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Mini-Review

Antisense oligodeoxynucleotides as antiviral agents*

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Antisense; Drug development; Oligonucleotide; Translation arrest; Transcription arrest

Introduction

A new paradigm is needed for the development of antiviral drugs. Most drugs that have been developed work according to a paradigm in which a small organic molecule, often of natural origin, fits into an enzyme active site. This interaction usually requires 3–4 hydrogen bonds that confer most of the specificity. Such drugs are discovered rarely in random screening programs.

Another paradigm is the incorporation into the putative drug molecule of a higher degree of information, that ensures greater specificity through more hydrogen bonds. Perhaps, the most specific interaction that has been discovered in biology is the Watson-Crick base-pairing found in DNA duplexes. This is the basis of the genetic code, that must be stable, selective and reproducible. It has also evolved over millennia. An informational drug might have encoded in it the complementary, or antisense, base sequence, to a target cellular nucleic acid sequence. In practical terms, this means using an oligodeoxynucleotide targeted at a sequence in the mRNA of the gene to bring about translation arrest (Cohen, 1989a).

This term was originally coined in relation to the expression in prokaryotes of antisense mRNA that is produced naturally as a regulatory process in relation to the expressed, or sense, mRNA (Inouye, 1988). The strategy of producing antisense mRNA through the medium of a plasmid vector has been used to regulate sense

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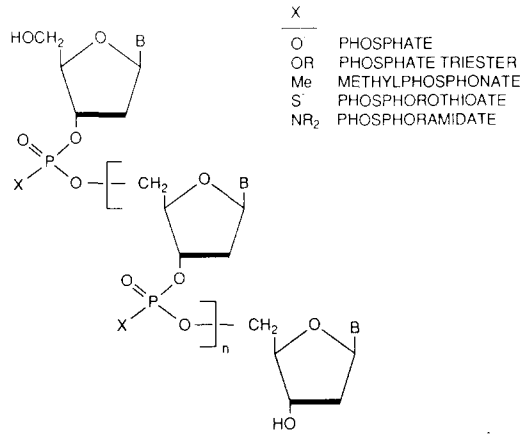

Fig. 1. The structure of an oligodeoxynucleotide showing some common modifications at the phosphodiester group (B = A, T, C, G).

mRNA expression, but is not anticipated to work effectively as a therapeutic approach. Notably there are difficulties in maintaining control of mRNA transcription (Kerr et al., 1988), and in eukaryotic cells, due to the presence of an RNA duplex unwindase activity (Bass and Weintraub, 1987; Rebagliati and Melton, 1987).

The concept of using a complementary oligodeoxynucleotide (or oligo for short) to hydrogen bond with a target cellular mRNA is deceptively simple. In practice many problems arise that can be traced to the assumptions built into the strategy. It is assumed that: (1) oligos will be stable in vivo; (2) oligos will enter cells; (3) oligos will hybridize effectively with their target sequences; and, (4) oligos will not bind to sites other than the unique complementary sequence. All of these assumptions require adjustment of the general strategy that will be considered in this article.

Strictly speaking, these assumptions cannot be dealt with separately since they are interconnected. The first assumption, that of stability, can be shown to be invalid for natural oligos with phosphodiester linkages (Fig. 1) due to the ubiquity of deoxyribonucleases (Wickstrom, 1986). Thus, great efforts have been made in the past few years to develop chemically modified oligo analogs in which the simplest substitution is made in the backbone, in order to confer nuclease-resistance without disrupting the ability to hybridize effectively. Examples of such analogs are phosphotriester (Miller et al., 1971), methylphosphonate (Miller et al., 1985), and phosphorothioate (Matsukura et al., 1987) (Fig. 1). Note that the former two are neutral molecules, and as such might be expected to be taken up more readily by cells than the phosphorothioates (or S-oligos) that are isoelectronic with the natural congeners. Changes in molecular structure can also be expected to have an effect on the ability of these substances to hybridize to form stable duplexes. This can be determined by measurement of their melting characteristics (Stein et al., 1988).

The minimum length of the oligo required for a unique sequence to exist in mammalian mRNA is approx. 14 bases. Of course, this is a statistical result, and

consequently to have a margin of safety most researchers use 15–30-mers. The viral genome is of course much smaller, but the specificity must be that of the viral genome in the presence of the human genome. It also transpires that the length of oligo required for effective duplex formation, particularly for modified analogs under conditions of less than ideal stringency, is also of this extent.

