INTEGRATED DNA TECHNOLOGIES

Antisense Technologies

Contents

Introduction

Antisense technologies are a suite of techniques that, together, form a very powerful weapon for studying gene function (functional genomics) and for discovering new and more specific treatments of diseases in humans, animals, and plants (antisense therapeutics). A conventional definition of antisense refers to the laboratory manipulation and/or modification of DNA or RNA so that its components (nucleotides) form a complementary copy of normal, or "sense," messenger RNA (mRNA). The binding, or hybridization, of antisense nucleic acid sequences to a specific mRNA target will, through a number of different mechanisms, interrupt normal cellular processing of the genetic message of a gene. This interruption, sometimes referred to as "knock-down" or "knock-out" depending upon whether or not the message is either partially or completely eliminated, allows researchers to determine the function of that gene.

In this review, a survey of the agents employed in antisense technologies will be presented along with a discussion of the various mechanisms they employ to achieve the goal of reducing or eliminating normal processing of a gene of interest. The focus will be on those techniques that employ oligonucleotides composed of both modified and unmodified DNA and/or RNA nucleotides. Another major antisense technology, called "RNA Interference", or RNAi, will be presented in more detail in another mini-review.

Antisense Oligonucleotides

Oligonucleotide-based antisense techniques represent the most common and, to date, the most successful approach to achieving suppression or elimination of a genetic message. The antisense effect of a synthetic oligonucleotide sequence was first demonstrated in the late 1970s by Zamecnik and Stephenson [1]. Using nucleotide sequences from the 5' and 3' ends of the 35S RNA of Rous sarcoma virus (RSV), Zamecnik and Stephenson identified a repeated sequence of 21 nucleotides (nt) that appeared to be crucial to viral integration. They synthesized a 13-mer oligonucleotide,

d(AATGGTAAAATGG), complement to a portion of this viral sequence. When this synthetic oligonucleotide sequence was introduced into cultured fibroblast cells infected with RSV, viral production was significantly inhibited. They correctly concluded that the oligonucleotide was inhibiting viral integration by hybridizing to the crucial sequences and blocking them. The term they introduced to describe such oligonucleotides was “hybridon.”

At the same time as this work was being done, other groups, notably Tennant et al. [2] and Miller et al. [3], were reporting similar effects for synthetic oligonucleotides in other systems. These results stimulated a rash of studies focusing on the ability of synthetic oligonucleotides to interfere with genetic processes. Many of theses studies failed to achieve the desired effect and it quickly became clear that there were a number of issues that needed to be addressed if synthetic oligonucleotides were to become generally useful reagents for these studies. The most immediately important of these issues was what can be called “persistence.” Synthetic oligonucleotides are foreign to the cells into which they are introduced and they immediately become prey for endogenous nucleases. If synthetic oligonucleotides were to attain the level of persistence in the cell that would be needed for them to accomplish their tasks, they would have to be protected from those endogenous nucleases. Following Kurreck [4], there are three possible sites on a nucleotide where protective modifications could be introduced (Figure 1). In both DNA and RNA nucleotides the base can be altered or changes can be effected

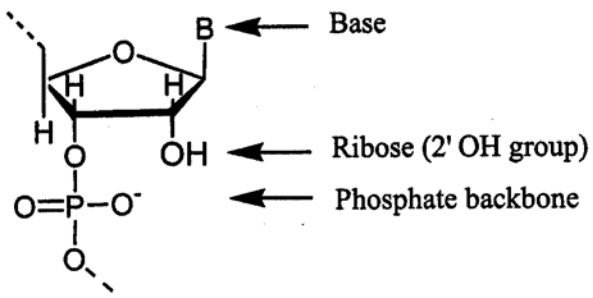

Figure 1. Possible sites for chemical modification of DNA or RNA nucleotides that will confer protection against the action of endogenous nucleases. Note that the 2’ OH site is only available in RNA. (Source: [4])

in the phosphate backbone. In RNA nucleotides the 2’ hydroxyl group, missing in DNA nucleotides, can also be modified. The “trick” involved in protective modifications of nucleotides is to introduce an alteration that is protective against nuclease degradation that does not, at the same time, eliminate the desired effect of the oligonucleotide sequence by blocking complementary hybridization or harming the cell.

