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In Vitro Splicing of the Ribosomal RNA Precursor of Tetrahymena: Involvement of a Guanosine Nucleotide in the Excision of the Intervening Sequence

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Summary

In previous studies of transcription and splicing of the ribosomal RNA precursor in isolated Tetrahymena nuclei, we found that the intervening sequence (IVS) was excised as a unique linear RNA molecule and was subsequently cyclized. In the present work, transcription at low monovalent cation concentration is found to inhibit splicing and to lead to the accumulation of a splicing intermediate. This intermediate contains splicing activity that either is tightly bound to the RNA or is part of the RNA molecule itself. The intermediate is able to complete the excision of the IVS when it is incubated with a monovalent cation (75 mM $(NH_4)_2SO_4$), a divalent cation (5–10 mM $MgCl_2$) and a guanosine compound (1 μM GTP, GDP, GMP or guanosine). ATP, UTP, CTP and guanosine compounds without 2′ and 3′ hydroxyl groups are inactive in causing excision of the IVS. Accurate excision of the IVS, cyclization of the IVS and (apparently) ligation of the 26S rRNA sequences bordering the IVS all take place under these conditions, suggesting that a single activity is responsible for all three reactions. During excision of the IVS, the 3′ hydroxyl of the guanosine moiety becomes linked to the 5′ end of the IVS RNA via a normal phosphodiester bond. When GTP is used to drive the reaction, it is added intact without hydrolysis. Based on these results, we propose that Tetrahymena pre-rRNA splicing occurs by a phosphoester transferase mechanism. According to this model, the guanosine cofactor provides the free 3′ hydroxyl necessary to initiate a series of three transfers that results in splicing of the pre-rRNA and cyclization of the excised IVS.

Introduction

Many eucaryotic tRNA, rRNA and mRNA genes are interrupted by stretches of noncoding DNA called intervening sequences (IVSs), which are transcribed as part of a precursor RNA and are subsequently removed by a cleavage–ligation reaction termed splicing. Although no splicing enzyme has yet been purified, some major features of the reaction mechanism have been described for the splicing of yeast tRNA precursors. In that case, splicing is accomplished by endonucleolytic excision of the IVS and ATP-dependent ligation of the two resulting tRNA half-molecules (Knapp et al., 1979; Peebles et al., 1979). The cleavage and ligation activities have been separated and partially purified, and appear to reside in different enzyme molecules (C. Peebles and J. Abelson, personal communication). It has heretofore been unknown whether the splicing of mRNA and rRNA precursors might follow pathways similar to that used for tRNA precursors, although from comparison of the nucleotide sequences at splice junctions it appears that at least the components that recognize the IVS are different for the three classes of transcripts.

In Tetrahymena pigmentosa strain 6 UM and in T. thermophila, all copies of the rDNA (rRNA gene) contain a 0.4 kb IVS (Cech and Rio, 1979; Din et al., 1979; Wild and Gall, 1979). When isolated Tetrahymena nuclei are incubated under conditions that allow elongation of rRNA chains initiated in vivo, the rRNA precursor is synthesized and spliced (Zaug and Cech, 1980). Transcription and splicing also occur in isolated nucleoli (Carin et al., 1980). The IVS is excised from the pre-rRNA as a linear 0.4 kb RNA molecule and is subsequently converted to a circular RNA (Grabowski et al., 1981). The same splicing pathway appears to occur in vivo, with both linear and circular forms of the excised IVS being present in nuclear RNA (S. Brehm and T. Cech, manuscript in preparation).

We have recently analyzed the sequence of the excised IVS RNA and have compared it with the sequence of the corresponding region of the rDNA, determined by N. Kan and J. Gall (manuscript in preparation). Both RNAase T1 fingerprint analysis of uniformly labeled IVS RNA and sequence gel analysis of 5′-end-labeled IVS RNA led to the same conclusion: the IVS RNA has a 5′ terminal pG (guanosine 5′-monophosphate) not found in the DNA sequence, and thereafter has a sequence colinear with that of the rDNA (A. Zaug and T. Cech, manuscript in preparation).

We now present evidence that excision of the IVS requires a guanosine residue, which is added to the 5′ end of the IVS during excision. We propose that this is the first in a series of phosphoester transfer events that ultimately leads to the splicing of the Tetrahymena rRNA precursor and the cyclization of the excised IVS. Such a mechanism is completely different from that involved in the splicing of yeast tRNA precursors.

Results

Trapping a Splicing Intermediate

The salt dependence of transcription and splicing of pre-rRNA in isolated nuclei is shown in Figure 1. Transcription proceeds at low concentrations of monovalent cation, but release of the IVS RNA is almost totally inhibited. When the RNA synthesized in low salt was subjected to heat denaturation or treated with proteinase K, no additional IVS RNA was released (Figure 1). In other experiments, more extensive treatment of the RNA with proteinase K or pronase, or

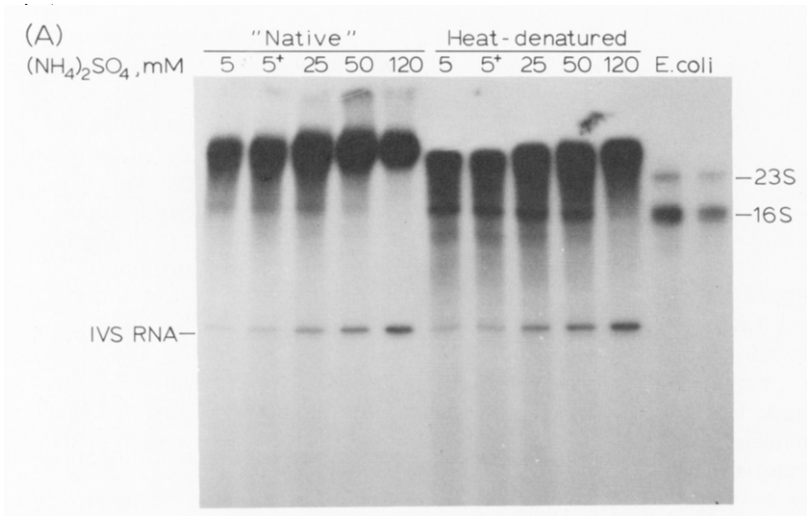
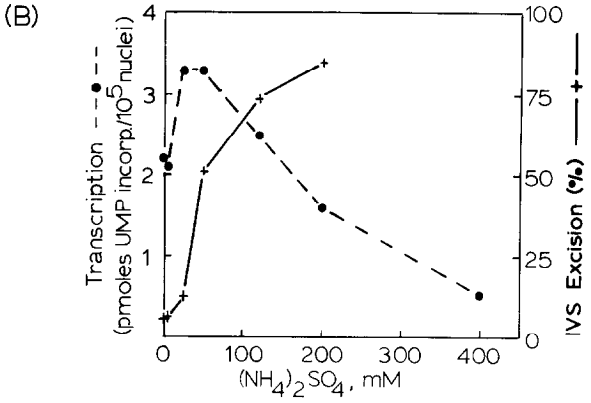


Figure 1. Salt Dependence of Transcription and Splicing of Pre-rRNA in Isolated T. thermophila Nuclei

(A) The rRNA transcribed at different $(NH_4)_2SO_4$ concentrations was analyzed by 2.5%–15% polyacrylamide gradient gel electrophoresis. Samples containing equal amounts of ^{32}P radioactivity were either layered directly onto the nondenaturing gel ("Native") or were first boiled for 5 min in electrophoresis buffer and cooled on ice (Heat-denatured). (Lane 5+) RNA transcribed in 5 mM $(NH_4)_2SO_4$ was subsequently treated with proteinase K to destroy any protein–RNA complexes that might have prevented release of the IVS. (Lane E. coli) rRNA size markers labeled with polynucleotide kinase.

