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Replication origins are associated with transcription initiation sequences in the mitochondrial genome of yeast

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Mitochondrial transcripts have been investigated in a series of spontaneous petite mutants of *Saccharomyces cerevisiae* endowed with mitochondrial genomes formed by short repeat units containing no genes, but either: (a) one of the seven *ori* sequences, the canonical origins of DNA replication (*ori*$^+$ mutants); or (b) partially deleted *ori* sequences, lacking GC-rich clusters A or C (*ori*$^-$ mutants); or (c) no canonical *ori* sequence, but only *ori*^{S} sequences, the surrogate origins of replication (*ori*$^\circ$ mutants). The results indicate that some *ori* sequences play a role in transcription initiation, and that the presence of cluster C and, more specifically, of an AT-rich sequence next to it, are essential for transcription to take place. Hybridization experiments with separated DNA strands have identified the template strand used in transcription as the strand containing the oligopyrimidine stretch of cluster C. S1 degradation of RNA-DNA hybrids indicated that transcription initiates at a TATTACTTATATATTT sequence next to the oligopyrimidine stretch of cluster C and proceeds in the cluster C→cluster A direction. The relevance of these results for the transcription of the wild-type mitochondrial genome is discussed.

Key words: mitochondrial DNA/transcription/yeast

Introduction

Many aspects of transcription of the mitochondrial genome of *Saccharomyces cerevisiae* have been investigated since its discovery by Wintersberger (1964). In particular, recent investigations (see Borst and Grivell, 1981, for a mini-review) have established a number of important points: the transcription maps of the genomes from different strains (Van Ommen *et al.*, 1979); the clockwise direction (on the standard map; see Figure 1) of transcription of mitochondrial genes, with the exception of the threonine tRNA gene (Li and Tzagoloff, 1979) and of an unidentified reading frame (*urf*) localized near the *oxi* 1 gene (Coruzzi *et al.*, 1981); the splicing pathways for some precursor mRNAs (Van Ommen *et al.*, 1979; Church *et al.*, 1979; Bonitz *et al.*, 1980; Osinga *et al.*, 1981). In addition, RNA polymerase and transcription complexes have been purified (Levens *et al.*, 1981a, 1981b) and evidence has been presented for the existence of transcription initiation at five or more sites (Levens *et al.*, 1981c). In spite of these advances, the precise number and location on the mitochondrial genome of the sequences involved in the initiation of transcription remain unknown, except for the two rRNA genes (Osinga and Tabak, 1982).

The present investigations were prompted by the discovery, the localization on the genome map and the determination of the primary structure of seven sequences involved in the initiation of DNA replication, the *ori* sequences (de Zamaroczy *et al.*, 1979, 1981; Goursot *et al.*, 1980; Bernardi *et al.*, 1980; Blanc and Dujon, 1980). These sequences are ~300 base pairs (bp) in length, and are characterized by three GC-rich clusters A, B, and C, containing most of the restriction sites (see Figure 2). The A-B cluster sequence can be folded in a secondary structure similar to those present in the origins of replication of the heavy strand of all mammalian mitochondrial genomes; on the other hand, the C cluster is formed by an oligopyrimidine stretch on one strand and an oligopurine stretch on the other, and is similar in its primary structure to a sequence found in the origins of replication of the heavy strand of mammalian mitochondrial genomes.

Since replication and transcription origins overlap or are contiguous in other genomes, in particular in the mitochondrial genome of HeLa cells (Cantatore and Attardi, 1980), we decided to test whether the *ori* sequences were also involved in the initiation of transcription in yeast mitochondria. To carry out these investigations, we took advantage of a collection of spontaneous petite mutants mainly derived from the same

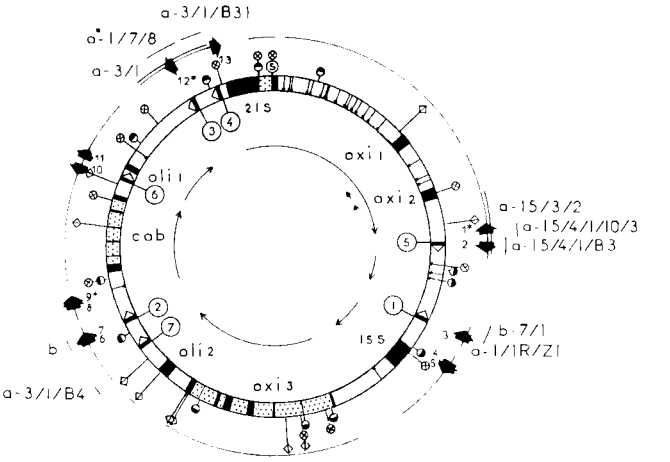

Fig. 1. Physical map of a mitochondrial genome unit of wild-type yeast strain A. The localization and orientation of *ori* sequences is shown (circled figures); triangles have their basis corresponding to cluster C (*Hph*I site of Figure 2) and point toward cluster A (*Ava*II site of Figure 2). This map is very similar to those of strains KL14/4A (Sanders *et al.*, 1977) and B (the latter, however, lacks *ori* 4). The map shows the localization of mitochondrial genes (dotted areas correspond to intervening sequences; thin radial lines to tRNA genes). Restriction sites: ⊗ *Hin*cII. ◇ *Eco*RI; ◇ *Hinf*I; ◓ *Hha*I, Ⓢ*Sal*I. (Modified from de Zamaroczy *et al.*, 1981). The internal circle shows the localization on the map of the mitochondrial transcripts (Van Ommen *et al.*, 1979); arrows indicate the clockwise direction of transcription, the small innermost arrows indicating the counter-clockwise direction of two transcripts (Li and Tzagoloff, 1979; Coruzzi *et al.*, 1981). The external circle shows the sequenced regions of the mitochondrial genome; the location and orientation of the TATTACTTATATATTT sequences are indicated by arrowheads pointing in the direction of the TAT end and numbered according to Table II; asterisks indicate sequences exhibiting the T→C change in position 4. The clockwise oriented sequences are present on the template strand; those having the opposite orientation are on the non-sense strand. The outermost thin lines give the localization of the repeat units of the mitochondrial genomes of spontaneous petites used in this work.

