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2′-*O*-Alkyl oligoribonucleotides as antisense probes

(modified RNA/affinity selection/ribonucleoprotein complexes/oligonucleotide synthesis/biotinylation)

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ABSTRACT **2′-*O*-Methyl oligoribonucleotides have recently been introduced as antisense probes for studying RNA processing and for affinity purification of RNA–protein complexes. To identify RNA analogues with improved properties for antisense analysis, 2′-*O*-alkyl oligoribonucleotides were synthesized in which the alkyl moiety was either the three-carbon linear allyl group or the five-carbon branched 3,3-dimethylallyl group. Both these analogues were found to be completely resistant to degradation by either DNA- or RNA-specific nucleases. Use of biotinylated derivatives of the probes to affinity-select ribonucleoprotein particles from crude HeLa cell nuclear extracts showed that the presence of the bulky 3,3-dimethylallyl group significantly reduces affinity selection, whereas the allyl derivative binds rapidly and stably to targeted sequences and affinity-selects efficiently. The allyl derivatives also showed an increase in the level of specific binding to targeted sequences compared with 2′-*O*-methyl probes of identical sequence. These properties indicate that the 2′-*O*-allyl oligoribonucleotides are particularly well suited for use as antisense probes.**

Chemically synthesized 2′-*O*-methyl oligoribonucleotides, as described by Ohtsuka and Morisawa and coworkers (1–6) and by Sproat *et al.* (7), are of interest as antisense probes since (*i*) an oligo(2′-*O*-methylribonucleotide)·RNA duplex has greater thermal stability than a corresponding DNA·RNA duplex (2), (*ii*) the oligo(2′-*O*-methylribonucleotide)·RNA duplex is not a substrate for RNase H cleavage (6), and (*iii*) the 2′-*O*-methyl oligoribonucleotides are resistant to nuclease degradation, thus facilitating their use in crude extracts containing high levels of endogenous RNase or DNase activity (7). These properties of antisense 2′-*O*-methyl oligoribonucleotides were exploited to map functional domains in the U2 small nuclear ribonucleoprotein (snRNP) that are required for the assembly of functional spliceosomes *in vitro* (8). In addition, when the ligand biotin is coupled to such antisense probes, they can be used to specifically and efficiently affinity-select targeted RNA–protein complexes from crude HeLa cell nuclear extracts by streptavidin-agarose chromatography (9, 10).

Recently, we have designed 2′-*O*-alkyl oligoribonucleotides that exhibit improved properties for use as antisense probes. Efficient antisense probes should ideally (*i*) be able to hybridize efficiently, stably, and specifically to targeted complementary sequences; (*ii*) be resistant to enzymatic degradation; (*iii*) be chemically stable and convenient to synthesize; and (*iv*) be able to interact minimally with nonspecific cellular factors such as nucleic acid-binding proteins. In addition, if the probes are to be of use for affinity chromatography, RNase H should not be able to cleave hybrids formed between the antisense probes and targeted RNA or DNA sequences.

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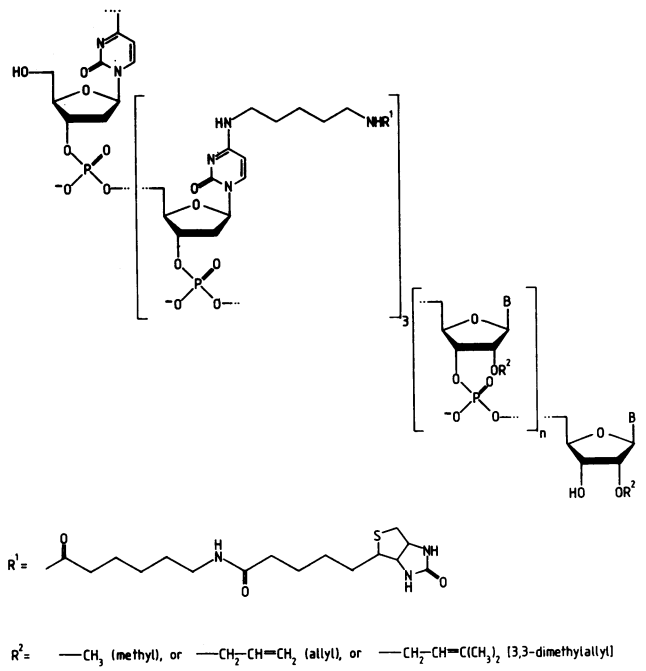

