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Map location of transcripts from *Torulopsis glabrata* mitochondrial DNA

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Unique transcripts for cytochrome b, ATPase subunits 6 and 9, cytochrome oxidase subunits 2 and 3 and S and L rRNA have been mapped by the S1 protection technique to the circular 19-kbp mitochondrial DNA (mtDNA) of the yeast *Torulopsis glabrata*. In contrast, a number of transcripts have been detected for the mosaic cytochrome oxidase subunit 1 gene with the largest being ~5000 nucleotides and the mature message having a length of 1760 nucleotides. Despite the presence in *T. glabrata* mtDNA of a sequence that hybridizes to the variant 1 gene of *Saccharomyces cerevisiae* mtDNA we have not detected a transcript of this region. Neither have we detected co-transcripts of adjacent genes in RNA from either glucose-repressed or derepressed cells. However, by comparison of RNA species from the two growth conditions, we have found that the ATPase subunit 6 transcript is lower in amount relative to other species in preparations from glucose-repressed cells. This information, together with the observation of separate transcripts and the knowledge that there are several species of mitochondrial RNA which can be capped by the guanylyl transferase catalysed addition of GMP, suggests that each of the genes investigated in the present study is separately transcribed.

Key words: glucose repression / mtDNA / transcripts / S1 nuclease mapping

Introduction

The circular 19-kb mitochondrial DNA (mtDNA) from the yeast *Torulopsis glabrata* contains sequences specifying small (S) and large (L) rRNAs, tRNAs (Clark-Walker *et al.*, 1980) and regions hybridizing to six polypeptide genic sequences from *Saccharomyces cerevisiae* mtDNA (Clark-Walker and Sriprakash, 1981, 1982). From these studies, two observations in particular have attracted our interest because they impinge on possible mechanisms of expression of the mitochondrial genome. Firstly, the six polypeptide genic sequences, together with the S and L rRNA regions, have the same orientation. Secondly, the hybridizable segments of ATPase subunits 6 and 9 are juxtaposed and have the same order as analogous sequences in the *Escherichia coli unc* or *atp* operon (Downie *et al.*, 1981; Gay and Walker, 1981).

Unique sequence orientation has also been found for a number of other yeast mitochondrial genomes (Clark-Walker and Sriprakash, 1981, 1982) including *S. cerevisiae* mtDNA (with the exception of one tRNA gene, Li and Tzagloff, 1979), despite extensive sequence rearrangements between some of these molecules. An explanation for preferred orientation could be that a constraint is imposed by transcription whereby polycistronic RNAs are produced from a limited number of promoters. Indeed, co-transcription of genes could be a feature of some yeast mtDNAs, especially those with genomes similar in size to mammalian mtDNA where large transcripts are known to be produced (Aloni and Attardi, 1971; Montoya *et al.*, 1982). Of particular interest in this respect are the juxtaposed ATPase sequences in *T. glabrata* mtDNA especially in view of the co-transcription of the *unc* operon genes in *E. coli*. We therefore examined mitochondrial RNA from *T. glabrata* to see initially if co-transcripts could be detected for the ATPase segment, and subsequently to determine whether transcripts or co-transcripts could be identified for the other large genic regions of the mtDNA. We report that although we have identified and mapped transcripts for all the large genic regions of mtDNA except *var1*, we have not found evidence for co-transcription in either glucose-repressed or derepressed cells.

Results

The restriction site map of *T. glabrata* mtDNA (Clark-Walker and Sriprakash, 1981) has been extended by the discovery of paired sites for *Eco*RV, *Pvu*II and *Sph*I. The map coordinates of these sites, together with those for other key sites used in constructing the transcript map, are listed in Table I. Also listed are the positions of the unique sites for the six restriction endonucleases *Sal*I, *Hpa*I, *Hha*I, *Xho*I, *Pst*I and *Kpn*I together with four *Hind*III sites used in establishing the original map.

Further to the mapping of genic sequences reported earlier, we have identified additional regions of the mitochondrial genome by hybridization with the *aap1* (ATPase-associated protein) and the *var1* genic sequences of *S. cerevisiae* mtDNA

Table I. Map coordinates of key restriction endonuclease sites in *T. glabrata* mtDNA

Restriction endonuclease site	Map coordinate
*Sal*I	0-18 990
*Sph*I	1220
*Hpa*I	2190
*Hind*III	2430
*Hha*I	2520
*Hind*III	4040
*Eco*RV	5440
*Xho*I	6800
*Bcl*I	7670
*Pvu*II	7820
*Hinf*I	8720
*Mbo*I	9570
*Eco*RV	10 450
*Hind*III	11 940
*Hind*III	13 290
*Pvu*II	13 670
*Pst*I	14 580
*Bcl*I	15 390
*Sph*I	16 340
*Kpn*I	16 400

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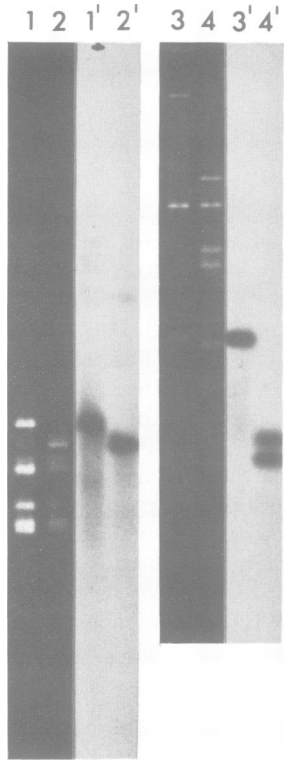

Fig. 1. Autoradiograms showing hybridization of *T. glabrata* mtDNA fragments with: **lanes 1'** and **2'** ^{32}P-labelled mtDNA from petite G5 (*aap*1 gene) and **lanes 3'** and **4'**, ^{32}P-labelled pBR *var*1. Digests have been generated with: **lane 1** and **2**, *Mbo*I before **(1)** and after **(2)** digestion with *Pvu*II; **lanes 3** and **4**, *Pst*I/*Sph*I before **(3)** and after **(4)** cleavage with *Bcl*I.

