Identification of BHB splicing motifs in intron-containing tRNAs from 18 archaea: evolutionary implications

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ABSTRACT

Most introns of archaeal tRNA genes (tDNAs) are located in the anticodon loop, between nucleotides 37 and 38, the unique location of their eukaryotic counterparts. However, in several Archaea, mostly in Crenarchaeota, introns have been found at many other positions of the tDNAs. In the present work, we revisit and extend all previous findings concerning the identification, exact location, size, and possible fit to the proposed bulge-helix-bulge structural motif (BHB, now renamed *hBHBh'*) of the sequences spanning intron–exon junctions in intron-containing tRNAs of 18 archaea. A total of 103 introns were found located at the usual position 37/38 and 33 introns at 14 other different positions, that is, in the anticodon stem and loop, in the D- and T-loops, in the V-arm, or in the amino acid arm. For introns located at 37/38 and elsewhere in the pre-tRNA, canonical *hBHBh'* motifs were not always found. Instead, a relaxed *hBH* or *HBh'* motif including the constant central 4-bp helix *H* flanked by one helix (*h* or *h'*) on either side generating only one bulge could be disclosed. Also, for introns located elsewhere than at position 37/38, the *hBHBh'* (or *HBh'*) structure competes with the three-dimensional structure of the mature tRNA, attesting to important structural rearrangements during the complex multistep maturation-splicing processes. A homotetramer-type of splicing endonuclease (like in all Crenarchaeota) instead of a homodimeric-type of enzyme (as in most Euryarchaeota) appears to best fit the requirement for splicing introns at relaxed *hBH* or *HBh'* motifs, and may represent the most primitive form of this enzyme.

Keywords: Archaea; Eukarya; evolution; intron; RNA processing; small RNA; splicing endonuclease

INTRODUCTION

Previously, we extracted, analyzed, and compared the nucleotide sequences of over 4000 tRNA genes (tDNAs) from 50 fully sequenced genomes, of which 13 originated from Archaea (Marck and Grosjean 2002). From the various features analyzed, Archaea appear as an intermediate domain between Eukarya and Bacteria, sharing many properties with either or both domains depending upon the

tDNA property considered. However, a unique feature of archaeal tDNAs is their ability to occasionally host introns at various locations other than the usual position within the anticodon loop (between nt 37 and 38) of the tRNA precursors. Although these unusually located introns in archaeal pre-tRNAs had already been observed in 1987, either within or 5' to the anticodon (Wich et al. 1987), identification of an enzyme-dependent spliceable intron outside the anticodon loop of pre-tRNA was made later by the group of Garrett. They identified an unusually located 18-nt-long intron in the variable loop of the precursor tRNA-Gly (CCC) of the extreme thermophile *Thermophilum pendens* (Kjems et al. 1989b). In this pre-tRNA, the exon–intron boundaries were able to fold into a structure similar but not identical to the characteristic bulge-helix-bulge (BHB) usually found in the intron–exon boundaries located at position 37/38 of pre-tRNAs (Kaine et al. 1983; Daniels et al. 1985; Daniels and Douglas 1986; Kaine 1987; Datta et al. 1989; Thompson et al. 1989; Thompson and Daniels 1990; Kleman-Leyer et al. 1997) and in the long Watson–Crick base-paired stems of a few rRNA precursors of hyperthermophilic Archaea (Kjems and Garrett 1988; Dalggaard and

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Abbreviations: 2D, two-dimensional; 3D, three-dimensional; A:C, A:G, C:T, and so on, mismatched base pairs; tDNA, tRNA gene; BHB, bulge-helix-bulge; *hBHBh'*, a canonical BHB splicing motif with the two outer helices having at least two Watson–Crick base pairs; *h*, the helix closing the 3-nt bulge on the exonic side; *h'*, the helix closing the 3-nt bulge on the intronic side; *hBH*, a relaxed BHB splicing motif in which the *h'* intronic helix has less than 2 bp; *HBh'*, a relaxed BHB splicing motif in which the *h* exonic helix has less than 2 bp. The universal, conventional numbering system for tRNA positions corresponds to that adopted in the tRNA sequence bank (Sprinzl et al. 1998).

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Garrett 1992; Burggraf et al. 1993; Durovic and Dennis 1994; Lykke-Andersen and Garrett 1994; Dennis et al. 1998; Itoh et al. 1998; Nomura et al. 1998; Ciammaruconi and Londei 2001). More recently, introns were discovered in the anticodon loop between nt 32 and 33 in the pre-tRNA-Pro (GGG) of *Methanobacterium thermoautotrophicum* (Smith et al. 1997), in the D-loop and anticodon stem of several tDNAs of *Sulfolobus solfataricus* (She et al. 2001), and in many other locations in tDNAs of *Pyrobaculum aerophilum* (Fitz-Gibbon et al. 2002).

The canonical BHB structural motif usually found at the exon–intron boundaries within the anticodon loop of archaeal pre-tRNAs consists of a 4-bp helix flanked on each 3' side with 3 nt in a bulge and was demonstrated to be the conformational structure most easily recognized by a unique archaeal splicing machinery (Thompson and Daniels 1988, 1990; Kleman-Leyer et al. 1997) that is supposed to work on both tRNA and rRNA precursors (Kjems et al. 1989a; Kjems and Garrett 1991; Lykke-Andersen et al. 1997; Tang et al. 2002; Salgia et al. 2003). A similar predicted BHB motif at intron–exon boundaries of messenger RNA precursors originating from three crenarchaeal species of thermophilic or hyperthermophilic archaea has been recently described, suggesting that the splicing of these pre-mRNAs probably depends on the same machinery as the one for archaeal pre-tRNAs and pre-rRNAs (Watanabe et al. 2002).

In the present work, we revisit and extend our earlier observations concerning the identification, exact location, size, and fit to the canonical BHB motif of all introns present in tDNAs of the 18 archaeal complete genomes available to date (September 2003). Beside the fact that we identified a novel intron position in the amino acid stem of several tDNAs of *P. aerophilum*, we noticed, together with others, that the nucleotide sequences of a few introns located between nt 37 and 38 and at other positions of the pre-tRNA sometimes exhibit a more relaxed BHB motif than the strict BHB rule as defined earlier for introns located at the canonical position 37/38 in pre-tRNAs (see references above). Examples of BHB two-dimensional (2D) structures presented in this work are taken from pre-tRNAs of two especially interesting archaea: *P. aerophilum*, a nitrate-reducing hyperthermophilic crenarchaeon ($T_{opt} = 100^{\circ}\text{C}$) the tDNAs of which contain the largest number of unusually located introns (19 altogether) and *Sulfolobus tokodaii*, an aerobic thermophilic crenarchaeon ($T_{opt} = 80^{\circ}\text{C}$) that contains a large number of introns exhibiting relaxed BHB motifs (15 altogether). The structural characteristics of these unusual archaeal introns in pre-tRNAs are discussed in relation to those described in pre-rRNAs (Lykke-Andersen et al. 1997) and pre-mRNAs of Archaea (Watanabe et al. 2002). Also, a correlation between the oligomeric structure of the splicing endonuclease and the type of BHB motif suggests a possible evolutionary scenario for the emergence and the evolution of the splicing machinery within the three domains of

life. The 800 aligned tDNAs sequences (with introns detailed) extracted from the 18 archaeal genomes are available upon request as Supplementary Material (send an e-mail message containing the keyword “tDNAs_18_Genomes” to christian.marck@cea.fr).

