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Sensitivity of intergenic regions of yeast mitochondrial DNA to single-strand-specific nucleases

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The reactivity of mitochondrial DNA (mtDNA) sequences from *Torulopsis glabrata* and *Saccharomyces cerevisiae* towards single-strand-specific nucleases has been examined. AT-rich stretches located in intergenic sequences from both yeasts were cleaved by nucleases when the sequences were contained in supercoiled plasmid DNA. In particular *ori/rep* sequences from the mtDNA of *S. cerevisiae* were shown to be sensitive to the single-strand-specific nucleases. The locations of the sensitive sites were related to the organisation of the sequence domains of *ori/rep* and the superhelicity of the DNA, as well as the presence of particular sequences. It is proposed that distortions of the DNA duplex could be generated in mtDNA molecules in vivo and that these distortions may provide a substrate for enzymes involved in transmission, recombination and/or transcription of mtDNA.

mtDNA; Intergenic sequence; Single-strand-specific nuclease; (Yeast)

1. INTRODUCTION

High nucleotide sequence homology has been observed between the coding regions of mitochondrial DNAs (mtDNAs) from various species. In contrast, non-coding regions of these mtDNAs show extreme sequence divergence [1,2].

In the mtDNA of *Saccharomyces cerevisiae* the intergenic regions consist of long AT-rich spacers, various GC clusters and eight *ori/rep* sequences [3]. These intergenic sequences are known to be involved in excision-deletion processes leading to respiratory deficient 'petite' mutants. It was demonstrated that these intergenic sequences are also involved in replication of yeast mtDNA [4,5]. However, recently it has been shown that several intergenic regions encompassing *ori/rep* are dispensable for expression of the respiratory phenotype [6,7]. These particular intergenic sequences appear to enhance transmission of mtDNA to progeny and may be inherited in yeast mtDNA for that reason. A candidate for the sequences which facilitate transmission may be *ori/rep* and its adjacent sequences [6,7].

In the yeast *Torulopsis glabrata* the mitochondrial intergenic regions consist only of a dozen short AT spacers including highly conserved oligonucleotide motifs (nonanucleotide, dodecanucleotide) [8] which are proposed to be the origin(s) of replication [9].

As biologically active regions of DNA frequently exhibit a dynamic secondary structure, as detected by sensitivity to single-strand-specific nucleases [10], various yeast mtDNA sequences were examined by this procedure in order to seek clues as to the possible functions of mitochondrial intergenic sequences. Here, the existence of nuclease sensitive structures induced by torsional stress in the mitochondrial intergenic sequences of two yeast species is reported.

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2. MATERIALS AND METHODS

2.1. *Plasmids*

Plasmids used in the following experiments are listed in table 1. The orientation of inserts in the plasmids is as follows. In pTGM1 the *Bgl*II site maps close to the *Pvu*II site in the vector, and the reverse in pTGM2. In pTGM19 the gene *cyt b* is oriented towards the vector *Eco*RI site. The precise map and sequences of *T. glabrata* mtDNA inserts can be found elsewhere [8]. Plasmid pSCM107 (*ori3*) is described in [12]. The *Ava*II sites in the insert of pTW1 (*ori1*) lie in the vicinity of the vector *Eco*RI site and the sequence of the insert can be found elsewhere [11]. The insert from pTW1 was recloned into the *Eco*RI and *Hin*dIII sites of pUC18 reversing the orientation of the insert and creating pBB5/18. pBB5/C was constructed from the pTW1 insert. The 0.46 kb *Hpa*II fragment containing the GC cluster C of *ori/rep* was cloned into the *Acc*I site of pUC13. The insert's *Mbo*II site is oriented towards the vector's *Hin*dIII site. The inserts are illustrated in figs 2 and 3. When some additional subfragments from the pTW1 insert were subcloned in pUC18/19, deletions and rearrangements in the inserts took place indicating that the constructs were unstable (not shown, and [11]).