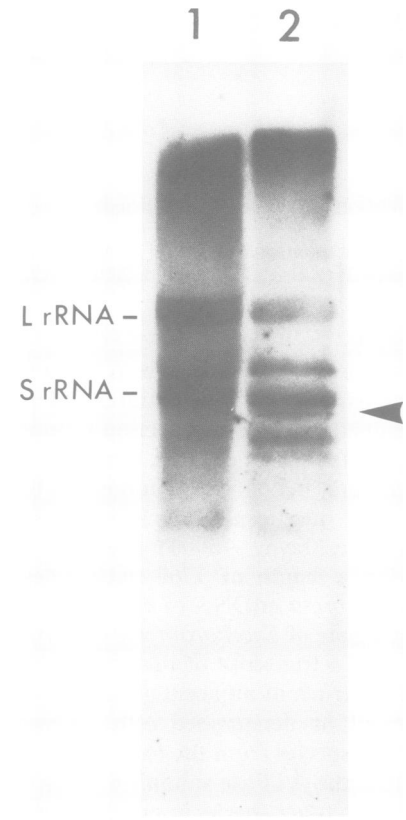

Fig. 2. Autoradiogram of mtRNA preparations from glucose-repressed **(lane 1)** and derepressed **(lane 2)** cells after hybridization with ^{32}P-labelled *T. glabrata* mtDNA. Glyoxal-denatured RNAs have been electrophoretically separated in 1.2% agarose and transferred to nitrocellulose prior to hybridization. The arrow marks the position of the ATPase subunit 6 transcript and the hybridizable region at the top of the autoradiogram is due to contaminating mtDNA. The positions of the S and L rRNAs are indicated on the side of the figure.

(Figure 1). Hybridization with the petite G5 (which contains the *aap*1 gene) to an *Mbo*I digest before and after *Pvu*II cleavage (lanes 1 and 2) showed that that *aap*1 sequence is located in the largest *Mbo*I band (1900 bp) that is cleaved by *Pvu*II to form a 1760-bp fragment. This fragment contains the ATPase subunit 6 and 9 genic sequences (Clark-Walker and Sriprakash, 1981).

A region hybridizing to the *var1* probe (plasmid pBR var1) was located by use of a *Pst*I/*Sph*I digest before and after cleavage with *Bcl*I (lanes 3 and 4). Hybridization is confined to a 1760-bp *Pst*I-*Sph*I band that is cleaved by *Bcl*I to yield two fragments of 950 bp (*Bcl*I-*Sph*I) and 810 bp (*Bcl*I-*Pst*I) both of which label with the probe. This positions the *var1* hybridizable sequence between the S and L rRNA genes as indicated on the map (Figure 8).

Examination of RNA preparations from glucose-repressed and derepressed cells was performed by glyoxal denaturation and separation of species by electrophoresis under low salt conditions. By using uniformly labelled mtDNA to display the RNA species we hoped to form some impression of the total number of transcripts (Figure 2). This number does not appear to be large at least for the major transcripts. Indeed, by use of specific probes (Figure 3), we can identify six transcripts that specify polypeptides and these RNAs, together with the S and L rRNAs previously described (Sriprakash and Clark-Walker, 1980), correspond to the predominant bands seen in Figure 2. Thus unique transcripts have been found for the S and L rRNAs, cytochrome b, ATPase subunits 6 and 9 and cytochrome oxidase subunits 2 and 3 whereas one major and at least three minor species are observed for cytochrome oxidase subunit 1 (only one minor band is visible in the figure). The size of these transcripts was estimated by comparison with denatured and glyoxalated DNA fragments from pBR322 run in parallel (Table II).

In comparing the RNA preparations from glucose-repressed and derepressed cells, the notable difference lies in the amount of ATPase subunit 6 transcript (arrow, Figure 2). This species, which has been identified by size and the S1 protection technique (see below), is under-represented in the preparation from glucose-repressed cells. Also under-represented in both RNA preparations are the S and L rRNAs which are the predominant species found by direct observation. One explanation for this result could be that the marked secondary structure of the rRNAs reduces the availability of sequences for hybridization to the mtDNA probe used to display the RNAs.

Initial experiments to determine both the size and location of transcripts with the S1 protection technique were performed with neutral gel rather than alkaline gel electrophoresis as less background hybridization occurred at the top of the filters. However, using values from neutral gels, we found that alignment of transcripts showed discrepancies with the restriction endonuclease site map. Subsequently, we obtained better agreement using values obtained from alkaline gel electrophoresis which results in lower estimates for transcript sizes (Figure 4, Table II). Different migration rates under the two conditions of electrophoresis is illustrated for the cyto-

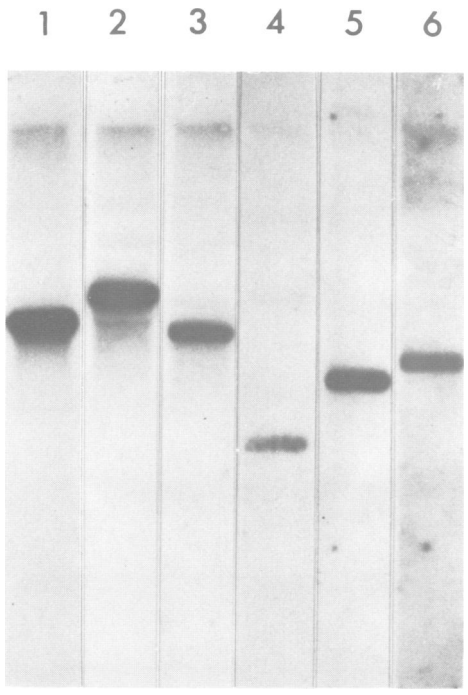

Fig. 3. Autoradiograms showing hybridization with specific probes to mtRNA from derepressed cells denatured with glyoxal, electrophoretically separated on 1.2% agarose and transferred to nitrocellulose. Probes used for hybridization are: **lanes 1**, DS400/N1, (cyt.b); **2**, DS6/A422, (cyt.ox.sub.1); **3**, DS14, (ATPase sub.6); **4**, DS400/A3, (ATPase sub.9); **5**, pSCM5 (cyt.ox.sub.2) and **6**, DS31, (cyt.ox.sub.3).

