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RNA synthesis in isolated yeast mitochondria

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ABSTRACT

Isolated yeast mitochondria incorporate added UTP into RNA. Amongst the products formed are the two rRNAs, 4S RNA and several components presumed to be mRNAs. In ω^+ strains (containing an intervening sequence in the 21S rRNA gene) besides mature 21S rRNA a transcript could be detected still containing nucleotide sequences transcribed from this intervening sequence. In ω^- strains (not containing this intervening sequence) also a longer form of the 21S rRNA could be observed. These results suggest that isolated yeast mitochondria are capable of carrying out RNA synthesis and processing, including splicing.

INTRODUCTION

Yeast mitochondria contain a 25 μm circular DNA (1). The expression of the few genes present on it, together with that of much greater number of nuclear genes is required for the biosynthesis of a functional mitochondrial inner membrane. After the construction of detailed restriction maps of the mtDNA of several Saccharomyces species (2) the genes for ribosomal RNAs (3) and 4S RNA (4) have been localised on these maps by means of hybridisation of the labelled RNAs to restriction fragments. More recently we have constructed a transcription map for more than 25 different minor species of yeast mtRNA. These transcripts could all be assigned to genetic loci known to be present on yeast mtDNA (5,6). This, coupled with the results of in vitro translation experiments (7,8) led to the suggestion that these transcripts represent mRNAs and intermediates in their synthesis (6, cf. also 9).

A striking finding of this study was the detection of several overlapping transcripts of many loci suggesting that RNA processing forms an intrinsic part of yeast mitochondrial RNA metabolism. Direct electron-microscopic evidence that some yeast mitochondrial RNAs are spliced was obtained by Bos et al. (10) for the large rRNA and by Grivell et al. (8) for the 18S transcript of the gene coding for cytochrome b apoprotein. A further

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finding of importance in the understanding yeast mtRNA metabolism is the detection of covalently closed circular RNAs most likely originating from excision events in the splicing process (11). Subsequent detailed studies of the splicing processes in yeast mitochondria have revealed an unexpectedly complex course of splicing events and the possible involvement of translation of introns in the regulation of mtRNA processing (12).

Encouraged by our previous finding that isolated mitochondria are capable of carrying out faithful translation of mtRNA (13,14) we have investigated the capacity of isolated yeast mitochondria for RNA synthesis.

METHODS

The yeast strains Saccharomyces carlsbergensis NCYC-74 and Saccharomyces cerevisiae KL14-4A were used in this study. Mitochondria were prepared from early logarithmic cultures as described earlier (12) under sterile conditions. Isolated mitochondria were incubated in a mixture (usually 0.5 ml) containing 20 mM Tris-HCl (pH 6.7), 150 mM KCl, 10 mM K-phosphate, 10 mM $MgCl_2$, 5 mM α-oxoglutarate, 3 mg/ml Bovine serum albumine, 5 mM phosphoenolpyruvate, 15 µg pyruvate kinase, 2.5 mM ATP, 0.3 mM GTP, 0.3 mM CTP, 0.1 mM labelled UTP (either [α-^{32}P]UTP or U[^{3}H]UTP), 60 mM mannitol and 1-4 mg mitochondrial protein. The reaction temperature was 30°. The reaction was terminated by the addition of 1 volume 10% TCA or, in case RNA was isolated, by the addition of 0.1 volume 20% sodium dodecylsulphate. mtRNA was isolated and fractionated on 1.8% agarose gels (6).

Digestion of mtDNA with restriction enzymes was according to Sanders et al. (2). Electrophoresis and transfer of the fragments to nitrocellulose filters, hybridisation and fluorography were done as described by van Ommen et al. (6). Tests for determining the extent of symmetrical transcription were carried out as described by Tabak et al. (15).

