The anticodon of the maize chloroplast gene for $tRNA^{Leu}_{UAA}$ is split by a large intron

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ABSTRACT

The maize chloroplast gene encoding $tRNA^{Leu}_{UAA}$ has been sequenced. It contains a 458 base pair intron between the first and second bases of the anticodon. The tRNA is 88 nucleotides long (the 3'-terminal CCA sequence included which, however, is not encoded by the gene) and differs in only four nucleotides (modified nucleotides are not considered) from the corresponding isoacceptor from bean chloroplasts. The unusual position of the intron in this maize chloroplast tRNA gene suggests a splicing model different from that generally accepted for eukaryotic split tRNA genes.

INTRODUCTION

Protein synthesis in green plant cells takes place in three well separated compartments: the nucleo-cytoplasm, the chloroplasts and the mitochondria. Organellar protein synthesis is however largely dependent on activities of the nucleo-cytoplasmic system, as is exemplified by the presence, in chloroplasts, of nuclear-encoded ribosomal proteins (1, 2) and aminoacyl-tRNA synthetases (3, 4). Whereas a number of plastid proteins are synthesized in the nucleo-cytoplasm and are transported into the plastid (1 - 7), there is no evidence that any of the RNA molecules found in plastids are transcripts of nuclear genes.

Hybridization of tRNAs to chloroplast DNA (8 - 11) and mapping of the genes on the maize (12) and spinach (13) chloroplast chromosomes suggest that the plastid genome codes for a full complement of tRNAs.

Chloroplast tRNAs can be aminoacylated by bacterial enzymes (14). This is not surprising since, in all the cases reported, sequence homology with bacterial isoacceptors is higher than 60%. However, all the plastid tRNA gene sequences reported so far (15 - 20) differ from bacterial tRNA genes in that the 3'-terminal CCA sequence is encoded by the bacterial but not by the chloroplast genome. This is a feature chloroplasts share with eukaryotic nuclear tRNA genes.

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Split chloroplast genes have been found in Chlamydomonas reinhardii for the 23S rRNA gene (21) and more recently in the $tRNA^{Ile}$ and $tRNA^{Ala}$ genes in the rDNA spacer region in maize (18). Introns have also been shown to exist in some mitochondrial genes (22 - 24) and they seem to be very common in eukaryotic nuclear genes. They are absent from bacterial genes. Their presence is thus restricted to eukaryotic cells including their organelles.

There are two main differences between the introns found in maize chloroplast tRNA genes and those in eukaryotic nuclear tRNA genes (25 - 33): a) in chloroplasts the introns are very long (949 base pairs in the case of the $tRNA^{Ile}$ gene and 806 base pairs in the case of the $tRNA^{Ala}$ gene) whereas they are much shorter (20 to 60 base pairs) in the nuclear tRNA genes in which they occur; b) in the nuclear genes they seem to be located in an invariant position in relation to the anticodon loop (between positions 37 and 38 of the mature tRNA, see ref. 34), whereas in chloroplasts there seems to be no general rule for locations in the anticodon loop. The precise location of the intron in the maize chloroplast $tRNA^{Ala}$ gene is not yet known, but it has been established recently in the case of the $tRNA^{Ile}$ gene to lie between nucleotides 38 and 39 (40) of the mature tRNA. We report here the presence in the maize chloroplast gene encoding $tRNA^{Leu}_{UAA}$ of a long (458 base pairs) intron located in the anticodon triplet between positions 34 and 35 of the mature tRNA.

MATERIALS AND METHODS

Maize chloroplast DNA fragment Bam 5 was cloned into RSF 1030 (35). The recombinant plasmid was designated pZmc 3119.

Isolation and labeling of nucleic acids, filter hybridizations and DNA sequencing have been described earlier (17).

RESULTS

Bam HI fragment 5 (Bam 5) of the maize chloroplast genome is located in the large single copy region of the chromosome (36). It is 6302 base pairs long and contains three Eco RI restriction sites (Fig. 1). Two of these are located 56 and 104 base pairs respectively from the terminal Bam HI sites. The third Eco RI restriction site is found at position 2245 from the left-hand Bam site and delimits Eco RI fragments "o" and "d" of the maize chloroplast Eco RI restriction map. The unique Sal I restriction site occurs at position 2821, delimiting Sal I fragments "D" and "A" of the maize chloroplast chromosome.

