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Reverse splicing of the *Tetrahymena* IVS: Evidence for multiple reaction sites in the 23S rRNA

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ABSTRACT

Group I introns in rRNA genes are clustered in highly conserved regions that include tRNA and mRNA binding sites. This pattern is consistent with insertion of group I introns by direct interaction with exposed regions of rRNA. Integration of the *Tetrahymena* group I intron (or intervening sequence, IVS) into large subunit rRNA via reverse splicing was investigated using *E. coli* 23S rRNA as a model substrate. The results show that sequences homologous to the splice junction in *Tetrahymena* are the preferred site of integration, but that many other sequences in the 23S rRNA provide secondary targets. Like the original splice junction, many new reaction sites are in regions of stable secondary structure. Reaction at the natural splice junction is observed in 50S subunits and to a lesser extent in 70S ribosomes. These results support the feasibility of intron transposition to new sites in rRNA genes via reverse splicing.

Keywords: group I splicing; intron evolution; intron transposition

INTRODUCTION

Group I introns have been found among widely divergent genomes. In many cases, sequence homologies among introns do not follow the species phylogeny, raising questions about their evolutionary origins (Sogin et al., 1986; Shub et al., 1988; Lonergan & Gray, 1994). It has been suggested that, although group I introns are likely to have shared a common ancestor, their modern distribution is the result of lateral transfer between species (Cech, 1988; Dujon, 1989; Belfort, 1990).

Intron mobility has been demonstrated at the DNA level in a number of lower eukaryotes and bacteriophage T4 (reviewed in Lambowitz & Belfort, 1993). Conversion of intron-less to intron-containing alleles, or intron homing, is initiated by cleavage of intron-less DNA near the insertion site by an intron-encoded endonuclease (Jacquier & Dujon, 1985; Macreadie et al., 1985). Homing endonucleases, however, typically require extensive sequence homology in the splice junction that limits transposition to new genes (Lambowitz & Belfort, 1993). In addition, many group I introns, including the *Tetrahymena* intervening sequence (IVS), do not encode a specific endonuclease.

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Reversal of the self-splicing reaction may serve as a first step in transposition of group I and group II introns to new sites at the RNA level (Cech, 1985; Sharp, 1985). Following integration of free intron into another RNA, reverse transcription and homologous recombination could result in incorporation of the intron into the genome (Sharp, 1985; Woodson & Cech, 1989). This model has been supported by demonstration of reverse self-splicing of the *Tetrahymena* IVS using oligoribonucleotide substrates in vitro (Woodson & Cech, 1989).

There is further evidence for RNA intermediates in the transposition of group II introns. An open reading frame within the yeast mitochondrial introns aI1 and aI2 encodes a reverse transcriptase (Kennell et al., 1993) that has been linked to insertion of introns into intron-less alleles, a process that also depends on splicing activity (Meunier et al., 1990). Transposition of group II introns to non-allelic sites in mitochondrial DNA requires splicing activity, and the sites of integration are related to the intron binding sites (IBS) of the original 5′ exon (Müller et al., 1993; Sellem et al., 1993). These observations are consistent with a reverse splicing step in movement of introns to new locations.

In ribosomal DNA, introns are clustered in regions of functional importance in translation (Garrett et al., 1991; Turmel et al., 1993). This is in contrast to other

variable regions in rRNA that are located in poorly conserved loops and may or may not lie on the surface of the ribosome. These include expansion segments, which are not removed from the mature rRNA, and internal transcribed spacers, which are excised but not religated (Gerbi, 1985; Burgin et al., 1990; Gray & Schnare, 1990). It has been proposed that the distribution of ribosomal group I introns is best accounted for by transposition events involving an RNA intermediate (Turmel et al., 1993), because their insertion sites correspond to regions of the rRNA, such as tRNA binding sites, that remain accessible in the ribosome (Noller, 1991).

Reverse self-splicing follows the pathway of the well-characterized forward reaction (Cech, 1990), but in the opposite direction (Fig. 1). The complete reaction results in a molecule that resembles a precursor RNA, and is stimulated by low GTP and high RNA concentrations (Woodson & Cech, 1989). The site of IVS integration is determined by base pairing between the internal guide sequence (IGS) and nucleotides 5′ of the splice junction that is analogous to the P1 helix in the forward reaction (Woodson & Cech, 1989). A shortened form of the intron, the L-15 IVS, can also integrate into oligomeric substrates (Woodson & Cech, 1989). The L-15 IVS lacks the first 15 nucleotides and is the product of autocyclization (Zaug & Cech, 1983).

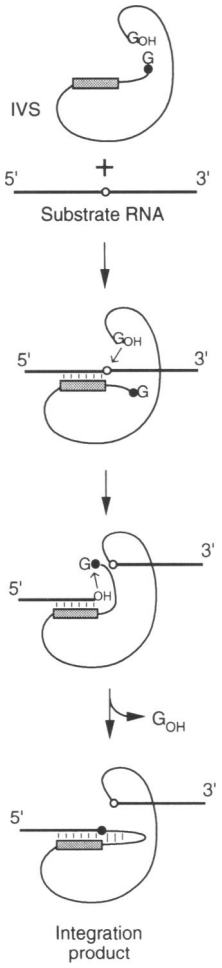

FIGURE 1. Mechanism of group I reverse splicing. Base pairing of the substrate (ligated exon) RNA with the internal guide sequence (IGS, shaded rectangle) is followed by attack of the 3′ terminal hydroxyl group of the IVS at the splice junction (open circle). In the second step, the 3′ hydroxyl of the 5′ exon reacts with the first phosphodiester bond of the intron (closed circle), displacing the 5′ guanosine. Adapted from Woodson and Cech (1989).

Because reverse splicing requires only a short recognition sequence (4–6 nucleotides) in the substrate (Woodson & Cech, 1989), many potential sites for integration are expected. However, secondary structure in the substrate RNA can prevent recognition of the target sequence. For example, integration of the *Tetrahymena* IVS into the natural site is inhibited by a ribosomal RNA hairpin flanking the splice junction (Woodson & Cech, 1991), although this inhibition is relieved in longer rRNA substrates (Woodson & Emerick, 1993).

In order to provide additional evidence for RNA-based transposition of introns among rRNA genes, we have tested the ability of the *Tetrahymena* IVS to integrate into the large subunit rRNA by reverse self-splicing. To facilitate interpretation of the data in terms of rRNA conformation, the *Escherichia coli* 23S rRNA was used as a substrate in this study, because its secondary structure and function are established (Noller, 1991). The results demonstrate that the *Tetrahymena* IVS can integrate into the *E. coli* 23S rRNA at a position homologous with the natural splice junction. In addition, the IVS also recognizes a large number of novel sites in domains II, IV, and V. These initial studies provide a foundation for further investigation of reverse splicing and RNA-catalyzed transposition of group I introns.

