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Characterization of Mutant Mitochondrial Plasmids of *Neurospora* spp. That Have Incorporated tRNAs by Reverse Transcription

ROBERT A. AKINS,† ROBERT L. KELLEY,‡ AND ALAN M. LAMBOWITZ*

Departments of Molecular Genetics and Biochemistry and the Biotechnology Center, The Ohio State University, Columbus, Ohio 43210

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The Mauriceville and Varkud mitochondrial plasmids of *Neurospora* spp. are closely related, closed-circular DNAs (3.6 and 3.7 kilobases, respectively) whose nucleotide sequences and genetic organization suggest relationships to mitochondrial introns and retroelements. We have characterized nine suppressive mutants of these plasmids that outcompete mitochondrial DNA and lead to impaired growth. All nine suppressive plasmids contain small insertions, corresponding to or including a mitochondrial tRNA ($tRNA^{Trp}$, $tRNA^{Gly}$, or $tRNA^{Val}$) or a tRNA-like sequence. The insertions are located at the position corresponding to the 5′ end of the major plasmid transcript or 24 nucleotides downstream near a cognate of the sequence at the major 5′ RNA end. The structure of the suppressive plasmids suggests that the tRNAs were inserted via an RNA intermediate. The 3′ end of the wild-type plasmid transcript can itself be folded into a secondary structure which has tRNA-like characteristics, similar to the tRNA-like structures at the 3′ ends of plant viral RNAs. This structure may play a role in replication of the plasmids by reverse transcription. Major transcripts of the suppressive plasmids begin at the 5′ end of the inserted mitochondrial tRNA sequence and are present in 25- to 100-fold-higher concentrations than are transcripts of wild-type plasmids. Mapping of 5′ RNA ends within the inserted mtDNA sequences identifies a short consensus sequence (PuNPuAG) which is present at the 5′ ends of a subset of mitochondrial tRNA genes. This sequence, together with sequences immediately upstream in the plasmids, forms a longer consensus sequence, which is similar to sequences at transcription initiation sites in *Neurospora* mitochondrial DNA. The suppressive behavior of the plasmids is likely to be directly related to the insertion of tRNAs leading to overproduction of plasmid transcripts.

The Mauriceville-1c strain of *Neurospora crassa* and the Varkud-1c strain of *Neurospora intermedia* contain closely related mitochondrial plasmids whose nucleotide sequences and genetic organization suggest relationships to mitochondrial introns and retrotransposons (10, 29, 33; R. A. Akins, D. M. Grant, L. L. Stohl, D. A. Bottorff, F. E. Nargang, and A. M. Lambowitz, J. Mol. Biol., in press). These plasmids are small, closed-circular DNAs, with monomeric circles having lengths of 3.6 and 3.7 kilobase pairs (kb), respectively (Fig. 1). In each case, some proportion of the plasmid is present in larger oligomers consisting of tandem head-to-tail repeats. The Mauriceville and Varkud plasmids have more than 97% positional identity to each other. They are localized entirely within mitochondria but have no substantial homology to mitochondrial DNA (mtDNA) or nuclear DNA. The plasmid-containing strains are more prone to senescence (1) but grow at wild-type rates and have no other obvious phenotypic characteristics that might be related to the presence of the plasmids (10; Akins et al., in press).

Both the Mauriceville and Varkud plasmids contain a 710-amino-acid open reading frame that has short blocks of amino acids that are characteristic of retrovirus reverse transcriptases and related proteins (29, 32, 33; Fig. 1, sequence blocks I through VII). The amino acid sequence of this open reading frame is strongly conserved between the two plasmids (701 of 710 amino acids [32; Akins et al., in press]), and it is likely to encode a polymerase required for propagation and/or expression of the plasmid, probably a reverse transcriptase (22). Both plasmids give major transcripts that are full-length linear RNAs in which every nucleotide in the plasmids is represented (33; Akins et al., in press). The synthesis of such full-length transcripts is an obligatory characteristic of elements that replicate or transpose by reverse transcription. Recently, we showed that mitochondria from the Mauriceville and Varkud strains contain a reverse transcriptase activity that is highly specific for endogenous plasmid RNA in preparations of ribonucleoprotein particles. This reverse transcriptase synthesizes a full-length minus-strand DNA, as expected for a reverse transcriptase involved in replication of the plasmids (22). Considered together, the characteristics of the plasmids suggest that they are retroelements that propagate as autonomous DNAs but replicate via an RNA intermediate and reverse transcription step. The only previously described examples of retroelements that propagate as autonomous DNA species are cauliflower mosaic virus and hepatitis B viruses (15, 37, 43, 45).

To investigate whether the Mauriceville and Varkud plasmids can function as mobile elements, we isolated mutants of the plasmid-containing strains whose growth is impaired as a result of deleterious behavior of the plasmids (1). Several of these mutants contain defective mtDNAs into which mitochondrial plasmid sequences had integrated. In three cases analyzed in detail, plasmid sequences at the plasmid-mtDNA junctions correspond precisely to 5′ ends of major transcripts of the plasmid, suggesting that the integrations occurred via an RNA intermediate and a reverse transcription step (1). The mutant strains also contain altered mitochondrial plasmids that have become suppressive and

* Corresponding author.
† Present address: Department of Biochemistry, Wayne State University Medical School, Detroit, MI 48201.
‡ Present address: Institute of Gas Technology, 3424 S. State St., Chicago, IL 60616.

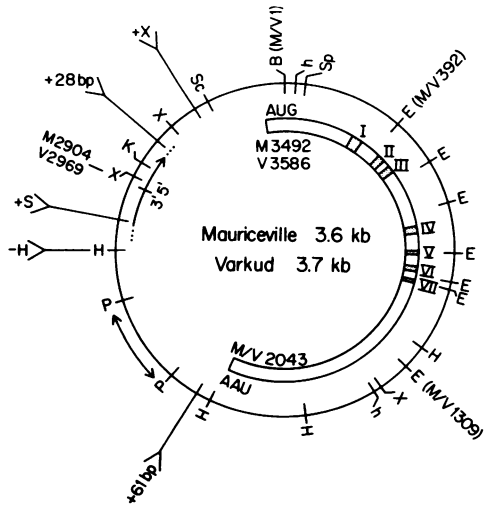


FIG. 1. Map of the Mauriceville and Varkud mitochondrial plasmids. The circular map shows restriction sites in common between the two plasmids, and differences in the Varkud plasmid are indicated to the outside of the circle (Akins et al., in press). +28 bp and +61 bp, Insertions in the Varkud plasmid; +X, +S, −H, additional or missing restriction enzyme cleavage sites in the Varkud plasmid. Restriction enzyme sites and the positions of the 61- and 28-bp insertions and deletions are based on sequence data (33; Akins et al, in press). The 710-amino-acid open reading frame is indicated inside the restriction map. Stippled areas in the open reading frame indicate short blocks of amino acids (I through VII) that are characteristic of reverse transcriptases (29). 3',5', Locations of the major 3' and 5' ends of the predominant plasmid transcripts. The 5'-end nucleotides are given in both Varkud (V) and Mauriceville (M) coordinates. Other abbreviations: B, *Bgl*II; E, *Eco*RI; h, *Hin*cII; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Spe*I; Sc, *Sac*I; Sp, *Sph*I; X, *Xba*I.

impair growth by outcompeting mtDNA. We showed previously that three of these suppressive plasmids had undergone insertion of a mitochondrial tRNA sequence at the position corresponding to the 5' end of the major plasmid transcript or 24 nucleotides (nt) downstream near a cognate of the sequence at the major 5' RNA end. The structure of the suppressive plasmids suggested that the tRNAs were linked to the plasmid transcripts at the RNA level and then reverse transcribed into plasmid DNA (1).

In the present work, we sequenced the region around the major 5' RNA start site in six additional suppressive mutants of the Mauriceville and Varkud plasmids and characterized transcripts of all nine mutant plasmids. We find that all the suppressive plasmids contain a mitochondrial tRNA or tRNA-like sequence inserted in a manner consistent with the previous hypothesis that the suppressive plasmids were generated via an RNA intermediate and reverse transcription step. In addition, we show that the 3' end of the wild-type plasmid transcript can itself be folded into a secondary structure which has tRNA-like characteristics, similar to the tRNA-like structures at the 3' ends of plant viral RNAs. This structure may play a role in replication of the plasmids by reverse transcription. Major transcripts of the suppressive plasmids begin at the 5' ends of the inserted mitochondrial tRNA sequences and are present in 25- to 100-fold-higher concentrations than are transcripts of wild-type plasmids. Mapping of 5' RNA ends within the inserted mtDNA sequence identifies a short consensus sequence (PuNPuAG), which is present at the 5' ends of a subset of mitochondrial tRNAs. This sequence, together with sequences immediately upstream in the plasmids, forms a longer consensus sequence which is similar to sequences at several *Neurospora* mtDNA transcription initiation sites identified by in vitro transcription (J. C. Kennell and A. M. Lambowitz, manuscript in preparation). The suppressive behavior of the plasmids is likely to be directly related to insertion of tRNAs leading to overproduction of plasmid transcripts, possibly via incorporation of a new promoter into the plasmids.

MATERIALS AND METHODS

Strains of *Neurospora* and growth conditions. Wild-type strains used in this study were *Neurospora crassa* 74-OR23-1*A* (designated 74*A*; FGSC 2489) and Mauriceville-1c (FGSC 2225) and *Neurospora intermedia* Varkud-1c (FGSC 1823). Senescent mutants used in this study were: M1-7, M2-8, M2-9, M3-24, V1-2, V3-8, V4-18, V5-36, V7-7, and V8-2. The isolation of these senescent mutants was described previously (1). The mutants are designated M (Mauriceville) or V (Varkud), followed by a number indicating the slant or race tube passage at which the mutant was isolated. Procedures for maintaining strains, preparing conidia, and growing cells in liquid culture were as previously described (1, 11, 24).

Wild-type 74*A* and the wild-type Mauriceville and Varkud strains were grown in liquid culture for 14 h at 25°C or 10 h at 37°C. As reported previously, some of the mutant strains show a significant temperature effect, such that the mutant plasmid accumulates to a greater extent at 37°C than at 25°C, and growth is impaired more severely at the higher temperature (1). Mutants were grown in liquid culture under the most stringent conditions that permitted sufficient growth for a mitochondrial preparation. Typically, mutants were grown at 37°C. As they became progressively impaired, it was necessary to grow mutants M2-8, M2-9, V1-2, V4-18, and V5-36 at 25°C. In the experiment shown in Fig. 6, strains were grown at temperatures indicated in the figure legend for analysis of mitochondrial RNAs by Northern hybridization. Growth times ranged from 20 to 30 h, depending on when the culture had reached sufficient density for a mitochondrial preparation. Liquid cultures of mutant strains were inoculated with up to three times the normal amount of conidia to compensate for poor germination.

