TARGETING

A chronic problem in the antisense field has been the difficulty in predicting effective targets for antisense oligos. Specifically, it is commonly found that S-DNAs are often and unpredictably inactive against many sequences in a selected RNA. This is likely due to relatively stable secondary structures in the RNA restricting access of the weak-binding S-DNAs. Even when suitable single-stranded regions in the RNA are available for nucleation of pairing the low-affinity S-DNAs often appear incapable of invading adjacent RNA secondary structures.

In contrast to the difficulty in predicting effective targets for the low-affinity S-DNAs, Morpholinos (particularly the new higher-affinity Morpholinos containing thymines instead of uracils) are generally effective against most sequences from the 5'cap to about 20 bases past the AUG translational start site of any selected mRNA. It is postulated that this good targeting predictability is a consequence of the high-affinity Morpholinos being far more successful than the low-affinity S-DNAs in invading RNA secondary structures.

The good targeting predictability of Morpholinos, even with mRNAs having a quite stable secondary structure, is demonstrated in Figures 6a and 6b. Figure 6a shows an mRNA construct comprising 85 bases of the leader sequence of Hepatitis B (HBV) mRNA joined to the amino acid coding sequence of firefly luciferase. The 7 numbered bold lines indicate target sites for 7 different Morpholino antisense oligos. Figure 6b shows the translational inhibition levels achieved by 1 microMolar concentrations of each of these 7 oligos as a function of their position along the mRNA, as well as corresponding values for another 5 oligos positioned further 3' to the translational start site. It is particularly noteworthy that oligos 3, 4 and 5 are seen to have effectively invaded the quite stable secondary structure within the HBV leader sequence.

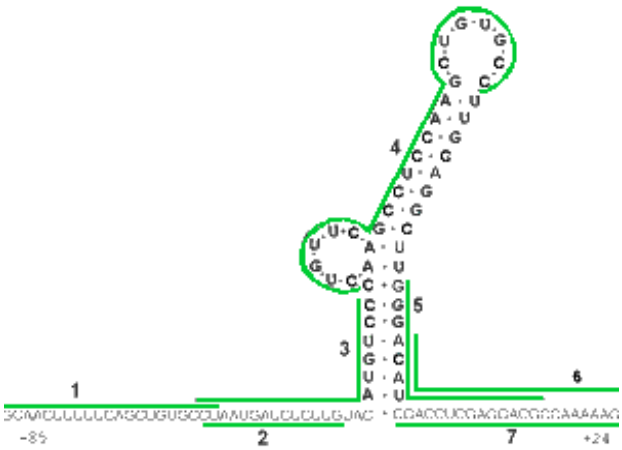

Figure 6. RNA Structure And Morpholino Antisense Activity

It should be noted that the low efficacy of Morpholinos targeted more than a few bases 3' to the AUG translational start site is probably a consequence of the robust unwindase activity associated with ribosomes after their full assembly at the AUG translational start site. As described in the next section, this lack of efficacy against sequences more than a few bases 3' to the translational start site contributes substantially to the dramatically greater specificity of Morpholinos relative to S-DNAs and chimeric oligos.

The results in Figure 6b suggest that by following rational and reliable targeting guidelines, Morpholino antisense oligos can be selected which have a high probability of being effective. These Targeting Guidelines are detailed below.

Targeting Guidelines

Properly targeted and selected Morpholino oligos generally show excellent activity in both cell-free translation systems and in scrape-delivered and osmotic-delivered cells. However, Morpholinos with inappropriately selected or targeted sequences will show poor or no activity.

Blocking Translation: The following guidelines generally lead to Morpholino oligos which are highly effective for blocking translation of their targeted mRNAs.

1. Select a target sequence in the post-spliced mRNA in the region from the 5'cap to about 25 bases 3' to the AUG translational start site.

As shown in Figure 7, Morpholinos (which function solely by a steric block mechanism) targeted more than about 30 bases 3' to the AUG translational start site do not block translation. It is noteworthy that this lack of translational inhibition at sites outside of these limits is one of the key factors underlying the exquisite specificity of Morpholino oligos.