RESULTS

The *Tetrahymena* IVS integrates into *E. coli* splice junction

To compare the ability of ribosomal sequences from *Tetrahymena* and bacteria to serve as substrates for reverse splicing, integration of the *Tetrahymena* IVS into a transcript derived from the *E. coli* 23S rRNA was examined. The *Tetrahymena* splice junction corresponds to nucleotide 1925 in the *E. coli* 23S sequence, and falls within a highly conserved region of the large subunit rRNA domain IV (Noller et al., 1981). To establish complementarity between the IVS and *E. coli* 23S rRNA, four nucleotides were mutated in the IGS (Fig. 2A), such that the expected site of reaction is between U1926 and A1927. This position was chosen to preserve the U·G pair at the 5′ splice site that is conserved among group I introns (Cech, 1988). The mutated IVS is designated "EC IVS" for ease of discussion, and has been

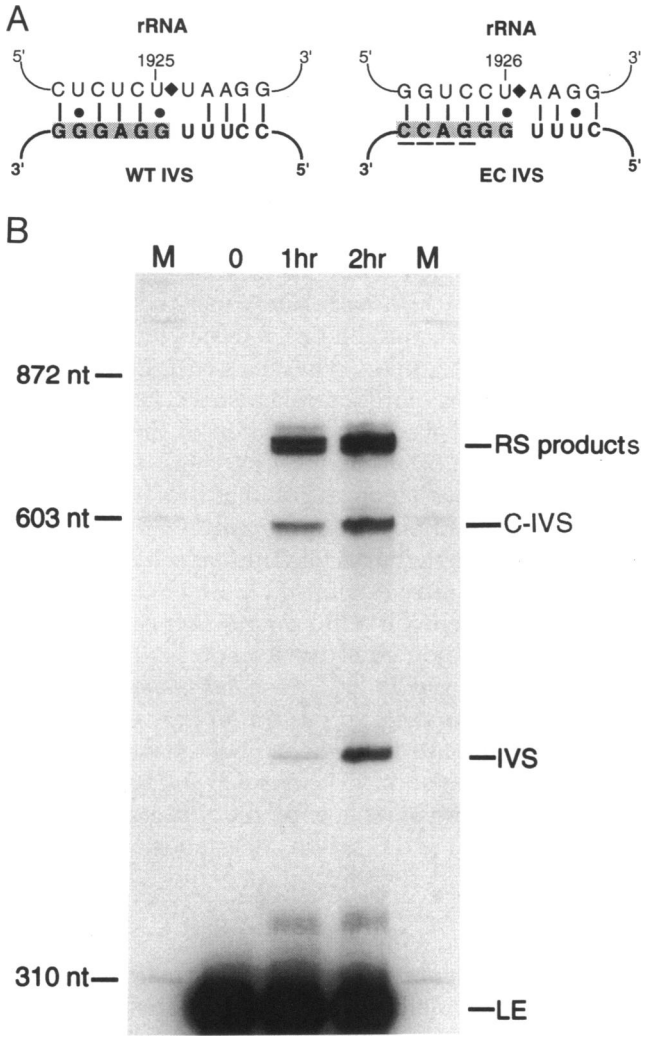

FIGURE 2. Reverse splicing of the *Tetrahymena* IVS with an *E. coli* rRNA substrate. **A:** Mutations in the internal guide sequence (IGS) of the *Tetrahymena* IVS (WT IVS) restore complementarity with the 5′ exon in the *E. coli* 23S rRNA (EC IVS). The splice junction has been shifted from 1925 to 1926 in *E. coli*. IVS, bold letters; exon, plain text; shaded box, IGS. Mutations are underlined. The splice junction is represented by (◆). **B:** Integration of EC IVS into a 295-nt *E. coli* rRNA substrate. Linear IVS (413 nt) and 5′ end-labeled ligated exon (LE, 295 nt) RNA were incubated under reverse splicing conditions at 42 °C for the times shown above the lanes and electrophoresed on an 8 M urea, 4% polyacrylamide gel. Lane M, ϕX174/Hae III DNA; RS products (708 nt), complete integration at positions 1926 (natural site) and 2030; C IVS, circular IVS. IVS RNA has become labeled by exchange of ^{32}P with the ligated exons.

shown to self-splice when inserted at this position of the 23S rRNA (Zhang et al., 1995).

Reverse splicing of EC IVS was tested with a 295-nt substrate (SW112-LE) that was derived from the *E. coli* 23S rRNA and contains most of domain IV (positions 1764–2046). Wild-type IVS has been shown to integrate into a 244-nt substrate that is homologous to SW112-LE and is derived from the *Tetrahymena* 26S rRNA (Woodson & Emerick, 1993). When end-labeled SW112-LE RNA was incubated with EC IVS under reverse splicing conditions, a labeled product of the correct size for complete intron integration was observed (Fig. 2B). The extent of reverse splicing is 2–5% for EC IVS, greater than previously observed in similar reactions with wild-type *Tetrahymena* RNAs (0.1%; Woodson & Emerick, 1993). This may be due to stabilization of the 5′ exon-IGS pairing by replacement of a U·G pair in the wild type with a C-G base pair in EC IVS. From these results, it was concluded that the *E. coli* 23S rRNA could be used as a model substrate for reverse splicing of the *Tetrahymena* IVS.

Detection of reverse splicing products by RT-PCR

We wished to develop a more sensitive method for the detection of integration products with long substrates (Fig. 3A). After reverse splicing, reverse transcription (RT) and PCR amplification (Saiki et al., 1988) were carried out in a single tube with no buffer changes, in order to minimize contamination with external templates. RNAs containing a 5′ integration junction (or 5′ splice site) were specifically amplified with a downstream primer complementary to the IVS and an upstream primer specific for 23S cDNA (Fig. 3A). Reaction conditions were optimized to permit amplification of small amounts of reverse splicing product in the presence of a high background of similar unreacted RNA.

This method was tested on reverse splicing substrates from *Tetrahymena* and *E. coli*. RT-PCR amplification of reverse splicing reactions containing either wild-type IVS and a *Tetrahymena* ligated exon substrate (SW012-LE; Fig. 3B) or EC IVS and an *E. coli* substrate (SW112-LE; Fig. 3C) gave rise to the expected DNA products. In Figure 3B, the 547-bp fragment corresponds to insertion of the IVS at the natural splice junction. This band was not detected by RT-PCR amplification of parallel reverse splicing reactions containing only IVS or ligated exon RNA. In Figure 3C, the 477-bp fragment is the size expected for IVS integration at U1926 in the *E. coli* rRNA substrate, and formation of the correct junction between the 5′ exon and the 5′ end of the IVS was verified by direct sequencing. A fragment approximately 580-bp long was also observed (Fig. 3C), which results from reaction of the IVS at position 2030 near the 3′ end of the substrate. The 340-bp product contains only IVS sequences and results from spurious single-primer amplification of free IVS RNA (data not shown).

The limit of detection for this method is estimated to be 1–5 fmol of reverse splicing product, as judged from mock reactions doped with authentic precursor RNA (data not shown). In similar experiments, addition of IVS to 23S rRNA in the presence of EDTA did not give rise to RT-PCR products (data not shown). In the presence of magnesium, the amount of RT-PCR product increased with the time of incubation up to 2 h (data not

