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Ribosomal RNA introns in archaea and evidence for RNA conformational changes associated with splicing

(extreme thermophile/23S rRNA/RNA rearrangement)

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ABSTRACT **The single 23S rRNA gene of the archaeon *Staphylothermus marinus* exhibits two introns which, at the RNA level, are located in highly conserved regions of domains IV and V. The RNA introns, which are 56 and 54 nucleotides long, respectively, can form single hairpin structures. *In vivo*, RNA splicing occurs efficiently, whereas *in vitro* pre-rRNA transcripts containing each intron were cleaved efficiently when incubated with archaeal cell extracts but were poorly ligated. The introns are cleaved by a mechanism which differs from the mechanisms of eukaryotic rRNA introns but resembles those of the rRNA intron of *Desulfurococcus mobilis* and the archaeal tRNA introns. The cleavage enzyme recognizes and cuts a putative bulge-helix-bulge structure that can form at the archaeal exon-intron junctions. Using a phylogenetic sequence comparison approach, we define the parts of this structural feature that are essential for cleavage. We also provide evidence for conformational changes occurring in the *S. marinus* 23S RNA, after cleavage, at both exon-exon junctions, which may account for the low yields of ligation observed *in vitro*.**

Introns occur widely among eukaryotic nuclei and organelles in genes for proteins, ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) (1, 2). Among archaea they have been detected in one rRNA gene (3) and in some tRNA genes (4–9). So far, they have not been detected in bacterial genomes, although they occur in protein genes of some bacteriophages (10, 11).

The only archaeal rRNA intron detected to date lies within the 23S RNA gene of *Desulfurococcus mobilis* (3). On excision, it yields a circular RNA which is large [622 base pairs (bp)] and stable and probably encodes a single protein (12). Although its splicing mechanism is special and differs in many respects from the mechanisms of eukaryotic group I introns, it shows similarities to the splicing of the intron in pre-tRNA^{Trp} of extreme halophiles (13) and other archaeal tRNA introns (4, 6–9); thus all can generate a bulge-helix-bulge structure at the exon-intron junction that may constitute a substrate for the cleavage enzyme (8, 9, 13, 14).

A possible basis for this similar mechanism of rRNA and tRNA cleavage is that the *D. mobilis* intron lies in a region of 23S RNA that appears isostructural with the D and anticodon arms of tRNA (12, 15). Therefore, to establish whether this intron and its location were an exception among archaeal pre-rRNAs, we searched for new rRNA introns among extreme thermophiles. The 23S rRNA genes from four recently discovered organisms were examined and one of them, from *Staphylothermus marinus* (16), exhibited two introns.[¶] Their mechanisms of cleavage are shown to be similar to those of the *D. mobilis* intron, and both require a rearrangement of base pairing, after cleavage, to generate the mature 23S RNA structure.

MATERIALS AND METHODS

Preparation of Cells, Cellular DNA, and RNA; Cloning and DNA Sequencing. Cells from *S. marinus*, *Pyrobaculum islandicum*, *Pyrococcus furiosus*, and *Pyrodictium occultum* were kindly provided by Karl Stetter, and cells from *D. mobilis*, *Thermoproteus tenax*, *Thermofilum pendens*, and *Sulfolobus* B12 were gifts from Wolfram Zillig. Isolation procedures for genomic DNA and total RNA were described earlier (17). Cloning was performed by replacing the internal *Bam*HI fragment of λ-EMBL3 (18) with genomic DNA cut with *Bam*HI. The rRNA genes were selected by using a mixture of randomly labeled RNA fragments from the 3′ half of 23S RNA from other extreme thermophiles (17, 19, 20). An ≈8-kbp *Bam*HI fragment was isolated from *S. marinus* and a 2.2-kbp *Bam*HI–*Pvu* II fragment, containing 1.8 kbp of the 23S RNA gene, was subcloned in M13mp19. Exonuclease *Bal*-31 (Amersham) was used to generate clones exhibiting increasingly large deletions from either end (21), such that continuous reading could be obtained on both DNA strands by using the dideoxynucleotide sequencing procedure (22). Southern blotting was performed under high stringency conditions (23).

