In vitro suicide inhibition of self-splicing of a group I intron from *Pneumocystis carinii* by an $N3' \rightarrow P5'$ phosphoramidate hexanucleotide

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ABSTRACT Binding enhancement by tertiary interactions is a strategy that takes advantage of the higher order folding of functionally important RNAs to bind short nucleic acid-based compounds tightly and more specifically than possible by simple base pairing. For example, tertiary interactions enhance binding of specific hexamers to a group I intron ribozyme from the opportunistic pathogen *Pneumocystis carinii* by 1,000- to 100,000-fold relative to binding by only base pairing. One such hexamer, d(AnTnGnAnCn)rU, contains an $N3' \rightarrow P5'$ phosphoramidate deoxyribose–phosphate backbone (n) that is resistant to chemical and enzymatic decay. Here, it is shown that this hexamer is also a suicide inhibitor of the intron’s self-splicing reaction *in vitro*. The hexamer is ligated in trans to the 3' exon of the precursor, producing dead-end products. At 4 mM Mg$^{2+}$, the fraction of trans-spliced product is greater than normally spliced product at hexamer concentrations as low as 200 nM. This provides an additional level of specificity for compounds that can exploit the catalytic potential of complexes with RNA targets.

Most human therapeutics have been discovered by screening natural products. Synthetic organic chemistry has made it possible to synthesize such natural products and derivatives thereof in large quantities, thus broadening the range of compounds that can be used clinically (1–3). Synthetic methodology coupled with the outpouring of protein structural information has also allowed rational design of completely new therapeutic compounds (4, 5). Similarly, the recent explosion in nucleic acid sequence information is providing a knowledge base for structure-based targeting of RNA. The first generation of such therapeutics consists of antisense nucleic acids that bind mRNA through Watson-Crick base pairing and thereby regulate translation (6, 7). Because of the long sequences employed, typically 15–20 nucleotides, potential disadvantages include high cost of synthesis (8) and lack of specificity (9). Cost of synthesis can be reduced and specificity increased by designing short antisense agents whose binding to RNA targets is enhanced by tertiary interactions (10, 11). Here we show that one such hexanucleotide can also be a suicide inhibitor (12, 13) of RNA function. This provides an additional design principle for increasing specificity of compounds that can exploit the catalytic potential of complexes with RNA targets.

Our model system is a large-subunit ribosomal RNA (rRNA) precursor from the opportunistic pathogen *Pneumocystis carinii*, which is a common cause of death in immunocompromised patients (14, 15). The rRNA precursor contains a group I self-splicing intron (10, 16) that provides a potential therapeutic target (16, 17) because self-splicing is required for assembly of active ribosomes (18). Hexamers that mimic this intron’s 5' exon can bind to the catalytic core of a ribozyme derived from the intron as much as 100,000-fold more tightly than expected if the hexamers bound by simple base pairing (10). Much of this binding enhancement by tertiary interactions (BETI) can be retained when these hexamers are modified to be resistant to nucleic degradation (11). In particular, d(AnTnGnAnCn)rU with $N3' \rightarrow P5'$ phosphoramidate (19) linkages (Fig. 1) binds 2,000-fold more tightly than expected for base pairing.

In this report, we show that d(AnTnGnAnCn)rU is ligated to the 3' exon of a truncated ribosomal RNA precursor in a reaction that mimics the second step of splicing (Fig. 2). At Mg$^{2+}$ concentrations lower than that required for optimal self-splicing *in vitro*, this trans-splicing can compete with the natural cis-splicing. Such trans-splicing produces dead-end products that would not lead to functional RNA. Thus, an oligonucleotide that is resistant to chemical and nucleic degradation can act as a suicide inhibitor of an RNA-catalyzed reaction *in vitro*. This suggests a strategy for enhancing specificity in targeting some RNAs.

MATERIALS AND METHODS

DNA and RNA Synthesis and Purification. The truncated *P. carinii* rRNA precursor (P-h), the derived ribozyme (P-8/4x), and hexanucleotides were synthesized and purified essentially as described (10, 20). Hexamers were 5' end radiolabeled, and the P-h RNA precursor was internally radiolabeled as described (10).

