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Replicator regions of the yeast mitochondrial DNA responsible for suppressiveness

(*Saccharomyces cerevisiae*/petite mutants/gene mapping/DNA sequence determination)

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ABSTRACT **Hypersuppressiveness is a heritable property of some ρ^- mutants (called HS) that, in crosses to ρ^+, give rise to about 100% ρ^- cells. The mtDNAs of all HS ρ^- mutants reveal a common organization: they all share a homologous region of about 300 base pairs (called *rep*) and the fragments retained are always short (*ca.* 1% of the wild-type genome) and tandemly repeated. Using one HS ρ^- mutant as an example, we show that, after crosses with ρ^+ strains, the mitochondrial genome of the progeny is indistinguishable from that of the HS parent. This suggests that HS mtDNA molecules have a decisive selective advantage for replication during the transient heteroplasmic stage that follows zygote formation, the *rep* regions playing a role in the control of replication initiation of the mtDNA molecules. The complete nucleotide sequence of one HS ρ^- mutant and its localization in the *oli1–rib3* segment of the ρ^+ mitochondrial genome are presented. Comparison of the nucleotide sequences of the *rep* regions of two different HS ρ^- mutants reveals that several *rep* sequences must exist in the wild-type genome, probably as a result of duplications of an originally unique ancestor.**

Soon after the discovery of ρ^- mutants by Ephrussi *et al.* (1), an intrinsic heritable property of these mutants, called suppressiveness, was found (2). Namely, in crosses of a ρ^- mutant with a wild-type strain (ρ^+) a given proportion of the zygotic clones consists entirely of ρ^- cells. In those clones therefore, although the ρ^+ mtDNA is originally present in the zygote, it is not transmitted to its progeny. The degree of suppressiveness, that is the proportion of the zygotic clones entirely composed of ρ^- cells varies widely from one mutant to the next (3–5).

Despite several studies (6–15) the mechanism of suppressiveness remains unclear. Several hypotheses have been advanced, assuming either a preferential replication of the ρ^- mtDNA molecules (6–10) or a particular recombination between ρ^+ and ρ^- mtDNA molecules leading to the genetic or even the physical destruction of the ρ^+ molecules (11–15). Evidence has been presented in favor of each hypothesis, leading us to suspect that the suppressiveness in different ρ^- mutants could be the final result of several distinct mechanisms. We have therefore decided as a first step to restrict our investigation to a particular class of ρ^- mutants showing the highest observable degree of suppressiveness (such ρ^- mutants are called "hypersuppressive" and abbreviated HS in this work) with the hope of narrowing the possible source of variability.

This paper reports the molecular analysis and the localization on the wild-type genome of one of the HS ρ^- mutants chosen as an example, the comparison with its progeny after crosses with ρ^+ strains, and the nucleotide sequence comparison of the DNA regions of homology with a second HS ρ^- mutant. The complete description of this study can be found in ref. 16 and will be published in detail elsewhere.

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MATERIALS AND METHODS

Strains. The HS ρ^- mutants KL14-4A/I21/HS416 (abbreviated as HS416) and KL14-4A/I21/HS1948 (abbreviated as HS1948) were isolated after ethidium bromide mutagenesis (conditions as described in ref. 12) from the strain KL14-4A/I21 *a his1 trp2 leu* ρ^+ ω^+ C^R_{321} O^R_1 P^R_{454}. The ρ^- progeny of HS416 were obtained from crosses with two different ρ^+ strains: KL14-2A α *ilv5 his1* and KL14-7D α *ilv5 his1*, isomitochondrial to KL14-4A (17). The strain IL8-8C/R53 α *his1 trp1* ρ^- ω^+ C^R_{321} E^R_{514}, whose mtDNA restriction map has been determined on a fine scale, was used to precisely localize the segment retained in the HS416 ρ^- mutant.

