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Insertion of an LrDNA gene fragment and of filler DNA at a mitochondrial exon-intron junction in *Podospora*

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ABSTRACT

A rearrangement of the mitochondrial genome of a long lived mutant of *Podospora anserina* is presented. It consists in the insertion of 191 bp of the LrDNA gene (coding for the large ribosomal RNA) at the junction between exon1 and intron α of gene *col* (coding for subunit 1 of cytochrome oxidase). This insertion is accompanied by a 53 bp deletion of the junction and the presence of extra A and T nucleotides at both sides of the inserted sequence. We discuss possible mechanisms of production of this rearrangement. The presence of extra nucleotides at the recombination junctions suggests that it may pass through a stage of free DNA ends originating from a DNA break at the junction between exon1 and intron α of gene *col*. The possibility that such a DNA break plays a major role in the instability of the mitochondrial genome is envisaged .

INTRODUCTION

In pluricellular organisms, mitochondrial DNA rearrangements are known to be responsible for several deleterious syndroms such as neuromuscular (1) or hematological (2) deseases in humans, male sterility in higher plants (3,4) and various types of mycelial degeneration in filamentous fungi (5,6).

In the filamentous fungus, *Podospora anserina*, senescence is correlated with rearrangements of the mitochondrial DNA and amplification of specific sequences of this genome as tandemly repeated circular molecules (sen-DNAs) (7,8,9). The most often amplified sequence in senescent cultures, called α sen-DNA or pl DNA, consists in the first intron (group II) of the mosaic gene *col* (coding for subunit 1 of cytochrome oxidase, Ref 10). Although the correlation between appearance of sen-DNA molecules and senescence is well established, the molecular mechanisms involved in this process are not understood. One way to better understand this relationship is to analyse mutants escaping senescence.

Several mutants have been described. Most of them are mitochondrial (11). They result either from rearrangements of the mitochondrial genome in the regions from which sen-DNAs originate, leading to the presence of several populations of molecules (12,13,14) or from the deletion of the α intronic region (11,15). We report here the molecular analysis of a new long lived mutant which differs from the previously described ones in the original structure of its mitochondrial genome. A fragment of 191 bp of the LrDNA gene (coding for the large ribosomal RNA) is inserted at the junction exon1-intron α of gene *col*. This insertion is accompanied by a 53 bp deletion of the junction and the presence of extra nucleotides (filler DNA) at both rearrangement points. Northern analysis of this mutant fails to detect any RNA corresponding to the free α sequence.

Relationships between this rearrangement and the senescence resistant phenotype are discussed. This mutant constitutes a supplementary argument in favour of a key role for the intronic α sequence and more particularly for its 5′ junction in the appearance and maintenance of the senescent state.

We are particularly interested by the possible mechanisms of production of this non homologous recombination and consider several hypotheses. The presence of filler DNA suggests that this rearrangement arose in a two step process involving free DNA ends in a first stage. The possibility that these DNA ends are generated by an endonuclease specific for the junction between exon1-intron α of gene *col* and the possibility that such a DNA break may play a major role in the instability of the mitochondrial genome in *Podospora* are envisaged.

MATERIALS AND METHODS

Materials. The juvenile, senescent and mutant cultures were derived from the *s* wild-type strain of mating-type (+). The culture conditions, life-span measurements, genetic analysis and the extraction of the mitochondrial DNA were as previously described (16,17).

Restriction analysis and Southern blotting. Restriction endonucleases were purchased from New England Biolabs, Boehringer Mannheim or Appligene. DNA-DNA hybridization was done as previously described (11).

Cloning. The fragments used for probes or for DNA sequence determinations were separed on a preparative agarose gel. They were eluted from the agarose and cloned into the pUC18 or M13 mp19 vectors.

DNA sequencing. Nucleotide sequences were determined by the dideoxyribonucleotide chain terminating method with ^{35}S-dATP (18).

