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Review

# Morpholino antisense oligomers: the case for an RNase H-independent structural type

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**Abstract**

RNase H-competent phosphorothioates (S-DNAs) have dominated the antisense field in large part because they offer reasonable resistance to nucleases, they afford good efficacy in cell-free test systems, they can be targeted against sites throughout the RNA transcript of a gene, and they are widely available from commercial sources at modest prices. However, these merits are counterbalanced by significant limitations, including: degradation by nucleases, poor in-cell targeting predictability, low sequence specificity, and a variety of non-antisense activities. In cell-free and cultured-cell systems where one wishes to block the translation of a messenger RNA coding for a normal protein, RNase H-independent morpholino antisense oligos provide complete resistance to nucleases, generally good targeting predictability, generally high in-cell efficacy, excellent sequence specificity, and very preliminary results suggest they may exhibit little non-antisense activity. © 1999 Elsevier Science B.V. All rights reserved.

*Keywords:* Antisense; Morpholino; RNase H-independent; Specificity; Efficacy; Delivery

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## 1. RNase H cleavage: origins of the broad acceptance of S-DNAs

A key requirement for effective antisense oligos is that they remain intact for many hours in the extracellular medium and within cells. The methylphosphonate-linked DNA analogs developed by Miller and Ts'o in the late 1970s constituted a major advance in the emerging antisense field by providing the first antisense type having good stability in biological systems [1]. However, concerns subsequently developed that methylphosphonates might be inadequate for many antisense applications, particularly therapeutics, because of their low efficacy – typically requiring concentrations in excess of 20 μM for good activity in a cell-free translation system [2]. Some time later phosphorothioate-linked DNA analogs (S-DNAs) were introduced [3]. These S-DNAs were enthusiastically embraced because they achieved good efficacy at concentrations a 100-fold lower than corresponding methylphosphonates [4]. The S-DNAs also had good water solubility, reasonable resistance to nucleases, and they were readily prepared on standard DNA synthesizers with only modest modification of the oxidation step.

The surprisingly high efficacy of the S-DNAs relative to methylphosphonates was not readily explained by their moderately higher target binding affinities. Investigations into this large discrepancy in efficacies led to the discovery that while methylphosphonates and most other antisense types act only by a *steric block* mechanism, DNA and S-DNA oligos instead act predominantly by an *RNase H-cleavage* mechanism wherein after the oligo pairs to its RNA target sequence the enzyme RNase H can cleave the paired RNA target sequence [5]. It was also discovered that those structural types which function only by a steric block mechanism (*RNase H-independent* types) are generally effective in blocking translation only when targeted against mRNA sequences in the region extending from the 5′ cap to a few bases past the AUG translational start site (see Fig. 2). In contrast, structural types which utilize an RNase H-cleavage mechanism (*RNase H-competent* types) could also be effective against target sequences elsewhere in the RNA transcript of a gene. Representative RNase H-competent and RNase H-independent antisense types are shown in Fig. 1.

The explanation for these differing targeting properties lies in the mechanism of protein translation. In eukaryotic systems an initiation complex recognizes and binds to the 5′ cap structure and then scans down the 5′ leader sequence until it encounters the AUG translational start site, at which point the full ribosome is assembled, followed by translation of the amino acid coding region of the mRNA. Antisense oligos apparently can physically block progression of the initiation complex down the mRNA leader and block assembly of the ribosome at the AUG translational start site. However, once ribosome assembly occurs at the translational start site that ribosome is capable of displacing almost any bound antisense oligo it encounters as it traverses the amino acid coding region of the mRNA. Presumably this oligo displacement is effected by the very robust ATP-driven unwindase activity of translating ribosomes [6].

Thus, most RNase H-independent antisense types can block translation only when targeted against sequences in the region from the 5′ cap to about 25 bases past the AUG translational start site of an mRNA. In contrast, RNase H-competent oligos can also be effective against sequences elsewhere in the RNA transcript by virtue of their effecting deg-

radation of the paired RNA target sequence by RNase H.

*To summarize*: Based on the higher efficacy and greater targeting versatility of S-DNAs relative to the early RNase H-independent oligos, many workers in the antisense field have concluded that RNase H competency is essential for good antisense activity. This, combined with their ready availability and low cost, have established S-DNAs as the structural type currently most used in antisense studies.

## 2. Limitations of S-DNAs

With continued study of S-DNAs it is now widely recognized that their good efficacy and targeting versatility are counterbalanced by a variety of disadvantages.

### 2.1. Degradation

S-DNA oligos are sensitive to nucleases, being degraded in biological systems over a period of hours [7]. Such instability can complicate interpretation of experimental results and may require either shorter-than-desired experiments or multiple dosing.

### 2.2. Cleavage of non-targeted RNA sequences

RNase H cleaves DNA/RNA and S-DNA/RNA duplexes as short as 5 or 6 base pairs in length and is highly active against such duplexes only 9–10 base pairs in length [8]. As a consequence, essentially every RNase H-competent oligo has the potential to form transient complexes with and induce cleavage of 'non-targeted' cellular sequences having partial homology to the intended target RNA. It seems reasonable to expect that this RNase H cleavage could compromise the sequence specificity of S-DNAs. In simple cell-free translation systems with added RNase H poor sequence specificity is indeed seen with S-DNAs [9]. This same RNase H cleavage might also be expected to cause disruptions in more complex cellular systems and in patients.

### 2.3. Promiscuous binding

While both DNA and S-DNA support RNAse H cleavage, because DNA oligos undergo rapid degradation in biological systems S-DNAs have become by default the choice for RNase H-competent antisense oligos. The problem this presents is that the pendent sulfurs in the phosphorothioate linkages of S-DNAs interact with a wide variety of proteins, including laminin, bFGF, protein kinase C, DNA polymerase, telomerase, fibrinogen, phospholipase $A_2$, HIV gp120, HIV reverse transcriptase, CD4, *Taq* polymerase, T4-polynucleotide kinase, fibronectin, many tyrosine kinases, and proton-vacuolar ATPase [10]. For this and other reasons S-DNAs can cause multiple non-antisense effects.

In addition, S-DNAs containing the sequence Pu-Pu-C-G-Py-Py have been shown to trigger B cell activation [11] and S-DNAs containing four or more contiguous guanines have been shown to form a tetrameric complex which can cause a variety of non-antisense effects [12]. S-DNAs within cells have also been reported to rapidly induce Sp1 transcription factor [13].

The non-antisense effects caused by S-DNAs can result in control oligos exhibiting biological activities on a par with that of the antisense oligos [14]. Further, because S-DNAs can effect multiple non-antisense activities it is difficult to confirm that a given biological response is truly due to an antisense mechanism – leading to considerable uncertainty and possible misinterpretations in antisense experiments utilizing S-DNAs [15].

*To summarize*: Because of their sensitivity to nucleases, limited sequence specificity, and multiple non-antisense effects it appears that S-DNAs are less than optimal antisense tools.