Media, Crosses, and Suppressiveness. Media and crosses are as in ref. 11. Suppressiveness of ρ^- mutants was determined and calculated as described in ref. 3.

mtDNA Preparation and Analysis. The mtDNA was prepared by a method adapted from ref. 18. Restriction endonuclease analyses were performed as described (16). DNA fragments were transferred to nitrocellulose filters according to refs. 19 and 20. The DNA fragments on nitrocellulose filters were hybridized either with ^{32}P-labeled cRNA synthesized as described in ref. 21 or with ^{32}P nick-translated DNA (22). The nucleotide sequence of DNA was determined according to the chemical method of Maxam and Gilbert (23).

RESULTS

HS416 as an Example of Hypersuppressive ρ^- Mutants. Among a collection of about 2000 ρ^- mutants isolated after ethidium bromide mutagenesis, 41 clones were found to be HS by a qualitative test for suppressiveness; i.e., in crosses to ρ^+ testers, they gave rise to close to 100% of entirely ρ^- zygotic clones. In constructing this collection, we avoided any selection for any particular markers or character in order to obtain a faithful representation of all the mutational events that could lead to stable ρ^- clones. Eleven of the HS ρ^- mutants were taken at random. All were found to be more than 95% suppressive by quantitative tests, and their mtDNAs were found to be composed of regular tandem repeats of a 600–1100 base pair (bp) unit containing an *Msp* I restriction site. One of them (HS416, which is 98–100% suppressive) has been chosen as an example in the study presented here. The HS416 mtDNA hybridizes with all the HS ρ^- mutants (16). From heteroduplex mapping (16) the sequence homologous to all HS ρ^- mutants is estimated to be around 300 bp long.

The mtDNA of HS416 was submitted to restriction cleavage

Abbreviations: HS, hypersuppressive; bp, base pair(s); kb, kilobase(s).

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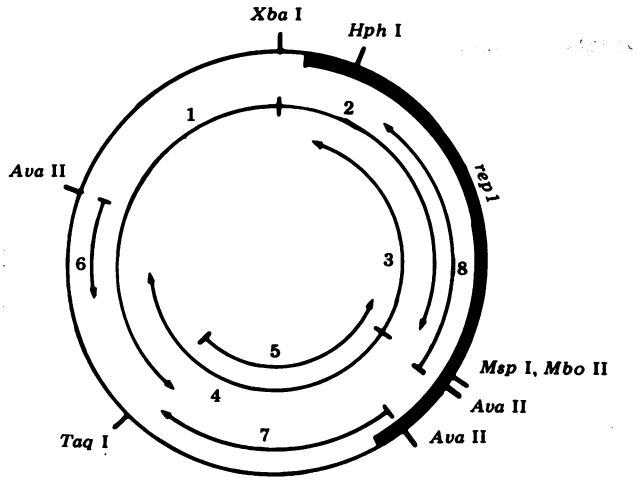

FIG. 1. Physical map of HS416 mtDNA and strategy for DNA sequence determination. The restriction map is depicted as a circle of a single repeat unit of HS416 mtDNA. The actual DNA is composed of regular tandem repetitions of this unit. The thick part of the map indicates the approximate location of the *rep1* region hybridizing with all HS ρ^- mutants. The restriction sites used for 5'-end labeling and the lengths of the nucleotide sequences determined from each fragment are indicated in the inner arcs. Completion of the sequence determination was ensured by sequencing large overlaps and across all restriction cuts (with the exception of *Xba* I). Fragments 1–4 are derived from the mtDNA of HS416 and fragments 5–8 from the recombinant plasmid pSCM107, carrying the complete repeat unit of HS416. No differences could be found in any overlapping regions.

analysis in order to determine the arrangement of the repeats and as a means of molecular mapping. With each of the enzymes *Msp* I, *Xba* I, *Hph* I, and *Mbo* II a single fragment of about 700 bp was found. This indicates that the HS416 mtDNA is a perfect tandem repeat of a 700-bp-long segment. Double digests allowed the construction of the map shown in Fig. 1. The region drawn with a thick line corresponds to the sequence that cross-hybridizes to other HS ρ^- mutants. For reasons discussed later it is called *rep1*.

