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Characterization of Modified Antisense Oligonucleotides in *Xenopus laevis* Embryos

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ABSTRACT

A wide variety of modified oligonucleotides have been tested as antisense agents. Each chemical modification produces a distinct profile of potency, toxicity, and specificity. Novel cationic phosphoramidate-modified antisense oligonucleotides have been developed recently that have unique and interesting properties. We compared the relative potency and specificity of a variety of established antisense oligonucleotides, including phosphorothioates (PS), 2′-*O*-methyl (2′OMe) RNAs, locked nucleic acids (LNAs), and neutral methoxyethyl (MEA) phosphoramidates with new cationic *N,N*-dimethylethylenediamine (DMED) phosphoramidate-modified antisense oligonucleotides. A series of oligonucleotides was synthesized that targeted two sites in the *Xenopus laevis* survivin gene and were introduced into *Xenopus* embryos by microinjection. Effects on survivin gene expression were examined using quantitative real-time PCR. Of the various modified oligonucleotide designs tested, LNA/PS chimeras (which showed the highest melting temperature) and DMED/phosphodiester chimeras (which showed protection of neighboring phosphate bonds) were potent in reducing gene expression. At 40 nM, overall specificity was superior for the LNA/PS-modified compounds compared with the DMED-modified oligonucleotides. However, at 400 nM, both of these compounds led to significant degradation of survivin mRNA, even when up to three mismatches were present in the heteroduplex.

INTRODUCTION

THE FIRST ANTISENSE EXPERIMENTS were conducted using unmodified DNA (Stephenson and Zamecnik, 1978), and a wide variety of chemical modifications have since been employed to improve antisense agents by conferring resistance to active cellular and serum nucleases. Nuclease-resistant modifications of the phosphate internucleoside linkage include phosphorothioates (PS) (Iyer et al., 1990), phosphoramidates (Dagle et al., 1991b), methylphosphonates (Miller et al., 1985), boranophosphates (Rait and Shaw, 1999), and phosphonoacetates (Sheehan et al., 2003). Other useful synthetic strategies include modifications of the sugar group, such as 2′-*O*-alkyl RNA (Monia et al., 1993; Sproat et al., 1989), locked nucleic acids (LNAs) (Sorensen et al., 2002; Wahlestedt et al., 2000), and 2′-deoxy-2′-fluoro-D-arabinose (Lok et al., 2002). It is also possible to entirely replace the natural ribose/phosphodiester backbone (Nielsen, 1995) with other polymers, such as morpholinos (Summerton and Weller, 1997) or peptide nucleic acids (PNAs) (Hanvey et al., 1992).

Antisense compounds can work by two distinct mechanisms of action, one in which the antisense polymer directs mRNA degradation and the second where the antisense polymer tightly binds to the RNA and interferes with translation or splicing by steric blockade (Bonham et al., 1995; Chiang et al., 1991; Johansson et al., 1994;

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Walder and Walder, 1988). RNase H degrades the RNA strand of an RNA/DNA heteroduplex and is used *in vivo* to cleave the target mRNA at the site of antisense oligonucleotide hybridization. Unfortunately, most of the modifications that confer nuclease resistance to an antisense polymer do not form a substrate for RNase H when hybridized with RNA. All known replacement backbones (such as morpholinos and PNAs), most phosphate modifications (such as phosphoramidates and methylphosphonates), and most 2′-ribose modifications do not form a substrate for RNase H when hybridized to a complementary RNA.

The useful properties of RNase H-inactive chemical modifications can still be exploited in RNase H-active antisense agents if modifications from both classes of chemistries are incorporated into a single chimeric antisense oligonucleotide. Various chimeric designs have been proposed and tested. The most commonly used design places RNase H-inactive modifications that have increased nuclease stability at the 5′-end and 3′-end with a central core of phosphodiester or PS DNA that serves as a domain for RNase H activation. End-modified chimeras, or gapmers, were first shown to work as RNase H triggers using 2′-*O*-methyl (2′OMe) RNA/DNA constructs *in vitro* (Inoue et al., 1987; Shibarhara et al., 1987). In both *Xenopus* embryos and human cultured cells, a minimum of five or six unmodified or PS-modified DNA residues are required in the central RNase H-activating domain to trigger RNase H-mediated degradation of the target mRNA (Dagle et al., 1991b; Monia et al., 1993).

We have reported previously that positively charged phosphoramidate-modified oligonucleotides show potential as antisense agents (Dagle et al., 2000). Here, we expand on this previous work and directly compare the relative potency and specificity of novel positive phosphoramidates with a series of alternative antisense chemistries in a *Xenopus* microinjection system. We also provide insights into the parameters that determine the overall activity of an antisense oligonucleotide.

MATERIALS AND METHODS

Oligonucleotide synthesis

Oligonucleotides were synthesized, purified, and characterized by Integrated DNA Technologies (Coralville, IA) using standard techniques. The oligonucleotides used in this investigation are shown in Table 1. The mRNA target chosen for these studies was survivin, an abundant, maternal *Xenopus* transcript encoding a protein thought to be involved in both cell cycle progression and apoptosis regulation (Murphy et al., 2002). Unmodified (PO), PS, 2′OMe, and beta-D-LNA modifications were synthesized using modifications of standard phosphoramidite chemistry. N,N-dimethylethylenediamine (DMED) (Pos) and 2-methoxyethylamine (Neu) phosphoramidate linkages were synthesized using hydrogen phosphonate chemistry followed by oxidative amidation, as previously described (Dagle et al., 1990). These compounds were purified first by reverse-phase HPLC, followed by polyacrylamide gel electrophoresis, and were subsequently dialyzed against water. Oligonucleotide quantitation was performed by UV spectroscopy, using modification-specific extinction coefficients. Oligonucleotides were then characterized by both capillary electrophoresis and electrospray ionization mass spectrometry. The majority of oligonucleotides used in this study (32 of 45) were ≥90% pure, with only two compounds having a purity between 80% and 85%.

Oligonucleotide analysis

Duplex stability studies were performed by thermal denaturation and monitored using UV spectroscopy, as previously described (Owczarzy et al., 2004). These studies were performed with both DNA/DNA duplexes and DNA/RNA heteroduplexes in 100 mM Na^+ at a pH of 7.0. The 3′ exonuclease stability of phosphodiester bonds adjacent to a modified phosphate linkage was determined using a model oligonucleotide: FAM-C-A-C-A-A-A-C-CT•G-T-T-C-T-T-G-G-C-A-G, where FAM is a 5′ carboxyfluorescein-aminohexylphosphate, and • represents the modified internucleoside linkage bond. Aliquots of each oligonucleotide (200 pmol) were incubated with increasing amounts of exonuclease I (up to 10 U), exonuclease III (up to 200 U), or snake venom phosphodiesterase (up to 32 μU) to ensure complete digestion. Electrospray ionization mass spectrometry was performed on the oligonucleotide fragments after a 4-hour incubation at 37°C.

Xenopus *embryo injections*

Adult *Xenopus laevis* were purchased from Xenopus I (Ann Arbor, MI) and housed in a thermally controlled environment in the Animal Care Unit at the University of Iowa. Eggs were obtained from hormonally treated females and were fertilized as previously described (Rebagliati et al., 1985). In the majority of experiments, single cell embryos were injected with 9.2–18.4 nL of a solution containing a total of 370 fmol of oligonucleotide, as previously described (Dagle et al., 1991b; Gutierrez et al., 1997; Heidenreich et al., 1997; Weeks et al., 1991). Because the diameter of an individual single cell *Xenopus* embryo can normally vary from 1.0 to 1.3 mm (a volume of 0.52–1.15 μL), the final concentration of the oligonucleotide in any given embryo could range from approximately 325 to 700 nM. For the sake of simplicity, we will assume an average intracellular volume of 0.92 μL for a single cell embryo, resulting in a final

