SUPPRESSION OF kalDNA-RELATED SENESCENCE IN NEUROSPORA

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ABSTRACT

Senescence in Neurospora, the progressive loss of growth potential culminating in death, is caused by the insertion of a linear mitochondrial plasmid kalDNA into the mitochondrial chromosomes. Investigation of the suppression of kalDNA-related senescence in Neurospora has revealed that there are three mechanisms of suppressing the kalDNA-related senescence in Neurospora. The first mechanism is that one allele of one nuclear gene from strain 83 inhibits the replication of the kalilo plasmid to a very low level in the mitochondria, there are no (or too few) IS-kalDNAs to affect the normal function of the mitochondria. The other allele of the gene from strain HB9006 permits the plasmid to replicate to high levels, thus the insertion activities of the plasmid destroy the mitochondrial function and consequently cause death of the host fungus. The second mechanism is the cytoplasmic segregation. The mitochondria in senescent strains are heterogenic, some containing AR-kalDNA or and IS-kalDNAs, some containing neither. When asci, ascospores or conidia are formed with the mitochondria lacking kalDNA from their parental cytoplasm containing kalDNA, the sexual or asexual progeny with normal mitochondria grow immortally, escaping the fate of death. The third mechanism is that of a nuclear gene with two alleles in ascus 13. One allele of this gene from HB9006 cannot suppress the deleterious function of IS-kalDNAs, resulting in vegetative death. The other allele of this gene, from strain 428, allows the plasmid to replicate normally and insert into the mitochondrial chromosomes generating IS-kalDNAs, but the allele somehow suppresses the deleterious effects of IS-kalDNAs, resulting in the host fungi growing normally, and carrying both AR-kalDNA and IS-kalDNAs in their mitochondria.

The kinetic studies of mitochondrial kalDNA in senescent Neurospora strains have revealed the general trend of kalDNA changes in the kalilo senescence process. The free form (AR-

kalDNA) of the plasmid is observed in all subcultures of senescent series, increases in quantity at the early stage of senescence until a peak is reached at the late stage, and then drops to a low level. The inserted form (IS-kalDNA) of the plasmid is not observed at the early stage, but observed at the late stage and increases in quantity until the last analyzable subcultures. The relationship of AR-kalDNA and IS-kalDNA, and their replication characteristics have been discussed. A model of accumulation of IS-kalDNA has been proposed.

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INTRODUCTION

A 1. NEUROSPORA LIFE CYCLE

The genus Neurospora belongs to the kingdom Fungi and is a member of the class Ascomycetes. The ascomycetes are the largest class of fungi and provide most of the species which have been widely used in genetics. The principal feature of this group is the ascus which encloses the products of meiosis, the ascospores; there are four or, following an extra mitotic division, eight in number. For example, in N. crassa, N. sitophila and N. intermedia a mature ascus contains eight ascospores. Neurospora possesses a mycelial form of cellular organization where the mycelium has septa which delineate hyphal compartments. Within each compartment are several nuclei. The septa have central pores through which the nuclei and cytoplasm can pass thus uniting the various hyphal compartments into a continuous protoplasmic system. This form of cellular organization is referred to as coenocytic.

A diagram of the life cycle of Neurospora is shown in Figure 1. The life cycle of N. crassa, N. sitophila and N. intermedia involves both sexual and asexual propagation (reviewed by Beadle, 1945). These species are all heterothallic and consequently require mycelia of opposite mating types to fuse in order to complete the life cycle. Mating type is determined by a mating type gene which is located on Linkage Group 1 (Perkins et al, 1982). The two mating types are designated A and a, determined by codominant alleles of the mating type gene. On suitable crossing medium, either mating type is capable of producing female sexual structures, protoperithecia. Protoperithecia, consist of coiled filaments of hyphae which become surrounded by a thick layer of hyphae. The coiled filaments are destined to become ascogenous hyphae. From each filament a sexual hypha, the trichogyne, is produced, which can fuse with a fertilizing cell of the opposite mating

type. The male cells may be either vegetative hyphae, or asexual spores called macroconidia or the less abundant microconidia. It has been shown that trichogyne growth and localization of the male fertilizing cell is a chemotactic response initiated by the presence of a pheromone released by the male fertilizing cell (Bistis, 1986). After fusion of the trichogyne and the male fertilizing cell, the nucleus from the male cell is transferred through the trichogyne into the ascogenous hyphae. The paternal and maternal nuclei undergo a number of synchronous mitotic divisions to form a small mass of dikaryotic ascogenous hyphae. At the same time the protoperithecium enlarges and becomes blackened with melanin and eventually forms the mature fruiting body, the perithecium. Nuclear fusion and karyogamy eventually occurs in the penultimate hyphal compartment of the ascogenous hyphae. Immediately after karyogamy, meiosis occurs and the four products of meiosis undergo a round of mitosis to give a total of eight nuclei which form the ascospores. At maturity the asci elongate and eject their spores through the ostiole of the perithecium. The ascospores germinate under high temperatures, 60°C, and form mycelia. On vegetative medium, aerial mycelium is formed and conidia are produced through mitosis. The conidia become airbone and give rise to new colonies that continue the life cycle.

The advantage of using Neurospora for genetic analysis is the availability of the products of each meiosis which remain together as a tetrad of ascospores. The segregation of different phenotypes in a tetrad can determine the pattern of inheritance of genes. Furthermore, the phenotypic ratios in either ordered or unordered tetrads provide information on various chromosome mutations such as nondisjunction, translocation, and inversion, on gene mutations, and on gene conversion. Reciprocal crosses are possible since any strain of Neurospora may be used as either a male or female parent. Thus, analysis of tetrads or

random ascospores from reciprocal crosses aids in distinguishing between nuclear and extranuclear inheritance. In Neurospora, the cytoplasm shows strict maternal inheritance, and therefore extranuclear inheritance can be distinguished from nuclear inheritance based on reciprocal differences in crosses.)

A 2. CYTOPLASMIC MUTATIONS OF FUNGI

A 2.1 Petite mutations of Saccharomyces cerevisiae

Mitochondrial genetics of S. cerevisiae was initiated when Ephrussi et al (1949) reported that some respiratory-deficient mutants, obtained after acriflavin exposure, showed non-Mendelian patterns of inheritance. These mutants are called "petites" or "vegetative petites". These strains are non-reverting pleiotropic mutants containing large deletions of the mtDNA (ρ^-) or no mtDNA at all (ρ^0) (Dujon, 1981). In contrast to filamentous fungi which die as a consequence of major mtDNA deletions, yeast is a facultative anaerobe and can survive complete loss or gross alterations of its mtDNA. For ρ^- mutants the fragment of mtDNA retained is variable and usually less than one third of the genome (Dujon, 1981). Since the total amount of mtDNA in a ρ^- mutant is similar to the wild type mtDNA from which it was derived, irrespective of the size of the deletion, it was suggested that the number of copies of the retained mtDNA must be amplified (Hollenberg et al, 1972; Nagley and Linnane, 1972; Faye et al, 1973; Fukuhara et al, 1974; Borst et al, 1976). Further investigation revealed that there are two major types of arrangements of the amplified conserved mtDNA sequences in ρ^- mutants (Dujon, 1981). Generally head to tail repeats of the conserved sequences are observed when the retained mtDNA is less than approximately 1 kb. In the other type of arrangement, the repeated unit is an inverted

duplication of the conserved sequence. Usually, these inverted repeats are not perfect. Some other ρ^- mutants are observed to contain mixtures of the repeats where the repetition of the conserved sequences may be direct or inverted along the same molecule. In addition to these two major arrangements of the retained mtDNA, rearrangements may be observed including internal inversions, deletions or illegitimate recombination between different ρ^- mutants (Dujon, 1981).

The degree of suppressiveness, that is the proportion of zygotic clones composed of ρ^- mutant cells, is characteristic of a given ρ^- mutant (Ephrussi and Grandchamp, 1965). Two models were proposed to explain the mode of action of the suppressive petite mtDNA in heteroplasmic crosses. These include a model based on the destructive recombination of wild type mtDNA molecules, thus giving rise to deleted molecules (Coen et al, 1970; Michaelis et al, 1973; Deutisch et al, 1974; Perlman and Birky, 1974; Slonimski and Lazowska, 1977). Although this has been suggested as a model for suppressiveness, the demonstration that the petite mtDNA in diploid progeny from a cross are both physically (Goursat et al, 1980; Blanc and Dujon, 1980) and genetically (Gingold, 1981) similar to those of the petite parent has focussed attention on the out-replication model. This model is based on a replicative advantage of petite mtDNAs (Slonimski et al, 1968; Rank, 1970a, 1970b; Rank and Bech Hansen, 1972; Carnevali and Leoni, 1981). The out-replication model has received much support from analysis of the nature of the mtDNA of very strongly suppressive petites. These petite mtDNA molecules usually consist of short repeats which include one of a small number of closely related sequences thought to represent origins of replication (de Zamaroizy et al, 1979; Bernardi et al, 1980; Blanc and Dujon 1980, 1981) It was suggested that the high degree of reiteration of origin of replication sequences in highly suppressive petites would confer a replicative advantage to

the petite mtDNAs in heteroplasmic zygotes. It was further suggested that ρ^- mutants showing lesser degrees of suppressiveness reflects the lower degree of reiteration of the origin of replication sequences. Further analysis of ρ^+ and ρ^- heteroplasmic zygotes was achieved by growing mating mixtures from petite by grande crosses in selective medium containing radioactively labelled uracil (Chambers and Gingold, 1986). In this medium only zygotes could grow and incorporate label into their newly synthesized mtDNA. MtDNA was isolated from the cultures, cut with restriction enzymes, and newly synthesized mtDNA restriction fragments visualized by autoradiography of gels. By focussing on bands unique to the petite and the grande mtDNAs, the authors were able to ascertain the relative amounts of label incorporated into the two mtDNA species and determined their relative level of synthesis in the zygote. It was discovered that the out-replication hypothesis does not hold true for all suppressive petites. Hypersuppressive petites did exhibit a competitive replication advantage over the ρ^+ mtDNA molecules, but less suppressive petites do not necessarily show this same pattern. Interestingly, a strain which was essentially nonsuppressive gave an indication of replicative superiority. Thus, the simple out-replication hypothesis does not offer an explanation for these inconsistencies. Therefore the possibility remains that the destructive recombination model may also play a role in the phenomenon. The key idea in this model is that the presence of a ρ^- molecule can cause crossover which lead to the destruction of the wild type DNA (Coen et al, 1970; Michaele et al, 1973; Deutsch et al, 1974; Perlman and Birky, 1974) The actual process which is occuring to cause suppressive behavior may be a combination of mechanisms. Clearly, some mechanism other than destructive recombination of wild type mtDNA or enhanced replication also could be involved.

A 2.2 The ragged mutation of Aspergillus amstelodami

Jinks (1956) discovered a cytoplasmically-inherited mutation of A. amstelodami referred to as "ragged". It was observed that these mutants maintain a state of senescence over long periods of time where vegetative death occurs in the hyphal tips giving the growing front of a characteristically ragged appearance (Jinks, 1956; 1959; Caten, 1972). The ragged phenotype arises spontaneously at a reasonable high frequency, and ragged mutants are characterized by cytochrome deficiencies and mtDNA rearrangements. Analysis of the mtDNA of ragged mutants revealed that there is a heterogeneous mixture of a tandemly repeated element and a full copy of the wild type mtDNA (Lazarus et al, 1980). This situation is analogous to the deletion /duplication seen in the petites, except for the presence of a normal copy of mtDNA. Presumably this molecule is lost as the culture ages. One region of the mtDNA is found in the tandemly repeated fragment of almost all ragged mutations. It is postulated to contain a mtDNA origin of replication which when tandemly duplicated offers a replication advantage on the ragged mtDNAs. However, the presence of ragged mtDNAs which do not contain this sequence and instead are found to have a different region of the mtDNA amplified (Lazarus and Kuntzel, 1981) suggests that mutated mtDNA somehow replaces the wild type mtDNA. The replacement could result from out-replication of aberrant mtDNAs, destructive recombination or a combination of both.

A 2.3 Senescence in Podospora anserina

The unavoidable decline in growth potential culminating in vegetative death in P. anserina was discovered to be maternally inherited (Rizet, 1953; 1954). This was confirmed by microinjection of juvenile cultures with cytoplasm isolated from senescent cultures, and

from hyphal fusion experiments (Marcou and Schecroun, 1959). Mycelial senescence is race specific (Rizet, 1957; Marcou, 1961) where race A expresses senescence more rapidly than other races.

It was shown that the 94 kb juvenile mtDNA is greatly diminished in senescent cultures and replaced by amplified multimeric sets of small circular DNA (Stahl et al, 1978; Cummings et al, 1979a). These circular DNAs are referred to as sen plasmids and consist of head-to-tail tandem repeats of specific regions of the mtDNA (Stahl et al, 1978; Cummings et al, 1979a; 1979b; 1980; Jamet-Vierny et al, 1980; Kuck et al, 1981; Osiewacz and Esser, 1984). The most frequent sen plasmid is alpha. Other sen plasmids, not seen as frequently as alpha senDNA, are referred to as beta, epsilon, theta and gamma (Stahl et al, 1978; Cummings et al, 1980; 1985; Jamet-vierny et al, 1980; Wright et al, 1982; Osiewacz and Esser, 1984). Confirmation that alpha senDNA is responsible for senescence in some strains came from information on a mutant that has shown no signs of senescence even after four years of culturing. Analysis of the mtDNA of this mutant revealed that the sequences of the intron, which normally excise to generate alpha senDNA, are absent in the mtDNA of this mutant (Vierny et al, 1982).

Senescent cultures of both race A and race s contain alpha senDNA sequences. These two races differ in the number of introns present in the COI gene. Race A has an extra class II intron referred to as intron A (Cummings and Wright, 1983). This intron does not share overall sequence homology with the intron which excises to generate alpha senDNA. It was proposed that the presence of two class II introns in the COI gene of race A, compared with one in race s, could account for the difference in the rates of senescence between these two races. This hypothesis was stimulated by the finding that these two group II introns

contain nucleotide sequence homologies with the retroviral reverse transcriptase of Rous Sarcoma virus and HTLV-1 Virus (Matsuura et al, 1986). Thus , if reverse transcription of these two class II introns is responsible for the amplification of sen plasmids, then race A would have twice as many copies of sen plasmids as race s. No autonomous sen plasmid homologous with intron A sequences has been identified suggesting that this is probably not the mechanism controlling the rate of production of the sen plasmids (Cummings et al, 1987). It was suggested that intron A may play a modulating role in the excision of alpha senDNA and that it cannot be excised except in the absence of alpha senDNA. Although amplification of alpha senDNA does not appear to depend on the reverse transcription of the class II intron, evidence that there is reverse trancriptase activity in senescent race A cultures and not in young cultures does suggest that alpha senDNA may be generated through an RNA intermediate (Steinhilber and Cummings, 1986).

A model to explain the amplification of senDNAs is based on the hypothesis that sen plasmids may show superior replication relative to the mtDNA. Lazdins and Cummings (1982) showed that cloned alpha, beta and gamma senDNAs, as well as those young mtDNA sequences which overlap and hybridize with senDNA sequences, confer origin of replication characteristics to the otherwise nonreplicating vector YIp5. It has also been shown that pBR322 containing alpha senDNA is able to replicate in P. anserina (Stahl et al, 1982). Together these results indicate that the amplification of the small circular senDNAs may depend on the presence of origin of replication sequences and identification of only five sen plasmids may define the regions of the mtDNA which contain origin of replication sequences.

The onset of senescence in P. anserina appears to depend on any one of a number of nuclear genes (Tudzynski and Esser, 1979; Esser and Tudzyski, 1980). These genes are pleiotropic because in addition to prolonging life, they alter mycelial morphology. The double mutant vivax incoloris is able to prevent senescence. Microinjection experiments showed that senescent mycelia were not able to infect vivax incoloris mutants. Moreover, ascospores from a cross between a senescent wild type female and a viv inc male mutant revealed that viv inc progeny did not express senescence whereas the wild type progeny were senescent (Tudzynski and Esser, 1979). It was suggested that the viv inc combination counteracts the senDNAs in their active form (Esser and Tudzyski, 1980).

Recently, several nuclear-mutated, long-lived strains of P. anserina have been identified and their mtDNA analyzed (Turker et al, 1987a,b,c; Turker and Cummings, 1987; Schulte et al, 1988; Osiewacz et al, 1989; Schulte et al, 1989; Silliker and Cummings, 1990). MtDNA rearrangement of longevity mutants and their plasmids share many characteristics with previously described rearrangements and plasmids from senescent cultures of P. anserina. There is a heterogeneous population of subgenomic mtDNA molecules in long-lived mutants (Schulte et al, 1989; Silliker and Cummings, 1990). An 11 bp consensus sequence has been described which seems to be important for the excision of all of the senDNAs except alpha senDNA (Turker et al, 1987). Many excision-junction sites of plasmids in these long-lived mutants have in common the 11 bp consensus sequence (Silliker and Cummings, 1990). It is perplexing that there are not greater differences between the mtDNA of long-lived and senescing cultures. The longevity plasmids occur along with plasmids previously described as senDNAs (Silliker and Cummings, 1990). These results suggest that the plasmids and rearrangements observed are the characteristic products of regular recombination events which occur in Podospora. The plasmids

themselves may not be responsible for a particular life span phenotype; the minor mtDNA species may be more important in determining whether a strain will be able to escape senescence (Silliker and Cummings, 1990).

A 2.4 Cytoplasmic Mutations of Neurospora

A 2.4.1 Group I Cytoplasmic Mutations of Neurospora

The first cytoplasmic mutation of N. crassa to be identified was "poky" (Mitchell and Mitchell, 1952). This mutant was characterized as initially exhibiting slow growth, showing a progressive increase in growth rate until a rate of wild type was reached. Young cultures of these mutants are deficient in cytochromes aa_3 and b (Hazkins et al, 1953). Additional mutants with similar phenotype were identified later (Bertrand and Pittenger, 1972a, 1972b; Bertrand et al, 1976). Most group I mutants are suppressed by a nuclear suppressor called f (Bertrand and Pittenger, 1972b). Lesions which give rise to pokys seem varied, although all seem to lead to deficiencies in small rRNA or small ribosomal subunits (Collins and Bertrand, 1978; Lambowitz et al, 1979). In the identification of six nuclear suppressors of poky, all were found to promote the assembly of the missing ribosomes (Bertrand and Kohout, 1977; Kohout and Bertrand, 1976). This has led to the idea that all group I mutants affect a single process, or function, perhaps the 19S rRNA, and that all suppressors of group I mutants affects small ribosomal subunits (Collins and Bertrand, 1978).

It has been shown that all pokys, regardless of their origin and in addition to any other mutation they have, contain a 4 bp deletion near the 5' end of the 19S rRNA, and that this

deletion causes an aberrant 19S rRNA to be synthesized which is 38-45 nucleotides shorter than the wild type (Akins and Lambowitz, 1984). The mechanism of poky phenotype has been hypothesized to be the impairment of transcription of the small rRNA, as the 4 bp deletion has been found to occur in the promoter sequence for the 19S rRNA gene (Kennel and Lambowitz, 1989).

A 2.4.2 Group II Cytoplasmic Mutations of Neurospora crassa

Group II mutants include mi-3 (Mitchell et al, 1953; Bertrand and Pittenger, 1972a) and exn-5 (Bertrand et al, 1976). Both have an initial lag in growth rate and are deficient in cytochrome aa3. These phenotypes are suppressed completely by the nuclear gene su-1 (Bertrand, 1971). The molecular defect in mi-3 has been identified as a missense mutation in the OXI-3 gene (Lemise and Nargang, 1986).

A 2.4.3 Group III Cytoplasmic Mutations of Neurospora crassa

The group III mutants are characterized by an irregular pattern of growth and no growth. Based on their stop-start growth pattern, these mutants were termed "stoppers". Stopper mutants are female sterile and deficient in cytochrome aa3 and b (Bertrand et al, 1980; DeVries et al, 1981). The molecular lesions in different stoppers have been found to be varied, however all stoppers have deleted mtDNAs which retain a large region that includes the Eco RI-1, -4, and -6 fragments. This region contains the rRNA genes and most of the tRNAs, and has been postulated to contain an origin of replication (Bertrand et al, 1980; Collins and Lambowitz, 1981; DeVries et al, 1981).

Stoppers often contain more than one type of mtDNA molecules. One mutant, stp, has been found to contain two populations of mtDNAs, a 21 kb molecule that spans the region mentioned above, and a 43 bp molecule that contains the rest of 65 kb mtDNA. During the stop phase of growth only the 21 kb molecule can be detected, however upon resumption of growth, the large molecules appears (Bertrand et al, 1980; Gross et al, 1984). The observation that the altered mtDNA molecules form the majority of the mtDNA population during the stop phase suggested that there is a competition between defective and intact mtDNAs such that cells in the stop phase should show strong selection for nuclear or extranuclear mutations that permit resumption of growth and visa versa for cells in the growth phase (Bertrand et al, 1980).

Two other stopper mutants have been studied extensively. The E-35 mutant contains two molecules with 4 and 20 kb deletions (DeVries et al, 1981), which seem to arise via a sequence specific event. The region that is deleted to form the molecule with the 4 kb deletion has been postulated to contain the gene for a subunit of NADH dehydrogenase in Neurospora mitochondria (DeVries et al, 1981). Another stopper, ER-3, has a mtDNA molecule with a 25 bp deletion which seems to occur via the same sequence specific mechanism as E-35 (Almasan and Mishra, 1988). Therefore, the mechanism by which stoppers form may be a specific one in Neurospora crassa.

A 3. SENESCENCE IN NEUROSPORA

Five variants having properties similar to the "stopper" extranuclear mutants of N. crassa were discovered by Rieck et al (1982) in a sample of N. intermedia strains collected from the Hawaiian island of Kauai. These variants were identified by their inability to grow the

length of a 500 mm race tube. They exhibited irreversible terminated growth, cytochrome aa3 and b deficiencies, abnormal respiration, and abnormal mitochondrial ribosome profiles. After a more intense survey was conducted using a serial subculture protocol, a total of 26 variants was identified. Most variants ceased to grow within 10 subcultures (Griffiths and Bertrand, 1984). The striking phenotypic similarities between these variant strains and the stopper cytoplasmic mutants of laboratory strains of N. crassa (Bertrand et al, 1968; Bertrand and Pittenger, 1969) promoted the assumption that their growth and cytochrome defects are caused by gross rearrangements of the mitochondrial chromosome (Bertrand et al, 1980; DeVries et al, 1981; Gross et al, 1984; Infanger and Bertrand, 1986). A comparison of a number of field-isolates of N. intermedia from Kauai indeed revealed differences between normal strains and the variants with respect to the size and number of restriction fragments of their mtDNAs (Rieck et al, 1982).

It was suspected that the cytoplasmic abnormalities of these variants are maternally inherited. Proof of the maternal inheritance was obtained from the analysis of ascospores from reciprocal crosses between variants and normal strains. It was shown that the phenotype of these variants differed from the classic "stopper" phenotype, in that juvenile cultures of a senescent strain, whether initiated from conidia or from an ascospore, were phenotypically normal. However, upon subculturing, these strains showed a progressive loss of growth potential culminating in death. This process leading to the vegetative death of these organisms was termed senescence. The process was repeatable, in that duplicates of cultures initiated from a common culture always were capable of a defined number of subcultures before death. Further, it was shown that the changes occuring in the cytoplasm throughout this process are heritable, and that progeny of a senescent parent had less growth potential than the progeny of presenescent parent. During the changes, the

cytoplasm was found to be heterogeneous for some undetermined factor of senescence. The phenomenon of senescence among a high proportion of natural isolates of N. intermedia from the Kauai island was termed "kalilo", a Hawaiian word meaning "dying", and affected strains were termed kalilo strains (Griffiths and Bertrand, 1984).

Analysis of mtDNA from the prototype senescent strain P561, revealed that insertion of a foreign nucleotide sequence into mtDNA, specifically into the intron of the 25S rRNA gene. The 8.6 kb transposon-like element was termed kalilo DNA (kalDNA) and the inserted form has been designated mtIS-kalDNA (Bertrand et al, 1985; Bertrand, 1986). Mitochondrial chromosomes carrying mtIS-kalDNA are thought to accumulate as a culture ages until aberrant mtDNAs dominate the cytoplasm. Sites of insertion of kalDNA were found to differ not only between senescent strains, but between senescent subcultures of individual strains. The displacement of normal mtDNA suggests that mtDNA molecules carrying mtIS-kalDNA are suppressive. Whether suppressiveness is at the level of mitochondrial division or at DNA level is not known. The insertion of kalDNA into mitochondrial chromosomes could result in "renegade" multiplication of mutant mitochondria during growth displacing normal mitochondria. The second possible way of displacement is that mtDNA molecules carrying mtIS-kalDNA may exhibit a more efficient rate of replication compared with normal mtDNA molecules. This hypothesis could depend on the presence of additional mitochondrial origin of replication sequences which could be associated with mtIS-kalDNA. The third is that the displacement of normal mtDNA molecules may involve unidirectional gene conversion. Manella and Lambowitz (1979) demonstrated that two sequences of mtDNA of N. crassa could spread through the mtDNA population in heteroplasmons by high frequency unidirectional gene conversion. Mitochondrial intron omega of some cultures of S. cerevisiae also can spread through the

mtDNA population (Jacquier and Dujon, 1985). The fourth possibility is that kalDNA continuously integrates into mitochondrial genomes and the insertion becomes faster in aged subcultures.

An 8.6 kb linear autonomously replicating DNA plasmid was found to be the progenitor of the mtDNA foreign insertion sequence in senescent strains. The plasmid is structurally identical to the mtIS-kalDNA and was found to be present in high copy number in the cytoplasm of senescent and presenescent strains. The free form of the plasmid has been designated mtAR-kalDNA. It was suggested that the plasmid was responsible for senescence because it was a mutator of mitochondrial genes, creating suppressive mtDNAs, and causing the death of affected strains. The plasmid was initially thought to be nuclear (Bertrand et al, 1986), but now has been demonstrated to be mitochondrial (Myers et al, 1989). A restriction map of the plasmid is presented in Figure 2.

Studies on the transmission of kalilo during sexual crosses have shown that when a presenescent culture is used as a female parent in a cross, the only form of kalilo which is found to be transmitted is mtAR-kalDNA; mtIS-kalDNA is never seen in the progeny. During somatic propagation of these cultures, inserts of kalilo arise, and generally persist until death. However, inserts are seen that are not lethal, and instead seem to be replaced by novel inserts that persist until the death of the organism. It has been suggested that inserts arise from de novo integration of mtAR-kalDNA into the mitochondrial chromosome and the novel inserts arise either from rearrangements of preceding inserts, from novel insertion events, or from the transposition of mtIS-kalDNA (Myers et al, 1989).

The kalilo plasmid has now been completely sequenced. It is 8632 bp in length, with perfect inverted repeats of 1361 bp. Using the mitochondrial genetic code, kalilo is found to encode two large ORFs, running in opposite orientations, of 893 and 811 aa. The large ORF shows critical homologies to the putative DNA polymerases of bacteriophage 29, the S1 plasmid of maize, and certain other viral DNA polymerase. The small ORF encodes a putative RNA polymerase which shows homology to the T3 and T7 RNA polymerase, and to the putative RNA polymerase of the S2 plasmid of maize (Chan et al, 1991). The kalilo plasmid has covalently linked 5' terminal proteins (Vierula et al, 1990). Structural and genetic similarities among linear plasmids from fungi and plants suggest that they all might be distantly related to each other and to bacterial viruses.

The insertional behavior of kalilo has been studied, and it has yielded some interesting observations. All insertions of kalilo which have been studied show a pentanucleotide match somewhere within the last approximately 20 bp of kalilo and mtDNA. Integration of the plasmid is via an unusual mechanism which creates long inverted repeats in the mtDNA flanking mtIS-kalDNA (Figure 3). The mechanism is not known, but possibilities have been hypothesized. Both ends of the plasmid might form an association with short segments of the mitochondrial chromosome. This association would involve homologous pairing between nucleotide sequences that are at least 5 bp long, generating novel origins of replication within mtDNA. Two double stranded copies of mtDNA that are generated by unscheduled replication at the point of plasmid/mtDNA association are in some manner joined to the ends of the kalilo plasmid by a process that recognizes the point of synaptonemal-like pairing between the ends of the plasmid and mtDNA (Chan et al, 1991). A summary of kalilo insertion sites is shown in Figure 4.