It should be noted that the mono-substituted backbone analogs (X= OMe, Me, S^-) are chiral, because they have four different substituents around the tetrahedral phosphorus atom. This leads to two stereoisomers at each P atom, or 2^n for n P atoms. In order to overcome this problem, and yet retain nuclease-resistance, some di-thio compounds have recently been synthesized (Neilsen et al., 1988; Farschtschi and Gorenstein, 1988; Dahl et al., 1989). Whether their other characteristics will make them useful analogs is yet undetermined. Other oligo analogs that have been developed as putative antisense agents are modified in the deoxyribose moiety (Rayner et al., 1989). This is a difficult tactic because of possible steric alterations in the effectiveness of the Watson-Crick base pairing interaction, that is the basis of the overall strategy.

Since exonucleases are predominant in vivo, it has been suggested that blocking the termini, particularly the 5′ end, should be sufficient to ensure that most oligos can reach their target intact. However, this ignores the fact that endonuclease activity, while minor, can be devastating for an antisense strategy, because the cleavage of an oligo will produce shorter oligos that can interact at secondary sites, and possibly produce toxic side effects, although they will hybridize less efficiently. For a long-lived drug molecule it seems evident that biological stability is essential, and so terminal blocking without endonuclease protection is deemed insufficient. In general, it is incumbent upon anyone suggesting a particular oligo analog to research its ability to resist nucleases, to hybridize effectively, and to bring about the desired viral inhibition.

Inhibition in model expression systems

For any oligo to be considered a serious candidate for antisense inhibition it must be able to exhibit translation arrest in a model expression system. One example will be given here, of a comparison made between the natural, the phosphorothioate and the α-oligo analogs (Fig. 2) microinjected into *Xenopus* oocytes (Cazenave et al., 1989).

Fig. 3 shows the results for 17-mers antisense against the β-globin mRNA in the normal phosphodiester and the phosphorothioate constructs. Two things should be emphasised: (1) the S-oligo was more effective in inhibiting β-globin expression on a comparative molar basis; (2) when the oligo was injected six hours prior to the β-globin mRNA, the natural oligo lost all inhibitory activity, while the S-oligo retained most of its activity. This latter result can be explained on the basis of the nuclease suspectibility of the natural oligo, and the nuclease-resistance of the S-oligo. The α-oligo was not effective at inhibiting translation in this system, and this was attributed to its inability to activate RNase-H (Gagnor et al., 1987; Verspierian

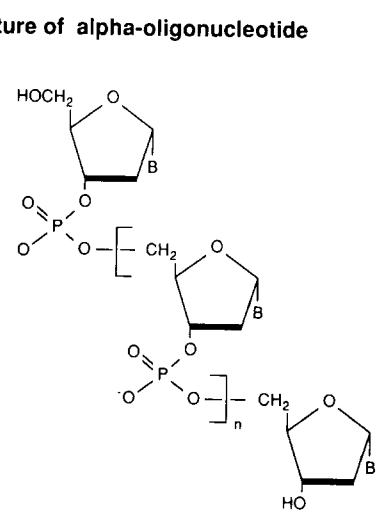

Fig. 2. Structure of the non-natural glycosidic α-configuration in an oligodeoxynucleotide.

et al., 1987), while the other two oligos do activate RNase-H (Walder and Walder, 1988; Cazenave et al., 1989). On the basis of these (Cazenave et al., 1989) and other experiments it can be concluded that the S-oligos are effective antisense inhibitors of expression.

Cellular uptake of oligodeoxynucleotides

In order to be effective inhibitors of translation, oligos must be able to penetrate the cell membrane. This has generally been considered unlikely for charged polyelectrolytes such as oligodeoxynucleotides. The uncharged oligo analogs were deliberately developed to overcome this limitation. Since we have reviewed this topic recently (Jaroszewski and Cohen, 1990), I will not enter into details here, except to say that fluorescently labelled or ^{35}S-labelled S-oligos have been shown to be taken up by cells, and that this process appears to involve a surface receptor protein of 85 kDa for which the charged analogs compete (Loke et al., 1990). By contrast, the methylphosphonate analogs do not compete for uptake, and presumably enter cells by passive diffusion. However, many factors appear to influence the uptake process (Jaroszewski and Cohen, 1990), including the type of cells studied (and presumably the nature and number of cellular receptors), the length and type of oligo analog, the fluorescent group attached, the medium used, and possibly even the sequence of the oligo, so that generalizations are difficult to make, except to say that oligos definitely enter cells. The larger question of oligo pharmacology in vivo is still a matter of active research.

