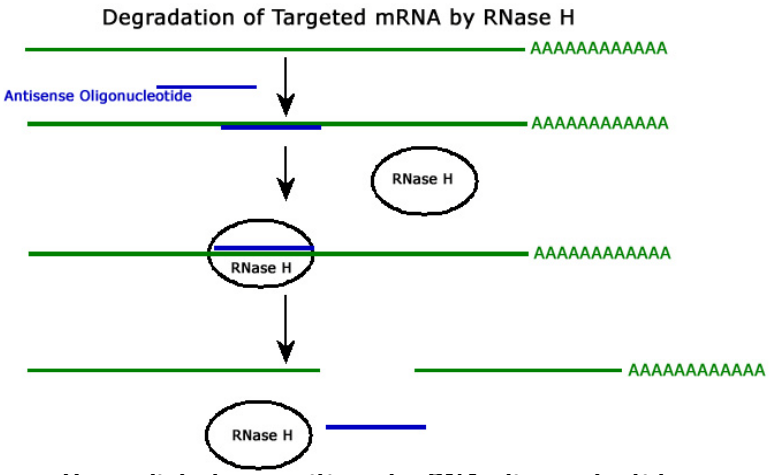
INTEGRATED DNA TECHNOLOGIES

Designing Antisense Oligonucleotides

Contents

Introduction

Antisense oligonucleotides have been used for a number of years to modify the expression of specific genes both *in vivo* and *in vitro* [1]. The most potent mode of antisense activity is through RNase H–mediated degradation of RNA (Figure 1). The RNase H–endonuclease specifically cleaves RNA only when it is hybridized as a heteroduplex with DNA. In general, oligonucleotides that cannot invoke RNase H pathways are 10–100 fold less potent than RNase H–activating oligonucleotides of identical sequence.



H– mediated recognition of mRNA:oligonucleotide hybrid.

Ideally, any researcher should be able to choose a specific target sequence of interest, order the synthesis of their designer antisense oligonucleotide, introduce it into their system of choice, and observe the effects. However, in practice, things are not this simple. The choice of sequence and chemistry of the antisense oligonucleotide is crucial to the success of the experiment [2].

Problem: Natural phosphodiester oligodeoxynucleotides (or oligoribonucleotides) are quickly digested by nucleases both *in vitro* and *in vivo*. Although multiple endo- and exonucleases exist and may be important *in vivo* [3], it appears that the bulk of biologically significant nucleolytic

activity in serum is seen as a 3' exonuclease [4] (Figure 2). Within the cell nucleolytic activity is seen as both 5’ and 5’ exonuclease [5] (Figure 3).

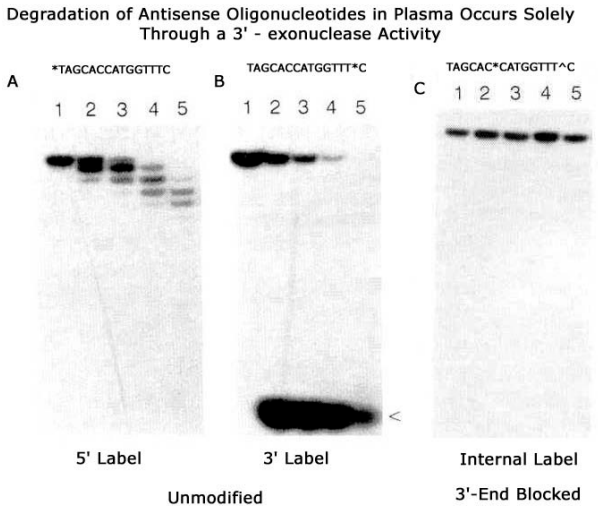


Figure 2. Substrate specificity and kinetics of degradation of antisense oligonucleotides by 3' exonuclease in plasma. 1=control, 2=15 minutes, 3=30 minutes, 4=1 hour, 5=2 hours, *=32P, ^=TCDM phosphotriester. Adapted from [4].

