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Association between variant plasmid formation and senescence in retroplasmid-containing strains of *Neurospora* spp.

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Abstract Serial transfer of *Neurospora* strains harboring the Mauriceville and Varkud mitochondrial retroplasmids frequently displays erratic growth and senescence. Growth impairment is associated with the formation of variant forms of the retroplasmids that can integrate into the mitochondrial genome, resulting in mtDNA rearrangements and eventual loss of respiratory function. Here, we evaluate the rate at which variant plasmids arise in subcultures of the Mauriceville strain of *N. crassa* and their association with the senescent phenotype. Although variant plasmid formation preceded senescence, subcultures were found to tolerate variant plasmids for variable lengths of time and no correlation could be made between the specific sequence inserted in the plasmids and the rate or frequency of senescence. In addition, many cultures were found to contain more than one variant plasmid. The lack of concordance between the timing of variant plasmid formation and growth cessation distinguishes these two events, and provides additional insight into the etiology of senescence. We also detected differences in the frequency of senescence between retroplasmid-containing strains of *N. crassa* and *N. intermedia* and report the isolation of a strain in which senescence occurs in the absence of variant plasmid formation or detectable alterations in mtDNA. Our findings indicate there are multiple pathways that lead to senescence and suggest there are host-specific mechanisms that suppress the deleterious effects of the variant plasmids.

Key words Mitochondria · Senescence · Plasmid · Reverse transcription

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Introduction

Mitochondrial plasmids are prevalent in fungal populations (Griffiths 1995). Surveys of natural isolates of *Neurospora*, the most thoroughly studied genus, estimate that slightly more than half of all strains harbor one or more autonomously-replicating mitochondrial (mt) plasmid (Yang and Griffiths 1993; Arganoza et al. 1994). Molecular characterization of DNA plasmids found in *Neurospora* species indicates that mt plasmids can be divided into discrete groups based on their genomic structure and further classified by DNA sequence similarities to other plasmids. Such comparisons indicate that there are at least four homology groups of *Neurospora* mitochondrial DNA plasmids having linear genomes and more than five known homology groups of circular DNAs (Griffiths 1995).

Among the circular plasmids, one homology group includes plasmids that replicate via reverse transcription (termed retroplasmids). The surveys of natural isolates demonstrate that closely related members of a homology group are often found in different species of *Neurospora* which, together with heterokaryon experiments that demonstrate the plasmids can be transferred across incompatibility barriers (Griffiths et al. 1990), provide a strong indication that mt plasmids are horizontally transmitted in nature (Arganoza et al. 1994, Griffiths 1995). These studies indicate the mt plasmids are met with little resistance in their new hosts and readily form associations with different nuclear backgrounds (see Rosewich and Kistler 2000 for a recent review). The extraordinary diversity and distribution of mt plasmids among *Neurospora spp.* is intriguing since the plasmids provide little benefit to their host and, in certain cases, can be quite detrimental.

Serial transfer of *Neurospora* strains containing the linear DNA plasmids Kalilo or Maranhar, or the circular retroplasmids, Mauriceville and Varkud, results in severe growth defects and senescence (defined here as growth cessation; Bertrand et al. 1985, 1986; Akins et al.

1986; Court et al. 1991). Characterization of senescent cultures containing these plasmids indicates that growth cessation is associated with the integration of the plasmid into the mitochondrial genome, which generates defective mtDNAs that displace or out-compete the wild-type mtDNA (reviewed in Griffiths 1995). As defective mtDNAs accumulate, mitochondrial function declines and cells lose the ability to respire. Although the outcome is the same, the frequency and rate of senescence differ among strains containing DNA plasmids (which replicate using a plasmid-encoded DNA-dependent DNA polymerase) and strains having retroplasmids (which replicate using a plasmid-encoded RNA-dependent DNA polymerase, or reverse transcriptase). In a study of senescence associated with the linear Kalilo DNA plasmid, 18 different plasmid-containing isolates were all found to senesce in relatively few serial transfers (approximately 5) under a variety of culture conditions (Griffiths et al. 1986). In contrast, an initial study of senescence of *Neurospora* strains having the Mauriceville and Varkud retroplasmids revealed that the rate and frequency of senescence are greatly reduced compared to the linear DNA plasmids and are generally dependent on the growth temperature, occurring at much higher frequencies at 37 °C than at 25 °C (Akins et al. 1986). Interestingly, differences in the rate and frequency of senescence were apparent between *N. intermedia* strains having the Varkud plasmid and *N. crassa* strains having the Mauriceville plasmid: all subcultures containing the Varkud plasmid serially transferred at 37 °C senesced whereas only a fraction of the Mauriceville subcultures senesced within an extended period of time. This variation could be attributed to differences between the plasmids; however, the Mauriceville and Varkud retroplasmids have more than 97% sequence identity and appear to replicate in an identical manner (Nargang et al. 1984; Akins et al. 1988; Kuiper and Lambowitz 1988).

Molecular characterization of plasmid-containing senescent cultures has provided a partial explanation for the variable frequency and rate of senescence observed between strains harboring different types of mitochondrial plasmids. The linear Kalilo or Maranhar plasmids appear to be capable of directly inserting into the mt genome, either at sites that share short regions of homology with the plasmids [i.e. 5 bp for characterized Kalilo inserts (Bertrand and Griffiths 1989)] or at non-homologous sites (Court et al. 1991). In contrast, the Mauriceville and Varkud retroplasmids do not appear to directly insert into the mtDNA; instead, prior to integration, the wild-type retroplasmids are first replaced by variant forms of the plasmid which contain DNA insertions that are complementary to mitochondrial RNAs (Fig. 1; Akins et al. 1986, 1989).