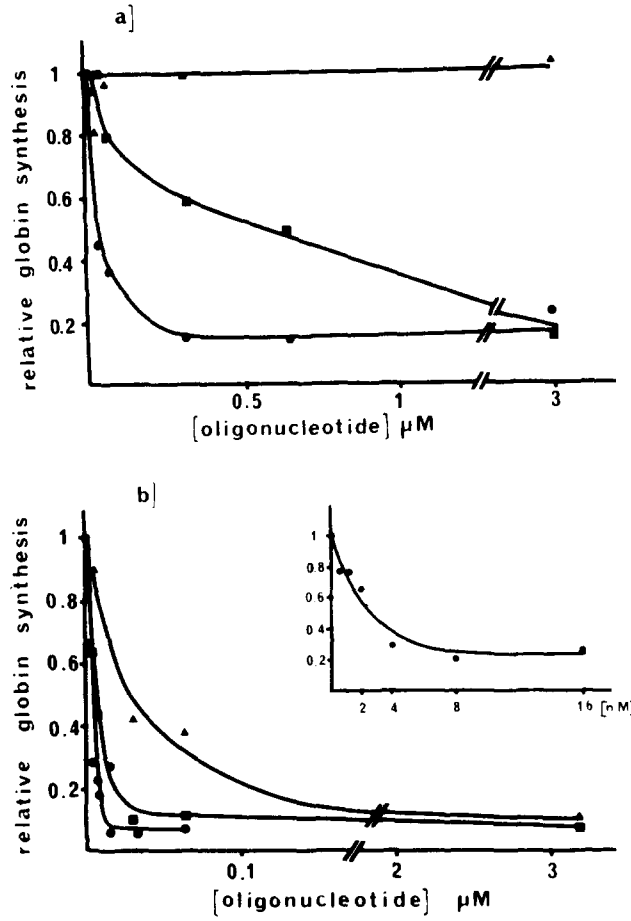

Fig. 3. Effect of a 17-mer antisense to the β-globin mRNA (a), and its phosphorothioate analog (b). Globin synthesis was determined from densitometer tracings of the autoradiographs, relative to expression determined in the absence of added oligodeoxynucleotide. ●, oligos coinjected with mRNA; ▲, oligos injected 6 h prior to mRNA; ■, oligos injected 6 h after mRNA. The inset in panel (b) is an enlargement of the lowest curve in (b) (from Cazenave et al., 1989). Reprinted by permission of Oxford University Press from Nucleic Acids Res., Vol. 17, p. 4266 (1989).

Oligos as antiviral agents

A partial list of the use of oligos as antisense inhibitors of translation in several viral systems is given in Table 1. The earliest application using manually synthesized natural oligos was that of Zamecnik and Stephenson (1978). The methyl-phosphonate analogs have been applied to several viruses, initially by Miller et al. (1985). The application that I will use as an example here is that of HIV. This is chosen not only because of its importance as a target in human disease, but also because it is perhaps the most extensively studied virus by the antisense oligo approach, and I have reviewed this topic recently (Cohen, 1990). The application

TABLE 1

Inhibition of virus expression by antisense oligonucleotides

Virus	Oligo analog	Reference
Rous sarcoma	Natural	Zamecnik et al., 1978
VSV	P-Me	Agris et al., 1986
	Natural	Lemaitre et al., 1987
	Natural	Wickstrom et al., 1986
HSV	P-Me	Smith et al., 1986
SV40	P-Me	Westermann et al., 1989; Miller et al., 1985
Influenza	Natural	Zerial et al., 1987
Sendai	Natural	Gupta et al., 1987
TMV	Natural	Crum et al., 1988
HIV	Natural	Zamecnik et al., 1986
	P-S	Matsukura et al., 1987, 1988, 1989a; Agrawal et al., 1988; Shibahara et al., 1989
	P-N	Agrawal et al., 1988
	P-Me	Sarin et al., 1988; Zaia et al., 1988

VSV, vesicular stomatitis virus; HSV, herpes simplex virus; SV40, simian virus 40; HIV, human immunodeficiency virus; TMV, tobacco mosaic virus; P-Me, methylphosphonate; P-S, phosphorothioate; P-N, phosphoramidate.

of antisense oligos to HIV also serves to illustrate the complexities that can arise in the application of a complex substance such as an oligodeoxynucleotide analog to a complex system such as HIV infected T-cells.

