DNA sequence of the excision sites of a mitochondrial plasmid from senescent *Podospora anserina*

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ABSTRACT

During senescence in *Podospora anserina*, specific gene regions of the mitochondrial genome are excised and amplified. The most prevalent, termed α -event senDNA, is a 2600 bp circular molecule which is excised from the contiguous Hae III fragments 23,14 region of the mitochondrial DNA restriction map. We have cloned α -DNA plasmid from races s^+ and A^+ as well as the genomic fragments Hae III 23,14 and have sequenced those regions which constitute the α -junction sites. We have found that one excision site (J1) is located 24 bp from the proximal Hae III 23 restriction site and the other (J2) 172 bp from the distal Hae III 14 site. Flanking the α -DNA sequences on the mitochondrial genome, there are 10 bp palindromic sequences: CAATATATTG, ending 3 bases from the J1 site, and ATTATATAAT which starts 8 bases from the J2 site. Neither of these 10 bp palindromes are present on the α -DNA plasmid. Abutting the J1 site on the α -DNA there is a 5 bp sequence (GTGCT) which is repeated 8 bp downstream. In joining the two distal J1 and J2 sites, a 7 bp repeat (ACGTGCG) is produced. These results are discussed within the context of site-specific recombination.

INTRODUCTION

For some years, *Podospora anserina* has been used as a model system for studying maternal inheritance of senescence (1-4). Several geographic isolates or races have been studied and each has a race-specific programmed rate of senescence. Moreover, evidence was obtained that a transmissible cytoplasmic factor was involved (5). Based on the effect of inhibitors of mitochondrial function as well as genetics, Belcour and Begel (6) put forth a model for senescence which implied that mitochondria played a suppressive-like role. This model was drawn largely from the petite mutation in the yeast *S. cerevisiae* where illegitimate site-specific recombination has been proposed to lead to the amplification of portions of the mitochondrial genome (see Bernardi (7)). At the same time, we (8) and others (9) showed that mitochondrial (mt)DNA from senescent cultures of *Podospora anserina* contained a multimeric set of small circular molecules rather than the 94 kilobase pair (Kb) circular molecules isolated from young cultures (10). Later work showed that different independent

senescent cultures gave rise to this same set or to other sets of small circular molecules rather than the 94 kilobase pair (Kb) circular molecules isolated from young cultures (10). Later work showed that different independent senescent cultures gave rise to this same set or to other sets of small circular mtDNA molecules. At least six such sets have been described and all have been shown to hybridize to the young mitochondrial genome (11-13). The most prevalent of these, termed α -event senDNA or α -DNA plasmid is a 2.6 Kb circular molecule derived from the Hae III fragments 23,14 segment of the restriction map (14,15). We showed that each of these sets contained or were part of specific gene regions of the mitochondrial genome with α -DNA plasmid containing most of the Oxi III gene (subunit I of cytochrome c oxidase) (15). Recently, we showed that α -event senDNA (as well as another senescent mt plasmid, termed β -event) could be transferred to the nuclear genome during senescence (16). Thus, these plasmid-like mitochondrial gene regions, in particular α -event senDNA, could be playing the role of the transmissible cytoplasmic factors referred to earlier.

From a molecular viewpoint, the most interesting feature of these mitochondrial plasmids which arise during senescence is their ability to excise and amplify. Numerous examples of this phenomenon exist in both procaryotes (17) and eucaryotes (18). Determining the nature of the excision and consequent junction sites is essential in understanding this process. By comparing the junction genomic region of the mitochondrial genome with the junction splice sites of the plasmid we are able to determine the DNA sequence at the distal sites of excision as well as at the spliced plasmid junction sites. Here, we report these DNA sequences from α -plasmid isolated and cloned from races s^+ and A^+ and the mt genomic sequences from race s^- .

METHODS

Podospira anserina Strains and Clones

Podospira anserina race $s(-)$ mating type was used for the isolation of mitochondrial DNA. Isolation of mtDNA was carried out as previously described (10,19). The Hae III 23,14 contiguous fragments were isolated from a partial digest of EcoRI fragment 4, ligated with EcoRI linkers, and as before (15) cloned into pBR325. Races $s(+)$ and $A(+)$ were used for the establishment of senescent cultures necessary for cloning α -DNA plasmid. α -DNA plasmid was cloned into the plasmid pSV010 which possesses a single Bgl II restriction site in a polylinker (20).

