Cell, Vol. 31, 147–157, November 1982, Copyright © 1982 by MIT

Self-Splicing RNA: Autoexcision and Autocyclization of the Ribosomal RNA Intervening Sequence of Tetrahymena

Kelly Kruger, Paula J. Grabowski, Arthur J. Zaug, Julie Sands, Daniel E. Gottschling and Thomas R. Cech
Department of Chemistry
University of Colorado
Boulder, Colorado 80309

Summary

In the macronuclear rRNA genes of Tetrahymena thermophila, a 413 bp intervening sequence (IVS) interrupts the 26S rRNA-coding region. A restriction fragment of the rDNA containing the IVS and portions of the adjacent rRNA sequences (exons) was inserted downstream from the *lac* UV5 promoter in a recombinant plasmid. Transcription of this template by purified Escherichia coli RNA polymerase in vitro produced a shortened version of the pre-rRNA, which was then deproteinized. When incubated with monovalent and divalent cations and a guanosine factor, this RNA underwent splicing. The reactions that were characterized included the precise excision of the IVS, attachment of guanosine to the 5′ end of the IVS, covalent cyclization of the IVS and ligation of the exons. We conclude that splicing activity is intrinsic to the structure of the RNA, and that enzymes, small nuclear RNAs and folding of the pre-rRNA into an RNP are unnecessary for these reactions. We propose that the IVS portion of the RNA has several enzyme-like properties that enable it to break and reform phosphodiester bonds. The finding of autocatalytic rearrangements of RNA molecules has implications for the mechanism and the evolution of other reactions that involve RNA.

Introduction

In some species of Tetrahymena, a genus of ciliated protozoa, all copies of the rDNA are interrupted by a 0.4 kb intervening sequence (IVS; Wild and Gall, 1979; Din and Engberg, 1979). The IVS is transcribed as part of the rRNA precursor and then removed in the nucleus by a cleavage–ligation process termed RNA splicing (Cech and Rio, 1979; Din et al., 1979). In these respects the Tetrahymena IVS resembles the intervening sequences that interrupt other eucaryotic genes for mRNA, tRNA and rRNA (reviewed by Abelson, 1979; Breathnach and Chambon, 1981; Cech et al., 1982). Because a large amount of pre-rRNA goes through the same splicing pathway and because the splicing reaction can be manipulated in isolated nuclei or nucleoli, it has been possible to examine many of the features of splicing in this system. The IVS is excised as a unique linear molecule that is subsequently converted to a circular form (Zaug and Cech, 1980; Carin et al., 1980; Grabowski et al., 1981). The linear IVS RNA has a 5′-terminal guanosine residue that is not encoded by the DNA (Zaug and Cech, 1982; Kan and Gall, 1982) but that is added to the RNA during excision (Cech et al., 1981). The requirement for the guanosine cofactor and the lack of an energy requirement for the reaction led us to propose a topoisomerase-like phosphoester transfer mechanism for pre-rRNA splicing (Cech et al., 1981). We have recently found that cyclization of the IVS also involves linked cleavage and ligation of RNA, providing strong support for the phosphoester transfer mechanism (A. J. Zaug, P. J. Grabowski and T. R. Cech, manuscript submitted).

While the mechanism of pre-rRNA splicing was being clarified, the nature of the activity responsible for splicing remained perplexing. We were able to purify an active form of the pre-rRNA that excised and cyclized its IVS without the addition of any protein (Cech et al., 1981). We called this RNA a splicing intermediate, because one explanation for its activity was that an enzyme was tightly associated with the RNA. The activity survived SDS–phenol extraction, boiling in SDS and extensive treatment with proteases, so the putative enzyme would have had to be unusually stable (Cech et al., 1981). We discussed another possibility, that the reactions could be mediated by the folded RNA molecule itself. More recently, we attempted to detect a protein on the RNA by radiochemical labeling or by a density shift of the RNA in a Cs_2SO_4 gradient. We found no evidence for any protein attached to the RNA (B. Bass, A. Zaug and T. Cech, unpublished results). Furthermore, S1 nuclease mapping experiments showed that the "splicing intermediate" can form a continuous RNA–DNA hybrid through both splice junctions (P. Grabowski, unpublished results), as would be expected if the "intermediate" were simply the unspliced pre-rRNA.

These results lent credence to the possibility that the splicing activity was intrinsic to the structure of the ribonucleic acid. We have now tested this hypothesis by synthesizing a portion of the pre-rRNA (the IVS and adjacent coding sequences) in a completely defined plasmid transcription system. We have found that these transcripts, without exposure to any eucaryotic protein, are able to excise and cyclize the IVS and apparently to ligate the exons. RNA cleavage and ligation activity is therefore intrinsic to the structure of the RNA molecule.

Results

Construction of Plasmids for In Vitro Transcription

The 1.6 kb Hind III fragment of T. thermophila rDNA contains the 413 bp IVS and adjacent 26S rRNA-coding sequences (Figure 1A). This fragment was isolated from the plasmid pTT116, which contains two 1.6 kb Hind III fragments cloned in tandem in pBR313. One of the 1.6 kb inserts has the "wild-type" se-

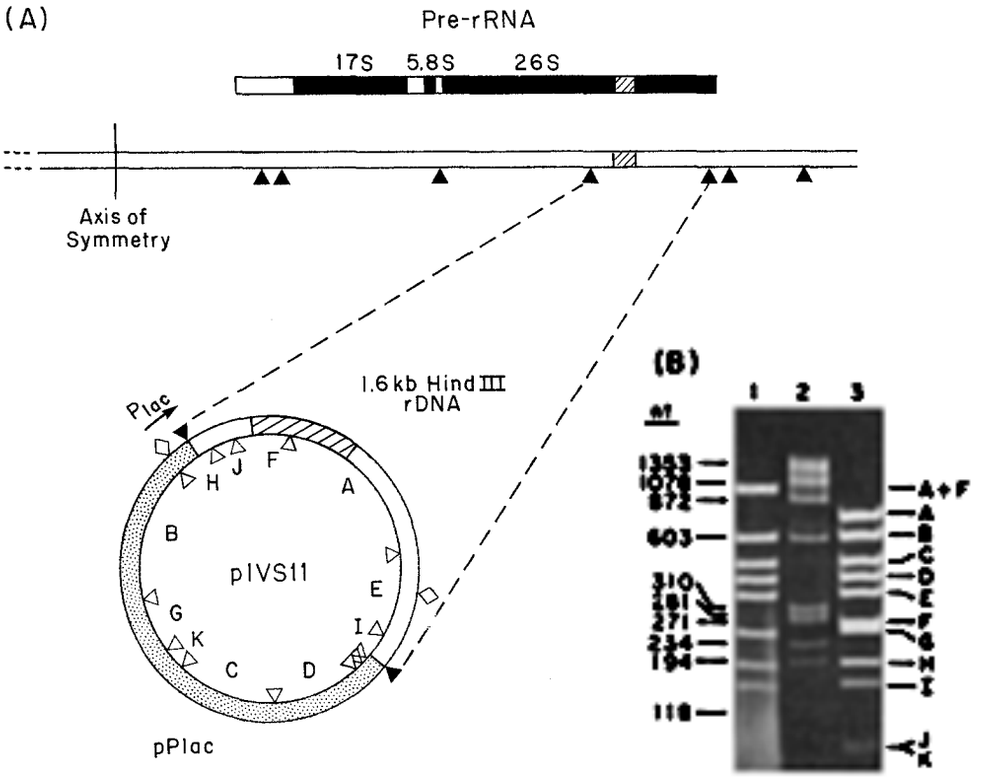


Figure 1. Plasmids Constructed for In Vitro Transcription of a Segment of the Tetrahymena rDNA

(A) A map of half of the palindromic rDNA of T. thermophila (Karrer and Gall, 1976; Cech and Rio, 1979; Din et al., 1979, 1982; Engberg et al., 1980). (Top) Diagram of the primary transcript. Open boxes: transcribed spacers. Solid boxes: mature rRNA sequences. Hatched box: the IVS. (pIVS11) Plasmid containing the 1.6 kb Hind III fragment of the rDNA inserted in the Hind III site of pPlac. Restriction endonuclease sites shown are Hind III (▲), Eco RI (◇) and Hae III (△). The larger Hae III fragments are designated by capital letters. pIVS13 has the same structure as pIVS11 except it is missing the Hae III site within the IVS.

