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Preferential recombination between GC clusters in yeast mitochondrial DNA

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Yeast mitochondrial DNA molecules have long, AT-rich intergenic spacers punctuated by short GC clusters. GC-rich elements have previously been characterized by others as preferred sites for intramolecular recombination leading to the formation of subgenomic *petite* molecules. In the present study we show that GC clusters are favored sites for intermolecular recombination between a *petite* and the wild-type *grande* genome. The *petite* studied retains 6.5 kb of mitochondrial DNA reiterated tandemly to form molecules consisting of repeated units. Genetic selection for integration of tandem 6.5 kb repeats of the *petite* into the *grande* genome yielded a novel recombination event. One of two crossovers in a double exchange event occurred as expected in the 6.5 kb of matching sequence between the genomes, whereas the second exchange involved a 44 bp GC cluster in the *petite* and another 44 bp GC cluster in the *grande* genome 700 bp proximal to the region of homology. Creation of a mitochondrial DNA molecule with a repetitive region led to secondary recombination events that generated a family of molecules with zero to several *petite* units. The finding that 44 bp GC clusters are preferred as sites for intermolecular exchange adds to the data on *petite* excision implicating these elements as recombinational hotspots in the yeast mitochondrial genome.

Key words: GC cluster/mitochondria/recombination/yeast

Introduction

The mitochondrial genome of the yeast *Saccharomyces cerevisiae* is a covalently closed circular DNA molecule of approximately 78 000 bp (de Zamaroczy and Bernardi, 1985), over 90% of which has been sequenced (de Zamaroczy and Bernardi, 1986a). Coding regions for subunits of inner membrane enzyme complexes and structural RNAs are separated by intergenic spacers composed almost exclusively of adenine–thymine (AT) base pairs (de Zamaroczy and Bernardi, 1987). However, at irregular intervals within the long stretches of AT sequence are short (10–60 bp) GC-rich clusters. GC clusters fall into several different sequence classes, with clusters of any one class dispersed throughout the genome (de Zamaroczy and Bernardi, 1986b). Members of one sequence group are associated with origins of replication (de Zamaroczy *et al.*, 1984). Pairs of GC clusters from all classes are more often the sites of recombination in the spontaneous formation of *petite* genomes than would have been predicted based on the overwhelming AT-richness of the intergenic spacer regions (de Zamaroczy *et al.*, 1983).

Petite genomes, generally termed ϱ^-, are subgenomic fragments of yeast mitochondrial DNA (mtDNA) that are reiterated either tandemly in a head-to-tail fashion or in a palindromic head-to-head, tail-to-tail mode, to form longer molecules containing variable numbers of repeats (Locker *et al.*, 1974). A newly formed ϱ^- can undergo replication and segregation from additional copies of the wild-type *grande*, ϱ^+, genome in the mitochondrion during several generations of growth, providing there is no selective pressure to retain both genome types within the organelle.

Some ϱ^- arising in respiratory-deficient haploid strains are retained because gene rearrangements in the *petite* allow the yeast to grow on non-fermentable medium (Dieckmann *et al.*, 1984; Mueller *et al.*, 1984). Haploids harboring both ϱ^+ and ϱ^- genomes have been termed 'heteroplasmic' because they contain more than one type of mtDNA molecule. Alternatively, ϱ^+ and ϱ^- genomes carried separately by haploid parents can interact when the strains are mated. In the zygote, mitochondria from the parents fuse, mixing the two genome types. Wild-type sequence carried on the ϱ^- genome can rescue point mutations in the ϱ^+ genome by a homologous double crossover exchange, similar to marker rescue in merodiploids of *Escherichia coli*.

In the present study, a haploid heteroplasmic strain was used as an alternative to a zygotic system to study recombination between ϱ^+ and ϱ^- genomes. We expected to observe exchanges in a 6.5 kb region of homology. However, we observed that only one of the two exchanges in a double crossover event occurred in the expected interval. The second crossover occurred preferentially between ectopically located, homologous GC clusters.

Results

The heteroplasmic mitochondrial system

The haploid heteroplasmic strain used in this work arose during genetic studies of a respiratory-deficient *pet* mutant. The nuclear *PET* genes encode 200–300 mitochondrial polypeptides which are translated on cytoplasmic ribosomes and imported into the organelle (Tzagoloff and Myers, 1986). A subset of the *PET* genes encode polypeptides that interact with the long, untranslated, 5′ leader sequences of specific mitochondrial mRNAs. Mutations in these genes either result in the inability of the mRNA to be translated or in the instability of the target mRNA. For example, *pet494* mutants have normal levels of mRNA for cytochrome oxidase subunit 3, however it is not translated (Mueller *et al.*, 1984). In the *cbp1* mutants used in the present study, the mRNA for cytochrome *b* is degraded (Dieckmann *et al.*, 1982). The first haploid heteroplasmic strains arose in response to selection for respiratory-competent revertants of *pet494* and *cbp1 pet* mutations (Dieckmann *et al.*, 1984; Mueller *et al.*, 1984).

