

Minireview

Morpholino antisense oligonucleotides: tools for investigating vertebrate development

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Abstract

Antisense oligonucleotides provide a promising approach to investigating gene function *in vivo*, but their ability to offer unambiguous insights into phenotypes has been debated. The recent use of morpholino antisense oligonucleotides in zebrafish embryos may prove a major advance, but rigorous controls are essential.

The escalating pace at which genome sequencing projects are completed has increased the need for high-throughput methods for controlling gene expression. One possible approach is the use of antisense oligonucleotides to bind mRNA and prevent protein synthesis. In theory, this strategy allows rapid progress from synthesis of oligomers to observation of phenotype [1]. In practice, antisense technology has been plagued by a propensity for nonspecific interactions, and these have slowed its wide application to biological investigations [2]. Recently, however, improvements in the chemical properties of oligonucleotides and in our understanding of their mechanism of action have combined to make their successful use more likely. Work from a number of laboratories now suggests that morpholino oligonucleotides can be microinjected into zebrafish [3-7], sea urchin [8] or *Xenopus* embryos [9], where they block gene expression and produce phenotypic effects during the early stages of development.

Morpholino oligonucleotides are nonionic DNA analogs available from Gene Tools LLC [10,11]. They possess altered backbone linkages compared with DNA or RNA (Figure 1). In spite of their altered backbone, morpholinos bind to complementary nucleic acid sequences by Watson-Crick base-pairing. This binding is no tighter than binding of analogous DNA and RNA oligomers, necessitating the use of relatively long 25-base morpholinos for antisense gene inhibition. The backbone makes morpholinos resistant to digestion by

nucleases. Also, because the backbone lacks negative charge, it is thought that morpholinos are less likely to interact non-selectively with cellular proteins; such interactions often

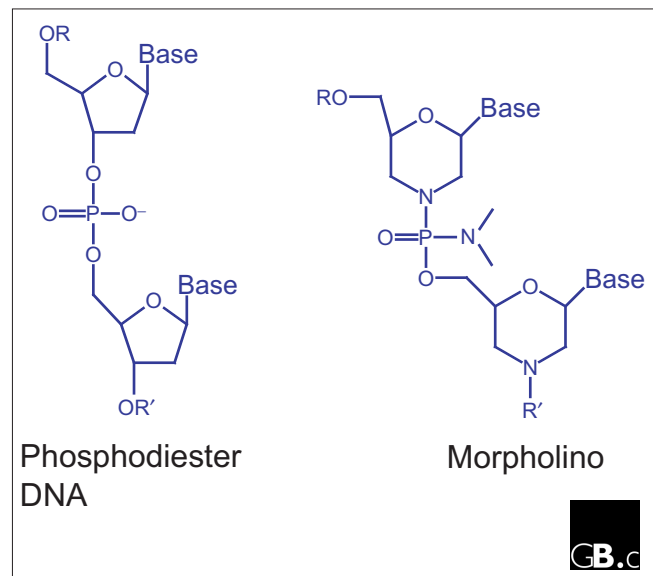


Figure 1
Structures of DNA and morpholino oligonucleotides. R and R' denote continuation of the oligomer chain in the 5' or 3' direction, respectively.

obscure the observation of informative phenotypes. The strengths of morpholinos as tools for investigating vertebrate development are well described in a recent review by Ekker [5]. Their greatest advantage is that phenotypes can be rapidly observed in Fo animals using a relatively inexpensive method.

A major obstacle to the use of antisense oligonucleotides is choosing a target sequence. Antisense oligonucleotides that contain DNA are able to form RNA-DNA hybrids. These hybrids can act as a substrate for RNase H, which promotes cleavage of the mRNA target. Because the RNA is degraded, any sequence within the coding region of the target gene has the potential to be a useful antisense site. Morpholinos, by contrast, form RNA-morpholino hybrids that are not substrates for RNase H, and thus the mRNA is not degraded. This is an important consideration, because morpholinos targeted to most of the coding region will be displaced by the ribosome as it translocates along the mRNA, and therefore will be ineffective in preventing translation. Morpholino oligonucleotides targeted to the 5'-untranslated region (UTR) or the start codon might work preferentially, by preventing the translation machinery from binding, but there is no guarantee that this is a general rule and it will need to be demonstrated empirically for each target gene.

Ekker and colleagues report that fluorescently labeled morpholino oligonucleotides can be injected into sphere-stage zebrafish embryos and achieve uniform distribution. Morpholino oligomers targeted to the start codon for green fluorescent protein (GFP) blocked GFP expression, whereas control oligomers that are complementary to GFP did not. The level of *GFP* mRNA was not changed, which is as expected if RNase H is not involved. These model experiments are significant because they establish the ability of morpholino oligomers to unambiguously block gene expression in a sequence-specific manner. Ekker and colleagues also report inhibition of several endogenous zebrafish genes and have begun to compile a database detailing the phenotypes produced by morpholino oligonucleotides [12].