FIG. 1. General structure of 2′-*O*-alkyl oligoribonucleotides. The structures of the three 2′-O-linked alkyl groups tested, methyl, allyl, and 3,3-dimethylallyl, are shown as R^2. For biotinylated probes (as illustrated), a long-chain biotin derivative (R^1), was attached to an aminoalkyl spacer arm, linked to the exocyclic amino group of the non-base-pairing 2′-deoxycytidines. By appropriate incorporation of such modified cytidines, biotin can be placed at either the 5′ or the 3′ terminus as required. To facilitate 5′ end-labeling of 5′-biotinylated oligonucleotides, an additional, nonbiotinylated 2′-*O*-methyluridine was routinely placed at the 5′ terminus.

In this report we present a characterization of two 2′-*O*-alkyl oligoribonucleotide derivatives, 2′-*O*-allyl and 2′-*O*-(3,3-dimethylallyl) RNA (Fig. 1). Based on these results we propose that the properties of the 2′-*O*-allyl oligoribonucleotides render them especially well suited for general use as antisense reagents.†

MATERIALS AND METHODS

RNasin was purchased from Promega. T7 RNA polymerase was purchased from Stratagene. T4 polynucleotide kinase, BAL-31 exonuclease, and mung bean nuclease were purchased from New England Biolabs. All other nucleases were purchased from Boehringer Mannheim. [γ-^{32}P]ATP (10 mCi/

Abbreviations: snRNP, small nuclear ribonucleoprotein; snRNA, small nuclear RNA.

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†The 2′-*O*-allyl and 2′-*O*-(3,3-dimethylallyl) oligoribonucleotides described in this report are the subject of a German patent application, no. P 40 11 473.2.

ml, 6000 Ci/mmol; 1 Ci = 37 GBq) was purchased from Amersham. Streptavidin-agarose was purchased from Sigma. X-ray film was purchased from both Kodak (X-Omat) and Fuji (RX).

Oligonucleotides. 2′-*O*-Alkyl oligoribonucleotides were synthesized on a solid phase from base-protected 5′-*O*-dimethoxytrityl-2′-*O*-alkylribonucleoside 3′-*O*-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite)s by using 5-(4-nitrophenyl)-1*H*-tetrazole as activator, as previously described for the 2′-*O*-methyl compounds (7). The various building blocks for the solid-phase synthesis were prepared according to our recently published procedure (11) using versatile intermediates. Controlled-pore glass supports were prepared as described (7). Deprotection, purification, and tetrabiotinylation (when performed) were done as described for the 2′-*O*-methyl analogues (7). We note that due to solubility properties, ethanol precipitation could not be used to concentrate the 2′-*O*-(3,3-dimethylallyl) oligoribonucleotides. For these probes purification was done by either gel filtration chromatography or dialysis. Oligonucleotides were 5′-end-labeled by using T4 polynucleotide kinase (7).

Analysis of Digested Oligonucleotides. Nuclease digestion products were analyzed by electrophoresis in 0.4-mm-thick, 15% polyacrylamide (30:1 acrylamide/*N*,*N*′-methylenebisacrylamine weight ratio)/8 M urea denaturing gels run in 1× TBE buffer (90 mM Tris base/90 mM boric acid/2 mM EDTA). Gels were typically run at 45 V/cm for 1.5–2.5 hr at 4°C.

Analysis of snRNPs. Native gel electrophoresis was done using 3.5% polyacrylamide (60:1:acrylamide/*N*,*N*′-methylenebisacrylamide)/0.4% agarose composite gels run in 50 mM Tris/glycine buffer (8). Affinity-selection experiments were performed basically as described (9, 10), with the following modifications: the buffer used to preblock streptavidin-agarose beads was (20 mM Hepes, pH 7.9/250 mM-KCl/0.05% Nonidet P-40 with tRNA at 100 μg/ml). One hundred microliters of streptavidin-agarose suspension was used per 100-μl incubation mixture containing HeLa cell nuclear extract (final extract protein concentration, 4 mg/ml). Oligonucleotides were incubated in nuclear extract containing 1.5 mM ATP, 5 mM creatine phosphate, 1.5 units of RNasin per μl, 0.4 M KCl, and 2 mM $MgCl_2$ (final concentrations) for 60–90 min at 30°C.

