Hyperactive Recombination in the Mitochondrial DNA of the *natural death* Nuclear Mutant of *Neurospora crassa*

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In *Neurospora crassa*, a recessive mutant allele of a nuclear gene, *nd* (natural death), causes rapid degeneration of the mitochondrial DNA, a process that is manifested phenotypically as an accelerated form of senescence in growing and stationary mycelia. To examine the mechanisms that are involved in the degradation of the mitochondrial chromosome, several mitochondrial DNA restriction fragments unique to the *natural-death* mutant were cloned and characterized through restriction, hybridization, and nucleotide sequence analyses. All of the cloned DNA pieces contained one to four rearrangements that were generated by unequal crossing-over between direct repeats of several different nucleotide sequences that occur in pairs and are dispersed throughout the mitochondrial chromosome of wild-type *Neurospora* strains. The most abundant repeats, a family of GC-rich sequences that includes the so-called *Pst*I palindromes, were not involved in the generation of deletions in the *nd* mutant. The implication of these results is that the *nd* allele hyperactivates a general system for homologous recombination in the mitochondria of *N. crassa*. Therefore, the *nd*⁺ allele either codes for a component of the complex of proteins that catalyzes recombination, and possibly repair and replication, of the mitochondrial chromosome or specifies a regulatory factor that controls the synthesis or activity of at least one enzyme or ancillary factor that is affiliated with mitochondrial DNA metabolism.

In *Neurospora crassa*, a recessive mutation in the *nd* (natural-death) locus on linkage group I causes a progressive deterioration of the growth and reproductive potentials of asexually propagated mycelia (57). Phenotypically, the process is manifested as an accelerated senescence syndrome and leads to cell death after two to four sequential (weekly) passages of asexual spores (conidia) to fresh agar slants (56, 57). Consequently, the mutant has been used as a model system for the study of cellular aging (38, 44, 47, 50, 52). The most recent of these studies has shown that the *nd* mutation causes the progressive and coordinate loss of several different mitochondrial functions in growing and stationary-phase mycelia (50, 56). The accelerated degeneration of the respiratory capacity of the cells is paralleled by the appearance of a rather heterogeneous population of grossly altered mitochondrial DNAs (mtDNAs) (56). These observations led to the proposal that the wild-type allele of the *nd* gene codes for a protein that protects the mitochondrial chromosome from intra- and intermolecular recombination or breakage events that potentially cause deletions, inversions, and other deleterious rearrangements (56).

In recent years, other senescence syndromes have been studied extensively in the filamentous fungi, but none of these is caused by mutation of a nuclear gene (reviewed in reference 35). Instead, the predisposition to senesce prematurely and the symptoms that are associated with these aging processes are inherited maternally. Furthermore, it has been shown repeatedly that the degeneration of growing fungal mycelia results from the suppressive displacement of wild-type mitochondrial chromosomes by mutationally altered mtDNAs (9–14, 16, 23, 27, 30, 36, 43). In contrast, the senescence phenotype that is caused by the *nd* mutation

segregates in Mendelian fashion in crosses (57), and the relative stability of *nd/nd*⁺ heterokaryons, which are presumably heteroplasmic, suggests that the abnormal mtDNAs that are engendered in the mutant might not be highly suppressive (56, 57).

In order to elucidate the nature of the mtDNA defects that are induced by mutation of the *nd* locus and to gain some insight into the activity of the product of this particular gene, we have cloned and analyzed several *Eco*RI restriction fragments that are unique to the mtDNA from senescent mycelia of the mutant. The results indicate that the recessive *nd* allele engenders a mitochondrial hyperrecombination phenotype and that the rapid degeneration of the mtDNA in the mutant results from an excessively high frequency of mispairing and crossing-over between repeats of a small number of different 70- to 701-bp-long nucleotide sequences that occur in pairs and are dispersed throughout the mitochondrial chromosome of *N. crassa*.

MATERIALS AND METHODS

Strains and growth conditions. The *nd* mutant was obtained from the Fungal Genetics Stock Center (Department of Microbiology, University of Kansas Medical Center, Kansas City, Kans.) in the form of a heterokaryon (strain FGSC 3572; hereafter designated *ndH*) containing a mixture of nuclei of the *nd al-2* and *nd*⁺ *al-2*⁺ genotypes. Procedures for isolating *nd* homokaryons and culture conditions were as described previously (24, 56). The normal control strains were *pan-2* or *nic-1 al-2* derivatives of the 74-OR23-1A (74A) standard laboratory wild-type strain of *N. crassa*.

Escherichia coli JM83r[−] and DH5α were used as host cells for cloning and grown according to standard protocols (54).

Isolation of mtDNA. Mitochondria were isolated from exponential-phase cultures, and mtDNA was extracted by

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FIG. 1. Cloning of unique *Eco*RI restriction fragments from the mtDNA of the *nd* mutant of *N. crassa*. (A) *Eco*RI restriction fragment patterns of the mtDNAs from wild-type strain 744 (lane 1), a two-times-subcultured *nd* homokaryon (lane 2), and the *ndH nd⁺/nd* maintainer heterokaryon (lane 3). Numbers on the right identify the restriction fragments of the wild-type mtDNA by order of size from the top (largest fragment, 20.54 kb) to the bottom (smallest fragment, 1.87 kb), and the two arrows on the left point to unusual restriction fragments that always appear in relatively high copy numbers in mtDNAs from senescing *nd* mutant cultures. The sizes of all the *Eco*RI fragments of wild-type mtDNA can be found in reference 61. (B) *Eco*RI restriction patterns of wild-type mtDNA (lane 1), *nd* mutant mtDNA (lane 2), and recombinant plasmids pBSR33 (lane 3), pBSR40 (lane 4), pBSR24 (lane 5), and pBSR49 (lane 6), which contained unique, high-copy-number fragments of *nd* mtDNA corresponding in size to those marked by arrows in panel A. (C) *Eco*RI restriction patterns of wild-type mtDNA (lane 1) and recombinant plasmid pBSR34 (lane 2), which contains a unique, low-copy-number restriction fragment of *nd* mtDNA. In panels B and C, the positions of the restriction fragments of wild-type mtDNA are marked on the left by the corresponding numbers, and the position of the pUC19 vector DNA is indicated by an arrow labeled vector.

the proteinase K procedure as previously described (11, 14, 56). Digestion of DNA with restriction enzymes was performed under the conditions recommended by the suppliers (GIBCO-BRL and New England Biolabs, Inc.), and the resultant fragments were separated by electrophoresis in 0.8% agarose gels at 30 to 50 V for 12 to 14 h in Tris-borate buffer (54). Gels were stained with ethidium bromide before photography.

