Fate of an Intervening Sequence Ribonucleic Acid: Excision and Cyclization of the *Tetrahymena* Ribosomal Ribonucleic Acid Intervening Sequence in Vivo†

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ABSTRACT: In previous studies of RNA splicing in vitro, we have shown that the intervening sequence (IVS) of the *Tetrahymena* rRNA precursor is excised as a unique linear RNA molecule and subsequently cyclized. In the present work, we have investigated the occurrence and stability of these RNA species in vivo. RNA was separated by gel electrophoresis, transferred to diazotized paper, and hybridized with ^{32}P-labeled DNA probes. RNA molecules containing the IVS were found to reside within the nucleus and not in the cytoplasm. The species found in the nucleus include both the linear and circular forms of the excised IVS RNA, as well as the unspliced precursor. On the basis of quantitation of the hybridization, the half-lives of the IVS-containing pre-rRNA and the excised IVS RNA in rapidly growing cells were estimated as 2 and 6 s, respectively. We conclude that splicing is not a rate-limiting step in rRNA maturation and that the IVS RNA is quickly degraded after its excision. When the deproteinized nuclear RNA was incubated at 37 °C in a Mg^{2+}-containing solution, a substantial portion of the linear IVS RNA was converted to the circular form. Autocyclization, previously characterized with IVS RNA produced by splicing in vitro, is therefore also a property of IVS RNA produced in vivo.

Many eucaryotic genes are interrupted by intervening sequences [see reviews by Abelson (1979), Breathnach & Chambon (1981), and Cech et al. (1982)]. The intervening sequences are transcribed as part of precursor RNA molecules and are subsequently removed by a cleavage–ligation process termed splicing. Because split genes are so common, the production of intervening sequence transcripts accounts for a substantial part of the RNA synthesis in eucaryotic cells. Little is known, however, about the ultimate fate of the excised intervening sequence (IVS)[1] RNAs.

The macronuclear rRNA genes of some species of *Tetrahymena* contain a 0.4-kb intervening sequence which is transcribed and spliced (Wild & Gall, 1979; Cech & Rio, 1979; Din et al., 1979) (see Figure 1). Studies of splicing in isolated nuclei have shown that the *Tetrahymena thermophila* IVS is excised as a unique linear molecule and subsequently cyclized (Zaug & Cech, 1980; Grabowski et al., 1981). The rate of cyclization is greatly enhanced at 37–40 °C compared to 30 °C in vitro. Cyclization is not a simple end-to-end ligation of the linear IVS RNA molecule. Instead, it is accomplished by cleavage of the linear IVS RNA 15 nt from its 5′ end and joining of the original 3′ end to the newly created 5′ end (Zaug & Cech, 1982; Zaug et al., 1983). Cyclization therefore occurs by a cleavage–ligation mechanism analogous to that involved in splicing the pre-rRNA.

The rRNA intervening sequences of *Tetrahymena pigmentosa* and *T. thermophila* do not contain long open reading frames (Wild & Sommer, 1980; Kan & Gall, 1982). The excised IVS RNA is therefore unlikely to function as a mRNA. In this respect, the *Tetrahymena* rRNA IVS's resemble the intervening sequences of other nuclear genes and differ from those of mitochondrial mRNA and rRNA genes (Lazowska et al., 1980; Dujon, 1980). One can conceive of other, nonmessenger functions for an excised intervening sequence (Crick, 1979). The circular form is particularly intriguing, since it might be expected to have increased stability due to its resistance to exonucleases.

To provide a basis for thinking about the function of the linear and circular IVS RNAs, we have examined their occurrence and lifetime in vivo. We find that both the linear and circular forms of the IVS RNA are present in the nucleus. Because the lifetime of the pre-rRNA was previously determined by pulse–chase kinetic experiments, we were able to use relative hybridization values to estimate lifetimes of the IVS-containing nuclear RNA species. We find that the excised IVS RNA in its linear and circular forms has a very short half-life (~6 s). Although these results do not eliminate the possibility that the circular IVS RNA has some function, they lead us to suggest that cyclization may be occurring as a step in the splicing mechanism and not because the circular RNA product is necessary.

Experimental Procedures

Growth and Starvation of Cells. *T. thermophila* strain B VII and *T. pigmentosa* strain 6UM were grown to densities of (1–3) × 10^5 cells/mL in 1% proteose peptone (Difco), 0.003% sequestrine (CIBA-Geigy), and 0.005% each of streptomycin and penicillin (Sigma). One-liter cultures of *T. thermophila* were grown in 2.8-L Fernbach flasks with shaking in a water bath at either 30 or 39 °C. *T. pigmentosa* were grown at 28 °C. Starvation of *T. thermophila* was accomplished by resuspending pelleted cells in 50 mM Tris-HCl (pH 7.5) and incubating for 16 h at the same temperature at which they were grown, as described by Cech & Brehm (1981).

Isolation of RNA. Isolation of nuclei and nuclear RNA was performed exactly as described by Zaug & Cech (1980), except that cytoplasmic RNA was extracted during the nuclei isolation. Cells were lysed in NP-40 and nuclei pelleted by centrifugation (10 min, 4000 rpm, Beckman JS-13 rotor). The

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[1] Abbreviations: IVS, intervening sequence; ETS, external transcribed spacer; kb, kilobase (1000 base pairs of DNA or bases of RNA); bp, base pair(s); nt, nucleotide(s); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; DBM, diazobenzyloxymethyl; Me_2SO, dimethyl sulfoxide.