(B) Quantitation of transcription and IVS excision. 10% of the total radioactivity released as free IVS RNA is taken to correspond to 100% excision (Zaug and Cech, 1980).



electrophoresis of the RNA on an 8 M urea, polyacrylamide gel at 65°C, failed to increase the amount of free IVS RNA (data not shown). It will become clear from the results presented below that the IVS is still present in the high molecular weight RNA synthesized under these conditions. We do not know whether the IVS is still an integral part of the precursor, or is associated with it by means of another molecule such as a splicing enzyme.

We hoped to use the unspliced pre-rRNA synthesized in vitro under low salt conditions as a substrate to assay for splicing activity in nuclear extracts. This approach proved to be impossible, however, because excision of the IVS occurred when the isolated RNA was incubated in transcription cocktail (see Experimental Procedures) at normal salt concentration in the absence of a nuclear extract. The pre-rRNA isolation procedure included two SDS-phenol extractions, sedimentation in a formamide-sucrose gradient, and in some cases protease treatment, so the presence of such activity in the RNA preparation was completely unexpected. The small RNA molecule produced in this "autoexcision" reaction was identified as the IVS RNA by its electrophoretic mobility relative to an IVS RNA marker, by its conversion to the circular form when the reaction was performed at 39°C (Figure 2) and by direct sequence analysis (see below).

Inhibition of splicing in isolated nuclei therefore leads to the accumulation of a splicing intermediate that can complete the excision of the IVS under favorable conditions. The activity of this intermediate may be due to a splicing enzyme tightly bound to the pre-rRNA, or it may be a novel case of an RNA-mediated reaction that requires no protein (see Discussion). In addition to IVS-excision activity, the intermediate has at least one type of ligation activity: that responsible for cyclization of the excised IVS RNA. The pre-rRNA also appears to be ligated in this system, as judged by the absence of a discrete 1070 nucleotide RNA fragment (extending from the 3′ end of the IVS to the 3′ end of the pre-rRNA) that would be produced by excision of the IVS without ligation of the resulting pre-rRNA half-molecules. If such a fragment were formed, it would be clearly resolved by gel electrophoresis (see, for example, Figure 2).

Requirements for IVS Excision

Three components of the transcription cocktail (see Experimental Procedures) proved to be necessary to cause excision of the IVS from the splicing intermediate. These components were a monovalent cation, a divalent cation and GTP. We determined the optimum concentration of each cofactor empirically by incubating the splicing intermediate (purified as described in Experimental Procedures) with the three cofactors, two of which were held at fixed concentration while the concentration of the third was varied. The products of the IVS-excision reaction in each case were analyzed by denaturing gel electrophoresis. One example, the magnesium ion dependence, is shown in Figure 3A. The percentage of IVS excised was calculated from data obtained from densitometric tracings of the autoradiographs (Figures 3B, 3C and

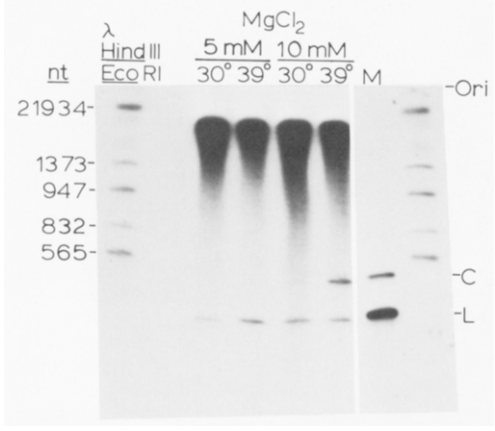


Figure 2. Transcription under Low Salt Conditions Produces an Unspliced Pre-rRNA That Is Active in Splicing and IVS Cyclization in Vitro

The splicing intermediate, labeled with ^{32}P during transcription, was purified as described in Experimental Procedures. It was then incubated with GTP and $(NH_4)_2SO_4$ at two concentrations of $MgCl_2$ and two temperatures, as indicated. The RNA products were analyzed by denaturing gel electrophoresis at 65°C. Denatured lambda DNA restriction fragments produced by a Hind III–Eco RI double digest were used as molecular weight markers (left lane). (Lane M) Markers for the linear (L) and circular (C) IVS were purified from RNA produced by high salt transcription in isolated nuclei as previously described (Grabowski et al., 1981).

3D). Excision of the IVS occurred at a maximal level with ≥75 mM $(NH_4)_2SO_4$, 5–10 mM $MgCl_2$ and ≥1 μM GTP.

The circular IVS is also formed under these conditions. Cyclization is enhanced at 39°C compared to 30°C, as shown in Figure 2, consistent with our earlier observations that IVS cyclization is temperature-dependent in vitro (Grabowski et al., 1981). The cyclization reaction has an absolute requirement for magnesium ion (Figure 3B; our unpublished observations), and is inhibited by high concentrations of ammonium sulfate (Figure 3D).

We found that ATP, CTP and UTP at concentrations as high as 10 μM did not substitute for GTP in the reaction (data not shown). (Some reaction was observed with 1 mM ATP, CTP or UTP, which could be explained by a 0.01%–0.10% contamination of GTP in these other nucleotides.) Several guanosine compounds other than 5′-GTP were active as cofactors in excising the IVS in vitro (Figure 4; Table 1). Guanosine, 5′-GMP and 5′-GDP showed similar levels of activity in the reaction compared with 5′-GTP (Table 2). Compounds lacking a 2′ or 3′ hydroxyl, as in the case of 2′-deoxyGTP, 2′,3′-dideoxyGTP and 3′-GMP, were inactive as cofactors in the reaction.

We therefore conclude: a guanosine compound containing both a free 2′ and a free 3′ hydroxyl is required for excision of the IVS in vitro; and the guanosine compound required for the reaction is not utilized as an energy cofactor. That is, since the number of phosphates at the 5′ position of the guanosine moiety can be varied from 0 to 3 without any observable change in the amount of IVS excised, phosphate or pyrophosphate cleavage is unlikely to be important in the reaction.