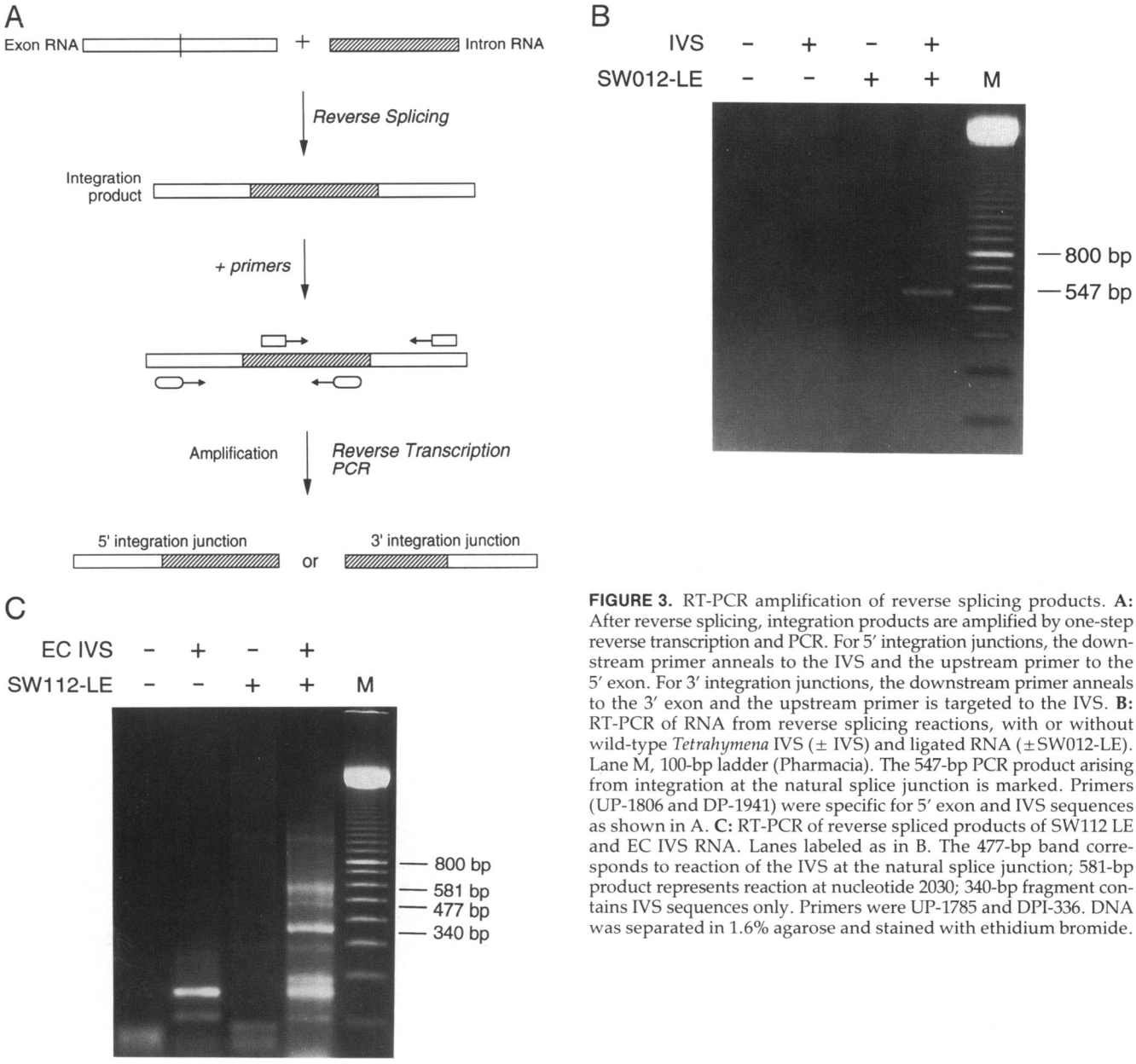

FIGURE 3. RT-PCR amplification of reverse splicing products. **A:** After reverse splicing, integration products are amplified by one-step reverse transcription and PCR. For 5′ integration junctions, the downstream primer anneals to the IVS and the upstream primer to the 5′ exon. For 3′ integration junctions, the downstream primer anneals to the 3′ exon and the upstream primer is targeted to the IVS. **B:** RT-PCR of RNA from reverse splicing reactions, with or without wild-type *Tetrahymena* IVS (± IVS) and ligated RNA (±SW012-LE). Lane M, 100-bp ladder (Pharmacia). The 547-bp PCR product arising from integration at the natural splice junction is marked. Primers (UP-1806 and DP-1941) were specific for 5′ exon and IVS sequences as shown in A. **C:** RT-PCR of reverse spliced products of SW112 LE and EC IVS RNA. Lanes labeled as in B. The 477-bp band corresponds to reaction of the IVS at the natural splice junction; 581-bp product represents reaction at nucleotide 2030; 340-bp fragment contains IVS sequences only. Primers were UP-1785 and DPI-336. DNA was separated in 1.6% agarose and stained with ethidium bromide.

shown). These observations support the interpretation that the amplified products are a result of IVS-catalyzed reactions.

Integration of the *Tetrahymena* IVS into 23S rRNA

Although reverse splicing occurs with rRNA fragments, the ability of the IVS to recognize the splice junction in mature rRNA had not yet been demonstrated. To this end, reverse splicing was carried out with total *E. coli* rRNA or only 23S rRNA. Products were reverse transcribed and 5′ integration junctions amplified as described above (Fig. 4A). In these experiments, the primer used for (−) strand synthesis (DP-1937) annealed to sequences spanning the original 3′ splice site in order to suppress amplification of unreacted IVS RNA. The upstream primer (UP-1666) was complementary to the 5′ exon as before. A 683-bp fragment corresponding to integration at the natural splice junction was obtained from reactions containing both IVS and rRNA substrate, but not in the absence of IVS RNA (Fig. 4A). Reactions containing just 23S rRNA substrate yielded more product than those with 16S+23S rRNA. This was most likely due to the lower molarity of 23S rRNA in the mixed sample and not to any inhibitory effect of the 16S sequences, because the amount of RT-PCR product increased when a larger amount of 16S+23S rRNA was used in the reaction (data not shown).

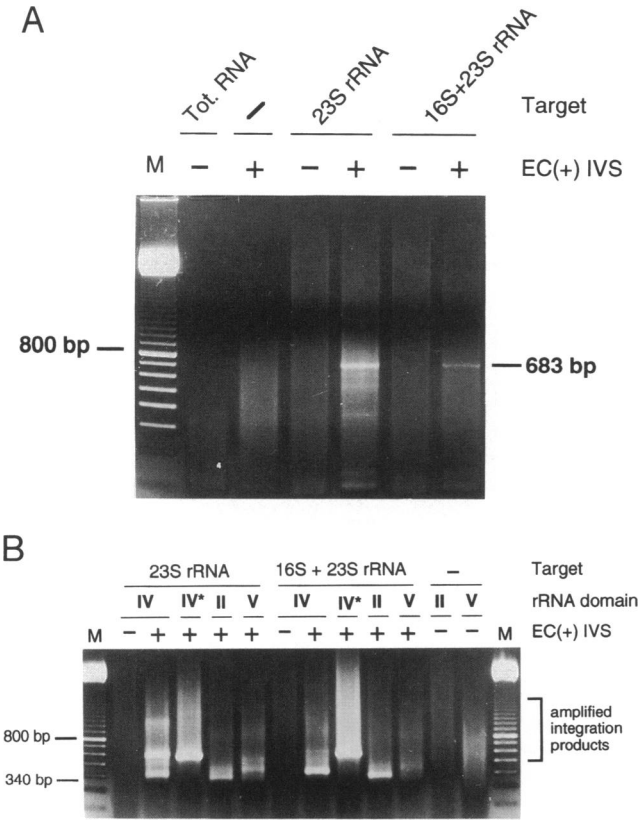

FIGURE 4. IVS integration into ribosomal RNA. 23S rRNA or 16S+23S rRNA from *E. coli* were incubated with (+) or without (−) IVS RNA (EC(+) IVS) under reverse splicing conditions, and amplified by RT-PCR. Tot. RNA, total *E. coli* RNA; −Target, no substrate RNA; M, 100-bp ladder. **A:** Integration products were amplified with an upstream primer specific for the 5′ exon (UP-1666) and a downstream primer (DP-1937) that anneals to the expected 3′ splice site. The 683-bp fragment represents IVS integration into position 1926 of the 23S rRNA. **B:** RT-PCR with primers specific for 5′ integration junction in domains II, IV, and V of the *E. coli* 23S rRNA, or 3′ integration junctions in domain IV (lanes IV*). Products corresponding to integration at the original splice junction are 596 bp (lane IV) or 573 bp (lane IV*). The 340-bp fragment represents amplification of IVS only. Upstream primers were UP-1666, lanes IV; UPI-94, IV*; UP-570, II; UP-2050, V. Downstream primers were DPI-336, lanes IV, II, and V; DP-2180, lanes IV*.

True IVS integration requires the formation of both 5′ and 3′ splice sites. Having already shown ligation of the 5′ splice site, the presence of a 3′ junction at U1926 was probed with an upstream primer (+ strand DNA) in the IVS (UPI-94) and a downstream primer (cDNA) complementary to the 3′ exon (DP- 2180) as outlined in Figure 3A. As shown in Figure 4B (lane IV*), a PCR product containing the original 3′ splice site is readily detected when either 23S or 16S+23S rRNA is used as substrate. Again, the specific PCR product was not observed when the IVS was not added to reverse splicing reactions (data not shown). Direct sequencing of the PCR fragment confirmed that the terminal nucleotide of the IVS (G414) is ligated to nucleotide 1927 of the 23S rRNA as expected. Together with formation of the 5′ junction, the data suggest that the IVS is fully integrated into the 23S rRNA, at a site homologous to the original splice junction in *Tetrahymena* rDNA.