RESULTS

Few archaea host tDNA introns at location others than “37/38”

Without exception, the archaea of which the complete genome sequence was analyzed contain introns in their tRNA genes (tDNA; Table 1; see also Marck and Grosjean 2002). However, most of them contain only 2–5 intron-bearing tDNAs out of a population of 34–47 tDNAs per genome. In all cases but one, intron-containing tDNAs are present as single-copy genes [two copies of the intron-containing tDNA-Glu (TTC) are present in *Methanopyrus kandleri*]. These introns are most frequently found at canonical position 37/38 in the anticodon loop (see below), the unique location in their eukaryotic counterparts. Of the 18 archaea considered, only six contain tDNAs harboring introns at position others than 37/38. These are the four crenarchaeota: *P. aerophilum*, *Aeropyrum pernix*, *S. solfataricus*, and *S. tokodaii*, and the two (out of 14) euryarchaeota: *M. kandleri* and *M. thermoautotrophicum*.

Altogether, we found 136 introns among the 800 tDNAs analyzed, of which 103 (75%) are located at position 37/38 and only 33 (25%) are distributed among 14 different positions within the pre-tRNA molecules: in the anticodon branch, in the variable arm, and in the D- and T-loops as well as in the amino acid arm (Fig. 1). In six cases, two introns are found within the same precursor tRNA (Table 1; indicated by 1/2 and 2/2 in Fig. 1). Among all archaea examined, the crenarchaeon *P. aerophilum* contains the largest number of tDNA introns (26 altogether), the largest proportion of introns located at noncanonical positions (19 cases) and the largest number of two introns within the same tDNA (three cases; Table 1). The three closely related species *Pyrococcus abyssi*, *Pyrococcus horikoshii*, and *Pyrococcus furiosus* contain tDNAs harboring introns only at the usual position 37/38 of the anticodon loop. Thus, not all thermophilic or hyperthermophilic archaea contain introns at nonconventional locations.

The majority of introns at “37/38” obey the canonical BHB splicing motif

Figure 2A lists a selection of 30 sequences of exon–intron junctions out of a total of 103 identified at position 37/38 in the 800 archaeal tDNAs analyzed (a complete list is available as Supplementary Material). The most often encountered 37/38 BHB splicing motif (67 out of 103; see Table 1) is the one that was initially defined by the group of Daniels and Garrett (Kjems et al. 1989a; Thompson and Daniels 1990).

TABLE 1. List of archaeal genomes analyzed

	Introns located at 37/38				Introns located at other positions						Double introns	Endo type	References
	tDNAs	tRNAs	Nr	Min size	BHB type		Nr	Min size	BHB type				
					hBHBh'	hBH			hBHBh'	HBh'			
Crenarchaeota													
<i>Pyrobaculum aerophilum</i> IM2	46	46	7	13	2	5	19	13	8	11	3	4x	Fitz-Gibbon et al. 2002
<i>Aeropyrum permix</i> K1	46	46	9	18	8	1	5	19	3	2	0	4x	Kawarabayasi et al. 1999
<i>Sulfolobus solfataricus</i> P2	46	46	17	12	4	13	3	15	0	3	1	4x	She et al. 2001
<i>Sulfolobus tokodaii</i> 7	46	46	20	11	8	12	4	16	1	3	0	4x	Kawarabayasi et al. 2001
Euryarchaeota													
<i>Methanopyrus kandleri</i> AV19	34	33	8	15	8	0	1 ^a	33	0	1	1	4x	Slesarev et al. 2002
<i>Methanobacterium thermoautotrophicum</i> delta H	39	37	3	16	3	0	1	32	1	0	1	4x	Smith et al. 1997
<i>Methanococcus jannaschii</i> DSM2661	36	34	2	33	2	0	—	—	—	—	—	4x	Bult et al. 1996
<i>Pyrococcus abyssi</i> GE5	46	46	2	31	2	0	—	—	—	—	—	4x	Cohen et al. 2003 ^b
<i>Pyrococcus horikoshii</i> (shinkai) OT3	46	46	2	31	2	0	—	—	—	—	—	4x	Kawarabayasi et al. 1998
<i>Pyrococcus furiosus</i>	46	46	2	32	2	0	—	—	—	—	—	4x	Robb et al. 2001
<i>Archaeoglobus fulgidus</i> DSM4304	46	46	5	15	4	1	—	—	—	—	—	2x	Klenk et al. 1997
<i>Halobacterium</i> sp. NRC-1	47	46	3	33	3	0	—	—	—	—	—	2x	Ng et al. 2000
<i>Thermoplasma acidophilum</i> DSM1728	46	46	4	12	2	2	—	—	—	—	—	2x	Ruepp et al. 2000
<i>Thermoplasma volcanium</i> GSS1	46	46	4	14	3	1	—	—	—	—	—	2x	Kawashima et al. 2000
<i>Ferroplasma acidamanus</i>	46	46 ^c	3	16	3	0	—	—	—	—	—	2x	^d
<i>Methanosarcina barkeri</i>	46	46	4	20	3	1	—	—	—	—	—	2x	^d
<i>Methanosarcina mazei</i> Go1 (DSMW 3647)	46	46	4	20	4	0	—	—	—	—	—	2x	Deppenmeier et al. 2002
<i>Methanosarcina acetivorans</i> C2A	46	46	4	20	4	0	—	—	—	—	—	2x	Galagan et al. 2002
total: 18 genomes	800	794	103	67	67	36	33	—	13	20	6		

The first column lists the archaeon names and strains for which the genomes have been fully sequenced (the corresponding references are listed in the last column). For each of these genomes, under headings "tDNAs" and "tRNAs" are listed the total number of tRNA genes (tDNA redundancy) and the corresponding number of isoacceptor tRNAs with different anticodons able to read the 61 sense codons (anticodon choice pattern; Marck and Grosjean 2002). The total number of introns located at position 37/38 or at other positions of the tRNA molecules, the size (in nucleotides) of the shortest intron identified in each family of intron-containing pre-tRNAs, and the number of splicing motifs harboring either the canonical or relaxed splicing motif as defined in the text are listed under the headings "Nr" and "Min size" and "Min size" and "splicing motif" ("BHB type"), respectively. Few archaeons contain tDNAs with two introns within the same pre-tRNA molecule; their numbers are listed under "Double introns." Under the heading "Endo type", "4x" means that the endonuclease is a homotetramer (α_4), and "2x" means it is a homodimer (β_2 ; see Materials and Methods).

^atDNA-Glu (TTC) of *M. kandleri* (two introns located at positions 37/38 and 21/22) is present in two nearly identical copies in the genome (C or T at position 17).

^bThe sequence data for *P. abyssi* were obtained from Genoscope at http://www.genoscope.cns.fr/Pab/Pabyssi_complete_genome.fasta.

^cThe missing tDNA-Cys of *F. acidimanus* (Marck and Grosjean 2002) was identified using tRNA-Scan-SE (Lowe and Eddy 1997) with lowest constraints (it contains an unusual A18-C55 base pair).