First, it should be pointed out that the results of such inhibition are not consistent in all the studies. Specifically the initial claim that natural oligos inhibit HIV (Zamecnik et al., 1986), has not been reproduced by others (Matsukura et al., 1987; Zamecnik et al., 1986; Shibahara et al., 1989; Matsukura et al., 1989). However, since the results are to some extent assay-dependent, and the target sequences differ in each case, some caution should be exercised in interpreting this apparent discrepancy. A similar discrepancy however seems to occur for the methylphosphonate oligo analogs (Matsukura et al., 1987; Sarin et al., 1988). It is agreed however that the S-oligos are the most effective inhibitors of HIV (Matsukura et al., 1987; Agrawal et al., 1988). Phosphoramidate oligos have also been reported to exhibit inhibitory activity (Agrawal et al., 1988).

The initial observation that the S-oligos inhibit HIV in an acute cytopathic infection system in a sequence non-specific manner (Fig. 4) (Matsukura et al., 1987; Matsukura et al., 1988), while fortuitous, was quite puzzling. Subsequent work revealed that antisense inhibition of HIV translation occurs in a chronic infection assay using an anti-*rev* 28-mer S-oligo sequence (Fig. 5) (Matsukura et al., 1989). This result has recently been confirmed by the use of *pol* and *gag*-directed oligo sequences in a different chronic assay using different cells (Kinchington et al., 1990). Formally, the antisense inhibition could have the retroviral RNA as its target, but it is considered most likely that the mRNA is the actual complement. The mechanism of the sequence non-dependent inhibition has been clarified by studies in which (1) the stage of inhibition has been shown to be prior to reverse transcription (Matsukura et al., 1987), and (2) concomitantly the S-homo-oligomers, such as

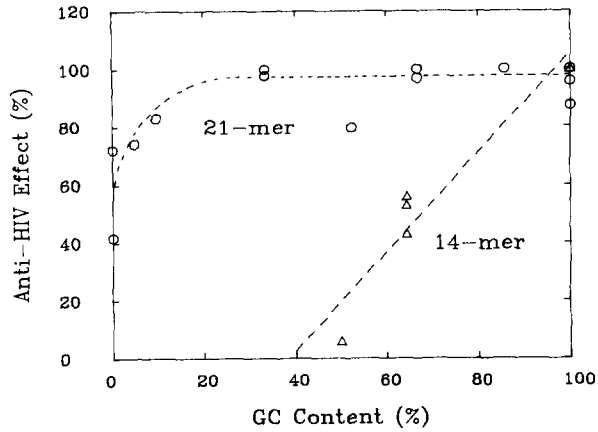

Fig. 4. Sequence non-specific inhibition in a cytopathic assay. The number of viable ATH8 cells after >7 days incubation plotted against the GC content for a series of S-oligo 21-mers (o) and 14-mers (Δ). Data points were obtained at concentrations of 1 μM for the 21-mers and 5 μM for the 14-mers (from Stein et al., 1989).

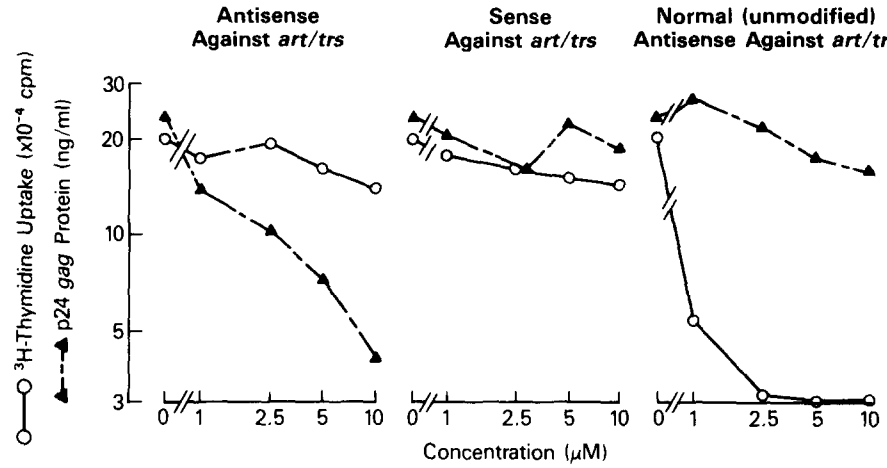

Fig. 5. Antisense inhibition of expression of HIV p28 *gag* protein in chronically infected H9 cells using the phosphorothioate 28-mer anti-*rev* sequence (left), and the absence of inhibition with the sense S-oligo and the normal antisense 28-mer; the reduction in [^{3}H]thymidine uptake in this case can be attributed to the production of cold thymidine due to hydrolysis of the normal oligo (from Matsukura et al., 1989).