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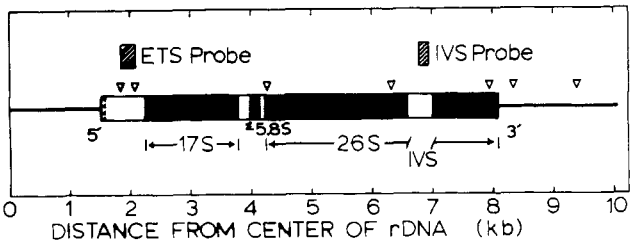

FIGURE 1: Transcription unit of *T. thermophila* rDNA. Half of the palindromic rDNA is shown. The wide bar represents the sequences coding for the primary transcript. Sequences present in mature rDNA are shaded. (▽) *Hin*dIII restriction sites. Data are taken from Engberg et al. (1980), Cech & Rio (1979), and Din et al. (1982).

supernatant was centrifuged a second time to remove remaining nuclei. It was then extracted with phenol and chloroform, and the cytoplasmic RNA was precipitated with 2 volumes of ethanol. The first nuclear pellet was resuspended, the nuclei were sedimented through sucrose, and the nuclear RNA was extracted (Zaug & Cech, 1980). Whole cell RNA was isolated by a method designed to minimize the possibility of any further splicing reaction during RNA preparation. Growing cells were poured onto sterile crushed ice and collected by centrifugation at 4 °C in precooled bottles. Cells were resuspended in 0.02 M Tris–HCl, pH 7.5, 0.1 M Na-$C_2H_3O_2$, and 0.05 M EDTA, lysed by addition of $NaDodSO_4$ to 0.5% final concentration, and extracted with phenol and chloroform as above. All these steps and the subsequent DNase treatment were performed on ice; neither splicing nor cyclization takes place in vitro at this temperature (unpublished results). Whole cell RNA was fractionated by sucrose gradient sedimentation in an EDTA-containing buffer, and a portion of each fraction was subjected to gel electrophoresis and blot hybridization.

Gel Electrophoresis of RNA. For glyoxal–agarose gel electrophoresis, 20-μg RNA samples were ethanol precipitated, washed with 95% ethanol, and dried. They were redissolved in a solution containing glyoxal and Me_2SO and placed in a 50 °C water bath for 1 h, as described by Carmichael & McMaster (1980). Immediately following the incubation, the samples were loaded onto a 1.5% agarose gel and electrophoresed at 85–90 mA for 30 min at room temperature. For 8 M urea–4% polyacrylamide gel electrophoresis, 20-μg RNA samples were loaded in sample buffer containing 8 M urea. The gels were run at 30–40 mA for 1.5–3.0 h in a 65 °C oven unless stated otherwise.

Transfer of RNA to DBM Paper. Glyoxal–agarose gels were soaked in 50 mM NaOH for 40 min and then neutralized by soaking for 30 min in 0.2 M $NaC_2H_3O_2$ (pH 4.0) containing 4 μg/mL ethidium bromide. After neutralization, gels were viewed under UV light and photographed. DBM paper was prepared from NBM paper (Schleicher & Schuell) according to the manufacturer's directions. Transfer of the RNA to DBM paper was essentially as described by Southern (1975).

The polyacrylamide gels containing 8 M urea were stained with ethidium bromide and photographed. The urea was then removed by three washes (15 min each) in twice-distilled H_2O. The gel was soaked in 40 mM NaOH and neutralized as described above. The RNA was electrophoretically transferred to DBM paper as described by Stellwag & Dahlberg (1980). The transfer was for 4–6 h at 275–300 mA at room temperature. The running buffer [0.2 M $NaC_2H_3O_2$ (pH 4.0)] was recirculated with a stir bar at the bottom of the tank.

Hybridization. Gel blots were prehybridized to remove any reactive diazonium groups and to eliminate nonspecific sites on the paper by incubation for at least 5 h at 42 °C in hybridization buffer [50% formamide (MCB), 750 mM NaCl, 75 mM sodium citrate, 50 mM sodium phosphate (pH 6.5), 0.2% $NaDodSO_4$, 0.02% each of bovine serum albumin, ficoll, and poly(vinylpyrrolidone), and 2.5 mg/mL denatured herring sperm DNA (Sigma D 2251)]. For the prewash, glycine was added to the buffer to a final concentration of 1%. All washing and hybridization steps were done in a Sears Seal-a-Meal bag. For hybridization, the paper was incubated with hybridization buffer (200 μL/cm² DBM paper) containing the nick-translated DNA probe (1×10^5 cpm per gel lane; boiled 5–10 min). Hybridization proceeded for 24 h with shaking at 39–42 °C. The DBM paper was then washed in 50% formamide, 50 mM sodium phosphate (pH 6.5), 750 mM NaCl, and 75 mM sodium citrate for 1 h at 42 °C with shaking, followed by a second wash in fresh solution for 5–24 h. The DBM paper was then dried and exposed to Kodak XAR-5 X-ray film with an intensifying screen at −70 °C.

Plasmid DNA. Plasmid pTpAA1, containing a 145-bp *Alu*I fragment of *T. pigmentosa* strain 6UM rDNA inserted in the *Bam*HI restriction site of pBR313, was obtained from Martha Wild. The insert includes nucleotides 188–332 of the 407-bp *T. pigmentosa* IVS (Wild & Sommer, 1980). Its sequence is homologous with that of *T. thermophila* (Kan & Gall, 1982) except for three small regions, which would produce mismatches of one, four, and six bases in a heteroduplex. Plasmid pRP1 (Engberg et al., 1980), containing a 241-bp *Hin*dIII fragment of *T. thermophila* rDNA inserted in the *Hin*dIII restriction site of pBR322, was obtained from Ron Pearlman. The fragment begins 250–310 bp downstream from the major transcription initiation site and ends 163 bp upstream from the 5′ end of mature 17S rRNA (N. Din and J. Engberg, personal communication; Niles et al., 1981). *Escherichia coli* hosts containing plasmids were grown at 39 °C to an optical density of 0.4–0.6. Chloramphenicol (Sigma) was added to a final concentration of 240 mg/mL for plasmid enrichment. Cells were allowed to continue growing for 16–18 h. Plasmid DNA was isolated from 1 L of cells by a scaled-up version of the rapid alkaline extraction method of Birnboim & Doly (1979).

Preparation of DNA Probes. Plasmid DNA was cleaved with the appropriate restriction endonuclease to release the insert. The resulting fragments were electrophoresed on a 3.5% polyacrylamide gel and stained with ethidium bromide. The desired fragment was cut from the gel while viewed under an ultraviolet source. Gel slices were crushed with a sterile spatula, and 3 mL of 500 mM $NH_4C_2H_3O_2$, 10 mM Mg-$(C_2H_3O_2)_2$, 1 mM EDTA, and 0.1% $NaDodSO_4$ was added to elute the DNA. The isolated fragment (2 μg) was labeled to a specific activity of $(0.7–2.5) \times 10^7$ cpm/μg by nick translation (Maniatis et al., 1975) using [α-^{32}P]dATP (New England Nuclear). Unincorporated dATP was removed by Sephadex G-50 column chromatography. Fractions containing the DNA were pooled and frozen.