The E655/N1-21 revertant of *cbp1-20* harbors both *grande* genomes and supressor *petite* genomes. The *petite*, named ϱ^{4-35}, was formed by the deletion of all of the mitochondrial genome between the proximal end of the leader sequence for the ATP synthase subunit 9 gene, *oli1*, and the coding sequence for cytochrome *b*, *cob*. As shown in Figure 1A, the 6.5 kb repeats of ϱ^{4-35} are arranged tandemly with the fusion of the *oli1* promoter to the *cob* coding sequence at every repeat boundary. The

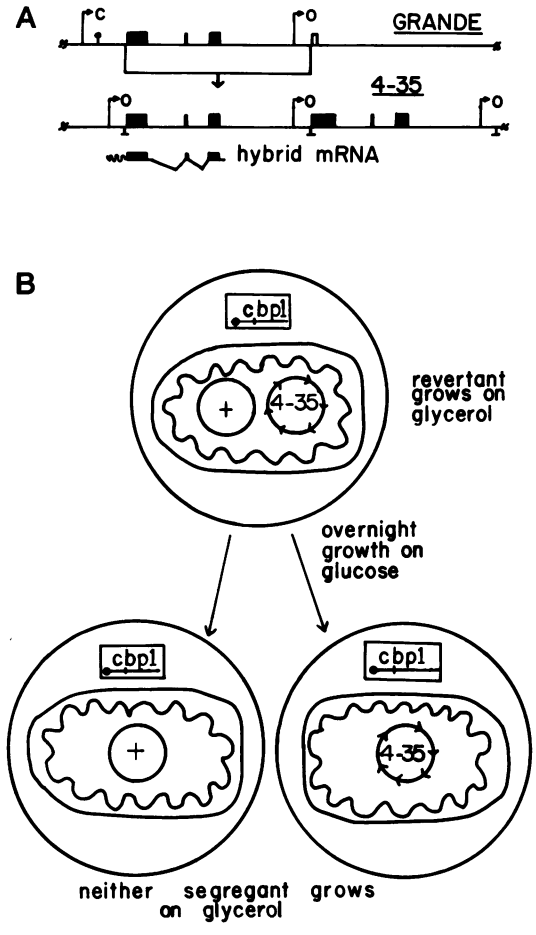

Fig. 1. The formation of ϱ^{4-35} and the heteroplasmic nature of E655/N1-21. (**A**) *oli1−cob* fusion. The top line depicts a portion of the *grande* genome. *tRNA^{glu}* (filled circle) and exons 1, 2, 3 (dark boxes) of cytochrome *b* are co-transcribed from the promoter shown as an elevated rightward arrow with superscript 'c', whereas ATP synthase subunit 9 gene, *oli1* (open box), is transcribed from the promoter with superscript 'o'. ϱ^{4-35} was formed by the deletion of all but the bracketed 6.5 kb of mtDNA. In the ϱ^{4-35} genome depicted on the second line, upside down T's mark the boundaries of the tandemly reiterated repeats. The *oli1* promoter and 600 bp untranslated leader sequence are fused to the *cob* coding sequence. The *oli1* leader in the hybrid mRNA is shown as a squiggle. (**B**) Segregation of *grande* and suppressor *petite*. ϱ^{4-35} is co-retained with the ϱ^+ genome in the E655/N1-21 strain. The *oli1−cob* fusion in ϱ^{4-35} suppresses the phenotype of the nuclear *cbp1* mutation by producing functional cytochrome *b*, while the ϱ^+ genome is responsible for the production of all other mitochondrially-encoded proteins and RNAs. When E655/N1-21 is grown on glucose medium, the ϱ^+ and ϱ^{4-35} genomes segregate, yielding colonies that are respiratory-deficient. Yeast cells are depicted as circles, nuclei as rectangles and mitochondria as ovals. mtDNA molecules are shown as smaller circles within the ovals.

oli1−cob gene fusion is transcribed and the mRNA is translated, providing cytochrome *b* in the absence of the CBP1 protein (Dieckmann *et al.*, 1984). The ϱ^+ genome provides the remaining complement of mitochondrially-encoded proteins and structural RNAs. As shown in Figure 1B, ϱ^{4-35} and ϱ^+ segregate from each other if the strain is grown on fermentable glucose medium.

Recombination between ϱ^+ and ϱ^- in the heteroplasmic strain

The heteroplasmic strain, E655/N1-21, offered a novel system in which we could select for crossing over between ϱ^+ and ϱ^- genomes. On YEPG, a rich medium containing the non-fermentable carbon source glycerol, the growth rate of the strain is half that of the wild-type, presumably due to the absence of a true origin of replication in ϱ^{4-35}. This hypothesis is supported by studies which show that there is one ϱ^{4-35} for every 15 ϱ^+ molecules in E655/N1-21 (Dieckmann *et al.*, 1984). We reasoned that if two or more ϱ^{4-35} repeats were inserted into the *grande* genome by a single or double crossover event, then the *oli1−cob* transcription fusion would be established in the *grande* genome (Figure 2 depicts a double crossover). The formation of the recombinant molecule would facilitate replication of the suppressor gene copy as part of the ϱ^+ genome, augmenting the growth rate of the strain. This would allow the recombinant to overgrow the culture of heteroplasmic cells in which it arises. In addition, it would create a unique mitochondrial genome with a tandem duplication of 6.5 kb.

