RNA (1997), 3:1016–1027. Cambridge University Press. Printed in the USA.

Association of a group I intron with its splice junction in 50S ribosomes: Implications for intron toxicity

TANIA NIKOLCHEVA[1] and SARAH A. WOODSON[1,2]

[1]Program in Molecular and Cell Biology, University of Maryland, College Park, Maryland 20742-4451, USA
[2]Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742-2021, USA

ABSTRACT

The effect of genetic context on splicing of group I introns is not well understood at present. The influence of ribosomal RNA conformation on splicing of rDNA introns in vivo was investigated using a heterologous system in which the *Tetrahymena* group I intron is inserted into the homologous position of the *Escherichia coli* 23S rRNA. Mutations that block splicing in *E. coli* result in accumulation of unspliced 23S rRNA that is assembled into 50S complexes, but not 70S ribosomes. The data indicate that accommodation of the intron structure on the surface of the 50S subunit inhibits interactions with the small ribosomal subunit. Spliced intron RNA also remains noncovalently bound to 50S subunits on sucrose gradients. This interaction appears to be mediated by base pairing between the intron guide sequence and the 23S rRNA, because the fraction of bound intron RNA is reduced by point mutations in the IGS or deletion of the P1 helix. Association of the intron with 50S subunits correlates with slow cell growth. The results suggest that group I introns have the potential to inhibit protein synthesis in prokaryotes by direct interactions with ribosomes.

Keywords: catalytic RNA; pre-rRNA processing; self-splicing

INTRODUCTION

Although it is often assumed that group I introns are a vestige of an ancient RNA world (Cech, 1985; Darnell & Doolittle, 1986), their persistence in modern genes remains unexplained. One view is that, like transposable elements, introns are simply "selfish" genetic elements that propagate independently of the host genome (reviewed in Cavalier-Smith, 1991; Lambowitz & Belfort, 1993; Belfort & Perlman, 1995). On the other hand, because group I introns are typically found in highly expressed and essential genes, it is presumed that their splicing and degradation pathways do not interfere with expression of other cellular genes (reviewed in Cech, 1990). The few direct comparisons of intron$^+$ and intron$^-$ strains have failed to reveal any clear selective advantage or disadvantage (Nielsen & Engberg, 1985; Bell-Pedersen et al., 1989; Johanningmeier & Heiss, 1993). Coordinated splicing and translation of group I introns in phage T4 may provide as yet unappreciated mechanisms of genetic regulation (Belfort, 1990).

Reprint requests to: Sarah A. Woodson, Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742-2021, USA; e-mail: sw74@umail.umd.edu.

Relatively little is known about the extent to which genetic context may affect the splicing of group I introns, and thus by extension, their genomic distribution. An interesting set of examples is the large number of group I introns found in the rDNA of lower eukaryotes. Unlike expansion segments (Gerbi, 1985) and internal transcribed spacers that are either not excised or excised without religation of the rRNA (Burgin et al., 1990), group I introns are nearly always inserted in conserved regions of the rDNA that are functionally important in the mature ribosome (Gerbi, 1985; Turmel et al., 1993; Johansen et al., 1996). Consequently, loss of splicing activity is expected to inhibit rRNA function.

Studies of the group I intron from *Tetrahymena thermophila* rDNA have shown that rRNA secondary structure inhibits self-splicing (Woodson & Cech, 1991; Emerick et al., 1996). The *Tetrahymena* intron is naturally located in domain IV of the 26S rRNA gene, immediately 3′ of a stable hairpin that is conserved among large subunit rRNAs (Fig. 1A; Noller et al., 1981). Formation of this hairpin in pre-rRNA inhibits 5′ splice site recognition (Woodson & Cech, 1991). In vivo, splicing is one of the earliest steps in pre-rRNA processing (Cech & Rio, 1979; Din & Engberg, 1979) and is estimated to be 20- to 50-fold more rapid in vivo than in

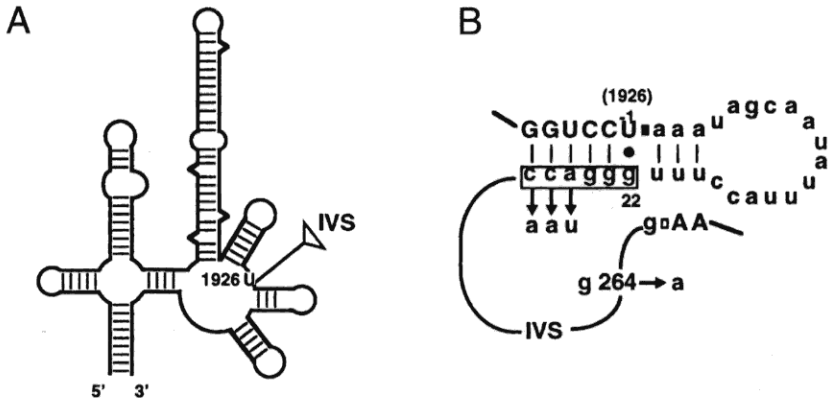

FIGURE 1. Insertion of the *Tetrahymena* intron in *E. coli* 23S rRNA. **A:** Phylogenetically predicted secondary structure in domain IV of the 23S rRNA, showing the intron insertion site after U1926. **B:** Diagram of the P1 stem-loop of the EC intron, showing point mutations at nt 25, 26, and 27. The G-site mutation in the intron (G264A) is indicated schematically. The intron sequence up to C27 is deleted in ΔIGS clones. Upper case, exons; lower case, intron (IVS); (●), 5′ splice site. The guide sequence (IGS) is boxed. U-1 is U1926 in the 23S sequence.

vitro (Brehm & Cech, 1983). To investigate splicing of introns in pre-rRNA, we have established a bacterial expression system in which the *Tetrahymena* intron was inserted into the homologous position of the *Escherichia coli* 23S rRNA (Zhang et al., 1995). Facilitation of splicing in vivo does not require a species-specific factor, such as a maturase protein, because splicing is as rapid in bacteria as it is in *Tetrahymena*.

Using this bacterial expression system, we have begun to investigate whether splicing is coordinated with folding and maturation of the pre-rRNA by asking whether the presence of a group I intron inhibits other steps in pre-rRNA processing and ribosome assembly. Unexpectedly, 50S ribosomal complexes from *E. coli* cells expressing the *Tetrahymena* intron were found to contain spliced and unspliced intron RNA. Unspliced 23S rRNA is assembled into 50S subunits, suggesting that the intron does not prevent binding of ribosomal proteins. However, the intron was not found in polysomes, suggesting that these subunits are inactive in translation. Noncovalent association of intron RNA with 50S subunits depends on base pairing between the internal guide sequence (IGS) of the intron and the 23S rRNA.

Overexpression of the intron inhibited the growth of *E. coli* cells, even though splicing was very efficient. In *E. coli*, the excised intron is stable, with an estimated half-life of up to 20 min (Zhang et al., 1995), whereas in *Tetrahymena*, the intron is degraded rapidly (Brehm & Cech, 1983). Mutations in the IGS that reduce the amount of intron bound to 50S subunits also reduce inhibition of cell growth by the intron RNA. These results suggest that group I introns are capable of inhibiting protein synthesis in prokaryotes by direct interactions with mature ribosomes.

