# 2 Nucleic Acid Therapeutics
## *An Introduction*

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## 1. INTRODUCTION

The development of simple, reliable tools for modifying gene expression "on demand" would represent a major technical advance for cell biologists. Because much progress has been made in understanding the molecular pathogenesis of many diseases, we may easily hypothesize that these same tools could be of tremendous importance to clinicians as well. For example, many genes responsible for cellular transformation have been identified. If the function of these genes were shown to be either completely or relatively tumor specific, they would become legitimate targets for therapeutic manipulation of their expression. More effective, less toxic cancer treatments could reasonably be expected to result if the strategy were successful.

The notion that gene expression could be modified through use of exogenous nucleic acids derives from studies by Paterson et al., who first used single-stranded DNA to inhibit translation of a complementary RNA in a cell free system in 1977 *(1)*. The following year, Zamecnik and Stephenson showed that a short (13mer) DNA oligodeoxynucleotide antisense to the Rous sarcoma virus could inhibit viral replication in culture *(2)*. Based on this work, the latter investigators are widely credited for having first suggested the therapeutic utility of antisense nucleic acids. In the mid-1980s, the existence of naturally occurring antisense RNAs and their role in regulating gene expression was demonstrated *(3–5)*.These observations were particularly important because they lent credibility to the belief that "antisense" was more than a laboratory phenomenon and

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encouraged belief in the hypothesis that reverse complementary ASNA could be utilized in living cells to manipulate gene expression. These seminal papers, and the many that have since followed, have stimulated the development of technologies employing nucleic acids to manipulate gene expression. As discussed immediately below, virtually all available methods rely on some type of nucleotide sequence recognition for targeting specificity but differ where and how they perturb the flow of genetic information.

Strategies for modulating gene expression may be thought of as being either "anti-gene" or anti-mRNA, reviewed in *(6,7)*. Anti-gene strategies focus primarily on gene targeting by homologous recombination *(8,9)*, or by triple-helix-forming oligodeoxynucleotides (TFOs) *(10,11)*. Because homologous recombination involves vector technology and, at least at the present time, is much too inefficient for clinical use, it is not considered further in this discussion. TFOs bind in the major groove of duplex DNA in a sequence-specific manner *(11)*. Gene targeting with these molecules is constrained by the fact that TFOs require runs of purines on one strand and pyrimidines on the other (~10–30 nts in length) for stable hybridization. The TFO can be composed of either polypurine or polypyrimidine bases, but hybridization always occurs on the purine strand of the duplex through formation of Hoogsteen bonds (Fig. 1).

Although successful use of this strategy for blocking transcription and inducing specific mutations in vitro and in vivo has been reported, the frequency of such events is considerably less than1%, and therefore this approach is also too inefficient for clinical use at this time.

ASNA transcription factor decoy molecules have also been employed to disrupt gene expression at the level of transcription *(12)*. For many technical reasons, including limited gene accessibility within the nuclear/chromosomal structure, the clinical application of these methods has not progressed at a very rapid rate. An alternative approach, using polyamides, or lexitropsins, has been described by Kielkopf and colleagues *(13–15)*. Polyamide ligands contain the aromatic amino acids pyrrole (Py), hydroxypyrrole (Hp), and imidazole (Im). These small molecules have the ability to diffuse into the nucleus where they can then contact double-stranded DNA in the minor groove. Pairs of such amino acids can be constructed that recognize all four Watson-Crick basepairs. It is theoretically possible then to construct polyamides that will recognize specific DNA sequences and squelch gene expression by preventing transcription in a manner analogous to TFOs (*see* Fig. 2).

However, these small molecules also share problems in common with TFOs. Included among these is the fact that recognition of longer sequences, as would be required for gene-specific recognition, require larger molecules, which are likely to have trouble gaining access to the nucleosome. In addition, maintaining the appropriate amino acid register for accurate sequence recognition is also a significant issue *(17)*. Accordingly, much remains to be accomplished by the

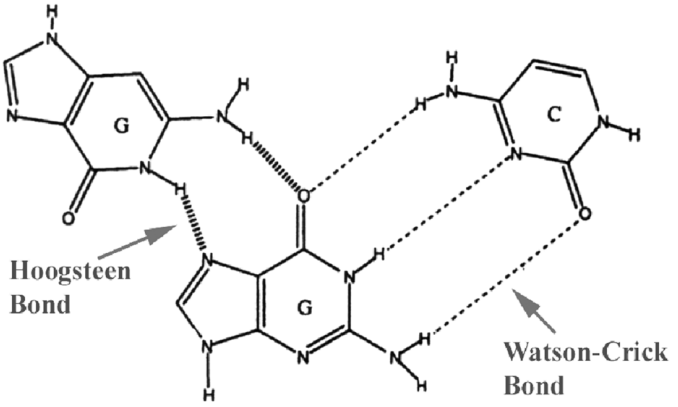


Fig.1. Triple helix formation at nucleotide level showing Watson-Crick and Hoogsteen Bond formation between duplex pairs and the third strand.

