REVIEWS ARTICLE

Nucleic Acid Therapeutics: State of the Art and Future Prospects

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As we approach the new millennium, a quick glance backwards reveals that truly astounding progress has been made in the identification of genes responsible for cell growth, development, and neoplastic transformation. With this knowledge has come a natural desire to "translate" this information into new therapeutic strategies for many of the common maladies that afflict mankind. These include in particular cardiovascular, gastrointestinal, neurologic, infectious, and neoplastic diseases. Attempts at inserting genes into cells that either replace, or counter the effects of, disease-causing genes has been one of the primary ways in which scientists have tried to exploit this new knowledge. This technically complex, as yet largely unrealized endeavor is scientists have tried to exploit this new knowledge. This common goal of these various strategies, which are turning out to be as technically demanding as more traditional gene therapy, is to identify disease related genes and target them for "silencing." Because the numbers of maladies that might be treated by this approach are genuinely enormous, this is clearly a most important field of endeavor. It will be the goal of this review to describe available strategies for "silencing," or perhaps more appropriately, perturbing gene expression. Given the expertise and experience of our laboratory, we will place special emphasis on the use of reverse complementary or so called "antisense" oligodeoxynucleotides (ODN) for this purpose. Problems associated with the use of antisense ODN for modifying gene expression are well known and they will be discussed, along with potential strategies for overcoming these problems. The prospects for ultimately using these materials successfully in the clinic will also be elaborated upon. This review is meant to be complete, but not exhaustive. Even a cursory examination of the literature data base reveals that since 1992 more than 1,300 manuscripts have been published which list antisense DNA or RNA among its key words. We therefore apologize in advance to colleagues whose work we do not cite, but which we nonetheless admire.

NUCLEIC ACID-BASED STRATEGIES FOR PERTURBING GENE EXPRESSION—A PRIMER

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. The following year Zamecnik and Stephenson showed that a short, 13-nucleotide (nt) DNA molecule antisense to the Rous sarcoma virus could inhibit viral replication in culture. The latter investigators are widely credited on this basis for having first suggested the therapeutic utility of antisense nucleic acids. In the early to mid 1980s, Simons and Kleckner and Mizuno et al. showed the existence of naturally occurring antisense RNAs in prokaryotes and showed that these molecules played a role in regulating expression of their corresponding genes. These observations were particularly important because the existence of naturally occurring antisense RNAs lent credibility to the belief that the use of reverse complementary nucleic acids was a "natural" mechanism for regulating gene expression, thereby raising hope that the process could be exploited in living cells to manipulate gene expression. The work of Izant and Weintraub further buttressed belief in this potential by demonstrating that expression of antisense RNA in eukaryotic cells could also modulate expression of the complementary gene. These seminal reports, and many others which quickly followed, have stimulated the rapid development of technologies using nucleic acids to manipulate gene expression. 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. Most simply, gene expression may be perturbed at the level of transcription or translation (Fig 1).

Inhibiting Gene Expression at the Transcriptional Level

Inhibition of gene expression at the level of transcription may be accomplished by at least three different methods. The "gold standard" exploits homologous recombination. This approach is designed to take advantage of naturally occurring cross-over events during DNA replication. In a typical system, a plasmid capable of infecting the desired target cells and expressing the desired sequence is constructed. The construct expresses a selectable gene marker, such as an aminoglycoside resistance gene, flanked by sequences complementary to the gene of interest in the genomic DNA. When the targeting plasmid is introduced into the cell of interest the vector and complementary portions of genomic DNA undergo rare (\(1:1000\) heterologous recombinations) cross-over events during the course of cell division. The cross-over results in insertion of the targeting sequence into the genomic DNA at the intended site resulting in effective destruction of the targeted gene (Fig 2A). Further, the inserted sequence remains under the control of the targeted gene’s promoter. Therefore, cells in which the desired event has occurred are selected by exposure to G418 (geneticin), which is toxic to cells in the absence of the aminoglycoside resistance gene. Those cells that survive the exposure must have incorporated and expressed the resistance gene and therefore the targeting cassette. If resistant cells are injected into a murine blastocyst, animals expressing the mutated gene will develop, assuming of course that loss of the targeted gene does not lead to an embryonic lethal condition. The effect of functional
absence of the targeted gene in a developing animal may then be
discerned, giving important insights into the biologic impor-
tance of the gene chosen for elimination. This method, com-
bined with appropriate animal breeding, is quite effective at
generating heterozygous or homozygous loss of function mu-
tants. Further, recent elegant modifications of this basic ap-
proach using bacteriophage recombinases such as Cre, which
recognize specific DNA elements known as loxP, have the
capability to significantly increase the efficiency and utility of
this approach.\textsuperscript{10} Cre, for example, has the ability to excise all
chromosomal DNA between loxP sites and then ligate the cut
ends, a process that can occur even in postmitotic cells.
Constructing a targeting vector with loxP sites and an inducible
promoter allows for temporal and tissue-specific gene targeting
when cells are exposed to Cre. Nevertheless, while homologous
recombination is extremely powerful, it is hampered by that fact
that it remains inherently inefficient, time consuming, and
expensive. It should also be noted that complementation of the
targeted gene’s function by an alternative gene may give a
misleading impression of the targeted gene’s function and
relative importance.\textsuperscript{11,12} Clearly, this is a method that is
presently restricted to use in cell lines and animal models.
Whether it is likely to have clinical relevance as a therapeutic
modality anytime in the foreseeable future is uncertain.

Another second option for disrupting gene expression at the level of
transcription uses synthetic ODN capable of hybridizing with
double-stranded DNA.\textsuperscript{13-15} Such hybrids are typically formed
within the major groove of the helix, but very recently a strategy
for hybridization within the minor groove has also been
reported.\textsuperscript{16} In either case, a triple-stranded molecule is pro-
duced, hence the origin of the term triple-helix forming
oligodeoxynucleotide (TFO) (Fig 2B). TFOs may act in two
ways. They may prevent binding of transcription factors to the
gene’s promoter and therefore inhibit transcription. Alterna-
tively, they may prevent duplex unwinding and, therefore,
transcription of genes within the triple-helical structure. TFO
sequence requirements are based on the need for each base
comprising the TFO to form two hydrogen bonds with its
complementary base in the duplex. This constrains TFOs to
hybridization with the purine bases composing polypurine-
polypyrimidine tracks within the DNA. The bonds formed
under these conditions are also referred to as Hoogsteen bonds
after the individual who first described them. They may form in
the parallel or antiparallel (reverse Hoogsteen) orientation
relative to the 5'-3' orientation of the purine strand depending
on the thermodynamics of the specific base interactions in-
volved. An A or a T in the TFO can bond with the A of an A · T
pair in the DNA duplex, while G can bond with the G of a G · C
pair. C can also bond with the G of a GC pair if protonated (C\textsuperscript{+}).
Accordingly, TFOs containing C form stable hybrids under
acidic conditions. Although this tendency can be modified
somewhat by methylation of the cytosine at the C-5 position,\textsuperscript{17}
C-containing TFOs are expected to be less active at physiologic
pH. In contrast, G- and T-containing TFOs can form stable
hybrids at physiologic pH.\textsuperscript{18} However, these TFOs are plagued
by the fact that physiologic concentrations of potassium inhibit
triplixx formation, although some very recent studies suggest
that TFOs substituted with 7-deazaxanthine can hybridize
efficiently with their target even in the presence of 140 mmol/L.
K\textsuperscript{+}\textsuperscript{19} G-containing TFOs also require divalent cations such as
Mg\textsuperscript{2+} for stability, while A-containing TFOs appear to require
Zn\textsuperscript{2+}.

Stability of triple helices is further dependent on a number of factors,
including the length of the TFO, with \sim 13 nucleotides
being suggested as a minimum for phosphorothioate com-
ounds,\textsuperscript{20} and the presence of any base mismatches. Such
mismatches are particularly problematic when they occur in the
middle of a strand because they interfere significantly with
“nucleation,” the relatively slow process whereby the initial
base-pair associations between the TFO and the strand being
targeted are brought about. In fact, a single mismatch in the
middle of the strands, or the presence of a single pyrimidine
base in the homopurine run, can decrease TFO affinity by 20-
to 30-fold. In addition to these considerations, it has also been
reported that helicases, enzymes which unwind duplexed DNA
for transcription and repair, easily disrupt triple-stranded DNA.\textsuperscript{21}

These problems have significantly hampered the use of TFOs as
reagents for studying gene function in intact cells, and make
them quite problematic as potential pharmaceuticals.\textsuperscript{14}

Despite the problems discussed above, a number of ap-
proaches have been investigated for optimizing the activity of
the TFO. One is to use an oligonucleotides that binds to
alternate strands of the duplexed DNA,\textsuperscript{22} a maneuver that
obviates the requirement for polypurine-polypyrrimidine se-
tance in the target DNA. Another is to increase the binding
affinity of the TFO by covalently linking the DNA to intercalat-
groups such as acridine\textsuperscript{23,24} and psoralens.\textsuperscript{25,26} Incorporation
of strand-cleaving moieties may also increase efficiency of
TFOs.\textsuperscript{27} Finally, work on expanding the third-strand binding
code may also enhance the utility of this approach. Recent
experiments from Wang et al\textsuperscript{28} and from Kochetkova et al\textsuperscript{29,30}
have provided evidence that triple-helix formation can occur in
living cells, suggesting that these difficulties may ultimately be
overcome. If shown to be practical, it has also been postulated
that TFOs may prove useful in the treatment of certain genetic
disorders such as sickle cell anemia and hemophilia B, where
their ability to induce mutations might be used to correct single
base–pair mistakes responsible for the disease.\textsuperscript{28,31-33} Because
this method may also inadvertently introduce undesired muta-
tions into the genome by the same mechanism, concerns have
been raised about using this approach in patients.

One final approach that has not undergone extensive develop-
ment, at least at the level of transcription, is the use of specific
nucleic acid sequences to act as “decoys” for transcription
factors (Fig 2C).\textsuperscript{34,35} Since transcription factor proteins recog-
nize and bind specific DNA sequences, the principal upon
which electrophoretic mobility shift assays are based, it is
possible to synthesize nucleic acids that will effectively com-
pete with the native DNA sequences for available transcription
factor proteins in vivo. If effective, the rate of transcription of
the genes dependent on the particular factor involved will
diminish. Unless single gene transcription factors can be
identified, it is difficult to conceive how this approach, though
potentially effective for controlling cell growth, can be made
gene specific. Transcription factor decoys will not be further
considered here, but the decoy concept will be mentioned again
briefly below because RNA decoys have also been used to block
translation.
**Inhibiting Gene Expression at the Translational Level**

Strategies for inhibiting translation are primarily directed toward impairing utilization of messenger RNA (Fig 3). Approaches that have this as a goal are what have traditionally been designated “antisense” strategies because of their reliance on the formation of reverse complementary (antisense) Watson-Crick base pairs between the targeting construct or vector, and the mRNA whose function is to be disrupted. It is the specificity of the Watson-Crick base pairing that allows a particular mRNA species to be selectively targeted. The antisense strategies rely on either introducing the reverse complementary nucleic acid sequence into the target cell, or on expressing the reverse complementary sequence in the target cell from a transfected viral or plasmid vector. The reverse complement may be DNA or RNA. The relative merits of each are discussed below. In theory, however, if hybridization between the target mRNA and the exogenous nucleotide sequence occurs, a duplex is created which, in effect, forms a “jam” that prevents the ribosomal complex from reading along the message. If the ribosomal complex can’t read the message, the appropriate tRNAs are not assembled and the encoded peptide is not made. This would appear to be a relatively foolproof mechanism for preventing mRNA utilization but, as was true for triple-stranded DNA molecules, RNA-RNA or RNA-DNA duplexes can be unwound by a variety of repair/editing enzymes such as helicase and RNA unwindase. In addition, the ribosomal complex itself has unwindase activity that likely permits “reading” of the complexly fold mRNA. In the case of ODN that are targeted downstream of the translation initiation site, it has been shown that the ribosomal complex can unravel the RNA/DNA duplex, allowing the complex to read through the block. Peptide assembly is thereby unperturbed.