RESULTS

Accumulation of 50S subunits in splicing-deficient mutants

Pre-rRNA containing the *Tetrahymena* intron was transcribed from a plasmid (pLK45-IVS, Fig. 2A) containing the *rrn*B operon downstream of the λ P_L promoter, which is under the control of a temperature-sensitive allele of the λ repressor (Powers & Noller, 1990). Plasmid-encoded 23S rRNA contains the erythromycin-resistance mutation A2058G, which enables it to be distinguished from chromosomally derived RNA in primer extension assays (Sigmund et al., 1988). The *Tetrahymena* splice junction is homologous to C1925 in the *E. coli* 23S rRNA. The intron sequences were inserted after 23S U1926 (Fig. 1A), because a U normally precedes the 5′ splice sites of group I introns (Cech, 1990). The IGS of the intron was mutated to restore complementarity with the *E. coli* 5′ exon (Fig. 1B), and is designated "EC" (Zhang et al., 1995).

Preliminary analysis of polysome pools after induction of transcription from the plasmid showed that

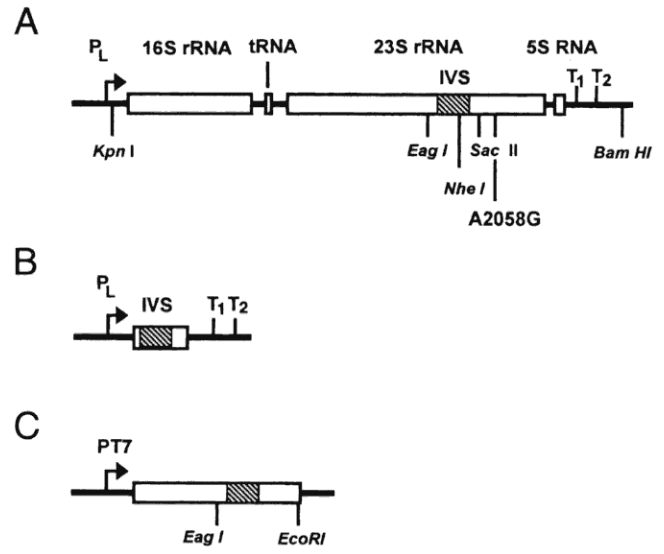

FIGURE 2. Plasmids for expression of the *Tetrahymena* intron. **A:** pLK45-IVS (Zhang et al., 1995). The hatched box represents the intron (IVS) in the *rrn*B operon of *E. coli* in pLK45 (Powers & Noller, 1990). A2058G mutation confers erythromycin resistance (Sigmund et al., 1988). Transcription is from the λ P_L promoter. **B:** pLKEC438 (Roman, 1996). The pre-RNA transcript contains 6 nt of 23S rRNA sequence in the 5′ exon and 19 nt in the 3′ exon. **C:** pTN112. The plasmid encodes 176 nt *E. coli* 23S sequence upstream and 90 nt downstream of the *Tetrahymena* intron, behind a promoter for T7 RNA polymerase.

similar fractions of plasmid-derived 23S rRNA were incorporated into active 50S subunits, when cells transformed with intron$^+$ and intron$^-$ plasmids were compared (E.S. Ramsay & S. Woodson, unpubl.). Thus, the presence of the rapidly spliced EC intron did not greatly impair the activity of the 23S rRNA from which it was excised. To further investigate formation of 50S subunits, ribosomes were isolated from transformed cells on 5–20% sucrose gradients after growth at 42 °C to induce transcription of the plasmid (Fig. 3; Table 1). The 50S peaks from strains with either no intron (pLK45; Fig. 3A) or active EC intron (pLK45-IVS; Fig. 3B) were of similar heights, although the 70S peak is larger in intron$^-$ cells.

We next wished to determine whether mutations that decrease self-splicing of the intron inhibit maturation of 50S subunits. The mutation G264A in the G-binding site of the intron (Michel et al., 1989) decreases the rate of in vivo splicing 100-fold (Zhang et al., 1995). Deletion of the IGS together with the G-binding site mutation (EC:G264A/ΔIGS; Fig. 1B) completely inhibits splicing in *E. coli* (Zhang et al., 1995). When ribosomes were isolated from cells with splicing-deficient 23S rRNA, the amount of the 50S peak increased significantly (Table 1). In cells containing the EC:G264A intron, the 50S peak was approximately the same height as the 70S peak (Fig. 3D). For the nonsplicing G264A/ΔIGS mutant, the 50S peak was much larger than the 70S (Fig. 3E). The increased size of the 50S peak is not merely the result of the G264A mutation, because a double mutation in the IGS (A25U, C26A), which destabilizes the P1 splice site helix and decreases splicing efficiency both in vitro and in vivo (data not shown), also caused increased accumulation of dissociated 50S subunits (Fig. 3C).

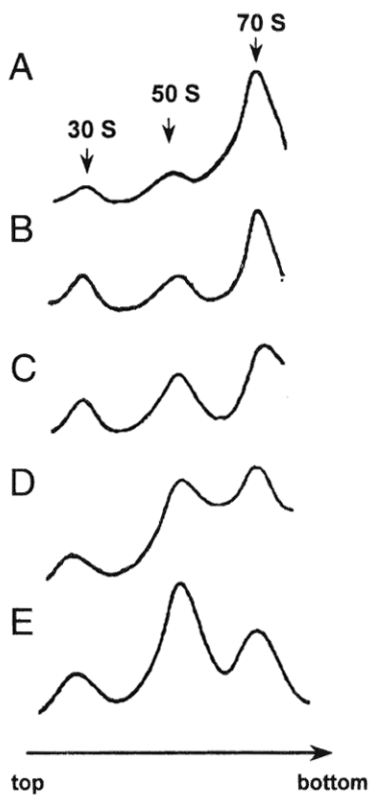

FIGURE 3. Sucrose gradient ultracentrifugation of *E. coli* ribosomes. Cells transformed with plasmids expressing intron$^-$ or intron$^+$ rRNA were harvested after 1 h induction at 42 °C (A_{600} = 0.3). Crude ribosomes were fractionated on 5–20% sucrose gradients as described in Materials and Methods, and the A_{260} versus gradient fraction plotted as shown. The *y*-axes were scaled so that the total area under each curve is constant. Cells were transformed with (**A**) pLK45 (intron$^-$); (**B**) pLK45-IVS; (**C**) pLK45-IVS:A25U,C26A; (**D**) pLK45-IVS:G264A; (**E**) pLK45-IVS:G264A,ΔIGS.

TABLE 1. Relative amounts of ribosomal complexes from transformed *E. coli*.[a]

Plasmid[b]	70S	50S	30S
Intron$^-$	72	19	9
Intron$^+$ (EC)	56	22	22
G264A	46	43	11
G264A/ΔIGS	29	53	17
C26A	66	19	16
C27A	62	22	15
A25U/C26A	48	36	16

[a]Area of each peak in sucrose gradients (%) relative to the total area under the curve, based on absorbance at 260 nm. Gradient fractionation of total ribosomes (wild type and plasmid-encoded) was performed as in Figure 3.

[b]Intron$^-$ 23S rRNA was transcribed from plasmid pLK45 (Powers & Noller, 1990). Intron$^+$ rRNA (EC) was transcribed from pLK45-IVS. All other RNAs were transcribed from derivatives of pLK45-IVS containing the additional intron mutations listed.