In the late 1960s Eckstein and colleagues successfully introduced what has been termed by a number of authors the first-generation antisense-motivated nucleotide

modification [5]. They replaced one of the non-bridging oxygen atoms in the phosphate backbone with a sulfur atom (Figure 2A). Called a phosphorothioate, this modification did achieve the goal of nuclease resistance as measured by an increased half-life for a phosphorothioated oligonucleotide of up to ten hours in human serum compared to about one hour for an unmodified oligonucleotide having the same sequence [6]. Moreover, Matsukura and colleagues demonstrated that phosphorothioated oligonucleotides were effective hybridons against HIV replication in cultured cells [7]. On the other hand, phosphorothioated oligonucleotides displayed slightly reduced hybridization kinetics and, much more importantly, a tendency toward unspecific binding with certain proteins that resulted in cytotoxicity at high concentrations. Thus, the additional consideration of dose-response was added to the mix of issues for antisense agents and the search for other, useful modifications continued.

The so-called second-generation class of modifications directly addressed the non-specific and cytotoxic issues raised by phosphororthioates by introducing RNA oligonucleotides with alkyl modifications at the 2' position of the ribose sugar (Figure 2B). The two most important of these modifications are 2'-O-methyl (OMe) and 2'-O-methoxy-ethyl (MOE) RNAs. Antisense oligonucleotides composed of or containing these modifications display nuclease resistance in concert with lower toxicity and slightly increased hybridization affinities. The major drawback of 2'-O-alkyl modifications is that antisense agents containing them are not available to the most powerful antisense mechanism- RNase H cleavage (see below). Thus, these agents are only effective through the steric block mechanism (see below). The inability of 2'-O-alkyl agents to induce RNase H cleavage of RNA has been used to an advantage, however. 2'-O-methyl oligonucleotides have been used to increase the expression of desired alternate splices in certain proteins by suppressing the undesired splice variant. This has been shown *in vitro* to promote expression of wild type β-globin over the mutant β-globin variant in β-thalassemia [8].

Since RNase H cleavage is the most desirable mechanism for antisense effect, and since 2'-O-alkyl modifications are desirable for nuclease resistance, a hybrid oligonucleotide construct incorporating both characteristics has appeared in the form of the "gapmer" antisense oligonucleotide. A gapmer contains a central block of deoxynucleotides sufficient to induce RNase H cleavage flanked by blocks of 2'-O-methyl modified ribonucleotides that protect the internal block from nuclease degradation. These "chimeric" oligonucleotides have also been promoted as an answer to yet another antisense issue. The phenomenon of irrelevant cleavage occurs because short stretches of nucleotides can bind promiscuously in most genomes. For example, as pointed out by Kurreck [4], a 15-mer can be viewed as a series of eight overlapping 8-mers. In a genome the size of the human genome (3.3×10^9 base pairs, bp), if we assume that each of the four bases occurs at random, any sequence of eight nucleotides can potentially bind 49,500 times, $(0.25)^8$, by chance alone. While the universe of potential random targets is significantly lower in an mRNA population, the potential for promiscuous binding and subsequent RNase H cleavage is still quite high. This theoretical potential became real in

