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Transfer RNA genes in pieces

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The short genes encoding transfer RNA (tRNA) molecules are highly conserved in both sequence and structure, reflecting the central role of tRNA in protein biosynthesis. The frequent occurrence of fragmented intron-containing tRNAs that require processing to form contiguous molecules is therefore surprising. Recent discoveries of permuted and split tRNA genes have added to the apparent creativity of nature regarding the organization of these fragmented genes. Here, we provide an overview of the various types of fragmented tRNA genes and examine the hypothesis that the integration of mobile genetic elements—including viruses and plasmids—established such genes in pieces.

Keywords: genome integration; intron; mobile elements; splicing; tRNA

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Introduction

Central to the process of protein biosynthesis are transfer RNAs (tRNAs), which translate individual codons of a messenger RNA (mRNA) into the corresponding amino acid, and thereby form the link between the genetic code and protein sequence. To fulfil this role, a subset of tRNA species exists for each of the canonical 20 amino acids, which is recognized and aminoacylated specifically by its cognate aminoacyl-tRNA synthetase (reviewed by Ibba & Söll, 2000). The resulting aminoacyl-tRNAs are then delivered to the translating ribosome where they are required to fit into its tRNA-binding sites (reviewed by Ramakrishnan, 2002). Therefore, all canonical tRNAs show a conserved cloverleaf-shaped secondary structure that folds into an L-shaped tertiary structure. The central function of tRNAs in these crucial cellular processes accounts for their highly conserved sequence and structure, as well as their restricted length, which averages 76 nucleotides (nt; Marck & Grosjean, 2002).

Here, we focus on the remarkably diverse arrangements of tRNA genes that are not directly transcribed into a standard contiguous tRNA. Instead, in these instances, the initial transcript is a disrupted precursor that is subsequently enzymatically processed to form mature tRNA. Given the small size of tRNA, it is remarkable to see the diverse ways in which these genes are disrupted. Here we give a synopsis of these fragmented tRNA genes and describe how the cell assembles the functional tRNAs. Finally, we present our view on the evolution of such 'tRNA genes in pieces'.

Introns

The most common and best-described disruption of tRNA genes is the presence of an intron, which was initially found to be inserted exclusively 1 nt after the anticodon-adjacent position 37 of eukaryotic tRNAs (Goodman *et al*, 1977; Valenzuela *et al*, 1978). This intron is removed by the eukaryal splicing endonuclease that recognizes the body (mature domain) of a tRNA molecule and measures the fixed distance to the splice sites (Reyes & Abelson, 1988). Subsequently, an RNA ligase joins the resultant tRNA halves and a 2'-phosphotransferase removes the 2'-phosphate from the splice junction.

By contrast, bacterial tRNAs are found to contain a different type of intron, the group I intron ribozyme, in their anticodon loops. This is able to self-splice either without the help of protein cofactors (Adams *et al*, 2004; Reinhold-Hurek & Shub, 1992) or with the help of splicing factors, including mitochondrial tyrosyl-tRNA synthetase (Paukstelis *et al*, 2008).

Finally, the analysis of archaeal genomes allowed the identification and investigation of several more varied introns inserted at numerous positions within the tRNA molecule (reviewed by Marck & Grosjean, 2003). It became clear that in certain members of the Archaea, introns are also located in the anticodon stem, the acceptor stem, the D-loop, the T-loop and the variable region. Common to all archaeal tRNA introns, a structural motif is formed at the intron–exon junction that is recognized and cleaved by the archaeal splicing endonuclease. This motif is known as the bulge–helix–bulge motif (BHB) and is characterized by a central conserved 4 base-pair (bp) helix flanked by two 3-nt bulges with an approximate twofold symmetry (Fig 1A,B; Diener & Moore, 1998; Xue *et al*, 2006). The eukaryal splicing endonuclease naturally excises introns based on their distance to the mature tRNA domain; however, it has also been shown to recognize and cleave BHB motifs both *in vitro* and *in vivo* (Di Segni *et al*, 2005; Fabbri *et al*, 1998). The activity of archaeal splicing endonucleases relies solely on the presence of these BHB motifs in a so-called 'mature-domain-independent fashion'. The nature of the archaeal RNA ligase is still unknown.

