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Commentary

Facilitating oligonucleotide delivery: Helping antisense deliver on its promise

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Recent years have witnessed the advent of innovative technologies for disrupting the expression of virtually any gene desired. From an operational point of view, disruptive strategies may be thought of as either antigene or anti-mRNA. Antigene strategies focus primarily on gene targeting by homologous recombination or by triple-helix-forming oligodeoxynucleotides (TFOs). Homologous recombination remains the "gold standard" of all the disruption methodologies since it physically destroys the gene of interest (1, 2). Nevertheless, its general application is considerably constrained by the fact that it requires the transfection of large DNA constructs and so it is inefficient, expensive, and restricted to a small number of cell types. TFOs hybridize in the major groove of DNA by Hoogsteen or reverse Hoogsteen bonding and can disturb gene function by preventing the binding of transcription factors (3, 4), by inhibiting duplex unwinding (5), or by inducing mutations in the targeted gene (6, 7). This non-vector-based approach to manipulating gene expression does not appear to be cell type restricted, but it is constrained by the need for polypurine/polypyrimidine target sequences. Nonetheless, genetic manipulation has been achieved via triple helix formation in the nuclei of intact cells (6, 7). A larger body of work has focused on the anti-mRNA or so-called "antisense" strategies, composed principally of the use of ribozymes and antisense oligodeoxynucleotides (AS ODNs). Antisense oligonucleotides have received the majority of attention because of their apparent ease of synthesis and use. In fact, several ODN reagents have reached clinical trials for a variety of indications, including leukemia, cancer, and AIDS (8).

The power of the antisense approach has been demonstrated in experiments in which critical biological information has been gathered by antisense technology and has been subsequently verified by other laboratories using other methodologies. For example, the biologic importance and function of many protooncogenes in hematopoietic cells has been accurately predicted by using AS ODNs (9–12), in some cases with the achievement of clinically relevant changes in gene expression and cellular phenotype (8, 13). A particularly important example of the utility of this approach was the AS ODN-generated suggestion that the c-mpl receptor would bind a ligand with specific effects on megakaryocyte development (14). This observation led directly to the cloning of thrombopoietin, the long-sought hematopoietic hormone, which is the predominant regulator of megakaryocytopoiesis and platelet development (15). In another example, an antisense ODN directed against the c-myb mRNA was shown to specifically inhibit the growth of human leukemia xenografts in immune-deficient mice, eventually leading to an ongoing clinical trial (8). Several examples of successful antigene studies could also be cited. A phosphodiester oligonucleotide designed for triplex formation at the c-*myc* promoter was shown to block expression of the gene in HeLa cells (16). Triplex-forming oligonucleotides tethered to a mutagen, psoralen, were used to direct base-pair-specific mutations to and thereby inactivate a reporter gene in monkey cells (6).

However, this technology, in spite of its successes, has been found to be highly variable in its efficiency. To the extent that many have tried to use AS ODNs and more than a few have been perplexed and frustrated by results that were noninformative at best—or even worse, misleading or unreproducible—it is easy to understand why this approach has become somewhat controversial. What are the causes that have led to this situation?

We perceive that two major stumbling blocks are slowing progress in this field. First, in order for an ODN to hybridize with its mRNA target, it must find an accessible sequence. Sequence accessibility is at least in part a function of mRNA physical structure, which is dictated in turn by internal base composition and associated proteins in the living cell. Attempts to describe the *in vivo* structure of RNA, in contrast to DNA, have been fraught with difficulty (17). Accordingly, mRNA targeting is to some extent a hit or miss process, accounting for many experiments in which the addition of an ODN yields no effect on expression. Hence, the ability to determine which regions of a given mRNA molecule are accessible for ODN targeting is a significant impediment to the application of this technique in many cell systems. The other major problem in this field is the ability to deliver ODN into cells and have them reach their target. Without this ability, it is clear that even an appropriately targeted sequence is not likely to be efficient. This problem of ODN delivery has been addressed by the report of Lewis and co-workers (18) in this issue.

Native phosphodiester ODNs and the widely used phosphorothioate modified ODNs, which contain a single sulfur substituting for oxygen at a nonbridging position at each phosphorus atom, are polyanions. Accordingly, they possess little or no ability to diffuse across cell membranes and are taken up by cells only through energy-dependent mechanisms. This appears to be accomplished primarily through a combination of adsorptive endocytosis and fluid-phase endocytosis, which may be triggered in part by the binding of the ODN to receptor-like proteins present on the surface of a wide variety of cells (19, 20). In HL60 cells, binding of phosphorothioate ODNs to the cell surface can be inhibited by both heparin and fibrinogen (C.A.S., J. Loike, and L. Benimetskaya, unpublished observations), suggesting that the binding site may map to an epitope of the CD11b or CD11c/CD18 fibrinogen receptor. After internalization, confocal and electron microscopy studies have indicated that the bulk of the ODNs enter the endosome/lysosome compartment. These vesicular structures may become acidified and acquire other enzymes that degrade the ODNs (20). Biologic inactivity is the predictable result of this process.