TABLE 1. OLIGONUCLEOTIDE SEQUENCES

PO-1	C A C A A A C C T G T T C T T G G C A G
PS-1	C*A*C*A*A*A*C*C*T*G*T*T*C*T*T*G*G*C*A*G
5-10-5 2′OMe[a] PO-1	mC mA mC mA mA A C C T G T T C T T mG mG mC mA mG
5-10-5 2′OMe PS-1	mC*mA*mC*mA*mA*A*C*C*T*G*T*T*C*T*T*mG*mG*mC*mA*mG
5-10-5 2′OMe MM1	mC*mA*mC*mA*mA*A*C*C*T*G*A*T*C*T*T*mG*mG*mC*mA*mG
5-10-5 2′OMe MM2	mC*mA*mC*mA*mA*A*C*G*T*G*A*T*C*T*T*mG*mG*mC*mA*mG
5-10-5 2′OMe MM3	mC*mA*mC*mA*mA*A*C*G*T*G*A*T*C*A*T*mG*mG*mC*mA*mG
5-10-5 2′OMe TMM1	mC*mA*mG*mA*mA*A*C*C*T*G*T*T*C*T*T*mG*mG*mC*mA*mG
5-10-5 2′OMe TMM2	mC*mA*mG*mA*mA*A*C*C*T*G*T*T*C*T*T*mG*mG*mC*mA*mG
7-10-7 2′OMe PS-1	mU*mG*mC*mA*mC*mA*mA*A*C*C*T*G*T*T*C*T*T*mG*mG*mC*mA*mG*mA*mG
2-17 Pos-1	C+A C A A A C C T G T T C T T G G C A+G
4-15 Pos-1	C+A C+A A A C C T G T T C T T G G+C A+G
6-13 Pos-1	C+A C+A A+A C C T G T T C T T+G G+C A+G
6-13 Pos-1 MM1	C+A C+A A+A C C T G A T C T T+G G+C A+G
6-13 Pos-1 MM2	C+A C+A A+A C G T G A T C T T+G G+C A+G
6-13 Pos-1 MM3	C+A C+A A+A C G T G A T C A T+G G+C A+G
6-13 Pos-1 TMM1	C+A G+A A+A C C T G T T C T T+G G+C A+G
6-13 Pos-1 TMM2	C+A G+A A+A C C T G T T C T T+G G+G A+G
6-13 Neu-1	C=A C=A A=A C C T G T T C T T=G G=C A=G
5-10-5 Neu-1	C=A=C=A=A=A C C T G T T C T T=G=G=C=A=G
3-14-3 LNA gPS-1	c a c A*A*A*C*C*T*G*T*T*C*T*T*G*G c a g-p
3-14-3 LNA PO-1	c a c A A A C C T G T T C T T G G c a g-p
4-12-4 LNA gPS-1	c a c a A*A*C*C*T*G*T*T*C*T*T*G*g c a g-p
5-10-5 LNA PO-1	c a c a a A C C T G T T C T T g g c a g-p
5-10-5 LNA gPS-1	c a c a a A*C*C*T*G*T*T*C*T*T g g c a g-p
5-10-5 LNA PS-1	c*a*c*a*a*A*C*C*T*G*T*T*C*T*T*g*g*c*a*g-p
5-10-5 LNA Pos-1	c a c a a A+C C T G T T C T T+g g c a g-p
6-8-6 LNA gPS-1	c a c a a a C*C*T*G*T*T*C*T t g g c a g-p
6-13 LNA PO-1	c A c A a A C C T G T T C T t G g C a G
6-13 LNA gPS-1	c A c A a A*C*C*T*G*T*T*C*T t G g C a G
5-10-5 LNA-1 MM1	c a c a a A*C*C*T*G*A*T*C*T*T g g c a g-p
5-10-5 LNA-1 MM2	c a c a a A*C*G*T*G*A*T*C*T*T g g c a g-p
5-10-5 LNA-1 MM3	c a c a a A*C*G*T*G*A*T*C*A*T g g c a g-p
5-10-5 LNA-1 TMM1	c a g a a A*C*C*T*G*T*T*C*T*T g g g a g-p
5-10-5 LNA-1 TMM2	c a g a a A*C*C*T*G*T*T*C*T*T g g g a g-p
PO-2	A G T A T T C C A G C A G G C G C T T T
PS-2	A*G*T*A*T*T*C*C*A*G*C*A*G*G*C*G*C*T*T*T
5-10-5 2′OMe PO-2	mA mG mU mA mU T C C A G C A G G C mG mC mU mU mU
5-10-5 2′OMe PS-2	mA*mG*mU*mA*mU*T*C*C*A*G*C*A*G*G*C*mG*mC*mU*mU*mU
6-13 Pos-2	A+G T+A T+T C C A G C A G G C+G C+T T+T
6-13 Neu-2	A=G T=A T=T C C A G C A G G C=G C=T T=T
5-10-5 Neu-2	A=G=T=A=T=T C C A G C A G G C=G=C=T=T=T
5-10-5 LNA gPS-2	a g t A t T*C*C*A*G*C*A*G*G*C g c t t t-p
PO Con	T C G G A C T C T G A G T T T C G G T T
PS Con	T*C*G*G*A*C*T*C*T*G*A*G*T*T*T*C*G*G*T*T
5-10-5 2′OMe PS Con	mU*mC*mG*mG*mA*C*T*C*T*G*A*G*T*T*T*mC*mG*mG*mU*mU
6-13 Pos Con	T+C G+G A+C T C T G A G T T T+C G+G T+T
6-13 Neu Con	T=C G=G A=C T C T G A G T T T=C G=G T=T
5-10-5 LNA gPS Con	t c g g a C*T*C*T*G*A*G*T*T*T c g g t t-p

[a]mN 2′ O-methyl sugar modification (2′OMe).

*Phosphorothioate linkage (PS).

+Dimethylethylenediamine phosphoramidate linkage (Pos).

=Methoxyethylamine phosphoramidate linkage (Neu).

Lower case signifies beta-D-locked nucleic acid (LNA) sugar modification.

Underlined residues represent mismatches with the survivin mRNA sequence.

oligonucleotide concentration of 400 nM. In selected experiments, serial dilutions of the stock oligonucleotide solutions were injected into embryos, creating a final oligonucleotide concentration of 40 nM or 4 nM. Embryos, in groups of 10, were harvested at 30, 60, and 240 minutes after injection by flash freezing on dry ice. All antisense experiments were repeated at least three times. All procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Iowa.

RNA isolation and analysis

Total RNA was isolated from groups of 10 embryos (treated as a single sample) using RNAqueous, a column-based RNA isolation kit from Ambion (Austin, TX). Total RNA was treated with DNAse I (Turbo DNAse Free, Ambion) and quantitated by UV spectroscopy. Approximately 40–50 μg total RNA was obtained for each group of 10 embryos. Total RNA (1 μg) from each sample was added to a reverse transcription (RT) reaction, using Superscript II reverse transcriptase (Invitrogen, San Diego, CA) according to the manufacturer's recommendation. An aliquot of the cDNA (equivalent to an input of 25 ng total RNA) was analyzed by real-time PCR in triplicate using Immolase polymerase (Bioline, Randolph, MA) on an ABI SDS 7700. The mean value obtained from the triplicate RT-PCR reactions was used to determine the number of target molecules.

The oligonucleotides used in the RT-PCR reaction to determine survivin mRNA levels include the primers AATGTACTCTGCCAAGAACAGG (forward) and GAGCAATGGTGTTGGTTGGAGAAG (reverse) and the probe FAM-CTTGAAGGAGCTGGAGGGC-TGGG-BHQ, where FAM is 6-carboxyfluorescein-aminohexylphosphate, and BHQ is Black Hole quencher-1. Prior to sample analysis, this assay was validated and shown to be quite robust, with a linear correlation between the number of starting target molecules (present in a plasmid) and cycle threshold over seven orders of magnitude ($r^2 \geq 0.999$). The number of survivin mRNA molecules in each sample was then normalized to that of 28S rRNA to account for any small variations in the amount of cDNA present in each amplification reaction. The oligonucleotides used in the RT-PCR reaction to determine 28S rRNA levels include the primers GGT-GTTGACGCGATGTGATTTCTG (forward) and TAG-ATGACGAGGCATTTGGCTACC (reverse) and the probe FAM-AAATTCAATGAAGCGCGGGTAAACG-GCGG-TAM, where FAM is 6-carboxyfluorescein-aminohexylphosphate, and TAM is 6-carboxytetramethylrhodamine. The amount of survivin mRNA in each sample was then compared with the amount present in an uninjected cohort of embryos. These data are presented graphically as mean with standard error bars.

RESULTS

Antisense activity of modified oligonucleotides

Traditional compounds. Oligonucleotides possessing PS internucleoside linkages and those with terminal 2′OMe modifications have been used extensively in antisense studies in a number of systems (Grunweller et al., 2003; Monia et al., 1996; Shen et al., 2001; Stein et al., 1997). These modifications have the advantage of at least partially counteracting the intracellular nucleases present in the biologic systems used. Antisense oligonucleotides, including unmodified (PO) or those modified with either PS linkages, 2′OMe PO, or both (2′OMe PS), targeting the mRNA encoding the antiapoptotic protein survivin were injected into single-cell *Xenopus* embryos to examine their relative activity as antisense compounds. The final intracellular concentration of oligonucleotide used in these studies was approximately 400 nM, a concentration equivalent to or less than that typically employed in many antisense studies using *Xenopus* (Choi and Han, 2005; Dagle et al., 2003; Heasman et al., 1994; Moreno et al., 2005). Similarly, modified control oligonucleotides (PO Con, PS Con, 5-10-5 2′OMe PS Con) were also injected. As shown in Figure 1A, all the antisense oligonucleotides tested showed significant activity compared with control compounds. PS-1 and PO-1 showed similar activity at the 30-minute time point; however, no further mRNA depletion was seen with PO-1, whereas a consistent decline in target message continued over the duration of the assay with PS-1. These data suggest that complete degradation of the unmodified oligonucleotide occurred in the first 30 minutes after injection, preventing any subsequent mRNA depletion.