Calculation of RNA Lifetimes from Steady-State Concentrations. The analysis requires that there is no wastage of rRNA transcripts, a fact established for both rapidly growing and starved cells (Sutton et al., 1979). Thus, each primary transcript is spliced, giving rise to one free IVS RNA molecule and one molecule of PRE 2. When we use an IVS probe, we are following the reaction series

$$\text{PRE 1} \xrightarrow{k_1} \text{IVS} \xrightarrow{k_d} \text{P} \qquad (1)$$

where k_1 and k_d are the first-order rate constants for excision of the IVS and degradation of the free IVS into products P,

respectively. Because we do not know whether all excised IVS molecules are cyclized in vivo, we take "IVS" to be the sum of the linear and circular forms. The constant k_d may therefore be affected by (or even determined by) the rate of cyclization. If no reverse reactions occur, then in the steady state

$$k_1[\text{PRE 1}] = k_d[\text{IVS}] \quad (2)$$

The ratio of the steady-state concentrations of free IVS and PRE 1 gives the relative half-lives of the RNA species:

$$\frac{[\text{IVS}]}{[\text{PRE 1}]} = \frac{k_1}{k_d} = \frac{t_{1/2}(\text{IVS})}{t_{1/2}(\text{PRE 1})} \quad (3)$$

The ratio [IVS]/[PRE 1] is obtained from the relative amount of hybridization of the IVS probe to these two RNAs on the same lane of gel blot, corrected for the molecular weight dependence of transfer-retention efficiency. It is assumed that, in a probe-excess hybridization, the amount of probe hybridization is proportional to the amount of complementary RNA on the filter. An analogous situation arises when we use the ETS probe to follow the removal of the ETS fragment from the pre-rRNA:

$$\text{PRE 2} \xrightarrow{k_2} \text{ETS} + \text{PRE 3}$$

The ratio of the steady-state concentrations of the free ETS fragment and PRE 2 gives the relative half-lives of these RNA species:

$$\frac{[\text{ETS}]}{[\text{PRE 2}]} = \frac{t_{1/2}(\text{ETS})}{t_{1/2}(\text{PRE 2})} \quad (4)$$

When we follow the mature rRNA sequences, the reaction is

$$\text{PRE 1} \xrightarrow{k_1} \text{PRE 2} \xrightarrow{k_2} \text{PRE 3} \xrightarrow{k_3} \text{rRNA} \quad (5)$$

If all steps are first order and no reverse reactions occur, then in the steady state

$$k_1[\text{PRE 1}] = k_2[\text{PRE 2}] = k_3[\text{PRE 3}] \quad (6)$$

or

$$\frac{[\text{PRE 1}]}{t_{1/2}(\text{PRE 1})} = \frac{[\text{PRE 2}]}{t_{1/2}(\text{PRE 2})} = \frac{[\text{PRE 3}]}{t_{1/2}(\text{PRE 3})} \quad (7)$$

The half-life of PRE 1 is then

$$t_{1/2}(\text{PRE 1}) = t_{1/2}(\text{PRE 2})\frac{[\text{PRE 1}]}{[\text{PRE 2}]} = t_{1/2}(\text{PRE 2})\frac{H(\text{IVS})}{H(\text{ETS})}\frac{\text{SA(ETS)}}{\text{SA(IVS)}}\frac{L(\text{ETS})}{L(\text{IVS})} \quad (8)$$

where $H(x)$ = the autoradiographic intensity of hybridization to precursor-size RNA by probe x, SA(x) = the specific activity of probe x, and $L(x)$ = the number of bases in the RNA that can hybridize to probe x. The corrections for probe specific activity and length are required here because hybridization with two different probes is being compared. Sample calculations for growing (log) cells were the following: $t_{1/2}$(PRE 2) = 24 s from Table II; $H(\text{IVS})/H(\text{ETS}) = 0.22$; SA(ETS)/SA(IVS) = 0.26; $L(\text{ETS})/L(\text{IVS}) = 241$ nt/145 nt = 1.66; therefore, $t_{1/2}(\text{PRE 1}) = 24 \times 0.22 \times 0.26 \times 1.66 = 2.3$ s.

Results

IVS Is Present in Several Species of Nuclear RNA. Nuclear RNA isolated from cells grown at either 30 or 39 °C was completely denatured in the presence of glyoxal and electrophoresed on a 1.5% agarose gel. The major classes of RNA seen by ethidium bromide staining (Figure 2A) have been previously identified. The "35S" pre-rRNA is actually a mixture of three species (Figure 3A; Cech & Rio, 1979). The 17S RNA is a mixture of the small ribosomal RNA and the two fragments produced by nicking 26S rRNA in vivo (Bostock et al., 1971; Eckert et al., 1978; Engberg et al., 1980). Any contaminating cytoplasmic rRNA would contribute to the 17S fraction.

When the RNA in the gel was transferred to DBM paper (Alwine et al., 1977) and hybridized with radioactive DNA specific for the IVS, the autoradiogram shown in Figure 2B was obtained. Hybridization of the IVS-specific probe occurred to RNA molecules with three discrete sizes. The species with the highest mobility (490 ● 30 nt) was identified as the excised IVS RNA. It coelectrophoresed with an IVS RNA marker, which had been labeled with ^{32}P during in vitro

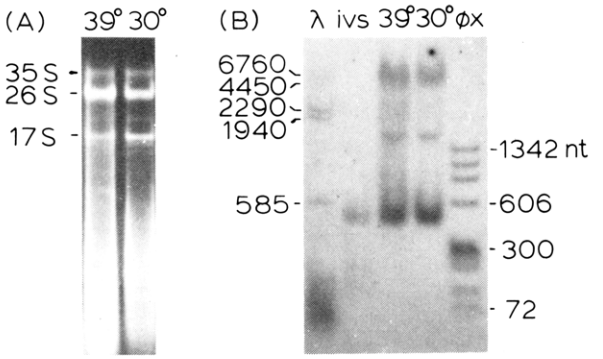

FIGURE 2: Hybridization of the IVS probe to nuclear RNA species separated by glyoxal–agarose gel electrophoresis. (A) A portion of the ethidium-stained gel, showing lanes containing RNA isolated from nuclei of cells grown at 30 and 39 °C. (B) Autoradiogram obtained by transfer of the RNA to DBM paper and hybridization with a DNA probe specific for the IVS. Molecular weight markers labeled with ^{32}P prior to electrophoresis: (λ) *Hin*dIII restriction fragments of phage λ DNA; (ivs) purified IVS RNA labeled during transcription in isolated nuclei; (φX) *Hae*III restriction fragments of φX174 DNA.

