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

Matthew Cotten, Berndt Oberhauser, Helmut Brunar[1], Armin Holzner[1], Georg Issakides[1], Christian R.Noe[1], Gotthold Schaffner, Ernst Wagner and Max L.Birnstiel

Institute for Molecular Pathology, Dr. Bohr Gasse 7, A-1030 Vienna and [1]Institut für Organische Chemie, Technische Universität Wien, Getreidemarkt 9, A-1060 Vienna, Austria

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-nitrophenyl)ethyl; PMSF: phenylmethylsulfonyl 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.
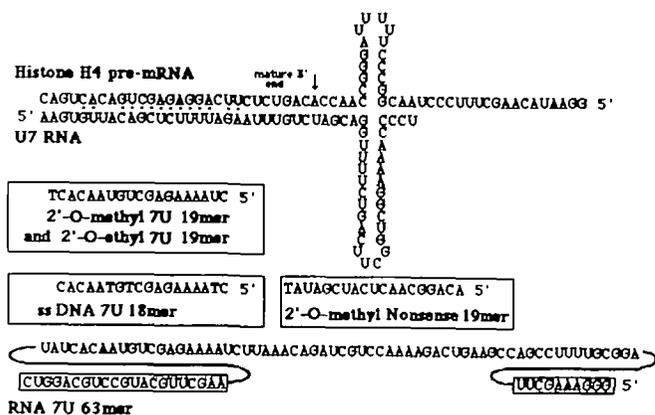
Figure 1. The sequences used in these experiments. The U7 sequence and the mouse histone H4 pre-mRNA have been described previously (7). The U7 RNA probably functions through a basepairing interaction with the purine-rich element in the pre-mRNA. The sequences of 7U 19mer (as 2'-O-methyl and 2'-O-ethyl 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.

Nuclear extracts (14) were prepared from mouse EBI cells (4) and *in vitro* histone procession reactions were performed as previously described (10) using an abbreviated mouse histone H4 RNA substrate (12).

Synthesis of normal antisense U7-RNA (7U) was performed by *in vitro* T7 polymerase transcription of a derivative of pTZ19 (Pharmacia) containing a mouse U7 sequence insert derived from synthetic DNA oligonucleotides (7).

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 ethanol precipitation and denaturing polyacrylamide gel electrophoresis.

2'-O-Methyl oligoribonucleotides: The 19mer 2'-methoxy oligo-nucleotides were synthesized on an ABI 380 B DNA-synthesizer using controlled-pore glass (CPG) solid support with a starting 3'-deoxynucleoside. 2'-O-Methyl nucleoside (2-cyanoethyl)-N,N-diisopropylphosphoramidites with the following protecting groups were used: A: N^6-phenoxyacetyl, 5'-O-DMTr; C: N^4-benzoyl, 5'-O-MMTr; G: N^2-phenoxyacetyl, 5'-O-DMTr; U: 5'-O-MMTr (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 carbondisulfide, 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-
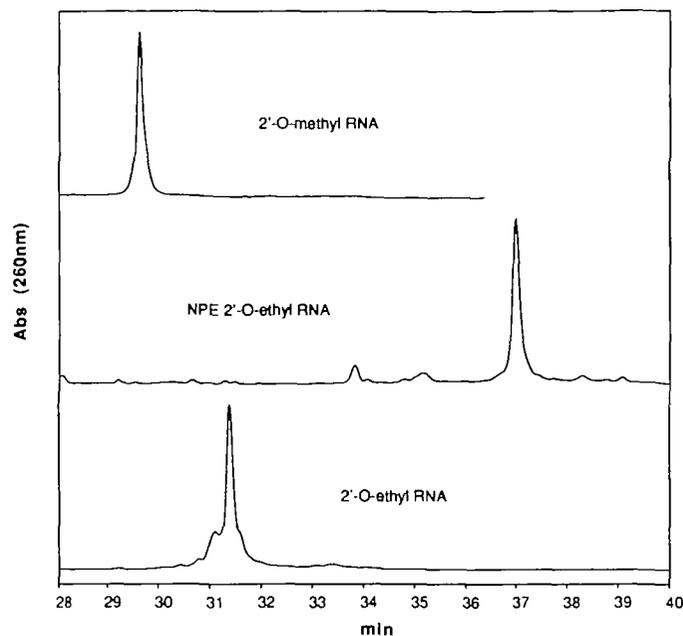
Figure 2. HPLC profiles (UV-detection 260 nm) of 2'-O-methyl-7U 19mer, ^NPEG-2'-O-ethyl-7U 19mer, 2'-O-ethyl-7U 19mer; column: 4×250 mm Nucleosil 100−5 C18 M6, gradient: 100 mM TEAA pH=6.5, 0.1 mM EDTA, 10−40 min 0−40%, 40−60 min 40−100% acetonitrile.