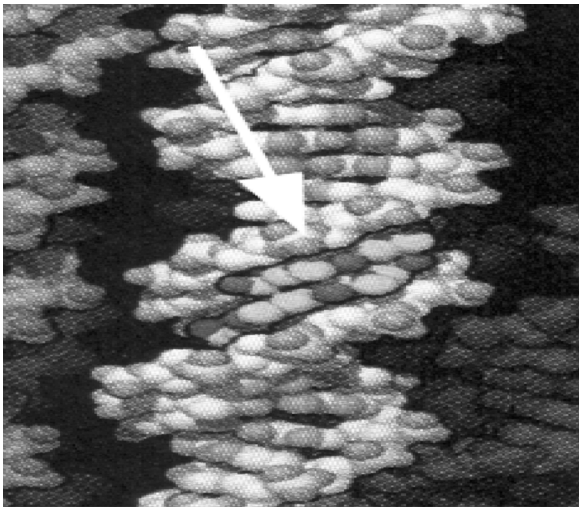

Fig. 2. Lexitropsin (polymide) molecule (arrow) binding in minor groove of DNA (adapted from ref. *16*).

scientists interested in this approach before attempts at clinical use can be contemplated.

A larger body of work has focused on destabilizing mRNA. This approach, although less favorable than antigene strategies from a stoichiometric point of view, is nonetheless attractive because mRNA, unlike a given gene's DNA, is theoretically accessible to attack while being transcribed, transported from the

nucleus, and translated. Two ASNA-based strategies have emerged for blocking translation. One employs oligonucleotides that act as alternate binding sites, or "decoys," for proteins that act as transcriptional activators, or as stabilizing elements that normally interact with a given mRNA *(18,19)*. By attracting away the desired protein, the decoy may prevent transcription or induce instability, and ultimately destruction, of the mRNA. The use of decoy RNAs for therapeutic purposes was initially suggested by Baltimore in 1988 *(20)*. The approach has gained credibility since the molecules are nontoxic and appear to have been successful for control of viral gene expression, including HIV, in vitro *(21,22)*. It is assumed in these studies that the decoy is directly interfering with HIV replication by sequestering REV, but this was not formally proven *(21)*. Its mechanism of action is therefore uncertain. Recent studies on human β-globin mRNA are of interest in this regard. Stability determinants for this mRNA species have been defined in sufficient detail so that it can be used as a model system for testing the hypothesis that altering mRNA stability with decoys will be a useful form of therapy *(23–25)*.

The other strategy for destabilizing mRNA is the more widely applied antisense strategy, using ribozymes, DNAzymes, antisense RNA, or antisense DNA (ODN). This approach to gene squelching has been the subject of numerous authoritative reviews *(6,26,27)*, but simply stated, delivering an antisense nucleic acid into a cell where the gene of interest is expressed should lead to hybridization between the antisense sequence and the targeted gene's mRNA. Stable mRNA-antisense duplexes cannot be translated, and depending on the chemical composition of the antisense molecule, can lead to the destruction of the mRNA by binding of endogenous nucleases, such as RNase H, or by intrinsic enzymatic activity engineered into the sequence (ribozymes and DNAzymes) (*see* Fig. 3).

Finally, a third and newly developing approach for targeting mRNA is called post-transcriptional gene silencing, or RNA interference (RNAi) *(28,29)* (Fig. 4). RNAi is the process by which double-stranded RNA (dsRNA) targets mRNA for destruction in a sequence-dependent manner. The mechanism of RNAi involves processing of dsRNA into 21–23 basepair (bp) fragments that hybridize with the target mRNA and initiate its destruction. The mechanism for RNAi is fast being elucidated, although many intriguing questions remain to be answered *(28)*. At this time, it appears likely that dsRNA is processed by an enzyme called Dicer *(30–32)* into approx 21–22 nt long double strands. These small cleavage products are then incorporated into a larger RnP complex that simultaneously scans the complementary mRNA sequence for homology to the small, now unwound, RNA fragment and then promotes its destruction through an enzymes integral to the complex *(33–35)*. RNAi has been employed successfully for gene silencing in a variety of experimental systems including petunias, tobacco plants, neurospora, *C. elegans*, insects, planaria, hydra, zebrafish. The use of long