## 3. Is RNase H competency necessary?

### 3.1. Efficacy

A key property of the RNase H-competent S-DNAs which led to their broad adoption by the antisense community was their greatly increased efficacy (likely a consequence of their RNase H competency) relative to methylphosphonates. However, since then at least two RNase H-independent types (PNAs [16] and morpholinos [17] shown in Fig. 1) have been developed which often match or exceed the efficacy

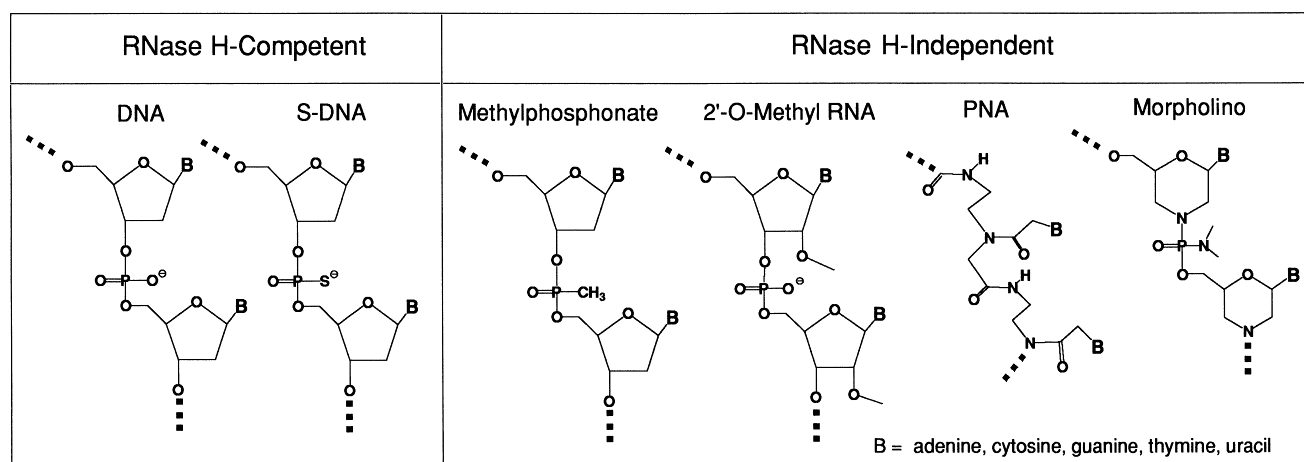


Fig. 1. Representative RNase H-competent and RNase H-independent types.

of S-DNAs in a cell-free translation system when said oligos are targeted to sequences in the region from the 5′ cap to about 25 bases 3′ to the AUG translational start site [9].

In our cultured cell test system these advanced RNase H-independent antisense types (morpholinos and PNAs) show an even greater efficacy advantage over the RNase H-competent S-DNAs. To illustrate, both S-DNA (RNase H-competent) and morpholino (RNase H-independent) antisense oligos, all of which had been shown to be highly active in a cell-free translation system, were scrape-loaded into HeLa cells and assessed for efficacy in blocking their respective RNA target sequences therein. In this study in cultured cells [18] the two different morpholinos had $IC_{50}$ values of about 60 nM and near quantitative target inhibition at 300 nM. In contrast, neither of the corresponding S-DNAs achieved significant target inhibition within cells at concentrations up to 3000 nM. In similar experiments we have found PNAs (another advanced RNase H-independent type) to exhibit a similar large efficacy advantage over S-DNAs in scrape-loaded cells (unpublished results).

In a different study involving inhibition of TNF-α, Kobzik and coworkers also found morpholinos to achieve appreciably higher efficacies than corresponding S-DNAs in cultured cells [19].

### 3.2. Targeting versatility

While S-DNAs and other RNase H-competent antisense oligos can target and destroy (via RNase H cleavage) sites throughout the RNA transcript of a gene, including splice sites, only RNase H-independent antisense oligos, such as morpholinos, can effect correction of splicing errors [20].

Both RNase H-competent and RNase H-independent types can be used to block translation of any specific mRNA by targeting the 5′ leader/translational start region of that mRNA.

It is commonly assumed that only S-DNA and other RNase H-competent oligos are suitable for studying point mutations and polymorphisms more than about 20 nucleotides 3′ to the translational start site in mRNAs. However, this perceived limitation of RNase H-independent oligos can be circumvented by using a gene switching strategy (P. Morcos, Methods Enzymol., in press). Typically this entails using cells containing a normal gene and transfecting in a plasmid containing a mutant or polymorphic form of that same gene. Key to this scheme is to use a leader sequence in the transfected gene's mRNA which differs by at least a few bases from the leader sequence of the endogenous gene's mRNA. One then uses a high-specificity RNase H-independent antisense oligo, such as a morpholino, to selectively block translation of either the endogenous or the exogenous mRNA, after which one assesses for phenotypic changes. By this means one can exploit the exceptional specificity of the RNase H-independent morpholino antisense oligos to carry out rigorous and well controlled studies of a wide variety of mutations and polymorphisms positioned anywhere in the mRNA.

### 3.3. Availability

Another factor which led to the widespread use of S-DNAs was their ready availability at moderate prices from commercial sources. While lack of commercial sources, high prices and slow delivery have in the past been significant barriers to use of advanced RNase H-independent antisense types, this situation is now changing. Both PNAs [21] and morpholinos [22] are now commercially available, and prices of morpholinos are now competitive with prices of advanced mixed-backbone S-DNAs (i.e., chimeras).

*To summarize*: Relative to S-DNAs, properly targeted morpholinos often achieve equal or better efficacy in cell-free systems and often achieve substantially better efficacy in cultured cells; of the two types only morpholinos can be used for correcting splicing errors; a new gene switching strategy gives morpholinos targeting versatility on a par with S-DNAs for selected applications; and morpholinos are now commercially available at moderate prices with reasonable delivery times.

## 4. Advantages of RNase H-independent morpholino antisense oligos

### 4.1. Predictable targeting

A problem which has plagued antisense research with S-DNAs is the difficulty of predicting which antisense sequences will be effective in cells. As a consequence, multiple S-DNAs may need to be prepared and empirically tested in order to identify an oligo with good in-cell activity [23,24]. Further, when one does find an effective oligo through such an empirical search it is not unusual to find that it is targeted in a region of the RNA, such as the 3′ untranslated region [25], which one would not normally expect to afford inhibition by an antisense mechanism. The poor showing of those many antisense oligos which do not show good efficacy in such searches has been attributed to their having restricted access to their RNA target sequences within cells due to secondary structure of the RNA [26] and/or due to proteins bound to the RNA. Alternatively, this lack of inhibitory activity by many S-DNAs could be due to some activity which disrupts RNA/S-DNA duplexes within cells [27] or due to S-DNAs being efficiently sequestered in a partially sequence-specific manner by some nuclear structure [28], or due to activation of Sp1 transcription factor [13] which acts to overshadow the translational inhibition by the S-DNA.

A need to sift through multiple S-DNAs in order to find one that effectively inhibits its target in cultured cells seriously limits the utility of S-DNAs as routine tools for the study of gene function and control. Further, in the absence of rational and reliable targeting rules for S-DNAs and in light of their partially sequence-specific non-antisense effects, the need to test multiple S-DNAs in order to find an effective one raises the specter that one may not be selecting for an accessible antisense target, but instead one may be selecting for an oligo sequence effective to generate some non-antisense activity which is then misinterpreted as the desired antisense effect [15].

In contrast to the difficulty in predicting effective targets for S-DNAs in cultured cells, we have found targeting of morpholinos (lacking undue self-complementarity) to be reasonably predictable both in cell-free systems and within cultured cells. This is illustrated by a targeting study using a target mRNA comprising an 85-base segment of the leader sequence of hepatitis B virus (HBV) fused to the coding sequence of firefly luciferase [18]. This HBV leader sequence presents a substantial targeting challenge because it contains a region of quite stable secondary structure extending from positions −47 to +3 (where +1 is the A of the AUG translational start site). Experimental procedures and the mRNA target used in this study are detailed in [18].

