Characterization of an unusual tRNA-like sequence found inserted in a *Neurospora* retroplasmid

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Received January 10, 2000; Revised and Accepted February 8, 2000 DDBJ/EMBL/GenBank accession nos AF213177, AF216708

ABSTRACT

We characterized an unusual tRNA-like sequence that had been found inserted in suppressive variants of the mitochondrial retroplasmid of *Neurospora intermedia* strain Varkud. We previously identified two forms of the tRNA-like sequence, one of 64 nt (TRL-64) and the other of 78 nt (TRL-78) containing a 14-nt internal insertion in the anticodon stem at a position expected for a nuclear tRNA intron. Here, we show that TRL-78 is encoded in Varkud mitochondrial (mt)DNA within a 7 kb sequence that is not present in *Neurospora crassa* wild-type 74A mtDNA. This 7-kb insertion also contains a perfectly duplicated tRNA⁷⁰ gene, segments of several mitochondrial plasmids and numerous GC-rich palindromic sequences that are repeated elsewhere in the mtDNA. The mtDNA-encoded copy of TRL-78 is transcribed and apparently undergoes 5′- and 3′-end processing and 3′ nucleotide addition by tRNA nucleotidyl transferase to yield a discrete tRNA-sized molecule. However, the 14 nt intron-like sequence in TRL-78, which is missing in the TRL-64 form, is not spliced detectably in vivo or in vitro. Our results show that TRL-78 is an unusual tRNA-like species that could be incorporated into suppressive retroplasmids by the same reverse transcription mechanism used to incorporate mt tRNAs. The tRNA-like sequence may have been derived from an intron-containing nuclear tRNA gene or it may serve some function, like mtRNA. Our results suggest that mt tRNAs or tRNA-like species may be integrated into mtDNA via reverse transcription, analogous to SINE elements in animal cells.

INTRODUCTION

The Mauriceville and Varkud strains of *Neurospora* harbor novel genetic elements termed mitochondrial retroplasmids (mRPs) (1,2). These mRPs exist predominantly as small circular DNA monomers of 3.6 and 3.7 kb for the Mauriceville (pMAU) and Varkud (pVAR) plasmids, respectively (Fig. 1), along with oligomers in which the monomers are organized in tandem head-to-tail repeats. The mRPs encode a reverse transcriptase (RT) and replicate via an RNA intermediate and reverse transcription step (3–5). The plasmid replication mechanism is analogous to that of certain RNA viruses in that the plasmid-encoded RT recognizes a 3′ tRNA-like structure of the mRP transcript and initiates synthesis of a full-length (−) strand cDNA de novo, beginning opposite the 3′ CCA. Based on this similarity, we and others have suggested that the plasmid replication mechanism may be transitional between RNA and DNA replication, perhaps arising at the time of transition from an RNA to DNA world (5,6).

The major steps in replication of the mRPs have been deduced from biochemical studies and characterization of intermediates. First, the double-stranded plasmid DNA is transcribed by the host *Neurospora* mitochondrial (mt)RNA polymerase from a promoter located ~260 bp upstream of the 5′-end of the plasmid transcript (7). Transcription around the plasmid coupled with RNA processing at a single site generates full-length linear RNAs that have a 3′ tRNA-like structure ending with two tandem CCA sequences (Fig. 1). These full-length transcripts serve both as mRNAs for the plasmid-encoded RT and as replication intermediates. In the first step of replication, the plasmid-encoded RT recognizes the plasmid transcript 3′ tRNA-like structure and/or 3′ CCA sequence and initiates cDNA synthesis de novo opposite the penultimate C residue (C-2) of the 3′ CCA (5,8). This de novo initiation, which is unprecedented for a DNA polymerase, appears to be the major mechanism used for initiation of cDNA synthesis both in vitro and in vivo (5,7). Some cDNA synthesis also
occurs by a template switching mechanism in which the 3′-end of a previously synthesized cDNA is used as a primer to initiate directly at the 3′-end of the mRP transcript (5,7,8). After synthesis of a full-length (−) strand cDNA by either mechanism, replication is presumably completed by (+) strand DNA synthesis and circularization to regenerate closed-circular plasmid DNA.

When the plasmid-containing strains are subjected to prolonged vegetative growth, suppressive variants of the plasmids arise that out-compete the wild-type plasmid and mtDNA, leading to progressively impaired growth and ultimately cell death (9–11). Remarkably, all these suppressive variants were found to have incorporated one of three mt tRNAs (tRNA^{Trp}, tRNA^{Gly} or tRNA^{Val}) or a tRNA-like sequence (TRL-64 or TRL-78) at the position corresponding to the 5′-end of the major plasmid transcript (Fig. 1). Because these inserted tRNA sequences include the 3′ nucleotide residues CC that are added post-transcriptionally by tRNA nucleotidyl transferase, we inferred that the insertion occurred via an RNA intermediate and reverse transcription step. A mechanism involving template switching between the plasmid transcript and the tRNA to generate a hybrid cDNA was supported by subsequent studies, which showed that the plasmid RT could carry out these reactions in vitro (12). It was also shown that the plasmid RT could initiate de novo at the 3′ CCA of tRNAs and then synthesize a full-length cDNA copy of the tRNA, so that template switching could occur either from the tRNA to the plasmid transcript or vice versa.

Insertion of the tRNA at a position corresponding to the 5′-end of the mRP transcripts leads to suppressive behavior of the plasmids by causing 25- to 100-fold overproduction of the mRP transcripts, which are presumed to be replication intermediates (10). The major transcripts of the suppressive plasmid are full-length linear RNAs that begin precisely at the 5′-end of the inserted tRNA sequence and are presumably generated by transcription beginning at the normal plasmid promoter, followed by site-specific cleavage at the 5′-end of the inserted tRNA by mt RNase P (10; S.Mohr and A.M.Lambowitz, in preparation). Overproduction of these transcripts could reflect either more efficient 5′-end processing as a result of the tRNA insertion or stabilization of the transcript by the 5′ tRNA or tRNA-like sequence.