Table II. Comparison of mitochondrial transcript sizes using glyoxal denaturation and S1 protection techniques

Mitochondrial gene[a]	Transcript size (nucleotides)		
	Glyoxal denaturation	S1 protection	
		Neutral gel	Alkaline gel
Cyt b	1400	1480	1360
Cyt. ox. subunit 1	4200	5000	
	3500	3900	–
	2900	2550	
	1760	1400	
		1220	
		660	
		580	
ATPase subunit 6	1250	1380	1300
ATPase subunit 9	500	465	460
Cyt. ox. subunit 2	890	930	830
Cyt. ox. subunit 3	985	1120	990
S rRNA	1400[b]	1760	1620
L rRNA	2700[b]	3180	3050

[a]Mitochondrial genes are listed in a clockwise direction.
[b]Sriprakash and Clark-Walker (1980).

chrome oxidase subunit 2 transcript using the same DNA size marker (Figure 4). Also illustrated in this figure (lane 2′) is the result of cleavage of the DNA with *Eco*RV prior to hybridization with mtRNA. The *Eco*RV site at map coordinate 10450 (Table I) is located in the middle of the transcribed sequence and results in the formation of two S1 nuclease-protected products of 425 and 400 nucleotides.

By similar experiments the size and location of transcripts for S and L rRNAs, cytochrome b and cytochrome oxidase

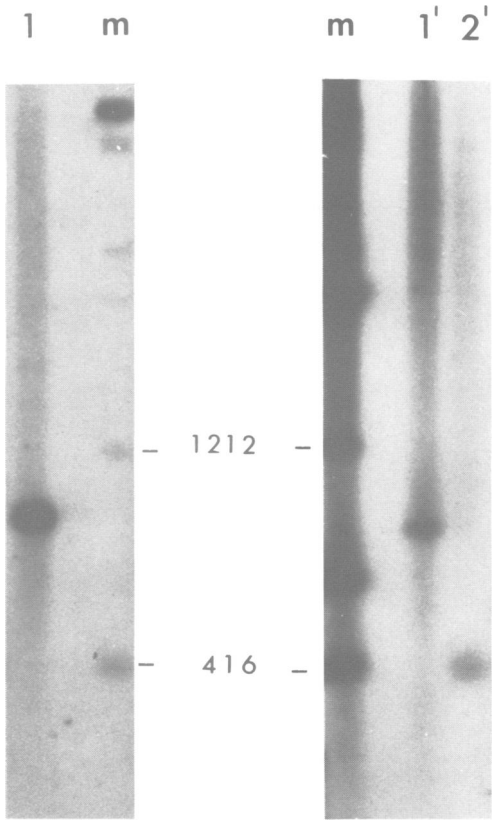

Fig. 4. Autoradiograms showing effect of electrophoresis under neutral **(left)** and alkaline conditions **(right)** following the S1 nuclease protection technique. Hybridization was performed with ^{32}P-labelled pSCM5 (cyt.ox.sub.2). **Lane m** contains a *BamI/BglII* digest of λ DNA end-labelled with ^{32}P, **lane 1** and **1′** are from an experiment with uncleaved *T. glabrata* mtDNA while in **lane 2′** the mtDNA has been cleaved with *Eco*RV prior to hybridization with mtRNA. The two products produced by *Eco*RV cleavage are not resolved. The size in nucleotides of two λ DNA fragments is included in the figure.

subunit 3 were determined as shown in Figure 5. The position of the S rRNA transcript (lane 1) was determined by cleavage with *HindIII* which results in the formation of a 1360-bp fragment (lane 2) protected from digestion with S1 nuclease. The 260 nucleotide remainder of the sequence resulting from *HindIII* digestion is not detected either because it is too small to be retained by the nitrocellulose or because it lacks sufficient homology with the P_2 mtDNA used as the probe.

For similar reasons, only one product of digestion is detected after *SalI* cleavage of mtDNA and subsequent hybridization to detect the L rRNA transcript (lanes 3 and 4, Figure 5). In contrast, digestion of mtDNA with *SphI* and subsequent hybridization with the cytochrome b probe revealed three bands (lanes 5 and 6). The two smallest bands of 900 and 460 nucleotides result from DNA-RNA hybrids produced by cleavage of the cytochrome b region with *SphI*, whereas the largest hybridizable band of 3870 nucleotides results from self-renaturation of the *SphI* fragment of mtDNA.

Likewise a band of 1350 nucleotides in the cytochrome oxidase subunit 3 experiment results from self-renaturation of the *HindIII* fragment of mtDNA (lane 8), whereas the 670 nucleotide protected fragment comes from a DNA-RNA hybrid, which has subsequently been shown to arise from the 5′ end of the cytochrome oxidase subunit 3 region (results not illustrated), while the 320 nucleotide 3′-terminal fragment was not detected for reasons outlined above.

