Ribozyme, Antisense RNA, and Antisense DNA Inhibition of U7 Small Nuclear Ribonucleoprotein-Mediated Histone Pre-mRNA Processing In Vitro

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A comparative analysis of ribozyme, antisense RNA, and antisense DNA inhibitors of the in vitro small nuclear ribonucleoprotein U7-dependent histone pre-mRNA processing reaction was performed. RNA molecules complementary to the U7 sequence inhibited in vitro processing of histone pre-mRNA at a sixfold excess over U7. Single-stranded DNA complementary to the entire U7 sequence inhibited the reaction at a 60-fold excess over U7, while a short, 18-nucleotide DNA molecule complementary to the 5' end of U7 inhibited the processing reaction at a 600-fold excess. A targeted ribozyme was capable of specifically cleaving the U7 small nuclear ribonucleoprotein in a nuclear extract and inhibited the U7-dependent processing reaction, but in our in vitro system it required a 1,000-fold excess over U7 for complete inhibition of processing.

A large array of experimental results demonstrates that complementary (antisense) DNA or RNA molecules can be used to interfere with the function of essential RNA molecules (reviewed in references 17, 25, 26, and 29). One mode of antisense DNA inhibition involves degradation of target RNA by RNase H cleavage of hybrid RNA. Antisense RNA molecules most likely function by forming a hybrid with the target RNA molecule, thereby blocking RNA processing, transport, or translation signals. However, there is evidence that double-stranded RNA molecules serve as substrates for adenosine deaminase activity (1), which could have disruptive consequences for the activity of target RNA molecules.

A third type of inhibitor has recently become available. RNA molecules with enzymatic activity (ribozymes) capable of cleaving specific target RNA molecules have been developed (13) based on the self-cleaving RNA sequences found in various plant viroids, virusoids, and satellite viruses (4, 7, 8, 14, 15, 24). These ribozymes are potentially very powerful inhibitors, combining catalytic inactivation of the target RNA with the possibility of in vivo synthesis from a gene. A direct comparison of these three inhibitors, ribozyme and antisense RNA and DNA, in a system with a known target RNA concentration has not been reported. We undertook such an analysis of inhibitors of the U7 small nuclear ribonucleoprotein (snRNP)-dependent histone pre-mRNA processing reaction.

snRNP U7 (5, 9, 18, 20, 22, 23) is required for maturation of replication variant histone transcripts (reviewed in references 3 and 19). The mRNA transcripts of these polymerase II genes lack the standard 3' polyadenylation and instead terminate in a highly conserved hairpin structure (reviewed in reference 2). During U7-dependent processing of these mRNA molecules, base-pairing interactions occur between the 5' end of the U7 snRNP and a purine-rich, highly conserved sequence element found at the 3' end of all members of this transcript family (21). Subsequently, cleavage of the message occurs to produce the mature 3' end and the message exits the nucleus en route to the translation machinery.

An in vitro system based on mammalian tissue culture cell

nuclear extracts has been developed which authentically processes histone pre-mRNA (10) in a U7-dependent fashion (5, 18, 20). Using this in vitro system, we compared the processing inhibition of antisense DNA and RNA and ribozyme molecules targeted to the U7 sequence. We found that antisense RNA was the most potent inhibitor of the processing reaction, blocking U7 activity (greater than 90%) when present at a sixfold excess over the small nuclear RNA. Full-length (65 nucleotides [nt]) antisense DNA inhibited the reaction to the same extent at a 60-fold excess over U7, while a short (18-nt) antisense DNA molecule required a 600-fold excess to attain this level of inhibition. The inhibitory activity of the U7-targeted ribozyme was similar to that of the short antisense DNA molecule, requiring a 1,000-fold excess over the target RNA for complete inhibition. The DNA and ribozyme inhibitors however, produced irreversible inhibition due to cleavage of the target RNA. Antisense RNA inhibition was reversible, presumably because of the dissociability of the RNA-RNA hybrid.

The final inhibition level achieved with each type of inhibitor was due to a combination of several effects. Certainly, the stability of the inhibitor in the extract plays a pivotal role. Furthermore, the strength of the hybrid formed between the inhibitor and the target RNA and the accessibility of the target RNA is important. We stress the functional nature of the inhibitory values that we determined; they result from the behavior of these molecules in a particular in vitro biological system. We are currently testing the inhibitors in vivo to determine whether the relative levels of inhibition we observed in vitro are generally true.