The formation of these so-called variant plasmids results from aberrant or promiscuous template switching by the plasmid-encoded reverse transcriptase during (−) strand cDNA synthesis (Chiang et al. 1994; Kennell et al. 1994). This leads to the incorporation of cDNA copies of mitochondrial RNAs, particularly tRNAs, directly downstream of the site corresponding to the 3′ end of the plasmid transcript (Akins et al. 1986;Chiang et al. 1994; Kennell et al. 1994; Stevenson et al. 2000). Transcripts from the variant plasmids have tRNA-like structures at both termini, which likely provides protection against mitochondrial exonucleases and, in turn, leads to the increased production of the retroplasmid DNA. Variant plasmids accumulate to high copy numbers in pre-senescent cultures, which presumably facilitates integration into the mitochondrial DNA. Neither the retroplasmids nor the linear DNA plasmids encode gene products that are specifically involved in integration (i.e. an integrase), rather plasmid integration appears to be a stochastic process that occurs via homologous recombination. Integration of the variant

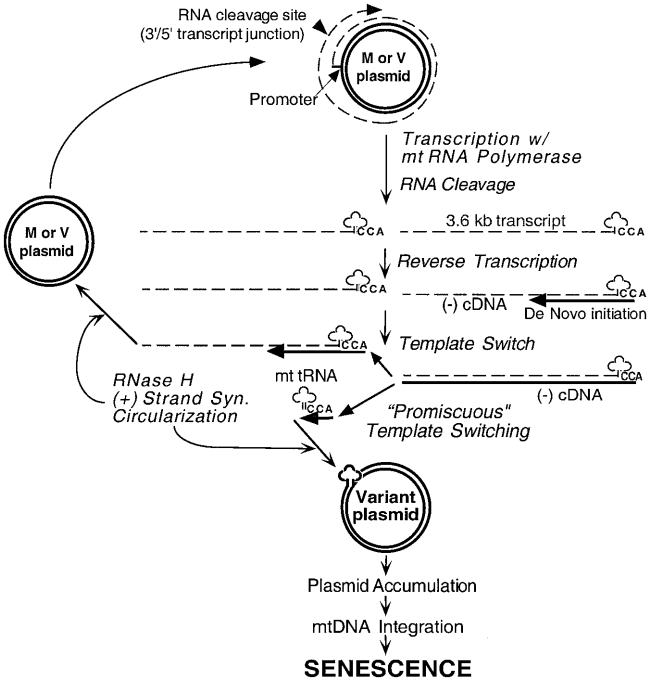

Fig. 1 Retroplasmid replication cycle and events leading to senescence. The Mauriceville (*M*) plasmid and closely related Varkud (*V*) plasmid are autonomously-replicating circular double-stranded DNAs of 3.6 and 3.7 kb respectively (Akins et al. 1988). The double-stranded DNA plasmid is transcribed by the mt RNA polymerase and the transcript (*dashed line*) is cleaved at a site ~260 bp downstream from the promoter, yielding a monomer-length RNA having a tRNA-like structure and 3′ CCA (Kennell et al. 1994). The plasmid-encoded RT initiates (−) strand cDNA synthesis either de novo, opposite the penultimate nucleotide of the transcript (Wang and Lambowitz 1993a), or by template switching from a previously synthesized cDNA (Kennell et al. 1994). Following (−) strand synthesis, the RNA template is either degraded by the mt RNase H (Wang and Lambowitz 1993b) or displaced during (+) strand synthesis. Variant forms of the plasmids are occasionally generated by the RT template switching to mt RNAs, which become incorporated into the plasmid. These so-called variant plasmids accumulate to high numbers and integrate into the mt genome by homologous recombination (Chiang et al. 1994) or through unresolved mechanisms (Akins et al. 1986). The resulting defective mtDNAs accumulate and lead to mitochondrial dysfunction and senescence

retroplasmids takes place at the site encoding the particular mt RNA that became incorporated into the plasmid (Chiang et al. 1994), or by unknown mechanisms at non-homologous regions of the mt genome (Akins et al. 1986, 1989). Following integration, similar events likely follow for both plasmid types, which presumably involve the selective amplification of the defective mtDNA or the mitochondria in which they reside (reviewed in Griffiths 1992). We recently reported an exception to this pathway, which occurs in a *N. crassa* strain that harbors a particular variant of the Mauriceville plasmid, termed pMS4416. Senescence of the MS4416 strain occurs in the absence of plasmid integration and concomitant alterations in mtDNA, and is associated with the over-replication of the pMS4416 plasmid, which interferes with the synthesis of mitochondrially encoded gene products (Stevenson et al. 2000).

To better understand the etiology of senescence of retroplasmid-containing strains, we examined the timing of variant plasmid formation and its relationship to senescence in individual subcultures. We found that the period of time between variant plasmid formation and senescence is quite variable and does not correlate with the specific sequence inserted in the variant plasmid. Surprisingly, a large proportion of subcultures of the Mauriceville strain were capable of tolerating variant plasmids for long periods of time and, in some cases, new variants were found to replace existing types. Our findings indicate that variant plasmid formation does not necessarily lead to growth cessation and suggest that the rate-limiting step in the pathway that leads to senescence most commonly involves the process of plasmid integration and/or the ability of the resulting defective mtDNAs to displace wild-type mtDNAs.