Cloning *nd* mutant mtDNA. mtDNA was isolated from the *nd* mutant and digested with *Eco*RI, and the resulting fragments were inserted into *Eco*RI-digested vector pUC19 (49). Transformation of *E. coli* JM83r⁻ with the recombinant plasmids was done by standard protocols (54). Chimeric plasmids were prepared from the isolated bacterial clones either by phenol extraction (54) or by the sodium dodecyl sulfate-NaOH procedure of Ausubel et al. (8). Only plasmids containing authentic mtDNA inserts, as determined by Southern hybridization with labeled wild-type mtDNA, of a size unique to the *nd* mutant (see Fig. 1) were chosen for further study. Plasmids pBSR24, pBSR40, and pBSR49 contained unique fragments of approximately the same size, 6,700 bp; pBSR33 contained an insert of about 4,000 bp; and pBSR34 had an insert of approximately 1,800 bp.

Southern hybridization analysis. Plasmids were labeled radioactively by the primer extension method of Feinberg and Vogelstein (31), according to the protocol provided with the labeling kit (Pharmacia LKB Biotechnology). Wild-type

mtDNA was isolated, digested with either *Eco*RI or *Hind*III, and analyzed by electrophoresis in 0.8% agarose gels. DNA was transferred to nylon membranes by diffusion (59) and hybridized with the denatured probes by standard protocols (54).

DNA sequencing. DNA sequencing was performed on double-stranded plasmids by the primer extension protocol of Agellon and Chen (1) and the Sanger et al. (55) dideoxy-chain termination method. Sequencing reagents were purchased from Pharmacia LKB Biotechnology. The derivation of the unique *Eco*RI fragments from noncontiguous segments of the mtDNA of wild-type *N. crassa* was ascertained by determination of the nucleotide sequences at their termini through the use of forward and reverse universal primers, which anneal to pUC19 sequences adjacent to the multiple cloning site. To obtain information about the internal structures of the cloned segments of *nd* mtDNA, either the appropriate restriction fragments were subcloned for sequencing, as described in Results, or the cloned *Eco*RI fragments were sequenced directly through the use of synthetic primers (Table 1) that were obtained from VetroGen, London, Ontario, Canada. DNA sequences were compared with a data bank of wild-type *N. crassa* mtDNA sequences with the Beckman Microgenie software (51) and with the GenBank nucleotide sequence data base through the BLAST family of programs (6). The accession numbers of pertinent published sequences are given in the figure legends.

TABLE 1. Special DNA-sequencing primers used for locating deletion junctions in the mtDNA of the *natural death* mutant of *N. crassa*

Primer	Purpose	Region in mtDNA ^a	Sequence
1	Junction 1 in pBSR40	HindIII-9 (nt 356–370)	TGCATGATACAATGA
2	Junction 1 in pBSR40	HindIII-9 (nt 544–559)	AGTGGCCTAGAGCTA
3	Junction in pBSR33	EcoRI-2 (nt 3912–1927)	ACATACCACAGGTCT
4	Junction in pBSR33	EcoRI-6 (nt 2820–2834)	GTTCTCATAGGCAAA
5	Junction 2 in pBSR40	EcoRI-3 (nt 4634–4648)	CATTAAGATAGTTAG
6	Junction 1 in pBSR40	EcoRI-1 (nt 780–794)	GGCCATATTGGCTCA
7	Junction in pBSR34	EcoRI-10 (nt 1661–1676)	GAGTGGCGTTTAAAC

^a nt, nucleotides.

RESULTS

Cloning of rearranged segments of *nd* mtDNA. Shotgun cloning of *EcoRI* fragments of *nd* mtDNA in pUC19 resulted in 51 ampicillin-resistant *E. coli* clones that contained recombinant plasmids. Of these, 34 plasmids hybridized strongly to labeled wild-type mtDNA (data not shown), and several contained fragments of mtDNA which were unique to the *nd* mutant on the basis of their migration in agarose gels. Among the plasmids that contained *EcoRI* inserts of *nd*-specific mtDNA, one, pBSR33, had an insert of about 4,000 bp that migrated to a position just below the *EcoRI*-4 fragment of the wild-type mtDNA (Fig. 1). An additional three plasmids, pBSR24, pBSR40, and pBSR49, all contained fragments that were more or less equivalent in size to the unique, 6,700-bp *EcoRI* fragment of *nd* mtDNA that migrated between fragments *EcoRI*-3 and *EcoRI*-4 of the wild-type mtDNA (Fig. 1). A fifth recombinant plasmid, pBSR36, also contained a 6,700-bp fragment of mtDNA, which later was found to have a restriction map identical to that of the insert in pBSR49 and was not examined further. The unique 4,000-bp and 6,700-bp *EcoRI* fragments were observed in the restriction patterns of the mtDNAs from all of the *nd* homokaryons that have been examined thus far; therefore, they are the products of frequently occurring rearrangements (56). A sixth unique chimeric plasmid, pBSR34, contained an mtDNA fragment that was slightly smaller than the 1,946-bp *EcoRI*-10 fragment of wild-type mtDNA (Fig. 1). Although this novel fragment is not visible in the sample of *nd* mtDNA that is shown in Fig. 1, it usually is distinctly discernible on ethidium bromide-stained gels of *EcoRI*-cut *nd* mtDNA and thus also appears to be the product of a frequently occurring rearrangement. Furthermore, it should be noted that the mtDNAs that have been prepared from different isolates of the *nd* mutant always lack *EcoRI*-10 (56; this study).