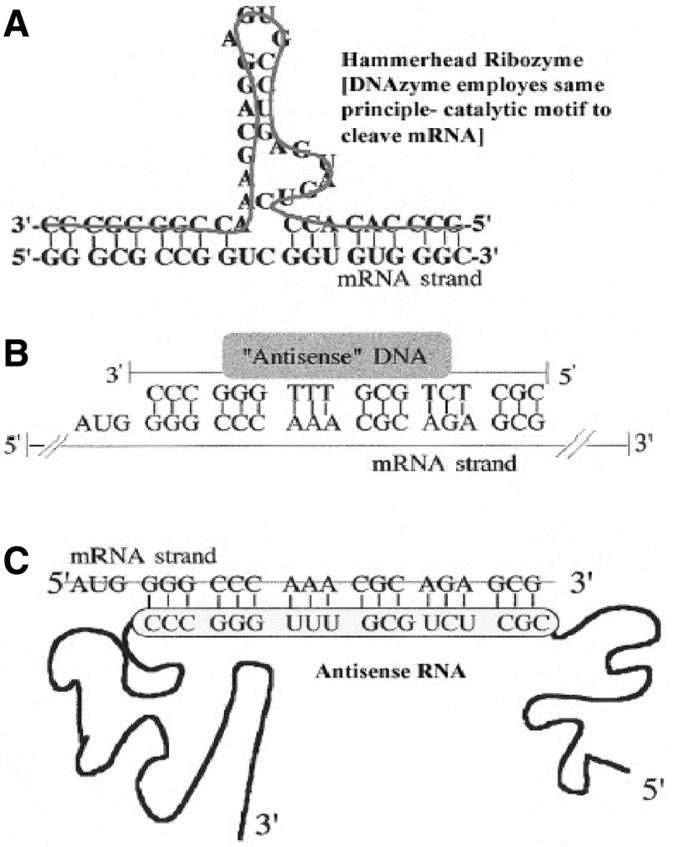


Fig. 3. Antisense strategies for squelching gene expression.

dsRNA to squelch expression in mammalian cells has been tried, largely without success *(36)*. It has been suggested that mammalian cells recognize these sequences as invading pathogens, which triggers an interferon response that leads to apoptosis and cell death *(37)*. However, a number of recent reports suggest that RNA double strands of approx 21–22 nts in length, called short interfering RNA (siRNA), may be able to squelch expression in mammalian somatic cells if appropriately modified to contain 3'-hydroxy and 5'-phosphate groups *(38–41)*. Although reports about the utility of this method for silencing mammalian genes continue to accumulate *(42–45)*, the ability to apply this method to all types of mammalian cells remains uncertain *(36)*. This is also true for traditional antisense experiments, and not surprisingly, the possibility of experimental artifacts being misinterpreted as specific gene targeting is being increasingly recognized as well *(46)*. Accordingly, it is highly likely that many