(B) DNA fragments produced by restriction endonuclease treatment of the recombinant plasmids, analyzed by electrophoresis on a 4% polyacrylamide gel. (Lane 1) Hae III digest of pIVS13. (Lane 2) Hae III digest of ϕX174 DNA. (Lane 3) Hae III digest of pIVS11. nt: nucleotides.

quence found in T. thermophila rDNA. The other, "variant" insert is identical except for a heterogeneous region near the middle of the IVS, where it is 7 bp longer (Kan and Gall, 1982). The seven extra base pairs destroy a Hae III restriction site present in wild-type rDNA. The variant sequence probably arose during cloning of the DNA in Escherichia coli, although it is possible that it represents a minor species of Tetrahymena rDNA.

The 1.6 kb fragments were subcloned into the Hind III site of pPlac, which contains the *lac* UV5 promoter–operator region. Clones pIVS11 and pIVS13 each had a single 1.6 kb Tetrahymena insert in the correct orientation with respect to the promoter, as determined by restriction mapping with Eco RI and Hae III. From the Hae III restriction endonuclease pattern (Figure 1B) it could also be seen that pIVS11 contained the wild-type IVS insert and pIVS13 contained the variant IVS. If the pIVS11 template were truncated at the Eco RI site, transcription by RNA polymerase should then produce RNA containing 35 bases of bacterial sequences, 261 bases of the 5′ exon (rRNA sequences preceding the IVS), the 413 base IVS and 624 bases of the 3′ exon (rRNA sequences following the IVS).

Purified plasmid DNA was transcribed in vitro with E. coli RNA polymerase under conditions that were chosen to be in the optimal range for this enzyme (see Experimental Procedures). The RNA made in vitro was then purified by SDS–phenol extraction and analyzed by denaturing polyacrylamide gel electrophoresis. As seen in Figure 2, lanes 1 and 3, transcription of pIVS11 and pIVS13 cleaved with Eco RI resulted in the production of a set of discrete high molecular weight RNAs, which are discussed below. In addition, there were two low molecular weight species that comigrated with the linear and circular forms of the excised IVS RNA. (In this and all subsequent experiments, we detected no difference between the transcripts derived fron the wild-type and the variant plasmid.) Transcription of supercoiled pIVS11 (Figure 2, lane 5) produced a broad smear of high molecular weight RNA, as expected, since the plasmid had no terminator for the *lac* UV5 promoter transcripts. Two discrete transcripts with the size of the IVS were again apparent. Proof that these RNAs are the linear and

circular IVSs is presented below (see Autoexcision of the IVS; Autocyclization of the IVS).

The deproteinized RNA preparations were also incubated at 39°C in a solution containing GTP, $MgCl_2$ and salt, conditions designed to optimize excision and cyclization of the IVS (Cech et al., 1981). As seen in Figure 2, lanes 4 and 6, the total amount of IVS RNA increased only slightly, but there was a marked decrease in the amount of linear IVS and increase in the amount of circular IVS. The results are consistent with the conclusion that the majority of the IVS is excised from the RNA during the 30 min transcription reaction, with very little cyclization taking place because of the low temperature (Grabowski et al., 1981).

Separation of Transcription and Splicing

In previous studies with isolated Tetrahymena nuclei, we found that at low monovalent cation concentrations pre-rRNA transcription proceeded but IVS excision was inhibited (Cech et al., 1981). Now it appeared that in the plasmid transcription system, low monovalent cation concentrations were not inhibiting IVS excision (Figure 2). We therefore tested plasmid transcription by E. coli RNA polymerase with the Tetrahymena nuclear transcription conditions (see Experimental Procedures). The amount of transcription after 30 min was the same as with the buffer system used in the experiment of Figure 2 (300 pmole GMP incorporated into RNA per pmole DNA template). As seen in Figure 3A, lanes 1 and 3, no IVS RNA was produced during a 30 or 60 min transcription reaction. When the RNA samples were deproteinized and then incubated at 30°C with GTP, $MgCl_2$ and salt, excised IVS RNA was produced (Figure 3A, lanes 2 and 4). The polyamines were the components of the Tetrahymena transcription mixture that were mainly responsible for the inhibition of splicing at low salt concentrations, although Ca^{2+} had some inhibitory influence (Figure 3A). Presumably, the polyamines bind to the RNA at low concentrations of monovalent cation and stabilize a structure of the RNA that is not competent to undergo splicing. The polyamines are displaced at high concentrations of monovalent cation. A recent example of such a salt-dependent influence of polyamines on a nucleic acid reaction is described by Krasnow and Cozzarelli (1982).

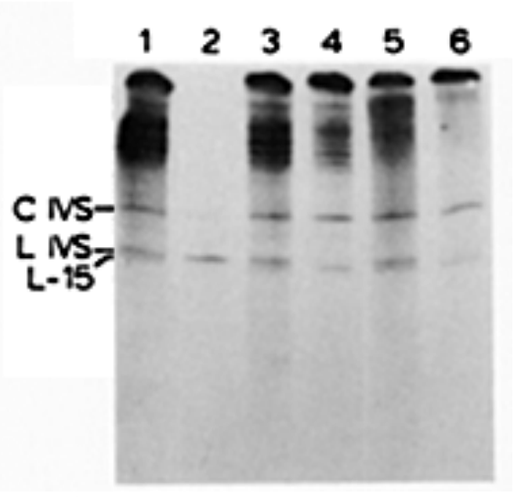


Figure 2. In Vitro Transcription of Plasmids pIVS11 and pIVS13 with Purified E. coli RNA Polymerase

RNA was transcribed with the standard E. coli RNA polymerase transcription buffer (see Experimental Procedures), subjected to electrophoresis on a 4% polyacrylamide, 8 M urea gel at 65°C and visualized by autoradiography. (Lane 1) Transcripts from pIVS13 that had been cleaved with Eco RI. (Lane 2) Markers for linear and circular IVS RNA purified from Tetrahymena nuclear transcripts. (Lane 3) Transcripts from pIVS11 that had been cleaved with Eco RI. (Lane 4) A portion of the RNA in lane 3 was incubated for 30 min with GTP, $MgCl_2$ and salt as described in the Experimental Procedures (In Vitro Splicing). (Lane 5) Transcripts from supercoiled pIVS11. (Lane 6) A portion of the RNA in lane 5 was incubated under in vitro splicing conditions, as described for lane 4. C IVS: circular form of the excised IVS RNA. L IVS: linear form of the excised IVS RNA, the immediate product of IVS excision. L-15: RNA with an electrophoretic mobility that is consistent with a size 15 nucleotides shorter than that of the L IVS. This RNA is not the direct product of splicing. Both nicked C IVS RNA and abortive cyclization products have this mobility (our unpublished results).

In an attempt to identify which of the high molecular weight RNA species contained the IVS, we transcribed RNA under the conditions inhibitory for splicing, fractionated it by polyacrylamide gel electrophoresis and recovered the fractions from the sliced gel. A portion of the RNA from each band was incubated with GTP, $MgCl_2$ and salt. As seen in Figure 3B, several of the large RNA species contained the IVS and were able to excise it when incubated under splicing conditions. One of these precursors (Figure 3B, lanes c) was 1350 nucleotides, the size expected for a run-off transcript from the Eco RI–truncated template. The smaller precursor (Figure 3B, lanes d; 1100 nucleotides) could be the result of premature termination of transcription. The other species all had molecular weights larger than expected. They may arise from the polymerase's turning around at the end of the DNA and transcribing the other strand for variable distances (Oostra et al., 1981).