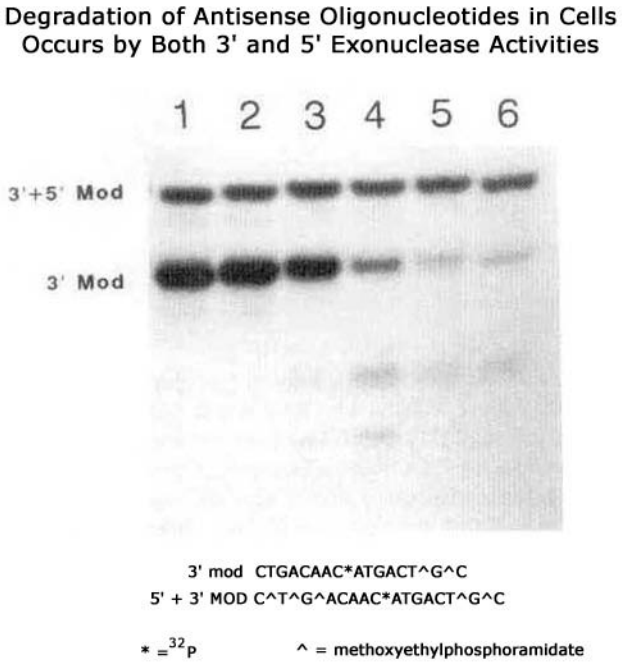


Figure 3. Pathways of degradation and mechanism of action of antisense oligonucleotides in *Xenopus laevis* embryos. 1 = control, 2 = 1 minute, 3 = 10 minutes, 4 = 20 minutes, 5 = 40 minutes, 6 = 60 minutes. Oligonucleotides modified at both the 3’ and 5’ ends by methoxyethylphosphoramidate are protected from exonuclease digestion. Oligonucleotides modified at only the 3’ end are digested by a 5’ exonuclease. Adapted from [5].

Solution: Synthetic antisense oligonucleotide probes differ from natural DNA or RNA in that they are modified to some form that is nuclease-resistant. Many such modifications have been conceived and tested, few of which work as well as intended. Ideally, these compounds should:

- Activate RNase H–degradation pathways.
- Be easy to make and inexpensive.
- Not be physiologically toxic.
- Not be easily degraded.
- Not disrupt normal Watson–Crick base-pairing.
- Not induce any unanticipated sequence-independent biologic effects.

Choosing a modified oligonucleotide

Many different modifications have been substituted by investigators into the native phosphodiester oligodeoxynucleotide polymer to limit nuclease sensitivity in an effort to enhance their utility as antisense agents. Short oligos have very brief intracellular half-lives regardless of modifications, but modified oligos with a length of 16 or so residues can have half-lives on the order of days [3]. Residue modifications can also affect the ability of an oligo to trigger RNase H mediated degradation of RNA following hybrid formation. As RNase H mediated destruction of RNA may be the primary mode of antisense action, this effect cannot be ignored. Human RNase H cleaves RNA in RNA:DNA hybrids. The enzyme requires divalent cations to be active. Peak activity occurs in the presence of 10mM Mg^{++}, 5mM Co^{++}, or 0.5mM Mn^{++}. Action of the enzyme leaves a 5'-phosphate and a 3'-hydroxyl [4].

Summary of Candidate Compounds for Potential
Use as Antisense Oligonucleotide Probes

Chemistry	Resistance To Nucleases	Activation of RNase H pathways	Comments
Phosphodiester	NO	YES	Natural DNA
Methylphosphonate.	YES	NO	Will work as chimera
Phosphorothioate	YES	YES	Stimulates many biologic responses in a sequence non-specific fashion
α-nucleoside	YES	NO	Will work as αβα chimera
2'-*O*-substituted RNA	YES	NO	Greater stability than DNA:RNA hybrids Will work as chimera
Phosphoramidite	YES	NO	Will work as chimera
Morpholino	YES	NO	Will work as steric blocker
Chimeras	YES	YES	Contain an internal core of unmodified phosphodiester DNA flanked by modified residues

Ribonuclease H–Mediated Antisense Activity

The RNase H class of endonucleases acts primarily in the nucleus, although activity can de detected in cytoplasm [6]. It is thought that the antisense oligonucleotide probe binds specifically to target mRNA, which initiates RNase H–mediated degradation of the double-

stranded antisense probe:mRNA hybrid [5] (Figure 5). RNA degradation products corresponding to the fragments expected from RNase H action can be detected in living cells treated with antisense agents [7]. The precise mechanism of action is not known but it has been proposed to be similar to that of DNase I [8]. Most DNAs that have been modified to be nuclease-resistant do not form a heteroduplex structure. One exception is the phosphorothioate-modified oligonucleotides discussed below.