In contrast, depletion of mRNA by PS-1 throughout the time course demonstrates continued presence and activity of the PS-modified oligonucleotide. Surprisingly, the addition of several 2′OMe ribonucleotides to the 3′- and 5′-termini of PO-1 to generate 5-10-5 2′OMe PO-1 had little effect on survivin mRNA levels at the 30-minute and 60-minute time points and only a slight improvement in antisense activity at the 4-hour time point. This finding is unexpected, given the significant improvement in antisense activity demonstrated in cultured mammalian cells after modification with 2′OMe moieties (Fisher et al., 1993) and may result from more active endonucleases present in the *Xenopus* system. An increase in mRNA degradation was observed in 5-10-5 2′OMe PS-1 compared with PS-1 at the later time points, suggesting an increase in nuclease stability provided by this modification. Consistent with several previous studies reporting nonspecific toxic effects with PS oligonucteotides (reviewed in Stein, 1996), we saw a reproducible decrease in survivin mRNA levels after injection with PS Con. This decrease was not observed with

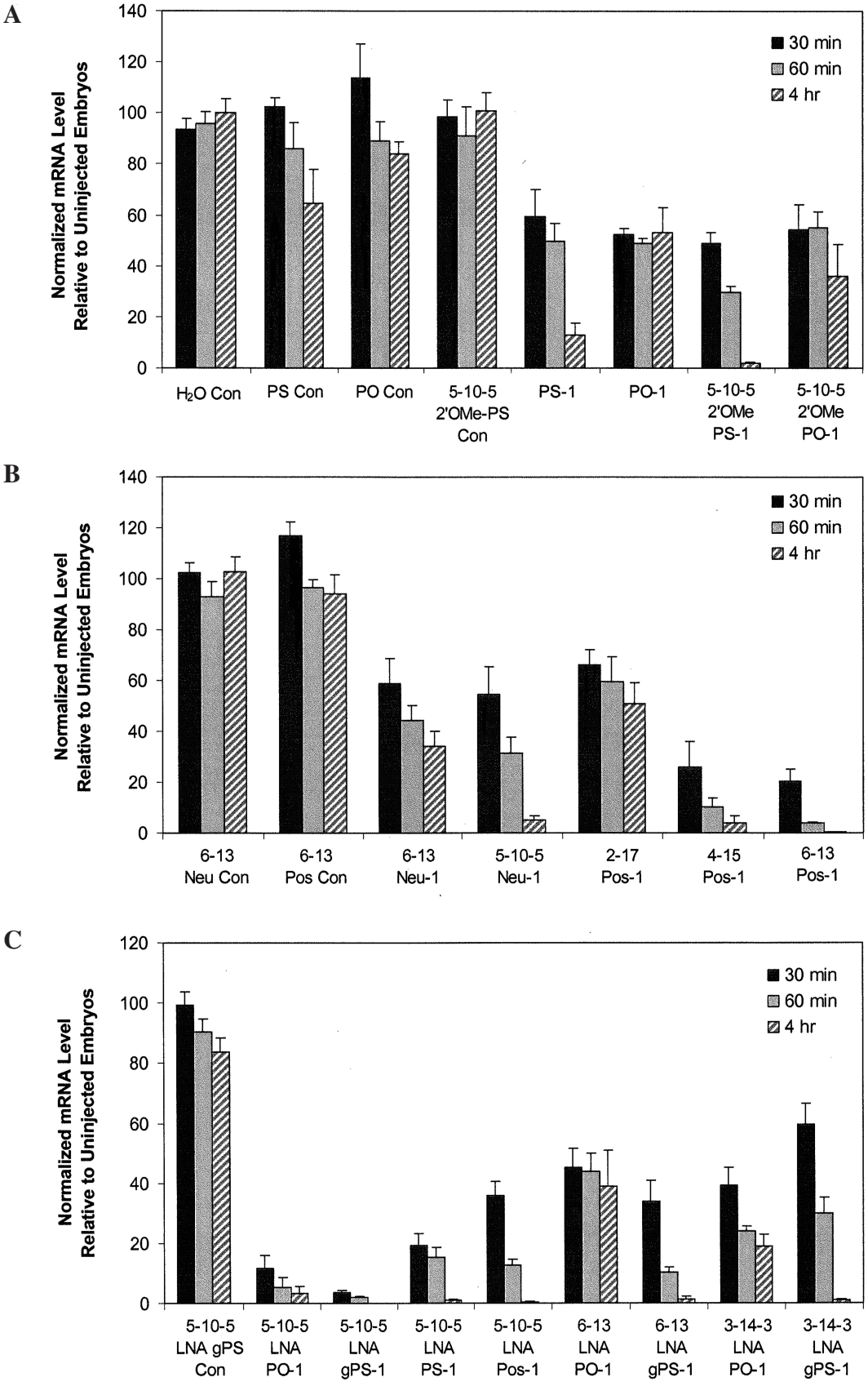
A
Normalized mRNA Level
Relative to Uninjected Embryos
140
120
100
80
60
40
20
0
30 min
60 min
4 hr
H2O Con
PS Con
PO Con
5-10-5 2'OMe-PS Con
PS-1
PO-1
5-10-5 2'OMe PS-1
5-10-5 2'OMe PO-1
B
Normalized mRNA Level
Relative to Uninjected Embryos
140
120
100
80
60
40
20
0
30 min
60 min
4 hr
6-13 Neu Con
6-13 Pos Con
6-13 Neu-1
5-10-5 Neu-1
2-17 Pos-1
4-15 Pos-1
6-13 Pos-1
C
Normalized mRNA Level
Relative to Uninjected Embryos
120
100
80
60
40
20
0
30 min
60 min
4 hr
5-10-5 LNA gPS Con
5-10-5 LNA PO-1
5-10-5 LNA gPS-1
5-10-5 LNA PS-1
5-10-5 LNA Pos-1
6-13 LNA PO-1
6-13 LNA gPS-1
3-14-3 LNA PO-1
3-14-3 LNA gPS-1

FIG. 1.

any other control oligonucleotide used in these studies. As *Xenopus* embryos are transcriptionally quiescent at this point in development, it would follow that overall mRNA stability is either directly or indirectly altered by the PS-modified oligonucleotides in a sequence-independent manner.

Phosphoramidate-modified compounds. The next class of compounds examined included oligonucleotides possessing phosphoramidate-modified internucleoside linkages. These modifications are unique compared with the other compounds tested in that they alter, to varying degrees, the net electrostatic charge carried by the oligonucleotide. The net negative charges of the oligonucleotides examined in this study are as follows: 6-13 Neu-1 = 13, 5-10- 5 Neu-1 = 9, 2-17 Pos-1 = 15, 4-15 Pos-1 = 11, and 6-13 Pos-1 = 7. In comparison, the net negative charge of most other oligonucleotides used in this study is 19. The only exceptions are LNA-modified oligonucleotides that have an estimated net negative charge of 21, resulting from the 3′-phosphate added to simplify the synthesis. The highest number of positively charged linkages examined in this study was limited to six, present in the 6-13 compound. More extensive DMED phosphoramidate modification, although synthetically feasible, negatively affects the ability to fully characterize the resulting oligonucleotides by both mass spectrometry and capillary electrophoresis. We and others have, however, used more extensively modified oligonucleotides, possessing either neutral (Dagle et al., 1991 b; Heasman et al., 1994) or cationic (Dagle et al., 2003; Veenstra et al., 2000) phosphoramidate linkages, to successfully alter gene expression during early *X. laevis* development. The antisurvivin phosphoramidate-modified oligonucleotides were injected into single cell embryos, and levels of survivin mRNA were determined at the time points indicated in Figure 1B. All the antisense compounds tested showed significant activity compared with the control oligonucleotides. Neither of the phosphoramidate-modified control oligonucleotides significantly altered survivin mRNA levels compared with the uninjected control embryos. The more heavily modified 5-10-5 Neu-1 showed increased antisense activity compared with 6-13 Neu-1. The advantage of the 5-10-5 oligonucleotide might be related to enhanced stability against nucleases, as the lesser modified compound would be predicted to have superior hybridization characteristics (Dagle et al., 1991a).

We also compared the antisense activity of a series of oligonucleotides possessing cationic phosphoramidate modifications, finding increased activity with increasing degree of modification. The activity of 2-17 Pos-1 was not significantly different from that of an unmodified oligonucleotide (Fig. 1A), with approximately 60% of the survivin mRNA remaining 4 hours after the injection of either compound. Antisense activity increased substantially with increasing cationic modification, with near complete elimination of the target message after injection with 6-13 Pos-1. Comparison of 6-13 Neu-1 with 6-13 Pos-1 is especially helpful in differentiating the component of antisense activity attributable to the phosphoramidate linkage from that component resulting from the alteration in charge density. This observation is consistent with our previous report demonstrating that a phosphoramidate-modified oligonucleotide bearing cationic alkyl groups is capable of directing mRNA degradation in *Xenopus* embryos after the midblastula transition (i.e., zygotic transcripts), whereas one carrying neutral phosphate modifications did not (Dagle et al., 2000). Notably, oligonucleotides 6-13 Neu-1, 5-10-5 Neu-1, 4-15 Pos-1, and 6-13 Pos-1 all show evidence of significant mRNA degradation throughout the entire 4-hour time course, an indication of stability to nuclease degradation.