The P-h RNA precursor was 3'-end radiolabeled by incubating 1 mM $[^{5'}S\cdot^{32}P]pCp$, 440 nM P-h RNA transcript, 10 mM MgCl$_2$, 5 $\mu$M ATP, 3 mM DTT, 250 ng of BSA, 50 mM Heps (pH 8.3), and 30 units of T4 RNA ligase in a total volume of 25 $\mu$l for 5 h at 37°C. The reaction mixture was passed through a Chromaspin G100 size-exclusion spin column (CLON-TECH) to remove unincorporated $[^{5'}S\cdot^{32}P]pCp$, and then added to 12.5 $\mu$l of 2× stop buffer (10 M urea, 3.1 mM EDTA, 10 mM Tris, and 9 mM boric acid at pH 8.4) and 2 $\mu$l glycerol. The labeled precursor was purified on a 5% polyacrylamide, 8M urea denaturing gel. The precursor band was excised from the gel and eluted by pulverizing at room temperature overnight in 1 ml sterile water with a sterile stir bar (the spin-soak procedure). The resultant solution was spin-filtered (Isolab) to remove gel particulate and ethanol precipitated twice to remove residual salts and urea.

Inhibition of Self-Splicing. Reactions were conducted in H$_2$Mg buffer consisting of 50 mM Heps (25 mM Na$^+$), 135 mM KCl, and x mM MgCl$_2$ at pH 7.5, where x refers to the amount of MgCl$_2$ in mM in the buffer (listed in the figures). For splicing reactions conducted with internally radiolabeled precursor RNA, about 180 mM RNA was annealed by heating at 55°C for 5 min in the appropriate buffer in a volume of 3 $\mu$l and then slow-cooling to 37°C. A 3-$\mu$l solution of buffer at 37°C containing either 2 mM pG and/or 60 $\mu$M d(AnTnGnAn-...
buffers (10). In these assays, 6.56 H5Mg, H4Mg, and H3Mg as the binding and electrophoresis determined by direct band-shift PAGE assays by using H15Mg, the reaction was quenched by the addition of 6 M urea gel. To check sequence specificity, the self-splicing products and reactants were separated on a 5% acrylamide, 8 M urea gel. The gel was dried under vacuum, and the bands were quantified with a Molecular Dynamics PhosphorImager. The intensity of each band was corrected for the number of adenines in each sequence. A final concentration of 1 mM pG was used in these assays because 3 M pG, although resulting in marginally more spliced product (1), also doubles the amount of 5’ exon–intron hydrolysis product (data not shown).

The fate of the hexamers, d(AnTnGnAnCn)rU and d(CnAnTnAn)rU, also was analyzed by using radiolabeled hexamer and unlabeled precursor in the presence and absence of pG cofactor. Approximately 300 nM unlabeled P-h precursor was annealed by heating at 55°C in 3 μl of the appropriate buffer for 5 min and slow-cooling to 37°C. A 3-μl solution of 8 nM 5′-end radiolabeled hexamer was in the same buffer at 37°C was added, and the reaction was allowed to proceed for 1 h. The reaction was quenched by the addition of 6 μl of 2× stop buffer, and the reactants and products were separated on a 10% polyacrylamide, 8 M urea gel. The gel was dried under vacuum, and the bands were quantified with a Molecular Dynamics PhosphorImager.

To directly monitor the cis- and trans-spliced products, the self-splicing reaction was analyzed as a function of d(AnTnGnAnCn)rU concentration by using radiolabeled hexamer and unlabeled precursor in the presence and absence of pG. Approximately 300 nM unlabeled P-h precursor was annealed by heating at 55°C for 5 min and then slowly cooled to 37°C. Approximately 8 nM 32P-radiolabeled 5′ exon mimic in 0.94 μl of the appropriate buffer at 37°C was added, and the solution was allowed to equilibrate for 90 min. The fraction of mimic bound was partitioned from unbound on a 37°C, 10% native polyacrylamide gel, which was made with the same buffer as the binding buffer. The gel was then dried under vacuum, and the bands were quantified with a Molecular Dynamics PhosphorImager. Dissociation constants were calculated as described (10).

