

Methylation interference experiments identify bases that are essential for distinct catalytic functions of a group I ribozyme

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Methylation interference experiments reveal bases involved in three different catalytic functions of the T4-phage derived sunY self-splicing intron. RNA molecules methylated at the N-7 position of the guanine at the cofactor binding site are inactive in cofactor-dependent splicing and 3' splice-site hydrolysis. In contrast, 5' splice-site hydrolysis occurs despite methylation at this position. Specific adenines that have been implicated in docking of the P1 stem to the catalytic core are shown to be important for cofactor-dependent splicing and essential for 5' splice-site hydrolysis. Similarly, methylation of bases in the P9.0 stem, as well as C56 in J5/4, interferes with 3' splice-site hydrolysis and with the splicing reaction. All of the bases identified as important for the overall splicing reaction are also identified as essential for either the 5' or 3' splice-site hydrolysis reactions, and vice versa. It is inferred that the bases implicated in 5' and 3' splice-site hydrolysis are involved in specific interactions of the 5' and 3' splice site, respectively, with the catalytic core.

Key words: catalytic RNA/dimethylsulfate/group I intron splicing/splice sites

Introduction

Self-splicing of group I intron RNA proceeds via two consecutive transesterification reactions. For initiation of splicing, an external guanosine cofactor binds to the cofactor binding site and acts as a nucleophile to attack the phosphate at the 5' splice site via its 3' hydroxyl group, thereby becoming covalently linked to the 5' end of the intron (reviewed in Cech, 1990). Therefore, the 5' splice site must be brought in close proximity to the guanosine cofactor binding site. A universally conserved guanine in stem P7 has been identified as a component of the cofactor binding site (Michel *et al.*, 1989). For the second step of splicing a conformational transition occurs in which the 3' terminal intron nucleotide, always a guanosine, has been shown to interact with the cofactor binding site in a manner similar to that of the free guanosine cofactor (Inoue *et al.*, 1986; Michel *et al.*, 1989; Been and Perrotta, 1991). This conformational rearrangement would bring the exon boundaries together, followed by attack of the 3' splice site by the 3' hydroxyl of the upstream exon, resulting in ligation of the exons.

Investigation of the splicing mechanism for the Tetrahymena rRNA intron led to the discovery of intron-

catalyzed splice-site hydrolysis (Zaug *et al.*, 1985; Inoue *et al.*, 1986). These cofactor-independent reactions take place preferentially at alkaline pH and cleave the RNA chain specifically at the two splice sites, resulting in 3' hydroxyl and 5' phosphate ends, as also observed for the cofactor-dependent splicing reaction. In contrast, uncatalyzed alkaline hydrolysis of RNA by free hydroxide ion leads to 2',3' cyclic phosphates. For intron-catalyzed splice-site hydrolysis, it was suggested that, instead of the 3' hydroxyl of guanosine or the 5' exon, the hydroxide ion serves as the nucleophile (Cech, 1987). Recent evidence, mostly from mutagenesis studies (e.g. van der Horst and Inoue, 1993), supports the view that the same intramolecular interactions are involved in splicing and splice-site hydrolysis.

The modification–interference approach has been employed to identify intra- and intermolecular interactions involving RNA (Herr *et al.*, 1979; Peattie and Herr, 1981). Basically, a sample of RNA is subjected to limited chemical modification, followed by a selection procedure in which the RNA molecules in the population that are competent for a specific function are separated from those that have lost the function. Bases that are modified in the total RNA population, but are unmodified in the functional subpopulation, are those whose modification interferes specifically with the given function. These bases can be identified conveniently in the usual ways. Here, we demonstrate the use of this approach to identify bases in a group I intron that are essential for three distinct catalytic functions: cofactor-dependent splicing and 5' and 3' splice-site hydrolysis.

Results

Experimental strategy

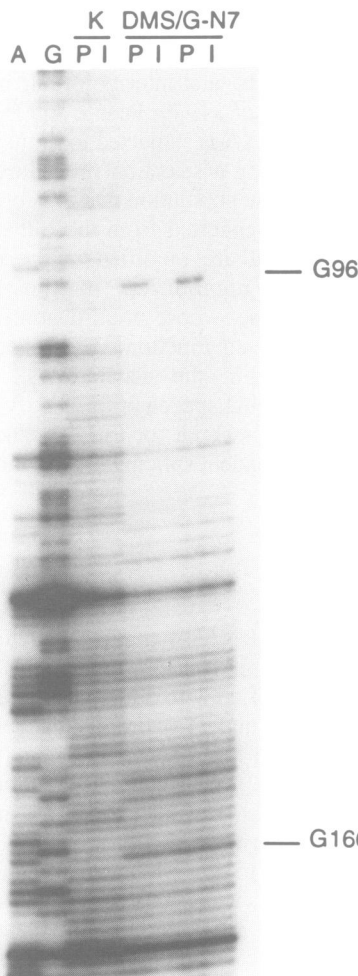
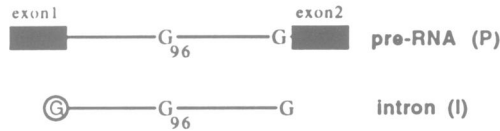
Gel-purified precursor RNA of a shortened form of the T4-phage derived sunY intron (Xu and Shub, 1989) was renatured and then modified with dimethylsulfate (DMS) in its native conformation under conditions that result in less than one methylation per molecule, on average (von Ahsen and Noller, 1993; see Materials and methods). DMS methylates the N-1 position of adenines, N-3 of cytosines and N-7 of guanines (Stern *et al.*, 1988). After modification, the RNA was subjected to conditions where either splicing (in the presence of the guanosine cofactor) or intron-catalyzed hydrolysis at the 5' or 3' splice sites (in the absence of cofactor) occurred. The cleaved products were separated by size from the unreacted molecules on a denaturing polyacrylamide gel and recovered. Only those molecules which retain their catalytic activity, despite the methylation reaction, are able to undergo splicing or splice-site hydrolysis and are thereby resolved from precursor RNA on the gel. Thus, these molecules will contain base modifications only at positions where methylation is tolerated. Bases that are modified in the total population, but unmodified in the