The time course of IVS excision was determined, for which the autoradiograph of the denaturing gel is shown in Figure 5A. The data were quantitated both by densitometric tracing of the autoradiograph and by scintillation counting of the sliced, dried gel (Figure 5B). The data obtained by the two different methods agreed favorably. As shown in Figure 5B, the reaction was half complete after 2 min. The maximum amount of IVS excision occurred after 10 min under these experimental conditions, which justifies the use of 30 min incubation times for the experiments described above.

Addition of a Guanosine Nucleotide to the 5′ End of the IVS during Its Excision

We tested the possibility that the guanosine required for IVS excision is the source of the extra G residue found on the 5′ end of the excised IVS RNA. We prepared the pre-rRNA splicing intermediate by in vitro transcription using ^{3}H-UTP as the labeled precursor. Gel electrophoresis followed by tritium fluorography (Figure 6A) revealed no free IVS RNA in the preparation.

When the splicing intermediate (either ^{3}H-labeled or unlabeled) was incubated with α-^{32}P-GTP under the conditions for IVS excision described above, ^{32}P radioactivity was bound only to one RNA species, which had the electrophoretic mobility of the excised linear IVS (Figure 6B). Southern hybridization of this RNA to Bgl II, Hae III, Hha I and Hind III restriction fragments of rDNA confirmed that it was the IVS (data not shown). No labeling of the IVS occurred in parallel incubations, in which α-^{32}P-ATP was substituted for the GTP or in which Mg^{2+} was excluded from the reaction. Each sample contained a large amount of unincorporated nucleoside triphosphate, a small fraction of which remained at the origin. In no case was there any detectable labeling of the pre-rRNA, which migrated well into the gel (Figures 6B and 6C). When the splicing intermediate was incubated with ^{32}P-GMP, the IVS RNA was again excised and labeled (data not shown).

The IVS RNA labeled during completion of splicing in vitro was recovered from polyacrylamide gels and analyzed by digestion with various enzymes (Figure 7). Treatment of the GMP-labeled IVS RNA with bacterial alkaline phosphatase led to the release of the label as inorganic phosphate, indicating the terminal location of the label. Treatment of the RNA with nuclease P1 produced labeled pG, while treatment with RNAase T1 or T2 produced labeled pGp. During splicing, therefore, the labeled GMP became linked to the 5′ end of the IVS RNA via a normal phosphodiester bond (3′ hydroxyl of GMP to 5′ phosphate of IVS

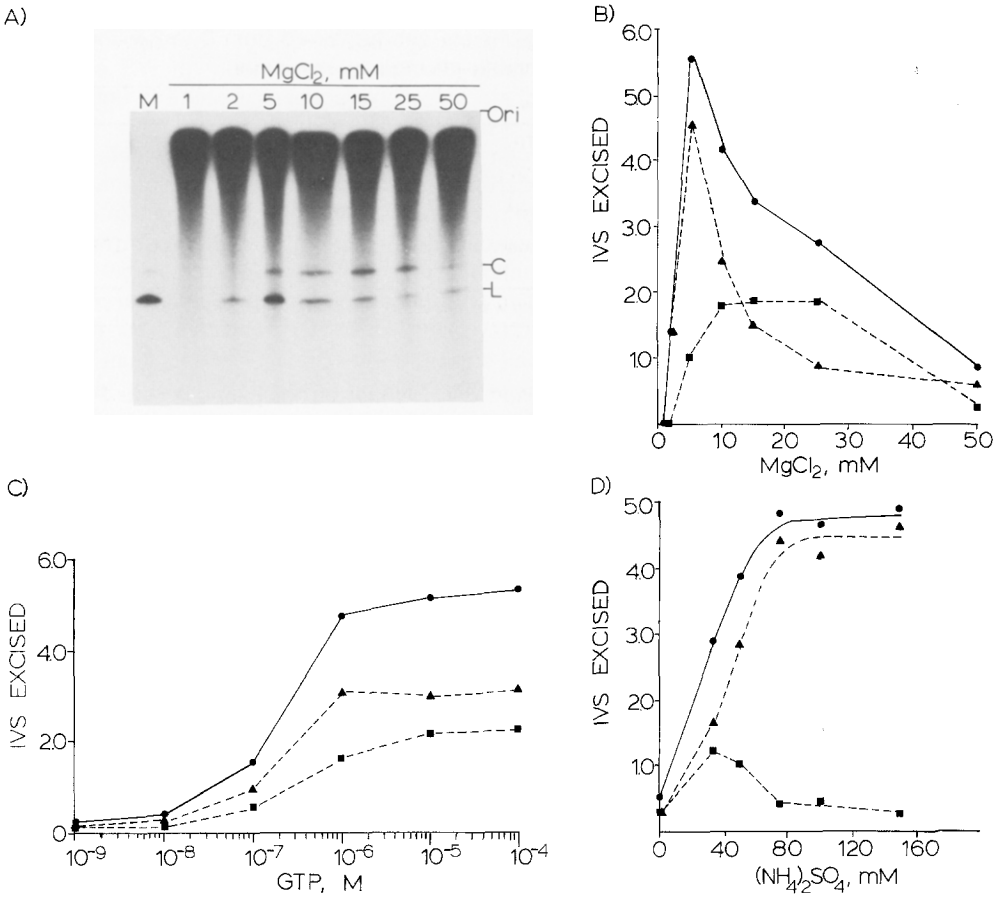


Figure 3. Three Cofactors Are Required for Excision of the IVS from the Purified Splicing Intermediate

(A) Denaturing gel electrophoresis of the RNA products of the IVS-excision reaction at increasing concentrations of $MgCl_2$, with the GTP and $(NH_4)_2SO_4$ concentrations held constant.

(B) Data obtained from densitometric tracings of the autoradiograph in (A) give the $MgCl_2$ dependence of IVS excision in vitro. The GTP dependence (C) and the $(NH_4)_2SO_4$ dependence (D) of the reaction were determined in analogous experiments. (●——●) Total excised IVS (linear and circular). (▲--▲) Excised linear IVS. (■--■) Excised circular IVS. Data are given as a percentage of the total RNA in each sample.

RNA). The same type of analysis was performed on the IVS RNA labeled with α-^{32}P-GTP during completion of splicing. In this case, treatment with nuclease P1 led to the release of the label as pppG (data not shown). The GTP, like the GMP, was therefore linked to the 5′ end of the IVS RNA via a normal phosphodiester bond. The data eliminated the possibility that the guanosine nucleotide is forming a cap structure such as that found on small nuclear RNAs, as well as the possibility that hydrolysis of GTP accompanies formation of the covalent bond.

The fraction of the excised IVS RNA molecules that were end-labeled with GTP during splicing was calculated from the ^{32}P to ^{3}H ratio of the IVS RNA. For example, gel slice 13 in Figure 6C contained 11,950 cpm ^{32}P (7.2×10^{-15} mole GTP) and 14,000 cpm ^{3}H (7.3×10^{-15} mole IVS RNA, based on 107 uridines per molecule [N. Kan and J. Gall, manuscript in preparation]). The molar ratio was therefore 7.2/7.3 = 0.99 GTP/IVS RNA. In another splicing reaction, the ratio was 1.35. We conclude that the excision of each IVS RNA molecule is accompanied by the addition of a guanosine nucleotide to its 5′ end.