The *EcoRI* and *HindIII* restriction maps of the mitochondrial chromosome of *N. crassa* are presented in Fig. 2 as a reference for interpretation of the DNA hybridization and nucleotide sequence data that are presented in the sections that follow. All the analyses are based on the assumption that the map represents the 63-kb nucleotide sequence of the standard wild-type *N. crassa* 744 mtDNA in a clockwise 5'→3' orientation.

Analysis of the mtDNA component of pBSR33. Hybridization of the random-primer ³²P-labeled products of pBSR33 with *EcoRI*- and *HindIII*-digested wild-type mtDNA gave the results presented in Fig. 3A. The insert in the plasmid was homologous to *EcoRI*-2, but it also hybridized weakly with the noncontiguous *EcoRI*-6 fragment. Three contiguous *HindIII* fragments, 3, 9 and probably 16b, also hybridized with pBSR33, and the noncontiguous *HindIII*-1 fragment

was recognized weakly by the probe. Sequencing of pBSR33 with the universal forward and reverse primers revealed that the 5' end of the insert of *nd* mtDNA corresponded to the 5' end of *EcoRI*-2, whereas the 3' end of the insert corresponded to the 3' end of *EcoRI*-6. Together with the results of the hybridization analysis, this observation implied that the unique *EcoRI* fragment that had been cloned in pBSR33 had been generated in the *nd* mutant by deletion of the segment of mtDNA that encompasses the 3' half of *EcoRI*-2, all of *EcoRI* fragments 10, 3, 5, 7a, 8, 9, 11, 7b, and 4, and the 5' terminus of *EcoRI*-6 (see Fig. 2).

Digestion of pBSR33 with *HindIII* revealed that it contained in intact form the segment of the mitochondrial chromosome that is represented by *HindIII* fragments 9 and 16b (see Fig. 2). *HindIII*-16b DNA contains an *XbaI* site, which was used in combination with the *XbaI* site in the polylinker sequence of the vector to subclone the 3'-terminal portion of the insert. Sequence analysis of the subcloned DNA with universal forward and reverse primers confirmed that one end of the subcloned DNA was homologous to *EcoRI*-2 and the other to *EcoRI*-6. Further sequencing with two synthetic primers (3 and 4) identified the anticipated deletion junction, which is shown in Fig. 4A and 5. The data confirmed that the *EcoRI* fragment of DNA that had been cloned in pBSR33 arose in the *nd* mutant by deletion of a 38-kb segment of DNA which extends clockwise from within *EcoRI*-2 through most of *EcoRI*-6 and contains primarily protein-coding genes (see Fig. 2). Furthermore, analysis of the sequences around the *EcoRI*-2/6 junction demonstrated that the deletion was caused by unequal crossing-over between two directly repeated copies of a 70-bp nucleotide sequence (rep70), one located in *EcoRI*-2 (rep70A) and the other located in *EcoRI*-6 (rep70B), as illustrated in Fig. 2, 4A, and 5. The function of the rep70 sequence is unknown, but one copy, rep70A, is located immediately upstream of the coding sequences for the NDH2 and NDH3 proteins and either includes the promoter or encodes the 5'-end processing signal for the RNA that is transcribed from these loci (48).

Analysis of the mtDNA component of pBSR40. When the labeled chimeric pBSR40 plasmid was hybridized with *EcoRI*-digested wild-type mtDNA, it annealed relatively strongly to fragments 1 and 3 and slightly to fragment 2 (Fig. 3B). Together, these three fragments constitute a continuous 40-kb segment of the wild-type mitochondrial chromosome that is much larger than the 6,000-bp *nd* mtDNA insert in pBSR40 (see Fig. 2). Hybridization of pBSR40 to *HindIII*-digested wild-type mtDNA resulted in strong labeling of at least one of the three *HindIII*-7 fragments and at least one of the two contiguous *HindIII*-11 fragments (Fig. 3B). Since *HindIII*-7c and -11a jointly overlap the 3' end of *EcoRI*-3 in

FIG. 2. Genetic and *EcoRI* and *HindIII* restriction fragment maps of the mtDNA of wild-type strain 744 of *N. crassa*. The map represents in a clockwise direction the 63-kb nucleotide sequence of the mitochondrial chromosome in the conventional 5'→3' direction and in the same orientation as the transcripts of the corresponding genes. The positions of the following genes are shown on the outer perimeter of the restriction map: *coI*, -II, and -III, coding for subunits 1, 2, and 3, respectively, of cytochrome oxidase; *cyb*, coding for the apoprotein of cytochrome *b*; *ndh1*, -2, -3, -5, and -6, coding for the respective subunits of the mitochondrial NAD dehydrogenase; *SrRNA* and *LrRNA*, genes coding for the small and large ribosomal subunit RNAs, respectively; *atp6* and -8, coding for the corresponding subunits of the mitochondrial ATPase; *S5*, coding for polypeptide 5 of the small subunit of mitochondrial ribosomes; *mal*, silent copy of a gene that specifies the hydrophobic "proteolipid" polypeptide of the mitochondrial ATPase (the active gene is located in the nucleus in *N. crassa*); and *urfN* and *urfU*, which are open reading frames of unknown function. Genes coding for tRNAs are indicated by dots. Direct repeats of nucleotide sequences are represented on the inner circumference on the map by symbols that are explained in the center of the figure. The A and B copies of each repeat are located in clockwise succession to each other starting from the *EcoRI* site at the apex of the circular map. Information for the construction of the map originated from references 61 and 22, and nucleotide sequences that were retrieved from the GenBank data base are indicated in the legends to Fig. 4 and 6.