Integration into other sites in 23S rRNA

To test the possibility that EC IVS integrates into other sites in the 23S rRNA, primers that anneal to domains II, IV, and V were used for RT-PCR in conjunction with a downstream IVS primer (DPI-336). These domains of the 23S rRNA are important for ribosome function, and encompass known insertion sites of group I introns (Noller, 1991). RNA from reverse splicing reactions was reverse transcribed and amplified as previously, except that the RNA was passed through a gel filtration column before RT-PCR to reduce the excess of unreacted IVS that can hybridize with the downstream primer.

As shown in Figure 4B, weak bands were detected when primers specific for domains II or V were used for RT-PCR. Products 500 bp or longer were considered as possible candidates for integration products, and represent sequences spanning a putative 5′ integration junction. The 340-bp product (Fig. 4B) is due to amplification of IVS RNA. When the upstream primer used for amplification annealed to sequences in domain V, approximately three fragments were detected in the agarose gel. Only a faint smear was obtained in reactions with a primer specific for domain II. The results suggest that the *Tetrahymena* IVS has the capacity to react with other regions in the 23S rRNA, but to a lesser extent than at the natural splice junction.

The 3′ integration junctions in domain V were also amplified using a downstream primer, DP-2695, that was complementary to sequences at the 3′ end of the 23S rRNA and an upstream primer within the IVS (UPI-94). Three primary bands 400, 500, and 1,100 bp in length were visible in agarose gels (data not shown). The 1.1-kb product corresponded to integration at the natural splice junction in domain IV. The 400-bp and 500-bp RT-PCR products were indicative of IVS integration near positions 2600 and 2500 in the 23S rRNA, respectively.

RT-PCR products from reverse splicing reactions containing rRNA were cloned and the sequences of individual exon–IVS junctions determined. Their locations relative to the secondary structure of the 23S rRNA are shown in Figure 5. A large number of reaction sites were observed in regions flanked by the PCR primers used in this study. In domain IV, full-length IVS (L IVS) reacted at the natural splice junction (5′ and 3′ junctions) and one other site, 1697, which is two nucleotides upstream of a group I intron in the mitochondria *rrn*L gene from *Podospora* (Cummings et al., 1989). The 3′ junction at position 1697 was not detected. This was perhaps due to a greater frequency of reaction at

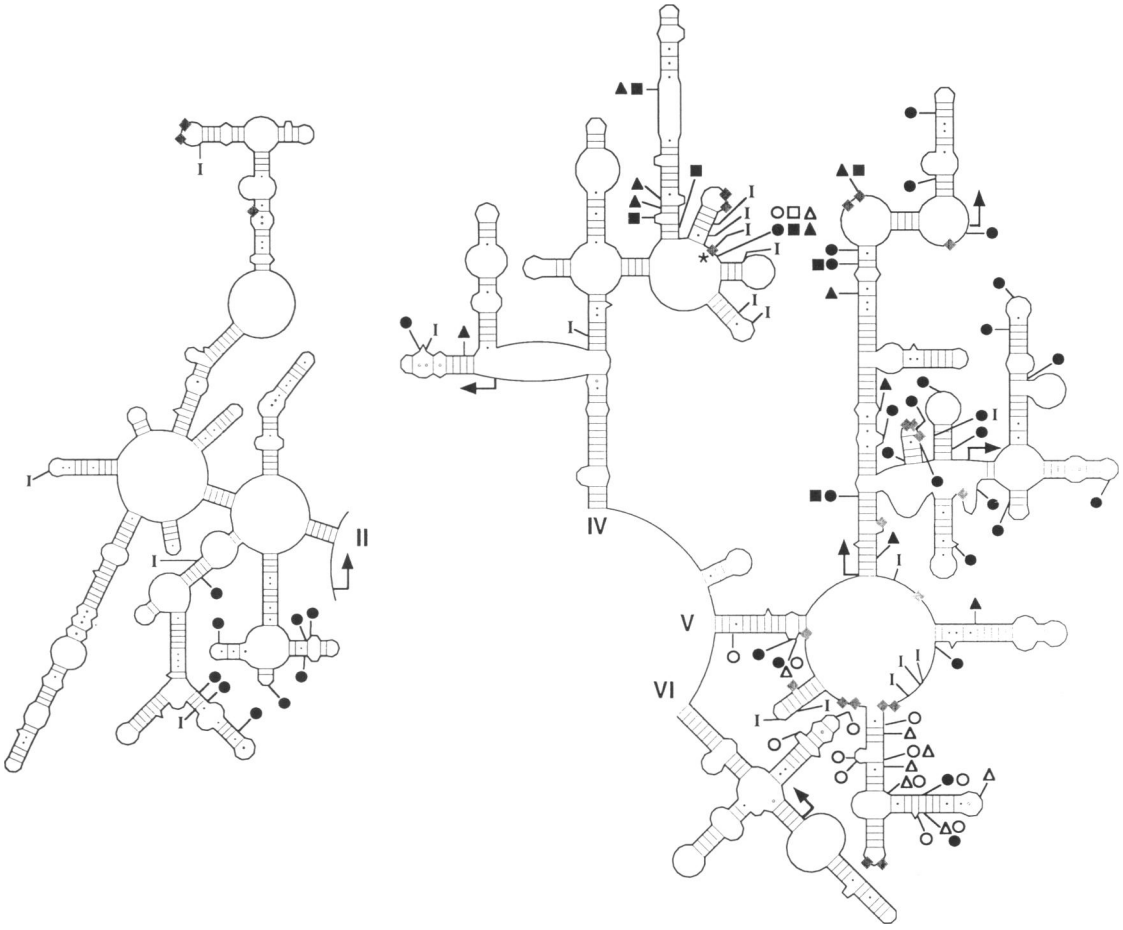

FIGURE 5. Reverse splicing reaction sites of ECIVS in 23S rRNA, as inferred from sequences of 5′ and 3′ junctions. Circle, naked 23S or 16+23S rRNA; triangle, 50S subunits; square, 70S ribosomes; filled symbols, 5′ junctions; open symbols, 3′ junctions; **I**, known insertion sites of group I introns (Liu et al., 1992; Turmel et al., 1993 and references therein; Aimi et al., 1994; Lonergan & Gray, 1994); asterisk, position of *Tetrahymena* splice junction. Shaded diamonds represent nucleotides involved in tRNA binding at the A, P, and E sites (Moazed & Noller, 1989, 1991). Arrows show the position and orientation of primers used for RT-PCR. Secondary structure of *E. coli* 23S rRNA drawn by Bryn Weiser (University of California, Santa Cruz) based on phylogenetic sequence comparisons (Noller et al., 1981; Gutell et al., 1993).

the natural splice junction. More likely, it was caused by better amplification of the shorter PCR product, which favors reaction sites closer to the primer binding site.

In domains V and VI, L IVS was found to react at 16 sites and the L-15 IVS at an additional 10 sites between nucleotides 2074 and 2613 in the 23S rRNA (Fig. 5). Seventeen 3′ integration junctions were also sequenced between nucleotides 2443 and 2660. Only a few overlap with the 5′ integration sites. This observation can again be explained by preferential amplification of shorter PCR fragments, a particular problem with one-step RT-PCR reactions. In domain II, nine novel 5′ integration junctions were found in sequences close to the primer binding site (position 570). The bias toward shorter amplification products probably precluded detection of integration events in the downstream portion of domain II, for example, near the GTPase center around position 1050.

