPs including [α-^{32}P]GTP, and HeLa nuclear extract from the HeLaScribe in vitro transcription system from Promega according to the manufacturer's instruction. Transcription reactions were incubated for 60 min at 30 °C, and the transcripts were resolved by denaturing PAGE followed by autoradiography.

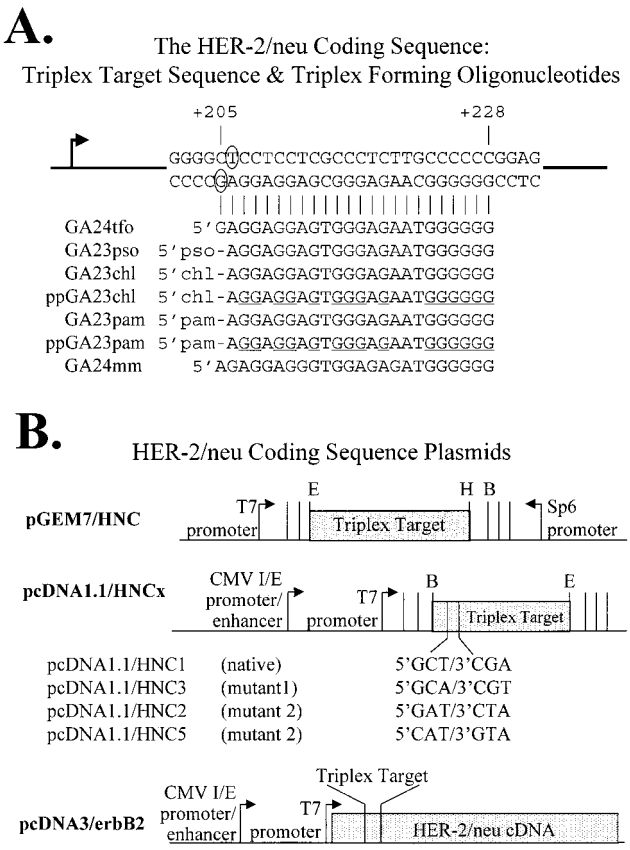

FIGURE 1: (A) Triplex target sequence in the human HER-2/neu coding sequence and triplex forming oligonucleotides. GA23pso photoreacts with thymine on the nontemplate strand at position +206 (circled). GA23chl reacts with guanine on the template strand at position +205 (circled). GA24mm is a control oligonucleotide with a scrambled sequence. (B) The triplex target sequence was cloned into several plasmid vectors for use in the in vitro transcription analysis. The pcDNA1.1/HNCx plasmids contain the triplex target with the native and mutated sequences indicated.

RESULTS

Triplex Target Region and Triplex-Forming Oligonucleotides. The regions of the HER-2/neu coding sequence containing the triplex target region and three derivative mutant sequences are illustrated in Figure 1. A nearly perfect homopurine−homopyrimidine tract at +205 to +228 was targeted for triplex formation with GA-motif TFOs that are antiparallel in orientation to the target purine strand (Figure 1A). The TFO designated as GA24tfo is a 24 base unmodified phosphodiester oligonucleotide designed to form reverse Hoogsteen G:G:C and A:A:T triplets with the target duplex. Thymines were aligned with the two C:G interruptions to potentially form T:C:G triplets (*24*). Triplex-directed covalent modification of the HER-2/neu target sequence was evaluated with a psoralen-modified oligonucleotide (GA23pso) and a chlorambucil conjugate (GA23chl). We also evaluated several other reactive oligonucleotide conjugates based on the design of the GA23chl oligonucleotide. Oligonucleotides were substituted at each guanine residue with the pyrazolopyrimidine analogue of guanine (ppG) to prevent the formation of potassium-induced G-quartets (ppGA23chl and ppGA23pam) (*6*). Oligonucleotides were also conjugated to phenylacetate mustard (GA23pam and ppGA23pam) (*22*),