The kalilo plasmid was found to give rise to multiple transcripts of 8.6, 4.4, 4.0, 1.3, 1.2 and 0.9 kb. These transcripts are all transcribed from a single, unique promoter located near the ends of the plasmid. The transcripts are not processed, but instead utilize optional transcriptional stop sites. Kalilo-specific RNA appears to be selectively unstable, and this may be a general property of linear plasmid DNA (Vickery, 1990).

The kalilo plasmid has been transferred from N. intermedia to N. crassa by mixed growth and mtIS-kalDNAs have been found in the mitochondria of N. crassa (Griffiths et al, 1990). A cryptic mitochondrial plasmid, not associated with senesence, has been transferred with kalilo to N. crassa. The two killer plasmids with similar structures, pGKL1 and pGKL2, are located and maintained in the cytoplasm outside the mitochondria in Klyveromyces lactis (Stam et al, 1986). They have been transferred to other genera (Gunge and Sakaguchi, 1981; Gunge et al, 1982; Sugisaki et al, 1985). Several other mitochondrial circular plasmids of Neurospora have been transferred to new mitochondria through "forced heterokaryon" formation (Collins, 1990) or more surprisingly through the male parent during sexual crosses (May and Taylor, 1989). It seems that the kalilo plasmid and other plasmids not only reside in mitochondria, but they also can penetrate the mitochondrial membrane and enter other mitochondria.

Studies of several senescent field-collected strains of N. crassa revealed that the genetic determinant for the predisposition to senesce is a 7 kb linear mitochondrial DNA plasmid, maranhar. The plasmid has inverted terminal repeats with proteins covalently bound at the 5' termini. The maranhar plasmid causes senescence by insertional mutagenesis of the mtDNA, creating molecules which are suppressive over wild type mtDNAs and lead to the death of the organism. The insertion of maranhar generates flanking inverted repeats of

mtDNA too. Kalilo and maranhar show no homology at the nucleotide level (Court et al, 1991), and they are capable of coexisting in a common cytoplasm of N. crassa (Griffiths et al, 1990). Thus, maranhar is functionally identical to kalilo.

A 4. OBJECTIVES OF THIS STUDIES

It has been observed that some Neurospora strains containing kalilo do not manifest senescence within the limits of the experiments (Griffiths and Bertrand, 1984; Myers, 1988; Griffiths, personal communication). It is of interest to understand the mechanism of suppression of kalilo-related senescence. The kalilo plasmid is structurally similar to other linear elements, but how it functions is not clear. The sequence of the plasmid is known, but only the two large ORFs, an apparent RNA polymerase and an DNA polymerse are found. These two proteins may be needed for plasmid maintenance, but as a number of other biological functions also may be required. Although eight species of kalilo-specific transcripts have been detected, and two of them are thought to correspond to the two large ORFs, functions of these transcripts are still unknown. Since the plasmid is small, and the number of possible functions encoded by the plasmid is small, it is reasonable to believe that the plasmid almost certainly relies upon the host genome for essential functions concerning its replication, integration into mitochondrial chromosome and expression of senescence. There could be nuclear genes which stop the replication of the plasmid resulting in elimination of the plasmid from the mitochondria. There could be nuclear genes that inhibit the replication of the plasmid from a high level to a very low level; the plasmid could still insert into mitochondrial genome, but not interfere the normal function of the mitochondria. There could be nuclear genes which permit toleration of high-copy levels of the plasmid, but prevent the plasmid from integrating into the mitochondrial

genome, resulting in the plasmid being a neutral parasite. There could be nuclear genes which tolerate high-copy level of both the free form and the inserted form of the plasmid, but prevent the deleterious function of IS-kalDNA from expression. The effects of all these kinds of nuclear genes will prevent plasmid expression, resulting in suppression of kalilo-related senescence in Neurospora.

It is of interest to determine the relationship between AR-kalDNA and IS-kalDNA during the senescence process in Neurospora. It has been observed that the aberrant mtDNA with IS-kalDNA accumulates at the late stages of the kalilo senescence in N. intermedia (Bertrand et al, 1985, Bertrand, 1986). Myers et al (1989) reported that IS-kalDNA increases whereas AR-kalDNA decreases as the kalilo strains proceed to senesce. The phenomenon of suppressive accumulation of aberrant mtDNAs has been observed in many experimental systems such as yeast petites (Faye et al, 1973; Locker et al, 1974), Neurospora (Bertrand et al, 1980; DeVries et at, 1981), and Aspergillus (Lazarus et al,1980; Lazarus and Kuntzel, 1981). It was hypothesized that the mitochondria with IS-kalDNA insertion show some kind of renegade multiplication, resulting in displacing normal mitochondria; the aberrant mitochondrial chromosome with IS-kalDNA somehow replicates faster than the normal mitochondrial chromosome and eventually displace the normal ones. Alternatively, the accumulation of IS-kalDNA might depend on the faster, continuous integration of higher level of the plasmid. Since mitochondrial chromosomes with IS-kalDNA could replicate the same as normal mitochondrial chromosomes, or replicate slower than the normal ones, experiments were designed to investigate the kinetics of both AR-kalDNA and IS-kalDNA in serial senescent Neurospora cultures. The replication characteristics of IS-kalDNA have been investigated in different nuclear backgrounds.

Figure 1. Life cycle of Neurospora showing both the sexual and asexual cycle. From Fincham et al, (1979).

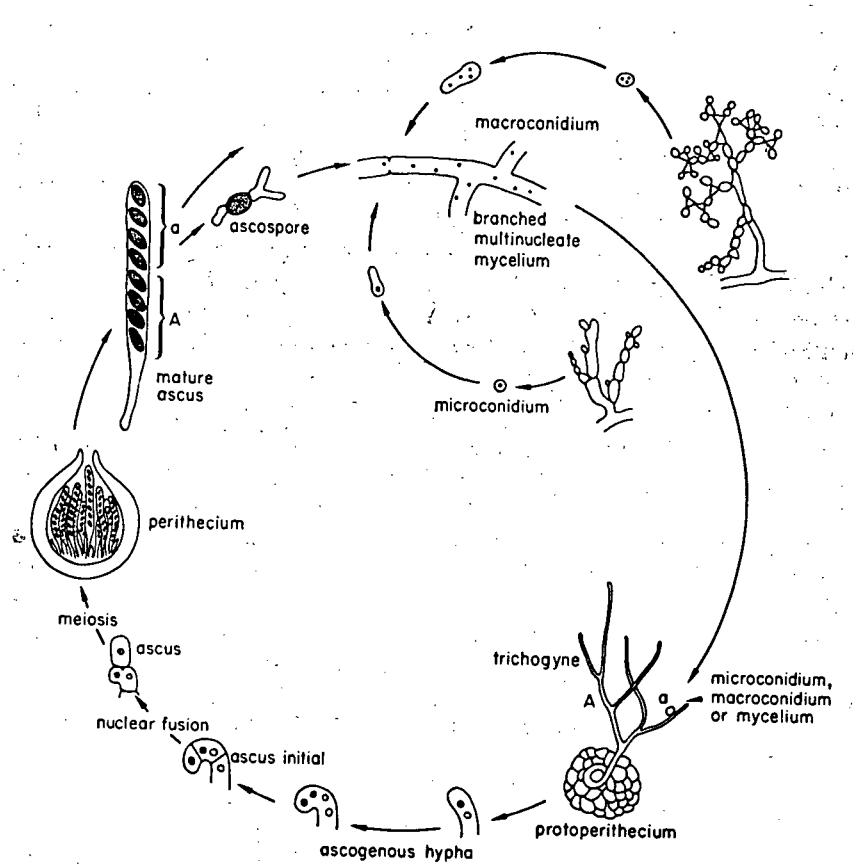

macroconidium
branched
multinucleate
mycelium
a
ascospore
A
mature
ascus
microconidium
perithecium
meiosis
ascus
nuclear fusion
ascus initial
ascogenous hypha
protoperithecium
trichogyne
A
a
microconidium,
macroconidium
or mycelium

Figure 2. Restriction map of kalilo DNA . The fragments created from the digestion of linear kalilo DNA with the enzymes Xba I, Eco RI, BgI II, Hind III and Pst I are shown. The fragments are named and are referred to in the text . The 1361 bp terminal repeats of the element are shown by the inverted arrowhead. Kalilo is 8632 bp long. The locations of the two ORFs are shown by dashed squares.

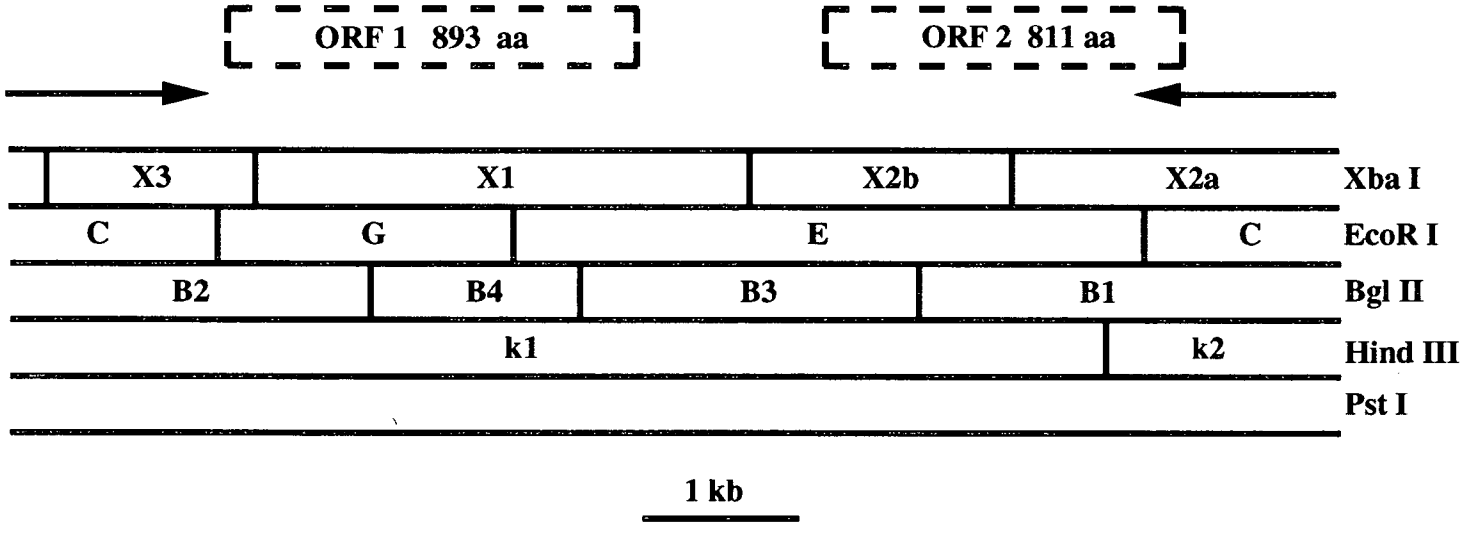
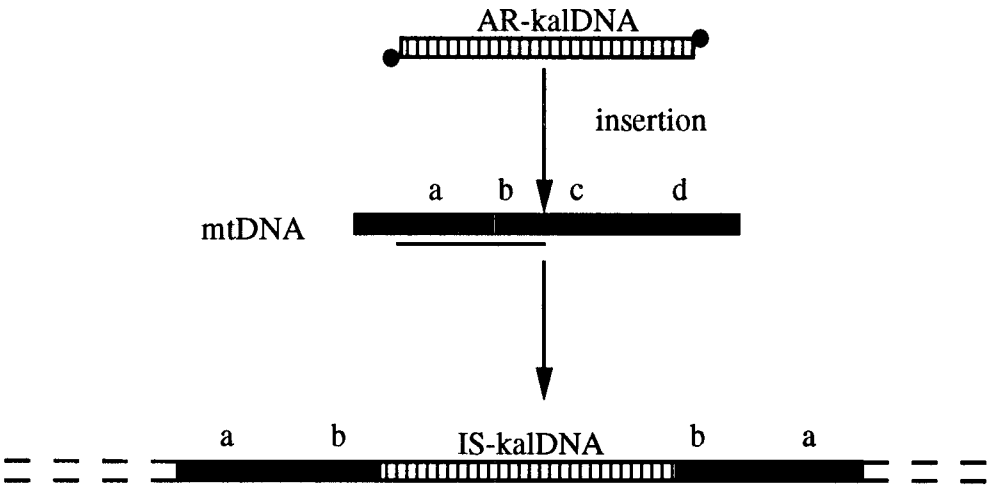
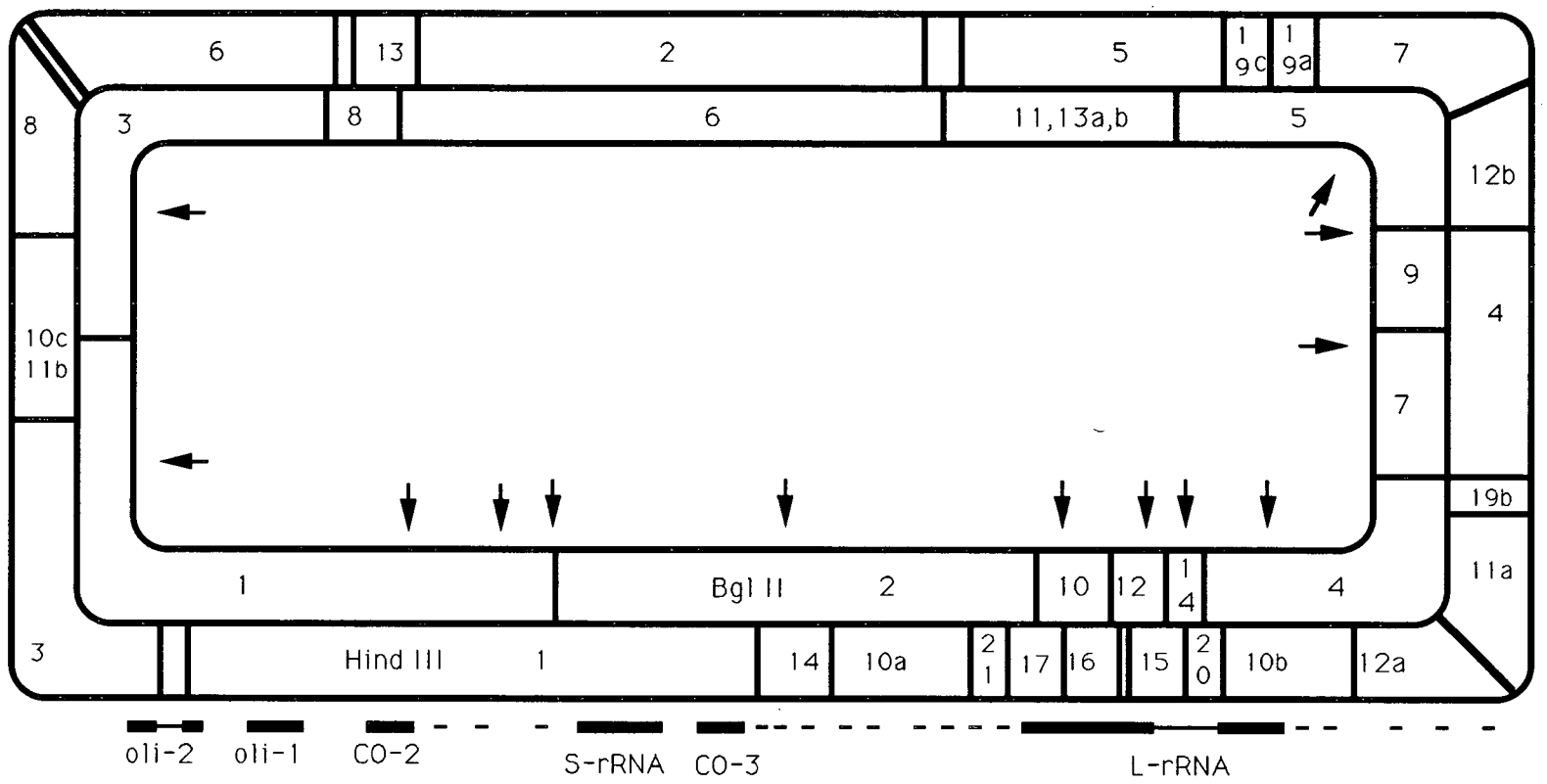

ORF 1 893 aa
ORF 2 811 aa
X3
X1
X2b
X2a
Xba I
C
G
E
C
EcoR I
B2
B4
B3
B1
Bgl II
k1
k2
Hind III
Pst I
1 kb

Figure 3. Formation of IS-kalDNA. The kalilo plasmid is represented as a squared line. The solid line represents part of mtDNA. It is shown inserting into the mtDNA at a point between markers b and c. After insertion, the mtDNA is represented as a long inverted repeat (this part mtDNA is underlined), and mtAR-kalDNA has become mtIS-kalDNA, the mitochondrial insertion sequence. The length of the these structures and the ultimate structure of the senescent mtDNA molecules are not known, and have been represented by the dashed lines.



IS-kalDNA with flanking inverted repeats of mtDNA

Figure 4. Previously identified insertion sites of mtIS-kalDNA. A restriction map of the mtDNA of Neurospora intermedia showing the sites of insertion of kalDNA in different strains, designated by the arrowheads. An Hind III and Bgl II map is shown. The positions of some of the mitochondrial genes are presented. The filled-in boxes represent the exon regions of each gene and the lines represent intron sequences. The mitochondrial tRNAs are indicated by dots. Sites marked with arrowheads have been identified by Bertrand (1986), Myers (1988), and Vickery (1990).


6
13
2
5
19c
19a
7
8
3
8
6
11,13a,b
5
12b
9
4
10c
11b
7
19b
11a
1
Bgl II
2
10
12
14
4
3
Hind III
1
14
10a
21
17
16
15
20
10b
12a
oli-2
oli-1
CO-2
S-rRNA
CO-3
L-rRNA

MATERIALS AND METHODS

B 1. STRAINS

Neurospora intermedia strains P561 and P605 are natural isolates collected from Kauai, Hawaii. Strain 1766 was isolated from Taiwan. All other strains were obtained from the Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical School, Kansas City Kansas. Ascospore derivatives C4, C5, C13 and C16 were from the cross P561 x 1766 described by Griffiths and Bertrand (1984) and Myers (1988).

Neurospora crassa Strain HB9006 is kalilo derivative of N. crassa, synthesized by transferring the kalilo plasmid into a a, nic-1, al-2 genotype. Laboratory strain 12-21-428 (428) is marked with A, ad-3A, al-2, pan-2 and cot, and 83 is marked with A, un-3, ad-3A and nic-2. 74-OR23-1 A (Oak Ridge) is wild type, and obtained from the Fungal Genetics Stock Center.

B 2. MEDIA AND GROWTH CONDITIONS

Vegetative culturing was performed exclusively on Vogel's minimal medium containing 2% glucose (Vogel, 1956). Serial subcultures were made in 10 x 75 mm tubes. Each series was subcultured by mass conidial transfer twice a week. For the growth of mycelium for nucleic acid isolation, liquid Vogel's medium was inoculated with approximately 10^6 conidia/ml and shaken at 200 rpm for a minimum of 16 hours. Crosses were performed on solidified Westergaard's crossing medium as described by Davis and deSerres (1970). Cross designations are such that the strain used as the female parent is written first and

followed by the strain used as the conidial parent. Mating type determination of ascospores was performed on crossing medium. Vegetative cultures and crosses were incubated at 25°C.

Subculturing was performed as described by Griffiths and Bertrand (1984). In subculture series derived from nature isolates, the original culture is numbered zero, for example P561-0. The same applies for ascospore series except that the ascospore isolation number is used in place of strain designation, for example 15-1-0. Serial subcultures were then numbered -1, -2, -3.....-n, for example, BC3-3-1, -2, -3 or 15-1-1, 15-1-2, ...15-1-20.

Conidial isolation for inoculation of liquid Vogel's was prepared by pouring conidial suspension through layers of cheese cloth. The appropriate volume of this suspension was then added to liquid Vogel's medium. Cultures were harvested by suction filtration through Whatman #1 filters in Buchner funnels.

Random ascospore isolation was performed by pouring ascospore suspensions of 30-40 ascospores/ml mixed with 2 ml of 1% agar on plating medium, subjecting to heat shock at 60°C for 0.5-1.0 hour, and incubating for three days. The germinating individual ascospores were isolated and subcultured.

Unordered asci were collected using the procedure of Newcombe and Griffiths (1972) slightly modified for use with crosses on solid medium (Myers, 1988).

All other procedures were standard for Neurospora and are described by Davis and deSerres (1970).

B 3. MITOCHONDRIAL DNA ISOLATION

Mitochondrial DNA was isolated according to the small scale mtDNA isolation of Myers (1988), with the addition of a proteinase K digestion prior to phenol/chloroform precipitation of protein, unless otherwise stated in the text. All procedures were carried out at 0-4°C, unless otherwise noted. 200 ml of liquid culture was harvested and stored on ice until needed. The mycelial pellet was ground with a half volume of acid-washed sand and suspended in 25 ml DNA isolation buffer (44 mM sucrose, 50 mM Tris-HCl, pH 7.6, 1 mM EDTA). The suspension was centrifuged at 2 krpm for 5 minutes in an SS-34 rotor. The supernatant was then centrifuged for 15 minutes at 15,000 rpm in an SS-34 rotor to pellet the mitochondria. The mitochondrial pellet was suspended in 3 ml 70% sucrose in T10E1 (10 mM Tris-HCl, pH 7.6, 1 mM EDTA), and layered with 2 ml 44% sucrose in T10E1. The flotation gradients were centrifuged at 45 krpm for 2 hours in an SW50.1 rotor. Mitochondria were collected from the interface beneath the 44% sucrose step gradient layer, and diluted to 3 ml with 2 ml T200E1 (200 mM Tris-HCl, pH 7.6, 1 mM EDTA) in microfuge tubes. The tubes were centrifuged to pellet the mitochondria. The mitochondrial pellets were pooled and resuspended in 500 ul of a solution of T200E1, 40 ul of 20% SDS was added to lyse the mitochondria, followed by the addition of 20 ul of a 10 mg/ml solution of proteinase K (Bethesda Research Laboratories). The mitochondrial DNA was incubated at 37°C overnight. Protein was extracted by the addition of a half volume of Tris-HCl saturated phenol, and a half volume of chloroform/isomyl alcohal (24:1). Tubes were mixed by inversion and centrifuged for 15 minutes at 10,000 rpm.

The aqueous phase was collected and this step was repeated once. Nucleic acids were precipitated from the aqueous phase by the addition of 2.5 volumes of ethanol containing 200 mM ammonium acetate, and incubated overnight at -20°C. The nucleic acids were pelleted and washed with 70% ethanol. The mtDNA was resuspended in 100-150 ul T10E1, with the addition of 1 ul of 20 mg/ml RNase A (SIGMA). RNA was digested at 55°C for an hour. DNA concentration was determined by UV absorption. Typically, the yield was 10-20 ug of mtDNA per 200 ml of liquid culture.

B 4. ENZYME DIGESTION AND GEL ELECTROPHORESIS

Restriction enzyme (Bethesda Research Laboratories, BRL) digestion of mtDNA was standard as described by Bethesda Research Laboratories. Digestion of 1 ug mtDNA was carried out for an hour at 37°C. Digestion of 1 ug mtDNA with Lambda exonuclease (BRL) was incubated for an hour at 37°C in a solution of 67 mM glycine KOH, ph 9.4, 2.5 mM $MgCl_2$, 50 ug/ml BSA and 10 units of Lambda exonuclease. Digestion of 1 ug mtDNA with exonuclease III (BRL) was incubated for an hour at 37°C in solution of 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM DTT, 50 ug/ml BSA and 50 units of exonuclease III.

3 ul of 5x loading buffer (5% SDS, 50% glycerol, 0.025% bromphenol blue) was added to the digestion reaction. Samples were loaded into wells of 0.8% agarose gels and separated by size at 40 volts for 16 hours. The buffer for gel electrophoresis was 1x TAE (40 mM Tris acetate, pH 7.6, 2 mM EDTA). Gels were stained with ethidium bromide and photographed (Polaroid 57) under short wave UV illumination.

B 5. LABELLING OF NUCLEIC ACID

Oligolabelling was carried out according to the protocol from D.J. Vickery. Reactions were to a final volume of 25 ul. 1.5 ul of random primers [Pharmacia poly $(dNTP)_6$, 90 OD units/ml 1 mM Tris-HCl pH 7.5, 1 mM EDTA] was mixed with 50-500 ng probe DNA to a final volume of 14.0 ul. This was incubated at 100°C for 5 minutes and then chilled on ice for 10 minutes. 2.5 ul of 0.5 mM dNTPs (dATP,dTTP,dDTP), 2.5 ul 10x Klenow fragment buffer, 5 ul 3000 Ci/mmol dCTP (50 uCi). 1 ul of Klenow fragment (5 units) were added and incubated at room temperature for minimun of two hours. The reaction were stopped by adding 1 ul of 0.5 M EDTA, 3 ul of 10 mg/ml tRNA and 100 ul TE buffer. Unincorporated nucleotides were removed by Sephadex G-50 (Pharmacia) spin column chromatography.

B 6. PROBES

Cloned kalDNA B3, B4, X3 and X2b in pUC18 were generously given by D.J. Vickery. Other fragments of kalDNA used as probes were isolated from 0.8% low melting agarose gels after electrophoresis separation.

B 7. BLOT HYBRIDIZATION

B 7.1. Southern Blot Analysis

Southern blot analysis was performed essentially as described by Southern (1975). DNA separated by gel electrophoresis was depurinated for 5 minutes in 0.25 M HCl, denatured

for 30-45 minutes in alkaline solution of 0.5 N NaOH, 1.5 M NaCl and then neutralized for 45 minutes in 1 M Tris-HCl, 3.0 M NaCl. DNA was transferred to Hybond filter with 2x SSC (1x = 0.15 M NaCl, 10 mM sodium citrate, pH 7.0) for 24 hours. After transfer, the filters were baked at 80°C for 2-3 hours.

DNA fragments were detected by hybridization to 32P labelled probes. Filters were prehybridized for 24 hours at 42°C with 40% deionized formamide, 1% SDS, 1x Denhardt's solution (100x = 2% BSA, 2% PVP, 2% ficoll), 1 M NaCl, and 0.4 mg/ml denatured herring sperm DNA (Bertrand, 1985). Hybridizations were carried out in the same buffer with the addition of labelled probe to 10^6 cpm/ml. Hybridization was for 24 hours at 42°C. After hybridization, filters were washed in 2x SSC at room temperature for 5 minutes, twice in 2x SSC, 1% SDS at 65°C for half an hour. After air drying, blots were wrapped in Saran Wrap and exposed to Kodak X-Omat RP film for the appropriate time.

B 7.2. Colony hybridization

About 10^4 conidia were suspended in 0.5 ml distilled water. The conidia were transferred to hybond filter (Bethesda Research Laboratories) through dot blot apparatus (BioRab). The filter with conidia was saturated with alkaline solution of 0.5 N NaOH and 1.5 M NaCl for 30 minutes at room temperature to lyse their cell membrane and denature DNA, and then neutralized for 30 minutes in solution of 1M Tris-HCl, ph 8.0 and 3.0 NaCl. After air drying, the filter was baked at 80°C for two hours. The following procedure was the same as the southern blot hybridization.

B 8. MEAUREMENT OF DNA COPY NUMBER

Transparent, positive films were made by taking pictures (Polaroid 55) of the photographs of ethidium bromide stained gels. Both transparent, positive films and autoradiographs were scanned with white light through a densitometer (Helena Laboratories) at 40 V of detector voltage and 4 mm per minute of scan speed. The relative quantity of each interested DNA fragment was calibrated by signal area given by the fragment. The relative copy number of fragments was calculated by dividing the signal squares by molecular weight of the fragment. The copy number ratio of fragments were calculated by dividing relative copy number over those of other fragments.

CHAPTER ONE

SUPPRESSION OF kalDNA-RELATED SENESCENCE IN NEUROSPORA

INTRODUCTION

This chapter describes experiments investigating the function of host nuclear genes that affect the function of the kalilo plasmid, and mechanisms by which the kalDNA-related senescence is suppressed. Since the plasmid is small, 8.6 kb, and has only two major ORFs, it almost certainly relies upon the host genome for essential functions concerning replication, insertion and expression. It is fundamental to understand such nuclear function for further insights into the senescence process.

