2'-O-methyl, 2'-O-ethyl oligoribonucleotides and phosphorothioate oligodeoxyribonucleotides as inhibitors of the in vitro U7 snRNP-dependent mRNA processing event

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ABSTRACT
We describe the synthesis of 2'-O-methyl, 2'-O-ethyl oligoribonucleotides and phosphorothioate oligodeoxyribonucleotides and demonstrate their utility as inhibitors of the in vitro U7 snRNP-dependent mRNA processing event. These 2'-O-modified compounds were designed to possess the binding affinity of an RNA molecule towards a complementary RNA target with an enhanced stability against nucleases. The 2'-O-methyl and 2'-O-ethyl antisense compounds function as potent inhibitors of the reaction at 1-10 nM, approximately 5-fold more effective than a natural antisense RNA molecule and requiring an approximate 5-fold excess over the target RNA for 80% inhibition of the processing reaction.

INTRODUCTION
The use of synthetic oligonucleotides as specific inhibitors of gene expression has attracted molecular biologists’ attentions. However, a major limit to the application of this strategy can be traced to the biological instability of short, single-stranded DNA molecules when they enter the living cell. An approach to reduce this bioerosion has been to synthesize unnatural nucleic acid analogs which continue to possess the inhibitory activity of natural nucleic acids yet have enhanced biostability. We are in the process of developing modified synthetic RNA molecules. Because the RNA-RNA duplex is more stable than the DNA-RNA hybrid, we have focused our attention on this sort of hybrid. However, in return for an increased hybrid stability, the chemical properties of RNA carries a high price in increased susceptibility to nuclease degradation. To counter this phenomenon we have investigated RNA oligonucleotides prepared from nucleotides which possess modifications at the 2' hydroxyl group, as a method of blocking at least one class of nucleases. We report here the biological inhibitory activity of two of these nucleic acid analogs, the 2'-O-methyl and 2'-O-ethyl derivatives of RNA.

We are studying the control of histone mRNA biosynthesis. One of the major control points in this complex synthesis pathway is the interaction of the U7 small nuclear ribonucleoprotein (snRNP) with the histone precursor mRNA. This interaction is required for endonucleolytic cleavage of the pre-mRNA to generate a mature-sized mRNA which is then transported to the cytoplasm (1). The 15-20 nucleotides near the 5' end of the U7 RNA include complementarities (Figure 1) to the histone pre-mRNA sequence (2-5) and these complementarities are essential for the processing event (3-7). Furthermore, it has been demonstrated, that during the cell cycle the accessibility of these same 5' nucleotides of the U7 snRNP are modulated by the cell in concert with DNA synthesis (8, 9). Because the processing of histone pre-mRNA by the U7 snRNP plays an important role in the cell cycle control of histone biosynthesis, we have developed an in vitro system for reproducing this processing event (10). This system uses a nuclear extract from rapidly dividing mammalian cells as a source of the processing factors (primarily the snRNP U7, see (11, 12). When a radioactive RNA molecule containing the processing signals of a histone pre-mRNA is added to this extract, a specific cleavage of the pre-mRNA occurs to generate a molecule identical to the in vivo cleaved product (10). Because this cleavage event requires the basepairing interaction between the U7 snRNP and the pre-mRNA (6) and the sequence of the U7 RNA is known (3-5), we have designed oligonucleotides (Figure 1) complementary to either the pre-mRNA or the U7 snRNA which, upon binding to their target sequence, interfere with the processing event (7). We have previously used this test system to compare the inhibitory activity of various natural RNA and DNA antisense molecules and ribozymes targeted to the U7 sequence (7, 13).

GENERAL METHODS
Abbreviations: DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene; DMAP: 4-(N,N-dimethylamino)pyridine; DMTr: dimethoxytrityl; DTT: dithiothreitol; EDTA: ethylenediaminetetraacetic acid; HEPES: 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid; MMTr: monomethoxytrityl; NPE: 2-(4-nitrophenoxy)ethyl; PMSF: phenylmerhylsulfonyl fluoride; RP-HPLC: reverse phase-HPLC; SDS: dodecylsulfonate Na-salt; TBE: 89 mM Tris, 89 mM borate, 0.1 mM EDTA buffer; TEAA: tetraethylammonium acetate buffer.
The synthesis of the oligonucleotides

Oligodeoxynucleotides: DNA synthesis was performed on an ABI 380 B DNA-synthesizer (Applied Biosystems) using standard methodology (15). The oligodeoxynucleotide was purified by denaturing polyacrylamide gel electrophoresis.