Integration into 70S ribosomes and 50S subunits

In order to test the effect of ribosomal proteins on reverse splicing, the intron was incubated with *E. coli* 70S ribosomes or 50S subunits under reverse splicing conditions. RT-PCR was used to amplify 5′ and 3′ integration junctions in 23S domains II, IV, and V. As shown in Figure 6, the 596-bp fragment represents reverse splicing at the natural splice junction in domain IV when EC IVS was incubated with deproteinized rRNA, 50S subunits, or 70S ribosomes. Less PCR product was obtained when 50S subunits and 70S ribosomes were used as a substrate, suggesting that ribosomal proteins protect the rRNA from cleavage by the IVS. The least amplified product was obtained with 70S ribosomes, compared to an equimolar amount of 50S substrate.

Despite the reduced yield of the RT-PCR reactions, amplified fragments were cloned and 5′ and 3′ integration junctions sequenced. The sites of IVS reaction with

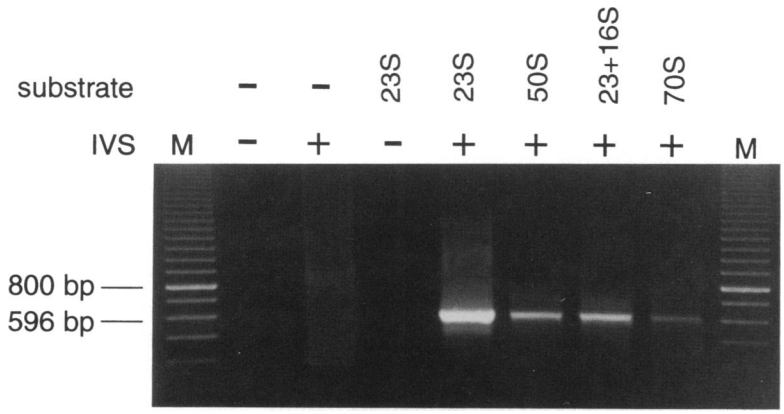

FIGURE 6. Reaction of EC IVS with 50S and 70S ribosomes. 23S rRNA (5 pmol), 16S+23S rRNA (3.3 pmol), 50S subunits (5 pmol), and 70S ribosomes (5 pmol) were incubated with (+) or without (−) EC IVS under reverse splicing conditions. M, 100-bp ladder. Products were amplified with a downstream primer (DPI-336) that anneals to the IVS and an upstream primer specific for 5′ exon (UP-1666). Reverse transcription and PCR were performed in two steps. The 596-bp fragment represents integration of the IVS into position 1926 of the 23S rRNA.

70S ribosomes and 50S subunits are also diagrammed in Figure 5. Correct 5′ and 3′ integration junctions with the L IVS at the natural splice junction (1926) were obtained with both 50S and 70S ribosomes. Seven additional reaction sites were observed in domain IV, along with five 5′ integration junctions and nine 3′ integration junctions in domain V (Fig. 5). No IVS-containing clones were obtained from RT-PCR reactions specific for domain II. Many of the IVS targets in 50S subunits, particularly those in domain V, overlapped with those found in naked 23S rRNA. Among the 42 IVS reaction sites in domain V, however, 12 were observed in reactions containing 50S and only 3 with 70S ribosomes. These results, in addition to the difficulty in amplifying integration products at the natural site (1926), suggest that many of the potential rRNA integration targets are masked in the 70S ribosome.

Comparison of sequences at the integration sites

Sequences upstream of putative integration sites were analyzed for base complementarity with the IGS that could promote reverse splicing at those positions (Table 1). Based on the relative intensities of PCR products and the number of clones obtained, the natural insertion site appears to be the preferred substrate for L IVS integration (Table 1A). Six other 5′ integration junctions with the L IVS sites were preceded by the consensus sequence $^{5'}$CCU. Integration junctions were found at 8 of the 24 $^{5'}$CCU trinucleotides within the areas covered by the PCR primers. In other products, a G or a U is typically 5′ of the cleavage site. The number of clones containing a G at the 5′ splice site was somewhat surprising. However, these sequences can often be aligned so that the G is paired with a U in the IVS. Perhaps the canonical U·G pair of group I introns can be accommodated by the active site in the opposite orientation, although this is not true for reactions involving G addition to the 5′ splice site (Doudna et al., 1989).

Ligation of 3′ exons to the IVS

Theoretical 5′ integration junctions were also obtained from experiments in which the sequences 3′ of the integration site were amplified (Table 1B). Again, base pairing between the 5′ exon and the IGS was important for selection of the reaction site, based on a comparison of clones obtained with the greatest frequency. However, a number of putative integration sites come after a C rather than a U. For example, nucleotides 2541 and 2613 are preceded by $^{5'}$GGUCCC (Table 1B), which is exactly complementary to the IGS in the intron. These clones were obtained with high frequency. Junctions with the 5′ end of the IVS were also obtained at these insertion sites (Table 1A).

Reaction with L-15 IVS

In many clones, nucleotides at the 5′ end of the IVS are deleted (Table 1C,D). These products correspond to reaction of the 23S rRNA with shortened forms of the IVS RNA (L-6 and L-15 IVS) that arise from cyclization (Zaug & Cech, 1983). Reactions involving L-6 or L-15 IVS frequently occur after $^{5'}$GGU or $^{5'}$GGUU, that base pair with the 3′ end of the IGS ($^{5'}$ACC). This half of the IGS specifies the L-15 and L-19 cyclization sites in the wild-type *Tetrahymena* IVS (Been & Cech, 1987).

Similar products could result from opening of circular IVS by addition of free 5′ exon RNA (Sullivan & Cech, 1985). Circle reopening results in ligation of the 5′ exon to the 5′ end of the IVS, but does not lead to joining of the 3′ exon to the 3′ end of the IVS. Unlike reverse splicing, this mechanism requires a free 3′ OH in the 5′ exon substrate, which could be produced by IVS-catalyzed hydrolysis of the substrate. To evaluate this possibility, the integrity of rRNA samples was estimated by denaturing PAGE and by primer extension. There was no significant breakdown of rRNA at L-15 reaction sites in the absence of IVS, but the 23S rRNA was cleaved when incubated with IVS RNA under the usual reverse splicing conditions (data not shown).

TABLE 1. Alignment of IVS integration junctions in the 23S rRNA.

A. 5′ integration junction amplified: L-IVS

pos. rRNA	5′ exon[a]	3′ exon[a]	IVS / IGS	frequency[b]	substrate[c]
1926 (WT)	UAACGGUCCU	AAGGUA	G·AAAUAGCAAUAUUUACCUUUGGGACCAAAA	7	23S, 50S, 70S
607	UAGCAAGGUU	aaccga	.....CUUUGGGACCAA..	1	23S
630	AGCCGAAGGG	aaaccg	.....UAUUUACCUUUGGGA.....	1	23S
646	AGUCUUAACU	gggcgu	.....CUUUGGGA.....	1	23S
700	CCAUGGGCAG	guugaa	.....CCUUUGGG.....	1	23S
1697	UAACUUCGGG	agaagg	.....ACCUUUGGGACC....	3	23S, 70S
1841	CCUGCCCGGU	gccgga	.....CCUUUGGGACCAA..	1	50S
1903	CCGGUAAACG	gcggcc	.....AAUAUUUACCUU.....	1	70S
2074	CCGUGAACCU	uuacua	.....UUGGGACCAAA.	2	23S
2105	CAUUGAGCCU	ugaugu	.....CCUUUGGGACCAAAA	3	23S, 70S
2107	UUGAGCCUUG	augugu	.....CUUUGGGACCAAAA	2	23S
2166	GAGCCGACCU	ugaaau	.....CCUUUGGGAC.....	1	23S
2237	GUGUCUGGUG	gguagu	.....AUAUUUACCUU.....	1	23S
2246	GGGUAGUUUG	acuggg	.....GGGACCAAAA	1	23S
2253	UUGACUGGGG	cggucu	.....AUUUACCUUUG.....	1	23S
2262	GCGGUCUCCU	ccuaaa	.....UUGGGACC....	1	23S
2269	CCUCCUAAAG	aguaac	.....ACCUUUGGGACC....	1	23S
2279	AGUAACGGAG	gagcac	.....UACCUUUGGG.....	1	23S
2360	GAGCGUGACG	gcgcga	.....UACCUUUGGG.....	2	23S
2379	GGUGCGAAAG	cagguc	.....ACCUUUGGGACC....	1	23S
2522	AUCACAUCCU	ggggcu	.....UUGGGACC....	1	23S
2540	AGUAGGUCCC	aagggu	.....ACCUUUGGGACCAA..	3	23S
2612	GUUCGGUCCC	uaucug	.....UUGGGACCAA..	2	23S
2613	UUCGGUCCCU	aucugc	.....UUGGGACCAA..	1	23S