Autoexcision of the IVS

In previous studies involving pre-rRNA from Tetrahymena nuclei, we showed that excision of the IVS is accompanied by addition of a guanosine nucleotide to its 5′ end (Cech et al., 1981). The ability to separate transcription from splicing provided a convenient way to check for G addition with the plasmid transcripts. RNA was transcribed from the plasmid in the presence of polyamines, with the use of tritiated nucleotides. The RNA was deproteinized, and residual nucleotides were removed by gel-filtration chromatography. The RNA was then incubated under splicing conditions with α-^{32}P-GTP, Mg^{2+} and salt, and chromatographed again to remove unincorporated GTP. Gel electrophoresis and autoradiography revealed one labeled RNA species, which comigrated with linear IVS RNA isolated from Tetrahymena nuclei (Figure 4A). Similar

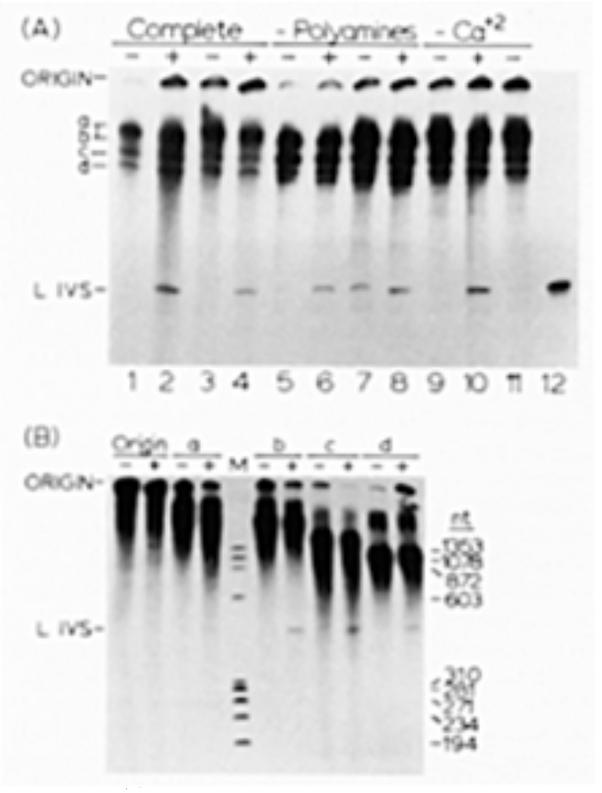


Figure 3. Separation of Transcription and Splicing

(A) RNA labeled during in vitro transcription of Eco RI–cleaved pIVS13 with E. coli RNA polymerase was subjected to electrophoresis on a 4% polyacrylamide, 8 M urea gel. (Lanes −) RNA purified from transcription reaction, no further treatment. (Lanes +) A portion of each purified RNA sample was incubated for 30 min with GTP, $MgCl_2$ and salt, as described in the Experimental Procedures (In Vitro Splicing). (Lanes 1 and 2) RNA transcribed during a 30 min reaction in complete Tetrahymena nuclear transcription mixture, as described in the Experimental Procedures. (Lanes 3 and 4) Same as lanes 1 and 2 except transcription was carried out for 60 min. (Lanes 5 and 6) RNA transcribed for 30 min in Tetrahymena nuclear transcription mixture from which all polyamines were omitted. (Lanes 7 and 8) Same as lanes 5 and 6 except transcription was carried out for 60 min. (Lanes 9 and 10) RNA transcribed for 30 min in Tetrahymena nuclear transcription mixture from which $CaCl_2$ was omitted. (Lane 11) Same as lane 9 except transcription was carried out for 60 min. (Lane 12) Marker for linear IVS RNA purified from Tetrahymena nuclear transcripts.

(B) In vitro splicing with electrophoretically separated species of high molecular weight RNA. RNA was transcribed from pIVS11 under conditions inhibitory for splicing, as in (A, lane 1). The RNA was separated by denaturing gel electrophoresis, and the RNA from the origin and from individual bands (corresponding approximately to those designated a–d in A) was recovered by crushing and soaking of the sliced gel. The RNA samples were then subjected to electrophoresis on a long 4% polyacrylamide, 8 M urea gel without additional treatment (lanes −) or after incubation under in vitro splicing conditions (lanes +). (Lane M) Molecular weight markers are end-labeled Hae III restriction fragments of ϕX174 DNA, denatured prior to electrophoresis.

labeling was obtained with γ-^{32}P-GTP, as would be expected if excision of the IVS from the plasmid transcript occurred by the same pathway described for the Tetrahymena pre-rRNA (Cech et al., 1981).

The GTP-labeled IVS RNA was sequenced by the enzymatic method of Donis-Keller et al. (1977). The results are shown in Figure 4B. The sequence of the first 39 nucleotides was unambiguous, and corresponded exactly to the known sequence of the IVS (Kan and Gall, 1982; Zaug and Cech, 1982). Thus autoexcision of the IVS is precise at the 5′ splice junction. Furthermore, the ability of RNAase T1 and alkali to cleave after the terminal G residue provides evidence that it is linked to the IVS by a normal 3′ → 5′ phosphodiester bond.

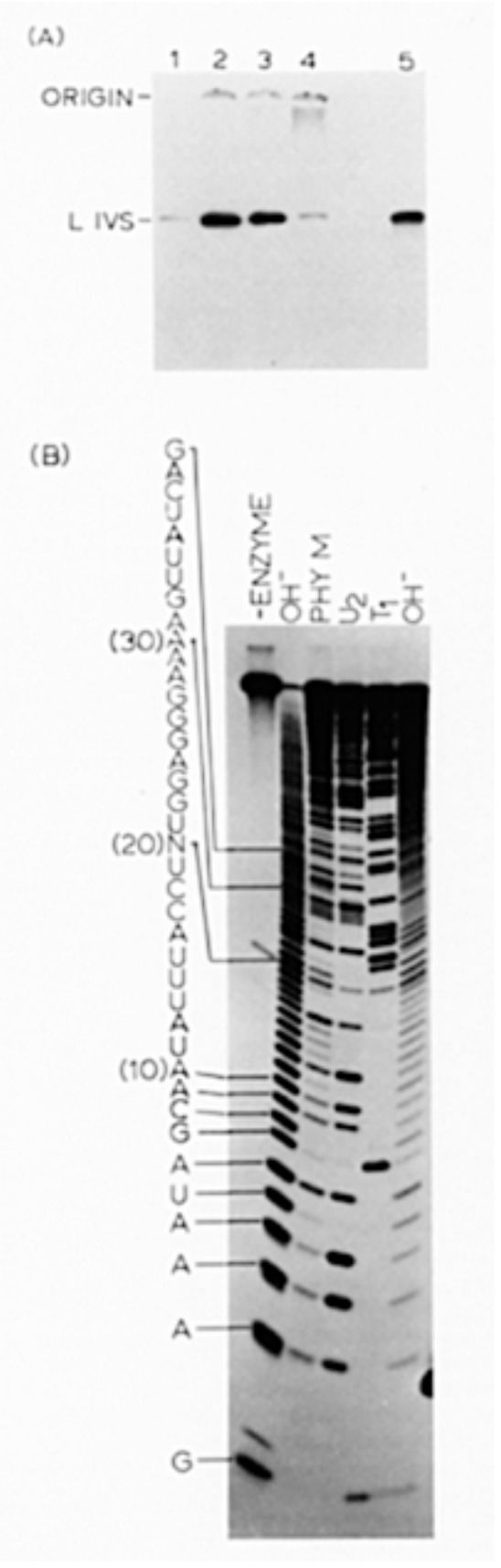
Autocyclization of the IVS

Internally labeled RNA was prepared by in vitro transcription of pIVS11 with α-^{32}P-GTP under conditions that allow IVS excision. The RNA was fractionated by gel electrophoresis, and the linear IVS RNA was eluted from the sliced gel. Incubation of this RNA at 39°C in a Mg^{2+}-containing buffer resulted in the conversion of more than half of the linear IVS to the slower mobility circular form (Figure 5, lanes 4 and 5). Linear IVS RNA produced by transcription–splicing in isolated Tetrahymena nuclei was incubated in parallel under the same conditions. Approximately the same amount of autocyclization was observed (Figure 5, lanes 1 and 2). In other experiments, essentially complete autocyclization has been observed with both the IVS derived from plasmid transcription and the IVS isolated from Tetrahymena nuclei.

The plasmid-derived linear and circular IVS RNAs were also analyzed by T1 fingerprinting (data not shown). The major spots were the same as those identified in the fingerprint of the IVS RNA from Tetrahymena nuclei (Zaug and Cech, 1982), with some additional spots attributed to the contaminating RNA species seen in Figure 5. The fingerprint of the linear IVS did not contain the 5′-terminal pGp identified in the fingerprint of the IVS from Tetrahymena nuclei (Zaug and Cech, 1982). Instead, there was a new spot with a very high mobility in the electrophoretic dimension that was identified as pppGp by secondary digestion. This was an expected result, because in the splicing of the plasmid-derived RNA the only guanosine cofactor available for the excision reaction was pppG and there was no exposure to a pyrophosphatase. The fingerprint of the linear IVS also provided evidence that IVS excision was precise at the 3′ end. The 3′ terminal oligonucleotide (Zaug and Cech, 1982) was clearly present in the fingerprint of the linear IVS (and missing in the fingerprint of the circular IVS, in which it is linked to other sequences and therefore forms a new spot).