Human RNase H enzymes will completely digest RNA in heteroduplex form and have optimal activity when the gap between phosphorothioate linked bases and phosphodiester linked bases is at least 6–8 residues [9]. However, when enzyme levels are limiting, it becomes clear that certain sites cleave preferentially [9, 10, 11, 12]. Cleavage rates of a single ribonucleotide residue embedded in DNA proceeds at A > U > C > G; the rate of cleavage at rA is over four times faster than at an rG [4] (Figure 3). Further, the RNA secondary and tertiary structures may present certain sites as topologically favored for enzymatic attack. The scientist must be certain that the target sequence is accessible within the folded RNA structure when designing antisense compounds.

Phosphorothioates

The easy-to-synthesize phosphorothioate oligonucleotides (Figure 4) assume the native Watson–Crick nucleotide hydrogen-bonding patterns, can activate RNase H–mediated degradation of cellular mRNA, and are nuclease-resistant [13, 14, 15]. The antisense effects of the phosphorothioates can be observed for over 48 hours after a single application to tissue culture cells [16]. This degree of stability is needed for *in vivo* work [17]. However, the actual stability of a phosphoro-thioate oligonucleotide in a specific experiment can vary with each sequence and cell line examined [18]. Although early work using these compounds was very encouraging, it has become clear that some of the most exciting results were actually due to sequence independent biological effects of phosphorothioate DNA (sulfated polyanion) and did not result from true antisense mechanisms [19, 20].

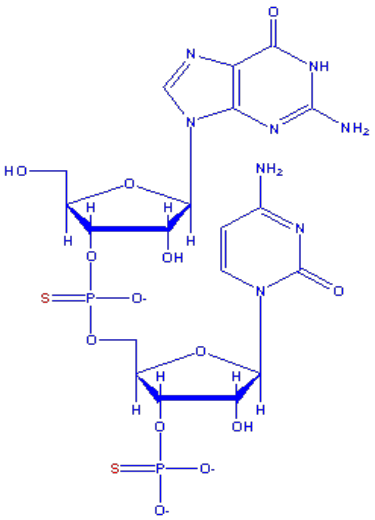


Figure 4. Phosphorothioate Oligonucleotide (GC)

The synthesis of chimeric oligos having modified 3'- and 5'- ends with a normal phosphodiester central core has been employed using phosphoramidites and methylphosphonates with good results. Similar chimeras have been evaluated for phosphorothioate-modified oligos in an attempt to lower the total thiol-modified content of the oligo and decrease side-effects/toxicity. Leaving a central core of as few as 3 phosphodiester linkages leaves the oligo relatively unprotected from *Bal*31 nuclease digestion [21]. Such end-blocked oligos may still be protected sufficiently to be useful in practice, however, as the predominant activity that destroys oligos in serum is a 3'-exonuclease. Chimeric structure has been shown to have decreased side effect profile *in vivo* [22, 23]. In spite of some disadvantages, phosphorothioates remain the most popular agent used in antisense studies. When they work, they work well and are cost effective

The phosphorothioate modification slightly decreases the relative binding affinity of an oligo, similar to most other phosphate modifiers, such as phosphoramidites or methylphosphonates. Other changes can be introduced into such oligos to increase their relative binding affinities and compensate for this decrease in T_m. The addition of C-5 propyne pyrimidines into phosphorothioate oligos can increase their binding affinity and thereby their potency such that they become effective in the 100nM range. This shift in the dose response curves might lower the effective dosage of these agents to the point that worries over toxic, non-specific side effects will disappear [16, 24, 25]. The use of C-5 propyne-modified oligos allow for a decrease in oligo length. Oligos as short as 11 bases can have potent antisense effects [26]. A similar enhancement can be achieved using C-7 modified purines - 7-deaza-2'-deoxyguanosine and 7-deaza-2'-deoxyadenosine [27]. Further improvement in oligo T_m and nuclease stability can be achieved through the combined use of both 2'-*O*-modification plus thiolation [28].