the wild-type mtDNA, the insert in pBSR40 was presumed to include this region of the mitochondrial chromosome (see Fig. 2). However, pBSR40 also hybridized to *HindIII*-1, which overlaps the 5' end of *EcoRI*-1, and to *HindIII*-9, which overlaps the 5' end of *EcoRI*-2. Since the three segments of mtDNA that are homologous to the unique *EcoRI* fragment in pBSR40 are normally widely separated from each other on the mitochondrial chromosome of wild-type *N. crassa*, the results indicated that at least two large deletions were involved with the generation of this peculiar segment of *nd* mtDNA. The first deletion presumably removed a large stretch of DNA from a point close to the 5' end of *EcoRI*-1 to a point close to the 5' end of *EcoRI*-2. The second deletion apparently had removed the 3' end of *EcoRI*-2, all of *EcoRI*-10, and a large portion of the 5' end of *EcoRI*-3. Therefore, it was anticipated that the 5' and 3' ends of the mtDNA fragment that had been cloned in pBSR40 corresponded to the 5' and 3' ends of *EcoRI*-1 and -3, respectively.

Sequencing of pBSR40 with the universal forward and reverse primers revealed that the 3' end of the insert corresponded to the 3' end of *EcoRI*-3, as expected, but the 5' end of the *nd* mtDNA insert surprisingly corresponded to the 5' end of *EcoRI*-2. Thus, in order for pBSR40 to hybridize with the wild-type *EcoRI*-1 fragment, some rearrangement of sequences beyond a simple deletion was indicated. Further sequencing with synthetic primers 1 and 2

identified a rearrangement junction that had resulted from a crossover between two repeats of a 701-bp nucleotide sequence that overlap the *EcoRI*-6/1 (rep701A) and *EcoRI*-1/2 (rep701B) boundaries. Each repeat includes a tRNA^{Met} gene and the N-terminal portion of the *ndh2* locus (2) (Fig. 2). The sequence of rep701A differs from that of rep701B by a single base pair at position 527, a feature that could be used as a marker for the conclusion that the deleterious crossover had occurred somewhere within the 127 bp that are proximal to the 3' termini of the two repeats and at a point that is located more than 500 bp inwards from the 5' terminus of the mtDNA insert in pBSR40, as shown in Fig. 4B and 5.

The above results prompted the search for a second rearrangement involving nucleotide sequences located in *EcoRI*-1 and *EcoRI*-3. An electrophoretic analysis of the products of restriction enzyme digests of pBSR40 revealed that the two successive *HindIII* restriction sites at the *HindIII*-21/7c and *HindIII*-7c/11a boundaries (see Fig. 2 and 5) were intact in the cloned fragment of *nd* mtDNA. Therefore, it was concluded that the anticipated second rearrangement junction was situated somewhere near the 5' end of *HindIII*-21. In order to locate this second junction, pBSR40 was digested with *HindIII* and religated. Because the *HindIII* site in the multiple cloning region was located at the 3' end of the insert, this approach removed all of the mtDNA except for a relatively large *EcoRI*-*HindIII* fragment that remained connected to the vector DNA. Sequencing of the

FIG. 3. Hybridization of cloned *EcoRI* fragments of *nd* mtDNA to Southern blots of the electrophoretically separated *EcoRI* (lanes 1) and *HindIII* (lanes 2) fragments of wild-type mtDNA. The radioactive probes were the segments of *nd* mtDNA that had been cloned in plasmids pBSR33 (A), pBSR40 (B), and pBSR34 (C). The numbers to the left and right of each panel mark the positions of the *EcoRI* and *HindIII* fragments, respectively, of the wild-type mtDNA, as determined from photographs of ethidium bromide-stained gels that were used to prepare the Southern transfers.

subcloned DNA with the standard reverse primer and synthetic primer 5 identified the predicted rearrangement junction between nucleotide sequences from *EcoRI*-1 and *EcoRI*-3, shown in Fig. 4B. The event that created this junction was a 34-kb deletion that resulted from an unequal crossover event between two identical direct repeats of the mitochondrial *tRNA^{Cys}* gene, one of which is located in *EcoRI*-1 (positions 2223 to 2298; rep75A), and the other of which is located in *EcoRI*-3 (positions 4403 to 4478; rep75B). It is important to note that the structure of the *EcoRI* fragment of *nd* mtDNA that was cloned in pBSR40 can be explained only by assuming that the crossover between rep75A and rep75B was an intra- or intermolecular event that preceded in time an intermolecular crossover that involved mispairing of the rep701A in the already recombinant molecule with a rep701B sequence of a different mtDNA molecule. These events are partially represented in Fig. 5.

The genesis of the unusual *EcoRI* fragment that had been cloned in pBSR40 was not limited to the two unequal crossover events involving mispairing of rep701 and rep75 sequences. A third rearrangement, which is described in the next section, was found near the 3' end of this and related pieces of *nd* mtDNA.

Analyses of the *EcoRI* inserts in pBSR24 and pBSR49. Digestion with different restriction enzymes and sequencing with primers 1 and 5 revealed that the *nd* mtDNA inserts in pBSR24 as well as pBSR49 each also contained the rep701 and rep75 rearrangement junctions that were identified in the insert of pBSR40. However, the three cloned segments of mtDNA differed slightly in size (Fig. 1). Electrophoretic analysis of the DNA fragments in *HindIII* plus *EcoRI* double digests of the three plasmids revealed that the 3'-terminal fragments, which presumably contained the 5' half of *HindIII*-11a, varied in size (Fig. 6A). Plasmid pBSR40 con-

tained about 200 bp more than pBSR24 and about 400 bp more than pBSR49. The 3'-terminal *HindIII*-*EcoRI* fragment of each insert was therefore subcloned and sequenced. The sequence of this segment from the mtDNA insert in pBSR24 was identical to that reported for the 3' terminus of the *EcoRI*-3 fragment of wild-type mtDNA (17, 19). However, it was noted that this sequence contains a direct repeat of 128 bp (rep128). The two copies of rep128 are separated by a 40-bp spacer and can be distinguished from each other by slight differences in the nucleotide sequences near their respective 5' and 3' ends (Fig. 6B).

