A leucine tRNA gene adjacent to the *qa* gene cluster of *Neurospora crassa*

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ABSTRACT

A single tRNA^{Leu} gene has been localized and sequenced from *Neurospora crassa*. It is located only 375 bp from the *qa* gene cluster and it is the only tRNA or 5S rRNA gene within this cloned 37 kb region. The gene encodes a tRNA^{Leu} with the anti-codon AAG, and unlike the other nuclear eukaryotic tRNA^{Leu}(AAG) gene sequenced (from *C. elegans*), contains an intervening sequence of 27 bp. The *Neurospora* tRNA^{Leu}(AAG) is 84% and 73% homologous respectively to the *C. elegans* and bovine tRNA^{Leu}(AAG), and is 84% homologous to a *Drosophila* tRNA^{Leu}(CAA). However, it is only 65% homologous to a yeast tRNA^{Leu}(CAA) and there is little conservation of intervening sequences or V-loop regions. The gene hybridizes to at least 16 other DNA fragments in the *Neurospora* genome. Its expression does not seem to be linked to that of the *qa* genes.

INTRODUCTION

Several instances have been reported in which an RNA polymerase III transcription unit has been localized close to a gene transcribed by RNA polymerase II (1-3). In one particular case, a leucine tRNA gene was identified 340 bp upstream of the yeast *leu2* gene, and it was proposed that the tRNA gene might be involved in controlling expression the *leu2* gene (2). We report here the identification of a leucine tRNA gene only 375 bp from one end of the inducible *qa* gene cluster of *Neurospora crassa* (4). The tRNA gene is the only class III gene within the cloned 37 kb region containing the *qa* gene cluster. The gene encodes the anticodon, AAG, and contains a 27 bp intervening sequence, in contrast to the *C. elegans* tRNA^{Leu}(AAG) gene (5) but in common with yeast (6) and *Drosophila* (7) tRNA^{Leu}(CAA) genes.

MATERIALS AND METHODS

In Vitro Transcription. For analytical reactions, 0.6 µg of supercoiled plasmid was incubated for 20 min at 30°C with 4 µl of transcription extract in a 25 µl reaction containing 2.5 µCi [α -³²P]UTP as described by Tyler and Giles (8). For tRNA splicing, after the transcription reaction, ATP was

added to 4 mM, UTP to 400 μ M and triton X-100 to 0.5%, and the reaction continued for 20 min at 30°C (8). RNA was extracted from the reaction mixtures and fractionated on 0.3 mm 7M urea/8% acrylamide gels (8). For preparation of 32 P-labelled tRNA for hybridization, 2 μ g of plasmid was incubated for 1 hour at 30°C with 8 μ l of extract in a 50 μ l reaction containing 100 μ Ci of [α - 32 P]UTP. 32 P-labelled tRNA was purified as described (8).

Filter hybridizations. Restriction endonuclease digests of the purified *Xba*I-*Cla*I fragment (see figure 2) were fractionated on a 10% glycerol/5% acrylamide gel, then transferred to diazobenzyloxymethyl paper as described by Geever et al. (9). The filter was hybridized with 32 P-labelled tRNA overnight at 42°C in 30% formamide, 0.75 M NaCl, 0.075 M Na₃ citrate (5X SSC), 0.1% glycine, 100 μ g/ml calf thymus DNA, 100 μ g/ml poly(U), 0.5% sodium dodecyl sulfate, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin, 0.02% ficoll.

For genomic hybridizations, 32 P nick-translated plasmid DNA was hybridized to size-fractionated restriction digests of *Neurospora* DNA on nitrocellulose filters as described by Geever et al. (9).

DNA sequencing. The DNA sequence determination was carried out on cloned wild-type (74A) DNA. DNA fragments were sequenced by the method of Maxam and Gilbert (10). All fragments were labelled at the 5' end using T4 polynucleotide kinase (New England Nuclear). Single end-labelled fragments were generated by digestion with a second restriction enzyme and purified by fractionation on a 5% acrylamide/10% glycerol gel. Sequenced fragments were fractionated on 8% and 20% acrylamide/7M urea gels and the gels exposed 1-2 days at -70°C without intensifying screens.