Unspliced 23S rRNA is assembled into 50S ribosomal subunits

To determine whether 50S ribosomal subunits in splicing-deficient strains contained plasmid-encoded 23S rRNA, rRNA was recovered from 50S and 70S pools, and the fraction of RNA derived from the plasmid was quantitated using a primer extension assay that detects the plasmid-specific base change A2058G (Sigmund et al., 1988; Zhang et al., 1995). The 50S fractions contained a high proportion of the plasmid-encoded rRNA, even from clones with a splicing-deficient intron. For the G264A and the G264A/ΔIGS mutants, 68–71% of the free 50S pool was derived from plasmid-encoded RNA (Fig. 4; Table 2). However, only background levels of 23S rRNA containing the nonsplicing G264A/ΔIGS intron were detected in 70S fractions (Fig. 4; Table 2). Therefore, mutations that block splicing result in loss of 23S rRNA translational activity. Taken together, the data indicate that defects in splicing of the *Tetrahymena* intron from the 23S rRNA do not prevent assembly of 50S complexes, but instead cause accumulation of stable, inactive 50S complexes containing plasmid-encoded RNA.

To determine whether the plasmid-encoded 23S rRNA in 50S complexes was spliced, RNA from su-

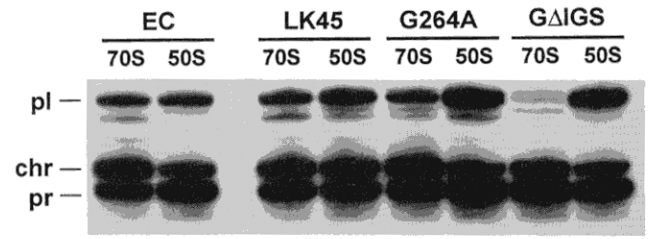

FIGURE 4. Quantitation of plasmid-encoded rRNA in ribosomes by primer extension. RNA was extracted from 70S and 50S complexes in peak fractions of sucrose gradients (Fig. 3), then analyzed by extension of ^{32}P-labeled primer CP2076 with reverse transcriptase (Zhang et al., 1995). Reactions contained 400 μM each dATP, dCTP, dGTP, and 200 μM ddTTP. EC, pLK45-IVS; G264A, pLK45-IVS:G264A; GΔIGS, pLK45-IVS:G264A,ΔIGS. pr, unextended primer; chr, product of chromosomal 23S rRNA (A2058); pl, product of plasmid-encoded 23S rRNA (2058G).

TABLE 2. Expression of plasmid-encoded 23S rRNA.[a]

Plasmid[b]	Total RNA	50S	70S	50S:70S[c]
−Intron	19[d]	23	16	1.4
+Intron (EC)	20	24	12	2.0
G264A	19[d]	68	12	5.9
G264A/ΔIGS	ND[e]	71	4	17.5
A25U	17	ND	ND	ND
C26A	19	17	10	1.7
C27A	14	11	7	1.6
C26A/C27A	12	25	9	2.8

[a]Percent of plasmid-encoded 23S rRNA relative to the total 23S rRNA (plasmid plus chromosomal) in each sample was quantitated from primer extensions as in Figure 4 and as described in Materials and Methods. Reproducibilty is typically ± 3%. Total RNA samples were isolated from *E. coli* cultures after 60 min at 42 °C. 50S and 70S RNA was prepared by organic extraction of complexes in 50S and 70S peaks of sucrose gradients.
[b]As in Table 1.
[c]Ratio of plasmid-encoded rRNA in 50S to 70S peaks.
[d]From Zhang et al. (1995).
[e]Not determined.

crose gradient fractions was analyzed by northern blots of agarose gels using a radiolabeled intron probe (Fig. 5). Because the EC intron is spliced very rapidly, unspliced 23S rRNA is barely detectable by hybridization (Zhang et al., 1995), although a band corresponding to the splicing intermediate (intron–3′ exon) is detected readily. For the splicing-deficient mutants, however, an intense band corresponding to unspliced rRNA was visible at the top of the gel. Free intron RNA was not detected for the inactive double mutant G264A/ΔIGS, consistent with its lack of splicing activity. The size of the unspliced RNA suggests that the internal and external transcribed spacers flanking the 23S coding sequence have been cleaved (Srivastava & Schlessinger, 1990).

Even less expected, however, was the observation that the majority of unspliced 23S rRNA is found in the 50S peak (Fig. 5). This demonstrates that the unexcised intron in domain IV does not prevent formation of 50S complexes. Because little unspliced 23S RNA remains at the top of the gradient, its incorporation into ribosomal complexes appears to be efficient. A small amount of unspliced 23S RNA was detected in the 70S and 30S fractions and could have resulted from overlap of peaks in the sucrose gradient, or, in the latter case, from a 32S assembly intermediate of the large subunit (Nierhaus et al., 1973). The sedimentation of 50S complexes containing unspliced 23S rRNA is indistinguishable from that of wild-type subunits at the resolution of these sucrose gradients. This suggests that most of the large subunit r-proteins are bound (Lindahl, 1975), although the data do not rule out minor differences in the protein composition or rRNA modification of intron-containing and wild-type subunits.

Association of the spliced intron with 50S subunits

Further examination of northern blots showed that significant amounts of the spliced intron RNA were also present in the 50S fractions (Fig. 5), indicating that the intron remains noncovalently associated with the large subunit after splicing. This was particularly evident for the rapidly spliced EC intron, of which up to 25% sedimented in the 50S fractions (Figs. 5, 6). Very little intron RNA was detected in 30S and 70S fractions, suggesting that this interaction is specific for 50S subunits.

The presence of excised intron RNA in the 50S fractions could be due to very slow dissociation of the

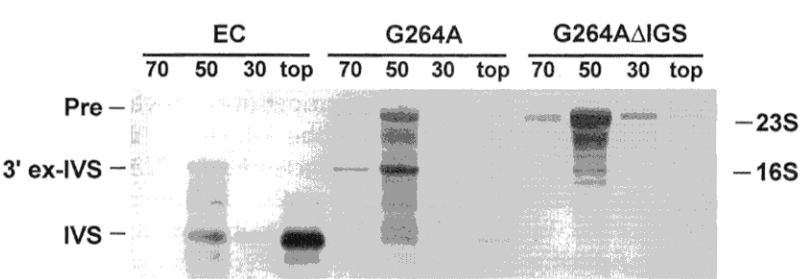

FIGURE 5. Northern hybridization analysis of ribosomal RNA from intron+ cells. RNA (3 μg) from peak fractions in sucrose gradients (Fig. 3) was separated on a 1.4% agarose gel after treatment with glyoxal, and then subjected to northern hybridization with ^{32}P-labeled intron probe. EC, cells transformed with pLK45-IVS; G264A, pLK45-IVS:G264A; G264AΔIGS, pLK45-IVS:G264A,ΔIGS. IVS, free intron; 3′ex-IVS, splicing intermediate; Pre, unspliced 23S rRNA. Positions of mature 23S and 16S rRNA were determined by staining the membrane with methylene blue. Products were compared to RNA molecular weight markers (not shown).