Cells in which recombination between ϱ^+ and ϱ^{4-35} had occurred were enriched for by growing E655/N1-21 for 200 generations on YEPG. After the enrichment procedure, single-colony isolates were tested for segregation of ϱ^+ from ϱ^{4-35} on glucose medium. In this way, a faster-growing, non-segregating derivative, E655/Int, was identified with a doubling time on YEPG at 30°C of 4.0 h, close to the 3.5 h generation time of the wild-type parent strain, and much less than the 6.0 h doubling time of E655/N1-21. To test whether the stability of the strain was due to the introduction of the *oli1−cob* fusion into the *grande* genome, the mtDNA from E655/Int was subjected to restriction digestion analysis.

Characterization of recombinant mitochondrial DNA

The region of the *grande* genome with homology to the 6.5 kb repeat of ϱ^{4-35} is flanked by two *Hha*I sites and has no internal sites (see Figure 2) (Morimoto and Rabinowitz, 1979). If, as expected, two ϱ^{4-35} repeats recombined into the ϱ^+ genome, then the wild-type 7.9 kb *Hha*I fragment should have increased in size by 6.5 kb to 14.4 kb. As shown in Figure 3A, the 7.9 kb *Hha*I band is not present in the DNA of the E655/Int strain. There is a new band visible in the expected size range (i.e. 14.4 kb). However, when this digest was electrophoresed on a 0.7% gel and compared to fragments of a *Sma*I digest of λ DNA (data not shown), this novel band migrated somewhat faster than the 12.2 kb *Sma*I fragment of λ. Curiously, this ~12 kb fragment is present in submolar quantities with respect to fragments of similar mol. wt. Other submolar bands at 5.5 and 19 kb are evident when more DNA is applied to the gel (data not shown). In addition to the variety of novel, submolar fragments, the wild-type 4.5 kb *Hha*I fragment which flanks the 7.9 kb fragment on the left in Figure 2 (top map), is missing. Clearly, the integration of ϱ^{4-35} repeats into ϱ^+ did not produce the expected results.

As the *Hha*I site between the 4.5 kb and 7.9 kb fragments appeared to be deleted by the recombination event, this region was investigated further. This *Hha*I site in wild-type mtDNA lies at −328 relative to the ATG of the cytochrome *b* gene and is contained within a 1670 bp *Mbo*I fragment covering the region from −1350 to +319 (see Figure 2). mtDNA from E655/Int was restricted with *Mbo*I and compared to the equivalent digest of ϱ^+ DNA. As shown in Figure 3B, the 1670 bp band is not present in the mtDNA of E655/Int. Instead, the appearance of a 1360 bp band suggests that a deletion of 300 bp at the left-hand or upstream recombination endpoint, including the *Hha*I site, has accompanied the integration of ϱ^{4-35} repeats.

To establish whether E655/Int was an anomaly, or harbored the preferred stable integration product, four additional in-