Important for the use of ribozymes in vivo, cleavage of U7 RNA by this ribozyme proceeded at a temperature (30°C) milder than that used previously (50°C; 13) and at a lower magnesium concentration (5 rather than 20 mM; 13). We were optimistic that the catalytic activity of a ribozyme (13, 24, 27, 28) might provide inhibition of the U7-dependent processing event that would far exceed that of the static, antisense RNA approach. We were surprised to find that the U7-targeted ribozyme inhibited the processing event no more efficiently than did a short antisense DNA molecule. On the other hand, while previous studies have demonstrated that targeted ribozymes can be used to cleave naked

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FIG. 1. Various sequences used in this study. (A) The mouse histone H4 pre-mRNA sequence is shown aligned with the U7 sequence. The potential base pairing between these two RNA molecules, the mature 3' end of the histone mRNA, and the histone palindrome are indicated. (B) The U7 sequence (as determined by Cotten et al. [5]) is shown aligned with the two ribozyme sequences used in this study. The catalytic sequence of the satellite RNA of tobacco ringspot viroid, as described by Haseloff and Gerlach (13), is shown in bold type. The cleavage site of each ribozyme is indicated. Note that the U7 sequence used in these studies lacked the trimethyl-G cap structure found on natural U7 small nuclear RNA and contained additional vector-derived nucleotides at the 5' and 3' ends (boxed in panels B and C). (C) The antisense DNA and RNA molecules used in this study. The DNA molecules contained 5 nt of an *Hind*III linker sequence at the 5' and 3' ends. The sense U7 DNA molecule contained the complement of the antisense U7 DNA molecule and also possessed *Hind*III ends. The antisense U7 molecule used in the study was synthesized from a plasmid construction identical to that used to synthesize sense U7 RNA, except that the U7 DNA oligonucleotides were cloned in the reverse orientation. In all of the panels, the 5' end of each sequence is indicated.

RNA substrates in protein-free systems (13, 24, 27, 28), this may be the first reported use of a ribozyme to cleave a snRNP and inhibit a biological process in a proteinaceous environment. This suggests that this class of inhibitor has the potential for in vivo applications.

MATERIALS AND METHODS

Processing reactions. The in vitro histone pre-mRNA processing system we used was that of Gick et al. (10). Essentially, a radioactive RNA (derived from in vitro SP6 polymerase transcription of a linearized DNA template) containing the conserved palindrome, cleavage site, and downstream spacer element of a mouse H4 histone gene (24a; Fig. 1) was incubated in a modified nuclear extract (6) of mouse hybridoma (EB1) cells. The U7 contents of the extracts were determined by RNase protection mapping; the extracts used in this study contained 0.3 fmol of U7 per μ l. In 20 mM EDTA, the precursor RNA (74 nt; see Fig. 3A, lane 1) was cleaved to yield a mature species (42 nt; see Fig. 3A, lane 2) plus a series of spacer RNA molecules (10). A

standard reaction contained 10,000 cpm (13 fmol) of 32 P-labeled precursor RNA, 15 μ l of nuclear extract in buffer D (20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.9], 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol), 12 U of RNasin (Promega Biotec), and 5 μ g of *Escherichia coli* tRNA in a final volume of 30 μ l. The processing reaction was allowed to proceed at 30°C for 2 h, after which it was stopped by addition of an equal volume of proteinase K (1 mg/ml)–1% sodium dodecyl sulfate (SDS)–300 mM NaCl–20 mM EDTA–20 mM Tris (pH 8) and incubated at 37°C for 20 min. The sample was then extracted with phenol-chloroform, ethanol precipitated, dissolved in 80% formamide–TBE (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA) (16) and resolved by electrophoresis through a 9.7% acrylamide–8.3 M urea–TBE gel. The labeled RNA was then detected by autoradiography.

Preparation of oligonucleotides. DNA oligonucleotides were synthesized by using phosphoramidite chemistry. After deprotection, the oligonucleotides were purified by gel electrophoresis (to isolate the full-length molecules) on acrylamide gels containing 8.3 M urea and 1 $\times$ TBE, followed by reverse-phase chromatography on a Millipore Sep-Pak C-18 column. Final DNA concentrations were determined by spectrophotometry.

RNA oligonucleotides were prepared by transcription of linearized DNA templates with either T7 polymerase (kindly supplied by Martin Nicklin) or SP6 polymerase (Boehringer Mannheim) with m⁷GpppG present at a 10-fold molar excess over GTP. After transcription, the material was treated with proteinase K–SDS, extracted with phenol-chloroform, ethanol precipitated, and resolved on an 8.3 M urea–TBE–acrylamide gel. The transcript was visualized by UV shadowing, excised, and eluted into HEB (0.75 M ammonium acetate, 10 mM magnesium acetate, 1% [vol/vol] phenol, 0.1% [wt/vol] SDS, 0.1 mM EDTA). The eluted RNA was extracted twice with phenol-chloroform and once with chloroform and precipitated with ethanol. The final material was dissolved in water, and the concentration was determined by spectrophotometry.

RNA ribozymes were prepared in a similar manner. DNA oligonucleotides encoding ribozyme sequences were cloned as complementary synthetic DNA oligonucleotides into pSPT19 (Boehringer Mannheim) linearized with *Hind*III–*Sa*II, and T7 polymerase transcriptions were performed with plasmid DNA molecules linearized with *Sma*I. All plasmid constructions were sequenced by standard dideoxy sequencing methods to determine whether they possessed the correct sequence insertion.

