

REVIEW

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Oligonucleotide-based strategies to reduce gene expression

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Abstract Research on embryonic development and differentiation provides a sensitive, but challenging opportunity to use a variety of techniques designed to modulate gene expression. Changes in the expression of a single gene can alter levels of other genes and provide information on developmentally regulated gene expression pathways. The morphological consequences of altered gene expression can link gene expression to developmental fate. Oligonucleotide-based approaches offer a variety of means to potentially disrupt normal gene expression. The basis for some of these approaches is presented in this review.

Key words oligonucleotides · antisense · triplex

Introduction

The potential for using oligonucleotides as a way to probe function has been recognized for quite a while. It is fair to say that the number of published reports that have used oligonucleotides with the aim of reducing the expression of a particular protein, although numerous, may represent a minority of the times that such experiments have been tried. This disparity in the attempted versus successful use of oligonucleotide-based approaches has polarized opinion on the reliability of these methods. Some of the failed attempts may have at their source unrealistic expectations for what can be accomplished using oligonucleotides or the failure to include controls that could have clarified the reason for failure of a particular sequence (Lebedeva and Stein, 2001).

Here we review some of the current oligonucleotide-based approaches, how they are thought to work inside a cell, and some of the problems that may be encountered. We rely heavily on our own experience using oligonucleotides in studies on *Xenopus laevis* oocytes and embryos, although the basic principles should be valid for most applications.