RESULTS

Identification and localization of a tRNA gene. In the course of examining transcription of the *qa-1F* gene of *Neurospora crassa* (11) by RNA gel blot hybridization, we observed that the 3.3 kb *Bam*HI-*Cla*I fragment containing the *qa-1F* gene also hybridized to a small molecular weight (4-5S) RNA species. To investigate the possibility that this fragment contained a 5S rRNA gene or a tRNA gene, a plasmid containing the fragment was used to program a *Neurospora* RNA polymerase III *in vitro* transcription system (8). As shown in Figure 1A track a, this plasmid directed the synthesis of two major transcripts of 128 nucleotides (nt) and 108 nt, plus a minor transcript at 81 nt. This pattern of transcripts resembled that produced from a

Figure 1. Transcription of pLH40 *in vitro*.

Panel A shows the products of *in vitro* transcription reactions. The reactions were programmed with 0.6 μ g pLH40 (*Bam*HI-*Cla*I fragment) DNA (tracks a and b), 0.6 μ g pMF3 (*tRNA*^{Phe} gene) DNA (tracks c and d) or 0.6 μ g pLH35 (*Bam*HI-*Xba*I fragment) DNA (track e). After the initial 20 min transcription reaction, the reactions shown in tracks b and d were continued for 20 min in the presence of 4 mM ATP, 0.5% triton X-100 and 400 μ M UTP (*tRNA* splicing conditions). Panel B shows the position of the *qa-1F* gene (11) with respect to the *Bam*HI, *Xba*I, and *Cla*I sites.

cloned *tRNA*^{Phe} gene (12) (track c) suggesting that the *Bam*HI-*Cla*I fragment contained a *tRNA* gene. Moreover, when the 128 nt and 108 nt transcripts were further incubated under conditions favoring *tRNA* processing and splicing (8), an 81 nt form accumulated (track b), suggesting the presence of an intervening sequence in the putative *tRNA* gene. Track d shows splicing of the *tRNA*^{Phe} transcripts under the same conditions. Since a plasmid (pLH35) containing the 2.0 kb *Bam*HI-*Xba*I fragment from the 5' end of the *Bam*HI-*Cla*I fragment did not direct synthesis of the *tRNA* (track e), the putative *tRNA* gene was tentatively localized to the 1.3 kb *Xba*I-*Cla*I region at the 3' end of the *qa-1F* gene.

In order to more precisely localize the gene, the *Xba*I-*Cla*I fragment was isolated, digested with a variety of restriction endonucleases, transferred to diazobenzyloxymethyl-paper and hybridized with the ³²P-labelled products of the *in vitro* transcription reaction. Figure 2A shows that the transcripts hybridized strongly to a 530 bp *Taq*I fragment, a 170 bp *Hinf*I fragment and to two *Hpa*I fragments 1090 bp and 260 bp. Accordingly the presumptive *tRNA* gene was localized as shown in Figure 2B, crossing the *Hpa*I site.

Figure 2. Localization of the Putative tRNA Gene.

Panel A: *in vitro* synthesized 32 P-labelled tRNA was hybridized to isolated *Xba*I-*Cla*I fragment DNA digested with the indicated restriction endonucleases. Panel B shows the deduced position and orientation (see text) of the putative tRNA gene.

To determine the direction of transcription, the *Bam*HI-*Cla*I plasmid was cleaved with *Hpa*I, digested with the 3' exonuclease activity of T4 DNA polymerase to expose different strand-specific single-stranded regions of the tRNA gene, redigested with *Bam*HI and the fragments hybridized with the *in vitro* synthesized 32 P-labeled transcripts. Since the transcripts hybridized to the leftward, *qa-1F* containing fragment (data not shown), the direction of transcription was inferred to be left to right, away from the *qa-1F* gene (Figure 2B).