In early reports, there were two groups of sexual progeny from crosses using senescent Kauaian strains as female parents. The first group, the majority, senesced within 20 subcultures. The second group grew normally until the 20th subculture, showing no signs of senescence. Some of the non-senescent ascospores were subcultured further. Some of them manifested vegetative death in further subculturing, some still grew normally. From unpublished results (Griffiths, personal communication), it was known that there were some tetrads from some senescent crosses in N. intermedia and N. crassa showing one to one segregation of vegetative death and immortal growth. But at that stage of understanding, the location of the kalilo plasmid and its structure were still not clear. Primary studies of the relationship between senescence and the plasmid in those tetrads did not provide enough information to interpret this phenomenon. Those published and

unpublished data suggested that there could be nuclear genes which suppress the kalDNA associated senescence in Neurospora.

Based on this information, mitochondrial DNA analyses were conducted for some of the non-senescent ascospores and tetrads. Backcross and test crosses were made and mtDNA analysis done on their sexual progeny. These studies revealed that there are three kinds of mechanisms of suppressing the senescence in Neurospora.

RESULTS

Kalilo plasmids have been transferred from N. intermedia to N. crassa (Griffiths et al, 1990). The strains of N. crassa containing the kalilo plasmids are senescent. The kalilo plasmids show similar behavior by inserting into N. crassa host mitochondrial genomes. In studies to investigate the behavior of the kalDNA in the new environment of N. crassa, the kalilo strain HB9006 was crossed as female to three laboratory strains 74-OR23-1A (Oak Ridge), 12-21-428 (A ad-3A al-2 pan-2 cot) and 83 (A un-3 ad-3A nic-2). A total of 16 unordered asci was isolated and subcultured (Griffiths, unpublished data).

From the cross HB9006-4 x Oak Ridge, ascospores of ten asci were isolated and subcultured for 20 transfers (Figure 5). All ascospores from asci 5, 6, 7, 8, and 10 showed senescence within several subcultures. All eight ascospores from asci 2, 4, and 9 showed no senescence within 20 subcultures. Two ascospores of ascus 3 and three ascospores of ascus 1 showed normal growth in 20 subcultures, the remaining ascospores showed senescence. Therefore there are three kinds of asci: the first , whose all ascospores

senesce, second those whose all ascospores do not senesce and grow normally to the 20th subculture, and third those in which some ascospores grow normally and some senesce.

From the cross HB9006-4 x 428, three asci, (11, 12, and 13,) were studied by subculturing. Only seven ascospores from each ascus germinated. All ascospores from ascus 12 showed senescence, 6 ascospores from ascus 11 senesced but one ascospore grew normally. Four of ascospores from ascus 13 showed senescence, and three of them did not senesce (Figure 6).

From the cross HB9006-4 x 83, ascospores of three asci, (14, 15, and 16) were isolated and subcultured. Four out of eight ascospores from asci 14 and 15 senesced, the remainder grew normally. All ascospores from ascus 16 showed normal growth (Figure 7).

The progeny of the crosses have different fates, both senescence and immortal growth. What happened to the asci and the ascospores which escaped the fate of death? Are there kalilo plasmids in their mitochondria? How do the plasmids behave, do they insert into mitochondrial genomes or simply replicate themselves as a neutral parasite? To investigate these questions, mtDNA analyses were conducted.

1.1 MtDNA ANALYSES OF THE ORIGINAL CROSSES

1.1.1 Tetrads From The Cross HB9006-4 x Oak Ridge

MtDNAs were prepared from early subcultures and late subcultures of the ascospores from asci 1, 2, 3, 4, and 9 (refer back to Figure 5). Uncut mtDNAs were separated and probed with the radioactively labelled kalDNA fragment B3. The EtBr-stained gel and the autoradiograph indicate that in ascus 1, ascospores 1-1, -5 and -7 do not contain kalDNA in either early or late subcultures. Ascospores 1-2, -3, -4, -6 and -8 contain the plasmids in their early subcultures, and both AR-kalDNA and IS-kalDNAs in their late subcultures. In ascus 3, ascospores 3-3 and -6 do not contain kalDNA in either their early or late subcultures, ascospores 3-1, -2, -4, -5, -7 and -8 contain the kalilo plasmids in their early subcultures, and both AR-kalDNA and IS-kalDNAs in their late subcultures. In asci 2, 4 and 9, all eight ascospores do not contain the kalDNA either in their early or late subcultures (Figure 8).

Asci 2, 4 and 9 from the cross show no kalilo DNA in their mitochondria. Their ascospores grew normally, showing no signs of senescence. In asci 1 and 3, the majority of their ascospores contained the kalilo plasmid in their early subcultures, and both forms of kalDNA in their late subcultures, two or three ascospores somehow did not contain the plasmid in their early subcultures. The ascospores with the plasmids in their mitochondria manifested vegetative death, and the other ascospores without the plasmid grew normally. Thus there is a strong co-relationship of senescence and the insertion activities of the plasmid. These data support the early conclusion that insertion of the plasmids into the mitochondrial genomes causes the death of Neurospora.

There is a high frequency of individual asci and ascospores lacking the plasmid, resulting in escaping from the senescent fate. All asci shared the same cytoplasm of their female parent HB9006, but some asci contained the plasmid in their cytoplasms, some did not.

Asci 1 and 3 inherited female parental cytoplasm with the plasmid; their ascospores showed irregular 3:5 or 2:6 segregation of the plasmid. What mechanism produced such phenomenon, nuclear genes, cytoplasmic segregation or mutations? Several possibilities can be considered.

1. There could be several nuclear genes, clearly segregating, which determine or influence the replication or existence of the plasmid. The recombination of these genes from both parents in ascospores could result in regular behavior of the plasmid, or in maintenance of the plasmid at a very low undetectable level, or elimination of the plasmid from cytoplasm.

2. Alternatively there could be two kinds of mitochondria in the female cytoplasm. Some mitochondria would contain the kalilo plasmid within them, some not. When perithecia are formed during crossing, some perithecia inherit their female parental cytoplasm without the plasmid in their mitochondria, resulting in all ascospores lacking the plasmid. Some perithecia inherit their female cytoplasm with the plasmid in their mitochondria, so their ascospores inherit the plasmid in their mitochondria. The mitochondria in some perithecia could be mixed, some ascospores inheriting mitochondria without the plasmid, some with the plasmid, resulting in irregular segregation like asci 1 and 3.

3. Mutations could occur in some genes in the nuclear or mitochondrial genomes. The products of these genes could be involved in replication and expression of the plasmid. The original alleles of these genes from HB9006 allow the plasmid to replicate and express, but mutated alleles would not. Such frequent mutations in genomes could result in the irregular segregation of ascospores with or without the plasmid. But it is difficult to imagine this kind of highly frequent mutations. These questions are investigated later.

1.1.2 Tetrads From The Cross HB9006-4 x 428

MtDNA analyses were performed to investigate the senescence mechanism in two asci 11, and 13, from this cross (refer to Figure 6). MtDNAs were prepared from early and late subcultures of the seven ascospores from ascus 11. The EtBr-stained gel and the autoradiograph show that ascospore 11-2 does not contain the kalDNA in its early or late subculture. Ascospores 11-1, -3, -4, -5, -6 and -7 contain the plasmid in their early subcultures, and both AR-kalDNA and IS-kalDNA in their late subcultures. This situation of segregation and activities of the plasmid are like those of asci 1 and 3 from the cross HB9006-4 x Oak Ridge.

MtDNAs were prepared from early and late subcultures from the seven ascospores of ascus 13. The EtBr-stained gel of uncut mtDNAs and the autoradiograph (Figure 9) show that there is a small band, smaller than mitochondrial genomes in all early and late subcultures. This DNA band hybridized with the kalDNA fragment B3 probe, indicating that this band is AR-kaDNA. The uncut mitochondrial DNA hybridized with the probe too in their early and late subcultures of all ascospores, indicating that kalDNA is inserted into the mitochondrial genomes. This ascus presents a new situation. Ascospores 13-1, -2, -3 and -4 contain both AR-kalDNA and IS-kalDNA in their early and late subcultures. These four ascospores showed vegetative death, just as other reported senescent strains did. But other ascospores 13-5, -6 and -7 contain both AR-kalDNA and IS-kalDNA in the first and the 20th subculture. These three ascospores did not senesce, growing normally until the 20th subculture. Did these three ascospores have not enough time to manifest death or were there factors to suppress their senescence?

To investigate this question, the late ascospore subcultures 13-5-20, 13-6-20 and 13-7-20 were subjected to further subculturing. They grew normally to the 38th subculture where subculturing was terminated. MtDNAs were prepared from the 38th subcultures of these ascospores. The mtDNAs from the first and the 38th subculture were digested with restriction enzyme Pst I. Because the kalilo plasmid does not have a Pst I target site, AR-kalDNA maintains its original structure after Pst I digestion. IS-kalDNAs contain fragments of mitochondrial genomes, are larger than AR-kalDNA, and move slower than AR-kalDNA when mtDNA fragments are separated by electrophoresis. The EtBr-stained gel and the autoradiograph (Figure 10) show that the control BC 4-1-20, which inherits HB9006 cytoplasm but lacks of detectable kalDNA, its mtDNA does not contain AR-kalDNA or IS-kalDNA, its mtDNA profile does not have any fragment larger than AR-kalDNA (8.6 kb). In ascospores 13-5, -6 and -7, there are several large fragments, which are not observed in the control. The brightest fragment (8.6 kb) of the larger fragments is AR-kalDNA, which is found in the early and the late subcultures of these ascospores. There are several large fragments, larger than AR-kalDNA, in the early and the late subcultures of these ascospores. They are different species of IS-kalDNAs, because they are not observed in the control, are larger than AR-kalDNA (8.6 kb) and hybridize with the kalDNA probe. These mtDNAs lose the same fragments, Pst I-6 and -7 of mitochondrial genomic DNA, and gain the apparently identical larger fragments L-3 and L-4. The comparison indicates that the IS-kalDNA fragments with the same molecular weights found in these ascospores are inserts at the same sites in the mitochondrial genomes (Pst I-6 and -7) or, less possible, different inserts but with close molecular weights. The three ascospores in the first subcultures contain five or six identical species of IS-kalDNAs. Ascospore 13-5 maintains AR-kalDNA and the original IS-kalDNAs during subculturing.

Ascospore 13-6 maintains only AR-kalDNA during further subculturing, but somehow loses the IS-kalDNAs or decreases to an undetectable level in the 38th subculture. Ascospore 13-7 maintains both AR-kalDNA and IS-kalDNAs during subculturing, but AR-kalDNA decreases significantly.

Four out of seven germinating ascospores from ascus 13 contained AR-kalDNA and IS-kalDNAs in their early and late subcultures, and senesced. Three other ascospores contained both AR-kalDNA and IS-kalDNAs in their early subcultures, and AR-kalDNA in late subcultures. Two of the three still contained IS-kalDNAs, but one of them lost IS-kalDNA in the 38th subculture. Unfortunately, the eighth ascospore with the same nuclear genome of ascospore 13-5 (based on their nuclear markers) did not germinate. From these results of the mtDNA analyses of ascus 13, it seems that there is a nuclear gene, which suppresses the deleterious function of IS-kalDNAs. Namely, under this nuclear background the kalilo plasmid can replicate autonomously and insert into mitochondrial genomes generating IS-kalDNAs, but IS-kalDNAs do not harm the mitochondrial function. The suppressive allele of this gene could be derived from genetic heterogeneity, or from new mutation in the parent, because this allele has not been detected in the other two asci from the same cross.

It is best to test this suppressive allele of the gene by crossing ascospores with the allele to ascospores without the allele, for example 13-1 x 13-6. But experiments to study the nuclear gene in this ascus were terminated, because a similar gene was found in strain 2360 his of N. intermedia (Griffiths et al, 1991).

The analysis of mtDNA of ascospore 13-6, showed that mtDNA of the first subculture contained five or six detectable inserts of the kalilo plasmids, but these inserts were not detected in mtDNA of the 38th subculture. They were somehow lost during the serial subculturing. The tests of nuclear markers (refer to Figure 6) indicate that ascospore 13-6 is sister of ascospore 13-7. This result suggest that these inserts do not provide advantageous replication to the mitochondrial genomes, rather, the mitochondrial genomes with these inserts replicate slowly compared to normal mitochondrial genomes or can not replicate.

1.1.3 Tetrads From The Cross HB9006-4 X 83

Three asci (14, 15 and 16) from the cross HB9006-4 X 83 were isolated and subjected to subculturing. MtDNA analyses were conducted from all ascospores to investigate suppression of senescence and kalDNA activities.

Ascus 14

MtDNAs were prepared from the first subcultures of ascospores of ascus 14 representing the early stage, and from the 4th subcultures of ascospores 14-1, -2, -3, and -4, and from the 20th subcultures of ascospores 14-5, -6, -7, and -8 representing the late stage. MtDNAs were digested with restriction enzyme Pst I. The EtBr-stained gel (Figure 11) shows that in the first subcultures, there are one to six large fragments in ascospores 14-1, -2, -3, -4, -5, and -6, but no such large fragments in ascospores 14-7 and -8, whose mtDNA profiles are like that of control mtDNA. In the late subcultures, there are three to six large fragments in ascospores 14-1, -2, -3, -4, and -6, no such large fragment in

ascospores 14-5, -7, and -8. The autoradiograph (Figure 11) shows that these large fragments hybridized with the probe of kalDNA fragment B3. The brightest and smallest one of them is AR-kalDNA. The fragments larger than AR-kalDNA are inferred to be different species of IS-kalDNAs. Ascospores 14-1, -2, -3, and -4 maintained both AR-kalDNA and their original IS-kalDNAs during serial subculturing, and these ascospores died within ten subcultures. Ascospores 14-5 lost both AR-kalDNA and two original IS-kalDNAs in the 20th subculture. Ascospore 14-6 maintained AR-kalDNA in the 20th subculture, and two new species of IS-kalDNAs were found in the 20th subculture. Ascospores 14-7 and -8 maintained their original situation of lacking kalDNA in their 20th subcultures. Ascospores 14-1, -2, -3, -4, and -5 had two apparently identical species of IS-kalDNAs, ascospores 14-2 and -3 had additional three apparently identical species of IS-kalDNAs.

The ascospores 14-5, -6, -7, and -8 grew normally until the 20th subcultures. Three of them contained no kalDNA, but one of them,14-6, contained AR-kalDNA and two species of IS-kalDNAs which is not observed in the first subculture. This ascospore series was subjected to further subculturing to the 38th subculture. MtDNA analysis was performed. Both EtBr-stained gel and autoradiograph (not shown) indicated that there was no detectable kalDNA in the mtDNA preparation from its 38th subculture. Therefore, the ascospore loses kalDNA gradually.

Ascospores 14-1, -2, -3, and -4 have the same nuclear markers a al nic. Ascospores 14-5, -6, -7, and -8 have the same nuclear markers un A ad nic (refer to Figure 7). Phenotypically this ascus shows the exact segregation of the nuclear markers on chromosome I. Ascospores 14-1 and -4, 14-2 and -3, 14-7 and -8 have the identical

patterns of Pst I digested mtDNAs respectively. Ascospores 14-5 and -6 have the common AR-kalDNA, but not IS-kalDNA. Based on the patterns of Pst I digested mtDNAs, ascospores 14-1 and -4, 14-2 and -3, 14-7 and -8, 14-5 and -6 must be ascospore pairs.

The analyses of mtDNAs from this tetrad show several points. The ascospores containing both AR-kalDNA and IS-kalDNAs in their early and late subcultures stopped vegetative growth. The ascospores containing AR-kalDNA or and IS-kalDNAs in their early subcultures but losing kalDNA in their late subcultures grew normally. The ascospores containing no detectable kalDNA in their early and late subcultures grew normally. These results demonstrate the strong correlation between senescence and insertion activities of the plasmid.

Ascospore 14-6 contained AR-kalDNA in the first subculture, there were two new species of IS-kalDNAs in the 20th subculture, indicating that the new IS-kalDNAs were generated during subculturing or increased the amount of the original two IS-kalDNAs from an undetectable level to a detectable level. But interestingly, both AR-kalDNA and the two species of IS-kalDNAs were lost or at an undetectable level in the 38th subculture (data not shown). Ascospore 14-5 contained AR-kalDNA and two species of IS-kalDNAs in the first subculture, but these were lost or decreased to an undetectable level in the 20th subculture. These results favour the notion that new IS-kalDNAs are generated by AR-kalDNA de novo insertion into mitochondrial genomes, and it is not necessary that IS-kalDNAs give advantageous replication to mitochondrial genome containing them.

Another interesting phenomenon is observed. Half of the ascospores of this ascus contained both AR-kalDNA and IS-kalDNAs in their first and late subcultures; and they

stopped vegetative growth within ten subcultures. The other half ascospores lacked AR-kalDNA and IS-kalDNAs in their early or late subcultures, and these ascospores escaped the senescent fate. This ascus showed one to one segregation of vegetative death and immortal growth among its ascospores. This suggests the segregation of a nuclear gene with two alleles; one allows the plasmid to replicate normally and insert into mitochondrial genomes, the other allele suppresses the high-copy number of the plasmid, namely, its product inhibits the replication to a very low level or stops the replication. Also, this phenomenon could be explained by cytoplasmic segregation during or close to meiosis, but this is unprecedented and seems unlikely because of the correlation with nuclear marker segregation.

Ascus 15

MtDNAs were prepared from the first subcultures of ascospores of ascus 15 representing the early stage, the fourth subcultures of ascospores 15-1, -2, -3, -4, and the 20th subcultures of ascospores 15-5, -6, -7, and -8 representing the late stage. MtDNAs were digested with restriction enzyme Pst I. The EtBr-stained gel (Figure 12) shows that there is a clear one-to-one segregation concerning the large fragments in this ascus. In the first subcultures, ascospores 15-1, -2, -3, and -4 have the identical pattern of Pst I digested mtDNA, more than 20 fragments, and ascospores 15-5, -6, -7, and -8 have the identical pattern of Pst I digested mtDNA too, but lack the three large fragments. At the late stage, ascospores 15-1, and -4 maintain all the original Pst I fragments found in their first subcultures, and ascospores 15-2 and -3 not only maintain the original Pst I fragments, but also obtain three larger fragments (L1-L3) and one larger fragment (L3) respectively. Ascospores 15-5, -6, -7, and -8 still maintain the original pattern of Pst I digested mtDNA,

with no larger fragment. The autoradiograph of the gel shows that there is an exact co-segregation of kalDNA. The most abundant and smallest fragment of the large fragments is AR-kalDNA. Other large fragments, larger than AR-kalDNA, are different species of IS-kalDNAs. In the first subcultures ascospores 15-1, -2, -3, and -4 contain both AR-kalDNA and two species of IS-kalDNAs, but ascospores 15-5, -6, -7, and -8 contain only a very low level of AR-kalDNA, which was observed in the original autoradiograph after longer exposure. At the late stage, ascospores 15-1, -2, -3, and -4 contain both AR-kalDNA and IS-kalDNAs, but ascospores 15-5, -6, -7, and -8 do not contain any detectable kalDNA even after longer exposure. Half of the ascospores, 15-1, -2, -3, and -4, containing AR-kalDNA and IS-kalDNAs at the early and the late stages, proceeded to senesce. The other half ascospores, 15-5, -6, -7, and -8, which contained AR-kalDNA in their early subcultures but lost AR-kalDNA in the late subcultures, escaped the fate of vegetative death. Again, this ascus demonstrated one to one segregation of vegetative death and immortal growth. The pattern of segregation suggests that there could be a nuclear gene which determines the fate of the plasmid. This gene has two alleles, the non-suppressive one from HB9006 and the suppressive one from 83. In the ascospores that inherited the non-suppressive allele, the kalilo plasmids could replicate autonomously to high copy number and insert into mitochondrial genomes. The other half ascospores inherited the suppressive allele, which inhibits the replication of the plasmid from a high copy number to an undetectable level or stops the replication. So that there is no or a low-copy number of the plasmids in mitochondria, and the plasmids could not destroy the mitochondrial function by inserting into mitochondrial genomes.

The two large fragments, IS-kalDNAs labelled L4 and L5, are observed in ascospores 15-1, -2, -3, and -4 at both the early and the late stages. The mtDNA fragments Pst I-6 and -7

are at low levels compared with other mtDNA fragments. Apparently AR-kalDNA inserts into mtDNA fragments Pst I-6 and -7, and generates the two species of IS-kalDNAs. Therefore, the species of IS-kalDNAs with the same molecular weights are generated by AR-kalDNA inserting into the same sites of mtDNA.

Ascospore 15-2 obtains additional three species of IS-kalDNAs, L1, L2, and L3, in its late subculture, and its mtDNA fragments Pst I-10, -11, and -12 are at lower levels compared with other mtDNA fragments. Ascospore 15-3 obtains an additional species of IS-kalDNA, L3, in its late subculture, and its mtDNA fragment Pst I-11 is at lower level. These results indicate that AR-kalDNA continues inserting into mitochondrial genomes after early IS-kalDNAs have been generated.

In the original autoradiograph, there are three, much larger fragments, G1, G2, and G3, which hybridized with the kalDNA probe, in ascospores 15-1, -2, -3, and -4 at both the early and the late stages. These large fragments are observed in asci 13 and 14 too. These giant fragments which are homologous with kalDNA have not been reported before, and will be addressed in another paper.

Ascus 16

MtDNAs were prepared from the first subcultures and the 20th subcultures of all ascospores of ascus 16 from the cross HB9006-4 x 83. There was no detectable kalilo DNA in the EtBr-stained gel and there was not a positive signal to the probe of kalDNA fragment B3 in their mtDNA preparations in the autoradiograph of longer exposure (not shown data). Ascus 16 is a sib of asci 14 and 15, and shares the same parental nuclear

background. Ascospores should show one to one segregation of vegetative death and immortal growth if parents contained non-suppressive and suppressive alleles of the gene, just like the segregation patterns shown by asci 14 and 15. This phenomenon could be produced by pre-meiotic cytoplasmic segregation resulting in a lack of kalDNA in the perithecial cytoplasm, thus all progeny ascospores escape death. Alternatively the ascospores of the ascus could be products of segregation of suppressors from both parents, so the replication of kalDNA is inhibited to an undetectable level. These two possibilities are investigated by testcrossing, described in the next section.

1.2 MtDNA ANALYSES OF ASCI FROM BACKCROSS

Two asci 14 and 15 from the cross HB9006-4 x 83 showed one to one segregation of vegetative death and immortal growth, which corresponds exactly to the segregation of the kalDNA. These results indicate that there could be a nuclear gene in these ascospores which determines that fate of the plasmid in their cytoplasms, and in turn, the activities of the plasmid determine the fate of a subcultural series.

To confirm that the nuclear background influences senescence and interacts with the kalDNA to determine the fate of a subcultural series, a backcross was performed. The fourth subculture of senescent strain HB9006 was used as the female parent, the 20th subculture of an escaping ascospore, 15-6, was used as the male parent. Five unordered asci, BC1, BC2, BC3, BC4, BC5, were isolated and subcultured (Figure 13). The five tetrads showed exact four to four segregations of their nuclear markers on the chromosome I. All 40 ascospores were screened for kalDNA by colony hybridization of their first subcultures. Ascospore colonies from ascus BC1 and BC5 did not have a positive

hybridization signal to the kalDNA fragment B3. Even though the colony hybridization is not sensitive, it still provides some basic information. During the subculturing, all eight ascospores of ascus BC1 and BC5 did not show signs of senescence, and grew normally. This correlates with the negative signal of the colony hybridization to the kalDNA fragment B3 probe. Most colonies of ascospores from ascus BC2, BC3, and BC4 had positive signals to the kalDNA fragment B3. Three ascospores from ascus BC2 died within ten subcultures and the remaining five ascospores grew normally to the 20th subculture. This 3 to 5 irregular segregation could be produced by post-meiotic cytoplasmic segregation resulting in a lack of kalDNA in an ascospore. Four ascospores from asci BC3 and BC4 died within ten subcultures, and the remaining 4 ascospores grew normally to the 20th subculture. Asci BC3 and BC4 demonstrate one to one segregation. MtDNA analyses were carried out to investigate changes at the DNA level.

Ascus BC3

MtDNAs were prepared from the first subculture of ascospores of ascus BC3, from the 20th subcultures of ascospores BC3-1, -2, -3, and -4, from the eighth subculture of BC3-5, and from the sixth subcultures of BC3-6, -7, and -8. MtDNAs were digested with restriction enzyme Pst I. The EtBr-stained gel and the autoradiograph (Figure 14) show that in the first subcultures, ascospores BC3-1, -2, and -3 contain a very high copy number of AR-kalDNA, but no IS-kalDNA. Ascospore BC3-4 contains neither. Ascospores BC3-5, -6, -7, and -8 contain AR-kalDNA and two species of IS-kalDNAs. In the late subcultures, there is no detectable kalDNA in ascospores BC3-1, -2, -3, and -4, and high copy number of AR-kalDNA and five species of IS-kalDNAs in ascospores BC3-5, -6, -7, and -8. The ascospores of this ascus showed one to one segregation of vegetative death

and immortal growth, which corresponds exactly to the one to one segregation of the kalDNA.

Ascospores BC3-1, -2, and -3 contained a high copy number of AR-kalDNA, around 15-20 copies of AR-kalDNA per mitochondrial genome, in their first subcultures, but no detectable AR-kalDNA in their 20th subcultures. The abundant AR-kalDNA somehow was lost during subculturing. On the other hand, AR-kalDNA in ascospores BC3-5, -6, -7, and -8 increased to a high level in their late subcultures, and three new species of IS-kalDNAs were generated. The smaller two species of IS-kalDNAs, found in their early subcultures of ascospores BC3-5, -6, -7, and -8, were observed in the first subcultures of asci 13, 14, and 15. These IS-kalDNAs with the same molecular weights in different ascospores are apparently identical inserts based on the loss of the same mtDNA fragments and the generation of the new fragments with the same molecular weights. Three new species of IS-kalDNAs found in the late subcultures of these four ascospores are apparently identical among these ascospores. The changes of AR-kalDNA and IS-kalDNAs in this ascus suggest that ascospores BC3-1, -2, -3, and -4 contain an allele of one nuclear gene, inherited from 15-6 (eventually strain 83), which inhibits or stops the replication of the kalilo plasmid. Ascospores BC3-5, -6, -7, and -8 contain another allele of the gene which is inherited from HB9006, allow the plasmid to replicate to a high level.

The co-segregation of senescence and kalDNA in this ascus confirms that there is a nuclear gene with two alleles in their parents. The suppressive allele from their male parent 15-6 inhibits or stop the replication of the plasmid. The other allele of the gene from their female parent HB9006 allows the plasmid to replicate to a high level.

Ascus BC4

MtDNAs were prepared from the first and the late subcultures from ascospores of ascus BC4. The mtDNAs were digested with restriction enzyme Pst I. The EtBr-stained gel (Figure 15) shows that in the first subculture, ascospore BC4-1 contains the same mtDNA fragments as control mtDNA without kalDNA, and no fragment larger than 6.5 kb. Ascospores BC4-2 and BC4-3 contain all mtDNA fragments and one 8.6 kb fragment. Ascospore BC 4-4 contains all mtDNA fragments and two large fragments (11.0 and 20.0 kb) at low levels. Ascospores BC4-5, -6, -7, and -8 contain all mtDNA fragments and three large fragments (8.6, 9.0, 10.7 kb), their mtDNA fragments Pst I-6 and -7 are at lower levels compared with the other four ascospores. In the late subcultures, ascospores BC4-1, -2, -3, and -4 contain all mtDNA fragments and one large fragment (11.0 kb). Ascospores BC4-5, -6, -7 and -8 contain six or seven large fragments (8.6 to 15.0 kb) and most mtDNA fragments. Their mtDNA fragments Pst I-2, -3, -6, -7, -9, and -10 disappear or are at lower levels. The autoradiograph (Figure 15) shows that the smallest fragment (8.6 kb) among these large fragments is AR-kalDNA, other large fragments in ascospores BC4-5, -6, -7, and -8 are different IS-kalDNAs. Three additional large fragments (15-20 kb) in the late subcultures of these four ascospores hybridized with the kalDNA probe.

But other large fragments (11.0 and 20.0 kb) found in ascospores BC4-1, -2, -3, and -4 in the original photograph did not hybridize with the kalDNA probe. These strange fragments are derived from another plasmid and are investigated in another paper. There are several faint giant fragments labelled G1, G2, and G3 observed in the original autoradiograph, and this phenomenon is observed in ascus BC3 too. These giant fragments are much larger than IS-kalDNA, and their molecular weights are 40-60 kb or even larger. They hybridize

with kalDNA probe, suggesting being homologous with kalDNA. These giant fragments are investigated in another paper.