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 deoxythymidine as the 3'-starting nucleotide. 2'-O-Ethyl nucleoside (2-cyanoethyl)-N,N-diisopropylphosphoramidites with the following protecting groups were used: A: N^6-phenoxyacetyl, 5'-O-DMTr; C: N^4-benzoyl, 5'-O-DMTr; G: N^2-phenoxyacetyl, O^6-NPE, 5'-O-DMTr; U: 5'-O-DMTr (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. amidite, 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 hindrance by the bulky 3'-ethoxy group. The mean coupling efficiency was 98.3% (calculated from the amount of released tritylcation 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 O^6-2-(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
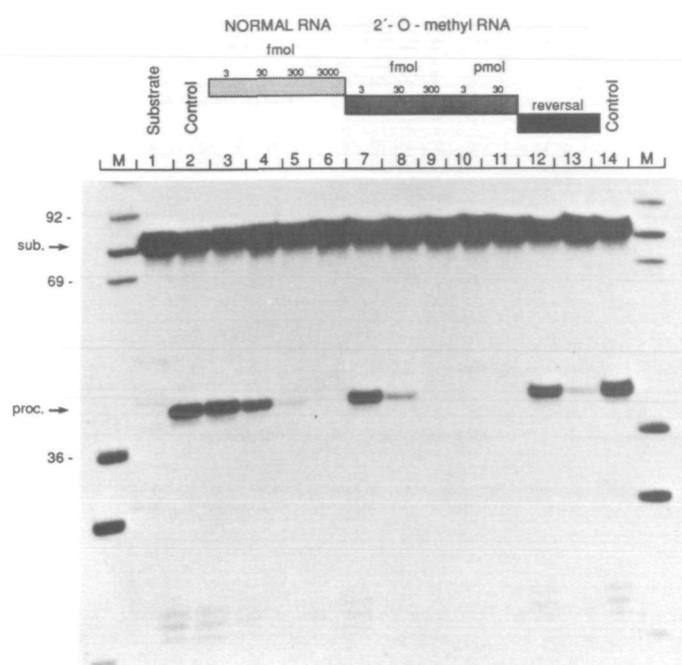
Figure 3. Comparison of 2'-O-methyl 19mer vs. 7U RNA. **Lane 1**, unreacted substrate; **lane 2**, control processing reaction; **lanes 3−6**, processing after a preincubation with 3, 30, 300, or 3000 fmoles 7U RNA; **lanes 7−11**, processing after a preincubation with 3, 30, 300, 3000, or 30,000 fmoles 2'-O-methyl-7U 19mer; **lane 12**, 300 fmoles of 2'-O-methyl-7U 19mer were preincubated with 3 pmoles U7 RNA (in buffer D) before addition to nuclear extract; **lane 13**, the nuclear extract was preincubated with 300 fmoles 2'-O-methyl-7U 19mer, afterwards, 3 pmoles of U7 RNA were added to the reaction; **lane 14**, control processing reaction; the addition of 3 pmoles U7 RNA has no effect on the processing reaction (7); **lanes M**, molecular weight standards: HpaII-cut pBR322 ^{32}P-labeled with the Klenow fragment of polymerase I and α-^{32}P-dCTP, with the size of some of the fragments indicated to the left of the figure. The abbreviations *sub.* and *proc.* indicate the positions of the pre-mRNA substrate, and the processed product.