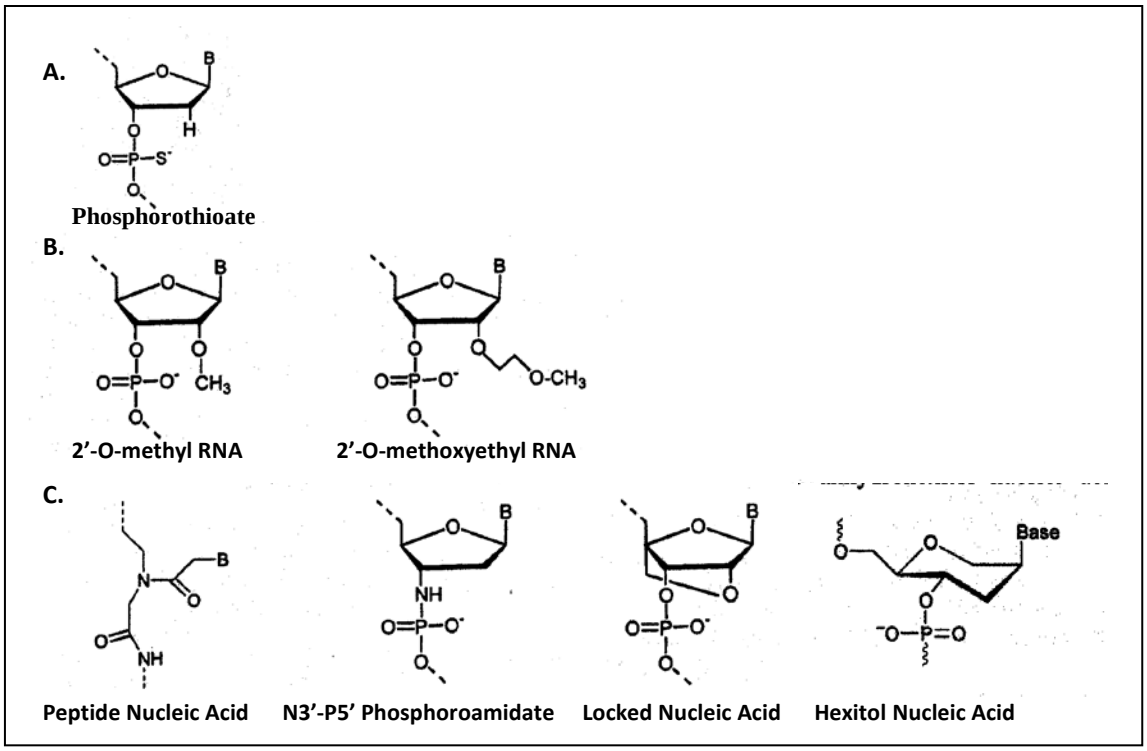

Figure 2. Representation of three generations of nucleotide modifications for use in antisense agents. A. the first generation phosphorothioate backbone modification. **B.** second generation ribonucleotides modified at the 2' hydroxyl by adding a methyl (OMe) or a methoxy-ethyl (MOE) group. **C.** Four of the third generation modifications involving a variety of sites including the entire backbone as in the peptide nucleic acid (PNA), a backbone substitution as in the N3'-P5' phosphoroamidate (PA), the conformational lock in the LNA, or the substituted ring in the hexitol nucleic acids (HNAs).

the case of a 20-mer phosphorothioate oligonucleotide targeted to the 3'-untranslated region (UTR) of the protein kinase C alpha gene (*PKCα*). Due to a strong similarity, this agent also knocked down the protein kinase C zeta (*PKCζ*) gene due to the presence of an 11bp sequence homology between the two genes that matched part of the 20-mer. Shorter targeted central sequences bounded by modified RNA nucleotides that are unable to induce RNase H cleavage solve this problem to a large extent.

While unmodified oligo-deoxynucleotides will routinely form desired DNA:DNA and DNA:RNA duplexes, synthesis of various modifications that confer enhanced high-affinity recognition of DNA and RNA targets has been an ongoing endeavor. A variety of nucleic acid analogs have been developed that display increased thermal stabilities when hybridized to with complementary DNAs or RNAs as compared to unmodified DNA:DNA and DNA:RNA duplexes. These are the third generation antisense oligonucleotide modifications. Among these analogs are peptide nucleic acids (PNAs) [9, 10], 2'-fluoro N3-P5'-phosphoramidites [11], 1', 5'- anhydrohexitol nucleic acids (HNAs) [12, 13], and locked nucleic acids [14, 15]. These structures are shown in Figure 2C. A

more thorough discussion of third generation modifications can be found in Herdewijn [16] and in Kurreck [4].