B. 3′ integration junction amplified[d]

pos. rRNA	5′ exon	3′ exon	IVS / IGS	frequency	substrate
1926 (WT)	UAACGGUCCU	AAGGUA	G·AAAUAGCAAUAUUUACCUUUGGGACCAAAA	11	23S, 50S, 70S
2443	aagguacucc	GGGGAU	.....ACCUUUGGGAC.....	2	50S
2460	acaggcugau	ACCGCC	.....UUUGGGACCAAA.	1	50S
2508	cucgaugucg	GCUCAU	.....CUUUGGGA.....	7	23S
2509	ucgaugucgg	CUCAUC	.....CUUUGGGA.....	1	50S
2512	augucggcuc	AUCACA	.....UUUGGGACCAA..	5	23S
2513	ugucggcuca	UCACAU	.....UUUGGGACCAA..	2	50S
2517	ggcucaucac	AUCCUG	.....UUUGGGACC....	2	23S, 50S
2522	aucacauccu	GGGGCU	.....ACCUUUGGGACC....	2	23S
2530	cuggggcuga	AGUAGG	.....ACCUUUGGGACCAAA.	1	50S
2540	aguaggucCC	AAGGGU	.....UUACCUUUGGGACCAA..	6	23S, 50S
2541	guaggucCCa	AGGGUA	.....UUUACCUUUGGGACCAA..	1	23S
2571	uuaaaguggu	ACGCGA	.....UGGGACCAAA.	1	23S
2573	aaaguggUac	GCGAGC	.....UACCUUUGGGACCAA..	1	23S
2612	guucggucCC	UAUCUG	.....UUGGGACCAA..	7	23S, 50S
2621	cuaucugccg	UGGGCG	.....UACCUUUGGGA.....	1	23S
2653	ggcugcuccu	AGUACG	.....CUUUGGGACC....	1	23S
2659	uccuaguacg	AGAGGA	.....ACCUUUGGG.....	1	23S

C. 5′ splice junction amplified: L-6 IVS

pos. rRNA	5′ exon	3′ exon	IVS / IGS	frequency	substrate
607	UAGCAAGGUU	aaccga	CAAUAUUUACCUUUGGGACCAA..	1	23S
1856	AAGGUUAAUU	gauggg	.....CUUUGGGA.....	1	50S
2115	UGAUGUGUAG	gauagg	.....ACCUUUG.....	1	50S
2321	UCAGGAGGUU	AGUGCA	.....GGGACCAA..	2	23S
2402	UCCGGUGGUU	cugaau	.....CCUUUGGGACCAAA.	1	23S

(*continued*)

TABLE 1. *Continued.*

D. 5'splice junction amplified: L-15 IVS

pos. rRNA	5′ exon	3′ exon	IVS (IGS)	frequency	substrate
6061	GUAGCAAGGU	uaaccg	ACCUUUGGGACCAAAA	2	23S
621	GAAUAGGGGA	gccgaa	UUGGGACCAA..	2	23S
680	CCCGAAACCC	ggugau	..CUUUGGGAC.....	1	23S
703	UGGGCAGGUU	gaaggu	......GGGACCAA..	1	23S
710	GUUGAAGGUU	ggguaa	......GGGACCAA..	1	23S
1704	GGGAGAAGGC	acgcug	......GGGACCAA..	1	50S
1838	ACGCCUGCCC	ggugcc	..CUUUGGGAC.....	1	70S
1840	GCCUGCCCGG	ugccgg	..CUUUGGGAC.....	1	50S
1856	AAGGUUAAUU	gauggg	..CUUUGGGA.....	2	50S, 70S
2074	CCGUGAACCU	uuacua	....UUGGGAC.....	1	70S
2101	UGAACAUUGA	gccuug	.....UGGGACCAAAA	1	50S
2115	UGAUGUGUAG	gauagg	.CCUUUGGG.....	1	70S
2129	GGUGGGAGGC	uuugaa	....UUGGGACCAA..	1	23S
2141	UGAAGUGUGG	acgcca	......GGGACCAAA.	1	23S
2233	GACAGUGUCU	gguggg	..CUUUGGGAC.....	1	50S
2255	GACUGGGGCG	gucucc	..CUUUGGG.....	1	23S
2302	GGCUAAUCCU	ggucgg	....UUGGGACC....	1	23S
2307	AUCCUGGUCG	gacauc	...UUUGGGACCAAA.	1	23S
2321	UCAGGAGGUU	agugca	...UUUGGGACCAAA.	1	23S
2386	AAGCAGGUCA	uaguga	..CUUUGGGACCAA..	1	23S
2402	UCCGGUGGUU	cugaau	.CCUUUGGGACCAAA.	1	23S
2496	GGUGUUUGGC	accucg	....UUGGGACCAAAA	1	23S
2522	AUCACAUCCU	ggggcu	ACCUUUGGGACC....	1	23S

[a] Sequences of the 23S rRNA adjacent to sites of ligation with either the 5′ or 3′ end of the IVS. Exon sequences in lower case were inferred assuming complete integration. Potential base pairs with the IGS were evaluated based on conservation with known IGS-dependent reactions of the *Tetrahymena* IVS (Been & Cech, 1986; Been & Cech, 1987; Barfod & Cech, 1989). Complementary sequences (including U·G pairs) in the exons and IVS are underlined. In a few cases, no alignment permitting three consecutive base pairs with the IGS was identified. [b] Number of clones obtained. [c] Substrates used in reverse splicing reactions from which clones were obtained. [d] L IVS is shown in the top line for simplicity.

Thus, some 5′ junctions may be the result of specific cleavage followed by addition of the 5′ exon to circular IVS RNA.

Self-splicing of novel integrants

It was of interest to determine whether the products of reverse splicing at new sites are able to undergo the forward splicing reaction. Position 2612 in domain V was a frequent site of IVS integration, and clones covering both 5′ and 3′ junctions were retrieved. The 5′ exon is perfectly complementary with the IGS, and contains a C-G pair at the 5′ splice site instead of the universally conserved U·G pair. A U to C substitution is expected to impair recognition of the 5′ splice site during self-splicing (Barfod & Cech, 1989; Doudna et al., 1989; Strobel & Cech, 1995).

A short precursor RNA (EC-2612) containing the 23S sequences from 2603 to 2612 in the 5′ exon and 2613 to 2622 in the 3′ exon was incubated under self-splicing (5 mM $MgCl_2$) or reverse self-splicing (25 mM $MgCl_2$) conditions plus 0.1 mM GTP. Splicing of EC-2612 was barely detectable in 5 mM $MgCl_2$ (data not shown). In high magnesium, the extent of splicing was 5%, as judged from accumulation of L IVS (Fig. 7) and by addition of radiolabeled GTP (data not shown). This is much lower than splicing of the ECIVS precursor containing a U at the 5′ splice site (80%). Although the EC2612 precursor self-splices poorly, it is capable of reacting under conditions of in vitro reverse splicing. Thus, even noncanonical products of reverse splicing retain some ability to catalyze intron excision.