Oligonucleotide approaches based on sequence-specific targeting of RNA

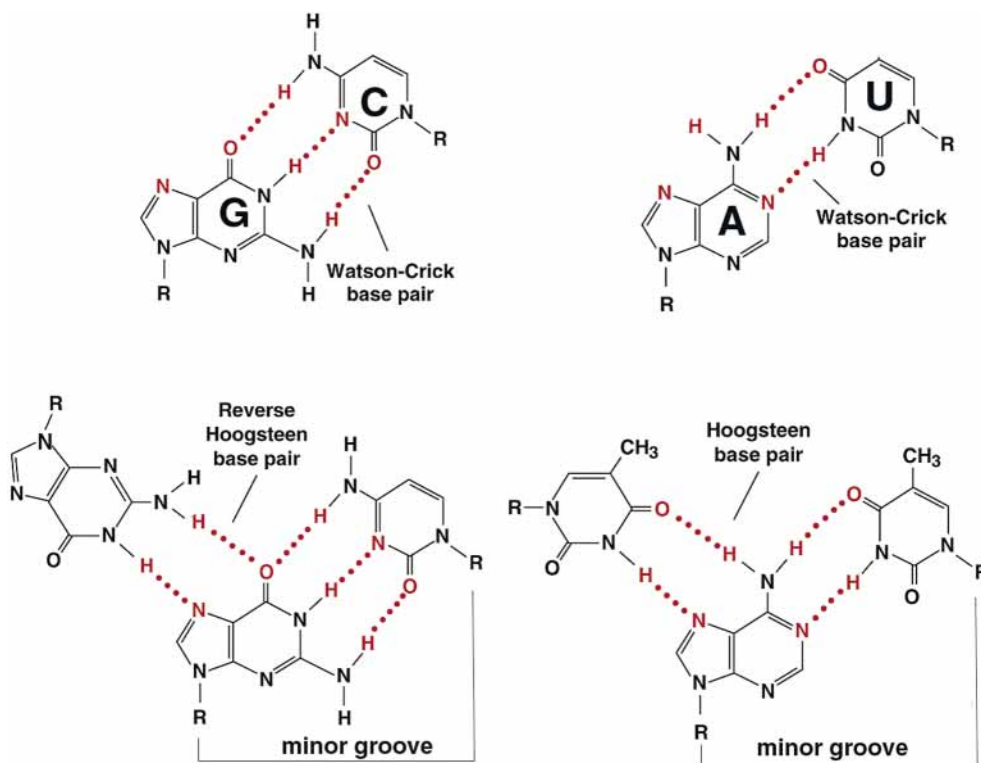
Antisense strategies

Oligonucleotide-based approaches, for the purposes of this review, take advantage of specific hydrogen bonds that form between the bases of nucleic acids (alternative uses of oligonucleotides, such as the activation of the immune system using CpG oligonucleotides, will not be covered here but interested readers can find information on this application in Krieg, 2001). Hydrogen bonds are a relatively weak interaction, and stability of the structure formed depends upon the sequential binding of bases. By far the most common use of oligonucleotides as inhibitors of gene expression is the so-called antisense approach. In this approach, oligonucleotides are synthesized that are complementary to the RNA of interest. Specificity is mediated through Watson:Crick base pairing (see Fig. 1) with the oligonucleotide binding so that its orientation is anti-parallel to the RNA that is being targeted. The minimum effective length of the oligonucleotide is related to its ability to form a stable duplex under cellular conditions and to carry out, mechanistically, the desired effect.

For *in vivo* applications, oligonucleotides must work, within a fairly narrow set of conditions. It is, of course, impractical to change the chemical composition or pH of a cell to accommodate oligonucleotide binding. In ad-

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Fig. 1 The hydrogen bonding interactions used in oligonucleotide base approaches: Target specificity is mediated through Watson-Crick base pairs for strategies targeting mRNA. When DNA is the target, strand displacement strategies (like PNA base approaches) use Watson-Crick base pairing, while triplex forming oligonucleotides use either Hoogsteen or reverse Hoogsteen base pairing in the major groove. Not shown, but discussed in the text, is the binding of polyamides in the minor groove of the DNA.



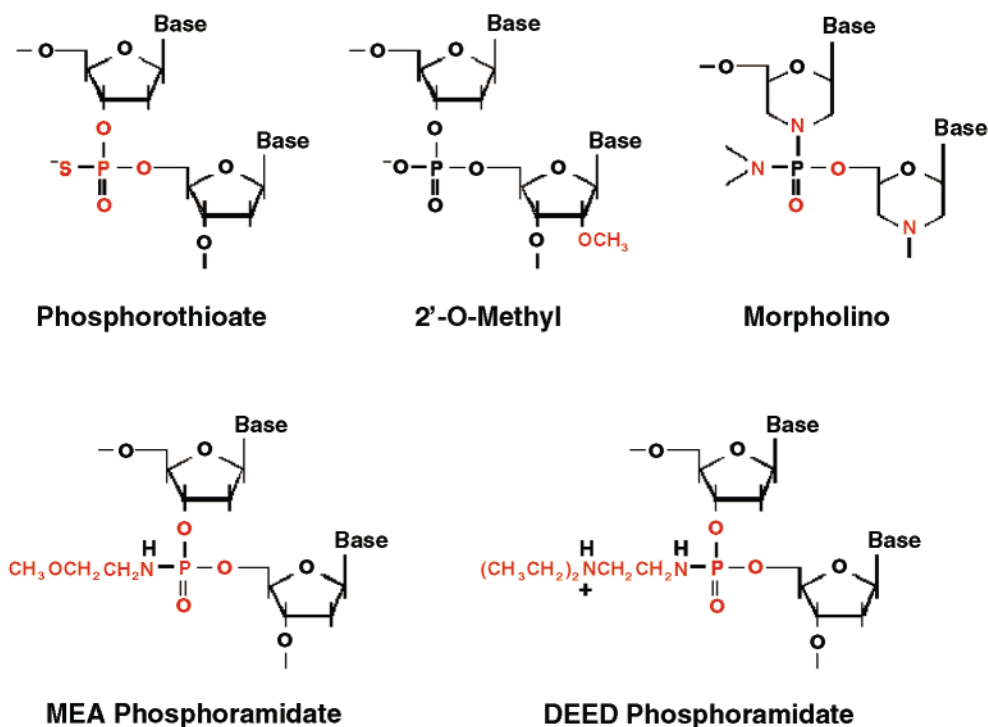
dition, temperature and oligonucleotide concentration must also be kept within fairly conservative limits to avoid changes due, not to the oligonucleotide, but to the incubation conditions of the experiment. Oligonucleotide delivery and stability are also important considerations.

