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Identification of phosphate groups important to self-splicing of the *Tetrahymena* rRNA intron as determined by phosphorothioate substitution

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ABSTRACT

The group I intron from the rRNA precursor of *Tetrahymena* undergoes self-splicing. The intron RNA catalyst contains about 400 phosphate groups. Their role in catalysis has been investigated using phosphorothioate substituted RNA. In such RNA one of the peripheral oxygens of the phosphodiesters is replaced with sulfur. Incorporation of adenosine 5′ phosphorothioate in either the 5′ or 3′ half of the ribozyme blocked splicing whereas incorporation of uridine 5′ phosphorothioate only blocked splicing if the substitution was in the 3′ half of the molecule. Modification-interference assays located two major and three minor inhibitory phosphorothioate substitutions suggesting that the corresponding phosphates play a significant role in self-splicing. These are all located in the most highly conserved region of the intron.

INTRODUCTION

The ribosomal RNA precursor of *Tetrahymena thermophila* contains an intron which is able to catalyze its own excision and the ligation of its flanking exons (1). The sole requirements for catalysis are guanosine (or its 5′ phosphorylated derivatives) and magnesium ions (1). Introns having the same characteristic RNA secondary structure as that of the *Tetrahymena* intron are known as Group I (2−5). Although splicing sometimes depends on proteins in vivo, it is likely that in all Group I introns, RNA is the primary catalyst of the two transesterification reactions involved in splicing. Ultimately therefore, catalysis reduces to the interaction of nitrogenous bases, ribose sugars, phosphate groups and magnesium ions. The role of the nitrogenous bases has been studied by mutational analysis and by probing the structure of the RNA with ribonucleases sensitive to either single or double stranded regions (6,7). These together with phylogenic comparisons have yielded a reliable model of the RNA's secondary structure. Studies on the role of the ribose sugars have shown that the 2′ and 3′ hydroxyl groups of the nucleophilic nucleoside play a crucial role in both catalysis and binding (8−11). The 2′ hydroxyl of the penultimate nucleoside of the 5′ exon may contribute to the binding of the 5′ exon (10).

The role in splicing played by the phosphate groups has however received less attention. This work uses phosphorothioate substituted RNA to show that certain phosphates play an important role in splicing. In phosphorothioate RNA, phosphate groups are replaced by phosphorothioate groups, with the result that one of the peripheral oxygens of the phosphodiester is replaced with sulfur. Phosphorothioate containing RNA is synthesized by including a nucleoside 5′-O-(1-thiotriphosphate) analog (NTPαS) in a transcription reaction instead of or as well as the standard nucleoside triphosphate (NTP). Deeney et al (12) had previously reported that RNA precursors containing adenosine or uridine 5′ phosphorothioates (NpsA or NpsU linkages) could not be spliced, whereas transcripts

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containing NpsG or NpsC linkages retained splicing activity. I have therefore concentrated solely on the inhibitory effects of ATPαS and UTPαS incorporation.

It was desirable to know whether inhibition was caused by phosphorothioate substitution at either a small number of specific sites or a large number of random sites throughout the molecule. Two approaches were taken: a bimolecular assay and a modification interference assay. In the first, the RNA precursor was synthesized in two halves, one of which contained natural phosphate groups and the other either NpsA or NpsU linkages. Since a mixture of the two unmodified ribozyme halves retained splicing activity, the tolerance of the ribozyme to phosphorothioate substitution in each half of the molecule could be investigated. Modification-interference permitted mapping at the resolution of the nucleotide and is described later.

EXPERIMENTAL PROCEDURES

Plasmid Construction

The transcription vector pIBI24 contains a T7 RNA polymerase promoter upstream of a series of multiple cloning sites including EcoRI (promoter proximal) and HindIII. An EcoRI HindIII intron fragment from an M13mp8 clone M.TET.14 (13) was cloned into pIBI24 to give pTT14. The fragment contained 7 and 17 bases of *Tetrahymena* 5′ and 3′ exon sequences respectively plus a small amount of flanking M13mp8 sequence (14). The plasmid pTL21 in which transcription starts at intron base 22 (Fig. 1) to yield the L-21 precursor was constructed from pTT14 using oligonucleotide directed mutagenesis to make the appropriate deletion (15). p3HF was constructed from pTT14 by digesting with EcoRI and BglII (to drop out the 5′ half of the ribozyme), filling in the 5′ single stranded regions with the Klenow fragment of DNA polymerase and religating.

RNA Transcription and Splicing

L-21 precursors and 3′ half RNA fragments were transcribed from pTL21 and p3HF respectively, linearized with PvuII. The PvuII site is 126 bases downstream of the 3′SS. 5′ RNA (with a 5′SS) and L-21 5′ RNA half fragments were transcribed from pTT14 and pTL21 respectively, both linearized with BglII. 10−20 μl transcription reactions contained 40 mM Tris pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 100 μg/μl BSA, 1 u/μl T7 RNA polymerase (United States Biochemical Corp.), 10 mM dithiothreitol, 1 u/μl RNasin (Promega Corp), 40−60 μg/ml DNA, 0.2 mM GTP, 0.4 mM remaining nucleoside triphosphates (unless stated otherwise) and nucleoside thiotriphosphates as required to give the desired ratio. Incubation was at 37°C for 1 hour. Uniform radiolabelling of RNA was accomplished by inclusion of 0.5 μCi ^{32}P-GTP (New England Nuclear). Transcription reactions were treated with phenol, ethanol precipitated and stored at −20°C in 10 mM tris pH 7.5, 0.1 mM EDTA.