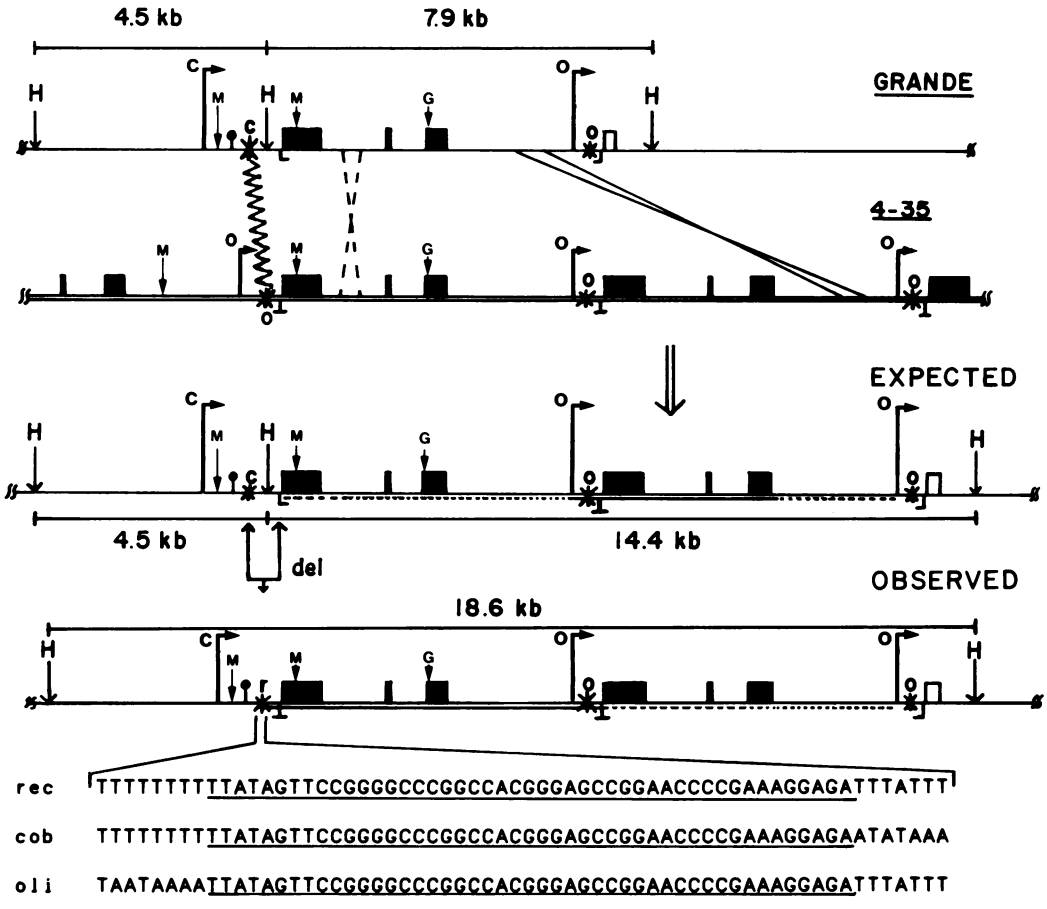

Fig. 2. Integration of ϱ^{4-35} into ϱ^{+}. The top two maps are equivalent to Figure 1A. The two sets of crossed lines between the maps suggest locations for homologous recombination events to introduce the *oli1–cob* fusion gene into the ϱ^{+} genome; the left-hand event is dashed to indicate that a homologous exchange was expected but not observed. The observed site-specific exchange between the GC clusters (*c = −696 *cob*, *o = −396 *oli1*) is shown as a jagged line. The expected integrated molecule is shown by the third map, whereas the observed recombination product is shown by the fourth map. In addition to the symbols explained in Figure 1A, the H's denote *Hha*I sites, the M's denote the *Mbo*I sites flanking the left-hand recombination site, and the G's denote the *Bgl*II sites. *r shows the position of the recombinant GC cluster on the fourth line. The extent of ϱ^{4-35} information incorporated into the *grande* is shown with a double line. The right-hand homologous crossover could have occurred anywhere between the *cob* coding sequence and the *oli1* promoter, thus the double line is dotted in this region. The 700 bp of ϱ^{+} deleted by the site-specific exchange is shown in brackets. 200–300 bp *Ssp*I fragments containing each of the three GC clusters were prepared from subclones of the *grande* genome (*c and *o) (Nobrega and Tzagoloff, 1980; Tzagoloff *et al.*, 1980), and from the mitochondrial DNA of E655/Int (*r). The *Ssp*I fragments were ligated to pSP65 and to M13mp18 at the *Sma*I site. For sequencing, inserts from the pSP65 vectors were prepared by restriction digestion with *Bam*HI + *Eco*RI, end-labeled with [γ-^{32}P]ATP, strand separated, and chemically modified by the method of Maxam and Gilbert (1980). Sequencing by the Sanger method (Sanger *et al.*, 1979), was performed on single-stranded M13 templates by elongation of the 17-mer -40 sequencing primer with Klenow fragment in the presence of [α-^{35}S]thio dATP. Only the regions of the *Ssp*I fragments containing the clusters are shown. Homology shared by all three clusters is underlined.

dependently derived integrants were obtained. All had *Hha*I and *Mbo*I restriction patterns of mtDNA equivalent to that of the original isolate, E655/Int (data not shown).

Sequence of the GC cluster recombination site in E655/Int mtDNA

In order to localize the apparent deletion more precisely, the region encoding the cytochrome *b* leader in the novel mitochondrial DNA of E655/Int was sequenced. The sequence revealed a totally unexpected recombination site. One GC cluster in a ϱ^{4-35} repeat (at −399 to −356 relative to *oli1* ATG), had recombined with a GC cluster in the *grande* genome found at −739 to −696 relative to the *cob* ATG. This *cob* cluster is 700 bp to the left of the region homologous to ϱ^{4-35} and is embedded in sequence heterologous to the sequence surrounding the ϱ^{4-35} *oli1* GC cluster. Recombination at this site deletes the upstream *cob* sequence from −696 to −28 and replaces it with *oli1* leader from −356 to −22, resulting in the apparent 300 bp reduction in size of the wild-type 1670 bp *Mbo*I fragment in E655/Int mtDNA (see Figure 2).

As neither the wild-type GC cluster at −696 of *cob* nor that at −356 of *oli1* had been fully sequenced in the mitochondrial genome from which the recombinants were derived (Nobrega and Tzagoloff, 1980; Tzagoloff *et al.*, 1980), the sequences of the two clusters involved in the aberrant left-hand recombination site of E655/Int mtDNA were determined and compared to that of the recombinant cluster (see the inset in Figure 2). The *cob*, *oli1*, and recombinant clusters have the same 44 bp sequence, suggesting that the site-specific crossover occurred within the matching interval. All three sequences are fully homologous to the consensus 44 bp sequence found at six other intergenic locations (Goursot *et al.*, 1982).