Recombinant plasmids and bacteriophage. Plasmid pV2 contains the Varkud mitochondrial plasmid cloned as a *Bgl*II fragment in the *Bam*HI site of pBR322. pLSE52 contains the 2.9-kb *Eco*RI fragment of the Mauriceville plasmid cloned in pBR322 (33). The following recombinant plasmids contain mitochondrial plasmids from the senescent strains indicated in parentheses cloned as *Sac*I fragments in Bluescribe vector [pBS(+); Stratagene, La Jolla, Calif.]: pM32435 (M3-24), pV381 (V3-8), and pV771 (V7-7). The following recombinant plasmids contain mitochondrial plasmids from the senescent strains indicated in parentheses cloned as *Bgl*II fragments in pBR322: pM282 (M2-8), pV128 (V1-2), and pV828 (V8-2). The following recombinant phage contain *Bgl*II fragments of the mitochondrial plasmids indicated in parentheses cloned in M13mp18 (34): phage M13mpM172 (M1-7), M13mpM292 (M2-9), M13mpM3245 (M3-24), M13mpV382b (V3-8), M13mpV4183 (V4-18), M13mpV5363d (V5-36), M13mpV771 (V7-7), and M13mpV822a (V8-2).

Isolation of mitochondria and mitochondrial nucleic acids. Mitochondria were isolated by the modified flotation gradient method (23). mtDNA and RNA were isolated by the UNSET-phenol-chloroform-isoamyl alcohol procedure (1,

16) and digested with RNase or DNase as previously described (Akins et al., in press).

DNA sequencing. Single-stranded M13 phage DNAs were sequenced by the dideoxy-chain termination method (39, 40) by using the universal M13 primer (−20) or oligomers (20-mers) synthesized by an Applied Biosystems DNA synthesizer. Double-stranded plasmid DNAs were sequenced by the same method, with a modification of the alkaline denaturation procedure (8; Promega Notes, July 1986; Promega Biotec, Madison, Wis.). End-labeled restriction fragments were sequenced by the method of Maxam and Gilbert (27). The Beckman Microgenie system was used for analysis and storage of data.

Northern hybridization. Mitochondrial RNAs (7 μg) were denatured with glyoxal (28), electrophoresed in a 1.4% agarose gel, photographed, and transferred to nitrocellulose as previously described (41). Hybridizations were overnight at 37°C in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate)–2.5 mM sodium phosphate buffer (pH 7.0)–0.2% sodium dodecyl sulfate–0.2 mg of sheared, denatured salmon sperm DNA per ml–1× Denhardt solution (12). Blots were washed twice in 2× SSC–0.1% sodium dodecyl sulfate (room temperature, 5 min) and three times in 0.1× SSC–0.1% sodium dodecyl sulfate (45°C, 15 min). Probes were ^{32}P labeled by nick translation (38).

RNase H digestion. To analyze the conformation of plasmid transcripts, mitochondrial RNAs were digested with RNase H in the presence of synthetic oligomer O-II. O-II is a 20-mer (GCATCGATTCCAACCTTAGAC) complementary to a sequence 306 to 325 nt upstream of the major 3′ end of both the Mauriceville and Varkud plasmid transcripts (Akins et al., in press). RNase H digestion products were analyzed by gel electrophoresis as described by Akins et al. (in press).

Primer extension mapping. Primer extension mapping of 5′ RNA ends was carried out by using synthetic oligomer O-I, a 20-mer (GCATCCTTTCTAGAACCCAA) complementary to a sequence 80 to 99 nt downstream of the major 5′ RNA end of both the Mauriceville and Varkud plasmid transcripts. The oligomer was 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (27). Reverse transcription was as described by Akins et al. (in press). Primer extension products were analyzed on sequencing gels containing 8% polyacrylamide and 8 M urea, next to dideoxy-sequencing ladders generated from the same primer, by using single-stranded DNAs of M13 phage clones of the same senescent plasmid as templates.

S1 nuclease mapping. 5′ ends of mutant plasmid transcripts were mapped by using a single-stranded, 5′-end-labeled *Xba*I-*Nla*III fragment. This fragment had to be isolated separately from each mutant plasmid that was analyzed. To obtain this fragment, recombinant plasmids pM282, pV128, pV381, pV771, and pV828 (10 to 20 μg) were digested with *Xba*I, incubated with calf intestine alkaline phosphatase (24 U, 60 min, 37°C; Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (27). The DNAs were digested with *Nla*III, and the single-stranded, 5′-end-labeled fragments were isolated by electrophoresis in sequencing gels containing 6% polyacrylamide and 8 M urea. The fragments were annealed to whole mitochondrial RNAs from the corresponding strains and digested with S1 nuclease, essentially as described previously for the wild-type Varkud plasmid transcript (Akins et al., in press). The reaction products were analyzed on sequencing gels containing 8% polyacrylamide and 8 M urea, next to Maxam-Gilbert sequencing ladders generated from the same ^{32}P-labeled *Xba*I-*Nla*III restriction fragments (27).

3′ ends of mutant plasmid transcripts were mapped by using a single-stranded 3′-end-labeled *Spe*I-*Xba*I fragment, which again had to be isolated separately from each mutant plasmid analyzed. To obtain this fragment, 10 μg of recombinant plasmid pV128, pV381, pV771, or pV828 was cut with *Spe*I and ^{32}P labeled by filling in with the Klenow fragment of DNA polymerase I with [α-^{32}P]dCTP plus unlabeled dTTP, dGTP, and dATP. The plasmids were then cut with *Xba*I, and single-stranded DNA fragments varying in length from 208 to 264 nt, depending on the size of the mtDNA inserts contained in the mutant plasmids, were purified by electrophoresis in a sequencing gel containing 6% polyacrylamide and 8 M urea. Hybridization to mitochondrial RNA and digestion with S1 nuclease were as described previously for wild-type Varkud plasmid transcripts (Akins et al., in press). S1 nuclease digestion products were analyzed on sequencing gels containing 8% polyacrylamide and 8 M urea, next to appropriate dideoxy-sequencing ladders. The sequencing ladders were generated from the same double-stranded, recombinant plasmids by the alkaline denaturation procedure (see above), with a *Spe*I-*Dde*I fragment of pV2, which had been 5′ end labeled at the *Spe*I site with [γ-^{32}P]ATP and T4 polynucleotide kinase (27), as a primer.

In vitro capping of mitochondrial RNAs with guanylyltransferase. *Neurospora* whole mitochondrial RNAs were capped with vaccinia virus guanylyltransferase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) essentially as described by Levens et al. (26) for yeast mitochondrial RNAs, except that heat denaturation of the RNA (3 min, 65°C) was used instead of denaturation with methyl mercuric hydroxide. RNAs were denatured with glyoxal and electrophoresed in a 1.4% agarose gel as described previously. Gels were dried under vacuum and autoradiographed. Good labeling of high-molecular-weight RNAs was dependent upon the quality of the commercial guanylyltransferase preparation, which was found to be highly variable, as judged by capping of in vitro transcripts synthesized by using bacteriophage T3 RNA polymerase.

RESULTS

Suppressive mutant plasmids contain inserted mitochondrial tRNA sequences. The isolation of *Neurospora* mutants containing suppressive mitochondrial plasmids was described previously (1). Briefly, the plasmid-containing strains were subjected to continuous vegetative growth in race tubes or serial transfer in slants at 37°C. Suppressive plasmids or defective mtDNAs are generated spontaneously during vegetative growth and then accumulate relative to mtDNA, leading to impaired growth of hyphal tips and eventually cell death. Mutants in which hyphal tips had stopped growing completely were analyzed by taking hyphae from an earlier section of the race tube or from an earlier transfer in slants. We showed previously (1) that 12 mutant strains isolated in this way contain suppressive plasmids that outcompete mtDNA, and we reported the nucleotide sequence around the major 5′ RNA start site in three of these plasmids. In the present work, we determined sequences around the major 5′ RNA start site in six additional mutant plasmids. The data for all nine plasmids are shown in Fig. 2, and a schematic diagram summarizing the major structural features of the plasmids is shown in Fig. 3.

The DNA sequencing showed that all the suppressive plasmids contain small insertions of mtDNA sequences and