2.2. *Mapping of nuclease-sensitive sites*

Approx. 5 μg of supercoiled plasmid DNA, prepared from CsCl gradients, was used per digestion. The reaction buffer used throughout was 0.05 M sodium acetate, pH 4.6, 1 mM $ZnSO_4$ plus 0.2 M NaCl (high concentration buffer) or 0.05 M NaCl (low concentration buffer). The DNA was digested with 30 units of mung bean nuclease or S_1 nuclease at 30°C for 20 min, then extracted with phenol/chlorophorm. In some experiments, DNA was preincubated with ethidium bromide (Et-Br). As the preliminary results did not show any difference between the nucleases (not shown), the experiments were mostly carried out with mung bean nuclease. After ethanol precipitation the DNA was cleaved with restriction enzymes and the digests fractionated by electrophoresis in 1.5% agarose. If necessary, gels were blotted onto nylon membranes and hybridized with plasmid inserts labelled with ^{32}P by the random primer method. Gel electrophoresis, blotting and labelling are described elsewhere [13].

Nuclease-sensitive sites were then determined from stained gels or from autoradiographs. DNA sizes were calibrated using standard markers: pBR322/*Hin*fI, lambda/*Hin*dIII, SPP-1/*Eco*RI (Bresa). Reference sites for mapping of the sensitive sites were: in pSCM107, the *Pvu*I and *Sca*I sites and the closest *Hin*fI site; in pTW1, pBB5/18 and pBB5/C, the unique sites of the polylinker sequence and the *Hpa*II site of the GC cluster B of *ori/rep*; in pTGM1 and pTGM2, the *Bgl*II and *Mbo*I sites in the vector, and the *Pvu*II and *Eco*RI sites in the insert; in pTGM19, the *Hha*I and *Eco*RI sites.

Table 1

Plasmids employed in ss-nuclease studies

Plasmid	Description and origin
pTGM1	pBR328 plasmid with the 3.9 kb *Eco*RI fragment of *T. glabrata* mtDNA (containing genes for *ATPase* subunits and *cyt ox* subunits) inserted in the *Eco*RI site (from Clark-Walker)
pTGM2	pBR328 plasmid with the same insert as in pTGM1 but in opposite orientation (from Clark-Walker)
pTGM19	pUR250 plasmid with the 0.8 kb *Bgl*II fragment of *T. glabrata* mtDNA (containing he 3′-end of the *cyt b* gene) inserted in the *Bam*HI site (from Clark-Walker)
pSCM107	pBR322 plasmid with a 0.7 kb insert containing a permuted *ori/rep* sequence of *S. cerevisiae* mtDNA inserted in the *Pst*I site (from Blanc)
pTW1	pUC9 plasmid with a 0.7 kb insert containing an *ori/rep* sequence of *Saccharomyces cerevisiae* mtDNA inserted in the *Hin*cI site (from Nagley)
pBB5/18	pUC18 plasmid with the same insert as pTW1, but in opposite orientation (this paper)
pBB5/C	pUC13 plasmid with the 0.46 kb *Hpa*II insert containing the C GC cluster of the pTW1 insert inserted in the *Acc*I site (this paper)

3. RESULTS

The single-strand-specific nuclease (ss-nuclease)-sensitive sites in each plasmid were mapped from at least two sites and from both sides. The experimental error in obtained DNA sizes was estimated to be ± 10 bp in most experiments (where the examined sizes were smaller than 400 bp), except in the case of pTGM1 and pTGM2 where the error was estimated to be ± 30 bp (here the examined sizes were around 900 bp). This is in accord with previous publications [14]. Linear plasmids did not exhibit specific nuclease-sensitive sites as exhibited by supercoiled plasmid DNA (e.g. fig.1A). All the sites considered in this paper are dependent on plasmid supercoiling.