T7 RNA Polymerase Transcription *in Vitro*. Templates for transcription were prepared by cloning, first, a 136-bp *Hae* III fragment containing intron 1 and, second, a 106-bp *Alu* I–*Hae* III fragment containing intron 2 in the unique *Stu* I site of an M13mp19 construct behind the T7 RNA polymerase promoter. The recombinant phage was linearized with *Pst* I and blunt ends were then produced by using the Klenow fragment of DNA polymerase (Amersham). Transcription using T7 RNA polymerase (24) produced RNA with the cloned sequence plus an extra GG at the 5′ end and CCN, CCNN, or CCNNN at the 3′ end.

Conditions for *in Vitro* Splicing. Crude cell extracts from all organisms were prepared as described earlier (12). A typical *in vitro* splicing mixture contained 1–5 μg of RNA which was randomly labeled with [α-^{32}P]GTP in 20 μl of standard splicing buffer (50 mM Tris·HCl, pH 7.5/5 mM EDTA/100 mM NaCl/2 mM spermidine) and 2 μl of crude cell extract from *S. marinus* cells containing about 25 μg of total protein. The mixture was incubated at 37°C for 30 min. For splicing of large amounts of nonradioactive RNA, the reaction volume was scaled up and the RNA concentration was maintained.

5′- and 3′-End-Labeling of RNA Fragments and RNA Sequencing. 5′-Phosphates were labeled by first removing 5′-

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[¶]The sequence reported in this paper has been deposited in the GenBank data base (accession no. M38363).

phosphates with alkaline phosphatase (12) and then labeling with 50 μCi of [γ-^{32}P]ATP (6000 Ci/mmol, NEN; 1 Ci = 37 GBq) and polynucleotide kinase (Amersham) (23). The 3' ends were labeled with [^{32}P]pCp by using T4 RNA ligase (Amersham) (25). End-labeled RNA was sequenced enzymatically (26). Primer-directed sequencing of RNA using reverse transcriptase and dideoxynucleotides was performed as described earlier (12).

RESULTS

Splicing *in Vivo*. An 8.0-kbp *Bam*HI fragment from *S. marinus* chromosomal DNA which hybridized to a 23S RNA-specific probe was cloned in λ-EMBL3. A 2.1-kbp *Bam*HI–*Pvu* II subclone was isolated which contained ≈1.8 kbp coding for domains II to VI of the 23S RNA and 0.3 kbp of the downstream region. It was sequenced on both strands and a comparison of the sequence of the noncoding strand with the sequences of other 23S RNAs revealed the presence of two intronlike inserts of 56 bp and 54 bp within domains IV and V, respectively (Fig. 1). The remainder of the gene, coding for domain I and part of domain II, was not sequenced. Corresponding *Bam*HI fragments were cloned from the chromosomal DNAs of *Pyrobaculum islandicum*, *Pyrococcus furiosus*, and *Pyrodictium occultum* and subclones starting within domain II and extending beyond domain VI were sequenced. No intronlike inserts were detected.

The number of 23S RNA genes and intronlike inserts in the genome of *S. marinus* were investigated by the Southern blotting procedure. Total cellular DNA was digested with *Bam*HI, *Eco*RI, *Hin*dIII, or *Pst* I and hybridized to a randomly labeled restriction fragment from the 23S RNA gene. Total DNA digested with each restriction enzyme yielded a single band (data not shown), which implies that only one copy of the gene, containing two inserts, occurs in the genome.