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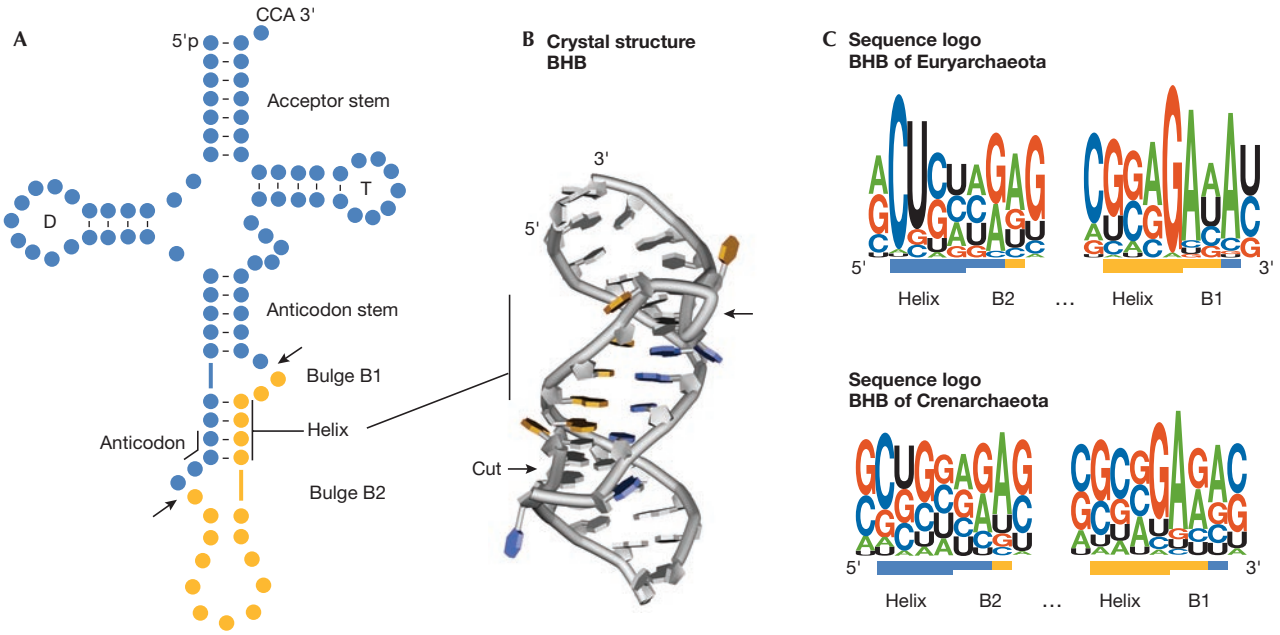


Fig 1 | Characteristics of the archaeal bulge–helix–bulge splicing motif. The colouring scheme in all three panels indicates the transfer RNA (tRNA; blue) and the intron (orange). Splice sites are indicated by arrows. (**A**) Schematic secondary structure of an intron-containing pre-tRNA. The conserved features of a tRNA are indicated, D and T indicate the D-loop and the T-loop respectively. (**B**) Crystal structure of a bulge–helix–bulge (BHB) motif taken from the complex structure with splicing endonuclease from *Archaeoglobus fulgidus* (Xue *et al*, 2006). (**C**) Sequence logo representation of 44 euryarchaeal (top) and 137 crenarchaeal (bottom) BHB motifs (extracted from Marck & Grosjean, 2003; Sugahara *et al*, 2007).

The extent of conservation of both sequence and structure of the BHB motif varies among the archaeal kingdoms. The members of the Euryarchaeota show a relatively conserved BHB motif, which is located mostly in the anticodon loop and is recognized by homomeric splicing endonucleases (Li *et al*, 1998; Lykke-Andersen & Garrett, 1997). By contrast, the members of the Crenarchaeota show more relaxed BHB motifs, which are also inserted in non-anticodon tRNA regions that are processed by a heterotetrameric splicing endonuclease (Calvin *et al*, 2005; Randau *et al*, 2005a; Tocchini-Valentini *et al*, 2005; Yoshinari *et al*, 2005). In these organisms, the structural splicing motif can sometimes be reduced to the central 4-bp helix flanked by only one 3-nt bulge and a loop of varying length. Sequence conservation of the BHB motif can be examined by a sequence logo representation of an alignment of archaeal BHB sequences in which the height of each letter stack represents the extent of conservation (Fig 1C; Crooks *et al*, 2004). The initial 2 nt of the euryarchaeal BHB helix are most often C and U; the conserved nucleotides interact with ribosomal RNA and precede the anticodon of the tRNA. The bulge containing two intron-derived nucleotides (B1 in Fig 1) shows a greater conservation with predominant guanosine and adenosine residues at the helix–bulge B1 junction (Fig 1C). This adenosine was shown to be the most important recognition element by the archaeal splicing endonuclease and is positioned at a sharp bend in a conserved cross-subunit arginine–nucleotide–arginine stack (Xue *et al*, 2006). Nevertheless, the overall conservation of the BHB sequence seems to be lower than originally thought (Kleman-Leyer *et al*, 1997). It should be noted that BHB motifs were also found at archaeal introns of both ribosomal RNA (rRNA) and mRNA (Kjems & Garrett, 1988; Tang *et al*, 2002; Watanabe *et al*, 2002).