Exon Ligation

To test whether the exons were ligated during the IVS excision reaction, we looked for RNA molecules that were protected from nuclease digestion by hybridization to a DNA fragment that spans the ligation



junction (Figure 6A). The approach was similar to the S1 nuclease mapping technique of Berk and Sharp (1977), but differed in that the RNA was labeled, while the DNA to which it was hybridized was unlabeled. We originally chose to have the label in RNA instead of DNA so that we could detect the splicing of the in vitro transcription products in the presence of excess unlabeled RNA that had already been spliced in vivo. Even in systems in which there is no preexisting spliced RNA, this approach has an advantage: the differentiation of free exons from ligated exons does not depend on the ability of S1 nuclease to cleave DNA at a small interruption in an RNA–DNA hybrid (Figure 6B).

RNA was synthesized by in vitro transcription of supercoiled pIVS11 with α-^{32}P-UTP under conditions that largely prevented IVS excision. The purified RNA was then incubated under conditions in which excision occurred or under conditions in which it was prevented (Figure 6C). These RNA samples were hybridized to the 468 bp Hind III Taq I fragment of T. pigmentosa 8ALP rDNA. (The rDNA of 8ALP is highly homologous to that of T. thermophila, but it contains no IVS [Wild and Gall, 1979; Wild and Sommer, 1980]. It therefore serves as a convenient source of a DNA molecule that is continuous through the ligation junction.) The RNA–DNA hybrids were digested with RNAase T1 or S1 nuclease to remove any single-stranded regions of RNA. The nuclease-resistant hybrids were then denatured and subjected to electrophoresis on a denaturing polyacrylamide gel with single-stranded DNA markers. As seen in Figure 6D, several high molecular weight RNAs were nuclease-resistant, whether or not they had been hybridized with the DNA. These bands, presumably double-stranded RNA produced during in vitro transcription, were not analyzed further. Two RNA species (215 ± 9 and 227 ± 9 nucleotides) that were rendered nuclease-resistant only after hybridization with the DNA were unique to the unspliced RNA samples. Their

Figure 4. Autoexcision of the IVS from the Plasmid Transcripts Involves Addition of a Guanosine Nucleotide to the 5′ End of the IVS RNA

(A) Unlabeled RNA was synthesized by in vitro transcription of supercoiled pIVS11 DNA under conditions inhibitory for splicing. The purified RNA was incubated with α-^{32}P-GTP, $MgCl_2$ and salt, then chromatographed by low-speed centrifugation through a small Sephadex G-50 column to remove unincorporated GTP. (Lanes 1–4) RNA from successive washes of the Sephadex column analyzed by denaturing gel electrophoresis and autoradiography (1 hr exposure of wet gel at room temperature). The fourth wash contained substantial amounts of unincorporated GTP, a small fraction of which adhered to nucleic acid near the origin of the gel. (Lane 5) Uniformly labeled linear IVS RNA purified from Tetrahymena nuclear transcripts, run as a marker.

(B) γ-^{32}P-GTP-end-labeled RNA recovered from a gel like that shown in (A) was subjected to RNA sequence analysis. Autoradiograph of the 20% polyacrylamide, 8 M urea sequencing gel is shown. (Lane −ENZYME) RNA incubated under RNAase T1 conditions with enzyme omitted. Other lanes contain RNA subjected to partial alkaline or RNAase hydrolysis, as indicated.

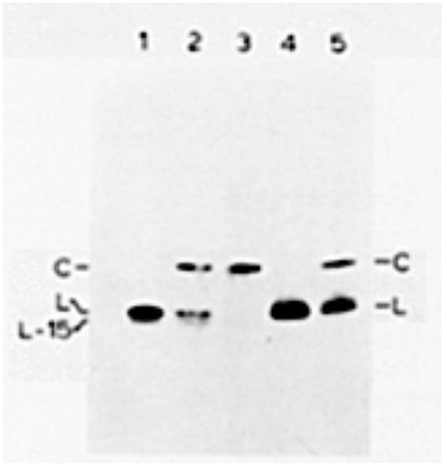


Figure 5. IVS RNA Excised from the Plasmid Transcripts Undergoes Autocyclization

Autoradiograph of a 4% polyacrylamide, 8 M urea gel. (Lane 1) Linear IVS (L) RNA produced by transcription–splicing in isolated Tetrahymena nuclei and purified by gel electrophoresis. RNA was uniformly labeled during transcription. (Lane 2) Tetrahymena linear IVS RNA (lane 1) incubated under autocyclization conditions (20 min at 40°C in 25 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$, 10 mM Tris–HCl [pH 7.5]). (Lane 3) Marker for the circular IVS (C) RNA, previously subjected to RNAase T1 fingerprint analysis (Zaug and Cech, 1982). The sample contains a small amount of nicked circular IVS RNA (L-15). (Lane 4) Linear IVS RNA produced by splicing of plVS11 RNA and purified by preparative gel electrophoresis. A contaminating plasmid transcript, slightly larger than the linear IVS, comprises ~20% of the RNA; fingerprint analysis showed that it was unrelated to the IVS. (Lane 5) Plasmid-derived linear IVS RNA (lane 4) incubated under autocyclization conditions, as described above.

sizes were approximately those expected for protection of the exon segments of the pre-rRNA (207 and 261 nucleotides; Figure 6B). The discrepancy in the size of the larger fragment could be due to a mismatch in the T. thermophila rRNA–T. pigmentosa rDNA hybrid located in the 5′ exon approximately 40 nucleotides downstream from the Hind III site. Two RNA species (409 ± 16 and 478 ± 19 nucleotides) that were barely detectable in the unspliced samples increased greatly in the spliced samples. The 478 nucleotide RNA is the size expected for protection of the ligated exons (Figure 6B). The 409 nucleotide RNA is large enough that it too must be continuous through the ligation junction; its smaller than expected size is consistent with the same region of mismatch postulated above. The small amount of the 409 and 478 nucleotide RNAs in the unspliced samples is explained by the small amount of splicing that occurred during in vitro transcription, as is evident from the presence of the excised IVS RNA in Figure 6C, lane −. In the S1 nuclease experiment, quantitative densitometry of the autoradiograph revealed that the increase in intensity of the 409 and 478 nucleotide bands in the spliced sample was 98% as large as the decrease in intensity of the 215 and 227 nucleotide bands. In the RNAase T1 experiment, the increase in intensity of the 409 nucleotide band in the spliced sample was 94% as large as the decrease in intensity of the 215 and 227 nucleotide bands.

Discussion

Self-Splicing RNA

We have shown that several of the cleavage and ligation reactions involved in the splicing of the Tetrahymena rRNA precursor do not require any enzyme. A segment of the rRNA precursor was transcribed in vitro from a purified plasmid DNA template. The only protein present was purified E. coli RNA polymerase, which was removed by SDS–phenol extraction prior to the splicing reaction. The purified RNA had the intrinsic ability to perform the three cleavage reactions and at least two of the ligation reactions that have been shown to be involved in Tetrahymena pre-rRNA splicing. These are excision of the IVS, covalent attachment of a guanosine nucleotide to the 5′ end of the IVS RNA and cyclization of the excised IVS, which is itself a cleavage–ligation reaction. The excision reaction occurred at precisely the same sites in the plasmid-derived RNA as with RNA synthesized in isolated Tetrahymena nuclei (Zaug and Cech, 1982) or RNA synthesized in vivo (Wild and Sommer, 1980; Kan and Gall, 1982). In a separate study (A. J. Zaug, P. J. Grabowski and T. R. Cech, manuscript submitted) we have shown that cyclization of IVS RNA both from Tetrahymena nuclei and from plasmid transcripts involves release of an oligonucleotide (15 nucleotides) from the 5′ end of the linear molecule. Thus the site of IVS cyclization is also faithfully reproduced by the plasmid-derived RNA.