Ascospores BC4-1 and -4 did not contain kalDNA in the first and the late subcultures. Ascospores BC4-2 and -3 contain only AR-kalDNA in their first subculture, but lose it in their late subcultures. Ascospores BC 4-5, -6, -7, and -8 contain both AR-kalDNA and two species of IS-kalDNAs in their first subcultures, and AR-kalDNA at high level and additional three species of IS-kalDNAs in their late subcultures. These ascospores demonstrated one to one segregation of the kalDNA at their late stage, which corresponds exactly to the one to one segregation of vegetative death and immortal growth. Again, this pattern of corresponding segregation of death and kalDNA suggests that there is one nuclear gene with two alleles. The allele from the male parent 15-6 stops or inhibits the replication of the plasmid in ascospores BC4-1, -2, -3, and -4, thus there is no or a very low level of the plasmid in the mitochondria under such a nuclear background. The allele of the gene from their female parent HB9006 permits the plasmid to replicate to detectable even enormous levels in ascospores BC4-5, -6, -7, and -8.

There are three new species of IS-kalDNAs detectable on the EtBr-stained gel in their late subcultures of ascospores BC4-5, -6, -7, and -8. This result favours that these new IS-kalDNAs are generated by de novo insertion of the plasmid into mitochondrial genomes.

There are two species of IS-kalDNAs in their first subcultures of ascospores BC4-5, -6, -7, and -8. The mitochondrial DNA fragments, Pst I-6 and -7, are at lower levels compared with the control mtDNA profile. This indicates that these two species of IS-kalDNAs were generated by AR-kalDNA inserting into mtDNA Pst I-6 and -7 fragments respectively.

Analysis of mtDNA profiles of their late subcultures of ascospores BC4-5, -6, -7, and -8 shows that an IS-kalDNA with the same weight is generated, and the same mtDNA fragment lost. This result favours the conclusion that most IS-kalDNAs with the same molecular weights in the same kind of mitochondria are in the same insertion sites in the mitochondrial genomes.

There are three large species of kalDNAs (15-20 kb) detected in the late subcultures but not in the first subcultures of ascospores BC4-5, -6, -7, and -8. They hybridized with the kalDNA probe. Thus it is reasonable to believe that these large fragments are new species of IS-kalDNAs. But there is a mystery. AR-kalDNA is 8.6 kb, the largest Pst I-cut mtDNA fragment, Pst I-1, is 6.5 kb. If the plasmid inserts into this fragment in a simple manner generating inverted repeats of mtDNA, the Pst I-digested mtDNA-kalDNA fragment could be 9.0 to 21.6 kb. But insertion sites are not in the fragment, rather in other smaller fragments. How these large fragments were generated will be discussed in Chapter 2.

Ascus BC2

Ascospores from ascus BC2 show an irregular three to five segregation, unlike the four to four segregation of ascus BC3 and BC4. To investigate what happened to this ascus, mtDNAs were prepared from the first subcultures and the late subcultures. MtDNAs were digested with restriction enzyme Pst I. The EtBr-stained gel (Figure 16) shows that in the first subcultures, seven ascospores contain both AR-kalDNA and two species of IS-kalDNAs in their mtDNAs. One ascospore (BC2-3) contains a lower level of kalDNA in its mtDNA preparation which was only detected when the hybridized filter was exposed for

longer time. In the late subcultures, there are both AR-kalDNA and five species of IS-kalDNAs in the ascospores BC2-1, -5, and -7, which died within 10 subcultures. There is neither kalDNA in ascospores BC2-2, -3, -4, -6, and -8, which grew normally to the 20th subcultures. This result demonstrates that there is strong correlation between senescence and inserting activities of kalDNA, supporting the idea that insertion of kalDNA into mitochondrial genomes is one linked step of causing death of the host fungi under this nuclear background. If there is one suppressor allele and one non-suppressor allele of one nuclear gene in their parents, four ascospores inheriting the suppressor allele of the gene should escape the fate of vegetative death, the other four ascospores inheriting the non-suppressor should die during subculturing, because AR-kalDNA can replicate autonomously to high copy number and generate IS-kalDNAs. The nuclear markers in this ascus show four to four segregation, and a crossover happened on the chromosome I between markers nic-1 and al. Ascospores BC2-1, and -3, BC-5, and -7 have the same markers respectively suggesting that these ascospores are ascospore pairs. They should inherit the non-suppressive allele of the gene and the other four ascospores inherit the suppressor. But only three out of these ascospores, BC2-1, -5, and -7, showed the vegetative death; BC2-3 lost kalDNA and escaped the fate. Under the non-suppressor nuclear background, one ascospore losing kalDNA during the vegetative subcultures, could have occured from cytoplasmic segregation, a chance loss.

Ascospores BC2-2, -4, -6, and -8 contained two apparently identical species of IS-kalDNAs in their first subcultures, but lost them in their late subcultures. Interestingly, ascospore BC2-2 contained a lower copy number of AR-kalDNA than that of IS-kalDNAs. This indicates that IS-kalDNAs do not rely completely on AR-kalDNA to exist in the mitochondria after IS-kalDNAs are generated, IS-kalDNAs can replicate with the host

mitochondrial genomes. The loss of these IS-kalDNAs in their late subcultures should be due to their replication slower than the mitochondrial genomes without them, and no insertion of AR-kalDNA.

The mtDNA profiles of this ascus show that IS-kalDNAs with the same molecular weights are seen, and the same corresponding mtDNA fragments disappeared in different mtDNA preparations. This phenomenon indicates that IS-kalDNAs with the same molecular weights in different ascospores are generated by AR-kalDNA insertion into the same sites of mitochondrial genomes. The IS-kalDNAs with different weights are in different inserting sites in the mitochondrial genomes.

1.3 FUNCTION OF THE SUPPRESSIVE NUCLEAR GENE

Analyses of three asci of the backcross show corresponding segregation of vegetative death and kalDNA inserting activities, demonstrating that there is one suppressive allele in strain 83. Four ascospores in asci 14, 15, BC2, BC3, and BC4 inherited the suppressive allele from strain 83. The product of the allele inhibits or stops the replication of the kalilo plasmid, thus there are few or no plasmids in the mitochondria to insert into mitochondrial genomes. Consequently the normal function of the mitochondria is maintained and ascospores escape vegetative death. The other four ascospores inherited the non-suppressive allele of the gene from strain HB9006, thus the plasmid can replicate to high levels and insert into mitochondrial genomes and cause senescence.

To investigate the function of the suppressive allele of the gene, namely its ability to inhibit or stop the replication of the kalilo plasmid, one further experiment was performed.

Ascospore BC3-3 contains AR-kalDNA at a very high level (about 20 copies per mitochondrial genome) in its first subculture, and no detectable kalDNA in its 20th subculture (Figure 14). The loss of AR-kalDNA was due to the suppressive allele in its nucleus. MtDNA of the 20th subculture was cut with Pst I and radioactively labelled, and probed the mtDNA of its first subculture. The autoradiograph (Figure 17, A section "mtDNA of BC3-3-20") shows that the abundant AR-kalDNA of the first subculture produces a very weak hybridization signal, indicating that there is AR-kalDNA at a very low level in the 20th subculture of this ascospore. This result suggests that the function of the suppressive allele is to inhibit the replication of the kalilo plasmid to a very low level, not to stop its replication and eliminate the plasmid from the mitochondria consequently. Also this figure (section "EtBr" and "kalilo") shows that ascospore BC3-3 contains a series of kalilo-associated plasmids; this phenomenon will be addressed in another paper.

1.4 TEST CROSSES

The backcross experiments confirm that there is a nuclear gene with two alleles, the suppressive allele is derived from strain 83, the non-suppressive allele is derived from HB9006. If the parental strains are homokaryotic, all the asci from the cross HB9006 X 83 should contain the suppressive allele in four ascospores, the non-suppressive allele in other four ascospores . Thus these asci should show one to one co-segregation of senescence and kalDNA. But ascus 16 did not manifest the co-segregation shown by asci 14 and 15, all eight ascospores grew normally within 20 subcultures, and there was not kalDNA in their mtDNA preparations. What mechanism resulted in this irregular phenomenon? Two asci BC3 and BC4 demonstrated the one to one co-segregation of senescence and kalDNA. But two asci BC1 and BC5 did not show the co-segregation,

rather, they are like ascus 16, lacking the kalDNA and growing normally. These non-senescent asci could also have been produced by cytoplasmic segregation or by heterogeneity of the suppressive gene in their parental nuclear genomes.

To distinguish the two possibilities, further test crosses were conducted. All ascospores from ascus 16 were used as male parents in crosses to two senescent ascospore series 5-1-1 A and 5-4-1 a (see Figure 5). Ascospores from eight asci, one from each cross, were isolated and subjected to subculturing for 20 transfers. Four asci showed one-to-one segregation of vegetative death and immortal growth, the other four asci showed no segregation, all ascospores growing normally (Figure 18). Colony hybridizations were done to screen ascospores of these eight asci at their late subculture for kalDNA. All the ascospores showing senescence gave positive signals (not shown), all the ascospores showing normal growth gave negative signals. This segregation pattern of asci from the test cross confirms that there is a suppressor allele in the male parent 83, and the lack of kalDNA in ascus 16 is resulted from the cytoplasmic segregation, not from the heterogenic nuclear genes of their parents, and the mitochondria in the female parent HB9006 are heterogenic for kalDNA.

In the ascus analyses of senescence from the cross HB9006-4 x Oak Ridge, there are three kinds of asci. In some asci, all their ascospores stopped growth, in some asci, all their ascospores showed immortal growth. These two kinds of asci could be explained by containing the non-suppressive allele of the nuclear gene from both their parents or cytoplasmic segregation under non-suppressive nuclear background. In some asci, five or six ascospores showed vegetative death, the remaining ascospores grew normally. Such irregular segregations were observed in ascus 11 and BC2. What mechanism produced

such a phenomenon? Did the normal growing ascospores result from cytoplasmic segregation, nuclear heterogeneity of their parents, or frequent mutations from non-suppressor to suppressor? To investigate the question, a non-senescent ascospore 3-3-4 a from ascus 3 was used as male parent in cross with a senescent ascospore 5-2-4 A as female parent (see Figure 5). Tetrads from the cross were isolated and germinated. Colony hybridization of conidia from each zero subcultures of these ascospores to the radioactively labelled kalDNA fragment B3 was done. All ascospores showed positive signals to the kalDNA probe (not shown). This result indicates that all ascospores from this cross contain kalDNA in their initial colonies. Ascospores from two of the asci were subjected to subculturing. All of them manifested vegetative death within 23 subcultures. If their parents are heterogenic for suppressor and non-suppressor, ascospores from each ascus of this test cross will show four to four segregation of vegetative death and immortal growth. If there is highly frequent mutation from non-suppressor to suppressor in the nuclear genomes of their parents, the ascospores from each ascus of this test cross should show four to four or some kind of irregular segregation of death and growth. The result supports the explanation that the non-senescent ascospores and asci are produced by cytoplasmic segregation resulting in a lack of kalDNA in cytoplasm of some ascospores and asci, and that mitochondria in its parental perithecium are heterogenic.

The above experiments dealt with the kalDNA activities in the strains or derivatives of N. crassa. An N. intermedia strain that escapes senescence was found during the kinetic studies (see Chapter two). Ascospore series C4 had been chosen to study the kinetics of kalDNA. One of several replicate subculture series of series C4 grew normally until the 50th subculture, and did not show any signs of senescence. Most of the replicate series stopped growth at the 18th to 24th subcultures. The escaper C4 was investigated for the

reasons by which it escaped vegetative death. MtDNAs were prepared from some representative subcultures of the series and digested with restriction enzyme Pst I. The EtBr-stained gel and the autoradiograph (Figure 19) show that mtDNAs from subcultures 2 to 10 contain AR-kalDNA, mtDNAs from subculture 14 contain both AR-kalDNA and two species of IS-kalDNAs (this sample was underloaded, refer to Figure 20), mtDNAs from subculture 18 to 42 contain neither. There are three possibilities for escape. One is contamination with normal-growing strains with the same mtDNA profile during subculturing, one is cytoplasmic segregation, and one is the heterogeneity in the nuclear genomes generated by mutation from non-suppressor to suppressor. P605 is the only normally growing control strain used and with the same mtDNA Pst I-cut profile; its mating type is A. The escaper C4 series could cross with P605, namely, its mating type is a. P605 is only N. intermedia strain used in our laboratory during all my experiments. This makes the possibility of contamination unlikely.

Subculture 20 of the escaper C4 was used as male parent in a cross to a senescent ascospore C2-5 A from the same cross P561 x 1766, which is very sick, only two subculture away from death. Seven intact unordered tetrads were isolated and germinated, 11 ascospores stopped growth just after germination, 20 ascospores did not germinate, and the remaining germinated and grew weakly. The two healthier-looking tetrads were subjected to subculturing. All of these 16 ascospores stopped vegetative growth at the first subculture. Colony hybridization of conidia from their zero subcultures was done to screen for kalDNA. All conidial colonies produced positive signals to kalDNA probe (not shown). This result demonstrates that there is no suppressor allele in the nuclear genome of the escaper C4. It suggests that the lack of kalDNA in the late subcultures of the escaper series resulted from cytoplasmic segregation. The loss of IS-kalDNAs in this series

suggests that the mitochondrial genomes inserted by AR-kalDNA sometimes seem to not replicate faster than the normal genomes, and mitochondria with IS-kalDNA do not multiply faster than the normal mitochondria. Again, the mitochondria in the parental cytoplasm must be heterogenic.

Figure 5. Lengths of subculture series of unordered ascus isolates from the cross HB9006-4 x Oak Ridge. The original culture from which a series arose is labelled 0, and subcultures are 1, 2 and so on. The last number occupied by O indicates the subculture which produced no viable conidia. Those series showing no signs of senescence in the 20th subculture were terminated there.

HB9006-4 a al-2 nic-1 x Oak Ridge A

ASCUS 1

```
  0 1     5       10      15      20
1 OOOOOOOOOOOOOOOOOOOO
2 OOOOOOOOOOOO
3 OOOOOOOOOO
4 OOOOOOOOOOOOO
5 OOOOOOOOOOOOOOOOOOOO
6 OOOOOOOOOOO
7 OOOOOOOOOOOOOOOOOOOO
8 OOOOOOOOOOO
```

ASCUS 2

```
1 OOOOOOOOOOOOOOOOOOOO
2 OOOOOOOOOOOOOOOOOOOO
3 OOOOOOOOOOOOOOOOOOOO
4 OOOOOOOOOOOOOOOOOOOO
5 OOOOOOOOOOOOOOOOOOOO
6 OOOOOOOOOOOOOOOOOOOO
7 OOOOOOOOOOOOOOOOOOOO
8 OOOOOOOOOOOOOOOOOOOO
```

ASCUS 3

```
1 OOOOOOO
2 OOOOOO
3 OOOOOOOOOOOOOOOOOOOO
4 OOOOOO
5 OOOOOO
6 OOOOOOOOOOOOOOOOOOOO
7 OOOOOOOOOO
8 OOOOOOOOOO
```

ASCUS 4

```
1 OOOOOOOOOOOOOOOOOOOO
2 OOOOOOOOOOOOOOOOOOOO
3 OOOOOOOOOOOOOOOOOOOO
4 OOOOOOOOOOOOOOOOOOOO
5 OOOOOOOOOOOOOOOOOOOO
6 OOOOOOOOOOOOOOOOOOOO
7 OOOOOOOOOOOOOOOOOOOO
8 OOOOOOOOOOOOOOOOOOOO
```

ASCUS 5

```
1 OOOOOOO
2 OOOOOOOOOOOOOO
3 OOOOOOOOOOOO
4 OOOOOOO
5 OOOOOOOOO
6 OOOOOOO
7 OOOOOOOOOOOOOO
8 OOOOOOOOOOOOOO
```

ASCUS 6

```
1 OOOOOO
2 OOOOOO
3 OOOOOO
4 OOOOOO
5 OOOOOO
6 OOOOOO
7 OOOOOO
8 OOOOOO
```

ASCUS 7

```
1 OOOOOO
2 OOOOO
3 OOOOO
4 OOOOO
5 OOOOOO
6 OOOOO
7 OOOOO
8 OOOOO
```

ASCUS 8

```
1 OOOOOO
2 OOOOOOO
3 OOOOOO
4 OOOOOOO
5 OOOOOO
6 OOOOOOOO
7 OOOOOOOO
8 OOOOOOO
```

ASCUS 9

```
1 OOOOOOOOOOOOOOOOOOOO
2 OOOOOOOOOOOOOOOOOOOO
3 OOOOOOOOOOOOOOOOOOOO
4 OOOOOOOOOOOOOOOOOOOO
5 OOOOOOOOOOOOOOOOOOOO
6 OOOOOOOOOOOOOOOOOOOO
7 OOOOOOOOOOOOOOOOOOOO
8 OOOOOOOOOOOOOOOOOOOO
```

ASCUS 10

```
1 OOOOOO
2 OOOOO
3 OOOOO
4 OOOOO
5 OOOOO
6 OOOOOO
7 OOOOO
8 OOOOOO
```

Figure 6. Lengths of subculture series of unordered ascus isolates from the cross HB9006-4 x 428. In this figure crosses (x) indicate germination failure.

HB9006-4 a al-2 nic-1 x 428 A ad-3 al-2 cot-1 pan-2

ASCUS 11

```
   0 1      5        10       15       20
1  OOOOOO
2  OOOOOOOOOOOOOOOOOOOO
3  OOOOOO
4  OOOOOO
5  OOOOOO
6  OOOOOO
7  OOOOOO
8  x
```

ASCUS 12

```
1  OOOOOO
2  OOOOOO
3  OOOOOO
4  OOOOOO
5  OOOOOO
6  OOOOOO
7  OOOOOO
8  x
```

ASCUS 13

```
 0 1      5        10       15       20      nuclear markers
1  OOOOOOO                                    +   +  pan  +  a
2  OOOOOOO                                    +   +  pan  +  a
3  OOOOOOO                                    +  nic pan  +  a
4  OOOOOOO                                    +  nic pan  +  a
5  OOOOOOOOOOOOOOOOOOOOO                     ad  +   +  cot A
6  OOOOOOOOOOOOOOOOOOOOO                     ad nic  +  cot A
7  OOOOOOOOOOOOOOOOOOOOO                     ad nic  +  cot A
8  x
```

Figure 7. Lengths of subculture series of unordered ascus isolates from the cross HB9006-4 x 83.

HB9006-4 a al-2 nic-1 x 83 A un-3 ad-3A nic-2

ASCUS 14

	01 5 10 15 20	nuclear markers
1	OOOOOO	+ a + + al nic
2	OOOOOO	+ a + + al nic
3	OOOOOO	+ a + + al nic
4	OOOOOO	+ a + + al nic
5	OOOOOOOOOOOOOOOOOOOO	un A ad nic + +
6	OOOOOOOOOOOOOOOOOOOO	un A ad nic + +
7	OOOOOOOOOOOOOOOOOOOO	un A ad nic + +
8	OOOOOOOOOOOOOOOOOOOO	un A ad nic + +

ASCUS 15

	01 5 10 15 20	
1	OOOOOO	+ a + + al nic
2	OOOOOO	+ a + + al nic
3	OOOOOO	+ a + + al nic
4	OOOOOO	+ a + + al nic
5	OOOOOOOOOOOOOOOOOOOO	un A ad nic + +
6	OOOOOOOOOOOOOOOOOOOO	un A ad nic + +
7	OOOOOOOOOOOOOOOOOOOO	un A ad nic + +
8	OOOOOOOOOOOOOOOOOOOO	un A ad nic + +

ASCUS 16

	0 1 5 10 15 20	
1	OOOOOOOOOOOOOOOOOOOO	+ a + + al nic
2	OOOOOOOOOOOOOOOOOOOO	+ a + + al nic
3	OOOOOOOOOOOOOOOOOOOO	+ a ad nic al nic
4	OOOOOOOOOOOOOOOOOOOO	+ a ad nic al nic
5	OOOOOOOOOOOOOOOOOOOO	un A + + + +
6	OOOOOOOOOOOOOOOOOOOO	un A + + + +
7	OOOOOOOOOOOOOOOOOOOO	un A ad nic + +
8	OOOOOOOOOOOOOOOOOOOO	un A ad nic + +

Figure 8. Gel electrophoresis analysis of uncut mtDNAs from the first and the 20th subculture of ascus 4 of the cross HB9006-4 x Oak Ridge. The two lanes labelled by "-" and "+" represent the nonsenescent male parent Oak Ridge and the senescent female parent HB9006-4 respectively. The numbers above the remaining lanes represent the ascospores of the ascus from which mtDNA was prepared. The autoradiograph shows the bands hybridizing with kalilo DNA fragment B3. In this figure the mtDNA is overloaded to facilitate the detection of kalDNA.

ASCUS 4

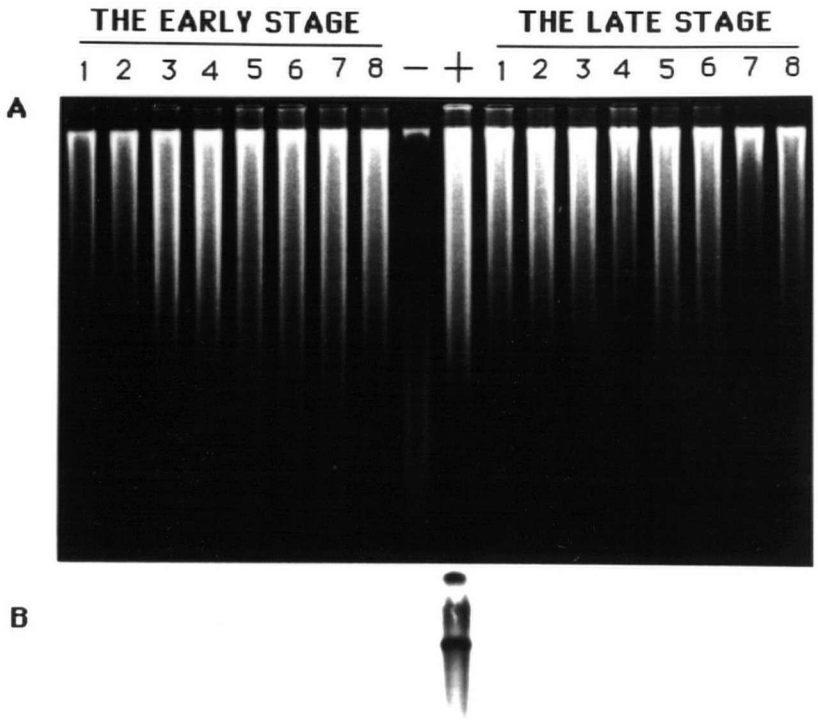


Figure 9. Gel electrophoresis analysis of uncut mtDNAs from the first and the 20th subculture of ascus 13 of the cross HB9006-4 x 428. The two lanes of the ethidium bromide stained gel and autoradiograph labelled "-" and "+" represent the nonsenescent male parent 428 and the senescent female parent HB9006-4 respectively. The numbers above the remaining lanes represent the ascospores of the ascus from which mtDNA was prepared. The autoradiograph shows the bands hybridizing with kalilo DNA fragment B3. The clear and small band is AR-kalDNA. The smearing band in the uncut mtDNA position is IS-kalDNA.

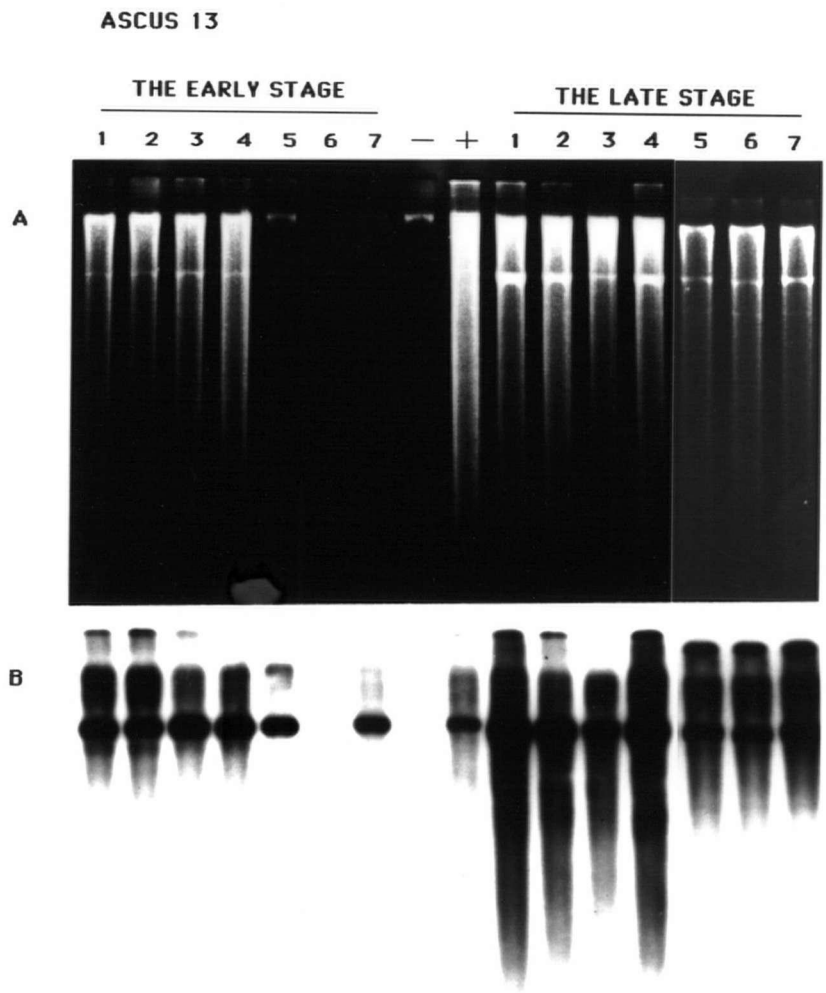

ASCUS 13
THE EARLY STAGE
THE LATE STAGE
1 2 3 4 5 6 7 — + 1 2 3 4 5 6 7
A
B

Figure 10. Gel electrophoresis analysis of Pst I digested mtDNAs from the first and the 38th subcultures of three ascospores from ascus 13 of the the cross HB9006-4 x 428. The numbers above the lanes of the ethidium bromide stained gel and autoradiograph represent the subcultures of the ascospores from which mtDNA was prepared. BC4-1-20 shares the same cytoplasm but lacks kalDNA and serves as control. The autoradiograph shows the bands hybridizing with kalilo DNA fragment B3. The bands labelled G1 and G2 are giant DNA fragments containing kalDNA. The bands labelled L1, L2, L3 and L4 are different species of IS-kalDNAs. The fragment labelled 1, 2 and so on are normal mtDNA fragments digested with Pst I.