LNA-modified antisense oligonucleotides. Recent studies in a variety of experimental systems have shown that oligonucleotides possessing LNA-modified nucleotides, which significantly increase duplex stability, are quite effective antisense compounds (Frieden et al., 2003; Kurreck et al., 2002). We initially tested three modification strategies, each with either phosphodiester or PS linkages in the central (RNase H active) region on the oligonucleotide, between LNA-modified flanking regions. The three strategies included (1) five consecutive LNA linkages on both termini flanking a central region of 10 unmodified nucleosides (5-10-5), (2) three alternating LNA

FIG. 1. Antisense activity of modified oligonucleotides. Oligonucleotides targeting survivin mRNA were injected into single cell *Xenopus* embryos to a final concentration of approximately 400 nM. After the indicated incubation times, survivin mRNA levels were determined by real-time PCR. Survivin mRNA levels were normalized to 28S rRNA and presented relative to survivin mRNA present in uninjected embryos. (**A**) Traditional antisense oligonucleotide. PO, unmodified oligonucleotides; PS, oligonucleotides possessing PS-modified internucleoside linkages; 2′OMe, oligonucleotides with terminal 2′-O-methyl ribose modifications. Con indicates a control oligonucleotide. (**B**) Phosphoramidate-modified oligonucleotides. Neu, neutral, terminal methoxyethylamine phosphoramidate internucleoside linkages; Pos, cationic, terminal DMEM phosphoramidate internucleoside linkages. (**C**) LNA-modified oligonucleotides. LNA, oligonucleotides with terminal beta-D-LNA deoxyribose modifications; PS, oligonucleotides possessing exclusively PS-modified internucleoside linkages; gPS, oligonucleotides possessing PS-modified internucleoside linkages only in the central (gap) region of the compound not modified by LNAs. Bars indicate the standard error of the mean (SEM). All oligonucleotide constructs are shown in Table 1.

and unmodified nucleosides on each termini flanking 9 unmodified nucleosides (6-13), and (3) three consecutive LNA linkages on both termini flanking a central region of 13 unmodified nucleosides (3-14-3). The 5-10-5 LNA-modified oligonucleotides tested include those possessing only phosphodiester (5-10-5 LNA PO), only phosphorothioate (5-10-5 LNA PS), or a combination of central PS with terminal phosphodiester (5-10-5 LNA gPS) internucleoside linkages. Finally, we examined the activity of one oligonucleotide with a cationic phosphoramidate linkage interfaced between the LNA-modified termini and the unmodified central region (5-10-5 LNA Pos). As shown in Figure 1C, all the LNA compounds studied possessed significant antisense activity, with at least 50% of the target message depleted within 30 minutes after injection. Overall, the LNA-modified compounds showed the highest level of antisense activity compared with all other classes of oligonucleotide modification examined. The 5-10-5 LNA oligonucleotides, as a group, were far more effective than the 6-13 or 3-14-3 LNA oligonucleotides. Both 5-10-5 LNA PO-1 and 5-10-5 LNA gPS-1 reduced target mRNA levels to $<20\%$ of starting levels within 30 minutes. The fully phosphorothioated LNA oligonucleotide (5-10-5 LNA PS-1) and the hybrid LNA compound with two cationic linkages (5-10-5 LNA Pos-1) showed slightly less activity at the early time points, but the target mRNA was essentially eliminated by 4 hours after injection for both. Not only were the 6-13 LNA oligonucleotides less effective than the 5-10-5 compounds, but also 6-13 LNA PO-1 showed a level and pattern of antisense activity quite similar to that of the oligonucleotides PO-1 and 2-17 Pos-1. These results suggest that 6-13 LNA PO-1 is completely degraded during the first 30 minutes of the assay, preventing further mRNA depletion, whereas 6-13 LNA gPS-1 remains intact and active during the entire 4-hour assay. A similar pattern is seen in the 3-14-3 series of oligonucleotides, where mRNA levels for 3-14-3 LNA PO-1 do not change between the 60-minute and 4-hour time points, whereas continued depletion is appreciated during the course of the assay for 3-14-3 LNA gPS-1. However, the difference in the magnitude of effect between the 6-13 and 3-14-3 LNA PO-1 compounds, each with six LNA modifications, demonstrates the importance of contiguous LNA residues when designing these antisense compounds.

The antisense activity of an LNA-modified oligonucleotide (5-10-5 LNA gPS-1) at varying concentrations was next compared with that of an oligonucleotide possessing cationic phosphoramidate linkages (6-13 Pos-1). These two compounds were chosen for comparison because they represent the most active members of their respective classes. The data presented in Figure 2 demonstrate little difference between the two compounds at the 4 nM level, with only a slight decrease in target mRNA levels after 4 hours seen with 6-13 Pos-1. At the 400 nM

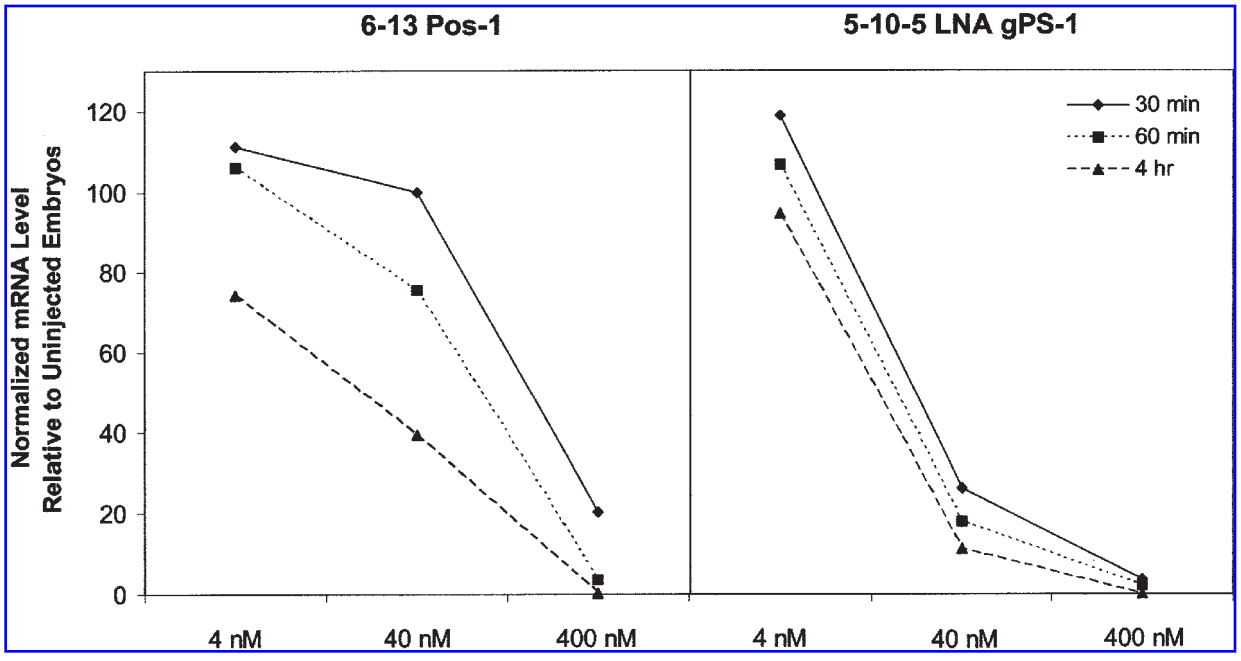

FIG. 2. Concentration dependence of potent antisense oligonucleotides. 6-13 Pos-1 or 5-10-5 LNA gPS-1 was injected into single cell *Xenopus* embryos to a final concentration of approximately 4, 40, or 400 nM. After the indicated incubation times, survivin mRNA levels were determined by real-time PCR. Survivin mRNA levels were normalized to 28S rRNA and presented relative to survivin mRNA present in uninjected embryos.

concentration, both oligonucleotides direct the nearly complete elimination of the target mRNA over the 4-hour time course, with a more rapid degradation directed by 5-10-5 LNA gPS-1. The major difference between the two compounds is seen at the 40 nM concentration (approximately 0.25 ng of injected oligonucleotide), where the 30-minute, 60-minute, and 4-hour time points show vastly different extents of mRNA depletion with 6-13 Pos-1 but similar and nearly complete degradation with 5-10-5 LNA gPS-1. This temporal pattern reveals slower and steady mRNA degradation with 6-13 Pos-1 compared with 5-10-5 LNA gPS-1, where the majority of activity is complete at the 30-minute time point following the 40 nM injection. The 5-10-5 LNA gPS-1 would thus be predicted to show either more rapid or more stable duplex formation than 6-13 Pos-1.