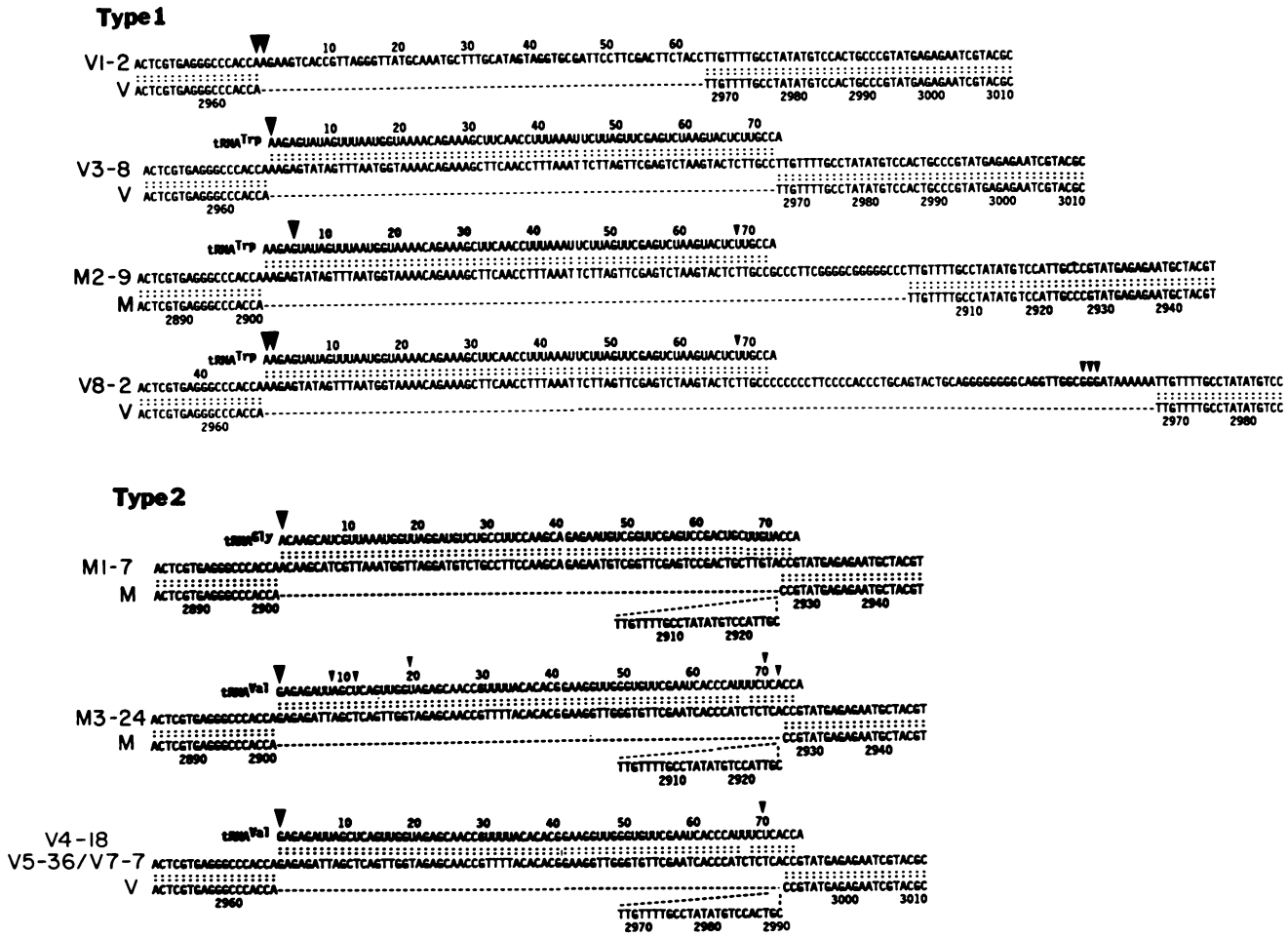


FIG. 2. Nucleotide sequence around the major 5′ RNA start site in nine mutant plasmids. Sequences of both DNA strands were determined from restriction fragments cloned in M13mp18, Bluescribe (pBS+), or pBR322. The sequences of $tRNA^{Trp}$ and $tRNA^{Val}$ were determined by RNA sequencing (18). The sequence of $tRNA^{Gly}$ was inferred from the DNA sequence of the gene (K. Browning, C. Breitenberger, M. Potter, and U. L. RajBhandary, personal communication). ▼▾, Positions of major and minor 5′ ends of plasmid transcripts, respectively, determined by primer extension experiments (Fig. 8). In V7-7 and one preparation of V8-2 RNA, the 5′ end at the beginning of the tRNA insert was less abundant than the downstream 5′ end (see Results and Fig. 9). In M2-9 only, the major 5′ end mapped by primer extension was at G(+5) in the tRNA insert. However, this is likely to be due to an artifact in the primer extension or to a secondary alteration in the plasmid, since the major 5′ end mapped by S1 nuclease digestion for the previous subculture (M2-8) was directly at the 5′ end of the tRNA insert (Fig. 9).

that all but one of these insertions correspond to or include a known mitochondrial tRNA sequence. Three plasmids contain $tRNA^{Trp}$ (M2-8/M2-9, V3-8, and V8-2; M2-8 and M2-9 are successive subcultures of the same mutant), four plasmids contain $tRNA^{Val}$ (M3-24, V4-18, V5-36, and V7-7), and one plasmid contains $tRNA^{Gly}$ (M1-7). The remaining plasmid, V1-2, contains a 64-nt insert that hybridizes to mtDNA restriction fragment *Eco*RI-8. This latter insert does not correspond to a known tRNA but can be folded into a cloverleaf secondary structure and has scattered homology to *Neurospora* mitochondrial $tRNA^{Trp}$ (Fig. 4). The insert in V1-2 may correspond to a deviant tRNA species, or more likely, a tRNA pseudogene. Interestingly, the homology between the V1-2 insert and $tRNA^{Trp}$ extends into the 3′-flanking region of the $tRNA^{Trp}$ gene (Fig. 4).

The mtDNA sequences are inserted at either of two positions in the plasmid. In type 1 plasmids, the insert is directly at the major 5′ end of the plasmid transcript (position M2904 or V2969). In type 2 plasmids, the insert is located 24 nt downstream (M2928 or V2993), near a cognate of the sequence surrounding the major 5′ RNA end (CCAPyTG; position M2921-2926 or V2986-2991; Fig. 2 and 3). In both type 1 and 2 plasmids, the insert is located immediately downstream of two tandem CCA sequences (position M2903 or V2968) which correspond to the major 3′ end of the plasmid transcript (Fig. 2 and 3). Three of four type 1 plasmids contain $tRNA^{Trp}$, and as discussed above, the remaining type 1 plasmid, V1-2, contains a tRNA-like insert with some homology to $tRNA^{Trp}$. Type 2 plasmids contain either $tRNA^{Val}$ or $tRNA^{Gly}$. In all type 2 plasmids, the same plasmid sequences, M2904 to M2927 or V2969 to V2992, have been deleted precisely (Fig. 2).

In each case, the inserted mitochondrial tRNA sequence is oriented head to tail with respect to the major plasmid transcript (Fig. 2 and 3). In seven plasmids, V1-2, V3-8, V7-7, M1-7, M3-24, V4-18, and V5-36, the insert begins precisely at the 5′ end of the tRNA and ends at the 3′ end, excluding the final A residue. In two plasmids, M2-9 and V8-2, the insert begins precisely at the 5′ end of the tRNA, extends through the terminal CC residues of the tRNA and

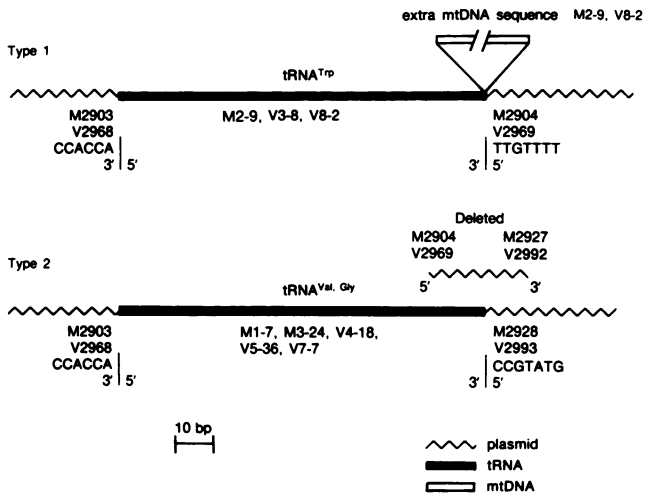


FIG. 3. Schematic representation of the structure of the mutant plasmids. In type 1 plasmids, tRNA sequences are inserted head to tail at a position corresponding to the major 5′ end of the plasmid transcript (M2904 or V2969). In type 2 plasmids, tRNA sequences are inserted 24 nt downstream (M2928 or V2993), adjacent to a cognate of the sequence at the major 5′ RNA start site. In both types of plasmids, the inserted tRNA sequence is always located immediately downstream of two tandem CCA sequences corresponding to the 3′ end of the major transcript of the plasmid (M2903 to V2968). In type 2 plasmids, plasmid sequence M2904 or M2927 or V2969 to V2992 has been deleted precisely. The inserted sequences begin precisely at the 5′ end of the tRNA and end at the 3′ end of the tRNA, excluding the terminal A residue. In type 1 plasmids M2-9 and V8-2, extra mtDNA sequences (19 and 56 bp, respectively) are present downstream of the tRNA sequence. The origin of these sequences is discussed in the text.

then contains additional mtDNA sequences of 19 and 56 base pairs (bp), respectively (Fig. 2 and 3). The origin of these additional sequences is discussed below. The finding that the tRNA sequences are always missing the terminal A residue may be related to the mechanism by which the tRNAs were inserted into the plasmids (see Discussion).

In eight of nine cases, the inserted mtDNA sequence ends with the dinucleotide CC (Fig. 2). In type 2 plasmids, we cannot distinguish whether these CC residues are derived from the tRNA insert or from the plasmid sequence at position M2928 or V2992. However, in the type 1 plasmids V3-8, V1-2, and M2-9, this ambiguity does not exist and the terminal CC residues must be derived from $tRNA^{Trp}$ or the other inserted mtDNA sequences. As noted previously, at least one of the C residues at the 3′ end of the $tRNA^{Trp}$ insert is not encoded in mtDNA and must have been added posttranscriptionally to the tRNA (1). The only insert that does not end in CC is the second half of the composite insertion in plasmid V8-2, which ends in a run of six A residues (Fig. 2).

In addition to the inserted mitochondrial tRNA sequences, plasmids M3-24 and V5-36 have relatively large deletions (522 and 345 nt, respectively) at other locations in the plasmid (R. A. Akins and A. M. Lambowitz, unpublished data). Further, since only the region around the major 5′ RNA start site has been sequenced, it is possible that some mutant plasmids contain additional alterations that do not lead to gross changes in size or restriction sites. Such additional alterations may contribute to differences in the behavior of plasmids having the same tRNA inserts (e.g., differences in transcription of V7-7 and V4-18 or V5-36).

Two variant plasmids contain complex insertions consisting of segments from noncontiguous regions of *Neurospora* mtDNA. In plasmids M2-9 and V8-2, the $tRNA^{Trp}$ insert is flanked by additional sequences of 19 and 56 nt, respectively, which differ between the plasmids (Fig. 2 and 5). Surprisingly, these sequences are not contiguous with the $tRNA^{Trp}$ gene in mtDNA from wild-type 74*A*, Mauriceville, or Varkud. A computer search of available mtDNA sequences from wild-type 74*A* revealed an 18- of 20-nt match between the sequence in M2-9 and a sequence in mtDNA restriction fragment *Hin*dIII-7b. This sequence is located 10 kb downstream of the $tRNA^{Trp}$ gene and separated by the genes encoding the large rRNA and other tRNAs. Similarly, the sequence in V8-2 matches a sequence in *Hin*dIII-12, which is located 150 bp upstream of the $tRNA^{Trp}$ gene. In both cases, deviations occur at or near the ends of the inserted mtDNA sequences (Fig. 5). Sequencing of the appropriate regions of Mauriceville and Varkud mtDNA showed that these deviations do not reflect strain-specific differences in mtDNA sequences, but it remains possible that better matches are present in other regions of *Neurospora* mtDNA for which the nucleotide sequence is presently unavailable.