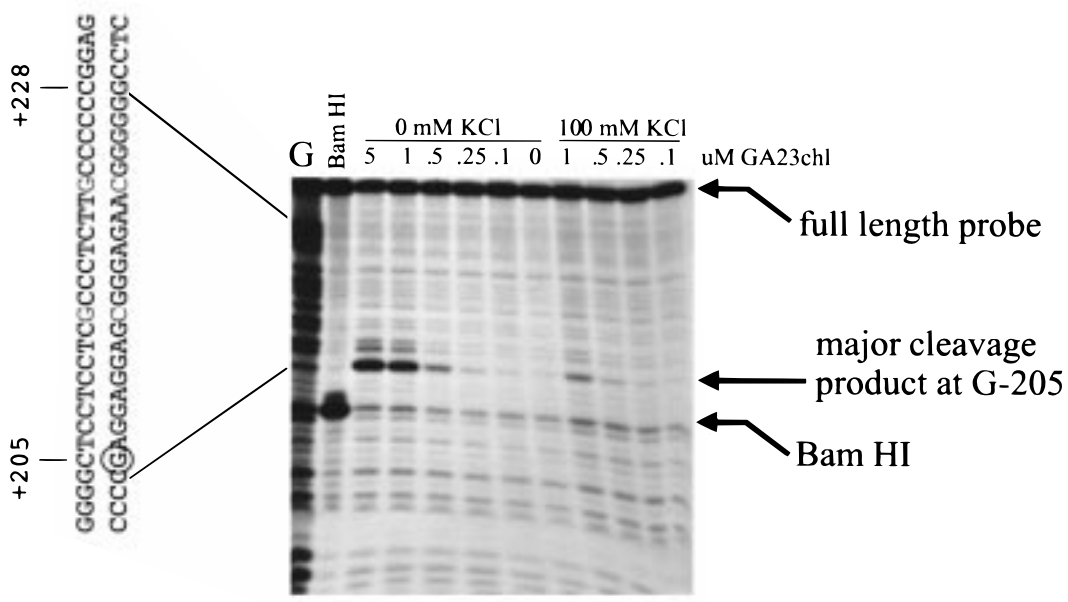

FIGURE 2: Triplex-directed alkylation of the triplex target site by the chlorambucil conjugate (GA23chl) was demonstrated by the piperidine cleavage reaction. A 57 bp *Hin*dIII/*Eco*RI fragment of the pcDNA/HNC1 plasmid was labeled on the bottom strand at the *Hin*dIII site. Triplex formation with GA23chl at the concentrations indicated took place in the presence or absence of 100 mM KCl. The site of the piperidine cleavage was identified by a Maxam−Gilbert G-reaction on the same labeled DNA substrate. A *Bam*HI digest near the beginning of the triple helix is also shown. The major cleavage reaction is at G+205.

a bifunctional chloroethylamine mustard which differs from chlorambucil by shortening the four-carbon chain of the benzenebutanoic acid group of chlorambucil to a two-carbon chain in the benzeneacetic acid group of phenylacetate mustard, conferring a slightly longer half-life in aqueous solution. A scrambled sequence oligonucleotide of identical base composition (GA24mm) and its 5′-psoralen conjugate (MM23pso, not shown) were included as controls for some experiments. The plasmid vectors constructed for the analysis of triplex formation and in vitro transcription are shown in Figure 1B.

Triplex Binding Studies. Gel mobility shift analysis was used to demonstrate the ability of the TFOs to bind to the HER-2/neu coding sequence. Triplex formation with GA24tfo occurs to near-completion at 37 °C in TBM, pH 7.4, at 100 nM concentration of the TFO. The apparent dissociation constant for GA24tfo is 32 nM. GA24mm did not interact with the target duplex at any concentration. Triplex formation with the unmodified TFO was also demonstrated by its ability to specifically protect the triplex target from digestion with the restriction endonuclease *Mnl*I (data not shown). Denaturing gel mobility shift analysis was used to evaluate the ability of psoralen oligonucleotides to form covalent photoadducts with the HER-2/neu coding sequence (data not shown). By labeling either the top (nontemplate) or the bottom (template) strands, monoadduct and cross-link formation involving the labeled strand was analyzed. GA23pso directed monoadduct formation with only the nontemplate strand of the native HER-2/neu coding sequence. In mutant 1, the T:A base pair at position +206 was inverted to an A:T base pair so that photoadduct formation would occur with the template strand only. In mutant 2, the C:G base pair at position +205 was changed to an A:T base pair, resulting in a 5′ApT step at the duplex−triplex junction that would be capable of forming an interstrand cross-link with the psoralen-modified oligonucleotide. Gel shift demonstrated that a monoadduct forms first with the bottom strand, and with the addition of further energy, an interstrand cross-link is formed with both the top and bottom strands.

The ability of the TFO−chlorambucil (TFO−chl) conjugate to bind to the triplex target and form a covalent adduct with the HER-2/neu coding sequence was evaluated in a similar manner with native and denaturing gel shift analysis. The base or bases alkylated by the chlorambucil may be precisely determined by converting the modified base to a single-strand break in the piperidine cleavage reaction (Figure 2). An *Eco*RI/*Hin*dIII fragment of the pcDNA1.1/HNC1 (native) plasmid containing the triplex target was labeled on the bottom strand. Triplex formation with various concentrations of GA23chl was followed by cleavage with piperidine and analysis on a sequencing gel with a Maxam−Gilbert G-reaction. It can be seen that the chlorambucil primarily alkylates the target guanine at +205 on the template strand. Minor reactions occur with guanines at +207 and +208 in the triple helix, but alkylation at other guanines is not observed. Identical reactions performed in the presence of 100 mM KCl demonstrate that triplex-directed DNA alkylation by the chlorambucil conjugate is markedly inhibited by the presence of potassium cation, probably due to a decreased ability of this GA-motif TFO to undergo triplex formation as discussed below. The specificity of triplex-directed DNA alkylation is well illustrated by this experiment. However, the alkylation efficiency approaches only approximately 50% of the labeled DNA substrate, and it requires a high concentration of TFO (5 μM) to achieve this degree of DNA alkylation. At lower concentrations of TFO, triplex-directed alkylation decreases markedly. We believe that this high concentration requirement is due to the kinetics of triplex formation relative to aziridinium cation formation by the chloroethylamine groups of the chlorambucil. At higher concentrations of TFO, triplex formation occurs more rapidly than the spontaneous formation of the aziridinium cation by the chlorambucil, leading to a reasonable (~50%) amount of alkylation. At lower concentrations, triplex