Standard splicing conditions for 3′ splicing were 30 mM tris pH 7.5, 10 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, 2.5 mM CpU for 1 hour at 42°C. When 5′SS cleavage was required, 0.2 mM GTP was added without CpU. Splicing was stopped by the addition of 1 volume of loading dye (95% formamide, 20 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol).

RNA purification and RNA electrophoresis

For the modification-interference assays, the RNA was separated on 7M urea, 5.5% polyacrylamide gels. RNA was located by autoradiography. Acrylamide strips were cut out, the RNA eluted overnight in 0.3 M Na-acetate, 20 mM tris pH 8.0, 2 mM EDTA at 4°C, ethanol precipitated and stored as above. Other splicing reactions were also run

on 7M urea, 5.5% polyacrylamide gels. All autoradiography was performed at room temperature without an intensifier screen. RNA was quantified using an UltroScan densitometer (Pharmacia).

Iodoethanol Cleavage

50 – 100 cps (determined with a hand held Geiger counter with the sample in a microfuge tube) of RNA was brought to a volume of 6 μl; 3 μl of loading dye (see above) containing 7% iodoethanol (Sigma) was added and the sample incubated for 3 minutes at 95°C and then placed on ice (16). Samples were run on a 7M urea 6% polyacrylamide gel. The gel was dried down and subjected to autoradiography at room temperature without an intensifier screen for 7 – 14 days (overnight exposures could be obtained at −70°C with an intensifier screen).

Nucleotide Purity

High purity nucleoside triphosphates were obtained from Pharmacia and the concentrations checked spectrophotometrically. ATPαS and UTPαS solutions were obtained from Du Pont New England Nuclear. The manufacturer reported that the ATPαS had 1.2% ATP contamination, the UTPαS had a negligible UTP contamination, and the ATPαS and UTPαS contained 0.1% of the unincorporable Rp isomer.

5′ end Labelling

5′ end labelling of RNA treated with bacterial alkaline phosphatase was not very efficient and resulted in considerable RNA degradation. Transcripts were therefore synthesized in the presence of similar amounts of guanosine and GTP. Sufficient guanosine was incorporated at the 5′ end to directly label an adequate fraction of the RNA using T4 kinase but unfortunately, pTL21 transcripts initiated with guanosine at two points, three bases apart; presumably at G_{22} and G_{25} as there is no G base at position −3 of the T7 promoter. (As both transcripts spliced equally well, this implies that the 5′ exon substrate substitute, CpU, can utilize an internal guide sequence truncated by 3 bases). p3HF transcripts were however successfully labelled this way as follows: transcription reactions were performed as described above but included 0.4 mM guanosine. The RNA was then phenolised, passed through a G-50 spun-column (17) and ethanol precipitated. Half the preparation was incubated in 10 μl of 50 mM tris pH 8.0, 10 mM $MgCl_2$, 1 mM spermidine, RNasin (1 u/μl), 5 mM DTT, 3 units T4 kinase with 50 μCi of gamma ^{32}P-ATP (5,000 Ci/mmol, Amersham) for 30 min. at 37°C. pTL21 transcripts were synthesized as described but the GTP concentration was lowered to 0.1 mM and the reactions performed in 15 μl with 80 μCi gamma ^{32}P-GTP (5,000 Ci/mmol, Amersham) to label the 5′ end (16).

RESULTS

In order to perform the modification interference assay more easily, splicing was assayed throughout this work by monitoring 3′ splice site cleavage of a 5′ truncated form of the RNA precursor. This was generated from plasmid pTL21. The RNA lacked the 5′ splice site and the first 21 bases of the intron but retained the 3′ splice-site (3′SS) and 126 bases of 3′ exon (Fig. 1). Normal splicing occurs as follows. The 5′ exon binds to an internal guide sequence 5′ GGAGGG 3′ (the first base here is G_{22} the start of the L-21 truncated precursor), the 3′ hydroxyl of a guanosine cofactor cleaves the 5′ splice-site via a transesterification reaction, the 3′ hydroxyl of the 5′ exon then attacks the 3′SS in another transesterification reaction to generate ligated exons and the released intron. The second reaction can be duplicated by mixing the 5′ exon in trans with either the complete RNA precursor, the intermediate consisting of intron plus 3′ exon, or the L-21 5′ truncated