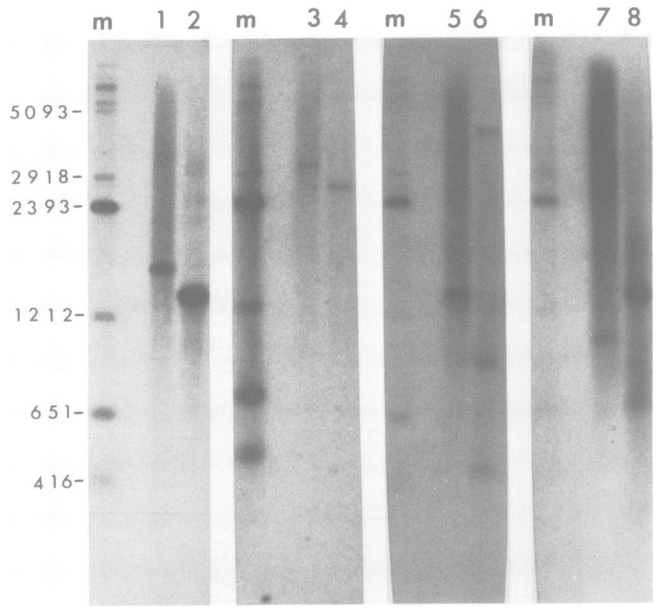

Fig. 5. Autoradiograms illustrating the mapping of transcripts by the S1 protection technique under alkaline gel conditions. Hybridization has been performed with: **lanes 1** and **2**, P2 (S rRNA); **3** and **4**, L rRNA; **5**, and **6**, DS400/N1, (cyt.b) and **7** and **8**, DS31, (cyt.ox.sub.3). Digestion of mtDNA prior to hybridization with mtRNA was performed with: **lane 2**, *Hind*III; **4**, *Sal*I; **6**, *Sph*I and **8**, *Hind*III whereas mtDNA was uncleaved in **lanes 1, 3, 5** and **7**. Each lane marked m contains ^{32}P end-labelled *Bam*I/*Bgl*II λ DNA fragments whose size in nucleotides is indicated on the left of the figure.

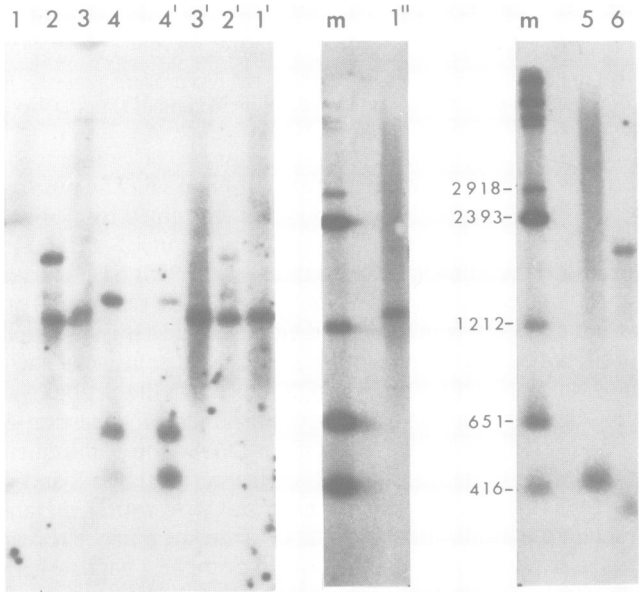

Fig. 6. Autoradiograms illustrating the mapping of transcripts by the S1 protection technique under alkaline gel conditions. Hybridization has been performed with: **lanes 1−4** and **1′−4′**, DS14, (ATPase subunit 6); **lane 1″**, G5 (*aap*1 gene) and **5** and **6**, DS400/A3, (ATPase subunit 9). Digestion of mtDNA prior to hybridization with mtRNA was performed with: **lane 2** and **2′**, *Mbo*I; **3** and **3′**, *Pvu*II; **4** and **4′**, *Hinf*I and **6**, *Mbo*I whereas mtDNA was uncleaved in **lanes 1, 1′, 1″** and **5**. Mitochondrial RNA from glucose-repressed cells has been used in **lanes 1−4** while **lanes 1′−4′** are from experiments using derepressed mtRNA. Lanes marked m contain ^{32}P end-labelled *Bam*I/*Bgl*II fragments of λ. These lanes have been removed from the first pattern because they are overexposed, nevertheless the calibration of this gel is identical to the others.

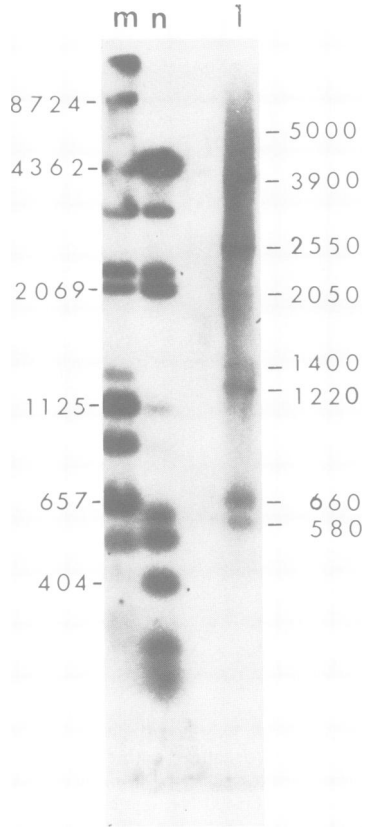

Fig. 7. Autoradiogram showing hybridization of DS6/A422 (cyt.ox.sub 1) to protected fragments following S1 digestion of hybrids formed between uncleaved mtDNA and mtRNA (**lane 1**). The size in nucleotides of abundant species are indicated on the right of the figure and have been obtained from the size of the end-labelled pBR322 fragments present in **lanes m** and **n**. The size of key fragments used to calibrate the autoradiogram are indicated on the left of the figure. Electrophoresis was carried out under neutral conditions.