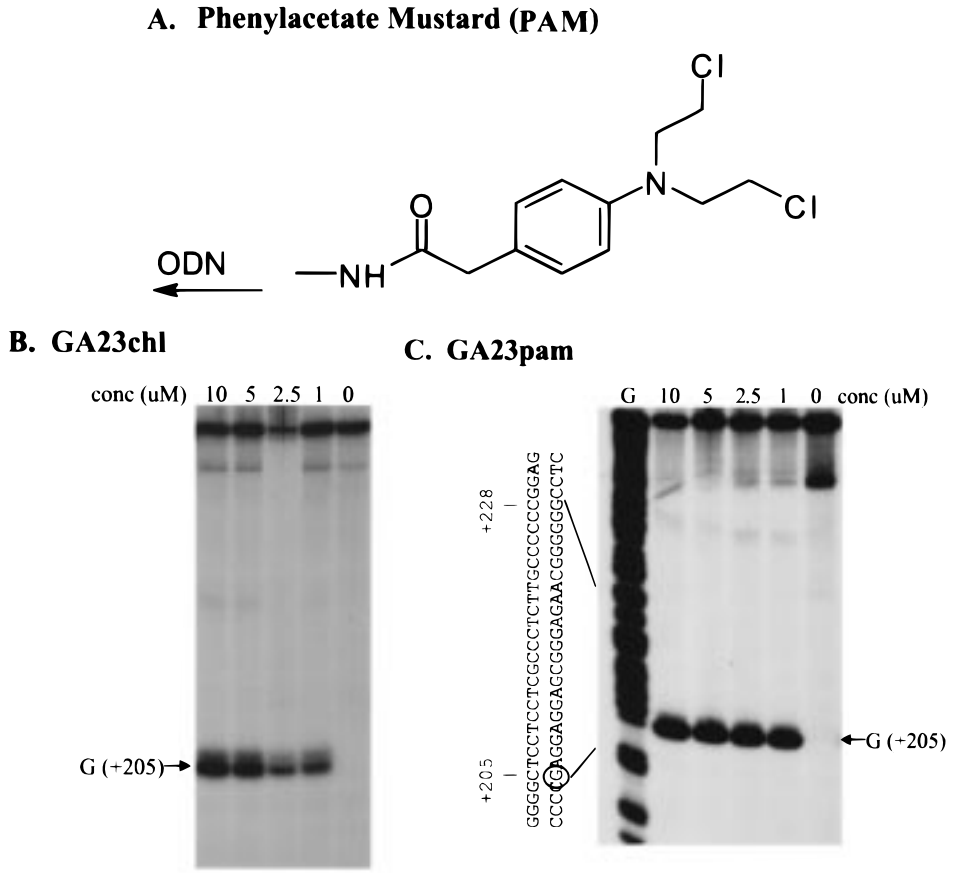

FIGURE 3: Concentration dependence of triplex-directed DNA alkylation with chlorambucil and phenylacetate mustard TFO conjugates was determined by piperidine cleavage. A 251 bp *Hin*dIII/*Eco*RV fragment of the pcDNA3/erbB2 plasmid was labeled on the bottom strand at the *Hin*dIII site. Approximately 5 ng was used in each reaction. (A) Structural formula of phenylacetate mustard, which is conjugated to the GA23 oligonucleotide by a C6 linker arm. (B and C) Piperidine cleavage analysis of triplex formation by GA23chl and GA23pam as a function of TFO concentration.

formation is slower than aziridinium cation formation, so that alkylation cannot be achieved by the time triplex formation occurs.