The suppressive plasmid found in the Varkud variant V1-2 was unusual in that it had incorporated a novel 64-nt sequence (TRL-64) that could fold into a cloverleaf structure but did not correspond to a conventional mt tRNA (9,10). We subsequently isolated two additional suppressive plasmids (V51-6a and V34-14) that incorporated a longer form of this tRNA-like sequence (TRL-78) containing a 14-nt insertion in the anticodon stem at a position reminiscent of a nuclear tRNA intron (11). Here, we show that TRL-78 is encoded in Varkud mtDNA within a 7-kb region that is not present in Neurospora crassa wild-type 74A mtDNA. This unusual 7-kb sequence also contains parts of three different mitochondrial plasmids and a duplicated copy of the mt tRNA^{Trp} gene. The genomic copy of the tRNA-like sequence is transcribed in the wild-type Varkud strain and processed to yield a discrete 78-nt RNA. This novel tRNA-like species may have integrated into mtDNA via reverse transcription and it may serve some function, like mtRNA, or it may have been derived from an intron-containing nuclear tRNA gene.

The accession number for the Varkud EcoRI-3 sequence is AF213177 and that for the Hanapepe EcoRI-4 sequence is AF216708.

MATERIALS AND METHODS

Neurospora strains and growth conditions

Wild-type strains used in this study were N.crassa 74-OR23-1VA (designated 74A, FGSC no. 2489) and Mauriceville-1c (FGSC no. 2225) and Neurospora intermedia Varkud-1c (FGSC no. 1823) and Hanapepe (FGSC no. 3720). Senescent mutant V1-2 was described previously (9). A collection of wild-type strains and natural isolates that was surveyed for the TRL sequence is
listed in Table 1. Procedures for maintaining the strains, preparing conidia and growing cells in liquid culture were as described (13). Wild-type strains were grown in liquid culture for 14 h at 25°C and the V1-2 mutant was grown for 20–30 h at 25°C.

<table>
<thead>
<tr>
<th>Strain</th>
<th>FGSC no.</th>
<th>TRL-78</th>
<th>Palindrome no. 6</th>
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<td>-</td>
</tr>
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<td>2499</td>
<td>-</td>
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<td>626</td>
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**Neurospora** wild-type strains and natural isolates were screened for the presence of the TRL sequence by PCR of whole cell DNA using primers corresponding to the 5'- and 3'-ends of the TRL sequence (V1-2 5' and V1-2 3'), as well as primers corresponding to its flanking sequences in the Varkud EcoRI-3 fragment (5'-up and 3'-down) (see Materials and Methods and Fig. 3 for the sequences and location of the primers, respectively). The PCR products were gel purified and sequenced. Control PCR with primers specific for pVAR verified that none of the strains indicated to contain TRL-78 was contaminated with Varkud or V1-2 DNA. Neurospora tetrasperma strain 85 lacks the GC-rich palindromic sequence (no. 6 in Fig. 3) found upstream of TRL-78 in Varkud and other strains.

### DNA oligonucleotides

Synthetic DNA oligonucleotides used in this study were: dCBam, 5'-GGG GAT C16; dTBam, 5'-CCG CGG ATC CT16; TRL-78 3'R, 5'-GGG AAT TCT AGG TGC GAT TCC TTT GAC TTC TA; TRL-78 5'R, 5'-GGG GAT CCG CAT AAC CCT AAC GGT GAC TTC TT; tRNA7p, 5'-GCA AGC TTG CTT TTT TAC C; V1-2 3', 5'-CCG CGG ATC AGG TGG AAC TGA; V1-2 5', 5'-CCG CGG ATC AGG TGG AAC TGA; V1-2 5', 5'-CCG CGG ATC CGG TAG AAG TGG AAC TGA; V1-2 3', 5'-CCG CGG ATC CGG TAG AAG TGG AAC TGA; V1-2 5', 5'-CCG CGG ATC CGG TAG AAG TGG AAC TGA.

### Recombinant plasmids

Plasmids p20-15 and p20-16 contain the TRL-64 and TRL-78 sequences cloned in pBluescript SK(+) (Stratagene, La Jolla, CA). To construct these plasmids, the TRL sequences were amplified from V1-2 mtDNA using primers V1-2 5' and V1-2 3'. The PCR products were then digested with EcoRI and BamHI and cloned between the corresponding sites of the vector, such that transcription with phage T3 RNA polymerase yields sense transcripts and transcription with phage T7 RNA polymerase yields antisense transcripts. Plasmid p7-2 contains the TRL-78 sequence from Varkud cloned similarly in pBluescript SK(+)

### Isolation of whole cell and mitochondrial nucleic acids

Nucleic acids were isolated by a modification of the method of Hoge et al. (14). Frozen conidial pads were ground in a mortar on dry ice, then resuspended in boiling lysis buffer (2 M sodium borate, 30 mM EDTA, 1% SDS, pH 9.0) and extracted three times with phenol/chloroform/isooamyl alcohol (25:24:1) (phenol-CIA). The nucleic acids were ethanol precipitated in the presence of 0.3 M sodium acetate, pH 4.8, washed twice with 70% ethanol and resuspended in 2 M LiCl. After incubation on ice for 2 h, RNA was sedimented at 15 000 g for 15 min at 4°C. The DNA in the supernatant was dialyzed overnight against 0.1x TE (1 mM Tris–HCl, pH 7.5, 0.1 mM EDTA), then ethanol precipitated in the presence of 0.3 M sodium acetate, pH 4.8, washed twice with 70% ethanol, dissolved in distilled H2O and stored at −20°C. Portions containing 1 mg RNA were stored in ethanol at −70°C. If necessary, residual RNA in the DNA preparations or DNA in the RNA preparations were removed by RNase or DNase digestion, respectively.

