A Novel Family of Mitochondrial Plasmids Associated with Longevity Mutants of Podospora anserina*

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We have identified a novel family of plasmids, each containing very short monomeric units, in Podospora anserina longevity mutants. These plasmids, termed small mitochondrial DNAs (sMt-DNAs), are derived from a highly ordered 368-base pair region of the mitochondrial genome. A total of five direct repeat sequences and seven significant regions of dyad symmetry (i.e. palindromes) were found within a 434-base pair mitochondrial sequence, which includes this 368-base pair region. Mitochondrial DNA rearrangements accompany the formation of these small plasmids indicating their derivation from a plastic region of the mitochondrial genome. A possible relationship between the direct repeat sequences, the palindromic regions, and the excision process is discussed.

Fungal mitochondrial plasmids have been associated with sudden stops in vegetative growth. For example, in Neurospora intermedia, senescence apparently results from the insertion of a 9.0-kbp foreign element that lacks homology with the mitochondrial genome (Bertrand et al., 1985). On the other hand, senescence in Podospora anserina is associated with the excision and amplification of specific mitochondrial sequences (Stahl et al., 1978; Cummings et al., 1979b; Jamet-Vierny et al., 1980; Wright et al., 1982). Similar phenomena are also involved in the "ragged" mutation in Aspergillus amstelodami (Lazarus and Kunzel, 1981) and the "stopper" mutation in Neurospora crassa (Mannella et al., 1979; de Vries et al., 1981). In Podospora, a 2.6-kbp plasmid, known as α-senDNA, is often observed in senescent cultures (Stahl et al., 1978; Cummings et al., 1979b). This plasmid is derived from a group II intron of the CO(I) gene (Osiwacz and Esser, 1984; Cummings et al., 1985), and it has the coding capacity for a protein with extensive amino acid homology to retroviral reverse transcriptase (Michel and Lang, 1985). Other senDNAs have also been observed (Cummings et al., 1980, 1985; Belcour et al., 1981; Wright et al., 1982), but they appear in senescent cultures relatively infrequently. How the senDNAs are connected with senescence is not known, but possibilities include interference of mitochondrial DNA function by preferential replication of the senDNAs, loss of essential gene functions due to genome rearrangement, or the appearance of large amounts of the putative reverse transcriptase coded for by the α-senDNA plasmid (Steinhilber and Cummings, 1986).

To determine the relationship between the excision and amplification of mitochondrial DNA and the senescence process in Podospora, a number of longevity mutants, defined as strains capable of growth renewal after one or more senescence crises (i.e. growth stoppages) were isolated. Most were derived as outgrowths from senescent cultures. The mutants were identified as strains with abnormal growth characteristics from a cross of the short-lived A race with the relatively long-lived s race. When these mutants were examined for the presence of amplified DNAs, more than half revealed the presence of a novel family of plasmids. These plasmids were derived from the mitochondrial genome and had very short monomeric unit sequences. They were therefore termed small mitochondrial DNAs (sMt-DNA). Three sMt-DNAs were identified; they were not observed in young, nonsenescent mycelia and were found to exist in the mitochondria as relatively large multimeric plasmids. DNA rearrangements, possibly promoted by sMt-DNA formation, were observed in mitochondrial DNA isolated from the mutant strains. Sequence analysis of the sMt-DNAs and the corresponding mitochondrial genome revealed five different excision sites. These excision sites were correlated with the presence of direct repeats and palindromes.

MATERIALS AND METHODS

Growth of Podospora and Isolation of DNA—These procedures were performed as described in Cummings et al. (1979a) and Wright and Cummings (1983). All strains were grown at 30 °C with the exception of the L14a2 mutant which was grown at 27 °C.