The *EcoRI* fragment in pBSR49 contained the smallest *HindIII*-*EcoRI* 3'-terminal component, because a segment of DNA equivalent to the combined lengths of one repeat and the spacer region (168 bp) was deleted by unequal crossing over between rep128A and rep128B (Fig. 6C). In contrast, the *HindIII*-*EcoRI* terminal segment of the insert in pBSR40 was enlarged by the addition of a third rep128 sequence through a crossover between mispaired rep128A and rep128B sequences from two different mtDNA molecules (Fig. 5 and 6C). It should be noted that the deletion in pBSR49 and the addition in pBSR40 are potentially the reciprocal products of an unequal crossover between the rep128A and rep128B sequences in two different mtDNA molecules, as shown in Fig. 6C.

Characterization of the mtDNA in pBSR34. On the basis of its migration in agarose gels, it was estimated that the insert in plasmid pBSR34 was approximately 100 bp shorter than the wild-type *EcoRI*-10 (Fig. 1). Hybridization with wild-type mtDNA revealed that pBSR34 had a strong homology to *EcoRI*-10 and *HindIII*-5 (Fig. 3C). As shown in Fig. 2, these two fragments overlap in wild-type mtDNA. The probe also hybridized slightly with at least one of four *HindIII* fragments, namely 15, 16a, 16b, or 16c, which are nearly equal in size. Overlap of the 3' end of *EcoRI*-10 with the 5'

A. pBSR33

EcoRI-2 4448 TTTATTTTATAAGCAGGAAATAACAGTATACGGAGCGGGTTATCACTAAGATGAGCGGGCAGGGGCTCATTTTGCCTATatgtccaatg 4539

EcoRI-6 2755 agggataaaaAAGCAGGAAATAACAGTATACGGAGCGGGTTATCACTAAGATGAGCGGGCAGGGGCTCATTTTGCCTATGAGAACAAAA 2845

nd recDNA 4448 TTTATTTTATAAGCAGGAAATAACAGTATACGGAGCGGGTTATCACTAAGATGAGCGGGCAGGGGCTCATTTTGCCTATGAGAACAAAA 4539

B. pBSR40

EcoRI-2 400 TTACATGGAGGTTTACTTCATATAACCAATATAACACAAGTATTCCAAATATTTATTTTTTGGATTAGTATATTAATATTACAATTAACAAGTTTCGATCCCATAAAA

EcoRI-1 265 ttacatggatGTTTACTTCATATAACCAATATAACACAAGTATTCCAAATATTTATTTTTTGGATTAGTATATTAATATTACAATTAACAAGTTTCGATCCCATAAAA

nd recDNA 400 TTACATGGAGGTTTACTTCATATAACCAATATAACACAAGTATTCCAAATATTTATTTTTTGGATTAGTATATTAATATTACAATTAACAAGTTTCGATCCCATAAAA

 AAATTTTATATTATGAGACATCCTAGGTTTATTAATAAGTGGCCTAGAGCTATAGGATATTCATACCATTATCCTcaatacaag 593 *EcoRI*-3 4399 gcaa

 AAATTTTATATTATGAGACATCCTAGGTTTATTAATAAGTGGCCTAGAGCTATAGGATATTCATACCATTATCCACTCCACTCC 457 (1756 bp) 2213 TCAC

 AAATTTTATATTATGAGACATCCTAGGTTTATTAATAAGTGGCCTAGAGCTATAGGATATTCATACCATTATCCACTCCACTCC 593 (1756 bp not sequenced) 2349 TCAC

 gggatcAGGATTACGATGTAATAGGTTTACCCTTTGACTGCAAATCTTAAGGATTAGGTTTCGATTCTACGTAATCCTTTAGGGTTAGCCTATA 4496 (3746 bp to rep128A)

 ATTATAAGGATTACGATGTAATAGGTTTACCCTTTGACTGCAAATCTTAAGGATTAGGTTTCGATTCTACGTAATCCTTTAGGGggttagcctt 2310

 ATTATAAGGATTACGATGTAATAGGTTTACCCTTTGACTGCAAATCTTAAGGATTAGGTTTCGATTCTACGTAATCCTTTAGGGTTAGCCTATA 2446 (3746 bp to rep128A)