The primary antisense issue with many third generation modifications is the desired mix of increased thermal stability in hybridization and enhanced target recognition. One of the earliest constructs to address these was the peptide nucleic acid (PNA). First introduced by Nielson et al. [17], PNAs are dramatic alterations in which the sugar phosphate backbone is replaced completely by polyamide linkages. While these constructs afford increased stability and favorable hybridization kinetics, they suffer from being unavailable to the RNase H cleavage mechanism, problematic solubilities, and delivery difficulties. Nonetheless, PNAs are the most studied constructs for antisense after phosphorothioates and 2'-O-alkyl RNAs and numerous successes have been reported [18].

The newest and most promising third generation modification is the locked nucleic acid (LNA). Introduced by Koshkin et al. [19], Obika et al. [15], and Singh et al. [14], an LNA is composed of nucleotides that are "locked" into a single conformation via a 2'-O, 4'-C methylene linkage in 1,2:5,6-di-O-isopropylene-α-D-allofuranose (Figure 2C). LNAs were immediately seen to display remarkably increased thermodynamic stability and enhanced nucleic acid recognition.

Ribozymes

Ribozymes are RNA enzymes that were first described in *Tetrahymena thermophilia* by Cech and colleagues in the early 1980s [20, 21]. The RNA processing capabilities of these enzymes were immediately seized on by those interested in their potential as antisense agents. A number of ribozymes have been characterized, including the most studied form called the hammerhead ribozyme. This enzyme was first isolated from viroid RNA by Uhlenbeck [22] and Haseloff and Gerlach [23]. An excellent discussion of hammerhead ribozymes is presented by Kurreck [4] and discussions of the nature and mechanisms of action of other ribozymes can be found in Doudna and Cech [24], James and Gibson [25], and Sun et al. [26].

RNA Interference (RNAi)

RNA interference (RNAi) was first described in *Caenorhabditis elegans* by Fire and colleagues [27]. They discovered that the introduction of long double-stranded RNAs (dsRNAs) into *C. elegans* cells led to a highly specific degradation of targeted RNAs. This phenomenon was found to be analogous to what had been termed post-translational gene silencing in plants and quelling in *Neurospora crassa* [28, 29, 30]. RNAi has generated enormous interest by both those who view it as a potentially powerful antisense tool and those who recognize it as an ancient eukaryotic cellular defense mechanism. As a result of this interest great strides have been made in understanding RNAi and in applying it to antisense research.

Antisense Mechanisms

To this point the discussion has focused on the various agents that have been or can be used for antisense research. Here, the various mechanisms through which they are known to act are presented. The overall goal in introducing an antisense agent into cells either *in vitro* or *in vivo* is to suppress or completely block the production of the gene product. This means that at some point in the transition from DNA sequence to amino acid sequence the normal transcription and translation apparatus must be affected. As shown in Figure 3 there are three points at which this can be achieved. Figure 3A shows the normal processing of a genetic message from DNA sequence to pre-messenger RNA (pre-mRNA) to mature messenger RNA (mRNA) to amino acid sequence. At step one the sense strand of the DNA is transcribed into a pre-mRNA. In step two the pre-mRNA is converted into a mature mRNA via the simultaneous action of three separate processes. These are 5' capping, intron excision, and poly-adenylation. Finally, in step three the mRNA is transported to the ribsomes for translation into the appropriate poly-peptide.