Delivery in many developing systems can be accomplished by direct injection into cells. When microinjection is not an option, delivery can be enhanced in some cases by using hydrophobic or cationic carriers. Unfortunately, there is not yet a universally reliable carrier. Some recent studies have successfully used localized electroporation (for a review see Weaver and Hogan, 2001). This approach may prove very useful in the future for delivery of charged oligonucleotides.

Stability *in vivo* depends upon resistance to endogenous nuclease. How much of a problem do endogenous nucleases present? The half life of an unmodified oligonucleotide in a *Xenopus* oocyte or embryo is only minutes (Dagle et al., 1991). In addition, the pool of deoxynucleotides in most cells is tightly regulated, and fluctuations due to the degradation of large amounts of oligonucleotides may interfere with the cell sensing of adequate deoxynucleotide levels. From the point of view of single stranded DNA, the most active nuclease *in vivo* is a 3' exonuclease, however, both 5' exonuclease and endonuclease are also present (Dagle et al., 1991). The result of degradation of oligonucleotide lowers the concentration available for hybridization to RNA, changes the rate that hybrids can form, and changes the ability

to target RNA over time. Modification of the phosphodiester bond that links nucleosides together can afford some relief from nuclease activity. Some of the modifications used for this purpose are indicated in Fig. 2. The most commonly used modification is the substitution of one of the non-bridging oxygens with a sulfur. The resulting phosphorothioate modification has, unfortunately, to some researchers become synonymous with the phrase anti-sense oligonucleotide, of which it is only one of many possible modifications. The internucleoside phosphate is a chiral center, and in almost all *in vivo* work where phosphorothioates have been used, a mix of Rp and Sp form is present at each linkage, with the Sp form being far more resistant to nuclease than the Rp form (Koziolkiewicz et al., 1997). The consequence is that at every linkage there is variable resistance to nuclease. Some of the other modifications that have been used include the use of 2'-O-methyl ribose and the inclusion of amidates like methoxyethylamine or diethylethylenediamine. Unlike the use of phosphorothioates or 2'-O-methyl modification, the amidation of the linkage can lead to charge changes, making the normally negatively charged phosphodiester linkage either neutral (methoxyethylamine) or positively charged (diethylethylenediamine) (Dagle et al., 1990; 2000). Additional modifications, including the construction of oligonucleotides using a 5' to 2' linkage, peptide-linked oligomers (Nielsen and Egholm, 1999) and morpholino oligomers (Summerton, 1999), take the modification further, changing the intrinsic chemistry of the backbone while

Fig. 2 Modifications of oligonucleotides that can inhibit nuclease digestion. Because nucleases that degrade oligonucleosides cleave at the phosphodiester bond that joins nucleotides together, modification of that bond (shown in red) inhibits oligonucleotide degradation.



still linking together purines and pyrimidine in the proper spacing to provide hybridization potential. The selection of which chemical form of oligonucleotide is best suited for a particular experiment depends upon how long the oligonucleotide must last inside the cell, the desired mechanism of action, and more practically, the available modes of delivery into the cells and the amount (and cost) of oligonucleotide needed.