The size and location of the ATPase subunit 6 and 9 transcripts are illustrated in Figure 6, which also shows the relative decrease in the amount of ATPase subunit 6 transcript in RNA prepared from glucose-repressed cells (lanes 1−4). The difference in hybridization intensity of the uncleaved samples (lanes 1 and 1′) is reflected in the relative levels of the fragments produced by *Hinf*I digestion of the DNA (lanes 4 and 4′). The smallest 470 nucleotide fragment in each case results from an RNA-DNA hybrid whereas the 610 nucleotide band, located entirely within the transcribed region, probably results from a mixture of double-stranded DNA and DNA-RNA hybrids. On the other hand, the largest hybridizable fragment results from self-renaturation of a 1460 nucleotide *Hinf*I fragment of mtDNA. Comparison of the intensities of these three bands in the two experiments clearly reveals that the level of the ATPase subunit 6 transcript is lower in the RNA obtained from glucose-repressed cells. Similar comparisons between glucose-repressed and derepressed RNA preparations were made for the other transcripts examined in this study (not illustrated) but no such differences were detected.

Localization of the ATPase subunit 6 transcript was obtained from prior digestion of DNA with *Mbo*I (lane 2), *Pvu*II (lane 3) and *Hinf*I (lane 4). The first two digestions revealed that the 1300 nucleotide transcript has arisen between the *Pvu*II site at map coordinate 7820 and the *Mbo*I site

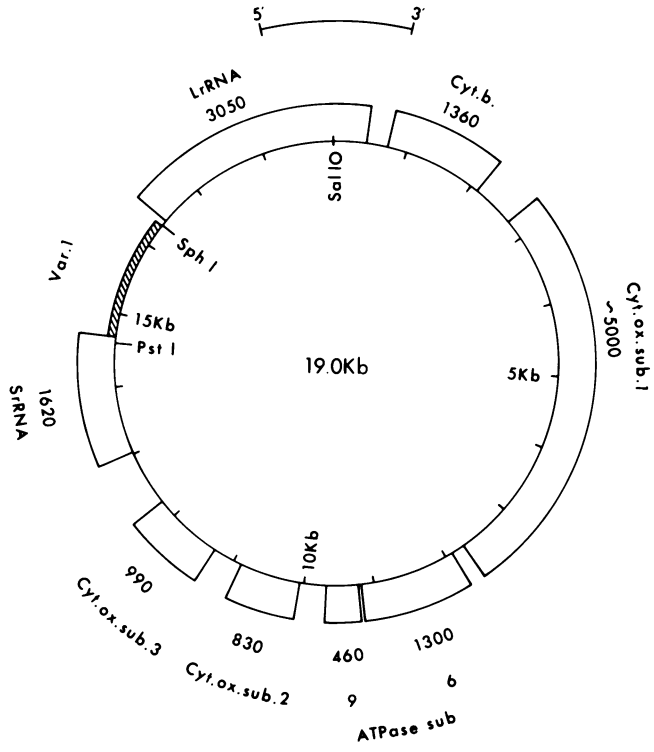

Fig. 8. Location and size of transcripts on the restriction endonuclease site map of *T. glabrata* mtDNA. The hybridizable region to the variant 1 probe is indicated by the hatched region. The divisions on the circle represent 1000 bp beginning with the *Sal*I site and the common orientation of all transcripts is indicated at the top of the map.

at 9570, while the *Hinf*I digestion results in a 470 nucleotide fragment from the 3′ end of the sequence. From other experiments the critical *Hinf*I site in the ATPase subunit 6 sequence has been positioned at coordinate 8720 (Table I) thereby enabling the transcript to be located on the map.

From data described above, the *aap1* hybridizable region is found in the same portion of the mitochondrial genome as the ATPase subunit 6 sequence. This juxtaposition of the two genic regions is complemented by the observation that the *aap1* probe also hybridizes to the ATPase subunit 6 transcript (lane 1″ Figure 6). On the other hand, a separate transcript is found for the ATPase subunit 9 region (lane 5). This transcript could be positioned by cleavage of the DNA with *Mbo*I which reduces the size of the protected fragment by 105 nucleotides (lane 6). The 1900 nucleotide band in lane 6 corresponds to the *Mbo*I fragment produced by renaturation of mtDNA.

In contrast to the results described in the preceding sections, whereby unique transcripts are found for each of the genic regions, multiple bands are observed on examination of cytochrome oxidase subunit 1 transcripts (Figure 7). The S1 nuclease-protected fragments vary in size from 580 to ~5000 nucleotides with the most prominent species being 3900 and 2550 nucleotides. Notable is the absence of the 1760 nucleotide band which is by far the most abundant species in direct examination of mtRNA by blotting and hybridization.

Using the results described above for the size and cleavage pattern of the different transcripts together with the coordinates of the key restriction endonuclease sites, we have constructed a map showing the location of the large genic transcripts for *T. glabrata* mtDNA (Figure 8). The position of the *var1* hybridizable region is also illustrated. Although we have examined mtRNA preparations from both glucose-repressed and derepressed conditions by direct hybridization and the S1 protection technique, we have not detected a transcript hybridizing to the *var1* probe.

Discussion

Examination of mtRNA from glucose-repressed or derepressed cells of *T. glabrata* by either transfer to nitrocellulose and hybridization with specific probes or with the S1 nuclease protection technique under both neutral and alkaline conditions has shown that unique transcripts are present for the S and L rRNA genes, cytochrome oxidase subunits 2 and 3, cytochrome b and the ATPase subunits 6 and 9. These results demonstrate that intervening sequences are absent from these genic regions.

On the other hand, for cytochrome oxidase subunit 1, there are a number of transcripts detected by the three procedures with the largest being ~5000 nucleotides. These results are in accord with the previously demonstrated mosaic nature of this gene and the size of the largest transcript is in general agreement with the length of the mtDNA region identified by hybridization to specific probes (Clark-Walker and Sriprakash, in preparation). The major species of cytochrome oxidase subunit 1 RNA observed on electrophoresis of glyoxal-denatured material is 1760 nucleotides. As this species is absent in S1 nuclease protection experiments we believe it to be the mature RNA after removal of the intervening sequences.

