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The initiation of DNA replication in the mitochondrial genome of yeast

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We report here the first direct demonstration that the active *ori* sequences of the mitochondrial genome of *Saccharomyces cerevisiae* are indeed origins of DNA replication, as previously postulated on the basis of compelling but indirect evidence. Basically, such sequences are formed by four regions: (i) GC clusters A and B, which are separated by a 29-bp AT stretch; (ii) a central 200-bp AT stretch, *l*; (iii) GC cluster C; (iv) a 16-bp AT stretch *r*, which comprises a site for transcription initiation. The *ori* sequences investigated, *ori* 1 and *ori* 5, have opposite orientations on the parental wild-type genome; *ori* 1 has but *ori* 5 does not have an additional 14-bp AT stretch *r'*, between cluster C and sequence *r*; they were carried by the genomes of two spontaneous petites. In both *ori* sequences, nascent DNA chains using as template the strand containing sequence *r* (the '*r* strand') start at the *r* end of cluster C, are elongated towards sequence *l*, and follow an RNA primer starting at sequence *r*. Nascent DNA chains copied on the 'non-*r* strand' start within cluster C, are elongated towards sequence *r*, and follow an RNA primer starting in sequence *l* just before cluster C. *Ori* 1 and 5 are, therefore, used as sites for RNA-primed bidirectional replication of mitochondrial DNA. Several aspects of this process are discussed.

Key words: DNA replication/mitochondrial genome/petite mutants/*Saccharomyces cerevisiae*

Introduction

Our laboratory has determined the number, the location, the orientation and the primary structure of the *ori* sequences present in the mitochondrial genome of wild-type *Saccharomyces cerevisiae* (Figure 1; de Zamaroczy *et al.*, 1979, 1981, in preparation; Goursot *et al.*, 1980; Bernardi *et al.*, 1980; Bernardi, 1982; Faugeron-Fonty *et al.*, 1984). So far, evidence for the involvement of *ori* sequences in DNA replication rests: (i) on their expected presence in the repeat units of the vast majority of spontaneous petites; (ii) on the correlation between the intact or altered state of the *ori* sequence and the level of transmission of the petite mitochondrial genome to the progeny of crosses with wild-type cells; (iii) on the expected association between the sites of initiation of DNA replication and RNA transcription; and (iv) on their similarity with *bona fide* replication origins.

At least one of the active *ori* sequences (*ori* 1, 2, 3 and 5) is present within each one of the tandem units of the mitochondrial genome of the vast majority of spontaneous cytoplasmic petite mutants (de Zamaroczy *et al.*, 1979, 1981, in preparation); in contrast the three inactive *ori* sequences, which exhibit the insertion of the extra GC cluster γ in sequence *r* (*ori* 4, 6 and 7), are only exceptionally or never found alone in petite genomes (Baldacci and Bernardi, 1982; Faugeron-Fonty *et al.*, 1984). A correlation exists between the transmission of the parental petite genome to the progeny from a petite x wild-type cross and the structure of the *ori* sequence harbored by it (de Zamaroczy *et al.*, 1979, 1981; Goursot *et al.*, 1980, 1982; Faugeron-Fonty *et al.*, 1983; Mangin *et al.*, 1983): *ori*$^+$ petites containing an unmodified *ori* sequence (without a γ cluster) exhibit high transmission levels (up to