^dPreliminary sequence data for *F. acidimanus* and *M. barkeri* were obtained from The DOE Joint Genome Institute (JGI) at <http://genome.ornl.gov/microbial/faci/> and <http://genome.ornl.gov/microbial/mba/>. Large and small contigs files were concatenated (1,932,066 bp and 5,133,054 bp, respectively).

cal BHB structure (Fig. 3A) as **hHB** for the upper part (where **h** is the exonic helix; upward arrow) and **HBh'** for the lower part (where **h'** is the intronic helix; downward arrow), the whole symmetric structure being designated as **hHBh'**. Notice that only introns at least 14 nt long will allow the formation of a minimal 2-bp helix **h'** (with 3 nt allowed for the loop). Also the anticodon (boxed in Fig. 3A,B,C) occupies the two last positions of strand **H1**, the forward strand of double helix **H**, and the first position of bulge **B1**. After cleavage by the archaeal endonuclease and subsequent enzymatic ligation of the two exonic ends and circularization of the intron, two hairpin loops, one for the seven-membered tRNA anticodon loop and the other for the circularized intron, will be generated (Salgia et al. 2003 and references therein).

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FIGURE 2. (Legend on next page)

canonical **hBHBh'** splicing motif. Among introns located at position 37/38, some are, in fact, too short to allow for the formation of an intronic double helix **h'**. The shortest 37/38 intron that we found, among the 18 archaeon sequences examined, is that of pre-tRNA-Ser (UGA) of *S. tokodaii* (11 bases, listed in Fig. 2A and shown in Fig. 3B). In this case, due to the quasi absence of the intronic helix **h'**, the 3-nt bulge **B1** cannot be generated. Only the exonic part of the BHB structure (indicated by the arrowed **hBH** in Fig. 3B) is present and we refer to these variants of the BHB motif as **hBH** to indicate the lack of **h'** helix. Following Li and Abelson (2000), we consider an intron located at position 37/38 to be a **hBH** type (and not a **hBHBh'**) if the **h'** helix within the intron is shorter than 2 bp. Figure 3C shows another example of an **hBH**-type intron in the pre-tRNA-Thr (UGU) of *S. tokodaii*. Here, the intron is longer (17 nt), but the **h'** helix cannot be formed due to the lack of complementary bases. Therefore, the canonical **hBHBh'** splicing motif in intron–exon junctions of archaeal pre-tRNAs can obviously be relaxed into a simpler structure, **hBH**, where only two helices (**h** and **H**) and one bulge (**B2**) are present (Fig. 3B,C).

A consensus sequence has been reported for the **hBHBh'** motif as part of the anticodon loop within the **H** helix (Kleman-Leyer et al. 1997). However, this consensus sequence is probably not essential for the endonuclease recognition because it results from the quasi-strict conservation of pyrimidine at position 32 (first nucleotide in the **H1** strand), uridine (but one C) at position 33 (second nucleotide) and purine at position 37 (second nucleotide of **B1**) in the anticodon loops of all archaeal tRNAs (see consensus in Fig. 2A; see also Table I in Marck and Grosjean 2002). In the 103 introns located at position 37/38, a few mispairings were observed in helix **H**: A:C (11 times), A:G (five times), C:T (three times), T:T (twice; Fig. 2A; Supplementary Material).

Relaxed splicing motifs predominate in intron–exon junctions at positions other than 37/38

All cases of introns identified at positions others than 37/38 (33 cases among 14 different positions in 800 tDNAs) are listed in Figure 2B (see also Fig. 1). As for the case of 37/38 introns, the intron–exon junction can fold either into the canonical **hBHBh'** motif (13 cases) or into a more relaxed motif, **HBh'** (20 cases), with an **h'** helix of at least 3 bp for the hyperthermophilic crenarchaeon *P. aerophilum* and 2 bp for the thermophilic ones (*S. sulfolobus* and *S. tokodaii*). Figure 3D illustrates the case of the pre-tRNA-Val (GAC) of *P. aerophilum*, in which the intron located at position 29/30 of the anticodon stem can be folded into the canonical **hBHBh'** motif, and Figure 3E illustrates the case of pre-tRNA-Lys (CUU) of *A. pernix*, in which the intron located at position 45/46 of the V-arm cannot fold into a perfect canonical splicing motif. However, it can fold into a relaxed **HBh'** motif. In this latter case, the intronic (rightmost) part of the intron–exon junction can be folded into a hairpin structure with 4 bp in the **h'** helix and a large loop, whereas the exonic (leftmost) part remains unpaired (dotted box).

In introns located at positions other than 37/38, apart from Watson–Crick base pairing in **H** and **h'** helices and a strong preference for GC base pairs in **h'**, no strict nucleotide conservation is observed (a single case of T:T mispairing was observed in the pre-tRNA-Glu (TTC) of *P. aerophilum*; see in Fig. 2B). However, we cannot exclude that selected nucleotide sequences, like the GNRA (Heus and Pardi 1991) or UNCG (Cheong et al. 1990) sequences within the intronic part (for review, see Michel and Costa 1998) may be important for stabilizing the loop of short hairpins. No intron shorter than 17 or 15 nt (for hyperthermophiles and thermophiles, respectively) is found for the **HBh'** motif compared to 11 nt in **hBH** introns, probably because of the requirement for a sufficiently stable **h'**

FIGURE 2. Intron–exon sequences in selected archaeal tDNAs sorted out as a function of the type of their splicing motifs. The details of the sequences of all introns presented in Figure 1 are given here. Additionally, all introns (any type) of *S. tokodaii* are illustrated. (A) Only 30 sequences of intron–exon junctions (out of a total of 103 sequences in the 800 tDNAs analyzed; see Supplementary Material) corresponding to intron located at position 37/38 are listed. The sequences shown extend from base 27 to base 43 of the anticodon arm. Uppercase letters correspond to exonic sequence and the lowercase letters to the intronic sequence. The bases in the **H** helix (under headings **H1** and **H2**; gray background) are in bold; complementary bases in **h** and **h'** helices (strands **h1**, **h'1**, **h'2** and **h2**) are in bold italics. Mismatches in **H** helix are shown with inverted case: lowercase in **H1** (exon) and uppercase in **H2** (intron). Those that exhibit an intronic **h'** helix of two or more base pairs are designated as canonical **hBHBh'**, and those with an intronic **h'** helix shorter than 2 bp as relaxed **hBH** (see examples of 2D structures in Fig. 3). Notice that, for the **hBHBh'** type, all elements of the splicing motif can be defined (**h1**, **H1**, **B1**, **h'1**, **loop**, **h'2**, **H2**, **B2**, and **h2**) whereas for the **hBH** type, only **h1**, **h2**, **H1**, **H2**, and **B2** can be defined; the intronic **h'1**, **h'2**, and **B1** elements cannot. At the bottom of A, is the consensus sequence of the splicing motif corresponding to the 30 sequences shown here or in all the 103 sequences (see Supplementary Material). Mismatched bases were not taken into account for consensus computation. For the degenerate alphabet used, see legend to Figure 1. (B) The 33 sequences of intron–exon junctions corresponding to introns located at positions other than 37/38 in the 800 tDNAs analyzed are listed as a function of the decreasing size of their **h** helix (exonic). The sequences shown extend from five bases upstream of the 5' end of strand **H1** down to five bases downstream from the 3' base of the intron. The same lettering conventions as above are used. Those that exhibit an **h** helix of two or more base pairs are designated as canonical **hBHBh'**, and those with an exonic **h** helix shorter than 2 bp as relaxed **HBh'**. In this latter case, only **H1**, **B1**, **h'1**, **h'2**, and **H2** are present and, at variance with the above **hBH** motif, no exonic **h1**, **h2**, and **B2** elements can be defined. Vertical linking lines at right show the six cases of two introns in the same pre-tRNA. Numbering at the extreme right of the figure corresponds to the figure numbers in which the 2D structures are shown. At the bottom of B is the consensus sequence of the splicing motif corresponding to the 33 sequences listed in this panel. (C) generalized consensus from sequences shown in A and B and from all 136 introns in tDNAs of the genomes of the 18 archaeons analyzed. Mismatched bases in **H** helix were not taken into account for consensus computation.