Isolation of mitochondrial RNA, Northern blots. Mitochondria were extracted from exponentially growing mycelium (liquid cultures grown during 24h), from a sucrose gradient, according

to the method used for preparation of yeast mitochondria (19). They were lysed in Tris-HCl 10 mM pH 7.5 (T.E), SDS 2% and the suspension was extracted twice with phenol. RNAs were precipitated by ethanol, resuspended in T.E + 10 mM Vanadyl ribonucleotide complex (Bethesda Research Lab). After DNase treatment, RNAs were electrophoresed through 1.4% agarose gels containing formaldehyde and transferred to nitrocellulose filters.

Labelling of cloned DNA probes was performed by nick-translation, labelling of oligonucleotides was performed at their 5'ends with ^{32}P-ATP in a reaction catalyzed by T4 polynucleotide kinase (Boehringer Mannheim).

RESULTS

I. Characterization of mutant mex16

Mutant *mex16* is a spontaneous mutant selected as an outgrowth of a senescent *s mt* + mycelium. It shows uninterrupted growth (more than 200 cm since its selection) unlike a wild type *s mt* + strain whose mean lifespan is 40cm. It exhibits a slow growth rate (reduced from one half compared to that of wild-type) and lacks the cytochrome aa3 absorption peak. It is sterile when used as the female parent and does not transmit any of these traits when used as male.

Restriction analysis of the mitochondrial DNA of *mex16* shows that with *Hae*III, it lacks fragment n°23 and presents an additional band of about 6 Kb in a stochiometric ratio (Fig.1). Fragment *Hae*III n°23 normally belongs to the intron α (Fig. 2).

Hybridization experiments on this DNA using the α sen-DNA as probe confirm the absence of fragment *Hae*III n°23 and show that the additional band hybridizes with α (Fig.1). As no other detectable difference was observable in the restriction pattern of *mex16*, we hypothezised that the *Hae*III site between fragments n°23 and 5, just in front of the junction between exon1 and intron α of gene *col*, was lost, leading to the disappearance of these two fragments (loss of fragment n°5 is not discernable by restriction analysis because there exists another fragment of the same size —see Fig. 2—), and the appearance of a new fragment including both of them.

We therefore cloned fragment *Bgl*IIn°5 that contains the junction between *Hae*III n°23 and 5, from the mitochondrial DNA of *mex16*. The restriction analysis of this clone confirmed the loss of the *Hae*III site and revealed the presence of a small insertion of about 150 bp at about the position of the lost H*ae*III site.

In order to determine the origin of this insertion, the *Bgl*II n°5 fragment from *mex16* was used to probe digested mitochondrial DNA from *mex16* and wild type. In addition to the expected fragments included within the *Bgl*II n°5 region, new fragments (*Hae*III n°25, *Bgl*II n°2, *Xho*I n°4, *Cla*I n°8) located within the *Eco*RI n°5 region (Fig. 2) hybridize with this probe in both wild type and *mex16*. Results with *Hae*III digested DNA are presented in Fig. 3. These data indicate therefore that mutant *mex16* carries upstream of the α intron, a small duplicated sequence of about 150 bp coming from the *Eco*RI n°5 region.

II. Sequence of the rearranged exon1-intron α junction in mutant mex16

To determine the exact structure of the rearrangement, the nucleotide sequence of the modified region was established. The presence of one *Sac*I and one *Hind*III site on each side of the insertion, allowed to subclone the fragment *Bgl*II n°5 from *mex16* into the polylinker of M13 and to sequence about 300 bp around the junction exon1-intron α of gene *col* in mutant *mex16*. Results are given in Fig. 4. They indicate several notable facts.