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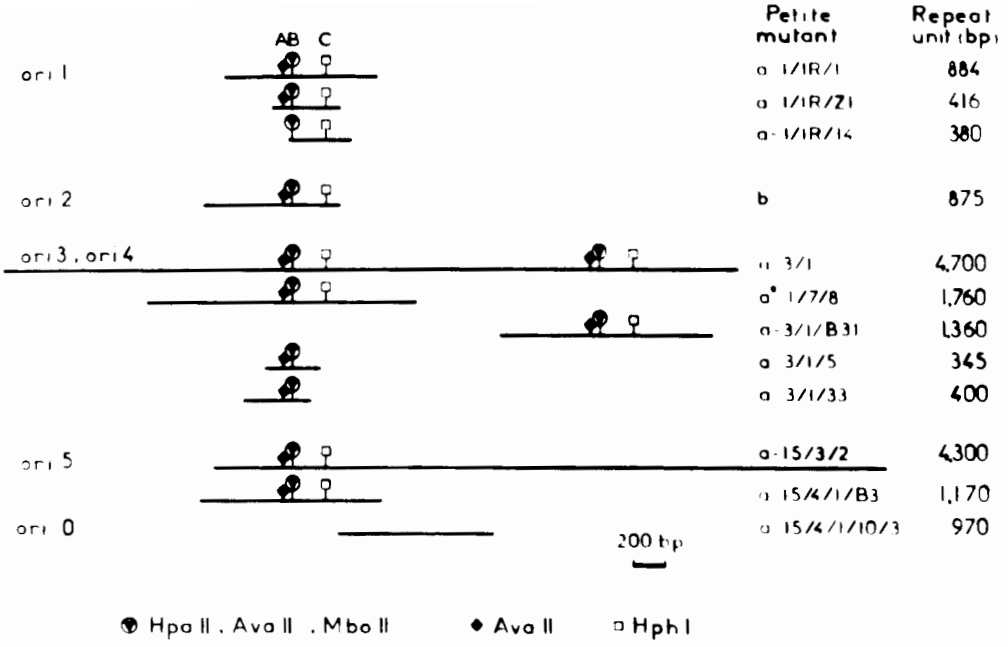

Fig. 2. Simplified restriction map of the repeat units of the mitochondrial genomes from the spontaneous petite mutants used. Only the restriction sites corresponding to the three GC clusters A, B, and C of *ori* sequences are indicated. Modified from de Zamaroczy *et al.* (1981).

wild-type strain A (Bernardi *et al.*, 1970, 1980; Faugeron-Fonty *et al.*, 1979) or from the closely related strain B. The mitochondrial genomes of these petite mutants were well characterized and many of them have been fully or largely sequenced in our laboratory. Simplified restriction maps of the repeat units of the mitochondrial genomes of the spontaneous petites used in this work are given in Figure 2, but more detailed maps are available (de Zamaroczy *et al.*, 1981). The common features of these genomes are that their repeat units are short and contain no known gene. As far as their *ori* sequences are concerned, these genomes belong to three classes (de Zamaroczy *et al.*, 1981): (a) *ori*$^+$ genomes, containing an intact *ori* sequence in their repeat units; (b) *ori*$^-$ genomes, in which part of the *ori* sequence has been deleted during the excision of the petite genome from the parental wild-type genome; (c) *ori*$^\circ$ genomes, containing no canonical *ori* sequence, but only a surrogate origin of DNA replication (Goursot *et al.*, 1982). As expected, these different classes of petites differ in the replicative ability of their mitochondrial genomes (Bernardi *et al.*, 1980; de Zamaroczy *et al.*, 1981).

Results

Figure 3A displays the electrophoretic patterns (after ethidium bromide staining) of mitochondrial RNAs from three spontaneous petite mutants, a-1/1R/Z1, a-1/1R/1, a-1/1R/14 (which contain the *ori* 1 sequence in the repeat unit of their mitochondrial genome; cluster A is absent in the case of a-1/1R/14; see Figure 2), and from their parental wild-type strain A. While the latter exhibits a characteristic mitochondrial RNA banding pattern, including the two rRNAs, the petite mutants show rather faint bands corresponding to the repeat units of the mitochondrial genome. Hybridization of ^{32}P-labelled mitochondrial DNA from petite a-1/1R/Z1 on the transferred RNAs of Figure 3A revealed (Figures 3B and 3C), in the case of the three petite RNAs, a main band corresponding in size to the repeat unit of the mitochondrial DNAs and weaker bands corresponding to oligomers of the repeat units. Some faint bands corresponding to intermediate sizes could also be seen. Identical results were obtained when using as probes the mitochondrial DNAs from petites a-1/1R/Z1 and a-1/1R/14 (not shown). In sharp