We also presented evidence that the pre-rRNA transcribed from the plasmid undergoes exon ligation. The complexity of the pattern of plasmid transcripts precluded direct observation of the fate of the exon sequences upon IVS excision. The ^{32}P-labeled transcripts were therefore hybridized with a DNA fragment that spans the splice junction, and the RNA–DNA hybrids were treated with RNAase T1 or with S1 nuclease. A protected RNA molecule the size of the ligated exons was observed only with the plasmid transcripts that had been through the splicing reaction (Figure 6). We have not yet examined the ligation of the exons at the nucleotide sequence level, so this ligation event is not as firmly established as the addition of guanosine to the linear IVS (Cech et al., 1981; Zaug and Cech, 1982) or the cyclization of the IVS (A. J. Zaug, P. J. Grabowski and T. R. Cech, manuscript submitted).

What sequences within the pre-rRNA contain cleavage–ligation activity? Autocyclization occurs with gel-purified IVS RNA, so that reaction must be totally independent of the exons. It is possible that all of the cleavage–ligation activity resides in the IVS. We do not know, however, whether cyclization and splicing are directed by the same RNA sequences (occur in the same active site), so we cannot rule out exon involvement in the latter reaction. We do know that autoexcision takes place with a transcript that contains only 261 nucleotides of the 5′ exon and 624

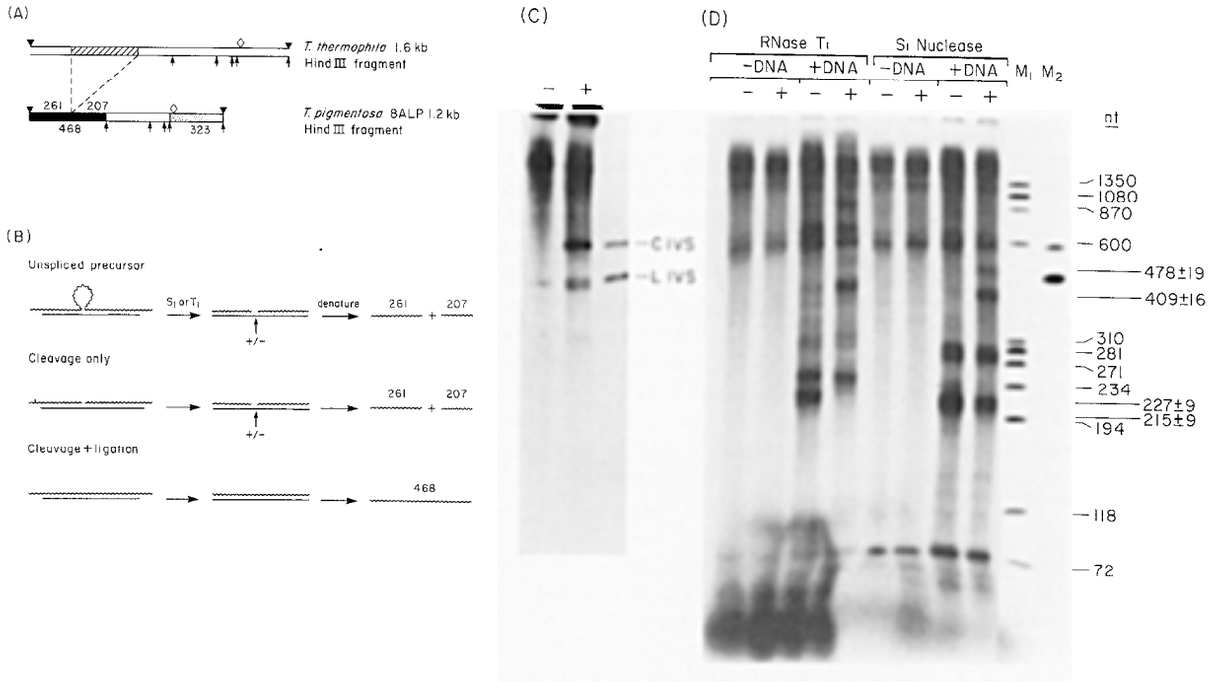


Figure 6. Exon Ligation

(A) Comparison of restriction sites in homologous regions of T. thermophila and T. pigmentosa 8ALP rDNAs, the latter of which does not contain an IVS. Restriction endonuclease sites Hind III (▼), Eco RI (◇) and Taq I (↑) are positioned according to the DNA sequence analysis of T. thermophila rDNA (Kan and Gall, 1982; N. Kan, personal communication). The larger Taq I fragments in T. pigmentosa 8ALP rDNA were mapped by Wild and Gall (1979), and their sizes were confirmed by size analysis of the fragments produced by Taq I cleavage of the 1.2 kb Hind III fragment (data not shown). The solid fragment (468 bp), which crosses the ligation junction, was used to probe for ligation of the exons during the IVS excision reaction. The stippled fragment (323 bp) was used in a control experiment.

(B) Expected products of RNAase T1 or S1 nuclease digestion of RNA–DNA hybrids formed with the 468 bp fragment of T. pigmentosa 8ALP rDNA. Solid lines: DNA. Wavy lines: RNA. +/−: possible site of DNA cleavage by S1 nuclease. If the RNA is being monitored, the results do not depend on the ability of the nuclease to cleave at such an interruption in the RNA–DNA hybrid.

(C) Purified RNA transcription products synthesized from pIVS11 that were used for the nuclease mapping experiments. (Lane +) RNA incubated under splicing conditions, as described in the Experimental Procedures, except that incubation was performed at 45°C in the presence of 1% SDS. (Lane −) RNA incubated under conditions in which IVS excision was prevented (same conditions as described above except with EDTA added to a final concentration of 20 mM to chelate the required Mg^{2+}, and RNA incubated on ice). (Right lane) Isolated linear and circular IVS RNA, used as markers. The RNA was subjected to electrophoresis on a 4% polyacrylamide, 8 M urea gel at room temperature.

(D) Spliced (lanes +) or unspliced (lanes −) RNA shown in (C) was hybridized with the 468 bp Hind III Taq I DNA fragment (lanes +DNA) or incubated under the same conditions without DNA (lanes −DNA). Samples were then digested with either RNAase T1 or S1 nuclease. The resulting hybrids were denatured and subjected to electrophoresis on a 4% polyacrylamide, 8 M urea gel at 65°C. Denatured ϕX174 Hae III DNA fragments (lane M_1) and isolated linear and circular IVS RNA (lane M_2) were used as markers. nt: nucleotides.

nucleotides of the 3′ exon (Figure 1A), and that excludes the natural termination region of the pre-rRNA (Niles et al., 1981; Din et al., 1982). Major portions of the exons are therefore dispensable. We are in the process of trimming the exons further to determine if they play any active role in autoexcision or in exon ligation. It is not known how much of the IVS is required for splicing or cyclization, but it is clear that some sequence variation is tolerated. We find that a cloned variant containing seven extra bases in the IVS has splicing and cyclization activity like that of the wild-type sequence. In addition the IVSs of T. thermophila and T. pigmentosa 6UM, both of which must function in vivo, differ by 14 single-base changes and three small deletions or insertions (Wild and Sommer, 1980; Kan and Gall, 1982). We have not yet investigated whether any of these sequence alterations affects the rate of RNA splicing.

Based on the ability of the RNA transcribed in vitro to undergo RNA splicing, several factors in addition to enzymes are eliminated as requirements for these reactions. These factors include modified nucleosides in the pre-rRNA, potential adapter RNAs such as small nuclear RNAs and the nucleolar proteins that organize the precursor into a ribosomal RNP in vivo. The half-time for excision of the IVS from pre-rRNA at 30°C is ~2 sec in vivo (S. L. Brehm and T. R. Cech, manuscript submitted) and ~2 min with deproteinized RNA in vitro (Cech et al., 1981). This rate difference could be due to a difference between the salts and other ions used in the in vitro reaction and those found in the cell. Furthermore, the RNA is isolated by harsh methods, including phenol extraction and ethanol precipitation, so it may be perturbed from its native structure. It remains possible that some of the factors listed above, particularly the folding of the RNA into an RNP, enhance the rate of the reaction in vivo by altering the conformation of the RNA. Because the difference

between the in vivo and in vitro reaction rates is only a factor of 60, we believe it is unlikely that the reaction is enzyme-catalyzed in vivo.