### In vitro transcription

Run-off transcripts were synthesized from restriction enzyme-digested recombinant plasmid DNA with phage T7 or T3 RNA polymerase (New England Biolabs, Beverly, MA), as described (16). For synthesis of 32P-labeled transcripts, the reaction mix contained 1 µCi/µl [α-32P]UTP (3000 Ci/mmol; Amersham-Pharmacia, Piscataway, NJ) and 500 µM each unlabeled NTP. Transcripts were treated with DNase I to remove the template DNA and purified through two Sephadex G-50 spin columns (Sigma-Aldrich, St Louis, MO).

### Southern and northern hybridization

For Southern hybridizations, mitochondrial or whole cell nucleic acids were separated by electrophoresis in a 0.6% agarose gel containing 1× TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0). After staining with ethidium bromide, the gel was blotted to Hybond-N (Amersham-Pharmacia) and the blots were hybridized overnight at 37°C with 32P-labeled DNA probes (>5×106 c.p.m.) in 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.2% SDS and 100 mg/ml sheared, denatured salmon sperm DNA (Sigma). The blots were then washed in 5× SSC, 0.2% SDS for 5 min at 50°C and, if necessary to reduce background, once in 0.5× SSC, 0.2% SDS for an additional 5 min at 50°C, prior to autoradiography.

For northern hybridizations, RNA species were either denatured with glyoxal and resolved in a 1.8% agarose gel containing...
phosphate buffer (17) or analyzed directly in a denaturing 5% polyacrylamide gel containing 0.5x TBE (0.045 M Tris–borate, 0.001 M EDTA). The agarose gels were stained with ethidium bromide and blotted to Hybond-N. Polyacrylamide gels were electroblotted to Hybond-N using a Trans-Blot Cell (Bio-Rad, Hercules, CA). Hybridization with 32P-labeled DNA restriction fragments or PCR product probes was carried out overnight at 42°C, as described above for Southern blots. Hybridization with the 5′-end-labeled DNA oligonucleotide tRNA16 was carried out overnight at 37°C in 5× SSC containing 0.2% SDS (18).

Densitometric scanning of ethidium bromide stained gels was carried out using the AlphaEase™ software package from Alpha Innotech (San Leandro, CA).

**DNA sequencing**

The EcoRI-3 fragment of Varkud mtDNA was cloned into pBluescript KS(+) (Stratagene) and sequenced by automated sequencing with fluorescent dyes at the University of Texas at Austin DNA sequencing facility. The sequencing with fluorescent dyes at the University of Texas at Austin DNA sequencing facility. The EcoRI-4 fragment of the Hanapepe mtDNA was cloned into pUC19 (Stratagene) and sequenced using the dideoxy chain termination method (19). Sequence comparisons with GenBank were done using the BLAST algorithm with the BLOSUM 62 matrix and an expect value of 10 (20) at the National Center for Biotechnology Information website.

**Screening of Neurospora strains and libraries for the TRL sequence**

*Neurospora* wild-type strains and natural isolates were screened for the presence of the TRL sequence by PCR of whole cell DNA (50–250 ng) using *Taq* DNA polymerase (Gibco BRL, Gaithersburg, MD) with 200 ng each of primers V1-2 5′ and V1-2 3′ or 5′-up and 3′-down. The PCR conditions were 94°C for 1 min, 30 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 45 s, then 10 min at 72°C.

*Neurospora crassa* cosmids pMOcosX and YAC libraries (Fungal Genetics Stock Center; 21,22) were screened similarly by PCR with primers V1-2 5′ using pooled DNA preparations from each microtiter dish. The PCR was carried out as above using 50 ng of DNA and 200 ng of each primer and gel-purified PCR products were sequenced directly at the University of Texas at Austin DNA sequencing facility.

For inverse PCR, 5 μg of Varkud whole cell DNA was digested with either *AluI*, *Avai*, *BsiI*, *RsaI* or *SalI* (Gibco BRL) and then religated. After extraction with phenol-CIA and ethanol precipitation, 0.01 μg of the ligated DNA was used as template for PCR as above using the primers TRL-78 5′R and TRL-78 3′R.

**Cloning of full-length cDNAs via RT–PCR**

TRL-78 RNA was purified in a denaturing 5% polyacrylamide gel and then tagged with poly(A) by incubating with 0.25 mM [α-32P]ATP (3000 Ci/mmol; Amersham-Pharmacia) and 30 U of poly(A) polymerase (Gibco BRL) in 50 mM Tris–HCl, pH 7.9, 0.25 M NaCl, 10 mM MgCl2, 2.5 mM MnCl2, 0.5 mg/ml bovine serum albumin (BSA) (Gibco BRL) for 30 min at 37°C. The tailed RNAs were reverse transcribed using a Superscript amplification kit (Gibco BRL) with primer dTBam and the resulting cDNAs were tailed with poly(dG) by incubating with 5 U of terminal deoxynucleotidyl transferase (Gibco BRL) and 200 μM dGTP in 500 mM potassium cacodylate, pH 7.2, 10 mM CoCl2 and 100 mM DTT for 1 h at 37°C. The tailed cDNAs were then amplified by PCR using primers dTBam and dCBam (94°C for 1 min, 30 cycles of 94°C for 1 min, 40°C for 1 min and 72°C for 1 min, then 10 min at 72°C). The PCR products were digested with *BamHI* and cloned in the corresponding site of pBS (+) (Stratagene). Alternatively, cDNAs were generated by tailing size-selected, gel-purified RNAs with poly(A) as above, reverse transcribing with dTBam and then amplifying directly using primers dTBam and V1-2 3′. The PCR products were digested with *BamHI* and EcoRI and cloned into the corresponding sites of pBS (+) (Stratagene).

