Recombination by sequence repeats with formation of suppressive or residual mitochondrial DNA in *Neurospora* 
(recombination hot spots/ illegitimate recombination/polymerase chain reaction)

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ABSTRACT Recombination junctions of several *Neurospora* mitochondrial DNA (mtDNA) mutants and their revertants were identified. Their nucleotide sequences and putative secondary structures were determined in order to understand the nature of the elements involved in intramolecular recombination. Multiple deletions, involving the same portion of *Neurospora* mtDNA, were identified in six independently isolated mutants. A 9-nucleotide repeat element, CCCCCCCCC, was found to be involved in these and other *Neurospora* mitochondrial recombination events. The repeat elements were clustered as hot spots on the *Neurospora* mtDNA and were associated with palindromic DNA sequences. The palindromes have a potential to generate hairpin structures. A much lower free energy of the putative hairpins at the 5′ end of the recombination site, and the possible formation of non-B-DNA structure by polypyrimidine tracks, may be important in the initiation of recombination. Using PCR, we found low levels of a specific mitochondrial deletion in certain *Neurospora* mutants. Their presence in low amounts in a population with a much larger number of normal mtDNA is unexpected. Contrary to earlier belief, this finding supports the view that deleted, smaller DNA molecules are not always suppressive relative to normal mtDNAs.

Intramolecular recombination of the mitochondrial DNA (mtDNA) may occur in animal, plant, and fungal cells (1–3). In *Neurospora crassa*, as well as in other fungi, these recombinational events can give rise to large deletions in the mitochondrial genome. They lead to mutant phenotypes characterized by grossly impaired mitochondrial function as seen in stopper mutants of *Neurospora* (3, 4), petite mutants of yeast (5), ragged mutants of *Aspergillus* (6), and senescent forms of *Podospora* (7).

Sequences involved in mtDNA recombination have been studied extensively in the yeast *Saccharomyces cerevisiae* (8). This was possible due to the availability of a large number of petite mutants (5); yeast, a facultative anaerobe, can survive with defective or no mtDNA. In contrast to yeast, *Neurospora* as an obligate aerobe has characteristics similar to higher eukaryotes, including humans. The deleted *Neurospora* mtDNAs are maintained as distinct replicating units (3, 4), which usually become suppressive by outnumbering the wild-type mtDNAs in heteroplasmons (multiple mitochondrial genotypes within an individual). In yeast and in most other organisms, there is little evidence for the maintenance of the mtDNA in a heteroplasmonic state. As in *Neurospora*, large mtDNA deletions and the presence of the resulting defective mtDNAs in a mixed population with normal mtDNA are associated with impaired mitochondrial function in humans. These lead to several myopathies including severe neurological disorders such as Kearn–Sayre syndrome and progressive external ophthalmoplegia (1, 9–11).

The facts that a detailed analysis of *Neurospora* mtDNA sequences is feasible and that intramolecular mtDNA recombination has important consequences in pathogenesis of a variety of organisms, including humans, prompted us to undertake this study. Here we present the identification, nucleotide sequence, and putative secondary structure of DNA segments involved in recombination of *Neurospora* mtDNA.

MATERIALS AND METHODS

Strains and Growth Conditions. The *Neurospora* strains were obtained from the Fungal Genetics Stock Center (FGSC) (Kansas City, KS). The wild-type strain was RL3-8A (FGSC 2218) and the spontaneous mitochondrial mutant strains were *abn*-1 (FGSC 1448), *abn*-2 (FGSC 1458) (12), and *stp*-B1 (FGSC 1574) (13). All other strains were generated in our laboratory. *Er-3* is an induced mutant (14), *abn*-1r, *abn*-2r, and *Er*-3r are apparent wild-type revertants. AA-4, AA-9, and AA-24 were obtained also as wild-type revertants from slow-growing mutants isolated during this study. All *Neurospora* cultures were grown as described earlier (4).

DNA Analysis. Methods for preparation of mitochondria, mtDNA, restriction digestion, nick-translation using [32P]dCTP, and other methods for cloning and hybridization were done as described (4, 15, 16). The mtDNA restriction fragment sizes and nomenclature used in this paper are as described earlier (4). The designation of recombiant plasmids was based on (i) original vector (pB, pBR322; pL, pBL30), (ii) the *Neurospora* strain or mutant (A1, *abn*-1; A2, *abn*-2; S1, *stp*-B1), and (iii) the size of the mtDNA insert in kilobase pairs (kbp) (i.e., pBA1-3.4 means pBR322 vector, *abn*-1 insert of 3.4 kbp).