3.1. T. glabrata *mitochondrial sequences*

In pTGM1, which carries approximately a quarter of the *T. glabrata* mitochondrial genome, two sensitive sites were detected in high salt buffer (fig.1A and B). Both were located within intergenic sequences and the location of these sites was identical in pTGM2 (fig.1B). One site was located 35 ± 30 bp from the end of the 3′-end of the gene *ATPase sub9* and another one mapped 155 ± 30 bp from the *Bgl*II site which is located between the genes *val tRNA* and *cyt ox sub3*) (fig.1B). In

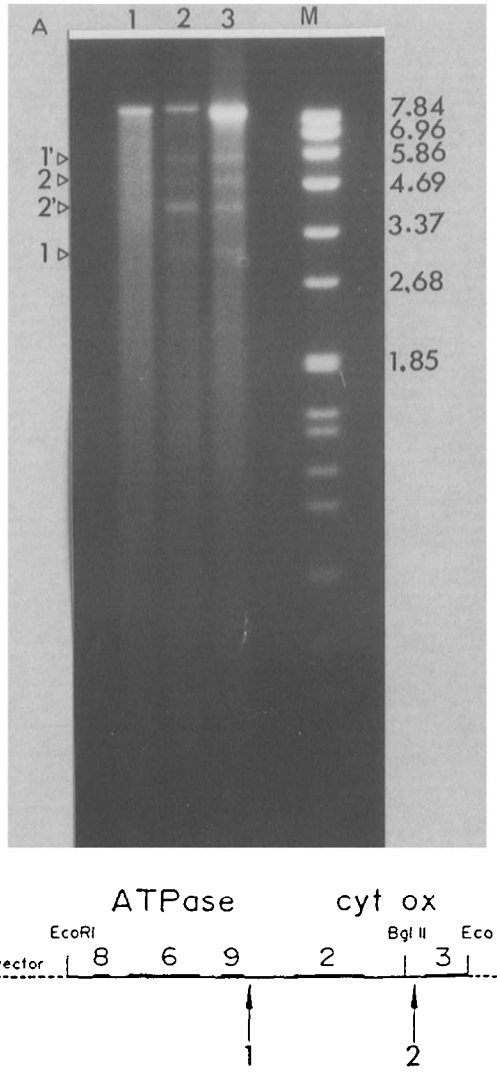

Fig.1. Sensitive sites in pTGM1 and pTGM2 are designated as 1 and 2, and 1′ and 2′, respectively. (A) Separation of DNA fragments from pTGM1. Lanes: 1, *Hin*dIII digest and subsequent mung bean nuclease treatment; 2, mung bean nuclease in the high salt buffer and subsequent digestion with *Hin*dIII; 3, mung bean nuclease in the low salt buffer and subsequent digestion with *Hin*dIII; M, marker fragments with sizes in kb. (B) The insert from pTGM1 and pTGM2 (3.9 kb) with arrows indicating the ss-nuclease-sensitive sites. Both sites are located in intergenic domains. The diagram summarizes data shown in A and data from several other experiments with higher resolution (see section 2).

low salt cleavage at these sites was intensified, but no new sites were observed (fig.1A).

In pTGM19 (which carries the 0.8 kb *Bgl*II fragment of *T. glabrata* mtDNA), one ss-nuclease-sensitive site was detected at the high salt concentration and located 105 ± 10 bp from the insert's *Hha*I site. A second weaker site appeared at low salt concentration and it was located within the same intergenic region between the genes *metf tRNA* and *pro tRNA* (fig.2). The three sensitive sites in pTGM1/2 and pTGM19 plasmids were located close to the oligonucleotide motifs thought to be involved in DNA replication.

3.2. S. cerevisiae *mitochondrial sequences*

Several sites sensitive to ss-nucleases were detected in plasmids carrying *ori/rep* sequences from *S. cerevisiae* mitochondrial DNA (pTW1, pBB5/18, pBB5/C (all *ori1*) and pSCM107 (*ori3*)). The ss-nuclease-sensitive sites were all located in the inserts, except in the case of the plasmid pBB5/C. Multiple sites of various intensities occurred in each case and their sensitivity was affected by salt concentration.

