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Miniribozymes, Small Derivatives of the *sunY* Intron, Are Catalytically Active

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The self-splicing *sunY* intron from bacteriophage T4 has the smallest conserved core secondary structure of any of the active group I introns. Here we show that several nonconserved regions can be deleted from this intron without complete loss of catalytic activity. The 3′ stems P9, P9.1, and P9.2 can be deleted while retaining 5′ cleaving activity. Two base-paired stems (P7.1 and P7.2) that are peculiar to the group IA introns can also be deleted; however, the activities of the resulting derivatives depend greatly on the choice of replacement sequences and their lengths. The smallest active derivative is less than 180 nucleotides long. These experiments help to define the minimum structural requirements for catalysis.

The *sunY* self-splicing intron from bacteriophage T4 is a member of the group IA class of introns, which has been defined by conserved sequences and secondary structure (9). The mechanism of self-splicing involves the attack of a free guanosine at the 5′ exon-intron junction, ligation of the exons, and circularization of the free intron (3). The catalytic core of *sunY* is quite small, making it particularly useful for studying structural requirements for enzymatic function in this class of introns.

The secondary structure of the wild-type *sunY* intron is shown in Fig. 1. The intron itself is 1,033 nucleotides long, but 788 of these residues are part of a large open reading frame in the loop of P9.1. The catalytic core of the intron is only 245 nucleotides long, well below the sizes of the cores of most known group I introns and the smallest of those known to self-splice. We have undertaken a deletion analysis of the *sunY* intron to eliminate nonessential regions of this ribozyme in order to obtain small, catalytically active derivatives.

Group IA introns typically have an extra stem-loop or pair of stem-loops inserted between the base-paired segments P7 and P3 (Fig. 1). These insertions range from 30 to 90 nucleotides long (7). Since the insertions are absent in many active group I introns, they are unlikely to be directly involved in catalysis. They may play a role in the proper folding of group IA introns or provide binding sites for proteins that facilitate splicing in vivo. Conversely, they may simply be extraneous elements that are excluded from the catalytic core when the intron is correctly folded. Models of the intron catalytic core tertiary structure, in which these sequences loop out the back of the enzyme away from the rest of the core, have been proposed (6).

We show here that P7.1 and P7.2 can be deleted from *sunY* without complete loss of catalytic activity. Sequences at the 3′ end of the *sunY* molecule can also be removed, with only modest effects on activity. Under conditions of increased salt and GTP concentrations, derivatives less than 20% the size of the wild-type intron can self-cleave with moderate efficiency. These are the smallest active group I intron derivatives found to date.

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MATERIALS AND METHODS

Construction of intron derivatives. The *sunY* intron and subsequent derivatives were constructed by cloning four overlapping pairs of synthetic oligonucleotides downstream of the T7 RNA polymerase promoter in a pBR322-derived plasmid. Recombinant plasmid DNA was prepared and sequenced through the insert by the method of Sanger et al. (8). Clones with the correct sequence were digested with the restriction enzyme *Bam*HI and transcribed in vitro with T7 RNA polymerase. Some preparations of RNA were labeled internally by including 0.2 μCi of [α-^{32}P]GTP per μl in the transcription reaction. RNA was purified either by electrophoresis on 6% polyacrylamide–7 M urea gels or on Sephadex G-75 spin columns. After phenol extraction and ethanol precipitation, the RNA was dried and suspended in deionized water at a concentration of 0.2 μg/μl.

Self-cleavage assays. Low-salt assays were performed by using 0.5 μM intron RNA and 4 mM GTP in a buffer containing 30 mM Tris hydrochloride (pH 7.4), 20 mM $MgCl_2$, 10 mM NH_4Cl, and 0.2 mM aurin trichloroacetic acid. High-salt assays were performed in the same manner except that each reaction included an additional 0.4 M KCl and 80 mM $MgCl_2$. The reaction volumes were each 5 μl. Reactions were incubated at 58°C since the parent RNA and all active derivatives showed optimal self-cleaving activity at this temperature (data not shown). Following incubation, reactions were mixed with 5 μl of loading dye containing 90% formamide, 10 mM Tris (pH 7.5), 1 mM EDTA, and 0.4% each of xylene cyanol and bromophenol blue. Reaction products were analyzed on 6% polyacrylamide–7 M urea gels. When the RNA was unlabeled, gels were stained with ethidium bromide to visualize products following electrophoresis. When the RNA was internally labeled with [α-^{32}P]GTP, gels were dried and autoradiographed.