Amplification of DNA. The PCR was performed in a 100-μl reaction mixture containing 67 mM Tris-HCl (pH 8.8), 6.7 mM MgCl2, 16.6 mM (NH4)2SO4, 10 mM 2-mercaptoethanol, each dNTP at 1 mM, bovine serum albumin (170 μg/ml), total genomic DNA (0.1–1 μg), 2–5 units of *Taq* DNA polymerase, and primers [at 50–100 μM for double-stranded DNA amplification or in a ratio of 1:50 for single-stranded (ss)DNA production]. Each PCR cycle consisted of denaturation for 1 min at 94°C, annealing for 2 min at 50°C, and extension for 3 min at 72°C. This cycle was repeated 30 times, using an automated thermal cycler (Perkin–Elmer/Cetus). The PCR primers CO2-283 and ND5-5328 were chosen to amplify the recombination junction. They were designated according to the genetic locus they define, ND5 or CO2, and the position of the nucleotide at the 5′ end of the primer corresponding to

Abbreviations: mtDNA, mitochondrial DNA; ssDNA, single-stranded DNA.

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RESULTS

Identification and Characterization of Recombination Junctions in mtDNA Mutants. *Neurospora* mutants with aberrant mtDNA were identified as those showing restriction fragment length polymorphisms on Southern blots when total DNA digests were probed with wild-type mtDNA. A number of mtDNA mutants, including *abn-1*, *abn-2*, ER-3, and *stp-B1* were chosen for detailed analyses of the recombination junction; the EcoRI digests of these putative mtDNA mutants (Fig. 1A) were probed with pBS1-2.2. As expected, two hybridization bands were identified in the wild-type mtDNA, corresponding to 4.85-kbp (E4) and 3.1-kbp (E6) DNA fragments. However, in mutants *abn-1*, *abn-2*, and ER-3, an additional hybridization band of 11.8 kbp corresponding to mtDNA was detected (Fig. 1B). In addition to the 11.8-kbp DNA fragment, ER-3 showed the presence of a 20-kbp (EcoRI-1) and a 3.1-kbp (EcoRI-6) band. These three contiguous DNA fragments, of 11.8, 20, and 3.1 kbp, defined the defective DNA in ER-3 (Figs. 1A and 2). Other DNA bands, present in lower amount, were wild-type specific, suggesting the presence of a mixed population of defective and wild-type mtDNA molecules in ER-3. Although *abn-1* and *abn-2* have the same 11.8-kbp fragment as ER-3, they do not share the other two EcoRI fragments. This polymorphism reflects secondary recombination events or more complex mtDNA rearrangements. A detailed analysis of the 11.8-kbp mtDNA fragments from *abn-1* and *abn-2* showed their restriction maps to be identical (Fig. 3A). Furthermore, the hybridization data presented in Fig. 3B showed that this 11.8-kbp DNA contained the recombination junction between the wild-type EcoRI-4 and HindIII-5 fragments, located >20 kbp apart on the mtDNA chromosome (see Fig. 2). Thus, the size and location of this deletion in *abn-1* and *abn-2* are the same as reported for ER-3 (4). Nevertheless, *abn-1* and *abn-2* show additional DNA bands, which indicates that their physical map is quite different from ER-3. This correlates with distinctive phenotypes in these mutant strains (12, 14).

In the mutant *stp-B1* an additional 2.2-kbp DNA band was identified. It represents a plasmid-like element derived from the mtDNA by excision and subsequent amplification of a contiguous DNA from EcoRI-4/6 wild-type mtDNA. Its detailed characterization including the recombination junction and entire nucleotide sequence was determined (13). In contrast to the above findings, AA9, *abn-2r*, and ER-3r displayed the pattern of restriction fragments expected for the wild type, suggesting the absence of recombinant mtDNA molecules.

*Fig. 1.* Identification of mtDNA deletions. (A) Gel electrophoresis of mtDNA EcoRI digests. Anomalous DNA fragments (arrows) correspond to strains *abn-1*, *abn-2*, ER-3 (2'), and *stp-B1* (2.2). Numbers designate wild-type-specific DNA fragments (left) and their molecular size (right). (B) Southern analysis. The pBS1-2.2 probe corresponds to wild-type EcoRI-4 (E4) and EcoRI-6 (E6) mtDNA fragments. The 11.8-kbp DNA band (2') contains the recombination junction in the mutants *abn-1*, *abn-2*, and ER-3. The 2.2-kbp DNA corresponds to plasmid-like DNA from *stp-B1*; the intense signal of hybridization is due to its high copy number.
**Amplification and Sequence Analysis of the Recombination Junctions.** A 218-bp DNA segment corresponding to the recombination junction was generated by the mutants abn-1, abn-2, and ER-3, but not by the wild-type RL3-8A during PCR amplification (Fig. 4A). Surprisingly, certain revertants (ER-3r and abn-2r) and isolate AA9 showed the same DNA amplification band of 218 bp (Fig. 4A) even though they looked identical to wild type as based on gel electrophoresis of mtDNA digests and Southern analysis (Fig. 1). Similar results were obtained for abn-1r, AA4, and AA24 (data not shown). The fact that these mutants and their revertant strains amplify the recombination junction confirms the presence of defective DNAs not detectable by other means.