functionally competent RNAs, are precisely those sites whose modification interferes with catalytic activity. It is important to emphasize that DMS modification was performed under ionic conditions in which the intron is catalytically active (Xu and Shub, 1989; Michel *et al.*, 1992; von Ahsen and Noller, 1993), so that the positions required for general folding of the RNA structure would be protected from modification. Thus, the positions that showed interference are expected to give information about functional interactions that change during the catalytic cycle.

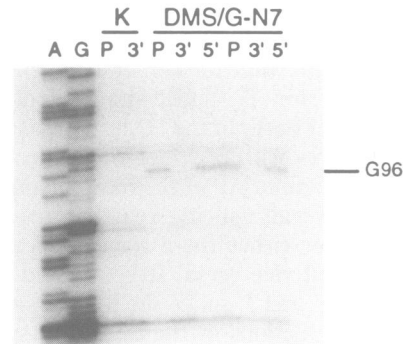
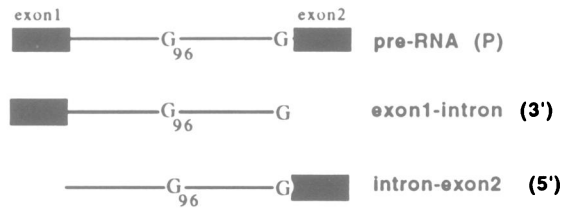
Requirement for an accessible N-7 position of the guanine at the cofactor binding site

The gel-isolated species (DMS-modified precursor RNA and cleaved products) were treated with NaBH₄ and aniline to detect guanines methylated at their N-7 positions (Peattie, 1979). Primer extension (Figure 1A) reveals that the N-7 position of the guanine at position 96, the guanosine cofactor binding site (Michel *et al.*, 1989), is hyperreactive towards DMS when the precursor RNA is in its native conformation. In contrast, methylated RNAs that retain splicing activity

A cofactor-dependent splicing



B splice-site hydrolysis



C guanosine binding to G-site

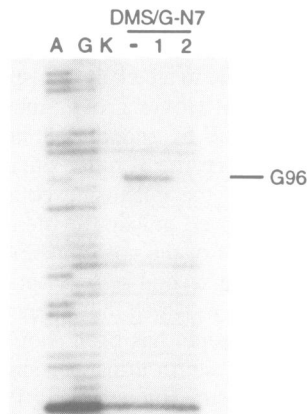


Fig. 1. Methylation interference experiment and footprinting analysis involving the N-7 position of G96 (cofactor binding site). (A) Methylation interference in cofactor-dependent splicing. Primer extension of modified precursor RNA and selected intron RNA separated on a 6% polyacrylamide gel is shown. Samples were incubated in the presence or absence of 100 μ M guanosine (see Materials and methods). Experiments with modified RNA were performed in duplicate. For detection of N-7 guanine methylations, aniline-induced strand scission was performed (Peattie, 1979). (B) Methylation interference in 5' and 3' splice-site hydrolysis. Samples were incubated in splicing buffer (pH 8.8) for 20 min at 37°C (without guanosine). Primer extension of precursor RNA and the two intermediates (intron-exon2 and exon1-intron) treated as in (A) is shown. (C) DMS footprinting of sunY1-13 intron RNA in the presence of 10 μ M (lane 1) or 4 mM (lane 2) guanosine. Abbreviations: A and G, sequencing lanes; K, control (no DMS); DMS, DMS-modified RNA; P, precursor RNA; I, intron RNA; 5', intron-exon2; 3', exon1-intron.

show complete selection against methylation at this position, suggesting that methylation of the N-7 position of G96 prevents splicing. It is believed that the 3' terminal base of the intron, as well as the free guanosine cofactor, interact with the cofactor binding site. However, we are unable to distinguish whether an accessible N-7 at this position is required for both steps. Because the sunY intron proceeds efficiently to the second step during splicing, we were unable to isolate sufficient amounts of sunY intron-exon2 intermediate to address this question (see below). It is worth noting the tolerance for methylation of the N-7 of guanine 166, a highly conserved base in J8/7, in the splicing reaction (Figure 1A).