The complete sequence of the HS416 mtDNA repeat unit has been determined from 5'-end-labeled restriction fragments (see Fig. 1). The complete sequence of the repeat unit (Fig. 2) is 701 bp long and shows an average G+C composition (11%) significantly lower than that of the average ρ^+ mitochondrial DNA (*ca.* 18%). Furthermore, the G·C pairs are unequally distributed: three short segments with high G+C contents are found (positions 20–36, 238–249, and 276–289) within the *rep1* region, which shows thereby an average composition of 19% G+C, whereas the rest of the sequence contains only 6% G+C. That the G+C-rich short sequences are themselves highly biased in favor of nonalternating G or C stretches is also remarkable. Some palindromic sequences as well as some short direct or inverted repeats are shown in Fig. 2, but because they almost all contain long stretches of alternating A-T, their significance remains to be established.

Localization of HS416 on the Mitochondrial Genome. Restriction cleavage products of ρ^+ mtDNA from the strain KL14-4A were transferred to nitrocellulose filters and hybridized with HS416 mtDNA or the cRNA transcribed from it. Intense hybridization with only one fragment of the ρ^+ genome (*Hha* I no. 5) can be seen (Fig. 3*A*). In similar experiments (data not shown) hybridization was found with the fragment *Hin*cII no. 7. Because these two fragments overlap each other (Fig. 4), an unambiguous localization of HS416 is obtained. For a more precise localization, hybridization to restriction fragments of a ρ^- mutant (IL8-8C/R53) whose mtDNA spans this region of the map (see Fig. 4) was performed. Hybridization was observed (Fig. 3*C*) with a band of 1450 bp, generated by *Hae* III restriction digestion, which actually corresponds to two restriction fragments. But, because only one of them (no. 2-2) contains a *Xba* I site (like HS416) and overlaps the fragments *Hha* I no. 5 and *Hin*cII no. 7, an unambigous localization of the segment retained in HS416 is again obtained. Finally, the hy-