The additional 19- and 56-nt sequences in plasmids M2-9 and V8-2 both have runs of pyrimidine residues near their 5′ ends and internal runs of G residues (Fig. 5). The dinucleotides GC or CC are found at both ends of the 19-nt sequence in M2-9 and at the 5′ end of the 56-nt sequence in V8-2. The V8-2 insert ends in a run of 6 A residues, which are not present in either the mtDNA or mitochondrial plasmid

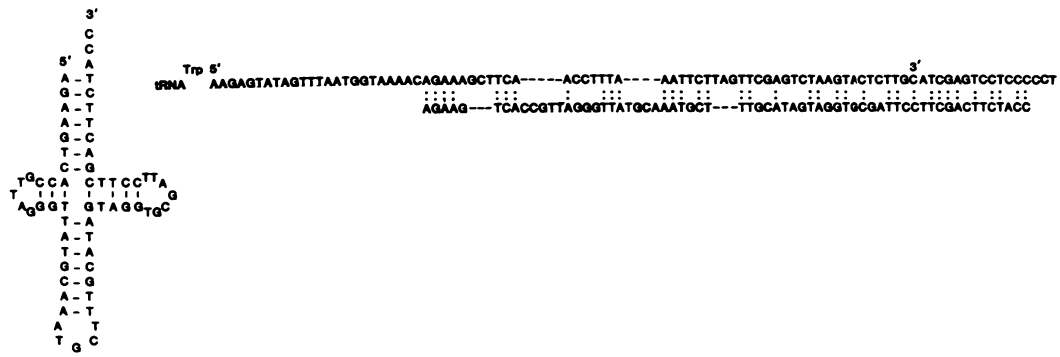


FIG. 4. The insert in mutant plasmid V1-2 is homologous to the $tRNA^{Trp}$ gene and can be folded into a cloverleaf structure. 5′ and 3′ nucleotides of the $tRNA^{Trp}$ transcript are shown on the top line.

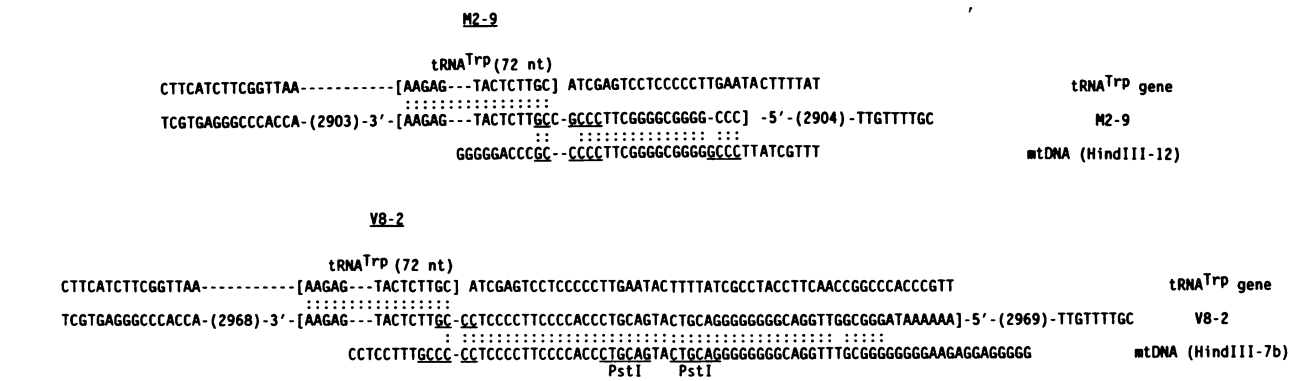


FIG. 5. The inserts in mutant plasmids M2-9 and V8-2 consist of tRNA^{Trp} plus additional mtDNA sequences that are not contiguous on the mitochondrial genome. Top lines indicate tRNA^{Trp} gene and flanking sequences in wild-type 74*A*, Mauriceville, and Varkud mtDNAs. Middle lines indicate sequences in mutant plasmids M2-9 and V8-2. Bottom lines indicate sequences from mtDNA restriction fragments *Hin*dIII-12 and *Hin*dIII-7b that match sequences flanking tRNA^{Trp} in the mutant plasmids. The mtDNA sequences were confirmed to be the same in wild-type 74*A*, Mauriceville, and Varkud. GC or CC sequences at the boundaries of inserted segments are underlined. *Pst*I sites in the *Pst*I palindromes of the insert in V8-2 are also underlined.

sequence. The 56-nt sequence in V8-2 also contains a *Pst*I palindrome, a repetitive sequence element in *Neurospora* mtDNA (47).

Transcripts of mutant plasmids. The inserted mitochondrial tRNA sequences presumably alter the replication or expression of the plasmids to cause their suppressive behavior. As a first step for investigating how this might occur, we characterized transcripts of the suppressive plasmids.

Figure 6 shows Northern hybridization analysis of whole mitochondrial RNAs from the Mauriceville and Varkud strains and from mutants containing suppressive plasmids. The RNAs were electrophoresed through 1.4% agarose gels, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled mitochondrial plasmid probe (recombinant plasmid pV2). The lane for the wild-type Mauriceville plasmid (M) shows a single prominent band of 3.6 kb. This RNA was characterized previously and shown to be a full-length, linear transcript whose major 5′ and 3′ ends are immediately adjacent on the plasmid DNA (33). The lane for the wild-type Varkud plasmid (V) shows prominent transcripts of 3.7 and 4.9 kb. The 3.7-kb RNA is a full-length linear RNA, equivalent to the 3.6-kb transcript of the Mauriceville plasmid, whereas the 4.9-kb Varkud RNA is a hybrid transcript consisting of the full-length 3.7-kb RNA plus a 5′ end extension of 1.2 kb that contains sequences from the mitochondrial small rRNA (Akins et al., in press). The ^{32}P-labeled plasmid probe also hybridizes to larger transcripts which may be RNA oligomers.

For most of the mutant plasmids, the Northern (RNA) blots show a single predominant transcript of the size expected for a full-length transcript of that plasmid (3.3 to 3.8 kb). This was also the case for plasmid V8-2, which is not shown in the figure. The only exception is V7-7, which shows two major transcripts longer than the monomer plasmid (5.0 and 5.2 kb), in addition to smaller amounts of full-length (3.8-kb) transcript (Fig. 6). The 5.2- and 5.0-kb transcripts may be hybrid RNAs analogous to the 4.9-kb RNA in the wild-type Varkud strain. Plasmids M3-24 and V5-36 contain relatively large internal deletions and consequently give full-length transcripts shorter than those for the other plasmids.

In addition to the predominant transcripts, the Northern hybridizations for the growth variants consistently showed a background of degraded RNAs that was not present for

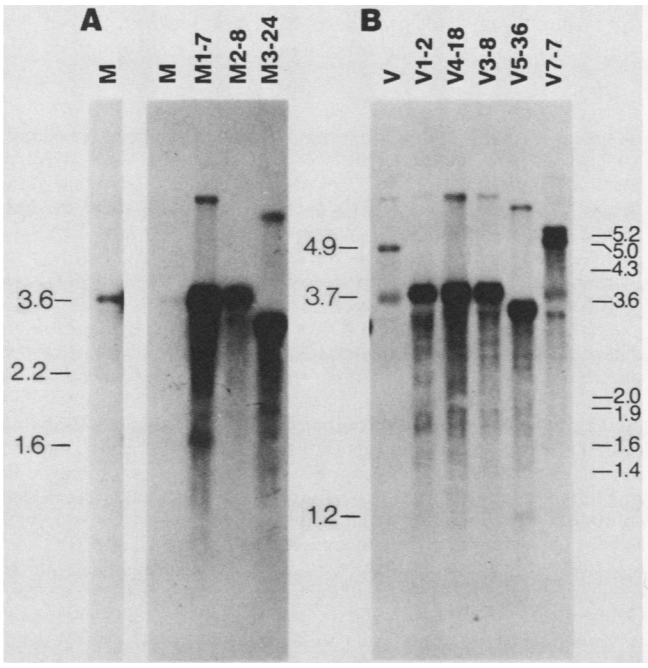
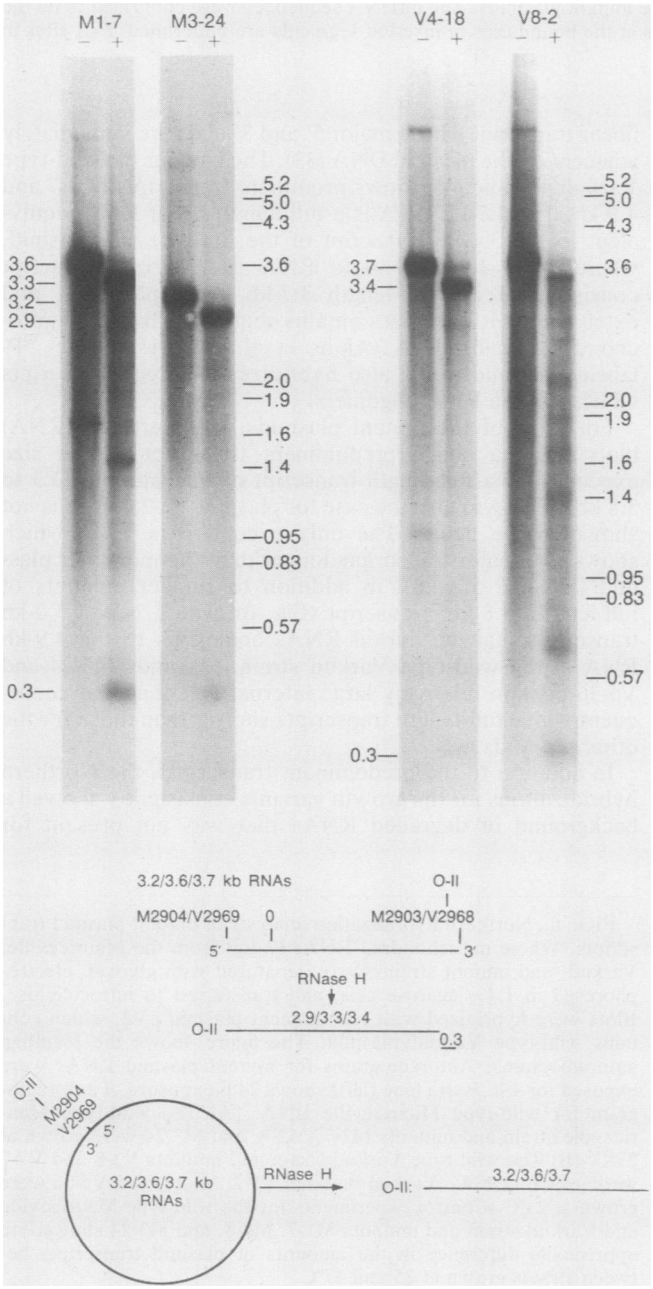