Hydrolysis at the 5' and 3' splice sites, first observed for the Tetrahymena intron (Zaug *et al.*, 1985; Inoue *et al.*, 1986), is a common property of group I introns. Modified sunY precursor RNA was subjected to hydrolysis conditions and the two intermediates, corresponding to either the 5' or 3' splice-site hydrolysis products, intron-exon2 or exon1-intron, respectively, were isolated. Inspection of the N-7 positions of G96 of the respective intermediates revealed that for hydrolysis at the 3' splice site there is complete interference, whereas 5' splice-site hydrolysis can clearly occur despite methylation of N-7 of G96 (Figure 1B). Selection against methylation by those RNA molecules that retain 3' splice-site hydrolysis activity can be explained by the interaction of the terminal intron nucleotide, always a guanosine, with the cofactor binding site in a manner analogous to binding of the free guanosine cofactor in the first step. By contrast, 5' splice-site hydrolysis is independent of interactions involving the cofactor binding site.

Although selection against methylation of G96 by splicing-competent introns shows that the cofactor binding site N-7 is required for splicing, it does not provide unambiguous evidence that the guanosine cofactor interacts with this position during splicing. Previously we showed that cofactor analogues, such as 3'-deoxyguanosine, protect the N-7 position of G96 from methylation. However, we were unable to demonstrate protection by the guanosine cofactor itself, because of its ensuing reaction at the 5' splice site (von Ahnen and Noller, 1993). Therefore this question was addressed directly in a separate footprinting experiment using a shortened form of the sunY intron. This ribozyme (sunY1-13, kindly provided by T.Cech, Boulder, CO) lacks the 5' and 3' splice sites and exon sequences; the remaining core exhibits catalytic activity (Heuer *et al.*, 1991). In the presence of 10 μ M or 4 mM guanosine, respectively, the N-7 position of G96 was partially or completely protected against methylation by DMS (Figure 1C). This experiment provides specific evidence for the interaction of the guanosine cofactor with the N-7 position of G96 in the cofactor binding site, supporting the binding mechanism proposed by Michel *et al.* (1989).

Identification of the bases required for interactions specifically involving the 5' or 3' splice sites

It is believed that 5' and 3' splice-site hydrolysis and the cofactor-dependent splicing reaction are catalyzed by the same active site in the catalytic core (Cech, 1990; Michel and Westhof, 1990). Using as selective criteria the ability to carry out the 5' or 3' splice-site hydrolysis reactions in addition to cofactor-dependent splicing, the methylation-

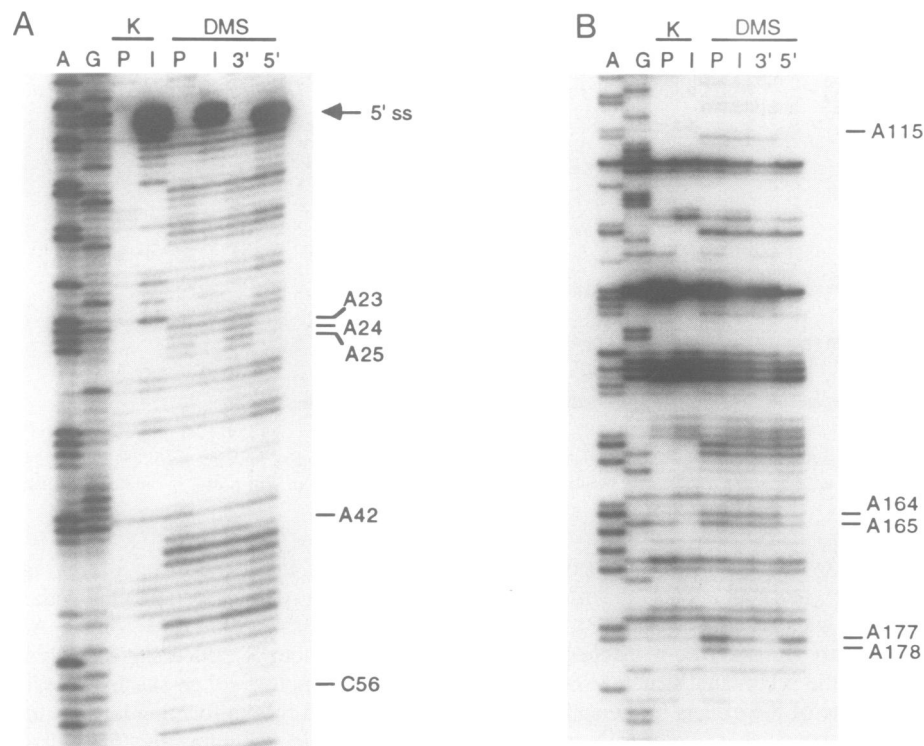


Fig. 2. Primer extension of modified precursor RNA and products of different catalytic reactions involving adenines and cytosines. The RNAs were incubated for 30 min at 37°C in the appropriate buffer and 100 μ M guanosine for cofactor-dependent splicing. (A) Autoradiograph showing primer extension of RNAs at the 5' region of the intron. Note that the product of 5' splice-site hydrolysis is one nucleotide shorter than the product of cofactor-dependent splicing because no guanosine is added to the 5' end of the intron. (B) Autoradiograph of primer extension of RNAs showing the central region of the sunY intron. The stem and loop region of P9.2 was excluded because the primer at the 3' end of the intron was too close to make confident statements about this region. P9.1a and P9.1b regions are not shown because of lack of interference. Abbreviations as in Figure 1.