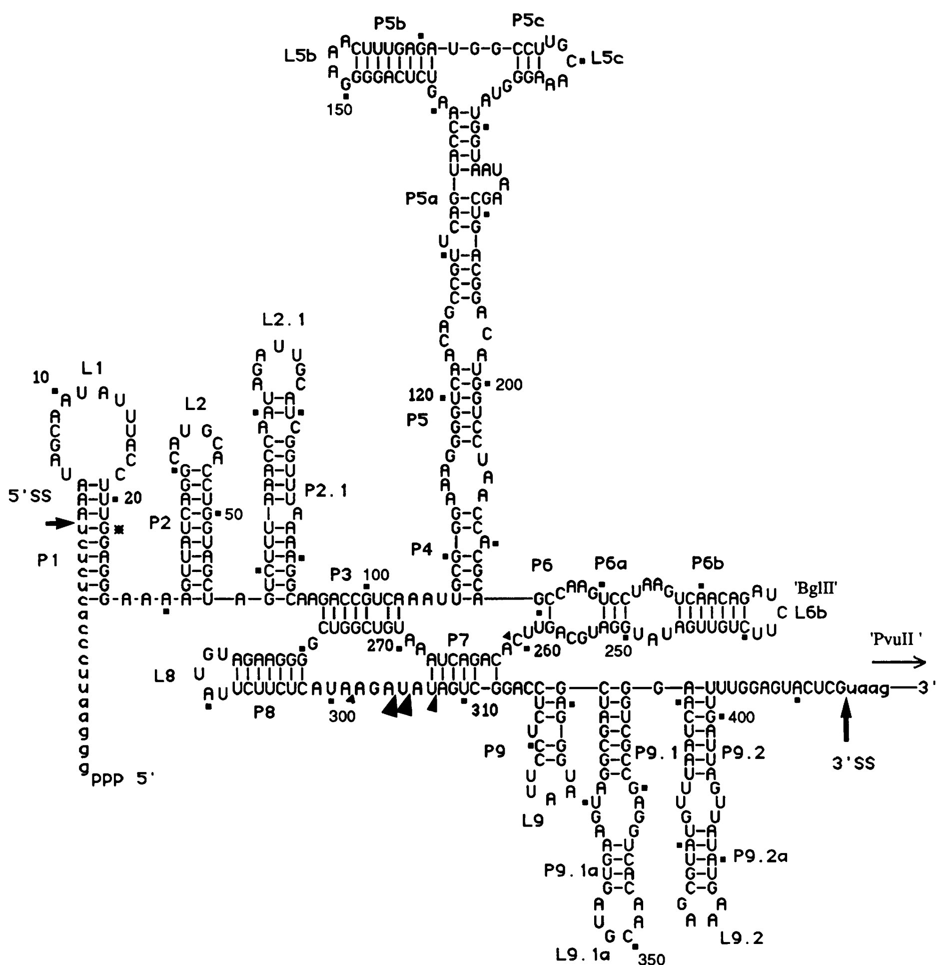

Fig.1. Secondary structure of the *Tetrahymena* group I intron: sites of phosphorothioate inhibition. Upper case is intron sequence, lower case exon sequence. The T7 RNA polymerase transcript from pTT14 is shown; the asterisk shows the first base of the L-21 precursor transcript (G_{22}), produced from pTL21. The PvuII site used to linearize many of the transcription templates is 126 bp downstream of the site corresponding to the 3'SS. Arrowheads indicate the sites where phosphorothioate substitution interfered with splicing: the larger the arrowhead, the greater the interference.

precursor. Even the dinucleotide CpU can act in trans as the 5′ exon (18). In the absence of other preferred 5′ exon substrates, the 3′ end of the 3′ exon is able to loop around, bind to the internal guide sequence like the 5′exon and attack the 3′SS to release a circular 3′ exon (13). The latter reaction occurs to some degree during transcription of pTL21. In this work the standard 3′SS splicing reaction contained CpU and the L-21 5′ truncated precursor.

Table 1. Inactivity of phosphorothioate containing RNA under a variety of reaction conditions.

Reaction	GTP	CpU	Spermidine	Mg^{++} (mM)	pH	Temp. °C	Time (hr)
1	+	−	−	5	7.5	37−50	4
2	−	+	−	5	7.5	42	4
3	+	−	+	5	7.5	50	2
4	−	+	+	5	7.5	42	4
5	+	−	−	25	7.5	50	2
6	−	+	−	25	7.5	42	4
7	+	−	−	25	9.0	50	2

L-21 RNA precursor containing either NpsA or NpsU linkages was incubated in 100 mM ammonium sulfate, 40 mM tris and the variables shown. GTP was 200 μM; CpU 2.5 mM; spermidine 2 mM.

Incorporation of either ATPαS or UTPαS into the intron RNA was reported to totally block splicing (12). As the complete assay conditions were not reported, a search was made for conditions which might nevertheless permit splicing of phosphorothioate modified RNA. These included variations in time, temperature, magnesium ion concentrations, pH and the presence of GTP (GTP can directly attack a 3′SS if provided in trans (19)). None of these conditions promoted splicing activity at the 3′SS (Table 1).