Recent computational studies predicted the simultaneous presence of two introns in certain crenarchaeal tRNAs and even three introns in $tRNA^{Pro}$ of *Thermofilum pendens* Hrk 5 (Sugahara *et al*, 2007). In this specific case, the pre-tRNA folds into a conformation that allows the splicing of a third intron only after the splicing of the first two introns (Fig 2B). The average length of reported archaeal tRNA introns is approximately 20–25 nt, and the individual length varies between 11 and 175 nt. It is likely that such long introns—longer than the tRNA—have a functional role. This is exemplified by the 104-bp intron of *Halobacterium volcanii* $tRNA^{Trp}$, which is suggested to mediate the 2'-O-methylation *in cis* of 2 nt of tRNA (Clouet-d'Orval *et al*, 2005).

Split tRNA genes

A unique type of tRNA gene disruption was found in the genome of *Nanoarchaeum equitans,* which is the only known organism to contain tRNA half-genes as well as four intron-containing tRNA genes (Randau *et al*, 2005b). Both a 5' tRNA half-gene and a 3' tRNA half-gene exist for six tRNA genes, which are distributed widely throughout the genome (Randau *et al*, 2005c). One exception is that two 5' $tRNA^{Glu}$ isoacceptor halves share the same 3' $tRNA^{Glu}$ half, which raises the interesting questions of whether and how transcription is controlled to guarantee the production of these molecules in a 1:1 ratio (from 2:1 half-genes). Each split tRNA gene is preceded by its own promoter, so that the initial transcripts are tRNA half-molecules that need to find each other in the cell (Fig 2C). This is guaranteed by a 12–14 bp GC-rich sequence after the 5' tRNA half that has a perfect reverse-complementary match to a sequence preceding its corresponding 3'-tRNA half. Once these sequences are annealed, a helix forms that is proposed to facilitate the folding of the tRNA