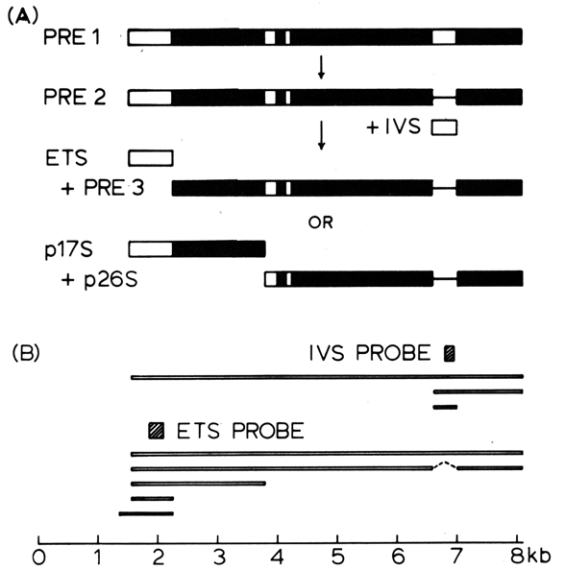

FIGURE 3: Identification of rRNA processing intermediates. (A) Early steps in the processing pathway. Mature rRNA sequences within the precursor are shown in black, while sequences removed during processing are shown in white. Evidence for the various species can be found in the following references: PRE 1, PRE 2, and PRE 3 (Cech & Rio, 1979); free IVS and free ETS fragments (this work); p17S (Sutton et al., 1979; a ~17S species with a triphosphate 5′ end was also described by Niles, 1978); p26S (Eckert et al., 1978; Engberg et al., 1980). (B) Assignment of gel blot hybridization signals to the processing intermediates. Assignments are based on the size of the hybridizing RNA and the identity of the probes which hybridized to it.

Table I: Relative Amounts of IVS in the Pre-rRNA and the Linear and Circular Excision Products in Growing and Starved Cells

cells	growth	expt no.[a]	rel hybridization with IVS probe[b] PRE 1	C	L	(C + L)/PRE 1	C/(C + L)
T. thermophila	30 °C, log	1	0.41	0.29	1.00[c]	3.1	0.22
		2	0.54	0.15	1.00[c]	2.1	0.13
		3	0.62	0.18	1.00[c]	1.9	0.15
		$\bar{X}$	0.52 ± 0.11	0.21 ± 0.08	1.00[c]	2.4 ± 0.7	0.17 ± 0.05
	30 °C, starved	1	0.16	0.05	0.41	2.9	0.11
		2	0.12	~0	0.23	1.9	~0
		3	0.12	0	0.21	1.8	0
		$\bar{X}$	0.13 ± 0.03	~0	0.28 ± 0.13	2.2 ± 0.7	~0
	39 °C, log	1	0.42	0.37	0.94	3.1	0.28
		4	0.24	0.15	0.94[c]	4.5	0.14
		$\bar{X}$	0.33 ± 0.09	0.26 ± 0.11	0.94	3.8 ± 0.7	0.21 ± 0.07
	39 °C, starved	1	0.14	0.31	0.84	8.0	0.28
		4	0.07	0.12	0.47	8.1	0.14
		$\bar{X}$	0.11 ± 0.04	0.21 ± 0.10	0.65 ± 0.19	8.0 ± 0.1	0.21 ± 0.07
T. pigmentosa	28 °C, log	2	0.03	0	0.62	21	0

[a] Experiment 2 raw data are shown in Figure 6; experiments 3 and 4 raw data are shown in Figure 7A,B. X is the mean value for the various experiments ± the range of values. [b] Determined by densitometry of autoradiograms. In each experiment, two or three X-ray films with different exposures were scanned to assure that signals were in the linear range of film response. Densitometer measurements of L and C IVS hybridization were multiplied by 4.2 to correct for their lower efficiency of transfer and/or retention on DBM paper relative to the precursor RNA (see text). [c] All values were normalized to the amount of hybridization to the linear IVS in 30 °C log-phase cells. There was no 30 °C log-phase cell RNA in experiment 4, so values were normalized to the amount of linear IVS in 39 °C log-phase cell RNA in experiment 1.

transcription. The species with the lowest mobility (6600 ± 1000 nt) was the size expected for the primary transcript. The band at 1600 ± 50 nt was not expected and might be a splicing intermediate. [If cleavage at the 5′ end of the IVS occurred prior to cleavage at the 3′ end, an intermediate containing the IVS linked to the 3′ exon would be produced. Its size would be 1513 nt (Din et al., 1982).] The same IVS-containing nuclear RNAs were present in cells grown at 30 and 39 °C. The assignment of the hybridizing RNA is summarized in Figure 3B. Similar results have been obtained by W. Eckert and collaborators (W. Eckert, personal communication).

Linear and Circular Forms of the Excised IVS Are Produced in Vivo. RNA was electrophoresed on 4% polyacrylamide–8 M urea gels at 65 °C (Grabowski et al., 1981) to provide good separation between the linear and circular forms of the excised IVS RNA. The RNA was then electrophoretically transferred from the gel to DBM paper as described under Experimental Procedures. The results of hybridization with the IVS-specific probe are shown in Figure 4. The nuclear RNA contained both linear and circular IVS RNA, identified by their comigration with ^{32}P-labeled IVS RNA markers prepared by in vitro transcription. Hybridization to the pre-rRNA and to the 1600-nt species also occurred, though these species were better resolved in the agarose gel system.

After a 12-h exposure, no hybridization was seen to cytoplasmic RNA that had been electrophoresed on wells adjacent to those containing the nuclear RNA (Figure 4). Even after a 1-week exposure of the film (not shown), no hybridization to the cytoplasmic RNA lanes was detected. Based on the length of the exposure time, we estimate that if any IVS RNA is present in the cytoplasm, its concentration is less than $^1/_{70}$ times its concentration in the nucleus.