Thermal stability of modified antisense compounds

To evaluate the thermodynamics of hybridization, we determined the melting temperatures (Tm) of duplexes containing several of the oligonucleotides employed in these antisense studies, as previously described (Owczarzy et al., 2004). Although the thermodynamic data present in Table 2 show both DNA/DNA and DNA/RNA duplexes, the DNA/RNA heteroduplex data are more relevant in discussions relating to antisense activity. In general, any modification of the phosphodiester bond caused decreased thermal stability of the DNA/RNA heteroduplex. Each PS modification decreased heteroduplex stability by 0.43°C, and the phosphoramidate modification decreased Tm by approximately 1°C per modification. Our observed decrease in DNA/DNA duplex stability of 0.45°C per PS residue approximates that previously reported for fully modified oligonucleotides possessing mixed diastereoisomers (Freier and Altmann, 1997). The observed instability has generally been attributed to the introduction of a chiral phosphorus as a result of these modifications, although the increased instability resulting from phosphoramidate modification compared with PS modification suggests additional steric contributions to destabilization. The decrease in DNA/DNA duplex stability observed with 5-10-5 2′OMe PO-1 (0.28°C per modified residue) was surprising, given the general trend toward increased stability of the modification previously reported and may represent a sequence-specific effect that has been observed with 2′ sugar substitutions (Freier and Altmann, 1997). The increased DNA/DNA duplex stability resulting from modification with LNA residues (1.47°C per modified residue) is lower than that previously reported for terminally modified LNA oligonucleotides (Kurreck et al., 2002), possibly representing a similar sequence-specific effect.

TABLE 2. OLIGONUCLEOTIDE MELTING TEMPERATURES

Oligonucleotide	*Complement*	*Tm (°C)*	*ΔTm (°C)*[a]	*ΔTm/mod (°C)*[a,b]
PO-1	DNA	61.76	—	—
	RNA	59.38	—	—
PS-1	DNA	53.27	−8.49	−0.45
	RNA	51.18	−8.21	−0.43
5-10-5 2′OMe PO-1	DNA	59.00	−2.76	−0.28
	RNA	66.44	+7.05	+0.70
5-10-5 2′OMe PS-1	DNA	51.09	−10.67	
	RNA	59.91	+0.53	
6-13 Neu-1	DNA	58.00	−3.76	−0.63
	RNA	53.71	−5.68	−0.95
5-10-5 Neu-1	DNA	53.26	−8.50	−0.85
	RNA	47.93	−11.45	−1.14
6-13 Pos-1	DNA	56.54	−5.22	−0.87
	RNA	52.81	−6.57	−1.10
5-10-5 LNA PO-1	DNA	76.42	+14.66	+1.47
	RNA	83.41	+24.03	+2.40
5-10-5 LNA gPS-1	DNA	70.75	+8.99	
	RNA	78.23	+18.84	
5-10-5 LNA PS-1	DNA	68.00	+6.24	
	RNA	75.10	+15.72	

[a]Compared with PO-1.

[b]ΔTm per modified nucleotide for oligonucleotides with one class of modification.

As predicted from the antisense data presented here, the Tm values for all the oligonucleotides tested were well above the 21–23°C incubation temperature used in the *in vivo* studies. There is more to antisense activity than hybridization, however, as most of the compounds tested showed a decreased Tm compared with an unmodified oligonucleotide but possessed increased antisense activity. In contrast, the 2′OMe and the LNA sugar modifications both increased the Tm of the heteroduplex, with the LNA oligonucleotides showing the most dramatic increase of 2.4°C per modification compared with 0.70°C per modification for the 2′OMe compounds. The enhanced hybridization properties of the LNA-modified oligonucleotides could completely explain the superior antisense activity of these compounds compared with all others examined.

The stability of heteroduplexes containing either the 2′OMe or LNA modifications is decreased by the introduction of PS bonds. In the case of 5-10-5 LNA gPS-1 and 5-10-5 LNA PS-1, the Tm of the DNA/DNA or DNA/RNA duplex can be approximated by subtracting the instability induced by each PS modification (from Table 1) from the Tm of 5-10-5 LNA PO-1 (from Table 1). The calculated Tms of 72.37°C ($76.42 - 0.45 \times 9$) for the duplex and 79.54°C ($83.41 - 0.43 \times 9$) for the heteroduplex containing 5-10-5 LNA gPS-1 are close to the experimentally observed values of 70.75°C and 78.23°C, respectively, and the calculated Tms of 67.87°C ($76.42 - 0.45 \times 19$) for the duplex and 75.24°C ($83.41 - 19 \times 0.43$) for the heteroduplex containing 5-10-5 LNA PS-1 are similar to the experimental values of 68.00°C and 75.10°C, respectively. The same mathematical approach can be used to estimate the thermal stability of oligonucleotides possessing both PS and 2′OMe modifications. The calculated Tms of 50.45°C for the DNA/DNA duplex and 58.27°C for the DNA/RNA heteroduplex containing 5-10-5 2′OMe PS-1 approach the experimentally observed values of 51.09°C and 59.91°C, respectively.

Notably, the Tm of the heteroduplex containing 6-13 Pos-1, which shows the highest antisense activity of all non-LNA oligonucleotides at 400 nM, is 52.81°C, which is 6.57°C lower than the heteroduplex containing an unmodified oligonucleotide (PO-1).

We next examined the relationship between heteroduplex stability and antisense activity using a series of LNA-modified oligonucleotides that possessed 3–6 terminal LNA modifications, with the remaining internal linkages modified as PS bonds. For example, 3-14-3 LNA gPS-1 contains 6 LNA modifications (3 on each terminus) and 13 PS bonds, whereas 6-8-6 LNA gPS-1 contains 12 LNA modifications (6 on each terminus) and 7 PS bonds. As shown in Figure 3, mRNA depletion increases with increasing Tm, up to the 5-10-5 modified

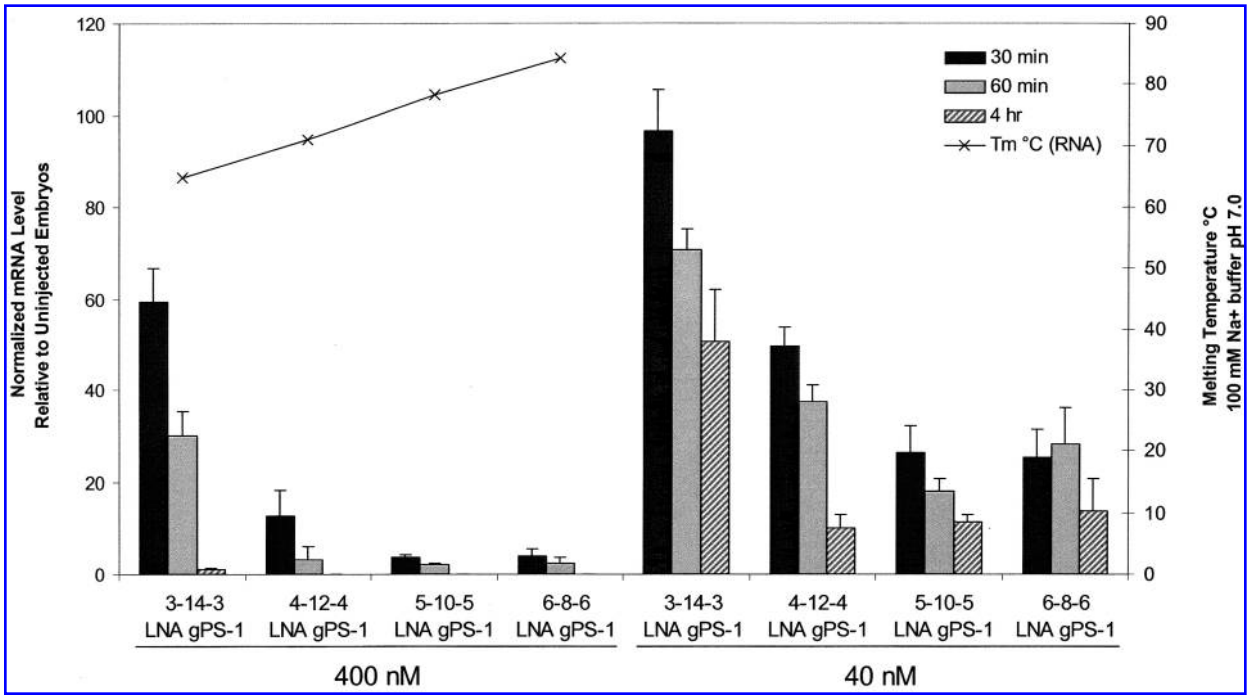

FIG. 3. Thermal stability and efficacy of LNA-modified oligonucleotides. Oligonucleotides (20-mer) possessing increasing numbers of LNA modifications (constructs are shown in Table 1) were injected into single cell *Xenopus* embryos to a final concentration of approximately 40 or 400 nM. After the indicated incubation times, survivin mRNA levels were determined by real-time PCR. Survivin mRNA levels were normalized to 28S rRNA and presented relative to survivin mRNA present in uninjected embryos. Bars indicate SEM. The Tm of each oligonucleotide with an RNA complement in 100 mM Na^+ (pH 7.0) is also shown.

oligonucleotide, at which point a plateau is reached. Further increases in heteroduplex stability, as seen with the 6-8-6 oligonucleotide, did not cause increased mRNA depletion. The relationship between duplex stability and mRNA degradation is most evident at the 30-minute time points after injection with 400 nM oligonucleotide, as the target mRNA is nearly completely eliminated at subsequent time points. In contrast, decreasing the oligonucleotide concentration to 40 nM demonstrates increased mRNA depletion at all time points throughout the course of the experiment. The equivalent antisense activity of the 5-10-5 and the 6-8-6 oligonucleotides is again seen after the 40 nM injections, possibly related to increased secondary structure (dimers and hairpins) of the more heavily modified LNA compounds. Although the increased thermal stability of heteroduplexes containing LNA oligonucleotides is most likely responsible for our observations, it is also possible that the oligonucleotides in the series become more resistant to nuclease degradation with increasing modification.

