

Utilization of Antisense Oligodeoxynucleotides with Embryonic Tissues in Culture

Raymond B. Runyan,* Christopher C. Wendler,* Laura A. Romano,*
Angelique S. Boyer,* John M. Dagle,† and Daniel L. Weeks‡

*Department of Cell Biology and Anatomy, University of Arizona, Tucson, Arizona; and †Department of Pediatrics, and ‡Department of Biochemistry, University of Iowa, Iowa City, Iowa

Experimental embryology has long used manipulation of interacting tissues to examine questions of tissue interaction and differentiation. The potential for specific manipulation of gene expression in such tissues has made the utilization of antisense techniques desirable. However, problems with this methodology have discouraged many investigators from using this approach. Selection of target sequences for antisense oligonucleotides, delivery of oligonucleotides into cells or tissues, and the type of modification of the oligonucleotide to be used all present concerns that must be addressed. This paper describes our approach to selection of target sequence and methods of delivery and describes the synthesis of a methoxyethylamidate-modified antisense oligonucleotide that has proved useful in our studies. This approach has enabled us to explore aspects of tissue interaction in the embryonic heart that would have been difficult to explore in a genetic model. © 1999 Academic Press

Experimentation with the chick embryo has a long history. One advantage of this organism is that staged embryonic tissues are easy to obtain and culture. Utilization of cells and tissues in culture and by reintroduction into the embryo forms a staple of experimental embryology. However, with the exception of work with a few selected strains of chickens, utilization of a difficult transgenic procedure, or viral strategies, genetic approaches are difficult in the chick. Therefore, we turned to antisense oligodeoxynucleotides as a way to explore the role of particular proteins in primary cultures of embryonic heart tissues. When successfully applied, antisense oligonucleotides can provide very specific and useful information. However, there are problems that have led a number of laboratories to abandon this approach and have discouraged others from attempting this technique. The objective of this

paper is to describe the experimental design and methodology that can provide the reader with the tools to pursue this approach.

LIMITATIONS OF ANTISENSE OLIGONUCLEOTIDE EXPERIMENTS

It is our perception that the most common source of experimental failure of the antisense oligonucleotide approach is an inappropriate experimental design. Antisense oligonucleotides are relatively short-lived reagents able to reduce levels of targeted mRNAs by forming an RNA:DNA hybrid that is the substrate for endogenous RNase H and thus reduce the *de novo* synthesis of a specific protein (1, 2). Among the variables that need to be considered are how efficiently a given cell takes up oligonucleotides, how stable the oligonucleotide is in the cell and/or culture medium, and the ability of the oligonucleotide to form a hybrid with the selected target region of the mRNA. In addition, genes with rapidly replenished mRNA levels and/or long-lived proteins may be difficult to disrupt with antisense oligonucleotide methodology. To be effective, an oligonucleotide must persist long enough or be delivered in large enough quantities to mediate a substantial loss of the targeted mRNA. If a mRNA is rapidly replaced by new synthesis, loss of an existing population may not produce a measurable effect. Further, targeted disruption of a mRNA is unlikely to be effective if the half-life of preexisting protein is long enough to enable an adequate amount of protein to persist through the period or event being analyzed. We typically use a heart explant assay in which the embryonic processes to be explored occur within a 12- to 24-h window of time after treatment (3; Romano and

Runyan, in press). Longer experimental protocols are likely to require reapplication of oligonucleotides to the cells on an empirically determined time schedule to be effective.