2'-O-Methyl oligoribonucleotides: The 19mer 2'-methoxy oligonucleotides were synthesized on an ABI 380 B DNA-synthesizer using controlled-pore glass (CPG) solid support with a starting 3'-deoxyxynucleoside. 2'-O-Methyl nucleoside (2-cyanoethyl)-N,N-diisopropylphosphoramidites with the following protecting groups were used: A: N6-phenoxycacetyl, 5'-O-DMTr; C: N4-benzoyl, 5'-O-DMTr; G: N7-phenoxycacetyl, O6-NPE, 5'-O-DMTr; U: 5'-O-MMTT Tr (16). Standard DNA methodology with increased coupling time (5 min instead of 3 min) was used. The oligonucleotide was deprotected and cleaved from the solid support with 25% aqueous NH4OH soln (15 h 55°C). After final detritylation the crude oligonucleotide was purified by denaturing polyacrylamide gel electrophoresis. For U7 inhibition, an aliquot was further purified on RP-HPLC (Fig. 2).

Phosphorothioate oligodeoxynucleotides: The synthesis of the phosphorothioate was accomplished by H-phosphonate methodology on an ABI 380 B DNA-synthesizer using the recommended procedures (17-19). The sulfurization was carried out with sulfur (5% in carbonsulfide, pyridine, triethylamine 12:12:1) on the solid support with the 5'-end still protected. After cleavage from the support and deprotection of the bases, the 5'-trityl phosphorothioate oligonucleotide was purified by RP-

Figure 1. The sequences used in these experiments. The U7 sequence and the mouse histone H4 pre-mRNA have been described previously (7). The U7 RNA probably functions through a basepairing interaction with the purine-rich element in the pre-mRNA. The sequences of 7U 19mer (as 2'-O-methyl and 2'-O-ethyl RNA) and 7U 18mer DNA, the control 2'-O-methyl oligonucleotide NS19mer (with no complementarities to U7), and the antisense U7 RNA 63mer (with additional vector sequences boxed) are also shown.

Figure 2. HPLC profiles (UV-detection 260 nm) of 2'-O-methyl-7U 19mer, NPE 2'-O-ethyl-7U 19mer, 2'-O-ethyl-7U 19mer, column: 4x250 mm Nucleosil 100-5 C18 M6, gradient: 40 mM TEAA pH=6.5, 0.1 mM EDTA, 10-40 min 0-40%, 40-60 min 40-100% acetonitrile.

HPLC. The main peak material was detritylated and again subjected to RP-HPLC purification.

2'-O-Ethyl oligoribonucleotides: The synthesis was performed on a Pharmacia Gene Assembler loaded with 0.2 µmol solid support (5 µm polystyrene based polymer-beads) with deoxthyrmidine as the 3'-starting nucleotide. 2'-O-Ethyl nucleoside (2-cyanoethyl)-N,N-diisopropylphosphoramidites with the following protecting groups were used: A: N6-phenoxycacetyl, 5'-O-DMTr; C: N4-benzoyl, 5'-O-DMTr; G: N7-phenoxycacetyl, O6-NPE, 5'-O-DMTr; U: 5'-O-MMTT Tr (16). The coupling conditions were as recommended for standard DNA-synthesis except for the coupling time, which was extended to 5 min (deprotection: 0.4 min 3% trichloroacetic acid in dichloroethane; coupling: 5.0 min 25 equiv. amide, 500 equiv. tetrazole in acetonitrile; capping: 0.4 min 10% acetic anhydride, 3% 4-(N,N-dimethylamino)pyridine (DMAP), 15% collidine in acetonitrile; oxidation: 0.1 min 0.01 M I2, 5% collidine in aqueous acetonitrile). This was necessary to counteract the reduced coupling efficiency due to steric hinderance by the bulky 3'-ethoxy group. The mean coupling efficiency was 98.3% (calculated from the amount of released thymidine during deprotection). Base deprotection and purification: After final detritylation the bound oligonucleotide was treated with 25% aqueous ammonia for 2 h at 55°C to cleave it from the support and to remove all protecting groups except the O6-2-(4-nitrophenyl)ethyl (NPE) group of guanosine. The solution was evaporated in vacuo and part of the crude NPE protected oligonucleotide was purified on RP-HPLC (Fig. 2) to serve as a negative control for the inhibition studies. To remove the NPE group, the oligonucleotide was treated for 24 h with 500 µl of 1M DBU in pyridine at 55°C (20). After neutralization of the DBU with 1.1 equivalents of acetic acid and partial evaporation in vacuo, the yellow solution was subjected to gel
filtration and the product was further purified by preparative polyacrylamide gel electrophoresis. The yield of purified 19mer was 240 μg (36 pmol, 18% based on the solid support, RP-HPLC profile Fig. 2).