To amplify non-size-selected RNAs, whole mtRNA was reverse transcribed using the primer V1-2 3′, which is complementary to the 3′-end of the TRL transcript, and the cDNA was tailed with poly(dG) using terminal deoxynucleotidyl transferase as above. The cDNA was then PCR amplified using the primers dCBam and V1-2 3′ and the products were digested with *BamHI* and cloned into the corresponding site of pBluescript KS(+) (Stratagene).

**RNase protection assay**

RNase protection assays were carried out to look for spliced TRL-64 RNAs in Varkud mitochondria. mtRNAs or sense transcripts of p20-15 and p20-16 as controls were ethanol precipitated with a >10-fold molar excess of 32P-labeled p20-16 antisense transcript, then redissolved in 30 μl of 80% formamide, 40 mM PIPES, pH 6.7, 0.4 M NaCl, 1 mM EDTA and annealed by incubating at 85°C for 5 min, followed by 40°C for 5 h. The annealed RNAs were added to 350 μl of digestion buffer (0.3 M NaCl, 10 mM Tris–HCl, pH 7.5, 5 mM EDTA) containing 1 μg/ml RNase T1 (Gibco BRL) plus 20 μg/ml RNase A (Boehringer, Indianapolis, IN) and incubated for 20 min at 40°C. Digestion was terminated by addition of 20 μl of 10% SDS and 25 μl proteinase K (2 mg/ml; Gibco BRL). The products were then incubated for 10 min at 37°C, extracted with phenol-CIA, ethanol precipitated in the presence of 0.3 M sodium acetate, pH 7.5, plus 2 μg plasmid DNA carrier and analyzed in a denaturing 8% polyacrylamide gel against RNA size markers.

**Primer extension assay**

A primer extension assay was also used to look for spliced RNAs that would give shorter primer extension products due to removal of the intron. For these assays, 15 μg of mitochondrial or whole cell RNA was ethanol-precipitated together with 20 ng of gel-purified, 5′-end-labeled DNA primer V1-2 3′. The pellet was dissolved in 40 μl of hybridization buffer (50 mM Tris–HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM MgCl2) and incubated for 5 min at 65°C, followed by 2 h at 42°C to anneal the primer. After addition of 20 U of AMV reverse transcriptase (Gibco BRL) and 0.5 mM each dATP, dCTP, dGTP and dTTP, primer extension was carried out for 1 h at 42°C. The reaction was terminated by adding 5 μl of formamide and the products were boiled for 2 min and analyzed in a denaturing 6% polyacrylamide gel.
probe did not hybridize to mtDNA from the Varkud strain from which the V1-2 strain was derived. The detect the single copy nuclear gene Fungal Genetics Stock Center; 21,22) under conditions that DNA libraries (the pMOcosX cosmid and YAC libraries; 39)

Southern hybridization of wild-type 74A to detect mtDNA sequences encoding the TRL sequence. EcoRI digests of mtDNAs from wild-type 74A, Mauriceville (M), the growth impaired mutant V1-2 and Varkud (V) were run in a 0.6% agarose gel, blotted to Hybond-N and hybridized with a 32P-labeled 84 bp BamHI-EcoRI fragment corresponding to cloned TRL-78 from p7-2. The ethidium bromide-stained gel is shown to the left and the autoradiogram to the right. The probe hybridized to the 2.8-kb EcoRI fragment of pV1-2 in the V1-2 strain and to the 9.2-kb mtDNA EcoRI-3 (E-3) fragment in both Varkud and V1-2. EcoRI fragments of wild-type 74A mtDNA are numbered to the left of the gel.

RESULTS

The TRL sequence is encoded in Varkud mtDNA

To investigate the origin of the TRL sequence found in the suppressive plasmids, a 32P-labeled DNA probe corresponding to the TRL sequence was hybridized to EcoRI digests of mtDNAs from different Neurospora strains. As shown in Figure 2, the probe hybridized not only to the expected 2.8-kb EcoRI fragment derived from pV1-2 in the V1-2 strain, but also to the EcoRI-3 fragment in both V1-2 and the wild-type Varkud strain from which the V1-2 strain was derived. The probe did not hybridize to mtDNA from the N. crassa wild-type 74A or Mauriceville strains. In an earlier work, a restriction fragment probe containing TRL-64 and its 5' and 3'-flanking sequences in the V1-2 plasmid was reported to hybridize to wild-type 74A mtDNA EcoRI-8 (9). However, this hybridization was found to be due to a 25/26 match between the flanking pVAR plasmid sequence and a sequence in EcoRI-8 and not to the TRL insert.

No other sequences related to TRL-64 could be detected by Southern hybridization of wild-type 74A or Varkud whole cell DNA nor by PCR of 74A whole cell DNA or two different 74A DNA libraries (the pMOcosX cosmid and YAC libraries; Fungal Genetics Stock Center; 21,22) under conditions that detect the single copy nuclear gene cyt-18 (see Materials and Methods). Inverse PCR of whole cell DNA from the Varkud strain gave products expected for the integrated mtDNA sequence, but no additional nuclear sequences (not shown).

Sequence of the Varkud mtDNA EcoRI-3 fragment

The EcoRI-3 fragment of Varkud mtDNA is homologous to the wild-type 74A EcoRI-9 fragment (GenBank accession no. X57636), but is ~7 kb larger, reflecting the presence of one or more insertions. We determined the complete DNA sequence of the Varkud mtDNA EcoRI-3 fragment, and its major features are summarized in Figure 3. The sequence shows that the Varkud EcoRI-3 fragment corresponds to the wild-type 74A EcoRI-9 fragment but with a 7053-bp insertion replacing the sequence between positions 1840 and 1900. In addition, two small insertions corresponding to GC-rich palindromic sequences (nos 3 and 17; Fig. 3) are present in the region homologous to wild-type 74A EcoRI-9. In all, five different types of GC-rich palindromic repeats (denoted I–V), which are exact copies of sequences located elsewhere in the mtDNA, were found throughout the Varkud EcoRI-3 sequence, including at one boundary of the 7-kb insertion (no. 4; see the legend to Fig. 3 for a summary of palindromic repeats).