FIGURE 3. Examples of splicing motifs present in archaeal pre-tRNAs bearing introns at positions 37/38 and others. (A,B,C) The cloverleaf sequences of three characteristic tDNAs harboring an intron between nt 37 and 38. (D,E) Introns located at unconventional positions. Small arrows show the intron splicing position, always located two bases downstream of the H1 and H2 strands of the central H helix. The central 4-bp H helix is highlighted with a gray background. (A) As exemplified with pre-tRNA-Lys (UUU) from *S. tokodaii*, this panel shows the most often encountered splicing motif **hBHh'** (67 times over a total of 103 cases; see Supplementary Material). In this case, the exon **h** helix (boxed) is formed by the antiparallel strands **h1** and **h2** of the anticodon stem. The central **H** helix (gray background) includes the first two bases of the anticodon (boxed). The third helix, **h'** (boxed), is formed within the intron (here, 25 nt in length). The three **h**, **H**, and **h'** helices allow for the formation of two bulges, **B1** and **B2**. The intron is always spliced symmetrically two bases downstream of strands **H1** and **H2** of helix **H**. (B) In the pre-tRNA-Ser (UGA) of *S. tokodaii*, the intron (11 nt) located between nt 37 and 38 is too short to allow the helix **h'** and the bulge **B1** to be formed. This type of relaxed splicing motif is referred to as relaxed **hBH**. (C) In the pre-tRNA-Thr (UGU) from *S. tokodaii*, although this pre-tRNA harbors a 17-nt-long intron, the intron-exon junction can only fold into a relaxed **hBH** type of splicing motif. No complementary bases are present within the intron, preventing the formation of the intronic **h'** helix. (D) Hypothetical folding of a 20-nt intron located at position 29/30 within the pre-tRNA-Val (GAC) of *P. aerophilum*. Bases 20–34 are replaced within the conventional cloverleaf scheme by asterisks and are drawn in an alternative 2D structure that fits the splicing motif requirement. In this pre-tRNA, formation of the canonical splicing motif **hBHh'** obviously competes with the formation of the regular D- and anticodon stems. (E) Hypothetical folding of a 36-nt intron located at position 45/46 in the pre-tRNA-Lys (CUU) of *A. pernix*. Here, bases 35–51 of the conventional cloverleaf are replaced by asterisks and are redrawn to fit a relaxed splicing motif **hBHh'**, in which a helix **h** cannot be formed because of the absence of the needed complementary bases (dotted box). This putative folding obviously competes with the formation of the regular anticodon and T-stems.

helix. Finally, the general consensus (Fig. 2C) computed in helix **H** and the two bulges **B1** and **B2** from all introns listed in Figure 2A,B (and in Supplementary Material) shows that virtually all positions can accept any base (except in two positions where A is totally absent in helix **H1** and conversely Y [C or T] is totally absent in helix **H2**; B means “no A” and V means “no T”).

Interestingly, the **HBh'** motif (lower part of Figs. 2B, 3E) appears to be the symmetric of the **hBH** motif (lower part of Figs. 2A, 3B), the helix **h'** that generates the bulge **B1**, being part of the intron, whereas in the case of the **hBH** type of motif, the exonic **h** helix generates the bulge **B2**. Therefore, from the enzymatic point of view, the two types of relaxed splicing motifs **hBH** (as listed in Fig. 2A) and motif **HBh'** (as listed in Fig. 2B) are probably similar, provided that helix **h** or **h'** is each sufficiently stable to compensate for the lack of the missing helix **h'** or **h** and to allow efficient recognition by the archaeal splicing endonuclease.

Introns located at noncanonical positions hinder the three-dimensional (3D) structure of pre-tRNAs

When present at position 37/38 in the anticodon loop, an intron between the 3' ultimate and penultimate residues of the anticodon loop should not hinder the 2D/3D structures of the archaeal pre-tRNA. Indeed, helix **h** corresponds to the pre-tRNA anticodon stem (exon) that simply extends the coaxially stacked D-stem within the cloverleaf structure of tRNA (see Fig. 3A,B,C), whereas helix **h'** is a hairpin terminating with a loop (intron) that will be removed and circularized after splicing, leaving a mature 7-nt anticodon loop. As a matter of fact, chemical and enzymatic probes revealed that the common tertiary structure seen in the crystal structure of yeast tRNA-Phe as model is maintained in intron-containing tRNAs and that the intron, including the splice sites, is the most exposed region of the molecule (Swerdlow and Guthrie 1984; Lee and Knapp 1985).

However, when the intron is located at any position other than 37/38 within the pre-tRNAs, the situation is different. For example, the formation of the **hBHBh'** splicing motif in pre-tRNA-Val (GAC) of *P. aerophilum* cannot be formed without complete unpairing of the D- and anticodon stems (indicated by asterisks in Fig. 3D). Similarly, the formation of a **HBh'** type of splicing motif (with 4 bp in the **h'** helix and a large loop) required for removal of the 36-nt intron located between nt 45 and 46 in the V-arm of pre-tRNA-Lys (CUU) of *A. pernix* cannot be formed without unpairing the anticodon and T-stems (indicated by asterisks in Fig. 3E). In fact, any intron located from positions 20b/21 to 32/33 (as indicated in Fig. 1) prevents the formation of the D- and anticodon stems, whereas those located from 38/39 to 45/46 prevent the pairing of the anticodon and T-stems.

A special case concerns the intron located at position 59/60 in the T-loop, like in pre-tRNA-Thr (TGT) of *P. aerophilum* (see Figs. 1, 2B), which allows the formation of

an almost perfect canonical **hBHBh'** structure. This location is equivalent to position 37/38 in the anticodon loop. Indeed, in the mature tRNA, both loops have 7 bases, each stem has 5 bp, and U33 is the homolog of U55 (discussed in Marck and Grosjean 2002). However, because the T-loop interacts with the D-loop in the 3D architecture of the tRNA, the presence of an intron in the T-loop obviously hinders the formation of a correct 3D folding of the pre-tRNA (not shown).

A novel intron location in the amino acid acceptor stem

A very intriguing situation was found with three tDNAs from the hyperthermophilic archaeon *P. aerophilum*. The tRNA structures found for tDNA-Asp (GTC), tDNA-Glu (CTC), and tDNA-Glu (TTC) by tRNAScan-SE (Lowe and Eddy 1997; see GtRDB, <http://rna.wustl.edu/GtRDB/>) or by the cloverleaf tDNA search (Marck and Grosjean 2002) predict three mismatched base pairs at the end of the amino acid acceptor stem (bp 1–72, 2–71, and 3–70). Because a well-paired acceptor stem required for proper functioning of tRNA at high temperatures (above 80°C) was always observed in archaeal tDNA (Marck and Grosjean 2002), we reasoned that such mispaired amino acid acceptor stems might be either posttranscriptionally edited into a perfect Watson–Crick helix (see Lavrov et al. 2000 and references therein) or resolved into a perfectly paired acceptor stem after splicing of a putative intron.