Inhibition of processing
In general, the inhibition assays were performed as previously described (7). A 7.5 or 15 μl aliquot of nuclear extract (in buffer D: 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 20% glycerol, 20 mM Hepes, pH 7.4) from a mouse hybridoma line (containing approximately 3 or 6 moles of U7 RNA) was preincubated with the test oligo in the presence of 5 mM MgCl₂ in a volume of 15 μl for 30 minutes on ice, 30 minutes at room temperature followed by 30 minutes at 30°C. After this preincubation, 15 μl of a reaction mixture containing tRNA, RNasin, EDTA (to final concentrations of 0.17 mg/ml, 400 units/ml and 20 mM) and approximately 10 fmol (10,000 CPM) of a 32P-labeled histone pre-mRNA were added to the sample. The reaction was allowed to proceed at 30°C for 2 hours, then proteinase K and SDS were added to 0.5 mg/ml and 0.5% respectively and the samples were incubated at 37°C for 30 minutes. The RNA from the sample was then treated with phenol/chloroform, precipitated with ethanol, dissolved in 80% formamide/0.5×TBE plus 0.025% bromphenol blue & 0.025% xylene cyanol and resolved on a preheated 10.7% acrylamide/8.3 M urea/TBE gel. The resulting radioactive pattern was visualized by exposure of X-ray film at -70°C. Various modifications of this protocol are described in individual figure legends.

**RESULTS**

The experiments were designed to test the efficiency of various oligonucleotide inhibitors of U7 function. We have previously shown that the 20 nucleotides at the 5'-end of the active U7 snRNP are accessible to micrococcal nuclease digestion (7, 8, 21) and that complexing these 5' nucleotides with a complementary RNA oligonucleotide or removing them with a coupled deoxyoligo binding/RNase H cleavage, blocks the processing reaction (7). We found that the most potent inhibitor of the reaction (on a molar basis) was an antisense RNA molecule complementary to 61 nt of the 63 nt U7 sequence and a major limit to the function of antisense inhibitors was rapid degradation of the inhibitor in the nuclease-rich extracts used for these experiments. Therefore, we sought to develop small oligonucleotides which incorporated the high binding affinity of an RNA molecule yet had enhanced resistance to nucleases. Modification of the ribose by the attachment of a 2'-O-methyl group blocks the action of both RNA- and DNA-specific nucleases (22–26). Therefore, we and others (22, 27–31, 38–40) have developed synthetic chemistry techniques which allow the synthesis of oligoribonucleotides bearing 2'-O-methyl modification. We have also produced 2'-O-ethyl modified RNA molecules with the idea that increased lipophilicity may both increase the stability against nucleases and enhance the intracellular uptake of the resulting oligoribonucleotides.

**Natural 63mer antisense RNA versus 2'-O-methyl 19mer antisense RNA**

For an initial test of a 2'-O-methyl oligoribonucleotide's inhibitory capacity, we compared processing inhibition between a 19 nucleotide 2'-O-methyl RNA molecule (2'-O-methyl-7U 19mer) and a naturally-synthesized RNA molecule containing the complement to 61 nt of the U7 sequence (7U RNA). In previous studies, we found this antisense RNA to be the most efficient inhibitor of in vitro processing, blocking the reaction completely when present at a 30-fold molar excess over the U7 RNA (7). Correspondingly the presence of 300 fmoles of 7U RNA blocks approximately 95% of the processing activity and 30 fmoles gives approximately 95% of the processing activity and 30 fmoles gives 80% inhibition at 30 fmoles.