FIG. 6. Northern hybridization analysis of mutant plasmid transcripts. Whole mitochondrial RNAs (7 μg) from the Mauriceville, Varkud, and mutant strains were denatured with glyoxal, electrophoresed in 1.4% agarose gels, and transferred to nitrocellulose. Blots were hybridized with recombinant plasmid pV2, which contains wild-type Varkud plasmid. The figure shows the resulting autoradiograms. Autoradiograms for mutant plasmid RNAs were exposed for 4 h. Extra lane (left) shows 24 h exposure of autoradiogram for wild-type Mauriceville RNA. (A) The wild-type Mauriceville strain and mutants M1-7, M2-8, and M3-24 were grown at 25°C. (B) The wild-type Varkud strain and mutants V3-8 and V7-7 were grown at 37°C. Varkud mutants V1-2, V4-18, and V5-36 were grown at 25°C. Control experiments for the wild-type Mauriceville and Varkud strain and mutants M1-7, M2-8, and M3-24 showed no appreciable difference in the amounts of plasmid transcripts between strains grown at 25 and 37°C.

wild-type strains. This background may reflect either increased degradation in vivo or higher nuclease activity released during isolation of RNAs from poorly growing mutant strains. Some mutants also show discrete smaller RNAs which are less abundant than full-length transcript: e.g., 1.6-kb RNA in M1-7, 2.2-kb RNA in M3-24, and 1.2-kb RNA in V5-36.

To investigate whether transcripts of the mutant plasmids are linear or circular RNA molecules, we carried out RNase H digestion in the presence of a synthetic 20-mer complementary to a sequence in the plasmid transcript. Figure 7 shows data for M1-7, M3-24, V4-18, and V8-2, and similar results (not shown) were obtained for V1-2 (25°C), V3-8 (37°C), and M2-8 (25°C). The mutants analyzed by RNase H digestion included one representative of each plasmid structure. The experimental procedure is outlined at the bottom of Fig. 7. Briefly, a circular RNA hybridized with the oligomer and digested with RNase H should give a single band corresponding to linearized RNA, whereas linear transcripts should give two fragments whose sizes add up to that of the original linear RNA. In each case, RNase H cut the plasmid transcript into two major fragments, as expected for a linear RNA molecule. In most cases, a light band (less than 5 to 10% of the predominant RNase H fragments) remained at the size expected for linearized RNA and could reflect either a small amount of linearized circular RNAs or incomplete digestion with RNase H. RNase H digestion of V8-2 RNA gave additional smaller bands, which could reflect the poorer quality of the RNA preparations obtained consistently from this strain. The data shown in Fig. 7 also show that the 1.6-kb RNA in M1-7 is digested by RNase H in the presence of oligomer O-II and must therefore correspond to a 3′ segment of the plasmid transcript.



The mutant plasmids have two major differences in their pattern of transcription. First, the steady-state concentrations of plasmid transcripts, calculated by scanning various exposures of autoradiograms, was consistently 25- to 100-fold higher for the mutant plasmids than for the wild-type plasmids (Fig. 6). Even allowing for the increased amounts of mutant plasmid DNA relative to wild type (two- to fivefold), this still represents a substantial increase in amount of transcript per plasmid. Second, with the exception of V7-7, the mutant plasmids do not synthesize the equivalent of the 4.9-kb Varkud plasmid transcript, which was shown previously to be a hybrid RNA having a 5′ leader derived from the mitochondrial small rRNA (Akins et al., in press).

Mapping of 5′ and 3′ ends of mutant plasmid transcripts. The 5′ ends of major transcripts of all the variant plasmids were mapped by primer extension by using a synthetic oligonucleotide primer complementary to a sequence near the 5′ end of the plasmid RNA (M2980-2999 or V3048-3067). Representative data are shown in Fig. 8, and major and minor 5′ ends detected by primer extension are indicated by large and small arrows in Fig. 2. The striking finding was that all of the variant plasmids have a major 5′ RNA end at or near the 5′ end of the inserted mitochondrial tRNA sequence. For several variant plasmids (V1-2, V3-8, M2-8, and

FIG. 7. Analysis of conformation of mutant plasmid transcripts by site-specific cleavage with RNase H. Oligomer O-II, which is complementary to a sequence approximately 0.3 kb upstream of the major 3′ end of the plasmid transcripts, was hybridized to whole mitochondrial RNA from each strain (V4-18 grown at 25°C and M1-7, M3-24, and V8-2 grown at 37°C). The DNA-RNA hybrids were digested with RNase H and analyzed by gel electrophoresis and Northern hybridization as described in Materials and Methods. Lanes labeled − and + show RNAs incubated with RNase H in the absence or presence of oligomer O-II, respectively. Sizes of predicted fragments for circular and linear RNAs are shown at the bottom. V4-18 and V8-2 (3.7 kb) gave linear RNA fragments of 3.4 and 0.3 kb. M1-7 (3.6 kb) gave linear RNA fragments of 3.3 and 0.3 kb. M3-24 (3.2 kb) gave linear RNA fragments of 2.9 and 0.3 kb. All of the digests show light bands remaining at the position corresponding to full-length plasmid RNA. These light bands could reflect a low proportion of circular RNA molecules or incomplete digestion with RNase H. Similar results were obtained for V1-2 (25°C), V3-8 (37°C), and M2-8 (25°C) (data not shown). The extra bands in the V8-2 digest may reflect the apparently poorer quality of RNA preparations that were obtained consistently from this strain. Controls incubated under the same conditions without RNase H did not show the indicated digestion products (data not shown).

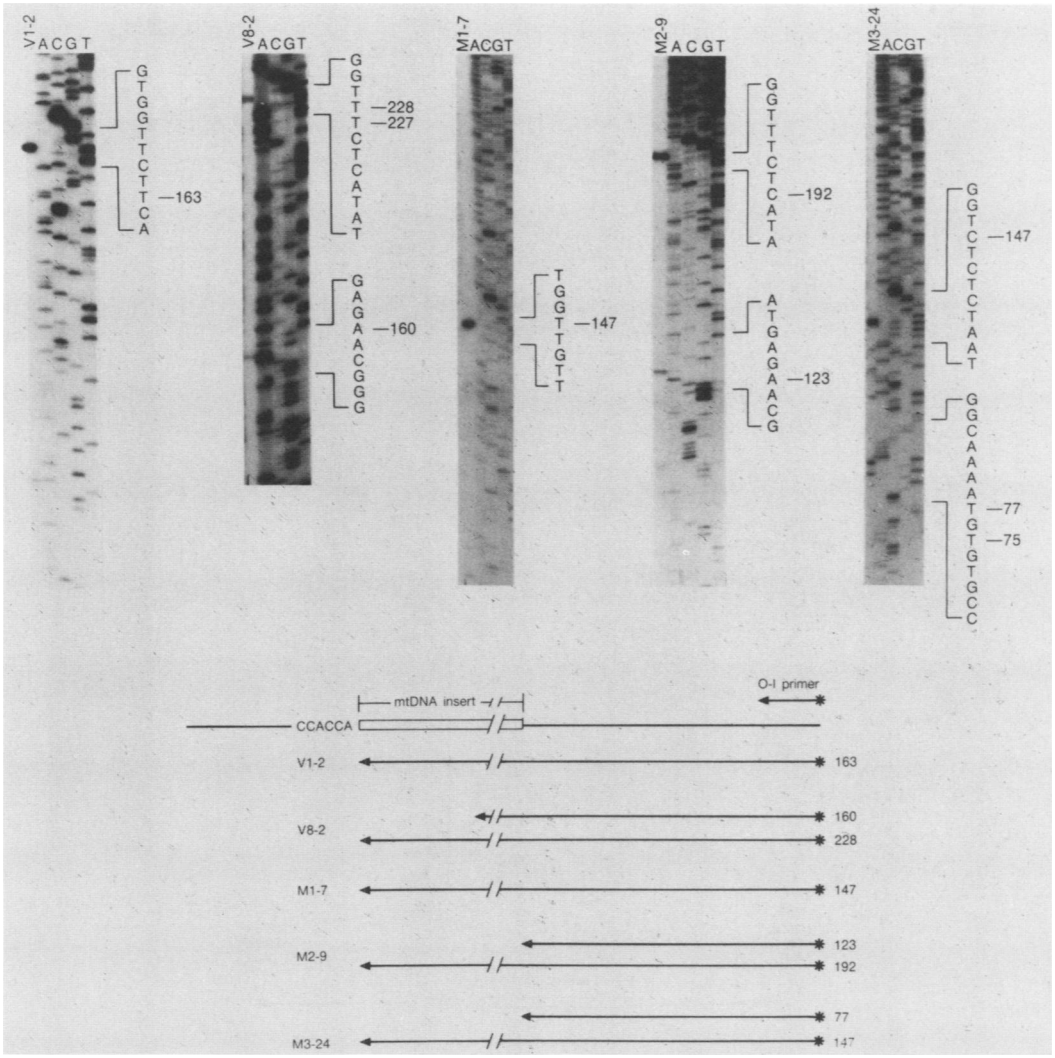


FIG. 8. Mapping of 5′ ends of mutant plasmid transcripts by primer extension. ^{32}P-labeled oligomer O-I, which is complementary to a sequence approximately 0.1 kb downstream of the 5′ end of wild-type plasmid transcripts (33; Akins et al., in press) was annealed to whole mitochondrial RNA from each mutant (M2-9 grown at 25°C and M1-7, M3-24, V1-2, and V8-2 grown at 37°C). Primer extension by avian myeloblastosis virus reverse transcriptase was carried out as described in Materials and Methods. Primer extension products were analyzed on 8% polyacrylamide, 8 M urea sequencing gels, next to dideoxy-sequencing ladders generated from the same primer (O-I), by using single stranded DNA from M13mp18 clones of the same variant plasmid as a template. Schematic at bottom shows position of 5′ RNA ends that were detected. V8-2 gives additional cDNAs of 108 to 110 nt that were run off the gel shown in the figure.