To establish whether the inserts were introns, the 23S RNA gene was sequenced across the putative exon-exon junctions. Two primers, P_1 (5'-GGGGACCTCGTTGATCC-3') and P_2 (5'-CCCGTTCCTCTCGTACT-3'), which are complementary to sequences downstream from putative introns 1 and 2, respectively, were annealed with total cellular RNA and upstream sequences were determined by the dideoxynucleotide procedure (22). Only sequences corresponding to ligated exons were detected, and no termination occurred at the ligation site (Fig. 2). We conclude that the inserts were, indeed, introns.

The splicing sites lie within regions of highly conserved sequence and secondary structure in domains IV and V, close to the sites of other known rRNA introns (Fig. 1). They occur after nucleotides A-2098 and C-2769 and both precede the

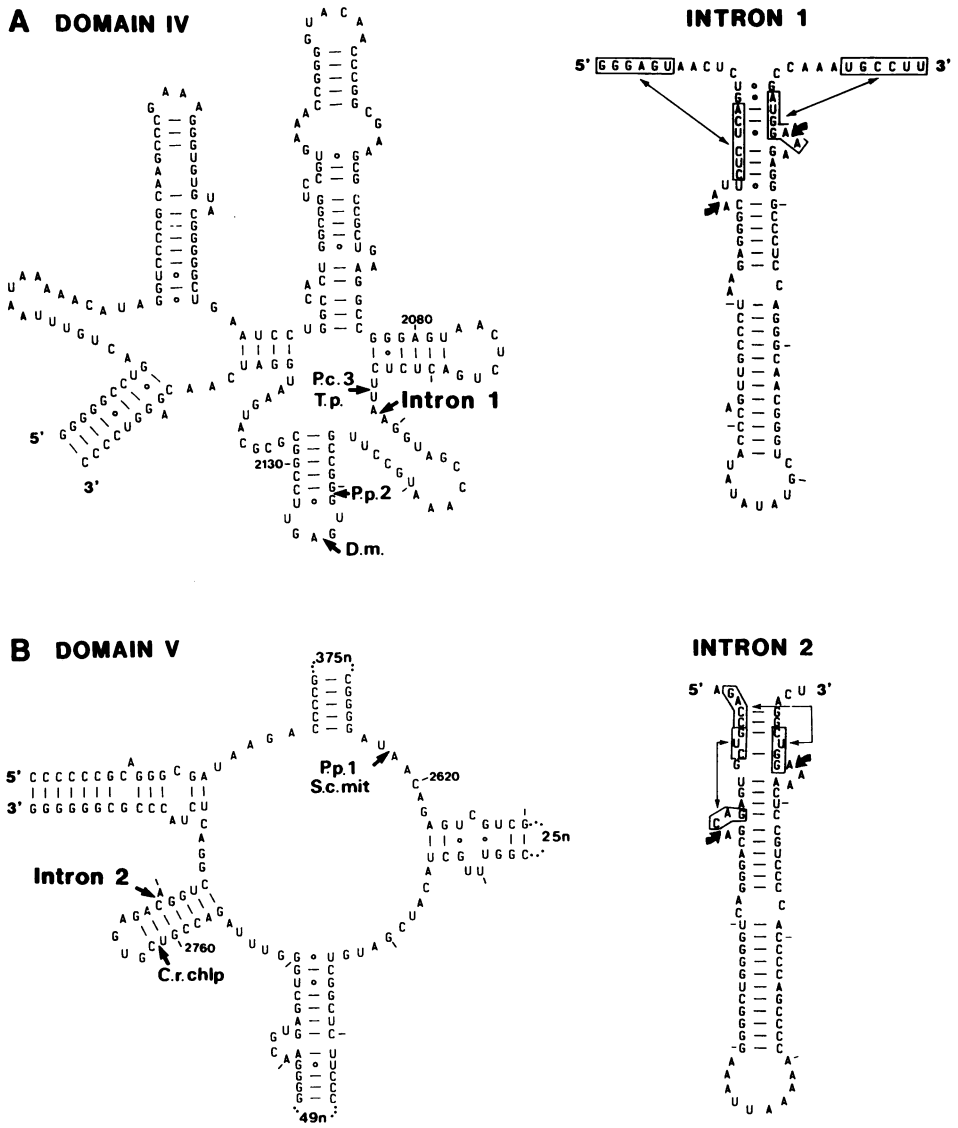

FIG. 1. Secondary structures within domain IV (*A*) and domain V (*B*) of the mature 23S RNA from *S. marinus*, showing the intron locations. The structures were derived from comparative sequence analyses and the nucleotide numbers of *D. mobilis* 23S RNA are used (19). Only base pairs which are phylogenetically supported in the mature rRNAs are indicated by lines or dots. Putative secondary structures are presented for each intron and exon-intron junction beside the respective domain; the cleavage sites are indicated by thick arrows. Those regions which we infer are involved in a base-pair rearrangement after cleavage are boxed and the sequences which are base paired in the mature 23S RNA are joined by arrows. A - denotes every 10th nucleotide in the vicinities of the splicing sites and in the introns. The locations of other known rRNA introns are also indicated with the following abbreviations: C.r.chlp, *Chlamydomonas reinhardii* chloroplast (27); D.m., *D. mobilis;* P.c.3, *Physarum carolina* (28); P.p.1 and -2, *Physarum polycephalum* introns 1 and 2 (29, 30); S.c.mit, *Saccharomyces cerevisiae* mitochondria (31); T.p., *Tetrahymena pigmentosa* (32).

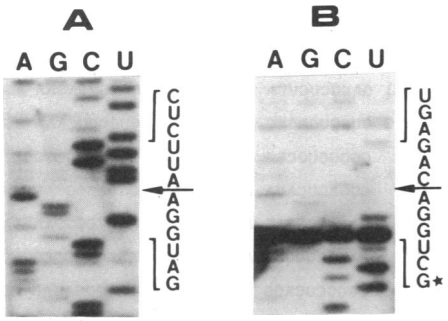