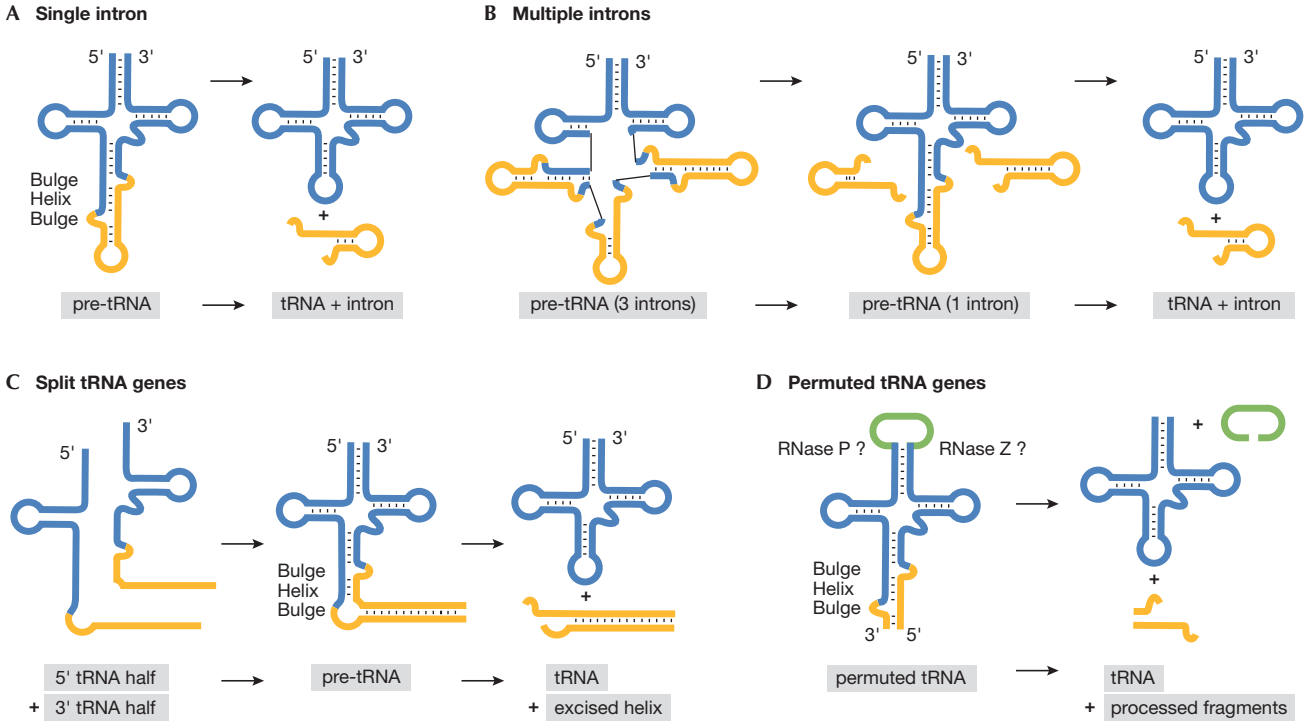


Fig 2 | Schematic overview of pre-transfer RNA processing of unusual transfer RNA gene products. (**A**) Processing of single introns in members of the Archaea. The conserved structural bulge–helix–bulge (BHB) motif is recognized by the splicing endonuclease. (**B**) Processing of up to three introns in *Thermofilum pendens*. Two BHB motif-containing elements are spliced out leading to the formation of pre-transfer RNA (pre-tRNA) with a third intron. (**C**) Split tRNAs in *Nanoarchaeum equitans* are assembled by long reverse-complementary sequences. The helix–tRNA junctions fold into the BHB motif, which are recognized by the splicing endonuclease. (**D**) Permuted tRNAs in *Cyanidioschyzon merolae* show a splicing motif on folding of the reversed tRNA sequences. The circular product is probably processed by RNase P and RNase Z to yield the mature 5' and 3' termini.

body. The junctions of these helices and the tRNAs form the intron-characteristic BHB motifs that are then recognized and spliced by the *N. equitans* splicing endonuclease (Randau *et al*, 2005a). This enzyme shows the heteromeric subunit conformation found in members of the Crenarchaeota that seems to be optimized for the recognition of non-canonical introns. As the six split tRNAs, as well as the four intron-containing tRNAs, are processed by the same splicing machinery, a common origin of both phenomena seems likely.

N. equitans shows another unique feature in the organization of its tRNA genes: it is the only known organism that has been shown to circumvent the need for RNase P, which is the otherwise universal ribozyme required for the removal of 5' leader sequences from pre-tRNA transcripts (Altman *et al*, 1989). In *N. equitans,* accurate promoter placement ensures that all tRNA genes begin transcription at the first nucleotide of the mature tRNA, resulting in leaderless tRNAs. Transcription initiation can only proceed with a purine; therefore, the three tRNAs that require a pyrimidine at position one contain an additional 5' purine base for tRNA genes. The apparent loss of RNase P in this organism is also supported by the detection of mature 5'-triphosphorylated tRNA species in total RNA extracts (Randau *et al*, 2008).

Permuted tRNA genes

Recently, another example of an unusual tRNA gene disruption was found in the nuclear genome of the red alga *Cyanidioschyzon merolae* (Soma *et al*, 2007). In total, 11 tRNA genes were identified in which the 3' region of the tRNA is located upstream of the 5' half of the tRNA gene. The two regions are separated by an intervening sequence 7–74 bp in length. On folding of the tRNA, this intervening sequence connects the mature termini of the tRNA in a circular fashion (Fig 2D). It is proposed that the enzymes usually involved in processing the 5' terminus (RNase P) and the 3' terminus (for example, RNase Z) of a pre-tRNA molecule are able to precisely cut and remove this sequence from the circular intermediate. Therefore, the first step in the maturation of this tRNA is the removal of the 5'-leader and 3'-trailer sequences unusually located in the anticodon region of the tRNA (Fig 2D). BHB motifs were proposed to form at the junctions of these sequences and the tRNA body, which would then be recognized and cleaved by the *C. merolae* splicing endonuclease. Subsequently, an RNA ligase would circularize these tRNA precursors. This mechanism raises several interesting questions. First is how these tRNAs are transcribed, as eukaryotic tRNAs typically contain RNA polymerase III elements located within the tRNA gene. These elements are known as A box and B box, and overlap with conserved regions of the D-arm and T-arm portions of the tRNA gene. This promoter arrangement is not possible in permuted tRNA genes, in which the T-arm lies unusually upstream of the D-arm. To compensate for the lack of the standard internal promoter, an upstream sequence has been proposed to be a potential external promoter resembling the archaeal