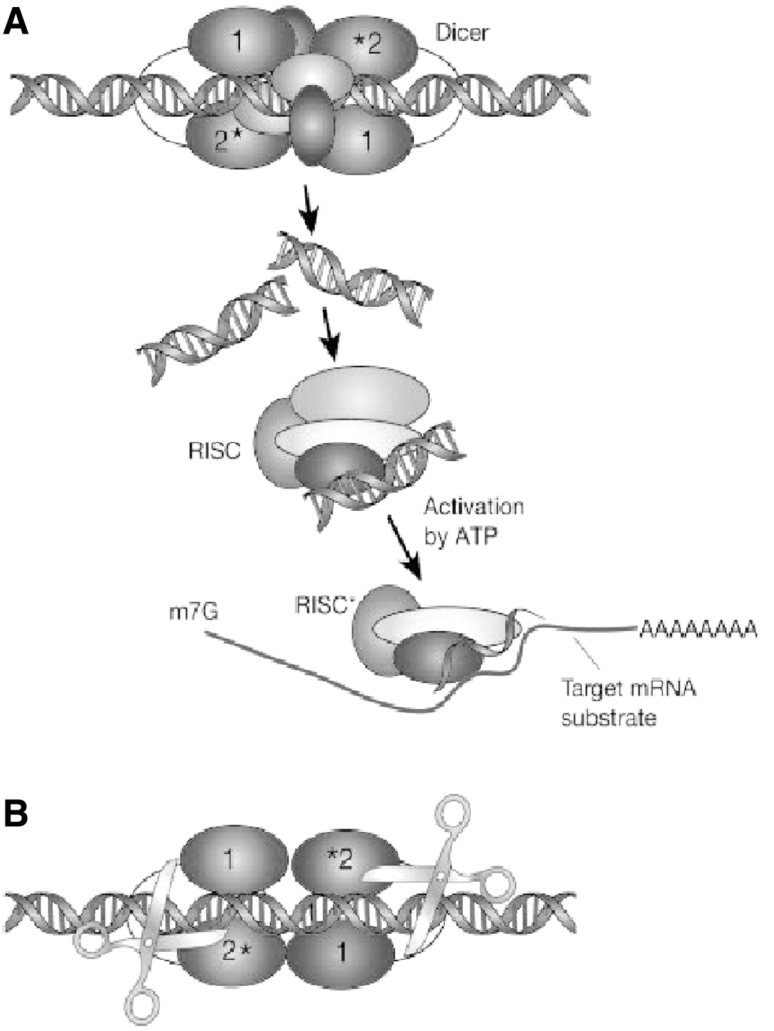


Fig. 4. Hypothetical RNAi mechanism (adapted from ref. *41*).

technical issues related to employing nucleic acid therapeutics in general will also apply to siRNA, including the need to identify mRNA accessible sequences in a predictable way *(47)*.

The power of nucleic-acid-mediated gene squelching has been demonstrated in experiments in which critical biological information has been gathered, and subsequently confirmed, using alternative or complementary experimental methods including the critical role of the *c-myb* gene in hematopoietic cell proliferation. These experiments were the motivation for an early example of "bench-to-bed-

side" research as they culminated in some of the first phase I/II antisense oligodeoxynucleotide studies for the treatment of leukemia here at the University of Pennsylvania *(48)*. A major conclusion of the work is that, at the least, expression of the targeted gene (*c-myb*) could be abrogated under clinically relevant conditions.

Nevertheless, despite many successes *(48–50)*, it is widely appreciated that the ability of antisense nucleic acids to modify gene expression is variable, and therefore wanting, in terms of reliability *(51–53)*. Several issues have been implicated as root cause of this problem, including molecule delivery to targeted cells *(51)* and identification of hybridization accessible sequence *(6)*. Sequence accessibility is determined by mRNA folding, which in turn is dictated by internal basepairing and the proteins that associate with the RNA in a living cell. Attempts to accurately predict the in vivo structure of RNA have been fraught with difficulty *(54)*. Accordingly, mRNA targeting with any hybridization-dependent targeting vehicle, including siRNA *(47)*, is largely a random process, accounting for many experiments where the addition of an antisense nucleic acid yields no effect on expression.

Recent work from this laboratory suggests that the self-quenching reporter molecules (SQRM) that we are developing will be useful for solving in vivo RNA structure *(55)*.

Another significant problem in this field is the limited ability to deliver ASNA into cells and have them reach their target *(51)*. Without this ability, it is clear that even an appropriately targeted sequence is not likely to be efficient. As a general rule, oligonucleotides are taken up primarily through a combination of adsorptive and fluid-phase endocytosis *(56–59)*. After internalization, confocal and electron microscopy studies have indicated that the bulk of the oligonucleotides enter the endosome/lysosome compartment, where most of the material either becomes trapped or degraded. Biologic inactivity is the predictable result of these events. Nevertheless, oligonucleotides can escape from the vesicles intact, enter the cytoplasm, and then diffuse into the nucleus, where they presumably acquire their mRNA or, in the case of decoys, protein target. In the last few years, delivery technologies have improved considerably, and it is likely that these and other newly evolving technologies can be used to delivery optimized nucleic acids to their cellular targets.

It is our hypothesis that development of effectively targeted, and efficiently delivered, nucleic acid molecules will lead to important advances in the diagnosis and treatment of human malignancies *(48)*. As was true for the field of monoclonal antibody therapies, where hype was followed by disappointment and then finally genuine triumph of the concept, we believe that breakthroughs in the area of nucleic-acid-mediated gene squelching will shortly be forthcoming and will more than justify the time and resources expended in developing the therapeutic use of these molecules.

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