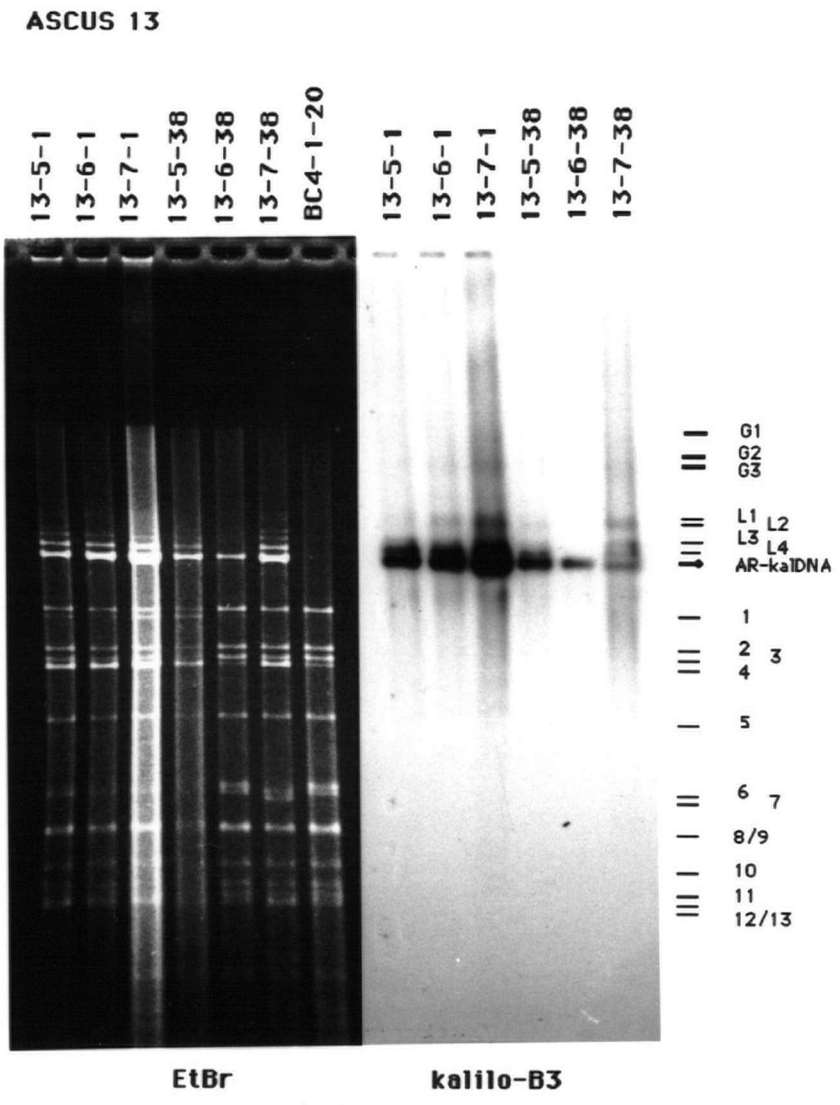

ASCUS 13
13-5-1
13-6-1
13-7-1
13-5-38
13-6-38
13-7-38
BC4-1-20
13-5-1
13-6-1
13-7-1
13-5-38
13-6-38
13-7-38
G1
G2
G3
L1
L2
L3
L4
AR-kalDNA
1
2
3
4
5
6
7
8/9
10
11
12/13
EtBr
kalilo-B3

Figure 11. Gel electrophoresis analysis of Pst I digested mtDNAs from the first and the late subcultures of ascospores from ascus 14 of the cross HB9006-4 x 83. The numbers above the lanes of the ethidium bromide stained gel and autoradiograph represent the subcultures of the ascospores from which mtDNA was prepared. The autoradiograph shows the bands hybridizing with kalilo DNA fragment B3. The lowest band is AR-kalDNA, other higher bands are different species of IS-kalDNAs.

ASCUS 14
EtBr
14-1-1
14-2-1
14-3-1
14-4-1
14-5-1
14-6-1
14-7-1
14-8-1
14-1-4
14-2-4
14-3-4
14-4-4
14-5-20
14-6-20
14-7-20
14-8-20
kallo-B3
14-1-1
14-2-1
14-3-1
14-4-1
14-5-1
14-6-1
14-7-1
14-8-1
14-1-4
14-2-4
14-3-4
14-4-4
14-5-20
14-6-20
14-7-20
14-8-20
BS-kalDNA
AR-tolDNA

Figure 12. Gel electrophoresis analysis of Pst I digested mtDNAs from the first and the late subcultures of ascospores from ascus 15 of the cross HB9006-4 x 83. The numbers above the lanes of the ethidium bromide stained gel and autoradiograph represent the subcultures of the ascospores from which mtDNA was prepared. The autoradiograph shows the bands hybridizing with the kalilo DNA fragment B3. The lowest band is AR-kalDNA, other higher bands are different species of IS-kalDNA.

ASCUS 15
EtBr
15-1-1
15-2-1
15-3-1
15-4-1
15-5-1
15-6-1
15-7-1
15-8-1
15-1-4
15-2-4
15-3-4
15-4-4
15-5-20
15-6-20
15-7-20
15-8-20
kallo-B3
15-1-1
15-2-1
15-3-1
15-4-1
15-5-1
15-6-1
15-7-1
15-8-1
15-1-4
15-2-4
15-3-4
15-4-4
15-5-20
15-6-20
15-7-20
15-8-20
G1
G2
G3
L1
L2
L3
AR
L4
L5
1
2
3
4
5
6
7
8
9
10
11
12
13

Figure 13. Lengths of subculture series of unordered ascus isolates from the back cross HB9006-4 x 15-6-20. In this figure, those series showing no signs of senescence in the 20th subculture were terminated there.

HB9006-4 a al-2 nic-1 x 15-6-20 A un-3 ad-3 nic-2

ASCUS BC1

	0 1 5 10 15 20	nuclear markers					
1	OOOOOOOOOOOOOOOOOOOO	+	a	+	+	+	+
2	OOOOOOOOOOOOOOOOOOOO	un	A	ad	nic	+	+
3	OOOOOOOOOOOOOOOOOOOO	un	A	ad	nic	+	+
4	OOOOOOOOOOOOOOOOOOOO	+	a	+	+	+	+
5	OOOOOOOOOOOOOOOOOOOO	+	a	+	+	al	nic
6	OOOOOOOOOOOOOOOOOOOO	+	a	+	+	al	nic
7	OOOOOOOOOOOOOOOOOOOO	un	A	ad	nic	al	nic
8	OOOOOOOOOOOOOOOOOOOO	un	A	ad	nic	al	nic

ASCUS BC2

1	OOOOOOOOO	+	a	+	+	+	+
2	OOOOOOOOOOOOOOOOOOOO	un	A	ad	nic	+	+
3	OOOOOOOOOOOOOOOOOOOO	+	a	+	+	+	+
4	OOOOOOOOOOOOOOOOOOOO	un	A	ad	nic	+	+
5	OOOOOOOO	+	a	+	+	al	nic
6	OOOOOOOOOOOOOOOOOOOO	un	A	ad	nic	al	nic
7	OOOOOOOO	+	a	+	+	al	nic
8	OOOOOOOOOOOOOOOOOOOO	un	A	ad	nic	al	nic

ASCUS BC3

1	OOOOOOOOOOOOOOOOOOOO	+	a	+	+	al	nic
2	OOOOOOOOOOOOOOOOOOOO	+	a	+	+	al	nic
3	OOOOOOOOOOOOOOOOOOOO	+	a	+	+	al	nic
4	OOOOOOOOOOOOOOOOOOOO	+	a	+	+	al	nic
5	OOOOOOOOOO	un	A	ad	nic	+	+
6	OOOOOOO	un	A	ad	nic	+	+
7	OOOOOOO	un	A	ad	nic	+	+
8	OOOOOOOOO	un	A	ad	nic	+	+

ASCUS BC4

1	OOOOOOOOOOOOOOOOOOOO	+	a	+	+	al	nic
2	OOOOOOOOOOOOOOOOOOOO	+	a	+	+	al	nic
3	OOOOOOOOOOOOOOOOOOOO	+	a	+	+	al	nic
4	OOOOOOOOOOOOOOOOOOOO	+	a	+	+	al	nic
5	OOOOOOO	un	A	ad	nic	+	+
6	OOOOOOOO	un	A	ad	nic	+	+
7	OOOOOOOO	un	A	ad	nic	+	+
8	OOOOOOO	un	A	ad	nic	+	+

ASCUS BC5

1	OOOOOOOOOOOOOOOOOOOO	+	a	+	+	+	+
2	OOOOOOOOOOOOOOOOOOOO	+	a	+	+	+	+
3	OOOOOOOOOOOOOOOOOOOO	un	A	ad	nic	+	+
4	OOOOOOOOOOOOOOOOOOOO	un	A	ad	nic	+	+
5	OOOOOOOOOOOOOOOOOOOO	+	a	+	+	al	nic
6	OOOOOOOOOOOOOOOOOOOO	+	a	+	+	al	nic
7	OOOOOOOOOOOOOOOOOOOO	un	A	ad	nic	al	nic
8	OOOOOOOOOOOOOOOOOOOO	un	A	ad	nic	al	nic

Figure 14. Gel electrophoresis analysis of Pst I digested mtDNAs from the first and the late subcultures of ascospores from ascus BC3 of the back cross HB9006-4 x 15-6-20. The numbers above the lanes of the ethidium bromide stained gel and autoradiograph represent the subcultures of the ascospores from which mtDNA was prepared. The autoradiograph shows the bands hybridizing with kalilo DNA fragment B3. The lowest band is AR-kalDNA. The bands labelled G1, G2, and G3 are giant DNA fragments containing kalDNA, which are observed in the original autoradiograph. The other bands between G3 and AR-kalDNA are different species of IS-kalDNAs.

ASCUS BC3
3-1-1
3-2-1
3-3-1
3-4-1
3-5-1
3-6-1
3-7-1
3-8-1
3-1-20
3-2-20
3-3-20
3-4-20
3-5-8
3-6-6
3-7-6
3-8-6
EtBr
3-1-1
3-2-1
3-3-1
3-4-1
3-5-1
3-6-1
3-7-1
3-8-1
3-1-20
3-2-20
3-3-20
3-4-20
3-5-8
3-6-6
3-7-6
3-8-6
kallo-B3
G1
G2
G3
IS-kalDNA
AR-kalDNA

Figure 15. Gel electrophoresis analysis of Pst I digested mtDNAs from the first and the late subcultures of ascospores from ascus BC4 of the back cross HB9006-4 x 15-6-20. The numbers above the lanes of the ethidium bromide stained gel and autoradiograph represent the subcultures of the ascospores from which mtDNA was prepared. The autoradiograph shows the bands hybridizing with the kalilo DNA fragment B3. The lowest band is AR-kalDNA. The bands labelled G1, G2 and G3 are giant DNA fragments containing kalDNA, which are observed in the original autoradiograph. The other bands between G3 and AR-kalDNA are different species of IS-kalDNAs. The fragments labelled 1, 2, and so on are the normal mtDNA Pst I-cut fragments.

ASCUS BC4
4-1-1
4-2-1
4-3-1
4-4-1
4-5-1
4-6-1
4-7-1
4-8-1
4-1-20
4-2-20
4-3-20
4-4-20
4-5-6
4-6-6
4-7-6
4-8-6
EtBr
kalilo-B3
4-1-1
4-2-1
4-3-1
4-4-1
4-5-1
4-6-1
4-7-1
4-8-1
4-1-20
4-2-20
4-3-20
4-4-20
4-5-6
4-6-6
4-7-6
4-8-6
G1
G2
G3
IS-kalDNA
AR-kalDNA
1
2 3 4
5
6 7
8/9
10
11
12/13

Figure 16. Gel electrophoresis analysis of Pst I digested mtDNAs from the first and the late subcultures of ascospores from ascus BC2 of the back cross HB9006-4 x 15-6-20. The numbers above the lanes of the ethidium bromide stained gel represent the subcultures of the ascospores from which mtDNA was prepared. The lowest band is AR-kalDNA and other labelled bands are different species of IS-kalDNAs.

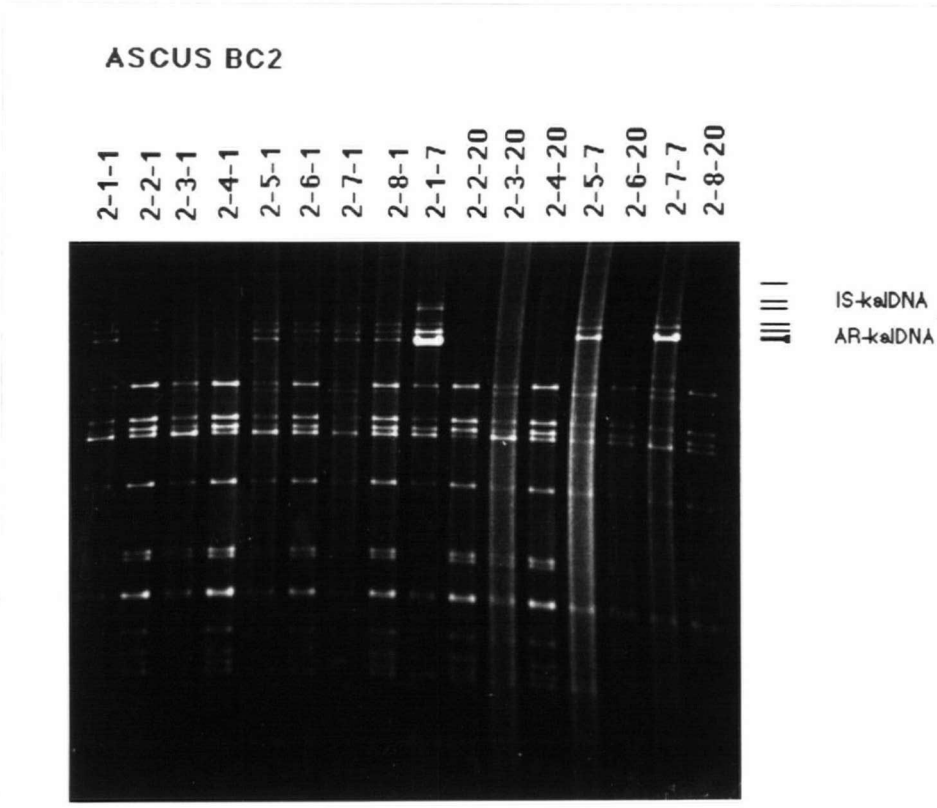

ASCUS BC2
2-1-1
2-2-1
2-3-1
2-4-1
2-5-1
2-6-1
2-7-1
2-8-1
2-1-7
2-2-20
2-3-20
2-4-20
2-5-7
2-6-20
2-7-7
2-8-20
IS-kalDNA
AR-kalDNA
EtBr

Figure 17. Gel electrophoresis analysis of 3' and 5' exonuclease-treated mtDNAs from the first subcultures of ascospores BC3-2 and BC3-3. The numbers and labels above the lanes of the ethidium bromide stained gel and autoradiograph represent the subcultures of the ascospores from which mtDNA was prepared and treated with exonuclease. The section labelled "EtBr" is the ethidium bromide stained gel. The largest band is AR-kalDNA, and the bands labelled 1, 2 and so on are numerous kalilo-derived plasmids. The section labelled "kalilo" is the autoradiograph of the gel hybridized with the intact kalilo DNA plasmid. The section labelled "mtDNA BC3-3-20" is the autoradiograph of the gel hybridized with Pst I-digested mtDNA of BC3-3-20.

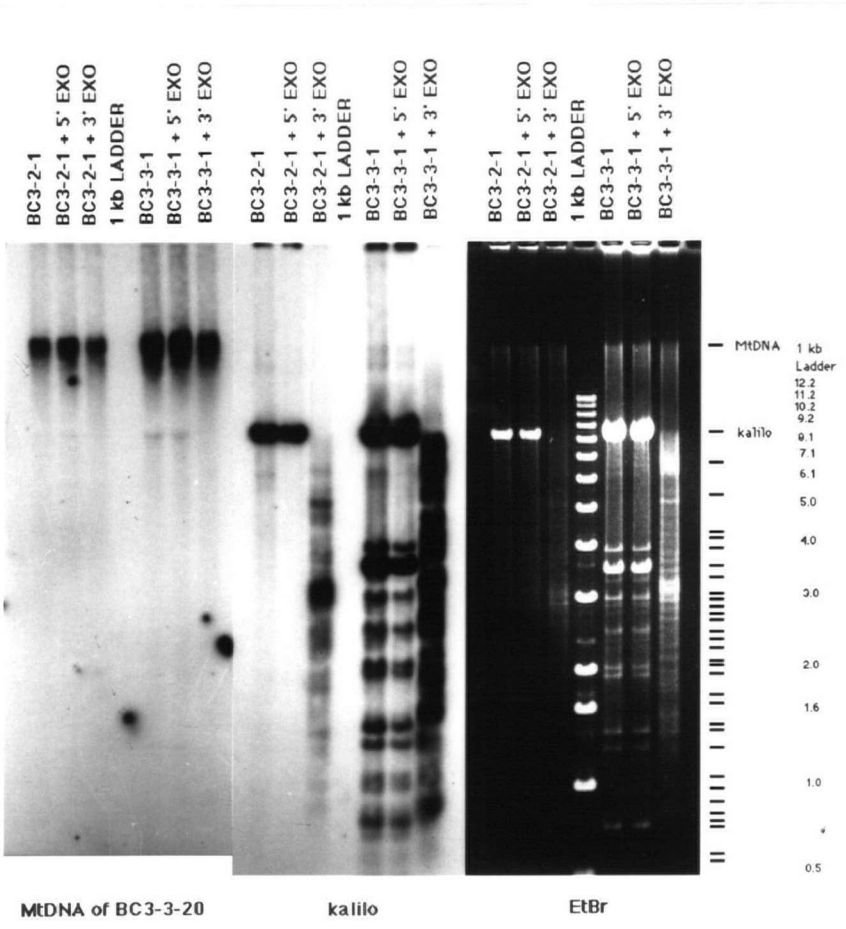
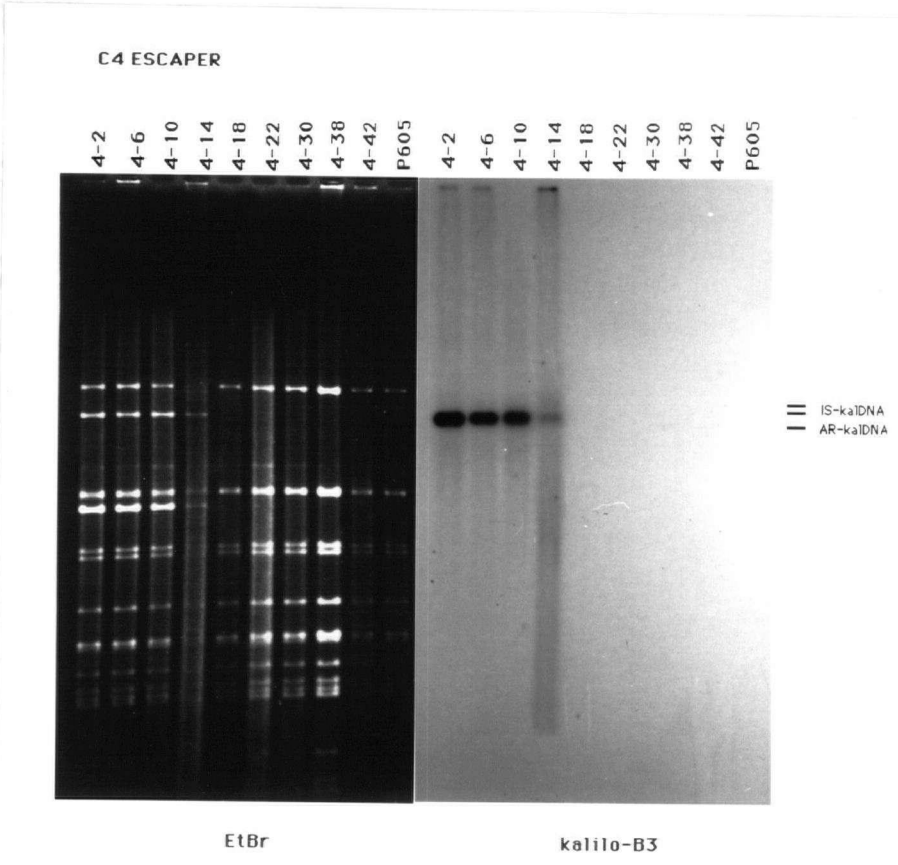

BC3-2-1
BC3-2-1 + 5' EXO
BC3-2-1 + 3' EXO
1 kb LADDER
BC3-3-1
BC3-3-1 + 5' EXO
BC3-3-1 + 3' EXO
MtDNA
kalilo
1 kb Ladder
12.2
11.2
10.2
9.2
9.1
7.1
6.1
5.0
4.0
3.0
2.0
1.6
1.0
0.5
MtDNA of BC3-3-20
kalilo
EtBr

Figure 18. Lengths of subcultures of unordered ascus isolates of the test cross on ascus 16. One unordered ascus was isolated from each cross and subcultured. The underlined numbers 1, 2, 3, 4, 5, 6, 7, and 8 represent ascospores of the asci. The numbers below the spore numbers are their life spans. Those ascospores showing no signs of senescence were terminated at the 20th subculture.

TEST CROSSES ON ASCUS 16

CROSSES	LIFE SPANS OF TETRAD SPORES							
	1	2	3	4	5	6	7	8
5-1-1 A x 16-1-2 a	11	11	11	11	10	10	10	10
5-1-1 A x 16-2-2 a	10	10	10	10	10	10	7	8
5-1-1 A x 16-3-2 a	12	12	11	11	11	11	11	11
5-1-1 A x 16-4-2 a	11	11	9	11	11	9	11	11
5-4-1 a x 16-5-2 A	20	20	20	20	12	11	11	11
5-4-1 a x 16-6-2 A	20	20	20	20	12	13	14	12
5-4-1 a x 16-7-2 A	20	20	20	20	10	11	9	10
5-4-1 a x 16-8-2 A	20	20	20	20	11	11	6	8

Figure 19. Gel electrophoresis analysis of Pst I digested mtDNAs from various subcultures of escaper series C4 derived from an ascospore from the cross P561 x 1766. The numbers above the lanes of the ethidium bromide stained gel and autoradiograph represent the control P605 and the subcultures of the series from which mtDNA was prepared. The autoradiograph shows the bands hybridizing with kalilo DNA fragment B3. The lowest band is AR-kalDNA and the upper two bands are different species of IS-kalDNAs.


C4 ESCAPER
4-2
4-6
4-10
4-14
4-18
4-22
4-30
4-38
4-42
P605
4-2
4-6
4-10
4-14
4-18
4-22
4-30
4-38
4-42
P605
IS-kalDNA
AR-kalDNA
EtBr
kalilo-B3

DISCUSSION

The results of these experiments which investigated the suppression of cytoplasmic senescence in Neurospora demonstrated that there are three mechanisms of escaping from the kalDNA-related senescence in Neurospora. The first mechanism is that one allele of one nuclear gene from strain 83 inhibits the replication of the kalilo plasmid to a very low level in the mitochondria, and there are no (or too few) IS-kalDNAs to affect the normal function of the mitochondria. The other allele of the gene from strain HB9006 permits the plasmid to replicate to high levels, thus the insertion activities of the plasmid destroy the mitochondrial function and consequently cause death of the host fungus.

The second mechanism is cytoplasmic segregation. The mitochondria in senescent strains are heterogenic, some containing AR-kalDNA or and IS-kalDNAs, some containing neither. When asci, ascospores or conidia are formed with the mitochondria lacking kalDNA from their parental cytoplasm containing kalDNA, the sexual or asexual progeny with normal mitochondria grow immortally, escaping the fate of death.

The third mechanism is that a nuclear gene with two alleles in ascus 13. One allele of this gene from HB9006 cannot suppress the deleterious function of IS-kalDNAs, resulting in vegetative death. The other allele of this gene, from strain 428, allows the plasmid to replicate normally and insert into mitochondrial genomes generating IS-kalDNAs, but the allele somehow suppresses the deleterious effects of IS-kalDNAs, resulting in the host fungi growing normally, and carrying both AR-kalDNA and IS-kalDNAs in their mitochondria.

The analyses of mtDNAs from asci 11 and 13 from the cross HB9006 x 428 demonstrate that there is a nuclear gene in ascus 13 from strain 428, which suppresses deleterious function of the plasmid. Such nuclear gene was not observed in ascus 11. There are basically two kinds of asci from the cross. HB9006 was suspected to be homokaryotic and proved to be in these experiments, because other crosses involved this parental strain and the test crosses of its progeny did not segregate other suppressive allele from it. The two kinds of asci must be due to the heterokaryotic nature of strain 428 concerning this suppressor gene. The suppressive allele of this gene should be derived from early mutation from non-suppressive to suppressive allele. Such a similar nuclear gene was found and proved to be true in strain 2360 his of N. intermedia (Griffiths et al, 1991). There could be other mechanism of suppressing senescence, such as combination of multiple genes and functional penetrance of some genes, but these possibilities seem remote in this case. Ascospores 13-5, -6 and -7 contained the suppressive allele of the nuclear gene. These ascospores maintained both forms of kalDNA in their 20th subcultures. This suggests that the suppressive allele suppress the deleterious function of kalDNA activities, but does not interfere with replication and insertion of the plasmid. MtDNA analyses of their further subcultures confirmed this conclusion. The same situation was found in N. intermedia strain 2360 his (Griffiths et al, 1991). Similarly, the maize mitochondrial plasmid S-1 and S-2 maintain their both forms in their host mitochondria (Schardl et al, 1984; 1985). Interestingly, ascospores 13-6 and 13-7 are sibs, sharing the same nuclear and mitochondrial genomes. In the 38th subcultures, ascospore 13-7 maintained both AR-kalDNA and IS-kalDNAs, but ascospore 13-6 maintained only AR-kalDNA and lost its original IS-kalDNAs. Thus the nuclear genotype seem not apparently affect the pattern of IS-kalDNAs, rather the loss of IS-kalDNAs from ascospore 13-6 should be due to that the mtDNA with IS-kalDNAs replicate slower than the mtDNA without IS-kalDNA, and AR-

kalDNA stops inserting activities. It is possible that, the generation and maintenance of IS-kalDNAs rely upon the constant insertion activities of AR-kalDNA. So far we have not found any strain with IS-kalDNAs but no AR-kalDNA in mitochondria. This favours the conclusion.

The results of mtDNA analyses of three asci from the cross HB9006 x 83 demonstrate that there is a nuclear gene which determines the level of AR-kalDNA in the mitochondria. The allele of this gene from HB9006 allows the plasmid to replicate to a high level, and high level of IS-kalDNAs is generated. The other allele of this gene from 83 inhibits replication of the plasmid to a very low level, usually undetectable one (in BC 3-3-20, one copy of the plasmid per 100-200 mitochondrial genomes). Under this nuclear background, IS-kalDNAs are not detected at late stages. All ascospores with the suppressive allele either lost or contained very low levels of kalDNA. The co-loss of both IS and AR forms of the kalilo plasmid could be explained in two ways. One possible way is that the replication of AR-kalDNA and IS-kalDNAs is dependent on some common factor determined by products of the gene. Another possible way is that maintenance of IS-kalDNAs depends on the continuous insertion of AR-kalDNA, which in turn depends on the products of the gene for its replication.

Some of these ascospores with the suppressive allele contained no detectable kalDNA in their first and late subcultures, some contained only AR-kalDNA or both forms of kalDNA in their first subcultures, and neither form in their late subcultures. It is clear now that the product of the suppressive allele inhibits the replication of AR-kalDNA to an undetectable level. The loss of IS-kalDNAs could be through the same process as AR-kalDNA. However, the mtDNA analysis of ascospore 14-6 makes this possibility unlikely. This

ascospore with the suppressive allele contained only detectable AR-kalDNA in the first subcultures, AR-kalDNA and two species of IS-kalDNAs in the 20th subculture, and no detectable kalDNA at all in the 38th subculture. The appearance of two new species of IS-kalDNAs in the 20th subculture is not consistent with the co-inhibition explanation, in other words, the product of the suppressive allele of the gene does not inhibit the generation and replication of IS-kalDNA. Rather, this indicates that the product of the suppressive allele does not interfere with the insertion activities of AR-kalDNA generating IS-kalDNAs; AR-kalDNA inserts into mitochondrial genomes continuously when AR-kalDNA reaches a certain level; IS-kalDNAs are lost or at an undetectable level when AR-kalDNA is inhibited to an undetectable level.

Do IS-kalDNAs replicate with mitochondrial genomes, slower or faster than the normal mitochondrial genomes without IS-kalDNAs? MtDNA analysis of ascospore BC2-2 with the suppressive gene (inhibitor) provides some insight. In the first subculture, the ascospore contained both IS-kalDNA and AR-kalDNA, and the copy number of IS-kalDNAs was several times higher than that of AR-kalDNA. In the 20th subculture there was no detectable kalDNA. The replication of AR-kalDNA had been inhibited in progress, and the level of AR-kalDNA had been decreased. If the IS-kalDNA could not replicate, completely relying upon the continuous insertion activities of AR-kalDNA, the relatively higher level of IS-kalDNAs should not be observed. All ascospores from ascus BC2 contain the same two species of IS-kalDNAs in their first subcultures. These two species of IS-kalDNAs are observed most frequently in sexual progeny from HB9006. The two common species of IS-kalDNAs favour the idea that these IS-kalDNAs are inherited from their female parent HB9006, in which they were generated through de novo insertion, and can replicate with mitochondrial chromosomes to some extent. Thus the loss of IS-

kalDNAs should be due to their slower replication than mitochondrial genomes without them, and slow and no insertion activities of AR-kalDNA. The loss of IS-kalDNAs in ascospore 13-6 without this suppressive allele supports this conclusion.

The above analyses of replication and insertion of the kalDNA are in some kind of nuclear suppressive environment. The studies of kalDNA activities under the suppressive nuclear background help to dissect the process of senescence. One nuclear gene in ascus 13 suppresses the deleterious function of IS-kalDNAs, and does not suppress its replication and insertion. One nuclear gene (inhibitor) from strain 83 inhibits its replication but not insertion. Both genes have not been mapped to genetic loci. From genetic studies, the suppressor in strain 83 could be on chromosome I.