In support of this last alternative, we noticed that: (1) the transcriptional promoter TATA element upstream of each of the three tDNAs considered, which should be normally located about 20–25 bases upstream of nt 1 of the mature tRNA (Thomm and Wich 1988), is, in fact, located farther away (59, 58, and 72 nt away, respectively, as shown in Fig. 4A, in which uppercase letters represent the mature tRNA and lowercase letters the intron and flanking sequences); (2) a **gccccgg** sequence upstream of the tRNA gene (designated **h'1**, in Fig. 4A) could pair with the exonic CCGGGGC sequence (designated **h'2**) in positions 66–72 of the unspliced tRNA; and (3) the nucleotide sequence of this loop can be folded into a canonical HBH motif **hBHBh'** (Fig. 4B). If this **hBHBh'** structure was spliced by the *P. aerophilum* splicing machinery, a 21-nt-long intron (box labeled “intron 3/4” in Fig. 4A) would be removed. After splicing, ligation of the two generated ends, and rearrangement of the tRNA secondary structure, a perfect Watson–Crick amino acid acceptor arm would be formed. Because these three tDNAs are highly homologous, the same sequential folding as proposed above should apply to each (Fig. 4A). However, the splicing motif in tDNA-Glu (CTC) is less perfect because only 1 bp closes the **B2** bulge (data not shown). Because the intron within the acceptor stem may hinder the recognition of precursor tRNA by the 3' endonuclease and by the RNase P, the splicing process, in this

FIGURE 4. A novel intron is located at position 3/4 in three tDNAs of *P. aerophilum*. (A) The sequences of the three tDNAs of *P. aerophilum* coding for tRNA-Asp (GUC) (*upper line*), tRNA-Glu (CUC) (*middle line*), and tRNA-Glu (UUC) (*lower line*) are shown. They extend from the putative upstream TATA promoter element (black bold) down to position 76 of the mature tRNA. Bases in uppercase letters belong to the mature tRNA, those in lowercase to the intron and mature tRNA flanking sequences. The bases making up the putative intron located between nt 3 and nt 4 of the amino acid acceptor stem, including the intronic helix **h**, are shown in lowercase letters (within the box labeled “intron 3/4”). The sequence elements forming the canonical splicing motif **hBHBh'** (**h1**, **H1**, **B1**, **h'1**, **loop**, **h'2**, **H2**, **B2**, and **h2**) are shown in bold (for a definition of these elements, see text and legend to Fig. 2). Bases forming the **H1** and **H2** strands are bold and highlighted with gray background. Bold italics are used to indicate the complementary bases forming the **h1** and **h2** antiparallel strands of the helix **h**, and the **h'1** and **h'2** antiparallel strands of the helix **h'**. Among these three tDNAs, only one, tDNA-Glu (TTC), harbors a second intron located between nt 58 and 59 (box labeled “intron 58/59”). (B) Hypothetical 2D structure of pre-tRNA-Glu (CUC) including the splicing motif within the amino acid acceptor stem. The sequence shown here, including the cloverleaf-like structure, extends from 31 bases upstream from tRNA base 1 down to base 73 at the 3' end of the tRNA. Plus signs are used to indicate Watson–Crick base pairing and filled circles to indicate wobble GU/UG pairings. The intron (in lowercase) is shown folded under a canonical **hBHBh'** splicing motif, using the same lettering conventions as in A. The small arrows indicate the splicing positions after nt 3 and before nt 4. The path of the nucleotidic chain is symbolized with thick gray lines. Remarkably, a peculiar **gccccgg** sequence is present in the 5' region of the three tRNA genes, which is an exact replica of bases 1–7 of the mature tRNA. This sequence, designated **h'1** is probably part of the pre-tRNA transcript, as it is located 20 bases 3' of the TATA element. It is shown here facing the antiparallel strand of the acceptor stem (bases 66–72, designated **h'2**) because it is not known whether a helix between complementary strands **h'1** and **h'2** can form.

case, would probably have to occur before the maturation of the 3' and 5' ends of the pre-tRNA (Krasilnikov et al. 2003; Zahler et al. 2003; for reviews, see Hall and Brown 2001; Mörl and Marchfelder 2001; see below for other cases of mutually exclusive maturation/splicing processes). A similar obligate removal of intron before 3' and 5' ends maturation probably also holds for introns located at other positions (especially in the T-loop; see Fig. 1) of several pre-tRNAs.

Few pre-tRNAs harbor two introns

In addition to the intron at position 3/4 of the amino acid stem, tDNA-Glu (TTC) of *P. aerophilum* harbors a second intron at position 58/59 (box labeled “intron 58/59” in Fig. 4A). Here again, a canonical motif **hBHBh'** structure can be formed (however with one mismatch in the **H** helix), without hindering the **HBh'** structure of the intron located at positions 3/4 of the amino acid arm (structure not shown).

It is not possible to predict whether splicing of these two introns proceeds in obligate or random order.

Five other cases of two introns within the same pre-tRNA have been reported (Figs. 1, 2). Among them, only in the case of the pre-tRNA-Pro (GGG) of *M. thermoautotrophicum* (the first reported case of two introns within the same pre-tRNA; Smith et al. 1997), can one predict an obligate order for splicing. In this pre-tRNA, the 32-nt-long intron located at positions 32/33 (Fig. 5) obviously has to be processed before the splicing of the second intron (16 nt in length) located at the usual position 37/38. Indeed, the **H1** strand of the **H** helix of the **hBHBh'** structure of the "second" intron (L-shaped box in the anticodon loop, Fig. 5) is interrupted between its first and second positions by the intron at positions 32/33. The canonical **hBHBh'** motif required to splice the second intron at position 37/38 can be formed only after splicing of the intron at position 32/33. This sequential removal of introns implies that there is enough plasticity of the RNA molecule within the whole anticodon stem and loop to allow major rearrangements between two successive intron splicing processes.