RESULTS

In the initial experiments designed to find optimal conditions for RNA synthesis in isolated yeast mitochondria we have used the amount of acid-precipitable counts as a measure for the incorporation of labelled UTP into RNA. Mitochondria suspended in a reaction medium, similar to that used for measuring protein synthesis (13) but supplemented with the substrates for RNA synthesis showed a high rate of UTP incorporation. This incorporation was linear with respect to the amount of mt protein added (up to 6 mg/ml) and linear with time up to 90 min (not shown). As is shown in Fig. 1 the

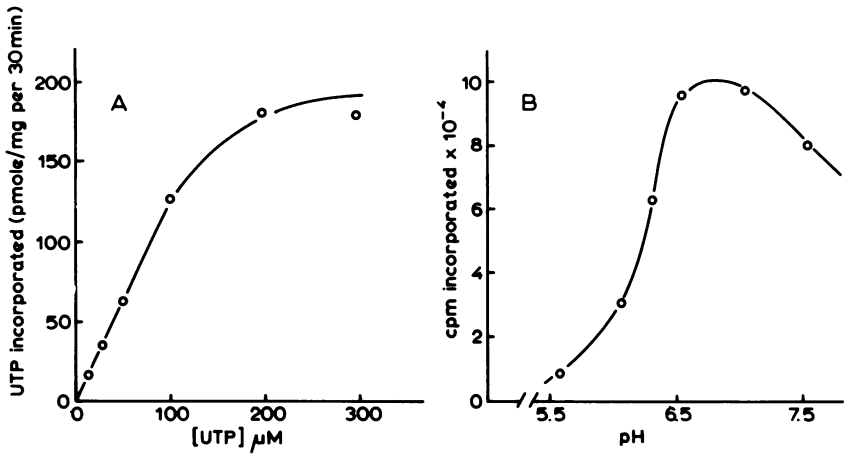

Fig. 1. Determination of the apparent Km for UTP (A) and the optimal pH (B). Experimental conditions were as described in Methods, using 5 mg mt protein except that the UTP concentration (A) or the pH (B) were varied as indicated.

apparent Km for UTP is approximately 75 μM and at the optimal pH of 6.7 the Vmax is approximately 200 pmoles UTP per mg protein per 30 min.

Table I summarizes the effects of several inhibitors on the rate of incorporation of UTP. Ethidium bromide and acriflavin inhibit the UTP incorporation already at low concentrations. This indicates that we are measuring a true mitochondrial activity and rules out bacterial contamination.

Table I. Effect of various inhibitors on mitochondrial RNA synthesis. RNA synthesis was measured as described in Methods for 20 min using 1.4 mg/ml (A and B) or 4.4 mg/ml (C) mitochondrial protein per incubation.

Addition or omission		cpm incorporated
A) None		33 000
Ethidium bromide	(4 μg/ml)	3 000
Acriflavin	(4 μg/ml)	2 800
Chloramphenicol	(20 μg/ml)	32 000
Erythromycin	(20 μg/ml)	34 000
Cycloheximide	(50 μg/ml)	35 000
B) None		51 000
Lomofungin	(10 μg/ml)	52 000
Thiolutin	(10 μg/ml)	53 000
C) None		67 690
- Mg^{++}		30 600
+ RNAase	(1 U)	69 300
+ Rifampicin	(20 μg/ml)	67 700

The RNAase resistance of the UTP incorporation shows that this process takes place in a compartment inaccessible to RNAase, probably intact mitochondria. Omission of Mg^{++} leads to a 50% reduction in the rate of the reaction. This is most likely due to the inactivation of the nucleoside triphosphate regenerating system. Inhibitors of nuclear transcription like thiolutin and lomofungin have no effect on the RNA synthesis in isolated mitochondria. The relative insensitivity of the transcription process towards Actinomycin D is probably caused by the inability of this substance to permeate the mitochondrial inner membrane. The absence of any inhibitory effect of rifampicin is caused by the inherent insensitivity of mitochondrial transcription to this inhibitor (16). Finally, chloramphenicol, an efficient inhibitor of mitochondrial translation is without effect on mitochondrial transcription suggesting no tight coupling between these two processes.

Table II shows that the labelled UTP is incorporated into material which can be specifically degraded by RNAase. Moreover it shows that under the conditions used, even during a 5 min pulse no appreciable amount of symmetrical transcription occurs. This makes total symmetrical transcription unlikely and suggests mode of transcription different from that proposed for mitochondria from HeLa cells (16). The incorporation is partly into RNA with a rapid turnover since approximately 15% of the incorporated label can chased out (Fig. 3).