Measurement of K_ms for GTP. The K_m for GTP for the parent RNA was measured by using unlabeled RNA, 1.2 μM [α-^{32}P]GTP, and cold GTP ranging from 10 μM to 2 mM in low-salt assay conditions. Reaction mixtures were incubated at 58°C for 2 min. The K_m for GTP for the mutant RNA was measured by using unlabeled RNA, 1.2 μM [α-^{32}P]GTP, and cold GTP ranging from 50 μM to 5 mM in high-salt assay conditions; incubation was at 58°C for 45 min. Reaction products were analyzed as described above. Gels were

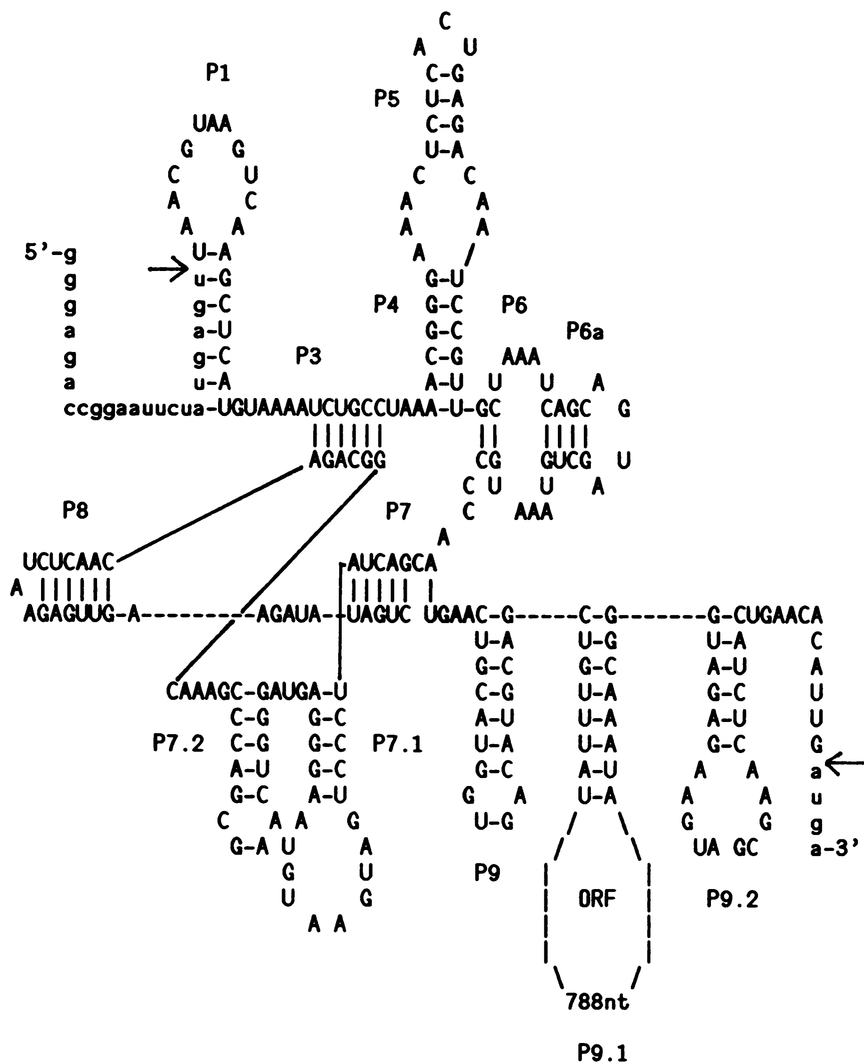

FIG. 1. Secondary structure of the *sunY* intron. Exon sequences are shown in lowercase letters; arrows indicate 5' and 3' cleavage sites. The 5' exon sequence is our synthesized intron. ORF, Open reading frame; nt, nucleotides.

quantitated directly by using a Betagen blot analyzer (Betagen Corp., Waltham, Mass.). The observed K_i for cold GTP equaled the K_m. Each K_m was the average of three independent experiments.