Fig. 2a shows the 5′ leader region and 24 bases of the amino acid coding sequence of this mRNA, and indicates with bold lines the target sequences for seven of the morpholino oligos tested in this study. Fig. 2b shows the linear positioning of the morpholino antisense oligos along this HBV-luciferase mRNA, as well as each oligo's percent inhibition achieved in a cell-free translation assay, with oligo present at a concentration of 1 μM and target mRNA present at 1 nM.

A representative subset of these morpholinos were tested in cultured cells stably transfected with a plasmid coding for the same HBV/luciferase mRNA construct. Fig. 2c shows inhibition of luciferase produc-

tion in cells scrape-loaded in the presence of 3 μM morpholino oligos, assessed as described in [29].

The results in Fig. 2b show that the tested morpholinos were reasonably effective along the entire 5′ leader and up to a few bases 3′ to the AUG translational start site. Oligos targeted to sites beginning more than 20 bases 3′ to the AUG translational start site showed no significant activity. Of particular note, the results demonstrate that oligos 3, 4, and 5 appear to have effectively invaded the quite stable secondary structure within the HBV leader sequence.

The results shown in Fig. 2c suggest that those morpholinos which are effective in a cell-free assay are generally also effective in cultured cells, while those morpholinos which are inactive in the cell-free assay (i.e., those targeted more than a few bases 3′ to the translational start site) are also inactive in cultured cells.

It should be noted that the $IC_{50}$ of morpholinos in the cell-free translation system are typically about 3–7-fold lower than the $IC_{50}$ in scrape-loaded cells. We postulate that this reflects limited entry of oligos through the very small [30] transient [29] holes believed to be generated in the cell membrane during the scrape-load procedure.

A possible basis for this relatively predictable targeting of morpholinos, even in regions of quite stable secondary structure, may be their high affinity for RNA. Fig. 3 illustrates the comparative affinities of S-DNA, DNA, and morpholino 20-mer oligos for their complementary RNA (assay conditions detailed in [9]).

I postulate that the apparent ability of long morpholino oligos to effectively invade RNA secondary

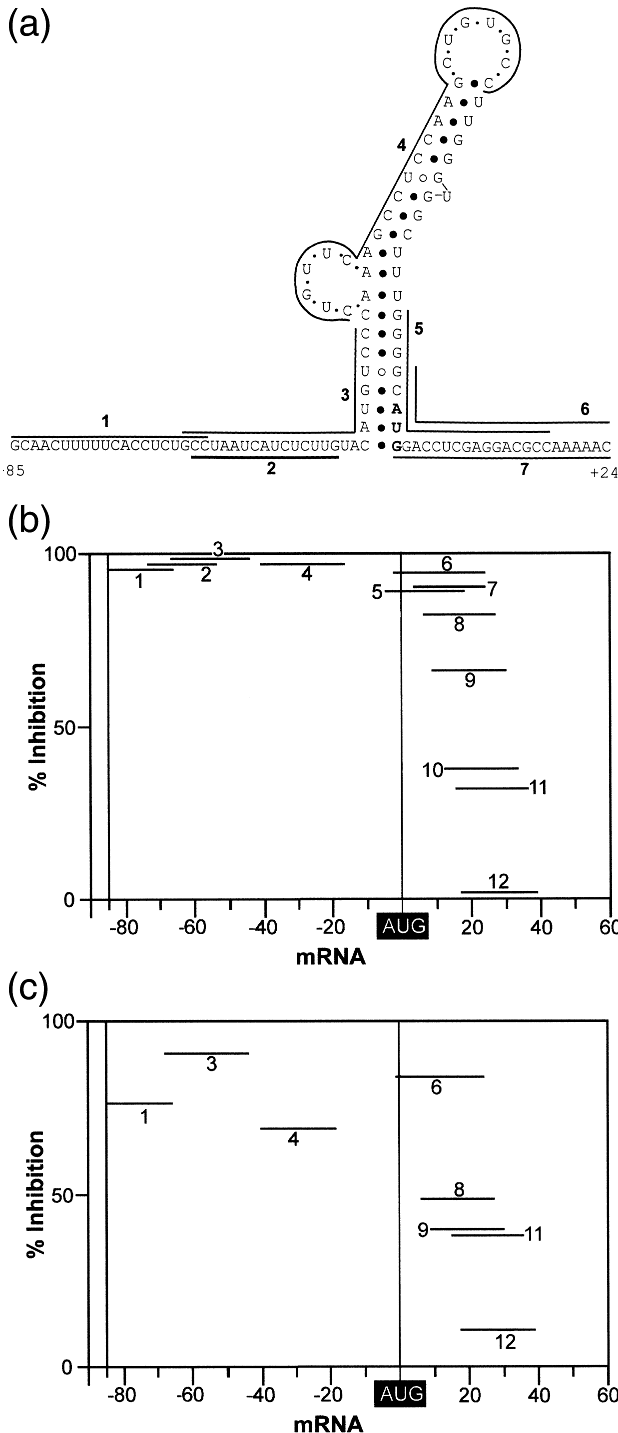


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Fig. 2. Translational inhibition as a function of target position on HBV/luciferase mRNA. (a) Leader and translational start region of mRNA with target sequences for oligos 1–7 indicated by numbered bold lines; (b) percent inhibition of luciferase synthesis in cell-free translation system by 1 μM morpholino oligos 1–12; (c) percent inhibition of luciferase synthesis in cultured HeLa cells expressing HBV/luciferase mRNA and scrape-loaded in the presence of 3 μM morpholino oligos 1, 3, 4, 6, 8, 9, 11, and 12. The sequence of the HBV/luciferase mRNA from −85 to +24 is: (−85) 5′-GCAAC-UUUUUCACCU-CUGCCUAA-UC-AUCUCUUGUA-CAUGUCCCAC-UGUUCAAGCC-UC-CAAGCUGU-GCCUUGGGUG-GCUUUGGGGC-**AUG**GAC-CUCG-AGGACGCCAA-AAAC (+24). The 14 oligos used in this experiment are targeted against the following sequences in this mRNA: oligo 1 (−85 to −66); 2 (−73 to −53); 3 (−68 to −44); 4 (−41 to −18); 5 (−5 to +18); 6 (−2 to +24); 7 (+3 to +24); 8 (+6 to +27); 9 (+9 to +30); 10(+12 to +33); 11 (+15 to +36); 12(+18 to+39); 13 (+192 to +216); 14 (+528 to +553). Oligos 12, 13, and 14 showed no significant activity in either the cell-free or cultured-cell tests.

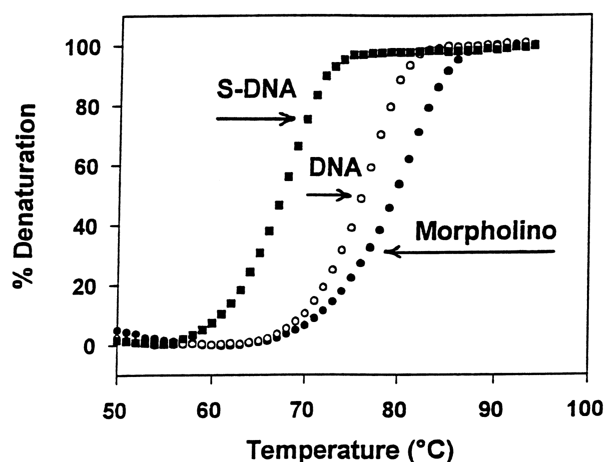


Fig. 3. Thermal transitions of 20-mer oligo/RNA duplexes. Oligo sequence: 5′-GGUGGUUCCUUCUCAGUCGG (T replaces U in DNA and S-DNA).

structures may be due at least in part to their high RNA binding affinities – which should favor displacement of short RNA/RNA duplexes to form appreciably longer high-stability RNA/morpholino duplexes. Conversely, S-DNAs, with their substantially lower affinity for RNA, are expected to be much less effective in invading RNA secondary structures.