For identification of the transcription products we have separated isolated mtRNA on agarose gels after labelling for 20 min. An autoradiogram of such a gel shown in Fig. 4 reveals that UTP has been incorporated into several species. Besides the intense labelling of 21S and 15S rRNA and 4S RNA

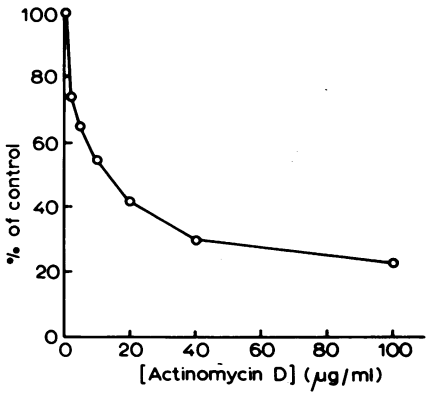

Fig. 2. Effect of Actinomycin D on the incorporation of UTP. Experimental conditions were as described in Methods. Actinomycin D concentrations as indicated in the figure.

Table II. Amount of selfcomplementary RNA formed during various incubation conditions. After RNA synthesis during the time indicated mtRNA was isolated as described in Methods. The amount of selfcomplementary RNA was determined as described by Tabak et al. (15). The conditions of the chase were as described in the legend to Fig. 3.

Labelling conditions	% selfcomplementary RNA
1. 5 min pulse	3
2. 5 min pulse plus 20 min chase	5
3. 30 min pulse	4
4. 30 min pulse plus 20 min chase	2

The material labelled under conditions 1, 2, 3 and 4 was degradable for 97.5, 95, 98 and 97% by RNAase respectively.

several other bands can be seen notably at 24S, around 18-19S and around 11-12S. The label incorporated into material longer than 24S disappears upon chasing and thus these species have a rapid turnover. This observation is consistent with the earlier proposed function of these long RNA species as precursors to various mature mtRNAs (6).

In order to characterize the transcriptional origin of the mtRNA synthesized in isolated mitochondria, we have hybridized total in vitro labelled RNA to separated restriction fragments of mtDNA. Fig. 5 shows that transcription is occurring from all regions of mtDNA. In this type of experiment internal competition of the hybridisation of newly synthesized is inversely proportional to the amount of preexisting (i.e.) stable transcripts. Thus transcribed regions with a fast turnover are preferentially detected e.g. a transcript of fragment 5 (see for map ref. 6). It may be pointed out that amongst the minor stable RNAs no transcript of this region

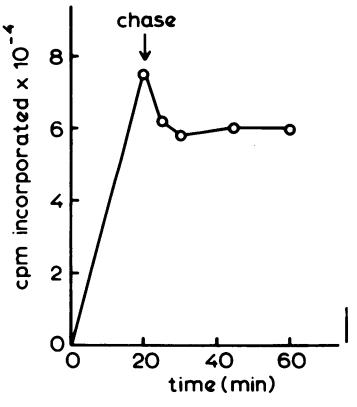

Fig. 3. Effect of a chase on the incorporation of labelled UTP. Experimental conditions were as described in Methods using 2 mg mt protein. After 20 min 5 mM unlabelled UTP was added.

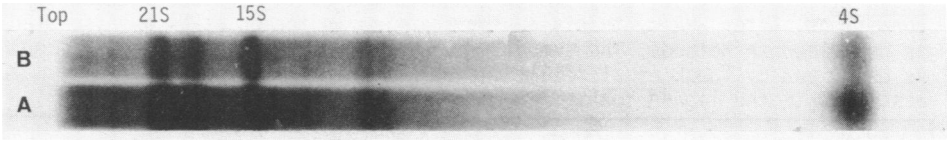

Fig. 4. Autoradiogram of mtRNA, isolated from mitochondria labelled with [α-^{32}P]UTP, separated on a 1.2% agarose gel. Experimental details as described in Methods. A = 20 min pulse; B = 20 min pulse + chase.

could be detected. These results could indicate that yeast mtDNA is much more completely transcribed than was detected thusfar (6,17). On the other hand a limited degree of erroneous transcription leading to unstable products that are rapidly degraded cannot be excluded.