C. pBSR34

EcoRI-10 1684 ACTCCCTCACTCTTAATAAAAAATAATAATAACCTGCCAGCATAGATATTATTGAAAAAATATTAGTTCACCGGTTAATTCATAGTTAATGGTGTAGTCATT

EcoRI-5 3596 gtaatggcgtTCTTAATAAAAAATAATAATAACCTGCCAGCATAGATATTATTGAAAAAATATTAGTTCACCGGTTAATTCATAGTTAATGGTGTAGTCATT

nd recDNA 1684 ACTCCCTCACTCTTAATAAAAAATAATAATAACCTGCCAGCATAGATATTATTGAAAAAATATTAGTTCACCGGTTAATTCATAGTTAATGGTGTAGTCATT

 TCGTGTGCTATTAGTAAATAATGAAGAAAGAAATTTgaatgttt 1832

 TCGTGTGCTATTAGTAAATAATGAAGAAAGAAATTC 3725

 TCGTGTGCTATTAGTAAATAATGAAGAAAGAAATTC 1823

FIG. 4. Nucleotide sequences of direct repeats in the mtDNA of *N. crassa* that are involved in the generation of deletions in the mtDNA of the *nd* mutant through unequal crossing over. Relative to the clockwise arrangement of the A and B copies of each repeat in Fig. 2, the first, or A, copy of each sequence is represented in the top line, the second, or B, copy is shown on the middle line, and the recombinant sequence that was found in the mtDNA from the *nd* mutant is shown on the bottom line. Each sequence is identified at the beginning by the *EcoRI* fragment of wild-type or *nd* mutant mtDNA in which it is located. The position of each sequence within the corresponding restriction fragment is given by numbers at the beginning and end of each sequence, which denote the distance of these sites in nucleotides from the nearest 5' *EcoRI* site, as defined in Fig. 2. Repeat-flanking sequences which appear in the recombinant *nd* mtDNA are represented by capital letters that are underlined or overlined with solid lines, whereas repeat-flanking nucleotide sequences that do not appear in the recombinant DNAs are represented by lowercase letters. Segments of the repeated nucleotide sequences are underlined or overlined with dashed lines, and crossovers are indicated in the center portion of each pair of repeats. Base pair differences that distinguish the A and B copies of each repeat from each other are marked by asterisks. (A) rep70 A and B, which recombined to form the deletion derivative of mtDNA that was cloned in pBSR33 (GenBank accession numbers X05115 [48] and L14439). (B) rep701 A and B, followed by the sequences of the A and B copies of rep75, all of which participated in the generation of two deletions that appear in the *EcoRI* fragments of *nd* mtDNA that were cloned in plasmids pBSR40, -24, and -49. The accession numbers of the sequences that contain rep701 DNA are X03793 (26) and X13337 (4), and those of the sequences that contain the A and B copies of rep75 are M26265 (17) and X01738 (28). (C) rep129 A and B, which recombined to form the deletion form of mtDNA that was cloned in plasmid pBSR34. The rep129 repeats are located in sequences that have accession numbers X03280 (18) and M26265 (17).

end of *HindIII*-16a (see Fig. 2) would explain this result. Many other *EcoRI* and *HindIII* fragments of the wild-type mtDNA also hybridized weakly with the pBSR34 probe, a circumstance that was attributed to the observation that

EcoRI-10 contains several GC-rich regions that are similar to the so-called *PstI* palindromes that are scattered throughout the mitochondrial genome (65). Collectively, the hybridization data implied that the fragment of mtDNA that had been

FIG. 5. Summary of unequal crossovers that cause high frequencies of deletions and some duplications in the mtDNA of the *natural death* mutant of *N. crassa*. A linear version of the circular *EcoRI* restriction map of the mtDNA of the standard wild-type strain 74A is shown in the middle of the figure, and the cloned recombinant *EcoRI* fragments of *nd* mtDNA, not drawn to scale, are represented above and below this map and identified by the names of the corresponding chimeric pBSR plasmids. Direct repeats of sequences that were involved in unequal crossing over are identified as in Fig. 2 by the following symbols: □, rep701; ▣, rep75; △, rep70; ●, rep129; ▽, rep128. Hybrid repeats that appear in the cloned segments of *nd* mtDNA are identified by numbers that designate the repeat and letters that identify the source of the 5' and 3' ends of the nucleotide sequence; e.g., 129 A/B denotes a recombinant rep129 sequence that has the 5'-flanking sequence of rep129A and the 3'-flanking sequence of rep129B. Lines that lead from the ends of cloned *nd* mtDNA fragments to restriction sites in the wild-type mtDNA map identify the *EcoRI* recognition sites that have been juxtaposed by deletions. Lines connecting pairs of repeats on the map for the wild-type mtDNA with single copies of the corresponding repeats on the maps of cloned *nd* mtDNA fragments identify unequal crossovers that resulted in deletions of the intervening segments of the mitochondrial chromosome. Segments of DNA that were juxtaposed in at least a fraction of the mtDNA molecules from the *nd* mutant by unequal crossovers but are distant from each other on the wild-type mitochondrial chromosome are identified within the bars that represent cloned DNAs by the numbers of the corresponding *EcoRI* fragments of the wild-type mtDNA. Relevant *HindIII* (H) and *XbaI* (X) restriction sites are represented above the bars. The underlined numbers that are shown above the bar labeled pBSR49 are the numbers of the *HindIII* restriction fragments that are shared by the mtDNA segments that were cloned in plasmids pBSR40, pBSR24, and pBSR49. The illustration of the events that caused rearrangements in the mtDNA segment that contains the rep128 sequences is incomplete because a detailed representation is provided in Fig. 6.

cloned in pBSR34 was formed in the *nd* mutant by deletion of a short nucleotide sequence from within *EcoRI*-10.

Restriction mapping of the *nd* mtDNA segment of pBSR34 disclosed that the *HindIII*-*EcoRI* segment corresponding to the 5' terminus of the wild-type *HindIII*-16a fragment was truncated by approximately 100 bp. Determination of the nucleotide sequence of the truncated fragment and its comparison with the 1,946-bp sequence of the wild-type *EcoRI*-10 fragment uncovered an apparent A to T transversion at position 1786 and termination of the *nd* mtDNA sequence in pBSR34 with an *EcoRI* restriction site corresponding to nucleotides 1817 to 1822 (Fig. 4C). A search for similar nucleotide sequences elsewhere in the mitochondrial chromosome led to the discovery of a 129-bp region (rep129B) at the 3' end (nucleotides 3596 to 3724, inclusive) of the 3,725-bp *EcoRI*-5 fragment (18) that is a direct repeat of the nucleotides between positions 1694 and 1822 (rep129A), inclusive, in the *EcoRI*-10 fragment (17). The two copies of the rep129 sequence differ from each other by an A/T mismatch at position 92, which corresponds to position

1786 in the partial sequence that is presented in Fig. 4C. The *EcoRI* site at the 3' end of the *EcoRI*-10 fragment of the wild-type mtDNA is located 124 bp downstream of the rep129A sequence, whereas *EcoRI*-5 extends only one nucleotide, a C, beyond rep129B. Since the sequence at the 3' terminus of the fragment of mtDNA that was cloned in pBSR34 exactly matches the 3'-terminal sequence of *EcoRI*-5, we concluded that it is the product of a 12.2-kb deletion that was generated in the *nd* mutant by unequal crossing-over between the mispaired A and B copies of the rep129 sequence, as indicated in Fig. 5.