Like all mono-substituted phosphate backbone modifiers, phosphorothioates are chiral. Chirally pure phosphorothioates can be synthesized. Pure Rp configuration oligos have higher binding affinity (higher T_m) than pure Sp or mixed congeners. The Rp species was found to have increased RNase-H-activating potential than Sp or chirally mixed oligos [29].

2' Substitutions

Substitutions at the ribose 2' position can significantly alter an oligo's nuclease stability and binding properties [30]. Hundreds of such modifications have been synthesized and tested for activity, including 2'-fluoro, 2'-*O*-methyl, 2'-*O*-allyl, 2'-*O*-propyl, and 2'-*O*-pentyl. These agents confer varying degrees of nuclease stability to an oligo. 2'-*O*-allyl analogs have a life span in serum of about 24 hours. 2'-*O*-methyl analogs (Figure 5) are some 10 fold less stable than the allyl class but are nonetheless more stable than native phosphodiester oligos [3, 31]. This degree of stability is not sufficient for most antisense applications. Oligos of this class do not activate RNase H [32], but can have antisense effects through a steric blocking mode of action. 2'-*O*-alkyl oligoribonucleotides have unambiguously been shown to be capable of inhibiting mRNA translation through steric effects *in vitro* [33]. The introduction of substitutions that increase binding affinity can enhance steric antisense effects. A C-5 propyne-pyrimidine 2'-*O*-allyl oligo was described which had a T_m of >90°C for its target RNA. This construct showed antisense effect after microinjection into CV1 cells with an IC_{50} of 5 µM. However, an RNase H-activating C-5 propyne phosphorothioate oligo of identical sequence had a T_m of only 79°C but showed an IC_{50} of 0.25 µM, a 20 fold increase in antisense potency [25]. RNase H activating capacity can be introduced into 2'-substituted oligos through the construction of 2'-modified / 2'-deoxy gap chimeras [34]. These chimeras can also be thioated to enhance nuclease resistance [28].

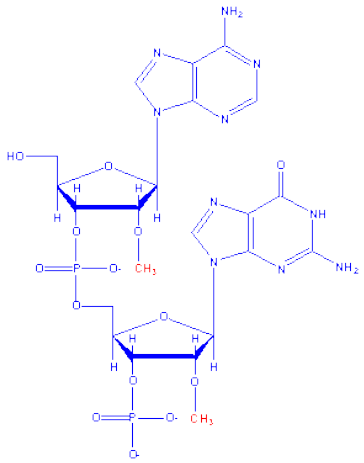


Figure 5. 2'-O-methyl oligonucleotide (AG)

Oligo-2'-fluoro-2'-deoxynucleotide phosphoramidites have been synthesized and these oligos appear to offer the best increase of T_m for this class of compounds [35]. Unfortunately, the addition of bulkier groups at the 2'-position is needed to confer nuclease resistance but these bulkier groups provide less benefit in improved binding affinities. The relative binding affinity of these compounds is: fluoro > methoxy > propoxy > pentoxy = deoxy. Conversely, the relative nuclease resistance of these compounds is: pentoxy > propoxy > methoxy > fluoro = deoxy [21]. Engineering a self-complementary hairpin onto the 3' -end can increase nuclease resistance of these compounds [36].

Griffey et al. [37] report the synthesis of a 2'-O-aminopropyl modified oligos.This substitution was more nuclease resistant than phosphorothioates and had slightly increased binding affinity compared to the standard phosphodiester oligo. "Gapmers", incorporating 2'-modified flanks with a phosphorothioate core, were synthesized and tested for antisense activity. The new constructs had 5-10 fold more activity (in tissue culture) than an otherwise identical homogenous phosphorothioate compound. One unusual 2'-substitution has recently been described. (2'*S*)-2'-deoxy-2'-C-methyl oligonucleotides are resistant to *Bal*31, P1, and mung bean nucleases and are stable in serum [38]. Relative binding affinities and potential for RNase H activation are not known. These compounds are now considered the reagents of choice for "second generation" compounds (Figure 6).