Northern hybridization analysis and preparation of RNA probes complementary to small nuclear RNA (snRNA) were done as previously described (9, 10).

RESULTS

Fig. 1 shows the structures of the 2′-*O*-alkyl groups tested, methyl, allyl, and 3,3-dimethylallyl. A major difference is that the allyl group is unbranched whereas the 3,3-dimethylallyl group has a branched terminus. For biotinylated oligonucleotides the biotin was attached on a flexible spacer arm to the exocyclic amino group of 2′-deoxycytidine (Fig. 1). The nuclease sensitivity of both 2′-*O*-allyl and 2′-*O*-(3,3-dimethylallyl) oligonucleotides, and of an oligo(2′-deoxyribonucleotide) of similar sequence, was assayed. Consistent with data previously reported for 2′-*O*-methyl oligoribonucleotides (7), both the allyl and dimethylallyl RNA derivatives were completely resistant to degradation by five separate RNA-specific nucleases or by DNase I (Fig. 2 *B* and *C*, lanes 8–13). In contrast, the control DNA oligonucleotide was degraded by DNase (Fig. 2*A*, lane 13). Analysis of chemically synthesized RNA oligonucleotides showed that all the RNases tested were able to digest RNA to completion under the same conditions (12). However, analysis of six nucleases that display dual DNA/RNA cleavage specificity revealed clear differences in the sensitivity of the allyl and dimethylallyl oligonucleotides (Fig. 2 *B* and *C*, lanes 2–7). The control DNA oligonucleotide was degraded by all these enzymes (Fig. 2*A*, lanes 2–7). The 2′-*O*-allyl oligoribonucleotide was resistant to S1 and mung bean nucleases and partially resistant to micrococcal nuclease but was cleaved by BAL-31 and P1 nucleases and by snake venom phosphodiesterase (Fig. 2*B*, lanes 2–7). This cleavage pattern is similar to that reported for 2′-*O*-methyl oligoribonucleotides (7), although careful comparison revealed that the 2′-*O*-allyl oligoribonucleotides were slightly more stable to micrococcal and P1 nucleases (data not shown). A more dramatic stabilization was observed with the 2′-*O*-(3,3-dimethylallyl) oligoribonucleotides. In this case, degradation was almost completely eliminated under the selected assay conditions with low levels of cleavage by micrococcal, BAL-31, and P1 nucleases and snake venom phosphodiesterase (Fig. 2*C*, lanes 2–7).

To assess the cleavage sensitivity of the 2′-*O*-allyl and 2′-*O*-(3,3-dimethylallyl) oligoribonucleotides, titrations were also performed with P1 nuclease and snake venom phosphodiesterase. In both cases, the two forms of oligonucleotide were incubated with various amounts of enzyme and the resulting products were analyzed in denaturing polyacrylamide gels that allowed separation of the undigested sub-