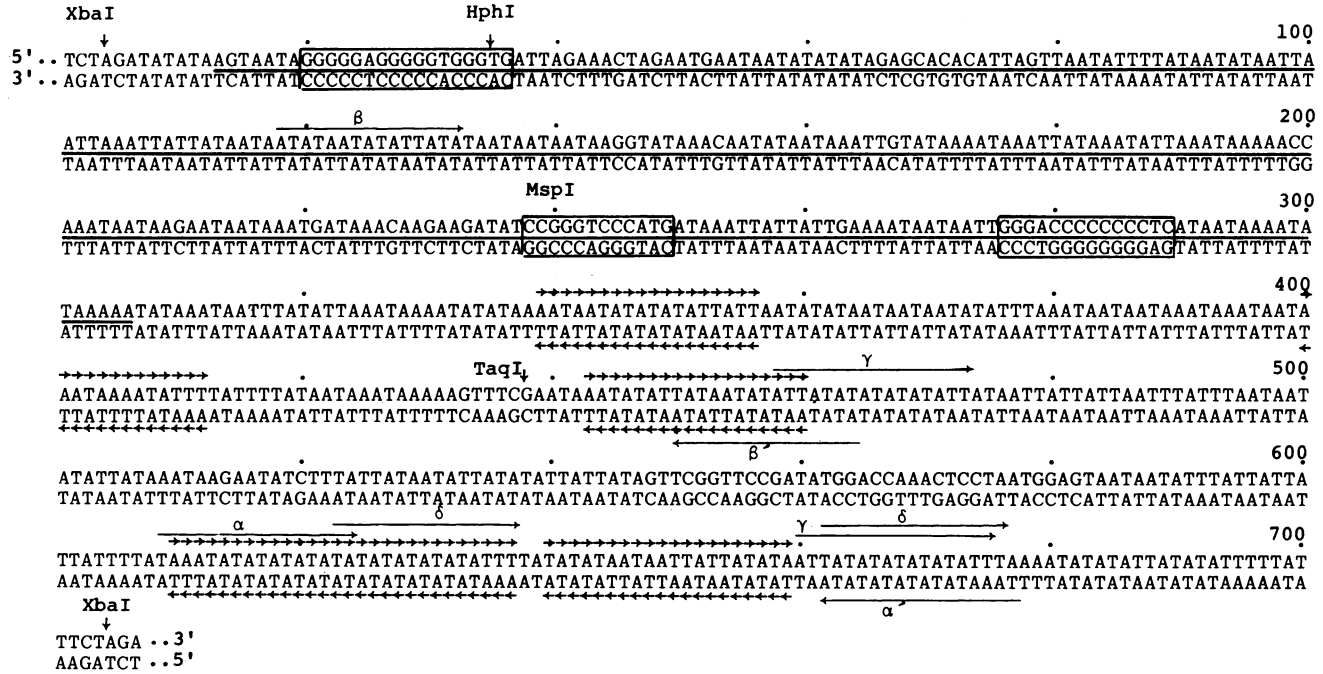

FIG. 2. Nucleotide sequence of the HS416 mtDNA repeat unit. The sequence is arbitrarily linearized at and numbered from the *Xba* I restriction site (the same site is shown also at the other end of the sequence). The thick line indicates the *rep1* region as determined from the homology with the other HS ρ^- mutants (see also Fig. 6). The three G+C-rich short sequences have been boxed. Palindromic sequences are indicated by double arrow stretches (⇄⇄⇄), and repeated sequences (direct or inverted) are shown by long arrows with Greek letters. Only exact repetitions of stretches 15 bp or longer have been considered.

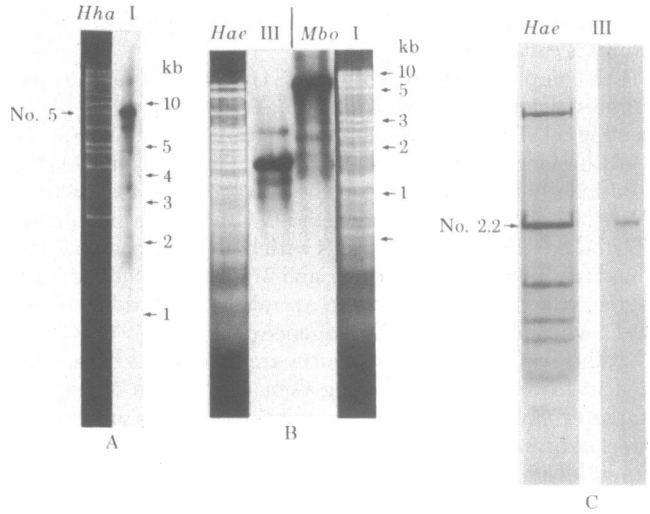

FIG. 3. Hybridization of HS416 mtDNA to restriction fragments of the ρ^+ or ρ^- mtDNAs. Restriction fragments of KL14-4A mtDNA (*A* and *B*) or IL8-8C/R53 mtDNA (*C*) were separated by electrophoresis on a 1% agarose gel (*A*) or on 2% agarose gels (*B* and *C*) and transferred to nitrocellulose filters. Hybridizations were carried out as follows. *A* and *B*: at 65°C for 15 hr in 1.5 M NaCl/0.15 M sodium citrate/5× Denhardt's solution (24)/0.1% sodium dodecyl sulfate in the presence of calf thymus DNA at 100 μg/ml and ^{32}P-labeled nick-translated HS416 mtDNA (*A*) or the fragment of HS416 mtDNA (*B*) corresponding to most of the *rep1* region (positions 47–240; see Fig. 2). *C*: at 37°C for 48 hr in 50% (vol/vol) formamide/0.75 M NaCl/0.075 M sodium citrate with a complementary RNA transcribed from HS416 mtDNA. kb, Kilobases.

bridization of HS416 mtDNA to fragments 1 and 9 of a *Hph* I digest of the IL8-8C/R53 mtDNA (data not shown) confirms the localization and determines the orientation of *rep1* relative to the mitochondrial map as shown on Fig. 4.