TYPES OF OLIGONUCLEOTIDES USED

The most common oligodeoxynucleotides used for antisense studies are the phosphorothioate nucleotides provided by a number of commercial suppliers. These oligodeoxynucleotides are easy to synthesize on most DNA synthesizers using conventional phosphoramidite chemistry. These molecules are modified so that one of the oxygen molecules of the phosphodiester backbone is replaced with a sulfur. This blocks the activity of exonucleases and provides a longer-lived molecule than a phosphodiester oligonucleotide. Phosphorothioate oligonucleotides have been widely used and appear to be useful in a number of experimental designs when appropriate controls are employed (4, 5). However, there are a number of questions about the specificity of this molecule and one should proceed with caution in the utilization of phosphorothioates. For example, one caveat to the use of this class of reagent is that the array of sulfur groups along the backbone mimics the charge distribution of heparan sulfate. This appears to have potential to interfere with the activity of heparan-binding growth factors such as fibroblast growth factor (FGF) and matrix molecules such as laminin and fibronectin (6–8). Recently, this class of molecules has been shown to bind and perturb vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) receptors on cells (9). A further caveat to this class of molecules is that the breakdown products of phosphorothioate oligonucleotides can be toxic to cells (10). Since many of these effects are sequence specific but unrelated to antisense function, experiments using this class of compounds should be interpreted carefully.

A variety of other oligonucleotide modifications can be found in the literature. Unlike the careful work of Stein and colleagues (6–9), relatively little work has been done to explore the advantages and disadvantages of many of these other modifications. We have been using the methoxyethylamidate modification for a number of years and feel comfortable with its specificity and utility (2, 3, 11). For experiments using external application of oligonucleotides, we have adopted the use of a combination of unmodified oligonucleotides and methoxyethylamidate-modified oligodeoxynucleotides.

Unmodified oligonucleotides are subject to rapid degradation, but they can be obtained quickly and cheaply. Commercial suppliers can provide oligonucleotides within 24 h at less cost per base than a laboratory can

produce them. When used in concentrations of 10–100 μM , or when delivered more efficiently in liposomes (see below), unmodified oligonucleotides can produce visible effects in a number of assays. Due to the time and expense incurred making modified oligonucleotides that can be ineffective, we turned to a strategy of screening sequences with unmodified oligonucleotides. Those sequences exhibiting a partial effect were then selected for testing as modified oligonucleotides. This strategy was previously used by Heasman *et al.* (12) for injected oligonucleotides in *Xenopus* oocytes. This technique became useful in explant cultures when liposome delivery of unmodified oligonucleotides proved to be effective. Occasionally, data obtained using unmodified oligodeoxynucleotides are so convincing that the subsequent utilization of modified oligonucleotides becomes unnecessary.

Oligonucleotides modified with methoxyethylamidate groups along the phosphodiester backbone have a longer half-life within the cell due to the protection from exonuclease activity (2). Since this modification is also inhibitory of the activity of RNase H, a hybrid form of oligonucleotide in which the ends of oligonucleotides are modified to protect from 5'- and 3'-exonucleotidase activity while an open stretch of seven or eight phosphodiester bonds is left in the middle was developed (2, 11, 13). This mixed backbone oligonucleotide allows RNase H to bind to the open (unmodified) region of the DNA. The hybrid oligonucleotide, although not as stable as a completely modified phosphorothioate oligonucleotide, provides an effective and relatively nontoxic mediator of targeted mRNA degradation. The half-life of this modified oligonucleotide is 600 times greater than that of an unmodified nucleotide as judged by comparison of labeled molecules in a *Xenopus* oocyte (2). When hybrid oligodeoxynucleotides bind to RNA in a DNA–RNA duplex, they mediate the targeted degradation of the RNA by RNase H (2). This releases the oligonucleotide to hybridize with additional targets and enables the serial degradation of multiple RNAs within a cell by each oligonucleotide.

APPLICATION OF OLIGONUCLEOTIDES TO TISSUES

Unlike experiments performed with *Xenopus* oocytes, where the size of the cell enables direct injection of oligonucleotides, many of our experiments rely on the ability of modified oligonucleotides to enter the cell through the membrane. Despite the greater ability of methoxyethylamidate-modified oligonucleotides to partition into a hydrophobic layer than unmodified oligonucleotides (2), this is an inefficient process. Experiments with heart tissue explants were performed with 1 nmol of oligonucleotide added per explant of

fewer than 10,000 cells (3). An estimated 10^{10} oligonucleotide molecules are delivered per cell in a small volume of medium using this protocol (1 μ l of a 1 mM solution/explant). Under these conditions, an adequate number of molecules (in combination with the potential for serial degradation) were delivered into the tissue to enable the specific degradation of transforming growth factor $\beta 3$ (TGF $\beta 3$) mRNA (3). The validity of these results has been verified over the years by a series of papers confirming isoform-specific function of TGF β s in the heart (14; 14a).