Isolation of Longevity Mutants—Longevity mutants were defined as strains capable of growth renewal following one or more senescence crises. A senescent crisis was defined as a growth stoppage for at least 1 week. The mutants NG-62, NG-101, and NG-358 were isolated as follows. Mycelia from a wild type A+ strain were treated with a physiological salt solution (10 mg/ml NaCl) containing 250 μg/ml N-methyl- N'-nitro- N-nitrosoguanidine (Sigma) for 1 h at room temperature. After this treatment, the mycelia were washed three times with physiological salt solution. Mycelial plugs were then removed and placed in race tubes. The mutant Na-1 was isolated by placing a wild type A+ mycelial plug in a race tube containing nalidixic acid (Sigma) at a concentration of 50 μg/ml. This strain grew continuously, albeit poorly, in the presence of the nalidixic acid. After the strain had grown the length of the race tube (35 cm), it was placed in a fresh race tube without nalidixic acid. It was maintained in the absence of this drug thereafter. The L14a2 mutant is an A+ strain isolated from experiments in which Podospora was grown continuously in liquid culture. These experiments, which yielded very long-lived strains, are described in detail elsewhere (Turker and Cummings, 1987). The longevity mutant Psen2X6, derived from a wild type s- strain, was isolated as a poorly growing outgrowth from a senescent region. Numerous mycelial plugs were taken from this outgrowth and the best growing culture was termed PsenIX. PsenIX
underwent a senescent crisis after 8 cm. An outgrowth derived from this senescence crisis was placed in a race tube and termed Psen2X6. The mutant strain A′(s)12, kindly provided by Dr. Leon Belcour (Centre National de la Recherche Scientifique, Gif sur Yvette, France), was derived from a cross of an A race strain with a sMt-1 strain. A′(s)85LI-1 and A′(s)86DI-5 were isolated by backcrossing the progeny of the A × s cross. In all cases, the strain containing the A mitochondrial genome was crossed as the female parent.

**Restriction Enzyme Analysis and Southern Blotting**—Restriction enzyme analysis was performed as described previously (Cummings *et al.*, 1980; Wright *et al.*, 1982). DNA-DNA hybridization was done according to Southern (1975) as modified by Wright *et al.* (1982).

**Coning of sMt-DNAs**—Middle DNA fractions, defined as DNA preparations with densities between 1.692 and 1.714 on CsCl-4.6-diamidino-2-phenylindole gradients (see below), from the longevity mutants NG-62, NG-101, Nal-1, and Psen2X6, were digested with the restriction endonuclease BglII (Bethesda Research Laboratories, Bethesda, MD) and separated on a preparative 1.2% agarose gel. The sMt-DNAs were electrophoresed from the agarose and cloned into the BamHI site of the pUC-18 cloning vector (Bethesda Research Laboratories).

**DNA Sequencing**—The chemical method of Maxam and Gilbert (1980) was followed throughout as described in Cummings *et al.* (1985).

### RESULTS

**Characterization of Amplified DNA Sequences in Longevity Mutants**—Mitochondrial DNA was isolated from 18 different longevity mutants and examined for amplified DNA sequences. Most of these mutants had grown from 2 (35 cm) to 16 (280 cm) times longer than the average seen for the wild type A strain and all had undergone at least one growth stoppage (i.e. senescence) before the DNA was isolated. These mutants were grouped into three classes: 1) stopper, strains that grow for a short time (<5 cm) between senescence crises; 2) occasional stopper, strains that grow for relatively long times (>5 cm) between senescence crises; and 3) continuous growth, a single mutant was identified that grows continuously without senescent crises. Pellets enriched for mitochondria were isolated from each strain, and DNA was purified on CsCl-4.6-diamidino-2-phenylindole gradients. On these gradients, mitochondrial DNA, α-senDNA, and nuclear DNA have densities of 1.692, 1.699, and 1.714, respectively (Cummings *et al.*, 1979b). The entire gradient fraction between the mitochondrial and nuclear DNAs, termed middle fraction, was collected from each longevity mutant and concentrated for analysis. The DNAs were digested four times, once each with the restriction endonucleases BglII, EcoRI, HaeIII, and PstI. They were then separated by electrophoresis on 1.2% agarose gels. The most striking observation was that the BglII middle fractions from nine of the mutants revealed a set of amplified, very small DNA fragments (<500 bp). These fragments were seen in five race A mutants, one s race mutant, and in three mutants derived from a cross between the two races. Fig. 1 shows this result for four of the mutants, along with young, nonsenescence controls. Three different size classes were observed and termed sMt-1, sMt-2, and sMt-3, in order of their size. The sMt-1 DNA element was observed in DNA from eight of the mutant strains, alone six times, with sMt-2 once, and with sMt-3 once. In two cases, sMt-2 was found alone, and, in one of these two cases, it was found in a strain which had yielded only sMt-1 in a previous isolate. The growth characteristics of the nine longevity mutants and the sMt-DNAs observed are given in Table I.