Mechanisms of action using antisense oligonucleotides

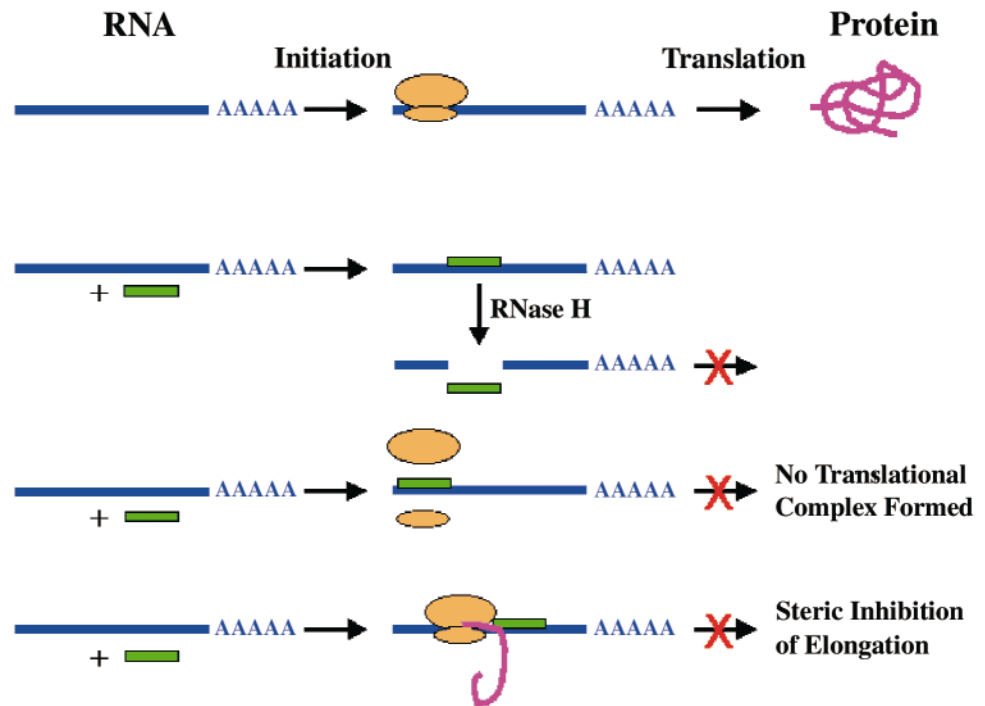
The three principle ways that antisense oligonucleotides have been used to disrupt protein production are shown schematically in Fig. 3. The primary mode of action for most antisense oligonucleotides is the creation of a substrate for endogenous RNase H, leading to directed cleavage of the RNA portion of the oligonucleotide: RNA duplex (Dash et al., 1987; Walder and Walder, 1988). RNase H is found in both the nucleus and the cytoplasm of all cells, and its name is derived from its ability to cleave RNA that is found in an RNA:DNA hybrid. Inside the cell, RNase H may participate in removal of primers during DNA synthesis, removal of misincorporated ribonucleotides, and in some transcription reactions. Post cleavage, the oligonucleotide can hybridize to another RNA transcript, enhancing the potential effect of any single molecule of oligonucleotide. When the mechanism of oligonucleotide action is linked to the formation of an RNase H substrate, chemical modification of the oligonucleotide must be carefully

planned. Many modifications inhibit the ability of RNase H to cleave the RNA that has formed a duplex with an oligonucleotide. So far, phosphodiester bonds (the normal linkage for DNA) or, with a slight loss in activity, phosphorothioates support RNase H cleavage, while the other modification shown in this review do not. Although there is some variation in RNase H activity found from different sources, eukaryotic RNase H are thought to generally require the DNA portion of the duplex to have five or six consecutive internucleoside linkages that can be recognized by RNase H (Dagle et al., 1990; 1991; 2000). Some oligonucleotide designers have taken these constraints into account, synthesizing oligonucleotides with nuclease resistant modifications at the 3' and 5' ends of the oligo, and six to eight unmodified or phosphorothioate modified linkages in the central portion. These chimeric oligonucleotides inhibit 3' and 5' exonuclease degradation while still serving as a substrate for RNase H (Dagle et al., 1990; 1991; 2000). For antisense oligonucleotides using RNase H, the clearest measure of effectiveness is the direct demonstration of specific reduction of the targeted RNA.

From their inception, oligonucleotides have been proposed to be capable of stopping translation by either hybrid arrest or inhibition the formation of the translation initiation complex. Until recently, there has been little convincing experimental support for these activities *in vivo*. The translational apparatus inside the cell must routinely deal with the resolution of RNA:RNA duplex structures formed naturally by mRNA. In general, the length of duplex formed by oligonucleotides used for

Fig. 3 Mechanism of action of antisense oligonucleotides.