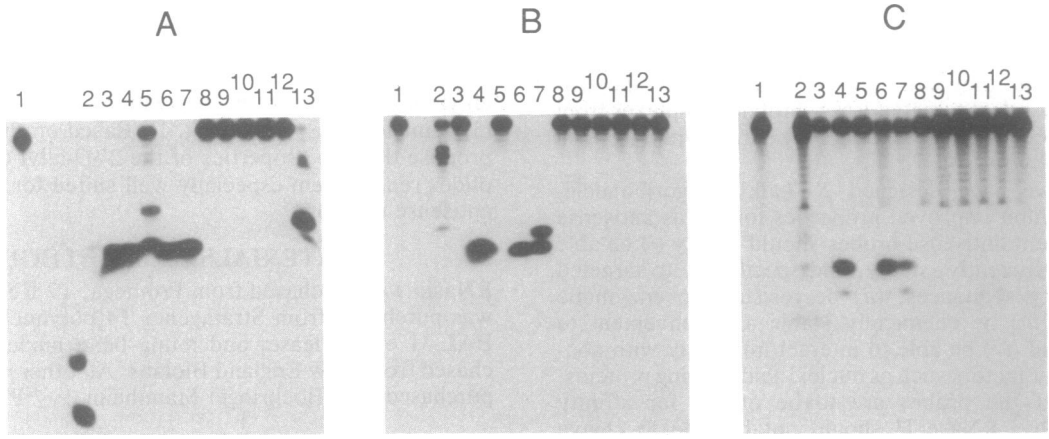

FIG. 2. Sensitivity of oligonucleotides to nuclease degradation. An oligo(2′-deoxyribonucleotide) (*A*), an oligo(2′-*O*-allylribonucleotide) (*B*), and an oligo[2′-*O*-(3,3-dimethylallyl)ribonucleotide] (*C*) were tested with a panel of nucleases. Lanes: 1, no nuclease (control); 2, micrococcal nuclease; 3, S1 nuclease; 4, BAL-31 nuclease; 5, mung bean nuclease; 6, snake venom phosphodiesterase; 7, P1 nuclease; 8, pancreatic RNase A; 9, RNase CL3; 10, RNase T1; 11, RNase T2; 12, RNase U2; 13, DNase I. Approximately 2 pmol of 5′-end-labeled oligonucleotide was digested in each assay. Assay conditions were as previously reported (7). Sequence of DNA oligonucleotide (*A*) was; 5′-GAACAGATACTACACTT-3′. Sequences of the 2′-*O*-alkyl oligoribonucleotides (*B* and *C*) were in each case 5′-IAACAIAUACUACACUU-3′.

strates. The 2′-*O*-(3,3-dimethylallyl) oligoribonucleotide was stable in the presence of a 1000-fold higher concentration of either snake venom or P1 nuclease than was required to digest at least 50% of the 2′-*O*-allyl oligoribonucleotide (Fig. 3). We note that the degree of steric hindrance to nuclease attack provided by the 2′-*O*-alkyl group is not linearly related to the length of the alkyl chain, as only a minor difference was observed in the cleavage of the 2′-*O*-allyl derivatives in comparison with the 2′-*O*-methyl derivatives (data not shown). It is possible, however, that the marked decrease in nuclease cleavage of the 2′-*O*-(3,3-dimethylallyl) oligoribonucleotide probe is due more to the branched structure of the alkyl group than to the increase in chain length.

To assess the usefulness of the allyl and dimethylallyl derivatives for antisense affinity chromatography of RNA–protein complexes, we constructed biotinylated derivatives targeted against the mammalian U4/U6 and/or U2 snRNPs (Fig. 1). As a positive control, biotinylated oligonucleotides of identical sequence made with 2′-*O*-methyl ribonucleotides were used. Such biotinylated 2′-*O*-methyl RNA probes allow efficient affinity selection of U2 and U4/U6 snRNPs from HeLa cell nuclear extracts (9, 10). The biotinylated 2′-*O*-allyl oligoribonucleotides were able to affinity-select both U2 and U4/U6 snRNPs from crude HeLa cell nuclear extracts as efficiently and specifically as the 2′-*O*-methyl probes (Fig. 4, compare lanes 2 and 4 with lanes 3 and 5). In contrast, the 2′-*O*-(3,3-dimethylallyl) probes gave inefficient selection over the range of concentrations tested, although without changing nonspecific background selection (Fig. 4, lanes 6–9). Our data indicate that the 2′-*O*-(3,3-dimethylallyl) oligoribonucleotides are unable to form stable hybrids with complementary RNA target sequences (Fig. 4 and data not shown).