Formation of the splicing motif also competes with other maturation processes

Switches between mutually exclusive folding patterns of the pre-tRNA allowing distinct maturation processes to occur

FIGURE 5. Ordered sequential intron splicing has to occur in certain pre-tRNAs harboring two introns. Pre-tRNA-Pro (GGG) of *M. thermoautotrophicum* contains two introns in the anticodon loop, one between nt 32 and 33 (32 nt in length) and the second one between nt 37 and 38 (16 nt in length). In the conventional cloverleaf scheme, bases 21–39 are replaced by asterisks. They are redrawn in an alternative 2D structure that fits the splicing motif requirement. The same lettering conventions as in Figures 1–4 are used. Only the hypothetical folding of the noncanonically located 32-nt intron is shown. In this pre-tRNA, the formation of the canonical splicing motif **hBHBh'** (allowing the intron at position 32/33 to be spliced) obviously competes with the formation of the splicing motif corresponding to the intron located at the usual position 37/38 (boxed). Only after removal of the intron at position 32/33, can the intron–exon junction corresponding to intron 37/38 be folded into another **hBHBh'** splicing motif (folding not shown).

in an obligate order have been recently described in the case of tRNA-Trp (CCA) of *Halobacterium volcanii*, which harbors a long intron composed of 104 bases. In this very peculiar pre-tRNA, the intron and flanking regions not only display the hallmark **hBHBh'** splicing motif but also a duplicate form of the hallmark C/D boxes that guide the 2'-O-methylations in RNA (d'Orval et al. 2001). Based on in vitro experiments, it has been suggested that the intronic part of *H. volcanii* tRNA-Trp guides the 2'-O-methylation in *cis* of both the 2' hydroxyriboses at positions 34 and 39 within the exon region of the precursor tRNA (Gaspin et al. 2000; d'Orval et al. 2001). The two ribose 2'-O-methylations clearly require structural rearrangements, implying that the intronic and exonic regions of the pre-tRNA-Trp generate the appropriate guide–target duplex for each independent methylation. Another structural arrangement is then required to generate the **hBHBh'** motif recognized by the splicing endonuclease. After intron excision, the two half exons of the methylated tRNA-Trp are ligated again by a tRNA ligase (for reviews, see Bachellerie et al. 2002; Omer et al. 2003). The formation of the splicing motif in this case should allow a correct 3D folding of the tRNA (see above).

It is noteworthy that no intron-containing tRNAs other than archaeal pre-tRNA-Trp display RNA C/D box in their introns (d'Orval et al. 2001; Dennis et al. 2001). However, not all archaeal pre-tRNA-Trp display the characteristic C/D' and C'/D motifs. They are absent in the shorter (33 nt) intron of pre-tRNA-Trp of *Methanococcus jannaschii* and in the pre-tRNA-Trp of *A. pernix* (of which the 37-nt intron is located at position 30/31, not 37/38). Pre-tRNA-Trp of *P. aerophilum* does not contain any intron (Marck and Grosjean 2002). It would be interesting to know whether a 2'-O-methyl ribose exists at position 34 of the mature tRNA-Trp of *M. jannaschii*, *A. pernix*, or *P. aerophilum*. In eukaryotic cells like yeast, formation of such 2'-O-methylcytosine-34 of tRNA-Trp is catalyzed by an intron-independent enzyme (Pintard et al. 2002). It could be that 2'-O-methylation of C-34 in anticodon of tRNA-Trp as well as at many other riboses of the tRNA molecules occurs via an intron-dependent methylation machinery in some archaea (for review, see Omer et al. 2003) whereas it might be catalyzed by intron-independent tRNA modifying enzymes in other archaea (Grosjean et al. 1995; for review, see Grosjean et al. 1997; Constantinesco et al. 1999).

DISCUSSION

Identification, comparative sequence analysis of the exon–intron junctions, and careful inspection of the fit to a conserved splicing motif in all intron-containing tDNAs in a given or phylogenetically related organisms should allow the better definition of the minimal core motif recognized by the archaeal splicing machinery. The hallmark of intron–exon boundaries in the majority of archaeal pre-tRNAs is the presence of a nucleotide sequence that can be folded into a characteristic bulge–helix–bulge, the so-called **HBH** motif, now renamed **hbHBh'**. The comparison of sequences of 136 such exon–intron boundaries in the tDNAs of 18 archaea allows us to raise the following issues.

Not all archaeal intron–exon boundaries can fold into a canonical **hbHBh'** motif

While examining all intron–exon junctions in the tDNAs of 18 archaea, we found that the most frequent **hbHBh'** splicing motifs are sometimes imperfect. First, for a few introns located at 37/38 (and 59/60 in a single case), the **h'** intronic helix is absent (less than 2 bp) either because the intron is too short or because no base-pairing complementarity exists between the putative strands **h1** and **h2**. Only the exonic **h** helix is always present (Fig. 2A,B). When the intron is located at 37/38, it corresponds to the anticodon stem whereas when it is located at position 59/60, it corresponds to the T-stem. Second, when the intron is located at any other place than positions 37/38 and 59/60, then the exonic helix **h** may be lacking, again due to the absence of base-pair complementarity. However, in such cases, a minimal **h'** intronic helix is always present [but for one case, that of tDNA-Glu (CTC) of *P. aerophilum*; see Fig. 2B]. Also, a complete absence of helix **h** (no complementary bases) or **h'** within the exon–intron boundaries of certain intron-containing pre-rRNAs has been described (see Burggraf et al. 1993; Lykke-Andersen and Garrett 1994). Altogether these observations argue against a universal perfect archaeal **hbHBh'** structure with at least 2 bp in helix **h** or **h'** as predicted from structural data (Li and Abelson 2000 and see below). Instead, they reveal the existence of more relaxed motifs, **hbH** and **HBh'** as defined in this work, that suggest less stringent structural requirements for archaeal splicing enzyme recognition, at least in some species, than has been supposed previously.

Only helix **H** and one of the two helices **h** or **h'** are major determinants for splicing in certain Archaea

The general trends that appear from the already published works together with the present thorough analysis of the nucleotide sequences of the intron–exon boundaries in 136 tDNAs from 18 complete archaeal genomes is that: (1) only the 4-bp helix **H** with no sequence preference is strictly

conserved—any base change within this central helix **H** was shown to abolish in vitro splicing activity (see Thompson and Daniels 1990; Ciammaruconi and Londei 2001)—however, in a few cases, a mismatch exists (see strands **H1** and **H2** in gray background in Fig. 2A,B); (2) at least one helix **h** or **h'** (**h1**, **h2**, **h'1**, and **h'2** strands boxed in Fig. 2A,B), generating a 3-nt bulge on one of the two sides of helix **H**, is always present, and their stability is probably important, as attested by their high G + C content in pre-tRNAs, especially from the hyperthermophilic archaea; and (3) as first demonstrated by Daniels' group (Thompson and Daniels 1988; Armbruster and Daniels 1997), there is no dependence on the 3D architecture of the tRNA molecule, because archaeal introns occur in many diverse locations (14 altogether; Fig. 1), provided that the conformational plasticity of the intron-containing pre-RNA molecules allows the formation of the minimal **hbH** or **HBh'** motif recognizable by the archaeal endonuclease (Kjems and Garrett 1991; this work). Notice that, in intron-containing pre-rRNAs and pre-mRNAs, at least one of the two helices **h** or **h'** is usually very long (Garrett et al. 1991; Nomura et al. 1998; Ciammaruconi and Londei 2001; Tang et al. 2002; Watanabe et al. 2002), whereas in pre-tRNAs harboring an intron at position 37/38 of the anticodon loop or at position 59/60 of the T-loop, the exonic stems **h** are extended by a coaxially stacked stem (D- or T-stem, respectively). For introns located at positions other than 37/38 (or 59/60), the possible influence of other parts of the folded pre-tRNA molecules on the stability of the splicing motif should not be underestimated. One main difference between cleavages at a **hbHBh'** splicing motif or at a relaxed **hbH** or **HBh'** variant is that, in the first case, ligation of the released fragments always generates two hairpin loops of seven residues each as in the anticodon- or T-loops of mature tRNAs (Salgia et al. 2003), whereas in the latter case, the released exons will be religated into a structure that is compatible with a subsequent folding of the RNA molecules into an appropriate functional structure (as a cloverleaf in the case of tRNAs).