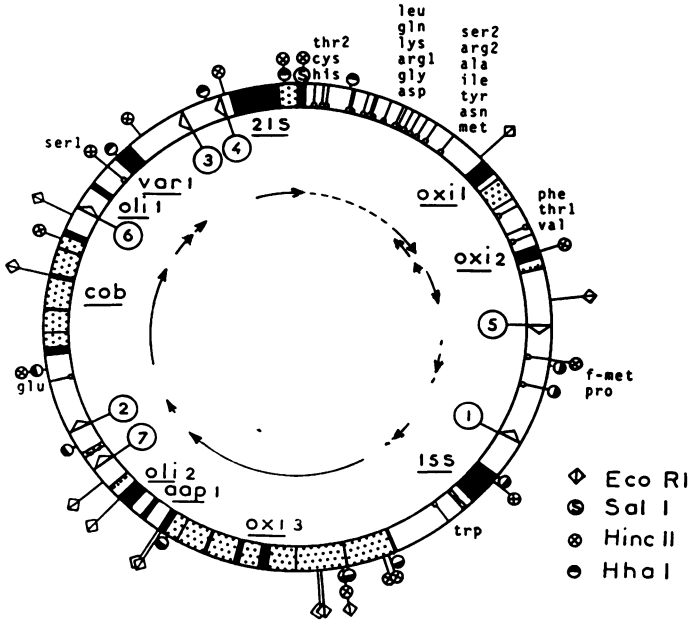

Figure 1. Physical map of a mitochondrial genome unit of wild-type yeast strain A from which spontaneous petite mutants containing *ori* 1 and *ori* 5 were obtained. Circled numbers indicate the location of clusters C of *ori* sequences; arrowheads point in the direction cluster C to cluster A. Black areas correspond to mitochondrial genes or their exons; dotted areas to intervening sequences and intergenic open reading frames; radial lines indicate tRNA genes. This map is from de Zamaroczy *et al.* (in preparation) with the addition of the open reading frames (*orf*) following (in the clockwise direction) *oxi* 2 and *oli* 2, respectively, (Osinga *et al.*, 1984b). The *orf* following the 15S RNA gene was described by Martin *et al.* (1983); that comprised between *ori* 7 and *ori* 2 has been studied in our laboratory (Colin *et al.*, in preparation). Some restriction sites are indicated. Inner arrows indicate the regions of the mitochondrial genome which have been shown to be transcribed; arrowheads show the direction of transcription. Transcripts comprise: the precursors of 21S rRNA, and of most tRNAs (broken line); the precursor mRNA from the *oxi* 1 gene; the only two stable RNAs transcribed counterclockwise (tRNAthr; Li and Tzagoloff, 1979; and the 1.5-kb RNA corresponding to the *orf* following the *oxi* 1 gene; Coruzzi *et al.*, 1981); the precursor mRNA from the *oxi* 2 gene; the 9S RNA of the tRNA synthesis locus (Miller and Martin, 1983); the precursor of 15S RNA; the precursor mRNAs from genes *oxi* 3; *aap1* and *oli* 2; the 0.9-kb RNA containing a small *orf* (Colin *et al.*, in preparation); the precursor mRNAs from the *cob* gene, the *oli* 1 gene and the *var* 1 gene. References not given above can be found in Osinga *et al.*, 1984b.