promoter organization. Second, as the occurrence of BHB-splicing motifs was previously thought to be an archaeal phenomenon, it will be of interest to determine to what extent the *C. merolae* splicing endonuclease resembles its archaeal counterpart, and to investigate how—and why—this peculiar algae adapted the described archaeal-like tRNA-processing elements.

Evolution of disrupted tRNA genes

The situations described above have led us to consider the evolution of the archaeal tRNA gene disruptions. Initially, one has to ask, what came first: did contiguous tRNA genes acquire introns and other gene disruptions over time, or did ancient fragmented tRNA genes evolve into connected versions?

There is substantial evidence that the modern tRNA gene originated from tRNA halves. The finding that a minihelix—that is, the acceptor stem plus T-arm—is an authentic substrate for several aminoacyl-tRNA synthetases provides what is possibly the best support for a hypothesis stating that this highly conserved part of the tRNA is older than the anticodon-arm–D-arm portion of the same molecule (Schimmel & Ribas de Pouplana, 1995; Weiner & Maizels, 1987; Widmann *et al*, 2005).

Nevertheless, it is questionable whether this origin of tRNA from two different hairpin portions is still reflected in modern fragmented tRNA genes. In this context, it is important to understand that these two coaxially stacked hairpins (minihelix and anticodon-arm–D-arm) do not comprise the exact regions of 5' and 3' half molecules separated in the anticodon loop. The tRNA halves created by canonical introns cannot fold to form the acceptor stem and the anticodon stem.

It has also been argued that the split tRNA genes of the supposedly early diverging *N. equitans* represent the ancient tRNA gene organization (Di Giulio, 2006). However, recent reinvestigation of the phylogenetic position of *N. equitans* placed this organism as a member of a fast-evolving euryarchaeal lineage with significant parasitic adaptation (Brochier *et al*, 2005; Das *et al*, 2006). Therefore, it seems likely that the split tRNA genes are derived from intron-containing genes that were separated in the process of genome rearrangement. This is in accordance with the observation that many conserved gene clusters are found separated in *N. equitans*, and that several split genes encoding conserved and usually contiguous proteins are present in its genome (Waters *et al*, 2003).

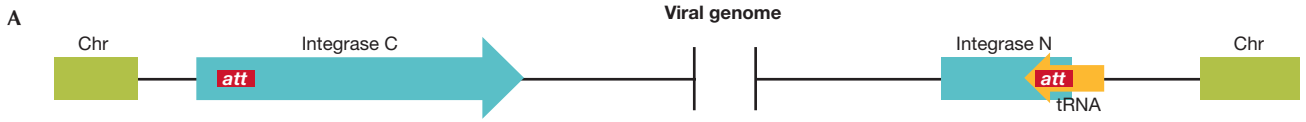
In our opinion, a scenario of late acquisition of introns by contiguous tRNA genes seems more plausible. Possibly the most convincing evidence for this derives from the identification of the highly variable intron-insertion sites in crenarchaeal tRNA genes. Here, the BHB-type introns do not simply disconnect two assumed ancient tRNA halves in the anticodon loop, but are inserted in different tRNA domains. For example, the introns in the acceptor stem of three tRNAs of the crenarchaeon *Pyrobaculum aerophilum* disconnect only the first 3 nt from the rest of the tRNA. Further evidence is provided by the simultaneous presence of two or three partly overlapping introns in tRNAs from *P. aerophilum, T. pendens* or *Methanopyrus kandleri,* which indicates a cumulative acquisition of these introns.