To further delineate the kinetics of triplex formation, we performed precisely timed triplex incubations with GA23pso at 37 °C, followed immediately by UV irradiation on ice for 10 min (data not shown). No triplex formation was observed while the reaction was on ice during the UV irradiation, so that the amount of monoadduct observed represents the amount of triplex formation that occurs during the 37 °C incubation. We found that the half-time for triplex formation at a 1 μM concentration of GA23pso and a 10 nM concentration of labeled target was approximately 10 min; at a GA23pso concentration of 100 nM, the half-time for triplex formation was approximately 40 min. It is therefore not surprising that there is a sharp concentration dependence observed with triplex-directed alkylation by chlorambucil, since the half-life of the reactive chloroethylamine groups of chlorambucil is approximately 25 min in aqueous solution (*22*). DNA alkylating agents with longer half-lives in aqueous solution would be expected to abrogate this problem. To this end, we have prepared GA23pam, a TFO conjugated to another nitrogen mustard, phenylacetate mustard (Figure 3A). Phenylacetate mustard undergoes spontaneous aziridinium cation formation with a half-time of approximately 55 min at 37 °C in aqueous solution (*22*). Piperidine cleavage reactions were performed comparing triplex-directed alkylation by GA23chl and GA23pam (Figure 3B,C). In contrast to the chlorambucil-conjugated TFO, with GA23pam there is no loss of triplex-directed alkylation over the concentration range of 10−1 μM.

Triplex formation by G-rich oligonucleotides is inhibited by high concentrations of potassium, which is the major intracellular monovalent cation; several modified bases have been substituted for guanine and evaluated for their ability to overcome potassium-mediated inhibition of triplex formation (*25*−*27*). We have evaluated a modified guanine base (8-aza-7-deazaguanine, the pyrazolopyrimidine analogue of guanine), which differs from guanine by switching the nitrogen at the N-7 position with the carbon at the C-8 position (*6*, *28*)(Figure 4A). In this way, self-association of guanine bases in the TFO is weakened by the loss of the hydrogen bond acceptor at the N-7 position, but Hoogsteen hydrogen bond formation is not affected. In native gel mobility shift analysis, triplex formation by guanine-containing alkylator conjugates (GA23chl and GA23pam at 1 μM final concentration) is markedly inhibited by the presence of 100 mM KCl (Figure 4B), while the oligonucleotides substituted at each guanine with the pyrazolopyrimidine guanine (ppGA23chl and ppGA23pam at 1 μM final concentration) have comparable binding in the presence or absence of KCl. Piperidine cleavage analysis demonstrates that triplex-directed alkylation by ppGA23chl is also similar in the presence or absence of 100 mM KCl (Figure 4C). This experiment also demonstrates that the efficiency of triplex-directed alkylation levels off at approximately 0.5 μM