RNase protection mapping. Samples were treated with proteinase K (1 mg/ml)–0.5% SDS–150 mM NaCl–10 mM EDTA–20 mM Tris (pH 8) at 56°C for 20 min, phenol-chloroform extracted, and ethanol precipitated in the presence of *E. coli* tRNA. Labeled RNA (normally, 20,000 cpm/25 fmol of antisense U7 RNA per 5 μ l of nuclear extract containing 1.5 fmol of endogenous U7 RNA) was added to the dried ethanol precipitate, and the sample was again dried and dissolved in 10 μ l of 80% deionized formamide–400 mM NaCl–20 mM PIPES (piperazine-N,N'-bis(2-ethanesulfonic acid; pH 6.5)–10 mM EDTA. Samples were overlaid with sterile paraffin oil, heated to 95°C for 3 min, and quickly transferred to a 45°C water bath for overnight incubation. In the morning, 0.3 ml of ice-cold 300 mM NaCl–30 mM Tris (pH 7.5)–10 mM EDTA–0.05 mg of RNase A per ml–80 U of RNase T1 per ml was added in a speedy motion, and the samples were incubated at room temperature for 45 min. Proteinase K and SDS were added to 1 mg/ml and 0.5%,

respectively, and incubation was continued at room temperature for 20 min, followed by 20 min at 56°C. The sample was extracted once with phenol-chloroform and precipitated with ethanol after addition of 10 µg of tRNA. The collected ethanol precipitate was dissolved in 80% formamide-TBE and resolved on a preheated 9.7% acrylamide-8.3 M urea-TBE gel.

RESULTS

Exposure of the 5' end of U7 and requirement of this region of U7 for the *in vitro* 3' processing reaction. When RNA molecules containing the 3' processing signal from a mouse H4 sequence are incubated in a nuclear extract in 20 mM EDTA, specific cleavage of the RNA occurs. The processed mRNA molecule has been shown to possess the same 3' terminus as mature histone mRNA isolated from *in vivo* sources (10). This RNA-processing assay, which is dependent on snRNP U7 (5, 18, 20), forms the basis for our studies on antisense inhibition of U7 activity. For examples of the processing substrate RNA and the cleavage product produced after incubation in a nuclear extract, see Fig. 3A, lanes 1 and 2.

The sea urchin U7 snRNP possesses a micrococcal nuclease-susceptible 5' RNA sequence (12) consistent with the necessity for this region to base pair with the precursor RNA during the processing reaction (21). It has been demonstrated that the U7-dependent mammalian histone-processing reaction is sensitive to micrococcal nuclease digestion (11, 18, 20). The region of mouse U7 RNA which is both essential for the processing reaction and susceptible to nuclease digestion was determined by treating processing-active nuclear extract for various times with micrococcal nuclease in the presence of calcium (Fig. 2). The nuclease was then inactivated by ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) chelation of the calcium. Each sample was divided in half. One portion was tested for pre-mRNA-processing activity; the RNA from a second portion was harvested and subjected to RNase protection analysis using an anti-U7 RNA probe.

Micrococcal nuclease treatment of the nuclear extract produced a gradual decline in processing activity leading to nearly complete cessation of processing after 120 min of digestion (Fig. 2A, lanes 3 to 7). When the state of U7 RNA was analyzed by RNase protection mapping, we found that coincident with the decline in processing activity was a shortening of the U7 RNA, removing initially 11 nt (Fig. 2B, lanes 3 and 4) and then an additional 10 nt (Fig. 2B, lanes 5 to 7). That the trimming of the U7 RNA molecule was from the 5' end could be determined from the digestion pattern. The two nucleases used in RNase protection mapping, RNase A and RNase T1, were unable to remove an A residue from the probe RNA; therefore, any U in the U7 RNA sequence produced a gap in the digestion pattern. The observed gaps in the pattern correspond only to the positions of U residues at the 5' end of U7 RNA.

The 5' end of mouse U7 snRNP, similar to that of sea urchin U7 snRNP (12), is therefore susceptible to micrococcal nuclease digestion. However, in contrast to the sea urchin snRNP, which exposes only the first 7 or 8 nt (12), the first 21 nt of mouse U7 snRNP are susceptible to removal by micrococcal nuclease. Note that these nucleotides include the sequences which bear complementarities to the histone pre-mRNA and most likely must be available for base pairing with this RNA.

Removal of the first 21 nt by treatment with a U7-complementary DNA oligonucleotide inhibits the processing reaction.

FIG. 2. Susceptibility of the 5' end of U7 snRNP to micrococcal nuclease digestion. Nuclear extract samples were incubated at 30°C with micrococcal nuclease (15 U/120 µl of nuclear extract) in 1 mM calcium. At the indicated times, samples were removed, EGTA was added to 4 mM, and the sample was divided into two portions; one portion was assayed for histone precursor processing in 20 mM EDTA (A). The second portion was immediately treated with proteinase K and SDS at 56°C, and the resulting RNA was subjected to RNase mapping with a labeled antisense U7 RNA probe (B). co, Material from a control processing reaction; ca, material from a sample incubated with calcium and no micrococcal nuclease. The time of micrococcal nuclease digestion is indicated at the top of each lane. M, molecular weight marker (pBR322 DNA digested with *Hpa*II and labeled with Klenow enzyme in the presence of [³²P]CTP); the sizes (in nucleotides) of some of the fragments are indicated to the right of each panel. The precursor (74 nt) and processed (42 nt) species of the histone mRNA substrate are indicated at the left of panel A. The RNA species protected by intact and truncated U7 RNAs are indicated at the left of panel B.