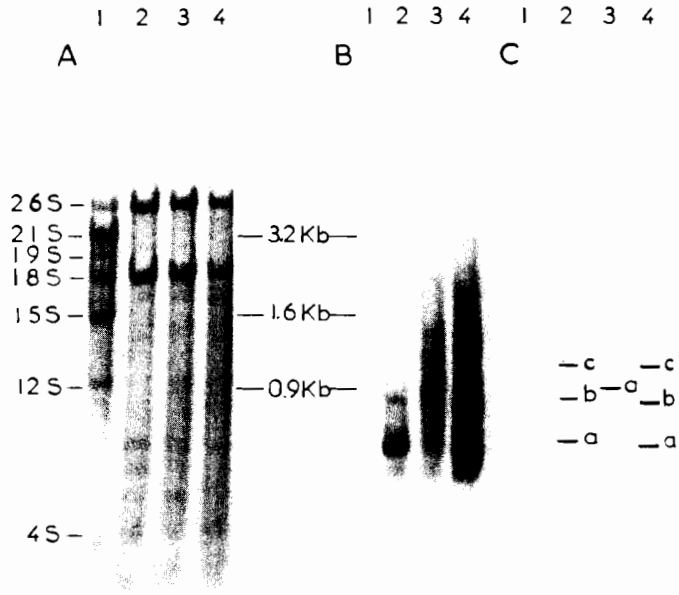

Fig. 3. A. Electrophoretic patterns, after ethidium bromide straining, of mitochondrial phenol extracts from wild-type strain A (**1**) and petites a-1/1R/1 (**2**), a-1/1R/1 (**3**) and a-1/1R/14 (**4**). About 15 μg of mitochondrial phenol extracts were treated with 4 Kunitz units of bovine pancreatic DNase, denatured and loaded on a 1.5% agarose gel (0.3 x 20 cm). The sedimentation coefficients given on the left correspond, respectively, to: 26S, cytoplasmic rRNA; 21S, mitochondrial rRNA; 19S, mitochondrial transcripts from several genes (*e.g., oxi* 3, *cob*); 18S, cytoplasmic rRNA; 15S, mitochondrial rRNA; 12S, cytoplasmic and mitochondrial transcripts; 4S, cytoplasmic and mitochondrial tRNAs. **B.** Autoradiograms obtained after hybridization of ^{32}P-labelled mitochondrial DNA (specific activity ~ 2 x 10^8 c.p.m./μg) from a-1/1R/Z1 on the RNAs from panel **A** blotted onto DBM paper. **C.** Scheme of hybridization patterns. Bands a – c correspond to sizes equal to the repeat unit of each petite genome, and its oligomers.

contrast, no hybridization was obtained with any of the probes on the RNAs from wild-type cells.

Results very similar or identical to those of Figure 3 were obtained by hybridizing ^{32}P-labelled mitochondrial DNAs from petite a-1/1R/Z1 and petite b on the mitochondrial RNAs from the same petites (Figures 4A and 4B). Both probes revealed main hybridization bands corresponding to the size of the repeat units of mitochondrial DNAs and weaker bands corresponding to dimers and trimers. In the first case, bands smaller than the repeat units (and their oligomers) were detected; similar results were seen in the experiments of Figure 3, when using longer exposures for autoradiography (not shown). Finally, mitochondrial DNA from petite b, like that from a-1/1R/Z1 (Figure 3B), did not show any hybridization on RNA from wild-type cells (not shown).

The hybridization of ^{32}P-labelled DNAs from petite a*-1/7/8 (containing *ori* 3) and a-3/1/B31 (containing *ori* 4) to DNase-treated phenol extracts of mitochondria from the corresponding cells revealed a faint smear in the first case and no detectable RNA in the case of a-3/1/B31 (Figure 5); both situations are very different from those of Figures 3 and 4.

The hybridization of ^{32}P-labelled mitochondrial DNA from petite a-5/4/1/B3, which contains an *ori* 5 sequence, to the corresponding RNA showed bands having the sizes of the repeat unit and its dimer and trimer; no hybridization was detected with RNA from wild-type cells (Figure 6A and 6B). In contrast, mitochondrial DNA from petite a-15/3/2, which

Fig. 5. Autoradiograms obtained after hybridization of ^{32}P-labelled mitochondrial DNAs from a*-1/7/8 (**1**) and a-3/1/B32 (**2**) on the corresponding mitochondrial phenol extracts. Other indications as in Figure 3B. Exposure for **lane 2** was three times longer than for **lane 1**.

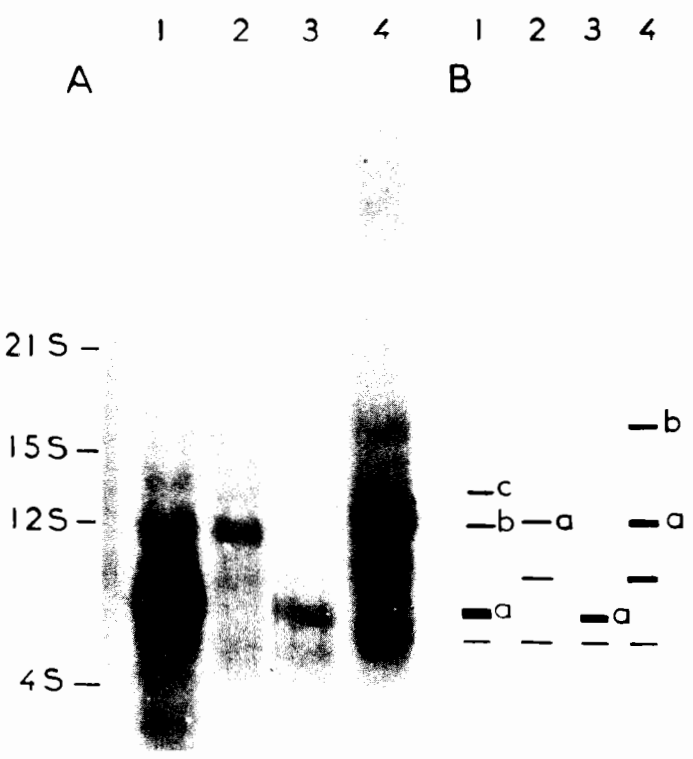

Fig. 4. A. Autoradiograms obtained after hybridization of ^{32}P-labelled mitochondrial DNA from a-1/1R/Z1 (**1** and **2**) and b (**3** and **4**) on the mitochondrial RNAs from a-1/1R/1 (**1** and **3**) and b (**2** and **4**). Other indications as in Figure 3B. **B.** Scheme of hybridization patterns. Bands a–c correspond to sizes equal to the repeat unit of each petite genome and its oligomers. Unlabelled bands correspond to sizes smaller than the repeat unit or its oligomers.