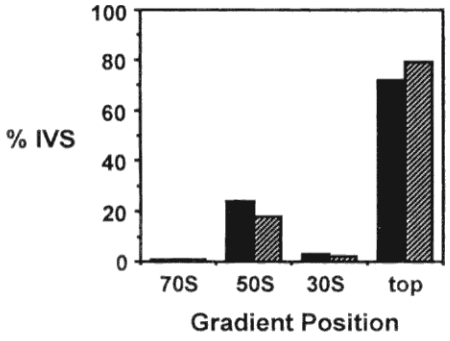

FIGURE 6. Co-sedimentation of spliced intron RNA with 50S ribosomal subunits. Percent spliced intron RNA detected in 70S, 50S, 30S peaks and top fractions of sucrose gradients, relative to the total for these fractions. Linear intron RNA was quantitated by extension of primer IP006, which anneals to the 5′ end of the intron, under conditions in which the amount of extension varies linearly with RNA concentration (Zhang et al., 1995). Solid, pLK45-IVS; hatched, pLKEC(438).

intron from the 23S rRNA after splicing. In vitro, dissociation of the spliced products is slightly slower than self-splicing at 30 °C (Emerick et al., 1996), and it is conceivable that interactions with ribosomal proteins could inhibit product dissociation even further. Alternatively, the intron RNA may be able to bind the 23S rRNA de novo. To discriminate between these two possibilities, we used a plasmid pLKEC(438) that expresses the EC intron as part of a very short non-rRNA precursor (Fig. 2B; Roman, 1996). This short precursor splices rapidly in vivo to yield linear intron RNA (Roman, 1996). Ribosomal subunits from *E. coli* transformed with pLKEC(438) or pLK45-IVS were isolated as above and the distribution of intron RNA in sucrose gradients was compared. The amount of free intron co-sedimenting with the 50S peak was nearly the same whether the intron was spliced from the complete pre-rRNA (pLK45-IVS) or from a short precursor (pLKEC(438); Fig. 6). From these results, we conclude that the EC intron is not only accommodated in the 50S when part of the 23S rRNA, but can also bind noncovalently to wild-type 50S ribosomal subunits in *E. coli*.

Association with 50S complexes requires the IGS

We next investigated whether the intron binds to 50S subunits through base pairing with the IGS, which is complementary to 23S rRNA at the spliced junction. The EC intron can integrate into the splice junction of the 23S rRNA in 50S subunits by reverse splicing in vitro (Roman & Woodson, 1995), demonstrating that these sequences are accessible to base pairing with the IGS. To test the requirement for base pairing between the 23S and EC intron RNA, single and double point mutations were introduced at positions 25, 26, and 27 of the intron (Fig. 1B). Mismatches at these positions are expected to destabilize intermolecular pairing between the intron and ligated exons, without weakening the intramolecular P1 helix to the extent that splicing is inhibited.

Intron RNA containing mutations in the IGS was expressed as part of the *rrn*B operon in *E. coli* as before. When ribosomal subunits from cells harboring the C26A mutation were analyzed, the amount of intron RNA in the 50S peak was decreased by about 67% relative to the parental EC clone (Fig. 7A,B). A decrease in association of the intron with the 50S subunit was also observed for other IGS mutations, such as A25U and C26A/C27A (Fig. 7C). The only exception is C27A, which is capable of making other pairing interactions with the 23S rRNA. Changes in the intron–50S interaction cannot be accounted for by decreased levels of transcription or reduced stability of the pre-rRNA, because the amount of 23S rRNA transcribed from the plasmid was similar in each case (Table 2), except for slightly lower expression of the C27A and C26A/C27A mutants.

To further test the IGS requirement, the P1 stem-loop was completely deleted (LKEC438: ΔP1; Roman, 1996). Only background levels of ΔP1 intron were detected in 50S fractions, indicating that their interaction was very destabilized or eliminated (Fig. 7A). The lack of ΔP1 intron in the 50S peak is not due to reduced expression of the RNA, because one obtains the same result when the data are normalized to the total amount of intron in the gradient (Fig. 7B). Overall, these results support the idea that the intron binds specifically to the 23S rRNA through base pairing with the IGS.

Splicing of *E. coli* pre-rRNAs containing mismatches in P1

An alternative explanation for the decreased association of IGS mutants with 50S subunits is that the pool of free intron RNA is reduced due to poor splicing. To address this possibility, splicing rates of pre-rRNAs containing base mismatches in the P1 helix were determined in vitro and in vivo. For in vitro studies, the same IGS mutations were introduced into a 679-nt pre-RNA containing the EC intron flanked by *E. coli* rRNA sequences (pTN112; Fig. 2C). Radiolabeled RNAs were synthesized with T7 RNA polymerase and incubated with GTP under standard self-splicing conditions (Fig. 8A). Self-splicing rates of the C26A and A25U mutant pre-rRNAs, which contained a single mismatch in P1, were decreased about 10-fold at 37 °C (Table 3). This is due to competition between P1 and an alternative stem-loop in the 5′ exon (T. Nikolcheva, unpubl.). Self-splicing products of pre-RNAs containing two mismatches in P1 (A25U/C26A and C26A/C27A) were not detected (data not shown).

Splicing of the pre-rRNA in vivo was examined by isolation of total RNA from transformed cells at vary-