Nucleotide sequence of the tRNA gene. To confirm the identity of the tRNA gene and to determine its specificity, 300 bp surrounding the *Hpa*I site were sequenced according to the strategy shown in Figure 3A. The sequence is shown in Figure 3B. By comparison with the invariant residues and secondary structures of tRNA sequences (13) and by using the sizes of the intervening sequence and V-loop predicted from the size of the *in vitro* transcription products, a leucine tRNA gene was identified. The gene has a 27 bp intervening sequence and an 11 bp V-loop. Transcription is away from the *qa-1F* gene, as predicted (see above). The anticodon is AAG, specific for leucine codons.

The tRNA^{Leu} gene hybridizes to at least 16 other tRNA genes in the *Neurospora* genome. To estimate the number of tRNA^{Leu} genes in the *Neurospora* genome, the *Xba*I-*Cla*I fragment containing the tRNA gene was hybridized to *Hind*III or *Eco*RI restriction endonuclease digests of *Neurospora* DNA. As

Figure 3. Nucleotide Sequence of the Leucine tRNA Gene.

A shows the sequencing strategy. Arrows indicate the direction and extent of the sequencing runs. All fragments were 5' end-labelled. The coding region is indicated by the shaded region. IVS indicates the intervening sequence.

B shows the nucleotide sequence. The coding region is shown in capitals while the flanking sequences and intervening sequences are shown in lower case. The anticodon and the T₀ RNA polymerase III terminator sequence are shown in italics. Double-underlined residues in the coding sequence differ from the *C. elegans* tRNA^{Leu} (AAG) sequence.

shown in Figure 4, at least 16 bands hybridized to the tRNA gene. Allowing for unresolved bands and bands lost due to poor transfer of small fragments (14), this indicates that there could be up to 21 individual sequences in the *Neurospora* genome hybridizing to this tRNA gene. It is unlikely that the multiple bands detected resulted from an unrelated repetitive sequence within the *Xba*I-*Cla*I fragment since ³²P nick-translated total *Neurospora* DNA hybridized only to the 170 bp *Hinf*I fragment containing the tRNA gene (data not shown).

DISCUSSION

A single leucine tRNA gene from *Neurospora crassa* has been identified and sequenced. The gene is located 375 bp downstream from the *qa-1F* regulatory gene at the end of the *qa* gene cluster on linkage group VII. The gene was identified by sequencing a region of DNA which directed the synthesis of tRNA-like transcripts *in vitro* and which hybridized to those transcripts. The gene was precisely localized and its anti-codon identified by examining the nucleotide sequence for residues and potential secondary structural features conserved among tRNAs, and by using the sizes of the extra arm

Figure 4.
Identification of Leucine tRNA Genes by
Hybridization to Genomic DNA.

N. crassa DNA was digested with *Eco*RI (A) or *Hind*III (B), fractionated by size, transferred to nitrocellulose and hybridized with the 1.3 kb *Xba*I-*Cla*I fragment containing the cloned tRNA^{Leu} gene (see Figure 2). The strongly hybridizing 12.5 kb *Eco*RI and 18 kb *Hind* III fragments correspond to the cloned gene.

and intervening sequence predicted from the *in vitro* transcription results. The anti-codon is AAG, which when modified to IAG (I is inosine) could recognize the leucine codons CUC, CUU and CUA (13). This is only the third eukaryotic nuclear tRNA^{Leu} sequenced with this anti-codon (5,13). The gene has an intervening sequence of 27 nucleotides and its position between residues 37 and 38 has been confirmed by directly sequencing the spliced *in vitro* transcript (8). The length of the extra arm or V-loop is also consistent with the gene encoding a leucine tRNA, since only leucine, tyrosine and serine tRNAs have been reported to have extra arms larger than 5 nucleotides (13).