S1 Nuclease Protection Experiments

S1 nuclease analysis was conducted essentially according to Berk and Sharp (21) as modified by Weaver and Weissman (22). mt RNA was isolated as previously reported (14,15). For the DNA-DNA heteroduplex, unlabeled Hae III fragment 23 was used in large excess and the final hybridization conditions were done in 65% formamide for 15 min rather than the 80% formamide and 4 hr hybridization as was done for the DNA-RNA experiments. S1 nuclease was obtained from Boeringer Mannheim and all restriction enzymes from BRL.

DNA Sequencing

The chemical method of Maxam and Gilbert (23) was used throughout. DNA fragments were first dephosphorylated and then labeled at their 5'-ends using polynucleotide kinase and ^{32}P - γ ATP (ICN). After labeling, uniquely labeled 5' ends were obtained by cleavage with an appropriate second enzyme. For the Hind III - Hinf I (D) fragment (see Figure 1), two separate cleavages were done, one with Msp I and the other with Dde I thus allowing completion and verification of the sequence.

RESULTS AND DISCUSSION

Sequencing Strategy

The entire circular restriction endonuclease maps and the localization of α -event senDNA have already been published (14,15). Only that region of the map containing the Hae III 23,14 fragments is presented here and only those sites pertinent to DNA sequencing are marked (Figure 1). α -event senDNA arises from inside the proximal Hae III 23 site on the left and the distal Hae III 14 site on the right. Fine structure mapping (not shown) indicated that the Hind III and Msp I sites near the so-called J1 region and the Hinf I site near the J2 region were ideal for determining the sequences of both α -DNA plasmids and the genomic DNA. These are referred to as A and C for the genomic and D for both plasmids. The only difference noted between the α -DNA plasmids from races s^+ and A^+ was that s had two closely spaced BamH I sites whereas A^+ had only one. For the plasmids, the Hind-Hinf and the Bam-Msp, Msp-Bgl fragments were labeled from their 5' ends, cleaved and sequenced. For the genomic DNA, only the Hind-Hae fragments and Hinf-Hae fragments were used. Appropriate cleavages were utilized to assure overlap in the DNA sequencing.

Localization of Excision Sites

The approximate location of the excision site within the Hae III 23 fragment was accomplished by S1 mapping. We had two purposes here. One was to determine

Figure 1. Restriction map of the Hae III 23, 14 genomic region of race s mtDNA and the corresponding race s α -DNA plasmid. The Hae III sites are shown at each terminus of the Hae III 23,14 fragment but the clone contained EcoRI linkers. As described in the text, fragments A and C were used as the source of genomic excision sites, J1 and J2 respectively. Fragment B was used as an initial source of each half of the molecule. Fragment D contained the J1 J2 splice junction for α -DNA plasmid from both race s and A.

the location of the J1 site and the other was to determine the coding strand. S1 mapping for both RNA-DNA and DNA-DNA duplexes was done using the Hind-Hinf labeled fragments (D) from either race s⁺ or A⁺ α -DNA plasmids. Only the fragment containing the ³²P- γ -ATP labeled at the Hind III 5' end hybridized to the RNA. Unlabeled genomic Hae III 23 fragment was used in the DNA-DNA hetero-duplex with the same ³²P- γ -ATP Hind III fragment. For sequence gel analysis, these S1 generated probes were placed side by side next to the same α -DNA plasmid fragment D and the genomic fragment A1. It can be noted (Figure 2) that both types of S1 duplexing experiments gave the same result. The J1 junction site lay between the Msp I site and Hae III 23 site. These S1 experiments are presented only to show the approximate location of the junction sites. Other details of S1 mapping of the RNA-DNA duplexes of α -DNA plasmid will be published elsewhere.

DNA Sequence of the Junction Site

As can be noted in the restriction maps (Figure 1), the α -DNA plasmid

Figure 2. Location of the J1-J2 splice junction by S1 nuclease digestion. Lane A represents the DNA-RNA protection and lane B the DNA-DNA protection. Note that both experiments yielded the same protection (J1-J2) site. The fully protected fragment in the DNA-RNA lane A represents another α -DNA plasmid transcript which will be reported elsewhere. Group α is the sequence ladder (8% polyacrylamide gel) for fragment D1 cleaved with Dde I endonuclease. Group 23,14 is the sequence ladder for fragment A1 cleaved with Msp I. For both, the Hind III 5'-labeled fragments were sequenced. Note that the sequence for both fragments is identical up to the Msp I site. Divergence occurs at 27 bp from this site. Appropriate control experiments showed only the expected probe sizes.