So far we have identified two nuclear genes which affect the activities of the plasmid. It is reasonable to believe that there are other nuclear genes which prevent the insertion activities. There are many mitochondrial plasmids with the similar structure (unpublished data). Most of them do not have the insertion activities as the kalilo and the maranhar plasmids, and have no homologous fragment in mitochondrial or nuclear genomes. As the studies of activities of these inserting plasmids continue, these nuclear genes will be found.

Both these suppressive nuclear genes were found in laboratory strains. From the view of evolution, it is reasonable that natural populations of Neurospora contain such kinds of suppressive genes, because the insertion activities of these plasmids could bring the end to their hosts. The hosts without suppressive genes will be eliminated from nature by selection. We would not detect these plasmids from natural collections if there were not diverse mechanisms of suppressing their harmful activities. A nature-collected senescent

strain P573 (Myers, 1988) showed suppressive function over the kalilo plasmid. The senescent mechanism in this strain was not associated with insertion activities (Griffiths, unpublished results). The AR-kalDNA had a inconsistent appearance in its subculture series. Ten random ascospores from the cross P573 x 1766 did not contain AR-kalDNA. One of them contained small kalDNA fragments in their mitochondrial genomes at a very low level. This result suggests that natural populations have mechanisms to deal with these harmful parasites. Other unpublished results (Griffiths, personal communication) indicate that there are nuclear genes from nature-collected strains, which suppress senescence caused by the insertion activities of the maranhar plasmid. Many plasmids found in the N. intermedia strain 3983 from China were eliminated or inhibited to an undetectable level in some ascospores from the cross 3983 x 3991 (unpublished data).

The results of the test crosses demonstrate that senescent strains can escape their death fate by cytoplasmic segregation during mitotic and meiotic processes. There is a high frequency of producing the non-senescent progeny in meiotic processes. It is reasonable to believe that there is the same frequency of producing such non-senescent progeny during their asexual propagation. The reason for discovering only one conidial escaper over thousands of transfers is that each subculture transfer carries thousands of conidia and they exchange cytoplasms through heterokaryon formation. Cytoplasmic segregation is a likely mechanism for natural senescent strains to escape their death.

In these studies, the insertion sites of IS-kalDNAs have not been located in detail. Several sites have been observed frequently, for example, the insertions in Pst I-6, -7, -2, -3, -9, and -10 fragments of mitochondrial genomes in N. crassa. Many ascospores from the same asci showed the same pattern of IS-kalDNAs, for example BC4-5, -6, -7, -8 and

BC3-5, -6, -7, -8. Many ascospores from different asci showed the same too, for example BC4-5, -6, -7, -8 and 14-2, -3. But there are many cases in which ascospores with the same nuclear genomes showed different patterns of IS-kalDNAs, for example 13-6 and 13-7. The studies of parallel subculture series (Vickery, 1990) showed that different transfer subcultures from the same asexual parents have different insertion sites. So thus the influence of nuclear genomes on the patterns of IS-kalDNAs is still not clear. In some cases, one IS-kalDNA accumulated in late subcultures, but the same IS-kalDNA was lost in another strain. The relationship between insertion sites and senescence has not been clear.

It has been observed that there is a high level of IS-kalDNAs at the late stages close to death. One fundamental question has not been answered. How do IS-kalDNAs accumulate to a high level at the late stages? There are two basic proposals. The first proposal is the renegade replication of IS-kalDNAs. Insertion of AR-kalDNA confers the mitochondrial genomes with IS-kalDNA a replication advantage over the normal genomes. The normal mitochondrial genomes would be replaced by the abnormal genomes. The second proposal is that the IS-kalDNAs are generated and maintained mainly by the continuous insertion of AR-kalDNA into the mitochondrial genomes, and the IS-kalDNAs replicate as the normal mitochondrial genomes or slower.

We can not study this question directly because we have not found any strain or derivative with only IS-kalDNAs and no AR-kalDNA, but accumulated data give some understanding.

If IS-kalDNAs replicate faster than the normal mitochondrial genomes, we should observe the following phenomena:

1. The same IS-kalDNAs should be detected in later subcultures after they are found in early subcultures of the same strains, namely the predominant insertion sites should not change during asexual propagation.
2. All individual conidia should have the same IS-kalDNAs of their asexual parents.
3. All sexual progeny should have the same IS-kalDNAs as their female parents when the female parents with detectable IS-kalDNAs were used.
4. The accumulation of IS-kalDNAs should not be related to quantitative changes of AR-kalDNA.
5. A senescent strain or derivative with only IS-kalDNAs should have been found.
6. The ratio of mitochondria over nucleus should increase in senescent subculture series. The reason for this predicted phenomenon is that the kalilo plasmid is mitochondrial; mitochondria with dominant abnormal genomes should multiply faster than the normal ones to replace the normal ones.

Our data do not support the idea. Many ascospores in my experiments lost their initial IS-kalDNAs, and new IS-kalDNAs were detected in their late subcultures. Ten subcultures from the same subcultures of ascospores showed different predominant IS-kalDNAs (Vickery, 1990). Ascospores from the cross P561 x 1766 showed more than ten different dominant species of IS-kalDNAs (Myers, 1988) while their female parent P561 has only one detectable species of IS-kalDNA. Many ascospores from the backcross contained two or three common species of IS-kalDNAs and several new species of IS-kalDNAs, and some contained only AR-kalDNA. The kinetic studies of kalDNA in senescent strains (see Chapter two) show that the level of IS-kalDNAs follows the quantitative changes of AR-kalDNA. Ascospore 13-6 with the non-suppressive nuclear gene for replication and insertion of the kalilo plasmid maintained AR-kalDNA but lost the original IS-kalDNAs in

the 38th subculture. Ascospore 14-6 with the inhibitive nuclear gene for the replication of AR-kalDNA contains only AR-kalDNA in the first subculture, two new species of IS-kalDNAs in the 20th subcultures, and no IS-kalDNAs in the 38th subculture. Many ascospores with the inhibitive allele contained IS-kalDNAs in their first subcultures, but lost them later. So far, we have not found a senescent strain or derivatives containing only IS-kalDNAs, IS-kalDNAs always are accompanied with AR-kalDNA. Preliminary observations from EM studies (Griffiths, personal communication) show that there are no significant differences of the ratios of mitochondria over nucleus between early and late stages of typical senescent Neurospora strains.

All data favour the second proposal that the accumulation of IS-kalDNAs is generated mainly by the continuous insertion of AR-kalDNA into the mitochondrial genomes. This question is probed further in Chapter two.

CHAPTER TWO

KINETICS OF kalDNA IN SENESCENT NEUROSPORA

INTRODUCTION

The senescent strains of N. intermedia from the Kauai island undergo a number of complex molecular events during the senescent process. While many of these changes resemble the mitochondrial aberrations that occur with cytoplasmic mutants such as stopper (Bertrand et al, 1980; De Vries et al, 1981), the kalilo senescence process has been shown to be highly programmed and repeatable in the wild isolates of kalilo containing strains of N. intermedia (Griffiths and Bertrand, 1984). Although it is known that almost any mitochondrial aberration can lead to the suppressive accumulation of defective DNAs, the observation that kalilo induced senescence is a highly repeatable process leads to the conclusion that there must be some aspect of kalilo senescence that is directed by the linear plasmid in addition to its maintenance functions.

It has been observed that aberrant mtDNA accumulated at the late stages of senescence in N. intermedia (Bertrand, 1986; Myers, 1988) as it did in other cytoplasmic mutants in N. crassa (Bertrand et al, 1980; Gross et al, 1981) and Podospora anserina (Stahl et al, 1978; Cummings et al, 1979a). It has been proposed that the defective mtDNA can out-replicate the normal mtDNA; the defective mtDNA replaces the normal mtDNA, resulting in defective mitochondrial function and death (Lazdins and Cummings, 1982). The accumulation mechanism of the aberrant mtDNA in kalilo senescent Neurospora has not been clear. There are basically two possible explanations. One is that the mtDNA with

insert kalDNA replicates faster than the mtDNA without the kalilo insert, and the mtDNA with IS-kalDNA replaces the normal mitochondrial genomes during the kalilo senescence process (Bertrand et al, 1986). The other is that the kalilo plasmid inserts into the mitochondrial genomes somehow faster at the late stages of the senescence process, resulting in a high level of the abnormal mtDNA. It has been shown that the intracellular location of the kalilo plasmid is within mitochondria (Myers et al, 1990) and the abnormal mtDNA is generated by the plasmid inserting into the mitochondrial genomes. Even though two possible explanations for the accumulation of the abnormal mtDNA have been proposed on limited studies, several questions still remain unknown: Does the kalilo plasmid continue its insertion activities after its first insertion? Do the mitochondrial genomes with IS-kalDNA replicate? Do the mitochondrial genomes with IS-kalDNA replicate like normal ones, and is their replication faster or slower than the normal ones? These questions should be answered before we can understand the mechanism of the accumulation of the abnormal mitochondrial genomes. The following kinetic studies of the AR and IS forms of kalDNA were designed to help understand the relationship of AR and IS forms of kalDNA during the senescence process, and the accumulation of the abnormal mitochondrial genomes.

The intracellular location of kalilo plasmid had been a mystery for several years. Bertrand (1986) reported that AR-kalDNA was nuclear or non-mitochondrial, but the kalilo senescence and the plasmid are inherited cytoplasmically (Rieck et al, 1982; Bertrand et al, 1986). Recently, however, Myers et al (1990) have reported that the plasmid is entirely mitochondrial. The reason for the confusion as to the cellular location of the kalilo plasmid is due to the presence of its 5' terminal proteins on the ends of the linear plasmid (Vierula et al, 1990). The linear plasmid was not discovered in the mitochondria because mtDNA is

not routinely proteinased during the isolation procedure. Most of the plasmid with its terminal proteins was eliminated from mtDNA preparations during the phenol/chloroform precipitation. There was still some AR-kalDNA at a very low level in the mtDNA preparations because its terminal proteins were broken or dropped from its ends during the mtDNA preparation, and this led to its discovery within mitochondria. There was some AR-kalDNA with its terminal proteins in the mtDNA preparation, and the AR-kalDNA with its terminal proteins did not move into the agarose gel during electrophoresis, rather it stayed in the well positions. This retardation phenomenon was observed in the mtDNA preparations without the proteinase K treatment or with proteinase K treatment but the digestion was incomplete (Bertrand et al. 1986; Vierula et al. 1990; observation). The linear plasmid location was incorrectly assigned to the non-mitochondrial compartment because of the characteristics of the various DNA and organelle isolation procedure: while it is possible to isolate nuclei-free mitochondria through the sucrose gradient procedure, it is not possible to isolate mitochondria-free nuclei (Bertrand et al, 1986). The standard protocol for the isolation of Neurospora nuclear DNA has an overnight proteinase K treatment that is capable of liberating the linear plasmid from the contaminating mitochondria.

In these kinetic studies, the mitochondrial AR-kalDNA and IS-kalDNA were quantified throughout the senescence process in Neurospora. The studies indicate that AR-kalDNA increases at early stages of senescence, reaches a peak and then decreases at a late stage when the senescent strains are close to death. IS-kalDNA appears at late stage, then increases steadily to its highest level at the latest stages of senescence. The other circular plasmids show kinetics paralleling to those of AR-kalDNA.

RESULTS

It was known that when kalilo senescent strains were used as female parents in crosses with non-senescent strains as male parents, ascospores from these crosses had different life spans. Some of the ascospores stopped vegetative growth within several subcultures, and some stopped vegetative growth only after more than 20 subcultures. In order to observe the changes of kalDNA, several representative ascospore series with long life spans were chosen for the kinetic studies. N. intermedia C4, C13, and C16 are ascospore series from the cross P561 x 1766 (Griffiths and Bertrand, 1984).

The kalilo plasmid does not have any Pst I-cut site. MtDNA digested with Pst I generates two kinds of DNA fragments which hybridize with kalDNA probe. The first one is AR-kalDNA, with molecular weight of 8.6 kb. The other is IS-kalDNA, larger than AR-kalDNA because it contains some mtDNA fragments adjacent to the insertion sites. Several mtDNA fragments were chosen as controls of the copy number of mitochondrial genomes. The quantities of kalDNA were measured by ratios of the copy number of kalDNA to that of the mitochondrial genomes. The relative quantities of DNA fragments were determined by scanning photographs and autoradiographs with a densitometer.

2.1 KINETICS OF MITOCHONDRIAL kalDNA

2.1.1 Series C4

In the original ascospore series C4 from the cross P561 x 1766, which died in 26 subcultures, some subcultures had been lost. When the early subcultures were subcultured

to fill the missing ones, they stopped vegetative growth before subculture 26. In most cases of subculturing, there were differences of several subcultures between the old and the new, and among the new ones. New subculture series were made by subculturing from the earliest subculture C4-1. Several new replicas of this series have 16 to 22 subcultures. One of the replicas with 18 subcultures was chosen to study the kinetics of kalDNA. MtDNA of subcultures C4-2, -4, -6, -8, -10, -12, -14, -16 and the non-senescent control P605 was prepared, and digested with restriction enzyme Pst I. The EtBr-stained gel (Figure 20) shows that the control, P605, contains more than 20 fragments in equal molarity, the largest one, Pst I-1, is 10.7 kb, and Pst I-3 and -4 are 5.4 kb. The profiles of Pst I digested mtDNAs of the controls N. intermedia and N. crassa (P605 in Figure 20 and 21, BC 4-1-20 in Figure 10) are different. MtDNA fragment Pst I-1 of N. intermedia is 10.7 kb, larger than AR-kalDNA, while Pst I-1 of N. crassa is 7.5 kb, smaller than AR-kalDNA. MtDNA from subcultures of series C4 contains several more fragments than the control. All mtDNA from subcultures C4-2, -4, -6, -8, -10, and -12 contain a fragment of 8.6 kb which increases in intensity in these subcultures, a faint fragment of 6.2 kb and a fragment of 5.0 kb in high quantity. MtDNA of subculture C4-14 contains one fragment of 9.5 kb more than that of its early subcultures. In mtDNA of subculture C4-16, its Pst I-1 fragment is at higher level than it should be compared with other mtDNA fragments. The new fragment observed in C4-14 still remains, but the 8.6 kb fragment is in lesser quantity and so are the other two small fragments of 6.2 and 5.0 kb, which are not observed in the control.

The autoradiograph (Figure 20) of the gel to the kalDNA fragment B3 (see its restriction map on page 24) shows that the 8.6 kb fragment observed in series C4 is the AR form of the plasmid. The 9.5 kb fragment observed in C4-14 and -16 is IS-kalDNA which first

appears in C4-12 (undetected in the EtBr-stained gel), and persists in late subcultures. The Pst I-1 fragment in C4-14 and -16 hybridized with the kalDNA probe, indicating that a new IS-kalDNA is generated in subcultures C14-14 and -16, and which co-migrates with mtDNA fragment Pst I-1. There are three or four large fragments of 40-50 kb in all subcultures of series C4. A faint hybridization band is observed below the AR-kalDNA band. The two small fragments of 6.2 and 5.0 kb do not hybridize with the kalDNA probe indicating that they are fragments derived from other plasmids.

The transparent, positive photograph of the EtBr-stained gel and the autoradiograph were used to measure the intensity of relevant fragments by means of a densitometer (Figure 21). The mtDNA fragments Pst I-1, -3, and -4 were used as control for the copy number of mitochondrial genomes. The quantities of AR-kalDNA and IS-kalDNA were measured by ratios of copy number of kalDNA to that of mtDNA control fragments. The measurements of kalDNA (Table 1, Figure 22) show that generally AR-kalDNA increases from subcultures 2 to 12, then decreases from subcultures 12 to 16, but there is variation in the quantity of AR-kalDNA in some subcultures. Before reaching a peak in subculture 12, the amount of AR-kalDNA in subculture 4 is less than that of subculture 2. In general, the quantity of AR-kalDNA increases steadily at the early stage of senescence up to a peak in a subculture several away from the death subculture, and then decreases drastically in the last subculture where DNA could be prepared. Comparable quantitative changes of AR-kalDNA and IS-kalDNA in this series have been observed in other duplicate mtDNA analyses of the series (data not shown). The first species of IS-kalDNA (9.5 kb) appears in subculture 12; which is maintained, increases in late subculture 14 and then decreases in subculture 16. The second species of IS-kalDNA (10.7 kb), co-migrating with mtDNA Pst I-1 fragment, appears in subculture 14 and increases in quantity in subculture 16 where

DNA could be prepared. IS-kalDNA appears in the subculture where AR-kalDNA peaks, and increases steadily in quantity until the last analyzable subculture.

The two small fragments of 6.2 kb and 5.0 kb are observed in mtDNA of all subcultures of series C4 but not in the control mtDNA, and do not hybridize with the kalDNA probe. The 6.2 kb fragment is in copy number less than that of mtDNA fragments, the 5.0 kb fragment is in copy number much higher than that of mtDNA fragments. They are not mitochondrial genomic fragments, rather they are fragments of other mitochondrial plasmids. These plasmids are investigated in another paper.

There are three or four giant fragments (40-50 kb) at very low level (undetectable in the EtBr stained gel), which hybridized with the kalDNA probe in all subcultures of series C4 (in the original autoradiograph). It is reasonable to believe that these giant fragments are not different species of IS-kalDNAs. Because the largest mtDNA fragment produced by Pst I digestion is 10.7 kb, if AR-kalDNA inserts into this largest fragment Pst I-1 and generates different inverted repeats of mtDNA, the Pst I digested fragments containing kalDNA and some mtDNA fragments will arrange from 9.0 to 30.0 kb. But quantification confirms that the Pst I-1 fragment is in equal copy number with other mtDNA fragments. These giant fragments could be different concatamerizations of AR-kalDNA. The structure of these kalDNA-related giant fragments are investigated in another paper.

There is a faint hybridization band of 7.0 kb in all subcultures of series C4, not detected in the EtBr-stained gel. This fragment could be a small plasmid at very low level, sharing some homologous regions with the kalilo plasmid. But from the smearing tail below the band and other similar observation, the most likely possibility is that this fragment is

produced by degradation of the kalilo plasmid. The reason for such degradation is that mtDNA preparation was routinely treated with proteinase K overnight after lysis of mitochondria. Some kind of exonucleases in mitochondria could be released and digest AR-kalDNA during the overnight incubation. This conclusion was confirmed by 3' and 5' exonucleases digestion of uncut mtDNA in which this band disappeared (not shown).

2.1.2 Series C16

The original ascospore series C16 from the cross P561 x 1766 died within 19 subcultures. When replicas of the series were made for kinetic studies, they were one or two subcultures different from the original one. One replica with 18 subcultures was chosen for kinetic studies. MtDNAs were prepared from even numbered subcultures and the control P605, and digested with restriction enzyme Pst I. The EtBr-stained gel (Figure 23) shows that the control P605 contains about 20 fragments, most fragments are in equal molarity but the Pst I-2 fragment is in lower molarity, which is derived from another plasmid. MtDNA from subculture C16-2, -4, -6, -8, and -10, contains two more fragments in different quantities, the large one is 8.6 kb and the small one is 5.0 kb. They are also observed in later subcultures C16-12, -14, and -16. They are the same fragments observed in series C4. MtDNA from subcultures C16-12, -14, and -16 contains one large fragment (13.0 kb) more than their earlier subcultures, and their mtDNA fragment Pst I-1 is at a higher quantity than those in the control and their earlier subcultures.

The autoradiograph (Figure 23) shows that several mtDNA fragments hybridized with the kalDNA fragment B3 in subcultures of series C16, but not in the control P605. The 8.6 kb fragment is AR-kalDNA observed in all subcultures of the series. There are three species

of IS-kalDNAs in subcultures C16-12, -14, and -16. The largest one (13.0 kb) is observed first in subculture 12, and increases steadily in quantity in the late subcultures 14 and 16. The second largest fragment (10.7 kb) co-migrating with mtDNA fragment Pst I-1 is observed first in subculture 12, and increases in quantity in late subcultures. The third IS-kalDNA (9.0 kb) which is not observed in the EtBr-stained gel appears first in subculture 12 and increases steadily in quantity in the late subcultures 14 and 16. There is a hybridization band (7.0 kb) with a smearing tail in all subcultures of the series. There are three or four giant fragments with molecular weight of 40-50 kb in very low quantity in all subcultures of the series, but they are not observed in the EtBr-stained gel. The two small 6.2 kb and 5.0 kb fragments do not hybridize with the kalDNA probe, indicating that they are fragments of other mitochondrial plasmids. They are investigated in another paper.

Measurements (Table 2, Figure 24) of relevant DNA fragments were done as in series C4. The quantity of AR-kalDNA shows variation in early subcultures and late subcultures. There is a peak of AR-kalDNA in subculture C16-14, then the quantity of AR-kalDNA decreases drastically in the last analyzable subculture. The 13.0 kb IS-kalDNA increases steadily, the 9.0 kb IS-kalDNA increases steadily too but still at very low level. The 10.7 kb IS-kalDNA increases drastically from subculture 12 to 14, but decreases a little in subculture 16. The total quantity of IS-kalDNAs increases by subculture. This result demonstrates that in general, the quantity of AR-kalDNA increases to a peak in late subculture and then decreases in the subculture close to death, whereas IS-kalDNA increases steadily until death. Similar quantitative changes of AR-kalDNA and IS-kalDNA in this series have been observed in other duplicate mtDNA analyses of the series (data not shown).

The copy number ratios of IS-kalDNA to mitochondrial restriction fragments are more than 1.0 in several subcultures of series C4 and C16, indicating that the copy number of IS-kalDNAs is more than that of mitochondrial genomes. If AR-kalDNA inserts into all mitochondrial genomes at one site, the maximal copy number ratio of an IS-kalDNA to mitochondrial genomes should be 1.0, not more than 1.0. This could be explained that AR-kalDNA inserts into several sites of mitochondrial genomes, and the abnormal mitochondrial genomes were cut by Pst I to generate close molecular weights of these kalDNA-mtDNA fragments, which then co-migrated to the same or very close position under electrophoresis. The second explanation for this phenomenon is that AR-kalDNA inserts into mtDNA at sites which are close to the inverted repeats of mtDNA generated by an earlier insertion. This could generate inverted repeats covering the original IS-kalDNA regions, so there are three copies of IS-kalDNA in one mitochondrial genome (Figure 25). Two of them have identical terminal inverted repeats of mtDNA. If there are recognition sites in their inverted repeats, restriction enzyme Pst I digestion will generate two identical copies of IS-kalDNA-mtDNA. If there is not a recognition site, Pst I digestion will generate a large fragment of kalDNA-mtDNA with three copies of IS-kalDNA. If AR-kalDNA inserts into each mitochondrial genome at such several sites, there will be a much higher ratio of IS-kalDNAs to mitochondrial genomes.

The original autoradiograph of this series shows that there are three or four giant DNA fragments (40 to 50 kb) in some mtDNA preparations, which are not detected in the EtBr-stained gel, but are detected by the radioactively labelled kalDNA fragment B3, and the same phenomenon was observed in series C4. These giant kalDNAs could be incompletely digested mitochondrial genomes with IS-kalDNA, but incompletely digested large DNA fragments generally give a smearing appearance on the gel, so then this explanation is

unlikely. These several giant bands containing kalDNA fragments gave clear signals over their background. The control mitochondrial genomes digested with the same restriction enzyme did not produce these giant DNA fragments. So it is reasonable to believe that they were generated by the multiple, close insertion of AR-kalDNA or different concatamerization of AR-kalDNA. The structures of these giant kalDNA fragments is investigated in another paper.

The 7.0 kb small fragments with a smearing tail observed on the autoradiograph is the same phenomenon observed in series C4. Its smearing tail supports the explanation that it was derived from degradation of the AR form of the plasmid during the overnight incubation with proteinase K.

2.1.3 Series C13

The original series C13 from the cross P561 x 1766 had 27 subcultures. The late subcultures had been lost. When the missing subcultures were filled by subculturing from early subculture, vegetative death occured much earlier than in the original series, giving new series with 12 to 16 subcultures. So this series for kinetic studies was initiated by subculturing from the original first subculture, which gave a new series with 12 subcultures.

MtDNAs were prepared from subcultures C13-2, -4, -6, -8, -10, and the control P605, and digested with restriction enzyme Pst I. The EtBr-stained gel (Figure 26) shows that the control P605 contains about 20 DNA fragments in equal molarity. All subcultures of series C13 contain more three fragments, and their molecular weights are 8.6, 6.2 and 5.0 kb.

Subculture C13-6 contains two more large fragments (10.2 kb and 11.5 kb); these two large fragments are not observed in the late subcultures C13-8 and -10. Rather, subcultures C13-8 and -10 contain a new large fragment (13.0 kb), and their mtDNA fragments Pst I-1 are in higher copy number compared with other mtDNA fragments. The autoradiograph shows that the 8.6 kb fragment is AR-kalDNA, the 10.2 kb and 11.5 kb large fragments in subculture C13-6 are different species of IS-kalDNAs. The 13.0 kb fragment in subcultures C13-8 and -10 is IS-kalDNA and a second species of IS-kalDNA in these two subcultures co-migrates with mtDNA fragment Pst I-1. There are two or three 40-50 kb giant fragments at very low level in all subcultures of this series (at highest level in subculture C13-8), which hybridized with the kalDNA probe.

The same techniques were used to measure the copy number ratios of the relevant fragments. The results (Table 3, Figure 27) shows that AR-kalDNA maintains a relatively constant level in the early subcultures C13-2, -4 and -6, reaches its highest level in subculture C13-8, then drops to a lower level in the last subculture where DNA could be prepared. IS-kalDNA appears at low level in subculture C13-6, then increased steadly in the late two subcultures. Similar quantitative changes of AR-kalDNA and IS-kalDNA in this series have been observed in other duplicate mtDNA analyses of the series (data not shown).

The three or four giant fragments found in the other two series are observed in this series too. These giant fragments are at their highest level in subculture C13-8 where AR-kalDNA is at its highest level (21.5 copies of AR-kalDNA per mitochondrial genome), while the ratios of two different IS-kalDNA to the mitochondrial genome are 0.31 and 0.68

respectively. These results support the idea that these giant kalDNA fragments are different concatamerization of the kalilo plasmid.

The ratio of the IS-kalDNA (10.7 kb) to mitochondrial genome in the latest subculture is 1.4, more than 1.0. This phenomenon could have resulted from several different insertion sites of mitochondrial genomes generating close molecular weights of kalDNA-mtDNA fragments, or from close, multiple insertions of the kalilo plasmid.

One interesting phenomenon found in this series is that the two inserts of IS-kalDNA found in subculture C13-6 are not detected or are at an undetectable level in the later two subcultures C13-8 and -10. Nonetheless, two new inserts of IS-kalDNA are detected. This result suggests that these early insertions of AR-kalDNA do not give a replicative advantage to the mitochondrial genomes bearing them. In other words, the abnormal mitochondrial genomes with IS-kalDNA do not replicate faster than the normal mitochondrial genomes. Otherwise, the early two inserts should reach higher levels in the later subcultures (Myers, 1988).

Analyses of other senescent series of N. intermedia and N. crassa gave similar results (not shown). In these studies of kalDNA kinetics, the general trend is that AR-kalDNA increases in early subcultures, reaches a peak in a late subculture and then decreases in the later subcultures close to the vegetative death. IS-kalDNA appears around the peak subculture of AR-kalDNA, increases steadily in the later subcultures, and reaches the highest level in the last analyzable subculture close to death.

2.2 KINETICS OF OTHER MITOCHONDRIAL PLASMIDS

There are several other mitochondrial plasmids not related to senescence in all progeny from the cross P561 x 1766 (the 6.2 and 5.0 kb fragments in Figure 20 and 23). They were inherited from the female parent P561 and not the male parent 1766. One of them is a circular plasmid, K2, which has different levels of concatamerization with a monomer of 5.0 kb and is not homologous to kalDNA. Pst I digestion of this plasmid and its derivatives produces a 5.0 kb fragment at a very high level (structural analysis in other paper).

The fate of these cryptic plasmids during senescence is of interest. The 5.0 kb fragment of the circular plasmids in series C16 was chosen for detailed kinetic studies. The EtBr-stained gel of series C16 (Figure 23) shows that there is a 5.0 kb fragment (labelled by an arrowhead) at a high level in all subcultures of the series. The autoradiograph (Figure 23) shows that this 5.0 kb fragment is not homologous with the kalDNA. MtDNA fragments Pst I-3 and -4 were used as copy number controls for mitochondrial genomes and the same techniques were used to measure the quantity of the 5.0 kb fragment. The results (Table 4, Figure 28) show that the 5.0 kb fragment of the circular plasmid increases in quantity from subculture C16-2 to C16-14 and then drops to a lower level from subculture C16-14 to C16-16. The quantitative changes of the circular plasmid K2 parallel the changes of the kalilo plasmid.