Taken together, the above observations allowed us to hypothesize the need of only one stable helix **h** or **h'**, together with the central 4-bp helix **H** to generate a spliceable substrate, at least in certain Archaea, mostly Crenarchaeota (see also discussion in Kjems and Garrett 1991). Based on this assumption, the motifs **hbH** and **HBh'** appear to be the symmetric of each other. Therefore, depending on binding of the splicing endonuclease to one or the other side of this “minimalist” splicing motif, one bulge between the helix **H** and **h** or **h'** will probably be cut first.

Detailed structural analysis of the minimal **hbH** or **HBh'** motif, associated or not with a purified recombinant archaeal endonuclease, is not yet available. However, the RNA stem-loop structure (TAR) that contains the helical stem region separated by a 3-nt bulge, which strongly resembles the **HBh'** structure, has been solved by NMR spectroscopy.

The only available **hBHBh'** structure at 1.77–4.46 Å average resolution determined by NMR spectroscopy is of a 38-nt RNA of a perfect **hBHBh'** motif as found in the mesophilic euryarchaeon *H. volcanii* pre-tRNA-Trp (CCA; Diener and Moore 1998). In this structure, the conformation of the two 3-nt bulges (in which the RNA molecule is cleaved) is stabilized by stacking interactions between bulge nucleotides and bases in the adjacent helices **H** and **h** or **h'**, both bulges being presented on the same minor groove face of the central 4-bp helix **H**. Interestingly, each of the two bases preceding the cleavage site within the bulge are flipped out. However, the geometry of one of the two bulges differs somewhat from that of the other one. It is therefore possible that one of the flipped nucleotides is preferred for endonuclease attack, even in a perfect **hBHBh'** splicing motif.

Interestingly, the **hBH** type of splicing motif as described above has some resemblance to the splicing substrate consensus “hBh” present in most yeast pre-tRNAs harboring introns at 37/38 (for review, see Culbertson and Winey 1989; Trotta and Abelson 1999). In this latter case, the intronic helix **h** is the same anticodon stem as for archaeal pre-tRNAs. It is flanked by a bulge (**B**), often larger than 3 nt, extended by another helix **h** (the equivalent of the conserved **H** of the archaeal pre-tRNAs). In this latter helix **h**, a conserved base pair between a pyrimidine of the 5' exon (position 32 in tRNA) immediately following the universal U-33 and the three bases of the anticodon and a base in the single-stranded loop of the intron (position –3, the so-called A-I base pair where A stands for anticodon and I for intron) is required for correct excision at the 3' splice site (Baldi et al. 1992; for review, see Fruscoloni et al. 2001).

Are the splicing motifs and oligomeric structure of the endonuclease evolutionary related?

The “approximately” twofold symmetry of this perfect **hBHBh'** splicing motif makes it well suited for interaction with each of the two types of RNA splicing endonuclease (RNase) EndA characterized so far: the homodimeric enzyme (β_2) with a monomer size in the range 288–353 residues or the homotetrameric enzyme (α_4) with a monomer size in the range 170–187 residues (see Table 1 and Materials and Methods). From in vitro RNA footprinting experiments on the complex formed between the pure recombinant homotetrameric endonuclease (α_4) from the euryarchaeon *M. jannaschii* and a synthetic RNA substrate bearing a perfect **hBHBh'** splicing motif (as in crenarchaeal *Desulfurococcus mobilis* rRNA), it has been demonstrated that only the nucleotides outside the minimal **BHB** sequence, thus including the two flanking helices **h** and **h'** adjoining the central **BHB** motif, were susceptible to chemical reagents; the backbone at the two 3-nt bulges and the central 4-bp helix **H** being protected by the endonuclease (Lykke-Andersen and Garrett 1997).

On the other hand, the α_4 -type of endonuclease of *M.*

jannaschii (Li et al. 1998) has been crystallized and its 3D structure obtained at 2.3-Å resolution. This homotetramer (α_4), with small subunits of less than 200 amino acid residues, is believed to assemble through dimer intermediates; only two of the four subunits playing the role of catalytic subunits, and the two others acting as structural dimerization subunits (Lykke-Andersen and Garrett 1997; Li et al. 1998; Bujnicki and Rychlewski 2000). This type of subunit organization is present in the endonucleases of all four Crenarchaeota and in 6 out of 14 Euryarchaeota examined (Table 1; Fig. 6). More recently, the crystal structure at 2.8 Å resolution of the other type of endonuclease from *Archaeoglobus fulgidus* has been obtained and its 3D architecture compared with that of the *M. jannaschii* endonuclease (Li and Abelson 2000). This enzyme is a homodimer (β_2) with each monomer being about twice as large as the homotetrameric enzyme. Each monomer bears two homologous repeats, with the C-terminal repeat being the most similar to the α subunit of the homotetrameric enzyme. This is also the part of the large subunit that has retained the catalytic function. The overall shape and subunit disposition of this homodimeric enzyme, which is found only in Euryarchaeota (eight out of 14 analyzed; Fig. 6; Table 1), is essentially the same as in the homotetrameric enzyme (Li and Abelson 2000).

Docking of the **hBHBh'** structure of a minimalist 38-nt RNA substrate as determined by NMR spectroscopy (Diener and Moore 1998) onto the X-ray structure of the homodimeric endonuclease of *M. jannaschii* favors the idea that the binding of the two 3-nt bulges **B** is required to activate the splicing reactions and that the minimal length of the **h** and **h'** is 2 nt (Li and Abelson 2000). These data are in agreement with the in vitro RNA footprinting experiments (Lykke-Andersen and Garrett 1997). Unfortunately, no crystal structure of the α_4 type of splicing endonuclease originating from an archaeon like *P. aerophilum* or *S. tokodaii* has yet been described. Such structural information should help to better understand putative variations in the substrate structural requirements of the endonucleases from evolutionarily related organisms. Indeed, among the 18 archaeal genomes investigated, only 6 of them harbor tDNA introns located at noncanonical positions (other than 37/38), and their splicing endonuclease is of the homotetrameric α_4 type. Regarding the Euryarchaeota/Crenarchaeota boundary, four of them (*P. aerophilum*, *A. pernix*, *S. sulfolobus*, and *S. tokodaii*) belong to the phylum Crenarchaeota whereas the two other (*M. kandleri* and *M. thermoplasticum*) belong to the phylum Euryarchaeota (Fig. 6; Table 1). Among the remaining 12 archaea that do not harbor introns elsewhere than at position 37/38, some have a homotetrameric (α_4) splicing enzyme (*M. jannaschii* and the three *Pyrococcus*) and the others have a homodimeric (β_2) enzyme. No known archaeon with a dimeric enzyme has any intron located at noncanonical positions. Other archaea are known (not investigated here) that do

FIGURE 6. Endonuclease-based phylogeny of pre-tRNA introns splicing motifs and related features. The homotetrameric α_4 endonuclease splicing at characteristic intron–exon junctions of archaeal pre-tRNAs (present in most Crenarchaeota and a few Euryarchaeota) is assumed to have evolved divergently towards a homodimeric β_2 enzyme in Euryarchaeota and into a heterotetrameric $\alpha, \beta, \gamma, \delta$ enzyme in Eukarya (see text). The monomer of the homotetrameric ancestral splicing endonuclease is designated by “ α ”; that of the homodimeric enzyme is designated by “ β ” and the four different subunits of the eukaryotic heterotetrameric enzyme by “ α , β , γ , and δ ”. Note that the size of the β subunit is about twice that of the α subunit and probably results from gene duplication and subsequent fusion of an ancestral α -like subunit gene (Lykke-Andersen and Garrett 1997; Bujnicki and Rychlewski 2000; Li and Abelson 2000).

have introns elsewhere than 37/38: *T. pendens*, a crenarchaeota, has an intron in the V-arm of its tDNA-Gly (CCC), but the nature of its splicing enzyme, homotetramer or homodimer, is not yet known (Kjems et al. 1989b).