Table I. Summary of bases that show interference by methylation at the N-1 of adenines and N-3 of cytosines in cofactor-dependent splicing or 5' or 3' splice-site hydrolysis

Base	Secondary structure location	Splicing ^a	5' hydrolysis ^b	3' hydrolysis ^c
A23–A25	J1/3	+	+	–
A42	J4/5	+	+	–
C56	J5/4	+	–	+
G96 (N-7)	P7 (G site)	+	–	+
A115	L7.1	+	+	–
A164	J8/7	+	+	–
A165	J8/7	±	+	–
A177, A178	J7/9	+	–	+

G site, cofactor binding site.

^aCofactor-dependent splicing. Isolated intron underwent first and second step of splicing.

^b5' splice-site hydrolysis resulting in intron–exon2.

^c3' splice-site hydrolysis resulting in exon1–intron.

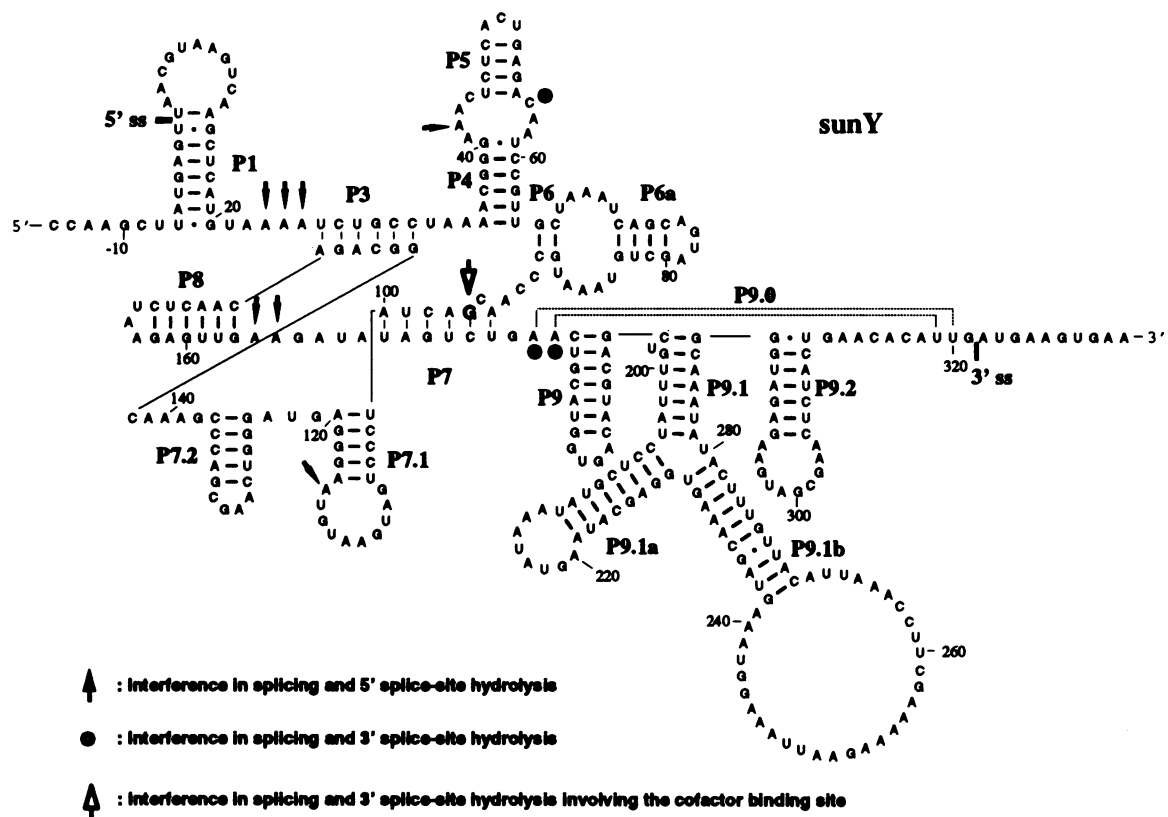


Fig. 3. Secondary structure of the sunY intron showing bases that interfere with cofactor-dependent splicing or 5' or 3' splice-site hydrolysis when methylated. Pairing P9.0 is shown as a dotted line. Pairing between G175 and C315 (Jaeger *et al.*, in press) is omitted for clarity. 5' ss, 5' splice site; 3' ss, 3' splice site.

interference approach thus allows the discrimination between bases that are essential for catalytic events specifically involving the 5' or 3' splice sites (Figure 2A and B). The bases that show interference are summarized in Table I and are graphically presented in the secondary structure of the sunY intron in Figure 3; some of the effects in the splicing reaction are subtle, although reproducible (see below). It was somewhat surprising to find no bases whose methylation interferes with all three processes, although the chemical probes used in these experiments do not, for example, address the possible importance of the Watson–Crick positions of uridines and guanines and the N-7 position of adenines. Instead, the interference patterns show a distinct

selectivity for either the 5' or 3' cleavage reactions. In all cases, splicing is affected in parallel with 5' or 3' hydrolysis. Thus, A23, A24, A25, A42, A115, A164 and A165 are essential for both 5' splice-site hydrolysis and the splicing reaction (but not 3' splice-site hydrolysis), consistent with their proposed role in forming tertiary interactions with the P1 stem, which contains the 5' splice site (Michel and Westhof, 1990; Young *et al.*, 1991; Herschlag, 1992; Pyle *et al.*, 1992). Conversely, methylation of C56, A177 and A178 interferes with 3' splice-site hydrolysis and the splicing reaction (but not 5' splice-site hydrolysis). The latter two bases have been shown to be involved in pairing of the 3' splice site with the catalytic core (P9.0; Burke *et al.*, 1990;