Relative Lifetimes of Various RNA Species. As described under Experimental Procedures, the half-lives of the various IVS-containing RNA species should be directly proportional to their steady-state concentrations. The steady-state concentrations should in turn be proportional to the saturation amount of hybridization if different size classes of RNA were transferred from the gel and retained by the DBM paper with equal efficiency. This last point was tested with a mixture of ^{32}P-labeled rRNA precursor and IVS RNA (Figure 5). It was found that 42% of the precursor and 10% of the linear IVS were transferred and retained on the DBM paper through

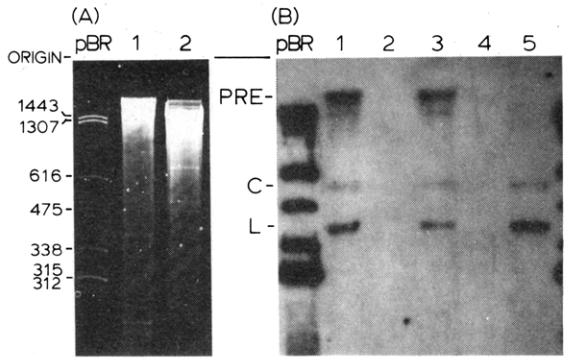

FIGURE 4: Hybridization of the IVS probe to nuclear and cytoplasmic RNA separated by 8 M urea–polyacrylamide gel electrophoresis. (A) A portion of the ethidium-stained gel. (B) Autoradiogram obtained by transfer of the RNA to DBM paper and hybridization with a DNA probe specific for the IVS. (Lane pBR) *Taq*I restriction fragments of pBR322 DNA, lengths (nt) listed at left. (Lane 1) Nuclear RNA and (lane 2) cytoplasmic RNA from *T. thermophila* growing at 30 °C. (Lane 3) Nuclear RNA and (lane 4) cytoplasmic RNA from cells growing at 39 °C. (Lane 5) ^{32}P-Labeled IVS RNA marker as in Figure 2.

the hybridization steps. There was not enough circular IVS RNA in the experiment of Figure 5 to permit direct comparison of linear and circular forms. From densitometry of the autoradiograms, however, it was concluded that both forms of the free IVS have ~10% efficiency of transfer and retention. On the basis of these results, all densitometer measurements of linear and circular IVS RNA hybridization have been multiplied by 4.2 to correct for their underrepresentation relative to the precursor. The corrected amounts of hybridization of the IVS probe to various nuclear RNA species are given in Table I. In both rapidly growing and starved *T. thermophila* cultured at 30 °C, there is 2.2–2.4-fold more free IVS than unspliced precursor in the nucleus. According to eq 3 (Experimental Procedures), the half-life of the primary transcript must therefore be 2.2–2.4 times shorter than that of the excised IVS.

Effect of Different Cell Growth Conditions on IVS RNA Metabolism. Nuclear RNA was isolated from *T. thermophila* growing rapidly at 30 °C and from cells starved for 16 h at 30 °C. The IVS-containing nuclear RNA species were then detected by hybridization, as shown in Figure 6. By com-

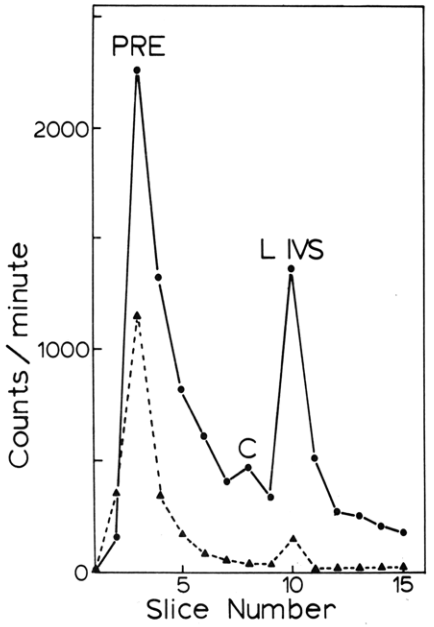

FIGURE 5: Transfer-retention efficiency of RNA electroblotted from a 8 M urea–polyacrylamide gel onto DBM paper. Purified pre-rRNA and excised IVS RNA, both labeled with ^{32}P during in vitro transcription, were mixed. Equal portions of the mixture were electrophoresed on three wells of an 8 M urea–4% polyacrylamide gel. One of the lanes was cut from the gel and dried onto paper. The other two were electrophoretically transferred to DBM paper; one was hybridized and washed along with the filter shown in Figure 6, while the other was mock-hybridized and washed. The dried gel and the two DBM paper strips were then cut into 3-mm slices (numbered from the origin), and the radioactivity was determined by liquid scintillation counting. (●) RNA in gel, not transferred. (▲) RNA remaining on DBM paper after transfer and hybridization (average of the two lanes, which were not significantly different).

paring lanes 1 and 3, it can be seen that both log-phase and starved cells have the same proportion of total IVS RNA to precursor, but the amount of each species is about 4-fold smaller in the nuclei of starved cells. About 17% of the IVS RNA in log-phase cells is circular, while no circular IVS RNA was detected in the starved cells. These differences were consistently observed, as evidenced by Figure 7 and the the compilation of data in Table I.

In previous studies of RNA splicing in isolated *T. thermophila* nuclei, it was found that cyclization of the IVS RNA occurred much more readily at 39 °C than at 30 °C (Grabowski et al., 1981). A comparison of panels A and B of Figure 7, however, shows that the nuclear RNA isolated from cells growing at 39 °C contains the same small amount of circular IVS RNA as that isolated from cells growing at 30 °C. There is an increase in the amount of circular IVS RNA in cells starved at 39 °C compared to those starved at 30 °C, but at both temperatures, the major part of the excised IVS RNA is still in the linear form.