- **Fig 1.** Gene expression may be disrupted, as indicated by the “X,” at the level of transcription, or translation. Oligonucleotides can inhibit transcription (1) by triple-helix formation with chromosomal DNA, or by acting as decoy’s for transcription factors (see Fig 2). Hybridization of an oligonucleotide to mRNA may inhibit translation (2) by hindering the ability of the ribosome complex to “read” the mRNA sequence, or by providing a substrate for RNase H (see Fig 3). 

- **Fig 2.** Strategies for inhibiting transcription. (A) Homologous recombination. Cross-over exchange between the targeting vector and genomic material during cell division is indicated. See text for more detailed explanation of events. (B) Triple-helix formation in the major groove between a polypyrimidine oligodeoxynucleotide (open pattern line) and polyurine sequence in double-stranded (black line) DNA. Textural representation of this event is indicated below the cartoon. (C) Decoy strategy. Double-stranded oligonucleotides compete with the native binding site for transcription factor protein (dark globular structures). 

- **Fig 3.** Strategies for inhibiting translation. Diagrammatic representations of (A) hammerhead ribozyme; (B) antisense oligodeoxynucleotide; (C) antisense RNA. Note that targeting specificity is conveyed in each case by Watson-Crick base pairing between complementary sequences.

- **Fig 4.** Chemical modifications of oligonucleotides. (A) Common modifications of the phosphodiester linkage, sugar, and bases moieties are depicted. Note that 2′-O methyl sugar modifications confer stability to single-stranded RNases but not DNases. They do not allow binding of RNase H either. The propynl pyrimidines demonstrate enhanced binding to RNA but cannot permeate membranes. (B) N3-SP phosphoramidates and peptide nucleic acid backbone modifications confer enhanced stability and RNA binding. However, the latter do not permeate membranes and neither activates RNase H.

Events that are triggered as a result of duplex formation are dependent on the nature of the antisense molecules used for mRNA targeting. Oligonucleotides of many, but not all, types (see below) support the binding of RNase H at sites of RNA-DNA duplex formation. Such binding is thought to be an important effector of antisense actions because once bound, RNase H, a ubiquitous nuclear enzyme required for DNA synthesis, functions as an endonuclease that recognizes and cleaves the RNA in the duplex. *Escherichia coli* RNase H requires a minimum of four RNA-DNA base pairs for enzyme binding, while human RNase H may require only one. Once initiated, destruction of the message by cleavage is assured. Of significant interest also is the fact that the DNA comprising the duplex is undamaged by the enzymatic attack. Therefore, it is free, at least theoretically, to hybridize with multiple RNA molecules, leading their destruction in a catalytic manner. Some chemical modifications, such as the phosphorothioates, are thought to activate RNase H very efficiently, while others do not support the activity of this enzyme at all (see below). It is also probably worth noting RNase H may produce unanticipated, non–sequence-dependent effects by cleaving transiently formed duplexes, or with sites of partial complementarity.

Although RNase H is generally thought to be critical for antisense effectiveness, not all studies support this contention. For example, Rosolen et al reported that overexpressing RNase H in U937 cells to a level 10 times greater than normal did not enhance the antisense effectiveness of a c-myc targeted ODN. One caveat in interpreting these experiments is that it was not human RNase H that was overexpressed. Similarly, Moulds et al studied antisense inhibition mechanisms using a microinjection assay and oligonucleotide modifications that were either permissive of, or did not support, RNase H binding. Their studies suggested that if a stable RNA-DNA duplex was formed translation of the targeted mRNA was completely inhibited. Based on these results they predicted that binding of RNase H to such a stable duplex would not further increase the efficiency of antisense inhibition of protein synthesis. These studies were somewhat artificial in that duplexes were preformed ex vivo and were then injected into the nucleus of the cell. Therefore, the direct physiologic relevance of these experiments is uncertain. More indirect is the fact that despite the apparent importance of RNase H for generating an antisense effect, few published reports have actually provided direct evidence of such attack by demonstrating that the predicted cleavage fragments have been generated. This may be because once the mRNA molecule is nicked, it is likely very rapidly destroyed.

The fate of RNA-RNA duplexes is less certain. As mentioned above, they may be unwound, in which case an antisense effect might not be expected. Alternatively, the dsRNA may serve as a substrate for editing enzymes such as double-stranded RNA adenosine deaminase (DRADA). When DRADA deaminate the adenosine, inosine is formed. The presence of inosine may tag the mRNA molecule for destruction. In any case, the message becomes unreadable. It is straightforward that either of these eventualities would contribute to an antisense effect. It is also straightforward that without physical destruction or modification of the targeted mRNA, strand unraveling would abrogate an antisense effect.
In an attempt to assure destruction of the mRNA target when using an RNA molecule for targeting, many researchers have been investigating the utility of ribozymes. Ribozymes are catalytic RNA molecules whose structures are based on naturally occurring site-specific, self-cleaving RNA molecules (Fig 3A). The catalytic moieties of ribozymes recognize specific nucleotide sequence, commonly GUX, where X = C, U, or A or, in some cases, NUX, where N = any nucleotide. Four major classes of naturally occurring ribozymes have been described, along with many artificially engineered types based on the folding and cleaving properties of the naturally occurring types. When the site-specific cleaving motifs of ribozymes are incorporated into single-stranded RNA molecules whose 5’ and 3’ ends have been designed to hybridize with specific sequence flanking an available catalytic cleavage site within an mRNA target, a trans-acting and specific mRNA cleaving molecule results. Such molecules are potentially very efficient because once they cleave their target, they are released from their mRNA target and are free to hybridize with another mRNA molecule. Like ODN then, they can destroy multiple mRNAs in a catalytic fashion.

The chemical and physical requirements of ribozyme-mediated catalysis are being carefully studied in hopes that more efficient molecules can be synthesized. In common with oligonucleotides are issues that bear on stability of the molecules and how to deliver them efficiently to cells. Structure/function considerations unique to these molecules include their speed of association with the target. Critically important as well is the ribozyme’s dependence on divalent cations for binding to, and cleavage of, their substrate. Hammerhead ribozymes cleave most efficiently in an environment containing greater than 500 mmol/L magnesium while the intracellular environment has been estimated to have a magnesium concentration of ~500 µmol/L. Length of the flanking antisense guide sequences are also important. For example, it has been shown that if flanking antisense recognition sequences extend beyond a certain length, ribozyme turnover is slowed and specificity of the reaction is decreased. Finally, the intracellular localization of the ribozyme has also been shown to be critical for activity because the ribozyme and its mRNA target clearly need to be in physical proximity. However, even physical colocalization of target mRNA and ribozyme is insufficient for complete cleavage as shown recently by Jones and Sullenger, who found only ~50% modification of an mRNA target by a ribozyme expressed is tandem off the same plasmid construct. The stability and activity of ribozyme constructs can also be profoundly influenced by secondary structure and protein interactions.

Finally, the nucleic acid decoy strategy mentioned briefly above has also been used to inhibit translation. This has been most extensively investigated in the context of attempts to inhibit replication of the human immunodeficiency virus (HIV). In this case, viral mRNAs encode proteins required for expression of viral genes. If expression of these genes can be inhibited, the virus fails to propagate. With this purpose in mind, a number of investigators have reported expressing RNA molecules corresponding to the HIV trans activation response (TAR) element or the Rev response element (RRE) in different cell types, and subsequently demonstrating that cells expressing such constructs were protected against infection by HIV-1. The vectors used for these studies were primarily neo–RRE and tRNA–RRE fusion gene constructs, suggesting that the carrier RNA to which the RRE element was fused was not critical for conveying protection. Rather, it was assumed that it is the RRE element functioning as a competitor, or decoy, for available Rev protein was responsible for protecting the cells. In the specific case of the Rev decoy some data were provided to support this assumption. Preliminary in vitro binding studies identified a 13-nt RNA sequence within the RRE that effectively bound Rev. In vivo expression of this sequence in the form of a tRNA fusion transcript was shown to inhibit HIV replication, and such inhibition was correlated with inhibition of Rev function. However, it must be noted that direct proof of decoy RNA and Rev protein interaction was not provided. Accordingly, while this approach to translational control of gene expression is certainly interesting, and may potentially work via the mechanism proposed, specificity at the single gene level remains an important issue that must be resolved.

OLIGONUCLEOTIDES—CONSIDERATIONS ON THE USE OF MODEL MOLECULES FOR TRANSIENT DISRUPTION OF GENE EXPRESSION

Chemical Considerations

Whether being used as an experimental reagent or pharmaceutical, ODN need to meet certain physical requirements to make them useful. First, ODN need to be able to cross cell membranes and then hybridize with their intended target. The ability of an ODN to form a stable hybrid is minimal if a function of the ODN’s binding affinity and sequence specificity. Binding affinity is a function of the number of hydrogen bonds formed between the ODN and the sequence to which it is targeted. This is measured objectively by determining the temperature at which 50% of the double-stranded material is dissociated into single strands and is known as the melting temperature or TM. The TM depends on the concentration of the oligonucleotide, the nature of the base pairs, and the ion strength of the solvent in which hybridization occurs. In the case of phosphodiester, this may be estimated from the following formula: 

\[ T_M = n(2°C) + m(4°C) \]

where \( n \) = number of dA · T pairs and \( m \) = number of dG · C base pairs. Thus, it may be seen that stability will increase directly with the proportion of dG · C base pairs. This is because G · C pair with three hydrogen bonds as opposed to the two bonds formed by dA · T pairs. At physiologic conditions (37°C, low salt) it is estimated that at least 12 bp need to form in order to form a stable hybrid with a phosphodiester backbone, although more recent studies from Wagner et al suggest that 7 nt is sufficient under certain conditions. It is worth noting that a single base mismatch, depending on its location, type, and surrounding sequence, can decrease binding affinity as much as 500-fold. mRNA associated proteins and tertiary structure also govern the ability of an ODN to hybridize with its target by physically blocking access to the region being targeted by the ODN. Finally, it is also clear that ODN should exert little in the way of non–sequence-related toxicity, and should remain stable in the extracellular and intracellular milieu in which they are situated. Meeting all these requirements in any one molecule has turned out to be a very difficult task because, as might be expected, satisfying one criterion is often accomplished at the
expense of another. If the object is to create a pharmaceutical agent, the more complex the molecule, the more expensive its synthesis. In an age of increasing cost consciousness, this too becomes an important consideration in the design of these molecules. For in-depth information and additional references on any of these issues the reader is referred to one of several outstanding reviews.82,83