ATPαS or UTPαS incorporation could have inhibited splicing in a cumulative manner or by inhibiting the function of key phosphate groups. In either case it was important to determine the relationship between splicing activity and the ratio of normal to phosphorothioate diester bonds. L-21 truncated RNA precursor was transcribed in the presence of various ratios of either ATP to ATPαS or UTP to UTPαS and then assayed for 3′SS splicing using the dinucleotide CpU as the 5′ exon (Fig. 2). I have assumed through this work that T7 RNA polymerase has approximately the same affinity for a nucleoside triphosphate as it has for its thiophosphate analog. The apparent K_M for the incorporation of ATPαS by T7 RNA polymerase is the same as that for the incorporation of ATP (20).

The degree of splicing was quantified and plotted against the ratio of NTP to NTPαS (Fig. 3). Unmodified RNA was 99% processed by the end of the incubation period of 1.25 hours. If one makes the hypothesis that there are three positions, incorporation of

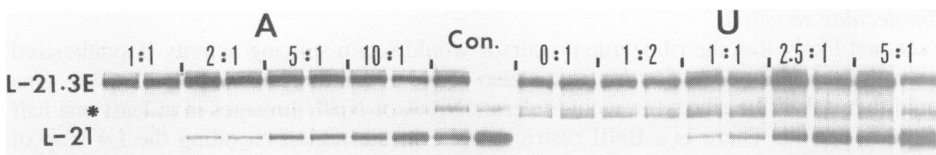

Fig.2. Splicing activity of RNA partially substituted with phosphorothioate. RNA was synthesized in the presence of varying ratios of NTP to NTPαS where N equals adenosine or uridine. For each ratio, the left most lane is the RNA after transcription and the right most, after 1.25 hr under splicing conditions, which were: 40 mM tris pH 7.5, 10 mM $MgCl_2$, 2.5 mM CpU at 42°C. L-21.3E is the L-21 precursor (517 bases), starting at base G_{22} and ending 126 bases downstream of the 3′SS. The products of the reaction are L-21 (393 bases) and CpU attached to the 3′exon (128 bases) (not shown). Con indicates an unmodified control reaction. * indicates an unknown contaminating RNA from the transcription reaction. About 1% splicing activity was detected in the reaction in which the ratio of UTP to UTPαS was 1:2.

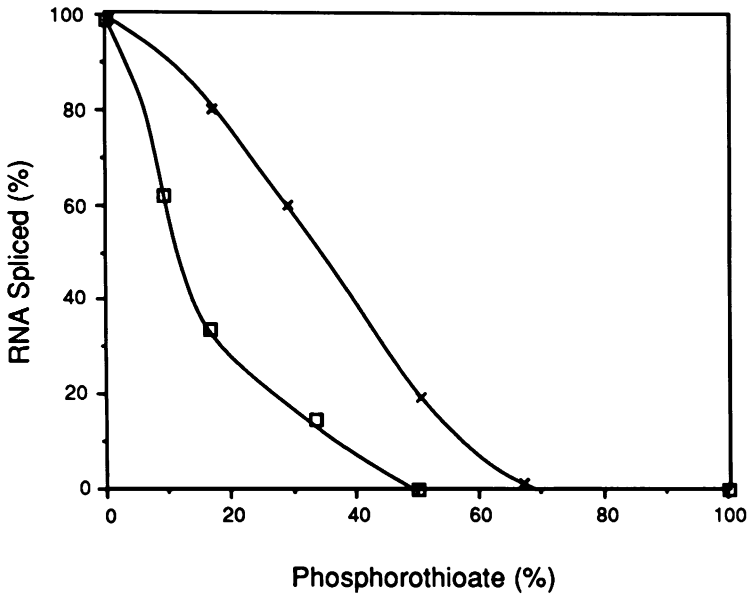

Fig.3. Relationship of splicing activity to phosphorothioate incorporation. The data from Fig. 2 was plotted, making the assumption that phosphorothioate analogues are incorporated as efficiently as normal nucleotide triphosphates. □ signifies ATPαS and X signifies UTPαS. '% RNA spliced' includes RNA spliced during either the transcription or the splicing reaction.

a phosphorothioate at any one of which totally blocks splicing, and applies this to the RNA synthesized using a ratio of UTP to UTPαS of 1:1 (Fig.3), one would expect no more than approximately 12.5% of the transcripts to splice (25% for two positions). As at least 20% of the transcripts reacted there must be very few points at which incorporation of UTPαS has a highly inhibitory effect. ATPαS incorporation was more inhibitory than incorporation of UTPαS. For example when the ratio of ATP to ATPαS was 1:1 there was no detectable splicing.