In hybridization experiments of *rep1* with *Hin*cII or *Hha* I digests of the ρ^+ mtDNA, in addition to the main band, weaker hybridizations are regularly observed. The specificity of these weak hybridizations has been tested by hybridizing with *Hae* III and *Mbo* I restriction digests of the ρ^+ mtDNA (Fig. 3*B*). In each case one fragment shows a major hybridization with *rep1*, and only a few others (out of *ca.* 70–100 in total) show weak but significant homology to the probe. This suggests that, in addition to the fragment containing *rep1*, other sequences related to *rep1* could exist in other regions of the mitochondrial genome (see *Comparison of the DNA Sequences* below).

In conclusion, the HS416 ρ^- mutant has retained a unique sequence of mtDNA located in the segment of the map limited by the genetic loci *oli1* and *rib3*, about 3 kb upstream from the 21S rRNA gene, in a region where no genetic markers were previously known. In addition, other regions of the mitochondrial genome show homology with the *rep1* sequence.

mtDNA of the HS416 ρ^- Mutant Is Not Distinguishable from That of Its Progeny in Crosses. The mtDNAs of different ρ^- diploid clones, taken at random among the progeny of crosses of HS416 with ρ^+ tester strains, have been analyzed by restriction enzyme digestions and hybridization. An example is presented on Fig. 5. It can be seen that in each case the *Msp* I digestion gives rise to a unique DNA band whose size is identical to the repeat unit of HS416 and which hybridizes with it. This shows that each ρ^- clone of the progeny contains a mtDNA with the same sequence as HS416 and also organized in tandem repetitions. The identity of the HS parent with its progeny is further confirmed by the fact that no difference could be found, with any of the restriction enzyme tested (data in ref. 16). The statistical significance of this result is, of course, limited by the number of clones tested but is sufficient to

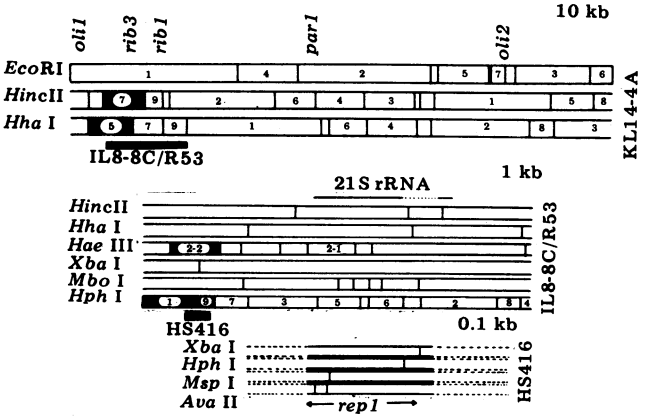

FIG. 4. Localization of HS416 on the physical map of the mitochondrial genome. The restriction maps of ρ^+ KL14-4A, of the ρ^- mutant IL8-8C/R53, and of the *rep1* region of HS416 are shown. For the strain KL14-4A, the map arrangement of the fragments and the positions of the genetic loci are according to refs. 25–27. For convenience the map has been arbitrarily linearized at the *Eco*RI site separating fragments 1 and 6. The physical map of IL8-8C/R53 has been determined from partial digestion and hybridization to specific fragments (unpublished data). Black fragments indicate their hybridization to the HS416 sequence but not necessarily over their entire length. The thick bars localize the segments retained in the ρ^- mutants IL8-8C/R53 and HS416.