DNA oligonucleotides complementary to the 5' portion of mouse (5, 20) or human (18) U7 RNA can inhibit the *in vitro* processing reaction. An 18-nt DNA molecule complementary to nucleotides 4 to 21 inhibited the processing reaction completely when present at a 6,000-fold molar excess over U7 (Fig. 3A, lane 6) and greater than 90% when present at a 600-fold molar excess over U7 (Fig. 3A, lane 5). At a 10-fold lower concentration, this oligonucleotide had little effect (lane 4). Preincubation with magnesium alone (lane 2) or with magnesium and a 6,00-fold excess of a control 18-nt DNA molecule with no complementarities to U7 RNA (nonsense, lane 7) had no effect on the processing reaction. The nuclear extracts used in this study contained sufficient endogenous RNase H activity; addition of bacterial RNase H did not lead to additional inhibition at any oligonucleotide concentration (data not shown).

When aliquots of the oligonucleotide-treated extracts were subjected to RNase protection mapping, it was apparent that coincident with the inhibition of processing activity was the removal of the 5' portion of U7 (Fig. 3B, lanes 5 and 6). Molecules with both an 11-nt resection and the expected 21-nt resection were observed. Incubation of the nuclear extract with magnesium alone (lane 3) or with magnesium and a nonsense 18-mer (lane 7) had no detectable effect on the U7 molecule. Therefore, consistent with the micrococcal nuclease digestion results (Fig. 2B), the 5' portion of U7 RNA is accessible to oligonucleotide-mediated RNase H cleavage.

Better inhibition of the processing reaction by a full-length anti-U7 DNA oligonucleotide than by the 18-nt molecule. Single-stranded DNA molecules with the sense or antisense (complementary) U7 sequence (Fig. 1B) were tested for inhibitory activity in the pre-mRNA processing reaction. The 65-mer sense DNA had no effect on the processing reaction with up to 30 pmol added (a 6,000-fold molar excess

FIG. 3. Blockage of U7-dependent activity by a U7-complementary oligonucleotide. Samples of nuclear extract were incubated in 5 mM magnesium chloride with the indicated quantities of an 18-nt nucleotide DNA molecule complementary to nt 4 to 21 of the U7 sequence (18-mer) or a nonsense 18-nt DNA (NS). Samples were incubated for 30 min on ice, 30 min at room temperature, and then 30 minutes at 30°C. A portion of each sample was removed for RNase protection mapping; to the remainder was added the radioactively labeled histone-processing substrate in 20 mM EDTA at 30°C for 2 h. (A) Histone pre-mRNA-processing activity. Lanes: m, molecular weight markers as described in the legend to Fig. 2; 1, untreated pre-mRNA; 2, control processing reaction; 3, processing reaction with a nuclear extract sample preincubated with magnesium and no oligonucleotide; 4 to 6, processing reactions with samples treated with the indicated quantities of an 18-mer oligonucleotide complementary to U7; 7, sample treated with a nonsense 18-mer. (B) RNase mapping of U7 RNA. Lanes: m, molecular weight markers as described in the legend to Fig. 2; p, RNase-mapping probe (antisense U7 RNA); t, probe hybridized with tRNA and subjected to RNase digestion; 2 to 7, counterparts of lanes 2 to 7 in panel A.

over endogenous U7; Fig. 4, lanes 6 to 9). However, the 65-mer antisense DNA molecule completely inhibited the reaction when 30 or 3 pmol was added to the reaction (Fig. 4, lanes 13 and 12, respectively) and produced approximately 90% inhibition when 300 fmol was added (Fig. 4, lane 11). No inhibition occurred with this molecule when 30 fmol was added to the reaction (lane 10). The long (65-mer) antisense DNA molecule inhibited at a 10-fold lower con-

FIG. 4. Comparison of DNA and RNA as inhibitors of U7 activity. Lanes 3 to 21 contained nuclear extract samples preincubated with 5 mM magnesium chloride and the indicated quantities of nucleic acid inhibitors as described in the legend to Fig. 3. At the end of the preincubation period, EDTA was added to 20 mM and histone pre-mRNA-processing activity was assayed. The amount of nuclear extract used in each sample corresponded to ca. 5 fmol of U7 RNA. Lanes: m, molecular weight markers as described in the legend to Fig. 2; 1, untreated processing substrate; 2 and 22, control processing reactions.

centration than that observed with the short (18-mer) DNA molecule. When RNase protection mapping was performed on extracts exposed to antisense DNA, we found that relative to treatment with the 18-mer DNA molecules, the same 5' nucleotides of U7 RNA were removed (data not shown). This suggests that the U7 RNA sequence that can hybridize with the short DNA molecule and be removed by RNase H activity is essentially the entirety of available U7 RNA. The remainder of the U7 RNA is most likely complexed with snRNP proteins and unavailable for RNase H cleavage (see Discussion).