Linear IVS RNA Produced in Vivo Has Cyclization Activity. We have previously found that linear IVS RNA, produced by transcription–splicing in isolated nuclei and then deproteinized, was able to circularize itself (Cech et al., 1982). To see if the linear IVS RNA produced in vivo had cyclization activity, we incubated samples of nuclear RNA (deproteinized by standard $NaDodSO_4$–phenol extraction as described under Experimental Procedures) with 10 mM $MgCl_2$ for 15 min at 37 °C. RNA samples subjected to this "autocyclization" treatment (designated "+" in Figures 6 and 7) were electrophoresed, blotted onto DBM paper, and hybridized with the IVS probe. Control samples (designated "–") were incubated at 20 °C instead of 37 °C, conditions which are unfavorable

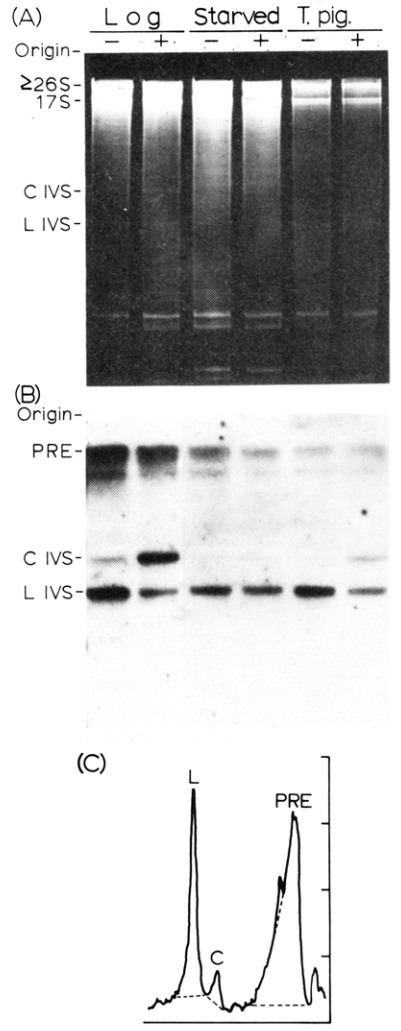

FIGURE 6: Hybridization of the IVS probe to nuclear RNA from rapidly growing (log) and starved *T. thermophila* and from rapidly growing *T. pigmentosa* 6UM. *T. thermophila* were grown at 30 °C and *T. pigmentosa* at 28 °C. Purified RNA was subjected to autocyclization conditions (+) or control conditions (–) as described in the text. (A) Ethidium-stained 8 M urea–4% polyacrylamide gel. (L IVS) Linear IVS RNA, faint band visible in all lanes. (C IVS) Circular IVS RNA, visible in second lane only. (B) Autoradiogram obtained after transfer of the RNA to DBM paper and hybridization with the IVS probe. For this photograph, the last four lanes were exposed more than the first two to aid in visualization. (C) An example of a densitometric scan [first lane in (B)].

for cyclization (Grabowski et al., 1981; P. Grabowski and T. Cech, unpublished results). The autocyclization treatment led to the cyclization of 51 ± 7% (mean ± range of three experiments) of the linear IVS RNA from *T. thermophila* that were growing rapidly at 30 °C; this is within the range of the maximum extent of autocyclization we observe with preparations of IVS RNA made by splicing in vitro (Kruger et al., 1982; Zaug et al., 1983). A much lower extent of autocyclization was observed with IVS RNA from cells growing at 39 °C and from starved cells. One explanation may be that the linear IVS RNA in these cells is predominantly nicked circles (399 nt) rather than the 414-nt linear form that is the direct product of excision. Nicked circles do not undergo autocyclization (P. Grabowski, unpublished results) and would be difficult to resolve from the linear excision product on the gel system used here.

The effect of the autocyclization treatment was specific for the free IVS RNA. The treatment did not affect other nuclear RNA species detected either by ethidium bromide staining or by hybridization with the IVS probe or the ETS probe.

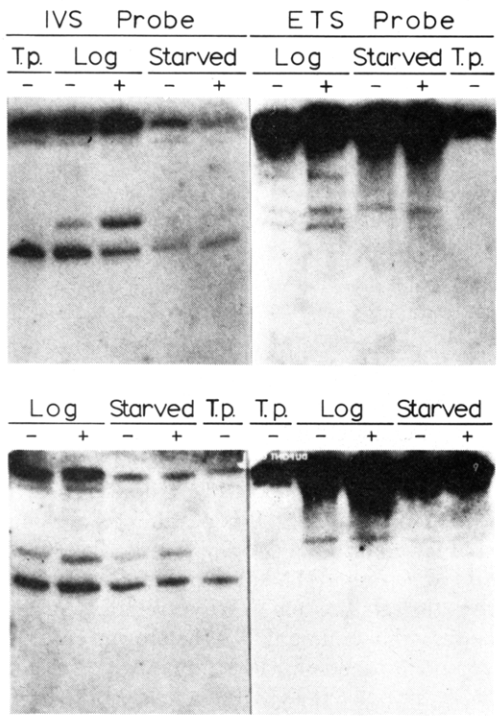

FIGURE 7: Comparison of hybridization with the IVS probe and the ETS probe. (A) Nuclear RNA from *T. thermophila* growing or starved at 30 °C. (B) Nuclear RNA from *T. thermophila* growing or starved at 39 °C. For both (A) and (B), duplicate samples were electrophoresed at room temperature on the same 8 M urea–4% polyacrylamide gel and transferred to DBM paper. The DBM paper was then cut in half. One-half was hybridized with the IVS probe and one-half with the ETS probe. Following the posthybridization washes, the two halves of each gel were placed side by side for autoradiography. (T.p.) *T. pigmentosa* RNA, same as in Figure 6.

Absolute Lifetimes of the Precursor and the Excised IVS RNA. Quantitation of hybridization signals has so far been used to estimate the *relative* lifetimes of the various IVS-containing RNAs (Table I). According to eq 8 (Experimental Procedures), the *absolute* half-life of the primary transcript PRE 1 can be determined if we know the absolute half-life of PRE 2 and the ratio of the steady-state concentrations of PRE 1 and PRE 2. The pulse–chase labeling study of Sutton et al. (1979) provides the basis for calculating $t_{1/2}$(PRE 2) for growing and starved *T. thermophila* at 30 °C, as outlined in Table II. (We avoid calculating half-lives for *T. thermophila* at 39 °C or for *T. pigmentosa*, because the necessary kinetic studies have not been performed for these cases.) The ratio [PRE 1]/[PRE 2] was determined by the split gel experiment of Figure 7. Duplicate gel blots were hybridized with the IVS probe (where the amount of hybridization to large RNA reflects the amount of PRE 1) or with the ETS probe (where the amount of hybridization to large RNA reflects primarily the amount of PRE 2). As summarized in Table II, these data allow the calculation of $t_{1/2}$(PRE 1) and, in one additional step, $t_{1/2}$(IVS). Because of the multiple steps and the assumptions involved in these calculations, we consider them accurate only within a factor of 2–3.