Bimolecular reactions

It seemed likely that the ribozyme precursor would retain splicing activity if synthesized in two halves (the phage T4 *td* intron is also active as a bimolecular complex (28)). One could then ask if the ribozyme would tolerate NpsA or NpsU linkages in at least one half of the molecule. There is a BglII restriction site in the region encoding the L4 loop of the P6 stem (Fig. 1). A plasmid p3HF was constructed by fusing this BglII site to the EcoRI site four bases downstream of the T7 promoter of the transcription vector pIBI24 (Fig. 1). Transcripts from p3HF linearized with PvuII contained 188 bases of the 3′ half of the intron and 126 bases of 3′exon. The 5′ half of the RNA was transcribed from pTL21, linearized with BglII to give an L-21 5′ fragment of 216 bases. A 5′ fragment with an intact 5′SS was generated from pTT14 linearized with BglII (Fig. 1)—this had 17 bases of 5′exon and 236 bases of intron. 5′ and 3′ RNA fragments were synthesized in which all NpA (or NpU) linkages were replaced by NpsA (or NpsU). These were then mixed with the complementary unmodified RNA fragment and assayed for splicing activity (Fig 4).

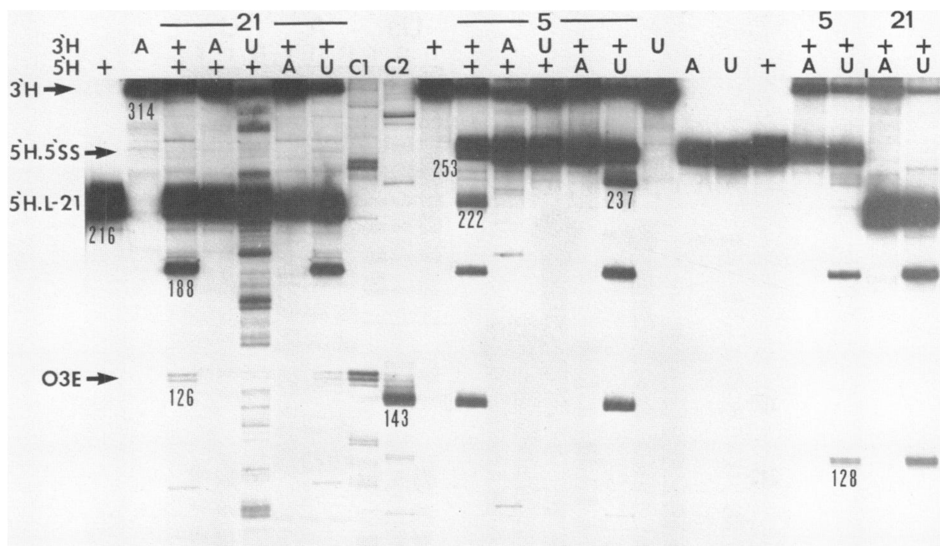

Fig.4. Bimolecular reactions of phosphorothioate substituted RNA. Reaction conditions were 30 mM tris pH 7.5, 10 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, 0.2 mM GTP. The concentration of each RNA half fragment was 10–50 nM. Incubations were for 30 min at 50°C and then 45 min. at 42°C. 5.0 mM CpU was included and GTP excluded in the last four reactions on the right. 5'H and 3'H indicate 5' and 3' half RNAs – the substitutions were as follows: A = ATPαS, U = UTPαS, + = unsubstituted. There are several control lanes with only one of the half RNAs. C_1 and C_2 are unimolecular controls of the L-21 precursor and full precursor respectively. 21 and 5 signify the bimolecular reactions which contained either the L-21 5' half molecule (5'H.L-21) or the full 5' half molecule containing a 5'SS (5'H.5'SS). The 3' fragment of 314 bases reacts to give 188 bases of intron RNA and either a circular 3' exon of 126 bases (03E) if reacted with L-21 5' half RNA, or ligated exons (143 bases) if reacted with complete 5' half RNA, or CpU linked to the 3'exon (128 bases) if reacted with CpU and either of the two 5' half RNAs. L-21 5' half RNA (216 bases) does not react: The complete 5' half RNA (253 bases) reacts to give 237 bases of intron RNA (see also below) and the 5' exon (17 bases) which eventually reacts to give ligated exons (143 bases). Other uncharacterized reactions also occured, probably at cryptic sites downstream of the 3' SS. Bands in the 5th lane are due to degradation not splicing.

In the normal splicing reaction, the excised intron circularizes as a result of a transesterification reaction between the end of the intron and the phosphodiester bond between bases 15 and 16. The circle can then reopen to give a linear molecule of 399 bases. In the bimolecular reaction the unmodified 188 and 237 base RNA molecules 'circularized' to produce a linear fragment of 410 bases (not shown) which upon 'reopening' yielded 5' and 3' fragments of 222 bases and 188 bases respectively. The reaction involving 5' RNA with a 5' SS contained NpsU linkages and unmodified 3' RNA underwent a low level of circularization (not shown) but no reopening. The transcription reactions generated several uncharacterized contaminating RNAs and so the gel has been cropped to exclude them—this also excludes bands in lanes C1 and C2 and the 'circular' RNAs described above.