RESULTS

Reactivity of Internally Radiolabeled Precursor as a Function of Mg2+ Concentration [Mg2+]. The effects of [Mg2+] on the formation of various products derived from internally radiolabeled precursor RNA in the presence and absence of 30 μM d(AnTnGnAnCn)rU and 1 mM pG are shown in Fig. 3. Fig. 3a shows that in the presence of d(AnTnGnAnCn)rU and pG, the 5′ exon–intron product reaches a maximum at 2 mM Mg2+, where it is 7.5-fold more prevalent than the completely excised intron product. Mg2+ concentrations higher than 3 mM, however, result in a predominance of completely excised intron. The 5′ exon–intron product could arise from either trans-splicing of d(AnTnGnAnCn)rU (Fig. 2) or hydrolysis of the precursor at the intron-3′ exon junction.
Fig. 3. Magnesium dependence of products from internally radio-
labeled precursor. Reactions were run for 1 h in HxMg buffer,
consisting of 50 mM Hepes (25 mM Na^+), in 135 mM KCl, and
x mM MgCl_{2}, where x is listed below the plots and above the
corresponding lanes of the gel. The gel shows a typical reaction with
1 mM pG and 30 μM d(AnTnGnAnCn)rU. Each plot is the average
of two independently run assays, and the error of each point is
typically ±6% of the average value. Circles represent the 5’
exon–intron product generated by either trans-splicing or intron–3’
exon junction hydrolysis. Squares represent the intron products formed
either by splicing or by hydrolysis at both the 5’ exon–intron and
intron–3’ exon junctions. Results are shown in the presence of 1 mM
pG and 30 μM d(AnTnGnAnCn)rU (a), in the absence of added
hexamer (b), in the absence of pG and in the presence of 30 μM
d(AnTnGnAnCn)rU (c), and in the absence of pG and d(AnTnGnAnC-
nCn)rU (d).

Fig. 3b shows results in the presence of 1 mM pG and
absence of d(AnTnGnAnCn)rU. When [Mg^{2+}] ≥ 4 mM, the
fraction of 5’ exon–intron band is the same in the presence
(Fig. 3a) and absence (Fig. 3b) of d(AnTnGnAnCn)rU. Thus,
when [Mg^{2+}] ≥ 4 mM, the 5’ exon–intron band is likely caused
by hydrolysis at the intron–3’ exon junction. At 2 and 3 mM
Mg^{2+}, however, much more 5’exon–intron product is formed
in the presence of d(AnTnGnAnCn)rU, suggesting it results
either from the trans-splicing reaction or from oligonucleotide-
induced hydrolysis at the intron–3’ exon junction. Either
mechanism results in the formation of oligonucleotide-
dependent dead-end products. At 2 mM Mg^{2+}, the fraction of
completely excised intron decreases by a factor of 2.5 on
adding 30 μM d(AnTnGnAnCn)rU (compare Fig. 3a and b),
suggesting that the dead-end products are, at least in part,
being formed at the expense of completely excised intron. Surprisingly, the fraction of completely excised intron is almost
0.6 when [Mg^{2+}] ≥ 5 mM in the presence of 30 μM d(AnTn-
GnAnCn)rU and 1 mM pG, while it is only 0.4 when pG is
present in the absence of d(AnTnGnAnCn)rU. One possible
reason for this is that d(AnTnGnAnCn)rU promotes hydro-
lysis at both the 5’ exon–intron and intron–3’ exon junctions,
thus releasing intron. Fig. 3c shows that in the presence of
d(AnTnGnAnCn)rU and absence of pG, the fraction of
excised intron approaches 0.2 at high [Mg^{2+}]. When added to
the 0.4 fraction generated in the presence of pG and absence of
d(AnTnGnAnCn)rU, this can account for the fraction
observed in the presence of both pG and d(AnTnGnAnCn)rU.

Fig. 3c also shows that in the absence of pG and presence of
30 μM d(AnTnGnAnCn)rU, the 5’exon–intron product max-
imizes at 2 mM Mg^{2+}. Evidently, formation of this product
does not depend on pG. At 3 mM [Mg^{2+}] ≤ 7 mM in the
presence of 1 mM pG and 30 μM d(AnTnGnAnCn)rU,
completely excised intron product is generated at the expense
of the 5’ exon–intron product (Fig. 3a).

Fig. 3d also shows that in the absence of pG and d(AnTnGn-
AnCn)rU, hydrolytic production of the 5’ exon–intron band at
2 mM Mg^{2+} is about 1/10 that in the presence of d(AnTnGn-
AnCn)rU. This is further evidence that the large production of
this band is not the result of simple hydrolysis at the intron–3’
exon splice junction.