Use of long morpholinos is also postulated to better assure there will be single-stranded regions in most target sequences to provide for nucleation of pairing.

### *4.2. Reliable efficacy in cultured cells*

As noted above, we have found that morpholino antisense oligos which exhibit good activity in a cell-free translation system also generally exhibit correspondingly good activity when scrape-loaded into cultured animal cells [18]. Similar correspondence between cell-free and in-cell activities of morpholinos has been reported by Kobzik and coworkers at Harvard [19] and by Kole and coworkers at the University of North Carolina (R. Kole, submitted for publication). While I have less experience with PNAs, nonetheless, the few PNAs we have tested also showed good correspondence between activity in a cell-free test system and activity in scrape-loaded cells (unpublished results).

For comparison we have also tested the in-cell efficacies of a number of S-DNAs, which had previously been shown to have excellent efficacies in our cell-free test system ($IC_{50}$ in the 10–30 nM range). In sharp contrast to the case for morpholinos and PNAs, the scrape-loaded S-DNAs typically show little in-cell efficacy in our test system, and then only at concentrations typically over 3000 nM [18]. In fact, at low to moderate concentrations both antisense and control S-DNAs often strongly increase production of the protein product of the targeted mRNA – possibly via activation of Sp1 transcription factor [13] which could then generate a net increase in the targeted mRNA.

Initially we suspected that perhaps the poor in-cell activity by scrape-loaded S-DNAs might be due to their multiple negative backbone charges preventing good cell entry during the scrape-load procedure. However, when fluorescein-labeled oligos were scrape-loaded into cells it was seen that this procedure achieves delivery of S-DNAs as well as or better than delivery of morpholinos [18].

It seems possible that the poor in-cell efficacy we have seen with S-DNAs and the good in-cell efficacy of morpholinos might be at least in part a consequence of the S-DNAs' sensitivity to nucleases [7] and the morpholinos' complete resistance to nucleases [31].

Another possible explanation for the apparent poor activity of S-DNAs in cells relates to RNase H. In our cell-free translation studies we add *Escherichia coli* RNase H (4 units/ml) because S-DNAs are only poorly active in reticulocyte lysates in the absence of added RNase H. Conceivably, in the HeLa cells, which we typically use for our in-cell studies, mammalian RNase H may be less abundant or less active and so the S-DNAs may afford much less activity than would be expected on the basis of their cell-free activities.

Still another possible reason for lower in-cell efficacies of S-DNAs may be that the S-DNAs' complexes with target sequences are being disrupted by some cellular factor, as postulated by Moulds et al. at Gilead Sciences [27], based on experiments wherein they pre-paired high-affinity anionic antisense oligos with their respective target RNAs and microinjected these duplexes into cells. They found that the anionic oligos were stripped off their target RNAs – evidenced by translation of the protein coded by that RNA. Conceivably such a cellular strand-separating factor might strip off anionic S-DNAs but fail to act

on corresponding non-ionic oligo/RNA duplexes, as would be formed when the non-ionic morpholinos bind their target RNA.

An additional contribution to the good in-cell efficacies of morpholinos and the poor in-cell efficacies we have encountered with S-DNAs in scrape-loaded cells may derive from their respective subcellular localization. Specifically, when low concentrations of fluorescein-labeled S-DNAs are scrape-loaded into cells fluorescence microscopy shows them to be largely sequestered in the nucleus. In contrast, fluorescein-labeled morpholinos scrape-loaded into cells are seen to distribute more evenly throughout the cell – though the concentration in the nucleus usually appears to be severalfold higher than in the cytosol. To appreciate the impact these subcellular distributions of oligo may have on antisense efficacies it should be noted that in the nucleus transcription and processing of a pre-mRNA, and transport of the resulting mature mRNA to the cytosol typically occur within minutes, while translation of the mRNA in the cytosol typically continues for hours to days. As a consequence, S-DNAs largely sequestered in the nucleus may have access to their target RNAs primarily in the brief time between transcription and export to the cytosol, while the more evenly distributed morpholinos should have access to their target RNAs both during the RNAs' brief sojourn in the nucleus and during their far longer residence in the cytosol. It seems quite possible that this difference in target access time might contribute to the high in-cell efficacies of morpholinos and PNAs and the low in-cell efficacies of S-DNAs.

It is noteworthy that in fluorescent microscopy studies of the subcellular distribution of oligos we have observed that addition of mounting medium to the cells can cause significant redistribution of the label relative to that in unperturbed cells – in particular, a transition from a perinuclear punctate pattern to a strongly nuclear pattern. Based on the composition of typical mounting media we suspect this redistribution of fluorescence within the cells may be due to osmotic shock and/or pH stress of the cells. To preclude such artifactual effects we view cells with an inverted fluorescent microscope. This allows the cells to remain bathed in normal growth medium during visualization and photography.

### *4.3. High specificity*

A key factor which lured many scientists (and investors) into the antisense field was the hope that a given antisense oligo could, with near perfect specificity, block its targeted mRNA while exerting essentially no other effects on the cell or patient. It was hoped such high specificity by antisense therapeutics would avoid the severe toxicities characteristic of present small-molecule antiviral and anticancer therapeutics [32]. However, these great expectations largely died for many scientists in the antisense field when: (a) it became widely believed that RNase H competency was essential for good efficacy; and (b) the specificity limitations of S-DNAs became widely appreciated.

Achieving reliably high antisense specificity in a predictable manner would also open the door for use of antisense oligos as dependable tools for studying the function and control of genes and for validating new therapeutic targets – commonly the first step in current strategies for small-molecule drug development.

At present there are two quite different approaches to achieving high antisense specificity – which I refer to as the 'shorter-is-better' and the 'longer-is-better' strategies.

#### *4.3.1. Shorter-is-better strategy*

In this strategy relatively short high-affinity oligos, typically of the RNase H-competent type, are used to target point mutations or unusual secondary structures in the selected RNA. When targeting a point mutation there is generally a one base difference between the targeted sequence (typically a mutant) and the non-targeted sequence (typically the wild type). Because there is only one base difference, clearly the shorter the antisense oligo the greater the differential between the antisense oligo's binding affinity for the target and non-target sequences. In order to achieve reasonable efficacies with such short sequences, modifications (such as propyne moieties on the pyrimidines [33]) are introduced to increase the oligo's binding affinity. Efficacy can be increased even further by going to a chimeric oligo wherein the weaker-binding RNase H-competent segment of the chimera is bounded on one or both sides by a higher-affinity stretch of derivatized RNA [34]. While this short/

high-affinity strategy can achieve impressive selectivity between the mutant and wild type sequences, statistical considerations suggest that such high-affinity oligos may inadvertently inactivate other sequences in the pool of cellular RNAs – as described in Section 4.3.2. As a consequence, these short high-affinity oligos may be unsuitable as general tools for most studies of function and control of new genes and for target validation programs. This is because in such cases one's objective is generally to predictably inhibit expression of a selected gene without significantly inhibiting the expression of any other gene in the cell.