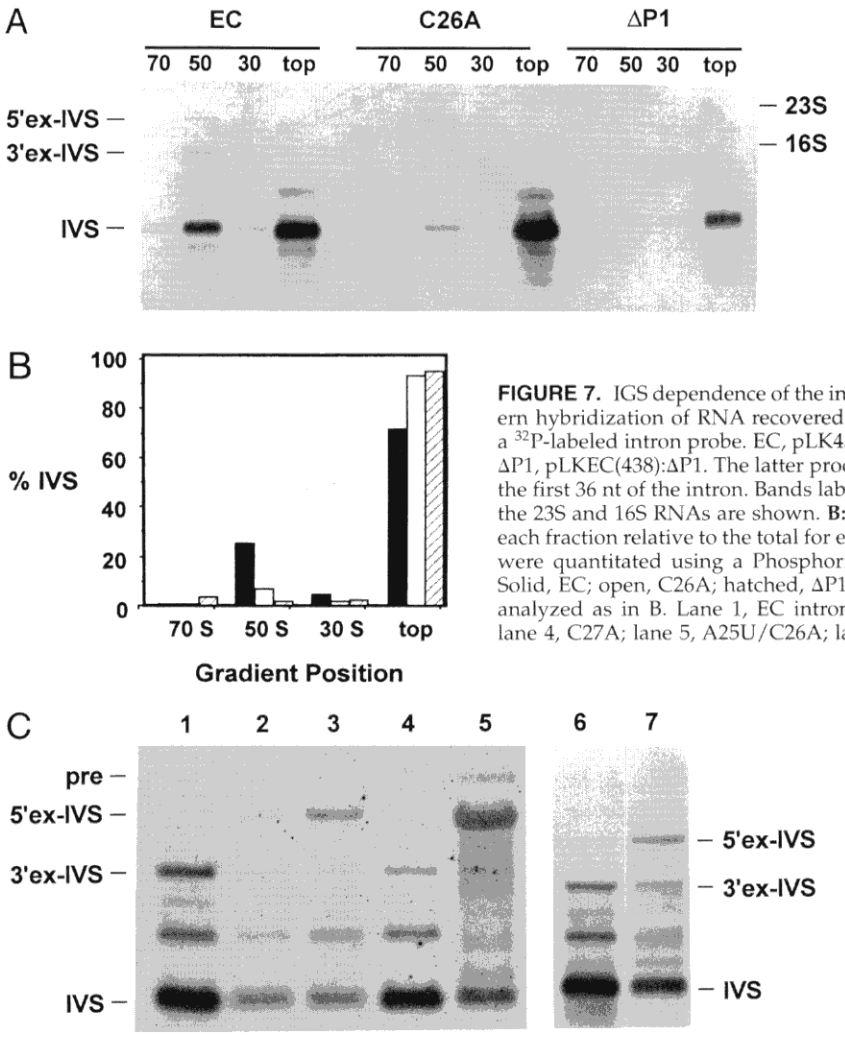

FIGURE 7. IGS dependence of the intron–50S association. **A:** Northern hybridization of RNA recovered from ribosomal subunits with a ^{32}P-labeled intron probe. EC, pLK45-IVS; C26A, pLK45-IVS:C26A; ΔP1, pLKEC(438):ΔP1. The latter produces a short transcript lacking the first 36 nt of the intron. Bands labeled as in Figure 4. Positions of the 23S and 16S RNAs are shown. **B:** Percent spliced intron RNA in each fraction relative to the total for each gradient. Data in Figure 7A were quantitated using a Phosphorimager (Molecular Dynamics). Solid, EC; open, C26A; hatched, ΔP1. **C:** RNA from 50S complexes, analyzed as in B. Lane 1, EC intron; lane 2, A25U; lane 3, C26A; lane 4, C27A; lane 5, A25U/C26A; lane 6, EC; lane 7, C26A/C27A.

ing times after induction of transcription of the plasmid, followed by northern hybridization with an intron cDNA probe. Very little unspliced pre-rRNA was detected for either the parental EC or the C26A clones (Fig. 8B), and a similar amount of spliced intron RNA accumulated for both. The C26A intron undergoes cyclization much more readily than the EC intron, as determined by northern blots of polyacrylamide gels (Fig. 8C). The L-15 and L-19 RNAs in Figure 8C result from site-specific hydrolysis of circular intron RNA (Zaug et al., 1983).

In vivo rates of splicing were estimated from the steady-state ratio of the unspliced pre-rRNA to full-length linear intron RNA and the rate of intron RNA decay (Brehm & Cech, 1983; Zhang et al., 1995). Rates of decay of linear introns containing IGS mutations were determined after addition of rifampicin to cultures (Fig. 8C). The amount of full-length linear intron and the ratio of intron to pre-rRNA were determined either from northern blots or from extension of primer IP006 (Zhang et al., 1995). Splicing rates for EC and C26A precursors obtained from quantitation of northern blots were surprisingly similar (15 and 13 min^{-1}), especially given the 10-fold difference in their reactivities in vitro (Table 3). Splicing of the double A25U/C26A mutant was reduced substantially in vivo, because a significant amount of pre-23S rRNA was detected.

For the parental EC intron, the rate of splicing determined from hybridization data is about twofold slower than that obtained from primer extensions (35 min^{-1}; Zhang et al., 1995). This is most likely due to overestimation of the amount of unspliced 23S rRNA due to hybridization background. For the C26A, however, only the hybridization data could be interpreted, because the circular intron gives rise to a primer extension product similar to that of the pre-rRNA. Assuming that hybridization gives comparable results for all clones, we conclude that the C26A intron is spliced rapidly in vivo, and that its reduced association with 50S subunits cannot be accounted for by a reduction in splicing efficiency. These results, together with the inability of the ΔP1 intron to bind 50S subunits, support our interpretation that the intron binds to the 23S RNA by base pairing with the IGS.