Regardless, some of the ODNs clearly escape from the vesicles intact, enter the cytoplasm, and then diffuse into the nucleus where they presumably acquire their mRNA, or gene, target. The processes that control release from the vesicles and regulate trafficking between the nucleus and cytoplasm are not well understood. It

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is very difficult to follow this trafficking by cell fractionation experiments because of the relatively low molecular weights and rapid diffusion of oligonucleotides. It is clear however that in addition to nuclear uptake, which is likely by passive diffusion through the nuclear pores (20), efflux from the nucleus also takes place. Therefore, sequestration of the ODNs in the endosome–lysosome compartment and efflux of ODNs from the nucleus are significant problems that must be overcome in order for this technique to work reproducibly and from cell type to cell type.

In this regard, it is interesting to note that almost all the triple-helix experiments that have so far been successful within mammalian cells have been characterized by the use of G-rich oligonucleotides designed to bind to DNA in the antiparallel purine triple-helix motif (21). It was thought that this mode of third-strand binding would be difficult to achieve inside cells because of the propensity of these G-rich oligomers to self-associate, especially in the presence of physiologic concentrations of potassium ions. Such self-association would impede oligonucleotide activity because it would theoretically reduce the concentration of oligomer available for DNA binding. However, the resulting unusual nucleic acid structures may have an enhanced ability to survive degradation in the endosomal pathway and may also provide a reservoir of ODN within the cell, as has been suggested (22).

To resolve the problems of uptake and trafficking, a large number of very clever strategies have been tried in order to augment the rate of cellular internalization of nucleic acids and to increase the rate at which they pass through the endosomal membrane. These strategies have met with variable levels of success and include, but are not limited to (*i*) coupling of oligomers to polycations such as polylysine (23), polyethylenime (24), or other "interpolyelectrolyte complexes" (25), (*ii*) use of transferrin/polylysine-conjugated DNA in the presence of the capsid of a replication-deficient adenovirus (26), (*iii*) conjugation of oligonucleotides to fusogenic peptides (27) or to a peptide fragment of the homeodomain of the *Drosophila* antennapedia protein (28), (*iv*) targeting of oligonucleotides to specific cell surface receptors, such as the folate (29), asialoglycoprotein receptors (30), and transferrin (31), (*v*) conjugation to cholesterol (32), and, most successfully (*vi*) complexation of oligonucleotides with cationic lipids (33). However, many of these lipids, which are in common laboratory use, suffer from problems of applicability ranging from lack of serum stability to cellular toxicity.

The work by Lewis *et al.* (18) tackles the delivery problem by synthesis and characterization of a new transfection reagent, GS2888 cytofectin, for oligonucleotide and DNA delivery into mammalian cells. GS2888 cytofectin is a formulation of dioleophosphatidylethanolamine as a fusogenic agent with a novel cationic lipid (GS2888) carrying an alkyl chain of optimized length. The reagent is reported to represent a major improvement over older generation material because it transfects ODN and plasmids into cells with high efficiency and low toxicity. It retains this ability in the presence of serum, a property that may or may not make it more useful for *in vivo* applications since, of course, serum is a byproduct of clotted plasma, which is not encountered in the living animal. How GS2888 accomplishes these effects is not fully detailed in the report but it appears to be able to appropriately destabilize the endosomal membrane, permitting the entry of phosphorothioate oligonucleotides to the cytoplasm, where they may diffuse into the nucleus. Even more remarkable, the figures accompanying the manuscript suggest that the ODN, once delivered to the nucleus, is not diffusing out as would ordinarily be expected. This might also explain its superiority compared to other lipid formulations, although how this is accomplished is again uncertain. In addition, GS2888 appears to permit phosphorothioate oligonucleotides to be antisense effector molecules at low nanomolar concentrations. This may entirely circumvent a major problem with these compounds, which is their propensity to interact with cellular macromolecules, especially proteins, in a non-sequence-specific fashion. This feature also may significantly lower the cost of ODN experiments and applications. The development of GS2888 cytofectin thus represents a welcome and potentially important addition to the array of lipid formulations that are available for transfection of DNA, whether as ODNs or as plasmids.

However, several caveats should temper our enthusiasm. First, the range of cell types susceptible to transfection with GS2888 cytofectin appears to be wide but not complete. It is also unclear what factors lead to success in some cell types and failure in others. Moreover, most cells tested so far have been transformed lines; further trials with primary cells, such as hematopoietic cells, are needed. Second, while the application of GS2888 to cell culture experiments has been clearly demonstrated, its utility for therapeutic applications remains to be determined. For such uses, it would seem that strategies for direct covalent modification of oligonucleotides may be preferable to comixture with transfection reagents. *In vivo* animal experiments (8, 13) will certainly address this issue.

The antisense approach has generated controversy with regard to mechanism of action, reliability, and ultimate therapeutic utility. Nevertheless, the potential power of this method remains undisputed. For this reason, we believe that efforts should be increased to decipher the mechanism of action of antisense ODN and to learn how they may be successfully used in the clinic. To the extent that GS2888 may facilitate these goals, we applaud the work of Lewis and colleagues. The community anxiously awaits the widespread availability of this reagent for further testing.

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