The 7-kb insertion contains a single copy of the TRL-78 sequence, which is identical to the TRL-78 sequence found in plasmids pV34-14 and pVS1-6a (Fig. 4), except that the mtDNA sequence lacks the 3' CC residues, which are presumably added post-transcriptionally by tRNA nucleotidyl transferase (see below). Compared to the TRL-64 sequence found in pV1-2, TRL-78 contains a 14-nucleotide insertion located 1 nt behind the anticondon, as expected for a tRNA intron (Fig. 4). TRL-64 and TRL-78 contain a number of conserved nucleotides found in tRNAs (circled in Fig. 4), but have significant deviations from the canonical tRNA structure (see Discussion).

In addition to TRL-78, the 7-kb insertion contains a mt tRNA sequence in the same transcription orientation 739 bp downstream of the TRL-78 sequence. This tRNA sequence is identical to the mt tRNA gene encoded in the EcoRI-1 fragment of wild-type 74A mtDNA (23) and we confirmed that the same tRNA gene is also present in the Varkud mtDNA EcoRI-1 fragment (not shown). Sequence identity between the duplicated tRNA genes begins 3 bp upstream of the 5’-end of the tRNA, but does not extend beyond the 3’-end, which in both cases corresponds to the A residue immediately upstream of the post-transcriptionally added 3’ CCA. The sequences flanking the tRNA gene in the 7-kb insertion have no unusual characteristics suggesting how this gene was duplicated. A sequence matching the N. crassa mtDNA promoter consensus sequence (10/10 match; 24,25) is present 792 bp upstream of the TRL-78 sequence in a position to transcribe both the TRL-78 and the duplicated tRNA gene.

In addition to the TRL-78 and tRNA genes, the 7-kb insert contains segments of three previously described mitochondrial plasmids: the linear plasmid Harbin-L (pHAR-L, nt 1875–2356, inserted at positions 3481–4051; 26), the linear plasmid Harbin-3 (pHAR-3, nt 3125–6581, inserted at positions 3481–4051; 26), the linear plasmid Maranhar (pMAR, nt 3125–6581, inserted at positions 3481–4051; 26), and the circular plasmid Fiji (pFiji, nt 4958–5008, inserted at positions 4155–4242; 27) and the inserted pHAR-3 sequence (inserted at positions 5378–8922; 28,29). The inserted pHAR-L and pMAR/pHAR-3 sequences include regions of the ORFs encoding the putative DNA polymerases of these plasmids. In both cases, however, the inserted ORFs are not complete and interrupted by a number of
GC-rich palindromes and other non-homologous sequences. Significantly, homology to the pMAR/pHAR-3 DNA polymerase ORF actually begins at position 9146 in the region corresponding to the wild-type 74A mtDNA (Fig. 3). This finding suggests that the mitochondrial plasmid insertion occurred prior to the divergence of *N. crassa* and *N. intermedia* and that most but not all of the inserted sequence was deleted in the line leading to *N. crassa* wild-type 74A.