It is probably easiest to approach this subject from the point of view of a DNA molecule and to consider the various modifications that might be made to satisfy the criteria mentioned above. Figure 4 shows two nucleotides of a hypothetical natural oligomer and the phosphodiester bridge that joins them. While many studies, including some from our own laboratory, have reported using natural DNA for antisense investigations,12,84-88 it is becoming increasingly common to use material that has been stabilized against attack from endonucleases and exonucleases. These omnipresent enzymes attack DNA molecules at the phosphodiester bridges and break them down to mononucleotides. First-generation antisense molecules were designed to make the internucleotide linkages more resistant to attack. This was accomplished primarily by replacing one of the nonbridging oxygen atoms in the phosphate group with either a sulfur or a methyl group. This type of modification forms a phosphorothioate89 or a methylphosphonate ODN, respectively.90

Methylphosphonates are neutral in charge and therefore lipophilic.89 Accordingly, in addition to being nuclease resistant, it was postulated that they may be taken up by cells in a more efficient manner, but this is controversial. Nevertheless, despite these properties, the methylphosphonates have not been widely used for at least three reasons. First, and perhaps most importantly, it has been found methylphosphonates do not allow RNase H–mediated cleavage of the mRNA to which the molecule may be hybridized. This appears to result in loss of significant antisense effect. Second, because they are hydrophobic they are difficult to get into solution. Third, the molecules are chiral at the methylphosphonate bridge and are therefore a mixture with respect to any given conformation. This likely lowers target mRNA affinity. Therefore, despite their otherwise useful properties, other modifications will be required to make this modification more useful. Some promising leads have been identified, including the use of methylphosphonate, dieter chimeric molecules.92

In distinct contrast to the methylphosphonates, phosphorothioates are very widely used in both the laboratory setting and the several clinical trials with antisense molecules that are now ongoing.93-95 This situation is a result of several desirable properties imparted to oligonucleotides synthesized with this modification. First, phosphorothioates are relatively nuclease resistant. In addition to their relative nuclease resistance, the negative charge imparted to the phosphate backbone renders the molecule hydrophilic and, therefore, water soluble. It is also noteworthy that phosphorothioates permit RNase H activity in the duplex. Nevertheless, this modification is also problematic. First, the polyanionic nature of these molecules impairs uptake because of the negative charge at the cell surface. Second, these molecules also have a chiral center at the internucleoside phosphorothioate. DeLong et al have synthesized dithioates in an attempt to solve this problem. Here both nonbridging oxygen atoms are sulfur substituted.96 Dithioates appear to have lower binding affinity for RNA but are capable of inhibiting HIV growth in culture.96 Third, many non–sequence-dependent effects of ODN are attributed to charge interactions between the phosphorothioate ODN and proteins found in the extracellular environment, on the cell surface, and intracellularly.97-99 Fibroblast growth factor (FGF) is a well-studied example. Guvaková et al100 have shown that phosphorothioates bind bFGF present on extracellular matrix, and block the binding of FGF to its surface receptor. Phosphorothioates have also been reported to bind DNA polymerases, numerous members of the protein kinase C family, and transcription factors. In addition to these considerations, phosphorothioates are known to activate complement and may impair clotting by binding to factors such as thrombin.101,102 Finally, high concentrations of phosphorothioates inhibit RNase H, thereby decreasing their effectiveness.103

A number of strategies have been introduced to overcome the limitations of phosphorothioates at the same time that their useful properties are preserved. Using end-capped phosphorothioates, where the 5′ and 3′ linkages are sulfated, appears to be a reasonable compromise in that exonuclease stability is conferred on the molecule and side effects associated with fully thioated molecules are correspondingly reduced.104,105 Mixed-backbone oligonucleotides (MBOs) are another example.106 Compounds of this type contain phosphorothioate moieties at the 3′ and 5′ ends for nuclease stability but with modified oligodeoxynucleotides or oligoribonucleotides in the central portion of the molecule to decrease the number of sulfur groups in the molecule. MBOs of this type have been reported to have improved properties compared with phosphorothioate oligodeoxynucleotides with respect to affinity to RNA, RNase H activation, and biological activity. Such molecules are also claimed to demonstrate more favorable pharmacological, in vivo degradation, and pharmacokinetic profiles.106

The chemical modifications that can be made to the phosphodiester linkage are essentially without limit, and many have been made. Two of the more interesting modifications currently under development are the N3′ → P5′ phosphoramidates and the peptide nucleic acids (PNAs). The phosphoramidate modification consists of substituting every 3′-oxygen for a 3′-amino group.107 This creates a highly nuclease-resistant molecule with an ability to form very stable duplexes with single-stranded DNA, and RNA, by Watson-Crick base pairing. The structure of complexes formed by phosphoramidates are quite similar to those of RNA oligomers. In contrast to natural phosphodiesters, the N3′ → P5′ phosphoramidates also form stable triplexes with double-stranded DNA under near-physiological conditions. Although the ability of the phosphoramidates to activate RNaseH is weak in comparison to natural DNA/RNA complexes, they do effectively block translation because of the stability of the DNA/RNA hybrids formed.108 Recent studies suggest that this modification may be useful for controlling cell proliferation109 and HIV viral replication.

In addition to modifications to the internucleoside bridge, examples of sugar and base alterations may also be cited. Changing the sugar’s glycosidic linkage from the naturally occurring β form, to the α anomeric form, where the base is projected in the opposite direction, has been found to increase nuclease stability significantly. However, this compromises
hybridization stability and ability to activate RNase H.\textsuperscript{110} Whether this is a fatal flaw appears to depend on the system being explored. Lavignon et al\textsuperscript{111} have reported that a 20-nt α-oligonucleotide targeted to the primer binding site (PBS) of a murine retrovirus inhibited viral spreading if cells were first permeabilized in the presence of the oligonucleotide. They speculated that antisense activity resulted from a decrease in initiation or inhibition of extension of the minus or plus DNA strands. Chimeric α, β anomic oligodeoxynucleotides have also been reported to be effective antisense compounds, as judged by the ability to inhibit in vitro translation of the pim-1 proto-oncogene because of restoration of the ability of the molecules to activate RNase H.\textsuperscript{112}

Sugars are also typically modified at the 2’ position with O-methyl, fluoro, O-propyl, O-allyl or other groups. These modifications increase affinity for RNA and impart some nuclease resistance. Nevertheless, these molecules do not support RNase H activity and, for this reason, do not appear to have significant activity in some assays.\textsuperscript{113} Therefore, a number of groups have used the 2’-O-methyl modification to flank natural diesters.\textsuperscript{113,114} Such chimeric molecules do activate RNase H if there are at least five internal natural nucleotides.\textsuperscript{113} Alteration of the C5 position of the pyrimidine bases producing the C5 propynyl substitutions have attracted notice because of their affinity for RNA, the stability of the hybrids formed, and their ability to activate RNase H.\textsuperscript{115} Whether the latter property is critical for their activity is in fact uncertain since some have reported that the tight hybrids formed by these compounds are very efficient at blocking translation.\textsuperscript{84} Again, however, these molecules must be used in conjunction with modified bridges because these modifications do not protect against nucleases. In addition, they require a carrier to get them across cell membranes or direct physical injection.\textsuperscript{116}

The PNAs represent a more radical approach to the nuclease resistance problem. Here, the phosphodiester linkage is completely replaced with a polyamide (peptide) backbone composed of (2-aminoethyl) glycine units.\textsuperscript{117} Such compounds are achiral and are completely nuclease resistant as they have no phosphodiester linkages. Since the bases attached to the PNA backbone are projected in space as they would be on a native backbone, the PNAs retain their ability to Watson-Crick base pair with single-stranded DNA or RNA. In addition, homopyrimidine PNAs can form triplexes with double-stranded DNA, and can also displace a duplexed DNA strand to bind with its complement.\textsuperscript{117,118} All of these properties are clearly useful for antisense gene inhibition. Nevertheless, compounds of this type also have problems. Because they cannot move freely across cell membranes they must be injected into cells or delivered with artificial vectors such as phospholipids.\textsuperscript{119} In addition to these problems, the PNAs do not activate RNase H. Accordingly, they most likely exert their antisense effect by blocking RNA elongation which, as in the case of methylphosphonates, may not be as efficient as destruction of the mRNA. Finally, PNAs are also sensitive to local ionic concentration and do not hybridize as well under physiologic conditions.

One additional chemical strategy that is also of interest is the use of circular DNA molecules. Circular DNA and RNA molecules are in fact quite abundant in nature, but it has only been recently that technical problems associated with their synthesis have been solved.\textsuperscript{120} Kool et al, the leaders in this area, have noted that these molecules have a number of attractive qualities which merit their development. First, since they have no 5’ or 3’ end they are resistant to exonuclease attack. In addition, they appear to have excellent binding affinity, sequence specificity, and are capable of activating RNase H. A circular molecule of ~20 to 30 nucleotides in length should be able to target a linear sequence of ~12 to 14 bases. If the circular DNA is composed predominantly of pyrimidines, it will not self-anneal and will therefore remain an open circle. Further, if sequence targeted is a purine-rich area, the circular DNA will be able to form Watson-Crick base pairs with one portion of the circle as well as a triple helix with the resulting duplex as the circular molecule winds around the DNA target. Therefore, an extremely stable structure is formed. Finally, the circular DNAs have excellent strand displacement activity which would, in theory, help them hybridize in areas of RNA that are folded. This could greatly increase “targetable sequence.” Whether this approach will work as well for mRNA targeting remains to be seen. Other, circular RNA/DNA chimeric ODN have been constructed.\textsuperscript{121-124} Experience with these modifications remains limited.

Picking the Right Tool for the Right Job—Oligo DNA Versus Oligo RNA

To inhibit translation, one must make a choice of whether to use a DNA or RNA molecule. Several factors may help facilitate this decision. DNA is inherently more stable than RNA, and is therefore much easier to apply to cells externally in the absence of a delivery vehicle. DNA is also easy to make on automated equipment, especially since the antisense ODN used for this purpose are typically from ~12 to 25 bases long. Placing functional groups on the DNA molecules to facilitate binding to, or destruction of, the mRNA and for tracking the oligonucleotide is also relatively easy. Finally, there appear to be few restrictions of the sequence that can be targeted.\textsuperscript{125} This is in marked contrast to antisense RNA, which must be delivered by vector; ribozymes, which must be targeted to cleavable sites; and parenthetically to TFOs, which must, at least for the moment, be targeted to polyuridine-polypyrimidine stretches of duplexed DNA. Nevertheless, while native DNA is clearly more resistant to nucleases present in serum or cells than RNA, it is still very much subject to degradation in either environment and must often be rendered more resistant by modifying the phosphodiester bridges between nucleosides, or the sugar moieties as was discussed above. In addition, the problems associated with transporting DNA into cells, and getting it to the proper locations for interacting with its target, are not at all trivial and are only now becoming understood.