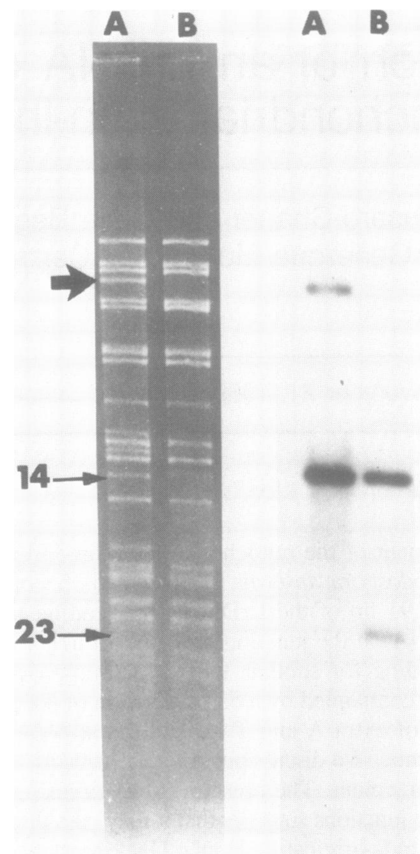

FIGURE 1 *Hae*III restriction pattern of *mex16* (A) and wild-type (B) mitochondrial DNA and corresponding hybridization with a ^{32}P-labelled probe, Pα, (sequence α inserted in pBR322).The thick arrow indicates the additional fragment in *mex16*; thin arrows, the positions of the *Hae*III n°14 and 23 fragments in wild type.

Firstly, a sequence of 191 bp corresponding to a sequence of the LrDNA exon1 gene (position 1912−2101), located about 30 Kb from intron α, in the *Eco*RI n°5 fragment (Fig. 2), is inserted upstream of the α sequence. The inserted sequence corresponds to a fragment of domain II of the 23SrRNA : 62 to a universally conserved structure and 129 to a non structured variable region (20).

Secondly, this insertion is accompanied by the deletion of 53 bp (30 exonic and 23 intronic) of the junction exon1 -intron α of gene *col*.

Thirdly, the insertion is flanked by short stretches (9+6) of A and T residues at both recombination junctions.

It can also be noted that a 7 bp direct repeat TATACAA is present at the insertion borders. This direct repeat overlaps the LrDNA sequence by several base pairs.

To verify that the extra A and T residues have been added at the insertion borders and are not present in the rDNA sequence of the mutant, the *Eco*RI n°5 fragment from *mex16* was cloned by a shotgun experiment and partly sequenced. This sequence revealed that the resident duplicated copy was identical in *mex16* and wild type and did not present the A and T residues. These are therefore foreign nucleotides which belong neither to the donor nor to the recipient sequence.

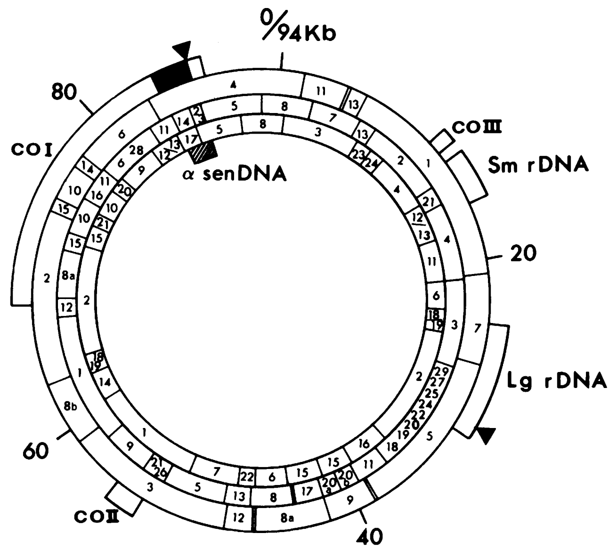

FIGURE 2 Restriction map of wild type mitochondrial DNA from *Podospora anserina*. From inside to outside are the *Bgl*II, *Hae*III, *Eco*RI restriction maps (from Turcker and Cummings; see Ref.34). The location of genes coding for the cytochrome oxidase subunits (*co1, co2, co3*), for the LrRNA and the SrRNA are given. The α sen-DNA is hatched and the α intron is darkened. The arrows show the position of the insert in *mex16* (at the junction exon1-intron α) and the region from where it originates (in the LrDNA gene).