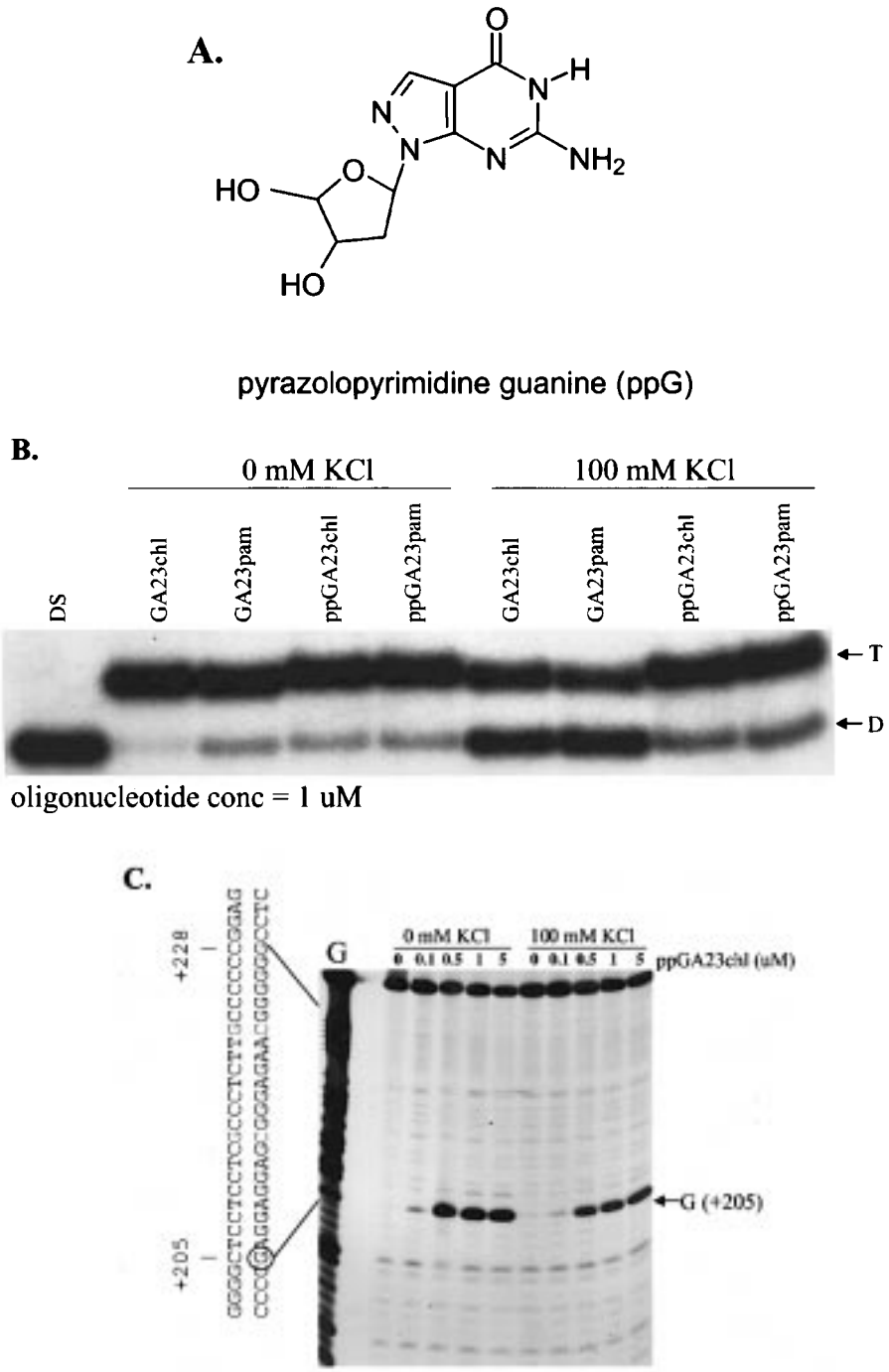

FIGURE 4: Triplex formation with the potassium-resistant base modification was evaluated by gel mobility shift and piperidine cleavage. (A) Structural formula of the pyrazolopyrimidine analogue of guanine. (B) Gel mobility shift assay of the TFO−alkylator conjugates in the presence or absence of 100 mM KCl. All oligonucleotides are at 1 μM final concentration. The 251 bp *Hin*dIII/*Eco*RV fragment containing the triplex target site is at 5 ng per reaction. (C) Triplex-directed DNA alkylation with the ppGA23chl oligonucleotide, as determined by the piperidine cleavage reaction.

ppGA23chl, suggesting that the ppG-substituted oligonucleotide has improved the kinetics (speed) of triplex formation as well.

In Vitro Transcription Analysis. To determine the ability of the TFO conjugates to inhibit transcription elongation, the triplex target sequence was cloned between the bacteriophage T7 and Sp6 promoters in the pGEM7/HNC plasmid (data not shown). In this way, run-off transcription with the T7 RNA polymerase will use the template (bottom) strand of the HER-2/neu triplex target as the template for RNA synthesis, while run-off transcription with Sp6 RNA polymerase will use the nontemplate (top) strand of the same triplex target. Triplex formation in the pGEM7/HNC plasmid with GA24tfo, GA23pso, and PYpso (a pyrimidine rich non-triplex-forming oligonucleotide−psoralen conjugate used as a control for the presence of a psoralenated oligonucleotide) was followed by UV irradiation and run-off transcription with T7 or Sp6 RNA polymerase. For T7 transcription, the pGEM7/HNC template was linearized with *Bam*HI so that run-off transcripts of 91 nucleotides (nt) are expected. Triplex formation with the unmodified TFO, GA24tfo, had no effect on T7 transcription, and all of the run-off transcripts were