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 fmoles of U7 RNA) was preincubated with the test oligo in the presence of 5 mM MgCl$_2$ (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 fmoles (10,000 CPM) of a ^{32}P-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 a partial reduction (Figure 3, lanes 2−5). In comparison, the 2'-O-methyl-7U 19mer functions to give complete inhibition at 300 fmoles and approximately 80% inhibition at 30 fmoles (Figure 3, lanes 6−10). As a control, the 2'-O-methyl-7U 19mer was prehybridized to a 10-fold excess of U7 RNA before adding it to the nuclear extract. We find that this maneuver results in a complete block of the inhibition (Figure 3, lane 12), which, with 300 fmoles of 2'-O-methyl-7U 19mer alone, is complete (Figure 3, lane 9). This demonstrates that the 2'-O-methyl inhibitor is functioning via base-pair hybridization with the target RNA and the inhibition is not due to unspecific effects. When the complement to the inhibitor is added after a preincubation of the inhibitor with the extract, we previously found that inhibition by antisense RNA could be reversed (7). When this same experiment is repeated with the 2'-O-methyl inhibitor, we find that only a small portion of the inhibition can be reversed
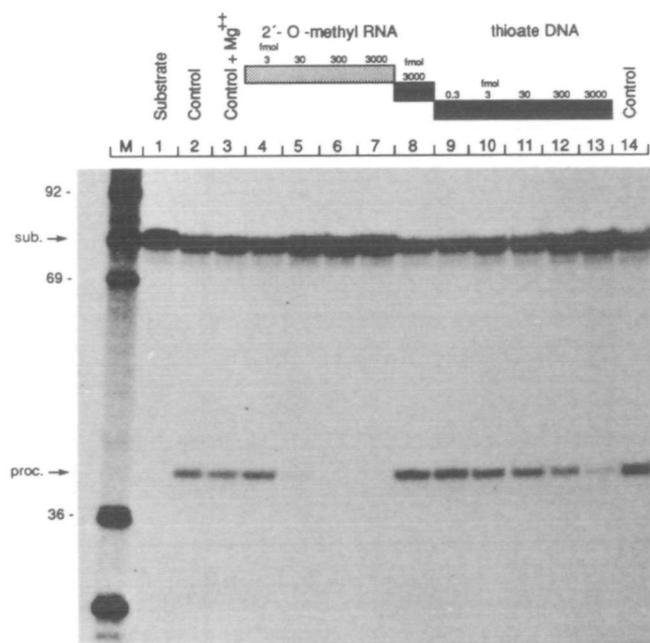
Figure 4. 2'-O-Methyl-7U 19mer vs. thioateDNA 7U 19mer. **Lane 1**, unreacted substrate; **lane 2**, control processing reaction; **lane 3**, processing reaction pretreated with magnesium; **lanes 4 – 7**, processing after a preincubation with 3, 30, 300, or 3000 fmoles 2'-O-methyl-7U 19mer; **lane 8**, processing after preincubation with 3 pmoles of a control 2'-O-methyl 19mer; **lanes 9 – 13**, processing after a preincubation with 0.3, 3, 30, 300, 3000, fmoles thioateDNA 7U 19mer; **lane 14**, control processing reaction, **lane M**, molecular weight standards as in Figure 1.

(Figure 3, lane 13). The cause and consequences of this phenomenon will be discussed below.

Phosphorothioate antisense DNA 19mer vs. 2'-O-methyl antisense RNA 19mer

We next tested a phosphorothioate DNA derivative of the 7U 19mer oligonucleotide. If the sole determinant of the enhanced inhibition by the 2'-O-methyl RNA molecule is due to stability against nucleases, then the use of a phosphorothioate derivative of the 7U 19mer oligonucleotide should also show an enhanced inhibition capacity. However, along with the increased nuclease resistance, the phosphorothioate modification displays a decreased binding affinity to its complement due to stereochemical considerations (see reference 32 for a discussion of this phenomenon). We tested, in the histone processing reaction, the inhibitory capacity of a phosphorothioate DNA 7U 19mer vs. the 2'-O-methyl-7U 19mer shown previously (Figure 3). Once again we find that the 2'-O-methyl-7U 19mer completely inhibits at 300 fmoles and inhibits to approximately 80% at 30 fmoles (Figure 4, lanes 4 – 7). We have included a sample with a control, nonsense 2'-O-methyl oligo, a 19mer with no complementarities to U7. This oligo (NS19mer) has no effect on the processing reaction when 3 pmoles are added to the reaction (Figure 4, lane 8). When the phosphorothioate DNA 7U 19mer is tested in this system, we find that minor inhibition of the reaction occurs at 300 fmoles and approximately 30% inhibition can be seen at 3 pmoles (Figure 4, lanes 9 – 13).