FIG. 2. Sequences complementary to the exon-exon boundary regions after excision of intron 1 (*A*) and intron 2 (*B*) and ligation *in vivo*. Sequences were obtained by annealing 0.5 pmol of 5'-end-labeled primer P_1 or P_2, respectively, to 20 μg of total cellular RNA. The primers were extended by using reverse transcriptase. Ligation sites are marked with arrows and the RNA sequences across these sites are shown. In *B* a putative guanosine modification, denoted by an asterisk, caused strong termination in all four lanes.

sequence AGGU. The introns differ in sequence but both can form 17- to 18-bp helices exhibiting small internal loops and A+U-rich terminal loops (Fig. 1).

The fate of the excised introns *in vivo* was investigated by annealing primers P_3 (5'-CCCAGCATATATATGGG-3') and P_4 (5'-GGCAGGGGTGGGGTCGGGG-3'), which are complementary to sequences within RNA introns 1 and 2, respectively, to total cellular RNA. Extension of these primers at the 5' end by reverse transcriptase revealed no longer products, which suggests that the introns are rapidly degraded after excision *in vivo* (data not shown).

Splicing *in Vitro*. Yields of the intron-containing pre-23S RNA isolated from cells were too low for *in vitro* splicing studies and, therefore, RNA transcripts 1 and 2, containing their respective introns and flanking exon sequences (Fig. 3), were synthesized by using the T7 RNA polymerase. RNA 1 was transcribed from a *Hae* III fragment and RNA 2 was derived from an *Alu* I–*Hae* III fragment. Neither transcript showed self-splicing activity when incubated over a wide range of solution and temperature conditions, including those tested for the rRNA intron of *D. mobilis* (12). However, incubation of both transcripts with a crude extract from *S. marinus* cells generated various cleavage products compatible with intron excision (Fig. 4, gels A). Cleavage occurred between 30°C and 80°C and was efficient at 75°C and 37°C for both RNAs, with a slightly higher rate of cleavage at the higher temperature. Cleavage was efficient between 50 and 150 mM NaCl, was more specific in the absence of Mg^{2+}, and was enhanced by spermidine (2 mM); NTPs had no effect.