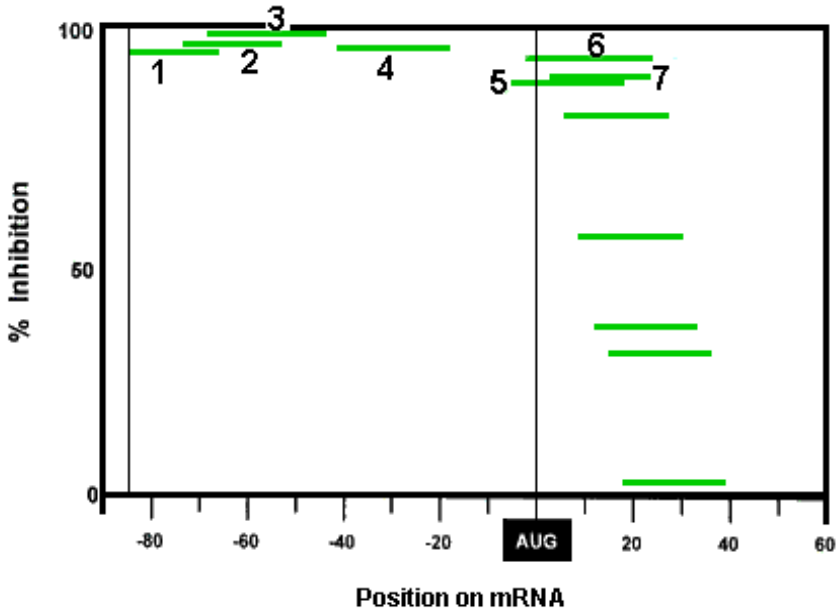

Figure 7. Antisense Activity Versus Target Position In mRNA

For most applications a simple and generally effective target site is recommended which is refered to as the "Translational Start Target" - which comprises the AUG translational start site and the 22 bases 3' to that site. Figure 8 reiterates the limits of the acceptable region in which to select a target sequence, as well as showing the recommended "Translation Start Target".

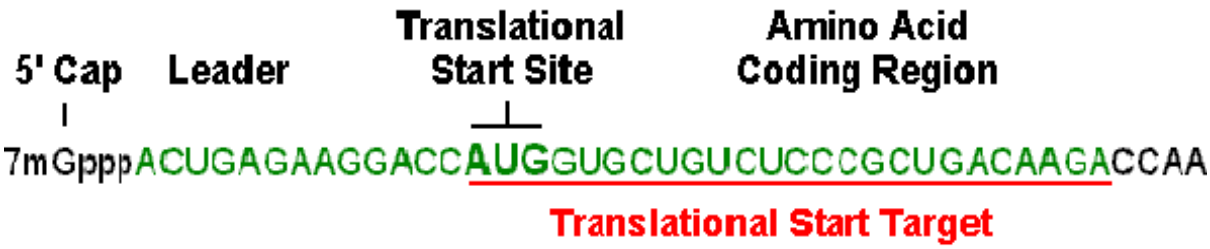

Figure 8. Efffective Targetable Region in mRNA

2. Make sure the selected sequence has little or no self-complementarity.

Preferably it should form no more than 4 contiguous intrastrand base pairs, and even 4 contiguous base-pairs are too many if they are all G-C pairs.

Figure 9 shows oligo A having an acceptable 4 contiguous base-pairs of self-complementarity, and oligo B, which should not be selected because it contains an unacceptable 5 contiguous base-pairs of self-complementarity.

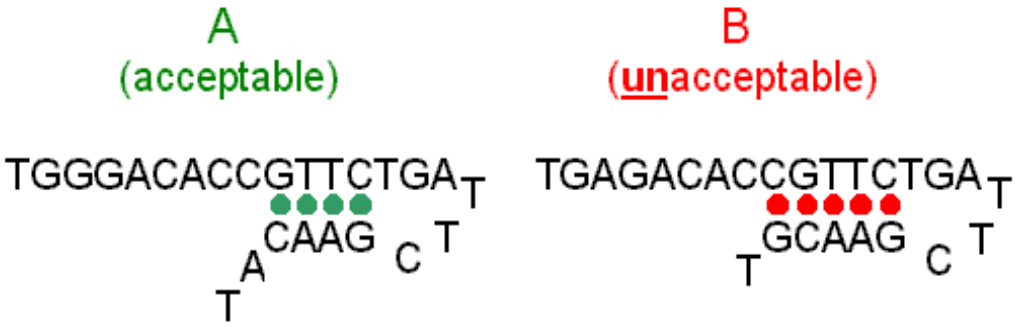

Figure 9. Oligos With Differing Self-complementarity

3. The oligo may show reduced water solubility if it contains over about 36% guanines or more than 3 contiguous guanines.

4. 25-Mers (the longest commercially available) are recommended for most applications. This is because efficacies increase substantially with increasing length and because long oligos best assure access to a single-stranded region in the target RNA, as is required for nucleation of pairing by the oligo.

Figure 10 demonstrates in a cell-free translation system the great efficacy gains to be had with increasing length of Morpholino oligos.