For the purposes of achieving antisense knock-down or knock-out the first target can be the transcription step in which an antisense agent is targeted to the DNA itself and prevents transcription of the primary message (Figure 3B). As noted by Dagle and Weeks [31], there are three ways in which this strategy can be carried out. These are minor groove binding polyamides, strand displacing PNAs, and major groove binding, triplex forming oligonucleotides. Introduced by White et al. [32,33], minor groove binding agents are pyrrole-imidazole polymers that achieve sequence-specific action through side-by-side pairing of pyrrole and imidazole amino acids with nucleotide base pairs in the minor groove of the DNA helix. Target specificity appears to be limited to short stretches of DNA, generally less than 7bp. PNA agents, on the other hand, are much longer and their mode of operation is to bind to the complementary strand of the DNA helix and displace the true complement. This process is aided by the fact that PNA:DNA duplexes are more stable than DNA:DNA duplexes so that the former duplex is thermodynamically favored over the latter duplex. The third method, major groove, triplex-forming oligonucleotides (TFO), also involves longer sequences but, instead of binding to one strand of the DNA helix and displacing the other, these agents create a stable triplex DNA. To date, two triplex-forming motifs have proved to be successful. Both involve interactions of the TFO with purine bases in a polypurine:polylyrimidine stretch of duplex DNA [31]. While TFOs have been shown to successfully inhibit transcription both *in vitro* and *in vivo*, the conditions for forming stable triplexes are problematic. The target dsDNA sequence is Watson-Crick bonded and the triplex forming oligodeoxynucleotides (TFOs) bind to the duplex via Hoogsteen hydrogen bonding; viz., T-A:T and C^{+}-G:C triplets. This strategy necessitates that only purine-pyrimidine dsDNA can be targeted and that the cytosines in the TFO must be protonated. Cytosine protonation is due to the requirement for acidic conditions in the assay. The recent introduction of locked nucleic acids (LNAs) may alleviate some of these problems, however. Sorensen et al. [34] reported that LNA-containing TFOs will stabilize triplex formation at physiologic pH. A 15-mer containing seven LNAs raised the