An interesting variation of the 'shorter-is-better' strategy has been pursued by scientists at Gilead. They have empirically identified a sequence in an RNA with a particular secondary structure which can be inhibited by very-high-affinity antisense oligos as short as 7 bases in length [35]. While statistical considerations would suggest poor specificity from such oligos, these workers report that these oligos do not inhibit their complementary sequence if that sequence is inserted at a different site in the RNA, presumably because effective inhibition is contingent on both the target sequence and the secondary structure encompassing that target sequence. Potentially this very-short/high-affinity/special-target strategy [36] could lead to relatively low cost antisense therapeutics – especially in light of a recent report that such short oligos can be rendered membrane permeable by addition of a suitable lipophilic moiety [37]. However, it seems unlikely they will soon constitute routine tools for studying gene function and control or for validating therapeutic targets because of the difficulty in routinely identifying suitable targets [26].

#### *4.3.2. Longer-is-better strategy*

Aside from their potential therapeutic applications (which is outside the scope of this review), probably the greatest value for antisense oligos would be their use as tools for studying gene function and control and for validating new therapeutic targets in small-molecule drug development programs. Such applications generally call for an antisense oligo which can predictably achieve near quantitative inhibition of its selected target RNA (high efficacy) while avoiding significant inhibition of any other of the host of inherent cellular RNA species (high specificity).

In this regard, conventional wisdom in the antisense field generally holds that one can design for high efficacy, at the cost of reduced specificity, or one can design for high specificity, at the cost of reduced efficacy, but that it is generally not possible to achieve both high efficacy and high specificity.

To the contrary, I contend that by using relatively long oligos of the proper structural type one can achieve both high efficacy and high specificity. To meet this dual efficacy/specificity challenge, I believe the antisense oligo should have a '*minimum inactivating length*' (MIL) sufficient to give a high probability that said oligo will inactivate essentially no inadvertent targets in the cellular RNA pool. Here I define the MIL as the shortest length of oligo of a given structural type which achieves substantial target inhibition at concentrations typically achieved in the cytosol/nuclear compartment of treated cells.

In regard to estimating the MIL value required for high specificity by a selected antisense structural type, one needs to have a reasonable estimate of the number of unique-sequence bases in the cell's RNA pool, as well as the approximate percentage of those bases which could potentially be targeted by that selected antisense structural type.

With respect to S-DNAs, it is difficult to estimate a required MIL value due to uncertainties regarding the fraction of bases in a representative RNA which are targetable. On the one hand, S-DNAs combined with RNase H have the potential to bind and degrade sequences anywhere along the length of an RNA strand. However, it is commonly found that a substantial fraction of S-DNAs fail to inhibit their targeted sequences within cells [23,24]. As a consequence, it is not possible to estimate with any assurance the fraction of bases in the cell's RNA pool which are potentially available for inactivation by S-DNAs within cells. Thus, it is not possible to estimate the MIL required for high specificity of S-DNAs.

While predicting effective targets for S-DNAs within cells can be difficult, this appears not to be the case for morpholinos. Specifically, in both cell-free translation systems and in cultured cells we have found morpholino oligos to be effective against the majority of sequences tested to date, even including sequences with quite stable secondary structures, in the region from the 5′ cap to about 25 bases 3′ to the AUG translational start site of mRNAs – as detailed

in Section 4.1. It should be noted that we know of two exceptions to this relatively good targeting predictability. Morpholinos with significant self-pairing potential (more than four contiguous intrastrand base pairs) and morpholinos with four or more contiguous guanines typically show poor activity, presumably due to intrastrand pairing and to interstrand complex formation, respectively.

In view of the relatively predictable targeting for morpholinos it should be possible to estimate a realistic lower limit for the MIL value of morpholinos adequate to assure high sequence specificity. To this end, I estimate that on the order of 3–5% of the genome is transcribed in any given cell type. Thus, in a typical line of cultured cells the RNA pool should comprise on the order of 120 million bases of unique-sequence RNA. From an assessment of a representative sampling of human RNA transcripts I estimate that the regions susceptible to inhibition by morpholinos comprise only about 2–5% of that RNA pool (the other 95–98% being introns and sequences more than about 25 bases 3′ to the translational start site). Accordingly, to achieve high sequence specificity a morpholino oligo only needs to distinguish its selected target sequence from no more than about 6 million bases of unique-sequence cell RNA (i.e., 5% of 120 000 000).

Table 1 was constructed based on an estimated RNA pool size of 120 million bases, of which 5% are targetable by morpholinos. Values were calculated by the equation given in the legend to Table 1, which factors in the additional sequences inherently present when the oligo length is greater than the MIL value. Table 1 lists the calculated number of inherent cellular RNA sequences expected to be inadvertently inactivated by a morpholino 25-mer antisense oligo having the indicated MIL values.

Table 1
Estimated numbers of inadvertent targets in the human RNA pool for an RNase H-independent morpholino 25-mer as a function of MIL

| MIL | Targets in pool |
|---|---|
| 7 | 6960 |
| 8 | 1650 |
| 9 | 390 |
| 10 | 92 |
| 11 | 21 |
| 12 | 5 |
| 13 | 1 |
| 14 | 0 |

$$\text{Inadvertent targets} = \frac{\text{pool complexity}}{4^{\text{MIL}}} \times (\text{oligo length} - \text{MIL} + 1).$$

Pool complexity: 6 000 000 for RNase H-independent oligo type.

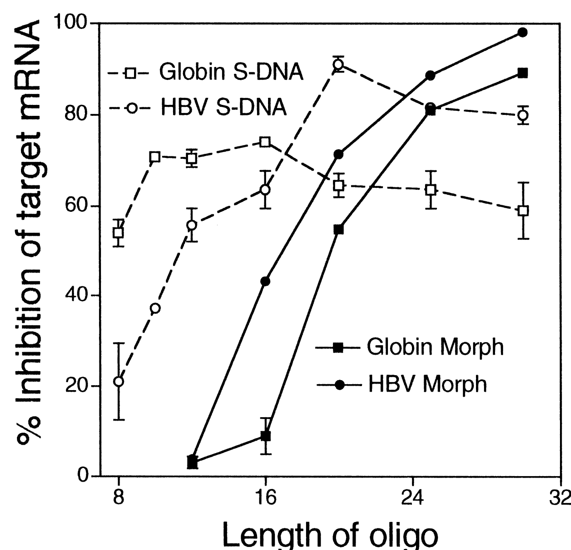


Fig. 4. Activity as a function of oligo length in a cell-free translation system with 300 nM oligo and 1 nM target mRNA. Globin refers to a globin/luciferase mRNA construct and HBV refers to a hepatitis B/luciferase mRNA construct, both detailed in [18]. The HBV target region comprises bases −85 to −56 having the sequence (−85) 5′-GCAACUUUUUCACCUCUGCCUAAUCAUCUC (−56), and oligos against this region comprise: 8-mer (−85 to −78); 10-mer (−85 to −76); 12-mer (−85 to −74); 16-mer (−85 to −70); 20-mer (−85 to −66); 25-mer (−85 to −61); 30-mer (−85 to −56). The globin target region comprises bases −30 to −1 having the sequence (−30) 5′-CUGGUCCAGUCCGACUGAGAAGGAACCACC (−1), and oligos against this region comprise: 8-mer (−8 to −1); 10-mer (−10 to −1); 12-mer (−12 to −1); 16-mer (−16 to −1); 20-mer (−20 to −1); 25-mer (−25 to −1); 30-mer (−30 to −1).

The values in Table 1 suggest that to achieve very high sequence specificity an RNase H-independent morpholino 25-mer should have an MIL of at least 13 or 14.