A sequence related to the 7-kb insertion in the Varkud *EcoRI-3* fragment was also found in the *EcoRI-4* fragment of *N. intermedia* strain Hanapepe. DNA sequencing of the Hanapepe insertion showed that positions 3776–6781 of the Varkud strain are missing and replaced with a different 87-nt sequence. This 87-nt sequence contains a region with homology to a sequence in the long terminal repeat of pHAR-3, which is not present in pMAR (either nt 2–38 or 7013–7049 inserted at Figure 3. Features of the *EcoRI-3* fragment of Varkud mtDNA revealed by DNA sequencing. The thick black line indicates sequence homologous to the wild-type *74A* mtDNA. The location of a 78-bp repeat that constitutes a previously identified polymorphism in different *N. crassa* strains (41) is indicated. The Varkud sequence corresponds to the short version of *EcoRI-9* having two copies of the 78-bp repeat (positions 112–189 and 190–267). The open box shows the 7053-bp sequence inserted in Varkud mtDNA, with the dashed segment indicating a remnant of the insertion that is retained in wild-type 74A mtDNA. The location and orientation of the TRL-78 and the tRNA^Trp^ sequences are indicated by black bars and arrows. The filled arrowhead shows the position of a putative *Neurospora* mtDNA promoter that could be used for transcription of TRL-78 and tRNA^Trp^ (41). The primer pairs *V1-2* 5′ and *V1-2* 3′ and 5′-up and 3′-down, which were used to survey the presence of TRL-78 in different *Neurospora* strains (Table 1), are shown above as open arrows. The 7-kb insert contains segments derived from the mitochondrial plasmids pHAR-L (vertically striped box), pFiji (gray box) and pMAR/pHAR-3 (diagonally striped boxes interspersed with sequences below the similarity threshold; see Materials and Methods). Nucleotide positions of the inserted plasmid sequences are shown above the boxes (pHAR-L, pFiji and pMAR; 26–28). The region corresponding to the pMAR/pHAR-3 DNA polymerase ORF is shown by a dashed arrow below. The region that is deleted and replaced with a different 87-nt sequence in the related Hanapepe strain of *N. intermedia* is indicated above. The positions of short GC-rich palindromic repeats are shown below the map. Five different palindromic sequences were found. Type I palindromes (5′-CCC CCT CCA GCT TCG CTG GAG GGGG-3′) are present at sites 1 (position 405), 4 (position 1840), 6 (position 2465), 11 (position 5826) and are also found in wild-type 74A mtDNA in ND3 intron 1 (positions 2806 and 3093), in the *EcoRI-8* fragment upstream of ORF-8 (position 28098), in ND4L intron 1 (position 6050), in *EcoRI-4* downstream of the *COII* gene (position 10255), in *EcoRI-11* (position 2466) and in *EcoRI-6* (position 11223). Type II palindromes (5′-CCC CCT TTT TCA AAT ACG AAA GGA GGG GG-3′) are present at sites 2 (position 1154), 5 (position 2150), 7 (position 2902), 8 (position 2968) and 12 (position 5657) and are also found in wild-type 74A in *EcoRI-9* (position 1232). Type III palindromes (5′-CCC CGC CCC CGA AGG GGG CGG GG-3′) are present at sites 9 (position 3754), 10 (position 3915) and 16 (position 7280) and are also found in wild-type 74A mtDNA downstream of the genes encoding tRNA^Lys^ (position 1458), tRNA^Ser1^ (position 4879) and tRNA^Ser2^ (position 2471) and in *EcoRI-6* (positions 55 and 573 in fig. 3 in 40). Type IV palindromes (5′-CCC CAC AAC GAC CCC ACA CAC CTA CGG TGT GTG GGC CGT TGT GGG G-3′) are present at sites 3 (position 1645), 14 (position 6342) and 15 (position 6464) and are also found in wild-type 74A mtDNA downstream of the *tRNAGln* gene (position 11248) and the *ND6* gene (position 4157) and in Varkud mtDNA downstream of the *ND1* intron ORF (position 1578; 54). A type V palindrome (5′-CCC CGG CCC CTC TTC GGA GGG GCC GGG G-3′) is found at site 17 and also in wild-type 74A mtDNA in *EcoRI-6* (position 9261). Nucleotide positions in wild-type 74A mtDNA are according to the compilation of Dr R. A. Collins (personal communication) and from figure 3 of Nargang et al. (40).
The remainder of the Varkud EcoRI-3 (E-3) and Hanapepe EcoRI-4 sequences are >99% identical (20 nt changes in 2556 bp) and the TRL-78 sequence along with flanking sequences at least 2505 bp upstream (up to the EcoRI site) and 1194 bp downstream are identical between the two N.intermedia strains.

Distribution of the TRL sequence in wild-type Neurospora strains and natural isolates

A collection of Neurospora wild-type strains and natural isolates was screened for the presence of the TRL sequence by PCR using primers corresponding to the 5′- and 3′-ends of the TRL sequence (V1-2 5′ and V 1 - 23′), as well as primers corresponding to sequences flanking TRL-78 in the Varkud mitochondrial EcoRI-3 fragment (5′-up and 3′-down; see Fig. 3). In the Varkud strain, the latter primers give a 250-bp product containing both TRL-78 and the upstream GC-rich palindrome (no. 6 in Fig. 3). The TRL-78 sequence was found to be present in a variety of N.crassa, Neurospora sitophila, N.intermedia and Neurospora tetrasperma strains and all except the N.tetrasperma strain 85 also contained the upstream type I palindrome (Table 1). Sequencing of the PCR products showed that, except for the missing GC-rich palindrome in strain 85, TRL-78 and its flanking sequences are identical in all these Neurospora strains.

Transcription of the tRNA-like sequence

To determine if the TRL-78 sequence encoded in Varkud mtDNA is expressed, we carried out northern hybridizations in which mtRNAs from wild-type 74A, V1-2 and Varkud were separated in a 1.8% agarose gel, blotted to Hybond-N and hybridized with a 32P-labeled TRL-78 probe (Fig. 5A). In the Varkud strain, the probe hybridized to a single transcript derived from the mtDNA-encoded copy of TRL-78, whereas in V1-2 the probe hybridized to a large range of transcripts derived from both the mtDNA-encoded TRL-78 sequence and from transcripts of pV1-2 and several smaller derivatives of that plasmid (S.Mohr and A.M.Lambowitz, in preparation) As expected, no transcripts corresponding to the tRNA nucleotidyl transferase (see Fig. 6). The insert at the bottom right shows an alternative structure for the 'anticodon stem' region of TRL-78.
Characterization of the TRL-78 transcript from Varkud mitochondria

To characterize TRL-78 transcripts from Varkud mitochondria, we obtained full-length cDNA clones by RT–PCR. In initial experiments, the TRL-78 RNA from Varkud mitochondria was gel purified and tagged with poly(A) using poly(A) polymerase (Fig. 6). Full-length cDNAs were then synthesized with Superscript RT using the oligonucleotide dTBAm and tagged with poly(dG) using terminal deoxynucleotidyl transferase. The tagged cDNAs were amplified by PCR using the primers dCBam and dTBam, cloned into pBlueSct and sequenced. All six sequenced clones corresponded to RNAs containing the 14-nt insert, with 5′- and 3′-ends corresponding precisely to those of the TRL transcript in the mRPs. Notably, the free TRL-78 RNAs end with two 3′ CC residues, which are not encoded in the mtDNA and were presumably added post-transcriptionally by tRNA nucleotidyl transferase. It is likely that the 3′-terminal A residue is also present, but since the transcripts were cloned via poly(A) tailing, this could not be assessed directly.