Bos et al. (18) have shown that in ω^+ strains, in which the gene for the large rRNA contains an insertion of approximately 1100 bp, a 24S transcript is present which most likely represents a precursor form of the 21S rRNA still containing the transcribed intervening sequence. Fig. 6 shows that such a transcript is synthesized in isolated mitochondria of strain KL14-4A. In this experiment (cf. Ref. 5 and 6) RNA, labelled for 20 min, was isolated and fractionated on an agarose gel analogous to the experiment described in fig. 4. The gel was sliced in 1 mm fractions and the RNA was eluted from the first 13 slices from the top. Each fraction was hybridized to restriction fragment DD_5, containing only base sequences representative for intervening sequence (see Ref. 18). It is clear that in fraction 8 (corresponding to approximately 24S) a positive hybridization is occurring

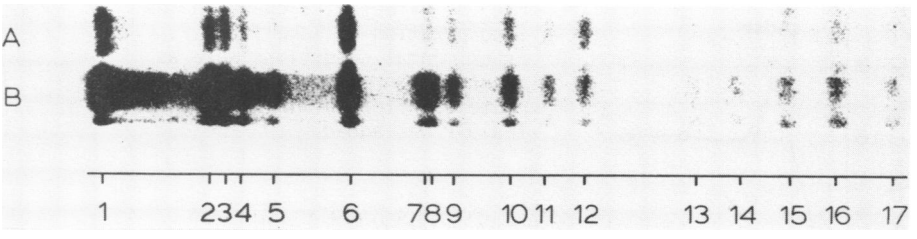

Fig. 5. Transcriptional origin of RNA, isolated from mitochondria with [α-^{32}P]UTP. S. carlsbergensis mtDNA was digested with Hind II+III plus EcoRI. Further experimental details are described in Methods. The resulting fragments are numbered according to decreasing size. (A) RNA pulse labelled for 20 min; (B) RNA pulse labelled for 20 min and chased for 20 min with 5 mM unlabelled UTP.

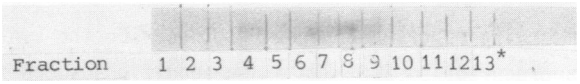

Fig. 6. Hybridization analysis of transcripts from the 21S rRNA gene in strain KL14-4A. See text for experimental details. The position of the 21S rRNA is indicated by the asterisk.

indicating the presence of longer form of 21S rRNA still containing the transcribed intervening sequence. From the fact that also a substantial amount of radioactivity is incorporated into mature 21S rRNA we conclude that isolated mitochondria are capable of splicing. We have repeated this experiment using mitochondria from S. carlsbergensis, an ω^- strain. As is evident from the result presented in fig. 7 in this case a longer form of the 21S rRNA is synthesized too. Since in this strain no intervening sequence is present in the gene for 21S rRNA, the mature 21S rRNA in S. carlsbergensis appears to be formed by processing of a precursor with extensions at the 5' and/or 3' end. The results are compatible with those obtained by Merten et al. (19).

DISCUSSION

In this paper we have demonstrated that isolated yeast mitochondria are capable of incorporating labelled UTP into RNA and that under the reaction conditions used proper processing including splicing of the transcript of the 21S rRNA gene occurs. These events take place inside the mitochondria as is evident from the insensitivity of the process towards added RNAase and the specific inhibition by known inhibitors of mitochondrial transcription. Although the experiments reported here only address to the processing events occurring after transcription of the 21S rRNA gene, preliminary experiments indicate that also the putative 18S mRNA for cytochrome b apoprotein is synthesized. Since this transcript is formed after extensive splicing (6) it is likely that also in this case correct processing of the initial transcript

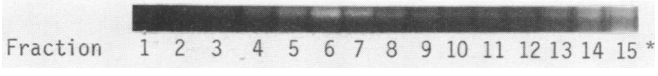

Fig. 7. Hybridization analysis of transcripts from the 21S rRNA gene in strain NCYC74. The experiment was carried out as described in the legend of fig. 6. The hybridization of the eluted RNA fractions to fragment TT_8 (Ref. 2) is shown. The position of the fraction containing 21S rRNA is indicated by the asterisk.

is occurring.

In the accompanying paper, Boerner et al. describe the synthesis and accumulation of a precursor to 21S rRNA under conditions suboptimal for transcription. The fact that we have not observed the accumulation of such a precursor is most likely due to the fact that under conditions used by us (high [ATP]) extensive processing occurs. Moreover, these investigators were able to detect a precursor to the 15S rRNA in a denaturing gel system which has superior resolution, especially in cases where the length differences are small such as with the precursor to 15S rRNA (100 bases) (see Ref. 20). These data reported by us together with the finding by Boerner et al. that the degree of processing of a precursor to 21S rRNA can be manipulated by the reaction conditions make the study of the details of mitochondrial RNA metabolism feasible in a simple experimental system.

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