Reverse transcription of intact RNA and cleavage products. Primer extension of a ^{32}P-end-labeled 49-mer DNA primer was performed essentially as described by Belfort et al. (2). The primer hybridized to the intron RNA such that its 3' end was 45 residues from the expected 5' cleavage site in P1.

RESULTS

Deletion of 3' sequences from the *sunY* core. We began our deletion analysis of the intron by removing sequences at the 3' end of the intron. Previous work on the group I intron from *Tetrahymena thermophila* showed that P9.1 and P9.2 are not required for 5' cleavage (10). We started by synthesizing a *sunY* intron derivative which was truncated after P9 to exclude P9.1, P9.2, and the open reading frame (Fig. 1). Since the 3' intron-exon junction was missing, the intron was capable of only the first step of self-splicing, which is guanosine-mediated cleavage at the 5' junction. The RNA was tested under a variety of conditions and was found to self-cleave only at the expected site in the low-salt buffer (Table 1). Self-cleavage in the high-salt buffer was faster, but cleavage at additional sites was seen. This self-cleaving molecule became the parent for further mutations in the intron.

We then constructed an intron in which P9 was deleted. This derivative was about 5% as active as the parent under high-salt conditions (Table 1). This destabilizing effect is similar to that observed when P9 is deleted from the *Tetrahymena* intron (1, 4).

Deletion of P7.1 and P7.2. We synthesized nine intron derivatives in which P7.1 and P7.2 were deleted and replaced by two, three, or four nucleotides connecting P7 to P3 (Table 1). Many group IB introns have only 2 nucleotides linking P7 and P3, usually AA, CA, GA, or UA (5' to 3') (7). We synthesized four derivatives containing these sequences between P7 and P3; however, these RNAs were found to be inactive under all conditions tested. Three more derivatives, in which P7.1 and P7.2 were replaced by AC, AAC, or AAAC (5' to 3'), were synthesized. These sequences are found immediately following P7.2 in wild-type *sunY* sequences. The RNAs were tested for self-cleavage, and the