Heterogeneity of repeat number in E655/Int mitochondrial DNA molecules

Once the novel recombination site had been determined, it was

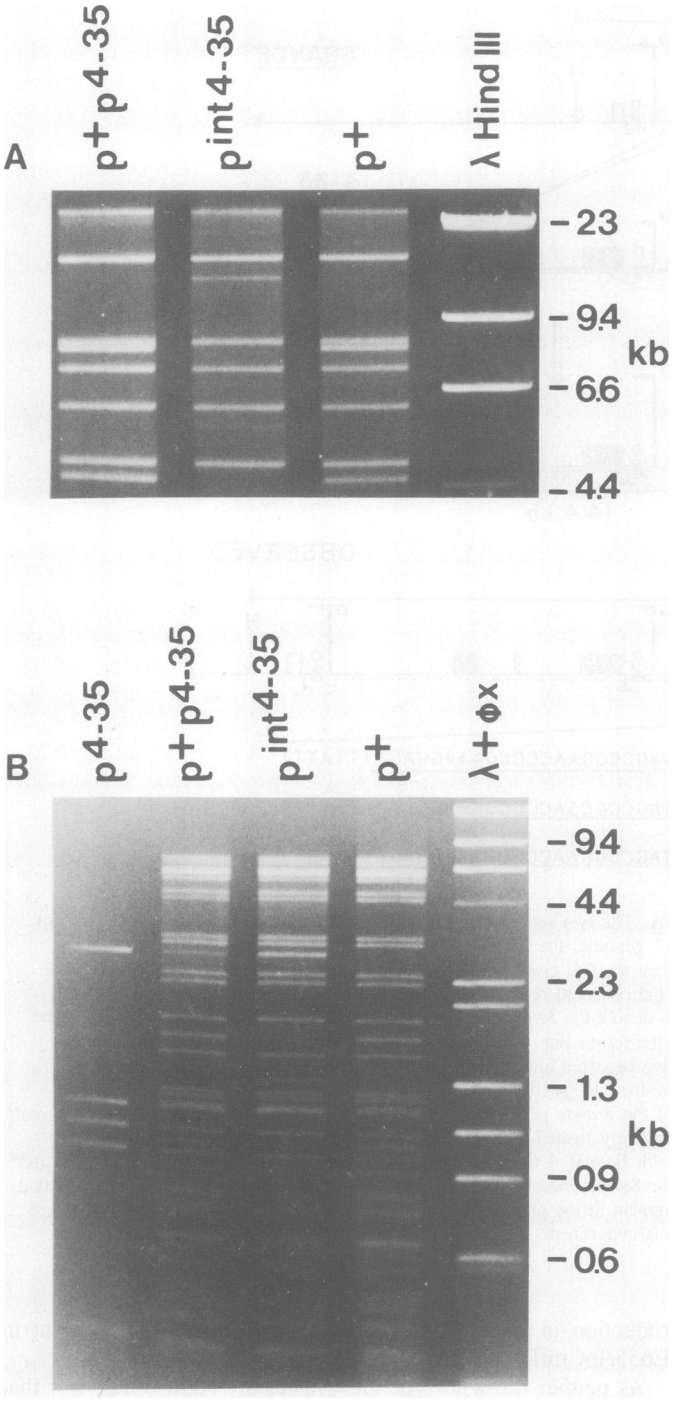

Fig. 3. Restriction analyses of mitochondrial DNA from E655/int. (**A**) *Hha*I digests of mitochondrial DNA. mtDNA from the segregating suppressor strain, E655/NI-21, **lane 2**; the non-segregating suppressor strain, E655/Int, **lane 3**; and the wild-type strain from which E655 was derived by mutation, D273-10B/A1, **lane 4**, was restricted with *Hha*I, electrophoresed on a 1% agarose gel prepared with Tris borate buffer, and compared to λ DNA restricted with *Hin*dIII, **lane 1**. The gel was stained with 0.5 μg/ml ethidium bromide, de-stained and photographed. (**B**) *Mbo*I digests of mtDNA from E655/Int. mtDNA from E655/4-35, a strain harboring only the ϱ^{4-35} genome, **lane 1**; from E655/N1-21, **lane 2**; from E655/Int, **lane 3**; and from D273-10B/A1, **lane 4**; was digested with *Mbo*I and compared to λ DNA restricted with *Hin*dIII mixed with $\phi\chi$ DNA restricted with *Hae*III, **lane 5**.