Materials and methods

Neurospora strains and growth conditions

The strains used in this study were *N. crassa* strain Mauriceville-1c (FGSC no. 2225) and *N. intermedia* strain Varkud (FGSC no. 1823). Subcultures described in this study were derived from the Mauriceville strain and are designated with an M preceding the culture number. Cultures were grown on minimal Vogel's growth agar containing 2% sucrose (Davis and de Serres 1970) in 12×75 mm glass tubes (conidial slants). Conidial slant cultures were grown at 37° or 25 °C and vegetative tissue was transferred by loop inoculation in a sterile hood twice per week.

Isolation of mitochondria

Strains were grown in flasks containing minimal Vogel's growth agar containing 2% sucrose for 7–10 days at 25 °C. Conidia were harvested and inoculated into 4-l flasks containing 2 l of Vogel's growth medium at a final concentration of 7.5×10^5 conidia/ml (Davis and de Serres 1970). Mycelia were harvested following 16–24 h of growth at 25 °C, and mitochondria were prepared by a modified flotation gradient procedure (Lambowitz 1979). Mitochondria were removed from the sucrose gradient, suspended in a solution containing 500 mM KCl, 25 mM $CaCl_2$, 25 mM Tris-HCl (pH 7.5), 5 mM DTT, pelleted by centrifugation and stored at −70 °C.

Isolation of nucleic acids

Mitochondrial nucleic acids were extracted by lysis of mitochondrial pellets in 1–5 ml of UNSET [8 M urea, 150 mM NaCl, 100 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2% SDS], followed by three or more consecutive phenol-CIA (25:24:1 phenol: chloroform: isoamyl alcohol) extractions and precipitation with isopropanol. To obtain mtDNA, total mt nucleic acid was digested at 37 °C for 10 min with RNase A (2 μg/ml) in a reaction buffer containing 5 mM Tris-HCl (pH 8.0), and 1 mM $MgCl_2$. Following digestion, reactions were extracted with phenol-CIA and precipitated with ethanol. To obtain DNA for PCR analysis, scant amounts of mycelia and conidia were placed in a microcentrifuge tube, heated in a microwave for 5 min and resuspended in TE. Cellular debris was removed by centrifugation (13,000 rpm) and a fraction of the cleared supernatant was used in each PCR reaction, essentially as described by Ferreira and Glass (1995).

Southern hybridizations

Five micrograms of total mtDNA was digested with *Eco*RI and restriction fragments were separated on 1.0% agarose gels containing 40 mM Tris-acetate, 1 mM EDTA and 0.1 μg/ml ethidium bromide. Images of the gels were recorded on an AlphaEase documentation machine (Alpha Innotech Co., San Leandro, Calif.) and selected bands were quantitated using the AlphaEase stand alone software. Gels were blotted following procedures described elsewhere (Sambrook et al. 1989). Gel-purified 2.7 kb *Eco*RI Mauriceville plasmid fragment was used to synthesize ^{32}P-labeled plasmid probes by a random primer technique, following the manufacturer's specifications (Promega, Madison, Wis.). The blots were hybridized for 16 h at 42 °C in a solution of 50% formamide, $5 \times$ SSC ($1 \times$ SSC is 150 mM NaCl, 15 mM sodium citrate), $5 \times$ Denhardt's reagent, 0.5% SDS and 200 μg/ml sheared, single-strand, herring sperm DNA. Blots were washed at 42 °C in a $2 \times$ SSC, 0.1% SDS solution for 30 min and in a $0.1 \times$ SSC, 0.1% SDS solution for 30 min, prior to autoradiography.

Polymerase chain reactions

Approximately 0.2–1.0 μg of total or mtDNA plus 200 ng of oligonucleotide primers M110-E (5′-GGGAATTCGAGTCGCAAGACTAT-3′>; having 19 nt of sequence identity to Mauriceville plasmid sequences 2801–2818) and MV5′-B (5′CGGGATCCAGTATGAGGGCTACTTCCTAC3′; having 23 nt of complementarity to Mauriceville plasmid sequences 2957–2979) were used in 50 μl reactions containing 50 mM Tris-HCl (pH 9.0), 20 mM ammonium sulfate, 1.5 mM $MgCl_2$, 200 μM concentration of each deoxynucleoside triphosphate and 1.0 U of Tfl Thermostable DNA Polymerase (Epicentre Technologies, Madison, Wis.). The reaction mixtures were cycled 10–25 times (1 cycle = 94° for 45 s, 50° for 2 min, 72° for 1 min) in a thermocycler (Perkin-Elmer Corp., Norwalk, Conn.) and the products were analyzed by agarose gel electrophoresis.

Cloning and sequencing

PCR products were isolated from gels, digested with appropriate restriction enzymes and cloned into pBluescribe or pBluescript II KS phagemid vectors (Stratagene, La Jolla, Calif.). The inserted regions were sequenced by the Sanger dideoxynucleotide chain-termination method (Sanger et al. 1977) with Sequenase enzyme (Amersham, Arlington Heights, Ill.), using forward and reverse primers.