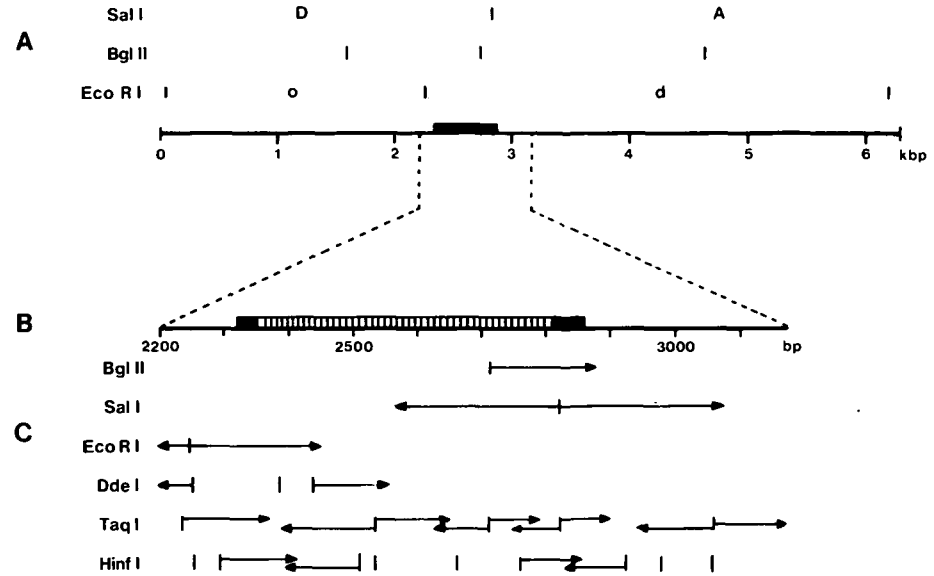

Figure 1: Physical map of Bam fragment 5 from maize chloroplast DNA and sequencing strategy of the region containing the $tRNA^{Leu}_{UAA}$ gene.
Part A shows the location of the restriction sites for Sal I (A and D represent parts of Sal fragments A and D respectively from the maize chloroplast genome), Bgl II and Eco RI ("d" and "o" represent Eco RI fragments d and o respectively). Small vertical bars show the restriction sites for the various enzymes. The solid horizontal line (between 2 and 3 kbp) corresponds to the $tRNA^{Leu}$ gene.
Part B shows an enlargement of the region containing the $tRNA^{Leu}$ gene: solid areas represent the regions which are found in the mature tRNA, the hatched area represents the intron.
Part C shows the sequencing strategy used for the determination of the DNA sequence of the $tRNA^{Leu}$ gene. The fragments were labeled on their 5'-ends using the T4 polynucleotide kinase; after redigestion with suitable restriction enzymes (for Bgl II, Sal I, Eco RI and Dde fragments) or strand-separation (for Taq I and Hinf I fragments) the nucleotide sequence was determined according to Maxam and Gilbert (42)

Bam 5 codes for at least four tRNAs (Steinmetz et al., manuscript in preparation). One of these is $tRNA^{Leu}_{1}$ ($tRNA^{Leu}_{UAA}$), as could be shown by tRNA catching experiments (12, 17) and by hybridization of radioactively labeled purified and identified tRNAs to Southern blots (37) carrying Bam HI-generated maize chloroplast DNA fragmens (12). The nucleotide sequence of Bam 5 revealed that the gene encoding $tRNA^{Leu}_{1}$ extends from position 2321 to position 2863 (Fig. 1 and 2), with a total length of 542 base pairs.

In the search for tRNA genes we screened for GTTC sequences which are indicative of the GTΨC sequence present in the T-arm of most tRNA species.