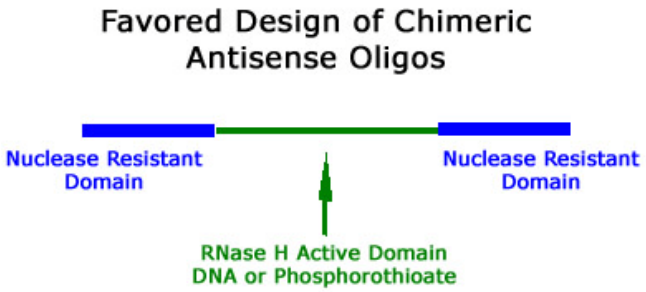


Figure 6. Recommended Chimeric Antisense Oligonucleotide Design

Phosphorothioates show increased binding to cellular proteins and components of the extracellular matrix as compared to natural phosphodiester oligonucleotides [19, 20]. This binding appears to be due to the polyanionic nature of these compounds; they behave similar to dextran sulfate and heparin sulfate. This binding can displace or mimic the binding of natural ligands to assorted proteins, such as receptors or adhesion molecules [39]. In fact, any of the heparin-binding class of proteins may also bind phosphorothioates [40, 41]. Phosphodiester DNA is a polyanion and may nonspecifically bind proteins, but due to nuclease action has such a shortened lifespan that the impact of this effect is most likely limited.

Chimeric oligonucleotides have been synthesized with phosphoramidite-modified 3'- and 5'-ends with a normal phosphodiester central core (Figure 7). Leaving a central core of as few as 3 phosphodiester linkages leaves the oligonucleotide relatively unprotected from *Bal*31 nuclease digestion [21]. However, since the predominant activity that destroys

oligonucleotides in serum is a 3'-exonuclease, end-blocked oligonucleotides are protected sufficiently to be useful in practice. Chimeric structure has been shown to decrease side effects *in vivo* [22, 23].

Lane	Oligo		Gap	Degradation
1-4	Controls		-	No
5	An2-8	C*T*G*$A_0C_0A_0A_0C_0A_0T_0G_0$A*C*T*G*C	3-8-4	Yes
6	An2-6	C*T*G*$A_0C_0A_0A_0C_0A_0T_0$G*A*C*T*G*C	4-6-5	Yes
7	An2-5	C*T*G*A*$C_0A_0A_0C_0A_0T_0$G*A*C*T*G*C	5-5-5	No
8	An2-4	C*T*G*A*C*$A_0A_0C_0A_0T_0$G*A*C*T*G*C	5-4-6	No

Figure 7. Pathways of degradation and mechanism of action of antiense oligonucleotides in *Xenopus Laevis* embryos. * Phosphorothioate internucleotide linkage, 0 phosphodiester internucleotide linkage. Central core of 6-8 phosphodiester linkages allow degradation by RNase H (Adapted from [5]).

Target-Site Selection

Finding the right target site may be easy or the most difficult step in the whole antisense process. It ultimately determines the success of any antisense investigation. This section discusses some of the things that can cause problems. Lest these appear daunting or even overwhelming, it should be pointed out that scientists at companies such as Isis and Sequitur claim that they achieve close to 100% success in finding useful antisense oligonucleotides for given targets.

Some investigators have found that about one out of ten phosphorothioate 20-mers will exhibit good activity and that this can be improved using superior chemical modifications [42]. If the oligomers are randomly selected, only a small fraction may be active [43, 44]. Sometimes only a single oligonucleotide, most often one complementary to the initiator AUG, will inhibit expression of a particular RNA. Obviously, testing as many oligonucleotides as possible improves the chance of identifying one that is particularly potent. This can be expedited by monitoring gene expression using RT-PCR in a 96-well format or some other high-throughput procedure such as micro-array technology [42].

Length. The stability of hybrids can depend on length, particularly for shorter oligonucleotides. Also, as the length of an oligonucleotide increases, it is less likely that it will encounter a complementary sequence other than the targeted RNA. On the other hand, increasing the length of the oligonucleotide increases the probability that it will bind a partially complementary sequence in a nontarget message, thereby activating RNase H, which requires only six or seven base pairs in a heteroduplex substrate for activation. The usual length for antisense oligonucleotides is around 20 bases, which is a convenient size for synthesis and long enough, on statistical grounds, to be unique in the human genome. In special cases, structural features in the target RNA may enable the use of shorter oligomers [24, 45]. However, most studies find a decrease or loss of antisense activity as length is reduced from twenty to ten bases [46].