Figure 29 is the EtBr-stained gel of uncut mtDNA from another series C16 replica. There are three giant circular plasmids and three small plasmids. The 8.6 kb fragment is AR-kalDNA, the K2 band is a circular plasmid, the 4.0 kb fragment is an unidentified plasmid. The three giant plasmids are different concatamerizations of the 5.0 kb circular plasmid K2

(structural details in another paper). This result demonstrates that all the plasmids show changes parallel with the kalilo plasmid during the senescence process.

Figure 30 is the EtBr-stained gel of uncut mtDNA from another series C4 replica with 22 subcultures. There are three giant circular plasmids and three small plasmids in all subcultures of the series but not in the control P605. The 8.6 kb fragment is AR-kalDNA. The 5.3 kb band is the circular plasmid K2 which gives rise to the other four plasmids. Again this series demonstrates that all other plasmids show similar patterns of changes to the kalilo plasmid during the senescence process.

These results suggest that replication of linear and circular DNA plasmids require some common factors from their mitochondria. When the function of mitochondria is destroyed or disordered by the insertion of AR-kalDNA into their genomes, the quantity of the common factors become lesser and lesser, so replication of these plasmids slows down to lower level, the quantity of plasmids is reduced in subcultures close to vegetative death.

Figure 20. Gel electrophoresis analysis of Pst I-digested mtDNAs from various subcultures of series C4 derived from ascospore 4 of the cross P561-1 x 1766. The first lane of the ethidium bromide stained gel and autoradiograph represents the nonsenescent control P605. The numbers above the remaining lanes represent the subcultures of the series from which mtDNA was prepared. The fragments labelled 1, 2 and so on are mtDNA-Pst I fragments of the control P605. The autoradiograph shows the bands hybridizing with the kalilo DNA fragment B3. The bands labelled G1, G2 and G3 are giant DNA fragments containing kalDNA. The strong band labelled AR-kalDNA is the kalilo plasmid. The band labelled "-" is degraded form of AR-kalDNA. Other labelled bands between the giant fragments and AR-kalDNA are different species of IS-kalDNAs.

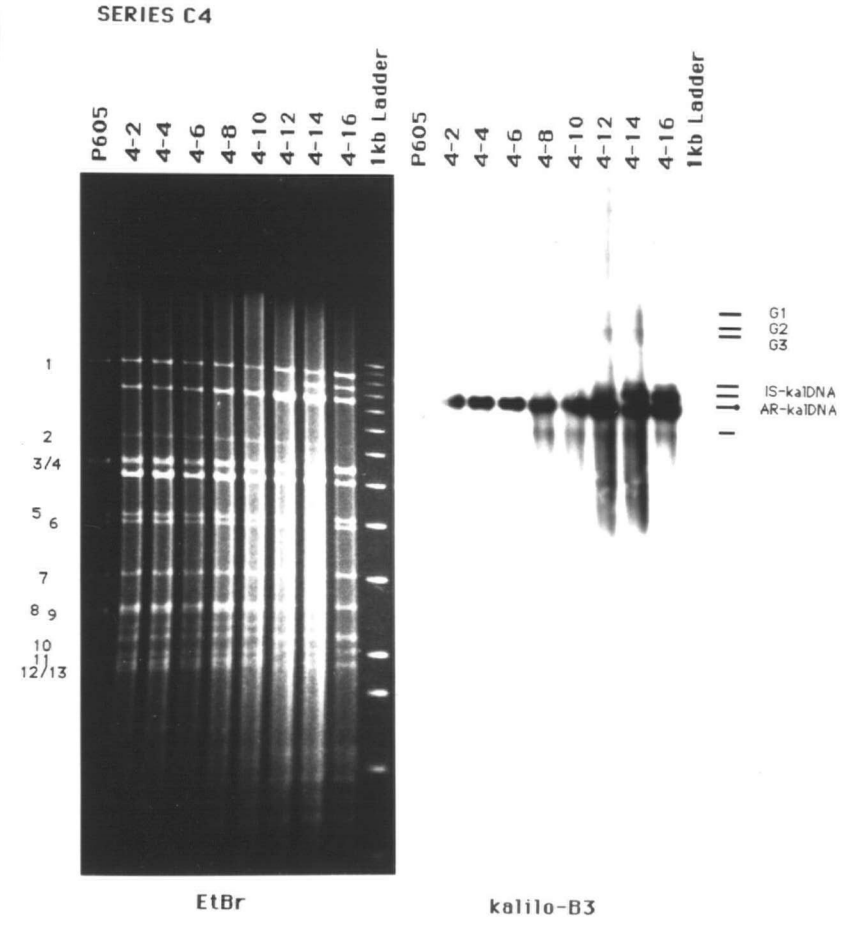

SERIES C4
P605
4-2
4-4
4-6
4-8
4-10
4-12
4-14
4-16
1kb Ladder
P605
4-2
4-4
4-6
4-8
4-10
4-12
4-14
4-16
1kb Ladder
1
2
3/4
5
6
7
8
9
10
11
12/13
G1
G2
G3
IS-kalDNA
AR-kalDNA
EtBr
kalilo-B3

Figure 21. Densitometer spectra of Pst I digested mtDNA from the second subculture of series C4 from ascospore 4 of the cross P561 x 1766. The relative copy numbers of interested fragments were calculated basing on the areas they cover.

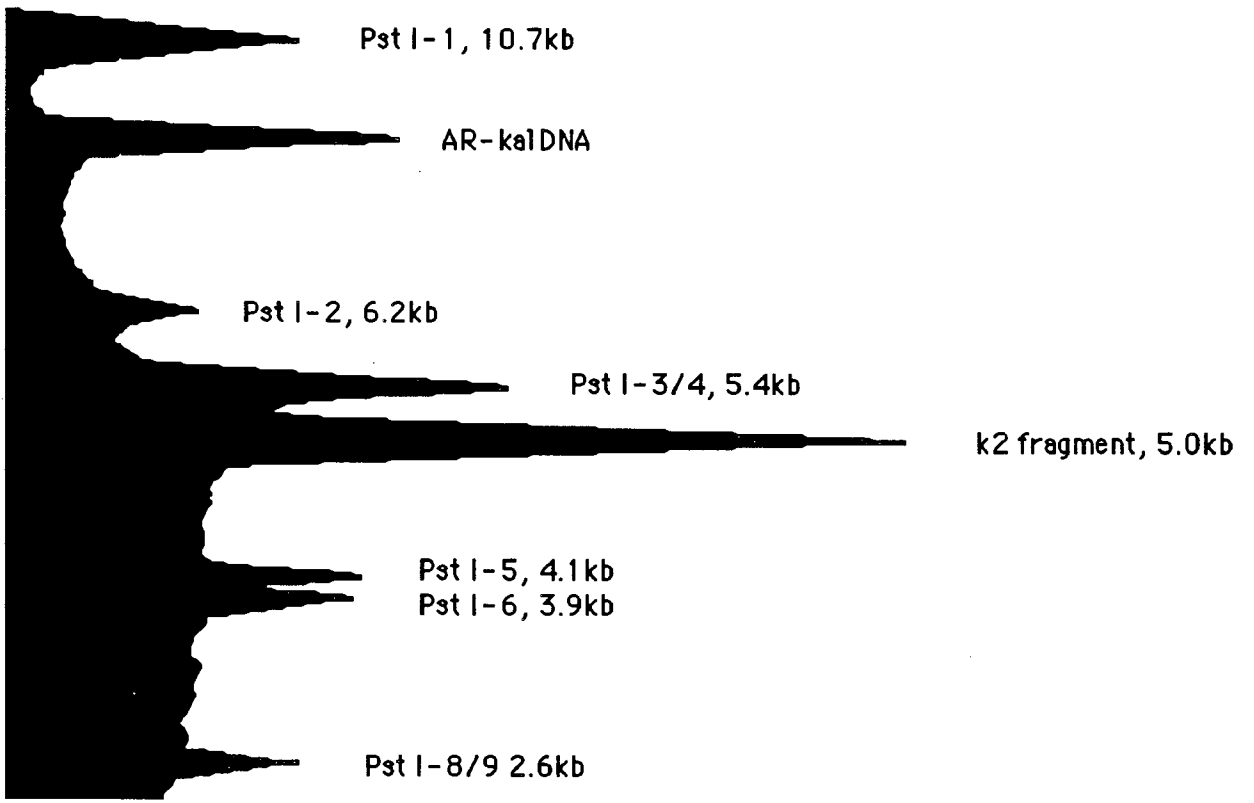

Pst I-1, 10.7kb
AR-kalDNA
Pst I-2, 6.2kb
Pst I-3/4, 5.4kb
k2 fragment, 5.0kb
Pst I-5, 4.1kb
Pst I-6, 3.9kb
Pst I-8/9 2.6kb

Table 1. The relative quantity of kalDNA in Series C4

Subcultures	2	4	6	8	10	12	14	16
AR-kalDNA	1.31	0.94	1.37	2.08	2.76	10.0	8.03	3.56
Total IS-kalDNA						0.20	2.13	2.41
(9.5 kb IS-kalDNA)						0.20	1.65	1.09
(10.7 kb IS-kalDNA)						0.0	0.48	1.32

Figure 22. Quantitative changes of kalDNA in senescent series C4 derived from ascospore 4 of the cross P561 x 1766. In this figure the relative copy numbers of kalDNA are ratios of kalDNA copy numbers over mitochondrial genomic numbers. The numbers below bars are various subcultures of the series from which mtDNA was prepared. The solid bars represent the quantities of AR-kalDNA, and the crossed bars represent the quantities of total IS-kalDNAs.

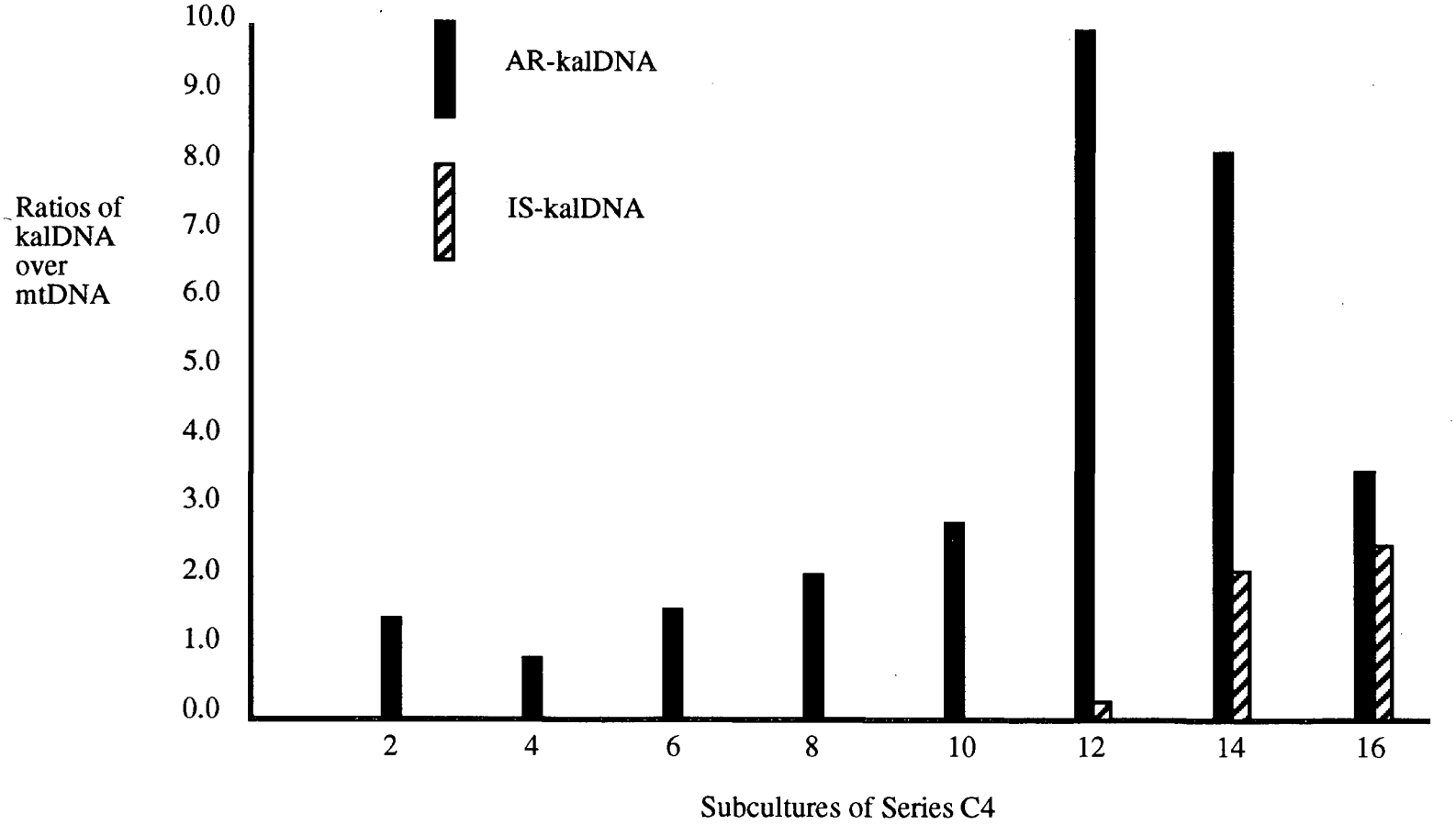

10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Ratios of kalDNA over mtDNA
AR-kalDNA
IS-kalDNA
2
4
6
8
10
12
14
16
Subcultures of Series C4

Figure 23. Gel electrophoresis analysis of Pst I-digested mtDNAs from various subcultures of series C16 derived from ascospore 16 of the cross P561-1 x 1766. The first lane of the ethidium bromide stained gel and autoradiograph represents the nonsenescent control P605. The numbers above the remaining lanes represent the subcultures of the series from which mtDNA was prepared. The fragments labelled 1, 2 and so on are mtDNA-Pst I fragments of the control P605. The autoradiograph shows the bands hybridizing with the kalilo DNA fragment B3. The bands labelled G1, G2 and G3 are giant DNA fragments containing kalDNA. The strong band labelled AR-kalDNA is the kalilo plasmid. The band labelled "-" is a degraded form of AR-kalDNA. Other labelled bands between the giant fragments and AR-kalDNA are different species of IS-kalDNAs.

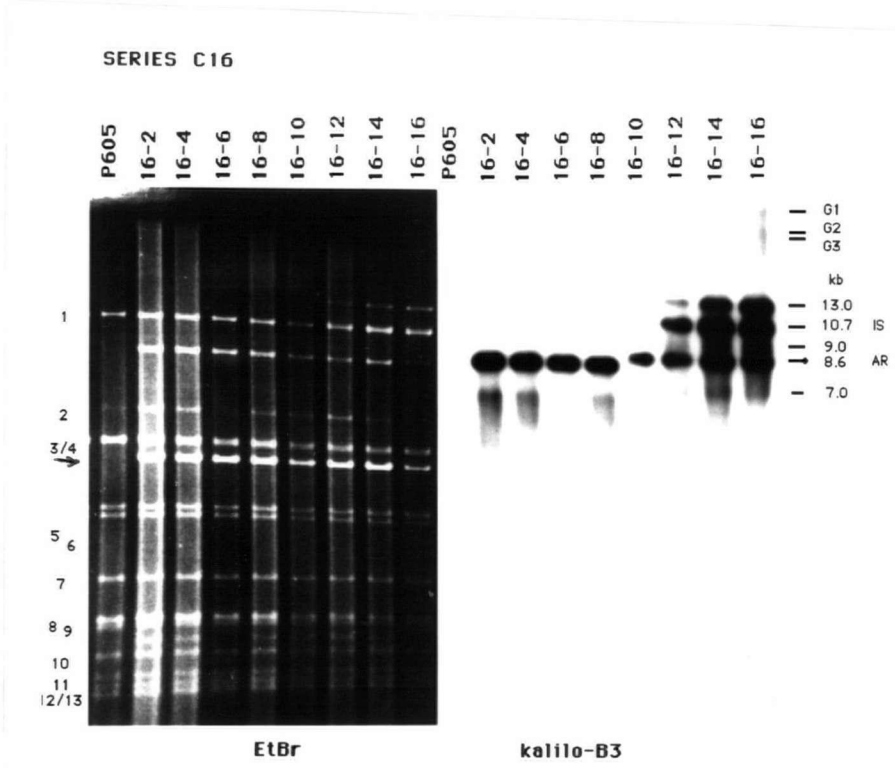

SERIES C16
P605
16-2
16-4
16-6
16-8
16-10
16-12
16-14
16-16
P605
16-2
16-4
16-6
16-8
16-10
16-12
16-14
16-16
1
2
3/4
5
6
7
8
9
10
11
12/13
G1
G2
G3
kb
13.0
10.7
IS
9.0
8.6
AR
7.0
EtBr
kalilo-B3

Table 2. The relative quantity of kalDNA in Series C16

Subcultures	2	4	6	8	10	12	14	16
AR-kalDNA	1.74	1.57	1.28	1.32	1.48	1.07	1.86	0.21
Total IS-kalDNA						0.77	2.51	2.87
(9.0 kb IS-kalDNA)						+	+	+
(10.7 kb IS-kalDNA)						0.62	2.08	1.90
(13.0 kb IS-kalDNA)						0.15	0.43	0.97

Figure 24. Quantitative changes of kalDNA in senescent series C16 derived from ascospore 16 of the cross P561 x 1766. In this figure the relative copy numbers of kalDNA are ratios of kalDNA copy numbers over mitochondrial genomic numbers. The numbers below bars are various subcultures of the series from which mtDNA was prepared. The solid bars represent the quantities of AR-kalDNA, and the crossed bars represent the quantities of total IS-kalDNAs.

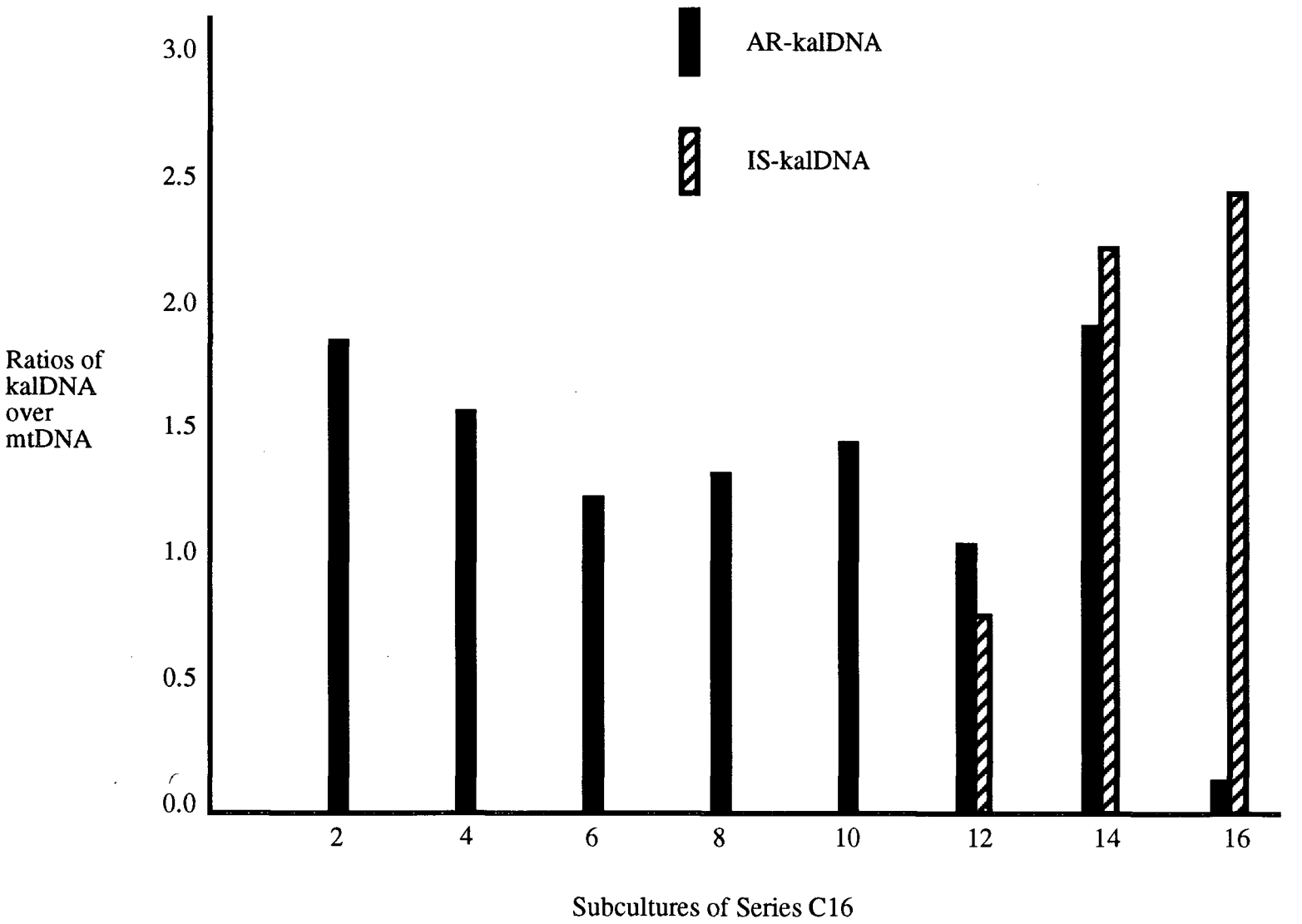
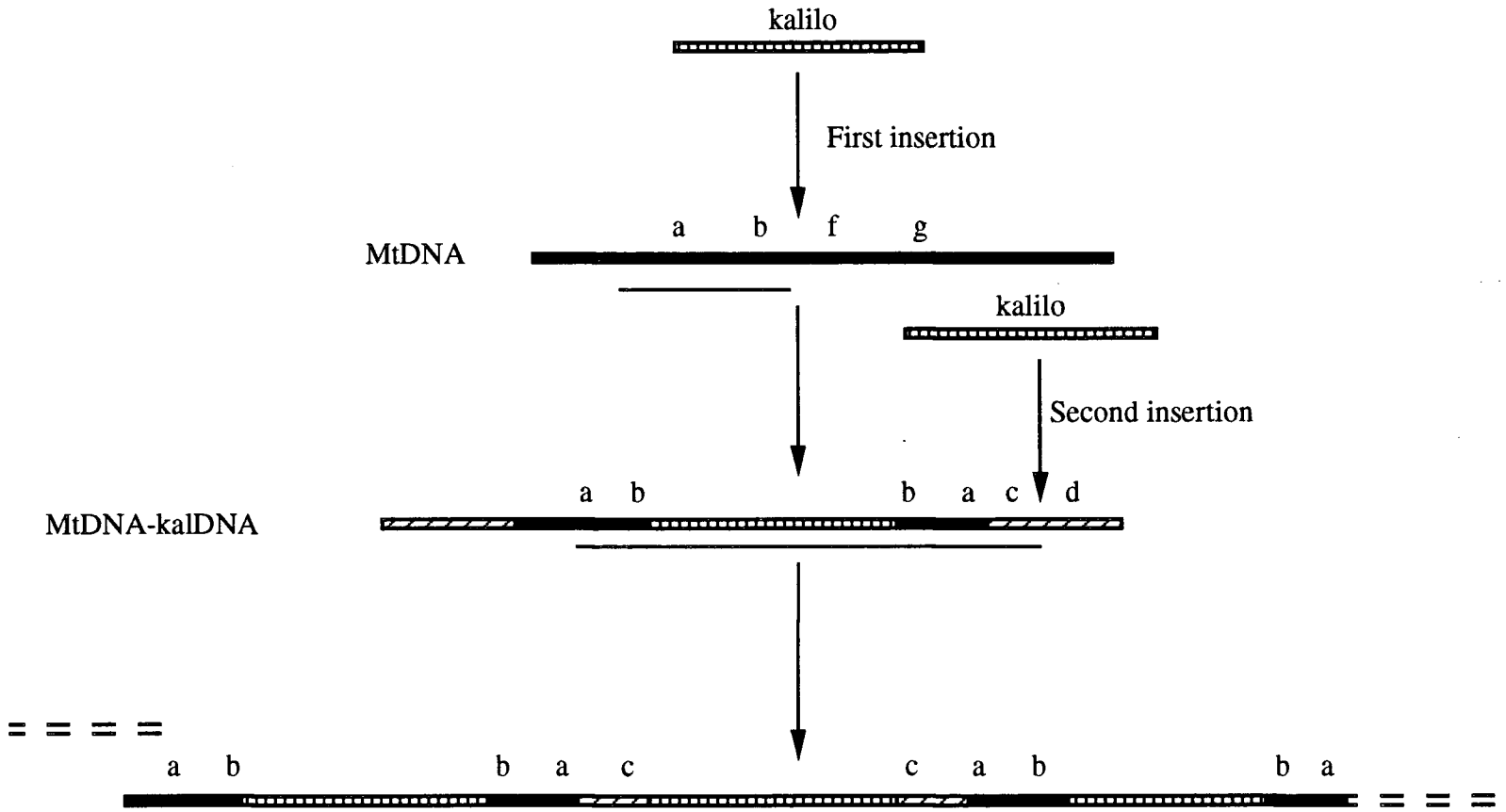

AR-kalDNA
IS-kalDNA
Ratios of kalDNA over mtDNA
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2
4
6
8
10
12
14
16
Subcultures of Series C16

Figure 25. Model for the multiple insertions of kalilo into mtDNA. AR-kalDNA, the free linear plasmid, is represented as a squared line. It inserts into mtDNA at the first site between b and c, and generates inverted repeats of mtDNA fragment covering a and b (solid line). It inserts into mtDNA at the second site between c and d of mtDNA with the first insert, generating inverted repeats of mtDNA fragment covering the first IS-kalDNA region, resulting in three copies of kalDNA in a copy of mtDNA.



MtDNA with multiple IS-kalDNAs

Figure 26. Gel electrophoresis analysis of Pst I-digested mtDNAs from various subcultures of series C13 derived from ascospore 13 of the cross P561-1 x 1766. The first lane of the ethidium bromide gel and autoradiograph represents the nonsenescent control P605. The numbers above the remaining lanes represent the subcultures of the series from which mtDNA was prepared. The fragments labelled 1, 2 and so on are mtDNA-Pst I fragments of the control P605. The autoradiograph shows the bands hybridizing with the kalilo DNA fragment B3. The bands labelled G1, G2 and G3 are giant DNA fragments containing kalDNA. The strong band labelled AR-kalDNA is the kalilo plasmid. Other labelled bands between the giant fragments and AR-kalDNA are different species of IS-kalDNAs.