V8-2), we confirmed the presence of a major 5′ RNA end mapping at the beginning of the inserted mitochondrial tRNA sequence by S1 nuclease analysis (Fig. 9). In each of the first three cases, the 5′ end mapped by S1 nuclease analysis was within 0 to 5 nt of the major 5′ end mapped by primer extension. In V8-2, the 5′ end mapped by S1 nuclease (band at 214 nt; Fig. 9) was 14 nt downstream of the major 5′ end mapped by primer extension. In the case of V7-7, the 5′ ends mapped by primer extension are assumed to correspond to 3.8-kb transcripts, rather than the longer 5.0- and 5.2-kb transcripts, which are also synthesized by this plasmid.

Several minor 5′ RNA ends were also detected by primer extension and S1 nuclease mapping. M2-9, which contains $tRNA^{Trp}$, as well as all the plasmids containing $tRNA^{Val}$ (M3-24, V4-18, V5-36, and V7-7), has an additional 5′ end near the 3′ end of the inserted tRNA sequence. In general, these minor 5′ ends could reflect minor transcription initiation sites or RNA cleavage sites. In V7-7 and one preparation of V8-2 RNA (Fig. 9), an additional downstream 5′ end was more abundant than that at the 5′ end of the tRNA sequence. In the case of V1-2 and all type 2 plasmids, some primer extension experiments showed minor 5′ ends mapping 20 to 40 nt upstream of the tRNA insert (data not shown).

If the major transcripts of the variant plasmids are full-length linear RNAs that begin at the 5′ end of the inserted tRNA sequence, then the transcripts should have the same 3′ end as wild-type plasmid transcript. In four cases (V1-2, V3-8, V8-2, and V7-7), we confirmed by S1 nuclease experiments that the major 3′ ends map to the CCA sequence shown previously to correspond to the 3′ end of the wild-type plasmid transcript (Fig. 10). No 3′ ends were detected at the position expected if the inserted tRNA sequence is linked downstream to the 3′ end of plasmid transcript. (The higher-molecular-weight bands in Fig. 10 correspond to fully

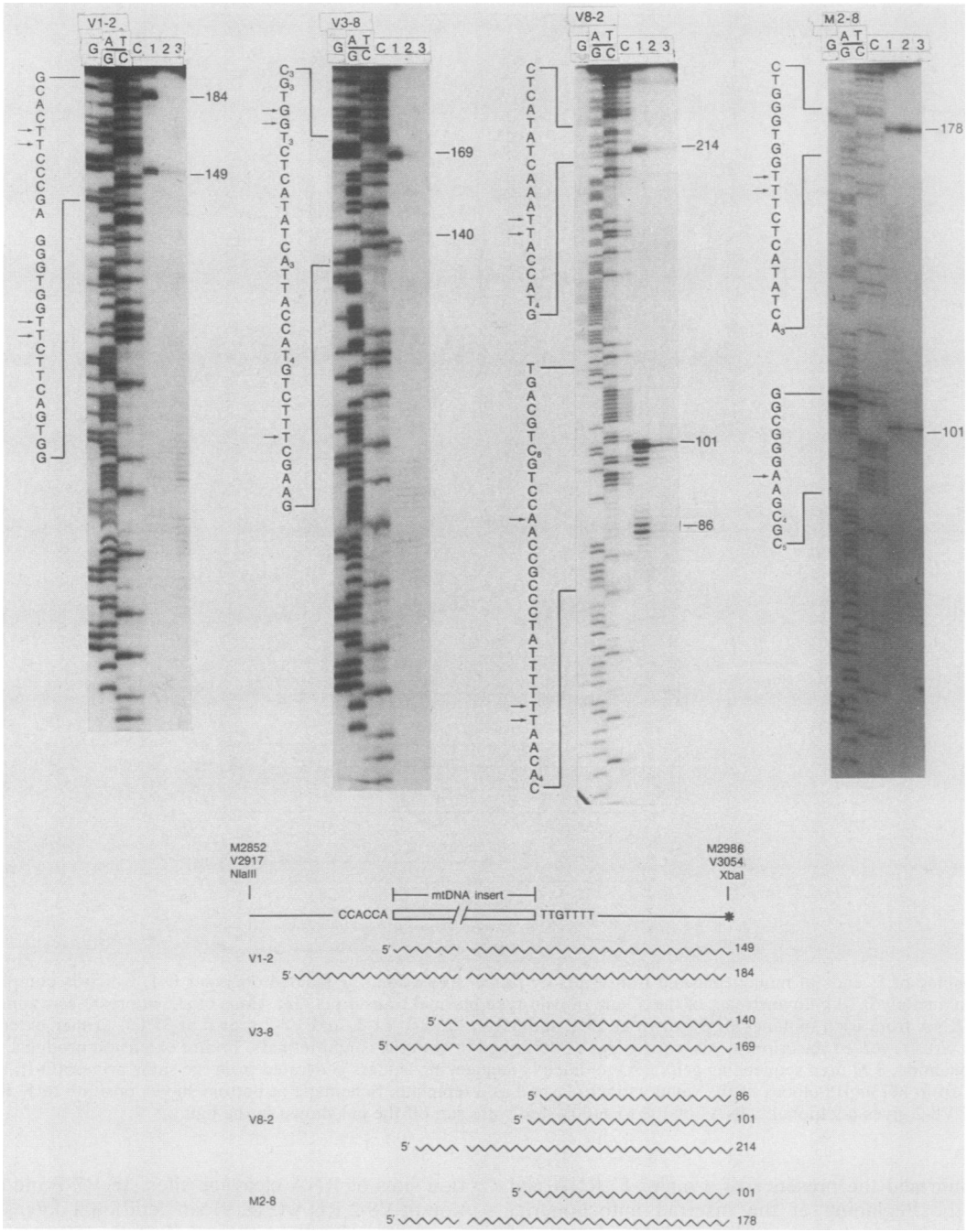


FIG. 9. S1 nuclease mapping of 5′ ends of mutant plasmid transcripts. Probes were single-stranded, 5′-end-labeled *Xba*I-*Nla*III fragments obtained from clones of each variant plasmid. Probes were annealed to whole mitochondrial RNA from the same strain (1, 0.3, and 0.1 μg in lanes 1, 2, and 3, respectively) and digested with S1 nuclease as described in Materials and Methods. M2-8 was grown at 25°C; V1-2, V3-8, and V8-2 were grown at 37°C. Protected fragments were analyzed on 8% polyacrylamide–8 M urea sequencing gels, next to Maxam-Gilbert sequencing ladders generated from the same 5′-end-labeled restriction fragments.

protected probe.) We conclude that major transcripts of the variant plasmids are full-length linear RNAs which begin at the 5′ end of the inserted tRNA sequence and have the same 3′ end as the wild-type plasmid transcript.

A consensus sequence at the 5′ end of the inserted tRNA sequences. The results described above show that all of the variant plasmids have a major transcript that begins at the 5′ end of the inserted tRNA sequence. Comparison of sequences corresponding to these major 5′ RNA ends identifies a short consensus sequence, PuNPuAG, which is present at the 5′ ends of $tRNA^{Trp}$, $tRNA^{Gly}$, and $tRNA^{Val}$, as well as the 5′ end of the tRNA-like insert in V1-2 (underlined in Fig. 11). A survey of 21 *Neurospora* mitochondrial tRNA sequences (17, 18, 42) showed that this 5′ consensus sequence is present in only four tRNA species, including the three listed above and mitochondrial $tRNA^{Tyr}$. The striking selection for tRNAs having this consensus sequence suggests strongly that it is a critical element for the

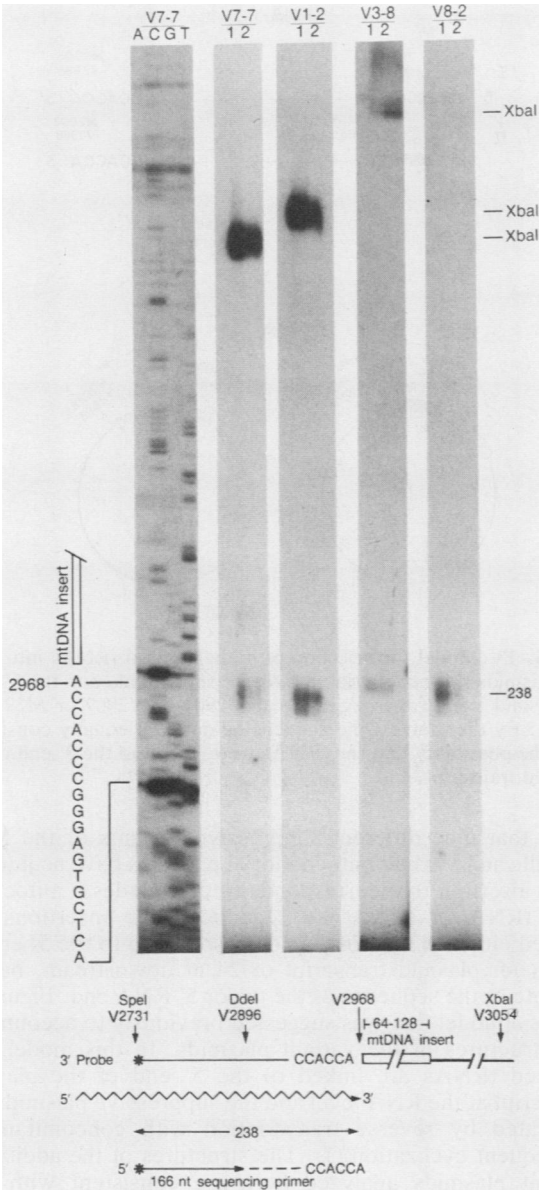


FIG. 10. S1 nuclease mapping of 3′ ends of mutant plasmid transcripts. Probes were single-stranded, 3′-end-labeled *SpeI-XbaI* fragments obtained from clones of each variant plasmid. Probes were annealed to whole mitochondrial RNA from each strain (0.3 to 0.1 μg in lanes 1 and 2, respectively) and digested with S1 nuclease as described in Materials and Methods. V1-2 was grown at 25°C; V3-8, V7-7, and V8-2 were grown at 37°C. Protected fragments were analyzed on 6% polyacrylamide–8 M urea sequencing gels, next to Maxam-Gilbert sequencing ladders generated from the same 3′-end-labeled restriction fragments. Only the sequencing ladder for V3-8 is shown, since all variant plasmid had identical sequence from the labeled *SpeI* site to position V2968. Some lanes show undigested probe DNA, which differs in size between the plasmids. This undigested DNA was present in RNA-free control lanes and most likely reflects incomplete separation of the DNA strands in the preparatory gel. Undigested probe DNA from V8-2 is too large to be seen in figure.

suppressive behavior of the plasmids. In general, these 5′ end sequences could correspond to promoters, enhancers, or RNA processing sites.