With respect to intervening sequences in this gene, it is interesting to note that one region has been found to hybridize to intron 4 of the cytochrome oxidase subunit 1 gene from *S. cerevisiae* mtDNA (Clark-Walker and Sriprakash, 1982). Recently it has been shown that a single base transition in this intron of the *S. cerevisiae* sequence can lead to the expression of an RNA maturase function (Dujardin *et al.*, 1982). This result raises the possibility that the intron 4 homologous region in the *T. glabrata* mitochondrial genome may code for an RNA maturase involved in processing the cytochrome oxidase subunit 1 primary transcript.

In contrast to the demonstration of transcripts for the genic regions mentioned above, we could not detect RNA hybridizing to the *var1* probe by any of the techniques even on long exposure of autoradiograms. One possible explanation for this result is that in *T. glabrata* the variant 1 protein is specified by a nuclear gene. An arrangement such as this would be comparable to the case of the ATPase subunit 9 gene in *Neurospora crassa* which has a nuclear location despite the occurrence of an intact sequence in the mtDNA (Van den Boogaart *et al.*, 1982).

As described above, discrete transcripts exist for five of the protein coding genes as well as the S and L rRNAs and co-transcripts (with one possible exception – see below) have not been detected even under conditions of glucose repression. This result suggests either that processing of co-transcripts is extremely rapid or that each genic region is separately transcribed. We favour the latter view as we can detect a number of mtRNAs which can be capped by guanylyl transferase catalysed addition of GMP (Sriprakash and Clark-Walker, 1983). This reaction is specific for di- or triphosphates at the 5′ terminus of RNA (Banerjee, 1980) and has been used to identify primary transcripts in mitochondria from *S. cerevisiae* (Levens *et al.*, 1981).

Table III. Location of transcripts on the restriction site map of *T. glabrata* mtDNA

Mitochondrial[a] gene	Restriction site and map coordinate	Size of protected fragments	Map position	
			5′ end	3′ end
Cyt b	*Sph*/1220	[3870]	760	2120
		900		
		460		
Cyt. ox. subunit 1	*Bcl*I/7670	b	~2700	~7700
ATPase subunit 6	*Hinf*I/8720	[1460]	7890	9190
		[610]		
		470		
ATPase subunit 9	*Mbo*I/9570	[1900]	9215	9675
		355		
Cyt. ox. subunit 2	*Eco*RV/10 450	425	10 050	10 880
		400		
Cyt. ox. sub. 3	*Hind*III/11 940	[1350]	11 270	12 260
		670		
S rRNA	*Hind*III/13 290	1360	13 030	14 650
L rRNA	*Sal*I/0	2600	16 390	450

[a]Mitochondrial genes are listed in a clockwise direction.
[b]Approximate 5′ and 3′ ends of the primary transcript have been ascertained from the observation that hybridization of the DS400/A422 probe occurs clockwise to the *Bcl*I site at 6760.
[]Denotes fragment derived from a DNA:DNA hybrid.

Table IV. Map distances separating mitochondrial transcripts

Mitochondrial genes	Separation (bp)[a]
Cyt b – cyt. ox. sub. 1	600[b]
Cyt. ox. sub. 1 – ATPase sub. 6	200[b]
ATPase sub. 6 – ATPase sub. 9	25
ATPase sub. 9 – cyt. ox. sub. 2	375
Cyt. ox. sub. 2 – cyt. ox. sub. 3	390
Cyt. ox. sub. 3 – S rRNA	770
S rRNA – L rRNA	1740
L rRNA – cyt b	310

[a]Estimates are based on the size of the transcripts determined with alkaline gels.
[b]The position of the 5000 nucleotide cytochrome oxidase subunit 1 transcript is inferred from the mtDNA hybridization mapping, therefore the gaps separating this transcript from flanking ones are only approximate.

It therefore appears that transcription of mtDNA in *T. glabrata* can occur from a number of separate promoters and it seems likely that each of the large genic regions could be bounded by sequences specifying initiation and termination of transcription. Thus, transcription in *T. glabrata* mitochondria could be analogous to the process producing separate transcripts for the S and L rRNAs from both the *S. cerevisiae* and *Kluveromyces lactis* mitochondrial genomes (Christianson *et al.*, 1982; Levens *et al.*, 1981; Osinga and Tabak, 1982; Osinga *et al.*, 1982).

If separate transcription is the case with *T. glabrata* mtDNA and for yeast mitochondrial genomes in general, we remain confronted with the need to explain the preferred sequence orientation in yeast mtDNA in the face of extensive sequence rearrangements between some of these molecules. We still consider it likely that preferred genic orientation is favoured by constraints of the transcription process rather than some limitation of the sequence rearrangement mechanism. Such constraints could be the avoidance of convergent transcription or the binding of DNA in a favoured orientation by a transcription and replication complex.

As mentioned above, there is one possible exception to the general observation that separate transcripts occur for each large genic sequence. This exception concerns the ATPase subunit 6 region because it has been found that both the DS14 and G5 probes hybridize to the same 1300 nucleotide transcript. As the G5 petite mtDNA contains the *aap1* gene (ATPase-associated protein) located 5′ to the *oli*2 gene in *S. cerevisiae* mtDNA (Macraedie *et al.*, 1982; Novitski *et al.*, 1983) the possibility exists that a similar gene is located 5′ proximal to the ATPase subunit 6 region in the *T. glabrata* mitochondrial genome. Sequence analysis of this region should reveal whether the single transcript contains separate or fused reading frames.