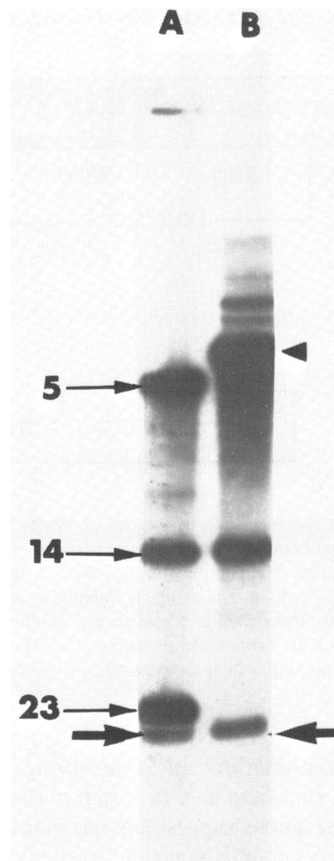

FIGURE 3 Hybridization of ^{32}P-labelled *Bgl*II n°5 fragment from *mex16* to *Hae*III-restricted mitochondrial DNA from wild type (A) and *mex16* (B). Thin arrows indicate the position of the *Hae*III n°5, 14, 23 fragments with which the *Bgl*II n°5 fragment normally hybridizes. indicates the position of the additional band in *mex16*. Shown by a thick arrow is the *Hae*III n°25 fragment which hybridizes with the insert present in the *Bgl*II n°5 fragment of *mex16*.

III. Absence of free α RNA molecules in mutant mex16.

Mitochondrial RNA from wild-type and *mex16* was analyzed by Northern hybridization experiments. The α intronic sequence and a synthetic 54 mer oligodeoxynucleotide, complementary to an exonic sequence located at the 3′ end of gene *col*, were used for detection of homologous RNAs. Fig. 5 shows that in wild type , the α probe hybridizes to two RNA species of 2.7 and 2.5 Kb, interpretated as two forms (branched and debranched lariat) of the excised intron, while the exonic probe hybridizes with the mature mRNA of 3.2 Kb of gene *col* (21). In *mex16,* both probes only hybridize with a high molecular weight RNA species, not detectable in wild type.

The splicing deficiency of the α intron is not surprising since 53 pb of the intron-exon junction are deleted, in particular the IBS elements (Fig. 4), whose pairing with intronic sequences is required for proper and efficient splicing of mitochondrial group II introns in *S. cerevisiae* (22). Furthermore, the inserted sequence disrupts the normally occuring reading frame between the intronic ORF and the upstream exon and therefore may cause a premature chain termination of the potentially encoded protein.

DISCUSSION

In this paper, we described an original rearrangement of the mitochondrial *col* gene resulting from a non homologous recombination event, in a long lived mutant of *Podospora*. This genomic rearrangement consists in 3 characteristics: 1) the insertion of a 191bp fragment of the LrDNA gene at the 5′ junction of intron α, 2) a 53 bp deletion of the junction α, 3) the addition of extranucleotides consisting in A + T residues at both insert borders. These data raise intriguing questions about the mechanisms involved in formation of this rearrangement. More than one mechanism is likely to be responsible for it.

In view of the nature of the insert (a fragment of rDNA) and its location (the exon-intron junction), the idea of a RNA intermediate can be envisaged. In *Neurospora*, hybrid RNA molecules consisting of the Varkud plasmid transcript plus a 5′ leader derived from the mitochondrial small rRNA have been observed (23). In *mex16*, recombination at the RNA level followed by reverse transcription, does not however account for the addition of the A + T residues at the insert junctions. This characteristic rather recalls non homologous rearrangements described in mammalian cells (reciprocal translocations, rearrangements in the immune system, in transfected DNA and in viral genomes....). As in *mex16,* a number of these rearrangements present extra nucleotides (termed filler DNA) at their junctions (24,25). In fungal mitochondria, the insertion of short stretches of nucleotides at recombination junctions is not unique to *mex16* and has also been observed in several other genomic rearrangements in *Podospora* (26,27) and in *Neurospora* (28).The presence of this peculiar type of junction in *Podospora* mitochondria suggests that, as in mammalian cells, free DNA ends to which extra nucleotides could be added, are likely intermediates in non homologous recombination mechanisms.