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As 2'-O-Methyl RNAs have proven to be efficient antisense inhibitors (Figure 3), we wondered if an ethyl group at the 2'-O-

position might both enhance the stability of the oligoribonucleotides and increase their binding selectivity. Therefore, the synthetic procedures that we initially developed for the preparation of the 2'-O-methyl RNA building blocks were applied, in a slightly modified form (additional NPE protective group on O6 of guanosine), to the synthesis of the 2'-O-ethyliboribonucleotide phosphoramidites (16).

Assembly of the 7U 19mer sequence occurred with high coupling efficiencies (greater than 98%). The standard treatment of the resin-bound 2'-O-ethyl-7U 19mer with ammonia, which mediates cleavage from the solid phase and deprotection, results in an 2'-O-ethyl-7U 19mer that still contains nitrophenylethyl (NPE) protective groups on the O6 oxygen of the three guanine bases of 7U 19mer. These additional protective groups are then removed by DBU/pyridine treatment (20). The resulting fully-deprotected 2'-O-ethyl-7U 19mer shows a shorter retention time on reverse phase HPLC compared to the NPE-protected species (see Fig. 2), but a still longer retention time than the 2'-O-methyl-7U 19mer compounds (Figure 2). This is consistent with the expected slightly increased lipophilicity of the 2'-O-ethyl- vs. the 2'-O-methyl-derivatives.

2'-O-Ethyl antisense RNA 19mer versus blocked-G, 2'-O-ethyl antisense RNA 19mer

We tested the behavior of a 2'-O-ethyl-7U 19mer possessing the NPE-protected G residues compared to the 2'-O-ethyl-7U 19mer possessing the NPE-protected G residues (Figure 5). The NPE-protected material serves as a control for the specificity of inhibition. Guanine groups modified with NPE are no longer able to form G-C base pairs. Since the G residues in 7U 19mer are distributed throughout the interior of the sequence (see Figure 1), the presence of NPE-G residues should block the hybridization of the inhibitor to the target U7 and hence, block the inhibition.

We find that the 2'-O-ethyl-7U 19mer inhibits the processing reaction completely at 300 fmoles and >95% at 30 fmoles. This
inhibitory activity is similar to that observed with the 2'-O-methyl compounds (Figure 3). However, the NPE 2’-O-ethyl-7U 19mer has no inhibitory activity even at the highest levels tested (3 pmole; Figure 5) demonstrating the role of guanosine base pairing in the inhibition event.

2’-O-Methyl-7U 19mer vs. 2’-O-ethyl-7U 19mer
A detailed comparison between the inhibitory activity of 2’-O-methyl-7U 19mer versus 2’-O-ethyl-7U 19mer was performed (Figure 6). In this experiment the nuclear extract concentration was decreased to 7.5 µl to allow a more sensitive analysis of the processing reaction. When the two inhibitors were tested at 3, 9, 30, 90 and 300 fmoles 2’-O-methyl-7U 19mer; lanes 3 – 12, processing after a preincubation with 3, 9, 30, 90 or 300 fmoles 2’-O-ethyl-7U 19mer; lane 13, processing reaction after addition of 30 fmoles 2’-O-methyl-7U 19mer without magnesium preincubation; lane 14, processing reaction after addition of 30 fmoles 2’-O-ethyl-7U 19mer without magnesium preincubation; lane 15, control processing reaction; lanes M, molecular weight standards as in Figure 1.

Quantifying U7 RNA levels in nuclear extracts before and after inhibitor treatment
There remains the possibility that in the presence of magnesium both the 2’-O-ethyl and the 2’-O-methyl oligos trigger a modification (or destruction) of the target U7 sequence which contribute to the inhibition (similar to RNase H activity with DNA/RNA hybrids). This may play a role in the irreversibility of the 2’-O-methyl inhibition seen in Figure 3. Although the RNA-RNA-like duplex present in a 2’-O-methyl or ethyl-U7 hybrid would not be expected to be a substrate for an enzymatic degradation of the U7 (such as the DNA-RNA hybrid is for RNase H), we observed that the preincubation of 2’-O-methyl or ethyl inhibitors with nuclear extracts in the presence of 5 mM Mg²⁺ leads to an enhanced inhibition and (with the 2’-O-methyl RNA) its irreversibility (Figures 3 and 6). To test if a modification of the U7 RNA had occurred which interferes with the ability of the U7 RNA to form base pair interactions, we used a sensitive RNase protection assay to determine the quantity of U7 and its hybridization capability, both before and after treatment with the various oligonucleotide inhibitors. After incubation with the various inhibitors, either in the presence of 5 mM Mg²⁺, or in
the absence, the EDTA concentration was raised to 20 mM, the protein of the sample was destroyed by proteinase K digestion and the RNA was harvested and hybridized to a radioactive 7U RNA. Subsequently the sample was treated with RNase A plus RNase T1 to destroy all non-duplex RNA and the protected RNA was resolved by gel electrophoresis (Figure 7).