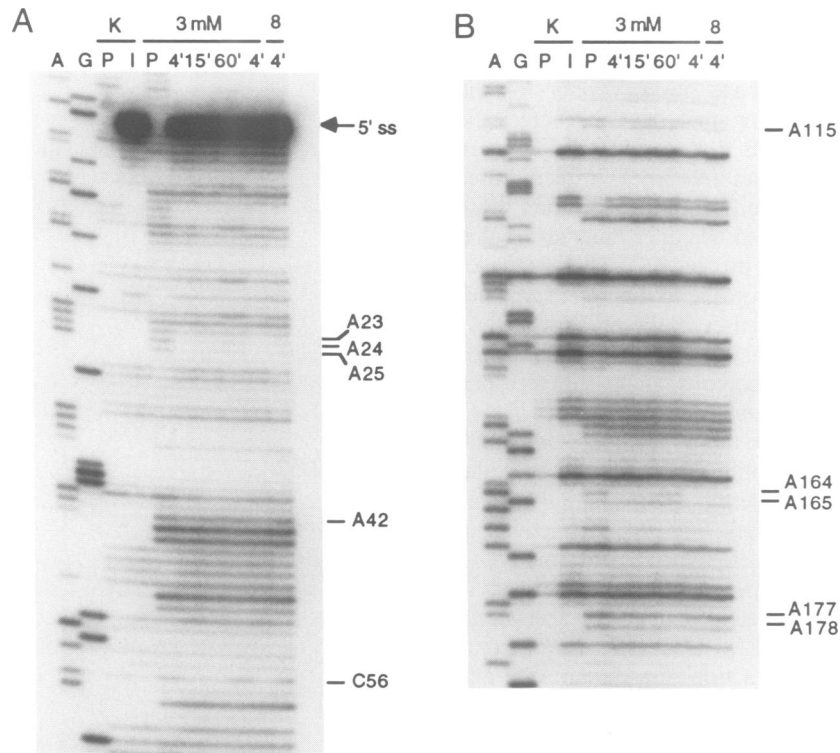


Fig. 4. Suppression of methylation interference in cofactor-dependent splicing by extending the incubation time or by increasing the magnesium ion concentration. The reactions were carried out essentially as in the experiment in Figure 2 for cofactor-dependent splicing except that time points were taken (lanes 5–8) and the magnesium concentration was raised to 8 mM (lane 10). Guanosine was added to a final concentration of 10 μ M. The last five lanes are all I (intron RNA). Other abbreviations are as in Figure 1. Samples in lane P and the K lanes were incubated for 15 min. (A) Primer extension of the 5' end of the intron. (B) The central region of the sunY intron.

Michel *et al.*, 1990a). Although other apparent differences can be seen in Figure 2 (e.g. around position 127 in Figure 2B), these are actually caused by differences in the degree of spontaneous reverse transcriptase stops, because they are also seen in the unmodified (K) lanes; accordingly, these effects are not subject to simple interpretation.

Time- and magnesium-dependent effects

We next investigated the interference pattern for cofactor splicing as a function of time of the splicing reaction, setting the guanosine concentration to 10 μ M to slow down the splicing process. The effects of a typical low magnesium ion concentration (3 mM) were also compared with that of an elevated level (8 mM), based on observations by others that raising the magnesium ion concentration can restore the catalytic activity of certain structural mutants.

RNA molecules were selected after 4', 15' and 60' splicing reactions and the products analyzed. By slowing down the splicing reaction, a stronger interference pattern, similar to that obtained for 5' or 3' splice-site hydrolysis, can now be seen at positions A42, A115 and A164 (and possibly A165) for the cofactor-dependent splicing reaction at the short incubation (4 min) time point (Figure 4A and B). All experiments were repeated at least three times and representative gels are shown. However, only the interference signal at position A165 in the cofactor-dependent splicing reaction was too subtle to make a confident statement about this position (in contrast to its involvement in 5' splice-site hydrolysis). Interference, evident at other positions in the experiment (Figure 2), can be suppressed by either extending the incubation time or elevating the magnesium ion concentration (e.g. C56, A115 and A164; Figure 4A and

Table II. Summary of bases that show interference in cofactor-dependent splicing and their strength in extended incubation time or elevated magnesium ion concentration

Base	Secondary structure location	Time or Mg ²⁺ suppression ^a	Neomycin enhancement ^b
A23	J1/3	+	+
A24, A25	J1/3	–	+
A42	J4/5	+	+
C56	J5/4	+	–
G96 (N-7)	P7 (G site)	–	–
A115	L7.1	+	+
A164	J8/7	+	–
A165	J8/7	±	–
A177, A178	J7/9	±	–

^aA plus (+) indicates that interference by methylation was suppressed by extending the incubation time during splicing or elevating the magnesium ion concentration.

^bThese bases show enhanced reactivity towards DMS in the presence of neomycin; compare with von Ahsen and Noller (1993).

B). In contrast, other positions, such as the A-rich junction J1/3 (except A23) and A177 and A178 (involved in P9.0 pairing), showed the same interference pattern under all conditions. These results are summarized in Table II.