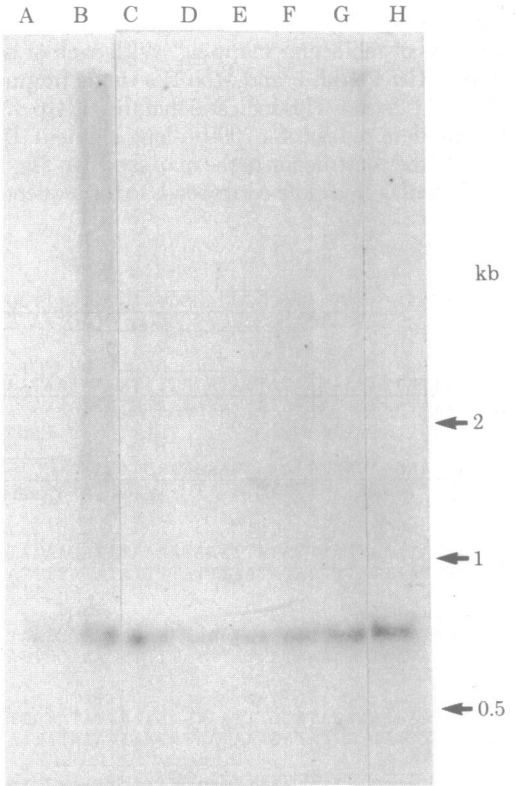

FIG. 5. Autoradiogram of restricted mtDNAs of the HS416 progeny hybridized with HS416 mtDNA. The mtDNAs of several homoplasmic diploid clones taken at random after segregation from the crosses HS416 × KL14-2A (B, E, and F) or HS416 × KL14-7D (C, D, G, and H) were purified by a small-scale procedure described elsewhere (28). All mtDNAs, including that of HS416 used as control (A), were then digested with *Msp* I restriction endonuclease and the fragments were electrophoresed on a 1.8% agarose gel, transferred to a nitrocellulose filter, and hybridized to HS416 mtDNA under conditions as for Fig. 3 *A* and *B*.

demonstrate that the large majority (if not all) of the progeny cells issued from hypersuppressive crosses are identical to the HS ρ^- parent itself.

Comparison of the DNA Sequences of *rep1* and *rep2*. From previous heteroduplex mapping (16), the other HS ρ^- mutants were shown to share sequence homologies with the *rep1* region of HS416 over a stretch of *ca.* 300 bp. Because we suspect the existence of other *rep* regions in the mitochondrial genome (Fig. 3), the nucleotide sequence of another HS ρ^- mutant (HS1948), independent from HS416, has been determined. The results are in Fig. 6. As expected, over a stretch of *ca.* 300 bp (corresponding to *rep1* in HS416) a high degree of sequence homology is found. However, the two sequences are not identical, differing by about 15%. The part of the sequence of HS1948 homologous to *rep1* is called *rep2*. The sequence divergences between *rep1* and *rep2* (mostly monosubstitutions but also additions or deletions of one or a few nucleotides) are remarkably scattered between relatively long segments of conserved sequences, suggesting that *rep1* and *rep2* result from independent spontaneous mutations of an original duplication of the same sequence in the ρ^+ strains. Apart from the *rep* regions, the sequences of the two HS ρ^- mtDNAs do not show higher homology than random A+T-rich sequences (only a part is shown on the figure).