sequences should be identical with those in Hae III fragment 23 as the left distal Hae III site is approached before divergence occurs at J1. Similarly, α -DNA sequences should be identical with Hae III fragment 14 until the right distal Hae III site is approached and then diverge at J2. Ligation at these two excision sites generates the α -DNA plasmid. The actual sequence of the A1, C and D fragments (Figure 1) were read by examining several fragments so that overlap was achieved. Sequences labeled at the 5' Hinf I site indicated on the right side of genomic DNA (fragment C) were identical with the Hinf I oriented sequence from the Hind-Hinf (D1) fragment generated from the Hae III

There are several interesting features of these excision site sequences. On each flank of the J1-J2 sites, there are 10 bp palindromes, neither of which are contained within the α -DNA sequences. These palindromes are non-identical except for an internal inverted repeat. While this structural arrangement could be fortuitous, these palindromic sequences might serve as recognition sites for putative DNA binding proteins which bring together the two distal ends of α -DNA.

Within the α -DNA, immediately 3' to the J1 excision site, there is a 5 bp sequence (GTGCG) which is directly repeated 8 bp downstream. In joining J1 to J2, this repeat becomes a 7 bp unit (ACGTGCG). These duplicated 7 bp sequences are present only in the α -DNA. A 7 bp unit could act as the recognition site for an excision enzyme complex as well as a target site for ligation. Other repeats are also present (CGTTT) on the 3' side of the J1 site but whether these or other sequences farther distant are significant is not known. Sequencing additional senDNA plasmid excision sites will be helpful. We have sequenced the entire region from the Hind III to the Hinf I sites (D1; 362 bp) and no other obvious repeats or palindromic elements are apparent. In addition, other than the one BamHI specific sequence noted earlier, no differences were noted between α -DNA plasmids obtained from two different races (A and s). Thus, these sequences are highly conserved.

Earlier, we indicated that these senDNA plasmids could be transferred to the nuclear genome during senescence (16). In addition, site-specific recombination has been put forth as a possible mechanism for the generation of these plasmids from the mitochondrial genome (6). For both of these, it is important to consider the significance of the palindromic and direct repeat sequences discussed above. Three examples are pertinent where site-specific recombination is known: transposons (24), integration and excision of λ bacteriophage (25) and the generation of complete immunoglobulin heavy-chain genes (26,27). Procaryotic transposons have direct or inverted repeats at each end of the transposon sequence which remain contiguous after transposition. The α -DNA terminal sequences (J1-J2) do not share such repeats. Site-specific recombination in λ -bacteriophage involves a 15 bp homologous sequence (or common core) on λ and bacterial host DNA (attP and attB, 24). We have noted no such common core on the flanking mitochondrial DNA sequences and the α -DNA plasmid. Thus it is difficult to explain excision-amplification and/or transposition of the senDNA plasmids on the basis of homologous sequences. The closest parallel to draw on is that mechanism proposed for immunoglobulin heavy-chain gene recombination. Here, the only conserved sequences noted are two blocks of short sequences 3' and 5' to the gene segments: a 7 bp imperfect palindrome (CACTGTC) and a nanomer (25, 26). The 7 bp unit was conserved on both flanks but the nanomeric sequence was different. Since complementary antiparallel pairing was not considered possible, Early et al (26), proposed a different sort of site-specific recombination. In this model, the two blocks of short flanking sequences were thought to be critical for interaction with specific DNA binding proteins. These protein complexes would bring and hold together the two distal ends of the immunoglobulin genes

prior to excision and recombination. The separation of the two blocks (sufficient for one or two turns of the DNA helix) was considered to be an integral feature of the model. In some respects, this model has similarities with the results presented here where 10 bp palindromes flank the ends. These could function as site-specific sequences for DNA binding proteins. The separation of these two palindromes from the splice junction is 10 bp (see Figure 3), or enough for one turn of the DNA helix (28). Whether this is significant for the binding and subsequent splicing of the excised ends of the plasmid is not clear. Clearly, care must be taken in attaching too much importance to structural similarities or dissimilarities with other recombination mechanisms. Thus far we have sequenced just one sendNA plasmid and compared its sequence with contiguous mitochondrial flanking sequences. We have no information as yet regarding other sendNA excision sites or the nuclear insertion sites of α - and β -DNA plasmids. Ascertaining these sequences should provide critical data for understanding excision and amplification. It can not be overemphasized that here we are dealing with putative mitochondrial mobile elements which may be playing a pivotal role in determining cellular senescence. This report presents the excision sites of one such element.

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