RNA molecules are attractive because they form more stable duplexes with their mRNA targets. In theory, this might lead to more efficient antisense effects. Nevertheless, because of the stability issues discussed above, antisense RNAs and ribozymes are typically expressed inside the cell from a vector designed for this purpose. This is problematic for all of the reasons that are now widely appreciated, including efficacy of transfection, expression, host cell range, and vector persistence.\textsuperscript{2} Despite these concerns, expressing antisense RNA or ribozymes from a vector is often the only practical approach one can take when...
long-term presence of the antisense sequence is desired, ie, when attempting to target an mRNA that encodes an abundant and long-lived protein. It should be noted that antisense RNA, especially in the form of ribozymes, has been delivered to cells externally.\(^{126}\) For this approach, the RNA molecules must be protected, eg, by stabilizing the phosphodiester bonds, and by packaging the material in liposomes.

**Size Does Matter**

Given the considerations discussed above, an oligonucleotide’s size becomes an important consideration. Antisense ODN are typically synthesized in lengths of 13 to 30 nucleotides. The origin of this convention arises from the fact that there are approximately three to four billion base pairs in the human genome. Statistical calculations based on this number suggest that the minimum ODN size needed to recognize a specific gene is between 12 and 15 bases in length.\(^{78,127}\) This number is convenient because as mentioned above, a phosphodiester oligomer needs to be \(~12\) nucleotides long to form a stable hybrid under physiologic conditions. Nevertheless, these basic considerations need to be modified based on a number of factors, in particular the chemistry of the oligonucleotide. The sulfur modification, for example, lowers the \(T_M\) so in comparison to a natural diester, a phosphorothioate targeted to the same sequence should be made longer by several bases to compensate. Unexpectedly, then, it has been reported that phosphorothioates may be effective and retain specificity with sequences only \(8\) nt in length.\(^{128}\) This finding may be explained in the following way. First, the experiments were performed in a frog oocyte system where the temperature is lower by several degrees in comparison to mammalian cells. Second, it has also been reported that sequence adjacent to the targeted region is also very important in allowing hybridization because such sequence dictates folding and, therefore, secondary and tertiary structure of the molecule.\(^{129-131}\) How targetable sequence may be found will be discussed in more detail below. Sequences longer than the minimal length to guarantee specificity and formation of a stable hybrid are also problematic. Longer sequence may form more stable hybrids through more extensive base pairing, but they are more expensive to synthesize and, somewhat paradoxically, may also suffer from lack of specificity. This is because short runs of complementary bases may hybridize if larger intervening sequences are looped out. Once duplexes form, any of the events discussed above can occur, leading to unintended loss of expression of a nontargeted mRNA. It is obvious then that simple rules, like many factors governing antisense experiments, are only a starting point for individualizing these factors to a particular set of experimental conditions one encounters in the system under study.

**Targeting mRNA-Sequence Selection**

It is straightforward that in order for an antisense molecule to perturb utilization of the mRNA to which it is targeted, the mRNA and the oligonucleotide have to hybridize with each other. As discussed above, hybridization efficiency is primarily dependent on affinity of the ODN for its complementary sequence. Nevertheless, other considerations also apply. It has been reported, for example, that ODN targeted to the 5’ end of a single-stranded loop have orders of magnitude higher affinity for their target than those targeted to the 3’ end.\(^{129}\) This observation may be explained by structural considerations, an intuitively obvious factor if one considers the highly complex folding that mRNA molecules may undertake. Such folding represents a major problem because it is largely unpredictable in vivo and can clearly render sequence inaccessible to the targeting ODN. A straightforward consequence of this situation is that the identifying sequence which is not buried in higher order structure and, therefore, which is accessible to the ODN, is a matter of chance.

A number of strategies have evolved to address the problem of oligo targeting. Because many investigators have reported success targeting around the initiator codons, or the transcriptional start site, this is often the initial target for many experiments. If this approach is unsuccessful, or if it is deemed desirable to target other regions of the mRNA, randomly selected sequence is then resorted to. This approach can be extremely frustrating because chance alone appears to dictate success. In response, it has been suggested that an mRNA “walk” be used as a means for identifying accessible sequence.\(^{132}\) In this approach a series of oligonucleotides are synthesized from the 5’ end of the molecule to the 3’ end. These are then tested for their ability to elicit an antisense effect.\(^{133}\) This method is effective, but because it is trial and error based is not particularly efficient (~25% success rate) and is potentially expensive if many sequences have to be tested before a useful one is found.

Several in vitro model systems for picking mRNA target sequence are being developed. For example, Mishra and Toule\(^{134}\) use an in vitro selection procedure designed to select random ODN sequences capable of hybridizing with a known structure, such a stem loop. Such sequences were called “aptastrucs” because they were likely, or apt, to bind to the structure. In this approach, a population of randomly synthesized oligonucleotides were mixed with the structure of interest and ODN sequences bound to it were selected and amplified. Selection was based on enzymatic digestion of nonduplexed ODN and polymerase chain reaction (PCR) amplification of those that survived. It is of interest that a DNA hairpin structure was used as the model example. Although the procedure was said to be appropriate for either DNA or RNA targets, there is no doubt that its effectiveness might be limited with the latter since predicting structure, as was just noted, is problematic. Computer modeling may be of some utility for predicting accessible RNA sites,\(^{135}\) but more recent studies support the notion that such structural predictions are of little of no use for picking target sequence.\(^{136}\)

Another strategy of interest has been reported by Rittner et al\(^{137}\) and is based on the observation that, at least in prokaryotic systems, the in vitro rate of hybrid formation between antisense RNA and its complement correlates with the antisense molecules effectiveness in vivo. Using HIV as a model, these workers synthesized a set of HIV-1–directed antisense RNAs with the same 5’-end but successively shortened 3’-ends produced by alkaline hydrolysis. The mixture was used to determine hybridization rates for individual chain lengths with a complementary HIV-1–derived RNA in vitro. They found that second order binding rate constants of individual antisense RNAs differed by more than 100-fold. Of interest, slow-
hybridizing and fast-hybridizing antisense RNAs differed by only two or three 3'-terminally located nucleotides in some cases. Of most importance, the binding rate constants determined in vitro for individual antisense RNA species correlated with the extent of inhibition of HIV-1 replication in vivo. Similar studies have been performed on bcr/abl mRNA with similar conclusions. A more complicated approach based on predicted structure and hybridization thermodynamics and been reported by Stull et al. It is of interest that the duplex formation kinetics were the most accurate predictors of ODN efficiency in this model, perhaps because this variable must be a function of target sequence availability.

Recently, Milner et al used a novel hybridization strategy to find oligodeoxynucleotides capable of hybridizing with specific mRNAs. An array of 1938 oligodeoxynucleotides, which ranged in length from monomers to 17 nt, was synthesized on the surface of a glass plate and used to determine the potential of any of the oligodeoxynucleotides to form heteroduplexes with rabbit β-globin mRNA. The oligodeoxynucleotides were complementary to the first 122 bases of mRNA comprising the 5' UTR and bases 1 to 69 of the first exon. These investigators reported that very few oligodeoxynucleotides showed significant heteroduplex formation with the target. Antisense activity, measured in a RNase H assay and by in vitro translation, correlated well with yield of heteroduplex on the array. The investigators point out that their results help to explain the variable success that is commonly experienced in the choice of antisense oligodeoxynucleotides. It is of interest that there were no obvious features in the mRNA sequence, or predicted secondary structure which adequately explained the variation in heteroduplex formation. The investigators suggest that their method may provide a simple though empirical method of selecting effective antisense oligodeoxynucleotides. However, the true test of the predictive value of this method must rest on the ability of the selected oligodeoxynucleotides to effectively interact with their target in vivo. Because RNA folding in vivo is likely to be quite different than that encountered in vitro, this is a critical point. An attempt to address this problem was recently reported by Ho et al, who used semi-random oligodeoxynucleotide libraries to probe a candidate mRNA molecule for RNase H cleavable sites. Oligos predicted to be effective were tested in a biological system where generally good correlation was found.

To address the problem of identifying accessible sequence in mRNA in vivo, we have synthesized reporter ODN composed of a stem-loop structure complexed to a fluorescent (F) moiety on one arm (EDANS) and a nonfluorescent quenching (Q) moiety (DABCYL) on the other. When these molecules hybridize with a complementary nucleotide sequence, the stem loop opens, the fluorophore and quenching moieties separate, and fluorescence is observed at 490 nm when the EDANS moiety is excited by UV light (336 nm). Such ODN have been dubbed “molecular beacons” (MB). We have investigated the utility of MB for demonstrating ODN-mRNA duplex formation in living cells. MB targeting myb or vav mRNA through complementary sequences in the loop region were constructed, and then initially tested in vitro. A threefold molar excess of target sequence was incubated with AS-myb-MB, AS-vav-MB, or their respective control (sense; 6-nt mismatch; complete mismatch) MB in a cell-free system. Quantitative fluorimetry showed that AS-MB generated a greater than 50-fold increase in signal intensity when compared with control MB. The specificity of hybridization in the presence of competing RNA was then tested. MB were incubated with 10 µg of K562 cell-derived total cellular RNA. An ~15-fold greater fluorescence was observed with AS MB than with any of the controls. Potential sensitivity of duplex detection in living cells using fluorescence microscopy was determined by microinjecting preformed MB-mRNA duplexes into K562 cells. Signal could be observed with as little as ~1 × 10^{-4} fg of complex when using a UV fluorescence lens–equipped microscope. Accordingly, detection of MB-mRNA hybridization for many genes should be possible. To test this directly, 550 µmol/L of each MB was microinjected into living K562 cells. Cellular fluorescence was detected with AS-MB but not with any controls. Accordingly, MB may well prove useful for studying the temporal and spatial kinetics of ODN/mRNA interactions in living cells.

**DELIVERY AND SUBCELLULAR TRAFFICKING OF OLIGONUCLEOTIDES**

Delivery and trafficking of oligonucleotides needs to be considered from both a cellular and subcellular point of view. Cellular delivery may be nonspecific, ie, all cells may have an opportunity to take up material or they may be targeted to a particular population. Subcellular trafficking depends on how the oligonucleotide molecule used is sorted within the cell. At the moment, factors that regulate sorting of these molecules is not well understood, but work with ribozymes at least suggests that this issue is critical for cleaving the mRNA target.

**Nucleic Acid Uptake and Trafficking**

It is probably best to first consider the uptake mechanism of naked nucleic acids. Although few studies have been performed with unmodified DNA, uptake of DNA does appear to be a natural phenomenon that has been postulated to represent a nucleic acid salvage mechanism for material excreted by apoptotic cells. A number of laboratories have examined oligonucleotide uptake using either native, methylphosphonate, or phosphorothioate DNA derivatives are uncharged molecules that have been reported to enter cells via passive diffusion, although this concept may represent an oversimplification. In contrast, native and phosphorothioate oligodeoxynucleotides are polyanionic molecules. This charge state makes it very difficult for them to passively diffuse across cell membranes. Not surprisingly then, ODN uptake appears to be primarily an active process dependent on time, concentration, energy, and temperature. Studies from our own laboratory suggest that the uptake mechanism is at least partially concentration dependent and that below a concentration of 1 µmol/L, uptake of phosphorothioate oligodeoxynucleotides is predominantly via a receptortlike mechanism, while at higher concentrations a fluid-phase endocytosis mechanism appears to predominate. Direct physical evidence that ODN may be found within clathrin-coated pits on the cytoplasmic membrane has also been reported. Several receptorlike proteins responsible for uptake have been described. For example, Loke et al reported using affinity chromatography to isolate an 80-kD surface protein that appeared to be responsible for transport. Geselowitz et al used photoactivat-
able cross-linkers to study oligonucleotide binding to HL60 cells and found that several proteins were labeled, the predomi-

nate species being a 75-kD membrane-associated protein. At least five major classes of receptorlike binding proteins were
described by Beltinger et al.147

Although there are suggestions that uptake of ODN may be more efficient in vivo that in vitro,153 a great deal of work is being
done to increase uptake because, as detailed above, ODN uptake is relatively inefficient. Increased cellular delivery will likely
lead to augmentation of antisense effectiveness, and several different strategies have been developed to enhance delivery of
these compounds. Microinjection has been used successfully by many laboratories but is of little use clinically.42,154,155 Other
commonly employed strategies may be classified as those which seek to physically modify the target cell, typically by permeabi-
lining the cell’s membrane, and those which seek to directly or indirectly modify the permeation properties of the ODN.