When the splicing intermediate was incubated under higher temperature conditions that promote both excision and cyclization of the IVS, a small amount of labeled linear IVS RNA was observed, but no labeled circular IVS RNA was observed (Figure 6B lane H). In other experiments in which either GTP or GMP was used, labeling of the linear IVS RNA was obtained at 39°C, but in no case was label detected in the gel at the position of circular IVS RNA. These results provide preliminary evidence that the guanosine nucleotide added to the 5′ end of the linear IVS RNA during excision is split off during cyclization.

Accuracy of IVS Excision in Vitro

We prepared 5′-end-labeled IVS RNA by allowing isolated splicing intermediate to complete excision of the IVS in vitro in the presence of ^{3}P-GTP. Its nucleotide sequence was then determined by partial digestion with base-specific RNAases followed by gel elec-

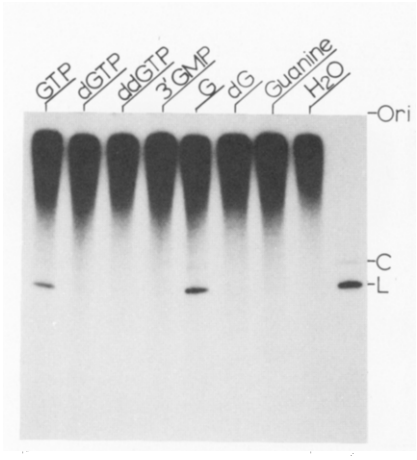


Figure 4. The IVS-Excision Reaction Requires a Guanosine Cofactor with a Specific Structure

Denaturing gel electrophoresis of the RNA products of the IVS-excision reaction, in which various guanosine compounds were tested for their activity as cofactors in the reaction. All compounds shown were tested at a final concentration of 2 μM. ddGTP: 2′,3′-dideoxyGTP. G: guanosine. dG: 2′-deoxyguanosine. (Lane H_2O) A control in which water was substituted by volume for the guanosine cofactor.

trophoresis (Donis-Keller et al., 1977; Simoncsits et al., 1977). From the sequencing gel (Figure 8A) 26 of the first 31 nucleotides could be identified. The RNA sequence was in total agreement with the DNA sequence of the IVS, except for the 5′ terminal G added to the RNA during splicing (Figure 8B). Thus the IVS that was end-labeled during excision in vitro has a discrete 5′ end that is exactly the one expected to be produced by accurate splicing. Furthermore, this experiment confirms that the GTP is linked directly to the RNA: the labeled products of the various RNAase treatments have the correct electrophoretic mobilities for oligonucleotides, which would not be the case if the labeled GTP were linked to a protein associated with the RNA.

Discussion

We have used transcription in isolated nuclei under low salt conditions to accumulate unspliced pre-rRNA. This RNA, purified by methods that normally result in complete deproteinization, has the inherent ability to excise its own IVS. A monovalent cation, a divalent cation and a guanosine compound are necessary and sufficient to cause the excision. What is the nature of the activity responsible for excision of the IVS and concomitant attachment of a guanosine nucleotide to its 5′ end? Either these events are catalyzed by a protein bound to the RNA, an unusual protein that retains activity through SDS-phenol extraction, boiling and protease treatment; or the events are mediated by the RNA molecule itself. We examine these possibilities in more detail in the next section.

The guanosine compound required for IVS excision becomes covalently attached to the 5′ end of the excised IVS RNA. It is not joined to the RNA by a phosphoanhydride bond, such as that formed by AMP in the adenylated nucleic acid intermediates produced by DNA ligase or RNA ligase (Kaufmann and Littauer, 1974; Lehman, 1974). Instead, based on the susceptibility of the linkage to cleavage by RNAases T1 and T2, the 3′ end of the guanosine moiety must form a normal phosphodiester bond with the 5′-terminal adenosine of the IVS. Presumably the phosphate group of the bond is contributed by the adenosine. When GTP is used as the cofactor, the excised IVS RNA has the sequence 5′–pppGpA . . . ; when GMP is used, the IVS has the sequence 5′–pGpA . . . . The IVS produced by linked transcription–splicing in isolated nuclei has a 5′-terminal monophosphate (A. Zaug and T. Cech, manuscript in preparation). The presence of the monophosphate could reflect a preference for GMP as the cofactor for splicing in nuclei. Alternatively, splicing could involve addition of GTP to the IVS RNA, with cleavage of inorganic pyrophosphate occurring later.

Table 1. Activity of Various Compounds in the Release of the IVS from the Pre-rRNA Splicing Intermediate

Active	Inactive
5′-GTP	5′-ATP, 5′-CTP, 5′-UTP
5′-GDP	Guanine
5′-GMP	3′-GMP
Guanosine	2′-Deoxyguanosine, 2′-deoxyGTP 2′,3′-DideoxyGTP

Activity was measured at a final concentration of 2 μM.

The circular form of the IVS RNA was originally found as a product of transcription and splicing in isolated nuclei, and it was not known whether cyclization was part of the splicing process or the result of an unrelated RNA-ligase activity in the nucleus (Grabowski et al., 1981). We now find that when purified, uniformly labeled pre-rRNA splicing intermediate is allowed to continue splicing in vitro, the circular as well as the linear form of the excised IVS RNA is produced. Cyclization therefore appears to be performed by an activity involved with splicing, and may in fact be an obligatory step in the process (see the model below).

RNA splicing requires both excision of the IVS and ligation of the resulting exons (mature RNA sequences that were interrupted by the IVS in the precursor). Incubation of the pre-rRNA splicing intermediate with the necessary cofactors results in excision of the IVS. Evidence for exon ligation, however, is indirect, based on the lack of a 3′ exon fragment that would be produced by cleavage in the absence of ligation. In the low salt conditions we used for synthesis of the pre-rRNA, accurate termination of transcription occurs (Leer et al., 1979); the 3′ exon should therefore

Table 2. Comparison of Guanosine Cofactors

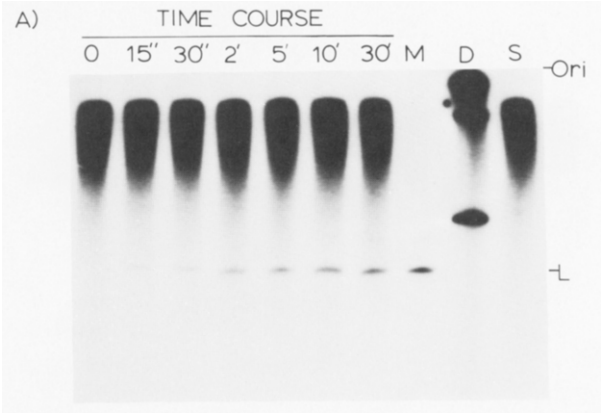
Cofactor	IVS Excised (%) 2.0 μM (a)	0.2 μM (b)	Increase in IVS Excision a/b
GTP	4.9	2.7	1.8
GDP	4.5	2.7	1.6
GMP	4.3	2.7	1.6
Guanosine	8.6	5.6	1.5
Mean ± SD			1.6 ± 0.1