Sizes of transcripts have been measured by three methods but only one procedure is thought to give reliable values. Estimates of RNA size based on the migration of DNA/RNA hybrids against a double-stranded DNA marker are, with the exception of the ATPase subunit 9 transcript, larger than the values obtained by the alkaline gel procedure where single-stranded DNA is compared against a single-stranded DNA marker (Table III). The discrepancy in size of transcripts between the two procedures does not show a linear relationship to overall length of RNA, as the largest departures occur with the cytochrome oxidase subunits 2 and 3. This result suggests that sequence-related conformation affects gel migration and that the DNA/RNA hybrids in general show slower migration perhaps because they are more rigid than double-stranded DNA possessing the same number of nucleotide pairs.

Likewise, discrepancies exist between estimates based on electrophoresis of RNA after glyoxal denaturation and those from the alkaline gel procedure. The most striking difference occurs with the S and L rRNAs where the glyoxal method leads to a large underestimation which probably arises from incomplete denaturation of these species (Sriprakash and Clark-Walker, 1980).

Therefore, we have used transcript sizes obtained by estimating lengths of equivalent single-stranded DNA rather than estimates based on direct sizing of RNA or from DNA/RNA hybrids. Values obtained by this method are also more consistent with the endonuclease site map of mtDNA than those from the other two procedures.

Using the values listed in Table II and the position of key restriction sites (Table III), we have positioned each of the large genic transcripts on the map, with the exception of the primary transcript from cytochrome oxidase subunit 1. The approximate position of this transcript is inferred from the mtDNA mapping whereby hybridization of the DS 6/A422 probe is known to occur clockwise of the *Bcl*I site at map coordinate 7670 (Clark-Walker and Sriprakash, 1981).

The size of gaps between the large genic transcripts have been obtained from the map and are listed in Table IV. In general, the gaps are 300–400 bp with exceptions being the 25 bp between the two ATPase subunits, the 770 bp between cytochrome oxidase subunit 3 and S rRNA and the 1740 bp region between the S and L rRNAs. In the latter case, we infer from the hybridization data that the major portion of this region, perhaps >1 kbp by analogy with the size of the *var*1 gene in *S. cerevisiae* mtDNA (Hudspeth *et al.*, 1982), is occupied by the *var*1 sequence while tRNAs are known to hybridize 5′ proximal to the L rRNA sequence (Clark-

Walker *et al.*, 1980). Likewise, tRNA hybridizable regions occur between the cytochrome oxidase subunits 2 and 3, cytochrome oxidase subunit 3 and S rRNA and L rRNA and cytochrome b. This location of tRNA sequences raises a question as to whether any of these genes are co-transcribed with large genic sequences as has been shown for the cytochrome oxidase subunit 3 and valine tRNA sequences in *S. cerevisiae* mitochondria (Thalenfeld *et al.*, 1983). Further studies are needed to answer this question and also to resolve whether groups of tRNA genes are co-transcribed in *T. glabrata*.

The remaining gaps between transcripts are too small to code for large polypeptide genes, which leads us to conclude that the mitochondrial genome of *T. glabrata* does not specify sequences equivalent to the unidentified reading frames observed in mammalian or *Aspergillus* mtDNA (Anderson *et al.*, 1981; Davies *et al.*, 1982).

Finally, in comparing the RNAs prepared from mitochondria obtained from cells grown in high glucose with those from ethanol, we have observed a difference in the relative amounts of ATPase subunit 6 RNA compared with the levels of other transcripts. In glucose-repressed cells, where the respiratory activity is only a third of the derepressed level (Clark-Walker and McArthur, 1978), there is a decrease in the amount of ATPase subunit 6 RNA. One explanation for the low amount of this transcript, which is related to the hypothesis that each genic region contains separate initiation and termination signals, is that the promoter sequence for this gene differs from others. We are at present investigating this proposition by examining the sequences at the 5′-flanking region of a number of genes in *T. glabrata* mtDNA.

Materials and methods

Yeast strain, culture conditions and preparation of mtDNA

T. glabrata CBS138 was the same strain as used in previous studies (Clark-Walker and Sriprakash, 1981). Cells derepressed for mitochondrial function were obtained by growth on ethanol-YP medium (Oakley and Clark-Walker, 1978). Glucose repression was achieved by growing cells in 4% glucose-YP to an optical density of 3–4 at 640 nm. Under these conditions, respiratory activity of cells is only one quarter to one third the level found in derepressed cells (Clark-Walker and McArthur, 1978). Mitochondrial DNA was prepared by CsCl buoyant density centrifugation in the presence of bisbenzimide H33258 as described previously (Clark-Walker *et al.*, 1981).

Specific probes

Fragments of mtDNA from *S. cerevisiae* containing defined portions of the genome were obtained from petite mutants or from plasmids. Details of the petite mutants are listed in Table I of Clark-Walker and Sriprakash (1981). In addition, we have used *S. cerevisiae* petite mutants G5 containing the *aap*1 gene (ATPase-associated protein) (Novitski *et al.*, 1983), petite P2 containing the S rRNA sequence (Sor and Fukuhara, 1980) and pBR*var*1 (R.A. Butow, personal communication) containing an ~1.1-kb portion of the *var*1 sequence cloned into the *Cla*I site of pBR322. In place of petite DS302 containing the cytochrome oxidase subunit 2 gene, we used pSCM5 which contains the large *Sau*3A fragment of DS302 (2500 bp) having 704 bp of the coding sequence, cloned into the *Bam*I site of pBR322. Details of this preparation have been described elsewhere (Clark-Walker *et al.*, in press). Labelling of probes with [α-^{32}P]dATP using random primers has been previously described (Clark-Walker and Sriprakash, 1981).