Experiments have been carried out to estimate MIL values in a cell-free translation system for both RNase H-competent S-DNAs and RNase H-independent morpholinos. In these activity versus oligo length experiments a set of both S-DNA and morpholino oligos ranging in length from 8 bases to 30 bases were targeted against the same region of

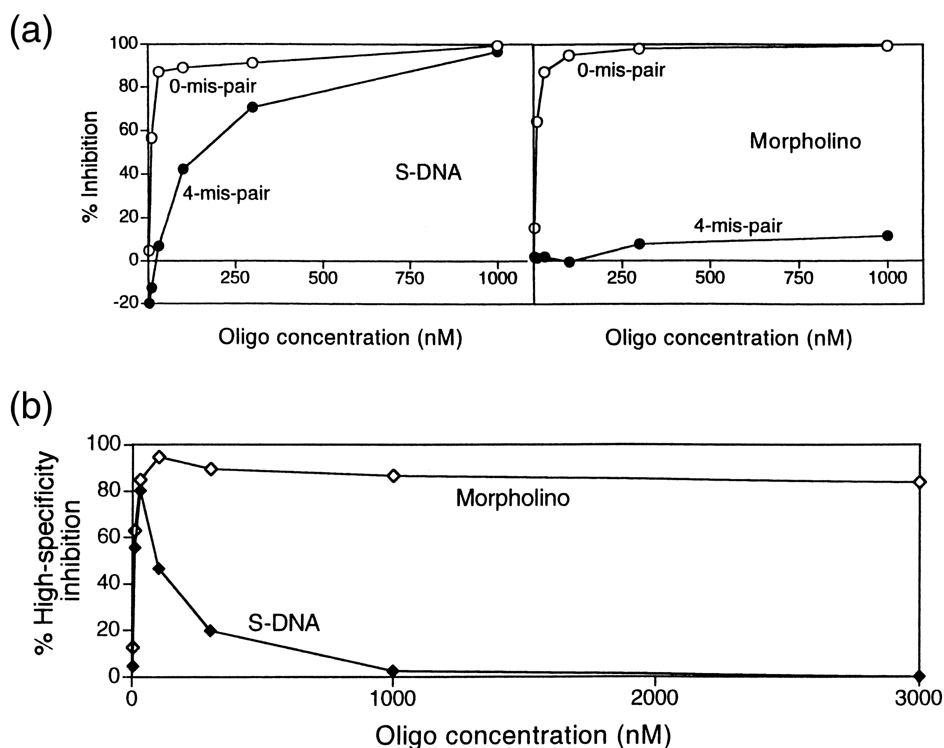


Fig. 5. Sequence-specific inhibition of globin/luciferase mRNA in a cell-free translation system. (a) Inhibition by 0-mispair oligos (open circles) and 4-mispair oligos (closed circles); (b) high specificity component of inhibition = (inhibition by 0-mispair oligo)−(inhibition by 4-mispair oligo); 0-mispair oligo: 5′-GGUGGUUCCUUCUCAGUCGGACUGG; 4-mispair oligo: 5′-GGU<u>C</u>GUU<u>G</u>CUU-CUCAGUC<u>C</u>GAC<u>A</u>GG.

rabbit α-globin leader sequence, and another set were targeted against the same region of HBV leader sequence. These two sets were assessed in a cell-free translation system for their abilities to inhibit translation of a downstream luciferase coding sequence. The experimental procedures and the RNA targets used in this length versus activity study are detailed in [18]. Fig. 4 shows the respective translational inhibition values at oligo concentrations of 300 nM.

The results in Fig. 4 suggest that under the conditions of this experiment morpholinos against these two target regions appear to have MIL values from about 14 to 17. Since their calculated minimum required MIL value for high sequence specificity in cultured cells is about 13 to 14 for morpholino 25-mers, this bodes well for such oligos achieving excellent sequence specificity. While a morpholino oligo in the 13–14 subunit length range should afford high specificity, if the oligo is only this length it will generally achieve only marginal efficacy. Therefore, to achieve high efficacy one needs to make the oligo appreciably longer than the MIL. We find that morpholino 25-mers generally achieve good efficacies in the nanomolar to low-micromolar concentration range [38]. Even with these relatively long oligos the values in Table 1 suggest most morpholino 25-mers should have very few or no inadvertent targets in a human cell line.

To test this predicted high specificity of morpholinos we have carried out a highly stringent specificity assay of morpholinos and S-DNAs in a cell-free translation system [18]. In these experiments two oligos of each structural type were used. One oligo of each type was perfectly complementary to its target mRNA (globin leader sequence) to provide a measure of total inhibition achieved by that oligo type. The other oligo of that type incorporated four mispairs to that same target sequence, with the longest run of perfect pairing comprising 10 contiguous base pairs, to provide a reasonable emulation of the estimated level of sequence homology likely to be encountered in the RNA pool within human cells.

Fig. 5a shows the percent inhibition values for these four oligos in the range from 1 nM to 1000 nM.

The difference between the inhibition value for the perfectly paired and the four mispaired oligos at each concentration provides a concentration-dependent measure of the high-specificity inhibition achieved by that structural type. Fig. 5b shows a plot of this high-specificity inhibition component over an extended concentration range (3–3000 nM).

In accord with the predictions from the MIL values in Table 1 and the length versus activity results in Fig. 4, the results in Fig. 5 demonstrate that in this stringent test of specificity the RNase H-independent morpholino 25-mer achieves both high efficacy ($IC_{50}$ less than 10 nM) and high sequence specificity (84–95%) over a broad 3000 nM concentration range.

In contrast, the RNase H-competent S-DNA 25-mer, which the results in Fig. 4 suggests has an MIL value of around 7–9, achieved only modest high-specificity inhibition (47–80%) in a narrow 90 nM concentration range.

*To summarize*: I postulate that both high specificity and high efficacy can be achieved by an antisense oligo: (a) whose actual MIL is at least as long as the MIL required to achieve high specificity; and (b) whose length is substantially longer than its actual MIL. The results in Fig. 5 suggest that morpholino 25-mers, which satisfy these MIL-related design criteria, do indeed meet the antisense field's long sought dual goals of high efficacy and high specificity over a broad concentration range.

### 4.4. Little non-antisense activity

As yet we have only limited information concerning possible non-antisense activity of morpholinos – but the very preliminary information we do have suggests morpholinos are free of some, and perhaps all of the non-antisense activities plaguing S-DNAs.

(a) In our in-cell test system low concentrations of S-DNAs, including both antisense and control sequences, often strongly stimulate instead of inhibit production of the protein product from the target mRNAs [18] – possibly due to the documented rapid induction of Sp1 transcription factor by S-DNAs [13]. In contrast, in our in-cell test system corresponding morpholino oligos generally do not significantly stimulate production of the protein product from the targeted mRNAs [18] and so presumably do not induce Sp1 transcription factor.

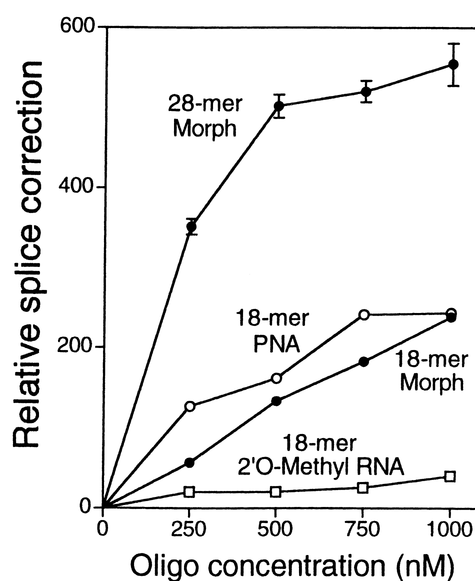


Fig. 6. Luciferase translated from corrected splicing of thalassemic globin/luciferase pre-mRNA in HeLa cells scrape-loaded with antisense oligos. Oligo sequences: 18-mer: 5′-CCTCTTACCTCAGTTACA; 28-mer: 5′-CCTCTTACCTCAGTTACAATTTTATATGC.