The cleavages involved in IVS autoexcision occur under physiological conditions (neutral pH, moderate temperature) and are highly specific (only 2 of the 1300 bases in the runoff transcript are cleaved). Enzyme-independent cleavage of RNA is not unprecedented. For example, specific cleavage of tRNA occurs in the presence of metal ion catalysts (Eichorn, 1981). The finding of enzyme-independent ligation reactions is more surprising. Phosphodiester bond formation involving a monophosphorylated donor is not a spontaneous reaction ($\Delta G^{\circ\prime} \simeq +9$ kcal/mole; Peller, 1976). When catalyzed by T4 RNA ligase, bond formation is coupled to the hydrolysis of ATP (reviewed by Higgins and Cozzarelli, 1979; Uhlenbeck and Gumport, 1982). The enzyme-independent ligation reactions described here—formation of a phosphodiester bond between the 3′ hydroxyl of the guanosine cofactor and the 5′ phosphate of the IVS and formation of a covalently closed circular IVS—require no ATP or GTP hydrolysis. The lack of an energy requirement can be explained by a phosphoester transfer mechanism, in which each ligation step is linked to a cleavage step (Cech et al., 1981; A. J. Zaug, P. J. Grabowski and T. R. Cech, manuscript submitted). Such reactions are expected to be reversible. Excision of the IVS could be driven forward by the large molar excess of guanosine cofactor relative to RNA. The reverse reaction could be prevented by destruction of one of the products (cyclization of the excised IVS), and in vivo, by the export of the mature, processed rRNA to the cytoplasm. It is therefore not necessary to invoke conformational rearrangements of the molecule upon cleavage at the splice junctions, although such rearrangements could certainly help drive the reaction forward.

Enzymatic RNA?

While the phosphoester transfer model allows us to explain the thermodynamics of the cleavage–ligation events, other problems remain. How is the activation energy for bond cleavage supplied, and how are the sites of the reaction designated? The answers to these questions must lie in the secondary and tertiary structure of the RNA. We envision the RNA molecule folded in such a way that one of its own bonds is weakened either by physical stretching or by the withdrawal of electrons. At the same time, the 3′ hydroxyl group of an "acceptor" nucleotide (for example, the guanosine cofactor) is positioned so as to mediate the phosphoester transfer.

We propose that the IVS RNA has the following enzyme-like properties. First, it lowers the activation energy for specific bond cleavage and formation events. Second, its activity depends on a precise structure. (Reactivity is lost at high temperatures or high concentrations of formamide or urea; our unpublished results.) Third, it has a specific binding site for the guanosine cofactor. Finally, two or more domains of the RNA form an active site or sites for the phosphoester transfer reactions. The active site is a cleft or hole that can exclude water, thereby preventing hydrolysis after each cleavage step. (Transfer RNA is an example of an RNA molecule with a precise structure and a hole; Sussman and Kim, 1976.)

There is one definitive property of an enzyme that the Tetrahymena IVS does not appear to possess: it is not a true catalyst. The kinetics of the autoexcision reaction are first order with respect to RNA concentration (B. Bass and T. Cech, unpublished data). Therefore, at least under in vitro reaction conditions, each IVS RNA molecule excises itself from the adjacent exon sequences and then cyclizes itself, but does not promote the splicing of other pre-rRNA molecules. Because the IVS RNA is not an enzyme but has some enzyme-like characteristics, we call it a ribozyme, an RNA molecule that has the intrinsic ability to break and form covalent bonds.

Other Ribozymes, Past and Present

We do not know how many other RNA splicing reactions are autocatalytic. The splicing of yeast pre-tRNAs occurs by a very different mechanism, probably involving 2′,3′-cyclic phosphates, and appears to require at least four enzymes (Peebles et al., 1979; Konarska et al., 1982; C. Peebles, P. Gegenheimer and J. Abelson, personal communication). Very little is known about the biochemistry of the splicing of other rRNA precursors or of mRNA precursors, making it difficult to assess whether these reactions might involve autocatalytic steps. In the case of mRNA precursors, deletions or rearrangements of nucleotides within intervening sequences but not near the splice junctions have generally been found not to prevent RNA splicing (reviewed by Breathnach and Chambon, 1981; Flint, 1983). Thus most of the nucleotide sequence of these IVSs does not appear to play a major role in excision. While mRNA precursors are unlikely to be self-splicing, it remains possible that they undergo such a reaction when complexed with small nuclear RNPs. If the RNA moiety of the small nuclear RNP bound a nucleotide cofactor or participated in catalysis in any way, it would be a ribozyme. In general, therefore, ribozymes could be either trans-acting or, like the Tetrahymena IVS, cis-acting.

Assuming that self-splicing RNA is or once was a general phenomenon, some additional points are worth noting. The autoexcision of an IVS may be reversible. The reverse reaction, integration of the IVS into another RNA molecule, would be favored under conditions of low GTP concentration and high RNA concentration. If the IVS could integrate into an RNA

molecule different from its original "host," the IVS would constitute an RNA transposon. Reverse transcription of such a recombined RNA product (see Goff et al., 1980; Van Arsdell et al., 1981; Hollis et al., 1982) would provide a pathway for introduction of an IVS into a gene that was formerly contiguous. We also note the possibility that single-stranded DNA might, under some conditions, be self-splicing. Such an event might take place when the DNA strands were separated for replication, and if it could be regulated, might provide a mechanism for DNA rearrangements during cellular differentiation.

The finding of self-splicing RNA adds a new dimension to discussions about possible roles for RNA early in evolution. In a primordial organism with a very limited genome and few enzymes, self-rearranging RNA might have allowed the creation of a diversity of sequences from a single RNA molecule. What we now see in the Tetrahymena pre-rRNA could be a vestige of this ancient process, evolved to the point where splicing is very rapid and absolutely precise.

Experimental Procedures

Plasmid Construction

Plasmid pTT116, constructed by E. Stephenson, was obtained from M. Wild. It contains two copies of the 1.6 kb Hind III fragment of T. thermophila rDNA in tandem in pBR313. Plasmid pPlac, which is pSV240 (Thummel et al., 1981) without the SV40 DNA insert, was obtained from D. Rio. pPlac contains nucleotides 2440–4362 of pBR322, including the gene encoding ampicillin resistance. Ligated to the Eco RI site at nucleotide 4362 is a 95 bp restriction fragment containing the E. coli *lac* UV5 promoter–operator region, followed by a Hind III site. The 1.6 kb Hind III fragment of pTT116 was purified for ligation into pPlac. Hind III–digested pTT116 was subjected to electrophoresis on a 1.0% low melting agarose gel. The DNA was stained with ethidium bromide and visualized under long-wavelength ultraviolet light, and the 1.6 kb DNA fragment was cut from the gel. The gel slice was incubated at 65°C for 10 min to melt the agarose and immediately extracted two times with buffer-saturated phenol and three times with ether. The DNA was ethanol-precipitated two times and resuspended in water. The pPlac DNA was digested with Hind III, extracted and precipitated as described above, and resuspended in water. A fivefold molar excess of the purified rDNA fragment was added to the linearized vector and incubated with T4 DNA ligase (New England Biolabs) overnight at 15°C. Transformation was performed by calcium shock (Mandel and Higa, 1970). The host was E. coli strain HB101 [F′ *lac*($i^q z^-$) *pro*A^+B^+], obtained fron J. Sadler. (HB101 is described by Boyer and Roulland-Dussoix; 1969.) This strain overproduces *lac* repressor. The transformants were selected by growth in the presence of ampicillin and screened by colony hybridization (Hanahan and Meselson, 1980) with nick-translated 1.6 kb Hind III fragment DNA as a probe.

Plasmid Growth and Purification

Plasmid-containing strains were grown in M9, and the plasmid was amplified overnight in chloramphenicol. Plasmid pIVS13 was purified by scaling up the rapid alkali extraction method of Birnboim and Doly (1979). Plasmid pIVS11 was purified by ethidium bromide–cesium chloride density gradient centrifugation. Isolated plasmid DNA was treated with ribonuclease (100 μg/ml RNAase A and 250 units/ml RNAase T1 for 1 hr at 37°C) and in some cases with restriction endonucleases (New England BioLabs; used according to the manufacturer's specifications). It was then extracted with phenol and chloroform, precipitated with ethanol and redissolved in 0.01 M Tris–HCl (pH 7.5), 0.001 M EDTA. Restricted DNA was checked by agarose gel electrophoresis to make certain that it was completely cleaved.