As proposed by Roth et al (24), the filler DNA could be generated by the addition of preformed blocks of nucleotides (RNA or DNA fragments) present in the cellular pool. The straight tendency towards high A + T content of this filler DNA would reflect the composition of the pool.

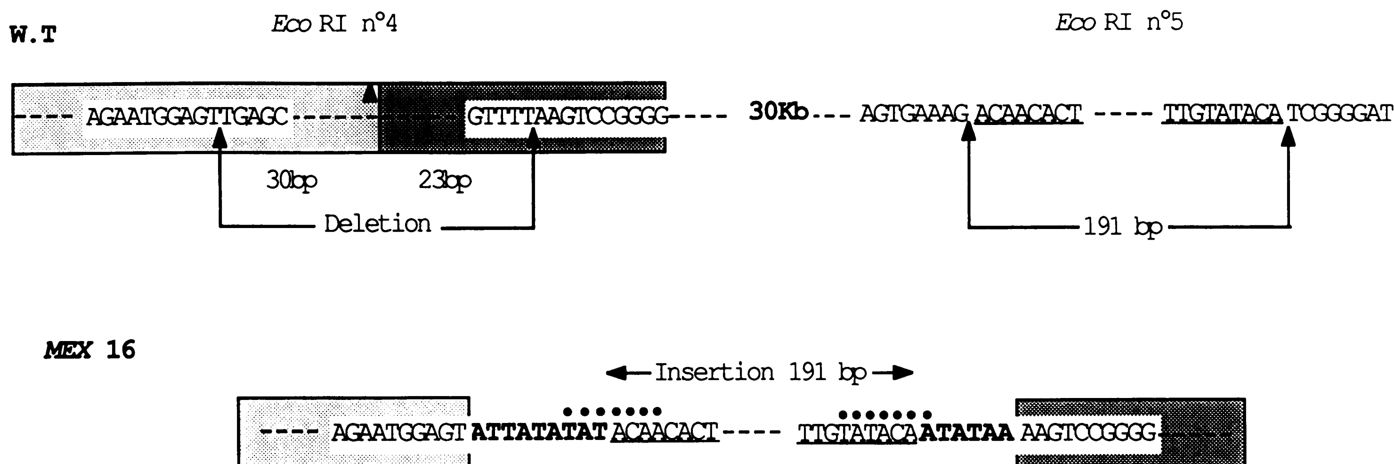

FIGURE 4 Schematic representation of the mitochondrial DNA from wild type (W.T) and the mutant *mex16*. For W.T, only part of the nucleotide sequence of the *Eco*RI n°4 fragment boarding the junction between exon1 and intron α of gene *co1* and part of the nucleotide sequence of the *Eco*RI n°5 fragment from which the insert comes are given. The complete sequence of intron α and the junction is given in Osiewacz et al (10) and in Cummings et al (35); the complete sequence of the LrDNA gene in *Eco*RI n°5 is given in Cummings et al (20). For *mex16*, only the nucleotide sequence of the rearranged exon-intron junction in the *Eco*RI n°4 fragment is shown. The *Eco*RI n°5 sequence is identical to that of W.T. Exon1 from *co1* is boxed and lightly shaded. Intron α is boxed and darkly shaded. indicates the position of the exonic IBS elements (22). The duplicated *Eco*RI n°5 sequence in *mex16* is underlined. The 53 bp deletion of *mex16* is indicated on the W.T. The extra-nucleotides at both extremities of the insert are printed in bold type. The 7 bp direct repeat at the insertion borders is indicated by points.

The possibility that free DNA ends are intermediates in non homologous recombination in *Podospora* mitochondria, implicates a DNA breakage as a first step in the recombination event. Several observations suggest that one of these breaks could be site specific and occur at the junction between exon1 and intron α of gene *co1*. Indeed, such a break could represent an initial step in the liberation of the α sen-DNA in wild type, it could also be at the origin of numerous mutants of *Podospora*, modified in the α region and which all share a rearranged 5′ junction of intron α. This is the case for mitochondrial mutants *mex1, mex5, mex7*, deleted for the exon1- intron α sequence junction (11) and for a nuclear mutant characterized by a premature vegetative death, in which the 5′ junction of intron α is systematically found reassociated with a specific distal fragment (Belcour, personal communication). It must be noted however, that whereas mitochondrial intron-encoded DNA endonucleases, specific for exon-exon junctions, have been observed in yeast (29,30,31), no endonuclease activity specific for exon-intron junctions has been found till now.