Movement of splice sites between open and closed complex

The P1 stem is believed to dock with the catalytic core to form a 'closed complex' (Bevilacqua *et al.*, 1992; Herschlag, 1992; Wang *et al.*, 1993). For cofactor-dependent splicing

and 5' splice-site hydrolysis our results show strong selection against methylation at positions A23, A24, A25, A42, A115, A164 and A165 (the latter base possibly only for the hydrolysis reaction), suggesting that these sites may be involved in tertiary interactions that maintain the P1 stem in the closed complex. The band intensities for these bases in the precursor RNA are significantly weaker than those of other reactive single-stranded adenines, suggesting that these positions are partially protected by higher order RNA–RNA interactions. According to this interpretation, the modest reactivities of the bases in the precursor RNA under native conditions would mean that part of the population exists in the closed complex and part in the open complex during the methylation reaction.

In such a scenario, if part of the population were stably trapped in the closed conformation, these molecules would escape modification at all of these positions, and this same part of the population would therefore retain its activity in the splicing reaction. To test this possibility, a modification–interference experiment was performed in which the modification was carried out in the absence of magnesium ions (0.5 mM EDTA). Under these conditions, Mg^{2+} -dependent tertiary interactions are destabilized and the bases in question become strongly reactive towards DMS. Cofactor-dependent splicing was then performed under the usual conditions (i.e. 3 mM Mg^{2+}). Figure 5 clearly shows interference for the same bases (A23–A25, A42, A115, A164 and possibly A165) for spliced intron RNA as observed when Mg^{2+} was present during modification. Because the closed complex is disrupted in the absence of Mg^{2+} ions, the possibility of pre-selection of molecules that exist in the closed complex can be excluded. In addition, the experiment (Figure 5) shows that methylation of several additional positions interferes with the splicing reaction. However, because these additional sites are completely inaccessible to DMS under native conditions, it is likely that their essential role is to stabilize the correct folding of the catalytic core [e.g. compare Figure 5 bases in J6/7 (Michel *et al.*, 1990b) and bases in J8/7 (Michel and Westhof, 1990)].

Discussion

Group I intron-catalyzed splicing involves at least three fundamental events that require interactions with the catalytic core of the ribozyme: (i) binding of the guanosine cofactor to the cofactor binding site; (ii) docking of the 5' cleavage site (in stem P1) close to the cofactor binding site for the first step of splicing; and (iii) a conformational rearrangement that results in docking of the 3' splice site with the catalytic core to allow exon ligation. Using a methylation–interference approach, we have identified bases that are important for three well-studied catalytic functions of the group I intron RNA: 5' and 3' splice-site hydrolysis and the splicing reaction itself. Using DMS as a probe, no single base was identified whose modification interferes with all three reactions, although it is thought that they all involve participation by the same catalytic center (Cech, 1990; Michel and Westhof, 1990). (It should be noted that our experimental conditions were chosen to exclude the modification of bases that are involved in structural stabilization of the core.) Instead, the essential bases can be assigned to two classes, according to which reactions were

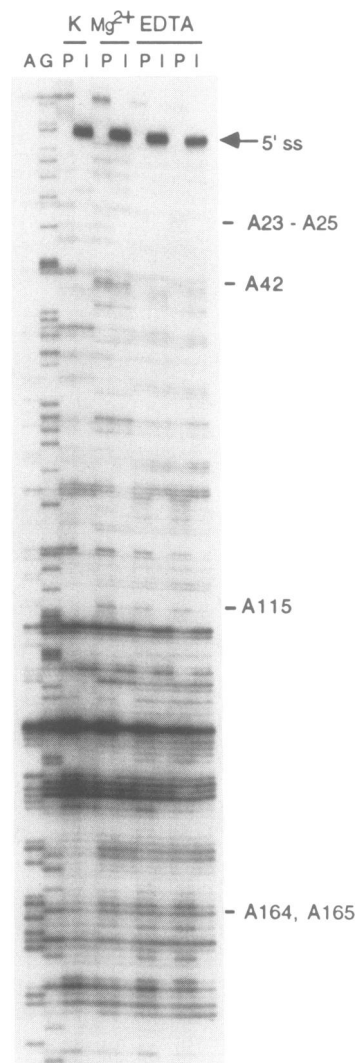


Fig. 5. Chemical modification in the presence of magnesium versus EDTA. A methylation interference approach was carried out as in the experiment in Figure 2 except that the initial modification of sunY precursor RNA was performed in the presence of EDTA and, for comparison, in magnesium-containing buffer (see Materials and methods). Note that in the presence of EDTA more bases are accessible to DMS modification. Chemical modification in the presence of EDTA and subsequent selection was performed in duplicate. Splicing reactions were performed in the presence of 10 μ M guanosine. Mg^{2+} , modification in the presence of magnesium (3 mM); EDTA, modification in the presence of 0.5 mM EDTA; other abbreviations are as in Figure 1.

abolished by their methylation: (i) 5' splice-site hydrolysis and cofactor-dependent splicing or (ii) 3' splice-site hydrolysis and cofactor-dependent splicing. If the same active site and basic catalytic mechanism are employed for all three reactions, the different effects of methylation are most simply explained by interference with intramolecular interactions involved in docking of the 5' and 3' splice sites with the catalytic core, and with interactions at the cofactor binding site.