Antisense oligonucleotides (green) form a hybrid with their RNA target (blue). There are at least three ways that the formation of the oligonucleotide:RNA duplex might prevent translation of a mRNA resulting in protein production (magenta). 1) The oligonucleotide:RNA duplex may form a substrate for endogenous RNase H, leading to mRNA cleavage. 2) The oligonucleotide:RNA duplex may prevent the productive assembly of the ribosomal complex (orange) preventing translation. 3) The oligonucleotide:RNA duplex may arrest a ribosomal complex already engaged in translation leading to a truncated protein. So far, most experimental data support mechanisms 1 and 2.



antisense studies is not stable enough to withstand the rather robust helicase activity of the ribosomal complex. This is in contrast to the expectation that a lengthier antisense RNA molecule might be able to form a sufficiently stable RNA:RNA duplex that would arrest translation. Of course, in some systems the formation of the RNA:RNA duplex has been shown to activate the RNAi pathway of mRNA degradation (Fire, 1999).

However, an exception may be made in the case of oligonucleotides that are modified to contain morpholino internucleoside linkages (Summerton, 1999). These oligonucleotides, when forming duplexes in the 5'UTR or in close proximity to the translational start site, have been shown to specifically reduce translation (Ecker and Larson, 2001; Xanthos et al., 2001). The design of morpholino oligonucleotides includes the use of slightly longer sequences and placement towards the 5' end of the mRNA. These two features are critical, the longer sequence enabling more stable heteroduplex formation and the site selection chosen to interfere with translation initiation. So far, studies have indicated that morpholinos that hybridize further down the mRNA are not particularly active, suggesting that, even with morpholinos, hybrid arrest of translation is a difficult proposition. These fully modified oligonucleotides have not been shown to lead to mRNA degradation in early trials, and thus, each oligo would potentially be limited to the regulation of a single RNA molecule. However, in most experiments, the molar level of oligonucleotide delivered to the cell far exceeds the RNA being targeted. The clearest measure of effectiveness for this class of oligonucleotide is the direct demonstration of specific reduction of pro-

tein produced from the targeted RNA. Recently, a whole issue of the journal *Genesis* (*Genesis* (2001) Vol. 30) was devoted to examples of the use of morpholinos as inhibitors of gene expression.

Appropriate target selection and experimental considerations

The best that can be accomplished with an antisense oligonucleotide is that new synthesis of a specific protein becomes undetectable. This effect must be balanced against the size and stability of existing pools of the protein, which would not be specifically changed by the oligonucleotide, and the amount of time that new synthesis must be suppressed to accomplish the experimental goal.

Target site selection is critical to the productive use of oligonucleotides. Some experimental failure of oligonucleotides, as judged from the lack of a discernable phenotype, can be traced back a lack of association with targeted mRNA. The ability of the oligonucleotide to bind to a specific sequence depends upon gaining access to that region of the RNA. Competition for the site comes from secondary structure (intramolecular base-pairing) and the association of other nucleic acids or protein with the RNA. There are not yet reliable predictive algorithms that will establish the ideal *in vivo* target site for antisense experiments, although there are several ongoing studies that have used empirical, computational, and statistical approaches to this problem (see, for example, Scherr et al., 2000). In addition, some re-

cent success at selecting appropriate target sites using microarrays has been reported (Sohail et al., 2001).

However, it often remains useful to sample several potential target regions, using as a first screen the capacity of the oligonucleotide to deplete mRNA and/or reduce protein levels. Attention to these early mechanistic events is quite important before trying to assign a phenotypic consequence to oligonucleotide treatment. For use in experiments like those performed to study early *Xenopus* development, the prescreening of effective targets can be done by injecting even unmodified oligonucleotides into *Xenopus* oocytes and looking for reduction of endogenous mRNA (if present in the maternal pool of mRNA) or reduction of a synthetic transcript that, as faithfully as possible, reflects the full size and structure of the intended target (for examples of this approach see Torpey et al., 1992; Raats et al., 1997; Veenstra et al., 2000). This preliminary experiment offers a relatively inexpensive prediction of an accessible target site *in vivo*. However, it is possible that the access to a specific mRNA target site may change during development or in specific cell types.