Results

In an initial study of senescence associated with the *Neurospora* retroplasmids carried out by Akins et al. (1986), subcultures of an *N. intermedia* strain harboring the Varkud plasmid invariably senesced when grown at 37 °C, whereas only a fraction (2 of 12 race tube cultures and 3 of 5 transfer cultures) of *N. crassa* subcultures having the Mauriceville plasmid senesced over an equivalent period of time. Characterization of pre-senescent cultures from both strains indicated that senescence likely occurred by the same mechanism, so the difference in the frequency of senescence between the strains suggested there might be host-specific factors that affect some step in the pathway that leads to senescence. We repeated these studies and serially transferred ten subcultures of the Varkud-1c and Mauriceville-1c strains onto slants twice a week and maintained the cultures at 37 °C. In our hands, all ten Varkud serial transfers senesced within a 25-week growth period (with senescence occurring at transfers 12–40; an average of 24.7 ± 8.3), whereas, only five of ten subcultures of the Mauriceville strain senesced after 50 transfers (with senescence occurring at transfers 17–42; an average of 32.4 ± 11.9) (Table 1). Our findings are in close agreement with the previous studies and support the hypothesis that there may be strain- or species-specific factors that govern the rate of senescence associated with mitochondrial retroplasmids.

To understand the variables that might affect the rate of senescence, we focused our studies on the Mauriceville subcultures (M1-M10), as they included both senescent and non-senescent cultures. Following steps taken in previous studies (which primarily characterized senescent Varkud subcultures; Akins et al. 1986; Chiang et al. 1994), we analyzed plasmid DNA and mtDNA from pre-senescent cultures in order to evaluate the molecular events associated with senescence. *Eco*RI restriction digestion of total mtDNA isolated from pre-senescent cultures revealed that four of five senescent cultures contained alterations in their mtDNA restriction profile relative to the original Mauriceville culture (M5, M7, M8 and M9; Fig. 2). In addition, most of the strains also had a highly abundant restriction fragment of approximately 2.7–2.8 kb, which is slightly larger than the major *Eco*RI restriction fragment of the wild-type Mauriceville plasmid. Southern hybridization revealed that the abundant restriction fragments were derived from the Mauriceville plasmid (Fig. 2).

These fragments were isolated, amplified by PCR, cloned and sequenced. The DNA sequence indicated that the variable size of the plasmid fragment in different senescent strains correlated with the length of additional sequences that were inserted directly downstream of the site corresponding to the 3′ end of the plasmid transcript (Tables 1, 2), which is identical to the site of insertion identified in previously characterized variant plasmids (Akins et al. 1986, 1989; Chiang et al. 1994; Stevenson et al. 2000). A longer exposure of the Southern blot revealed additional hybridization bands in lanes containing mtDNAs from subcultures M5, M7, M8 and M9 (not shown; bands are indicated by arrowheads in Fig. 2, left panel), which are associated with the integration of the variant plasmids into the mitochondrial genome. These observations are consistent with previous findings, which demonstrated that senescent strains are commonly associated with mtDNA rearrangements and deletions, resulting from variant plasmid integration (Akins et al. 1986, 1989; Chiang et al. 1994). Differences in restriction profiles of these cultures are likely to be due to the particular site of integration or secondary recombination events that occur following plasmid integration.

Of the five senescent cultures, subcultures M6 and M8 had interesting features that had not previously been observed among senescent cultures. The restriction profile of mtDNA isolated from the M6 culture did not contain any alterations characteristic of plasmid integration (Fig. 2, lane 3, left panel) and Southern analysis

Table 1 Subcultures of the Mauriceville strain subjected to serial transfer

Subculture	Transfer growth stopped	Transfer variant plasmid detected[a]	Transfers to growth cessation[b]	Plasmid insertions[c]	mtDNA alteration(s)
M1	–	None	n.a.	–	–
M2	–	11–18	–	$tRNA^{Trp}$ +	–
M3	–	11–19/30–47	–	$tRNA^{Trp}$ + /$tRNA^{Trp}$	–
M4	–	29–33/40–45	–	$tRNA^{Trp}$/$tRNA^{Trp}$ +	–
M5	17	5–9	8–12	$tRNA^{Val}$	Eco-2
M6	22	None	n.a.	None	None
M7	42	30–38	4–12	$tRNA^{Pro}$ +	Eco-1, -6
M8	40	31–37	3–9	$tRNA^{Trp}$ +	Eco-8, -9
M9	41	12–22/20–29	12–21	$tRNA^{Trp}$ +	All but E1, E4
M10	–	28–35	–	$tRNA^{Trp}$	–

[a] The range is determined by PCR analysis of total DNA isolated from selected transfer cultutures

[b] Refers to the number of transfers between the detection of the variant plasmid and the senescent culture. (*n.a.* not applicable)

[c] The identity of sequences inserted at the site corresponding to the 3′/5′ transcript junction. All inserted sequences include a cDNA copy of a mitochondrial tRNA and, in many cases, additional sequences 3′ to the tRNA that are homologous to other regions in the mitochondrial genome [indicated by (+) and shown in Table 2]