To determine if increasing the duplex stability of a 2′OMe-modified oligonucleotide also has a positive effect on antisense activity, we synthesized and tested 7-10-7 2′OMe PS-1, a 24-mer containing 2 additional nt on both the 5′- and 3′-termini of 5-10-5 2′OMe PS-1. Increasing the length of the oligonucleotide to increase thermal stability was chosen over the strategy of increasing the extent of modification, as was done with the LNA compounds, because of the much smaller effect of each individual modification on Tm seen with the 2′OMe-modified compounds. Increasing the length of the 2′OMe PS-1 oligonucleotide to 24 increased the Tm from 59.91°C to 67.26°C, although no increase in antisense activity was demonstrated with 7-10-7 2′OMe PS-1 compared with 5-10-5 2′OMe PS-1 when examined at either 400 nM or 40 nM (data not shown).

Altering an oligonucleotide, either by chemical modification or by changing the length of the polymer, clearly affects hybridization properties and, thus, effective concentration of target duplex. This issue is important to consider in experiments using *Xenopus* embryos, as the extent of mRNA depletion appears to be directly related to duplex concentration (increasing the amount of antisense oligonucleotide injected consistently results in an increase in target mRNA degradation). Care must be taken when enhancing antisense activity solely by increasing duplex stability, however, as an outcome of this strategy may be an increase in the depletion of related transcripts (i.e., nonspecific mRNA degradation).

Effect of mismatches on antisense activity and specificity

Several of the oligonucleotides tested show significant antisense activity in *Xenopus* embryos. We determined the relative specificity of action of three of the more active compounds, 5-10-5 2′OMe PS-1, 6-13 Pos-1, and 5-10-5 LNA gPS-1, by constructing oligonucleotides containing sequence mismatches to the actual survivin transcript. The sequences of compounds investigated in this study are listed in Table 1. Oligonucleotides designated MM1 possess an A instead of a T at nucleotide 11 (T11A), creating an A:A mismatch in the heteroduplex. Those designated MM2 possess T11A in addition to C8G, and MM3 have T11A, C8G, and T14A. All these MM sequence substitutions are located in the central (RNase H active) region of the oligonucleotide. Oligonucleotides designated TMM have sequence changes in the termini of the oligonucleotide, outside the central anionic domain. TMM1 has a G substituted for C at the third nucleotide (C3G), whereas TMM2 has both C3G and C18G sequence changes. Survivin mRNA levels were determined after injection of these oligonucleotides into single cell embryos (Fig. 4). Final oligonucleotide concentrations of 400 nM (Fig. 4, top) and 40 nM (Fig. 4, bottom) were examined. Wild-type (WT) indicates the parent oligonucleotides that are perfectly matched to the survivin mRNA sequence. At 400 nM, all the oligonucleotides demonstrated some attenuation of mRNA degradation with increasing numbers of mismatched basepairs. Oligonucleotide 5-10-5 2′OMe PS-1, the least active of the three initial compounds tested, also showed the least degradation of survivin mRNA as the number of mismatches increased. Attenuated mRNA degradation was observed with the 5-10-5 2′OMe PS-1 compounds possessing more than one mismatch at either 40 or 400 nM, with $>70\%$ of the starting RNA remaining. The 6-13 Pos-1 and 5-10-5 LNA gPS-1 mismatched oligonucleotides, at 400 nM, also showed decreased mRNA depletion as the number of mismatches increased.

Overall, the specificity of the LNA oligonucleotides (defined as the amount of RNA remaining after treatment with the mismatch oligonucleotide divided by the amount of RNA remaining after treatment with the WT oligonucleotide) was higher than that of the phosphoramidate-modified compounds. An interesting exception was seen with the MM3 oligonucleotides, where 6-13 Pos-1 MM3 reduced mRNA levels by 53%, and 5-10-5 LNA gPS-1 MM3 depleted $>98\%$ of the survivin mRNA by the end of the 4-hour incubation. Both of the oligonucleotides possessing modified ribose residues (2′OMe and LNA) showed greater perturbations in antisense activity with multiple internal mismatches compared with terminal mismatches (MM2 vs. TMM2) at the 400 nM concentration. Destabilizing one end of the duplex with a mismatch in the LNA-rich region had approximately the same effect as a central mismatch (TMM1 vs. MM1). A mismatch in the LNA region at both termini of the oligonucleotide caused mRNA depletion equivalent to three central mismatches (TMM2 vs. MM3).

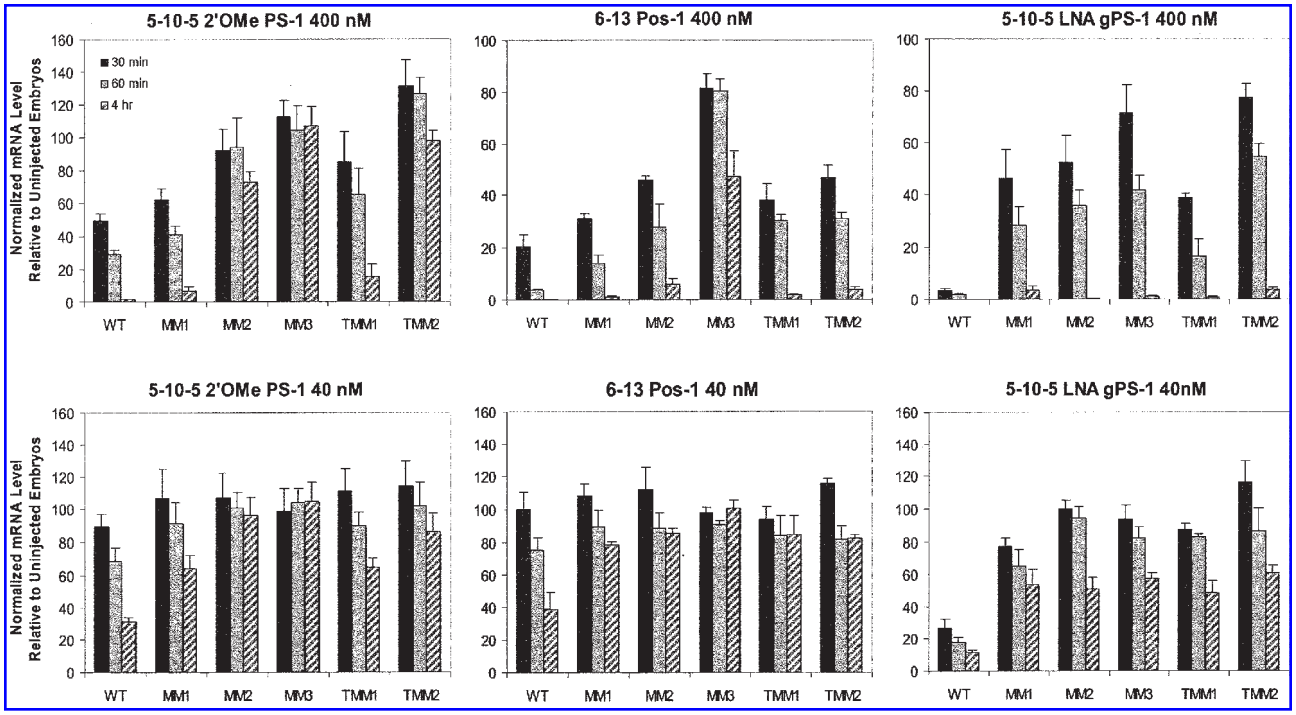

FIG. 4. Specificity of modified antisense oligonucleotides. Antisense activity was compared among three classes of antisense oligonucleotides containing modifications on the 5′- and 3′-termini. In addition, the 2′OMe and LNA compounds possessed PS-modified internucleoside linkages. WT, perfect basepairing between the oligonucleotide and the RNA complement; MM1, MM2, and MM3, one to three mismatches in the central region of the duplex; TMM1 and TMM2, one or two mismatches in the terminal region of the heteroduplex (see text for full description). Top, antisense activity at 400 nM. Bottom, activity at 40 nM. Bars indicate SEM. Oligonucleotide constructs are shown in Table 1.