An ancient endonuclease from Crenarchaeota might be at the origin of the present-day endonucleases in the three biological domains

The above considerations on the oligomeric structure diversity of endonucleases together with our intron sequence analyses led us to hypothesize that the more primitive homotetrameric α_4 enzyme might tolerate less perfect **hBHBh'** structures of the **hBH** or **HBh'** types and have less stringent structural needs than its homodimeric (β_2) successor, which requires more perfect **hBHBh'** structures. Assuming that the endonuclease of Crenarchaeota still behaves as the enzyme that was present in the last universal common ancestor before the divergence into the three major forms of life (Archaea, Bacteria, and Eukarya), one can imagine the following scenario for the evolution and appearance of the different forms of the present-day endonucleases (Fig. 6). Evolution may have started from a homotetrameric α_4 -type of RNA splicing endonuclease. This primitive form of enzyme was probably able to splice intron-containing RNAs at a simple structural motif like the

hBH or **BHh'** located at any position of an RNA precursor. After divergence of Crenarchaeota to the Euryarchaeota kingdom, a more stringent homodimeric form of tRNA-endonuclease (β_2) appeared that might have resulted from gene duplication and fusion followed by mutations. This newly formed enzyme could have progressively evolved into a site-specific endonuclease that cleaves only pre-tRNAs in the anticodon loop at position 37/38. With the appearance of the Eukarya, the same primitive α_4 precursor might have divergently evolved into an even more stringent and specialized heterotetrameric tRNA-endonucleases encompassing four different but related α , β , γ , and δ subunits (Bujnicki and Rychlewski 2000). This enzyme would now cleave pre-tRNAs exclusively at position 37/38 within a correct 3D folding of the tRNA molecule (see Di Nicola Negri et al. 1997; Trotta et al. 1997; for review, see Belfort and Weiner 1997; Trotta and Abelson 1999).

In support of this last hypothesis, it could well be that the archaeal relaxed **hBH** motif is an archaic form of the present day eukaryotic 3' splicing motif “**hBh**” (see above), in which the universal pyrimidine-32 in the anticodon loop must now pair with a conserved purine at position -3 from the 3' side of the intron (the so-called “A-I” base pair; Baldi et al. 1992). It is also interesting to note that the eukaryal endonuclease has still retained its ability to splice the archaeal recognition signal (**hBHBh'**, in fact **hBH** or **HBh'**

motif) within a tRNA^{arch-euka} whereas the purified endonuclease from the crenarchaeon *S. solfataricus* can also accurately splice the intron within the same chimeric pre-tRNA (Fabbri et al. 1998). Moreover, with certain chimeric pre-tRNAs, higher eukaryotic tRNA splicing endonucleases, such as the one from *Xenopus laevis*, can also cleave in a mode that is independent of the mature domain of the tRNA when substrates are able to form the perfect **hBHBh'** splicing motif, the A-I base pair now playing a pivotal role in this process (Fruscoloni et al. 2001). Last but not least, from thorough analyses of tRNA populations in 60 fully sequenced genomes, Xue et al. (2003) recently argued that both Eukarya and Bacteria probably originated from the phylum Crenarchaeota, whereas the Euryarchaeota evolved from the same ancestor of Crenarchaeota, but independently of Eukarya and Bacteria. Our proposal concerning the independent evolution of the endonucleases found in the three biological domains fits well with this idea.

MATERIALS AND METHODS

Search for introns in archaeal tDNAs

Compilation of all intron sequences and their corresponding locations within tDNA was first performed by using automatic procedures (tRNA-ScanSE [Lowe and Eddy 1997] or cloverleaf tDNA search [Marck and Grosjean 2002]). This allowed us to easily identify all introns present at the usual position 37/38 of the anticodon loop. Data from the literature (see references in Table 1), as well as those posted on "GtRDB: The genomic tRNA database" (<http://rna.wustl.edu/GtRDB/>) and the genome-specific websites (see GOLD website; <http://igweb.integratedgenomics.com/GOLD/>) were also taken into account. To locate introns at positions other than the canonical position 37/38 in archaeal tDNAs and missed by the above mentioned procedures, we specifically searched and visually inspected the cloverleaf fit of each individual tDNA that looked "abnormal" on the base of its size. The different criteria used to identify "abnormally located" introns in such "abnormally long" tDNAs were the following (see details in Marck and Grosjean 2002): (1) there exist rules that define the cloverleaf structure of archaeal tRNAs; (2) there is little or no tDNA redundancy in archaeal genomes; (3) a complete tDNA population able to translate all sense codons according to the known wobbling rules has to exist in every genome analyzed; and (4) like those located at position 37/38, any putative spliceable introns located elsewhere than at position 37/38 should harbor a minimal BHB structure (the central H helix and at least one bulge; see Results). All introns located at unusual positions in tDNA are referred to as "noncanonical introns." A complete list of all intron sequences (canonical or noncanonical) and their exact locations within the tDNAs of the 18 archaea examined in this work is given as Supplementary Material.

Endonuclease type determination

Identification of the type of endonuclease, homotetrameric like in *M. jannaschii* (Li et al. 1998) or homodimeric like in *A. fulgidus* (Li and Abelson 2000), in each genome analyzed was performed by inspection of the size of the protein using a Blast search on Gen-

Bank (Altschul et al. 1997) with the *M. jannaschii* protein as entry. Homotetramer-type of endonucleases have a monomer size in the range 170–187 residues (here referred to as the α -type subunit), whereas homodimer-type endonucleases have a monomer size in the range 288–353 residues (here referred to as the β -type subunit; see also Lykke-Andersen and Garrett 1997).

Nomenclature

We have redefined the canonical "BHB" splicing motif (Kjems et al. 1989a; Thompson and Daniels 1990; for review, see Trotta and Abelson 1999) that consists of a central 4-bp helix (H) flanked by two 3-nt bulges (B) as **hBHBh'**, where **h** and **h'** are the two outer double helices closing the bulges. The BHB model does not specify the length of these helices, but it has been estimated, from a docking analysis of the NMR structure of a **hBHBh'** RNA substrate (Diener and Moore 1998) onto the crystal structure of the *A. fulgidus* homodimeric endonuclease, that a minimal length of 2 bp is required for the splicing to occur (Li and Abelson 2000). We have retained the same minimal requirement and refer to the splicing motif as **hBHBh'** if both **h** and **h'** helices are at least 2 bp long, whereas we refer to it as **hBH** or **HBh'** if the **h'** or **h** helix is shorter than 2 bp, respectively. The one-letter code used for nucleic acids consensus sequences (Cornish-Bowden 1985) is given in the legend to Figure 1.

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