2'-O-Ethyl RNA as a new class of antisense compounds

As 2'-O-Methyl RNAs have proven to be efficient antisense inhibitors (Figure 3), we wondered if an ethyl group at the 2'-O-
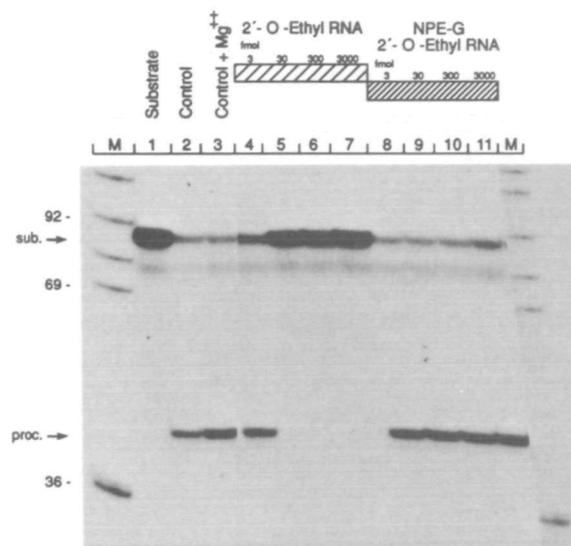
Figure 5. 2'-O-Ethyl-7U 19mer vs. NPEG-2'-O-ethyl-7U 19mer. **Lane 1**, unreacted substrate; **lane 2**, control processing reaction; **lane 3**, processing reaction pretreated with magnesium; **lanes 4 – 7**, processing after a preincubation with 3, 30, 300, or 3000 fmoles 2'-O-ethyl-7U 19mer; **lanes 8 – 11**, processing after a preincubation with 3, 30, 300 or 3000 fmoles NPEG-2'-O-ethyl-7U 19mer; **lanes M**, molecular weight standards as in Figure 1.

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 protecive group on O^6 of guanosine), to the synthesis of the 2'-O-ethylribonucleotide 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 O^6 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 compared to the 2'-O-ethyl 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 NPEG 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
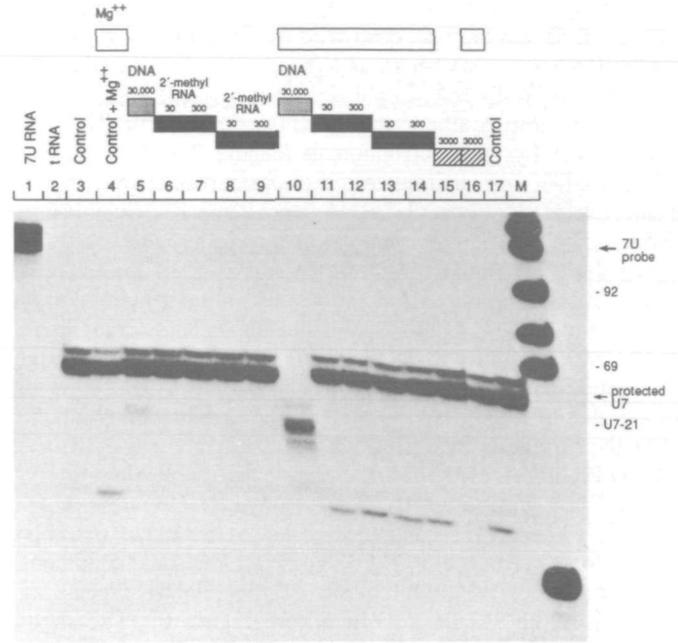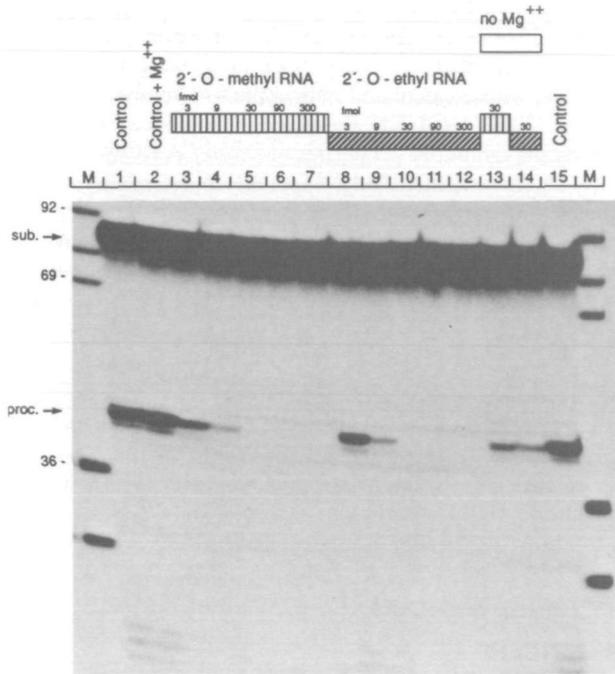
Figure 6. 2'-O-Ethyl inhibition versus 2'-O-methyl inhibition in the presence and absence of magnesium. **Lane 1,** control processing reaction; **lane 2,** processing reaction pretreated with magnesium; **lanes 3−7,** processing after a preincubation with 3, 9, 30, 90 or 300 fmoles 2'-O-methyl-7U 19mer; **lanes 8−12,** 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.