S-dC14, have been shown to be effective linear competitive inhibitors of HIV reverse transcriptase (Majumdar et al., 1989; Molling et al., 1990). Thus, two distinct mechanisms have been shown to occur in interactions of S-oligos with HIV (Fig. 6), which illustrates the fact that a single substitution in the backbone of an oligodeoxynucleotide can cause significant alterations in its biological properties, and hence the need for caution in interpreting results in such complex biological systems.

Since the interaction of S-oligos with RT are clearly selective, while being

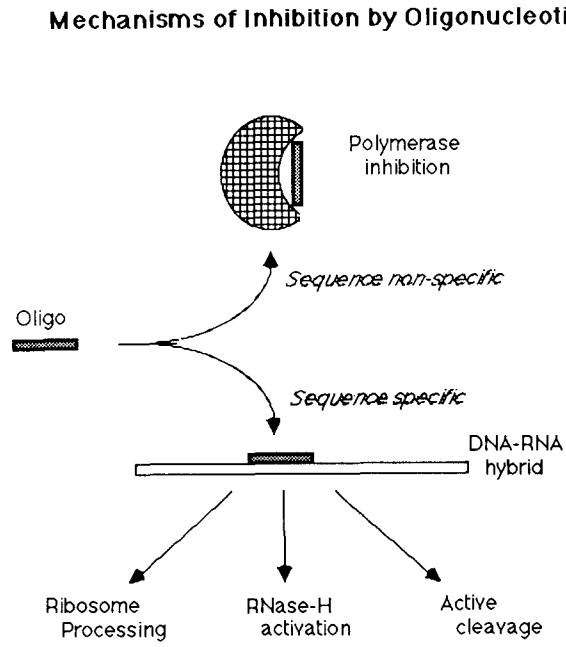

Fig. 6. Representation of two mechanisms of inhibition of HIV by S-oligos. Inhibition of reverse transcriptase is sequence non-specific, while inhibition of mRNA expression is an antisense sequence specific mechanism.

sequence non-specific, these compounds were tested against other polymerases. They were found to be specific for the inhibition of HSV-2 polymerase, while being relatively non-inhibitory to HSV-1 and human polymerases (Gao et al., 1989). This activity has now been extended to the inhibition of the HSV-2 itself in in vitro studies (Gao et al., 1990). The molecular basis for the selective interaction of S-oligos with HIV RT, and HSV-2 polymerase is unknown, but should be the subject of further investigations. However, it is a serendipitous finding, albeit with a non-antisense mechanism.

Future directions

Three aspects of the antisense approach suggest themselves for future exploration: (1) other DNA backbone modifications and other oligo analogs; (2) triplex formation between DNA duplexes and oligodeoxynucleotides (Fig. 7); (3) antisense autocatalytic ribo-oligomers, or ribozymes (Fig. 8). Each of these topics will be covered briefly.

In relation to (1), many other backbone modifications can be imagined (Cohen, 1989b), but few chimeric analogs have been prepared in sufficient quantity and examined in any detail so far. There is of course the possibility of combining different backbone modified analogs, to produce specific extents of charge for example

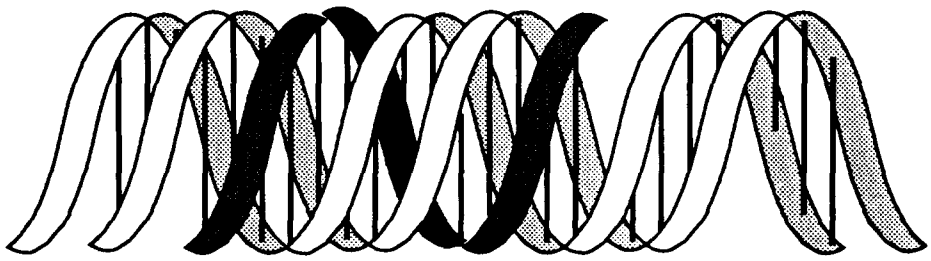

Fig. 7. Formation of a triple-stranded structure, or triplex, with a third oligo strand binding in the major groove of the DNA duplex.

by combining charged and neutral analogs. Thus, it it possible to make a long oligo with a small net charge. The effect of such combinations on the criteria as hybridization and cell uptake are a matter of some interest.