The properties of the 2′-*O*-allyl oligoribonucleotides were compared with those of the previously described 2′-*O*-methyl probes. First, the specificity of binding to targeted complementary RNA sequences was examined by a nondenaturing gel assay (8). 2′-*O*-Methyl or 2′-*O*-allyl oligoribonucleotides complementary to U2 snRNA were 5′-end-labeled and incubated with HeLa cell nuclear extracts (Fig. 5). As previously reported, the 2′-*O*-methyl probe binds stably to U2 snRNP in this assay but also binds to other, non-U2-related components in the nuclear extract (8). With the 2′-*O*-allyl oligoribonucleotide, however, the observed stable binding to U2 snRNP was increased relative to the amount of non-U2-specific binding (Fig. 5). The level of non-U2 snRNP related binding increased at higher concentrations of probe but was consistently lower than that observed with equivalent 2′-*O*-methyl oligoribonucleotides (unpublished observations). Additional experiments showed little U2 snRNP binding with a 5′-end-labeled 2′-*O*-(3,3-dimethylallyl) oligoribonucleotide, again indicating that it is unable to form stable hybrids with

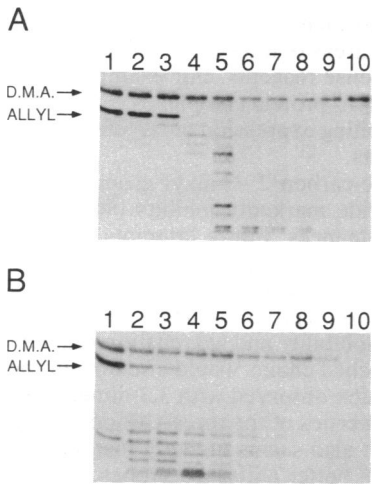

FIG. 3. Titration of snake venom phosphodiesterase (*A*) and P1 nuclease (*B*). Digestions were done with a mixture of 2′-*O*-allyl and 2′-*O*-(3,3-dimethylallyl) (D.M.A.) oligoribonucleotides. All assays were done in a final volume of 20 μl containing glycogen at 50 μg/ml and were incubated at 30°C for 1 hr. Enzyme concentrations (μg per assay mixture) were as follows. Snake venom phosphodiesterase (*A*): 0, 2×10^{-5}, 2×10^{-4}, 2×10^{-3}, 2×10^{-2}, 4×10^{-2}, 0.2, 0.4, 2, and 4 μg (lanes 1–10, respectively). P1 nuclease (*B*): 0, 2×10^{-6}, 4×10^{-6}, 2×10^{-5}, 4×10^{-5}, 2×10^{-4}, 4×10^{-4}, 2×10^{-3}, 2×10^{-2}, and 0.2 μg (lanes 1–10, respectively). Enzyme dilutions were done with 10 mM Tris, pH 7.6/1 mM EDTA containing bovine serum albumin at 10 μg/ml. Sequences of 2′-*O*-alkyl oligoribonucleotides are given in the legend to Fig. 2. Each was present at a final concentration of 0.3–3 pmol per reaction mixture.

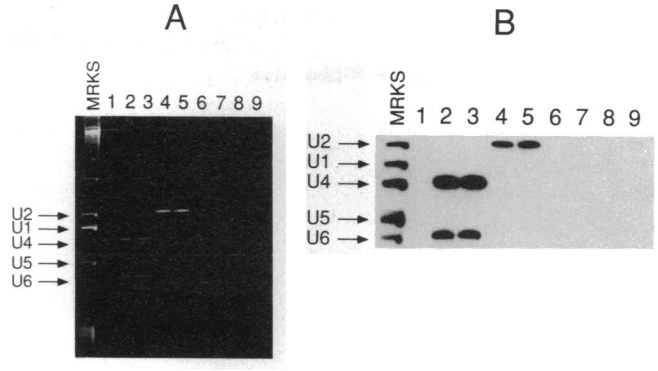

FIG. 4. Affinity selection of snRNPs from HeLa cell nuclear extracts by biotinylated antisense 2′-*O*-alkyl oligoribonucleotides. RNA species recovered from streptavidin-agarose pellets after affinity selection were detected both by ethidium bromide staining (*A*) and by Northern hybridization (*B*) using RNA probes complementary to U1, U2, U4, U5, and U6 snRNAs. Lanes: 1, no oligonucleotide (control); 2, anti-U6, 2′-*O*-methyl; 3, anti-U6, 2′-*O*-allyl; 4, anti-U2, 2′-*O*-methyl; 5, anti-U2, 2′-*O*-allyl; 6–9, increasing concentration of anti-U2, 2′-*O*-(3,3-dimethylallyl). MRKS, total HeLa nuclear RNA. Oligonucleotide concentrations used were 50–200 pmol per reaction mixture, corresponding to the empirically determined optimum concentrations. Sequences were as follows: anti-U6 snRNA (2′-*O*-methyl and 2′-*O*-allyl), 5′-U(dC-biotin)$_4$AUCIUUCCAAUUUUAIUAUAU-3′; anti-U2 snRNA (2′-*O*-methyl, 2′-*O*-allyl), as in legend to Fig. 2 but with four additional 3′ biotinylated residues. For the 2′-*O*-(3,3-dimethylallyl) anti-U2 probe the sequence was 5′-U(dC-biotin)$_4$AIAACAIAUACUACACUUIA-3′.