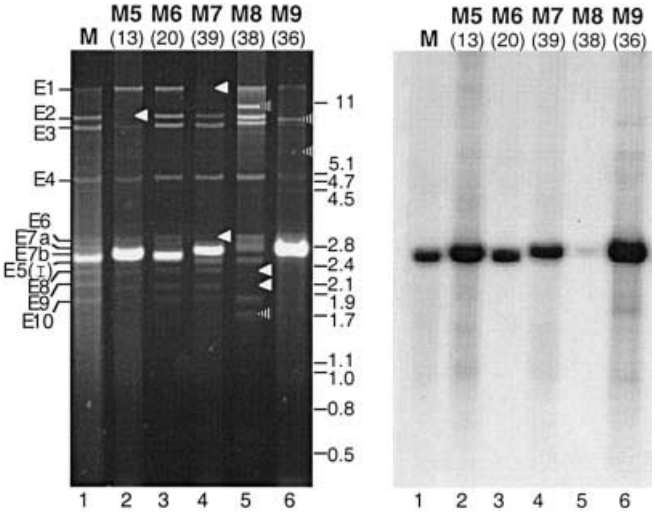

Fig. 2 Southern hybridization analysis of mtDNA isolated from pre-senescent subcultures. Total mtDNA isolated from the Mauriceville strain (*M*) and subculture M5 (transfer 13), M6 (transfer 20), M7 (transfer 39), M8 (transfer 38) and M9 (transfer 36) were digested with *Eco*RI and analyzed by electrophoresis in a 1.0% agarose gel. *Left panel* is an ethidium bromide-stained gel and *right panel* is a Southern hybridization with a ^{32}P-labeled plasmid probe. *Eco*RI restriction digest bands from the Mauriceville mtDNA are indicated on the left of the gel (E1–E10) and the size of *λ-Pst*I restriction fragments used as a marker is indicated to the right (in kb). Restriction bands that are absent or greatly reduced in certain subcultures are indicated with *white arrowheads*, whereas additional bands are indicated with *striped arrowheads*

failed to identify additional mitochondrial bands indicative of plasmid insertion into mtDNA (Fig. 2, lane 3, right panel and data not shown). In addition, the major *Eco*RI plasmid restriction fragment from a pre-senescent transfer culture of the M6 subculture co-migrated with the equivalent fragment from the wild-type Mauriceville plasmid and PCR analysis gave no indication that this culture contained a variant plasmid (see below). The sequence of the PCR product revealed the region corresponding to the 3′/5′ plasmid transcript junction is identical to that of the wild-type Mauriceville plasmid indicating the M6 plasmid does not contain an insert. Although it remains possible that the plasmid in the M6 strain has mutations in other regions that were not examined, these observations suggest that senescence of the M6 subculture occurred by a different mechanism from that associated with previously characterized senescent cultures harboring mitochondrial retroplasmids. This is supported by genetic analysis of this isolate, which indicates that the senescence phenotype may be related to a nuclear gene mutation and may not be dependent on the presence of the Mauriceville plasmid (J.C. Kennell, unpublished observations).

In contrast to the M6 strain, the M8 subculture had noticeable differences in the mtDNA restriction profile, yet pre-senescent cultures had greatly reduced levels of the variant plasmid relative to the amount of variant plasmids associated with other senescent cultures. Densitometric quantification of ethidium bromide-stained

Table 2 Sequence and identity of insertions in selected variant plasmids

Culture	tRNA insertion[a]	Additional inserted sequences	Homology	3′ flank[b]
M1-(50)	No insert			
M2-(50)	AAG…tRNA^{Trp}…GCC	ATCGAAAGTAGGTGCCGGTTCGATGCCGGT CTAGTTACGGAAGT- AAGTAGGTGCCGGT TCGATGCCGGTCTAGTTC	Eco-1[f]	2904
M3-(29)	AAG…tRNA^{Trp}…GCC[c]	GCCCCTCCTCTTGCCCCACCCTGCAGTACTG CAGGGGGGTGGGG-AAGGG GAGGGGGGTTGCGAA	Eco-1[g]	2904
M3-(48)	AAG…tRNA^{Trp}…GCC			2930[k]
M4-(39)	AAG…tRNA^{Trp}…GCC[d]			2904
M4-(46)	AAG…tRNA^{Trp}… tCC[e]	ACGAATACGGCCCACACACGA AGGTGTGTGGGGTATTCGTGGGCC	Eco-1, -4[h]	2904
M5-(13)	GAG…tRNA^{Val}…ACC			2904
M6-(20)	No insert			
M7-(39)	CGG…tRNA^{Pro}…ACC	TTGAGAACTAACTCAGGTTATT	?	2904
M8-(38)	AAG…tRNA^{Trp}…GCC	CACGAATACCCACACACACTTCG TGTGTGTGGGCCGTATTCGT GGGGTCC	Eco-1[i]	2904
M9-(36)	AAG…tRNA^{Trp}…GCC	CAGGGACGTAGGGTAACGGCC TACGCCTCGGGAGTT	Eco-11[j]	2904
M10-(50)	AAG…tRNA^{Trp}…GCC			2930[k]

[a] Insertions include a complete cDNA copy of a mitochondrial tRNA, minus the terminal A residue (only three terminal 5′ and 3′ nucleotides are shown)
[b] Indicates the Mauriceville plasmid sequence 3′ to the inserted sequences, based on the numbering of Nargang et al. (1984).
[c] Contains an additional G residue at position 31
[d] Contains a C→G transversion at position 30
[e] Contains a G→T transversion at position 71
[f] Has a 31-nt duplication (underlined) that is 100% homologous to a region within tRNA^{Asp}
[g] 100% homologous to a *Pst*I palindrome (*Pst*I site underlined; Yin et al. 1981) located between *co3* and tRNA^{Lys}
[h] 100% homologous to a region between tRNA^{Gly} and tRNA^{Asp}
[i] 100% homologous to a region 3′ to tRNA^{Ile-1}
[j] 100% homologous to a region 5′ to *atp8*
[k] Classified as a type 2 plasmid, lacking Mauriceville plasmid sequences 2904–2927 (Akins et al. 1989)