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      2250       2260       2270       2280       2290       2300       2310       2320
5'TAATGAATTC AATGATTCAA AAAAAACTAA GAGATGGATT AAATTATACA AGGAATCCTG GTTTCAAAGA AAAGTAAAAT
3'ATTACTTAAG TTACTAAGTT TTTTTTGATT CTCTACCTAA TTTAATATGT TCCTTAGGAC CAAAGTTTCT TTTCATTTTA

      2330       2340       2350       2360       2370       2380       2390       2400
GGGGATATGG CGAAATCGGT AGACGCTACG GACTTGATTG TATTGAGCCT TGGTATGGAA ACCTGCTAAG TGGTAACTTC
CCCCTATACC GCTTTAGCCA TCTGCGATGC CTGAACTAAC ATAACTCGGA ACCATACCTT TGGACGATTC ACCATTGAAG

      2410       2420       2430       2440       2450       2460       2470       2480
CAAATTCAGA GAAACCCTGG AATGAAAAAT GGGCAATCCT GAGCCAAATC CCTTTTTTGA AAAACAAGTG GTTCTCAAAC
GTTTAAGTCT CTTTGGGACC TTACTTTTTA CCCGTTAGGA CTCGGTTTAG GGAAAAAACT TTTTGTTCAC CAAGAGTTTG

      2490       2500       2510       2520       2530       2540       2550       2560
TAGAACCCAA AGGAAAAGGA TAGGTGCAGA GACTCAATGG AAGCTGTTCT AACGAATCGA AGTAATAACG ATTAATCACA
ATCTTGGGTT TCCTTTTCCT ATCCACGTCT CTGAGTTACC TTCGACAAGA TTGCTTAGCT TCATTATTGC TAATTAGTGT

      2570       2580       2590       2600       2610       2620       2630       2640
GAACCCATAT TATAATATAG GTTCTTTATT TTATTTTTAG AATGAAATTA GGAATGATTA TGAAATAGAA AATTCATAAT
CTTGGGTATA ATATTATATC CAAGAAATAA AATAAAAATC TTACTTTAAT CCTTACTAAT ACTTTATCTT TTAAGTATTA

      2650       2660       2670       2680       2690       2700       2710       2720
TTTTTTTTAG AATTATTGTG AATCTATTCC AATCAAATAT TGAGTAATCA AATCCTTCAA TTCATTGTTT TCGAGATCTT
AAAAAAAATC TTAATAACAC TTAGATAAGG TTAGTTTATA ACTCATTAGT TTAGGAAGTT AAGTAACAAA AGCTCTAGAA

      2730       2740       2750       2760       2770       2780       2790       2800
TTAATTTTAA AAAGTGGATT AATCGGACGA GGATAAAGAG AGAGTCCCAT TCTACATGTC AATACTGACA ACAATGAAAT
AATTAAAATT TTTCACCTAA TTAGCCTGCT CCTATTTCTC TCTCAGGGTA AGATGTACAG TTATGACTGT TGTTACTTTA

      2810       2820       2830       2840       2850       2860       2870       2880
TTCTAGTAAA AGGAAAATCC GTCGACTTTA TAAGTCGTGA GGGTTCAAGT CCCTCTATCC CCAAACCCTC TTTTATTCCC 3'
AAGATCATTT TCCTTTTAGG CAGCTGAAAT ATTCAGCACT CCCAAGTTCA GGGAGATAGG GGTTTGGGAG AAAATAAGGG 5'
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Figure 2: Nucleotide sequence of the $tRNA^{Leu}_{UAA}$ gene.
The numbers above the sequence refer to the nucleotide number in the Bam 5 sequence. Boxed sequences (2321-2355 and 2814-2863) represent the $tDNA^{Leu}_{UAA}$ sequence (non coding strand). The sequence located between the boxed regions (2356-2813) corresponds to the 458 base pair intron. The dotted region represents an open reading frame with the initiation codon underlined (2566-2568) and the termination codon at position 2386-2388. Possible promoter signals for transcription are underlined (2768-2774 and 2788-2795) (see text)