In Vitro Transcription

Plasmid DNA (0.5–1.0 μg; 0.2–0.4 pmole) was diluted into 40 μl of transcription buffer. Two different buffers were used. The standard E. coli RNA polymerase transcription buffer consisted of 20 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 40 mM Tris–HCl (pH 8.0). The Tetrahymena nuclear transcription buffer, a simplified version of that described by Cech et al. (1982), consisted of 5 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM 2-mercaptoethanol, 1 mM spermidine, 1 mM putrescine, 0.1 mM spermine, 50 mM Tris–HCl (pH 8.0). After the addition of 2 μl E. coli RNA polymerase (8 pmole; 60% active; purified by the method of Burgess and Jendrisak, 1975) the solution was preincubated for 10 min at 37°C and then for 2 min at 30°C. Transcription was initiated by the addition of 10 μl of 5× concentrated nucleoside triphosphates (dissolved in transcription buffer) to give final concentrations of 200–400 μM each of ATP, CTP and UTP, and 20 μM GTP. In some experiments a portion of the GTP was α-^{32}P-GTP (New England Nuclear; ~500 Ci/mmole; 10–200 μCi per reaction). After 1 min, 1 μl heparin (2.5 mg/ml) was added to bind excess polymerase. Reactions were stopped on ice with the addition of EDTA to a final concentration of 20 mM. The volume was increased with 200 μl of 0.25 M $NaC_2H_3O_2$, 0.05 M Tris–HCl (pH 7.5), 0.001 M EDTA, 0.3% SDS, and the solution was extracted one or two times with buffer-saturated phenol and one time with chloroform–isoamyl alcohol. Nucleic acids were precipitated with 2 volumes of ethanol overnight at −20°C. Incorporation of ^{32}P-GMP into RNA, determined by binding to Whatman DE81 filters, was typically ~25% of the input radioactivity for a 30 min transcription reaction. If the purpose of the transcription was preparative rather than analytical, the nucleic acids were subsequently treated with DNAase as described by Zaug and Cech (1980).

In Vitro Splicing

Purified ^{32}P-labeled RNA was incubated with 100 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 0.1 mM GTP, 50 mM Tris–HCl (pH 7.5) for 30 min at 37°C unless stated otherwise. To obtain end-labeled linear IVS RNA, we chromatographed RNA produced by transcription in the presence of unlabeled or tritiated nucleoside triphosphates on Sephadex G-50 to remove residual GTP, and then incubated it as described above except with 2 μM α- or γ-^{32}P-GTP. In vitro splicing reactions were stopped by the addition of EDTA (20 mM final concentration) and 2 volumes of ethanol to precipitate the RNA.

Gel Electrophoresis of RNA

RNA was analyzed by electrophoresis through 4% polyacrylamide, 8 M urea gels run in an oven at 65°C to ensure complete denaturation. Electrophoresis and autoradiography were performed as described by Cech et al. (1981).

RNA Sequencing and Fingerprinting

IVS RNA was purified by gel electrophoresis as described by Grabowski et al. (1981). IVS RNA end-labeled with ^{32}P-GTP during splicing was sequenced by the enzymatic method (Donis-Keller et al., 1977) as described previously (Cech et al., 1981). IVS RNA uniformly labeled with α-^{32}P-GTP during in vitro transcription–splicing was analyzed by RNAase T1 fingerprinting as described by Zaug and Cech (1982).

RNA–DNA Hybridization

RNA synthesized in vitro from pIVS11 under the Tetrahymena nuclear transcription conditions was treated with DNAase I (40 μg/ml; 5 min at room temperature) immediately prior to stopping the transcription reaction. The RNA was then phenol-extracted and passed through a Sephadex G50–150 column to remove oligodeoxyribonucleotides and unincorporated ribonucleoside triphosphates. The success of the DNAase treatment was checked by gel electrophoresis and ethidium bromide staining of the RNA. The ^{32}P-labeled RNA (150,000 cpm;

3.2 ng) was reextracted, ethanol-precipitated, washed in 70% ethanol, 30% of 0.05 M $NaC_2H_3O_2$, 0.05 M Tris–HCl (pH 7.5) and dried. It was resuspended in 2 μl H_2O and added to 20 μl of 1.2× hybridization buffer. The 1.2× hybridization buffer contained 84% deionized formamide, 0.1 M PIPES (pH 7.8), 0.024 M NaCl, 0.012 M disodium EDTA. DNA restriction fragments were isolated from pTp8002 (which contains the T. pigmentosa 8ALP 1.2 kb Hind III fragment; Wild and Gall, 1979) by the method of Dretzen et al. (1981). Salt-free DNA (2 μl; ~0.5 μg/μl) was added to the hybridization buffer containing the RNA. The final Na^+ concentration was ~0.17 M (Thomas et al., 1976). The 24 μl solution was sealed in a siliclad glass micropipette and heated to 70°C for 8 min to denature the RNA completely and to ensure strand separation of the DNA. The temperature was 22°C higher than the strand-separation temperature of the DNA fragment (t_{ss}) calculated according to the method of Thomas et al. (1976). The sealed micropipette was then transferred to a constant temperature bath at 48°C (t_{ss}), and hybridization was carried out for 4 hr.

Nuclease Digestion of RNA–DNA Hybrids

Hybridizations were terminated by rapid dilution into 10 volumes of ice-cold RNAase T1 or S1 nuclease digestion buffer. RNAase T1 buffer contained 0.05 M Tris–HCl (pH 7.5), 1.0 mM EDTA and 20 μg/ml yeast tRNA. The S1 buffer used was identical with that described by Berk and Sharp (1977) except for the substitution of 20 μg/ml tRNA or poly(A) for denatured DNA. Nuclease digestions were performed at 37°C for 15 min. An amount of enzyme that gave complete degradation of purified Tetrahymena pre-rRNA under the same conditions was used for digestion of the RNA–DNA hybrids. RNAase T1 (Sanyo) and S1 nuclease (New England Nuclear) were used at final concentrations of 5 U/ml and 1250 U/ml, respectively. S1 nuclease reactions were stopped by adjusting the concentrations to 17 mM EDTA, 83 mM Tris–HCl (pH 7.5) and 83 μg/ml tRNA; RNAase T1 reactions were stopped by adjusting the concentrations to 8.3 mM $ZnCl_2$ and 83 μg/ml tRNA. Each sample was brought to a concentration of 1% in SDS, extracted and ethanol-precipitated. In a control experiment, the same unspliced and spliced RNAs used for the experiment of Figure 6D were hybridized to the 323 bp Taq I fragment not containing the ligation junction (Figure 6A). After digestion with S1 nuclease, there was one major RNA species whose protection depended on prior hybridization to the DNA fragment. It was present in equal amounts in the unspliced and spliced RNA samples, and its size (299 ± 12 nucleotides) was very close to that of the Taq fragment. In a parallel experiment with RNAase T1, three DNA-protected RNA species (428 ± 17, 346 ± 14 and 298 ± 12 nucleotides) were observed. The presence of the two larger RNAs can be explained by secondary structure in the RNA adjacent to the RNA–DNA hybrid that protects the RNA from cleavage by the G-specific RNAase T1 but not from cleavage by nonspecific S1 nuclease. The 428 and 298 nucleotide RNAs were present in equal amounts in the unspliced and spliced RNA samples; the 346 nucleotide RNA increased in the spliced RNA, but the amount of increase was only 27% of that observed for the bands attributed to ligated exons in the experiments with the 468 bp Hind III–Taq I fragment (Figure 6D). Based on this control experiment, we concluded that under the conditions we used, S1 nuclease is able to trim the entire RNA tail from an RNA–DNA hybrid; RNAase T1 may in some cases leave a substantial tail, resulting in an upper limit for the size of the RNA–DNA hybrid.

Acknowledgments

We are grateful to Marv Caruthers, Larry Gold, Rick Hallick, Phil Sharp, Olke Uhlenbeck and many other colleagues for helping us think about the possibility of self-splicing RNA. We thank Mark Krasnow for a helpful discussion about polyamines. We thank Carol Cech for providing the RNA polymerase; Don Rio and Bob Tjian for pPlac; and Richard Gourse and Martha Wild for pTp8002. This work was supported by grants from the National Institutes of Health; the American Cancer Society; and the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health. T. R. C. was supported by a Research Career Development Award from the National Cancer Institute, Department of Health and Human Services.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August *12*, 1982

References

Abelson, J. (1979). RNA processing and the intervening sequence problem. Ann. Rev. Biochem. *48*, 1035–1069.