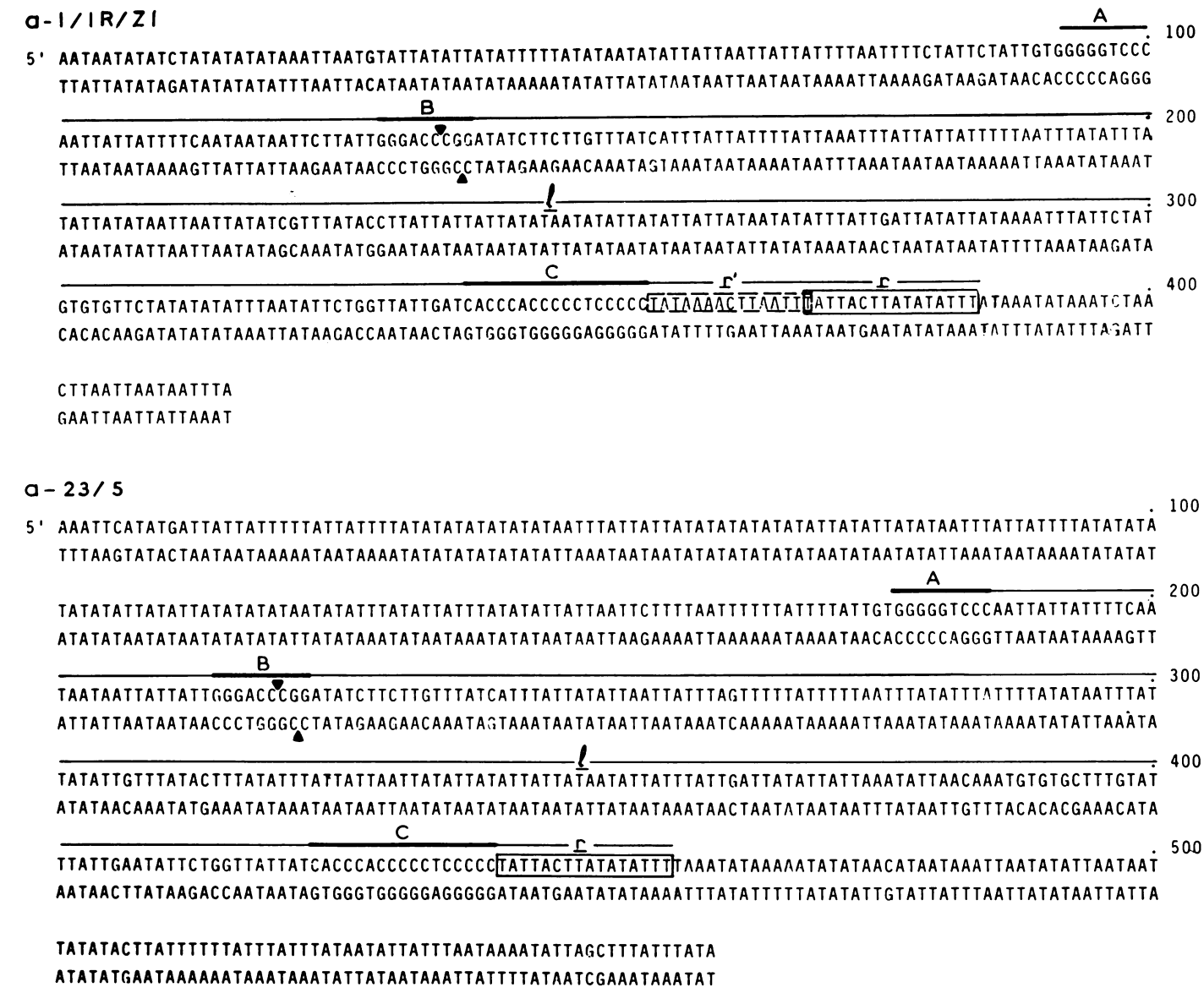

Fig. 2. Primary structures of the repeat units of mitochondrial genomes from the petites used in this work. *Ori* sequences are indicated by a continuous line; A, B and C are the three GC clusters. The *r* sequence (box) includes the transcription starting point (Baldacci and Bernardi, 1982); *r'*, (box) indicates an AT stretch, located between cluster C and sequence *r*, only present in *ori* 1 and *ori* 2; *l* is the AT stretch separating GC clusters B and C. Black triangles indicate the *Hpa*II sites used for cleaving the repeat unit and for 5' end-labelling. The repeat units are written so to allow an easy comparison of the *ori* sequences, but have opposite orientations in the wild-type genome (see Figure 1). The first 9 bp of the a-1/1R/Z1 repeat unit and the first 5 bp of the a-23/5 repeat unit correspond to the respective excision sequences (de Zamaroczy *et al.*, 1983). The primary structure of the repeat units were established by Gaillard *et al.* (1979) for a-1/1R/Z1 and by de Zamaroczy *et al.* (1981, 1983, in preparation) and in the present work for a-23/5.

99%). In contrast, (i) *ori*$^-$ petites deleted in cluster C and sequence *r*, (ii) *ori*^{r} petites having *ori* sequences with inverted orientations, and (iii) *ori*° petites lacking *ori* sequences altogether, all exhibit low transmissions. *Ori* sequences contain on one strand, a 16-bp AT stretch, *r*, which comprises a sequence for the initiation of transcription which is interrupted by the insertion of cluster γ and is not operational in *ori* 4, 6 and 7 (Baldacci and Bernardi, 1982). This sequence is also present in other transcription initiation sites of the mitochondrial genome (Baldacci and Bernardi, 1982) such as those for rRNAs (Osinga and Tabak, 1982) and those for other mitochondrial genes (Christianson and Rabinowitz, 1983). Direct evidence of RNAs initiated at *ori* sequences was obtained by *in vitro* capping of mitochondrial transcripts (Christianson and Rabinowitz, 1983) and by *in vitro* transcription analysis (Osinga *et al.*, 1984a). The *ori* sequences of the mitochondrial genome of yeast show structural similarities with those of the heavy strands of mammalian mitochondrial genomes (Bernardi *et al.*, 1980; de Zamaroczy *et al.*, 1981 and in preparation). Independent results on *ori* 3 and *ori* 5 were also obtained by others (Blanc and Dujon, 1980).

Here we report that active *ori* sequences of the mitochondrial genome of yeast are origins of bidirectional DNA replication, since two sites located in GC cluster C are starting points for nascent DNA strands; there are preceded by short primer RNA sequences. Two *ori* sequences, *ori* 1 and *ori* 5, having opposite orientations on the genome (Figure 1), and differing by the presence or absence of AT sequence *r'*, located between cluster C and sequence *r*, were investigated. A preliminary report on part of this work was published elsewhere (Baldacci and Bernardi, 1983).