Integration at tRNA genes

What causes tRNA genes to maintain introns? We believe that to answer this question it is desirable to investigate the interactions of mobile genetic elements such as conjugative plasmids and viruses—both of which integrate into tRNA genes—with their hosts. Viruses are described as the most abundant biological entity on Earth, clearly outnumbering their hosts (Bergh *et al*, 1989). Although much less is known about archaeal viruses than their bacterial counterparts, their abundance is evident and many archaeal viruses have been isolated from hydrothermal environments, which are also home to the tRNA-intron-rich extremophiles discussed above (Rice *et al*, 2001). The viruses and virus-like particles with crenarchaeal hosts all show unusual and diverse morphologies, and are known to infect the genera *Sulfolobus*, *Acidianus*, *Thermoproteus* and *Pyrobaculum*. The remarkable range of these viruses is exemplified by electron-microscopic studies of the viral particles found in two hot springs at Yellowstone National Park in the USA (Rachel *et al*, 2002). Potential hosts include the archaeal genera *Thermophilum*, *Thermoproteus*, *Pyrobaculum*, *Thermospheaera* and *Archaeoglobus*, as well as members of the archaeal groups Desulfurococcales, Korarchaeota and Nanoarchaeota (Rachel *et al*, 2002).

Many mobile genetic elements integrate into the archaeal chromosome by site-specific recombination occurring within a short region of perfect homology between the host chromosome (*attB* sequence) and the viral or plasmid DNA (*attP* sequence; Reiter *et al*, 1989). Their typical targets are tRNA genes, and this concept is considered to have been conserved during the evolution of integrating viruses of the Archaea, Bacteria and Eukaryota (Reiter *et al*, 1989; Winckler *et al*, 2005). The best-studied archaeal model for viral integration focuses on members of the family *Fuselloviridae*. Here, full-length copies of spindle-shaped virus (SSV) genomes are found integrated into the tRNA genes of their host. The viral-encoded SSV1 integrase acts as a site-specific endonuclease and ligase (She *et al*, 2004). On insertion into the host genome, the integrase gene is partitioned into an amino-terminal portion that overlaps with the reconstituted tRNA gene target and a carboxy-terminal portion with a direct repeat of the tRNA fragment-attachment site (Fig 3). The *attB* site of SSV1 is a 44-bp fragment of a $tRNA^{Arg}$ gene (Reiter *et al*, 1989), SSV2 contains a 48-bp fragment identical to a portion of the host $tRNA^{Gly}$ gene and SSV RH (isolated from the Ragged Hills area of Yellowstone National Park) contains a 59-bp *attB* site that is identical to a host $tRNA^{Leu}$ gene (Wiedenheft *et al*, 2004). In most cases, the *attB* sites comprise the 3' region of the tRNA with its highly conserved T-arm and the anticodon loop, which is the site of most introns (Fig 3B). One interesting example is the integration of the SSV K1 genome, which was shown to target the $tRNA^{Asp}$ gene and, surprisingly, also two $tRNA^{Glu}$ genes of *Sulfolobus solfataricus* (Fig 3B). The *attB* sites of $tRNA^{Asp}$ and the two $tRNA^{Glu}$ differ only in 4 nt, and the viral *attP* sequence comprises exactly the 49-nt of the 3' terminal $tRNA^{Asp}$. However, if integration into $tRNA^{Glu}$ occurs, the reconstituted tRNA gene acquires the viral *attB* site and is mutated (Fig 3B). This process introduces four mutations that alter the anticodon and the discriminator base of the tRNA, and switches its identity from $tRNA^{Glu}$ to $tRNA^{Asp}$. As the attachment site is misinterpreted in the original paper (Wiedenheft *et al*, 2004), it has been adjusted to account for the correct orientation of the tRNA gene (Fig 3B). It would be interesting to investigate whether these chimeric tRNAs are functional in protein biosynthesis. This imprecise integration not only provides a fascinating insight into the evolutionary relationship of these two tRNA species but could also be an obvious reason why the host organism would attempt to prevent such viral integration.

Many diverse archaeal viruses have been discovered, and shown to be inserted into the 5'-distal and 3'-distal regions of tRNA genes (Krupovic & Bamford, 2008; Pagaling *et al*, 2007). Interestingly, there are also examples of viruses that contain tRNA genes, and even tRNA genes containing introns (Haring *et al*, 2005).