DISCUSSION

Hypersuppressiveness of ρ^- mutants is defined by the criterion that close to 100% of the zygotic clones are entirely composed of ρ^- cells; i.e., although each zygote has received from the ρ^+ parent a population of mtDNA molecules, these molecules are not transmitted to its progeny. *A priori*, several mechanisms may be responsible for this. They lead to different predictions as to the nature of the sequences retained in the ρ^- cells of the progeny. Hypotheses based on the recombination between the ρ^+ and ρ^- molecules postulate that either a rearrangment of sequence of the ρ^- or its integration at an illegitimate point of the ρ^+ molecules leads to the genetic destruction of the ρ^+ molecules and eventually to their instability, generating a whole collection of new ρ^- molecules. On the other hand, hypotheses assuming a selective advantage for replication or segregation of the HS ρ^- mtDNA molecules predict that the progeny will be homogeneous and identical to the HS parent. Our results, using HS416 as an example, clearly show that the progeny (at least a large majority of it) of the HS ρ^- mutant in crosses is not distinguishable from the HS parent itself. Thus a faithful replication of these mtDNA molecules must have taken place while the ρ^+ sequences have not been replicated. Hypotheses of nonrandom (or directed) segregation of mtDNA molecules such that all buds would receive only HS mtDNA molecules could obviously also lead to bias in favor of ρ^- cells identical to the HS parent. However, no evidence for such a "directed" segregation has been found so far in mitochondrial crosses. Furthermore, mitotic segregation alone does not easily account for the extension of the phenomenon to the entire progeny of each zygote because, if their proportion remained constant in the zygote, the ρ^+ mtDNA molecules would eventually lead to the formation of homoplasmic ρ^+ cell lines within each zygotic clone. We conclude, therefore, that hypersuppressiveness results from the preferential replication of HS mtDNA molecules during the transient heteroplasmic stage that follows zygote formation.

The reason for the preferential replication of HS mtDNA should lie in features common to all HS ρ^- mutants: small size of the repeat unit and homologous *rep* sequences. Because other ρ^- mutants can retain mtDNA segments of similar sizes without exhibiting an HS property, one must conclude that the *rep* segments are critical for the preferential replication. The *rep* segments must therefore contain the sequence(s) that initiate or control the mtDNA replication. The exact limit and nature of these initiation sequences or replication origins are not yet precisely determined but should be sought among the sequences that do not show variability in the different *rep* segments (see Fig. 6).

Our results show that the wild-type genome contains several *rep* sequences. One of them, *rep1*, has been precisely localized in this work (Fig. 4). Preliminary results of experiments to determine the localization of the other *rep* regions suggest a hybridization of *rep* sequences around the *par1* region on the one hand and in the region flanked by the loci *oli1* and *oli2* on the other hand. Because the *rep1* and *rep2* sequences diverge by 10–15% at positions interspersed among conserved regions, we suggest that the different *rep* sequences of the mitochondrial genome arose from the duplication of a unique ancestral *rep* sequence, the different copies having then diverged from each other by spontaneous mutations.

The idea that the *rep* regions control the replication of mtDNA easily accounts for the replication of the ρ^+ mtDNA molecules [using the different *rep*(s)] and of the ρ^- mutants that happened to have retained one *rep* region. However, the fact that other ρ^- mutants do not retain any of the *rep* sequences (*rep*^{0}), although they are obviously able to replicate their mtDNA, suggests that, in the absence of *rep*, mtDNA replication can be initiated at other sequences or by a different mechanism that does not require a specific sequence. However, crosses of a HS ρ^- to a *rep*^{0} ρ^- with similar size of the repeat unit clearly show that the progeny is entirely composed of cells