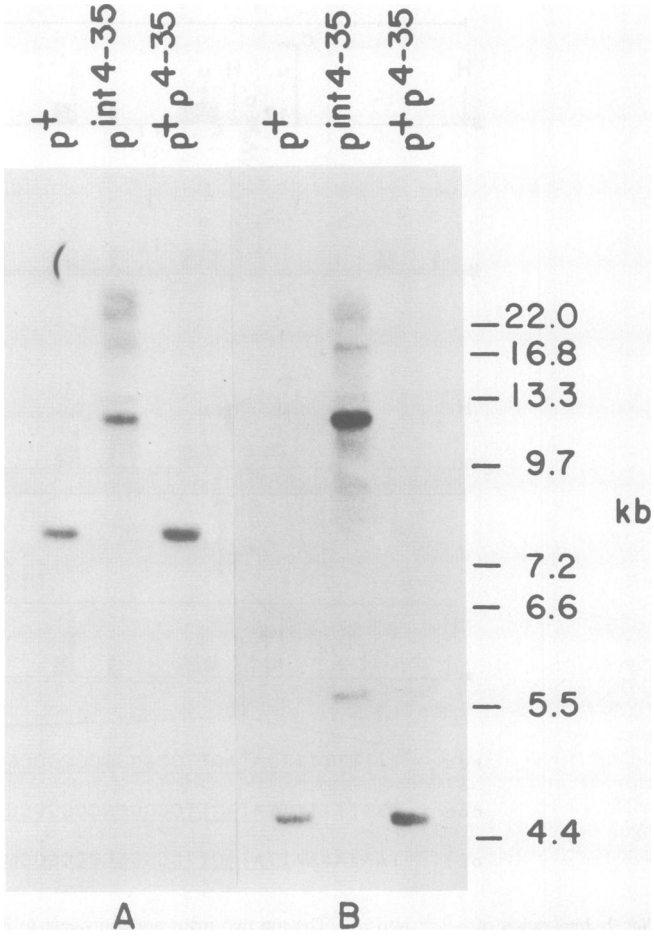

Fig. 4. Southern blot analyses of E655/Int mitochondrial DNA. Mitochondrial DNA from D273-10B/A1, **lanes 1** and **4**; E655/Int, **lanes 2** and **5**; and E655/N1-21, **lanes 3** and **6**; was restricted with *Hha*I, electrophoresed in 0.7% agarose and blotted to nitrocellulose (Southern, 1975). The Southern transfer was hybridized with a nick-translated probe prepared from a *Hin*fI–*Mbo*I fragment covering nucleotides +55 to +319 relative to the ATG of *cob*, (probe A, **lanes 1–3**), and a probe prepared from a *Mbo*I–*Aha*III fragment covering nucleotides −1350 to −887 (probe B, **lanes 4–6**). The positions of a mixture of *Bam*HI and *Bgl*II fragments of λ detected by ethidium bromide staining are shown on the right.

apparent that the wild-type 7.9 and 4.5 kb *Hha*I fragments should have been replaced by a larger fragment (≥18.6 kb) containing at least two ϱ^{4-35} repeats (see Figure 2, bottom line). A very faint band was observed in the 19 kb range, however a 12 kb fragment was the most abundant novel band in E655/Int mtDNA (Figure 3A). To verify the identity of the novel *Hha*I restriction fragments and to increase the sensitivity for detection of submolar high mol. wt species, a *Hha*I digest was blotted on nitrocellulose and hybridized with high specific activity, ^{32}P-labeled, nick-translated probes. As shown in Figure 4A, not only the 12 kb band is identified by a probe derived from within the ϱ^{4-35} homologous region of wild-type mtDNA, but higher mol. wt bands of approximately 19, 25 and 32 kb are also apparent. If a mtDNA molecule retained only one copy of ϱ^{4-35} homology, the recombinant *Hha*I band would be 12.1 kb. Molecules with two, three, or four repeats would have *Hha*I bands of 18.6 kb, 25 kb and 32 kb respectively. Hybridizing an equivalent blot with a probe derived from a region to the left of the recombinant GC cluster (Figure 4B), verified the fusion of the 4.5 kb and 7.9 kb wild-type *Hha*I fragments in the integrant, as this probe hybridized to the same set of novel, submolar bands that are seen in A. In addition, this probe hybridized to a 5.5 kb band that was visible as a lightly staining submolar

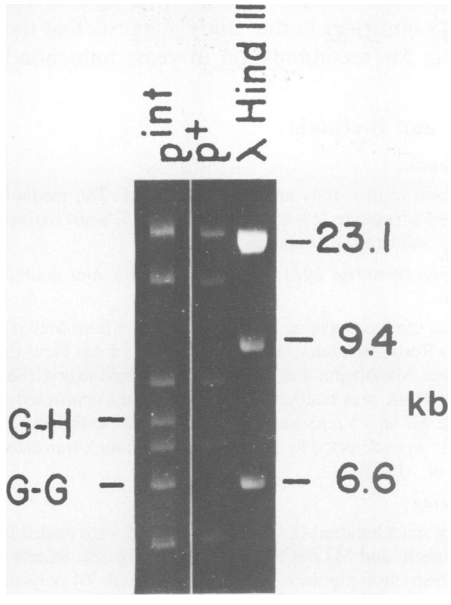

Fig. 5. Average number of 6.5 kb ϱ^{4-35} repeats per DNA molecule in E655/Int. Mitochondrial DNA from E655/Int, **lane 1**; and from D273-10B/A1, **lane 2**; was restricted with *Hha*I + *Bgl*II, and electrophoresed in a 0.7% agarose gel, and compared to a *Hin*dIII digest of λ DNA, **lane 3**. The gel was stained with ethidium bromide, de-stained, and photographed. The *Bgl*II−*Bgl*II 6.5 kb fragment unique to E655/Int mtDNA is labeled G−G. The G−H fragment is one of two unique *Bgl*II−*Hha*I fragments flanking the repeats. The other *Bgl*II−*Hha*I fragment is too small to appear in this figure.