Preparation of mtRNA

Cells from 4 l of culture were resuspended in 200 ml of 100 mM Tris pH 9.3, 100 mM β-mercaptoethanol and 20 mM EDTA and incubated at room temperature for 15 min. After washing twice in support buffer containing 0.5 M NaCl, 20 mM imidazole/HCl pH 6.5, 1 mM EDTA, the cells were resuspended in 50 ml of the support buffer with the addition of 4 ml of snail enzyme (Industrie Biologique Francaise). This mixture was incubated at 37°C until 80–90% of the cells were converted to protoplasts, as revealed by the decrease in optical density at 640 nm after a 1 in 1000 dilution in water. The protoplasts were washed twice with cold support buffer, resuspended in 50 ml of the same buffer, ruptured in the French Press at 2000 p.s.i. and a mitochondrial enriched fraction collected by differential centrifugation as described earlier (Clark-Walker, 1972).

To prepare RNA, this mitochondrial enriched fraction was resuspended by vigorous vortexing in 10 ml of 4 M guanidine thiocyanate, 50 mM Tris-HCl pH 7.6, 10 mM EDTA, 2% Sarkosyl and 140 mM β-mercaptoethanol. After centrifugation at 15 000 *g* for 10 min, the supernatant was layered over 3 ml of 5.7 M CsCl in 100 mM EDTA pH 7.0 and centrifuged in the Beckman SW41 rotor at 35 000 r.p.m. for 17 h. The supernatant was aspirated and the tube cut ~1 cm from the bottom. The RNA was dissolved in 200 μl of water then precipitated by addition of 2 volumes of ethanol. After centrifugation, the pellet was washed 3 times with 70% ethanol and finally the RNA was dissolved in 200 μl distilled water. RNA thus obtained was free of nuclease contamination. The mitochondrial RNA preparation from derepressed cells was relatively free of cytoplasmic RNA upon examination by electrophoresis whereas that from glucose-repressed cells was contaminated by ~50% cytoplasmic RNA. No attempt to remove this contamination was made as its presence did not affect the interpretation of the results.

RNA analysis

RNA (1–2 μg) was glyoxalated as described by McMaster and Carmichael (1977), then resolved by electrophoresis in 1.2% Agarose gel (20 x 20 x 0.3 cm) in 10 mM phosphate buffer for 2 h at 100 V. Staining with ethidium bromide and/or methylene blue (data not shown) clearly showed that the RNA isolated from glucose-repressed cells was contaminated with cytoplasmic RNA. For a direct comparison of the mtRNA species in the glucose-repressed and derepressed preparations, electrophoretically separated RNA was transferred to nitrocellulose (Thomas, 1980) and hybridized to purified *T. glabrata* mtDNA labelled with [^{32}P]dATP. To identify individual transcripts, RNA was blotted onto nitrocellulose and hybridized to specific probes as described earlier (Clark-Walker and Sriprakash, 1981). As size markers we used single-stranded DNA obtained by heat denaturation of the plasmid pBR322 DNA after digestion with: (a) *Bam*HI + *Pst*I; (b) *Pvu*II + *Eco*RI, and (c) *Hin*fI; the sizes of these fragments were obtained from the sequence analysis of this plasmid (Sutcliffe, 1978).

Mapping of transcripts with S1 nuclease

Estimation of both size and location of transcripts was made by the S1 nuclease protection technique (Berk and Sharp, 1978) with some modifications. Approximately 0.05 μg DNA in 20 μl was digested with the appropriate restriction endonuclease and the enzyme inactivated by heating at 65°C for 5 min. The DNA was precipitated by ethanol, collected by centrifugation and dried in a vacuum desiccator. An undigested sample of DNA was treated by the same procedure and carried in parallel through the following steps. The dried DNA was dissolved in 25 μl H_2O and transferred to a micro-tube of ~100 μl capacity. A 10- to 20-fold excess of RNA was added followed by 4 μl of hybridization buffer containing 100 mM Hepes pH 7.0, 2.5 M NaCl, 10 mM EDTA and 0.05% SDS and the final volume brought to 40 μl by the addition of water. The tube was capped and the sample denatured at 95°C for 3 min then incubated at 65°C for 150 min. After hybridization, the sample was transferred to an Eppendorf centrifuge tube containing 310 μl H_2O, 40 μl of S1 digestion buffer containing 1.5 M NaCl, 0.5 M sodium acetate pH 4.6, 30 mM $ZnSO_4$ and 0.1% SDS and 10 μl S1 nuclease (Boehringer Mannheim, ~10^4 units). The S1 nuclease digestion was performed at 37°C for 30 min and terminated by the addition of 1 ml chilled ethanol. After storage at −20°C for 30 min, the precipitate was recovered by centrifugation, washed with 100 μl of 70% ethanol, followed by a second wash with absolute alcohol then taken to dryness in a vacuum desiccator. The dried sample was dissolved in 15 μl of 1 mM Tris/HCl pH 7.0, 0.1 mM EDTA for neutral gel electrophoresis or 20 μl of alkali gel loading solution containing 50 mM NaOH, 1 mM EDTA, 2% w/v Ficoll and 0.02% bromocresol green. The electrolyte for alkaline gel electrophoresis contains 30 mM NaOH and 1 mM EDTA. Labelled marker DNA samples were run in parallel on the gel and, after electrophoresis and blotting to nitrocellulose, the S1 nuclease-resistant fragments were revealed by hybridization to the appropriate probe as previously described (Clark-Walker and Sriprakash, 1981).

Size markers

Calibration of gels was performed using DNA fragments of known size obtained by digestion of pBR322 as described previously or by *Bam*I/*Bgl*II digestion of λ DNA. Fragments were observed either by hybridization to uniformly labelled DNA or by end labelling of the digested DNA with [^{32}P]dATP catalysed by the large fragment of DNA polymerase. Sizes of the *Bam*I/*Bgl*II fragments of λ DNA are 7770, 6529, 5545, 5093, 2918, 2393, 1212, 651 and 416 bp.

Other methods

Restriction endonuclease digestion, electrophoresis, transfer of DNA to nitrocellulose, hybridization and autoradiography have been described

previously (Clark-Walker *et al.*, 1980, Clark-Walker and Sriprakash, 1981).

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