Splicing and Cyclization Do Not Occur during RNA Preparation. Excision and cyclization of the IVS do not require any enzyme or other protein in vitro (Kruger et al., 1982). We were therefore concerned that these reactions might be continuing during harvesting of the cells, isolation of the nuclei, or purification of the RNA, which includes DNase treatment in a Mg^{2+}-containing buffer. A whole cell RNA preparation scheme was therefore devised to avoid exposure of the RNA to temperatures much above 0 °C while

Table II: Half-Lives and Steady-State Concentrations of Various Nuclear RNA Species

	growing (log) cells (30 °C)		starved cells (30 °C)	
RNA	$t_{1/2}$ (s)	molecules/cell	$t_{1/2}$ (s)	molecules/cell
PRE 1 + 2 + 3[a]	60	54×10^4	720	37×10^4
PRE 2[b]	24	26×10^4	720	37×10^4
PRE 3[b]	24	26×10^4		ND[f]
PRE 1[c]	2.3	2.5×10^4	10	0.5×10^4
free IVS (C + L)[d]	5.6	6.1×10^4	30	1.5×10^4
free ETS (~700 nt)[e]	1.5	1.6×10^4	30	1.5×10^4

[a] Parameters for the pre-rRNA population taken as a whole (Sutton et al., 1979). [b] For growing cells, it has been shown that the steady-state concentrations of PRE 2 and PRE 3 are equal and are much greater than that of PRE 1 (Cech & Rio, 1979). It follows from eq 7 (Experimental Procedures) that $t_{1/2}$(PRE 3) = $t_{1/2}$(PRE 2) $\gg t_{1/2}$(PRE 1). It can then be shown that half-lives of 24 s for both PRE 2 and PRE 3 would give an apparent half-life of 1 min for the sum [PRE 1] + [PRE 2] + [PRE 3]. In starved cells, the pre-rRNA is predominantly PRE 2 (T. Cech and D. Rio, unpublished results). [c] Calculated from the relative amount of hybridization to pre-rRNA by the IVS probe vs. the ETS probe by using eq 8 (Experimental Procedures). [d] Calculated by using eq 3 and the values of [IVS]/[PRE 1] from Table I. [e] Calculated by using eq 4. Hybridization signals of the free ETS RNA were corrected by the same 4.2× factor used for free IVS RNA. [f] ND, not detected.

in the presence of Mg^{2+} (see Experimental Procedures). Such a preparation of log-phase cell RNA was analyzed by gel blot hybridization with the IVS probe (data not shown). The proportions of PRE 1, linear (L) IVS, and circular (C) IVS were similar to those seen in the log-phase nuclear RNA preparations described above. We therefore concluded that neither splicing nor cyclization was occurring to a significant extent during preparation of the RNA.

Endonucleolytic Processing of the External Transcribed Spacer. The ETS probe hybridized mainly to high molecular weight nuclear RNA, as shown in the right-hand half of Figure 7A,B. When the high molecular weight RNA was separated on a glyoxal–agarose gel (data not shown), we could detect two IVS-hybridizing species: the major band had the size of pre-rRNA, while a less intense band had a size of 2500 ± 150 nt. This is the size of the pre-17S rRNA discussed by Sutton et al. (1979) and diagrammed in Figure 3.

Several smaller RNA species also contained the ETS sequence (Figure 7). One species, which occurred in preparations of nuclear RNA from both rapidly growing and starved cells cultured at either 30 or 39 °C, had an estimated size of 650–720 nt. This is the size expected for the entire external transcribed spacer if it were removed from the precursor by a single endonucleolytic cleavage. [The distance from the 5′ end of the primary transcript to the 5′ end of the mature 17S rRNA is 660 nt, based on measurements of R loops (Cech & Rio, 1979). Recent mapping studies of the rDNA of *Tetrahymena pyriformis* (Niles et al., 1981) and *T. thermophila* (N. Din and J. Engberg, personal communication) give values of 713 and 654 nt, respectively.] The estimated half-life of this ~700-nt ETS fragment, based on its hybridization strength relative to the pre-rRNA, is 1.5 s in rapid-growing cells (Table II). Two other discrete ETS-hybridizing species (550 and ~900 nt) were detected only in the nuclear RNA of 30 °C log-phase cells. Several free ETS fragments in this size range have been independently identified by W. Eckert and collaborators (personal communication). None of these

RNA species has been accurately mapped on the rDNA.

Discussion

IVS-Containing RNA Synthesized in Vivo. We have transferred RNA from gels to diazotized paper and performed hybridization with radioactive DNA (Alwine et al., 1977) to detect the various IVS-containing RNA species in preparations of nuclear RNA from *T. thermophila* and *T. pigmentosa* 6UM. The largest species detected had the properties of unspliced pre-rRNA (PRE 1). This RNA species was previously characterized by electron microscopic analysis of R-loop hybrids and by S1 nuclease mapping (Din et al., 1979; Cech & Rio, 1979). Two low molecular weight species were identified as the linear and circular forms of the excised IVS RNA. The identification was based on their hybridization to the IVS probe and on their electrophoretic mobilities relative to IVS RNAs synthesized in vitro (Grabowski et al., 1981; Zaug & Cech, 1982). In addition, the autocyclization reaction is definitive for the linear IVS RNA (see below). When these RNAs were originally characterized as products of transcription and splicing in isolated nuclei, it was not known whether they were produced by splicing in vivo. We now conclude that splicing in vivo as well as in vitro involves endonucleolytic cleavage to give a linear IVS excision product which can be cyclized.

Excised IVS RNAs have been tentatively identified in a few other systems. *Drosophila* nuclei contain RNA of the correct size to be a linear IVS excised from pre-rRNA containing type II insertions (Kidd & Glover, 1981), but rRNA splicing has not been firmly established in this system. Some yeast mitochondrial rRNAs are spliced, and excised IVS's have been detected by hybridization (Merten et al., 1980; Bos et al., 1980; Green et al., 1981). In none of these other rRNA cases has a circular IVS been demonstrated. Circular RNAs containing intron sequences from yeast mitochondrial mRNA precursors have been identified (Halbreich et al., 1980).

How Quickly Does Splicing Occur? The pre-rRNA is spliced very rapidly ($t_{1/2} \simeq 2$ s) in cells in log-phase growth. On the basis of this half-life, unspliced pre-rRNA would account for 5% [calculated as $(2.5 \times 10^4)/(54 \times 10^4)$] of the total pre-rRNA population. This value is in accord with the low frequency of R-loop structures formed by unspliced vs. spliced pre-rRNA (Cech & Rio, 1979). Therefore, although a number of assumptions were used in calculating the value of 2 s (Table II), it must be accurate to within a factor of 2 or 3. It is clear that splicing occurs much more rapidly than subsequent endonucleolytic processing events, which have half-times of ~24 s.