band in ethidium stained gels. This band corresponds to molecules in which all of the ϱ^{4-35} repeats are deleted, the expected result if a site-specific recombination between the outermost GC clusters of any set of repeats occurred either in 'pop-out' intramolecular fashion or by intermolecular exchange. Therefore, after the initial integration event introduced some integral number of ϱ^{4-35} repeat units, secondary recombination led to the formation of a family of molecules with zero to several repeats. Molecules represented by the 12 kb *Hha*I fragment have lost the *oli1*−*cob* suppressor gene. A molecule of this type retains a *cob* gene with a scrambled composition; however, studies have shown that the transcripts of this *cob* gene are unstable in the *cbp1* background (Dieckmann and Mittelmeier, 1987). Therefore, *oli1*−*cob* genes can only be provided by molecules with two or more repeats.

As it is difficult to isolate full length mitochondrial DNA, large restriction fragments are often under-represented. Molecules with five or more repeats may exist but cannot be detected with the DNA isolation and detection techniques used, and thus we cannot estimate the molarity, number of repeat units, or *oli1*−*cob* fusion gene copies by analysis of the *Hha*I digest. To better estimate the number of *oli1*−*cob* fusion gene copies, E655/Int mtDNA was digested with *Hha*I and *Bgl*II. *Bgl*II cuts mtDNA once within the ϱ^{4-35} repeat, therefore the junction between any two repeats is represented in the digest as one 6.5 kb *Bgl*II−*Bgl*II fragment. As shown in Figure 5, the 6.5 kb *Bgl*II band unique to E655/Int mtDNA is at first approximation equimolar with all other non-repeat bands. This result suggests that there is an average of one ϱ^{4-35} junction, or two repeats, per mtDNA molecule. Molecules containing at least one *petite* junction (i.e. represented by the 18.6 kb, 25 kb and 32 kb *Hha*I bands) ensure the presence of equimolar amounts of the *oli1*−*cob* fusion relative to the other mitochondrial genes.

Discussion

The genetic system utilized in this study was set up to select for products of homologous crossing over between two different mitochondrial DNA molecules. We expected that a single copy of *petite* homology in the *grande* genome would be replaced by two or more 6.5 kb tandem ϱ^{4-35} repeats. The recombinants we did obtain can be viewed as products of double crossovers or of two sequential single events. If the recombination pathway involved two single crossovers separated in time, then the cointegrate intermediates were resolved before mtDNA was prepared from E655/Int for restriction digestion analysis. Whatever the mechanism, it resulted in a recombinant molecule in which the right-hand event was a homologous crossover, and, in all five isolates examined, the left-hand event was a site-specific exchange between 44 bp GC clusters embedded in heterologous sequence.

It is unclear why the expected product of homologous crossing over was not obtained. We do not have an estimate for the rate of this process, and it is difficult to estimate the frequency with which the selected events occurred. Therefore, we do not know whether the observed recombination occurred more frequently than homologous crossing over, or whether homologous crossing over did occur but the products were unstable. Instability might occur, for example, if the repeats were eliminated by a secondary recombination event, or if the repeated region stimulated the spontaneous formation of *petites* from other locations in the genome. The expected product would differ from that observed only in containing 700 bp of ϱ^+ information to the left of the repeated region; it is far from clear why this difference would affect the stability of the genome. Continued analysis of the mitochondrial DNA from independently derived, faster growing revertants in the present system may yield homologous recombinants at a lower frequency relative to the special class with one site-specific exchange. In addition, setting up an analogous system in a region of the mitochondrial genome devoid of 44 bp clusters may allow us to measure the rate of homologous crossing over.

Granted that some event other than a simple homologous crossing over could take place, why should this have occurred specifically at the GC clusters? As discussed below, these clusters have previously been implicated in the processes of recombination and replication, suggesting that mitochondria contain factors that either recognize the primary sequence of the cluster or bind to a stem-loop structure that the clusters could form. Cruciform structures formed in the DNA by the self-annealing of the individual strands within the GC palindromes might be recognized by enzymes involved in the resolution of recombination intermediates, such as those known to resolve Holliday junctions (Holliday, 1964; Symington and Kolodner, 1985). Alternatively, the clusters may be recognized by proteins involved in replication, generating new DNA strands at these sites. New strand synthesis or the breakage of DNA strands at the GC clusters would result in the generation of free ends that could invade a recipient double helix at sites homologous to the ends (Orr-Weaver *et al.*, 1981). If recombinogenic ends were produced only rarely at GC clusters, they could account for the recovery of the observed recombinants.