Approximately 10-fold greater effectiveness of antisense RNA than DNA as an inhibitor of the processing reaction. Antisense U7 RNA molecules generated by *in vitro* SP6 or T7 transcription could also be used to inhibit the *in vitro* processing reaction. A nuclear extract sample was preincubated with various amounts of an RNA molecule containing the antisense U7 sequence (Fig. 4, lanes 18 to 21). Complete inhibition of the processing reaction was obtained with 3 pmol of antisense U7 RNA (Fig. 4, lane 21), and greater than 90% inhibition was obtained with both 300 and 30 fmol of antisense U7 RNA (a 60- or 6-fold molar excess over endogenous U7). The presence of 3 fmol of antisense U7 RNA, a slightly less than equal molar quantity than the 5 fmol of U7 in this extract sample, had little effect on the reaction (lane 18). Surprisingly, the U7 RNA sequence itself had little or no effect on the processing activity, with up to 3 pmol added (Fig. 4, lanes 14 to 17). Comparing the antisense DNA with the antisense RNA mode of inhibition, we found that the short DNA molecule inhibited the processing reaction >90% at a 600-fold excess over the U7 target, a full-length antisense DNA inhibited the reaction to the same extent at a 60-fold excess, and the full-length antisense RNA inhibited the reaction to the same extent at a 6-fold excess.

Irreversibility of DNA inhibition and reversibility of antisense RNA inhibition in nuclear extracts from EBI mouse cells. When setting out to inhibit the processing reaction *in vivo*, it may be important to determine whether the antisense inhibition is reversible, especially when the inhibition relies on a nucleic acid-RNA interaction that, in effect, competes with the RNA-RNA interaction of the histone pre-mRNA-U7 RNA contacts. Certainly, the RNase H cleavage of RNA

base paired with DNA produced irreversible inhibition of the U7-dependent reaction. However, we were interested in determining whether the antisense RNA inhibition occurs only by hybrid formation and should therefore be reversible as the hybrid dissociates. Alternately, it is possible that a double-strand-specific enzymatic activity (1) might modify the U7 sequences complexed with antisense RNA, or formation of a double-stranded RNA molecule might displace essential U7 snRNPs, making the antisense RNA inhibition irreversible. To test this idea, we took advantage of the observation that large excesses of sense U7 RNA or DNA could be added to the processing extract without interfering with the processing reaction (Fig. 4, lanes 9 and 17). Therefore, we could preincubate a nuclear extract with inhibiting concentrations of antisense DNA or RNA and then complex the inhibitor with large excesses of the inhibitor's complement before testing the processing activity by addition of the processing substrate and EDTA. If the inhibition were simply due to hybrid formation, then the initial inhibition would be relieved by subsequent addition of the complement to the inhibitor. However, if the inhibition were due to some degradation or alteration in the U7 snRNP, then complexing the antisense molecule after preincubation should not restore processing activity.

We found that 90% of the inhibition by antisense DNA was irreversible. A nuclear extract was incubated with a quantity of antisense U7 DNA sufficient to inhibit the reaction (3 pmol; Fig. 5, lane 3). When antisense U7 DNA was first allowed to hybridize with a 10-fold excess of sense U7 DNA before addition to the nuclear extract, no inhibition occurred (Fig. 5; lane 7). However, when the nuclear extract was first incubated with the inhibiting DNA oligonucleotide in the presence of magnesium and later the complement of the inhibitor was added at a 3-fold (lane 4), 10-fold (lane 5), or 33-fold excess (lane 6), we found that only a fraction (less than 10%) of the processing activity was restored. When the sense U7 oligonucleotide was added by itself in similar quantities, it had no effect on the processing reaction (Fig. 5, lanes 8 and 9), whereas incubation with antisense U7 DNA oligonucleotides led to truncation of U7 RNA (Fig. 3B, lanes 5 and 6).

Inhibition of the reaction by antisense U7 RNA (Fig. 5, lane 10) could be completely blocked by incubating the antisense U7 RNA with a 10-fold excess of sense U7 RNA before exposure to the nuclear extract (lane 12). However, in contrast to the DNA inhibitor, inhibition by antisense U7 RNA could be reversed by adding excess U7 RNA after 30 min of preincubation of antisense U7 RNA with the processing extract (lane 11). The presence of 30 pmol of U7 RNA had no effect on the processing reaction (Fig. 5, lane 13). Inhibition of U7 snRNP activity by antisense RNA, therefore, is unlikely to involve any cleavage or irreversible disruption of the target snRNP and probably occurs by hybrid arrest.

Ability of U7-targeted ribozymes to cleave U7 RNA and inhibit the processing reaction under mild physiological conditions. Recently, techniques have been developed which allow generation of RNA molecules capable of selectively cleaving target RNA molecules (13). These RNA enzymes or ribozymes contain a portion of the catalytic RNA sequence of the satellite RNA of tobacco ringspot virus flanked by a pair of sequences complementary to the target sequence (Fig. 1). Using the strategy of Haseloff and Gerlach (13), we designed ribozymes targeted to either the GUC ending at position 29 or the CUC ending at position 15 in the U7 sequence.