DISCUSSION

Even though natural death is a nonconditional lethal Mendelian trait, the haploid nuclei carrying the recessive *nd* allele can coexist indefinitely in heterokaryons with compatible wild-type maintainer nuclei (56, 57). However, when *nd* homokaryons are reisolated from such heterokaryons, the resultant cultures rapidly develop respiratory defects (56)

FIG. 6. Unequal crossing over between the rep128 A and B sequences in the *Hind*III-11a region of the mtDNA of the *nd* mutant. (A) Ethidium bromide-stained agarose gel illustrating the three different sizes of *Hind*III-11a-derived *Hind*III-*Eco*RI fragments (bracket labeled 11a) that were cloned from the same sample of *nd* mtDNA in plasmids pBSR40 (lane 1), pBSR24 (lane 2), and pBSR49 (lane 3). The capital letters at the left mark three similarly sized fragments that are common to the three recombinant plasmids: A, pUC19; B, *Hind*III-7c; and C, the large *Eco*RI-*Hind*III fragment that is situated at the 5' end of the mtDNA insert in each plasmid (see Fig. 5). The *Hind*III-21 fragment of mtDNA, which is also present in each of the three cloned DNAs, is not visible. (B) Nucleotide sequence of the segment of the wild-type mtDNA that contains the rep128 A and B sequences. Base pair differences that distinguish the A and B repeats from each other and served to identify hybrid repeat sequences are marked by asterisks. The sequence that contains both rep128 copies has accession number X04512 (19). (C) Diagrammatic illustration of a reciprocal unequal crossover event that might have been involved in the generation of an "insertion" of an extra copy of rep128 together with a spacer sequence into the *Hind*III-11a segment of the *Eco*RI fragment of *nd* mtDNA that was cloned in pBSR49 (three copies of rep128 separated by two spacer sequences) and deletion of one copy of the repeat together with the spacer sequence from the *Eco*RI fragment of *nd* mtDNA that was cloned in pBSR40 (one A/B hybrid copy of rep128).

and die after a brief period of vegetative growth (56, 57). In an earlier study, we showed that the degeneration and death of *nd* cultures are associated consistently with an extraordinarily rapid "degradation" of large segments of the mitochondrial chromosome (56). Paradoxically, at least two well-defined sections of the mtDNA appeared to be immune to degradation. The results presented in this article offer a full explanation for the high degrees of lability and stability of the different sectors of the mitochondrial chromosome in the mutant.

The analyses of five unique *Eco*RI restriction fragments, all derived from the mtDNA of a single senescing *nd* culture, demonstrate that the apparent fragmentation of the mtDNA is caused by a remarkably high frequency of homologous "mispairing" and recombination between nucleotide sequences that are at least 70 bp long and occur naturally more than once in the mitochondrial chromosome of *N. crassa*. A corollary to these observations is that segments of mtDNA that are stable in *nd* homokaryons do not contain long nucleotide sequences that are repeated elsewhere in the mitochondrial chromosome. Indeed, a computer search of the mtDNA has revealed that at least *Eco*RI fragments 4, 7a, and 8, which remain relatively intact in the mutant (56), do

not contain nucleotide sequences longer than 35 bp that are repeated elsewhere in the mtDNA. At the same time, there are additional repeats, particularly in *Eco*RI fragments 6 and 9, for which recombinant restriction fragments have not been cloned yet, although they are implicated in recombinogenic activity by virtue of the fact that these restriction fragments disappear from the mtDNA of degenerating *nd* cultures. It should be noted that the products of crossing-over in correctly paired homologous segments of any two mtDNA molecules are not detectable in this system. Thus, our observations do not strictly discriminate between the activation of a site-specific or a general system for recombination. However, the diversity in the sequences of the repeats that are recombinationally active strongly suggests that the *nd* mutation affects a general system for homologous chromosome pairing and crossing-over in the mitochondrion. Thus, the mutant appears to degenerate and die because its mtDNA is "degraded" by unequal crossing-over events that occur at a rate that outpaces the maintenance of intact mtDNA molecules through replication. Since no single class of abnormal mtDNA molecules prevails in the degenerating cultures, the suppressive amplification of deletion chromosomes does not appear to be the primary cause for

senescence in this mutant, although it could be a contributing factor.

Since mtDNA rearrangements occur very rarely in wild-type strains (2, 12, 15, 27, 36), the magnitude of the recombinogenic activity in the mitochondria of the *nd* mutant is illustrated by the fact that two of the five novel *EcoRI* fragments that were characterized each contained three different rearrangements, one registered two instances of unequal crossing over, and only two contained single, albeit different, deletion junctions. All of these rearrangements had occurred within 3 weeks after extraction of the *nd* homokaryon from the maintainer heterokaryon, while it takes many months, often years, of growth for the appearance of a single rearrangement in the entire mitochondrial chromosome of wild-type strains (12, 15). Interestingly, all the rearrangements that have been found so far in the *nd* mutant are deletions arising from crossing over between mispaired long direct repeats that are relatively scantily represented in the mtDNA of *N. crassa*. Mismatching and crossing over between such repeats apparently does occur occasionally in wild-type strains and may be one of the primary causes for the formation of the truncated circular mtDNAs that are characteristic of *stopper* cytoplasmic mutants (2, 5, 12, 26, 36). For example, a reciprocal crossover between the two rep701 sequences which are located at opposite ends of *EcoRI*-1 (Fig. 2) apparently resulted in the formation of the two complementary truncated circular mtDNAs of the 1Arl55(11)2,107A *stopper* mutant described by Gross et al. (36). The direct repeats of the same sequence were involved in the crossover events that generated the deletion junction that is common to the three different fragments of *nd* mtDNA that were cloned in pBSR40, pBSR24, and pBSR49. The product of the reciprocal event of the crossover that is evident in these three clones was not found, presumably because the resultant *EcoRI* fragment is too long to be cloned in the bacterial pUC vectors. However, evidence for the reciprocity of crossover events is demonstrated by the deletion and addition derivatives of *HindIII*-11a that were cloned in pBSR49 and pBSR40 and are potentially the products of a reciprocal exchange between a rep128A sequence of an mtDNA unit that was mispaired with a rep128B sequence of a different mtDNA molecule.