Physical disruption of target cell membranes may be accomplished by electroporation156,157 or by use of agents such as streptolysin, which permeabi-
zine cell membrane.46,156,157 These methods are clearly only appropriate for ex vivo use, such as bone marrow purging. Both
methods are physically destructive to cells and their ultimate clinical utility remains uncertain. Calcium has been used in recent studies to assist uptake of
AS-ODN into the cellular milieu.158 Attempts to determine the mechanism of Ca⁺⁺/AS-ODN interactions are currently being
examined because double-stranded DNA is known to bind Ca⁺⁺, while inverted repeat hairpin conformations that are
capable of base pairing (palindromes) seem to bind magnesium and zinc.159

An alternative strategy to physical permeation of the target cell is to package the DNA in an artificial “viral vector” which
delivers the oligonucleotide into the cell. Such vectors are typically cationic lipids, which may either be manufactured to
contain the DNA molecule within a lamellar structure (lipo-
some), or mixed with the DNA, allowing the material to coat the DNA by charge interaction.\textsuperscript{116,160-162} Cationic peptides are being developed for a similar purpose.\textsuperscript{163} Whether packaging the DNA in a liposome is superior to merely coating it with cationic lipid is uncertain. In either event, application of the cationic amphiphile condenses the DNA, and imparts an overall positive charge that facilitates attachment to the anionic animal cell membrane while the lipid tails enhance subsequent passage through the lipid bilayer of the cell membrane. At the same time, the DNA is also protected from nuclease attack. While it had been originally assumed that DNA delivered by cationic lipids were “dumped” immediately into the cytoplasm by fusion of the lipid with the cell membrane, this is now known to be incorrect. Rather, it appears that these materials are taken up by endocytosis but, once inside a vesicle, the cationic lipid destabilizes the endosomal membrane. How this is accomplished remains uncertain and is likely complex.

Recent structural studies by Radler et al\textsuperscript{164} revealed that upon addition of either linear \(\lambda\)-phage or plasmid DNA to cationic lipids, an unexpected topological transition from liposomes to optically birefringent liquid-crystalline condensed globules is observed. Study of such structures is expected to shed light on how DNA is inserted through cell membranes. Other studies have suggested that vesicle destabilization induces flip-flop of anionic lipids from the cytoplasmic-facing monolayer, which diffuse into the DNA/lipid complex. DNA is released from the cationic lipid and released into cytoplasm.\textsuperscript{165,166} In the case of polyamines, water is attracted into the vesicle causing it to swell and burst. Once free in the cytoplasm the DNA diffuses into the nucleus. Numerous strategies for increasing the efficiency of cationic lipid delivery of antisense vectors have been reported. These include fusogenic peptides from appropriate virus in the lipid complex.\textsuperscript{167} Other conjugates such as cholesterol or by complexing with a specific antibody to allow targeting to a particular cell type.\textsuperscript{170} In recent studies on uptake into primary hematopoietic cells, Kronenwett et al\textsuperscript{171} reported on the utility, 85% within 1 hour,\textsuperscript{174} rapid clearance of antibody conjugated material, or biologic sequestering in the endosome lysosome compartment\textsuperscript{172,175} also limit effectiveness of this approach and still need to be overcome.

Subcellular Trafficking

Once across the membrane, the intracellular distribution of oligonucleotides is still somewhat controversial and is no doubt at least somewhat dependent on the chemistry of the molecules being studied. Fluorescent-labeled ODN microinjected into the cytoplasm of cells are found to rapidly accumulate in the nucleus.\textsuperscript{155} However, when fluorescent-labeled ODN are placed in tissue culture media, the labeled molecules accumulate in vacuoles, presumably endosomes and lysosomes, within the cytoplasm forming a punctuate perinuclear pattern.\textsuperscript{176} These results are in accord with most studies examining uptake of oligonucleotides from the extracellular environment. In contrast to ODN localization when the compounds are introduced by microinjection, there is no visible fluorescence in the nucleus itself, indicating that the release of ODN from these vacuoles is an inefficient process. Our own studies have visualized this process at the ultrastructural level\textsuperscript{147} and show that oligonucleotides progress through the lysosomal, endosomal compartments.

If the oligonucleotides escape the endosome/lysosome compartment, it appears that they migrate rapidly into the nucleus.\textsuperscript{146,147,177} The mechanism appears to be one of diffusion\textsuperscript{147,177} with subsequent trapping of the oligonucleotide, apparently by binding to a unique set of nuclear binding proteins.\textsuperscript{177} The fate of oligonucleotides localized to the nucleus is an important issue in terms of the bioavailability of these compounds. Should the majority of the oligonucleotide become tightly complexed to nuclear protein they may become biounavailable and, therefore, inactive. Alternatively, modifications designed to prevent nuclear import might also have favorable qualities because such molecules would likely reach higher cytoplasmic concentrations and should, therefore, have a greater chance of associating with ribosomal mRNA. This, of course, presumes that molecules with the desired modifications will be released intact from the vesicles in which they were imported. Accordingly, strategies aimed at increasing release of ODN from endosomal structures, such as the use of fusogenic peptides derived from the influenza hemagglutinin envelope protein, may also be successful for similar reasons.\textsuperscript{178-180}

Recent studies with ribozyme molecules support the hypothesis that intracellular sorting and localization of the antisense molecules may be critical for their activity. In addition to targeting to a cell type, it is likely that subcellular targeting may also be an important consideration.\textsuperscript{181} Sullenger and Cech\textsuperscript{69} critically examined this possibility by determining whether colocalization of ribozymes with their intended mRNA target would increase their efficiency. To perform their experiments, the investigators used a packaging cell line infected with a retrovirus (B2A) containing a copy of the lacZ gene. This line was then infected again with a B2A retrovirus engineered to express a lacZ ribozyme. It was then hypothesized that B2A transcripts destined to be packaged in replicating virus would be processed together in the same nuclear tracts while those that were fated to be distributed in the cytoplasm as mRNAs would be processed in different tracts. If colocalizing the ribozyme
with its target (lacZ mRNA) was important for activity of the ribozyme, this could be determined by measuring a decrease in titer of B-gal virus while B-gal activity, the result of translating lacZ mRNA, would be relatively unaffected. This, in fact, proved to be the case as the ribozyme reduced the titer of infectious virus containing lacZ by 90%, but had no effect on B-gal activity in the cells. These results convincingly argued that ribozymes needed to be in physical proximity of their target, and there is little reason to suspect that this would not be true for oligonucleotides as well.

Bertrand et al\textsuperscript{70} have also performed similar experiments and have reached similar conclusions. Their study was also designed to better understand the influence of RNA transcript context on RNA localization and catalytic RNA efficacy in vivo. Towards this end, they constructed and characterized several expression cassettes useful for transcribing short RNAs with well-defined 5' and 3' appended flanking sequences. These cassettes contain promoter sequences from the human U1 snRNA, U6 snRNA, or rRNA Meti genes, fused to various processing/stabilizing sequences. The levels of expression and the subcellular localization of the resulting RNAs were determined and compared with those obtained from Pol II promoters normally linked to mRNA production, which include a cap and polyadenylation signal. The rRNA, U1, and U6 transcripts were nuclear in localization and expressed at the highest levels, while the standard Pol II promoted transcripts were cytoplasmic and present at lower levels. The ability of these cassettes to confer ribozyme activity in vivo was tested with two assays. First, an SIV-growth hormone reporter gene was transiently transfected into human embryonic kidney cells expressing an anti-SIV ribozyme. Second, cultured T lymphocytes expressing an anti-HIV ribozyme were challenged with HIV. In both cases, the ribozymes were effective only when expressed as capped, polyadenylated RNAs transcribed from Pol II cassettes that generate a cytoplasmically localized ribozyme that facilitates colocalization with its target. The inability of the other cassettes to support ribozyme-mediated inhibitory activity against their cytoplasmic target is very likely due to the resulting nuclear localization of these ribozymes. These studies show that the ribozyme expression cassette determines its intracellular localization and, hence, its corresponding functional activity.

**PHARMACODYNAMICS OF OLIGODEOXYNUCLEOTIDE DRUGS**

To examine the pharmacokinetics and metabolism of AS-ODN, extensive investigations have been carried out in a variety of animal systems, and in a few human trials as well. Most reports examine the kinetics of intravenous or intraperitoneal administration. Of interest is that the material appears to be absorbed from the gastrointestinal tract as well as other routes.\textsuperscript{182} Using a phosphorothioate modified (20 nt) ODN, Agrawal et al\textsuperscript{114,183} examined the effects of intravenous and intraperitoneal administration in mice. In these studies, approximately 30% of the administered dose was excreted in the urine over the first 24-hour period while ODN accumulated preferentially in the liver and kidney. Similar results were reported in subsequent studies.\textsuperscript{184-187} ODN accumulate in the kidney and liver at concentrations that exceed plasma levels. In mice, the brain apparently is a privileged site into which very little ODN accumulates. In monkeys, the plasma half-life of ODN is similar to that of rodents.\textsuperscript{185} The organs showing the highest accumulation of ODN were kidney, liver, thymus, bone marrow, lymph nodes, salivary glands, lungs, and pancreas. Moderate accumulation was found in muscle, gastrointestinal tract, and trachea. The lowest accumulations were found in brain, spinal cord, cartilage, skin and prostate.

More recent studies in nude mice have confirmed these earlier reports, and made comparison between the pharmacokinetics and tissue distribution of phosphorothioate (PS), methyllphosphonate (MP), and phosphorodithioate (PS2) oligonucleotide analogs.\textsuperscript{96} In these experiments, radiolabeled 15-nt oligonucleotides complementary to the AUG region of K-ras were used in nude mice bearing a K-ras-dependent human pancreatic tumor. The oligonucleotide analogs were administered as a single dose in the tail vein. A rapid distribution phase with t1/2 values of 1 minute or less and an elimination phase with average t1/2 values of 24 to 35 minutes was observed. Volumes of distribution (Vd) were 3.2, 4.8, and 6.3 mL for PS2, MP, and PS, respectively, in comparison to 3.6 mL for sucrose, a fluid-phase marker. In general agreement with previous studies, relative tissue drug levels obtained at 1 and 24 hours after administration were kidney > liver > spleen > tumor > muscle. Total kidney and liver oligonucleotide accumulation was approximately 7% to 15% of the initial dose, with tumor accumulating 2% to 3%. Intact compound was recovered from all tissues, including tumor, as assessed by high-pressure reversed-phase HPLC coupled to radiometric detection. Importantly, integrity of the oligonucleotides ranged from 73% in blood to 43% to 46% in kidney and liver. Kidney and liver appear to be the primary sites of metabolism.