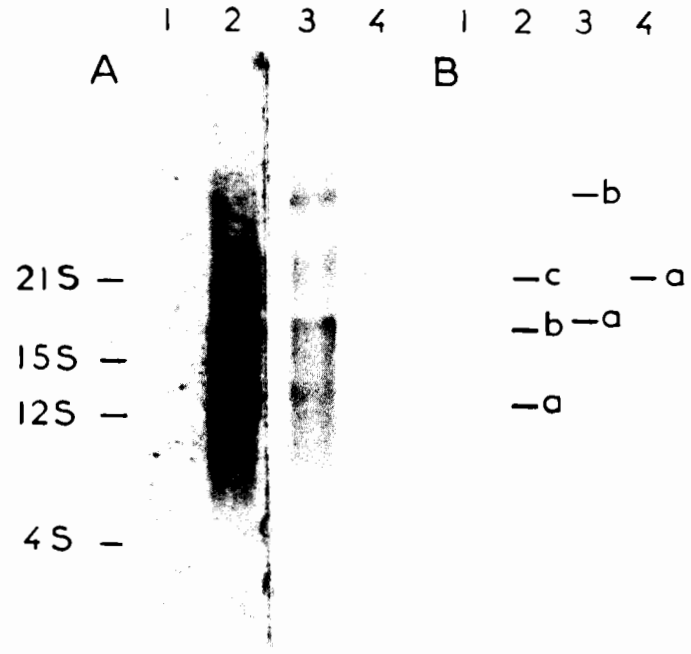

Fig. 6. A. Autoradiograms obtained after hybridization of ^{32}P-labelled mitochondrial DNA from a-15/4/1/B3 on RNA extracts from wild-type cells (**1**) and from the same petite (**2**); and after hybridization of mitochondrial DNA from a-15/3/2 on RNA extracts from the same petite (**3**) and from wild-type cells (**4**). **B.** Scheme of hybridization patterns, indicating the main hybridization bands; in the case of column (**2**), these correspond to the size of the repeat unit, and its oligomers. Contaminating cytoplasmic rRNA show up as clear bands.

also contains an *ori* 5 sequence but has a much longer repeat unit, (Figures 1 and 2), showed a main hybridization band of 2.4 kb (also evident on ethidium bromide stained gels; not shown) and another band of >5 kb; when hybridized to mitochondrial RNA from wild-type cells, the DNA from a-15/3/2 showed a single band of 3.2 kb. Transcription of mitochondrial genomes containing *ori* 6 and *ori* 7 were not tested because they were not available in our collection of spontaneous petites.

The mitochondrial DNAs from two *ori*° petites, containing no canonical *ori* sequence, (a-15/4/1/10/3 and a-3/1/B4, derived from two regions close to *ori* 5 and *ori* 7, respectively; Figures 1 and 2), did not yield any detectable hybridization with mitochondrial phenol extracts obtained from the same petites or from wild-type cells (not shown).

Ori⁻ petites lacking cluster C and the neighboring sequences in their *ori* 1 (b-7/1; C.Le Van Kim, personal communication) or *ori* 3 sequences (a-3/1/5 and a-3/1/33, both derived from a-3/1; see Figure 2) were hybridized with their mitochondrial phenol extracts. Petite b-7/1 showed no detectable hybridization with the separated strands of a-1/1R/Z1 (see below and Figure 7). The other two petites showed a much fainter smear than a*-1/7/8 (a petite also containing *ori* 3) when hybridized with ^{32}P-labelled homologous DNAs. This result appears, however, to be artefactual since it was not reproducible and not confirmed by hybridizations with the template strand (see below). Table I summarizes all the results presented above. Other experiments aimed at identifying the transcribed strand, the orientation of transcription and the precise initiation site of the transcript.

Autoradiograms obtained after hybridization of ^{32}P-labelled separated strands of petite a-1/1R/Z1 on mitochondrial phenol extracts from three petites, a-1/1R/Z1, b, and b-7/1 indicated that only the strand containing the oligopyrimidine stretch of cluster C gave a positive result in the first two cases but not in the last (Figure 7A). Hybridization experiments with this strand and extracts from petites a*-1/7/8, a-3/1/33, and a-3/1/B31 showed a faint smear hybridization in the first case and negative results in the other two (not shown).

The origin of transcription of mitochondrial RNA on the repeat unit of the a-1/1R/Z1 DNA was determined as follows. The mitochondrial DNA was digested with *Hpa*II, whose single site on the repeat unit is localized in cluster B of the *ori* sequence, and labelled at its 5′ ends. The strand used as template for transcription was then hybridized with the homologous mitochondrial phenol extract and the hybrid so formed was digested with S1 nuclease. The protected DNA was run in parallel with a partial chemical hydrolysate of the same template strand (Figure 7B), and shown to comprise the region between the *Hpa*II site of cluster B and a TATTACTTATATATTT sequence next to the oligopyrimidine stretch of cluster C (Figure 8). This 16-bp sequence was also present, at the same location relative to cluster C, in the *ori* 2 sequence, and contiguous to cluster C in the other *ori* sequences (Figure 8). The shift of the sequence in *ori* 1 and *ori* 2 seems to be due to the insertion of a 11-bp sequence (Figure 8). In the case of *ori* 4, 6, and 7, the 16-bp sequence contains the extra GC cluster γ (de Zamaroczy *et al.*, 1981). Interestingly (see Discussion), only the sequence present in *ori* 5 was identical with those of *ori* 1 and 2; that of *ori* 3 contained four base changes at positions 3, 7, 15, and 16; those of *ori* 4 and 7 contained one base change at position 7 (G replacing T), just before the γ cluster; and the sequence of *ori* 6 had a base deletion at position 8. This latter point should, however, be considered with caution since *ori* 6 is a sequence found by us in a region sequenced by Nobrega and Tzagoloff (1980) and known to present some uncertainties. Finally, two additional shorter copies of the sequence were found in *ori* 2 and *ori* 3 (Figure 8).

A comparison of the 16-bp sequence corresponding to the transcription initiation in *ori* 1 with the transcription initiation sequences of 15.5S and 21S rRNA genes as determined by Osinga and Tabak (1982) showed that 10 bp (positions