On the basis of an elongation rate of 40 nt/s (Cech et al., 1982), it would require 27 s for an RNA polymerase to traverse the 1100 nt between the 3′ end of the IVS and the 3′ end of the pre-rRNA. If splicing occurs with a half-time of ~2 s, most precursor molecules are spliced before transcription is terminated. Because the IVS is located near the 3′ end of the primary transcript, any nascent chain that contains the IVS will have an electrophoretic mobility similar to that of a full-length primary transcript. Therefore, the hybridization results (e.g., Figure 2) in no way rule out the possibility that splicing occurs on unterminated RNA molecules. However, in the S1 nuclease mapping study of Engberg et al. (1980), it was clear that some full-length pre-rRNA molecules (~20% of the total) had not yet been spliced. Perhaps not all pre-rRNA molecules are spliced with the same rate constant.

When *Tetrahymena* are starved, there is a 12-fold reduction in the rate at which pre-rRNA is processed [Sutton et al., 1979; see also Eckert & Kaffenberger (1980)]. If this were due to a decreased rate of splicing, starved cells would provide a valuable source of unspliced pre-rRNA. We find that the rate of splicing does appear to be reduced somewhat in starved cells, but it is apparent from Table II that the major bottleneck in processing is the removal of the ETS. This occurs so slowly in starved cells that little PRE 3 is formed; instead, PRE 2 is cleaved between the 17S and 26S rRNA sequences to give a pre-17S rRNA (Figure 3). Starvation, therefore, affects splicing much less than it affects the subsequent RNA processing events.

The rate of splicing of *Tetrahymena* pre-rRNA is much greater than the splicing rate of mRNA precursors. The estimated half-times for excision of intervening sequences from β-globin, immunoglobin, and ovomucoid mRNA precursors are in the range of 2–20 min (Curtis et al., 1977; Schibler et al., 1978; Perry & Kelley, 1979; Gilmore-Hebert & Wall, 1979; Tsai et al., 1980). It is possible that a longer time is required for pre-mRNA splicing because that process, unlike *Tetrahymena* pre-rRNA splicing, requires enzymes. Alternatively, the rate-limiting step for pre-mRNA splicing could be the assembly of an hnRNP (heterogeneous nuclear ribonucleoprotein particle), which might be the mandatory substrate for the splicing activity.

Lifetime of the Excised IVS. The excised IVS is rapidly degraded in the nucleus, and no significant portion of it reaches the cytoplasm. For rapidly growing cells, we calculate that the excised IVS has a half-life of ~6 s and a corresponding steady-state concentration of $\sim 6 \times 10^4$ molecules/cell. On the basis of this concentration, 10 μg of nuclear RNA should contain an amount of linear IVS RNA of the order of 10 ng, which is consistent with the fluorescence intensity of the IVS band seen in ethidium-stained gels (Figure 6A)

It is useful to compare the stability of the excised IVS to that of the free ETS, another fragment of the pre-rRNA that is discarded during processing. The free IVS is ~4-fold more stable than the free ETS in growing cells, and the two RNAs are about equally stable in starved cells. If both molecules were subject to random endonucleolytic degradation, some difference in their half-lives would be expected because of their different size, secondary structure, base composition, etc. Based on their approximately equal stability, we conclude that the excised IVS, like the ETS fragment, is not "marked" as a molecule to be preserved but rather to be degraded.

We have not attempted to assign separate lifetimes to the linear and circular forms of the IVS RNA. Such an assignment could be made if we knew what fraction of the excised IVS molecules were cyclized before being degraded. The smaller the portion of the IVS molecules that are cyclized, the longer must be the half-life of the circular molecule. Even if only 20% of the IVS molecules are cyclized, their half-life is only ~6 s. It is important to note that all such calculations depend upon the assumption that the RNA is depleted by one pathway with a single first-order rate constant. If there is heterogeneity in the population, a subset of the linear or circular IVS RNA molecules could be much more stable.

Cyclization of the IVS. Cyclization of the linear IVS RNA has a pronounced temperature dependence in vitro. Both in isolated nuclei (Grabowski et al., 1981) and in a purified in vitro system (Cech et al., 1981), cyclization is more rapid and more complete at 39 °C than at 30 °C. We now find that in vivo the steady-state concentration of circular compared to linear IVS is not significantly increased when cells are grown at 39 °C compared to 30 °C (Table I). There is an increase in the amount of circular IVS in cells starved at 39 °C com-

pared to 30 °C, but the linear form still predominates. The amount of circular IVS in vitro depends on its rate of formation; little if any degradation of IVS RNA occurs. The amount of circular IVS in vivo is a function of its rate of degradation as well as synthesis. If both rates increase at 39 °C in vivo, the steady-state concentration of circular IVS RNA may be relatively unaffected.

In a recent study (Kruger et al., 1982), we found that linear IVS RNA produced by splicing in vitro can cyclize itself in the absence of any protein. We therefore concluded that the cyclization activity is intrinsic to the structure of the IVS RNA molecule. The present finding, that IVS RNA produced by splicing in vivo and purified by $NaDodSO_4$–phenol extraction cyclizes when incubated with 10 mM $MgCl_2$ at 37 °C, adds support to the conclusion that the activity is intrinsic to the IVS. Furthermore, we now add that if the IVS RNA produced in vivo differs in any way from that produced in vitro (e.g., because of nucleoside modifications that might occur in vivo), the difference does not prevent autocyclization.

We have argued that *Tetrahymena* pre-rRNA splicing occurs by a series of reversible phosphoester transfers (Cech et al., 1981; Kruger et al., 1982; Zaug et al., 1983). The reverse reaction, reintegration of the IVS, could be prevented by the destruction of the excised IVS reaction product. Nucleolytic degradation is one possible pathway for IVS degradation and may predominate under some cell growth conditions such as low temperature. Under other conditions, cyclization may provide a more rapid pathway for inactivation of the IVS RNA. Because cyclization requires only divalent cations and no other cofactors or enzymes, it should be a very dependable reaction, occurring independent of the general level of metabolic activity of the cell.

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