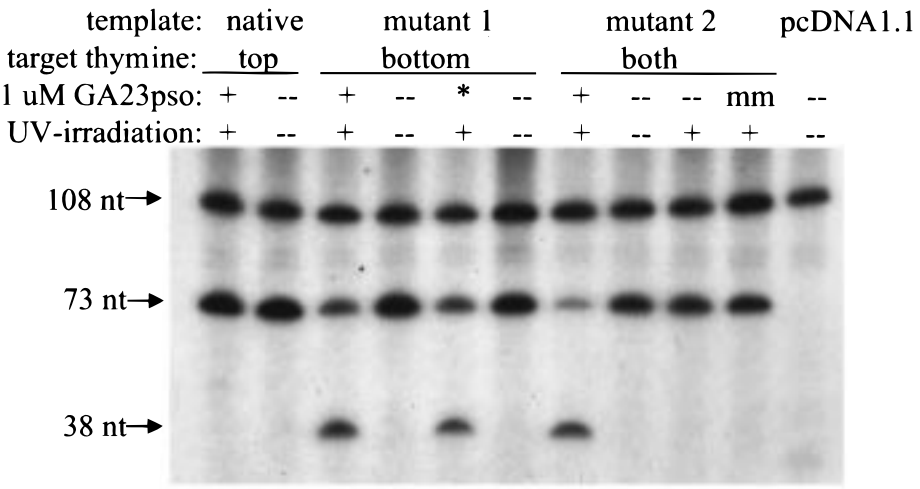

FIGURE 5: Inhibition of T7 transcription elongation resulting from psoralen photoadduct formation with the transcription template strand. The pcDNA1.1/HNCx plasmids were linearized with *Eco*RI so that run-off transcription would yield a 73 nt transcript. The identical plasmid backbone (pcDNA1.1) without a triplex target was linearized with *Xba*I to yield a 108 nt run-off transcript; 1 μg of each linearized plasmid template was included in each transcription reaction, so that the pcDNA1.1 template would serve as an internal control for non-template-specific effects of the oligonucleotides and UV irradiation on the transcription reaction. The plasmids were treated with GA23pso (1 μM) plus UV irradiation (10 min) where indicated. Treatments with UV irradiation alone and MM23pso plus UV irradiation were included as controls. Monoadduct formation with thymine on the bottom strand in mutant 1 and cross-link formation with thymines on both strands in mutant 2 lead to mostly truncated transcripts at 38 nt. (The asterisk indicates treatment with GA22pso, a 22 base TFO−5′-psoralen conjugate that was used to evaluate monoadduct formation by positioning the psoralen on the 5′ rather than the 3′ side of the target thymine on the template strand.)

full-length. Likewise, full-length transcripts were observed when the pGEM7/HNC plasmid was incubated with the psoralen control oligonucleotide plus UV radiation or with the GA23pso oligonucleotide in the absence of UV radiation. With this template, transcripts of 53 nt are expected if transcription is arrested at the site of psoralen photoadduct formation. When the plasmid is incubated with GA23pso followed by UV radiation, a few truncated transcripts of the expected size are seen, but >95% of the transcripts are full-length. In contrast, marked inhibition of Sp6 transcription elongation was observed after psoralen photoadduct formation with thymine +206. For these reactions, the pGEM7/HNC plasmid was linearized with *Xba*I so that full-length transcripts of 101 nt are expected. Truncation of transcription at thymine +206 will yield transcripts of 79 nt. Sp6 run-off transcription of the *Eco*RI-digested plasmid yields an 89 nt transcript and was used as a size marker. Again, treatment with GA24tfo, PYpso + UV, and GA23pso without UV irradiation had no effect on the size of the transcripts. However, triplex-directed photoadduct formation by GA23pso + UV yielded a large proportion (~80%) of truncated 79 nt transcripts. Thus, the transcripts appear to be truncated at the target thymine near the *Eco*RI site rather than at the beginning of the triplex helix. These experiments suggest that transcription elongation is not inhibited by triplex formation itself, but rather by the site-specific and strand-specific covalent DNA adduct formed by the psoralen.

To further demonstrate the need for covalent adduct formation with the template strand, T7 run-off transcription analysis was performed with the native and mutant HER-2/neu triplex targets (Figure 5). For these assays, the pcDNA1.1/HNCx plasmids were linearized downstream of the triplex target sequence with *Eco*RI. Full-length run-off transcripts at the *Eco*RI site would be 73 nt, and transcripts truncated at a photoadduct formed at the duplex−triplex junction would be 38 nt. In addition, the plasmid backbone (pcDNA1.1) without a triplex target was linearized with *Xba*I so that run-off transcripts would be 108 nt in length; this template was included in each reaction as a control to exclude any non-target-specific effects from the presence of TFO−pso and UV irradiation. Figure 5 demonstrates nearly equivalent band intensity in all lanes for the 108 nt control transcript. In contrast, the transcription reactions with mutant 1 and mutant 2 treated with GA23pso and UV irradiation demonstrate a marked decrease in full-length transcripts with the appearance of a 38 nt truncated transcript, indicating that transcription elongation through the covalent photoadduct on the template strand is prevented. It is apparent that cross-link formation with mutant 2 is a more efficient inhibitor of transcription elongation than monoadduct formation with mutant 1. The slightly lower inhibitory effect on transcription elongation observed with monoadduct formation with the bottom strand may also be due to a slightly lower efficiency of photoadduct formation with the target thymine resulting from an imperfect base triplet (A:T:A instead of A:A:T) introduced by the A:T to T:A mutation. In this experiment, monoadduct formation with the native HER-2/neu coding sequence, which contains a target thymine base only on the nontemplate strand, did not lead to any truncated transcripts. These data demonstrate that covalent adduct formation with the transcription template strand is necessary for triplex-mediated inhibition of bacteriophage RNA polymerase elongation.

Triplex-directed covalent modification also inhibited transcription elongation by eukaryotic RNAP II (Figure 6). For these studies, the pcDNA1.1/HNC1 vector was digested with *Dra*I, and the DNA fragment containing the CMV I/E promoter with 228 bp of plasmid downstream of the transcription start site was isolated. RNAP II transcription was performed using a HeLa nuclear extract, so that labeled run-off transcripts of 228 nucleotides would be generated. The pcDNA1.1/HNC1 vector contains the HER-2/neu triplex