Conformational and thermodynamic considerations. The major problem lies with the secondary and tertiary folding that can make much of the RNA inaccessible to a molecule as large as an oligonucleotide. Even those sequences that appear to be accessible may already be involved in intramolecular hydrogen bonding, stacking interactions, or in solvation that would be disrupted by hybridization of an oligonucleotide. Consequently, hybridization-induced rearrangement of the existing RNA structure may carry a prohibitive thermodynamic penalty. On the other hand, single-stranded sequences within the RNA may be preordered by stacking into helical conformations that are particularly favorable for hybridization. The exceptional stability of hybrids formed between the loops of two hairpins (kissing interactions) is well known and is important in the association of natural antisense RNAs with their targets. Factors such as these may account for the preferential hybridization of oligonucleotides to the 5' rather than 3' side of loops [47, 48, 49]. The difficulty of predicting accessible binding sites is illustrated by a study comparing experimentally determined RNase H–accessible sites in four RNA species with those predicted by known secondary or tertiary structure structure [50]. Overall, most cleavage reactions occurred at single-stranded sites in the RNA. While some single-stranded sites were good targets, many were not, and many good sites were located in double-stranded regions.

Even though the rules for base-pairing are very simple, additional subtleties govern the hybridization of oligonucleotides to RNA that are not well understood. The behavior of oligonucleotides is very dependent on the terminal nucleotides. Moreover, small changes in the length or a shift in binding site of one or two nucleotides can profoundly affect the kinetics of hybrid formation [51, 52, 53, 54, 55]. Even a few base changes that do not change the thermodynamic stability of the duplex may greatly change the kinetics of hybridization [56, 57, 58] . These effects may partially account for the efficacy of different antisense oligonucleotides *in vivo*.

Sophisticated modeling programs such as Foldsplit [59, 60] can often yield more active antisense oligonucleotides than those obtained by random selection [58]. Such modeling programs have predicted that, on average, there is about one good target site per 1000 bases in RNA.

Targeting protein-binding sites. Sequences in RNA that interact with proteins, ribosomes, spliceosomes, and other large entities are also likely to be accessible to oligonucleotides,

assuming no unwinding activity is required. Early on, the cap, initiator codon, and 3’-end were selected as targets [61]. Many later studies have also found that the initiator codon is a good target and has become something of an industry standard despite the occasional failure. Antisense oligonucleotides have also been used to redirect splicing to prevent formation of the functional mRNA [62]. Successful targeting of splice sites requires that the oligonucleotide gain access to the nucleus whereas inhibition of translation may be accomplished by hybridization in the cytoplasm. In some cases, it is possible to use oligomers that do not induce cleavage, particularly if they hybridize strongly such as the morpholino analogues or 2'-O-methyl derivatives [63, 64, 65, 66].

Testing libraries of oligomers. cDNA libraries may be made more efficacious for screening by cutting the cDNA into fragments of approximately ten bases [50]. RNase H is often used to identify those sequences showing greatest activity [50, 56, 57, 67, 68]. These studies can yield both kinetic and equilibrium hybridization data and have been shown to be predictive of activity in cells [56, 68, 69]. These experiments can also be performed in cell extracts to more closely mimic the structure and protein binding of the RNA in cells [70]. A potential problem using libraries of random oligonucleotides is that members of the library may interact with each other inhibiting hybridization with the target [47]. Alternatively, they could assist each other to hybridize to the target through cooperative binding [71, 72, 73]. This problem can be avoided by using arrays of immobilized oligonucleotides [53].

Sequence Checks

Before synthesizing an antisense oligonucleotide, the investigator should check the sequence for various features that could affect its activity. For instance, if the sequence complements nontarget RNAs, the probe may not be useful. In addition, the oligonucleotide should be examined for self-complementary sequences that might interfere with hybridization to the target. Certain sequence motifs have potent biological effects unrelated to antisense activity. This was unrecognized by early investigators and is still the biggest pitfall for the unwary researcher.