The above results indicate that d(AnTnGnAnCn)rU inter-
feres with self-splicing at 2–3 mM Mg^{2+} either by trans-splicing
or by oligonucleotide-induced hydrolysis at the intron–3’
exon junction or both. An increase in the 5’ exon–intron product at
2 mM Mg^{2+} does not occur on adding up to 30 μM of the

Fig. 4. Magnesium dependence of trans-splicing with 4 nM 5’
end-labeled d(AnTnGnAnCn)rU and 150 nM unlabeled precursor in
the presence (Upper) and absence (Lower) of pG. The gel corre-
sponds to the Upper plot. Reactions were run for 1 h in HxMg buffer,
consisting of 50 mM Hepes (25 mM Na^+), in 135 mM KCl, and
x mM MgCl_{2}, where x is listed below the plot and above the
corresponding lanes of the gel. ○ represents the hexamer-3’
exon trans-spliced product at 33 nt, and △ represents the unidentified
≈350-nt product.
control hexamer d(CnAnGnTnAn)rU instead of d(AnTnGn-AnCn)rU (data not shown), suggesting the effects are dependent on sequence complementarity between the oligonucleotide and the intron’s internal guide sequence (Fig. 2).

In contrast to the results at 37°C, at 50°C the 5′ exon–intron product predominates in H15Mg buffer (data not shown), suggesting the 5′ exon–intron product is favored under conditions that are expected to destabilize the group I intron structure.

**Reactivity of 5′ End Radiolabeled Hexamer as a Function of [Mg++]**. To directly monitor the trans-splicing product, 5′ radiolabeled d(AnTnGnAnCn)rU or d(CnAnGnUnAn)rU was added to solutions of unlabeled precursor. Fig. 4 shows that the 5′ exon mimic, d(AnTnGnAnCn)rU, is incorporated into two products; one is 33 nt in length corresponding to the expected trans-spliced product, and one is ~350 nt. Formation of the trans-spliced product in the presence of pG is maximal at 4–5 mM Mg2+ and then gradually decreases with increasing Mg2+ (Fig. 4, Upper plot), which corresponds to a gradual increase in formation of the 350-mer product. This trend also holds when the assay is conducted in the absence of pG (Fig. 4, Lower plot), indicating that pG is not required for formation of either product. The control, d(CnAnGnTnAn)rU, is not reactive in 2, 3, or 15 mM Mg2+, as expected, showing that the reactions are sequence-dependent.

**Reactivity of 3′ End Radiolabeled Precursor as a Function of d(AnTnGnAnCn)rU Concentration at 4 mM Mg2+**. To directly monitor both cis- and trans-splicing products, a 3′ end radiolabeled precursor was used. With 3′ end radiolabeled precursor, Mg2+-dependent trans-splicing reaches a plateau at 4 mM Mg2+ (data not shown). Therefore, the dependence of d(AnTnGnAnCn)rU concentration on trans-splicing with 3′ end radiolabeled precursor was analyzed at 4 mM Mg2+ (Fig. 5). The second-fastest migrating band in Fig. 5 is the 33-mer trans-spliced product, and its formation depends on oligonucleotide concentration, as expected. Note that the 27-mer 3′ exon hydrolysis product is distinguishable from the 33-mer trans-spliced product, and its formation is independent of oligonucleotide concentration. The trans-spliced product plateau at about 500 nM d(AnTnGnAnCn)rU, and the amount of trans-spliced product is greater than the amount of properly spliced product at concentrations of d(AnTnGnAnCn)rU as low as 200 nM. Because the Kd for d(AnTnGnAnCn)rU binding to the internal guide mimic, r(GGUCAU), is 34 μM (incorrectly reported as 340 μM in Table 1 of ref. 11) under the more stabilizing conditions of H15Mg buffer, the exon mimic is likely binding to the precursor at least partially through tertiary interactions. A trans-spliced product is not formed with up to 30 μM of the control oligonucleotide, d(CnAnGn-TnAn)rU, indicating that the reaction is sequence-specific (data not shown).