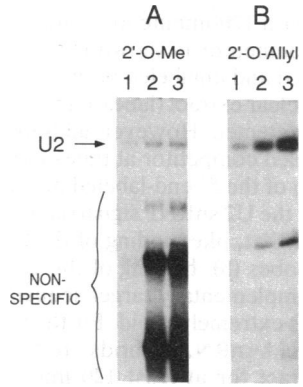

FIG. 5. Specificity of oligonucleotide binding to complementary target sequences. The 5′-end-labeled 2′-*O*-methyl (*A*) or 2′-*O*-allyl (*B*) oligoribonucleotides complementary to U2 snRNA were incubated with HeLa cell nuclear extract and analyzed by nondenaturing polyacrylamide/agarose composite-gel electrophoresis as previously described (8). The sequences of the oligonucleotides are given in the legend to Fig. 2. Each oligonucleotide was tested at 5 pmol (lanes 1), 10 pmol (lanes 2), and 20 pmol (lanes 3) per 20-μl reaction mixture.

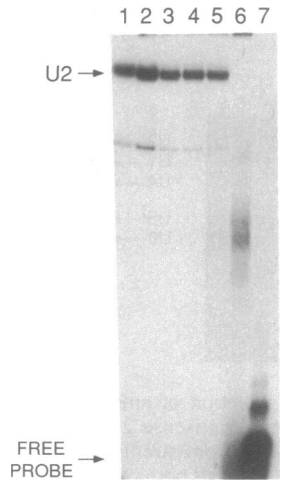

FIG. 6. Stability of 2′-*O*-allyl oligoribonucleotide/U2 snRNA hybrids assayed by competition. A 5′-end-labeled 2′-*O*-allyl oligoribonucleotide (3 pmol per reaction mixture) complementary to U2 snRNA was incubated with HeLa nuclear extract and analyzed in a native polyacrylamide/agarose composite gel. Lane 1, incubation without competitor for 2 hr at 30°C; lanes 2–5, same incubation conditions except that ≈300 pmol of unlabeled but otherwise identical oligonucleotide was added 5, 30, 60, or 90 min, respectively, after addition of the labeled probe. Lane 6, probe and excess unlabeled oligonucleotide were mixed prior to addition to the extract. Lane 7, labeled probe-incubated in the absence of HeLa nuclear extract. The sequence of the oligonucleotide is shown in the legend to Fig. 2.

complementary target sequences (data not shown). As most of the non-U2-related complexes formed with the 2′-*O*-methyl probes probably arise through interactions with protein components in nuclear extracts (8), the improved specificity found with the 2′-*O*-allyl oligoribonucleotides may be due to the bulkier 2′-*O*-allyl group reducing protein binding through steric hindrance.

The stability of the hybrids formed between 2′-*O*-allyl oligoribonucleotides and complementary target sequences was examined by a competition assay (Fig. 6). A 5′-end-labeled 2′-*O*-allyl oligoribonucleotide complementary to U2 snRNP was incubated in HeLa cell nuclear extract and challenged after various times of incubation with a 100-fold molar excess of an identical but unlabeled probe. The level of U2 snRNP binding by the labeled probe was detected by nondenaturing polyacrylamide gel electrophoresis (Fig. 5). The U2 snRNP signal after a 120-minute incubation in the absence of nonradioactive competitor is shown in lane 1. As a negative control, the labeled and unlabeled probes were mixed prior to addition to the nuclear extract (lane 6). In this case, essentially no U2 signal was detected. However, addition of an equivalent amount of unlabeled competitor at times ranging from 5 to 90 min after addition of the 5′-end-labeled probe resulted in little or no reduction in the U2 snRNP signal (lanes 2–5). From these data we conclude that, like binding of the 2′-*O*-methyl oligoribonucleotide probes (8), binding of the 2′-*O*-allyl oligoribonucleotides to complementary target sequences in HeLa cell nuclear extracts is extremely rapid. Further, we conclude that once 2′-*O*-allyl RNA·snRNA hybrids are formed they remain stable in the extract for at least 120 min, as no significant duplex exchange was observed upon subsequent addition of unlabeled competitor.