To look further for the presence of spliced transcripts, RNAs in the gel region between 60 and 80 nt were tagged with poly(A) and cloned via an analogous RT–PCR procedure using the primers dCBam and V1-2 5′-end-labeled. Every one of 90 cloned cDNAs analyzed was found to contain the 14-nt insertion and 3′-ends corresponding precisely to those of the TRL transcript in the mRPs. To exclude the possibility that we failed to see the spliced transcript because we were using size-selected RNAs, we carried out an additional cloning experiment using total mtRNA. In this experiment, the mRNA was reverse transcribed from primer V1-2 3′-end, which is complementary to the 3′-end of the TRL transcript, and the resulting cDNAs were tagged with poly(dG), PCR amplified using the primers dCBam and V1-2 3′-end and cloned into pBlueSct (see Material and Methods). All 10 clones analyzed had the same 5′-end and contained the 14-nt insert.
In other experiments, we were unable to detect the ‘spliced’ TRL-64 RNA in Varkud mitochondria using either RNase protection or primer extension assays (not shown; see Materials and Methods). Further, we were unable to splice TRL-78 transcripts in vitro either under self-splicing conditions or using mitochondrial lysates. Finally, the TRL-78 in vitro transcript could not be cut or spliced using yeast tRNA splicing enzymes (M.Steiger and E.Phizicky, personal communication), which have been shown to splice heterologous intron-containing tRNA species from vertebrates and plants (30). We conclude that there is little if any TRL-64 transcript present in Varkud mitochondria and that the 14-nt insert is not recognized as an intron by nuclear or mitochondrial enzymes.

DISCUSSION

In the present work we have characterized an unusual tRNA-like sequence found inserted in suppressive variants of the Varkud mitochondrial plasmid. We had previously found two different forms of the tRNA-like sequence: TRL-64 inserted in the suppressive plasmid pV1-2 (9) and TRL-78 inserted in pV34-14 and pV51-6a (11). TRL-78 is identical to TRL-64 except for a 14-nt internal insertion at the position expected for a nuclear tRNA intron. Here we show that TRL-78 is encoded in the EcoRI-3 fragment of Varkud mtDNA. The mtDNA-encoded copy is transcribed and is apparently processed at both its 5′- and 3′-ends to yield discrete tRNA-sized molecules. Although the 14-nt insert is not spliced, the 3′-end of TRL-78 RNA is recognized as a substrate for nucleotide addition by tRNA nucleotidyl transferase and we show elsewhere that the 5′-end of TRL-64 in the V1-2 plasmid is a cleavage site for mitochondrial RNase P (S.Mohr and A.M.Lambowitz, in preparation). The finding that TRL-78 is present as a discrete tRNA-sized transcript supports the hypothesis that it was incorporated into the pVAR plasmid by the same template switching mechanism used to incorporate mt tRNAs (see Introduction). Further, the finding that the mtDNA-encoded form contains the 14-nt internal sequence indicates that this sequence must have been deleted in the TRL-64 sequence that is deleted in TRL-64 (see Results).

In other experiments, we were unable to detect the ‘spliced’ TRL-64 RNA in Varkud mitochondria using either RNase protection or primer extension assays (not shown; see Materials and Methods). Further, we were unable to splice TRL-78 transcripts in vitro either under self-splicing conditions or using mitochondrial lysates. Finally, the TRL-78 in vitro transcript could not be cut or spliced using yeast tRNA splicing enzymes (M. Steiger and E. Phizicky, personal communication), which have been shown to splice heterologous intron-containing tRNA species from vertebrates and plants (30). We conclude that there is little if any TRL-64 transcript present in Varkud mitochondria and that the 14-nt insert is not recognized as an intron by nuclear or mitochondrial enzymes.
the correct dimensions for either the stem or loop and is missing all of the conserved bases. The anticodon stem in TRL-64 can be drawn with 5–8 bp, depending on whether the distal AU residues are paired, and it lacks all of the expected bases, with the possible exception of U37, which is also found in N. crassa mt RNAAla (32). There is no variable arm nor are there the requisite numbers of unpaired nucleotides separating the different stems. Given these deviations, it is likely that recognition of the TRL RNA by the mitochondrial RNase P and tRNA nucleotidyl transferase primarily involves the acceptor/TΨC arm, as found for bacterial and yeast tRNA nucleotidyl transfersases and human RNase P (reviewed in 33).

In the TRL-78 form, the additional 14-nt sequence is inserted 1 nt behind the anticodon, the same position found for nuclear tRNA introns (34). The insert is within the size range found for nuclear tRNA introns (8–60 nt) and can be drawn base paired to the anticodon, as for nuclear tRNA introns (Fig. 4). We initially imagined that the 14-nt insert was an intron because it was deleted in the TRL-64 form inserted in pV1-2. However, we could find no evidence for splicing of the 14-nt sequence in Varkud mitochondria in vivo nor in mitochondrial extracts in vitro. Further, the TRL-78 RNA was not a substrate for yeast nuclear tRNA splicing enzymes (M. Steiger and E. Pitzicky, personal communication). We cannot exclude the possibility that splicing occurs at a very low level, below the limit of detection in our assays. However, an alternative possibility is that the 14-nt sequence was deleted at the DNA level, perhaps in the process of cDNA synthesis during plasmid replication or insertion of the TRL species by template switching. Such a mechanism could have involved template jumping of the mRP RT between U residues flanking the deletion. A similar mechanism has been suggested for other deletions in the mRPs (35).

The TRL-78 sequence is encoded in the EcoRI-3 fragment of Varkud mtDNA in a 7-kb insertion that is not present in wild-type 74A mtDNA (Fig. 3). The N. intermedia strain Hanapepe contains a related 4-kb insertion, which also encodes TRL-78, and TRL-78 was detected by PCR in the same sequence context in varieties of other N. crassa, N. sitophila, N. intermedia and N. tetrasperma strains (Table 1). DNA sequencing showed that in addition to TRL-78, the 7-kb insertion in the Varkud strain contains a perfectly duplicated tRNA\textsuperscript{Trp} gene, segments of three different mitochondrial plasmids, pHAR-L, pFiji and pMAR/pHAR-3, and a number of GC-rich palindromic sequences that are repeated elsewhere in the mtDNA. The latter are thought to be analogous to GC-rich clusters, which have been shown to be mobile in yeast mtDNA (36,37). Likewise, both linear and circular mitochondrial plasmids have previously been shown to integrate into Neurospora mtDNA (9,11,38–40).