The ssDNA generated during PCR amplification was used for sequencing of the recombination junction. The nucleotide sequence analysis presented in Fig. 4B identifies a 9-nucleotide sequence (CCCCGCCCC) present in the recombination junction of abn-1, abn-2, and ER-3. The same recombination site was identified for the three suppressive mutants ER-3, abn-1, and abn-2 as well as for the six residual mutants analyzed. The comparison of this DNA sequence with the wild-type mtDNA shows that as a consequence of recombination >20 kbp of DNA between two 9-nucleotide repeat elements was deleted. The repeats are located in the EcoRI-4 and HindIII-5 mtDNA restriction fragments (Fig. 2). Clustering of the recombination sites for several other Neuro-

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**Fig. 3.** Mapping of the recombination junction in abn-1. (A) General strategy for cloning the recombination junction. Restriction digests (HindIII) of the 11.8-kbp and wild-type EcoRI-2 DNA fragments were identical, except for a 3.4-kbp (pBA1-3.4) instead of the expected 3.1-kbp DNA band. To characterize the junction pBA1-1.6 was selected for further analysis. Arrows designate the sites of recombination generating a deletion of >20 kbp. R, H, and P, EcoRI, HindIII, and Pst I restriction sites, respectively. (B) Southern analysis. pBA1-3.4 was used as a probe for hybridization with restriction digests from wild-type mtDNA. As shown by the arrows, pBA1-3.4 hybridizes to EcoRI-4 (HindIII-1) and EcoRI-2 (HindIII-5) situated at different sites on the mtDNA chromosome (see Fig. 2). Numbers on the left designate mtDNA restriction fragments, and those on the right designate their respective sizes.
spora mitochondrial mutants is also shown in Fig. 2. The location of these sites on the mtDNA map is presented for mutants E35 (20) and pSNM (21).

Secondary Structure of Recombination Junctions. Possible secondary structures of the DNA fragments participating in abn-1 recombination are presented in Fig. 5A. These DNA fragments have inverted repeats with the potential to form hairpin (or cruciform) stem-loop secondary structures. The mtDNA located at the 5' end of deletion (ND5) has an extensive Pst I palindrome, containing two closely spaced Pst I sites (23). The Gibbs free energy for the potential hairpin structure was calculated to be $-59$ kcal/mol (1 cal = 4.184 J). The possible hairpin structure at the 3' end (CO2), had $\Delta G$ of only $-14$ kcal (Fig. 5A). Similar secondary structures were found in E35, pSNM, and stp-BI, the only Neurospora mtDNA mutants studied earlier (13, 20, 21) for which the DNA sequence of the recombination junction is known (Fig. 5B and C). In all cases, the recombinational event is consistent with a cleavage at exposed single-stranded nucleotides (unpaired or mismatched) in the stem-loop structure. The cleavage is produced at the 9-nucleotide repeat element (CCCCNCCCCC) involved in recombination.

The nucleotide sequence preceding the CCCCCGCCCC repeats in abn-1 (abn-2 or ER-3) is situated within a long stretch of polypyrimidines/polypurines (Figs. 4B and 5A). In ND5, 23 of 25 nucleotides are pyrimidines (mostly C). A similar characteristic is found in another Neurospora mutant, E35 (20). These polypyrimidine/polypurine sequences could form alternative secondary structures. Such putative structures (Fig. 5D), with a non-B-DNA conformation, are more likely to form at the 5' end of the recombination site (ND5). They could represent a (C, C) hairpin or a triple-helix H-DNA (Fig. 5D).

**DISCUSSION**

Our data show that the majority of recombination sites are clustered in the EcoRI-4 fragment of the Neurospora mtDNA (Fig. 2). Other hot spots are located in HindIII-9/10a, HindIII-3, and HindIII-7a DNA fragments. Three mtDNA mutants, abn-1, abn-2, and ER-3, and six additional isolates had identical recombination sites. Similarly 11 of 29 human mtDNAs isolated from muscle cells of different patients with mitochondrial myopathies, showed an identical recombination event (11). Moreover, both the Neurospora as well as the human mtDNA deletions started in the ND5 gene and lost about one-third of the original length of the mtDNA.