**Mg2+ Dependence of d(AnTnGnAnCn)rU Binding to the P-8/4x Ribozyme.** To examine the effects of Mg2+ concentration on the binding of d(AnTnGnAnCn)rU to the catalytic core, binding was measured to the P-8/4x ribozyme, which is precursor truncated to remove 5′ and 3′ splice sites (11). The dissociation constant at 3 mM Mg2+ is roughly 2-fold and 6-fold larger than those at 4 or 5 and 15 mM Mg2+, respectively (Table 1).

**Table 1.** McCl2 dependence of binding d(AnTnGnAnCn)rU and P-8/4x

<table>
<thead>
<tr>
<th>[MgCl2], mM</th>
<th>k_d, nM</th>
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<tr>
<td>3</td>
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</tr>
<tr>
<td>4</td>
<td>94</td>
</tr>
<tr>
<td>5</td>
<td>99</td>
</tr>
<tr>
<td>15</td>
<td>31 (16)*</td>
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</tbody>
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Assays were run in HxMg buffer, consisting of 50 mM Hepes (25 mM Na+) at pH 7.5, 135 mM KCl, and x mM MgCl2. Each reported value is the average of at least two independent assays. The dissociation constant, K_d, was determined by direct band-shift gel electrophoresis. *K_d was determined by competition band-shift gel electrophoresis (11).

**DISCUSSION**

P. carinii is one of a class of mammalian opportunistic pathogens that contain conserved group I introns (21). The functional importance of introns combined with their absence in mammalian hosts makes them potentially important targets for pharmacological intervention (17, 22). Hexamers that mimic the 5′ exon of a P. carinii ribosomal RNA group I intron bind tightly to a derived ribozyme through base pairing and tertiary interactions (10, 11). The ribozyme, however, lacks the 5′ exon sequence that is endogenous to the P. carinii ribosomal RNA precursor. Therefore, it was unknown whether mimics can compete with the endogenous 5′ exon for the 5′ exon-binding pocket (see Fig. 2).

The reactivity of the exogenous 5′ exon mimic d(AnTnGn-AnCn)rU with precursor RNA indicates that the mimic binds to the catalytic core of the group I intron in the presence of the endogenous exons at about 2 mM Mg2+ for the assay with internally labeled precursor and about 4 mM Mg2+ for the other assays. Such Mg2+ concentrations are likely near physiological (23, 24). The difference in the Mg2+ dependence of trans-splicing between assays may be the result of structural heterogeneity of the precursor that differs because of different protocols for preparing unlabeled, internally radiolabeled, and...
Indeed, by using internally radiolabeled precursor, when internal guide sequence (proteins (37). Evidently, phosphoramidates are able to mimic oligonucleotides are known to bind tightly to RNA binding states inherent in functional RNAs. Other phosphoramidate linkages are potentially useful for designing oligonucleotides that target base pairing, tertiary interactions, and transition linkages are potentially useful for designing oligonucleotides in the natural Tetrahymena thermophila dynamics suggest that it is significantly weaker than the form such an upstream structure, but the predicted thermodynamics state near to that of the self-splicing reaction in vitro. The irreversibility of forming the end-dead inhibition products results in an increase in specificity relative to oligonucleotides that reversibly bind to the target.

Targeting group I introns as a general therapeutic strategy is attractive because of the advantages mentioned above and because these intron inhibitors require a minimum amount of design; only knowledge of the 5’ exon sequence is required, and then modifications for nucleic stability must be analyzed. Also, there is a need for more effective treatments against a variety of intron-containing pathogens (15), including the fungi Candida albicans (38) and Aspergillus nidulans (39). Furthermore, there are other functionally important RNA structures that might be targeted by exploiting tertiary interactions and activity with therapeutic oligonucleotides or with even simpler compounds containing a reactive group such as the cis-diol in d(AnTnGnAnCn)rU. Key elements in the success of such strategies are identification of functional structures, assessment of their specific biological activities, and understanding their structures to at least a low level of resolution. The enormous amount of sequence data being generated for RNAs will greatly expedite this process.

One potential limitation of this specific example is the relatively large amount of therapeutic that probably must be delivered to ribosomal RNA precursors, because this will be an abundant RNA. Also, designing suicide inhibitors requires catalytic potential between the RNA target and its bound therapeutic. Both of these limitations are likely to be obviated in coming years as new RNAs are identified as potential targets.

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