The BglII-digested sMt-DNAs were cloned into the pUC-18 vector and homology between the sMt-DNAs was examined by Southern blotting the BglII-digested middle fractions and hybridizing to 32P-labeled psMt-1 and psMt-3 DNA probes. The psMt-1 probe hybridized strongly to the other sMt-1 DNAs, but weakly to the sMt-2 and sMt-3 DNAs (not shown). Fig. 1, a′-f′, shows that the psMt-3 probe hybridized to all of the sMt-DNAs. Taken together, these results suggest that there is indeed sequence homology between the sMt-DNAs. To determine if the sMt-DNAs existed in multimeric forms, as previously shown for the senDNAs (Cummings *et al.*, 1979b; Belcour *et al.*, 1981), the mitochondrial, nuclear, and middle fraction DNAs from the NG-101 mutant was digested with an enzyme which does not digest the sMt-DNAs and hybridized to the psMt-1 probe. Fig. 2a shows that a number of hybridization bands were visualized in the middle fraction DNA, suggesting that the sMt-1 DNA exists as free plasmid with multiple copies per plasmid. A similar result was obtained for the sMt-2 DNA found in the Psen2X6 mutant (not shown). We also analyzed the middle fraction DNAs from most of the longevity mutants which yielded the sMt-DNAs for the presence of the α-senDNA plasmid. With a single exception, these middle fractions all revealed the α-senDNA plasmid, although in varying amounts. This exception was the continuous growth mutant, L14a2, which had no detectable α-senDNA (data not shown).

**The sMt-DNAs Arise during Vegetative Growth from the Mitochondrial Genome**—For several of the longevity mutants...
The middle fraction (a) mitochondrial fraction (b), and nuclear fraction (c) DNAs from the longevity mutant NG-101 were digested with the restriction endonuclease EcoRI and separated by agarose gel electrophoresis. These DNAs were then blot-hybridized to 32P-labeled psMt-1. The blot-hybridized middle fraction DNA shown in lane a was exposed for 1 week to highlight the lower molecular weight bands. This resulted in overexposure of the top portion of this lane. This portion of the lane resolved into four bands when exposed for 1 day (not shown). The hybridization band seen in lane b is 6.0 kbp.

**Fig. 2.** The sMt-DNAs exist as multimers in the middle fraction DNA. The middle fraction (a), mitochondrial fraction (b), and nuclear fraction (c) DNAs from the longevity mutant NG-101 were digested with the restriction endonuclease EcoRI and separated by agarose gel electrophoresis. These DNAs were then blot-hybridized to 32P-labeled psMt-1. The blot-hybridized middle fraction DNA shown in lane a was exposed for 1 week to highlight the lower molecular weight bands. This resulted in overexposure of the top portion of this lane. This portion of the lane resolved into four bands when exposed for 1 day (not shown). The hybridization band seen in lane b is 6.0 kbp.

**Fig. 3.** The sMt-DNAs arise during vegetative growth. Middle fraction DNAs from the A'(s)12 stock plate (a), A'(s)12 after growth of 4 cm in a race tube (b), the A'(s)86DI-5 stock plate (c), A'(s)86DI-5 after growth of 23 cm in a race tube (d), L14a2 after growth of 70 cm in a race tube (e), and L14a2 after growth of 140 cm in a race tube (f) were digested with the restriction endonuclease BglII, separated by agarose gel electrophoresis, and stained with ethidium bromide. A large amount of α-senDNA in lane a is indicated. The right-hand panel (a'–f') shows these DNAs blot-hybridized to 32P-labeled psMt-1.