Berk, A. J. and Sharp, P. A. (1977). Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell *12*, 721–732.

Birnboim, M. C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids Res. *7*, 1513–1523.

Boyer, H. W. and Roulland-Dussoix, D. (1969). A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. 41, 459–472.

Breathnach, R. and Chambon, P. (1981). Organization and expression of eucaryotic split genes coding for proteins. Ann. Rev. Biochem. *50*, 349–383.

Burgess, R. R. and Jendrisak, J. J. (1975). A procedure for the rapid, large-scale purification of *Escherichia coli* DNA-dependent RNA polymerase involving polymin P precipitation and DNA-cellulose chromatography. Biochemistry *14*, 4634–4638.

Carin, M., Jensen, B. F., Jentsch, K. D., Leer, J. C., Nielson, O. F. and Westergaard, O. (1980). In vitro splicing of the ribosomal RNA precursor in isolated nucleoli from Tetrahymena. Nucl. Acids Res. *8*, 5551–5566.

Cech, T. R. and Rio, D. C. (1979). Localization of transcribed regions on the extrachromosomal ribosomal RNA genes of Tetrahymena thermophila by R-loop mapping. Proc. Nat. Acad. Sci. USA *76*, 5051–5055.

Cech, T. R., Zaug, A. J. and Grabowski, P. J. (1981). In vitro splicing of the ribosomal RNA precursor of Tetrahymena: involvment of a guanosine nucleotide in the excision of the intervening sequence. Cell *27*, 487–496.

Cech, T. R., Zaug, A. J., Grabowski, P. J. and Brehm, S. L. (1982). Transcription and splicing of the ribosomal RNA precursor of Tetrahymena. In The Cell Nucleus, *10*, H. Busch and L. Rothblum, eds. (New York: Academic Press), pp. 171–204.

Din, N. and Engberg, J. (1979). Extrachromosomal rRNA genes in *Tetrahymena*: structure and evolution. J. Mol. Biol. *134*, 555–574,

Din, N., Engberg, J., Kaffenberger, W. and Eckert, W. (1979). The intervening sequence in the 26S rRNA coding region of T. thermophila is transcribed within the largest stable precursor for rRNA. Cell *18*, 525–532.

Din, N., Engberg, J. and Gall, J. G. (1982). The nucleotide sequence at the transcription termination site of the ribosomal RNA gene in Tetrahymena thermophila. Nucl. Acids Res. 10, 1503–1513.

Donis-Keller, H., Maxam, A. M. and Gilbert, W. (1977). Mapping adenines, guanines, and pyrimidines in RNA. Nucl. Acids Res. *4*, 2527–2538.

Dretzen, G., Bellard, M., Sassone-Corsi, P. and Chambon, P. (1981). A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. Anal. Biochem. *112*, 295–298.

Eichorn, G. L. (1981). The effect of metal ions on the structure and function of nucleic acids. In Metal Ions in Genetic Information Transfer. Advances in Inorganic Biochemistry, *3*, G. Eichorn and L. Marzilli, eds. (New York: Elsevier), pp. 1–46.

Engberg, J., Din , N., Eckert, W. A., Kaffenberger, W. and Pearlman,

R. E. (1980). Detailed transcription map of the extrachromosomal ribosomal RNA genes in Tetrahymena thermophila. J. Mol. Biol. *142,* 289–313.

Flint, J. (1983). RNA splicing in vitro. In Enzymes of Nucleic Acid Synthesis and Modification, *2,* S. T. Jacob, ed. (Boca Raton, Fla: CRC Press), in press.

Goff, S. P., Gilboa, E., Witte, O. N. and Baltimore, D. (1980). Structure of the Abelson murine leukemia virus genome and the homologous cellular gene: studies with cloned viral DNA. Cell *22,* 777–785.

Grabowski, P. J., Zaug, A. J. and Cech, T. R. (1981). The intervening sequence of the ribosomal RNA precursor is converted to a circular RNA in isolated nuclei of Tetrahymena. Cell *23,* 467–476.

Hanahan, D. and Meselson, M. (1980). Plasmid screening at high colony density. Gene *10,* 63–67.

Higgins, N. P. and Cozzarelli, N. R. (1979). DNA-joining enzymes: a review. Meth. Enzymol. *68,* 50–71.

Hollis, G. F., Heiter, P. A., McBride, O. W., Swan, D. and Leder, P. (1982). Processed genes: a dispersed human immunoglobulin gene bearing evidence of RNA-type processing. Nature *296,* 321–325.

Kan, N. C. and Gall, J. G. (1982). The intervening sequence of the ribosomal RNA gene is highly conserved between two *Tetrahymena* species. Nucl. Acids Res. *10,* 2809–2822.

Karrer, K. M. and Gall, J. G. (1976). The macronuclear ribosomal DNA of *Tetrahymena pyriformis* is a palindrome. J. Mol. Biol. *104,* 421–453.

Konarska, M., Filipowicz, W. and Gross, H. J. (1982). RNA ligation via 2′-phosphomonoester, 3′,5′-phosphodiester linkage: requirement of 2′,3′-cyclic phosphate termini and involvement of a 5′-hydroxyl polynucleotide kinase. Proc. Nat. Acad. Sci. USA *79,* 1474–1478.

Krasnow, M. A. and Cozzarelli, N. R. (1982). Catenation of DNA rings by topoisomerases. Mechanism of control by spermidine. J. Biol. Chem. *257,* 2687–2693.

Mandel, M. and Higa, A. (1970). Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. *53,* 159–162.

Niles, E. G., Cunningham, K. and Jain, R. (1981). Structure of the *Tetrahymena pyriformis* rRNA gene. Nucleotide sequence of the transcription termination region. J. Biol. Chem. *256,* 12857–12860.

Oostra, B. A., Arnberg, A. C., Ab, G. and Gruber, M. (1981). Terminal strand-switching of *E. coli* RNA polymerase transcribing a truncated DNA fragment. Biochim. Biophys. Acta *655,* 446–448.

Peebles, C. L., Ogden, R. C., Knapp, G. and Abelson, J. (1979). Splicing of yeast tRNA precursors: a two-stage reaction. Cell *18,* 27–35.

Peller, L. (1976). On the free-energy changes in the synthesis and degradation of nucleic acids. Biochemistry *15,* 141–146.

Sussman, J. L. and Kim S.-H. (1976). Three-dimensional structure of a transfer RNA in two crystal forms. Science *192,* 853–858.

Thomas, M., White, R. L. and Davis, R. W. (1976). Hybridization of RNA to double-stranded DNA: formation of R-loops. Proc. Nat. Acad. Sci. USA *73,* 2294–2298.

Thummel, C. S., Burgess, T. L. and Tjian, R. (1981). Properties of simian virus 40 small t antigen overproduced in bacteria. J. Virology *37,* 683–697.

Uhlenbeck, O. C. and Gumport, R. I. (1982). T4 RNA ligase. In The Enzymes, *15,* 3rd ed., P. D. Boyer, ed. (New York: Academic Press), pp. 31–58.

Van Arsdell, S. W., Denison, R. A., Bernstein, L. B., Weiner, A. M., Manser, T. and Gesteland, R. F. (1981). Direct repeats flank three small nuclear RNA pseudogenes in the human genome. Cell *26,* 11–17.

Wild, M. A. and Gall, J. G. (1979). An intervening sequence in the gene coding for 25S ribosomal RNA of Tetrahymena pigmentosa. Cell *16,* 565–573.

Wild, M. A. and Sommer, R. (1980). Sequence of a ribosomal RNA gene intron from Tetrahymena. Nature *283,* 693–694.

Zaug, A. J. and Cech, T. R. (1980). In vitro splicing of the ribosomal RNA precursor in nuclei of Tetrahymena. Cell *19,* 331–338.

Zaug, A. J. and Cech, T. R. (1982). The intervening sequence excised from the ribosomal RNA precursor of *Tetrahymena* contains a 5′-terminal guanosine residue not encoded by the DNA. Nucl. Acids Res. *10,* 2823–2838.