restriction fragments of the M8 pre-senescent culture indicated that the stoichiometry of the 2.8 kb plasmid band and mtDNA fragments are approximately the same (Fig. 2, lane 5), suggesting that following integration into the mtDNA, the autonomously replicating form of the plasmid may not have been maintained. To assess if the plasmid remains free from the mtDNA in these cultures, a Southern hybridization was carried out using a ^{32}P-labeled plasmid probe to a blot containing undigested mtDNA isolated from M8 cultures. The hybridization pattern demonstrated that the majority of the variant plasmid was not associated with the mtDNA, indicating that the plasmid remains autonomously replicating, albeit at a greatly reduced level (not shown).

The association between variant plasmid formation and growth cessation

The first step in the pathway that leads to senescence associated with the *Neurospora* retroplasmids appears to be the formation of variant plasmids by aberrant or promiscuous template switching by the plasmid RT during replication (Fig. 1; Akins et al. 1986; Kennell et al. 1994). Consequently, the rate of senescence in retroplasmid-containing strains first depends on the time of variant plasmid formation within a subculture transfer series. To assess the association between the appearance of variant plasmids and senescence, total DNA was isolated from approximately every tenth transfer of the Mauriceville subcultures and variant forms of the Mauriceville plasmid were identified using PCR. The region flanking the site corresponding to the 3′/5′ transcript junction was amplified as it is the site of cDNA insertion in all characterized variant plasmids. Figure 3 shows the partial results of this analysis and includes the first transfer identified in each subculture associated with an amplified product of increased size. Sequence analysis of selected amplified products indicated that the increased size was due to sequences inserted precisely at the 3′/5′ transcript junction site, demonstrating that PCR is a reliable method to evaluate variant plasmid formation. In addition, since the PCR assay can be performed on DNA isolated from mycelia and/or conidia taken from transfer cultures and does not demand additional growth that is normally required for the isolation of mitochondria for mtDNA analysis, it provides a more accurate method for assessing the status of the retroplasmids in growth-impaired tissue.

The transfer at which variant forms of the plasmid were first detected varied widely among senescent subcultures M5, M7, M8, and M9. For M5, a variant appeared between transfers 5 and 9, whereas for M7 and M8 a variant plasmid was not apparent until after the 30th transfer. In the M9 subculture series, a variant appeared between transfers 12 and 22, and a second variant appeared between transfers 20 and 29 and remained until the cultures senesced. Among these subcultures, the number of transfers that took place

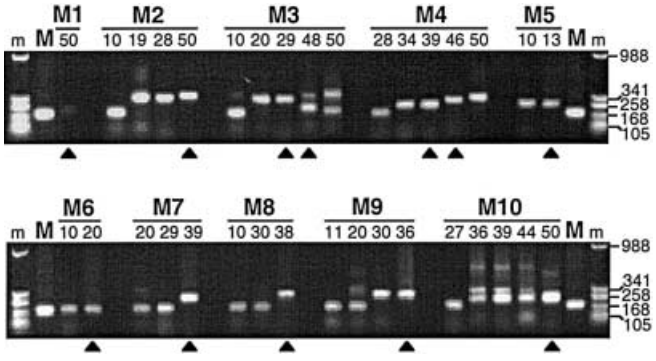

Fig. 3 PCR analysis of variant plasmids in Mauriceville subcultures. The region flanking the site corresponding to the 3′ end of the Mauriceville plasmid transcript was amplified from total DNA isolated from selected transfer cultures of ten subculture transfer series (M1–M10) of the Mauriceville strain. The passage number of the transfers is indicated below the subculture designation. Portions of the PCR products were separated by electrophoresis in a 2% agarose gel, stained with ethidium bromide and visualized using a UV transilluminator. The products in each panel are flanked by a PCR product derived from a clone of the Mauriceville plasmid (*M*) and *Sau*3AI restriction fragments of pBluescribe vector used as DNA size markers (*m*). The size of the restriction fragments is indicated on the right (in bp). *Arrowheads* below certain lanes indicate DNAs that were cloned and sequenced (see Table 2)

between the appearance of a variant plasmid and senescence ranged from 4 to 21 transfers (Table 1).

Excluding subculture M6, which did not form a variant plasmid, the appearance of a variant plasmid always preceded senescence; however, a correlation between the time of variant plasmid formation and senescence was not evident. More significantly, among the five subcultures that failed to senesce by the 50th transfer, all but subculture M1 were found to contain variant plasmids (Fig. 3; Tables 1, 2). The transfer at which a variant plasmid first appeared in subcultures M2, M3, M4 and M10 had approximately the same degree of variability as was detected among senescent cultures, ranging between transfer 11 and 28. The appearance of multiple variant plasmids within a transfer series was also apparent in at least three of the non-senescent subcultures (e.g. M3, M4, M10; Fig. 3, Tables 1, 2), and less abundant PCR products were detected at some point in practically all subculture transfer series. Taken together, these studies indicate that even though the formation of variant plasmids appears to be a prerequisite for retroplasmid-associated senescence, the appearance of a variant plasmid in subcultures of the Mauriceville strain is not the sole determinant of when, or even if, senescence occurs.