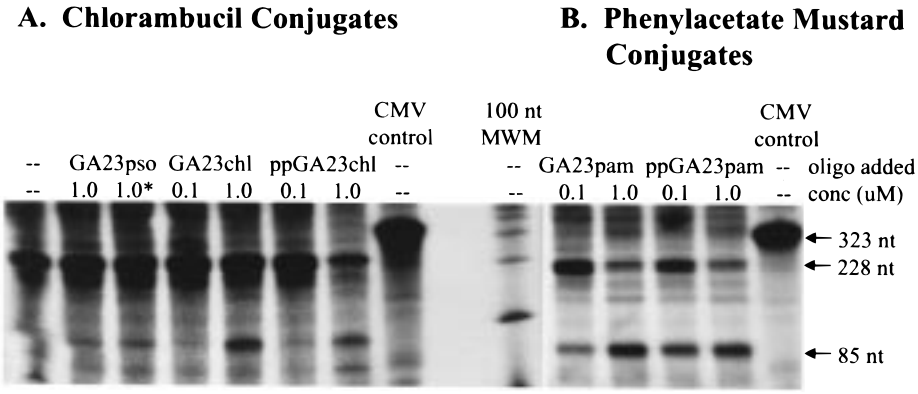

FIGURE 6: Eukaryotic transcription elongation through the native HER-2/neu coding sequence is inhibited by covalent adduct formation with the TFO−alkylator conjugates. (A) The *Dra*I fragment of the pcDNA/HNC1 plasmid was treated with GA23pso (the asterisk indicates UV irradiation), GA23chl, and ppGA23chl at the concentrations indicated and then used as a substrate for in vitro transcription with HeLa nuclear extract. (B) The same *Dra*I fragment was treated with the phenylacetate mustard conjugates, GA23pam and ppGA23pam, followed by in vitro transcription with HeLa nuclear extract. MWM, molecular weight marker.

target sequence beginning 85 bp downstream of the CMV transcription start site, so that triplex-directed covalent adduct formation with thymine +206 or guanine at +205 would yield run-off transcripts of 85−86 nucleotides. Inhibition of transcription elongation was very inefficient with GA23pso with or without UV irradiation whereas the chlorambucil conjugates yielded a concentration-dependent inhibition of RNAP II transcription elongation (Figure 6A). It can be seen that GA23chl at a final concentration of 1 μM leads to a small number of truncated transcripts relative to full-length transcripts. The amount of transcription inhibition correlates roughly with the degree of alkylation of G+205 seen at this same concentration in the piperidine cleavage reactions of Figures 2 and 3. The phenylacetate mustard conjugates inhibited RNAP II transcription elongation to a much greater extent, again in a concentration-dependent manner (Figure 6B). Partial inhibition of transcription elongation was demonstrated at 0.1 μM, while nearly complete inhibition was observed with a 1 μM concentration of TFO. Nearly all of the transcripts were truncated at 85 nt, indicating transcription arrest by the guanine adduct. Again, the degree of transcriptional inhibition correlates roughly with the higher degree of triplex-directed alkylation seen at these concentrations in the piperidine cleavage reactions (Figures 3 and 4). The ppG base modification also enhanced the ability of the oligonucleotide−alkylator conjugates (ppGA23chl and ppGA23pam versus GA23chl and GA23pam) to inhibit transcription when triplex formation in potassium-containing buffers preceded the in vitro transcription reaction (not shown). Thus, in both phage polymerase and eukaryotic polymerase transcription, triplex-mediated inhibition of transcription elongation through the HER-2/neu coding sequence required the formation of a covalent adduct with the template strand, and the degree of transcriptional inhibition correlated directly with the amount of adduct formed.

DISCUSSION

The overall objective of this work was to develop an antigene TFO that would be capable of inhibiting the expression of the HER-2/neu gene. The choice of a target in the coding sequence was based on considerations of target site accessibility and expansion of targeting to include the transcribed regions of the genome. Since the TFOs targeted to the HER-2/neu promoter region may need to displace previously bound transcription factors to be effective, triplex formation in the coding sequence may allow improved accessibility, particularly in cells in which the HER-2/neu gene is actively transcribed.

Triplex formation in the coding sequence of several genes has been described. In general, triplex formation by unmodified phosphodiester oligonucleotides has not been capable of inhibiting transcription elongation in vitro, likely due to displacement of the TFO by the RNA polymerase (*16*, *29*). Young and colleagues first demonstrated that triplex formation in the transcribed sequence of a G-free cassette induced a transient block in transcription elongation (*16*). Skoog and Maher demonstrated that triplex-mediated inhibition of an artificial bacteriophage promoter was disrupted by transcription of either strand of the triplex target, suggesting that the triple helix forming oligonucleotide was displaced by the elongating RNA polymerase (*29*). The work of Duval−Valentin corroborated and expanded these findings, demonstrating that a temporary and partial block of *E. coli* RNA polymerase was induced by a triple helix downstream of the promoter (*17*). This partial, temporary inhibition of transcription elongation was dependent on the temperature used in the in vitro transcription reaction as well as the reaction time, indicating that the stability of the triple helix is a critical factor in preventing the progression of RNA polymerase.