Methylation of G96 at its N-7 position abolishes 3' splice-site hydrolysis, as well as the overall splicing reaction. The importance of this base (or, more precisely, its analogue in the Tetrahymena group I intron) was first shown by Michel *et al.* (1989), who demonstrated by site-directed mutagenesis

experiments that it is involved in the binding of the guanosine cofactor. Recently it was shown that the N-7 position of G96 is protected from DMS attack by binding the cofactor analogues 3'-deoxyguanosine or arginine (von Ahsen and Noller, 1993). However, the results of this study provide direct evidence for the functional importance of the N-7 position of G96 in the splicing reaction and are consistent with the proposal (Michel *et al.*, 1989) that its functional role is to accept a hydrogen bond from the 2-amino group of the guanosine cofactor. It is also believed to bind the 3' terminal guanine of the intron using the same non-Watson-Crick interaction, one of the interactions that accompanies docking of the 3' splice site with the catalytic core. Interference with 3' splice-site hydrolysis (but non-interference with 5' splice-site hydrolysis) by methylation of G96 provides support for this proposal.

In their model for the 3-D folding of the core of the *Tetrahymena* intron, Michel and Westhof (1990) proposed a number of tertiary interactions to stabilize the interaction of the 5' splice site with the core. One suggestion involved the hydrogen bonding of a ribose 2'-OH group in the upper P1 stem to the N-1 position of A42 in J4/5 (for simplicity, sunY numbering is used). Subsequently, Pyle *et al.* (1992) identified a tertiary interaction between the 2'-OH of A(-3) upstream from the 5' splice site and the N-1 position of A302 in *Tetrahymena* group I intron, the likely homologue of A165 in the sunY structure. The sunY counterparts of both of these adenine N-1 positions, as well as that in A164, have been identified in the methylation-interference studies presented here as essential for splicing and for 5' splice-site hydrolysis, but not for 3' splice-site hydrolysis. In addition, adenines 23-25 have been identified as essential for these same two reactions; these bases have been proposed to act as a hinge to promote docking of the P1 stem with the catalytic core (Young *et al.*, 1991; Herschlag, 1992). Methylation of base A115 also interferes with these two processes; A115 occurs in the variable loop L7.1 and might therefore represent an interaction important only for a subset of group I introns that contain this stem-loop structure.

For the second step of splicing, in addition to the requirement for tertiary pairing between G321 and the 3'-terminal base of the intron with G96 in the cofactor binding site, Watson-Crick pairing between bases in the J7/9 region (A177 and A178 in sunY) and the 3' splice-site region (U319 and U320 in sunY) has been shown (Burke *et al.*, 1990; Michel *et al.*, 1990a). Here it is found that methylation of A177 and A178 interferes with both 3' splice-site hydrolysis and the complete splicing reaction, in close agreement with previous findings. In addition, C56 in J5/4 is implicated in the 3' splice-site reaction; C56 is located close to the position of the 3' splice site in a 3-D model for the sunY intron depicting its conformation during the second step of splicing (Michel *et al.*, 1992; Jaeger *et al.*, in press).

The close agreement between these findings and previous results strongly supports the possibility that the 5' and 3' splice-site hydrolysis reactions utilize the same intramolecular RNA interactions that are used in the first and second step, respectively, of the splicing reaction. Accordingly, the newly implicated bases discussed here are candidates for previously unidentified functionally important tertiary interactions. Moreover, the results of the interference experiments make a clear prediction as to whether a given base is important for the first or second step of the splicing reaction.

For some of the positions, where interference with both the splicing reaction and the 5' splice-site hydrolysis reaction is observed, the degree of interference can be overcome in the splicing reaction by either extending the incubation time or raising the magnesium ion concentration. This indicates that interactions involving these bases contribute to the stability of the catalytic complex, but are not absolutely required. Support for this interpretation comes from previous studies (Pyle *et al.*, 1992; Williams *et al.*, 1992). However, for adenines 24-25 in J1/3 interference persists under all conditions, suggesting that methylation of these sites interferes more directly with docking of the 5' splice site with the catalytic core.

Our results suggest that docking of the P1 stem is supported by a set of interactions involving the J1/3, J4/5, J8/7 and L7.1 regions of the sunY intron (Figure 3). Disruption of any one of this set of interactions results in decreased catalytic activity, although it does not abolish it (with the possible exception of J1/3). Similarly, for C56, whose methylation interferes with both the splicing and 3' splice-site hydrolysis reactions, interference with the splicing reaction is suppressed at increased magnesium ion concentrations or by extending the incubation time.

It is concluded that, for 5' splice-site hydrolysis, the P1 stem must be held tightly to the catalytic core by tertiary interactions. Even extending the incubation time to as long as 90 min failed to overcome the effect of the methylation of bases believed to be involved in these interactions on the 5' splice-site hydrolysis reaction (data not shown). This suggests that disruption of these tertiary interactions has a more severe effect on hydrolysis than on cofactor-dependent splicing; recent evidence supports this conclusion (Caprara and Waring, 1993; van der Horst and Inoue, 1993). It was suggested that the hydroxide ion serves as a nucleophile in the hydrolysis reaction because the reaction rate increases as a function of pH (Cech, 1987). The fact that the N-7 position of G96 can be methylated without affecting the 5' splice-site hydrolysis reaction excludes its involvement in this reaction. However, it has been reported that a G264A:C311U mutant in *Tetrahymena* (corresponding to the mutation of the G96:C174 pair in the sunY intron) showed a decreased rate of 5' splice-site hydrolysis (Legault *et al.*, 1992). Possible explanations for this are that the guanine of the cofactor binding site (via a position other than N-7) stabilizes a specific structure required for orientation of the nucleophile, or of a metal ion, which in turn might coordinate the oxygen of the target phosphate (Piccirilli *et al.*, 1993).