Each guanosine compound was tested at two final concentrations, the concentration at which GTP gives maximal IVS excision (a) and the concentration at which GTP gives half-maximal excision (b). Values have been corrected for the 1% IVS in the control, in which an equal volume of water was substituted for the cofactor.

be a discrete size fragment. Based on recent mapping of the 3′ end of the pre-rRNA at the nucleotide sequence level (N. Din, J. Engberg and J. Gall, personal communication), the size of this RNA would be 1070 nucleotides. Because gel electrophoresis (see, for example, Figure 2) revealed no such RNA, it is likely that the splicing intermediate is also capable of exon ligation. Alternatively, the two exons may be held together in the splicing complex without having been ligated. A more direct test of ligation may be provided by S1-nuclease-protection studies of hybrids formed between the in vitro splicing product and the rDNA.

Structure of the Splicing Intermediate

The specificity of the reactions performed by the splicing intermediate, and the absolute dependence of these reactions on a guanosine compound, make it seem very likely that an enzyme is associated with the RNA. The activity is not destroyed or removed, however, when the splicing intermediate is subjected to the following treatments: boiling in the presence of SDS and 2-mercaptoethanol, SDS-phenol extraction at room temperature or at 65°C, sedimentation in a 60% formamide sucrose gradient or treatment with proteases according to a variety of protocols described in Experimental Procedures. Although such results are normally taken as strong evidence against protein involvement, some known nucleic acid enzymes are resistant to inactivation by heat, SDS or protease treatment. For example, the single-strand-specific endonuclease activity of Bal 31 nuclease is highly resistant to digestion by pronase (1 mg/ml for 10 min at 37°C; H. B. Gray, Jr. and C. F. Wei, personal communication) and persists in the presence of 5% SDS when incubated in a high salt buffer containing calcium and magnesium (Gray et al., 1975). Furthermore, attachment of a protein to a large RNA molecule could account for its surviving the phenol extraction, and might explain its resistance to protease. Attempts to detect a protein on the splicing intermediate by labeling with radioactive amino acids are in progress.



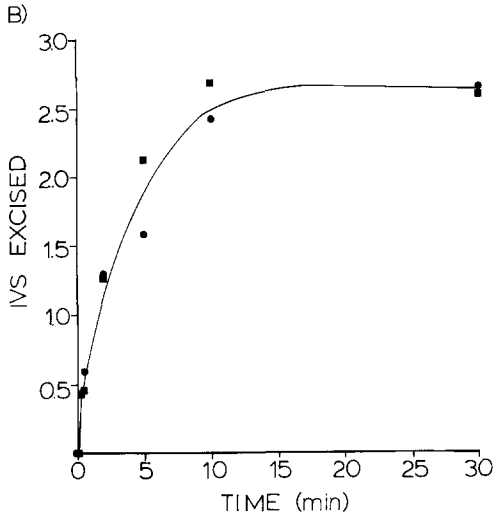


Figure 5. Time Course of the IVS-Excision Reaction

(A) Denaturing gel electrophoresis of the RNA products of the IVS-excision reaction monitored as a function of time. (Lane S) The untreated starting material. (Lane D) ^{32}P-end-labeled DNA marker.
(B) The amount of excised IVS RNA, given as a percentage of the total labeled RNA, was determined from densitometric tracings of the autoradiograph in (A) (●——●), and by scintillation counting of the sliced, dried gel (■——■).

The resistance of the splicing activity to phenol extraction, SDS and proteases can also be interpreted in a more straightforward manner. The rRNA precursor might be able to undergo splicing without the participation of a protein enzyme. A portion of the RNA chain could be folded in such a way that it formed an active site or sites that bound the guanosine cofactor and catalyzed the various bond-cleavage and ligation events. If one of the RNA molecules produced in the reaction (for example, the free IVS) retained its activity and catalyzed additional splicing events, then it would be an example of an RNA enzyme.

Another unresolved question about the splicing intermediate concerns the means by which the IVS is attached to the rest of the pre-rRNA. The IVS was not released from the high molecular weight RNA by any of the treatments mentioned in the preceding paragraph, or by electrophoresis in an 8 M urea gel at 65°C. These results provide strong evidence that the

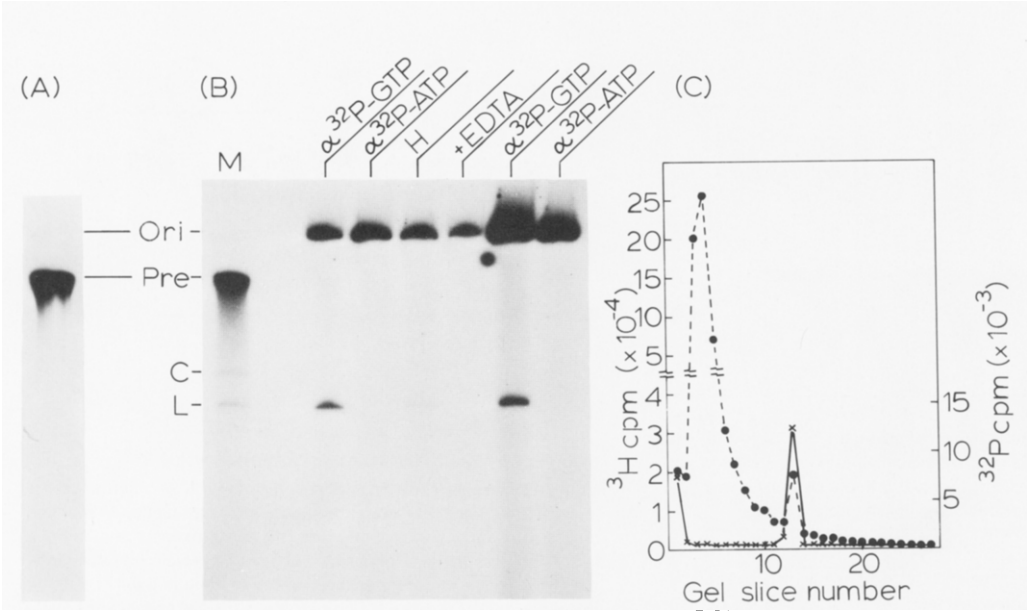


Figure 6. Addition of a Guanosine Nucleotide to the IVS during Its Excision

(A) Pre-rRNA splicing intermediate synthesized in nuclei under the low salt conditions with ^{3}H-UTP as the labeled precursor. RNA was subjected to electrophoresis on a 4% polyacrylamide, 8 M urea gel, which was then subjected to tritium fluorography. Note the absence of free IVS RNA.