An alternative type of failed experiment comes from phenotypes that are uninterpretable or non-specific. The pre-screening of potential antisense sequences by using the NCBI Blast, "Search for short nearly exact matches" algorithm and the specific organism under the options section of the program can uncover some unexpected (and unintended) additional targets. Target site choice should avoid sequences that are common to a family of molecules, thus, a nucleotide binding site or DNA binding domain conserved among a class of proteins would normally be avoided in an antisense study. In addition, antisense oligonucleotides that have self-complementary regions or are very G rich can form complexes that prevent effective hybridization to RNA targets.

As mentioned above, the concentration of oligonucleotide used *in vivo* must be kept in a fairly narrow range to avoid side effects. These side effects can be easily noted in *Xenopus* embryos, where one often sees reduction, irregular, or even cessation of cell cleavage, confirming the longstanding observation that there are many ways to disrupt development and kill an early embryo. In addition, oligonucleotides may interact with molecules other than the targeted RNA that can complicate interpretation of data. For example, it is well established that phosphorothioate-modified oligonucleotides can bind to the heparin binding class of growth factors (these include FGF; Guvakova et al., 1995). Thus, biological activity that is mediated by these growth factors can be unintentionally altered by the presence of this class of modified oligonucleotide.

What constitutes appropriate controls when using oligonucleotides? Since the mechanism of action of the oligonucleotide is either RNA turnover or inhibition of protein production, the specific demonstration of loss of RNA or protein is critical to productive interpretation

of any phenotypic changes. In the ideal case, RNA or protein production may be reduced to undetectable levels, however, practical information may be gained by less severe reduction. The interpretation of any developmental consequences is on more solid footing when the extent of protein reduction is clear. As with any treatment of embryos or cells with an exogenous compound, the lowest effective concentration of oligonucleotide that can reproducibly reduce RNA levels or protein production should be used. This level will depend upon both sequence and chemical modification. A titrated oligonucleotide dose can often separate non-specific and specific effects. As mentioned earlier, the non-specific interactions of oligonucleotides make treatment with control oligonucleotides of comparable sequence and similar modification another useful control. Where possible, the rescue of oligonucleotide treatment, re-adding either protein or RNA to the embryo, provides a compelling argument for specific oligonucleotide effect. In cases where RNA rescue is attempted, elimination of the target site by altering the sequence of the rescue RNA can be accomplished by taking advantage of codon redundancy, if the target sequence lies in the coding region of the mRNA, or elimination of the targeted region, if the sequence targeted is in the 5' or 3'UTR (see examples in Raats et al., 1997; Veenstra et al., 2000). Another possible source of rescue RNA is to use mRNA derived from the homologous gene of another organism. This approach, of course, presents its own set of concerns (expression level and efficacy).

In some cases, specifically in studies done in *Xenopus* oocytes, the rapid turnover of unmodified oligonucleotides allowed rescue of function through injection of synthetic forms of the targeted RNA. In studies done to examine the function of small nuclear RNA, the endogenous pool of RNA was degraded using unmodified oligonucleotides. After waiting for injected oligonucleotides to be degraded, a synthetic substitute for the targeted RNA was introduced (Peculis and Stietz, 1993).

Targeting duplex DNA: Triplex-based strategies

Less commonly used, but an approach with remarkable potential is an oligonucleotide-based strategy for altering gene expression based on direct binding to DNA. There are at least three different strategies actively under study. These include the study of minor groove binding polyamides, strand displacing PNAs, and major groove binding, triplex forming oligonucleotides (Fig. 4). The aim with these techniques is to block RNA polymerase transit, occupy the binding sites of transcription factors, or induce sequence specific damage to DNA.