CG-containing sequences. Unmethylated CG sequences occur more frequently in bacterial DNA than they do in eukaryotic DNA. Consequently, their presence may be used as a signal of bacterial infection by the immune system [74]. Oligonucleotides containing CG can act as immunostimulators by causing proliferation of B lymphocytes; by activating macrophages, dendritic cells, and T cells; and by inducing cytokine release [20, 74, 75, 76, 77, 78]. These CG-mediated immune effects depend on the sequences flanking the CG dimer, and are strongest with the purine.purine.CG.pyrimidine.pyrimidine motif [20]. These CG effects occur with phosphorothioates as well as with phosphodiesters [79], and may be responsible for some of the activities of oligonucleotides reported *in vivo*. The easiest solution is to choose oligonucleotides that do not contain CG, particularly those with flanking sequences that favor immune stimulation. An alternative is to replace the C in CG sequences with 5-methylcytidine [80]. Although it increases the expense of synthesis, this 5-methylcytidine substitution prevents immune stimulation without affecting hybridization.

Tetraplexes. In addition to forming duplexes and hairpins by Watson–Crick base pairing, some oligonucleotides can form structures comprising three, four, or more strands. In particular, formation of tetraplexes with potent biological activity has caused some problems in the antisense field [81, 82]. Investigators should carefully examine all oligonucleotides very rich in a particular nucleoside, particularly if they show repeated sequences or have multiple occurrences of two or more adjacent identical bases. Oligomers with multiple repeats of two or more consecutive Gs or Cs may form tetraplexes and other non–Watson–Crick structures. Not all oligomers with such features will necessarily form these higher order structures, particularly in physiological conditions. Nonetheless, such sequences raise warning flags and there is a well-documented danger in ascribing biological effects to an antisense mechanism without careful investigation.

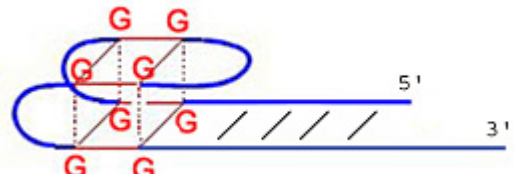


Figure 8. Possible Guanine Tetraplex Structure

The most extensively studied tetraplexes are formed by oligonucleotides containing multiple adjacent guanine residues (Figure 8). These may occur in a single run of around four residues but they can also be found in repeated GG or GGG motifs that occur in close proximity [83, 84]. Even if they do not form tetraplexes, G-rich sequences with multiple GG dimers may form other unusual structures depending on sequence context [85]. Tetraplex-forming runs of Gs seem to have an affinity for various proteins and when included in synthetic oligonucleotides, they produce a multitude of biological effects. For example, researchers have identified tetraplexes that bind to thrombin [86] and to the HIV envelope protein [87]. Other tetraplexes have been shown to bind to transcription factors [88] or to produce antiproliferative effects by protein binding [89]. The ability to form tetraplexes can be blocked by replacing guanosine residues with 7-deazaG [90] or 6-thioG [91]. It should also be noted that a phosphorothioate oligonucleotide containing only C residues was shown to have activity similar to one containing a G-tetraplex [82].

Other motifs. Investigators have suggested that stretches of purines in the target might stabilize the heteroduplex formed [92]. From examining the the sequences of active antisense oligonucleotides in many published studies, investigators have proposed that selecting a target containing the sequence GGGA gives a much better chance of success [93].

Conclusions

Antisense oligonucleotides can provide a tremendous amount of fundamental data to committed investigators. Systematic down regulation of particular gene products can be key in determining a gene's biological function and its role in a metabolic or regulatory pathway. Similarly, antisense technology provides a valuable tool for discerning genetic contributions to disease. The potential for antisense-based oligonucleotide drugs is only now beginning to be realized. Isis Pharmaceuticals recently brought to market Vitravene™, an antisense oligonucleotide drug for CMV retinitis. A number of other antisense oligonucleotides are currently in clinical trials, including those for treating malignancies and for targeting diseases such as psoriasis, hepatitis C, and rheumatoid arthritis. It is only through the proper design of

antisense oligonucleotides that these useful tools have come to yield a plethora of meaningful results, both in pure and applied research.

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