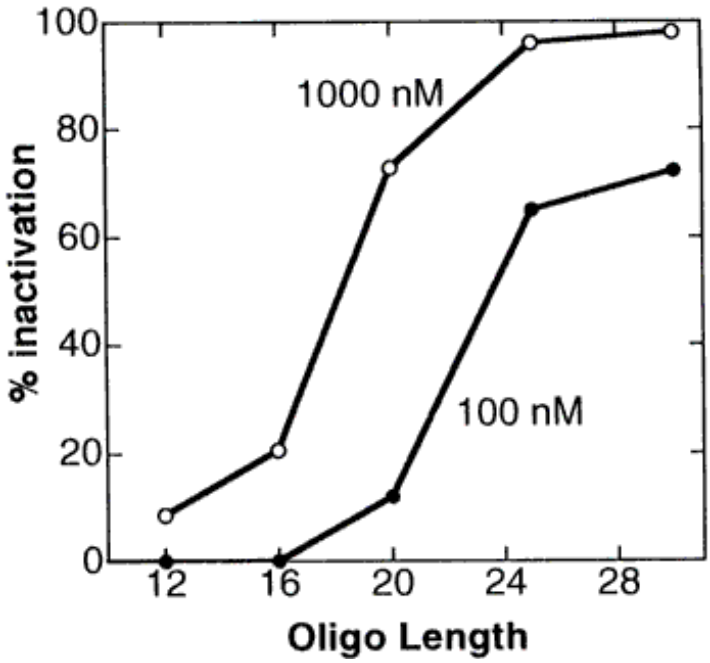

Figure 10. Length Versus Activity For Morpholino Oligos

It should be noted that this length versus activity study was carried out with Morpholino oligos containing uracils. In 1998 GENE TOOLS switched

from uracils to thymines - which increases the target binding affinity such that a "new" thymine-containing 25-mer has about the same efficacy as an "old" uracil-containing 28-mer. From this study it can be seen that short Morpholinos (shorter than about 20 subunits) exhibit only modest efficacy, while longer Morpholinos (available in lengths up to 25-mer) are far more effective. In fact, properly targeted 25-mer Morpholinos are generally more effective than any commercially available S-DNAs, chimerics, and PNAs.

Contrary to conventional wisdom in the antisense field, increasing the length of Morpholinos causes no significant loss in their specificity.

To encourage researchers to use longer higher-efficacy Morpholinos they are priced independent of length. Thus, 300 nanoMoles of a high-efficacy 25-mer costs no more than 300 nanoMoles of a low-efficacy 18-mer.

Blocking Nuclear Processing: The following guidelines give Morpholino oligos which have proven effective in blocking nuclear processing events, particularly splicing. However, it should be appreciated that some nuclear processing events occur quite soon after RNA transcription and so antisense oligos have only a brief time in which to find and bind their targeted nuclear processing sequence before said sequence carries out its function. Accordingly, effective blocking of such sequences may require substantially higher oligo concentrations then would be required for blocking translation of that RNA.

1. Select a target sequence which encompasses the selected nuclear processing sequence.

2. Make sure the selected sequence has little or no self-complementarity - preferably it should form no more than 4 contiguous intrastrand base pairs.

3. The oligo may show reduced water solubility if it contains over about 36% guanines or more than 3 contiguous guanines.

4. **25-Mers (the longest comercially available) are recommended for most applications.** This is because longer oligos provide substantially higher efficacies (with no increase in price and no loss of specificity). Long oligos also best assure access to a single-stranded region in the target RNA, as is required for nucleation of pairing by the oligo.

Figure 11 demonstrates in a splice correction assay [Kang, Cho & Kole, 1998] the efficacy gain in going from Morpholino and PNA 18-mers (the longest commercially-available PNAs) to a 28-mer Morpholino (note: currently Morpholinos are available only in lengths up to 25-mer).

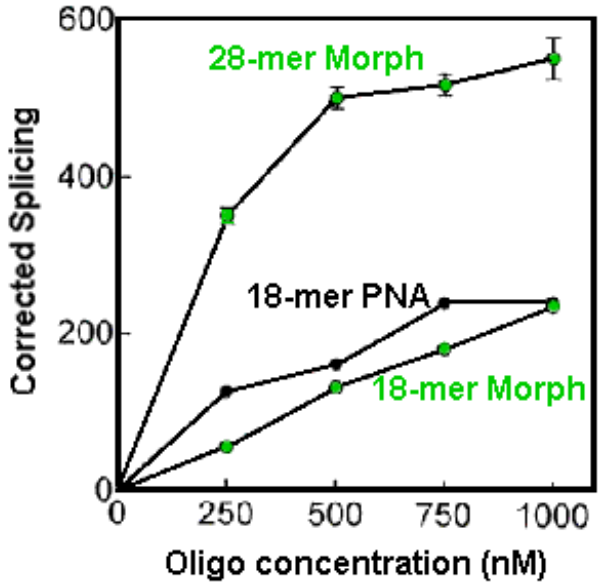

Figure 11. Splice Correction Assay

From this figure [Paul Morcos, in press - Methods in Enzymology] it can be seen that a 56% increase in the length of the Morpholino oligo affords an impressive 180% increase in efficacy of splice correction at 250 nMolar oligo concentration. In this context it should be noted that, contrary to conventional wisdom in the antisense field, increasing the length of Morpholinos causes no significant loss in their specificity.