The Varkud EcoRI-3 fragment, in which the 7-kb segment is inserted, is related to the N. crassa wild-type 74A EcoRI-9 fragment. The latter does not contain any complete mtDNA genes (41) and may be a region that tolerates or is susceptible to insertions, perhaps because of DNA topology. The mitochondrial plasmid insertions in Varkud mtDNA appear to be relatively old, since the pHAR-L and pMAR/pHAR-3 ORFs are no longer continuous and since the plasmid sequences are riddled with GC-rich palindromes, which are likely inserted from elsewhere in Neurospora mtDNA. Moreover, a remnant of the pMAR/pHAR-3 sequences is present in wild-type 74A mtDNA, suggesting that the insertion occurred prior to the divergence of N. crassa and N. intermedia and that most of the mitochondrial plasmid sequence was deleted subsequently in the line leading to N. crassa 74A.

The phylogenetic distribution of TRL-78 and its flanking sequences suggests that it was also present prior to the divergence of different Neurospora species. Although we cannot exclude the possibility that the TRL-78 sequence was inserted subsequently and disseminated via hyphal fusion, this seems less likely given the wide geographic distribution of strains containing TRL-78 and the expected low frequency of vegetative transmission between incompatible Neurospora strains and species. Our findings do not distinguish between TRL-78 being inserted separately or as part of a larger sequence. In addition, it is possible that TRL-78 is present in only a minor mtDNA population in some strains.

The tRNA\textsuperscript{Trp} insertion appears to have involved duplication of the tRNA\textsuperscript{Trp} gene in EcoRI-1. In general, duplication of the tRNA\textsuperscript{Trp} sequence could have occurred by DNA recombination or via reverse transcription and insertion of the resulting cDNA. The inserted tRNA\textsuperscript{Trp} sequence lacks the 3′ CCA, which is added post-transcriptionally and would ordinarily be indicative of integration via an RNA intermediate. However, it is difficult to account for precise insertion of the tRNA\textsuperscript{Trp} sequence by a DNA recombination event in the absence of substantial flanking sequence homology. Although it is possible that homologous flanking sequences diverged subsequently, we note that tRNA\textsuperscript{Trp} is one of only three tRNA species found incorporated in suppressive mutant plasmids, suggesting that it is a preferred substrate of the mRP RT (9,10). Thus, it seems more likely that insertion involved the cDNA copy of an RNA intermediate that lacked the 3′ CCA or that the 3′ CCA was initially associated with the duplicated sequence and diverged subsequently. A possible explanation is that the 3′ CC is selected against in mtDNA because it interferes with normal 3′-end processing (42). Although the 3′ CCA is the preferred initiation site for mRP RT, initiation can occur just upstream of the 3′ CCA in some instances (8,12). Based on previously described mechanisms for mRP integration (9,11), a cDNA copy of tRNA\textsuperscript{Trp} could potentially integrate directly or as part of a hybrid cDNA that is generated by template switching with a mitochondrial transcript and then integrates at the linked mtDNA sequence by homologous recombination. A similar scenario can also account for the integration of TRL-78, which likewise appears to be a preferred substrate for mRP RT. One possibility is that TRL-78 is the remnant of a nuclear tRNA sequence that integrated into mtDNA. There is ample precedent for integration of foreign DNA sequences into mtDNAs, and an RNA intermediate has been proposed in some cases (43–45). It is also possible that TRL-78 was derived from a functional RNA that was originally encoded elsewhere in the mtDNA or imported as an RNA species and then integrated via a cDNA into the Varkud EcoRI-3 fragment. The mtDNA integration of mt tRNAs or tRNA-like species via a cDNA intermediate would be analogous to integration of SINE elements in animal cells (46). A prediction is that such integrations in mRP-containing strains would involve RNA species, like tRNA\textsuperscript{Trp} and TRL-78, which are preferred templates for mRP RT.

Finally, we note that in some respects TRL-78 resembles bacterial mtRNAs, which function as both an amino acid acceptor and a mRNA (47). Thus, as in mtRNAs, the acceptor and TΨC stems are conserved, while the D-arm is diverged and the anticodon arm has an insertion, albeit the insertion in
TRL-78 is much smaller than in mtRNAs. All known mtRNAs have a G3:U70 wobble pair in the acceptor stem, which is an important recognition determinant for tRNA\textsubscript{Ala}, and they append a short polypeptide beginning with alanine. Although TRL-78 lacks the G3:U70 pair, this GU pair is also lacking in animal mt tRNA\textsubscript{Ala} (48) and recent experiments have shown that it is possible to change the amino acid acceptor identity of Escherichia coli mtRNA from alanine to histidine \textit{in vitro} (49). More generally, novel aminoacylatable RNA species may exist that it is possible to change the amino acid acceptor identity of such RNA species. However, if TRL-78 does have a function, it must not be essential, since it is not present in all Neurospora strains.

ACKNOWLEDGEMENTS

We thank Ms Michelle Steiger and Dr Eric Phizicky (University of Rochester School of Medicine, Rochester, NY) for carrying out assays with yeast nuclear tRNA splicing enzymes. Mrs Georg Mohr and Roland Saldanha for comments on the manuscript and Mr James Williams and Mr John M. Stryker for technical assistance. S.M. was a recipient of a post-doctoral fellowship from the Deutsche Forschungsgemeinschaft and L.W. was a recipient of an Ohio State University post-doctoral fellowship. This work was supported by NIH grant GM37949 to A.M.L. and by operating grants to H.B. from the Natural Sciences and Engineering Research Council of Canada.

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