DISCUSSION

Base pairing of the 5′ exon with the IGS (Fig. 2A) is required for 5′ splice site recognition in self-splicing of the *Tetrahymena* IVS (Been & Cech, 1986; Waring et al., 1986). In reverse splicing, the same pairing determines the position of the splice junction (Woodson & Cech, 1989). Comparison of the most frequently obtained splice junctions in this study demonstrates that pairing with the IGS is important. The results, however, also indicate a surprising degree of flexibility in selection of 5′ junctions, many of which differ significantly from the original 5′ exon ($^{5'}$GGUCCU). Base pairing

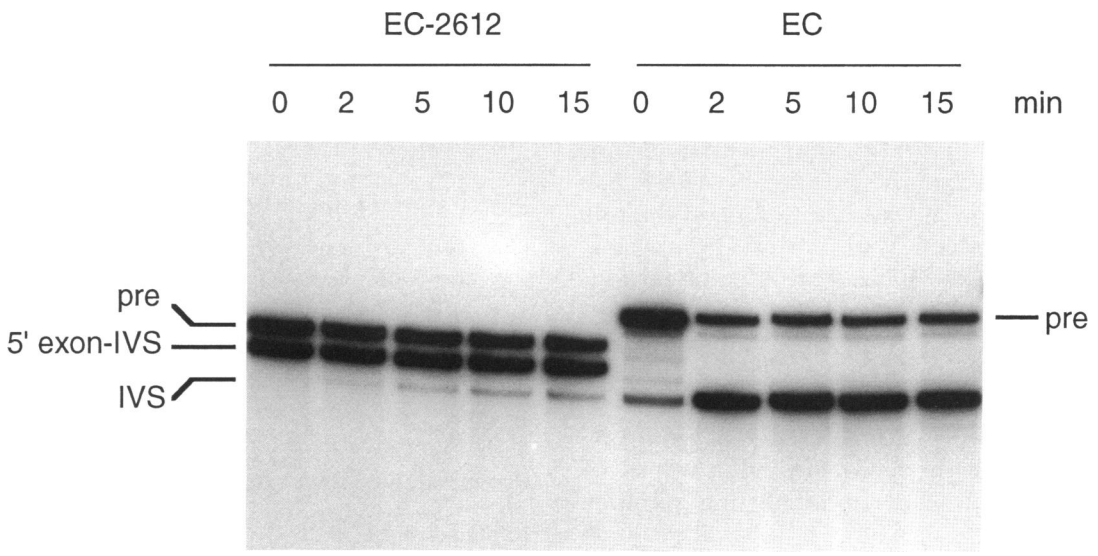

FIGURE 7. Splicing of a novel integration product. Radiolabeled precursor RNA was incubated in 25 mM $MgCl_2$ and 0.1 mM GTP at 30 °C as described in the Materials and methods for the times indicated above the lanes. Precursor EC-2612 (433 nt) corresponds to the product of IVS integration at 23S position 2612; precursor EC to insertion of the IVS at the original splice junction (1926). Pre, precursor; 5′ exon-IVS, 3′ splice site hydrolysis product; IVS, linear IVS RNA. The ligated exons have run off the bottom of the 4% polyacrylamide gel. 5′-exon-IVS at zero time is the result of hydrolysis during thermal renaturation (Woodson & Cech, 1991).

between the 3′ exon and the IGS (P10) has been proposed to assist in alignment of splice sites (Davies et al., 1982). For the *Tetrahymena* IVS, P10 appears to play only a minor role in selection of integration targets, in agreement with results with oligonucleotide substrates (Woodson & Cech, 1989).

Role of U·G pair in forward and reverse splicing

IVS insertions after a C was observed in a number of clones, including two sites in the 23S rRNA that are perfectly complementary to the IGS. The U·G pair at the 5′ splice site is an important recognition element for the first step of self-splicing, but a C-G pair does not inhibit exon ligation (Barfod & Cech, 1989; Doudna & Szostak, 1989). One explanation is that only the first step of integration has occurred, which is the reverse of exon ligation. Amplification of the 3′ splice site does not distinguish the IVS–3′ exon intermediate from fully integrated IVS. However, we also observe 5′ integration junctions at 2540 and 2612 (Table 1). More recent experiments provide evidence for complete reverse splicing at these sites (J. Roman & S. Woodson, unpubl.). Furthermore, a ligated exon RNA containing a C at the splice junction is a good substrate for reverse splicing in vitro, and the extent of the reaction is similar to that of the wild-type substrate (data not shown). A C-G pair appears to be an acceptable substitute for the normal wobble pair in the reverse reaction, in contrast to poor forward splicing of an integrant at position 2612 and to published work on the ribozyme-catalyzed endonuclease reaction (Knitt et al., 1994; Pyle et al., 1994).

Comparison to known group I insertion sites

The natural sites of group I introns in ribosomal RNA genes are concentrated in functional regions of the rRNA that are expected to remain exposed in the ribosome (Noller, 1991). For example, many of the known introns in large rRNA genes are located in or near the peptidyl transferase center in domain V, but introns have also been found in other functionally important areas such as the GTPase center in domain II (see Fig. 5B). In domain IV, nucleotides near the *Tetrahymena* splice junction (*E. coli* position 1925) have been implicated in subunit association and binding of P-site tRNA (Moazed & Noller, 1989). At present, seven known group I introns (Liu et al., 1992; Turmel et al., 1993; Lonergan & Gray, 1994) and an archaeal intron (Kjems & Garret, 1991) have been found at five sites near this position, suggesting that this is a particularly favorable location for rRNA introns. Our data show that the *Tetrahymena* IVS can integrate into position 1926 by reverse splicing, demonstrating that this sequence remains accessible to attack by a ribozyme in 50S subunits. Moreover, this position is less reactive in 70S ribosomes, as expected for nucleotides at the subunit interface.

Although the natural splice junction is not protected by ribosomal proteins, one might still expect the rRNA structure to block IVS integration. Pairing of the 5′ exon at the natural splice junction with the IGS requires dis-

ruption of a stable and conserved hairpin in the mature 23S rRNA. In very short (25 nt) ligated exons, formation of this hairpin inhibits reverse splicing (Woodson & Cech, 1991), thus interactions with the IVS alone are not sufficiently energetic to overcome the intramolecular secondary structure. Because longer transcripts (Woodson & Emerick, 1993) and the full-length 23S rRNA are substrates for reverse splicing, this hairpin must be destabilized by other interactions in the ribosome.

Reverse splicing in rRNA

The extent to which the natural locations of group I introns are predicted by the availability of the host rRNA for reverse splicing remains an interesting question. The current work reveals a large number of products arising from attack of the IVS at positions other than the natural splice junction. A few reactions sites are very near to known group I insertion sites, such as U2262, which is the homologous position of an *rrn*L intron in *Chlamydomonas reinhardtii* chloroplast, and C1697, which is a few bases from the site of an intron in *Podospora* mitochondria. Many reactions in domains IV and V occurred near tRNA binding sites (Moazed & Noller, 1989). Interestingly, reaction with 23S rRNA was also observed near conserved sequences that are not yet known to contain a group I intron, including the tRNA E-site (position 2110), a trinucleotide loop at 2050, and the α-sarcin loop (2660).

These results should be viewed with caution, because the data do not represent a complete sampling of reverse splicing products. In particular, short DNA fragments appear to have been amplified more readily than longer products. Nonetheless, the frequency at which a given integration junction is found in randomly selected clones is probably a rough reflection of the reverse splicing products in that region of the 23S rRNA. The failure to detect reaction at a particular site is uninformative. Positive clones demonstrate that attack of the IVS RNA at a given sequence is feasible.