In humans, studies have been performed on patients with leukemia\textsuperscript{101,188} and acquired immunodeficiency syndrome (AIDS).\textsuperscript{189} In six HIV patients, pharmacokinetic and toxicology data were gathered after 2-hour intravenous infusions (0.1 mg/kg) of 35S-labeled phosphorothioate oligonucleotide.\textsuperscript{189} Plasma disappearance curves could be described by the sum of two exponential, with half-life values of 0.18 ± 0.04 and 26.71 ± 1.67 hours. The radioactivity in plasma was further evaluated by polyacrylamide gel electrophoresis, showing the presence of both intact oligonucleotide and lower molecular weight metabolites. Urinary excretion represented the major pathway of elimination, with ~50% of the administered dose excreted within 24 hours and ~70% eliminated over 96 hours after dosing. Intact as well as degraded material was found in the urine. Of interest, the half lives of the oligonucleotide were shorter than those observed in experimental animals. The mechanisms responsible for the differences were not clear but could obviously be caused by species differences in metabolic capacity, renal clearance, and serum protein binding. In the first reported case where phosphorothioate ODN were infused into a patient with ALL, only slight increases in γ-glutamyl transpeptidase (γGTP) values, transient metallic taste, nausea, and vomiting were described.\textsuperscript{188} As with any new pharmaceutical agent, the potential side effects of antisense ODN are important to consider and determine. ODN, depending on their target, may disturb the expression of genes not only in neoplastic cells but also in normal, healthy cells. This raises the issue of differential sensitivity.
between normal and diseased cells. It is clear that if a gene common to both populations is being targeted, the normal cells must be more resistant to loss of the target gene’s function than the diseased cell. We might also expect some non-specific side effects of infused ODN which are not related to their ability to interact specifically with nucleotides or proteins. For example, one potential side effect of treatment with phosphorothioate ODN may be thrombocytopenia. Mice receiving high doses of phosphorothioate ODN manifest a decrease in platelet counts. It is hypothesized that the thrombocytopenia is related to the polyanionic charge of the ODN. Cardiovascular toxicity and deaths in monkeys after intravenous infusions of phosphorothioate ODNs has also been reported. Adverse events were noted only at large doses (5 to 10 mg/kg) and after rapid bolus administration. Slow infusion of similar doses appeared to be well tolerated. The toxic effects were believed likely to be due to complement activation, but release of anaphylatoxins, autotoxins, eicosanoids, leukotrienes, or cytokines are also possibilities.

POTENTIAL APPLICATIONS OF THE ANTISENSE STRATEGY IN CLINICAL HEMATOLOGY

Although oligonucleotides have been reported as having activity against HIV, cytomegalovirus (CMV) infections of the eye, and most recently Crohn’s Disease, this portion of the review will focus on potential uses of oligonucleotides in the treatment of hematologic disorders. These applications are of interest because they capitalize on “antisense,” as well as aptameric effects. The former have found the greatest application in the treatment of hematologic neoplasms, while the latter have been used as thombin inhibitors for anticoagulation purposes.

Chronic Myelogenous Leukemia (CML) as a Paradigm for Nucleic Acid Therapeutics

As of this writing, oligonucleotides are being used as ex vivo bone marrow purging agents, an approach pioneered by my own group and as potential drugs for direct in vivo administration. Most development and clinical experience has been gained with the ex vivo application, largely because of cost considerations. Marrow purging requires relatively small amounts of material in comparison to direct in vivo administration. It is also true that most development has occurred in the context of attempting to treat CML. The latter disease has been a lightning rod for development of this approach because of the presence of a tumor-specific mRNA target that all agree is important in the pathogenesis of the disease. However, as will be detailed below, choice of gene target is not always obvious and many factors must be taken into account in developing an effective therapeutic oligonucleotide in this and other diseases.

If one considers the molecular pathogenesis of CML, a number of potentially interesting gene targets to which oligonucleotides might be directed present themselves. First and foremost is the \textit{bcr/abl} mRNA itself. \textit{Bcr/abl} mRNA is the product of a neogene created by a reciprocal translocation involving the \textit{c-abl} gene on chromosome 9 and the \textit{bcr} gene on chromosome 22 (recently reviewed in Melo and colleagues). This translocation results in the formation of the Philadelphia chromosome and the neogene \textit{bcr/abl}. There are three major \textit{bcr/abl} variants depending on where the invariant portion of \textit{abl} is fused with \textit{bcr}. These are designated b1a2 (p185), b2a2, and b3a2 (both encode p210). A fourth variant e19a2 (p230), which is associated with a chronic neutrophilia, has been recently described. Each of these genes may be considered tumor specific and are likely both necessary and required for the CML transformation of hematopoietic cells. Because they are tumor specific, and pathogenetically important, they are an obvious oligonucleotide target in this disease.

Szczylak et al were among the first to suggest that oligonucleotides targeting \textit{bcr/abl} might be of therapeutic utility. These workers found that when primary leukemic blast cells were exposed to synthetic 18-nt oligodeoxynucleotides complementary to \textit{b2a2 or b3a2 bcr/abl} junctions, leukemic cell colony formation in vitro was significantly suppressed. In contrast, granulocyte-macrophage colony formation from normal marrow progenitors was unaffected by the same oligonucleotides. Suppression of colony growth was found to be sequence specific as well in that mismatched oligonucleotides had no effect on colony formation and oligonucleotides targeting one breakpoint did not inhibit growth of cells expressing the other. Finally, when equal proportions of normal marrow progenitors and blast cells were mixed, exposed to the oligodeoxynucleotides, and assayed for residual colony formation, the majority of residual cells were normal. These findings suggested that \textit{bcr/abl} targeted oligonucleotides represented a means for specifically killing CML cells. This work was followed by a number of reports from the same group, and others, which reported very similar findings.

Not all investigators have been able to reproduce the results described above. O’Brien et al, for example, examined the effects of antisense oligonucleotides of various lengths, sequences, and chemistry on the proliferation of eight different cell lines, five derived from patients with CML and three from other sources. They found that phosphodiester oligonucleotides had little antiproliferative activity in their system, presumably because of degradation by nucleases present in fetal calf serum or rapid intracellular breakdown. Phosphorothioate oligonucleotides antisense, but not sense, to the B2A2 and B3A2 breakpoints significantly inhibited the proliferation of three CML cell lines (BV173, LAMA84, and KYO1). However, the antiproliferative effect appeared to be independent of the type of breakpoint expressed by each cell line, suggesting that the inhibition was sequence dependent but not sequence specific. Other investigators have reported that inhibition of CML cell growth with oligonucleotides is not sequence dependent, suggesting that an aptameric effect is responsible.

Ribozymes have been applied against this target and appear to give rise to more specific, but still imperfect, cleavage of their target. An explanation for some of the controversy surrounding the ability of this breakpoint to be specifically cleaved may be found in recent work from the Szczakel laboratory. It was previously reported that rapid association of antisense sequence with its target is the most critical determinant of antisense efficacy. In the course of designing oligonucleotides and ribozymes directed to the \textit{bcr/abl} breakpoint, kinetic probing combined with calculations of the local folding potential indicated that the \textit{bcr/abl} fusion point sequences are not easily accessible for complementary nucleic acid hybridization. The study suggested that selective and
efficient antisense sequences should be directed against the \( bcr \) portion neighboring the fusion point as well as the first eight nucleotides of the \( abl \) sequence. To effectively inhibit this target then, relatively high doses of oligonucleotides may be required, and these in turn may produce nonspecific effects. In addition, if ribozymes with unfavorable kinetics are used to target the sequence, inhibition is likely to be inefficient and, therefore, difficult to measure.

Despite the caveats addressed above, Zhao et al\(^\text{214} \) reported a clever and apparently successful strategy for inhibiting growth of CML cells with an anti-\( bcr/abl \) ribozyme delivered by a retroviral vector that also delivered a methotrexate resistance gene. The hypothesis underlying these experiments was that transfer of a vector that combined a drug-resistance gene with anti-BCR/ABL antisense sequences might allow for posttransplant chemotherapy to decrease persistent disease while rendering inadvertently transplanted CML stem and progenitor cells functionally normal. A retroviral vector (LasBD) that combined a methotrexate-resistance gene and antisense sequences directed at the b3a2 BCR/ABL breakpoint was constructed. Cells engineered to express \( bcr/abl \) were transduced with LasBD and selected in methotrexate (MTX) for 14 days. Expression of the antisense sequences was reported to reduce \( BCR/ABL \) mRNA and p210(BCR/ABL) protein levels by 6- to 10-fold in most cells with apparent normalization of cell growth. LasBD also rendered 20% to 30% of primary \( Ph^+ \) and \( Ph^- \) CD34(+) cells MTX-resistant and decreased \( BCR/ABL \) mRNA levels in MTX-resistant \( Ph^- \) CD34(+) cells by 10-fold. LasBD decreased tumorigenicity of 32D \( BCR/ABL \) cells in vivo by 3 to 4 logs. Based on these results, the investigators concluded that the methotrexate resistance gene in the LasBD vector could protect normal hematopoietic cells from MTX-mediated toxicity, whereas the AS sequences in LasBD could suppress expression of the \( BCR/ABL \) gene and restore normal function of \( BCR/ABL \) cDNA-containing cells. Although the system described was highly constrained, these results were nonetheless very encouraging.

Regardless of the controversy over whether \( bcr/abl \) mRNA can be effectively destroyed, and whether such destruction leads to cell death, several groups have proceeded to determine the potential therapeutic utility of antisense \( bcr/abl \) nucleic acids for inhibiting CML cell growth. The general strategy has been to transplant CML blast cells into severe combined immunodeficient (SCID) mice and then to examine the effects of oligonucleotides on leukemic cell growth and animal survival, an approach originally reported by our group in 1992.\(^\text{195} \) For example, Skorski et al\(^\text{215} \) injected SCID mice with \( Ph^+ \) CML blast crisis cell line (BV173) and monitored expression of \( bcr/abl \) transcripts in bone marrow, spleen, peripheral blood, liver, and lungs. Once disease was established in these mice, treatment with a 26-nt \( bcr/abl \) AS-ODN at a dose of 1 mg/d for 9 days decreased \( bcr/abl \) mRNA levels in mouse tissues and induced the disappearance of CD10\(^+ \) and clonogenic leukemic cells. Further, antisense treated mice survived 18 to 23 weeks while control and sense treated mice were dead 8 to 13 weeks after leukemic cell injection.