FIG. 5. Irreversibility of U7 snRNP inhibition by DNA oligonucleotides and reversibility of inhibition with RNA. Nuclear extracts were preincubated with 3 pmol of antisense U7 DNA (lanes 3 to 6) in 5 mM magnesium chloride for 30 min at 30°C. The complement to the inhibitor was then added in a 3-fold (lane 4), 10-fold (lane 5), or 33-fold molar excess (lane 6). The inhibitor and complement were then allowed to anneal for 30 min before addition of EDTA (to 20 mM) and the processing substrate. That 30 min of incubation was sufficient to hybridize all of the inhibitor is demonstrated by the samples in lane 7, in which 3 pmol of antisense U7 DNA and 30 pmol of its complement were incubated at the same ionic strength and temperature before their addition to the nuclear extract for 30 min of incubation. In lanes 8 and 9, nuclear extracts were preincubated with 30 or 100 pmol of sense U7 DNA. Similarly, nuclear extracts were treated with 3 pmol of antisense U7 RNA (lanes 10 and 11). After 30 min of preincubation, 30 pmol of U7 RNA was added to the sample (lane 11). In lane 12, 3 pmol of antisense U7 RNA and 30 pmol of U7 RNA were prehybridized for 30 min before addition to the nuclear extract. In lane 13, the nuclear extract was preincubated with 30 pmol of sense U7 RNA. In all samples, after the initial preincubation with various test inhibitors, EDTA was added to 20 mM and the processing reaction proceeded for 2 h at 30°C; the samples were then treated as described above and resolved by electrophoresis and autoradiography. Lanes: m, molecular weight markers as described in the legend to Fig. 2; 1, untreated substrate RNA; 2 and 14, control processing reactions.

When the GUC-targeted ribozyme was tested for cleavage activity against naked U7 RNA, we found that the ribozyme was capable of cleaving the target RNA at the predicted site at 37°C in the presence of 5 mM magnesium (Fig. 6) and complete cleavage of 10 fmol of U7 RNA occurred with quantities of ribozyme down to 15 fmol (Fig. 6, lane 6). Little cleavage of the target occurred under these conditions with less than 15 fmol of the ribozyme (lanes 7 to 9). When U7 RNA was incubated with magnesium without ribozyme, no cleavage was observed (Fig. 6, lane 2) nor was cleavage by ribozyme observed when 10 mM EDTA was substituted for the magnesium (data not shown). All reported uses of this type of targeted ribozyme have relied on a higher temperature (50°C) and a higher magnesium concentration (20 mM), either of which conditions interferes nonspecifically with the *in vitro* processing event. Because we would like to use these cleavage devices within mammalian cells, it is useful that significant activity can be obtained under these physiological conditions.

When the GUC-targeted ribozyme was tested for cleavage activity against U7 in its native, protein-ensconced form, we found that the U7 snRNP was completely protected against ribozyme activity (data not shown). Neither inhibition of the processing reaction nor cleavage of U7 RNA could be detected with quantities of ribozyme up to 15 pmol added to

FIG. 6. Cleavage of U7 RNA at a specific site with a U7-targeted ribozyme. Samples of labeled U7 RNA (lanes 1 and 10; 10 fmol, 10,000 cpm) were incubated at 37°C for 2 h with 5 mM magnesium–100 mM NaCl–20 mM Tris (pH 8.0) alone (lane 2) or with the indicated quantities of ribozyme GUC (lanes 3 to 9). After incubation, EDTA was added to 10 mM and the samples were dried, dissolved in 80% formamide–TBE, and resolved by electrophoresis on a prewarmed 9.7% acrylamide–8.3 M urea–TBE gel. Lane m, Molecular weight markers as described in the legend to Fig. 2. Intact U7 RNA, the 3' cleavage product, and the 5' cleavage product are indicated at the left.

the reaction (a 300-fold excess over U7 snRNP; data not shown). One might have predicted this outcome on the basis of our knowledge of the sea urchin U7 snRNP structure, in which the sequence motif containing the GUC forms the center of an Sm protein-binding site (12). The GUC in mouse U7 is most likely covered with protein and inaccessible to ribozyme cleavage.

A number of trinucleotides other than GUC can function as ribozyme cleavage sites (14). A second ribozyme was designed to cleave at the CUC sequence 15 nt from the 5' end of U7. This sequence is accessible to digestion by micrococcal nuclease (Fig. 2B) and is available for hybridization to complementary DNA oligonucleotides (Fig. 3 to 5). The complementarities between the ribozyme and the substrate were increased to 11 or 12 nt on either side of the cleavage site (note that the GUC-targeted ribozyme contains eight possible base pairs on either side of the cleavage site). When this ribozyme was tested for cleavage capacity against naked U7 RNA, we found that like the GUC-targeted ribozyme, this ribozyme cleaved U7 RNA at the predicted site (Fig. 7). We describe here a set of reactions at a much higher substrate concentration with a 10-fold substrate excess over the ribozyme. The ribozyme functioned with 20 mM Mg^{2+} (Fig. 7, lanes 4, 5, 10, and 11) or 2 mM Mg^{2+} (lanes 3 and 9). No cleavage was detected in 0.2 mM Mg^{2+} (lanes 2 and 8) or in the presence of 10 mM EDTA (lanes 6 and 12). We found a slight enhancement of cleavage activity at 45°C relative to 37°C at the higher Mg^{2+} concentration (Fig. 7, compare lanes 4 and 5 with lanes 10 and 11). Activity similar to that at 37°C could be observed at 30°C, whereas there was a sharp drop in activity above 45°C (data not shown). Approximately five substrate molecules were