Interestingly, the consensus sequence at the 5′ end of the

Insert	Sequence
	M2903 5′- V2968
$tRNA^{Gly}$ (M1-7)	C C A C C A A C A A G C A
$tRNA^{Trp}$ (V3-8, V8-2)	C C A C C A *A A G A G A T
$tRNA^{Val}$ (V4-18, V5-36, V7-7)	C C A C C A *G A G A G A T
tRNA-like (V1-2)	C C A C C A *A *G A A G T C
Consensus 1	C C A A/G N A/G A G
N.C. small rRNA	T T *A *G A G A T G T A A T
N.C. large rRNA	T *T *A *G *A *A *A *T *G *T *A *A *T
N.C. cob	T T A *G *A *G *A *G G G A G T
Consensus 2	Y Y A A/G N A/G A G/T G
Yeast	A T A T A A G T *A
Human HSP	C *C *A *A A G A C
Human LSP	C C *A *A *A A G A
Mouse HSP-2	T T *A A C A A G
Mouse LSP	C T *A G A A G A A TT

FIG. 11. Consensus sequence at the 5′ ends of mutant plasmid transcripts. Asterisks indicate 5′-terminal nucleotides. 5′-terminal nucleotides in the mutant plasmids were determined by primer extension experiments (Fig. 8). Data shown are for all mutant plasmids except M2-9, where for unknown reasons the major 5′ RNA end mapped by primer extension was at G (+5). This is likely due to an artifact in the primer extension or to a secondary alteration in the plasmid, since the major 5′ end mapped by S1 nuclease digestion for the previous subculture (M2-8) was directly at the 5′ end of the tRNA (Fig. 9). Transcription initiation sites at the 5′ ends of *Neurospora crassa* (N.C.) mitochondrial small and large rRNAs and *cob* mRNA were determined by in vitro transcription (Kennell and Lambowitz, manuscript in preparation). The 5′ end positions indicated for these transcription initiation sites are those determined previously by primer extension and/or S1 nuclease mapping of RNAs isolated from wild-type mitochondria (2, 3, 16). mtDNA promoter consensus sequences for yeast (9, 35, 36) humans (4), and mice (6) are shown for comparison. HSP, Heavy-strand promoter; LSP, light-strand promoter.

tRNA inserts, together with the CCA immediately upstream in the plasmid, forms a longer consensus sequence [CCA(A/G)N(A/G)AG; Fig. 11, consensus 1], which is similar to sequences at several *Neurospora* mtDNA transcription initiation sites identified by in vitro transcription (5′ ends of the mitochondrial small and large rRNAs and *cob* mRNA; Fig. 11 and Kennell and Lambowitz, manuscript in preparation). This mtDNA sequence (consensus 2), which was believed previously to be an RNA processing site (3), is similar to promoter elements in human and mouse mtDNA (4, 6) and is proposed to be an element of a *Neurospora* mtDNA promoter (Kennell and Lambowitz, manuscript in preparation). Although we have been unable to obtain definitive evidence that the sequence in the variant plasmids functions as a promoter (see Discussion), incorporation of a new promoter into the plasmids could account for both the increased concentration of plasmid transcripts and the suppressive behavior of the plasmids.

A tRNA-like structure at the 3′ end of the plasmid transcript. We noted previously that the major transcripts of the wild-type Mauriceville and Varkud plasmids have 3′ ends terminating in CCA, which is characteristic of tRNAs, and can be folded into a series of small hairpins reminiscent of

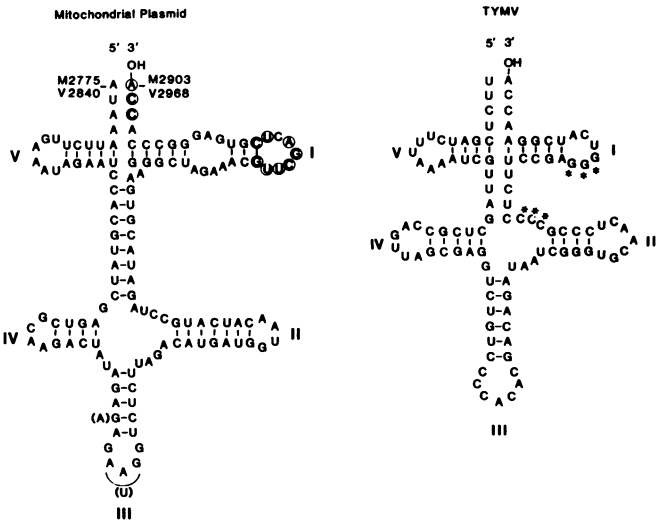


FIG. 12. Comparison of hypothetical secondary structure at the 3′ end of the Mauriceville plasmid transcript and the tRNA-like structure at the 3′ end of turnip yellow mosaic virus (14). Circled nucleotide positions in stem I of the mitochondrial plasmid structure indicate nucleotides that are conserved in aminoacyl-acceptor stems of all tRNAs. The plasmid matches the conserved nucleotide at all these positions. Nucleotides in parentheses in domain III indicate differences in the Varkud plasmid. One of these differences would disrupt the stem of domain III. *, Nucleotides involved in potential pseudoknots.

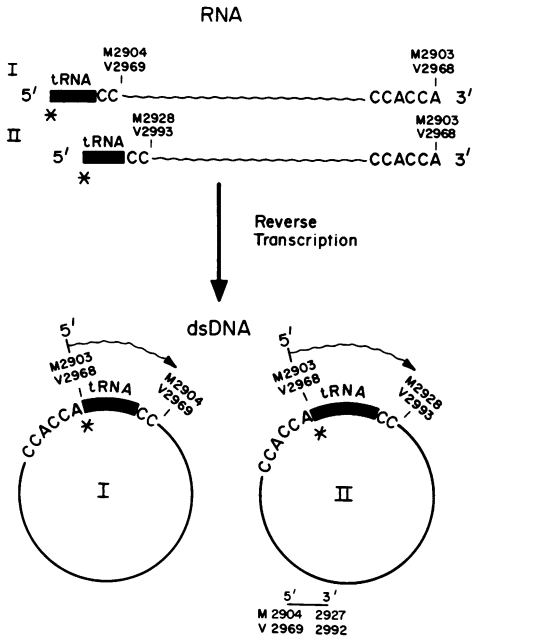


FIG. 13. Model for insertion of mitochondrial tRNAs into variant plasmids. Mitochondrial tRNAs are shown linked to the 5′ ends of plasmid transcripts beginning at M2904 or V2969 or M2928 to V2993. By themselves, the sequencing data are equally consistent with the possibility that the tRNAs were linked to the 3′ end of the plasmid transcript.

the tRNA-like structures at the 3′ ends of plant RNA viruses (1). The hypothetical secondary structure at the 3′ end of the plasmid transcript is shown in Fig. 12, along with the 3′-terminal tRNA-like structure of turnip yellow mosaic virus for comparison. The two structures have a similar overall geometry consisting of five stem-loops terminating in CCA. Further, stem I of the mitochondrial plasmid structure has several characteristics that resemble closely a tRNA aminoacyl-acceptor stem drawn in the conventional L-shaped tertiary structure. These include the 3′-terminal CCA sequence, an overall length of 11 nt for the base-paired region (compared with 12 nt in tRNAs), a perfect match for the highly conserved UUCG sequence in the loop at the end of the stem, and perfect matches for all conserved nucleotides in tRNAs (Fig. 12, circled). In the viral structure, the aminoacyl-acceptor stem is formed by a pseudoknot between the loop of stem I and nucleotides at the base of stem II (14; Fig. 12, asterisks), whereas the cognate acceptor stem in the mitochondrial plasmid structure contains a bulge after the first 5 bp and does not depend upon formation of a pseudoknot. The consequences of a bulged acceptor stem are unclear, but it is possible that all or part of this bulge is base paired with other sequences via tertiary interactions. Finally, the tRNA-like character of this structure has been supported by recent preliminary results showing that the 3′ end of the Mauriceville plasmid transcript is a substrate for AMP addition by *Escherichia coli* tRNA-nucleotidyl transferase (A. Theobald, M. Deutscher, M. Kuiper, and A. Lambowitz, unpublished data). This tRNA-like structure may play a role in replication of the plasmid by reverse transcription (see Discussion and reference 22).

DISCUSSION

Suppressive plasmids contain mitochondrial tRNAs incorporated by reverse transcription. In the present work, we show that nine different suppressive variants of the Mauriceville and Varkud mitochondrial plasmids have acquired a small insertion that corresponds to or includes a mitochondrial tRNA or tRNA-like sequence. The insertions are located either at the position corresponding to the 5′ end of the major plasmid transcript or 24 nt downstream, near a cognate of the sequence at the major 5′ RNA end. Figure 13 shows a model that was suggested previously to account for the structures of the variant plasmids. In this model, the inserted tRNAs are linked to the 5′ end of the plasmid transcript at the RNA level and the suppressive plasmids are generated by reverse transcription with concomitant or subsequent cyclization (1). The structures of the additional variant plasmids analyzed here are consistent with this model or with two additional models involving reverse transcription (see below).

A major feature of these models has been supported by recent biochemical experiments showing that mitochondria from the Mauriceville and Varkud strains contain a reverse transcriptase activity that is highly specific for the plasmid transcript in ribonucleoprotein particle preparations. This reverse transcriptase synthesizes a full-length DNA copy of the plasmid transcript (minus-strand DNA) beginning directly at the 3′ end of the transcript, and we suggested that synthesis of this minus-strand DNA may be the first step in replication of the plasmids by reverse transcription (22). The 3′ end of the plasmid transcript, which is the site of initiation of minus-strand DNA synthesis, has tRNA-like characteristics similar to the 3′ ends of plant RNA viruses (Fig. 12). By analogy with plant RNA viruses (see references 13 and 30), we suggest that this 3′-terminal tRNA-like structure may function as a recognition site for the polymerase that initiates minus-strand synthesis, in this case a reverse transcriptase (22).