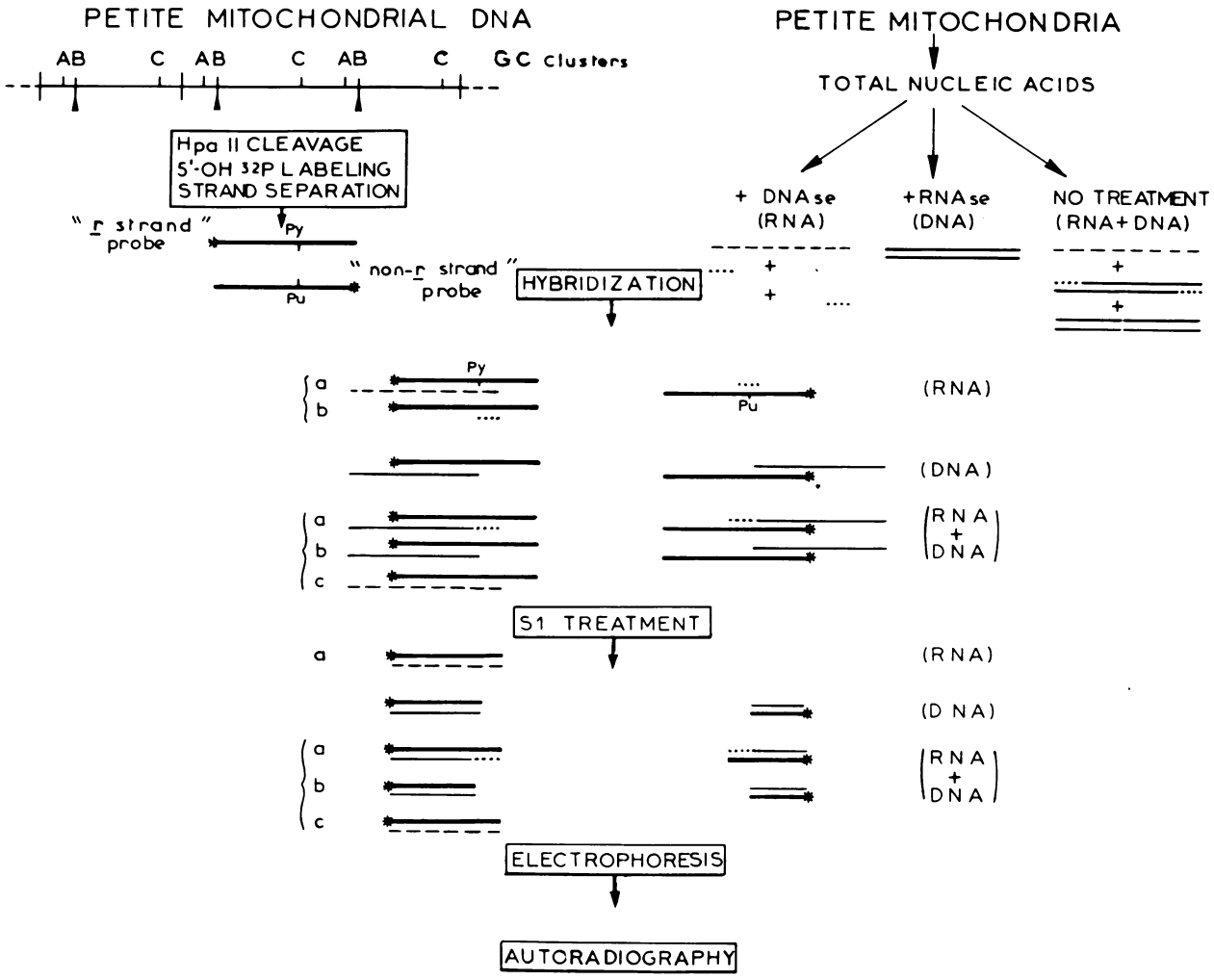

Fig. 3. Experimental strategy used to localize the 5′ ends of transcripts and of nascent DNA chains from mitochondria of petites a-1/1R/Z1 and a-23/5. Broken lines indicate RNA molecules, continuous lines DNA molecules, dots ribonucleotides joined to DNA molecules. Probes are shown by thick lines, asterisks marking the 5′ ^{32}P-labelled ends. The '*r* strand' probe is the strand containing pyrimidines (Py) in cluster C; the 'non-*r* strand' probe is the strand containing purines (Pu) in cluster C. RNA and/or DNA molecules which hybridize to the probes are indicated in parentheses. Possible hybrids are indicated for each probe and each treatment. After S1 nuclease treatment, only hybrids containing the 5′ ^{32}P-labeled end of the probes are shown.

Results

The supersuppressive, spontaneous petites used in this work were a-1/1R/Z1 (Gaillard and Bernardi, 1979; Faugeron-Fonty *et al.*, 1979) and a-23/5 (Mangin *et al.*, 1983). The repeat units of the mitochondrial genome of the former were 416 bp long and contained *ori* 1; those of the latter one were 560 bp long and contained *ori* 5. The primary structures of the repeat units are shown in Figure 2.

The experimental strategy basically consisted of mapping the 5′ ends of RNA and DNA molecules from the mitochondria of the two petite mutants on the repeat units of their mitochondrial genome. This was done (Figure 3) by splitting the repeat units of both genomes at a unique *Hpa*II site located in cluster B of the *ori* sequence, labelling the 5′ ends of these fragments, separating the two strands, and by using them as probes. In hybridization experiments these were used as probes with total nucleic acids, RNA, and DNA respectively, obtained from the mitochondria of the corresponding petites. The hybrids were digested with S1 nuclease, and the labelled S1 nuclease-resistant DNA strands were electrophoresed on sequencing gels. Each step was carefully controlled (see Materials and methods).

Hybridization of probes obtained by *Hpa*II degradation of the repeat units (Figure 4) of the mitochondrial genomes under study with mitochondrial RNAs obtained from the respective petites confirmed our previous observations (Baldacci and Bernardi, 1982), namely: (i) that the mitochondrial genomes of supersuppressive petites are transcribed; (ii) that this transcription is asymmetrical, only the '*r* strand' containing pyrimidines in cluster C, being transcribed; (iii) that the direction of transcription is from cluster C to cluster A; (iv) that the 5′ ends of the transcripts are located within sequence *r* (Figures 4 and 5).