Herschlag (1992) first suggested that the P1 stem folds to the intron core, alternating between an open and closed complex. In a time-resolved fluorescence study, Bevilacqua *et al.* (1992) obtained evidence for such movement of the P1 stem. Recently Wang *et al.* (1993) obtained further evidence for this movement from RNA-RNA crosslinking. The modest reactivity of bases, at certain positions where we found methylation-interference in the precursor RNA under native conditions, is interpreted as an indication that part of the population is in the closed complex and part is in the open complex during chemical probing. This hypothesis is supported by the fact that, in the presence of aminoglycoside antibiotics (e.g. neomycin) which appear to affect interaction of P1 with the catalytic core, several bases, whose methylation interferes with 5' splicing (the A-rich J1/3

region, A42 and the newly identified position A115), show enhanced reactivity towards DMS (compare Table II and von Ahsen and Noller, 1993). It is suggested that the binding of antibiotics stabilizes the precursor RNA in the open conformation, thereby increasing the accessibility of these bases to DMS.

For the second step of splicing, the 3' splice site is directed to the core of the ribozyme by another set of tertiary interactions. Because this step can occur independently of a processed 5' splice site (see also Inoue *et al.*, 1985), it is not unreasonable to imagine that the 3' splice site could also alternate between an open and closed complex. The fact that A177 and A178 are methylated in the precursor RNA indicates that the majority of the RNA molecules are in the open complex. Thus, the data suggest that the P9.0 interaction is formed only in conjunction with events involving catalysis at the 3' splice site.

The modification–interference approach, used here to probe dynamic interactions of ribozyme catalysis, may also be helpful in understanding other reactions of group I introns, such as intron circularization or the G-exchange reaction (Price *et al.*, 1987; Kay *et al.*, 1988), as well as in the study of other RNA-catalyzed reactions to better understand the dynamic interactions of RNA domains.

Materials and methods

In vitro transcription and chemical modification

In this study a shortened form of the T4-phage derived sunY intron was used (Xu and Shub, 1989) with a deletion in L9.1 (von Ahsen and Noller, 1993). *In vitro* transcribed and gel-purified sunY precursor RNA (von Ahsen *et al.*, 1992) was renatured as described previously (Michel *et al.*, 1992) and preincubated for 10 min at 37°C in 80 mM potassium cacodylate, pH 7.2, 4 mM MgCl₂, 50 mM NH₄Cl. A 50 pmol RNA was probed in a 100 μl reaction volume with 2 μl DMS (diluted 1:10 in EtOH) and incubated at 37°C for 5 min. For modification of unstructured RNA, MgCl₂ and NH₄Cl were omitted and 0.5 mM EDTA was used instead. After incubation, the reaction was stopped by the addition of 1 μl 0.6 M β-mercaptoethanol. The samples were then precipitated, centrifuged and dissolved in 10–20 μl H₂O. For footprinting guanosine binding to the sunY1-13 ribozyme (linearized with *Ear1*; Heuer *et al.*, 1991), DMS modification was performed as described by von Ahsen and Noller (1993).

Splicing/hydrolysis reaction and separation of reactive from unreactive RNA molecules

Equal aliquots (5–10 pmol) of modified RNA were incubated with the appropriate buffer. For cofactor-dependent splicing, 40 mM Tris–Cl pH 7.4, 3 mM MgCl₂ (8 mM MgCl₂ when indicated), 50 mM NH₄Cl and 0.1 mM spermidine were used. For splice-site hydrolysis the same buffer was used but the pH of the buffer was raised to pH 8.8. For splicing reactions, samples were preincubated without cofactor for 10 min at 37°C, then put on ice. Cofactor guanosine was added at a final concentration of 10 or 100 μM, depending on the experiment, and incubated at 37°C in a 10 μl reaction volume for 20 min, if not indicated otherwise in the figures. The reactions were stopped by the addition of an equal volume of 8 M urea and dyes (0.025% bromophenol blue and xylene cyanol). Samples were separated on a 5% denaturing polyacrylamide gel (4% to separate products of the hydrolysis reaction) and the products localized by UV shadowing or staining with ethidium bromide. RNA was recovered from gel slices as described previously (von Ahsen *et al.*, 1992) and precipitated in the presence of 5 μg tRNA as carrier. RNA was dissolved in 5–10 μl H₂O and methylated adenines and cytidines were detected by primer extension. For detection of the N-7 positions of guanines, aniline-induced strand scission was performed (Peattie, 1979).

Detection of methylated positions by primer extension

Isolated and modified (and unmodified) RNA was subjected to primer extension using [α -³²P]dTTP to label cDNA as described (Stern *et al.*, 1988). For some experiments the primer was 5' end-labeled to reduce background. For detection of adenines and guanines (sequencing lanes), dideoxythymidine and dideoxycytosine were added to the respective tubes

in primer extension. Products were separated on a 6% denaturing polyacrylamide gel and visualized by autoradiography.

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