Polyamides refer to pyrrole-imidazole polymers that bind cooperatively as anti-parallel dimers in the minor groove of DNA. Sequence-specific interaction is gained through the side-by-side pairing of pyrrole and imidazo-

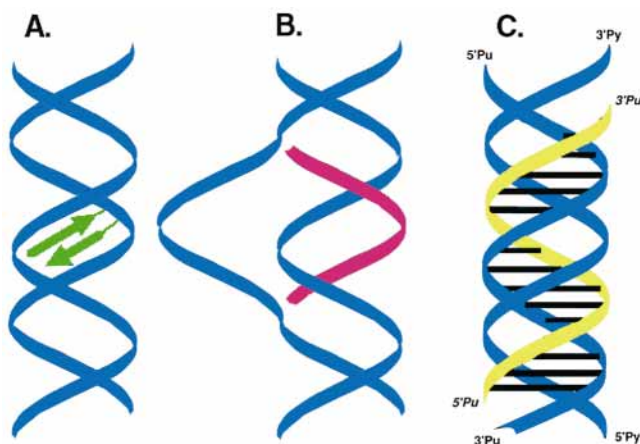


Fig. 4 Oligonucleotide targeting of double-stranded DNA. There are three styles of DNA targeting currently being tested. They are (A) binding in the minor groove of the DNA using polyamides, (B) strand displacement using PNA and (C) binding in the major groove of the duplex through Hoogsteen or Reverse Hoogsteen base pairs by triplex forming oligonucleotides.

le amino acids to nucleotide base pairs in the minor groove (White et al., 1997; 1998). There have been a number of reports characterizing the specificity of this interaction. The target is generally between 5 to 7 base pairs of DNA, as affinity of the polyamides for larger targets is reduced (Dickinson et al., 1998). Whether this method continues to be developed for more widespread *in vivo* work remains to be seen.

PNA (peptide nucleic acids) have a structure where the sugar-phosphate backbone of nucleic acid has been replaced by 2-aminoethyl glycine carbonyl units (see (Nielsen and Egholm, 1999 for a current review). The mechanism of action for PNA-based approaches is one of strand displacement. Under many conditions, the PNA:DNA duplex is more stable than a DNA:DNA duplex (Egholm et al., 1993). PNAs are very stable inside cells but, like all of the DNA targeting strategies, have seen limited successful use in experiments involving embryos or differentiating cells.

Alternatively, under certain conditions, triplex-forming oligonucleotides (TFOs) will bind in the major groove of a DNA duplex. There are two known binding motifs that allow TFO binding to a DNA duplex. Both involve interactions of the bases of the TFO with the purine bases of a polypurine:polypyrimidine stretch of duplex DNA. In the pyrimidine motif an ODN binds parallel to the purine strand of the DNA duplex (T binds to the A of an A:T duplex and C⁺ binds to the G of a G:C duplex). The C residue requires protonation for triplex formation, an event that requires an acidic pH (4.5–6) and therefore limits *in vivo* utility. Base modifications, such as 5-methyl Cytosine, may help alleviate this pH constraint, although little *in vivo* evidence for successful use of this strategy exists. In contrast, the purine motif is pH independent and may be better for

in vivo applications. In this scheme, the purine-rich TFO binds antiparallel to the purine stand of the duplex (either A or T bind to the A of an A:T duplex and G binds to G of a G:C duplex; Beal and Dervan, 1991). The major drawback to using this approach *in vivo* is the tendency of G-rich ODNs to self-associate into quartets at physiologic potassium concentrations. The self-association problem has been overcome by chemical modifications, specifically the amidation of the oligonucleotide during its synthesis using the cationic diethyl-ethylenediamine (DEED) (Dagle and Weeks, 1996; Bailey et al., 1998; Bailey and Weeks, 2000). Chemical modification of triplex forming oligonucleotides can include every internucleoside linkage, as there is no need for an enzyme like RNase H to recognize and act upon the three-stranded substrate that forms.