(B) Splicing intermediate (isolated as in [A], but unlabeled) was incubated with ^{32}P-GTP or ^{32}P-ATP. (Lane α-^{32}P-GTP) RNA incubated with 40 μCi α-^{32}P-GTP (5 μM) for 30 min at 30°C in 10 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$ and 40 mM Tris (pH 7.5). RNA splicing intermediate was at a concentration of ~1 nM. (Lane α-^{32}P-ATP) Same conditions, with GTP replaced by 40 μCi ATP. (Lane H) α-^{32}P-GTP incubation at 39°C instead of 30°C, conditions that favor cyclization of the excised IVS. (Lane EDTA) same as α-^{32}P-GTP incubation, except $MgCl_2$ replaced by EDTA. The two lanes to the right show the reaction with a different preparation of pre-rRNA splicing intermediate. (Lane M) Markers for the pre-rRNA and the circular and linear IVS, which were produced from the splicing intermediate (uniformly labeled with ^{32}P during transcription) by incubation as described above, except with 1 mM unlabeled GTP.

(C) Tritiated splicing intermediate shown in (A) was allowed to complete excision of the IVS in the presence of α-^{32}P-GTP. After gel electrophoresis, the gel was dried and sliced into one-eighth-inch fractions. The slices were solubilized, and the ^{3}H (● – – ●) and ^{32}P (×——×) radioactivities were determined by liquid scintillation counting.

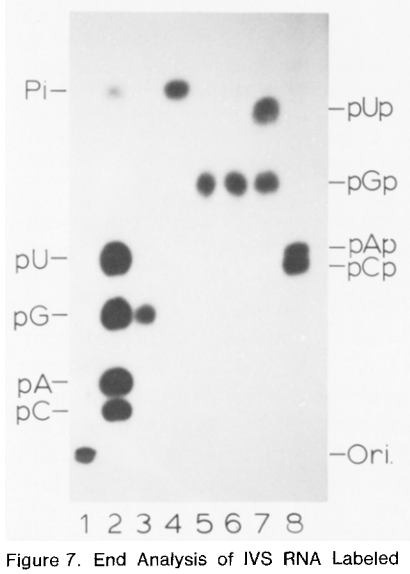


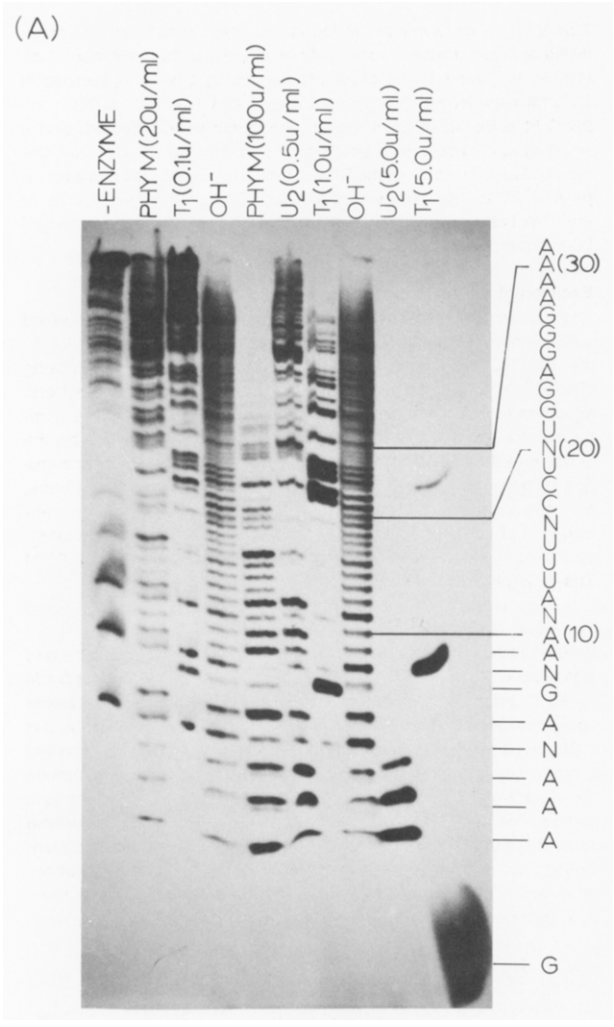
Figure 7. End Analysis of IVS RNA Labeled with 5′-GMP during Excision

Tritiated splicing intermediate was incubated with ^{32}P-GMP, salt and $MgCl_2$ to allow excision of the IVS to be completed. The IVS produced during this reaction was purified by polyacrylamide gel electrophoresis. It was then analyzed by high-voltage paper electrophoresis after no enzymatic incubation (lane 1), or after digestion to completion with nuclease P1 (lane 3), bacterial alkaline phosphatase (lane 4) or RNAase T2 (lane 5). (Lane 2) Uniformly labeled IVS RNA digested with nuclease P1 provided markers for the four pNs. (Lanes 6, 7, 8) Markers for the four pNps, as indicated.

IVS is covalently bound to the pre-rRNA. The simplest possibility is that the IVS is still an integral part of the pre-rRNA polynucleotide chain. Because there may be an unusually stable protein associated with the splicing intermediate (see above), it is also possible that the IVS has already been partially excised from the pre-rRNA and remains bound to it via a protein linkage (for example, as shown in Figure 9A). Such an intermediate would be similar to the covalent complex of a DNA topoisomerase with DNA that is formed when a nicking–closing reaction is interrupted in vitro (Depew et al., 1978).

A Model for Pre-rRNA Splicing

The seemingly incongruous features of pre-rRNA splicing include the addition of guanosine to the IVS during excision, the cyclization of the IVS, the apparent lack of an ATP requirement for cyclization or exon ligation and the possibility that a single activity accomplishes excision of the IVS and the two ligations. These features are largely reconciled by the model presented in Figure 9. According to this model, the splicing enzyme is a phosphoester transferase. The guanosine cofactor provides a free 3′ hydroxyl, to which the 5′ end of the IVS is transferred. A second phosphoester transfer releases the IVS and joins the two exons, while a third transfer cyclizes the IVS and releases the enzyme and the guanosine cofactor. This



(B)

```
     5'                                                    3'
DNA: ...CTCTCTAAATAGCAATATTTACCTTTGGAGGGAAAAGTT...CTCGTAAGGT...
IVS RNA:    pppG AAANAGNAANAUUUNCCUNUGGAGGGAAAA...
```

Figure 8. Nucleotide Sequence Analysis of the IVS RNA Labeled with GTP during Excision

(A) IVS RNA was prepared as described in the legend to Figure 7, except that the labeled cofactor was α-^{32}P-GTP. The IVS RNA was then digested to various extents with RNAase T1 (cleaves after G), U2 (cleaves after A) or Phy M (cleaves after A and U), and subjected to electrophoresis on a sequencing gel. (Lanes OH$^-$) Partial alkaline hydrolysis produced a "ladder" showing the positions of all the oligonucleotides. (Lane −ENZYME) RNA incubated without RNAase was cleaved to some extent, preventing the identification of some of the nucleotides (N). The diffuse band formed by the mononucleotide (pppGp) is most easily seen in the RNAase T1 digest that proceeded almost to completion.