FIG. 7. Ribozyme cleavage at a more accessible U7 site. Labeled U7 RNA was diluted to 10,000 cpm/pmol of RNA with unlabeled U7 RNA. Incubations with CUC-targeted ribozyme were performed in 100 mM NaCl–20 mM Tris (pH 7.5) at either 37 or 45°C with 0.2, 2, or 20 mM magnesium chloride or 1 or 10 mM EDTA as indicated. After 3 h of incubation, EDTA was added to 40 mM and the samples were dried, dissolved in 80% formamide–TBE, and resolved by electrophoresis as described in the legend to Fig. 6. Lanes: m, molecular weight markers as described in the legend to Fig. 2; 1, untreated U7 RNA; 7 and 13, U7 RNA incubated with 20 mM Mg^{2+} and no ribozyme at 37 or 45°C; 2 to 6, U7 RNA (1 pmol) incubated with 10 fmol of ribozyme CUC at 37°C with the indicated Mg^{2+} and EDTA concentrations (millimolar); 8 to 12, the same as lanes 2 to 6, except that incubation was at 45°C. Intact U7 RNA and the expected 5' and 3' cleavage products are indicated at the right.

cleaved per ribozyme molecule in a 3-h reaction at 37 and 45°C when this substrate concentration (0.17 μ M) was used.

The CUC ribozyme was tested for U7-inhibitory activity. Nuclear extract samples were treated with the CUC-targeted ribozyme in 5 mM magnesium at 30°C. The samples were divided into two portions; one portion was tested for processing activity (Fig. 8A), and the second portion was subjected to RNase mapping to assay the state of U7 RNA (Fig. 8B). Exposure of the nuclear extract to 100 (lane 6) or 10 (lane 7) fmol of ribozyme had no greater inhibitory effect on the processing than did incubation with Mg^{2+} alone (lane 3). However, we observed partial inhibition of processing activity after preincubation with 1 pmol of the ribozyme (lane 5) and complete inhibition of the processing reaction when an extract was treated with 9 pmol of ribozyme (Fig. 8A, lane 4). Coincident with this inhibition, cleavage of U7 RNA occurred, truncating the U7 molecule by the predicted 15 nt (Fig. 8B, lanes 4 and 5), and cleavage of U7 RNA was observed only in samples which displayed impaired histone-processing activity (lanes 4 and 5). Interpretation of the ribozyme-mediated inhibition is complicated by the observation that complete inhibition of the processing reaction occurred although uncleaved U7 RNA remained in the extract. It is possible that only a portion of the U7 snRNP in our extracts was active in the processing reaction and this

FIG. 8. Inhibition of the histone pre-mRNA processing reaction by a U7 RNA-specific ribozyme. Nuclear extract samples (15 μ l, corresponding to ca. 5 fmol of U7) were incubated with 5 mM magnesium (lanes 3) or magnesium plus the indicated quantity of ribozyme CUC for 2 h at room temperature in 20 mM HEPES (pH 7.9)–20% glycerol–100 mM KCl–0.2 mM EDTA–0.5 mM dithiothreitol. EDTA was then added to 20 mM, and the samples were split into two portions. One portion was assayed for histone pre-mRNA processing (A), and the second portion was subjected to RNase mapping to determine the state of U7 RNA (B). Lanes: m, molecular weight markers as described in the legend to Fig. 2. Lanes in panel A: 1, processing substrate; 2, control processing reaction; 3, processing reaction after preincubation with magnesium; 4 to 7, processing reaction after incubation with magnesium and the indicated quantities of ribozyme CUC. Lanes in panel B: 1, RNase protection probe (antisense U7 RNA); 2 to 7, RNase protection analysis of the counterparts to samples 2 to 7 in panel A.

same portion was susceptible to ribozyme attack. Therefore, ribozyme cleavage of one-third of the U7 population led to complete inhibition of processing. It is also possible that inhibition of processing occurred by both cleavage of U7 RNA and hybrid formation between the ribozyme and U7 RNA without U7 RNA cleavage. However, when samples of ribozyme-inhibited nuclear extracts were incubated with a DNA oligonucleotide complementary to the ribozyme in sufficient excess to bind and elicit RNase H destruction of the ribozyme, less than 20% of the processing activity was

restored (data not shown), similar to the irreversible inhibition observed with the antisense DNA molecules (Fig. 5, lanes 3 to 5). This suggests that most of the observed inhibition of processing was due to an irreversible event, most likely ribozyme cleavage of U7 RNA.