The various cleavage products were extracted from the denaturing polyacrylamide gels (Fig. 4, gels A), labeled at their 5' or 3' termini, and sequenced by using ribonucleases (26). Owing to the highly stable structures, we were unable to get complete terminal sequences for all the fragments. Nev-

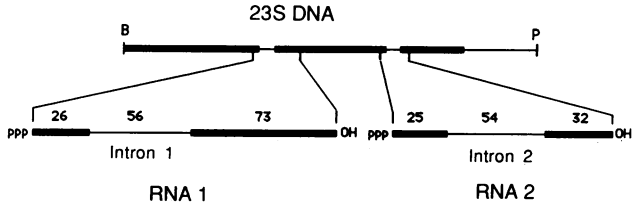

FIG. 3. Construction of the transcripts RNA 1 and RNA 2 *in vitro*. The upper line shows the organization of the 2.2-kbp *Bam*HI(B)–*Pvu* II(P) DNA fragment which was sequenced. Below, the compositions of the derived transcripts, RNA 1 and 2, are illustrated. Thick lines denote sequences corresponding to mature 23S RNA. The number of nucleotides in each transcript is indicated.

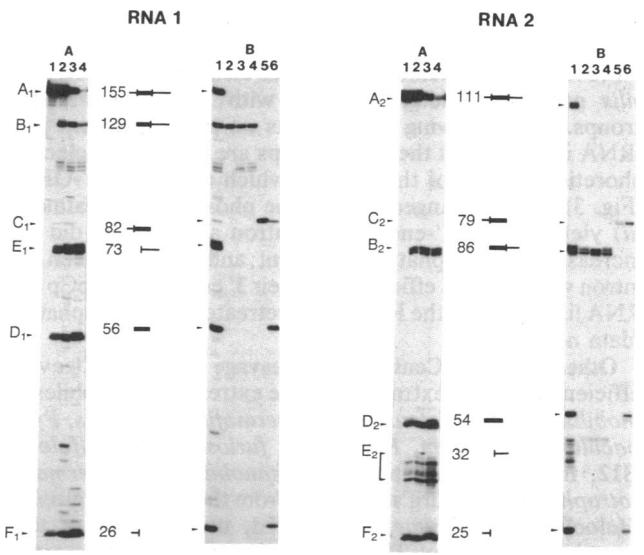

FIG. 4. Autoradiograms showing the *in vitro* cleavage products of RNA 1 and RNA 2 fractionated on denaturing 10% polyacrylamide gels. Reactions were performed by mixing randomly labeled transcripts with crude cell extracts of *S. marinus* and incubating at 37°C in standard cleavage buffer (50 mM Tris·HCl, pH 7.5/5 mM EDTA/100 mM NaCl/2 mM spermidine) unless otherwise stated. Gels A: Lanes 1, control RNA without cell extract. Lanes 2–4 contained cell extract. Lanes 2, 10 mM Mg^{2+} added and EDTA omitted; lanes 3, standard buffer; lanes 4, 5 mM spermidine. Gels B: Purified cleavage intermediates B_1 and B_2 (intron-3'-exon) or C_1 and C_2 (5'-exon-intron), from RNAs 1 and 2, were incubated at 37°C in standard cleavage buffer. Lanes 1, cleaved RNA marker; lanes 2, intermediate B without cell extract. Lanes 3 and 4 contained cell extract. Lanes 3, intermediate B; lanes 4, intermediate B plus 5 μg of unlabeled RNA. Lanes 5, control, intermediate C with no cell extract; and lanes 6, intermediate C with cell extract. The nucleotide lengths of the cleavage products are given and their compositions are indicated schematically; thick and thin lines correspond to introns and exons, respectively.

ertheless, by sequencing both ends of each fragment the cleavage sites could be assigned unambiguously (data not shown). Most of the possible cleavage products were detected, including the free intron but not the ligated exons; their identities are illustrated in Fig. 4. For RNA 1, the electrophoretic mobilities correlate with size, whereas for RNA 2, intron-containing bands migrated anomalously, probably due to incomplete denaturation of the putative 9 adjacent G·C base pairs of the intron (Fig. 1*B*). The 3' exon of RNA 2 (E_2) yielded multiple bands, which may reflect residual conformational heterogeneity.