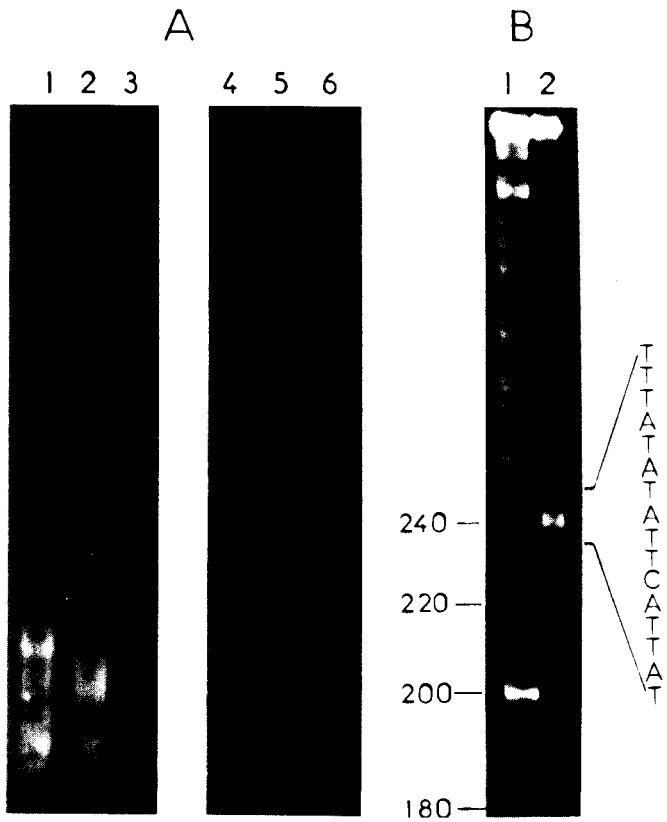

Fig. 7. A. Autoradiograms obtained after hybridization of separate strands ^{32}P-5′-end-labelled at the *Hpa*II site of cluster B (see Figure 2) of mitochondrial DNA from a-1/1R/Z1 on mitochondrial phenol extracts from petites a-1/1R/Z1, b and b-7/1. **1, 2, 3** and **4, 5, 6** indicate the hybridizations with the strands containing the oligopyrimidine and the oligopurine stretches of cluster C from *ori* 1, respectively. End-labelling and strand separation were performed according to the procedure of Maxam and Gilbert (1980). **B.** Autoradiograms of the transcribed strand of a-1/1R/Z1 5′-end-labelled at the *Hpa*II site of cluster B (see Figure 2): (**1**) after partial chemical degradation at A and G; (**2**) after hybridization with homologous mitochondrial phenol extracts and S1 nuclease digestion (see Results for a description of this experiment). The two samples were electrophoresed in parallel. Figures on the left indicate fragment sizes in bp; the sizes of S1 protected fragment correspond to the position of the TATTACT-TATATTT sequence (compare with Figure 8).

Table I. Transcription of mitochondrial genomes from spontaneous petite mutants

A. *ori*⁺ petites	Transcription	B. *ori*⁻ petites	Transcription
ori 1	+; bands	*ori* 1 A⁻	+; bands
ori 2	+; bands	*ori* 1 C⁻	–
ori 3	±; smear	*ori* 3 C⁻	–
ori 4	–		
ori 5	+; bands	C. *ori*° petites	
ori 6	n.d.	a-15/4/1/10/3	–
ori 7	n.d.	a-3/1/B4	–

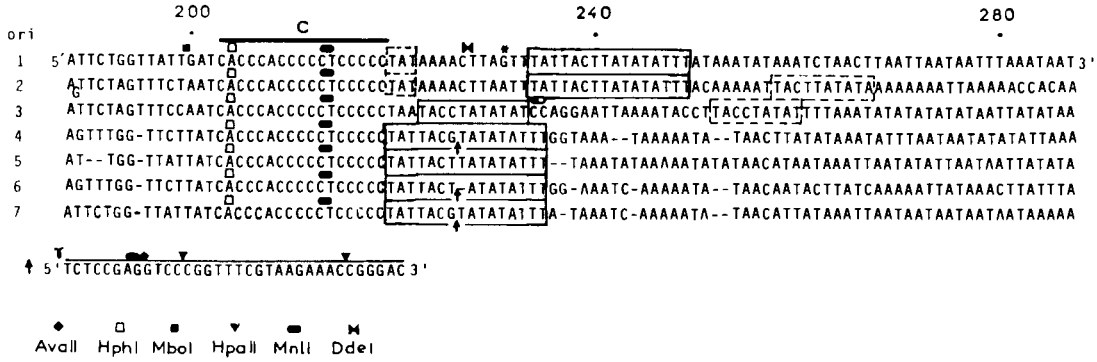

Fig. 8. Primary structure of the *ori* sequences in the neighborhood of oligopyrimidine stretch of cluster C. The positions of γ-clusters (arrows), of the TATTACTTATATTT sequences (solid line boxes), of the additional partial copies of them in *ori* 2 and *ori* 3 (broken line boxes) are indicated. Numbering is from the *Hpa*II site of cluster B (see Figure 2), so as to allow a comparison with the data of Figure 7B. Restriction sites are mapped. Modified from de Zamaroczy *et al.* (1981) on the basis of new results (G.Faugeron-Fonty and M.de Zamaroczy, personal communication).

4 – 13) were identical (Figure 9). In fact, in the specific case of *ori* 1, homology extended over 25 bp (with 2 and 4 base differences relative to 15.5S and 21S initiation sequences, respectively) including the first six transcribed nucleotides. These findings prompted a computer search through the known sequences located outside mitochondrial genes and open reading frames of introns (~40 kb) for similar sequences. This was done by looking for the 10-bp sequence comprised between positions 4 and 13 of the 16-bp sequence (including a variant containing a C instead of a T at position 7) as well as for the complementary sequence. A total of 13 such sequences were found; these were localized on the genome map and positioned on one or the other strand (Table II and Figure 1). In addition to the sequences present in *ori* 1, 2, 3, and 5 (*ori* sequences containing cluster γ were not included in the list) and to the sequences of Osinga and Tabak (1982), only six sequences were found in the 40 kb explored, four of them in the genomes of *ori*° petites sequences by Goursot *et al.* (1982). These sequences were often in the proximity of *ori*^{S} sequence, somehow mimicking the situation found in *ori* sequences. These *ori*° petites included a-15/4/1/10/3.