To improve on these results, a strategy using oligonucleotides with traditional chemotherapy, either in the context of ex vivo bone marrow purging,\(^\text{216} \) or treating CML-bearing SCID mice directly with “low-dose” cyclophosphamide and \( bcr/abl \) targeted oligonucleotides\(^\text{217} \) has been reported. With regard to marrow purging, Skorski et al reported that a 1:1 mix of \( Ph^+ \) leukemic cells that had been exposed to a combination of low-dose mafosfamide and \( bcr/abl \) AS-ODN appeared to be effectively (\( \sim \)100%) purged of \( Ph^+ \) cells by clonogenic assay. Indeed, \( bcr/abl \) transcripts were only found in untreated cells, in cells treated with mafosfamide, or with AS-ODN alone, but not in those treated with combination therapy. This strategy was extended to direct in vivo treatment where CML-bearing SCID mice were treated intravenously with \( bcr/abl \) oligonucleotides and 25% of what would normally be a therapeutically effective dose of cyclophosphamide. This treatment strategy was reported to result in cure of 50% of the animals.

**Alternative Gene Targets in CML**

Although \( bcr/abl \) is an obvious target for antigene therapies, a number of considerations suggest that it may not be as ideal a target as first considerations would suggest. It must be recalled that since anti-mRNA strategies based on the use of oligodeoxynucleotides are transient interventions, the target has to be expressed when oligonucleotides are physically present in the cell. In addition, it is straightforward that the strategy will only be effective if the target cell fails to tolerate transient downregulation of the targeted mRNA. There is reasonable evidence to suggest that \( bcr/abl \) mRNA is not expressed in CML stem cells, despite the fact that the translocation is present.\(^\text{219} \) Because CML is a stem cell disorder it is reasonable to hypothesize that the candidate gene for perturbation must be expressed at the stem cell level to be effective. If this is not the case, a constant supply of CML progenitors will be derived from the untreated stem cell. In addition to this important consideration, it is not at all clear that transient perturbation of \( bcr/abl \) expression results in cell death.\(^\text{219,220} \) Finally, given the known redundancy of signaling pathways, one might also be concerned that \( bcr/abl \)-mediated activation of Ras might have alternative pathways in primary malignant cells. For all these reasons then, we chose to pursue nonobvious mRNA targets.

**Transcription Factors**

\( c-myb \). Myb protein is encoded by the \( c-myb \) protooncogene, a member of a transcription factor family composed of at least two other highly homologous genes designated A-myb and B-myb.\(^\text{221,222} \) Located on chromosome 6q in humans, \( c-myb \)'s predominant transcript encodes a 7-kD nuclear binding protein (Myb) which recognizes the core consensus sequence 5'-PyAAC/G/PyG-3'.\(^\text{223} \) The protein plays a major role in regulating G1/S transition in cycling hematopoietic cells, and likely functions as a transactivator of a number of important cellular genes such as the Kit receptor,\(^\text{85,224} \) CD4,\(^\text{225} \) and CD34.\(^\text{226} \)

Attempts to exploit the \( c-myb \) gene as an antisense ODN target in CML was an outgrowth of studies which sought to define the role of Myb protein in regulating normal human hematopoiesis.\(^\text{227} \) These studies showed that exposing normal bone marrow mononuclear cells (MNC) to \( c-myb \) antisense ODN resulted in a decrease in cloning efficiency and progenitor cell proliferation. The effect was lineage indifferent because \( c-myb \) antisense DNA inhibited granulocyte-macrophage colony-
forming units (CFU-GM), BFU-E (erythroid), and CFU-Meg (megakaryocyte). In contrast, c-myc ODN with the corresponding sense sequence had no consistent effect on hematopoietic colony formation when compared with growth in control cultures. Finally, inhibition of colony formation was also dose related. Inhibition of the targeted mRNA was also demonstrated. Sequence-specific, dose-related biologic effects accompanied by a specific decrease or total elimination of the targeted mRNA were strong pieces of evidence to suggest that the effects we were observing were due to an “antisense” mechanism. As mentioned above, the mechanism responsible for inhibition of hematopoietic cell growth appeared to be an inducible block in G1/S transition.228 In other investigations it was also determined that hematopoietic progenitor cells appeared to require Myb protein during specific stages of development, in particular when they were actively cycling.229 as might be expected given the above functional description of Myb protein. Accordingly, Myb appeared to be critical for normal hematopoietic cell development, a finding that was largely confirmed using the technique of homologous recombination.230

Myb protein is also required for leukemic hematopoiesis. Evidence to support this statement includes the fact that Myb is widely expressed in malignant cell lines, and inhibition of Myb with oligonucleotides inhibits malignant cell line growth.231 Growth of cells derived from primary patient material is also inhibited.234 However, to be useful as a therapeutic target, leukemic cells would have to be more dependent on Myb protein than their normal counterparts, and such data have been provided in vitro84,194 as well as in vivo in an SCID mouse model using human leukemic cells.195

Why does downregulating Myb kill leukemic cells preferentially? Our initial studies on the function of the c-kit receptor in hematopoietic cells suggested that c-kit might be an Myb-regulated gene. Additional studies have since confirmed this.224,232,233 Because c-kit encodes a critical hematopoietic cell tyrosine kinase receptor,234 we have hypothesized that dysregulation of c-kit expression may be an important mechanism of action of Myb AS ODN. In support of this hypothesis it has been shown that when hematopoietic cells are deprived of c-kit R ligand (Steel factor) they undergo apoptosis.235 It has also recently been shown that when CD56bright natural killer (NK) cells, which express c-Kit, are deprived of their ligand (Steel factor), they too undergo apoptosis, perhaps because bcl-2 is downregulated.236 Malignant myeloid hematopoietic cells, in particular CML cells, also express c-kit and respond to Steel factor. Accordingly, we postulate that perturbation of Myb expression in malignant hematopoietic cells may force them to enter an apoptotic pathway by downregulating c-kit. Preliminary studies of K562 cells exposed to c-myc antisense ODN show that such cells do undergo nuclear degenerative changes characteristic of apoptosis. Of necessity, we must also postulate that normal progenitor cells, at least at some level of development, are more tolerant of this transient disturbance. Since neither Steel nor White Spotting (W) mice (which lack the Kit ligand or its receptor, respectively) are aplastic, this is a tenable hypothesis.

c-myc Work from Sawyers et al237,238 has suggested that in the absence of functional Myc, bcr/abl is unable to transform primary mouse marrow cells. Based on these studies, Skorski et al have targeted c-myc and bcr-abl simultaneously in an effort to improve on results obtained by targeting bcr/abl alone.240 In vitro CFU assays and SCID mouse studies were conducted. The latter were of particular interest because they represent a potentially relevant treatment model. In these studies, animals were injected with primary blast crisis CML cells and were then treated systematically with equal doses of bcr-abl, c-myc, or bcr-abl + c-myc targeted phosphorothioate oligodeoxynucleotides. Compared with mice treated with individual compounds, the disease process appeared to be better controlled in the group treated with both oligonucleotides, although the animals were not cured. The investigators suggested that targeting multiple cooperating oncogenes might be a useful strategy for increasing the effectiveness of oligonucleotide therapeutics.

Receptors and signaling proteins. Other potential mRNA targets for treating CML might also be envisioned. These may be found among the surface receptors and signaling proteins constitutive to hematopoietic cells. For example, we have previously suggested that the Kit receptor might serve this purpose.85 As noted above, Kit appears to be a c-myc–regulated gene, and downregulation of Kit in hematopoietic cells is associated with induction of apoptosis, perhaps an important mechanistic component of anti-Myb’s ability to kill malignant cells. Directly comparing the ability of anti-Myb and anti-Kit targeted oligonucleotides to inhibit the growth of leukemic cells suggests this possibility while at the same time supporting the potential of Kit-targeted oligonucleotides in the treatment of CML and other leukemias.

A newer target that may prove to be of even greater utility in patients with hematologic malignancies is signaling protein encoded by the vav proto-oncogene.231 Some Vav is expressed exclusively in hematopoietic cells where it is assumed to play a role in signaling. The importance of this role is uncertain, in part because of conflicting functional studies that have used different strategies for abrogating Vav gene expression in murine embryonic stem cells.232,233 Our studies with vav-targeted oligonucleotides lend some support to each of the conflicting reports cited above and imply that vav would be a therapeutically attractive target in CML.12 We have found that while vav appears to be required for erythropoiesis in both normal and malignant hematopoietic cells, malignant myeloid cell growth, in particular myeloid cells derived from CML patients, does appear to be dependent on Vav expression.12 The rationale for Vav as an antisense target is therefore anchored in the fact that it is differentially used in normal and malignant cells.

Early Clinical Experience With Oligonucleotide Drugs in the Treatment of CML

Early clinical experience with oligonucleotides has been reported by several groups. In the context of CML, we have reported, in preliminary communication, results of our clinical trials to evaluate the effectiveness of phosphorothioate modified ODN antisense to the c-myc gene as marrow purging agents for chronic phase (CP) or accelerated phase (AP) CML patients, and a phase I intravenous infusion study for blast crisis (BC) patients, and patients with other refractory leukemias.93 ODN purging was performed for 24 hours on CD34+ marrow cells. Patients received busulfan and cytoxan, followed by re-infusion of previously cryopreserved ODN purged MNC. In the pilot
marrow purging study 7 CP and 1 AP CML patients have been treated. Seven of 8 patients engrafted. In 4 of 6 evaluable CP patients, metaphases were 85% to 100% normal 3 months after engraftment, suggesting that a significant purge had taken place in the marrow graft. Five CP patients have shown marked, sustained, hematologic improvement with essential normalization of their blood counts. Follow-up ranges from 6 months to ~2 years. In an attempt to further increase purging efficiency we incubated patient MNC for 72 hours in the AS-ODN. Although PCR and LTCIC studies suggested a very efficient purge had occurred, engraftment in five patients was poor. In the phase I systemic infection study there were 18 refractory leukemia patients (2 patients were treated at two different dose levels; 13 had AP or BC CML). Myb AS-ODN was delivered by continuous infusion at dose levels ranging between (0.3 mg/kg/d × 7 days) to (2.0 mg/kg/d × 7 days). No recurrent dose-related toxicity has been noted, although idiosyncratic toxicities, not clearly drug related, were observed (1 transient renal insufficiency; 1 pericarditis). One BC patient survived ~14 months with transient restoration of CP disease. These studies show that ODN may be administered safely to leukemic patients. Whether patients treated on either study derived clinical benefit is uncertain, but the results of these studies suggest to us that ODN may eventually demonstrate therapeutic utility in the treatment of human leukemias.

Some clinical experience with bcr-abl–targeted antisense oligonucleotides in CML has also been reported. de Fabritis et al.202 treated a patient with CML in accelerated phase with autologous bone marrow transplantation. Before reinfusion, cells were purified in vitro with a 26-nt phosphorothioate antisense oligodeoxynucleotide specific for the B2A2 junction. This treatment resulted in a 24% and 41% reduction of CFU-GM and CD34+ cells, respectively. The patient was successfully engrafted with the purified marrow cells after 17 and 25 days for platelets and neutrophils, respectively. Using fluorescence in situ hybridization in interphase nuclei, some Ph+ cells were found after the autograft. The patient was reported to be in a complete hematologic remission at 9 months posttreatment.