B

	Acceptor stem	D-stem	D-loop	D-stem	Anticodon stem	Anticodon loop	Anticodon stem	Variable arm	T-stem	T-loop	T-stem	Acceptor stem
	>>>>>>	..>>>>	....	<<<<.	>>>>>	.....	<<<<<	....	>>>>>	.....	<<<<<	<<<<<<<.
tRNA-Leu	GCGGGGG	TGCCCG	AGCTAGGGCAA	AGGGG	GCGGA	CTGAGGC	TCCGC	TGGCGAAGGCCTGC	ACGGG	TTCAAAT	CCCGT	CCCCCGCA
P2 attP				GGG	GCGGA	CTGAGGC	TCCGC	TGGCGAAGGCCTGC	ACGGG	TTCAAAT	CCCGT	CCCCCGCA
tRNA-Arg	GGACCCG	TAGCTC	AGCCAGGATG	GAGCG	CCGGC	CTCCGGA	GCCGG	AGGtC	CCGGG	TTCAAAT	CCCGG	CGGGTCCG
SSV1 attP					GC	CTCCGGA	GCCGG	AGGtC	CCGGG	TTCAAAT	CCCGG	CGGGTCCG
tRNA-Gly	GCGGCCG	TAGTCT	AGGCTGGATTA	GGACG	CCGGC	CTCCCAA	GCCGG	TGAtC	CCGGG	TTCAAAT	CCCGG	CGGGCGCA
SSV2 attP				G	CCGGC	CTCCCAA	GCCGG	TGAtC	CCGGG	TTCAAAT	CCCGG	CGGCCCCA
tRNA-Asp	GGCGCGG	TAGTCT	AGTGGTGTA	GGATG	CGGGC	CTGTCGA	GCCGC	TGAC	CCGGG	TTCAAAT	CCCGG	CCGCGGCG
tRNA-Glu	GCCGCGG	TGGTTT	AGCCCGGTCA	GAATG	CGGGC	CTCTCGA	GCCGG	AGGC	CCGGG	TTCAAAT	CCCGG	CCGGGGCA
tRNA-Glu	GCCGCGG	TGGTTT	AGCCCGGTCA	GAATG	CGGGC	CTTTCGA	GCCGG	AGGC	CCGGG	TTCAAAT	CCCGG	CCGCGGCA
K1 attP				ATG	GGGGC	CTGTCGA	GCCGC	AGGC	CCGGG	TTCAAAT	CCCGG	CCGCGGCG

Fig 3 | Integration of archaeal viruses into transfer RNA genes and attachment sites. (**A**) Schematic presentation of a spindle-shaped virus (SSV) genome integrated in its host chromosome (Chr). The amino-terminus of the partitioned integrase gene (integrase N) restores the 3' terminal portion of the targeted tRNA gene (orange) with its attachment site (*att*). A direct repeat of this sequence is present in the carboxy-terminal integrase fragment (integrase C). (**B**) Four examples of attachment sites of SSV viruses and the corresponding transfer RNA (tRNA) sequences of the *Sulfolobus* host. The secondary structure of the tRNA is indicated as helices ('<' and '>' symbols) and unpaired bases (dots). The integration of SSV K1 mutates potential $tRNA^{Glu}$ targets at four positions (yellow), which changes the identity of the tRNA species from Glu to Asp (modified from Wiedenheft *et al*, 2004).

The highly conserved tRNA and rRNA genes are ideal integration targets, and, frequently, introns can be found in these genes. It can be easily argued that the presence of an intron in a tRNA gene is a valuable protection mechanism against the integration of viruses and other autonomous genetic elements. Similarly, split and permuted tRNA genes would be able to prevent such integration if the *attB* sites are disrupted. This scenario would explain the direct evolutionary pressure for maintaining disrupted tRNA genes instead of mutating them into a contiguous version. As the different *attP* sites are specific for the various individual mobile elements, only the directly targeted tRNA genes would benefit from the disruption of their corresponding *attB* sites. Following this line of thought, an organism would contain more introns either if it was a preferred target for an increased number of mobile elements or if the integrative elements were of particular harm to the organism. One example might be the extremely small and condensed genome of *N. equitans*; in this organism, the obvious need for genome reduction would benefit from a lack of integration.

A different perspective is that the formation of direct repeats of tRNA fragments on integration of viral particles might provide a mechanism for the generation of the more unusual tRNA gene disruptions discussed above. To understand the details of how permutations and split tRNAs might arise from insertion and excision events, it is desirable to investigate mobile genetic elements in organisms with a high tRNA-intron rate. Additional plasmids and viruses carrying integrase genes are no doubt awaiting discovery, and will aid our understanding of the full extent of their impact on genome evolution.

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