One of these sequences was found in a region which could be folded into a perfect T-stem and -loop. It was identical to that found in the corresponding region in the bean chloroplast $tRNA^{Leu}_{1}$ (38). Further homology with the bean isoacceptor was found in the flanking regions of this T-arm. Total continuous homology with the bean $tRNA^{Leu}_{1}$ extended over 50 nucleotides, starting at the 3'-end and ending after the first base of the anticodon (between positions 34 and 35). Only three differences (base substitutions) from the bean isoacceptor were found in the region corresponding to the extra arm of the tRNA. Beyond the anticodon, the sequence of the maize $tRNA^{Leu}_{1}$ gene differed completely from that of the tRNA of bean but when we searched for the sequence complementary to the 3'-end of the acceptor stem (CCCCA), we were able to find the second half of this $tRNA^{Leu}_{1}$ some 450 base pairs further upstream. Again, this half showed, with one exception,

perfect identity with the 5'-half of the bean isoacceptor.

Since the two halves were separated by a long sequence not found in the mature tRNA, it became clear that the maize chloroplast $tRNA^{Leu}_{1}$ gene is split by an intervening sequence located between the first and second nucleotides of the anticodon triplet.

The sequence of the $tRNA^{Leu}_{1}$ gene (Fig. 2 and Fig. 3) shows that, as in the case of the seven other chloroplasts tRNA genes reported so far (15 - 20), the 3'-terminal CCA sequence is not encoded in the gene. The cloverleaf structure (Fig. 3) of the deduced tRNA sequence shows that $tRNA^{Leu}_{1}$ is 88 nucleotides long (CCA included). It has a 3 base pair D-stem, an 11 nucleotide D-loop and a 16 nucleotide long extra arm. The anticodon is UAA, as in the case of $tRNA^{Leu}_{1}$ of bean chloroplast (38). The intron (Fig.2,4 and 5), which starts at position 2656 of the Bam 5 sequence, or after position 34 of the tRNA (34), is 458 base pairs long. It shows three interesting features: a) the first and last nucleotides of the intron are "G"s; b) five A-U pairs, two U-G pairs and three pairs of noncomplementary bases can be found in the next ten positions in the two strands of the intron (Fig. 4 and 5). This latter feature would not provide a strong secondary structure in the splicing region and might serve for recognition by a processing enzyme; c) the overall A & T content of the intron is high (68%).

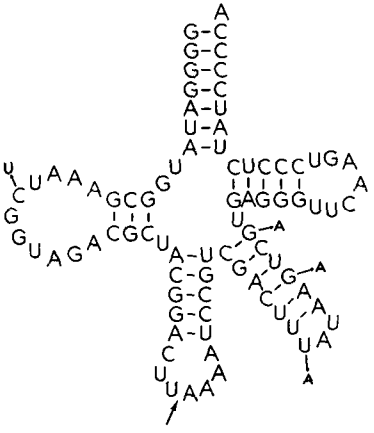

Figure 3: Cloverleaf structure of the maize chloroplast $tRNA^{Leu}_{UAA}$ as deduced from the tDNA sequence.
Modified nucleotides as well as the 3'-terminal CCA sequence are not shown. Small arrows point to differences with the bean chloroplast isoacceptor (see text). The heavy arrow indicates the position of the intron.

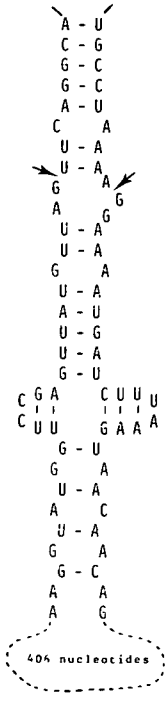

Figure 4: Possible secondary structure of the flanking regions of the intron. Start and end of the intron are marked with arrows. See also Figure 5.

DISCUSSION

Is this $tRNA^{Leu}_{UAA}$ gene expressed in vivo? Or is there another $tRNA^{Leu}_{1}$ gene in the maize chloroplast chromosome?