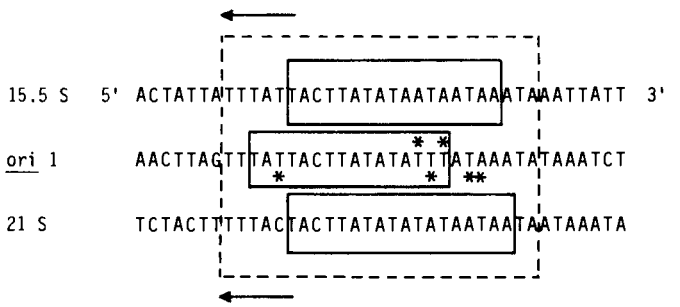

Fig. 9. Comparison of the transcription initiation sequences of *ori* 1 and of the 15.5S and 21S rRNA genes (Osinga and Tabak, 1982). Solid line boxes indicate the transcription initation sequences (as read on the coding strand to insure consistency with Figure 8); the broken line box indicates the region of homology among the three sequences; asterisks indicate base differences, arrows the start of rRNA transcripts.

Discussion

The results summarized in Table I lead to two major conclusions. The first is that some *ori* sequences are used for the initiation of transcription in spontaneous petite mutants. While *ori*$^{+}$ mutants carrying intact *ori* 1, 2, 3, and 5 do transcribe, *ori*° petites do not show any detectable transcription. The case of *ori*$^{-}$ petites provides a more precise indication of the sequence required for transcription to take place, since *ori*$^{-}$ petites lacking cluster C, and the surrounding regions, in their *ori* 1 or *ori* 3 sequences, are not transcribed; in contrast, the lack of cluster A does not impair detectable transcription. The results obtained with the petite genome carrying *ori* 4 narrow down even further the sequence requirements, since this genome carries an extra GC cluster γ, and also exhibits a base-pair change, in the sequence where transcription initiation is indicated to take place by other experiments (see below); it is impossible to judge at present whether the presence of cluster β in the long sequence separating clusters B and C (de Zamaroczy *et al.*, 1981) in *ori* 4 also contributes to the inactivation of transcription initiation. A comparison of petites carrying *ori* 4 with others carrying *ori* 6 (which also contains both extra clusters) or *ori* 7 (which only contains cluster γ) should provide an answer.

Table II. Properties of 'initiation' sequences[a]

Sequence number[b]	Sequence[c]	Strand[d]	Map location[e]	Reference[f]
	TATTACTTATATATTT			
(1)	-T----C-------A-	–	before *ori* 5	(1)
(2)	----------	+	*ori* 5	
(3)	----------	–	*ori* 1	
(4)	----------A-	+	near 15.5S	(1)
(5)	----------A-A	+	15.5S	(2)
(6)	A----------AAA	+	*ori* 2a[g]	
(7)	----------	+	*ori* 2	
(8)	C-AA----------A	+	before glu tRNA	(1)
(9)	------C------A-A	+	before glu tRNA	(1)
(10)	GTA----------AC	–	before *ori* 6	(3)
(11)	ATA----------AA-	+	after *ori* 6	(3)
(12)	--A---C-------CC	–	*ori*	
(13)	--C----------A-	+	21S	(2)

[a]Sequences interrupted by the γ cluster are not listed.
[b]See Figure 1 for precise location.
[c]The first line is the sequence of *ori* 5 taken as a reference.
[d]+ indicates the sense strand used in the transcription of most mitochondrial genes.
[e]Before and after refer to a clockwise reading of the map.
[f]References are: (1) Goursot *et al.*, 1982; (2) Osinga and Tabak, 1982; (3) Nobrega and Tzagoloff, 1980.
[g]This is the additional copy of the sequence mentioned in the Text.

We have two comments on this first point. (a) The results of Table I are the first to establish a correlation between structural features of the mitochondrial genome and its transcriptional behavior. Indeed, while it has been known for some time that some petite genomes are transcribed, whereas others are not (see, for instance, Morimoto *et al.*, 1979), no explanation for such differences was available. (b) There is an evident correlation between transcriptional activity and replication efficiency. While *ori* A^- petites are only weakly impaired in replication efficiency, as judged from suppressivity (de Zamaroczy *et al.*, 1981), and do not exhibit any major change in transcription, *ori* C^- and *ori*° petites replicate very poorly (de Zamaroczy *et al.*, 1981; Goursot *et al.*, 1981) and their transcription is undetectable. It would be very interesting to check whether the lack of transcription exhibited by the petite carrying *ori* 4 is accompanied by a poor replication efficiency, but this could not be tested because petite a-3/1/B31 is diploid. In any case, it is worthwhile mentioning that *ori* 4 is dispensable in wild-type cells, as shown by its absence in strain B (G.Faugeron-Fonty, personal communication), and that a-3/1/B31 is the only petite that we have found in extensive screening to carry *ori* 4 alone; similarly, we have not found so far any spontaneous petite genome carrying *ori* 6 or *ori* 7 alone, as if all the genomes characterized by the presence of cluster γ replicate inefficiently and are regularly competed out by the parental wild-type genome.

The second major conclusion concerns the different transcriptional behavior of petite genomes. Three different situations have been found. (a) *Ori*$^+$ petites carrying *ori* 1, 2, and 5 exhibit transcripts having the size of the repeat units of their mitochondrial genomes; lower amounts of dimeric and trimeric transcripts have been detected in all cases, as well as of discrete transcripts having sizes lower than the repeat units or their oligomers. The simplest interpretation of these findings is that there is a transcription termination signal just upstream of the initiation sequence; alternatively, the arrest of transcription is caused by the presence of another RNA polymerase molecule on the initiation sequence. In either case, some enzyme molecules seem to overcome this barrier to fall off at the corresponding location on the following repeat units, so originating dimer and trimer transcripts. The presence of discrete transcripts lower in size than the repeat unit or its oligomers (Figure 4), may either mean that some other secondary termination sequences are occasionally used or that the original transcripts have undergone processing. (b) In contrast to the *ori*$^+$ petites just discussed, a*-1/7/8, a petite containing *ori* 3, exhibits a weak transcription with a continuous distribution of sizes. In view of the results obtained with *ori* 4, it is tempting to suggest that this weak transcription is somehow associated with the four base changes found in the sequence of transcription initiation. On the other hand, the continuous distribution of sizes suggest that transcripts starting and/or stopping at a number of sites within the repeat unit (perhaps always present as a background noise) become apparent in this poorly transcribing genome. (c) The transcript sizes of a-15/3/2 are of special interest for several reasons. The repeat unit of this petite genome (Figure 10) corresponds to the 3′ trailing end of the *oxi* 2 transcript, which has been mapped by Van Ommen *et al.* (1979). As already mentioned, a-15/3/2 is the only *ori*$^+$ petite whose major transcript does not have the size of the repeat unit of the mitochondrial genome (4.3 kb) but is only 2.4 kb. This is exactly (Figure 10) the size of the segment