Slightly different patterns of antisense activity and specificity are seen at the 40-nM oligonucleotide concentration. Oligonucleotide 5-10-5 2′OMe PS-1 continues to show target mRNA depletion with both the WT compound and those possessing only one mismatch, both MM1 and TMM1. In contrast, the 5-10-5 2′OMe PS-1 oligonucleotides with more than one mismatch showed little mRNA depletion. The phosphoramidate-modified 6-13 Pos-1 oligonucleotide (WT) showed ongoing mRNA depletion over the 4-hour time course at 40 nM. The 6-13 Pos-1 mismatched oligonucleotides demonstrated reduced mRNA depletion, with >75% of the target message remaining after 4 hours. At 40 nM, the 5-10-5 LNA gPS-1 oligonucleotide continued to be the most potent antisense agent of the three tested. Although significant mRNA depletion is seen with all the mismatched LNA oligonucleotides, the specificity of these compounds at a concentration of 40 nM is superior to that of the other compounds tested.

Attenuated antisense activity of any mismatched oligonucleotide would be expected to result from decreased stability of the corresponding heteroduplex. To further investigate the differences in antisense patterns observed with the 5-10-5 2′OMe PS-1, 6-13 Pos-1, and 5-10-5 LNA gPS-1 compounds, we determined the Tms of the mismatched oligonucleotides with both DNA and RNA complementary strands *in vitro*. These data (Table 3) demonstrate that 5-10-5 LNA gPS-1 and all the corresponding mismatched LNA-modified oligonucleotides have Tms of at least 30°C greater than the incubation temperature of the developing embryos. Each internal mismatch decreases the Tm of the 5-10-5 LNA gPS-1 by 6–7°C. Whereas the first terminal mismatch of 5-10-5 LNA gPS-1 (TMM1) decreases the Tm with an RNA complement by 6.9°C, the second terminal mismatch (TMM2) destabilizes the duplex by an additional 11.4°C. This is not surprising, as mismatches present in the LNA-modified region are expected to have a larger effect on duplex stability than those in the central region. Both the 2′OMe PS-1 and 6-13 Pos-1 oligonucleotides demonstrated Tm values much lower than that of the 5-10-5 LNA gPS-1 compound. In addition, each internal mismatch had a greater effect on thermal stability, such that 2′OMe PS-1 MM3 has a Tm of 33.2°C and 6-13 Pos-1 MM3 has a Tm of 19.6°C, approximating incubation conditions. Unlike the oligonucleotides possessing modified ribose residues, each terminal mismatch in the 6-13 Pos-1 series (TMM1 and TMM2) has a decreased effect on duplex stability compared with those mismatches in the central region of the compound (MM1-MM3).

TABLE 3. MISMATCH MELTING TEMPERATURES

		LNA 5-10-5 gPS		*2′OMe 5-10-5 PS*		*Pos 6-13*	
Sequence	*Comp*	*Tm*	ΔTm[a]	*Tm*	ΔTm[a]	*Tm*	ΔTm[a]
WT	DNA	70.8	—	51.1	—	56.5	—
	RNA	78.2	—	59.9	—	52.8	—
MM1	DNA	65.0	−5.7	46.2	−4.9	51.3	−5.2
	RNA	71.3	−6.9	53.2	−6.7	44.4	−8.4
MM2	DNA	59.7	−11.0	40.2	−10.9	45.5	−11.1
	RNA	64.5	−13.7	43.1	−16.9	29.5	−23.3
MM3	DNA	53.3	−17.5	31.4	−19.7	37.6	−18.9
	RNA	59.1	−19.2	33.2	−26.7	19.6	−33.2
TMM1	DNA	62.3	−8.5	46.2	−4.9	54.0	−2.5
	RNA	71.4	−6.9	54.5	−5.4	52.1	−0.8
TMM2	DNA	52.2	−18.5	37.7	−13.4	49.0	−7.5
	RNA	59.9	−18.3	45.6	−14.4	47.5	−5.3

[a]Relative to wild-type melting temperature.

The significant nonspecific antisense activity of the various 6-13 Pos-1 mismatch oligonucleotides at 400 nM was surprising given our previous studies showing a high degree of specificity of phosphoramidate-modified antisense oligonucleotides targeting closely related cyclin molecules in developing *Xenopus* embryos (Weeks et al., 1991). In the previous study, neutral phosphoramidate-modified oligonucleotides were used to target the degradation of cyclin B mRNA variants without altering the levels of cyclin A mRNA, despite 15 of 17 basepair matches. The three main differences between the current survivin study and the prior cyclin report, other than the targets, are (1) the oligonucleotide length (20-mer vs. 17-mer), (2) the oligonucleotide structure (alternating vs. contiguous phosphate modifications), and (3) the charge of the phosphate modification (cationic vs. neutral).

Nuclease stability of modified antisense compounds

Antisense oligonucleotides must survive in a hostile intracellular environment in order to hybridize and direct mRNA degradation via RNase H. To further define the mechanisms behind our observed patterns of antisense activity, we determined the effect of a single, modified phosphate linkage on the stability of adjacent unmodified phosphodiester bonds to digestion by nucleases. Because most compounds used in this study are heavily modified and, thus, not readily radiolabeled, we developed an *in vitro* assay to measure stability against nucleolytic degradation. A model 20-mer DNA oligonucleotide (Fig. 5) was synthesized with a carboxyfluorescein (FAM) moiety on the 5′-terminus and with one modified phosphodiester bond (either PS or phosphoramidate) between residues 9 and 10. This oligonucleotide was incubated with one of three 3′-exonucleases (exonuclease I, exonuclease III, or snake venom phosphodiesterase) to an extent that completely degraded an unmodified oligonucleotide. Oligonucleotide fragments generated by this extensive nuclease treatment were analyzed by electrospray ionization mass spectrometry. A single PS stops the progression of all three exonucleases at the site of modification, resulting in a FAM-modified 10-mer (Fig. 5). When the modified linkage is a neutral methoxyethyl-phosphoramidate bond (Neu), digestion by both exonuclease I and snake venom phosphodiesterase is inhibited by the modified bond, resulting in a 10-mer. The major digestion product with exonuclease III, however, is an 11-10-mer, demonstrating the protection of the 3′-adjacent phosphodiester bond. The 10-mer (produced by digestion up to the modified bond) is present as a minor product of exonuclease III digestion, represented by lower case letters. Thus, in this assay, a phosphoramidate bond is superior to a PS bond in protecting neighboring phosphodiesters. Furthermore, when the internucleoside linkage is a cationic, DMED phosphoramidate bond (Pos), extended protection is seen against digestion of phosphodiesters by all three nucleases examined. Notably, a minor digestion product of the digestion with exonuclease I is a 12-mer, demonstrating the protection of two adjacent 3′-phosphodiesters by the modified linkage. Structural or steric effects can readily explain the differences in the degradation patterns between the PS-modified phosphoramidate-modified oligonucleotides examined. The enhanced protection afforded by the cationic phosphoramidate compared with the neutral phosphoramidate can be explained either by structural differences or by the potential charge present on the pendant alkylamine moiety.

Parent Compound	FAM-C-A-C-A-A-A-C-C-T•G-T-T-C-T-T-G-G-C-A-G	
Fragments Following Complete Digestion:		Fragment % (ESI)
Phosphorothiate (PS)		
Exo I	FAM-C-A-C-A-A-A-C-C-T•G	100%
Exo III	FAM-C-A-C-A-A-A-C-C-T•G	100%
SVPD	FAM-C-A-C-A-A-A-C-C-T•G	100%
Neutral Phosphoramidate (Neu)		
Exo I	FAM-C-A-C-A-A-A-C-C-T•G	100%
Exo III	FAM-C-A-C-A-A-A-C-C-T•G-T	86%
	fam-c-a-c-a-a-a-c-c-t•g	14%
SVPD	FAM-C-A-C-A-A-A-C-C-T•G	100%
Cationic Phosphoramidate (Pos)		
Exo I	FAM-C-A-C-A-A-A-C-C-T•G-T	76%
	fam-c-a-c-a-a-a-c-c-t•g	16%
	fam-c-a-c-a-a-a-c-c-t•g-t-t	8%
Exo III	FAM-C-A-C-A-A-A-C-C-T•G-T	100%
SVPD	FAM-C-A-C-A-A-A-C-C-T•G	89%
	fam-c-a-c-a-a-a-c-c-t•g-t	11%

• represents a single modified phosphate bond
FAM respresents a carboxyfluorescein-aminohexyl-phosphate modification
Lower case sequences represent minor degradation products

FIG. 5. Effect of phosphate modification on 3′-exonucleolytic degradation. Oligonucleotides were synthesized possessing a 5′-carboxyfluorescein-aminohexylphosphate (FAM) modification and a single modification of the internucleoside linkage between residues 9 and 10 (closed circles). These compounds were incubated with various concentrations of the indicated 3′-exonucleases. The oligonucleotide fragments generated were analyzed by electrospray ionization mass spectrometry. Exo I, exonuclease I; Exo III, exonuclease III; SVPD, snake venom phosphodiesterase; ESI, electrospray ionization mass spectrometry.