The hypothesis that tRNAs were inserted into the sup-

pressive plasmids at the RNA level is supported by the following evidence. (i) The inserted sequences begin precisely at the 5′ end of the tRNA and contain the entire tRNA sequence, excluding the terminal A residue. (ii) The CC residues at the ends of the inserted tRNA sequences include at least some nucleotides that are added posttranscriptionally (see Results). (iii) Sites of tRNA insertion in the plasmid correspond precisely to the 5′ end of the major transcript of the plasmid or a putative minor RNA end 24 nt downstream. (iv) In all five type 2 plasmids where the tRNA sequence is inserted at the downstream site, sequences between the upstream and downstream sites have been deleted precisely. As shown in Fig. 13 and discussed previously, the precise deletion of these sequences in different variant plasmids can be explained by linkage of the tRNAs to plasmid transcripts beginning at the downstream RNA start site and terminating at the same CCA sequence, which is the normal 3′ end of the plasmid transcripts (1).

An important prediction of the model shown in Fig. 13 has been supported by recent studies showing that the 4.9-kb Varkud plasmid transcript is a hybrid RNA consisting of the full-length 3.7-kb Varkud plasmid transcript plus a 1.2-kb 5′ end extension that is derived from the 5′ end of the mitochondrial small rRNA (Akins et al., in press). Since a template for this hybrid RNA could not be detected, this finding suggests that the plasmids have some mechanism for linking heterologous RNAs to the 5′ ends of their major transcripts. Although plasmid transcripts containing 5′-linked tRNAs could not be detected in wild-type strains (e.g., by primer extension experiments), such transcripts could be present in low concentrations and then selected by virtue of giving rise to suppressive plasmid DNAs.

The synthesis of hybrid RNAs has been described previously in several experimental systems and can occur by at least four different mechanisms: (i) *trans*-splicing (31, 44); (ii) copy choice by RNA polymerase (19, 20, 25); (iii) RNA priming of transcription, a mechanism used by certain animal viruses (21); and (iv) direct RNA-RNA ligation. *trans*-splicing seems unlikely in the case of the plasmid tRNA-inserts, since the sequences at the junctions do not correspond to splice sites of known introns. A copy choice mechanism could not account for insertion of a tRNA containing a posttranscriptionally added nucleotide. The two remaining mechanisms, RNA priming of transcription and direct RNA-RNA ligation, are difficult to distinguish, and neither can be excluded. However, the finding that two of the insertions (M2-9 and V8-2) are composites consisting of $tRNA^{Trp}$ linked to short stretches (19 and 56 nt, respectively) of unrelated mtDNA sequences seems most readily explained by sequential RNA-RNA ligations. Direct RNA-RNA ligation could also account for the finding that the inserted tRNAs lack the terminal A residue, whose cleavage may be coupled to the ligation reaction.

There appears to be considerable specificity in the process by which tRNAs were incorporated into the suppressive plasmids. All type 1 plasmids, in which the tRNA is inserted at the 5′ end of the major plasmid transcript, contain $tRNA^{Trp}$ or a sequence homologous to $tRNA^{Trp}$, whereas type 2 plasmids, in which the tRNA is inserted 24 nt downstream, contain either $tRNA^{Gly}$ or $tRNA^{Val}$. Further, all of the inserted tRNA sequences, including those that are part of composite insertions, end in the dinucleotide CC. The tRNA-like insert in V1-2 and the second half of the composite insertion in M2-9 also end in CC. The only exception is the second half of the composite insertion in V8-2, which ends in a run of A residues. Although in type 2 plasmid, the origin of the terminal CC residues is ambiguous, in type 1 plasmids, the CC residues are clearly derived from the 3′ end of the inserted tRNA or mtDNA sequence (Fig. 2). The repeated occurrence of CC at the boundaries of the inserted segments, even those that are not tRNAs, suggests that it is an important determinant of the specificity of the joining reaction. One possibility is that the tRNAs are linked to the plasmid transcript by a ligase activity which ordinarily functions in cyclization of plasmid RNA or DNA and which recognizes the tRNA-like structure and/or CCA sequence at the 3′ end of the plasmid transcript.

The recent finding of a plasmid-associated reverse transcriptase that synthesizes full-length minus-strand DNA suggests at least two additional mechanisms whereby tRNAs could be incorporated into the suppressive plasmid DNAs. First, as discussed previously (1), the tRNA may have been linked head to tail to the 3′ end of the plasmid transcript and the 3′ terminus of the tRNA may have been used as the site of initiation of minus-strand synthesis in place of the normal 3′-terminal tRNA-like structure. This mechanism has the major virtue of accounting readily for loss of terminal A residue by initiation of minus-strand synthesis opposite the penultimate C residue of the 3′-terminal CCA sequence, as in plant RNA viruses (30). In this case, the precise 24-nt deletion in type 2 plasmids could be accounted for by linkage of the tRNA to the 3′ ends of plasmid transcripts beginning at the downstream RNA start site or by precise termination of minus-strand synthesis 24 nt from the normal end. The second possible mechanism is that the inserted tRNAs were primers for plus-strand DNA synthesis by using as template the full-length minus-strand DNA synthesized in the first step of the replication process. This mechanism might involve cyclization of the newly synthesized minus-strand DNA followed by hybridization of the 3′ end of the tRNA to the 5′ end of minus-strand DNA, leaving the 3′ end of the tRNA in position to prime plus-strand synthesis. In this case, the precise deletion of 24 nt in type 2 plasmids would have to be accounted for by binding or slippage of the primer tRNA to the downstream position. A drawback of this mechanism is that it does not account readily for the loss of the terminal A residue from the tRNA sequence. In addition, neither of the above mechanisms accounts readily for the composite insertions containing sequences from different regions of the mitochondrial genome without additional ad hoc hypotheses. Nevertheless, it deserves emphasis that both mechanisms are viable possibilities, and additional biochemical experiments will be required to distinguish them from the model in Fig. 13.

Suppressiveness is correlated with increased concentrations of plasmid transcripts. The presence of the inserted tRNA sequence appears to profoundly affect transcription of the plasmids, resulting in a change in the major 5′ RNA start site and a 25- to 100-fold increase in the concentration of plasmid transcripts. The increased concentration of plasmid transcripts may be related to the suppressive behavior of the plasmids in several ways. First, assuming that the plasmids replicate by reverse transcription, the higher concentrations of plasmid transcript could contribute directly to the increased rate of replication. Second, the higher concentrations of plasmid transcripts may lead to overproduction of a plasmid-encoded protein or proteins required for replication of the plasmid. Finally, the inserted tRNA sequences could introduce or activate a promoter associated with a conventional DNA replication origin, thereby accounting both for the suppressive behavior of the plasmids and the increased concentration of plasmid transcripts (cf. references 5, 7, 35).

There is no direct evidence that the variant plasmid transcripts, which have extra tRNA sequences attached to their 5′ ends, can function as mRNAs. However, even in the case of wild-type plasmid, the 710-amino-acid open reading frame is 0.6 kb downstream of the 5′ end of the major 3.6- and 3.7-kb plasmid transcripts and may normally require initiation at an internal AUG. In that case, the additional 64- to 128-nt sequences at the 5′ ends of the variant plasmid transcripts may not prevent translation of the long open reading frame.

Inserted mitochondrial tRNAs may contribute promoters, enhancers, or RNA processing sites. In general, the increased concentration of plasmid transcripts in the suppressive mutants could reflect an increased rate of transcription or a decreased rate of RNA turnover or both. Major transcripts of the suppressive plasmid begin at or near the 5′ end of the inserted mitochondrial tRNA sequence and identify a short consensus sequence PuNPuAG, which is present at the 5′ ends of the three tRNA species inserted in the mutant plasmids, as well as the 5′ end of the tRNA-like insert in V1-2 (underlined in Fig. 11). This consensus sequence is found at the 5′ ends of only 4 of 21 sequenced *Neurospora* mitochondrial tRNAs, and 3 of these tRNAs are found in the suppressive plasmids. The striking selection of tRNAs having the same consensus sequence at their 5′ ends suggests strongly that it is related to the suppressive behavior of the plasmids.

In general, the consensus sequence at the 5′ end of the tRNAs could correspond to an element of a promoter or RNA-processing site, or the inserted tRNAs could act as enhancers increasing the efficiency of a promoter located elsewhere in the plasmid. Incorporation of a promoter or enhancer into the variant plasmids could account directly for the overproduction of plasmid transcripts. Alternatively, if the consensus sequence is an RNA-processing site, the increased concentration of transcripts could reflect more efficient processing at this position and/or increased stability of the transcript as a result of the tRNA structure at its 5′ end.

Interestingly, the sequence at the 5′ ends of the inserted tRNAs, together with the CCA sequence immediately upstream in the plasmid, forms a longer consensus sequence [CCA(A/G)N(A/G)AG; consensus 1; Fig. 11], which is similar to the consensus sequence for *Neurospora* mtDNA transcription initiation sites identified by in vitro transcription [consensus 2; PyPyA(A/G)N(A/G)A(G/T)G, Fig. 11; Kennell and Lambowitz, manuscript in preparation]. The latter consensus sequence, which is derived from transcription initiation sites at the 5′ ends of the mitochondrial small and large rRNAs and *cob* pre-mRNA, is similar to that for promoter elements in human and mouse mtDNA (Fig. 11; references 4 and 6) and is proposed to be an element of a *Neurospora* mtDNA promoter (Kennell and Lambowitz, manuscript in preparation).

The findings described above raise the possibility that the consensus sequence at the 5′ end of the inserted tRNAs also functions as a promoter element in the plasmids. In vitro capping of whole mitochondrial RNAs from mutants M2-8 and M3-24 with vaccinia viruses guanylyltransferase and [α-^{32}P]GTP gives capped RNAs comigrating with the major transcripts of the plasmids (data not shown), consistent with the possibility that they are primary transcripts. Thus far, however, we have been unable to obtain supporting evidence for this hypothesis either by 5′ end mapping of the capped RNAs, which is difficult because of the inefficiency of the capping reaction, or by in vitro transcription, which might be unsuccessful for a number of reasons.

The insertion of a tRNA sequences bringing a promoter into the mitochondrial plasmids with resulting increase in transcription would be reminiscent of short interspersed repeated sequences in mammalian nuclear genomes. These repetitive elements generally contain a tRNA-like sequence at their 5′ ends, sometimes with 3′-flanking sequences or poly(A) tracts, and the tRNA-like sequences contain internal promoters that are used for transcription of the elements. Like the suppressive mitochondrial plasmids, it is believed that short interspersed repeated sequences were derived by reverse transcription of tRNAs along with flanking sequences and poly(A) (46).

ACKNOWLEDGMENTS

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LITERATURE CITED

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