Cleavage Mechanism. Fractionation of stable 5'-exon-intron and intron-3'-exon intermediates (Fig. 4, gels A) enabled us to test for formation of the bulge-helix-bulge feature at the exon-intron junction, since the 5'-exon-intron product can generate a stable 5'-cleavage bulge while the intron-3'-exon cannot form the 3'-cleavage bulge. These intermediates were isolated from both RNA 1 and 2, purified, and incubated with crude cell extract. The results in gels B of Fig. 4 demonstrate that the 5'-exon-introns (intermediates C_1 and C_2) are, indeed, cleaved, while the intron-3'-exons (intermediates B_1 and B_2) are not, even after adding excess RNA transcript. This supports the idea that the bulge-helix-bulge structure is a substrate for the cleavage enzyme.

Finally, we investigated whether each pair of exons was ligated and whether the introns were circularized. Purified 5' and 3' exons from RNA 1 and RNA 2, and their introns (Fig. 4, gels A), were extracted and incubated with crude cell extract under various conditions (± Mg^2, ± spermidine, ±

NTP) and examined electrophoretically. Neither exon ligation nor intron circularization was detected (data not shown).

Previously, we demonstrated that cleavage of the *D. mobilis* pre-rRNA yields products with 5'-OH and 3'-PO_4 groups. The following experiments on the *S. marinus* pre-rRNA indicate that the same groups are formed: (*i*) electrophoretic mobility of the 3' exon which exhibits a 3'-OH_4– (Fig. 3) was unchanged by alkaline phosphatase treatment; (*ii*) yields of the 5'-end-labeled intron and 3'-exon did not increase after phosphatase treatment; and (*iii*) the 5' exon and intron were labeled efficiently at their 3' ends by [^{32}P]pCp and RNA ligase only if the RNA was pretreated with phosphatase (data not shown).

Other Archaea Contain the Cleavage Enzyme. Cleavage efficiencies of cell extracts from the extreme thermophiles *D. mobilis, Thermoproteus tenax, Thermofilum pendens, Pyrobaculum islandicum, Pyrococcus furiosus*, and *Sulfolobus* B12, from the methanogen *Methanobacterium thermoautotrophicum* Marburg strain, and from the extreme halophile *Halococcus morrhuae* were tested, using 25 μg of total protein in each assay. The extreme thermophile extracts were tested in standard splicing buffer containing 10 mM $MgCl_2$ instead of EDTA for 20 min at 56°C, while the methanogen extract was tested in the same buffer at 37°C and the extreme halophile was tested with and without 5 M NaCl at 37°C. All except the *H. morrhuae* extract produced cleavage (data not shown). None of the digests was complete and, in general, the yield of the 5'-exon-intron was lower than that of the intron-3'-exon, which is compatible with the former intermediate of *S. marinus* being more susceptible to cleavage (see above).

DISCUSSION

The discovery of two additional small rRNA introns among the archaea shows that the *D. mobilis* rRNA intron (3) was not an exception. Moreover, the exon junctions of the *S. marinus* rRNA do not apparently generate tRNA-like structures and, therefore, the presence of such a structure at the splicing site of the *D. mobilis* 23S RNA (12) is neither a prerequisite for archaeal rRNA introns nor an exclusive basis for any mechanistic similarities between archaeal rRNA- and tRNA-intron splicing.