Hybridization of the '*r* strand' probes with mitochondrial DNAs showed that the 5′ end of the unlabelled, newly synthesized DNA strand is located at the *r* end of cluster C, in both *ori* 1 and *ori* 5. This signal was practically not detectable in the hybridization of the same probe with total mitochondrial nucleic acids, where 5′ ends coincide with those of RNA [see (iv) above]. These results indicate that an RNA primer is initiated at sequence *r* and that the RNA-DNA transitions are at the boundary of cluster C and sequence *r* or *r*′. Indeed, if the probe was detecting unlinked RNA and DNA molecules, one should see the DNA signal in hybridizations with total

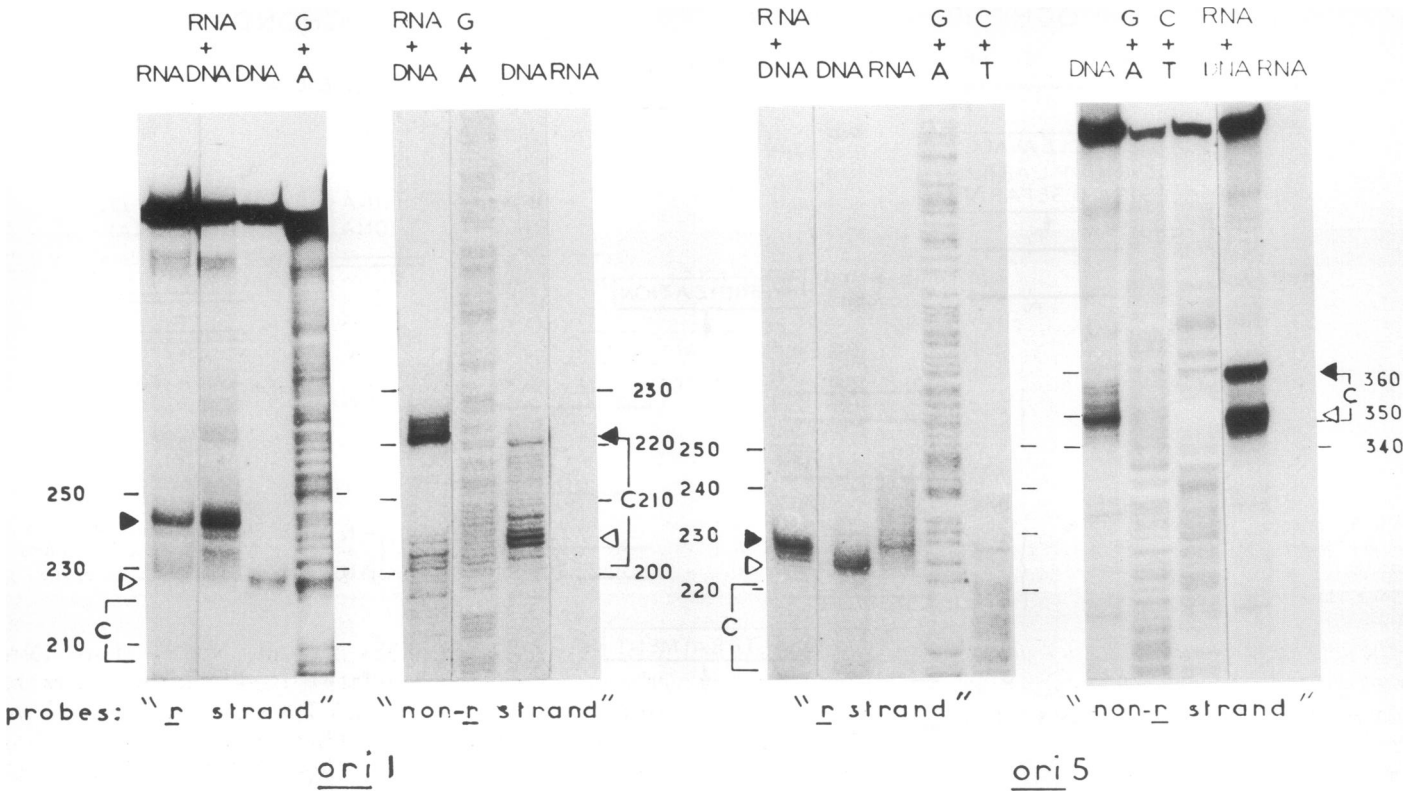

Fig. 4. Autoradiograms of S1 nuclease-resistant DNA strands obtained after hybridization of mitochondrial nucleic acids from a-1/1R/Z1 (*ori* 1) and a-23/5 (*ori* 5) with homologous mitochondrial DNAs cleaved by *Hpa*II, 5′ ^{32}P-labelled and strand-separated. Results obtained with the '*r* strand' (namely the transcribed strand) probe and 'non-*r* strand' probe are shown for each mutant. Headings:RNA indicates hybrids obtained with DNase-treated mitochondrial nucleic acids; DNA those obtained with RNase-treated mitochondrial nucleic acids; RNA + DNA results obtained with untreated mitochondrial nucleic acids; G + A and C + T are ladders of partial chemical degradations specific for purines or pyrimidines present in the probes. The position of cluster C is indicated; closed triangles indicate signals due to RNA molecules; open triangles signals due to DNA molecules. Numbers indicate fragment sizes, namely distances of 3′ ends from 5′-labelled ends. The top of the gels show a strong band, except where no protection of the probe was available (the 'non-*r* strands' were not protected by mitochondrial RNA). Such bands correspond to the size of the repeat unit of the probe (as shown by the co-migration with abundant fragments of sequencing ladders) and are apparent in the two outside panels; they are due to the protection of the monomeric probe by the bulk of nucleic acids from petite mitochondria.