**OLIGONUCLEOTIDES IN THE TREATMENT OF ACUTE LEUKEMIA AND LYMPHOMA**

It is obvious that the number of potential gene targets for the hematologic and other malignant disorders is enormous, and the literature is replete with examples of “antisense knockouts” that attempt to demonstrate the utility of such targets under a variety of experimental conditions. Many of these reports use cell lines, as opposed to primary cells, and one could also argue with the completeness of the controls used to demonstrate that an antisense effect has actually been achieved. Two of these targets bear particular mention because oligonucleotides designed to inhibit their function have been employed in clinical trials. These are, respectively, p53 and bcl-2.

p53 is a tumor suppressor gene that is mutated in many forms of neoplasia, including the blast phase of CML and acute leukemia.247 Though perhaps a nonobvious target for inactivation, the group at the University of Nebraska has pursued this gene’s mRNA as an antisense target in a variety of hematologic malignancies because of results obtained in vitro culture systems. This group has reported that when leukemia cell lines and primary patient cells are exposed to p53-targeted oligodeoxynucleotides, inhibition of colony growth may be observed.248,249 Treatment of normal bone marrow cells with p53 oligos has no effect on colony formation, therefore suggesting differential sensitivity. The mechanism for this effect is uncertain, but the preclinical results were believed to be promising enough to lead these investigators to use a p53-targeted oligodeoxynucleotide for bone marrow purging249 and for systemic therapy of treatment refractory leukemias.188,250 In the most recent study, a 20-nt phosphorothioate oligonucleotide complementary to p53 mRNA [OL(1)p53] was used in a phase I dose-escalating trial conducted to determine the toxicity of the oligodeoxynucleotide after systemic administration to patients with hematologic malignancies. A total of 16 patients with either refractory acute myelogenous leukemia (n = 6) or advanced myelodysplastic syndrome (n = 10) were treated. Patients were given OL(1)p53 at doses of 0.05 to 0.25 mg/kg/h for 10 days by continuous intravenous infusion. No specific toxicity directly related to the administration of the compound was observed. One patient developed transient nonoliguric renal failure. One patient died of anthracycline-induced cardiac failure. Approximately 36% of the administered dose of OL(1)p53 was recovered intact in the urine. Plasma concentrations and area under the plasma concentration curves were linearly correlated with dose. Leukemic cell growth in vitro was inhibited compared with pretreatment samples. There were no clinical complete responses. The investigators concluded that this particular phosphorothioate oligonucleotide can be administered systemically without complications and speculated that it might prove useful in combination with currently available chemotherapy agents for the treatment of malignancies. Whether any of the effects reported was due to an antisense mechanism is uncertain because this was not specifically looked for.

The bcl-2 gene was first discovered because of its involvement in the t(14;18) chromosomal translocations commonly found in lymphomas. The translocation results in deregulation of bcl-2 gene expression and cause inappropriately high levels of bcl-2 protein production (recently reviewed in Reed et al.251). Bcl-2 blocks the apoptotic process that normally contributes to cell death and thereby facilitates neoplastic cell expansion. Overproduction of the bcl-2 protein also prevents cell death induced by nearly all cytotoxic anticancer drugs and radiation, thus contributing to treatment failures in patients with some types of cancer. Based on these observations a number of groups have been interested in blocking bcl-2 function with antisense oligonucleotides and ribozymes, in particular for the treatment of lymphomas.

Within the past year Webb et al treated nine patients with refractory non-Hodgkin’s lymphoma with a bcl-2–targeted phosphorothioate oligodeoxynucleotide based on preclinical studies which suggested that the oligonucleotide could cause disease regression in an animal model.95,252 The oligonucleotide used was targeted to a portion of the bcl-2 mRNA open reading frame of the bcl-2 mRNA and was delivered as a daily subcutaneous infusion for 2 weeks. Toxicity was scored by the common toxicity criteria, and tumor response was assessed by computed tomography scan. This study was also more rigorously controlled in terms of mechanism because an antisense
effect was specifically assessed by quantification of \textit{bcl-2} expression, and \textit{bcl-2} protein levels in treated patients as assessed by flow cytometry. During the course of the study, the daily dose of \textit{bcl-2} antisense was increased incrementally from 4.6 mg/m\textsuperscript{2} to 73.6 mg/m\textsuperscript{2}. Save for local inflammation at the site of infusion, no treatment-related toxic effects occurred. In two patients on the study, computed tomography scans showed a reduction in tumor size which were stated to be “minor” and “major,” respectively. In two patients, the number of circulating lymphoma cells was also found to be decreased during treatment. Other indicators of response, such as a decrease in LDH, were also reported. \textit{Bcl-2} protein levels were measured by flow cytometry in five patients and were found to be decreased in two. The investigators concluded that in these patients the antisense therapy led to an improvement in symptoms, and was accompanied by some objective biochemical and radiological evidence of tumor response. Based on these favorable responses the investigators also speculated that \textit{bcl-2} antisense therapy might also prime cells for improved chemotherapeutic response. In common with the experience of others then, the antisense compounds appear to be relatively nontoxic and there is at least the suggestion that in some patients useful responses may be observed. No doubt however, these are likely due to a combination of aptameric as well as antisense effects.

**OLIGONUCLEOTIDES AS ANTICOAGULANT DRUGS**

As mentioned above, it is well known that proteins can bind to DNA via sequence specific recognition motifs and by charge interactions. Phosphorothioates in particular are noted to have these properties and one consequence is the prolongation of clotting times in patients receiving relatively large and rapid bolus injections of these materials. These facts have been exploited by some to screen for novel inhibitors of clotting protein function. An example relevant to this review is the use of oligonucleotide inhibitors of thrombin.\textsuperscript{255} The substrate specificity of thrombin is dependent on two electropositive surfaces which interact with fibrinogen and heparin, respectively. Knowing this, Tasset et al\textsuperscript{255} used a combinatorial screening methodology to identify single-stranded oligonucleotides manifesting high-affinity binding to human thrombin. With this approach they reported finding a 29-nucleotide DNA sequence that bound to human thrombin with a \textit{kd} of approximately 0.5 nmol/L. The antithrombin oligonucleotide inhibited thrombin-catalyzed fibrin clot formation in vitro likely by binding to the heparin-binding exosite. Additional inhibitors of the intrinsic clotting pathway have also been described, the mechanism for which appear to binding to fibrinogen.\textsuperscript{254} Given the relative expense of oligonucleotides in comparison to heparin or similar drugs it is uncertain whether oligonucleotides could be made cost efficient, although there are clearly circumstances where the availability of such drugs, for example in patients with heparin-induced thrombocytopenia, might be of clinical use.

**FUTURE PROSPECTS FOR “ANTISENSE” THERAPY**

The prospects for developing effective nucleic acid therapeutics are ultimately dependent on solving the problems that were alluded to in the preceding discussion. The rationale for moving forward with this approach is compelling. However, clearly there is frustration with these techniques as exemplified in particular with the antisense oligonucleotide strategy.\textsuperscript{99,255-258} Some of this is caused by unreasonable expectations based on naive assumptions about how these molecules work. While the theory is straightforward, the complexity of the task should now be much more apparent. We have tried to discuss these problems in a straightforward manner in the course of reviewing this approach to gene therapy. Nevertheless, emphasizing those issues we believe to be of paramount importance may be useful for moving the field forward.

First, delivery of oligonucleotides remains an important problem, and parenthetically, one which is not dissimilar to that which is faced by investigators who are seeking to deliver viral vectors into a broad range of cell types. In our view, an efficient delivery system would likely make available oligonucleotide chemistries effective and might increase the effectiveness of these molecules in a dramatic way. In this regard it is useful to recall that the oligonucleotide must not only cross cell membranes, but must then sort to a location where they may then begin to interact with their mRNA target. The ability to deliver ODN into cells and have them reach their target in a bioavailable form must be further investigated.\textsuperscript{259} Without this ability, it is clear that even an appropriately targeted sequence is not likely to be efficient. Recently described strategies for introducing ODN into cells, including various cationic lipid formulations, may address this problem.\textsuperscript{116,196,260} Colleagues attempting to deliver vector sequence to cells share this problem.

Second, finding single-stranded mRNA within the bowl of “spaghetti and meatballs” that may serve as a useful analogy for a highly folded RNA molecule and its associated proteins remains a major limiting step. It is obvious that if the targeted sequence is buried within the core of a structurally complicated mRNA molecule, or rendered inaccessible by an associated protein, then hybridization of the oligonucleotide and mRNA may not be possible. Cells have certainly evolved mechanisms for unwinding and editing RNA, and it may be possible to exploit these mechanisms for solving this problem. Colleagues with an interest in RNA biology might make important contributions to this area by addressing this critical issue.

We also need to consider the issue of sequence-dependent effects which are unrelated to hybridization with a specific message. These non-antisense, so-called aptameric effects may be related to the chemistry of the oligonucleotide used and continue to confound the issue of specific gene inhibition. Reports that four consecutive guanines (G-4 tract) within a larger phosphorothioate oligomer have a non-antisense antiproliferative effect is but one example.\textsuperscript{261,262} In our hands this has not been an issue,\textsuperscript{88} suggesting that cell type used may be an important issue. The products of ODN degradation such as 2’-deoxyadenosine or high concentrations of thymidine are toxic to cells and inhibit cell proliferation.\textsuperscript{263} Micromolar concentrations of dAMP or dGMP can have pronounced cytotoxic or cytostatic effects on cells, particularly those derived from hematopoietic tissues.\textsuperscript{264} In addition, phosphorothioate analogs may bind to ribosomes resulting in nonspecific inhibition of protein synthesis,\textsuperscript{265} as well as effects on cell growth, cell morphology, enzyme activities, and even viral proliferation.\textsuperscript{103,266-269} Hybridization through partially matched sequences has also been raised as a problem.\textsuperscript{41} The latter seems
a less important consideration because it has been shown that as few as two base mismatches result in loss of antisense effectiveness. However, having said this, it is useful to point out that aptameric effects may be as useful as specific anti-mRNA effects in the context of achieving a desired biological effect. In this context, the contribution of an aptamer is welcome. It is only when investigating the biology of a specific gene that these effects need to be clearly defined and understood.

Other issues which bedevil this field, such as the problem of reproducibility of experiments, attest to the need for careful experimental technique and attention to detail. Factors such as differences in nucleic acid synthesis, handling, storage, and purification are often overlooked in failed experiments. In many laboratories verification of the sequence ordered and its stability under the conditions that it is being used are simply not carried out. Finally, differences in experimental conditions, cell use, and familiarity with the methodology may also be contributory factors. These comments are applicable to all experimental methods.

Many patients suffering from hematologic, as well as nonhematologic malignancies remain incurable. These conditions, as well as others alluded to above, afflict a substantial number of individuals, often in their most productive years. Treating patients with highly toxic drugs and ionizing radiation is unpleasant for all connected with this process, especially when there is little hope for cure. Nucleic acid therapeutics, with their promise of exquisite molecular specificity and concordant limited toxicity, continue to be highly attractive pharmaceutical prospects for this reason. We have no doubt then that the goal of making useful “antisense” drugs will be achieved. The pace at which this will occur is dependent, as always, on the ability of the field to continue to attract talented investigators, and agencies disposed to funding their research.

ACKNOWLEDGMENT

We thank Drs. Arthur Kreig, Cy Stein, and Peter Glazer for helpful comments during the preparation of this manuscript. The editorial assistance of Elizabeth R. Bien is also gratefully acknowledged.

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Nucleic Acid Therapeutics: State of the Art and Future Prospects

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