It is also possible to make oligo conjugates with groups attached at either terminus, and this topic has recently been reviewed (Goodchild et al., 1990). The attached group can be present for the purpose of (1) blocking exonucleases, (2) increasing stability of duplex formation due to intercalation, (3) acting as a fluorescent probe, or (4) reacting with and attaching to or destroying the complementary strand. While many groups have been attached, only a few attempts have been made to specifically increase the activity of a known antisense effect by these means (Verspieren et al, 1987; Mori et al., 1989). The attachment of multiple groups, for example one at each end to confer increased cellular uptake and reactivity at the target site, is another versatile aspect of the oligo approach.

It has been known for some time that polynucleotides form triple stranded structures, termed triplexes (Fig. 7), as well as duplexes (Felsenfeld, 1957). These involve the binding of the third strand in the major groove of the B-form, with base triple formation between T with AT and C^+ or G with GC. Triplex formation has

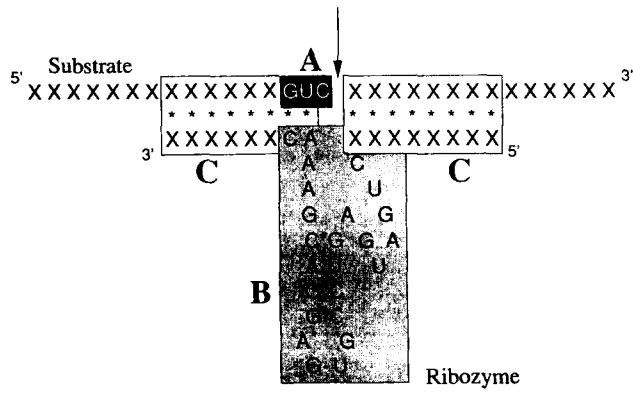

Fig. 8. Structure of a catalytic RNA or ribozyme. The three structural domains are: A, target sequence in mRNA substrate adjacent to site of cleavage; B, the highly conserved sequence in the ribozyme; C, the flanking sequences that comprise the antisense segments (from Haseloff and Gerlach, 1988). Reprinted by permission from Nature, Vol. 334, p. 587. © 1988 Macmillan Magazines Limited.

been shown to occur between oligos and the DNA duplex, and this has been developed as a major approach in transcription arrest (Dervan et al., 1989). It should be emphasised that the code for triplex formation by an oligo is not the same as the standard Watson-Crick base pairing scheme. An oligo has been shown to inhibit transcription of *myc* in a cell-free system by triplex formation (Cooney et al., 1988) and more recently in HIV (McShan et al., 1990). The use of oligos to replace transcription factors that bind to specific sequences on the DNA duplex is another adaptation of the triplex approach (Dervan et al., 1989). The development of nuclease-resistant oligo analogs that will nevertheless still exhibit triplex formation with DNA is a challenge of this approach.

mRNA splicing has been shown to take place by autocatalytic hydrolysis and trans-esterification of the RNA precursors, in which the 2′-OH of a specific sequence represents an active site, equivalent to that of an enzyme (Zaug and Cech, 1986). This approach has been adapted to the design of so-called hammerhead ribozymes (Haseloff and Gerlach, 1988) that could be produced inside the cell by the introduction of viral or plasmid vectors, and could then attack and hydrolyse a specific target RNA sequence, whether in viral RNA or mRNA (Fig. 8). This has recently been demonstrated in the case of HIV (Sarver et al., 1990). However, the development of ribozymes as a therapeutic modality faces many difficulties, not the least of which is that the ribozyme itself is potentially subject to degradation by the abundant ribonucleases in vivo.

Conclusion

There are many avenues that diverge from the concept of an oligonucleotide as an informational drug containing the instructions for its mode of action in the base sequence. Oligodeoxynucleotide analogs can cause translation arrest, transcription arrest, and enzyme inhibition. They can bind specifically to their complementary sequence, or selectively to a protein site. In considering such means of obtaining therapeutic selectivity we may be entering a new era of antiviral agents.

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