nucleic acids.

In the case of hybridizations of the 'non-*r* strand' probes, no S1 nuclease-resistant signal is obtained with DNase-treated mitochondrial phenol extracts, the transcription of the 'non-*r* strand' being absent, as already mentioned in (ii) above. In contrast, both untreated and RNase-treated mitochondrial phenol extracts produced S1 nuclease-resistant hybrids. Since the untreated samples show two major bands, one of which disappears in RNase-treated samples, we conclude that the latter is due to covalently joined RNA-DNA molecules. In both *ori* 1 and *ori* 5, the start of the RNA primer and the RNA-DNA transition of nascent chains replicated on the 'non-*r* strand' are located just before the *l* end of cluster C and inside cluster C, respectively (Figure 5).

Discussion

The initiation of bidirectional DNA replication in ori sequences

The present results provide the first direct evidence that *ori* 1 and *ori* 5 contain sites for the bidirectional replication of mitochondrial DNA. RNA-primed starting points of the nascent DNA chains are identical in both *ori* sequences. On the '*r* strand', DNA chains start just before the *r* end of cluster C, whereas on the 'non-*r* strand', DNA chains start towards the *r* end of cluster C (see Figure 5). The starting points of RNA primers are identical on the 'non-*r* strand', where they are located just before the *l* end of cluster C. Whereas on the '*r* strand', they are located towards the cluster C end of sequence *r*. Because of the presence of sequence *r′* in *ori* 1, and of its absence in *ori* 5, the primer is longer in the first case by the length of the *r′* sequence, 14 nucleotides.

The enzymes involved in the priming of DNA replication

What enzymes are involved in the priming of DNA replication on the two template strands? The sequences where the primers start are different in primary structure and are definitely seen to be different by mitochondrial RNA polymerase which can use one of them, that on the *r* strand, but not the other, as a starting site for the transcription of long RNAs (Baldacci and Bernardi, 1982). Whether the RNA polymerase activity and the primase activity are carried by the same enzymatic complex, or by two complexes which differ completely or differ because one of them contains an additional element, remains to be seen.

Since primers copied on the *r* strand start at a sequence which is present in several other locations of the wild-type genome (Baldacci and Bernardi, 1982) and is used for transcription initiation of mitochondrial genes (Osinga and Tabak, 1982; Christianson and Rabinowitz, 1983), it is difficult to escape the conclusion that nascent chains copied on the '*r* strand' are initiated by mitochondrial RNA polymerase. In contrast, chains copied on the 'non-*r* strand' are more likely to be initiated by a different enzyme, a primase, as is the case in other genomes (Staudenbauer *et al.*, 1980).