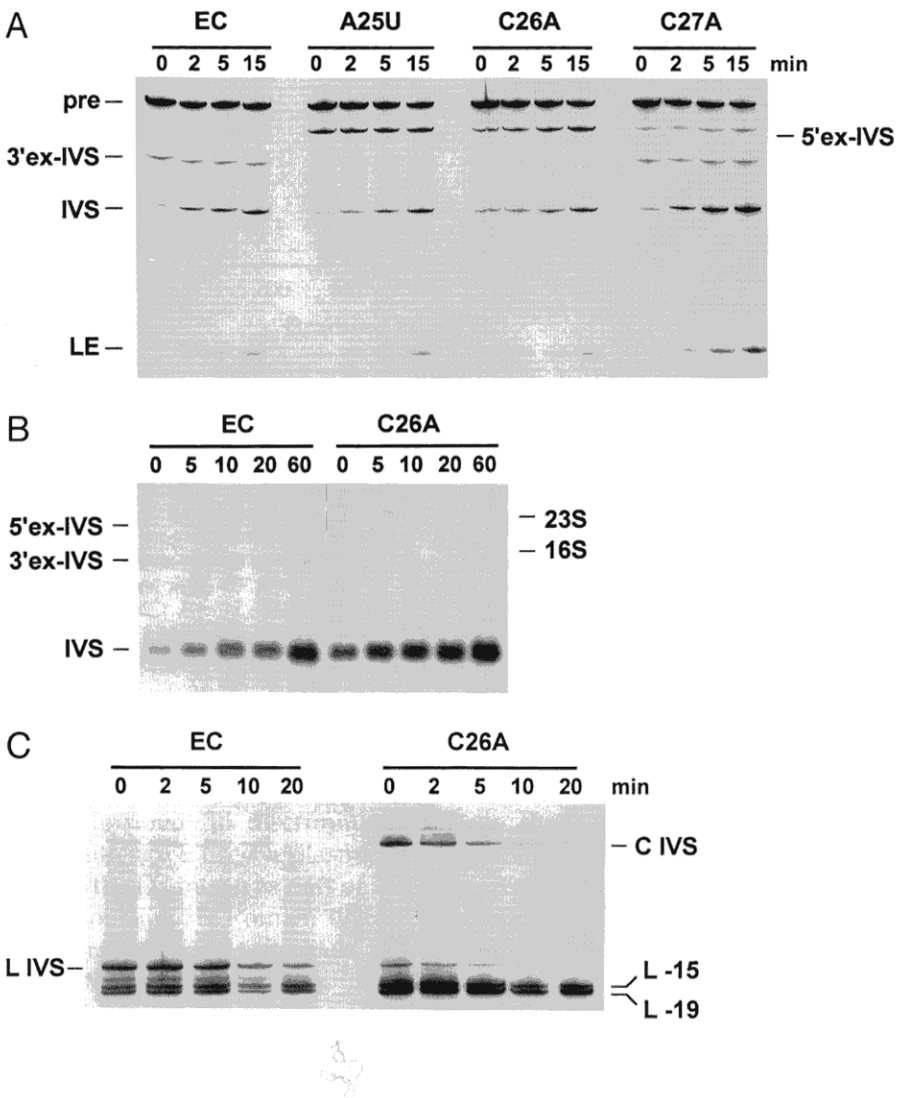

FIGURE 8. Splicing of pre-rRNAs containing mutations in the IGS. **A:** In vitro self-splicing of 679-nt pre-rRNA containing the EC intron and exons derived from *E. coli* 23S rRNA (domain IV). RNA was transcribed from pTN112 as described in Materials and Methods, and incubated at 37 °C in the presence of 0.2 M GTP under standard splicing conditions for the times shown above each lane (min). Aliquots were run on a 4% denaturing polyacrylamide gel. Pre, pre-rRNA; 3'ex-IVS, splicing intermediate; IVS, linear intron; LE, ligated exons; 5' ex-IVS, product of 3' splice site hydrolysis. **B:** In vivo splicing of pre-rRNA. Total RNA was isolated from transformed cells at 0, 5, 10, 20, and 60 min after induction of plasmid expression from pLK45-IVS. The RNA was separated on 1.4% agarose and subjected to northern hybridization with ^{32}P-labeled intron probe. Positions of 23S and 16S rRNAs are indicated. **C:** Half-life of mutant intron RNA. Northern blot of total RNA isolated at times shown, after inhibition of transcription by addition of 0.8 mg/mL rifampicin to cultures at 42 °C (Belfort et al., 1990). The RNA was separated on a 4% denaturing polyacrylamide gel and analyzed by hybridization with an intron probe as above. L IVS, full-length linear intron; L-15 and L-19, linear intron lacking the first 15 or 19 nt; C IVS, circular intron RNA.

TABLE 3. Self-splicing of heterologous *E. coli* pre-rRNAs.[a]

	k_{obs} (min^{-1})	
Pre-rRNA	In vitro	In vivo
EC	0.16 ± 0.01[b]	15
A25U	0.019 ± 0.00	ND[c]
C26A	0.018 ± 0.00	13
C27A	0.15 ± 0.01	14

[a]Splicing rate constants (k_{obs}) were measured as described in Materials and Methods and Figure 8. In vitro self-splicing reactions were performed with a 679-nt precursor containing the EC intron flanked by *E. coli* 23S exons. Self-splicing conditions were 100 mM $(NH_4)_2SO4$, 50 mM HEPES, pH 7.5, 6 mM $MgCl_2$, 1 mM EDTA, 0.2 M GTP, 37 °C. In vivo splicing rates in *E. coli* were calculated as described previously (Zhang et al., 1995), except that the unspliced pre-23S and free intron RNAs were quantitated by hybridization with a ^{32}P-labeled intron cDNA probe.

[b]Standard deviation from the mean. Values are the average of 2–3 trials.

[c]ND, not done.

Effect of IGS mutations on intron toxicity

In earlier experiments, we observed that overexpression of the *Tetrahymena* intron inhibits cell growth, to the extent that *E. coli* transformants are not obtained when the *Tetrahymena* intron is inserted into a plasmid containing the natural P1/P2 promoter of the *rrn*B operon (S. Woodson, unpubl.). This growth inhibition correlates with expression of the RNA, because cells transformed with inducible expression plasmids, such as pLK45-IVS, are viable under conditions in which transcription is repressed (Zhang et al., 1995). Growth rates of transformed cells with (pLK45-IVS) or without (pLK45) the EC intron were compared under conditions in which transcription of the *rrn*B operon on the plasmid was either induced (42 °C) or repressed (30 °C). At 30 °C, intron$^+$ cells had growth rates similar to the intron$^-$ control, whereas at 42 °C, intron$^+$ cultures grew noticeably slower 60 min after induction (Fig. 9A).

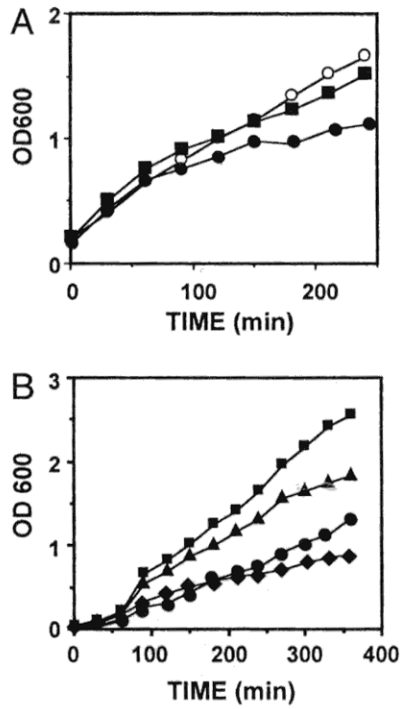

FIGURE 9. Growth of cells transformed with intron$^+$ or intron plasmids. **A:** Transformed DH1 cells were grown at 30 °C to early logarithmic phase and half of the culture was shifted to 42 °C to induce plasmid rRNA expression. Absorption at 600 nm was measured at 30-min intervals after induction. Open circles, noninduced pLK45-IVS; filled circles, induced pLK45-IVS; filled squares, induced pLK45 (intron$^-$). **B:** Effects of IGS mutations on cell growth. All cultures were grown at 42 °C. Filled circles, pLK45-IVS (EC); triangles, pLK45-IVS:C26A; squares, pLK45 (intron$^-$); diamonds, pLKEC(438).

Growth inhibition also correlated with the degree of splicing activity. Cells transformed with plasmids containing poorly spliced mutants (G264A and G264A/ΔIGS) were found to grow much more readily under inducing conditions than those expressing catalytically active intron RNA (data not shown). Although accumulation of inactive 50S subunits containing unspliced RNA is not deleterious, high levels of spliced intron RNA are apparently harmful to *E. coli*.

To determine whether growth inhibition was correlated with the ability of the intron to base pair with the 23S rRNA, growth rates of strains with mutations in the IGS were compared. At 42 °C, cells transformed with plasmids containing the C26A mutation grew at a rate intermediate between that of the parental EC clone and the intron$^-$ plasmid (Fig. 9B). This is consistent with the hypothesis that binding of the intron RNA to ribosomes contributes to its toxicity, because the C26A mutation destabilizes the interaction between the intron RNA with the 50S subunits (Fig. 7A) without loss of splicing activity (Fig. 8B). We also find that cells transformed with pLKEC(438), in which the intron is expressed in a short transcript, grew as poorly at 42 °C as those transformed with pLK45-IVS. This demonstrates that the intron RNA itself can inhibit cell growth, perhaps by binding to wild-type 50S subunits (Fig. 6).

Quantitation of intron RNA in *E. coli*

In order to estimate the amount of spliced intron available to bind to 50S subunits, we measured the total amount of intron RNA in *E. coli* cells 20 min after induction of transcription. Dot blots of total RNA from intron$^-$ and intron$^+$ strains were hybridized to either intron or 23S probes (data not shown). Total RNA samples from cells transformed with pLK45-IVS were compared to standard curves prepared with either purified 23S rRNA or intron RNA transcribed in vitro. As described in Materials and Methods, these data were used to determine the molar ratio of spliced intron to 23S rRNA, which was approximately 1:7. Assuming 25% of the free intron is bound to 50S subunits, we estimate that only 3–4% of 50S subunits in the cell are complexed with intron RNA under these conditions. Although this value could increase after longer periods of induction, it seems unlikely that noncovalent interactions with 50S subunits are sufficient to account for the decrease in growth rates. This suggests that the intron catalytically inactivates either the 23S or perhaps another RNA. Further experiments are required to address this possibility.