In an effort to enhance the delivery of oligonucleotides into cells, and reduce the amount of oligonucleotide needed per explant, several protocols were explored. Fluoresceinated, modified oligonucleotides were prepared to compare the efficacy of delivery into tissue culture cells and primary explants. A protocol for delivery of plasmids into cells using an adenovirus carrier in combination with DEAE-dextran and LipofectAMINE (Gibco-BRL) proved ineffective with oligonucleotides. However, we found that LipofectAMINE alone significantly enhanced the delivery of oligonucleotides into cells in culture. Comparison was then made between several liposome types with varying charges including LipofectAMINE and Lipofectin (Gibco-BRL) among others. LipofectAMINE was the only liposome material that was effective. However, when tested on explant cultures on collagen gels, the liposomes bound to the extracellular collagen matrix and remained sequestered away from the explant. To avoid this problem, we adopted a protocol where oligonucleotides in liposomes (6 μ g DNA and 12 μ g LipofectAMINE/ml in medium 199) were incubated with the tissue (heart 5–6 explants) in 1 ml of medium for 30 min prior to explanting onto the collagen gel. This protocol proved to be very effective with unmodified oligonucleotides (see below) but we were unable to prepare mixtures with modified oligonucleotides that were not toxic to the cells. We concluded that the efficiency of liposome delivery, combined with the extended half-life of modified oligonucleotides, produced this toxic effect by introducing too much oligonucleotide. Accordingly, we reverted to a procedure using direct application of modified oligonucleotides in a small volume of water or buffer to our explanted tissues (3).

SELECTION OF TARGET SEQUENCES FOR ANTISENSE OLIGONUCLEOTIDES

A significant problem in the design of antisense oligonucleotides has been in choosing a target sequence. One common hypothesis is that the region of the AUG translation start site presents an accessible target that is quite effective. In our experience, this region is often good but not uniformly so. For example, when we tar-

geted the start sites of TGF β isoforms in the chick embryo, the antisense oligonucleotide for the TGF $\beta 3$ molecule was quite effective. A second oligonucleotide toward the TGF $\beta 2$ isoform had no effect. While we performed the appropriate control to show that TGF $\beta 3$ message was degraded within the targeted tissue, we failed to confirm that TGF $\beta 2$ message was similarly degraded (3). Only later, after the TGF $\beta 2$ null mouse proved to have heart defects (15) did we reexamine the role of TGF $\beta 2$ in the chick heart. Since antiligand antibodies show specific and distinguishable effects in our cultures we can only conclude that the TGF $\beta 2$ -specific oligonucleotides used in the earlier study were ineffective (14a).

Since ineffective antisense sequences are rarely, if ever, published, the rules for design of antisense oligonucleotides are not well understood. Various laboratories have published successful interventions at the start site, in the 3' UTR, and at various locations along the mRNA. Where the genomic structure is known, it has been suggested that oligonucleotides that bridge splice sites are effective. Based on the original modification procedure used by Dagle *et al.* (11), we routinely select 16-mers. This provides a compromise between uniqueness of sequence and ease of use. By habit, we tend to select sequences with a 50% GC ratio but in a series of experiments targeting $G\alpha_1$ subunits we selected oligonucleotides that were 75% AT-rich because of the limited variation seen between isoforms. These oligonucleotides were effective, suggesting that there is room for further exploration.