Defining the Cleavage Substrate. Extracts from extreme thermophile cells that lack rRNA introns can induce splicing of *D. mobilis* rRNA transcripts and we inferred, therefore, that the cleavage and ligation enzymes have other cellular functions (12). This inference is reinforced by the finding here that cell extracts from several extreme thermophiles and a methanogen have similar activities. Daniels and coworkers (13) noted that a bulge-helix-bulge structure that had been proposed earlier as a processing site on the long stems of archaeal 16S and 23S RNAs (33) could also form at the exon-intron junctions of all archaeal pre-tRNAs (4–8, 14) and the pre-23S RNA of *D. mobilis*. The exon-intron junctions of the *S. marinus* introns can generate the same feature, and experimental support is provided for their formation. To test the generality of this concept, we undertook a phylogenetic analysis of the 16 available archaeal pre-tRNA and pre-23S RNA sequences that contain introns.

Sequence alignments across the exon-intron boundaries are presented in Fig. 5*A*. There are no strictly conserved nucleotides, although there is a low level of conservation (≈70%) for the motif 5'-R*A . . . intron . . . GAR*A-3' with cleavage sites (*) located in the bulges (Fig. 5*B*). Examination of each putative base pair revealed that the two pairs flanking each bulge, eight in total, are well supported by multiple compensating base changes with very few mismatches (Fig. 5*B*). In contrast, base pairs 1 and 2 and 11 and 12 in helices I and III, respectively, were subject to multiple mismatches, which implies either that they do not form or, if they do form