We had isolated DNA at growth points prior to those shown in Table I, allowing for an examination of these earlier isolates to determine if the sMt-DNAs had previously been present. Fig. 3 shows that in all cases tested the sMt-DNAs were not originally present, indicating that they arose during vegetative growth. To determine the origin of the sMt-DNAs, mitochondrial and nuclear DNA fractions were isolated from young A' and s- strains and from the longevity mutant NG-101. These DNAs were digested with EcoRI, Southern-blotted, and hybridized to the psMt-1 probe. All three mitochondrial DNAs showed a single 6.0-kbp molecular weight band, whereas no hybridization was seen to the nuclear DNAs (see Fig. 2 for NG-101). This single high molecular weight band was identified as the mitochondrial restriction fragment E7 (see Fig. 4) by hybridizing the psMt-1 probe to previously cloned mitochondrial DNAs. Although the sMt-3 DNA was also localized to the E7 region, psMt-1 and psMt-3 hybridized to different BglII restriction fragments within the cloned E7 fragment (data not shown). Based on the hybridization data, we tentatively localized the sMt-1 and sMt-3 DNAs to the B6 and B11 restriction fragments, respectively, of the mitochondrial genome (Fig. 4). It was assumed that, although there was some overlap around this BglII site, it was insufficient to be detected by these hybridization experiments. To be certain that the sMt-DNAs were located within the mitochondria, we repurified mitochondria from the L14a2 longevity mutant on a sucrose gradient (Wright and Cummings, 1983). DNA isolated from these purified mitochondria yielded the sMt-1 plasmid.

**Sequence Analysis of the sMt-DNAs—**A detailed sequence analysis of the sMt-DNAs was undertaken by sequencing the following: psMt-1 from NG-62, NG-101, and Psen2X6, psMt-2 from Nal-1 and Psen2X6, psMt-3 from NG-62, and the corresponding region of the mitochondrial genome. This sequence is presented in Fig. 5. We note that in all cases when cloned sMt-DNAs with greater than one copy per insert were sequenced they were found in head to tail conformations. This result is consistent with the data suggesting multimeric plasmids. There were no differences found among the sMt-1 and -2 DNAs derived from the s- strain Psen2X6 and those derived from the A' strains. The 5' and 3' excision sites for all the sMt-DNAs are illustrated in Fig. 5. It can be seen that sMt-2 (114 bp) is completely contained within sMt-1 (368 bp), and sMt-3 (67 bp) is completely contained within sMt-2. Only the 3' excision site for sMt-2 and sMt-3 is shared between the sMt-DNAs. The 20-bp region separating the 3' excision sites for sMt-2,3 and sMt-1 is unusually GC-rich (70%) as compared with the rest of the sMt-DNA sequence (40%). A computer analysis of the sMt-DNAs and the surrounding regions revealed two significant components which may play a role in excision of these elements: direct repeats of DNA sequences and dyad symmetries (i.e. palindromes).

Five direct repeat pairs were noted and are underlined in Fig. 5. Two of these repeats appear upstream of the 5' and 3' excision sites for the sMt-1 and -2 DNAs (direct repeat pairs 3 and 4, respectively, in Fig. 5). We have also found that the 13-bp repeat AACCTCCTCCTCG, repeated at the sMt-1 5' and 3' excision sites, occurs at a third location approximately 1600 bp upstream of the 3' excision site (not shown). Of the five direct repeat pairs noted in Fig. 5, three have one of the pairs located in the 27-bp sequence immediately upstream of the 5' excision site.

Five potential short range palindromes were found in the sMt-DNAs and the region upstream from the 5' excision site of sMt-1. A number of alternate structures were revealed by computer analysis, and Fig. 6, a–e, shows representative structures and free energies for each palindrome unit. Significantly, four of the five excision sites fell within these regions of secondary structure. All three 5' excision sites occurred at base pair mismatches. A linear map depicting the location of the five palindromes is also shown in Fig. 6. The palindromes illustrated in Fig. 6, c–e, are clustered together as compared...
FIG. 4. Restriction map of race A. P. anserina mitochondrial DNA digested with EcoRI. The relative positions of \( \alpha \)-senDNA, \( \beta \)-senDNA, \( \epsilon \)-senDNA, and the sMt-DNA plasmids are illustrated. The expanded region begins at the 5' end of the restriction fragment E7 and ends at the first BglII site of E1. No difference has been found in this region between race A and race s. The bars labeled a and c refer to BglII fragments B6 and B11, respectively. The bar labeled b indicates the small XhoI fragment of E7. The localization of the sMt-1 and sMt-3 DNAs from blot hybridization data is illustrated.