Evidence for the presence of a single $tRNA^{Leu}_{1}$ gene in the maize chloroplast chromosome comes from hybridization of individual tRNAs to Southern blots carrying plastid DNA fragments obtained by digestion with Bam HI and other restriction endonucleases (12). When $tRNA^{Leu}_{1}$, purified by polyacrylamide gel electrophoresis and RPC-5 chromatography, is hybridized to Bam HI fragments of plastid DNA, only Bam 5 shows hybridization (12). The presence of a gene encoding $tRNA^{Leu}_{1}$ was also demonstrated by the tRNA-catching experiment (12, 17) which showed that Bam 5 contains genes for at least 4 tRNAs: $tRNA^{Ser}_{3}$, $tRNA^{Leu}_{1}$, $tRNA^{Phe}$ and $tRNA^{Thr}_{2}$. These four tRNA genes were found in the DNA sequence of Bam 5 which has been determined entirely (Steinmetz et al., manuscript in preparation). The UUA codon, which is recognized by $tRNA^{Leu}_{UAA}$, is used in maize plastids (39) and this tRNA was identified as

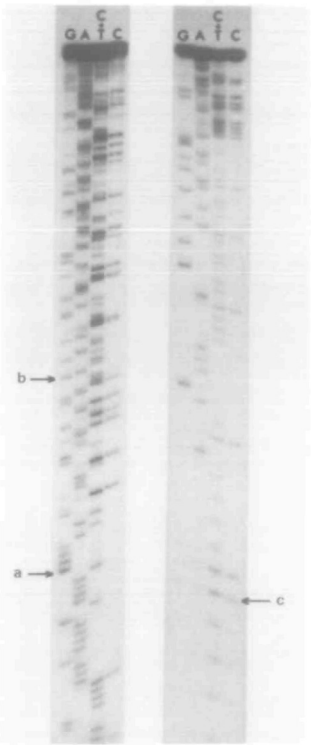

Figure 5: Autoradiographs showing sequencing gels of the regions flanking the intron.
Left Panel: 8% polyacrylamide gel showing the 5'-end of the tDNA^{Leu} (a) and the start of the intron (b). The sequence was obtained from the upper strand of the Taq I fragment starting at position 2236 (see Figure 1).
Right Panel: 20% polyacrylamide gel showing the end of the intron (c). This sequence (lower strand in Figure 2) was obtained from the lower strand (on strand separation gel) of the Taq I fragment which ends at position 2822 (this Taq I site corresponds to the Say I site).

that for leucine by its capacity to be charged with this amino acid.

What is the evidence for the unusual position of the intron?

Although the RNA sequence of the maize chloroplast tRNA$^{Leu}_{1}$ has not been determined, there is little doubt about the precise location of the intron in the gene for the following reasons: a) the proposed cloverleaf structure (Fig. 3) shows the expected very high homology with the sequence of bean chloroplast tRNA$^{Leu}_{1}$ (38) which also has the leucine anticodon UAA (see Fig. 3); b) the tRNA$^{Leu}_{1}$ used in the hybridization studies (12) has been doubly purified (two dimensional polyacrylamide gel electrophoresis and RPC-5 chromatography). No contamination with another tRNA species could be detected upon aminoacylation with other amino acids than leucine; c) if the intron occurred at the same position as in the eukaryotic tRNA genes, the anticodon would be UGA which is the anticodon for serine, not leucine, in the chloroplast system (39) as elsewhere.

Comparison of the positions of introns in chloroplast and nuclear tRNA genes.

In all eukaryotic nuclear tRNA genes so far known to contain introns,

these introns seem to be located in an invariant position, one nucleotide away from the anticodon toward the 3'-end (25-33). Although very little is known so far about structural features involved in this site-specific cleavage,it suggests that a single type of processing enzyme is responsible for the excision of the extra sequences in eukaryotes.

The situation seems to be different in chloroplasts. Split chloroplast tRNA genes have been found so far only in maize, but it seems likely that they are also present in some plastid tRNA genes of other higher plants. From the few data available, however, it appears that although the introns are located in the anticodon loop, they are not all found at the same place in this loop. Koch et al. (18) have described introns in the maize chloroplast genes for $tRNA^{Ala}$ and $tRNA^{Ile}$: the precise locations of these introns, however, could not be determined by these authors, but it was clear that the genes are split in the anticodon loop (on the 3'-side), outside of the anticodon triplet. Guillemaut et al. (40) have shown, by determining the sequence of $tRNA^{Ile}$ from maize chloroplasts, that the intron in the $tRNA^{Ile}$ gene is located between the anticodon loop and stem on the 3'-side (between positions 38 and 39 of the mature tRNA).