The control, untreated nuclear extract samples display the 63 nt band expected for intact U7 RNA (lanes 3 and 17). Quantitation of the radioactivity in this band allows us to say that there are approximately 3 fmols of U7 RNA in 7.5 μl of this nuclear extract. Incubating the extract with Mg2+ alone triggers a mild reduction in the U7 quantity as well as the appearance of small amount of a U7 subfragment (Figure 7, lane 4). A small amount of target destruction of U7 occurs with the DNA 7U 18mer in the absence of Mg2+ (lane 5) with the U7 destruction complete when the sample is incubated with 5 mM Mg2+ (lane 10; see also (7) Figure 3). However, there is no detectable alteration in the U7 RNA with either the 2'-O-methyl-7U 19mer or 2'-O-ethyl-7U 19mer of the control 2'-O-methyl NS19mer in either the presence or absence of Mg2+. These are the same conditions that generate complete inhibition of the histone processing reaction for the 7U 19mer oligos (see Figure 6). The slight reduction in U7 levels and the appearance of the U7 subfragment seen in lanes 11-14 are no greater than the alterations observed with Mg2+ incubation in the absence of oligonucleotide (lane 4). We feel safe in concluding that the potent inhibitory activity of 2'-O-methyl and ethyl oligoribonucleotides is not accompanied by an enzymatic alteration of the target RNA. However, there were subtle alterations in the U7 RNA or the snRNP which block processing activity that are undetectable by the RNase protection assay.

**DISCUSSION**

We are exploring the use of 2'-O-modified oligoribonucleotides as gene specific inhibitors. We have developed new procedures for the synthesis of 2'-O-methyl and 2'-O-ethyl protected ribonucleoside building blocks (16). We demonstrate here that both 2'-O-methyl and 2'-O-ethyl oligoribonucleotides are superior to unmodified antisense RNA in inhibiting an RNA processing event. The inhibition requires only a slight excess of the inhibitor over the target RNA (approximately 5-fold) and is largely irreversible under conditions that allow the reversal of a natural antisense RNA inhibition (see Figure 3). The irreversible nature of the inhibition does not appear to be accompanied by a modification in the target RNA such as the adenosine deaminase activity previously described (34-36). A likely explanation for this irreversibility may lie, simply, with the reduced degradation of non-hybridized 2'-O-methyl-RNA in the extract. The presumed high melting temperature of the inhibitor/U7 hybrid (37) and difficulty of adding enough complement to the inhibitor to drive the inhibitor/U7 dissociation reaction makes it nearly impossible to reverse the binding under physiological conditions. This irreversibility bodes well for in vivo applications of this class of inhibitor.

During the course of our work, a publication appeared demonstrating the advantages of large aliphatic groups at the 2'-O-position (31). It was demonstrated that a 2'-O-Acetyl oligoribonucleotide possesses the nuclease resistance properties of the 2'-O-methyl oligoribonucleotides but in addition, shows an increase in the level of specific binding to its complement within a nuclear extract (31). We are currently testing this parameter with the 2'-O-ethyl derivatives. Furthermore, all of the experiments reported here were done with 19mer oligoribonucleotides, whose calculated hybrid melting temperature is far above the 30°C used for these inhibitory studies. We are examining the inhibitory properties of shorter derivatives of both the ethyl and methyl-modified compounds to determine if shorter versions of either compound can be useful for the U7 snRNP inhibition. Of course, our ultimate aim is to use these inhibitors in vivo, where shorter oligoribonucleotides may display an advantage in both target specificity and cell penetration (33).

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