To assess if the particular sequence inserted in the variant plasmids could be correlated with the rate or frequency of senescence, plasmid DNAs or PCR products derived from senescent and non-senescent subcultures were cloned and sequenced. Table 2 shows the sequence of the variant plasmid inserts and their identity (if known). Among the senescent cultures having variant plasmids, two were found to contain inserts that were

homologous to the *Neurospora* mitochondrial $tRNA^{Trp}$ (M8 and M9). The capture of $tRNA^{Trp}$ is common among variant plasmids, occurring in 5 of 13 previously reported variant plasmids (Akins et al. 1988; Chiang et al. 1994; Stevenson et al., 2000). A pre-senescent M5 subculture contained a variant plasmid with an insert homologous to $tRNA^{Val}$ and the variant plasmid in subculture M7 contained sequences homologous to $tRNA^{Pro}$. The insert in the M5 variant plasmid is identical to an inserted sequence found within a previously characterized variant (M3–24; Akins et al. 1986) while the variant plasmid in subculture M7 is the first reported to have homology to $tRNA^{Pro}$. In addition to homologies to mt tRNAs, variant plasmids in subcultures M7, M8, and M9 contain additional sequences downstream of the region homologous to mt tRNAs. The M7 plasmid insert (from transfer 39) contains 22 nt of additional sequence of unknown identity, while variant plasmids from M8 (transfer 38) and M9 (transfer 36) have 50 and 36 nt of additional sequence that are identical to regions within the *Eco*RI-1 and *Eco*RI-11 mtDNA restriction fragments, respectively. Chimeric inserts are common among variant plasmids, and are likely the result of two or more successive template switching events (Akins et al. 1988; Kennell et al. 1994; Stevenson et al. 2000).

Variant plasmids recovered from transfer cultures that did not senesce within 50 passages had the same general features as those detected within the senescent cultures (Table 2). Of the six variant plasmids characterized from the non-senescent cultures, all were found to contain copies of mt $tRNA^{Trp}$ and three of the six had additional sequences 3′ to the tRNA insertion. The additional sequences are all homologous to regions of the mtDNA, with one containing a large direct repeat [M2-(50)], while another contains a *Pst*I palindrome (Yin et al. 1981) of approximately 54 nt. When compared to the insertions associated with variant plasmids in subcultures that senesced, no obvious feature could be identified that distinguished the variant plasmids recovered in non-senescent cultures from those associated with senescent cultures. This suggests that the sequence of the inserted region of the variant plasmids is not an important determinant of senescence.

Discussion

We determined if a correlation exists between the formation of variant plasmids and the rate and/or frequency of senescence among subcultures of the Mauriceville strain of *N. crassa*. In most cases, we found that variant plasmid formation preceded growth cessation; however, little concordance was observed between the time that variant plasmids were first detected and the rate at which cultures senesced. Surprisingly, four of ten subcultures studied were found to tolerate variant plasmids for at least 15 weeks of vegetative growth without signs of growth decline, demonstrating that the formation of variant plasmids does not necessarily lead to growth suppression. These findings indicate that the formation of variant plasmids occurs at higher frequencies than previously expected and provide new insight into the process of senescence associated with retroplasmid-containing strains.

We also report the isolation of a strain (M6) in which senescence occurs in the absence of variant plasmid formation or detectable alterations in mtDNA. Preliminary genetic analysis indicates that the senescent phenotype is associated with a nuclear mutation. Recently, a nuclear senescent mutant was isolated among a natural, plasmid-free isolate of *Neurospora* (Navaraj et al. 2000), demonstrating that there are pathways that lead to senescence that do not involve mitochondrial plasmids. In addition, we previously reported that in cultures containing an over-replicating variant of the Mauriceville plasmid senescence can occur without alterations in mtDNA (Stevenson et al. 2000).

These findings, combined with other studies of senescence in *Neurospora*, suggest at least three separate pathways can cause growth cessation: (1) mitochondrial DNA mutations, which for plasmid-containing strains result from the integration of plasmids into the mitochondrial genome; (2) plasmid over-replication, which interferes with mitochondrial protein synthesis; (3) nuclear gene mutations that impair mitochondrial function. Although the mechanism by which nuclear mutants disrupt mitochondrial function is not fully understood, the common feature of all three pathways is the generation of dysfunctional mitochondria that displace wild-type organelles (see below).

The ability of the Mauriceville subcultures examined in this study to tolerate variant plasmids could have been associated with spontaneous mutations that were selected during vegetative growth, as appears to be the case with the long-lived derivative strain of the MS4416 strain (Stevenson et al. 2000); yet this seems unlikely, due to the high frequency of non-senescent cultures. Alternatively, the variable rate of senescence detected among subcultures of the Mauriceville strain could have been related to the process by which vegetative tissue was propagated. For example, transfer culture inocula might have had insufficient amounts of newly formed variant plasmids for the plasmids to become established in the new cultures. However, we found the frequency of senescence of Mauriceville subcultures of *N. crassa* was significantly lower than Varkud subcultures of *N. intermedia* that were subjected to the same transfer regime, and previous studies demonstrated that this disparity is also observed in race tube cultures that permit continual growth (Akins et al. 1986). Since the etiology of senescence appears to be the same for both the Mauriceville and Varkud strains, differences in the rate and frequency of senescence are not likely associated with the frequency of spontaneous mutations or culture conditions, and our studies indicate that these differences cannot be explained by the rate at which variant plasmids are generated.