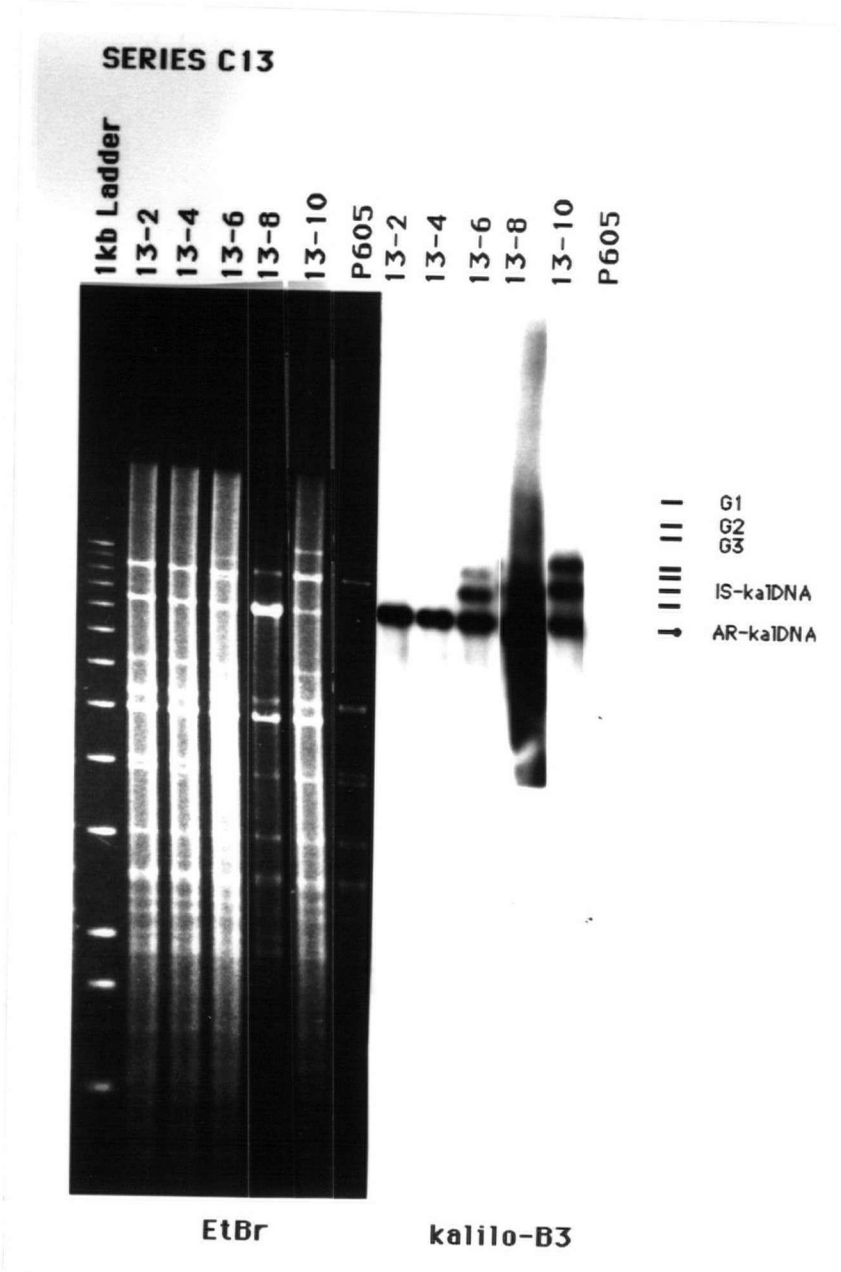

SERIES C13
1kb Ladder
13-2
13-4
13-6
13-8
13-10
P605
13-2
13-4
13-6
13-8
13-10
P605
G1
G2
G3
IS-kalDNA
AR-kalDNA
EtBr
kalilo-B3

Table 3. The relative quantity of kalDNA in Series C13

Subcultures	2	4	6	8	10
AR-kalDNA	1.04	2.17	1.36	21.5	1.80
Total IS-kalDNA			0.58	0.99	2.08
(10.2 kb IS-kalDNA)			0.40	0.0	0.0
(10.7 kb IS-kalDNA)			0.0	0.68	1.40
(11.5 kb IS-kalDNA)			0.18	0.0	0.0
(13.0 kb IS-kalDNA)			0.0	0.31	0.68

Figure 27. Quantitative changes of kalDNA in senescent series C13 derived from ascospore 13 of the cross P561 x 1766. In this figure the relative copy numbers of kalDNA are ratios of kalDNA copy numbers over mitochondrial genomic numbers. The numbers below bars are various subcultures of the series from which mtDNA was prepared. The solid bars represent the quantities of AR-kalDNA, and the crossed bars represent the quantities of total IS-kalDNAs.

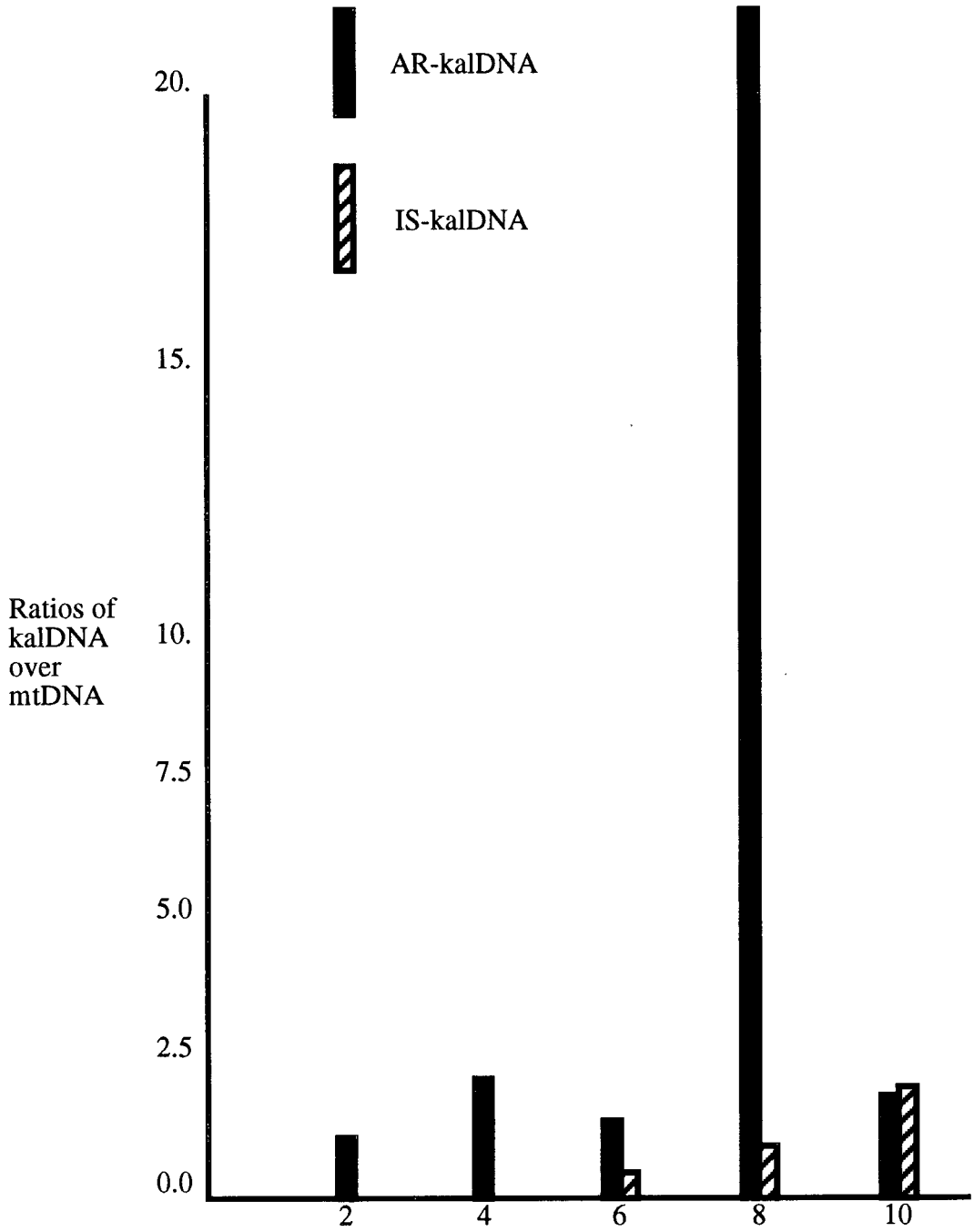

AR-kalDNA
IS-kalDNA
20.
15.
10.
7.5
5.0
2.5
0.0
Ratios of kalDNA over mtDNA
2
4
6
8
10
Subcultures of Series C13

Table 4. The relative quantity of plasmid K2 in Series C16

Subcultures	2	4	6	8	10	12	14	16
K2 fragment	2.0	2.3	3.3	3.77	5.6	5.86	6.9	3.78

Figure 28. Quantitative changes of plasmid K2 in senescent series C16 derived from ascospore 16 of the cross P561 x 1766. In this figure the relative copy numbers of K2 are ratios of K2 (5.0 kb fragment) copy numbers over mitochondrial genomic numbers. The numbers below bars are various subcultures of the series from which mtDNA was prepared.

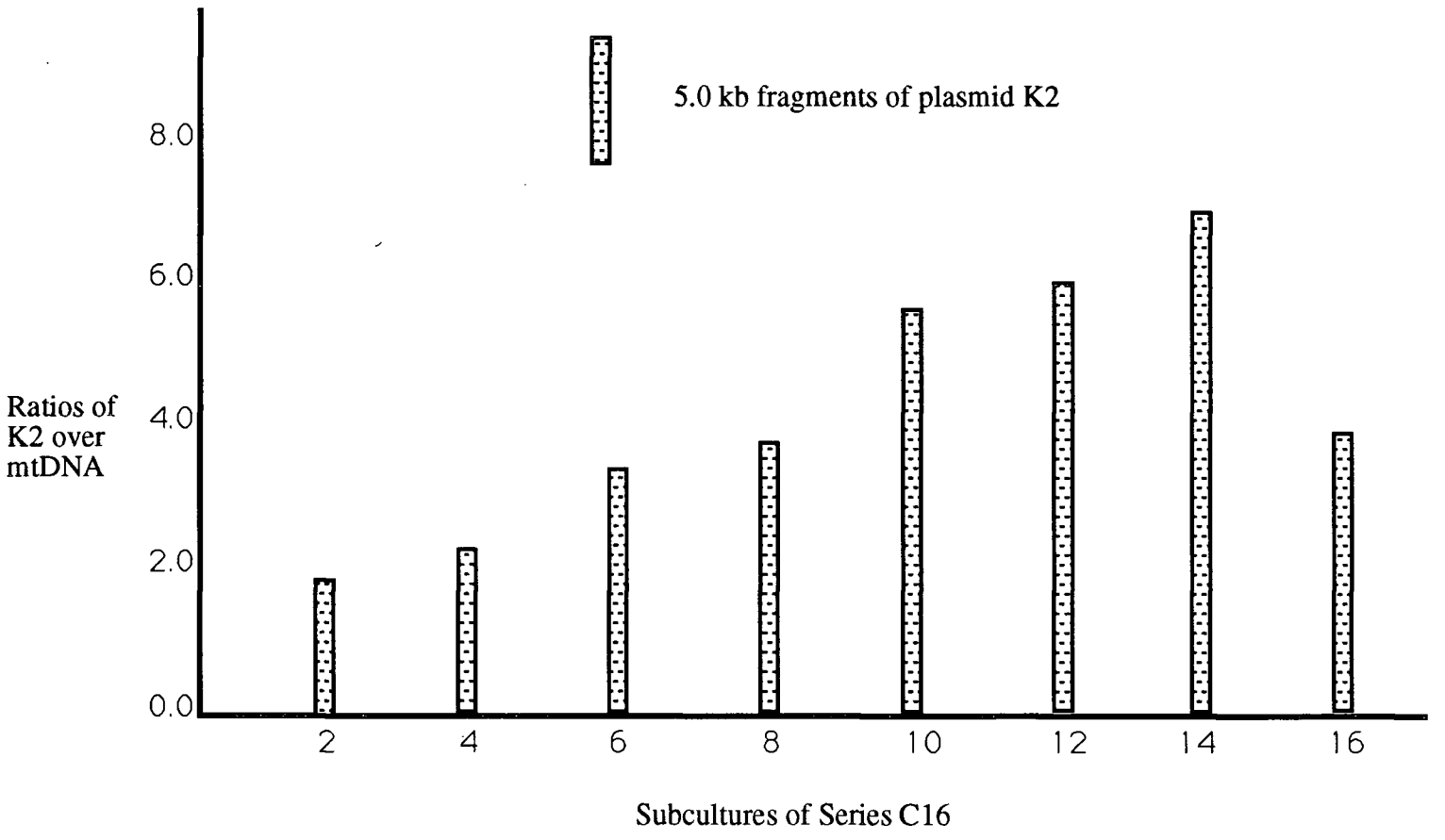
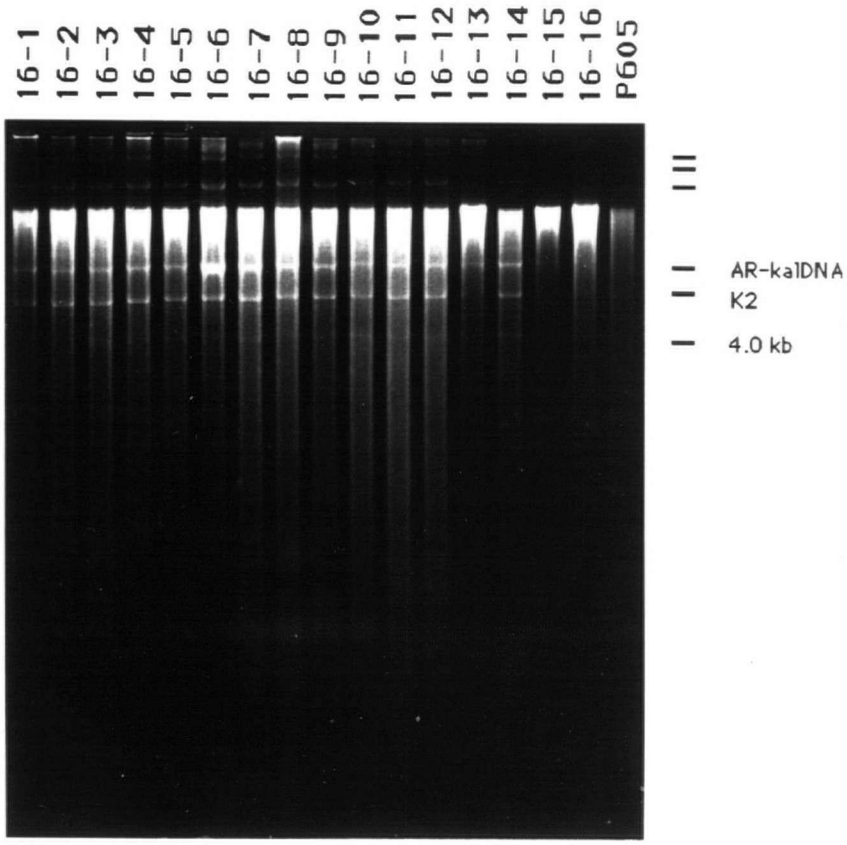

5.0 kb fragments of plasmid K2
Ratios of K2 over mtDNA
8.0
6.0
4.0
2.0
0.0
2
4
6
8
10
12
14
16
Subcultures of Series C16

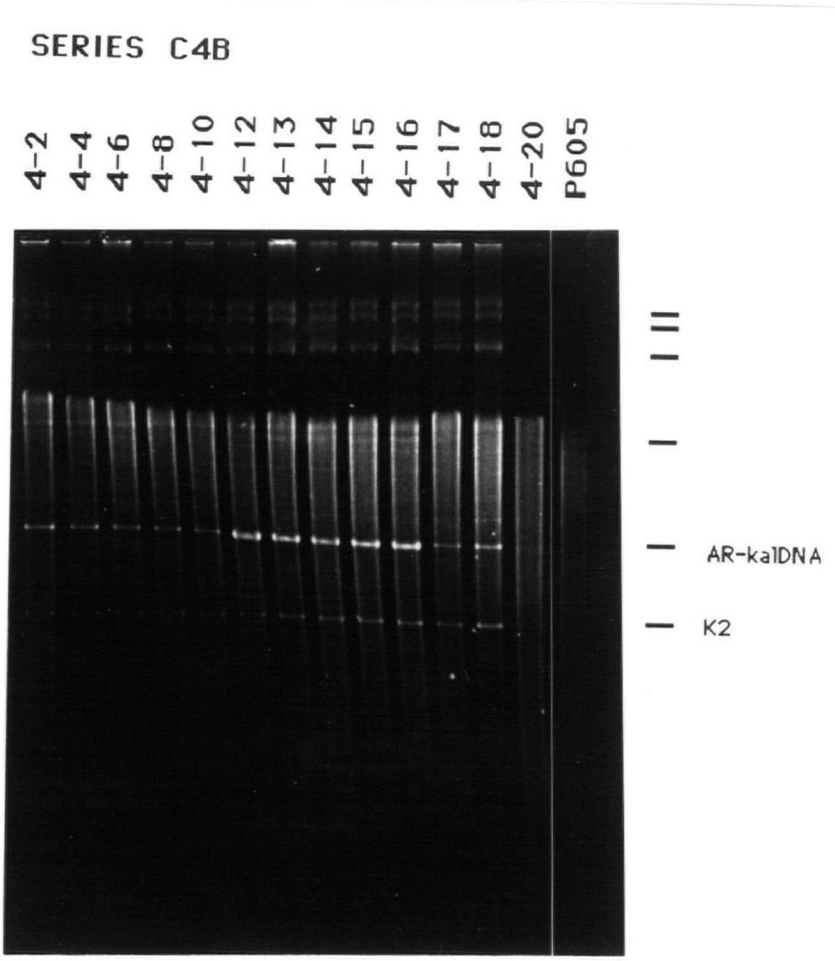
Figure 29. Gel electrophoresis analysis of uncut DNAs from various subcultures of a series derived from ascospore 16 of the cross P561 x 1766. The last lane of the ethidium bromide stained gel represents the nonsenescent control P605. The numbers above the remaining lanes represent the subcultures of the series from which mtDNA was prepared. The three labelled bands moving slower than the uncut mtDNA and the one moving faster than the uncut mtDNA are different multimeric forms of the circular plasmid K2. The band labelled "- 4.0 kb" is an unidentified plasmid.

SERIES C16B



EtBr

Figure 30. Gel electrophoresis analysis of uncut DNAs from various subcultures of a series derived from ascospore 4 of the cross P561 x 1766. The last lane of the ethidium bromide stained gel represents the nonsenescent control P605. The numbers above the remaining lanes represent the subcultures of the series from which mtDNA was prepared. The three labelled bands moving slower than the uncut mtDNA and the one moving faster than the uncut mtDNA are different multimeric forms of the circular plasmid K2.


SERIES C4B
4-2
4-4
4-6
4-8
4-10
4-12
4-13
4-14
4-15
4-16
4-17
4-18
4-20
P605
III
II
I
AR-kalDNA
K2
EtBr

DISCUSSION

The kinetic studies of mitochondrial kalDNA in senescent Neurospora strains demonstrate the general trends of kalDNA in the kalilo senescence process. These studies provide some information about the relationship of AR and IS forms of kalDNA and characteristics of their replication. AR-kalDNA is observed in all subcultures of these series, increases in quantity at early stages of senescence until a peak is reached at a late stage and then drops to a low level. IS-kalDNA is not observed at early stages, but is at late stages and increases in quantity until the last subculture where DNA could be prepared.

The appearance of IS-kalDNA at the late stages could result from two possible factors. One is that there is undetectable IS-kalDNA in the first subcultures, these IS-kalDNAs were inherited from their female parent's cytoplasm. The mitochondrial genomes with these IS-kalDNAs replicate faster than the normal mitochondrial genomes, become dominant mitochondrial genomes and eventually replace the normal ones. Many data do not support this hypothesis. The three ascospore derivatives C4, C13 and C16 had the common female parent P561, which contains a detectable species of IS-kalDNA (9.5 kb, Pst I fragment, data not shown). These three ascospores contain a total of five species of IS-kalDNAs in their late subcultures, one is Pst I-cut 9.5 kb IS-kalDNA, which is observed only in series C4. Myers (1988) demonstrated that ascospores from the same cross contain more than ten species of IS-kalDNAs in their late subcultures, rather than the one found in their female parent P561-1. It is not easy to explain that when the mitochondria of their female parent contain a species of IS-kalDNA at a detectable level, then the ascospores from the cross contain the IS-kalDNA at an undetectable level in their first subcultures, and the IS-kalDNA replicates faster later in the same mitochondrial and nuclear background. Vickery

(1990) demonstrated that ten subcultures from the same ascospores without detectable IS-kalDNA show vegetative death with different life spans and contain different species of IS-kalDNAs. Subculture C13-6 contains two species of IS-kalDNAs but these two species are lost in the later subcultures. Two species of IS-kalDNAs (9.5 kb in series C4 and 10.7 kb in series C16) decrease a little in their late subcultures. The losses of initial IS-kalDNA were reported before (Myers, 1988). The losses of initial IS-kalDNA were frequently observed in the suppression studies (see Chapter one).

The another possibility of generating IS-kalDNA in late subcultures is that generation and accumulation of IS-kalDNAs are due to the de novo, continuous insertion activities of AR-kalDNA, the sites and time of insertion depend upon their nuclear background, growth environment and their interaction. The increase of IS-kalDNAs coupled with the decrease of AR-kalDNA is expected from the explanation. All above data are compatible with this explanation. The decrease of AR-kalDNA after the peak subcultures would be due to faster insertion of AR-kalDNA in late subcultures than that in early ones, resulting in an increase of IS-kalDNA in late subcultures. This explanation is strongly supported from the suppression studies (see Chapter one). The ascospores (BC2-2, -4, -6, -8; 14-5, -6) with the suppressive gene contain both AR-kalDNA and IS-kalDNA in their first subcultures, but AR-kalDNA is inhibited to a very low level in late subcultures and then IS-kalDNA is lost.

It is observed that there is a quantitative peak of AR-kalDNA at late stages of the senescence process in senescent Neurospora. The reason for its faster replication of the kalilo plasmid remains unclear. Since the quantitative peak of AR-kalDNA occurs while a low level of IS-kalDNA is observed. It is possible that the faster replication of the plasmid

at late stages could have resulted from stimulation by nuclear and mitochondrial genomes with low levels of IS-kalDNA. When the abnormal mitochondrial genomes reach a higher level on a genomic background at late stages, the stimulation becomes weaker and then the replication of the kalilo plasmid slows down, resulting in a decrease of both of AR-kalDNA and total kalDNA. There are cases that challenge this explanation. In the suppression studies, ascospores BC3-1, -2, and -3 with the inhibitor gene contain enormous amounts of AR-kalDNA in their first subcultures, ascospores BC2-2, -4, -6, and -8 with the same inhibitor gene contain detectable AR-kalDNA and IS-kalDNA, ascospores 13-5, -6, and -7 with the suppressor gene contain both AR and IS forms of kalDNA in their first subcultures, but AR-kalDNA decreases in their 38th subcultures. All these ascospores contain one of two suppressive genes and have different nuclear genomes. It is reasonable to believe that the stimulation is a complex process; it could be a process of multiple factors and pathways.

In these kinetic studies of mitochondrial kalDNA in the three series, the steady increase of IS-kalDNA and decrease of AR-kalDNA from a peak at late stages of the senescence process is obvious and consistent, but the quantity of AR-kalDNA does not show constant increase by subcultures before their AR-kalDNA peaks are reached. Some subcultures contain less AR-kalDNA than their earlier subcultures. In the suppression studies, it has been proved that the mitochondria of senescent strains are heterogenic, some mitochondria containing less or more kalDNA within them, some containing none, resulting in escape from death. Bulk conidia were inoculated to grow mycelium for mtDNA preparations. The variation of AR-kalDNA quantity in early stage could be mainly due to the random sampling of conidia in subculturing, and not to the nature of AR-kalDNA itself.

The kinetics of the circular plasmids of K2 in the same mitochondria in series C16, show parallels with those of AR-kalDNA. This suggests that both linear and circular mitochondrial plasmids share some common pathway of replication. The stimulation by mitochondrial and nuclear genomes affects not only the linear kalilo plasmid but also the circular plasmids.In the suppression studies (Chapter One), both AR-kalDNA and the 5.0 kb fragment of plasmid K2 are always observed in the first subcultures of ascospores with the suppressive nuclear gene (inhibitor), but both are inhibited to an undetectable level in the 20th subcultures. Maybe the kalilo plasmid and these circular plasmids have some unknown special relationship, because both of them have been transferred from N. intermedia to N. crassa (Griffiths et al, 1990) and both were lost from the escaper series C4 (see Chapter one).

It is reasonable to believe that all mitochondria in kalilo senescent strains are infected with the kalilo plasmid, and a majority or all of mitochondrial genomes were inserted by the plasmid in the terminal subcultures, because the function of the mitochondria is disrupted resulting in vegetative death, and the ratio of IS-kalDNA to mitochondrial genomes is always greater than one in these cultures. In the suppression studies, it has been proved that mitochondria in the cytoplasms of senescent strains are heterogenic, some containing the plasmid, some not, even at stages with high levels of AR-kalDNA, for example the C4 escaper from subculture C4-14 (see Chapter one). So there must be some way by which mitochondria with kalDNA replace the mitochondria without them during the senescence process. The replacement could be done by faster multiplication of the mitochondria with kalDNA than those without kalDNA, in other words, there should be higher ratios of mitochondria per nucleus as senescence proceeds. Unpublished data (Griffiths, personal communication) suggest that there is no phenomenon of faster mitochondrial multiplication.

It seems more possible that the replacement is through plasmid infection. In other words, the kalilo plasmid can penetrate the mitochondrial membrane of their nature hosts, enter into cytosol and then re-enter other mitochondria.

The ability of the plasmid to exist outside the mitochondria is supported by the following facts. The kalilo plasmid and the circular plasmid K2 have been transferred from N. intermedia to N. crassa though "mixed growth" and the plasmids maintain their original function in the new host mitochondria (Griffiths et al. 1990). The two circular mitochondrial plasmids, Varkud (3.8 kb) and VS, in N. intermedia have been transferred to new host mitochondria through "forced" heterokaryon formation (Collins and Saville, 1990). A different Neurospora mitochondrial circular plasmid can be transmitted independently of the mitochondrial chromosome at low, but evolutionarily significant, frequencies by the male parent during sexual crosses (May and Taylor, 1989). The two killer plasmids, pGKL1 and pGKL2, can be located and maintained in the cytoplasm outside the mitochondria in Kluyveromyces lactis (Stam et al. 1986). They have been transferred to Saccharomyces cerevisiae by cell fusion (Gunge and Sakaguchi, 1981), and by mixing protoplasts of S. cerevisiae with the linear plasmids (Gunge et al, 1982), and to K. fragilis and Candida pseudotropicalis by illegitimate mating (Sugisaki et at. 1985). A Brassica mitochondrial linear plasmid has been transferred between mitochondria during protoplast fusion (Kemble et al. 1988), and the plasmid could be transferred through pollen to egg cell in the female at an average rate of 50% (Erickson et al. 1989). It seems that these small circular and linear plasmids not only live in mitochondria, but they also can, in some cases, penetrate the mitochondrial membrane, cross the cell membrane and mitochondrial membrane, and live in new host mitochondria.

CONCLUSION

The objectives of this studies is to investigate the mechanisms by which suppress the kalilo senescence in Neurospora, and investigate the relationship of AR-kalDNA and IS-kalDNA during the senescence process. The major finding reported in this thesis are summarized below:

1. There are three mechanisms of suppressing kalilo senescence in Neurospora.
    a. One nuclear gene from strain 83 inhibits replication of the plasmid to a very low level in the mitochondria, there are no (or too few) IS-kalDNAs to affect the normal functions of the mitochondria.
    b. The mitochondria in senescent kalilo strains are heterogenic, some containing AR-kalDNA or and IS-kalDNA, some containing neither. When asci, ascospores or conidia are formed with the mitochondria lacking kalDNA from their parental cytoplasm containing kalDNA, the sexual or asexual progeny with normal mitochondria grow immortally, escaping the fate of death.
    c. One nuclear gene from strain 428 allows the kalilo plasmid to replicate normally and insert into mitochondrial chromosomes, generating IS-kalDNA. But the gene suppresses the deleterious effects of IS-kalDNA, resulting in the host fungi growing normally and carrying both AR-kalDNA and IS-kalDNA in their mitochondria.

2. The insertion activities of the kalilo plasmid is an important factor of causing host fungi to die. There is strong correlation between insertion activities of the kalilo plasmid and host death.

3. The kalilo plasmid reside mainly in the mitochondria, and can cross the mitochondrial membranes into cytosol, then enter other mitochondria.

4. AR-kalDNA is observed in all subcultures of senescent kalilo strains, increases in quantity at early stages of senescence until a peak is reached at a late stage and then drops to a low level.

5. IS-kalDNA is not observed at early stages, but appears at late stages and increases in quantity until the last analyzable subculture.

6. Kalilo and other circular plasmids (K2 series) share some common factors for their replication. They show the parallel kinetics in senescent kalilo strains, and are inhibited to undetectable levels under the suppressive nuclear background.

7. Many new species of IS-kalDNAs are generated during subculturing, but some of them are lost later under the suppressive or the non-suppressive unclear backgrounds.

Based on all available data, a hypothesis is proposed:

1. The generation of IS-kalDNAs is through the de novo insertion of AR-kalDNA.
2. The IS-kalDNA can replicate with their vector mitochondrial genomes.
3. The mitochondrial genomes with IS-kalDNAs replicate slower than the normal ones without IS-kalDNA.

4. The accumulation of IS-kalDNAs in generated mainly by faster insertion of AR-kalDNA and slow replication of the mitochondrial genomes carrying them.

5. The insertion activities of the kalilo plasmid depend on the interaction of the nature of the plasmid itself, the quantitative level of the plasmid, nuclear background and growth conditions.

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