The most striking result was that UTPαS incorporation into the 3' half of the molecule blocked both 5' and 3' splicing whereas UTPαS incorporation into the 5' half of the molecule permitted both 5' and 3' splicing (the presence of fragments of either 222 or 237 bases is indicative of 5' splicing—see legend to Fig. 4). More than 50% of the RNA reacted under the conditions used. Neither of the reactions involving NpsA substituted RNA showed any detectable cleavage, indicating that the ribozyme cannot tolerate complete substitution of all NpsA linkages in either half of the molecule. As the concentration of NpsA substituted L-21 5' half RNA was low, a splicing assay was repeated with 8 times more RNA; no

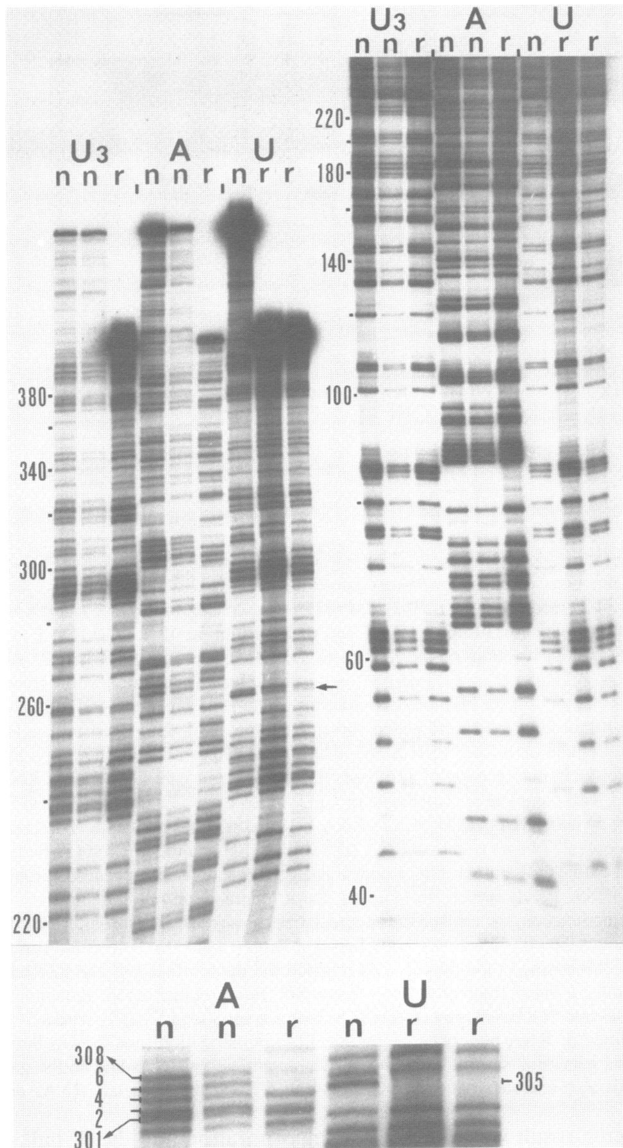

Fig.5. Modification-interference assay of L-21 truncated precursor. Transcription reactions contained 10:1 ATP to ATPαS and either 10:1 or 3:1 (labelled U_3) UTP to UTPαS. The A reaction was about 40% complete. The 10:1 and 3:1 U reactions were 70% and 15% complete respectively. **n** signifies non-reacted material and **r** reacted material. To aid sample comparison two different amounts of RNA were loaded for the non-reacted A, the non-reacted 3:1 U and the reacted 10:1 U samples. The sequence is numbered as shown in Fig. 1 (dots are every 20 bases). A region around 300 has been magnified (the 3:1 U reaction has been omitted to facilitate labelling of the A reactions).

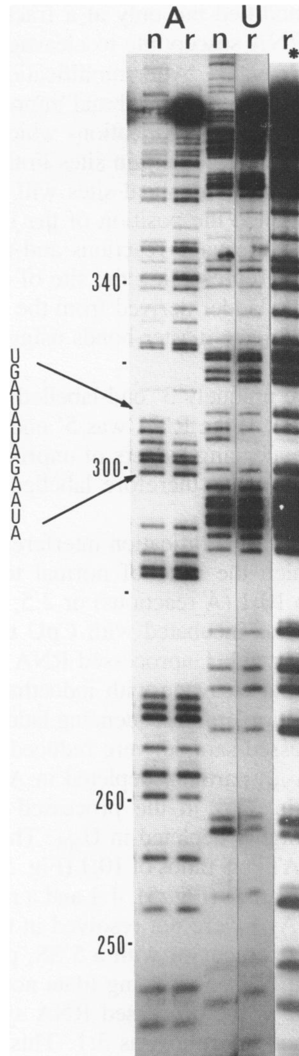

Fig.6. Bimolecular modification interference assay. * The second reacted RNA for the U experiment is the same as the first reacted RNA but run on another gel and exposed for a longer time.

splicing activity was observed (data not shown). Control reactions, using unsubstituted RNA, in which the concentration of 5′SS containing 5′ half molecules was reduced 10 fold, still went to greater than 50% completion.