DISCUSSION

We undertook a comparative analysis of antisense DNA and RNA and ribozyme inhibitors of the U7 snRNP-mediated histone pre-mRNA processing event. We report here that a 65-nt antisense DNA molecule was 10-fold more effective than a short 18-nt DNA molecule. This, of course, would easily have been predicted on the basis of an expected increase in hybrid stability for the longer molecule. However, the structure of the U7 snRNP particle probably limits either RNase H degradation or hybridization of complementary nucleic acids to 20 to 22 nt at the 5' end of U7. Similar quantities of U7 RNA were removed by RNase H activity after treatment with either the long or the short DNA molecule. The inhibitory activity may be enhanced if one supposes a certain amount of exonuclease activity in the extract. The longer oligonucleotide possesses extra, nonessential nucleotides flanking the essential hybrid-forming sequences; these may serve as a nuclease buffer to increase the half-life of the effective portion of the oligonucleotide. Indeed, when the lifetimes of radiolabeled inhibitors were measured in the presence of magnesium in this nuclear extract, we found that all three types of inhibitors were rapidly degraded, with half-lives in the range of 10 to 20 min for the RNA molecules and less than 10 min for the single-stranded DNA oligonucleotides (data not shown). A small fraction of antisense RNA was stable in the extract for long periods; presumably this was antisense RNA hybridized to U7 RNA, and this may have been responsible for the greater inhibitory activity of this molecule. Certainly, degradation appeared to be the major limit to inhibitor function, with the most effective inhibitor (antisense RNA) having the greatest longevity in the extract.

We found that antisense RNA inhibited U7 snRNP activity with an efficiency (on a molar basis) 10-fold greater than that of a similar antisense DNA molecule. The greater inhibitory capacity of the U7 RNA may simply be due to the greater stability of an RNA-RNA hybrid than a DNA-RNA hybrid. Furthermore, a stable RNA-RNA duplex may be formed which remains inhibitory to the processing reaction in the presence of EDTA. However, it is possible that certain U7 snRNPs enhance interaction with antisense U7 RNA and not with antisense U7 DNA; antisense RNA may bear a greater semblance to histone pre-mRNA than does antisense DNA.

The antisense DNA approach has the advantage of inhibiting the processing reaction in an irreversible manner, whereas the antisense RNA molecule inhibits reversibly. The reversibility of the inhibition means that antisense RNA essentially acts as an uncleavable competitor with histone pre-mRNA. Although we can effectively compete with a single pre-mRNA precursor in an *in vitro* system, inhibition within the nucleus may be more complex: antisense U7 RNA must compete with the pre-mRNAs produced by approximately 40 replication variant genes (30) active during the S phase. In light of this, an inhibitor which can catalytically inactivate U7 RNA during periods of the cell cycle lacking large quantities of histone precursor may prove to be a superior *in vivo* inhibitor.

The inhibition results described in this report allow us to draw some conclusions about the structure of the mamma-

lian U7 snRNP. The 5' end of the snRNP (which should be available for interaction with pre-mRNA) is accessible to micrococcal nuclease attack (Fig. 2B; see also reference 20) and RNase H digestion after hybridization to complementary DNA molecules. Exposure of the small nuclear RNA is limited to the first 20 nt; with both methods, no more than this is removed. Furthermore, RNase mapping of U7 after exposure of a nuclear extract to the full-length antisense DNA revealed the same truncation pattern (data not shown). Either some component of the snRNP structure limits RNase H cleavage to the same U7 RNA sequences or the snRNP structure limits hybridization of both the long and short oligonucleotides to the same RNA sequences. The RNA sequence surrounding the GUC at position 28 (the target of the GUC ribozyme) is also protected in some manner. Since this sequence is amid the signal identified as the Sm-binding site in sea urchin U7 (12) and the mouse U7 snRNP is immunoprecipitable by Sm antiserum (10), it is most likely that similar proteins are bound to mouse U7.

The performance of the ribozyme as an inhibitor was disappointing. In principal, the increased stability of an RNA-RNA duplex combined with the potential catalytic activity of the ribozyme should have produced a potent eradicator of U7 RNA. We did not see even the static antisense RNA inhibition that one might expect from this compound. It is possible that the presence of the viroid sequence between the two hybridization arms of the ribozyme could have a major disruptive effect on stability, making the essentially 23-base-pair hybrid less stable than two adjacent 11- and 12-base-pair hybrids. Two explanations for the poor ribozyme performance can be formulated. (i) Magnesium-activated nucleases are abundant in the nuclear extracts used here (O. Gick et al., manuscript in preparation). Simple bioerosion could account for the limited activity of the ribozyme. Indeed, when the stability of ribozyme activity was tested under the conditions used to inhibit U7 RNA activity, we found that 50% of the ribozyme activity disappeared in the first 15 min, with 90% of the activity gone after 2 h of incubation. A modified ribozyme with either additional secondary structure or altered chemical composition might be used to increase the stability of the inhibitor. (ii) It is also possible that the U7 snRNP is a particularly difficult RNA substrate for the ribozyme to cleave. Certainly, the U7 snRNP requires *in vivo* stability and most of the U7 RNA is protected from nuclease activity (Fig. 2), most likely by bound protein molecules. These bound proteins may affect the accessibility of the substrate to ribozyme attack. As our experience with ribozymes increases, we may find that other RNA targets, including mRNA sequences, are more accessible to ribozyme cleavage. We are presently testing ribozymes *in vivo* against both U7 snRNP and possibly more accessible mRNA targets. The ability to cleave a snRNP and inhibit a snRNP-mediated processing reaction gives us a certain optimism that ribozyme inhibitors, with the appropriate modifications, might be useful molecular biology tools.

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