In the case of plasmid pSCM107 (containing a permuted version of *ori3*) two sensitive sites appeared at the high salt concentration and were located within the rearranged *ori/rep* sequence ('270 bp region') (figs 3A and 4). However in pTW1, containing a normal *ori/rep* (*ori1*), no sites were observed within the 270 bp *ori/rep* region at high salt concentration. Instead two sites were located within the insert sequences flanking *ori/rep* sequence (fig.3C). At low salt concentration new sites, of different intensity, were induced in the inserts of both plasmids. The location of sites in pTW1 was: 58 ± 10, 118 ± 10, 420 ± 10 and 704 ± 10 base pairs from the first base of the GC cluster 'b' of the insert (fig.3D). The plasmid pSCM107 exhibited the sensitive sites located 20 ± 10, 260 ± 10, 415 ± 10 and 590 ± 10 base pairs from the first base pair of the GC cluster C in the insert (fig.3B). The location of site 1 within the *ori/rep* sequence in pSCM107 (fig.3A and B) appears to be the same as that of site 3 located in *ori/rep* of pTW1 (fig.3D) within the error of our measurements (± 10 bp). However, whereas site 1 in pSCM107 was sensitive in high salt, site 3 in pTW1 was sensitive only in low salt. The position of sites in pBB5/18 (insert reversed) was identical to those observed in pTW1 (fig.3C and D). In pBB5/C which contains the GC cluster C of *ori/rep* with adjacent regions subcloned from pTW1, this site (3 in pTW1) was not sensitive to

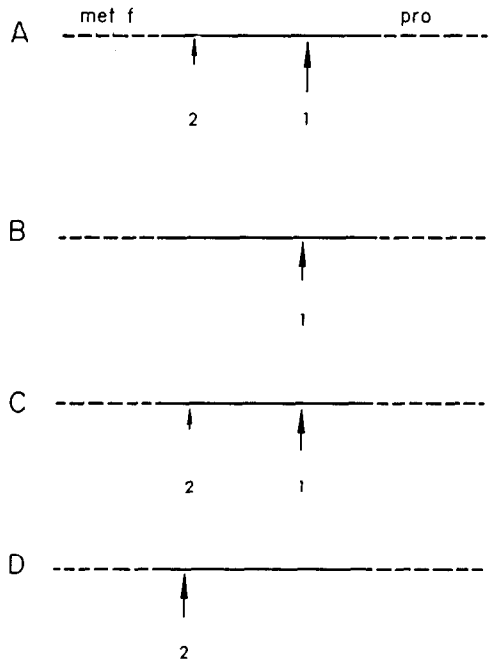

Fig.2. The insert from pTGM19 (0.8 kb) with arrows indicating the relative sensitivity to mung bean nuclease in the presence and absence of Et-Br. The sites are designated as 1 and 2. (A) Sites in the low salt buffer without Et-Br, (B) sites in the high salt buffer without Et-Br, (C) sites in the high salt buffer with 0.0001 mg Et-Br/ml, (D) sites in the high salt buffer with 0.001 or 0.01 mg Et-Br/ml.

the ss-nucleases at all. A sensitive site in this plasmid appeared in the position located 545 ± 10 bp from the *PstI* site in a close proximity to the *HgiIII* site in the vector (fig.3E). It is interesting to point out that a sensitive site was not detected in the vicinity of the hypothetical cruciform structure formed by the A and B GC clusters in *ori/rep* [10]. In both plasmids (pTW1 and pSCM107) the region flanking cluster C was sensitive towards the ss-nucleases, but the location of sites was different. In pSCM107 one of these sites (site 4) was located within the 270 bp *ori/rep* region (fig.3B). The sensitive site within the 270 bp *ori/rep* sequence was located close to the oligonucleotide motif presumed to be involved in mtDNA replication.