The presence of short repeat elements at the deletion ends as reported here is found in a variety of other mitochondrial systems: fungal (8, 28), plant (2), insect (29), and human (9–11), as well as in bacteria (30). At the same time, the Neurospora recombination junctions are characterized by long inverted repeats, capable of forming strong stem-loop hairpin (or cruciform) structures. The 5' end of the recombination site is characterized by a Pst I palindrome; this could generate a much stronger hairpin than the palindrome present at the 3' end. The polarity in hairpin strength, found in these mutants as well as stp-BI, E35, and pSNM, suggests the role of DNA secondary structures in the initiation of recombination, possibly through a scanning mechanism.

In addition, long regions of homopurine/homopyrimidine stretches present at the site of Neurospora mtDNA recombination (this study; refs. 4 and 20) have been recently shown in all human mtDNA recombinants analyzed (11). This strengthens the view that these structures display an altered DNA conformation. Besides the possibility of a simple hairpin structure, there is a potential to form a non-B-DNA structure, such as a (C, C) hairpin (24, 25) or H-DNA triple helix (26, 27). The common feature of any of these secondary

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**Fig. 5.** Potential secondary structures at the recombination junctions. (A) Hairpin loops in abn-1, abn-2, and ER-3. Vertical bar highlights the 9-nucleotide repeat CCCCCGCCCCC, and arrows designate potential cleavage sites to generate the recombinant fragments. There are two alternative cuts in ND5 (H5a or H5b) and CO2 (E4a or E4b). The hairpin at the 5' end of the deletion is much stronger than the one at the 3' end; it also contains a tandem of Pst I (P) sites (23). The Gibbs free energy values for these structures are given by $\Delta G$. (B and C) Hairpin loops in other mutants, stp-BI (B) and E35 (C). (D) Non-B-DNA structures. Alternative secondary structures are possible at the 5' end of deletion (ND5) through the formation of nonstandard DNA bonds: C-C*, (C, C) hairpins; G-G H-DNA (24–27).
structures is the extrusion of ssDNA. Once generated, such a DNA secondary structure could facilitate the initiation of the recombination event.

The most likely mechanism responsible for intramolecular recombination is considered to be an illegitimate recombination event (31). The same mechanism is believed to generate deletions at nuclear loci in many organisms, including mammals; better characterized are the hypoxanthine phosphoribosyltransferase, adenosine phosphoribosyltransferase, retinoblastoma, Duchenne muscular dystrophy, or steroid sulfatase loci (32–35). In addition, insertion and excision of mammalian viruses like simian virus 40 or polyomavirus (36) as well as DNA amplification in the mammalian system (37) are believed to take place by the same mechanism. The illegitimate recombination may be mediated either by slipped mispairing of direct repeats during the replication process (38) or by topoisomerase-catalyzed DNA breakage and rejoining (39). Such a role of topoisomerases has been shown for vaccinia virus, phage M13, bacteriophage T4, and phage λ DNA (39–41). These two mechanisms might be replication dependent since the topoisomerases are found as part of the replication complex. In fact, the topoisomerase-mediated recombination in phage M13 usually occurs at the replication origin (40). Moreover, multiple deletions in human mtDNA were mapped to the end of the D region (42). Alternatively, a nuclease could be involved in specific DNA cleavage for the initiation of the recombination event. A Neurospora endo–exonuclease has been recently shown to cleave in vitro mtDNA sequences similar to the ones we have characterized (43).

The Neurospora mutants analyzed resemble the heteroplasmic states of human mtDNA found in muscle cells with myopathy (9–11). It is generally thought that the selective maintenance of defective mtDNAs, as the basis for their suppressiveness, is due to the replicative advantage of a smaller DNA molecule (1, 4). However, our finding of the residual defective mtDNAs in several Neurospora mutants or revertants suggests that the copy number of these recombinant mtDNAs is highly variable. Further studies are necessary to investigate the nature of nuclear or mitochondrial factors controlling the copy number of these defective mtDNA molecules. A detailed analysis of recombination events in Neurospora may provide a useful model for the study of mtDNA recombination, deletion, and amplification, relevant to higher eukaryotes, including humans. This could be a convenient system, since there are no mammalian cell lines available with mitochondrial deletions or with mtDNAs maintained in a heteroplasmic state.

Note. After this work was completed, using PCR, it was found that normal heart, muscle, and brain from older human individuals contain low levels of a specific mitochondrial deletion (44). The amount of the defective DNA found in adult individuals contrasts with its high proportion in skeletal muscle of patients with Kearns-Sayre syndrome and ophthalmoplegia (9–11). This report of defective human mtDNA being present at low levels in a mixed population with normal mtDNA strengthens the view that deleted, smaller DNA molecules are not always suppressive in a heteroplasm with normal mtDNA.

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