This work adds to previous evidence that GC clusters of several sequence types are recombinogenic. More than half of the ϱ^- arising spontaneously have deletion endpoints in repeated GC clusters, though the intergenic regions of yeast mtDNA are 90% AT base pairs (Marotta *et al.*, 1982; de Zamaroczy and Bernardi, 1987). The 44 bp cluster type, which includes the *cob* and

Table I. Names and genotypes of yeast strains

Name	Genotype	Reference
D273-10B/A1	α,ϱ^+,*met6*	Tzagoloff *et al.*, 1976
E655	α,ϱ^+,*met6,cbp1-20*	Dieckmann *et al.*, 1984
E655/N1-21	$\alpha,\varrho^+,\varrho^{-(4-35)}$,*met6,cbp1-20*	Dieckmann *et al.*, 1984
E655/4-35	$\alpha,\varrho^{-(4-35)}$,*met6,cbp1-20*	Dieckmann *et al.*, 1984
E655/Int	$\alpha,\varrho^{+(\text{int } 4-35)}$,*met6,cbp1-20*	This study

oli1 and six other homologous clusters (Goursot *et al.*, 1982), is one of the more common excision endpoints (de Zamaroczy *et al.*, 1983). Moreover, an optional GC cluster found in the *var1* coding sequence is involved in gene conversion events (Strausberg *et al.*, 1978). The GC clusters favored as excision endpoints for the formation of ϱ^-, and the *var1* cluster type, can form putative stem-looped structures (de Zamaroczy *et al.*, 1984; Goursot *et al.*, 1982; Zassenhaus and Butow, 1984). If a mitochondrial factor introduces nicks or double strand breaks at the 44 bp clusters, then breaks at the *oli1* and *cob* clusters may have stimulated the site-specific recombination between these sites in the formation of the novel mitochondrial DNA molecule in E655/Int.

GC clusters have also been implicated in the initiation of DNA replication. The wild-type yeast mitochondrial genome contains four active origins of replication, *ori* (Baldacci and Bernardi, 1982). Many *petite* genomes, including ϱ^{4-35}, do not contain one of these origins, and yet are able to replicate, suggesting that some other site can serve as a surrogate origin (Goursot *et al.*, 1982; Fangman and Dujon, 1984). It has been suggested that 44 bp GC clusters are involved in surrogate initiation, as some ϱ^- containing these clusters can compete with ϱ^+ in transmission to progeny (Goursot *et al.*, 1982). In ϱ^+, replication initiation generates DNA strands with transient 5′ ends in an *ori* GC cluster (Baldacci *et al.*, 1984). If replication of ϱ^{4-35}, which does not have an active *ori*, produced transient 5′ ends at the 44 bp cluster, then at some frequency a new 5′ end might invade a recipient duplex at a homologous site, e.g. the *cob* GC cluster in the ϱ^+ genome, producing the observed recombinants.

The DNA molecules in the recombinants appear to be in a dynamic equilibrium with respect to the copy number of the repeating unit. The Southern blot data of Figure 4 revealed several high mol. wt submolar *Hha*I fragments from the repeated region in the recombinant mtDNA from E655/Int. We presume that the initial recombination event introduced some integral number of ϱ^{4-35} repeats into the ϱ^+ genome. The heterogeneity in copy number of ϱ^{4-35} repeats may have arisen as the result of secondary homologous recombinant events, either between offset repeats in two different molecules or between repeats in the same molecule. Secondary site-specific exchanges are also occurring between the outermost 44 bp GC clusters flanking the repeated region, as evidenced by the presence of genomes in which all of the repeats have been excised (Figure 4). Recombination between these two clusters must be favored in molecules with multiple repeats, because this excision is not common enough in ϱ^+ strains (which can be thought of as having one repeat) to observe a novel restriction fragment in *Hha*I digests.

If the length of homologous sequence between two molecules was the only factor governing the frequency of genetic exchange, we would expect 150 exchanges in a 6.5 kb match for every exchange in a 44 bp match. If the frequency of homologous crossovers is not depressed due to the instability of the recombinant products, then the preferential crossing over between 44 bp GC clusters observed in this study suggests that these elements are hotspots for recombination in yeast mitochondrial DNA.

Materials and methods

Strains and media

The strains used in this study are listed in Table I. The media are: YPD, 1% yeast extract, 2% peptone, 2% glucose. YEPG, 1% yeast extract, 2% peptone, 3% glycerol. Solid media contain 2% agar.

Isolation of mitochondrial DNA, restriction digests, and Southern blot hybridizations

The procedures used in preparing mitochondrial DNA have been published (Bonitz *et al.*, 1980). Restriction enzymes were purchased from New England Biolabs and Boehringer Mannheim. For the Southern hybridization (Southern, 1975), mitochondrial DNA was restricted with *Hha*I, electrophoresed in a horizontal 0.7% agarose gel at 5 V/cm and transferred to nitrocellulose. Specific restriction fragments were detected by hybridization with nick-translated DNA probes (Maniatis *et al.*, 1975).

DNA sequencing

The regions of mitochondrial DNA to be sequenced were cloned into both pSP65 (Promega Biotech) and M13mp18 (Messing, 1983). The inserts in pSP65 were prepared by restriction digestion, 5′ end-labeled with T4 polynucleotide kinase (Pharmacia) and [γ-^{32}P]ATP (ICN), strand separated and sequenced by the method of Maxam and Gilbert (1980). To clarify sequence in GC-rich regions, the inserts in M13mp18 were sequenced by the method of Sanger (Sanger *et al.*, 1979).

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