(B) The RNA sequence determined from the gel in (A) is compared with the sequence of the IVS region of the rDNA (N. Kan and J. Gall, manuscript in preparation). Arrows: the IVS boundaries in the DNA.

model invokes a single enzymatic activity to accomplish excision and cyclization of the IVS and ligation of the exons, thereby simplifying the problem of how all three reactions could be performed by the activity associated with the splicing intermediate. Because

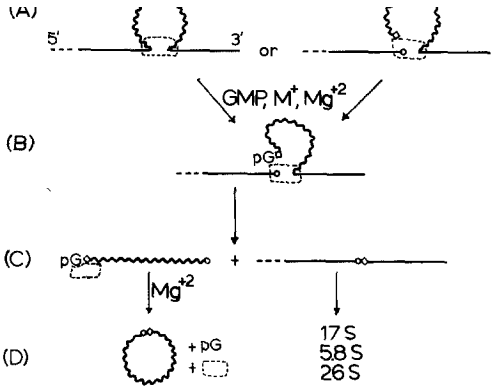


Figure 9. A Strand-Transfer Model for RNA Splicing

(A) The diagrams show two of several possible structures for the intermediate that accumulates when the splicing reaction is inhibited by low salt concentration. Solid lines: pre-rRNA exons. Wavy line: the IVS. Dashed rectangle: the splicing enzyme (a protein or a domain of the RNA). In the structure to the left, the enzyme is tightly bound to the substrate RNA; in the structure to the right, the enzyme has made one chain scission and is covalently bound to one or both of the termini. All strand cleavages produce 3′ hydroxyl (○) and 5′ phosphoryl (◇) termini.

(B) In the presence of the guanosine compound (shown as 5′-GMP) and monovalent and divalent cations, the first phosphoester-transfer reaction is completed. The 5′ end of the IVS RNA is linked to the 3′ hydroxyl of the guanosine cofactor.

(C) A second phosphoester transfer involves cleavage of the RNA at the 5′ end of the distal exon and rejoining of this phosphate to the 3′ hydroxyl of the proximal exon. Splicing of the pre-rRNA is now complete. The IVS is released as a linear molecule with an added 5′-terminal guanosine nucleotide (see text). Cyclization activity is tightly associated with the excised IVS (P. Grabowski, unpublished results).

(D) A third phosphoester transfer involves cleavage of the IVS near its 5′ end and rejoining with a new 3′ hydroxyl group. In this case, the 3′ hydroxyl group is at the end of the same strand of RNA, so that the reaction produces a circle. Recent results indicate that the guanosine nucleotide added during excision is released upon cyclization; it is not released as a free mononucleotide, but as part of a larger molecule that has not yet been identified (A. Zaug, unpublished results). Release of splicing activity could occur only if it were a separate molecule.

cleavage and ligation are linked, the proposed mechanism circumvents the need for ATP or GTP hydrolysis.

The linear excised IVS RNA has a 3′-terminal G–OH (A. Zaug and T. Cech, manuscript in preparation). Cyclization of the IVS, like the first phosphoester transfer event, therefore involves an attack by the 3′ hydroxyl of a guanosine residue. A guanosine at the 3′ end of the IVS is a feature common to rRNA and mRNA intervening sequences (see, for example, Lerner et al., 1980; Wild and Sommer, 1980; Nomiyama et al., 1981). It is possible that this guanosine is conserved because it is required for IVS cyclization, though the only other case in which cyclization has yet been observed involves yeast mitochondrial mRNA intervening sequences (Arnberg et al., 1980; Halbreich et al., 1980).

The proposed phosphoester-transferase activity is similar to that of DNA nicking–closing enzymes, except that the 5′ phosphate produced by the nicking step is joined to a different 3′ hydroxyl group. DNA

nicking–closing events catalyzed by E. coli DNA topoisomerase I or the NalA subunit of DNA gyrase have no energy requirement (Kornberg, 1980). The phosphoester transferase also bears some similarity to the A and A* proteins involved in ϕX174 DNA replication (Eisenberg et al., 1977; van der Ende et al., 1981). In both cases, the enzyme introduces a nick at a specific place in a nucleic acid molecule, producing a 3′ hydroxyl; remains bound to the nucleic acid; and while bound can catalyze a cleavage–ligation reaction that results in production of a circular nucleic acid molecule.

The model does not address the problems of recognition of the IVS and formation of the splicing complex. These processes may be mediated by an adapter RNA such as a tRNA (Gourse and Gerbi, 1980) or a small nucleolar RNA (Reddy et al., 1979; Wise and Weiner, 1980), both of which have some sequence complementarity to exon sequences on both sides of the IVS.

Experimental Procedures

Nucleotides and Enzymes

Unlabeled nucleoside triphosphates were purchased from P-L Biochemicals, and for some experiments were further purified as described by McClure et al. (1978). Other nucleotides, nucleosides and bases were of the highest quality available from Sigma and were used without further purification. ^{32}P-labeled nucleoside triphosphates were purchased from New England Nuclear. Labeled nucleoside 3′,5′-bisphosphates (pNps) were made from the corresponding unlabeled 3′-monophosphates with polynucleotide kinase and γ-^{32}P-ATP (both from New England Nuclear). We made 5′-^{32}P-GMP from pGp using nuclease P1 (Sigma) to remove the 3′ phosphate; it was then purified by thin-layer chromatography on PEI-cellulose. RNAases T1, T2 and U2 were obtained from Sankyo, and bacterial alkaline phosphatase was obtained from Bethesda Research Laboratories. RNAase Phy M was a gift from N. Pace.

Transcription and Splicing in Isolated Nuclei

Growth of cells (T. thermophila strain B VII) and isolation of nuclei were carried out as described by Zaug and Cech (1980). Transcription reactions contained 1 to 2 × 10^7 nuclei in 0.45 ml of transcription cocktail (53 mM Tris–HCl [pH 8.0], 5.3 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM putrescine, 1 mM spermidine, 0.1 mM spermine, 2 mM 2-mercaptoethanol, 0.048 mM each of ATP, CTP, UTP and GTP, 0.2–0.4 mM aurintricarboxylic acid (nuclease inhibitor) and 40 μg/ml α-amanitin). In most experiments one or all four of the nucleoside triphosphates were either α-^{32}P- or ^{3}H-labeled. In addition, each reaction contained $(NH_4)_2SO_4$ at a concentration of 5 mM to prevent splicing or 120 mM to optimize the excision of the IVS. The mixture was incubated for 30 min at 30°C with occasional shaking, at which time the reaction was stopped by the addition of EDTA and SDS to final concentrations of 20 mM and 1%, respectively. The incorporation varied from 2%–20% of the input radioactivity, as determined by Whatman DE81 filter binding.