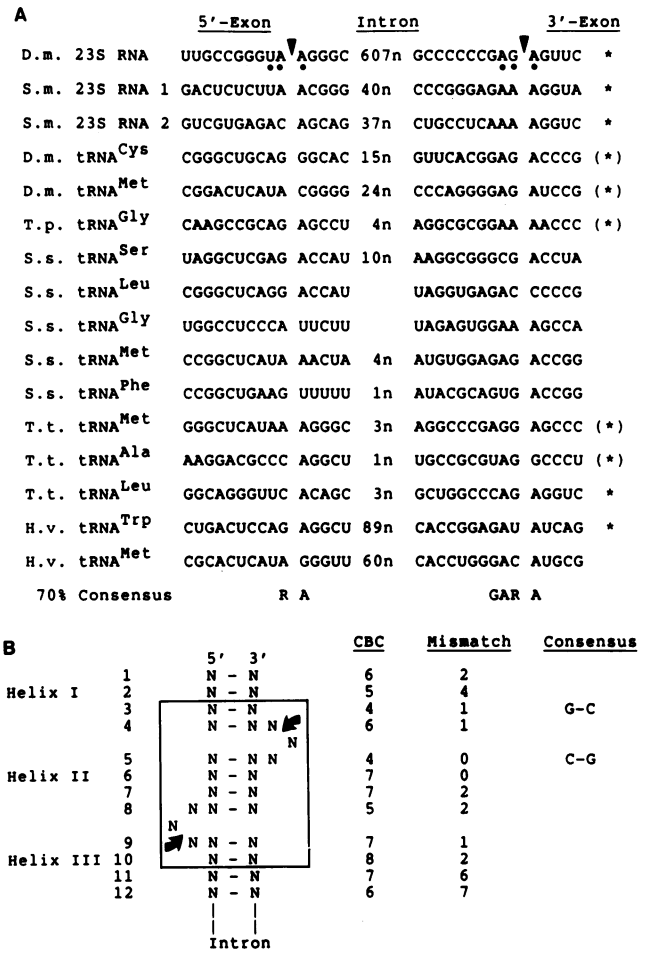

FIG. 5. (*A*) Alignment of sequences at the exon-intron junctions of the archaeal rRNA and tRNA introns. D.m., *D. mobilis*; S.m., *S. marinus*; T.p., *Thermofilum pendens*; S.s., *Sulfolobus solfataricus*; T.t., *Thermoproteus tenax*; H.v., *Halobacterium volcanii*; n, nucleotides. Two arrowheads on the upper line denote positions of the cleavage sites and the nucleotides with dots underneath are bulged in the secondary structure shown below. For organisms marked with an * the cleavage sites have been determined accurately, while those marked with (*) have been determined within ±1 nucleotide. The lack of an asterisk indicates that the sites are derived from sequence alignments. Nucleotides common to more than 70% of the sequences are presented below as a consensus sequence. (*B*) Phylogenetic sequence comparison evidence for the formation of the consensus structure at the exon-intron boundary. Ns denote any of the sequences given in *A* and the arrows indicate the cleavage sites. Base pairs are numbered 1–12 and the numbers of compensating base changes (CBC) and mismatches are indicated for each base pair. The conserved base pair region which receives strong phylogenetic support is boxed. Literature references for the sequences which are not given in the text are D.m. tRNAs, ref. 8 and unpublished results; T.p. tRNA, ref. 9; S.s. tRNAs, ref. 4; T.t. tRNAs, ref. 6; and H.v. tRNAs, refs. 5 and 34.

in some pre-RNAs, that their presence is not critical for substrate recognition and cleavage. Thus, the boxed area in Fig. 5*B* represents the essential structural core of the substrate.

A more extensive analysis of the structures of the exon-intron regions revealed two main types of helices I and III. Most of the pre-tRNAs exhibit a stable helix I and, generally, a short helix III, while the pre-rRNAs from *D. mobilis* and *S. marinus*, and the pre-tRNA^{Gly} from *Thermofilum pendens* exhibit a short or irregular helix I and a long stable helix III. This suggests that either a stable helix I or a stable helix III is required, together with helix II, to generate a functional

substrate. These two substrate classes also correlate with the type of cleavage intermediate that accumulates *in vitro*.

Pre-RNAs exhibiting a strong helix I/weak helix III accumulate the 5′ exon-intron and are exemplified by pre-tRNA^{Trp} from *Halobacterium volcanii* (13). When the 3′ bulge is cut first, helices II and III will dissociate, thereby disrupting the 5′ bulge and preventing its cleavage. In contrast, prior cutting of the 5′ bulge will not appreciably destabilize helices I and II or the 3′ bulge. The second class of pre-RNAs with a weak helix I/strong helix III accumulates the intron-3′-exon, and is typified by rRNA intron 2 of *S. marinus*. Here, prior cutting of the 5′ bulge will lead to dissociation of helices I and II, loss of the 5′ exon, and disruption of the 3′ bulge, while prior cutting of the 3′ bulge will not appreciably destabilize helix II, helix III, or the 5′ bulge.

In summary, (*i*) the cleavage substrate constitutes a conserved core structure consisting of eight base pairs and two bulges at the exon-intron junction (boxed in Fig. 5*B*); (*ii*) a long stable helix is required on one side of the substrate; and (*iii*) the bulges are not cut in a given order *in vitro*.

23S RNA Rearranges After Intron Excision. Initially, we were sceptical about formation of the bulge-helix-bulge structure in pre-23S RNA of *D. mobilis* because it would require a substantial RNA rearrangement, after cleavage, to generate the mature 23S RNA (8, 19). However, subsequently, we found an intron-containing pre-tRNA (9), in addition to two described earlier for *Thermoproteus tenax* (6), and now the pre-rRNA from *S. marinus*, all of which form a bulge-helix-bulge feature at the expense of the mature RNA structure (35, 36). We also provided experimental support for formation of the bulge-helix-bulge structure in the pre-rRNA of *D. mobilis* (8). Thus, for six of the known archaeal exon-intron junctions both the bulge-helix-bulge feature (Fig. 5*B*) and the locally rearranged structure of the mature RNA, after splicing, are strongly supported phylogenetically (19, 37). Formation of these alternative conformers is also supported by free energy calculations (ref. 13; J.K., unpublished results).

The putative conformational change may account for the low yields of ligated exons produced *in vitro* where, under suboptimal conditions, there may be time for the RNA to rearrange prior to ligation, which could render the ligation reaction more difficult.

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