Figure 7. RNase mapping of U7 levels before and after inhibitor treatment. Nuclear extract samples (7.5 μl) were incubated in buffer D7.5/11.5 containing the indicated quantities of ssDNA 7U 19mer, 2'-O-ethyl or 2'-O-methyl-7U 19mer or 2'-O-methyl NS19mer oligoribonucleotides. For samples indicated 'Mg^{2+}' (4, 10−14, 16) the samples included 5 mM MgCl$_2$ and were incubated for 30' on ice, 30' at room temperature, 30' at 30°C followed by addition of 20 mM EDTA and the preparation for RNase protection mapping. For the remaining samples (3, 5−9, 15) the oligonucleotide inhibitor (as indicated) was incubated with the sample in buffer D7.5/11.5 (which contains 0.13 mM EDTA and no divalent cations) for 20 minutes on ice followed by addition of 20 mM EDTA and processing for RNase protection mapping. Mapping was performed with an antisense mouse U7 RNA probe as described previously (7). Buffer D7.5/11.5 is buffer D diluted by a factor of 7.5/11.5. **Lane 1,** 7U probe; **lane 2,** 7U hybridized to 20 μg tRNA; **lanes 3 and 17,** control: extract treated with no oligo, no Mg^{2+}; **lane 4,** extract treated with Mg^{2+}; **lane 5,** extract treated with 30 pmoles ssDNA 7U 19mer; **lanes 6 and 7,** extract treated with 30 and 300 fmoles 2'-O-methyl-7U 19mer; **lanes 8 and 9;** extract treated with 30 and 300 fmoles 2'-O-ethyl-7U 19mer; **lane 10,** extract treated with 30 pmoles ssDNA 7U 19mer plus Mg^{2+}; **lanes 11 and 12,** extract treated with 30 and 300 fmoles 2'-O-methyl-7U 19mer plus Mg^{2+}; **lanes 13 and 14;** extract treated with 30 and 300 fmoles 2'-O-ethyl-7U 19mer plus Mg^{2+}; **lane 15,** extract treated with 3 pmoles control 2'-O-methyl NS19mer; **lane 16,** extract treated with 3 pmoles control 2'-O-methyl NS19mer plus Mg^{2+}; **lanes M,** molecular weight standards as in Figure 1. The truncated U7 species generated by DNA oligo/RNase H cleavage are indicated (U7-21).

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 pmoles; 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, we found virtually identical inhibition activity (Figure 6, lanes 3−12). A slight modification of the assay was performed to analyze the inhibitory activity in the absence of magnesium. When the two inhibitors were added directly to the extract in the absence of the divalent cation and the processing substrate RNA was then promptly added in the presence of 20 mM EDTA, we found that the 2'-O-ethyl inhibitor is slightly more active (compare in Figure 6, lanes 13 and 14). However this difference is subtle. Both inhibitors show an enhancement in their activity at 30 fmoles when preincubated with the extract plus magnesium (compare lanes 5 and 13 for the 2'-O-methyl and lanes 10 and 14 for the 2'-O-ethyl).

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 Mg^{2+} 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 Mg^{2+}, 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 fmoles of U7 RNA in 7.5 μl of this nuclear extract. Incubating the extract with Mg^{2+} 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 Mg^{2+} (lane 5) with the U7 destruction complete when the sample is incubated with 5 mM Mg^{2+} (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 Mg^{2+}. 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 Mg^{2+} 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 could be 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 is 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-allyl 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 have been done with 19mer oligonucleotides, 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 oligonucleotides may display an advantage in both target specificity and cell penetration (33).

ACKNOWLEDGEMENTS

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