To identify the most effective DNA sequences, we adopted a scanning-testing procedure previously reported by Heasman and colleagues for injection into frog oocytes (12). This consists of an array of four or more unmodified oligonucleotides selected to match the mRNA sequence at various points. These are ordered from a commercial supplier as gel-purified oligonucleotides. The unmodified oligonucleotides are resuspended in a small volume and desalted on a NAP 5 column (Pharmacia) in distilled water. After concentration, they are mixed with LipofectAMINE at 6 μ g/12 μ l of lipid and diluted to 1 ml in a tissue culture medium. Each oligonucleotide is added to heart explant cultures and the cultures are observed for changes compared with untreated and control oligonucleotide-treated cultures. Since the half-life of unmodified oligonucleotides is short, the results obtained with unmodified oligonucleotides are often limited. However, partial effects are indicative of sequences that can be pursued with modified oligonucleotides. A recent study of five oligonucleotides against the transcription factor *Mox1* in the avian heart showed two to be especially effective (one bridging the start site and another 20 bases into the coding sequence) while one was only moderately effective and two were ineffective (Wendler and Runyan,

unpublished). In contrast, four distinct sequences against the transcription factor *Slug* were all effective (3a).

CONTROLS

One of the major reasons that antisense oligonucleotides have been held in poor regard by reviewers and study sections has to do with inadequate controls. As shown by Woolf *et al.* (16), there is reason to question the specificity of antisense oligonucleotide effects. Although calculations of sequence specificity suggest that a 16-mer should be adequate, it is likely that more common partial homologies, especially with unmodified oligonucleotides and phosphorothioate oligonucleotides, may produce effects by hybrid arrest or by RNase H-mediated degradation. The ideal antisense experiment includes at least two specific and effective antisense sequences, one or more ineffective control sequences, demonstration of specific loss of message and protein, and rescue by replacement of the lost protein or message. In many experiments, rescue is not feasible but a minimum level of proof requires two independent and effective antisense sequences and demonstration of specific loss of message or protein. Although the most common control sequence for antisense experiments is a sense sequence, this choice can be a poor one. We and others (17) have seen partial effects produced by sense control sequences. Though the basis for these effects is unclear, one can speculate that sense sequences may interfere with hairpin structure within a mRNA. Such interference might lead to a loss of function within the cell. In our hands, more reliable controls include reversed, but uncomplemented, sequences or scrambled sequences. In the case of methoxyethylamidate-modified oligonucleotides described below, the limited size of the RNase H binding site enables a control oligonucleotide to be designed by reversing just two or three of the internal bases within the oligonucleotide (2).

PREPARATION OF METHOXYETHYLAMIDATE-MODIFIED OLIGONUCLEOTIDES

DNA Synthesizer

Any DNA synthesizer capable of H-phosphonate chemistry is adequate for production of modified oligonucleotides. Although phosphoramidite chemistry is more efficient and more widely supported, it is useful only for the production of phosphodiester- and phosphorothioate-modified oligonucleotides. Many synthesizers can be reprogrammed for the alternative chemistry using the manufacturer's instructions. We use a MilliGen/Bioscience Cyclone Plus Synthesizer with the program provided

by the manufacturer. Although the manufacturer no longer supports DNA synthesizers, the apparatus remains useful for the production of modified oligonucleotides. We have previously used an ABI Synthesizer with good results. Since the commercial production of oligonucleotides on a cheaper scale has bypassed many university DNA facilities, functional DNA synthesizers can be found on many campuses that can be exploited for production of modified oligonucleotides.

Chemicals

The only chemistry supplier currently supporting H-phosphonate chemistry is Glenn Research (Sterling, VA). This manufacturer has good technical support and can be reached at (800) 327-4536 and <http://www.glenres.com>. Solutions used for H-phosphonate chemistry vary between synthesizers and Glenn Research will provide the appropriate reagents when the synthesizer is specified.