Oligonucleotides conjugated to psoralen have been previously investigated as antigene agents to enhance the ability of TFOs to inhibit transcription elongation (*17*, *23*, *30*). In general, these studies have made use of bis-adduct formation with triplex target sequences adjacent to 5′TpA/3′ApT steps. Since the HER-2/neu gene does not contain a triplex target sequence adjacent to such a step, we originally hypothesized that triplex formation stabilized by intercalation of the psoralen at the duplex/triplex junction and monoadduct formation, even with the nontemplate strand, would possibly inhibit transcription elongation. However, our results indicate that transcription elongation by both bacteriophage polymerases and eukaryotic RNAP II is efficiently inhibited only by covalent adduct formation with the template strand. More recent work with N3′→P5′ phosphoramidate TFOs (*18*) and TFO−acridine conjugates (*19*) has demonstrated that a triple helix stabilized by the improved binding characteristics of

the phosphoramidate linkage or the intercalated acridine is capable of inhibiting transcription elongation in vitro. The ability of these oligonucleotides to inhibit transcription elongation was strongly dependent on the stability of the triple helix, and the percentage of truncated transcripts observed with acridine intercalation was highly dependent on the temperature of the in vitro transcription reaction. Our results are in broad agreement with these observations, in that an unmodified TFO was incapable of inhibiting transcription elongation. We performed our transcription reactions as shown here under conditions of time and temperature that were optimized for in vitro transcription. Over a temperature range of 20−37 °C, we observed no difference in the ability of the unmodified triplex-forming oligonucleotide or the psoralen conjugate to prevent T7 RNA polymerase elongation. In addition, when the T7 transcription reactions were terminated after very short times over a range from 30 s to 10 min, no differences were observed in the presence or absence of the TFO. We suggest that the inability of the unmodified TFO to inhibit transcription elongation is due to its displacement by the elongating RNA polymerase, a conclusion that is supported by our findings with Sp6 transcription in which elongation is terminated at the site of covalent adduct formation rather than at the beginning of the triple helix.

TFOs coupled to DNA-modifying drugs have also been previously described (*6*, *16*, *22*, *31*−*36*). Site-specific targeting of the drug has been possible, and demonstration of biological effects using this strategy has included the ability of N^4,N^4-ethano-2′-deoxycytidine-containing oligonucleotides to inhibit RNA polymerase elongation in vitro in a G-free cassette (*16*), and the ability of 4-(3-aminopropyl)-(*N*-2-chloroethyl-*N*-methyl)aniline-conjugated oligonucleotides to suppress gene expression from the *c-fos* promoter in a reporter plasmid (*31*). Moreover, Lampe and co-workers suggest that chlorambucil in a TFO−chl conjugate lacks sufficient DNA binding affinity to interact on its own, and their data indicate that the initial binding of an oligonucleotide−chlorambucil conjugate to DNA is mediated by the TFO with little or no contribution from the chlorambucil (*36*). Our results are in agreement with those of Lampe and colleagues. We observed minor reactivity of the TFO−alkylator conjugates with guanines adjacent to the target guanine and internal to the triple helix (G+205 was the target, and minor reactions were observed with G+207 and G+208), but we observed no reactivity of the chlorambucil at any other sites in the plasmid vectors, even when micromolar concentrations of the oligonucleotide−alkylator conjugates were used.

The ability of the TFO−alkylator conjugates to inhibit transcription elongation appears to depend directly on the formation of a covalent adduct with the template strand, and factors which affect the efficiency of alkylation have the greatest effects on inhibiting transcription elongation. We found that the most important factor is the rapidity of triple helix formation relative to the half-life of the alkylating agent in aqueous solution. Indeed, although triplex formation by unmodified oligonucleotides is demonstrated at less than 10^{-7} M oligonucleotide, the rate of triplex formation at this concentration is relatively slow, and efficient triplex-directed alkylation generally required oligonucleotide−alkylator conjugate concentrations of 10^{-6} M or greater. Efficient inhibition of transcription elongation was also observed at oligonucleotide−alkylator conjugate concentrations of 10^{-6} M or greater. The use of a somewhat more stable alkylating agent (phenylacetate mustard as opposed to chlorambucil) improved the alkylation efficiency. If the time required for the oligonucleotide to reach the target sequence in the nucleus of a living cell is considered, an even more stable alkylating agent may be required. Another obstacle to triplex formation in living cells is the high intracellular concentration of potassium. We demonstrated a significant improvement in the ability of the TFO−alkylator conjugates to resist the inhibitory effects of potassium ion on triplex formation, triplex-directed DNA alkylation, and inhibition of in vitro transcription by using the pyrazolopyrimidine guanine base modification.

These studies provide a foundation for the rational design of an antigene TFO to inhibit gene expression. This work demonstrates that the coding sequence of a gene remains a potential target for triplex formation, and including the coding sequence will expand the number of triplex targets available in genes of interest. This work also suggests that in the absence of other triplex-stabilizing strategies, an effective TFO inhibitor of transcription elongation should covalently react with the transcription template strand.

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