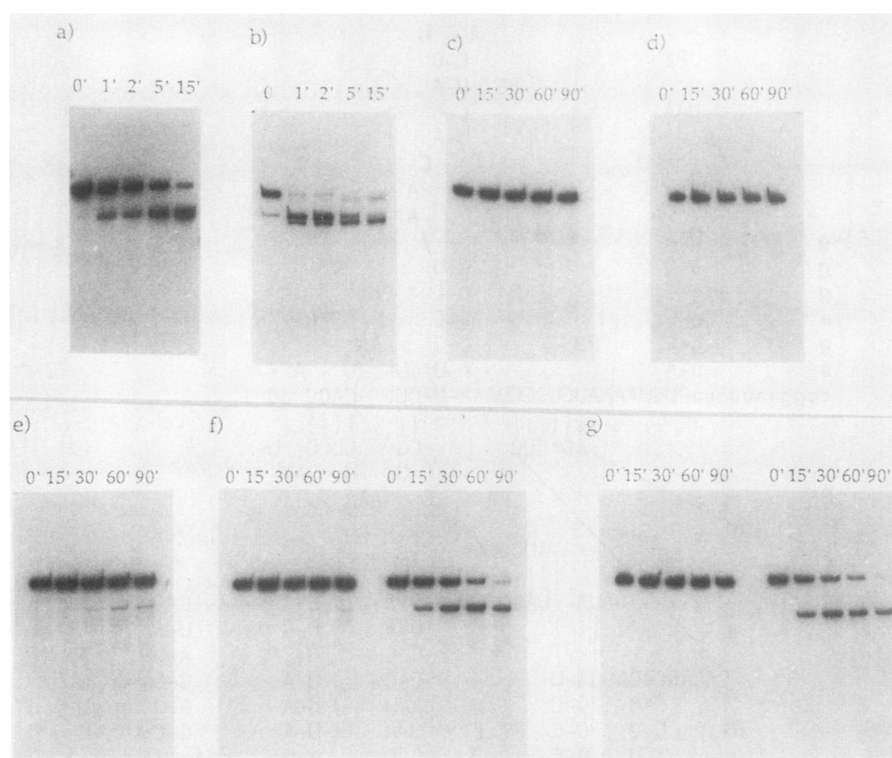

FIG. 2. Time courses (in minutes) for self-cleavage of parent and derivative introns under different conditions. Gels were quantitated directly by using a Betagen blot analyzer to determine half times for cleavage. Panels show introns as follows: a, parent (low salt); b, parent (high salt); c, with AA in place of P7.1 and P7.2 (high salt); d, with CA in place of P7.1 and P7.2 (high salt); e, with AAA in place of P7.1 and P7.2 (high salt); f, with AAC in place of P7.1 and P7.2 (left five lanes, low salt; right five lanes, high salt); g, with AAAC in place of P7.1 and P7.2 (left five lanes, low salt; right five lanes, high salt).

TABLE 1. Catalytic activities of wild-type and variant *sunY* introns

Plasmid	RNA		Cleavage half time[a]
	Size (nucleotides)	Sequence	
pJD901	222	Wild type	~0.5 min
pJD903	202	P9 deleted	9 min
pJD904	182	P7.1 and P7.2 deleted and replaced by AA	Inactive
pJD905	182	P7.1 and P7.2 deleted and replaced by CA	Inactive
pJD906	181	P7.1 and P7.2 deleted and replaced by GA	Inactive
pJD907	182	P7.1 and P7.2 deleted and replaced by UA	Inactive
pJD911	181	P7.1 and P7.2 deleted and replaced by AC	Inactive
pJD912	183	p7.1 AND P7.2 deleted and replaced by AAC	45 min
pJD913	184	P7.1 AND P7.2 deleted and replaced by AAAC	30 min
pJD924	183	P7.1 and P7.2 deleted and replaced by AAA	~5 h
pJD917	164	P9 deleted; P7.1 and P7.2 deleted and replaced by AAAC	>5 h

[a] Measured under high-salt conditions (10 mM NH_4Cl, 30 mM Tris hydrochloride, 100 mM $MgCl_2$, 400 mM KCl). Inactive, No detectable self-cleavage under 10 different reaction conditions.

AAC and AAAC derivatives were found to have significant activities in high-salt buffer.

Since all of the 2-nucleotide linking sequences were inactive, the effect of the sequence of the link between P7 and P3 was unclear. We therefore constructed another mutant in which AAA replaced P7.1 and P7.2. This RNA was found to have only a very low level of activity in the high-salt buffer (Fig. 2e), showing that both the sequence and its length are important.

Kinetic of self-cleaving for active derivatives. The self-cleaving activities of the parent *sunY* molecule and its functional derivatives differed depending on the kinds and amounts of salts included in the reaction (Fig. 2). The parent intron was active in the low-salt buffer (10 mM NH_4Cl, 30 mM Tris hydrochloride, 20 mM $MgCl_2$), with a half time for 5′ cleavage of 4 min. A half time for cleavage of about 30 s was observed in the high-salt buffer (10 mM NH_4Cl, 30 mM Tris hydrochloride, 100 mM $MgCl_2$, 400 mM KCl), and cleavage at two additional sites was observed. The sites

TABLE 2. Wild-type and mutant K_m values for guanosine

Plasmid	RNA		K_m[a] for GTP
	Size (nucleotides)	Sequence	
pJD901	222	Wild type	0.85 ± 0.2
pJD912	183	P7.1 and P7.2 deleted and replaced by AAC	1.2 ± 0.2

[a] Average of three experiments. Values (millimolar) are reported as means ± standard deviations.

were mapped by primer extension to nucleotides 28 (U), 22 (U; the expected site in P1), and 4 (A), counting from the 5′ end of the intact RNA shown in Fig. 1 (data not shown).