Experiments are actually in progress to test the occurence of such an activity in *Podospora* mitochondria. If free DNA ends resulting from a specific intron-exon cleavage really exist, they would have been trimmed by a nuclease in mutant *mex16* before being joined via filler DNA. The mechanisms leading to insertion of the invading LrDNA segment are subject to speculation and the nature of the intermediate that integrates (DNA, cDNA, RNA) remains on open question. Although no precise model could be advanced from these data, one can mention that proposed by Schwartz-Sommer et al (32) and Pritchard et al (33) which assumes that for some LINE elements, the transcript integrates within a preexisting nick in the chromosomal DNA, allowing subsequent priming of reverse transcription.

As for the other mitochondrial mutants modified in the α intronic sequence (11,14), mutant *mex16* does not allow us to conclude wether the long lived phenotype is correlated with the absence of cytochrome oxidase or with the loss of the integrity of the α sequence. Only the selection of other mutants, specifically deficient in cytochrome oxidase activity or in the

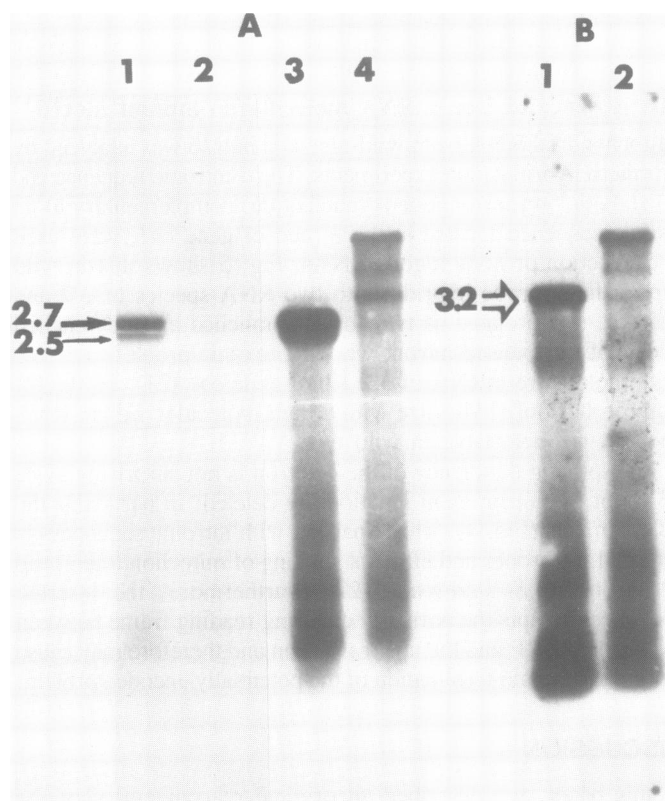

FIGURE 5 A : Hybridization of the ^{32}P- labelled probe, Pα (see legend to Fig. 1), to mitochondrial RNAs extracted from wild.type (1,3) and *mex16* (2,4) strains. For 1 and 2, the autoradiogram was exposed 5 hours at -80°C. Thick and thin arrows are respectively interpretated as the lariat and the debranched forms of the α RNA (21). For 3 and 4, the autoradiogram was exposed 2 days at -80°C. B : Hybridization of a ^{32}P-end labelled exonic oligonucleotidic probe to the same blot as in A after dehybridization, 1 : wild.type, 2 : *mex16*. The open arrow corresponds to the mature *co1* transcript (21). Molecular weights are indicated in Kb.

intronic sequence will help us to clarify this question.

However, the accumulation of independent mutations, selected for a long lived phenotype and localized in this region seems to be significant and argues for a key role of the α sequence, in particular of its 5′ intronic junction, in the appearance and maintenance of the senescent state in *Podospora*.

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