Comparison of two target regions

We have compared the antisense activity of several modified oligonucleotides with a target sequence near the 5′-end of the *X. laevis* survivin mRNA (site 1: bases 8–27, where the A of the AUG initiation codon is base 1). To determine if the relative antisense activity of these compounds at one target site could predict activity at a different site in the mRNA, we tested several modified oligonucleotides that hybridize to the 3′-end of the survivin transcript (site 2: bases 423–442, where the A of the TGA termination codon is base 483). This comparison is important in generalizing the results already presented. The G-C content of both of these sequences is identical at 50%.

Because most genes in *Xenopus* possess two closely related paralogs (Bisbee et al., 1977), antisense oligonucleotides must be chosen in regions of identity between the two. Site 1 and site 2 were chosen in the present study because no sequence polymorphisms were found in these regions, not only when examining available *X. laevis* sequence data but also when sequences from *Xenopus tropicalis* (a related diploid, amphibian model organism) were considered. There were no consistent differences in the extent of mRNA degradation between the two sites with any of the oligonucleotide modifications examined (Fig. 6).

DISCUSSION

We have examined the biologic and physical characteristics of several antisense oligonucleotides generated using a number of popular synthetic strategies, resulting in modifications of the phosphodiester linkage (negatively charged PS, neutral methoxyethyl (MEA) phosphoramidate, and cationic DMED phosphoramidate) and sugar residue (2′OMe and LNAs). *X. laevis* embryos were chosen as the experimental system for these studies primarily because their large size allows direct microinjection of the antisense compounds, eliminating confounding issues related to cellular uptake. *X. laevis* oocytes and embryos have proven extremely useful in the development of antisense oligonucleotides, allowing

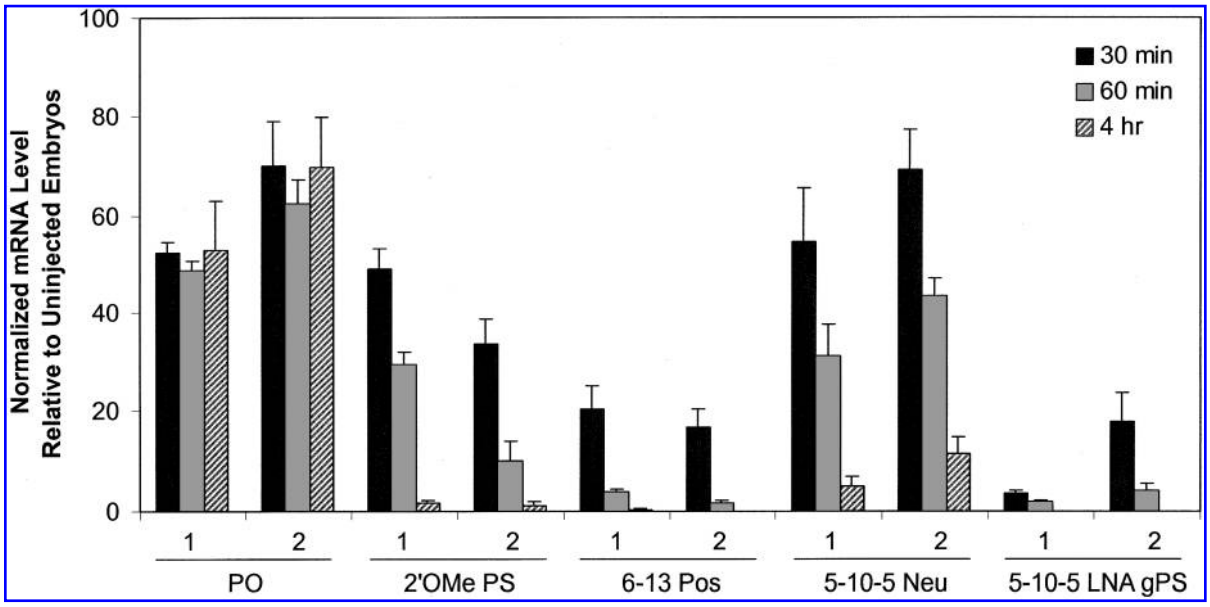

FIG. 6. Comparison of two target regions. Antisense oligonucleotides targeting two regions of the survivin transcript were injected into single cell *Xenopus* embryos to a final concentration of approximately 400 nM. After the indicated incubation times, survivin mRNA levels were determined by real-time PCR. Survivin mRNA levels were normalized to 28S rRNA and presented relative to survivin mRNA present in uninjected embryos. Site 1 is located near the initiation codon, and site 2 is near the termination codon. Bars indicate SEM. The oligonucleotide sequences and modifications are shown in Table 1.

both examination of the relative activity of various compounds and, more importantly, characterization of the biologic obstacles that limit the antisense activity of a given compound. Understanding the basic biologic and biophysical mechanisms governing antisense oligonucleotide efficacy requires the introduction of a known amount of compound at a known point in time. It is more difficult to directly compare potency and specificity of different antisense agents in standard cell culture systems, where significant alterations in cellular uptake or transfection efficiency, brought about by a specific modification, may mask potential improvements in oligonucleotide stability, heteroduplex formation, or RNase H-directed mRNA degradation.

The LNA compounds not only were the most active antisense oligonucleotides examined but also showed the highest degree of specificity. This paradox can be explained by the mismatch discrimination properties of LNA-modified oligonucleotides, which has been exploited in the field of nucleic acid probe technology.

We saw surprisingly little benefit of adding the 2′OMe ribose modification when the central, anionic region of the oligonucleotide contained phosphodiesters (PO-1 vs. 5-10-5 2′OMe PO-1). It is possible that the very active nucleases present in *Xenopus* embryos rapidly degraded the 2′OMe PO-1 compound, negating any possible beneficial effect of the modified termini. In contrast, the 2′OMe modification improved antisense activity when the oligonucleotide contained PS bonds in the central region (PS-1 vs. 5-10-5 2′OMe PS-1). Overall, the 2′OMe PS-modified oligonucleotides showed a level of antisense activity and specificity similar to the cationic phosphoramidate-modified compounds. The 2′OMe compounds, as well as the LNA oligonucleotides, have an advantage in being commercially available (both monomers and synthesized oligonucleotides) from a number of sources.

The oligonucleotides containing cationic phosphoramidate linkages demonstrated potent antisense activity and were expected to have a high degree of specificity, based on data obtained with compounds possessing neutral phosphoramidate bonds. Whether cationic or neutral, each phosphoramidate modification causes a decrease in duplex stability by 0.63–1.14°C. In general, a lower Tm would be predicted to improve discrimination between perfectly matched complements and those with mismatches. In conditions of high oligonucleotide concentration and 3 mismatches with the target, oligonucleotides possessing cationic linkages did, in fact, show higher specificity than the LNA-modified compounds. We speculate that increased stability to intracellular nucleases, and thus increased effective oligonucleotide concentration, is responsible for the level of antisense activity observed. Because PS modification significantly enhanced the antisense activity of both the 5-10-5 2′OMe compounds and the 5-10-5 LNA-modified oligonucleotides, we speculate that oligonucleotides possessing a PS central region flanked by phosphoramidate-modified

termini would show increased potency compared with 6-13 Pos-1. Although we have been unable to make this compound using standard synthetic techniques, we are currently examining alternative strategies to generate this class of potentially interesting compounds.

We have demonstrated the protection of unmodified phosphodiester bonds by an adjacent phosphoramidate linkage, with a cationic modification providing a higher degree of stability against nucleases than a neutral modification. The active sites of many nucleases contain either cationic amino acids or bound divalent cations, both of which would be electrostatically repulsed by cationic moieties present on the oligonucleotide substrate. This mechanism is consistent with our observations that despite a lower Tm, 6-13 Pos-1 proved to be a more potent antisense oligonucleotide than 6-13 Neu-1. It is also possible that a cationic DNA strand alters the conformation of a heteroduplex, making it a better substrate for RNase H. Finally, the altered charge density of the oligonucleotide could change the intracellular localization of the compound, making it more available for the antisense pathway.

The concentrations of oligonucleotide examined in this study showed a range of antisense activity from complete mRNA elimination with the more potent compounds at 400 nM to very little effect of any compound at 4 nM, which is appropriate for the time frame of interest. Our goal was to complete the antisense activity assay prior to the midblastula transition, when zygotic transcription commences (Newport and Kirschner, 1982). At that time, survivin mRNA undergoes a natural, rapid decrease in abundance that could confound the end point of our assay (Murphy et al., 2002). In reality, however, few antisense applications require the complete elimination of the target mRNA within a few hours. It is likely that most applications of this technology will demand a more gradual reduction of target mRNA over several hours to days, favoring the use of lower concentrations of more stable and specific agents.

ACKNOWLEDGMENT

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