Isolation of RNA

After in vitro transcription, the lysed nuclei were diluted with 1–3 ml of 0.25 M sodium acetate, 0.05 M Tris–HCl (pH 7.5). The lysate was extracted twice with phenol and once with chloroform and treated with DNAase as described previously (Zaug and Cech, 1980).

Preparation of the Splicing Intermediate

RNA synthesized in isolated nuclei under the low salt conditions and purified as described above was ethanol-precipitated. The RNA from ~2 × 10^7 nuclei was redissolved in 0.05 ml of 0.05 M Tris–HCl (pH 7.5). It was then denatured in formamide and sedimented in a denaturing sucrose gradient essentially as described by Anderson et al. (1974). We used 12 ml gradients containing 5%–20% sucrose in 60% formamide, which were centrifuged for 11.5 hr at 36,000 rpm, 25°C in a Beckman SW41 rotor. Fractions were collected, and a small aliquot of each was spotted on a DE81 filter to quantitate the labeled RNA. The bulk of the RNA synthesized in vitro formed a broad peak at 5S to 16S, and was discarded. The approximately 25% of the RNA that sedimented at >16S was collected by ethanol precipitation and used as the splicing intermediate.

Excision of the IVS from the Splicing Intermediate

The standard IVS-excision reaction involved incubation of the purified splicing intermediate in 10 μl of 100 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 30 mM Tris–HCl (pH 7.5), 0.2 mM GTP for 30 min at 30°C. Prior to being divided into 10 μl samples, the splicing intermediate was mixed with all of the reaction components except one, either GTP or the component to be varied. The reactions were stopped on ice by the addition of 20 mM EDTA. In the experiments in which the concentration of one cofactor was varied, that component was added back, after the reactions were stopped, in amounts to equalize its concentration in all reaction tubes. The RNA was then ethanol-precipitated, and the precipitates were washed, dried and resuspended in 0.05 M Tris–HCl (pH 7.5) before gel electrophoresis.

Gel Electrophoresis of RNA

Unless stated otherwise, slab gels contained 4% polyacrylamide and 8 M urea. RNA in urea-containing sample buffer was heated for 5 min at 65°C prior to loading on the gel. The electrophoresis buffer contained 89 mM Tris–HCl, 89 mM boric acid, 2.5 mM EDTA (pH 8.3). Electrophoresis was carried out for 2 hr at 35 mA constant current at room temperature, or in an oven at 65°C to ensure complete denaturation. After soaking out the urea, we routinely dried the gels and autoradiographed them overnight at −70°C using Kodak XRP-5 or XAR-5 x-ray film and a DuPont Cronex intensifying screen. Densitometry of x-ray films was performed at 480 nm with the gel scanner accessory of the Cary 219 spectrophotometer. Peak areas were determined with a Numonics electronic planimeter.

End Analysis of RNA

RNAase T2 digestions of IVS RNA were carried out for 1 hr at 37°C in 10 μl of 0.01 M Tris–HCl, 0.001 M EDTA (pH 7.5) with 5 U enzyme. Nuclease P1 digestions were done as for T2, except at 60°C with 2 U enzyme. Bacterial alkaline phosphatase incubations were done in 10 μl of 0.01 M Tris–HCl (pH 8.0) for 1 hr at 37°C with 100 U enzyme. Reaction mixtures were fractionated by high-voltage paper electrophoresis on Whatman 3MM paper (Barrell, 1971). The samples were spotted 10 cm from the end of a piece of 3MM paper and allowed to dry completely. A small spot of dye mixture containing 1% each of acid fuchsin, methyl orange and xylene cyanol was put on each side of the paper. Electrophoresis was carried out at 2500 V in 5% acetic acid, 0.3% pyridine, 1 mM EDTA (pH 3.5) until the xylene cyanol had migrated 10 cm from the origin.

RNA Sequence Analysis

RNA was sequenced according to the procedures of Donis-Keller et al. (1977) and Donis-Keller (1980), with slight modifications. End-labeled IVS RNA in a solution containing 250 μg/ml tRNA was partially digested with RNAase T1 or U2 at pH 3.5 for 15 min at 50°C. Enzyme to substrate ratios (units per microgram of tRNA) were 2 × 10^{-2}, 4 × 10^{-3} and 4 × 10^{-4} for T1 and 2 × 10^{-2} and 2 × 10^{-3} for U2. Partial digestions with RNAase Phy M (Donis-Keller, 1980) were done at pH 5.0 with enzyme to substrate ratios of 4 × 10^{-1} and 8 × 10^{-2}. Partial alkaline hydrolysis at pH 9.0 was carried out in sealed silated capillary tubes at 90°C for either 45 or 60 min. Samples were loaded on a preelectrophoresed 0.4 mm thick 20% polyacrylamide, 7 M urea gel and run at 30 W (1200–1600 V) for 3 to 4 hr.

Protease Treatments

The isolated unspliced pre-rRNA was incubated in the presence of 10 mg/ml pronase (Sigma) or 0.4 mg/ml proteinase K (EM Biochem-

icals) in a buffer containing 50 mM Tris–HCl (pH 7.5), 1 mM EDTA and 0.2% SDS (buffer A). The incubations were performed in 10 μl reaction mixtures at 37°C for 60 min. Incubation of the precursor in buffer alone constituted the control. Immediately after the protease treatments, the reactions were adjusted to splicing conditions on ice by the addition of a concentrated stock solution of splicing buffer (1× buffer: 10 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, 30 mM Tris–HCl [pH 7.5] and 0.1 mM GTP) and incubated at 30°C for 30 min. (The protease was not removed prior to the splicing reaction, since the splicing activity appears to be lost or inactivated by chloroform extraction at this point.) In addition, the above protease treatments were performed after the splicing intermediate was boiled for 5 min in buffer A. The reactions were then adjusted to 5 mg/ml pronase or 0.2 mg/ml proteinase K and incubated for 1 hr at 37°C in 90 μl volumes. The splicing reaction was performed without the removal of the protease as described above (except for the larger volume). We assayed the proteases used in these treatments using known quantities of ^{14}C-labeled proteins (Bethesda Research Laboratories). Under the conditions specified above, pronase routinely digested 82% of a 1 mg sample of a ^{14}C-labeled protein–bovine serum albumin mixture, and proteinase K digested 93% of the protein sample, as determined by trichloroacetic acid solubility.

Acknowledgments

We thank Nancy Kan and Nanni Din for sharing unpublished DNA sequences; Norm Pace for advice on RNA sequencing; and Nicholas Cozzarelli for a helpful discussion. This work was supported by grants from the National Institutes of Health; the Council on Research and Creative Work of the University of Colorado; and the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health. T. R. C. was supported by an NIH Research Career Development Award.

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Received September 24, 1981; revised October 23, 1981

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