Modification-interference

The modification-interference assay was developed for the analysis of RNA processing by Conway and Wickens (21) and Rymond and Rosbash (22). A subset of chemical groups

in the RNA to be studied is modified but only at a fraction of the sites available. The modification must render the RNA susceptible to cleavage by a specific chemical while unmodified groups remain uncleavable. After modification the RNA is incubated in the appropriate processing reaction. The processed and unprocessed material are separated, purified and then chemically cleaved. Modifications which block processing will not be present in processed RNA and therefore certain sites in the processed RNA will remain chemically uncleaved; in contrast the identical sites will be cleaved in the unprocessed material. If the RNA is end-labelled the position of the inhibitory modifications can be located by limiting the chemical cleavage reactions and then resolving the products on a sequencing gel system. Bands, representing the site of an inhibitory modification will be absent in the RNA sequencing ladder derived from the processed RNA. A method for specifically cleaving phosphorothioate diester bonds using iodoethanol has recently been developed by Gish and Eckstein (16).

The intron RNA could not be uniquely 3′ end-labelled because the run-off transcripts did not have precise 3′ ends (13). If the RNA was 5′ end-labelled it was not possible to assay 5′ splicing because the sequencing ladders of unprocessed and processed RNA did not start at the same point. RNA was therefore labelled at the 5′ end and 3′ splicing performed.

On the basis of the data in Fig. 2, modification interference was performed using RNA transcribed in reactions in which the ratio of normal to phosphorothioate nucleoside triphosphate varied from 5:1 to 10:1 (A reactions) or 2.5:1 to 10:1 (U reactions). 5′end-labelled L-21 precursor RNA was incubated with CpU to promote 3′ splicing and the reaction loaded onto a denaturing gel. Unprocessed RNA and cleaved L-21 intron RNA were purified, subjected to partial cleavage with iodoethanol and the RNA resolved on a sequencing gel (Fig. 5). Inspection of the sequencing ladder showed that a small number of bands present in the unprocessed sample were reduced in the processed RNA. NpsA RNA was highly depleted in A_{306}, partially depleted in A_{308} and marginally depleted in A_{302} and A_{261}. The increase in A_{314} in the processed RNA was not seen in other experiments. NpsU RNA was highly depleted in U_{305}. These trends were observed with RNA synthesized using ATP to ATPαS ratios of 10:1 (Fig. 5), 6:1 and 5:1 (data not shown) and UTP to UTPαS ratios of 10:1, 3:1 (Fig. 5), 4:1 and a repeat of 10:1 (data not shown). The first five A bands (A_{24} to A_{31}) were not resolved in these experiments; however in a experiment using the full RNA precursor with a 5′SS, phosphorothioate incorporation in these positions did not inhibit 5′ or 3′ splicing (data not shown). Relative to U_{259} and other bands, U_{258} was depleted in the processed RNA synthesized using 10:1 UTP to UTPαS but barely changed when the ratio was 3:1. This anomaly was pursued further in a series of control reactions.

There was no significant difference in the cleavage banding pattern between RNA which was subjected to splicing conditions but remained unprocessed and RNA which was never incubated in splicing conditions—similar observations were made by Conway and Wickens (1987) using a different modification interference assay. The extent of cleavage by iodoethanol was difficult to control particularly if the sequence of the entire RNA molecule was required. In order to load comparable amounts of processed and unprocessed RNA, different percentages of the total yield of purified RNA had to be loaded (eg: 10% and 50%). To assess the consequences of some of the possible variables, preparations of RNA, synthesized in the presence of either 5:1 ATP to ATPαS or 2.5:1 UTP to UTPαS, were cleaved over a five fold range in RNA concentration, a four fold range in iodoethanol

concentration, in the presence of an approximately 100 fold excess of unlabelled tRNA and in 5mM DTT. The presence of either tRNA or 5mM DTT slowed cleavage of the NpsA RNA but had less effect on the NpsU RNA. The sequence ladders were inspected for bands whose intensities with respect to those of their neighbors varied with the different treatments. There were a few small fluctuations but U_{258} varied significantly over a 2 to 3 fold range. For this reason and the discrepancy between the two U reactions shown in Fig. 5, the apparent depletion in U_{258} in the processed RNA is probably primarily an artefact.

Bimolecular modification interference assays

The modification interference assay was also performed using the bimolecular reactions and phosphorothioate substituted 3′ half RNA molecules. 5′ end labelled 3′ half RNA was synthesized using either 5:1 ATP to ATPαS or 2.5:1 UTP to UTPαS. The RNA was incubated under 3′ splicing conditions with unmodified 5′ half RNA. The reactions went to 15 and 20 percent completion which was somewhat lower than the unimolecular reactions. In general the modification interference pattern corresponded extremely well to that obtained in the unimolecular reactions (Fig. 6).