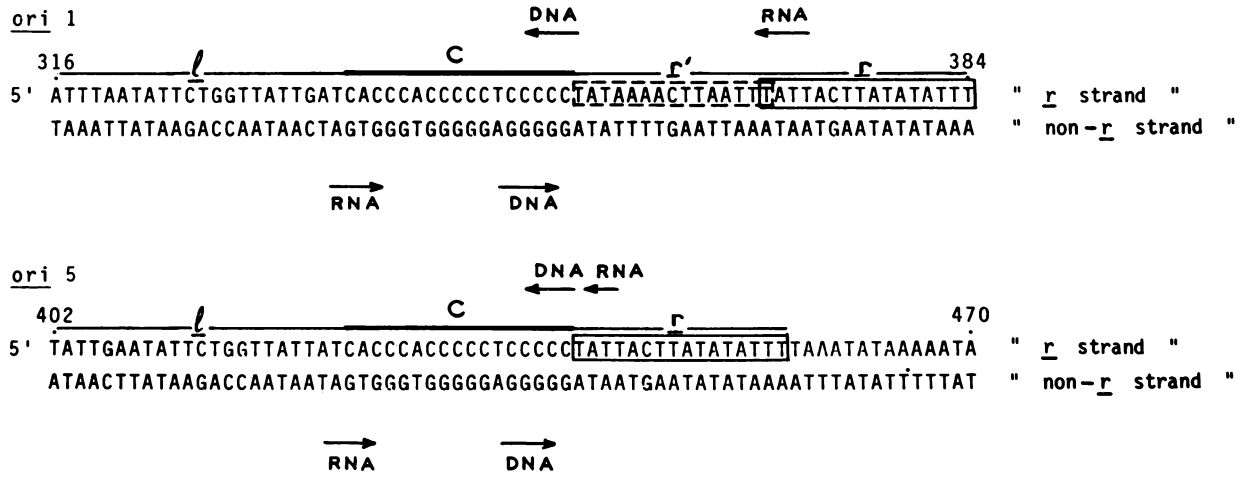

Fig. 5. Scheme of the results of Figure 4. Numbers refer to nucleotide sequences (Figure 2) of a-1/1R/Z1 (*ori* 1) and a-23/5 (*ori* 5). Cluster C and sequences *r* (and *r'* in *ori* 1) and the end of sequence *l* are shown. Arrows correspond to the positions of starting points for RNA and DNA synthesis for each strand of the two *ori* sequences. Arrows start at the positions observed in Figure 4. Because of the use of the S1 nuclease mapping, such positions are good within a few nucleotides.

The initiation of replication on the two strands of ori sequences

Since replication initiation may be due to two different enzymes, it is conceivable that the frequency of replication initiation is different on the two strands, and also that initiation on the 'non-*r* strand' requires previous opening of cluster C by transcripts and/or nascent DNA chains copied on the '*r* strand'. Incidentally, this explanation (inspired by the transcriptional activation proposed for other genomes; Hobom, 1981), suggests that the inactivation of the *r* sequence by the insertion of cluster γ may also block replication using the 'non-*r* strand' as template. In any case, if replication of one strand lags behind that of the other, the replication of the lagging strand would become the rate-limiting factor in the replication of the tandem repeat units of spontaneous petite genome.

The same phenomena would lead to a different consequence in the case of the wild-type genome, since here two active *ori* sequences have one orientation and the other two (or the other one in many strains; Faugeron-Fonty *et al.*, 1984) the opposite orientation. One would, therefore, expect that each newly synthesized strand is the result of replication initiations on both '*r* strands' and 'non-*r* strands' of *ori* sequences. Unless such initiations are suppressed for some *ori* sequences, this implies that nascent DNA chains starting at different *ori* sequences must be ligated into full-sized newly synthesized strands.

An even more complex situation may prevail in the spontaneous *ori*^{r} petites (and in many of the ethidium bromide-induced petites), where repeat units are arranged as short inverted tandem dimers. In this case (Mangin *et al.*, 1983; Faugeron-Fonty *et al.*, 1983), we know from the very low suppressivity of *ori*^{r} petites (<5%) that replication is extremely poor, since *ori*^{r} genomes are competed out by wild-type genomes. Problems here might arise from the production of long transcripts starting at sequence *r* of the *ori* sequence in petites, but not in wild-type cells (see below), or from secondary structures formed between subsequent inverted repeats.

Long transcripts and short primers initiated at r sequences

An important question concerns the relationship between the transcription of long RNA molecules and of short RNA primers at the same site on the '*r* strand'. The *r* sequences are used as starting points of stable RNA transcripts which have at least the length of the repeat units in petite genomes, unless a termination signal is met (Baldacci and Bernardi, 1982). In contrast, the map of transcripts of the wild-type genome (Figure 1) shows gaps in the regions immediately downstream of *ori* sequences (Baldacci and Bernardi, 1982). Either such transcripts are not made in the wild-type cells or, more probably, they are made but are rapidly degraded or cut short so that they are used just for transcriptional activation and/or as primers. If such is the case, the question may be asked as to whether the wild-type mitochondrial genome itself does not contribute a polypeptide which is used in this particular processing of the RNA chains starting at the *r* sequences. If so, the lack of protein synthesis in petites would account for the lack of a physiological processing of such RNA chains. An alternative possibility would be the synthesis in wild-type mitochondria of an RNA used in such processing, similar to that described by Miller and Martin (1983). In this case, the loss of this hypothetical RNA in petites would be associated with the absence of the corresponding gene.

The initiation of DNA replication in ori sequences from yeast and mammalian mitochondrial genomes

The replication initiation of the mitochondrial genome of yeast may be compared with that found in mammalian mitochondrial DNA. In the best known case of mouse L cells (Clayton, 1982): (i) replication is unidirectional starting from two *ori* sequences, one for each strand; (ii) the nascent H and L strands are RNA primed (Tapper and Clayton, 1982); (iii) the primer sequence of newly synthesized H strand (rArArGrArGrGrArGrGrGrG) corresponds to the transcription of an oligopyrimidine stretch, which is conserved in the D-loop regions of mammalian mitochondrial DNAs (sequence CSB-2 of Wong *et al.*, 1983); (iv) the *ori* sequence for H strand synthesis has a leading function relative to the replication of the L strand, the latter beginning only after the newly synthesized H strand reaches the L origin.