DISCUSSION

Although group I introns are often found in rDNA, the degree to which splicing may be coupled to other steps in pre-rRNA processing or to assembly of ribosomal subunits is not known. By inserting the *Tetrahymena* intron at the homologous position of the *E. coli* 23S rRNA, we have shown that spliced 23S rRNA is active, as judged by it's presence in polysome pools. In addition, unspliced 23S rRNA is assembled into 50S complexes, although these intron-containing complexes are inactive in translation. These experiments also revealed that the intron can associate noncovalently with wild-type large ribosomal subunits, and that this interaction most likely involves base pairing between the IGS of the intron and the 23S rRNA at the splice junction. Noncovalent binding of the intron to 50S ribosomes correlates with slow growth of transformed cells.

The fact that the unspliced intron does not block subunit assembly was initially surprising, because the intron is inserted in a highly conserved region of domain IV (Noller et al., 1981). The intron structure apparently folds independently of the 23S sequences in vivo and does not occlude binding of ribosomal proteins. In fact, nucleotides near the *Tetrahymena* splice junction (position 1925 in *E. coli*) are close to P-site tRNAs, have been implicated in peptidyl transferase

activity (reviewed in Noller, 1991), and are proposed to form part of the interface between the large and small subunits (Frank et al., 1995; Leviev et al., 1995; Joseph & Noller, 1996). Because nucleotides near the splice junction are accessible to tRNAs, it seems reasonable that the intron could be accommodated on the surface of the 50S subunit, as suggested by our results. Furthermore, insertion of a 413-nt intron at this position of the 23S rRNA would be expected to sterically block interactions between the large and small subunits. This agrees with our observation that little unspliced 23S RNA or free intron is found in the 70S peak of sucrose gradients, and unspliced 23S rRNA is not found in polysomes.

Nearly all known rDNA introns are inserted in conserved sequences that interact with tRNAs or elongation factors in the ribosome, and are likely to remain solvent accessible (Gerbi, 1985; Turmel et al., 1993; Woodson, 1996). Consequently, many group I introns may be able to bind directly to ribosomes, perhaps in a mode analogous to tRNA. The ability of the *Tetrahymena* intron to base pair with the 23S rRNA was suggested by an earlier demonstration that the *Tetrahymena* intron can integrate into the 23S rRNA by reverse splicing when 50S subunits are provided as a substrate (Roman & Woodson, 1995). In those experiments, it was presumed that binding of the intron is infrequent and unstable, because the efficiency of reverse splicing is generally low. Here we find that an appreciable fraction (25%) of the intron RNA is bound to large ribosomal subunits in a complex that remains stable over many hours of ultracentrifugation. We were unable to form a stable intron–50S complex in vitro, despite varied attempts (E.S. Ramsay & S. Woodson, unpubl.). This suggests that either the intron binds nascent 23S rRNA in vivo before 50S assembly is complete, or that this interaction requires other proteins such as translation initiation factors. Indeed, the active plasmid-encoded 23S rRNA found in polysomes may reflect transcripts in which the spliced intron dissociated at an early stage of 50S assembly.

We have also shown that the spliced intron interferes with *E. coli* metabolism if allowed to accumulate. Inhibition of cell growth is positively correlated with the amount of intron bound to 50S complexes and inversely correlated with splicing activity. A simple interpretation is that the intron inhibits translation by simply binding to 50S subunits and sterically blocking their interaction with the pre-initiation complex. In preliminary experiments, a pool of 50S subunits enriched for unspliced 23S rRNA (~40%) exhibited very low peptidyl transferase activity in assays using fMet-tRNA^{fMet} and puromycin, suggesting that the intron also inhibits binding of peptidyl tRNA or peptide bond formation (J. Roman, E.S. Ramsay, & S. Woodson, unpubl.). Because only 3–4% of 50S subunits are complexed with intron RNA, however, steric interference seems insufficient to explain the observed decrease in growth rates, even if this value were to increase several fold after long periods of induction. Thus, we consider at least two general ways in which the intron could inhibit protein synthesis catalytically.

First, the intron could modify the 23S rRNA directly, either by reverse splicing (Roman & Woodson, 1995) or by cleavage of the 23S rRNA via the well-known riboendonuclease reaction (Zaug et al., 1986). Recent work by Butow and co-workers has shown that a null mutation in a subunit of the yeast mitochondrial exonuclease permits accumulation of the omega group I intron from the mitochondrial rDNA (Margossian et al., 1996). The omega intron recleaves the 23 S rRNA at the splice junction, resulting in loss of mitochondrial function.

Second, the intron could interfere with final maturation of 50S subunits. Assembly of the large subunit in vivo passes through an intermediate 50S complex that contains all the ribosomal proteins, but is inactive (Lindahl, 1975). These complexes can be activated in vitro by incubation at 50 °C (Dohme & Nierhaus, 1976), suggesting that a conformational change is required that could be blocked by the intron. In addition, the intron could inhibit final maturation of the 5′ and 3′ ends of the 23S rRNA, which is proposed to occur after initiation of protein synthesis and appears to enhance 50S activity (Srivastava & Schlessinger, 1988). Detailed characterization of intron-containing ribosomal complexes awaits in vitro reconstitution or an effective means of separating them from wild-type subunits.

All of the models above are consistent with the observed correlation between intron–50S association and reduced cell growth. However, we do not exclude the possibility that the *Tetrahymena* intron restricts bacterial growth by cleaving another essential transcript. The substrate recognition sequence is only 5–6 nt and appears frequently in the *E. coli* genome. The intron could even encode a peptide that interacts with ribosomes (Tenson & Mankin, 1995), because there are several reading frames (≤23 codons) that are preceded by weak Shine–Dalgarno sequences. Additional experiments are required to discriminate among these possibilities.

Regardless of the mechanism of intron inhibition, this report opens a new perspective on the selective pressures that influence the evolution of group I introns. First, although splicing activity of the pre-rRNA must be maintained, it need not be 100% efficient. Slight decreases in splicing activity, which in this case lead to accumulation of inactive 23S rRNA, do not compromise cell viability seriously. On the other hand, large amounts of a catalytically active ribozyme can exert a dominant negative effect on cell growth (this work and Margossian et al., 1996). This presumably does not occur in *Tetrahymena* because the intron is degraded rapidly in the nucleus (Brehm & Cech, 1983). RNA

decay mechanisms may be equally or perhaps even more important than splicing mechanisms for determining the spread and maintenance of self-splicing introns in diverse organisms. In this light, it is even conceivable that the co-evolution of host-encoded maturase proteins and the devolution of self-splicing activity provide another strategy by which cells limit the catalytic potency of intron RNA.

MATERIALS AND METHODS

Plasmids and strains

pLK45 (Powers & Noller, 1990), pLK45-IVS, pLK45-IVS: G264A and pLK45-IVS: G264A, ΔIGS (Zhang et al., 1995), and pLKEC(438) (Roman, 1996) have been described. *E. coli* strain DH1 (F^-, recA1, endA1, gyrA96, thi1, hsdR17 r_k^- m_k^+, supE44, l^-) transformed with pCI857 (Remaut et al., 1983) and pLK45 or a derivative were used for all in vivo experiments.

pTN112 is a derivative of pTZ19U and was created by introducing two new restriction sites 75 bp (*Sma* I) and 86 bp (*Eco*R I) from the 3′ splice site by PCR amplification of pSW112 (Zhang et al., 1995) with a suitably modified downstream primer. The resulting PCR fragment was ligated into the *Nhe* I and *Eco*R I sites of pSW112, resulting in deletion of downstream 23S and terminator sequences. The TN112 T7 RNA polymerase transcript is 679 nt when the DNA is linearized with *Eco*R I.