Synthesis of Modified Oligonucleotides

Modified, hybrid methoxyethylamidate-modified oligonucleotides are made by the sequential production of a 3' 5-mer with four modified bonds, a middle sequence with seven unmodified phosphodiester bonds, and a 5' sequence that again is modified to block exonuclease degradation of the phosphodiester backbone. The preparation of these oligonucleotides is as described by Dagle *et al.* (11) and listed below in stepwise fashion. The instructions here are for a 0.2- μ mol synthesis which provides sufficient material for 25-35 explant treatments. Note that all steps performed with the column removed from the synthesizer should be performed under a fume hood with gloves, eye protection, and a laboratory coat.

1. Load the appropriate CPG beads corresponding to the 3' end of the desired sequence into a synthesis column and rinse with acetonitrile to flush excess CPGs. Program the first five bases, 3' to 5', into the machine for the production of the terminal five bases. Program the synthesizer to leave the trityl (DMT group) on and not to oxidize the product.

2. Once the initial sequence has been completed remove the column from the synthesizer and dry under a vacuum for 5 min. Fix the column between a syringe loaded with modification buffer (3 ml carbon tetrachloride and 330 μ l methoxyethylamine) and an empty syringe. (Syringes must be organic stable without a rubber-tipped plunger.) Under a fume hood, push the solvent back and forth through the column every few minutes. Allow 5 min for each modified residue. This procedure modifies the phosphodiester bonds within the sequence to include the methoxyethylamidate group.

3. Flush the column with 5 ml of 2% triethylamine (TEA) in acetonitrile, dry for 5 min, and reapply to the

synthesizer. Enter the next sequence (eight bases), beginning with the 5' base of the previous sequence, into the synthesizer (3' to 5'). This sequence is to include the open phosphodiester bond sequence recognized by RNase H. Programming includes normal oxidation and trityl group removal.

4. Once the middle section is complete and is oxidized reprogram the synthesizer to add the remaining four nucleotides to the sequence (five bases including the terminal base of the previous sequence). Program the synthesizer to leave the trityl group on, without oxidation, after the completion of synthesis.

5. Flush the column once again with modification buffer between two syringes to modify the 5'-terminal linkages (5 min per residue). Wash the completed sequence with 5 ml of 2% TEA in acetonitrile.

6. Transfer the DNA synthesis column containing the modified oligonucleotides to a new 5-ml syringe with the plunger depressed. Fill a second syringe with 3 ml of fresh 30% ammonium hydroxide and attach to the column. Alternately depress the syringes to force the ammonium hydroxide over the column. Rest the column for 45 min and then flush the solution through the column three or four more times. Rest the column for another 45 min and flush again before drawing the solution into one of the syringes. Then decant this solution containing the oligonucleotides into a screw-top centrifuge tube and leave at room temperature for 24 h. Cool the oligonucleotide-containing solution and add 30 μ l of TEA to protect the 5'-DMT group. Evaporate the solution to dryness on a Savant Speed Vac or similar rotary evaporation unit.

Purification of Modified Oligonucleotides

The efficiency of H-phosphonate chemistry is approximately 95–96% at each coupling. Therefore, the completed synthesis of a 16-mer has a significant component of failure sequences and these must be removed in a two-step HPLC process. The first step takes advantage of the 5'-DMT group left on the final synthetic product for purification by reverse-phase chromatography. A second run is then performed after detritylation to obtain product with the DMT group removed. The purified product is then run over a desalting column to remove contaminating organics before use. The specific steps to be taken are listed below. We use a Waters high-performance liquid chromatograph with a two-pump system with a Delta Pak column, C₁₈, 300 Å, 5 μ m, 3.9 \times 150 mm (Waters), and a 254-nm detector. The pellet can be stored at -20°C at any stage except after step 4.

1. Dissolve pellet from 0.2- μ mol synthesis in 200 μ l of 100 mM triethylammonium acetate (TEAA), pH 6.5 (Eluant A). Centrifuge briefly and transfer supernatant.