DISCUSSION

The group I intron from the rRNA precursor of *Tetrahymena* undergoes self-splicing in vitro in the absence of any protein. Binding sites for the splice-site substrates, the catalytic site(s) and the stability of the overall ribozyme structure must therefore be entirely provided by the interactions of nitrogenous bases, ribose sugars, phosphate groups and magnesium ions. Using a crude and a fine assay I have identified some of the phosphate groups within the core of the RNA that are likely to play an important role in splicing. Bimolecular reactions showed that splicing could be inhibited by substituting phosphorothioate groups 5′ to uridine in the 3′ half of the molecule but not the 5′ half. The modification-interference assay suggests that phosphorothioate substitution of the phosphate 5′ to U_{305} contributed significantly to this effect. However the virtual inactivity of the RNA synthesised in the presence of 1:2 UTP to UTPαS (Fig. 2) indicates that the U_{305} phosphorothioate could not be solely responsible for the inactivity of fully substituted NpsU RNA. Incorporation of a phosphorothioate group at the 3′SS (GpsU) could also have had a major effect. This could not be addressed in this work, but it is known that substitution of a phosphorothioate group at the 5′SS does not inhibit splicing (23) unless the intron and assay are modified (24).

Bimolecular analysis showed that inhibitory NpsA linkages mapped in both 5′ and 3′ halves of the molecule. Modification-interference assays only identified specific sites of inhibition in the 3′ half of the molecule. Buzayan et al (25) have investigated the effects of phosphorothioate substitution on autolytic processing of a small satellite RNA of tobacco ringspot virus. Only ATPαS incorporation significantly blocked cleavage. While this was primarily attributed to the presence of a phosphorothioate at the CpsA cleavage site, inhibition at other sites was not ruled out.

Given the similarity of the 5′ and 3′ transesterification reactions, it is likely that a similar modification-interference pattern will be seen for 5′ splicing. A single experiment using ATPαS only, revealed no extra inhibitory phosphorothioate groups specific to the 5′SS (data not shown). As the processed RNA underwent both 5′ and 3′ splicing, the experiment would not have identified phosphorothioate substitutions which permitted 5′ splicing but blocked 3′ splicing. At the gel resolution obtained there was only a single band at the

apparent position of A_{301} and A_{302}. This was reduced in intensity in the processed RNA but the relative reduction in the bands could not be determined.

T7 RNA polymerase only polymerizes the Sp isomer of nucleoside alpha-thiotriphosphates and in so doing inverts the phosphorothioate group to the Rp isomer (20). This work has therefore only studied the consequence of replacing the pro-R oxygen of phosphate groups with sulfur.

Significance of the sites of the inhibitory phosphorothioate diesters

The two major inhibitory phosphorothioate substitutions in the entire ribozyme map adjacent to each other. There are also minor inhibitory effects in neighboring phosphates. Furthermore both phosphates map in the middle of one of the only two highly conserved sequences (known as R, bases 257 to 270 and S, bases 301 to 312) in group I introns. These are thought to be close to, or part of the catalytic site of the ribozyme. The 3′ half of sequence S is base paired with part of sequence R (pairing P7 in Fig. 1); the 5′ half is thought to be primarily single stranded. U_{305} and A_{306} lie at the junction of the single and double stranded regions. U_{305} and A_{306} are conserved in 37 and 35 respectively of 42 group IB introns (5). It is possible that U_{305} is paired with A_{269}, making A_{306} a single 'bulged' nucleotide. Sequence analysis suggests that this structure is probably not a conserved feature of all group IB introns (5). Furthermore it is not likely to be stable because A_{269}, A_{308} and A_{306} are partially susceptible to modification by chemicals which react with unpaired A bases (7). Mutational analysis has led to the suggestion that splicing may require destabilising the P7 paired region (26).

Mode of inhibition by phosphorothioate substitution

ATPαS incorporation inhibited splicing in an apparently gradual and accumulative manner in the 5′ half of the molecule. Any incremental inhibition in the 3′ half of the molecule was overshadowed by inhibition at a small number of specific sites. The causes of these two types of inhibition probably differ in nature rather than merely in degree. The substitution of a sulfur for an oxygen could have various consequences. Sulfur is somewhat larger and therefore could disrupt RNA tertiary structure in a gradual and accumulative way if present in several adjacent phosphates. Helix destabilisation is unlikely to explain the accumulative effect of ATPαS incorporation because RNA duplexes of poly r(A−U) (alternating copolymer of A and U) and totally phosphorothioate substituted poly r(A−U) have the same T_m (27).

Modification of the phosphate groups at the specific sites detected by modification interference probably disrupts a critical region of tertiary structure. One such region might be the part of the core structure that interacts with the internal guide sequence. This region has not yet been identified, but it may be close to the sequence spanned by U_{305} and A_{308} (29). The sulfur of a phosphorothioate group binds less well to magnesium than the oxygen of a phosphate group (30). Sulfur substitution also alters the charge distribution around a phosphate group although there is some debate as to the extent and nature of this alteration (31). The peripheral oxygens of phosphate groups play an important role in tRNA tertiary structure. They contribute to the stability of a characteristic sharp turn structure, found in the anticodon and pseudouridine loops (32). They also contribute to the binding of several magnesium ions: some oxygens interacting directly, and others indirectly via water molecules (some of the magnesium ions are coordinated to adjacent phosphate groups) (33). The phosphate groups singled out by the modification-interference assay may well play such a role in self-splicing. Disruption of a single interaction between a phosphate

group's peripheral oxygen and either the rest of the ribozyme or a magnesium ion is unlikely to totally block self-splicing but would possibly be detected in the modification interference assay.

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