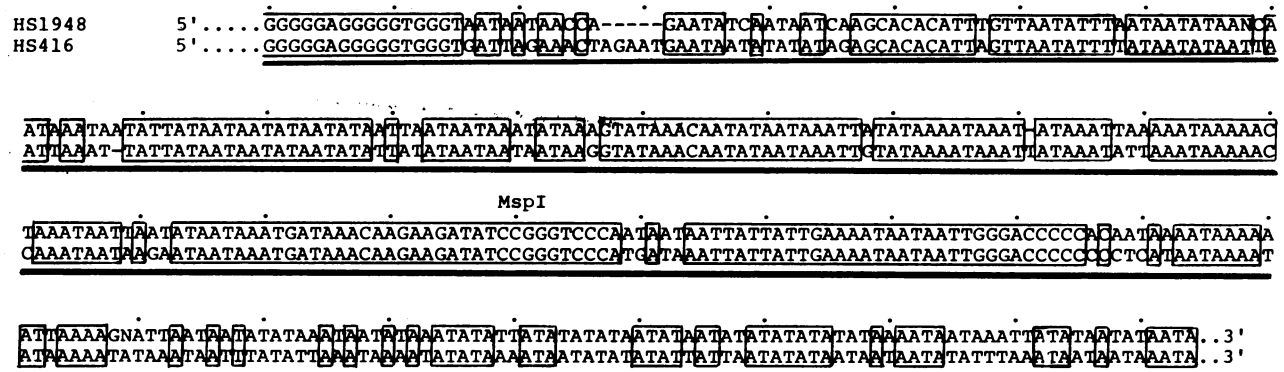

FIG. 6. Comparison of the nucleotide sequence of HS1948 and HS416 in and around their regions of homology (*rep*). The two sequences have been aligned to provide the maximum of homology (boxed sequences) with the minimum postulated additions or deletions of one or a few nucleotides (−). The thick line indicates the *rep* regions.

with the HS ρ^- mtDNA (G. Dujardin, personal communication). We conclude therefore that, in the absence of *rep*, the mtDNA molecules are normally replicated as long as they are in a homoplasmic cell but fail to replicate (or do it with low efficiency) if in "competition" with other molecules carrying a *rep* sequence. Thus, the *rep* regions must carry sequences with high affinity for a factor initiating mtDNA replication that is present in a limiting amount in the normal yeast mitochondria. Such a hypothesis immediately provides a mechanism to control the quantity of mtDNA present in a yeast cell by simply controlling the limiting quantity of the initiation factors or signals synthesized from nuclear genes, the replication itself going on all the time. This seems in agreement with the accumulation of mtDNA under conditions in which nuclear division is stopped (29–32).

So far several attempts to find a general correlation between the various degrees of suppressiveness with structural or genetic features of the mtDNA of different ρ^- mutants have been unsuccessful. On the contrary, upon deliberate restriction of our analysis to hypersuppressiveness, the characteristic features of a homogeneous and discrete class of ρ^- mutants have emerged; both the retention of a *rep* sequence and a unit size of about 1% of the ρ^+ mtDNA are required for a ρ^- mutant to be HS. If only one of these factors is missing the ρ^- mutant is not HS. For example ρ^- mutants such as IL9-8A/D122 [whose repeat unit, 800 bp long, has been sequenced and found not to contain any *rep* (33)] or such as IL8-8C/R53 (which, on the contrary, contains *rep1* but has a repeat unit of *ca.* 13,000 bp) are either neutral or of low suppressiveness. Because the total amount of mtDNA is roughly equal in ρ^+ and in ρ^- cells, the number of copies of the repeat unit per ρ^- cell (the input in a cross) will be, to a first approximation, inversely proportional to the size of the repeat unit. In the case of HS mutants versus ρ^+ the input ratio will be close to 100:1. We regard this fact, in conjunction with the *rep* sequences, to be the reason for the HS property. In other cases, however, the input being always greater for a ρ^- than for a ρ^+, after zygote formation, the random mitotic segregation of mtDNA molecules alone should lead, if the repeat units of a ρ^- are in a dynamic equilibrium, to a certain degree of suppressiveness. Therefore in our minds, the low or moderately suppressive ρ^- mutants are in fact the true "neutral," because no mechanisms other than mtDNA segregation need to be invoked. The various degrees of moderate suppressiveness may then well be the combined result of the different inputs and of the presence or absence of the *rep* sequences. However, for the particular class of the totally nonsuppressive ρ^- mutants (classically called neutral) a different mechanism involving high frequency of recombination seems to play an essential role (see ref. 34).

In any case, the present recognition and purification of the *rep* sequences by *in vivo* cloning into mini-replicons offer the possibility of a direct investigation of these questions in the near future.

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