The concept that the *nd* mutation might stimulate intra- as well as intermolecular recombination is also supported by the fact that only one of the two overlapping deletions that were involved in the generation of the two rearrangement junctions that are common to the *EcoRI* fragments that were cloned in pBSR24, pBSR40, and pBSR49 (Fig. 5) could possibly have been generated by an intramolecular crossover event—the second deletion must have resulted from mismatching of repeats that were physically located on separate mtDNA molecules. These observations, together with the fact that the repeats that are involved in the generation of deletions are quite diverse in sequence and size, indicate that mtDNA recombination in general, rather than site-specific recombination, is enhanced by the nuclear *nd* mutation. Hence, it is highly probable that the *nd* locus codes for a factor, most likely a protein, that directly or indirectly affects mtDNA crossing over or pairing, even though it has a moderating effect on these activities in its wild-type form.

By far the most common repeats in the mtDNA of *N. crassa* are a heterogeneous group of cryptic GC-rich, 30- to 80-bp-long palindromic nucleotide sequences, some of which contain two closely spaced *PstI* restriction sites (65). These sequences are situated almost exclusively in the noncoding segments of mtDNA that are located between genes, and

numerous copies are widely distributed throughout the mitochondrial chromosome. Characterizations of the amplified small circular mtDNA derivatives from several *stopper* cytoplasmic mutants (2, 26) and of the continuously growing *stp-ruv* variant of 1Arl55(11)2,107A (37) indicate that recombinogenic chromosome breaks within nucleotide sequences that potentially form cruciform structures, particularly the so-called *PstI* palindromes (65), can cause deletions in the mtDNA of *N. crassa* (5, 26). Similar events appear to induce deletions of fairly large, nonessential segments of DNA from the Mauriceville and Varkud circular mitochondrial plasmids (3).

Endonucleases that cleave DNA at cruciform structures are ubiquitous (25, 39, 40, 60, 63, 64). These observations, together with cognizance of the fact that the segments which disappear most rapidly from the mtDNA of *natural death* homokaryons contain the highest densities of GC-rich palindromes, led to the proposal that the *nd*⁺ allele could code for a protein that protects the mtDNA from attack by a site-specific endonuclease (56). However, analyses of the cloned mtDNA fragments from the mutant suggest that the involvement of GC-rich palindromes in the *nd*-activated recombination process may be negligible relative to that of other, much less abundantly repeated, nucleotide sequences. Thus, it is conceivable that mitochondria have two distinct systems for recombination: system I, which is the general system mentioned above, and system II, which is site specific and potentially recognizes cruciform structures and possibly only GC-rich sequences, such as the *PstI* palindromes. In this scheme, inactivation of the *nd* product either does not affect or perhaps even suppresses system II activity. However, the results of our studies provide no new insights into system II other than the recognition that its activity is not enhanced by the *nd* mutation.

Mutations that cause hyperactive recombination in other systems have been described and generally involve enzymes which play a role in DNA replication or repair. In some hyperrecombination mutants of *E. coli*, affected genes include *polA* (DNA polymerase I [29, 45, 58, 66]), *lig* (DNA ligase [29, 45, 58, 66]), and *uvrD* (DNA helicase [7, 41, 45, 66]). Similarly, mutations affecting DNA topoisomerases were shown to cause nuclear gene hyperrecombination phenotypes in *Saccharomyces cerevisiae* (21, 62). Of particular interest, however, is the *pif1* nuclear gene of *S. cerevisiae*, which codes for an mtDNA helicase (33, 42) that is required for mtDNA repair and recombination (32, 34). Significantly, *pif1* mutants are deficient in mitochondrial recombination, whereas the *nd* mutant clearly manifests a mitochondrial hyperrecombination phenotype. Therefore, the *nd* gene most likely either codes for a mitochondrial enzyme with one of the above activities or specifies a regulatory protein that controls the synthesis of one or more of the proteins that are involved in mtDNA metabolism. The *pif1* and *nd* genes could be functionally related to the nuclear plastome mutator (*pm*) genes of higher plants, such as *Oenothera* (20) and *Arabidopsis* (46, 53) spp. Recessive mutant *pm* alleles characteristically effect very high mutation rates in the chloroplast genome, but their role in chloroplast DNA metabolism is unknown.

While it is generally accepted that the majority of the enzymes and ancillary factors that are involved in mtDNA metabolism are encoded by nuclear genes, it has also become increasingly evident that many of these proteins are different from those that have equivalent functions in the nuclear compartment. In spite of a rather extensive and detailed body of information on the transmission, structure,

mutation, recombination, expression, and functions of mitochondrial genes, one of the perspectives that is still missing from the collage of concepts on mtDNA metabolism is a clear understanding of the nature and activities of many of the nuclear gene products that are involved in the transactions of the mitochondrial genome. Molecular and genetic characterization of the *nd* and similar mutants undoubtedly will engender many of the missing pieces of information that are needed for the formulation of a global model for the maintenance and biological role of the mitochondrial genome.

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