2. Perform an analytical run on the oligonucleotide

to evaluate the synthesis and determine retention time. Take 10 μ l of suspended oligonucleotide solution and dilute with 40 μ l of Eluant A. Inject 10 μ l to column of chromatograph running at 1 ml/min with a linear gradient. The gradient is initially begun at 95% Eluant A and 5% Eluant B [95:5 (v/v) acetonitrile:HPLC-grade H₂O] and runs to 60% Eluant A and 40% Eluant B at 40 min. Failure sequences will elute as a large peak and the desired product will elute as a retained second peak.

3. Run preparative purification with remaining undiluted material. Collect desired peak in a fraction collector, pool peak fractions, and dry down to a pellet.

4. Remove 5'-DMT group by resuspending the pellet in 100 μ l 80% acetic acid for 30 min and dry down again.

5. Resuspend in 500 μ l of 1% TEA and concentrate to dryness. This step protects the oligomer from acid exposure.

6. Resuspend the pellet in 200 μ l of Eluant A and use 10 μ l as before to perform an analytical HPLC run. This will enable an evaluation of the success of the detritylation and determine the retention time of the peak.

7. Run a preparative purification with the remaining material. Concentrate the first peak and dry down again.

8. Resuspend the pellet in 500 μ l in 10 mM KCl and apply to a NAP 5 column (Pharmacia) that was previously washed with 15 ml H₂O. Elute the column with 1 ml H₂O and concentrate to 500 μ l.

9. Wash the NAP 5 column again with 15 ml H₂O and reapply the oligonucleotide solution. Elute with 1 ml, dry down the solution, and resuspend the oligonucleotides in water or saline solution at 1 mM for topical application to tissues.

TROUBLESHOOTING

Production

The yield of this preparation is usually sufficient for 25–35 treatments of 1 μ l/explant. Yields are considerably reduced with chemicals that have been left in the synthesizer. In high-moisture areas, it is critical to use the chemistry very quickly. One set of H-phosphonate chemistry is sufficient for eight oligonucleotides (0.2- μ mol size) and we try to use the chemicals within a few days of setting up the synthesizer.

Delivery

Initial uptake of oligonucleotides in tissues and cells can be evaluated by visualization of 3'-fluorescinated oligonucleotides with a confocal microscope or other fluorescence detection device. Fluorescinated oligonucleotides can be made by the

utilization of fluorescent CPG nucleotide beads (obtained from Glenn Research) to start the oligonucleotide synthesis. It should be noted that the degradation of oligonucleotides within tissues or media makes studies using localization of marker after any period essentially meaningless. Studies suggest that the majority of oligonucleotides are taken up by cells within 30 min (18). Transport into cells is thought to be via receptors and/or pinocytosis (19, 20). Delivery of modified oligonucleotides into tissues can be enhanced by reducing the volume and increasing the pericellular concentration. Examination of the recent literature shows that lipid delivery is a widely used method [e.g., (21, 22)]. Though we attempted to titrate levels of liposomes and modified oligonucleotide for some time, we were unable to obtain a result as satisfactory as the direct application of modified oligonucleotides to our cultured explants. Other investigators may find this combination to be effective under different experimental conditions.

Effectiveness

For a variety of reasons, different antisense oligonucleotide sequences are not equally effective. Reasons for this are unclear but the usual explanation is thought to lie in the accessibility of the targeted sequence within the mRNA. We have found several occasions where a cocktail of antisense sequences is more effective than individual sequences alone. This may prove to be a useful procedure in some experiments but proof of specificity in the loss of mRNA or protein is even more critical. Since unmodified oligonucleotides are degraded within minutes and modified oligonucleotides appear to be degraded within hours, protocols that include the reapplication of oligonucleotides may be needed for long-term processes (5). We have less experience with these types of protocols but we would argue that an evaluation of mRNA and protein half-lives can be critical to the success of this approach.

CONCLUDING REMARKS

We are convinced that careful application of antisense oligonucleotide technology can provide functional information economically and at a faster rate than can be generated by knockout technologies. In nongenetic systems, functional information may be difficult to obtain by any other means. The procedures described here should enable the reader to use modified oligonucleotides in a manner likely to produce results in his or her own experimental tissues.

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