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![Restriction map of race A](image)

and b. The GC-rich region separating the two 3' excision sites appears interesting in that it can potentially base pair with sequences overlapping either the region upstream of the 5' excision site of sMt-1 (Fig. 6f) or sequences overlapping the Fig. 6b palindrom (Fig. 6g). We also note that the sequence AGGAGGAGA (position 207-216), which falls within the Fig. 6b palindrom, can base pair with the sequence TCCTCCCT. This latter sequence falls immediately downstream of the 5' excision site for sMt-2.

The sMt-DNA sequences were also examined for the possibility of an open reading frame. Stop codons were found in all six possible reading frames with the longest possible open reading frame being 17 amino acids.

Mitochondrial DNA Rearrangements Occur in Regions Surrounding the sMt-DNAs—The mitochondrial DNAs from the nine sMt-DNA-bearing longevity mutants were digested with the restriction enzyme BglII and separated on 1.2% agarose gels. Fig. 7, b-g, shows extensive rearrangements for five of these mutants as compared with a wild type control (Fig. 7a). The other four sMt-DNA-bearing mutants also had mitochondrial DNA rearrangements (not shown). Lanes f and g in Fig. 7 show digested DNAs taken from the A- (a)12 mutant at different growth points (see Table I). It should be noted that three of the nine longevity mutants not exhibiting the sMt-DNAs in their middle fractions also did not exhibit mitochon-
Longevity mutants of *Podospora* were selected for their ability to resume growth following one or more senescent crises. In one-half of these mutants, we have identified a novel family of mitochondrial plasmids. We define these amplified DNAs as plasmids because they can be physically separated from nuclear and mitochondrial DNA on a CsCl gradient, they exist in a variety of multimeric sizes, and they are not released from BgIII-digested co-purified mitochondrial DNA. They are defined as mitochondrial plasmids because they co-purify with mitochondria on a sucrose gradient and they are derived from the mitochondrial genome. Although these plasmids, termed sMt-DNAs, exist in multimeric forms, sMt-1, -2, and -3 are derived from only 368, 114, and 67 bp, respectively, of the mitochondrial genome. They are therefore comparable in size to monomeric units of 89 and 70 bp, exclusively AT in composition (Fangman and Dujon, 1984), and a similar 68-bp unit (van Kreijl and Bos, 1977), which have been reported in yeast petite mutants. Both of the smaller elements, sMt-2 and sMt-3, are completely contained within sMt-1. The mitochondrial region from which the sMt-DNAs are derived will be discussed with regard to the role of DNA secondary structures and direct repeats in the excision process.

In considering the excision process, we note the data which indicate that the sMt-DNAs arose during vegetative growth. We also note that a variety of mitochondrial DNA rearrangements have occurred affecting regions linked to the sMt-DNA sequences, although we have not yet identified those fragments, if any, which result from sMt-DNA excision. However, we can state that the contiguous B6 and B11 regions are unusually plastic with regard to DNA recombination. In a more recent work we have extended this region of plasticity to the 5' end of E7 and slightly downstream of the 3' end of E7. The finding of both wild type and rearranged fragments in DNA from a given strain is not necessarily surprising since almost all of the stopper longevity mutants examined have mixtures of both mutant and wild type mitochondrial genomes (Cummings et al., 1986; Belecour and Vierny, 1986).