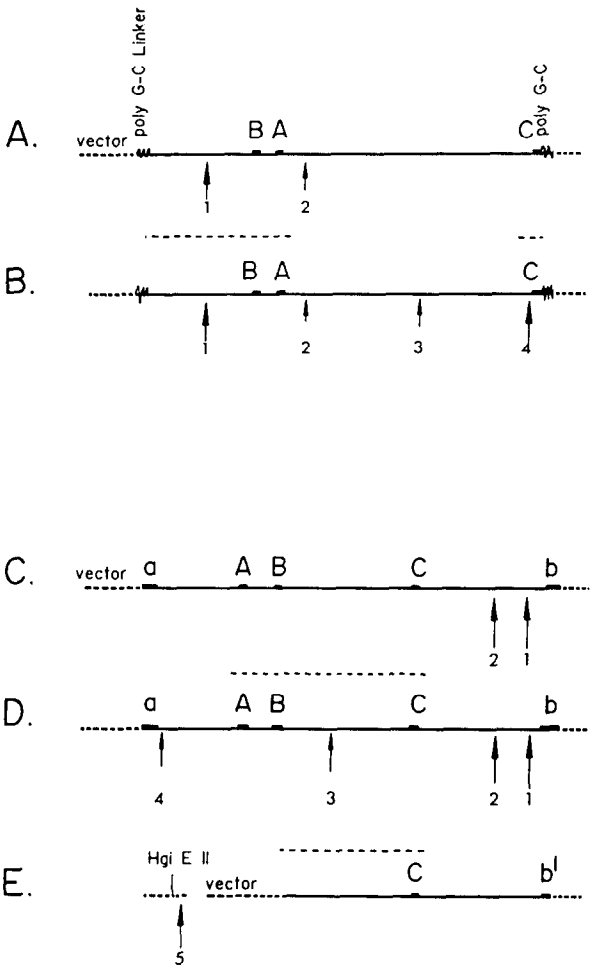

Fig.3. Mung bean nuclease-sensitive sites in inserts containing *ori/rep* and a rearranged *ori/rep* sequence. The broken line represents the *ori/rep* sequence. Arrow length designates the relative intensity of sensitive sites. GC-rich clusters in *ori/rep* are designated as A, B and C and GC clusters in adjacent regions as a' and b'. (A and B) The permuted *ori/rep* sequence (*ori3*) with adjacent regions (0.7 kb) of pSCM107. (A) High salt buffer, (B) low salt buffer. The sites are designated 1, 2, 3 and 4. (C and D) The *ori/rep* (*ori1*) with accompanying domains (0.7 kb), of pTW1 and pBB5/18. (C) High salt buffer, (D) low salt buffer. The sensitive sites are designated 1, 2, 3 and 4. (E) A fragment subcloned from the pTW1 insert (*ori1*, 0.46 kb). The sole ss-nuclease-sensitive site was located in the vector at both salt concentrations.

3.3. *Ethidium-bromide experiments*

Ethidium-bromide (Et-Br) changes the superhelicity of DNA by intercalating between the bases and unwinding the DNA. Ethidium first removes the natural negative supercoils and then introduces positive supercoils [15]. The effect of superhelicity on the occurrence of sensitive sites was examined with mung bean nuclease in the presence of Et-Br.

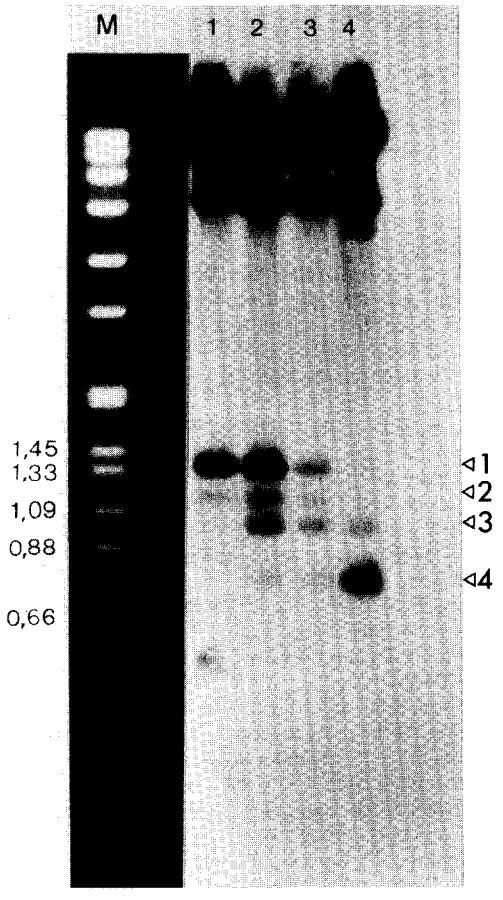

Fig.4. Autoradiograph illustrating the influence of Et-Br on the occurrence of nuclease-sensitive sites in pSCM107. The plasmid DNA was digested by mung bean nuclease in high salt buffer and subsequently with *ClaI*. The fragments were then hybridized with pSCM107 insert DNA. The sensitive sites are located in the insert (permuted *ori/rep*). Lanes: 1, without Et-Br; 2, 0.0001 mg of Et-Br/ml; 3, 0.001 mg of Et-Br/ml; 4, 0.01 mg of Et-Br/ml; M, marker fragments in kb. Sensitive sites are numbered from 1 to 4 and these numbers correspond to those in fig.3A.