(b) S-DNAs containing Pu Pu C G Py Py sequences often mediate B cell activation [40]. In contrast, the same-sequence morpholinos do not activate B cells (A. Krieg, personal communication).

(c) S-DNAs have been reported to have acute $LD_{50}$ values in mice in the range of 100 mg/kg to about 500 mg/kg [39]. In a small ranging study with four mice no acute toxicity was seen following intraperitoneal injection of a morpholino 22-mer at a dose of 800 mg/kg.

## 5. Positive test system

A long standing limitation in antisense research has been that the available test systems rely on down-regulation. This includes such a crude measure as inhibition of cell growth, as well as assays for inhibition of the synthesis or activity of a particular protein, and assays for degradation of a particular RNA (useful only with RNase H-competent oligos). The difficulty in these negative test systems is that a variety of non-antisense effects can also lead to

down-regulation or what appears to be down-regulation. Even when a reasonable set of control oligos are incorporated into the experiment there can still be significant uncertainty as to whether the observed biological effect is really due to an antisense mechanism. This is because non-antisense effects can also exhibit some degree of sequence specificity – while not being truly specific for the selected target RNA [15,39,41].

The limitations inherent in negative test systems have recently been overcome by the introduction of a positive antisense test strategy by Kole and co-workers [20,42,43]. Kole's 'splice-corrector' strategy entails targeting an RNase H-independent antisense oligo against a mutant site in the pre-mRNA which leads to erroneous splicing. Blockage of that mutant site by the antisense oligo corrects the splicing error, leading to correctly spliced mRNA, which is then translated to give the desired protein. An early version of this test system entailed up-regulation of β-globin and employed readout of both the correctly spliced mRNA and the final β-globin product [42]. A more recent version [43] affords a more convenient luminescence readout from firefly luciferase. Using this new test system, Fig. 6 shows representative results from an experiment measuring relative light units from luciferase synthesized in cultured cells scrape-loaded in the presence of varying concentrations of three different types of RNase H-independent splice-corrector antisense oligos (P. Morcos, Methods Enzymol., in press).

Experiments utilizing this new splice-correction assay should be largely free of the ambiguity which has plagued the antisense field. As such, we find this system to be particularly useful for studies focused on developing effective cytosol/nuclear delivery of antisense oligos. It should be noted that only RNase H-independent oligos can be used in these splice-correction assays because RNase H-competent oligos destroy the mutant pre-mRNA.

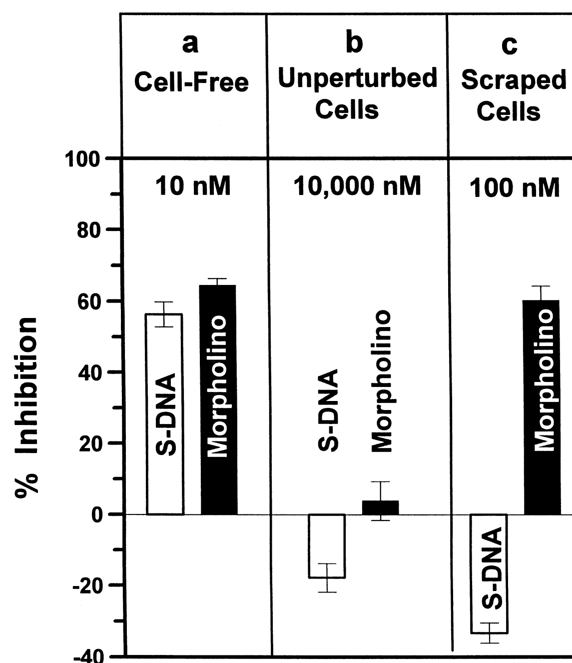


Fig. 7. Translational inhibition by 25-mer antisense oligos targeted against globin/luciferase mRNA: (a) in cell-free translation system with 10 nM oligo and 1 nM mRNA; (b) in unperturbed HeLa cells expressing globin/luciferase mRNA and treated with 10000 nM oligo for 16 h; (c) in HeLa cells expressing globin/luciferase mRNA and scrape-loaded in the presence of 100 nM antisense oligo. Oligo sequence: 5′-GGUGGUUCCUUCUCAGUCGGACUGG.

## 6. Delivery

### 6.1. Delivery into cultured cells

In the 1980s a number of antisense experiments with cultured cells suggested that antisense oligos readily enter cultured animal cells and have good access to their targeted RNAs therein. These early results led scientists in the antisense field to believe that antisense oligos, and particularly non-ionic types, could readily diffuse across cell membranes. However, by the early 1990s reality reared its ugly head in the form of a number of careful experiments whose results indicated that neither ionic nor non-ionic antisense oligos can diffuse across cell membranes at any reasonable rate [44,45]. Instead, much evidence suggests that antisense oligos enter cultured cells primarily via endocytosis and subsequently most or all of the oligos are degraded, remain trapped in the endosome/lysosome compartment, or are exocytosed from the cell [46].

These sobering findings stimulated broad ranging efforts to develop methods effective for delivering antisense oligos into the cytosol/nuclear compartment of cells. Probably the most used of the resulting delivery methods entails complexing anionic oligos

with liposomes, followed by fusion of these oligo/liposome complexes with cells [47,48]. Another method, which can be used with both ionic and non-ionic antisense types, entails generation of pores in the plasma membrane of cells using streptolysin O under serum-free conditions, and then after oligo has entered the cells, closing those pores by adding serum [49].

A third method which we use extensively entails adding oligos to adherent cells, scraping the cells from the plate, and transferring the scraped cells to another plate where they are allowed to readhere [29]. It is believed that when the cells are scraped from the plate, desmosomes connecting the cells to the plate are pulled out of the cell membranes, leaving very small transient holes in the cytoplasmic membrane [30]. This allows oligos to enter the cytoplasm for about a minute [29] before the holes reseal. This scrape-load procedure is fast, easy, and cheap, it works well in the presence and absence of serum; it causes only minimal damage to cells, and it is suitable for delivering both ionic and non-ionic antisense types. The utility of this scrape-load method in cultured cells is demonstrated in Fig. 7 [18], which shows representative translation inhibition results obtained with S-DNA and morpholino 25-mer antisense oligos in: (a) a cell-free translation system using a low concentration of antisense oligo; (b) in unperturbed cells treated with a very high concentration of antisense oligo; and (c) in cells scrape-loaded with a moderate concentration of antisense oligo.

These results demonstrate that oligos which are very active in a cell-free translation system ($IC_{50}$ of less than 10 nM) fail to show significant activity in unperturbed cells treated with a far higher concentration (10 000 nM) for 16 h. However, the morpholino oligo again shows good activity when cells are scrape-loaded with just one hundredth of that concentration.

The above scrape-load method is restricted to use with adherent cells. Another fast and simple method for introducing antisense oligos into the cytosol of both adherent and non-adherent cells entails pinocytotic loading of cells with a hypertonic solution including sucrose, polyethylene glycol, and the oligo to be delivered into the cytosol of the cell. After a 10 min incubation the hypertonic solution is removed and replaced with a hypotonic solution – which causes lysis of the pinosomes within the cell [50], thereby releasing the antisense oligo into the cytosol of the cell. A kit for such pinocytotic loading of cells was recently introduced by Molecular Probes (Eugene, OR).