A sequence analysis of the mitochondrial region giving rise to the sMt-DNAs has revealed an unusually ordered structure. Within a 434-bp sequence, we have identified five separate excision sites, seven significant instances of dyad symmetry, and five pairs of direct repeats. Two of these repeat pairs occur upstream of the 5' and 3' excision sites for sMt-1 and

2 M. S. Turker, J. Domenico, and D. J. Cummings, manuscript in preparation.

3 M. S. Turker and D. J. Cummings, unpublished observations.
2, suggesting homologous recombination as a mechanism of excision. Other examples of repeated sequences critical to DNA excisions have been described previously by us and by others. Direct repeats are found at the 5' and 3' excision sites of two other excision-amplification plasmids of Podospora, β-senDNA (9.8 kbp) and ε-senDNA (5.5 kbp) (Cummings et al., 1985). In the cyanobacterium Anabaena, rearrangement of the 11 kbp nitrogen fixation gene unit is associated with an 11-bp sequence repeated at the 5' and 3' excision sites (Gelden et al., 1985). In Neurospora crassa, the direct repeat of two tRNA^Met sequences has been implicated as a hot spot for intramolecular recombination (Gross et al., 1984). Finally, in the AT-rich var1 mitochondrial gene of yeast, a GC-rich sequence has been implicated as a potential promoter of recombination (Butow et al., 1985).

Although direct repeat pairs appear to play a role in the excision of the sMt-DNAs, they may act in concert with a second novel component, regions of dyad symmetries. Of the five major short palindromes revealed by a computer analysis, four were associated with excision sites. All three 5' excision sites were found to occur at a base pair mismatch, suggesting a recognition site. Only the 3' excision site of sMt-1 does not have a palindromic association with it. This 3' excision site, however, can potentially be brought near its 5' excision site. This would occur by base-pairing the GC-rich sequence preceding this excision site with a complementary sequence found upstream of the sMt-1 5' excision site (see Fig. 6f). The one palindrome we have not associated with an excision site (see Fig. 6b) is interesting because a portion of its sequence also has the potential to base pair with the GC-rich sequence mentioned above. In particular, we note the sequence AGGAGGAGGA which occurs within this palindrome. The complementary sequence TTCCTCCT occurs within the 13-bp repeat preceding the 5' and 3' excision sites for sMt-1. We therefore raise the possibility that portions of both of these TTCCTCCT sequences can simultaneously base pair at the AGGAGGAGGA sequence. This would bring the 5' and 3' sMt-1 excision sites into juxtaposition, allowing for the presumed excision and ligation events to proceed. Occasionally, base-pairing could occur with the TACTCTT sequence found at the 5' excision site for sMt-2, instead of the TTCCTCCT sequence found at the 5' excision site for sMt-1, allowing for the formation of this plasmid.

Considered as a whole, the data presented in this paper indicate that excision events lead to the formation of the sMt-DNA plasmids and that both direct repeats and dyad symmetries are involved in this process. The salient point to be made about this highly ordered sequence is that the potential to form a variety of secondary structures exists. Therefore a multistep process may precede the excision event. For example, the formation of secondary structures may bring excision site direct repeats into juxtaposition. However, this model does not explain the formation of the sMt-3 DNA plasmid. We note that, in our laboratory, approximately 25 kbp of Podospora mitochondrial DNA has been sequenced to date4 (Cummings et al., 1985), and no other region has been discovered with a similar complexity. We speculate that such a highly ordered sequence must have evolved to perform one or more specific functions and that one of these functions is the formation of the sMt-DNA plasmids. Assuming that the sMt-DNAs exhibit an autonomous replicating sequence function, as previously demonstrated for the senDNAs (Lazdins and Cummings, 1982), we speculate that the palindrome depicted in Fig. 6e is the likely candidate for this origin of replication. Its stem-loop structure is similar to other mitochondrial replication origins (de Zamaroczy et al., 1984), and it is the only ordered unit common to all three sMt-DNAs. Consistent with this hypothesis is the identification of an apparent origin of replication in the smallest sMt-DNA, sMt-3.

Finally, we note that the sMt-DNAs and, in particular sMt-1, were found relatively frequently in longevity mutants and that they have never been observed in Podospora strains prior to senescence. This result suggests a relationship between at least one type of longevity phenotype and the sMt-DNAs. If one accepts that a-senDNA is the causal agent of senescence, as has been argued in this laboratory and others (Osiiewicz and Esser, 1984; Belcour and Vierny, 1986; Wright et al., 1982), then the sMt-DNAs may act as antagonists to inhibit a-senDNA function. This possibility would predict an inverse relationship between a-senDNA and the sMt-DNAs in senescent strains and longevity mutants, respectively. We are currently testing this hypothesis.

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