DISCUSSION

Based on the utility of antisense oligoribonucleotides made of 2′-*O*-methyl RNA in the study of RNA processing and the purification of RNP complexes (8–10), we have investigated whether attachment of different types of alkyl groups to the 2′ hydroxyl of ribose might improve the properties of the resulting polymers for antisense applications. In particular, nuclease resistance and efficiency, stability, and specificity of binding to complementary RNA target sequences were tested.

The replacement of 2′-*O*-methyl with 2′-*O*-(3,3-dimethylallyl) (see Fig. 1), a branched, five-carbon alkyl group, dramatically increased the resistance of the resulting oligoribonucleotide polymers to nuclease degradation. However, this bulky alkyl group also resulted in a severe reduction in hybridization to complementary RNA sequences. This renders such oligonucleotides unsuitable for application as antisense probes. In contrast, the replacement of 2′-*O*-methyl with 2′-*O*-allyl, an unbranched three-carbon alkyl group (see Fig. 1), produced oligoribonucleotides that are in several respects superior to 2′-*O*-methyl RNA as antisense probes. The 2′-*O*-allyl oligoribonucleotides are resistant to digestion by either RNA- or DNA-specific nucleases and slightly more resistant to nucleases with dual RNA/DNA specificity than the 2′-*O*-methyl oligoribonucleotides. A major advantage is that the 2′-*O*-allyl oligoribonucleotides show a marked increase in the fraction of the probe that hybridizes to targeted complementary RNA sequences rather than to nontargeted factors in crude nuclear extracts (Fig. 5). This probably results, at least in part, from a higher level of steric hindrance imposed upon nucleic acid-binding proteins by the three-carbon allyl group as compared with the single-carbon methyl group. This decrease in nonspecific binding is not accompanied by any apparent decrease in the ability of the 2′-*O*-allyl RNA probes to bind rapidly and stably to targeted RNA sequences (Figs. 4 and 6). It therefore appears that the 2′-*O*-allyl oligoribonucleotides retain the useful properties of the previously described 2′-*O*-methyl RNA probes, including ready coupling to biotin or other prosthetic groups (7), while additionally displaying improved binding specificity and slightly greater stability. As the chemical synthesis of 2′-*O*-allyl-containing phosphoramidites entails no greater difficulty or expense than preparation of the 2′-*O*-methyl derivatives (11), and in certain respects is simpler, we propose that 2′-*O*-allyl oligoribonucleotides will have significant applications in many areas of molecular biology requiring the use of efficient antisense reagents. For example, in addition to use in studying RNA–protein complexes, such probes may prove useful in inhibiting expression of targeted cellular genes or as antiviral agents.

Since a five-carbon 2′-*O*-alkyl group in an antisense oligoribonucleotide markedly inhibits binding to complementary RNA sequences, future attempts to prepare alkylated RNA probes that are superior to the 2′-*O*-allyl oligoribonucleotides should concentrate on either four-carbon or alternative three-carbon groups. Longer alkyl groups are likely to pose serious solubility and aggregation problems, even if the use of unbranched chains would circumvent the inhibition of hybrid formation observed with 3,3-dimethylallyl. Given the favorable properties of the three-carbon 2′-*O*-allyl oligoribonucleotides, it also seems unlikely that shorter alkyl chains would provide better antisense probes. It is possible therefore that the 2′-*O*-allyl oligoribonucleotides are already close to the optimum structure of alkylated RNA-based antisense probes. Further improvements in binding specificity and stability may be possible, however, through additional modifications to the structure of the nucleotide bases and/or the phosphodiester backbone.

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