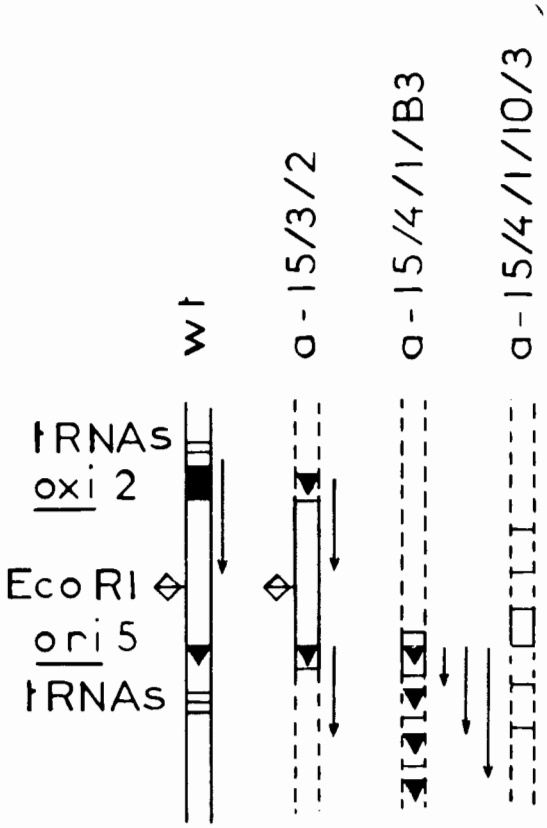

Fig. 10. Maps of the *ori* 2 region of the mitochondrial genomes of wild-type cells and of the repeat units of three petites originally excised from this region. Vertical arrows indicate transcripts; other symbols as in Figure 1. See text for further explanations.

comprised between the *ori* 5 sequence and the end of the *oxi* 2 transcript, on the following repeat unit; this end has been mapped by Thalenfeld and Tzagoloff (1979) and corresponds to a GC cluster in the DNA sequence. This finding very strongly suggests that *ori* 5 is indeed used as the initiation sequence and that a 'physiological' termination signal of the wild-type genome is used to stop transcription on the petite genome. The larger transcript (>5 kb) found in a-15/3/2 might be due to the termination signal present one more repeat unit downstream; if so, the transcript should have a size of 4.3 + 2.4 = 6.7 kb. Interestingly, if the DNA segment corresponding to the *oxi* 2 transcript is deleted, as in petite a-15/4/1/B3, transcripts corresponding to the repeat unit size, to its dimer and trimer are produced. If only a DNA segment comprised between *ori* 5 and the *oxi* 2 termination signal (but not containing any of these two sequences) is conserved as in the repeat unit of *ori*° petite a-15/4/1/10/3, transcription is no longer detected. Also in agreement with the interpretation given above is the finding that the mitochondrial DNA from a-15/3/2, unlike any of the other petite DNAs studied here and, in particular, of those from a-15/4/1/B3 and a-15/4/1/10/3, hybridizes on the *oxi* 2 transcript, 3.2 kb in size, from wild-type cells (Figure 6). It is perhaps worth stressing that the transcription of the DNA region corresponding to the *oxi* 2 transcript is not initiated on the 'physiological' initiation sequence, which obviously is upstream and not downstream of the *oxi* 2 gene.

The present work also indicates which strand of the *ori* sequence is transcribed, which is the direction and where the transcription initiation sequence is located. When the separated strands of mitochondrial DNA from a-1/1R/Z1 were hybridized on mitochondrial RNAs from a-1/1R/Z1

and b, two petites containing *ori* sequences having opposite orientations on the wild-type genome map, only the strand containing the oligopyrimidine stretch of cluster C showed a positive result in both cases. In other words, whether the C→A orientation of *ori* sequences on the wild-type genome map is counter-clockwise, as in *ori* 1, or clockwise as in *ori* 2, it is always the strand carrying the oligopyrimidine stretch of cluster C which is used as a template for transcription. This result is in agreement with our finding of the oligopurine stretch of cluster C of *ori* 6 and *ori* 7 on the non-coding strand sequence published by Nobrega and Tzagoloff (1980) and Macino and Tzagoloff (1980).

Concerning the initiation sequence and the direction of transcription, the S1 protection experiment has provided precise information. The transcription initiation has been localized a few nucleotides upstream of cluster C at the level of a particular sequence, which has been found to be present in the transcription initiation sequences of the two mitochondrial RNA genes of *S. cerevisiae* and to be partially conserved in the transcription initiation sequence of the small mitochondrial rRNA gene of *Kluyveromyces lactis*, a petite negative yeast (Osinga and Tabak, 1982). As already mentioned, the fact that this sequence is interrupted by cluster γ and presents a base change in the *ori* 4 sequence (as well as in *ori* 6 and *ori* 7) may be the cause for the lack of transcription of petite a-3/1/B31. The protection experiment also indicates that the direction of transcription runs from cluster C towards cluster A. This is in agreement with the conclusion one can draw from the transcription of a-15/3/2

A final point concerns the relevance of the present results as far as the transcription initiation in the wild-type genome is concerned. The strand used as a template in the petites containing *ori* 2 and *ori* 5 and the direction of transcription from these sequences are the same as for the physiological clockwise transcription of most mitochondrial genes in the wild-type genome. Furthermore, these *ori* sequences contain, just upstream of the oligopyrimidine stretch of cluster C, a sequence very largely or completely identical to those used as transcription initiation sites of rRNA genes (Osinga and Tabak, 1982) and of petite genomes containing *ori* 1. These points obviously raise the question whether *ori* 2 and *ori* 5 are also used in the physiological transcription of the wild-type genome; the same question also applies to the other two *ori* sequences showing the same orientation as *ori* 2 and *ori* 5, namely *ori* 6 and *ori* 7, as well as to the sequences of Table II sharing the same orientation, namely sequences (4), (6), (8), (9), and (11). At present the only reasonable answer is that this possibility should be considered as definitely open for *ori* 2, *ori* 5, and perhaps for some of the other sequences of Table II. Incidentally, four of these present a very peculiar tandem arrangement, (4) with the initiation sequence (5) of the 15.5S rRNA, (6) with the sequence (7) of *ori* 2, and (8) with (9). The possibility discussed above appears to be more uncertain for *ori* 6 and *ori* 7, which contain a γ cluster likely to be the explanation for the transcriptional inactivity of *ori* 4.