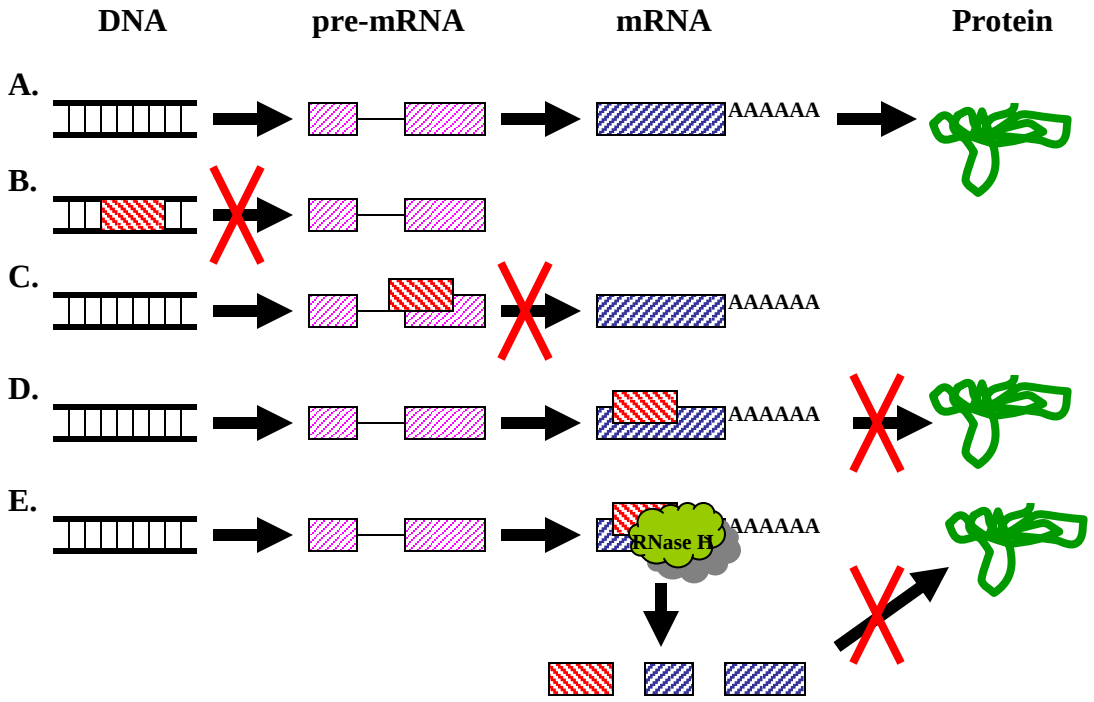

Figure 3. Summary of the strategies available for antisense knock-down or knock-out of a specific genetic message. A. The normal process of transcription and translation. **B.** Prevention of transcription by DNA-targeted agents. **C.** Prevention of mature mRNA formation by pre-mRNA targeting. **D.** Prevention of translation by interruption of the translational apparatus. **E.** Prevention of translation by RNase H digestion of the mRNA.

temperature for triplex to duplex transformation from 33°C to ~60°C at pH 6.8 [35] Sorensen et al. note, however, that an all-LNA TFO will not form triplexes under any conditions.

The next level of antisense attack focuses on the processing of the pre-mRNA and, in particular, the intron excision mechanism (Figure 3C). Here virtually any oligonucleotide-based agent will work in theory. All that is required is sequence-specific binding of the oligonucleotide agent to the pre-mRNA in such a way as to prevent intron excision. However, any agent that is capable of targeting a specific pre-mRNA sequence will work in either of the mature mRNA processes shown in Figure 3D and 3E and, of these, 3E has proved to be the most powerful of all antisense mechanisms.

In Figure 3D, the antisense agent is targeted to the mature mRNA and interferes with the transcription apparatus in one of two ways. Either the presence of the oligonucleotide prevents formation of the ribosomal complex or it acts as a steric blocker downstream to cause truncation of the poly-peptide. While this has been demonstrated *in vitro*, there is a significant issue with the actual operation of such a

mechanism *in vivo*. Any antisense oligonucleotide capable of duplexing with a mature mRNA will result in the formation of either RNA:RNA duplexes or RNA:DNA duplexes depending upon the nature of the oligonucleotide. In the former case, there is an active translational apparatus in the cell that routinely deals with RNA:RNA duplexes that naturally form in mRNAs [31]. Short RNA oligonucleotides would not be stable in the presence of the helicase enzymes in the ribosomal complex and longer RNA oligonucleotides may activate the RNAi pathway. The lone exception so far validated is the use of morpholino oligonucleotides [36]. These oligonucleotides are modified to contain altered internucleoside linkages (Figure 2C). When placed near the 5' end of the mRNA, morpholino oligonucleotides have been shown to specifically reduce translation [37, 38].

Finally, the most used and validated antisense mechanism is that of RNase H degradation of the mRNA (Figure 3E). RNase H is an endogenous enzyme that specifically cleaves the RNA moiety of an RNA:DNA duplex [39, 40, 41]. RNase H is found in both the nucleus and the cytoplasm of all cells and its normal function is to remove RNA primers from Okazaki fragments during DNA replication. Because of the normal function of RNase H, the oligonucleotides that will elicit an intentional and specific RNase H response must be carefully constructed. The favored design is a chimeric oligonucleotide with a central block composed of DNA, either with or without phosphorothioate modifications, and nuclease resistant 5' and 3' flanking blocks, usually 2'-O-methyl RNA but a wide range of 2' modifications have been used [42].

RNase H activation antisense has proved not only to be a powerful weapon in assessing gene function but is emerging as the method of choice for antisense therapeutics as well. Kurreck [4] lists a total of fifteen antisense oligonucleotides that are either approved or in clinical trails for use against diseases ranging from cancer to athsma. Of these, more than two-thirds utilize the RNase H mechanism [42].

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