The tRNA^{Leu} gene we have sequenced does not appear to occur in a tRNA gene cluster as *in vitro* transcription results indicate no other 5S rRNA or tRNA genes within the cloned 37 kb region containing the gene. However, it does hybridize to at least 16 and possibly up to 21 other DNA fragments in the genome. These fragments presumably contain other tRNA^{Leu} genes. While these may include pseudogenes, we do not know if they include any *Neurospora* tRNA^{Leu} genes with anticodons other than AAG. The sequence homology between isoaccepting tRNA's recognizing different anticodons varies from 63% to 85% in other species (13) and under the hybridization conditions used for the experiment shown in Figure 4 (hybridization in 6XSSC at 68°C, wash in 0.3 X SSC at 68°C) only sequences greater than 80% homologous to the tRNA gene would have been detected.

Figure 5.
Potential Secondary Structure for the Unspliced
Neurospora tRNA^{Leu} Precursor.

Arrows indicate the sites of splicing.
The anti-codon is indicated by a bar.

When compared to other eukaryotic leucine tRNAs there is significant sequence homology. The *Neurospora* tRNA^{Leu} (AAG) is 84% and 73% homologous respectively to the *C. elegans* (5) and bovine (13) tRNA^{Leu} (AAG) sequences. Despite the high degree of sequence homology between the *Neurospora* and *C. elegans* genes (Figure 4B), the *C. elegans* gene does not contain an intervening sequence. A comparison of the *Neurospora* tRNA^{Leu} (AAG) to the only *Drosophila* leucine tRNA gene sequenced (tRNA^{Leu} (CAA)) (7) indicates 84% homology, with identical matches for nucleotides 6-29 and 48-66, and differences only within the amino acid acceptor stem and the anticodon stem. However, the comparison to the same yeast tRNA^{Leu} gene indicated only 65% homology. In contrast, the *Neurospora* tRNA^{Phe} gene is 91% homologous to the same yeast tRNA gene (12). The *Neurospora* leucine tRNA gene sequence also shows only 63% and 54% homologies to the RNA sequences of a *Schizosaccharomyces pombe* and a *Torulopsis utilis* leucine tRNA (13). Taken together, these comparisons indicate that the yeast sequences show the least homology to the *Neurospora* gene of any other eukaryotic sequences.

Both the *Drosophila* and yeast tRNA^{Leu} genes contain intervening sequences between nucleotides 37 and 38. When the intervening sequence in the *Neurospora* gene is compared to those in the *Drosophila* and yeast genes there is little sequence homology. However, the intervening sequence of the *Neurospora* gene does have 6 residues complementary to the anticodon loop and could form the secondary structure shown in Fig. 5, like that proposed for the intervening sequence of the yeast tRNA^{Leu} (6). The intervening sequence of the *Drosophila* gene can also form such a structure (7).

It is intriguing that the tRNA gene is located so close to the inducible *qa* gene cluster. However, we have no evidence that expression of the *qa* genes and the tRNA gene are related. Neither transcription of the tRNA gene *in vitro* nor splicing of the transcripts are affected by quinic acid or by the presence of *qa* regulatory mutations in the strains from which the transcription extracts were derived. Further, the use of leucine CUU, CUC and CUA codons in the sequenced *qa* genes is not greater than in other sequenced *Neurospora* genes. In fact, while these codons constitute on average 68% and 53% of the leucine codons of *qa* structural genes (15; R. Geever and B. Rutledge, personal communication) and regulatory genes (L. Huiet, unpublished data) respectively, they constitute 88% on average of the leucine codons of the four other sequenced *Neurospora* genes (16,17, J. Fincham, personal communication). While these data are by no means conclusive nor exhaustive, it seems most likely that since CUC, CUU and CUA are the most commonly used leucine codons in *Neurospora*, the occurrence of a tRNA^{Leu} gene recognizing these codons close to the *qa* gene cluster is purely fortuitous.

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