The significance of the different locations of introns in chloroplast tRNA genes is unknown. It suggests, however, that the splicing mechanism in chloroplasts is different from that found in eukaryotic nuclear tRNA genes, and that in chloroplasts splicing might be performed by several splicing enzymes, each with a different specificity, or by a single enzyme which recognizes yet undetermined structures common to all the precursors.

What is the function of the intron?

The unusual length of the introns in chloroplast tRNA genes as compared to the small (20 to 60 base pairs) introns in eukaryotic nuclear tRNA genes increases the possibility that the former might contain sequences coding for proteins. An open reading frame that could code for a 123 amino acid-long polypeptide has indeed been identified by Koch et al. (18) in the intron of the $tRNA^{Ile}$ gene and one for a 45 amino acid-long polypeptide can be discerned in the $tRNA^{Ala}$ intron.

No open reading frame can be found on the part of the RNA transcript which comprises the intron in the case of the maize chloroplast $tRNA^{Leu}_1$. However an open reading frame that could code for a 60 amino acid-long polypeptide is found on a possible transcript derived from the opposite strand (see Figure 2, lower strand: from position 2568 to position 2389). This open reading frame shows a lower A & T content (60.6%) than its leader sequence

(85%). There is no evidence so far that this is a real gene. Several translation products could be obtained in the E. coli linked transcription-translation system (K. Koller, personal communication) using the isolated Bam 5 fragment. Whether one of the in vitro synthesized small polypeptides is derived from the intron region has not yet been determined. A Shine-Dalgarno (41) sequence, which seems to be present in at least some chloroplast protein genes (39, Steinmetz et al., unpublished), cannot be found within the 12 nucleotides upstream of the possible initiation codon , although possible promoter sequences for transcription could be detected (see Figure 2). These data suggest that the possible transcript might not be translated.

The significance of the introns in tRNA genes is as unclear as is their origin. Koch et al. (18) have suggested that they may be remnants of transposable elements. These authors found similarities in the two introns they studied but we could not detect sequence homologies between the intron found in the $tRNA^{Leu}_1$ gene and the introns from the $tRNA^{Ile}$ and $tRNA^{Ala}$ genes.

Comparison of maize $tRNA^{Leu}_1$ with $tRNAs^{Leu}$ from other organisms.

Maize chloroplast $tRNA^{Leu}_1$, as deduced from the tDNA sequence, shows a very high degree of sequence homology with the $tRNA^{Leu}_1$ from bean chloroplasts (post-transcriptionally modified nucleotides are not considered in this comparison). Base substitutions can be found in four positions: maize chloroplast $tRNA^{Leu}_1$ contains a C in position 17, whereas in bean chloroplast $tRNA^{Leu}_1$ a U is found at that same position; the three other base substitutions are found in the extra arm at position 47:3 (A→U substitution), at positions 47:8 and 47:11 (A→G substitutions).

The sequence homology of chloroplast $tRNA^{Leu}_1$ with E. coli isoacceptors (34) can be as high as 65% ($tRNA^{Leu}_5$) or 54% ($tRNA^{Leu}_1$). Since the sequence homology of $tRNA^{Leu}_1$ with the other chloroplast isoacceptors for leucine from the same organism varies between 50 and 65%, it is interesting to note that they are as divergent from another as they are from E. coli isoacceptors.

Whereas the D-loop of tRNAs for histidine (17), isoleucine and alanine (18) of maize chloroplasts are reduced, the D-loop is normal in the $tRNA^{Leu}_1$ as well as in some other maize chloroplast tRNAs that have been sequenced (Steinmetz et al., manuscript in preparation). A reduced D-loop thus cannot be considered a general feature of maize plastid tRNAs.

Our findings confirm the unique position of plastid tRNAs on the evolutionary scale. They are predominantly prokaryote-like with regard to

nucleotide sequences but the presence of introns and the absence of the 3'-terminal CCA encoding sequence in the genome are features seen in some form in nuclear genes for tRNAs in eukaryotes. The large sizes of the introns and their variable locations further distinguish plastid from nuclear genes for tRNAs.

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