The *Tetrahymena* IVS is clearly capable of cleaving many sites within bacterial rRNA. The apparent promiscuity of IVS-catalyzed reactions may be a consequence of high magnesium concentration and temperature during reverse splicing incubations. Nonetheless, all 5′ and 3′ junctions listed in Figure 7 represent an IGS-dependent reaction, if not complete IVS integration events. Presumably, an even greater number of sequences were exposed, but were not complementary to the EC IVS guide sequence. Small variations in the guide sequence would be expected to greatly increase the possibilities for intron integration. From these experiments we conclude that transient integration of a self-splicing intron into abundant RNAs, such as rRNA, is quite feasible, and that the scope of potential target sites is large. This increases the likelihood that reverse splicing played a role in transposition of introns during evolution.

MATERIALS AND METHODS

Preparation of linear IVS

Plasmid TZIVSΔ24EC (Zhang et al., 1995) was obtained by site-directed mutagenesis of pTZIVSΔ24 (Woodson & Cech, 1991). pTZIVSΔ24EC DNA was digested with *Hin*d III and transcribed with T7 RNA polymerase. Linear IVS RNA (L IVS) was prepared as described previously (Woodson & Cech, 1989).

Preparation of RNA substrates

Ligated exon substrate RNA was transcribed from pSW112LE DNA digested with *Sst* II and purified by gel filtration (Chromaspin TE-100 column, Clontech). SW112-LE RNA was 5′ end-labeled and gel purified as described previously (Woodson & Emerick, 1993). pSW112LE (Zhang et al., 1995) includes 163 nucleotides 5′ of the splice junction and 132 nucleotides in the 3′ exon from *E. coli* 23S rRNA (positions 1764–2046).

Ribosomes and ribosomal subunits from *E. coli* (JM83) were prepared as described by Spedding (1990). 70S ribosomes and 50S subunits were recovered from a 10–30% sucrose gradient, precipitated with 0.7 volumes ethanol, and resuspended in 10 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 60 mM NH_4Cl, 3 mM β-mercaptoethanol, and stored at −80 °C. *E. coli* 23S rRNA and 16S+23S rRNA were obtained after phenol and chloroform extraction of 50S subunits and 70S ribosomes, respectively. Total RNA was isolated as described previously (Belfort et al., 1990).

Reverse splicing reactions

Linear IVS (5 pmol) and ligated exon RNA (5 pmol SW112-LE, 5 pmol 50S and 70S ribosomes or 5 μg total RNA, 23S rRNA, or 16S+23S rRNA) were incubated in 50 mM HEPES, pH 7.5, 100 mM ammonium sulfate, 50 mM $MgCl_2$, and 1 mM EDTA in a final volume of 10 μL (Woodson & Cech, 1989). Reverse self-splicing was carried out for 2 h at 42 °C and stopped with 10 μL cold 50 mM EDTA. Reactions containing rRNAs were diluted with TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) to a final volume of 80–90 μL and passed through a Chromaspin TE-1000 gel filtration column (Clontech) to eliminate excess unreacted IVS. Mixtures containing ribosomal subunits were extracted with phenol and chloroform before gel filtration chromatography. Eluted RNA was precipitated with ethanol.

RT-PCR amplification

Pellets from reverse splicing reactions were resuspended in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 50 pmol each primer. This mixture was heated to 94 °C for 1 min and cooled quickly on ice. Deoxynucleotide triphosphates were added to a final concentration of 0.2 mM each, along with 0.75 mM

$MnSO_4$ and 1.5 mM $MgCl_2$. The 50-μL reaction was incubated with 2U Retrotherm-RT (Epicentre Technologies) at 70 °C for 15 min (reverse transcription), followed by 35 cycles of PCR amplification (94 °C for 1 min; 55 °C for 2 min; 70 °C for 3 min). Products were analyzed on 1.6% agarose gel (1:1, Sea Kem LE:Nu Sieve GTG, FMC).

For reverse transcription using random hexamers, RNA from reverse splicing reactions was incubated in a 10-μL reaction containing 50 pmol of random hexamers (Oligos Etc.), 25 mM Tris-HCl, pH 7.5, 60 mM NaCl, 10 mM DTT, 3 mM magnesium acetate, 0.5 mM deoxynucleotide triphosphates, and 2U of AMV Reverse Transcriptase (Life Sciences) at 42 °C for 1 h and terminated by incubating at 95 °C for 2 min. Before primer extension, annealing was performed at 65 °C for 10 min (in the absence of magnesium, nucleotides, and enzyme) and cooled quickly on ice.

A 2–4-μL aliquot from the random primed reverse transcription was submitted to DNA amplification using Taq DNA polymerase from Promega (Saiki et al., 1988). The 50-μL reaction contained 50 mM KCl, 10 mM Tris-HCl, pH 9, 0.1% Triton X-100, 5 μg gelatin, 0.2 mM each deoxynucleoside triphosphate, 1 mM $MgCl_2$, 1 μM each downstream and upstream primers, and 2.5 U Taq DNA polymerase. One cycle of 95 °C for 1 min; 55 °C for 1 min; 72 °C for 5 min was followed by 30 cycles of 95 °C for 1 min; 55 °C for 1 min; 72 °C for 2 min. The final incubation at 72 °C was for 5 min. Typically, 10–20 μL of each reaction was analyzed on a 1.6% agarose gel stained with ethidium bromide.

Primers

The primers used for RT-PCR were purchased from Oligos Etc. and Keystone Laboratories. The nomenclature used for the primers is as follows: UP, upstream; DP, downstream; I, intron; and SS, splice site. The numbers indicate the position in the rRNA or the IVS where the 5′ end of the primer anneals. UPI-94, GGAATTCAAGACCGTCAAATTGCGGGAA; DPI-336, GCGCAGGATCCGCTAGCTCCCATTAAGGAGAG; UP-570, CGCCTCAAGCTTGTATAATGGGTCAGC; UP-1666, CGCCTCGAATTCGAACTAGGCAAAATG; UP-1785, AACACAGCACTGTGCAAACACG; UP-1806, AGCATTG TGACGGCCTCAACA; DP-1937, GCGCAGGATCCTCGC TACCTTCGAGTACTCC; UP-2050, CGCCTCAAGCTTCA AGACGGAAAGACC; UP-2265, GGAATTCTAAAGAGTA ACGGAGGAGCAC; DP-1941, GCATTTGGCTACCTTAC GAGTA; DP-2180, CGGGATCCAGGGTGGTATTTCAAG GACGG; DP-2675, CGGGATCCACCCGAACACCAGTGA TGCGT.

Analysis of RT-PCR products

Desired amplification products were directly purified using powdered glass resin (Promega Wizard PCR Preps), or first separated by agarose gel electrophoresis. DNA fragments were subcloned into pTZ18U (Bio-Rad) after digestion with *Eco*R I and *Bam*H I, which recognize restriction sites included in the PCR primers. Clones containing the unique *Bgl* II site in the IVS were sequenced (Sequenase 2.0, USB). In some cases, RT-PCR products were sequenced without subcloning (Circumvent, New England Biolabs).

Self-splicing of EC-2612

pTZIVSΔ24EC was used as a template for PCR amplification of ECIVS sequences with primers containing exon sequences that flank position 2612 in *E. coli* 23S rRNA. Upstream and downstream primers included nucleotides 2603–2612 and 2613–2622, respectively. The amplified fragment was cloned into pTZ18U (BioRad) after digestion with *Eco*R I and *Bam*H I, whose recognition sites were also included in the PCR primers. The final clone was verified by DNA sequencing (Sequenase 2.0, US Biochemicals).

Uniformly radiolabeled precursor RNA was prepared by in vitro T7 RNA polymerase transcription of linear (*Hin*d III) pTZIVSΔ24EC (EC IVS) and pJR2612 (EC-2612) DNA as described previously (Woodson & Cech, 1991). Splicing reactions were carried out at 30 °C in 100 mM $(NH_4)_2SO_4$, 50 mM HEPES, pH 7.5, 0.1 mM GTP, and 5 mM or 25 mM $MgCl_2$. RNA was renatured at 95 °C before addition of GTP (Walstrum & Uhlenbeck, 1989). Splicing products were separated on a 4% polyacrylamide sequencing gel.

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