The question of usage of *ori* sequences as transcription initiation sequences in the wild-type genome should also be raised for the *ori* sequences having the opposite orientation, namely *ori* 1, 3, and 4, as well as for the corresponding sequences of Table II, namely (1) and (10). Here, the only sequence known to act as an efficient transcription initiation site, is *ori* 1; *ori* 3 is poor in this respect, *ori* 4 is inactive, and sequence (1) is present in an *ori*° petite, a-15/4/1/10/3, shown here not to be transcribed; nothing can be said at present about sequence (10). These data suggest, by comparison with those concerning the other strand, that the non-sense strand is also transcribed but from a more limited number of sequences and in a poorer way.

This conclusion, which is in agreement with the results of Li and Tzagoloff (1979), Coruzzi *et al.* (1981), Linnane *et al.* (1980), and Beilharz *et al.* (1982), puts the transcription of the mitochondrial genome of yeast in line with that of the animal mitochondrial genome. Similarly, it is conceivable that replication of this mitochondrial DNA proceeds unidirectionally from *ori* sequences having different orientations. On the basis of the present results and our finding of individual *ori* sequences in spontaneous petite genomes, a working hypothesis worth exploring could be that *ori* 2 and *ori* 5 are used for the replication of one strand and *ori* 1 and *ori* 3 for the replication of the other strand; if so, replication of yeast mitochondrial DNA would be somehow analogous with the replication of unicircular dimers of mammalian mitochondrial DNA (Clayton, 1982).

An independent, though indirect, additional argument in favour of the use of *ori* sequences as transcription initiation sites comes from the observations: (a) that the existing gaps on the transcription map (Figure 1) coincide with the positions of the *ori* sequences; and (b) that, with the exception (accounted for) of a-15/3/2, the petite genomes carrying *ori* 1, *ori* 2, and *ori* 5 fail to hybridize on the transcripts from the wild-type genome. Possible explanations for these two observations are: (a) that the 5′-proximal segments of mitochondrial transcripts from *ori* 1, 2, and 5 are the first to be processed and degraded using signals apparently also used in the petite genome; and (b) that very few transcripts are produced starting at the other *ori* sequences.

However tentative, the above considerations are of interest because they suggest new approaches to solve the problem of transcription initiation in the wild-type mitochondrial genome of yeast. Indeed, further studies of the transcription of spontaneous petite genomes and the sequencing of strategically located wild-type genome regions, whose primary structure is not yet known, should provide further indications in favour of the idea (Prunell and Bernardi, 1977) confirmed by the data of Levens *et al.* (1981c), Osinga and Tabak (1982) and of the present work, that multiple transcription initiation sites are used in the wild-type mitochondrial genome of yeast.

Materials and methods

Mitochondrial DNA was prepared from early stationary phase cells according to procedures described elsewhere (Faugeron-Fonty *et al.*, 1979).

To prepare mitochondrial RNA, protoplasts were prepared according to Faye *et al.* (1974) by digestion of cell walls with Zymoliase (Miles, Elkhart, MD) and lysed in the presence of vanadium adenosine (Burger and Birkenmeier, 1979) used as a ribonuclease inhibitor. Mitochondrial RNA was extracted by the method of Locker (1979). Mitochondrial phenol extracts were routinely treated with chromatographically purified pancreatic deoxyribonuclease (Sigma, St. Louis, MO) and further purified (Maxwell *et al.*, 1977) by passage through 4-aminophenyl phosphophenyl uridine 2′,3′ phosphate immobilized on agarose (Miles). The DNase activity recovered from the column was tested on ^{3}H-labelled DNA. The absence of residual contaminating RNase activity was tested by incubating mitochondrial RNA preparations from wild-type cells, showing high mol. wt. RNA bands, with the purified DNase. RNase-treated mitochondrial phenol extracts (previously treated with purified DNase) did not exhibit any hybridization with homologous ^{32}P-labelled mitochondrial DNA. When previous DNase treatment was not applied to the RNase-treated extracts, a very low level of contamination with mitochondrial DNA was detected, but the hybridization pattern was different from that obtained with RNA. When homologous mitochondrial DNA was added to the RNA samples, which were then submitted to RNA treatment,

the expected hybridization patterns were obtained, indicating the absence of contaminating DNase activity in the RNase preparation.

RNA samples were denatured in the presence of 1 M glyoxal (Mc Master *et al.*, 1977) and loaded on 1.5% agarose gels. Electrophoresis was carried out (4 h, 4°C, 9 V/cm) in 10 mM phosphate buffer, pH 6.8, recycled during the run. Fractionated RNA was then transferred by blotting to diazobenzyloxymethyl (DBM) paper (Alwine *et al.*, 1977) and hybridized to ^{32}P-labelled mitochondrial DNAs (Rigby *et al.*, 1977; Wahl *et al.*, 1979). Mol. wt. markers were mitochondrial rRNAs, assumed to have sizes equal to 3.2 kb and 1.6 kb, respectively, and a 12S RNA species from the wild-type strain cells, assumed to have a size of 0.9 kb (Van Ommen *et al.*, 1980). All mitochondrial RNA preparations were contaminated by cytoplasmic rRNAs; as expected, these did not hybridize any mitochondrial DNA and appeared, in fact, as clear bands on some hybridization patterns (Figure 6).

5′ End labelling, strand separation, and limited DNA cleavage at guanines and adenines were performed following the methods of Maxam and Gilbert (1980). S1 nuclease was purchased from Boehringer (Mannheim, FRG) and used according to the procedure of Berk and Sharp (1977), modified by Weaver and Weissman (1979).

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