All these properties parallel those we have just reported if one recalls that the *ori* sequences for the two DNA strands are overlapping in yeast whereas they are separated in mam-

malian cells. Indeed, in both mitochondrial genomes: (i) initiation of DNA replication on either strand involves RNA priming; (ii) the sequences involved in RNA priming of nascent DNA chains copied on the L or *r* strand are the homologous (de Zamaroczy *et al.*, 1981; in preparation) oligopyrimidine stretches of sequence CSB-2 and cluster C, respectively; and (iii) replication and/or transcription on one strand is required to activate replication on the other strand.

In conclusion, these investigations establish that DNA replication of the yeast mitochondrial genome is an RNA-primed, bidirectional process. They suggest that different enzymes (RNA polymerase and a primase) are used for RNA priming of the two nascent chains and that the initiation of DNA synthesis of one strand is activated by transcription and/or replication on the other strand. They also suggest the involvement of mitochondrial factors in the processing of transcripts started on the *r* strand. Finally they indicate the very strong basic similarities between the apparently different replication processes of the yeast and mammalian mitochondrial genomes.

Materials and methods

Yeast strains

Two spontaneous, cytoplasmic, supersuppressive, petite mutants derived from wild-type strain A (Bernardi *et al.*, 1970) were used: a-1/1R/Z1 (Gaillard and Bernardi, 1979; Faugeron-Fonty *et al.*, 1979) and a-23/5 (Mangin *et al.*, 1983; de Zamaroczy *et al.*, 1983).

Mitochondria and mitochondrial DNA

These were prepared as previously described (Faugeron-Fonty *et al.*, 1979; Baldacci and Bernardi, 1982). Total nucleic acids were obtained from mitochondria by phenol extraction. Aliquots of extracts were subjected to RNase A (Boehringer, Mannheim, FRG) or DNase I (Miles, Elkhart, IN) digestion. After enzyme treatment, mitochondrial nucleic acids were phenol-extracted again and precipitated.

DNA probes

Mitochondrial DNAs were digested with restriction enzymes (New England Biolabs, Beverly, MA). Digests were then dephosphorylated with alkaline bacterial phosphatase and rephosphorylated with T4 polynucleotide kinase using [^{32}P]ATP. Both enzymes were purchased from P.L. Biochemicals, Milwaukee, WI; radioactive ATP was obtained from Amersham, UK. Strand separation of digested, labelled DNA, elution from the gels and sequencing were performed according to Maxam and Gilbert (1980).

Hybridization

Aliquots of single strand probes ($50-100 \times 10^3$ c.p.m.) were hybridized with an excess of mitochondrial nucleic acids ($15-30$ μg) in the presence of 80% formamide, following the method of Favarolo *et al.* (1980). Hybridization temperature was chosen according to Casey and Davidson (1977); higher hybridization temperatures only reduced the intensity of signals but did not change hybridization patterns. After hybridization, samples were treated with S1 nuclease (Miles or P.L. Biochemicals) according to Favarolo *et al.* (1980). Samples were then precipitated, resuspended in a small volume ($1-2$ μl) of formamide and loaded on sequencing gels. Three 0.3 mm thick gels (6, 8 and 20% polyacrylamide) were used to investigate S1 nuclease-resistant hybrids.

Control experiments

(i) DNA-DNA hybrids were made using the labelled single strand probes mentioned above and other restriction fragments from the corresponding cold DNAs to verify that S1 nuclease treatment produced S1 nuclease-resistant hybrids having the expected size; these experiments were also used to determine the end point of S1 nuclease digestion; (ii) labelled, single-stranded probes were directly digested with S1 nuclease, in order to check the absence of self-annealed, resistant regions; (iii) the absence of degradation fragments in the probes was verified by gel electrophoresis of labelled, single-stranded probes; (iv) mitochondrial phenol extracts were tested by electrophoresis on agarose gels; when 5′-labelling of dephosphorylated, untreated, RNase-treated and DNase-treated extracts were used, low mol. wt. RNA fragments were found to be abundant, whereas RNA and DNA molecules of monomeric size were not abundant but clearly detectable; (v) RNase and DNase were tested for activity and lack of cross-contamination under the conditions used; RNase contamination of DNase was tested by DNase digestion of mitochondrial phenol extracts from wild-type yeast cells, the high mol. wt. RNAs contained in them being degraded by trace amounts of RNase; (vi) since at the pH optimum, of S1 nuclease, pH 4.3, some depurination was observed in the polypurine stretches of our probes, this enzyme was used at pH 5.2 in a larger amount ($500-1500$ units); under these conditions depurination was avoided; (vii) different temperatures and times were tried in hybridization experiments and conditions were chosen so as to minimize RNA degradation; likewise, the shortest possible times were used in all enzymatic treatments; (viii) in the case of *ori*° petite mutants (i.e., petites not containing any of the seven canonical *ori* sequences, but containing one or more GC cluster(s) called *ori*^{S}; Goursot *et al.*, 1982) preliminary results do not show the presence of strong S1 nuclease-resistant bands, so confirming that the presence of GC clusters is not conducive by itself to the production of S1 nuclease-resistant bands.

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