IGS mutations were introduced into pTN112 using "megaprimer" PCR mutagenesis (Picard et al., 1994). For in vivo experiments, DNA fragments containing the IGS mutations were subcloned in pLK45-IVS as described (Zhang et al., 1995).

Ribosome isolation and fractionation

Two-hundred milliliters transformed cells were grown at 30 °C to $OD_{600} = 0.07$, shifted to 42 °C, and harvested at $A_{600} = 0.3$ after addition of 100 mg/L chloramphenicol and rapid chilling of the cultures. The cells were lysed in a buffer containing 10 mM Tris-HCl, pH 7.75, 15 mM Mg-acetate, 0.5 mg lysozyme, and 200 U/mL recombinant RNasin (Promega) by the freeze/thaw procedure (modified from Ron et al., 1966). Cell debris was removed by centrifugation (20 min, 12,000 rpm). Lysates were loaded on linear 5–20% (w/v) sucrose gradients in 20 mM Tris-HCl, pH 7.5, 10 mM Mg-acetate, 100 mM NH_4Cl, and 1 mM DTT, and centrifuged for 15 h at 4 °C in an SW28 rotor (Beckman) at 18,000 rpm. Ribosomes were recovered from fractions at the center of each peak by ethanol precipitation (Spedding, 1990). Ribosomal RNA was isolated by extraction with phenol saturated with TE, phenol:chloroform:isoamyl alcohol 24:1 (CIA) (1:1) and CIA, followed by ethanol precipitation.

Northern hybridization

Three micrograms total RNA or rRNA recovered from sucrose gradients were denatured with glyoxal (Sambrook et al., 1989) and electrophoresed on a 1.4% agarose gel, or 5 μg RNA was separated on a 5% denaturing polyacrylamide gel. The RNA was transferred to a Nytran membrane (Schleicher & Schuler) via capillary action and hybridized with an intron probe prepared by nick-translation in the presence of α-[^{32}P] dATP. The membranes were visualized with autoradiography and the intensity of each band quantitated using storage phosphorescence (Molecular Dynamics).

Primer extensions

^{32}P-end-labeled primer (0.1 pmol CP2076 or IP006) was annealed to 0.25 μg RNA and extended with AMV reverse transcriptase in the presence of ddTTP or ddCTP, respectively, as described (Zhang et al., 1995). The products were separated on 20% polyacrylamide gels and quantitated using a Phosphorimager. CP2076 anneals to the 23S rRNA 3′ of A2058 and IP006 anneals to the 5′ end of the intron. The cpm of each product was normalized to the total cpm in the lane.

Self-splicing reactions

Pre-RNA was transcribed by T7 RNA polymerase from pTN112 DNA linearized with *Eco*R I, in the presence of α[^{32}P]-ATP. The pre-RNA was purified by a size-exclusion spin column (TE-100, Clontech), then renatured by heating at 95 °C for 1 min and cooling in the presence of splicing buffer (100 mM $(NH_4)_2SO_4$, 50 mM HEPES, pH 7.5, 6 mM $MgCl_2$, 1 mM EDTA) as described (Emerick & Woodson, 1993). Splicing reactions were performed in the presence of 0.2 M GTP at 37 °C, and the splicing rates determined from semi-logarithmic plots of $(1 - f_{sp})$ versus time over the first 2 min, where f_{sp} is the fraction of spliced products, normalized to the amount of precursor consumed after 2 h incubation.

Isolation of total RNA and in vivo splicing rates

E. coli cultures were harvested at specified times after induction of plasmid expression at 42 °C as described previously (Zhang et al., 1995). Total RNA was isolated by freeze/thaw lysis and extraction with phenol and chloroform (Belfort et al., 1990; Zhang et al., 1995), or by purification on silica resin (5 Prime 3 Prime, Inc.). The RNA was digested with 5 U RQ1 DNase I (Promega) for 2 h at 4 °C, followed by organic extraction and precipitation with ethanol.

Rates of splicing in vivo were calculated from the steady-state ratio of linear intron to pre-rRNA and the rate of decay of the linear intron as described (Zhang et al., 1995), except that values were based on quantitation of radioactive northern blots. The steady-state ratio of linear intron to pre-23S rRNA after 20 min of induction was calculated from:

$$\frac{F_{L\,IVS}}{f_{pre}} = \left(\frac{C_{IVS}}{C_{pre}}\right)\left(\frac{C'_{L\,IVS}}{C'_{IVS}}\right)$$

where C_{IVS} and C_{pre} are the intensity of the free intron and pre-23S RNA bands on northern blots of agarose gels, and $C'_{L\,IVS}$ and C'_{IVS} are the amounts of full-length linear intron (L IVS) and total intron RNAs, respectively, as resolved on 4% polyacrylamide gels.

The half-life of intron RNA was determined by adding 0.8 mg/mL rifampicin to cultures 20 min after induction of plasmid expression at 42 °C. Twenty milliliters of culture were

harvested at 0, 2, 5, 10, and 20 min and total RNA isolated as above. The RNA was subjected to either northern hybridization or to primer extension with IP006 (Zhang et al., 1995), and the rate of decay determined from the fraction of linear intron remaining versus time.

Quantitation of intron and 23S rRNA by dot-blot hybridization

Total *E. coli* RNA or RNA standards were denatured with glyoxal and transferred to a Nytran membrane with a dot-blot apparatus (BioRad). The membrane was hybridized with either an intron or a 23S probe labeled with α-[^{32}P] dATP, and the intensity of each spot quantitated using a Phosphorimager. To construct a standard curve for intron RNA, in vitro-transcribed TN112 pre-rRNA was subjected to 60-min standard splicing conditions, and then applied to the membrane in amounts ranging from 1 to 50 ng. The standard curve was obtained from a linear fit to cpm versus ng intron RNA. A standard curve for 23S rRNA was obtained in a similar manner, using 1–50 ng RNA extracted from purified *E. coli* 50S subunits. Total *E. coli* RNA samples were taken from cells transformed with pLK45-IVS and grown 20 min at 42 °C before harvest. Ratio (w/w) of intron:total RNA was measured using 0.5–3 μg samples, and was 0.0093 ± 0.0007. Ratio (w/w) of 23S:total RNA was determined using 1–100 ng samples, and was 0.467 ± 0.035. Values are the average of three trials. Molar ratio of intron to 23S rRNA was calculated according to:

$$\frac{\text{IVS (mol)}}{\text{23S (mol)}} = \frac{\text{IVS (ng)}}{\text{total RNA (ng)}} \times \frac{\text{total RNA (ng)}}{\text{23S (ng)}} \times \frac{2900\text{ nt}}{414\text{ nt}} = 0.14.$$

ACKNOWLEDGMENTS

We thank Edward Ramsay for technical assistance with purification of ribosomes, and Judibelle Roman for gift of pLKEC(438) and for communication of unpublished work. This work was supported by the National Institutes of Health (GM46686). S.W. acknowledges the support of the Pew Scholars Program in the Biomedical Sciences and the Camille Dreyfus Teacher-Scholar program.

Received May 5, 1997; returned for revision May 23, 1997; revised manuscript received June 16, 1997

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