Aside from issues of physiological pH and salt conditions, the formation of triplexes inside the cell depends upon having triplex binding sites and having those sites accessible to oligonucleotide binding. Access to a site can be lost if a DNA binding protein already occupies the major groove or if the target site is covered by a nucleosome. Although it is clear that some sites that have been used *in vivo* must be accessible, the mere presence of a purine-rich stretch on one strand of the DNA duplex does not guarantee triplex formation (Brown and Fox, 1996). One might expect that a gene being actively transcribed would have 'looser' chromatin structure and serve as a better candidate for triplex formation. Although some researchers have concluded that transcriptionally active genes are more prone to triplex formation, others disagree (Oh and Hanawalt, 1999).

What happens when oligonucleotides form triplexes with DNA? The effect of triplex formation on DNA replication has been examined. *In vitro* studies on the synthesis of DNA using a variety of DNA polymerases demonstrated that triplexes could inhibit synthesis (Hacia et al., 1994; Samadashwily and Mirkin, 1994). However, the conclusion that *in vivo* replication of DNA will be inhibited by triplex formation may be premature, as Maine and Kodadek (1994) have demonstrated, DNA helicase is undeterred in separating DNA strands in the presence of triplex structure. In addition, treating tissue culture cells with TFOs does not lead to cell cycle arrest, as would be expected if DNA replication were halted. It appears that, although purified DNA polymerase may be inhibited by triplex formation, the replication enzyme complex *in vivo* does not seem affected.

TFOs have been reported to inhibit transcription both *in vitro* (Cooney et al., 1988; Durland et al., 1991; Duval-Valentin et al., 1992; Maher, 1992; Noonberg et al., 1994; Kim and Miller, 1995) and *in vivo* (Ing et al., 1993; Kovacs et al., 1996; Bailey and Weeks, 2000). The mode of action depends upon the targeted site. For instance, if the target site includes the recognition sequence for a transcription factor then the TFOs compete with the transcription factor for access to the major groove of

the DNA. If the target site is close to the TATA box or between the TATA box and transcription start it may disrupt the assembly of the initiation complex. Finally, if the target site is in the transcribed region of the gene, formation of a triplex may cause premature termination of transcription. Examples of each mode of action have been reported (Maher, 1992; Ing et al., 1993; Wang et al., 1996; Bailey et al., 1998; Bailey and Weeks, 2000; Faria et al., 2000).

Perhaps one of the most exciting prospects for triplex forming oligonucleotides is the possibility of generating site-specific mutations. Several laboratories have used triplex formation to deliver DNA damaging agents to specific DNA sequences. Alkylating compounds, intercalators, photoactivatable cross linkers, and ^{125}I (to promote damage through ionizing radiation) have all been attached to oligonucleotides used to form triplexes (for a review see Giovannangeli and Helene, 1997). The damage seen includes single-strand nicking, double-stranded breaks, and photochemical cross-linking of the two duplex strands (Moser and Dervan, 1987; Perrouault et al., 1990; Takasugi et al., 1991; Oh and Hanawalt, 1999; Panyutin and Neumann, 1994). Under some circumstances, triplex formation leads to mutations even when DNA damaging agents are not attached to the TFO. Under these circumstances, the triplex is apparently recognized as DNA damage and the damage repair pathways are activated. The 'correction' of the perceived damage may itself cause the mutation through errors in repair or when an appropriate donor DNA fragment is available through homologous recombination stimulated by triplex formation (Vasquez and Wilson, 1998; Vasquez et al., 1999; 2000; Faruqi et al., 2000; Luo et al., 2000). Full stimulation requires a TFO that binds well *in vivo*, the presence of a donor fragment, active nucleotide excision repair pathway, and active homologous recombination pathway (Faruqi et al., 2000; Luo et al., 2000; Datta et al., 2001).

Concluding remarks

Oligonucleotide-based approaches have a variety of very attractive features as methods that can investigate the function of developmentally important genes. When thoughtfully applied, these approaches can provide an extremely useful complement to overexpression, misexpression, and dominant negative studies. In addition, continued development of novel forms of oligonucleotides and refined uses of existing modified oligonucleotides should enhance their place in the experimental repertoire of molecular and developmental biologists.

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