### *6.2. Delivery in vivo: the final challenge?*

While several effective methods are now available for delivering antisense oligos into cultured cells, none of the above methods are suitable for delivering antisense oligos into cells in vivo. However, contrary to expectations from cell culture work there are reports in the literature that S-DNA antisense oligos injected into animals efficiently gain access to their targeted RNAs within cells – even though the same S-DNAs are unable to achieve the corresponding access in cells in culture. Probably the best documented reports of in vivo antisense activity by S-DNAs are those of Monia and coworkers [51,52].

In spite of such reports that antisense oligos can readily enter the appropriate compartments of cells in vivo, because of the difficulty in confirming a true antisense mechanism with S-DNAs [15,39,41] and the uncertainties inherent in work with whole animals, I believe it is prudent to continue efforts to develop methods for delivering antisense oligos into the cytosol of cells by methods likely to be suitable for application in vivo. Below are two strategies which appear particularly promising for delivery of antisense oligos into the proper subcellular compartment of cells in vivo.

#### *6.2.1. Amphiphilic peptides*

Alain Prochiantz and coworkers in France discovered that a fruit fly transcription factor protein is excreted by cells and efficiently reenters adjacent cells, apparently by directly crossing the plasma membrane into the cytosol, after which that protein migrates to the nucleus. They subsequently discovered that an amphiphilic 16 amino acid sequence in that protein is largely responsible for this transmembrane transport activity [53]. Several other groups have also identified proteins which are excreted and then appear to pass directly across the plasma membrane into recipient cells – including VP22 from herpes simplex virus [54] and galaparan [55].

Pooga and coworkers have recently used two such

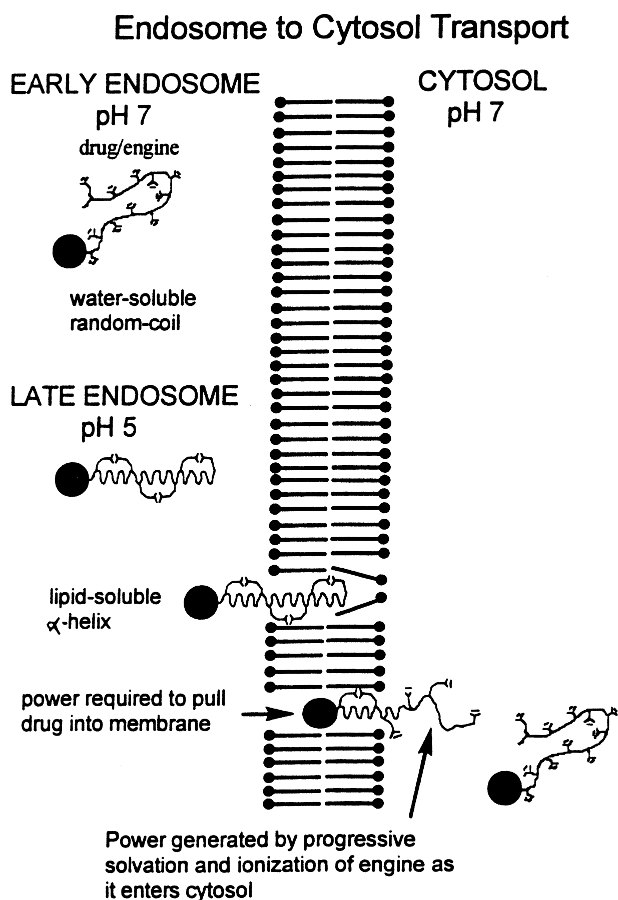


Fig. 8. Mechanism of drug transport from endosome to cytosol by molecular transport engine.

transporter peptides for delivering PNA antisense oligos into biological systems [56]. Specifically, they linked a PNA oligo to an amphiphilic transporter peptide and added this construct to cultured cells. They report that two such constructs utilizing two different amphiphilic transporter peptides efficiently entered cultured cells and achieved an antisense effect therein. They subsequently injected these constructs into rats. Based on an observed biological response in the rats, they infer that these antisense-peptide constructs successfully achieved entry into the proper subcellular compartment and exhibited an antisense effect therein. While these quite recent in vivo results have not yet been rigorously confirmed, they nonetheless appear promising because they were carried out with an antisense type (PNA) which appears not to elicit the numerous non-antisense effects commonly seen with S-DNAs.

*6.2.2. Molecular transport engine*

In a rather different delivery approach I and Dwight Weller have designed from first principles a class of molecular engines for transporting drugs from endosomes to the cytosol of cells [57,58]. The power source for these engines is the pH differential between the late endosome (pH about 5.5) and the cytosol (pH about 7). To convert this pH differential into useful power for drug transport the engines are designed to undergo reversible pH-mediated transitions between a water-soluble form and a lipid-soluble form. The engines contain carboxylic acid groups positioned along a peptide backbone, plus interspersed lipophilic moieties required to fine tune the transition pH and optimize lipophilicity under acidic conditions. A key requirement for achieving good lipophilicity under low-pH conditions is that neighboring carboxylic moieties be properly positioned so as to form doubly H-bonded dimers at low pH – thereby largely masking their polar character.

Fig. 8 illustrates the mechanism by which these molecular transport engines effect passage from the endosome to the cytosol. First, the drug-engine construct in its ionic water-soluble form is endocytosed, after which proton pumps embedded in the endosomal membrane acidify the endosome. When the pH is reduced sufficiently the engine converts from its high-pH ionic water-soluble form to its low-pH non-ionic lipid-soluble α-helical form, which partitions from the aqueous endosomal compartment into the lipid bilayer of the endosomal membrane. Because the engine in its low-pH α-helical form is longer than the membrane is thick (about 36 Å), continuing entry of the engine into the membrane results in the distal end of the engine contacting the pH 7 cytosol, whereupon the engine is actively drawn into the cytosol as it converts back to its high-pH water-soluble form. During its entry into the cytosol the motive force for pulling the attached drug into and through the endosomal membrane is generated by solvation and ionization of the engine at the membrane/cytosol interface.

To date a variety of studies, including solubility, octanol/water partitioning, circular dichroism, transport between low-pH and high-pH compartments separated by a lipid bilayer, and transport directly across the plasma membrane of cells briefly suspended in pH 5.5 medium, indicate that such molec-

ular engines function in the desired manner (Moulton et al., in preparation). Preliminary results with cultured cells also suggest that such an engine may transport a morpholino oligo into the cytosol of cells [57], but these studies (still in progress) are not yet definitive.

It is envisioned that in vivo these oligo-engine constructs will enter cells of the body by the normal endocytotic route, after which the engine will transport the oligo from the endosomes to the cytosol/nuclear compartment. While engines constructed from D-amino acids appear to persist within cells for long periods of time, our preliminary results suggest that engines constructed from L-amino acids are rapidly degraded in the cytosol, presumably by proteosomes to generate innocuous natural amino acids. Thus, it is hoped such engines will carry out their transport function and then be rapidly disposed of in a manner which generates only innocuous natural products.

*To summarize*: Morpholino antisense oligos appear to meet the requirements for use as effective and predictable tools for studying gene function and control in cultured cells and for validating targets in drug development programs. If and when effective delivery is achieved in vivo, oligos of this type may hold promise as effective, specific, and broadly applicable antisense therapeutics.

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single base from both of the CpG motifs abolished antiviral effect despite little change in the Tm for hybridization to the supposed mRNA target (79)'.

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