The derivatives were virtually inactive under low-salt conditions or with up to 1 M NH_4Cl or NaCl. However, in buffer including 0.4 M KCl and 100 mM $MgCl_2$, half times for cleavage were 45 and 30 min for the AAC and AAAC derivatives, respectively (Fig. 2). These molecules cleaved only at the expected nucleotide 22 (U) in P1, as determined by primer extension (data not shown).

In order to assess the effect of guanosine binding of deleting P7.1 and P7.2 from the intron, K_m values for guanosine were determined for the parent *sunY* sequence (truncated after P9) and for the derivative in which P7.1 and P7.2 were replaced by AAC. The K_ms for the parent version and the derivative were approximately 0.85 and 1.2 mM, respectively (Table 2).

DISCUSSION

Our results indicate that some catalytic activity is retained even when several nonconserved regions of the group IA self-splicing intron *sunY* are deleted from the molecule. Sequences downstrem of the P9 stem were deleted to produce a molecule that catalyzes only the 5′ exon cleavage reaction. Deletion of the P9 stem yielded an intron which was about 5% as active as the parent, as determined by the rate of self-cleavage under high-salt conditions. P9 may make contacts with other residues in the intron that stabilize the active conformation.

We found that the P7.1 and P7.2 stems could be deleted as long as they were replaced by a short string of nucleotide of the proper lengths and sequences. Length was the most important factor since none of the molecules with only two nucleotides as replacements were active. Molecules with longer replacement segments were active, but the two with a C rather than an A preceding P3 were much more efficient for self-cleaving. We propose that if the nucleotide preceding P3 is an A, it base pairs with the U on the opposite strand, which extends the length of the P3 stem. P3 is a highly conserved structural element in the group I introns; extension of the P3 helix could potentially disrupt helical stacking or other important tertiary interactions.

The intron derivatives are clearly less stable than the parent molecule since they require much higher salt concentrations for activity. This implies that the P7.1 and P7.2 stem-loops make contacts with other sequences in the intron core, as has been recently suggested (F. Michel, manuscript in preparation). The preference of the mutant introns for potassium ions rather than ammonium or sodium ions suggests that the size of the cation is important for proper RNA tertiary structure (5). The parent intron self-cleaved at three sites under high-salt conditions, at residues 4, 22, and 28 from the 5′ end of the RNA. Nucleotide 22 (U) is the normal cleavage site in P1. Sequences flanking the two alternate sites are partially complementary to the 3′ strand of P1 and can probably form alternate P1 stems in high-salt buffer.

Kinetic parameters for the parent *sunY* derivative are quite different from those for the *Tetrahymena* group I intron. The K_m for guanosine for the parent RNA in low-salt buffer was approximately 0.85 mM, which is at least 40-fold higher than the comparable value for the *Tetrahymena* intron. The K_m for guanosine in high-salt buffer may be significantly lower, but the reaction was not linear long enough for a reasonable measurement to be made. The K_m for guanosine for one of the smaller derivatives measured under high-salt conditions was about 1.2 mM. However, even when the mutant was saturated for guanosine, self-cleaving proceeded more slowly than for the parent molecule, implying a k_{cat} effect.

Smaller derivatives of the intron retain little catalytic activity. A molecule in which the P7.1 and P7.2 insertions and P9 were all deleted had a very low level of self-cleaving activity at high-salt concentrations. Since both single deletions have much higher salt optima than the parent RNA and therefore appear to be less stable than the parent enzyme, it is not too surprising that the double deletion is so unstable as to be almost inactive. Further size decreases may require detailed analysis of the length and sequence requirements of individual stems and loops.

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