Pathways investigated by Ekker and colleagues using morpholinos include developmental signaling through the Sonic hedgehog ligand and the effect of the *VEGF-A* angiogenic growth factor gene expression on angiogenesis. Overall, the Ekker laboratory have observed interesting phenotypes in 16 out of the 17 genes targeted [3-5,12]. Hammerschmidt and colleagues [6] have investigated morpholinos targeted to the type I serine/threonine kinase receptor gene *Alk8/Lost-a-fin*. Lin and coworkers [7] have reported that inhibition with morpholinos of *fez*, which encodes a zinc-finger protein, can reduce expression of *dlx2*, which encodes a homeodomain-containing protein, in the ventral forebrain. Where expression levels were examined, significant 'knockdowns' of gene expression (up to 90%) were achieved [3].

In organisms other than zebrafish, Angerer and colleagues have used morpholinos designed to block the translation of SpKr1, a transcription factor target for β -catenin regulation, in sea urchin embryos [8]. As with the work by Nasevicius *et al.* [4], a 'knockdown' of GFP expression was used as a positive control, and introduction of 4 μ M anti-*SpKr1* morpholino resulted in failure of the endoderm to differentiate. Heasman *et al.* have studied β -catenin signaling in *Xenopus* [9]. Injection at the 2- or 4-cell stage blocks dorsal-axis formation, while injection at the 8-cell stage blocks head formation [9]. Finally, Erickson and colleagues have used electroporation to deliver morpholinos directed against *FoxD3* to chick embryos. FoxD3 is a winged-helix-class transcription factor, and introduction of morpholinos alters progress of neural-crest cells, a result consistent with the localization of FoxD3 [13]. These experiments [3-9,13] illustrate the potential for morpholinos to finely dissect the temporal progression of development. When blocking a gene of unknown function or when observing an unanticipated result, it is important to keep in mind, however, that the novel phenotype is not necessarily due to reduction of expression of the target gene. This caveat is also present for standard genetic knockouts, and simply implies that results should be interpreted cautiously.

These data are provocative and suggest that, at least in these animal models, morpholino antisense oligomers may become routine tools for generating mutant phenotypes. It is essential to realize, however, that antisense technology rarely, if ever, duplicates complete 'loss-of-function' mutations. We also caution that antisense reagents have been widely misused in the past. Controls have been lacking, results are often marginal, and observed phenotypes have often been found to be due to a wide variety of unexpected non-antisense mechanisms. The causes of these non-antisense effects include unintended interactions with proteins and binding to non-target nucleic acid sequences [14]. It is therefore imperative that the methodology be applied carefully to avoid repeating the past mistakes that have slowed the progress of antisense technology.

As with any antisense experiment, rigorous controls for non-specific effects are critical to interpreting phenotypes properly. Experiments using morpholino oligonucleotides should always test at least one mismatch and one scrambled control oligomer, and the results of these tests should be reported. Dose-response assays to determine the margin between the induction of a specific phenotype and the onset of toxicity are also important. For instance, this margin can be less than twofold, emphasizing the need for carefully controlled dose determination and precisely quantitated delivery. Whenever possible, the levels of both the target protein and one or more control proteins should be evaluated in samples treated with experimental and control morpholinos. In addition, Ekker suggests that results be confirmed through mRNA rescue and/or by comparison to phenotypes of existing mutants [5].

The high success rate for the inhibition of gene expression by morpholino oligonucleotides is surprising. The dogma in the antisense field is that targeting the ATG start site is not a certain recipe for success, and that as many as 40 oligonucleotides may need to be tested to identify one that efficiently inhibits gene expression [14-18]. If targeting the start codon works so well, why do more investigators not do this rather than resorting to elaborate screens? Morpholinos may be more effective than other antisense chemistries, but why? The latter question will need to be addressed by systematic investigation of their properties and comparison with other types of oligonucleotide. The dramatically altered morpholino backbone may perhaps bind to mRNA more effectively or act as a better block to translation. If so, morpholinos could be superior agents for gene inhibition relative to other types of oligomer that might block translation, such as locked nucleic acid (LNA), peptide nucleic acid (PNA), or 2'-modified RNA [19]. On the other hand, other types of oligomer might work as well as, or better than, morpholinos, but their potential may be less apparent because they have not been tested in favorable experimental systems.

Misleading observations resulting from nonspecific interactions have confounded much previous research using oligonucleotides [2,20,21]. These difficulties have led many researchers to be skeptical of the use of oligonucleotides as a tool for basic research. Work published during the past 18 months suggests, however, that morpholino oligomers may have properties that allow researchers routinely to generate instructive phenotypes. The results are exciting, with the implication that morpholino oligomers could provide a generally applicable tool for chemical genetics and functional genomics. To realize this potential, the properties that govern the efficacy of these reagents must be determined. This information will enable researchers to optimize the methodology and achieve maximum 'knockdown' of a given target while minimizing confounding, nonspecific effects. Users of morpholino oligomers must understand the problems that have plagued the antisense field in the past, learn from these setbacks, and perform appropriate control experiments. These control experiments, at least as much as the production of interesting phenotypes, will determine whether morpholino oligonucleotides are the breakthrough that recent research suggests.

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