Taken together, these findings suggest there may be host-specific factors that influence plasmid integration into the mitochondrial genome or subsequent steps that lead to mitochondrial dysfunction. Interestingly, the frequency and rate of senescence associated with the Maranhar linear DNA plasmid of *N. crassa* also appear to be lower than those reported for *N. intermedia* isolates containing the similar, but not homologous, Kalilo plasmid (Griffiths et al. 1986; Court et al. 1991). Whether this reflects differences between the plasmids or species-specific differences in the ability to tolerate mitochondrial parasitic elements remains to be determined.

All variant plasmids described to date contain a near full-length copy of a mitochondrial tRNA [or a tRNA-like sequence (Akins et al. 1986; Chiang et al. 1994)] and, in many cases, additional mtDNA sequences which are positioned downstream of the tRNA insertions. Since one mechanism of plasmid integration involves homologous recombination, integration frequencies could be influenced by the size of the cDNA insert and extent of homology with the mt genome. It is also possible that the particular tRNA inserted into the plasmid is important if the site of integration in the mitochondrial genome were a critical determinant of senescence. However, we found that the inserted sequences in the variant plasmids associated with senescent and non-senescent cultures were very similar in both length and identity; thus it is unlikely that the nature of the inserted sequences plays a major role in determining the rate or frequency of senescence. Interestingly, PCR analysis suggests that new variant plasmids continually arise during growth and, in at least three of the subcultures examined, new variants were found to replace existing plasmids. This suggests that new variant plasmids occasionally out-compete the plasmids from which they were derived. Alternatively, the ascendance of new variants could relate to the process of serial transfer, which likely selects for tissue containing plasmids that are less suppressive to growth.

Previously, we showed that senescence associated with the integration of variant plasmids into mtDNA was only reproducible for transfer cultures that were very close to senescence (Stevenson et al. 2000). Taken together with results presented here that indicate that the appearance of a variant plasmid is not directly correlated with the rate or frequency of senescence in Mauriceville subcultures, the data suggest that the rate-limiting step in the pathway that leads to growth cessation associated with retroplasmid-containing cultures likely involves the process of plasmid integration and/or the ability of the resulting defective mtDNAs to accumulate and displace wild-type mtDNAs. Little is known about how 'suppressive' mtDNAs come to dominate senescent cultures, and this phenomenon is not restricted to senescent strains and includes other mitochondrial mutants such as *poky* and *stopper* mutants (Griffiths 1992). Bertrand (1995) has proposed a model for suppressiveness in obligate aerobic fungi (which include *Neurospora spp.*), in which the accumulation of defective mitochondria is a consequence of a cellular response to perturbations in cytochrome-mediated redox function or oxidative phosphorylation, which leads to an accelerated rate of mitochondrial biogenesis. This model implies that impaired mitochondrial function is limited to organelles that contain mutant mtDNAs and these defective mitochondria remain separate from wild-type organelles, resulting in the selective amplification of dysfunctional mitochondria. Accordingly, the unpredictability of senescence in retroplasmid-containing strains likely reflects both the stochastic process of plasmid integration as well as the degree to which plasmid insertions affect mitochondrial function.

Integration of variant plasmids at tRNA loci via homologous recombination, or at non-coding regions, would likely have a minimal effect on the expression of mitochondrial genes, and integrated forms of the Varkud retroplasmid, as well as the Kalilo plasmid, have been detected in non-senescent *Neurospora* cultures (Myers et al. 1989; Chiang et al. 1994). Consequently, multiple integration events may be necessary within the same mitochondrion in order to generate highly defective mtDNAs. Integration of the retroplasmids at separate sites within the same molecule or in different mtDNAs within the same mitochondrion would facilitate the generation of 'stopper-like' molecules that lack large regions of the mt genome following recombination between plasmid inserts. Consistent with this hypothesis, mtDNA restriction digest profiles from many retroplasmid-containing senescent cultures show the selective loss of one or more restriction fragment, while other mtDNA fragments remain at stoichiometric levels (Akins et al. 1986; Fig. 2, this report), similar to what is observed among stopper mutants (Bertrand et al. 1980; Almasan and Mishra 1988).

It was previously suggested that the formation of retroplasmids that contain inserts homologous to regions in mtDNA could have been a primitive mechanism by which retro elements integrated into the mtDNA (Chiang et al. 1994). The apparent increase in the frequency of integration brought about by the capture of mt tRNAs may, by itself, be innocuous; however, in combination with less frequent integrations at non-homologous sites, it could lead to recombination events that generate suppressive mtDNAs that are detrimental to the survival of the host.

Analysis of insertions at non-homologous sites indicates that the mechanism of integration most likely involves cDNA replication intermediates (Akins et al. 1986) and may not depend on the formation of variant plasmids. Models for the integration of the linear DNA plasmids, Kalilo and Maranhar, also invoke replication intermediates (Chan et al. 1991; Court et al. 1991), which suggests that plasmid integration events are an inadvertent consequence of plasmid replication and could potentially occur with all types of mitochondrial plasmids. Due to the prevalence of mt plasmids in filamentous fungi, particularly *Neurospora* species, it seems reasonable that mechanisms might have evolved to limit

plasmid integration and/or the production of suppressive mtDNAs which, in turn, could provide a permissive environment for mitochondrial plasmids. Further study of the *N. crassa* cultures that maintain variant plasmids for extended periods of time without causing growth suppression may provide additional evidence that such mechanisms exist.

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