Experiments were performed in the high salt buffer. Marked changes in position and intensity of the sites were observed when the concentration of Et-Br was increased (figs 2 and 4). The positions of induced sites corresponded to the position of the sites induced by the low salt concentration, but the relative intensity of the sites was different. The relative intensity also varied with increasing Et-Br concentration (figs 2 and 4). Multiple sensitive sites occurred for each tested plasmid even in Et-Br experiments.

4. DISCUSSION

The mapping strategy employed here detected double-strand breaks. The precise base cleavages of each site were not determined, so each detected site could exist as a 'cluster' of cleavages. The discrete banding patterns indicated that cleavages occurred at specific sites. All of the segments of yeast mitochondrial DNA examined exhibited sites sensitive to ss-nucleases under torsional stress. These sites were located exclusively in AT-rich intergenic regions and sensitive sites in the plasmid vectors were not detected. These observations agree with the previous reports that sensitive sites can correlate with early denaturation regions which often correspond to AT-rich sequences [16]. AT-rich sequences have been suggested to be structurally more variable, flexible and deformable [17]. Sensitivity to ss-nucleases suggests localised alterations of the secondary structure, but the basis for ss-nuclease recognition is still not completely clear [18,19]. Even if the intergenic spacers of the mtDNA of *T. glabrata* do not contain any GC clusters they exhibit a similar AT sequence pattern as in mtDNA of *S. cerevisiae*. This may provide a structural basis for the observed induction of ss-nuclease sensitive sites.

The induction of nuclease-sensitive sites is not completely sequence related (fig.3). A site between the GC clusters B and C was sensitive in pTW1 (site 3) and pSCM107 (site 1), but not in pBB5/C. Moreover, site 1 in pSCM107 was sensitive in high salt but site 3 in pTW1 was sensitive only in low salt. Similarly, the region flanking the GC cluster C was sensitive only in pSCM107 (site 4). These results suggest that a structural transition in DNA can be dependent on sequences that are distanced from those directly detected by the ss-nucleases. Similar phenomena were reported by Sullivan and Lilley [17].

Multiple sensitive sites characterized with different relative intensities occurred in some of the plasmids (figs 1–4). Previously it was shown that more than one nuclease-sensitive site can occur in a given DNA domain, and the sensitivity and frequency of these sites depend upon the superhelical density of the DNA [20] and also on accompanying sequences [17]. The results underscore the importance of salt concentration and superhelicity of the DNA in the induction of nuclease-sensitive sites

and frequency of their occurrence, and similar conclusions can be found elsewhere [21]. The influence of superhelicity on appearance and intensity of sensitive sites suggests that the examined sequences exhibit flexible structural properties.

The reactivity of these yeast mtDNA intergenic sequences towards the ss-nucleases in vitro raises the possibility that yeast mtDNA in vivo could suffer local structural changes in the intergenic sequences which are important in the function of those sequences. For example, in petite mutants it was shown that the intact primary sequence of *ori/rep* was not a sufficient requirement for enhanced transmission [23]. It is possible that *ori/rep* becomes functional in transmission only when specific secondary structure changes are induced in it, and that this induction is influenced by flanking sequences. The duplex distortions may be necessary for recognition by other elements involved in the process of enhanced transmission [6,7,12]. Other consequences of altered structures in mitochondrial intergenic sequences may include: (i) the creation of hot-spots for generation of mutant petite genomes, caused by open duplex structures sensitive to endogenous nucleases [4,22] and/or (ii) the provision of 'premelted' structures for DNA replication in the vicinity of *ori/rep* in *S. cerevisiae* and oligonucleotide sequences in *T. glabrata* [5,9,24].

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