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Mitochondrial RNA metabolism during mitochondriogenesis in yeast

NITHYAKALYANI RAGHAVAN and J. JAYARAMAN*
Department of Biochemistry, School of Biological Sciences, Madurai Kamaraj University, Madurai 625 021, India

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Abstract. Mitochondrial transcription has been studied as a function of mitochondriogenesis in yeast cells. Two systems have been used: synchronously growing cells and cells subjected to glucose repression followed by derepression. Maximal RNA synthesis has been found in the S phase of the cell cycle and during the 'repressed' phase in asynchronous cells. Activities of RNA polymerase, poly A polymerase and incorporation of [^{32}P]-into RNA *in vitro* are maximal at the same period. Gel analysis reveals the presence of some high molecular weight RNA species which are likely to be precursors. When chase experiments are carried out in the presence of excess glucose, the high molecular weight species remain unaffected, suggesting that RNA processing may be an important site of action of glucose repression.

Keywords. Mitochondrial RNA; mitochondrial RNA polymerase; glucose repression-de-repression; synchronous cultures.

Introduction

Evidence that during mitochondriogenesis in yeast, the assembly of the electron transport chain components occurs in a sequential order rather than in a concerted fashion, have been obtained by the following observations reported from this laboratory, (i) By the judicious use of the differentially acting antibiotics, cycloheximide (CHI) and chloramphenicol (CAP), it was shown that the mitochondrially and cytosolically synthesised protein components can be accumulated independent of each other and then reconstituted in vitro (Chandrasekaran et al., 1980). (ii) A second addition of glucose at the onset of derepression of enzyme activities keeps the mitochondrial functions in the repressed state, but the levels of cytosolically synthesised mitochondrial components remain at the same level or even enhanced (Chandrasekaran et al., 1978). (iii) Using immunoprecipitation techniques, it has been shown that the mitochondrially synthesised subunits of cytochrome C oxidase follow a partial order (Chandrasekaran et al., 1980). (iv) That the two protein synthesizing systems do not act in concert was also shown in synchronous cultures in which the cytosolically synthesised proteins F1-ATPase and 3 subunits of cytochrome oxidase accumulated in the cytosol during the S-phase and were sequentially integrated into the mitochondrial membrane at the G2 phase (Somasundaram and Jayaraman, 1981a, b). In this context it was of relevance to investigate the mitochondrial transcriptional

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*To whom correspondence should be addressed.
Abbreviations used: CHI, Cycloheximide; CAP, chloramphenicol; TCA, tricarboxylic acid.

events which should occur prior to mitochondriogenesis, as defined by the expression of the electron transport enzyme activities.

Mitochondria synthesise all their RNA and no import occurs into the organelle (Borst and Grivell, 1978). Mitochondrial transcription per se is fairly well-documented (Christianson et al., 1982; Kelly and Phillips, 1983). Neupert and his group (Neupert and Rucker, 1976; Neupert, 1977) have proposed a nuclear trigger to initiate mitochondriogenesis and molecules like cAMP, hemin and GDP have been variously proposed as likely candidates (Chandrakumar and Padmanabhan, 1980; Ohashi and Schatz, 1980; Somasundaram et al., 1980). Initiation of transcription should be the first event in mitochondriogenesis and consequently, establishing the stage of growth of yeast cells at which this process occurs, would be essential to locate and identify this 'trigger molecule'. It was with this approach we have studied the mitochondrial RNA metabolism in yeast cells during mitochondriogenesis.

Materials and methods

Culture and growth conditions

Saccharomyces cerevisiae NCIM 3095, a diploid strain, obtained from the National Collection of Industrial Microorganisms, Poona, was used in these studies and stock cultures were maintained in nutrient agar slants. Cells were grown at 28±1°C for 10–12 h with aeration in a liquid medium containing glucose (1 %), yeast extract (0·4 %), KH_2PO_4 (0·3%), $MgSO_4 \cdot 7H_2O$ (0·05%), $CaCl_2$ (0·04%) and $(NH_4)_2SO_4$ (0·04%). The harvested cells were used in experiments involving synchronous cultures or glucose repression/derepression system.

Cells were synchronously grown following the method of Mitchison and Vincent (1965) and the behaviour of these cultures were same as described in earlier communications (Somasundaram and Jayaraman, 1981 a,b). Good synchrony was obtained upto 3–4 generations. In our studies we have used the second generation cells.

Conditions of glucose repression/derepression have been described earlier in detail (Jayaraman *et al*., 1966). Briefly, the harvested cells were resuspended in the same medium at a concentration of about 4 mg wet wt cells/ml medium and allowed to grow at 28°C with shaking. Under these conditions, during the first $2\frac{1}{2}$ h (designated as repression phase), the respiratory rate as well as the levels of several mitochondrial enzyme activities and components decrease 4–5 fold, whereas the cells grow exponentially. After $2\frac{1}{2}$ h, the cells enter the diauxic growth (glucose in the medium having been exhausted) and exhibit a rapid increase in various mitochondrial parameters (designated derepression phase). Having established in preliminary studies that cell number and turbidity at 600 nm were linear, for routine experiments, turbidity was followed.

Respiratory activity was monitored using Clark type oxygen electrode (Model YSI 4004) (Borst and Grivell, 1978). Protein was assayed by the method of Lowry *et al.* (1951). RNA was estimated by measuring the absorbance at 260 nm and 280 nm (1 A_{260nm} = 40μg RNA) (Jayaraman, 1981). Mitochondrial RNA polymerase was prepared according to the method of Scragg (1974) by solubilisation from isolated mitochondria and was assayed following the method of Kuntzel and Schafer (1971).

Poly A polymerase was partially purified by the method of Rose *et al.* (1976) and assayed following the method of Jeyaraj *et al.* (1982).

Isolation of mitochondrial RNA

Mitochondria were prepared according to the method of Jayaraman *et al*. (1966) in an isolation buffer containing 0·25 M sucrose, 10 mM Tris-HCl, pH 7·4, 3 mM EDTA, 4 μg/ml heparin and 0·1 % diethyl pyrocarbonate. The mitochondria were lysed in a solution containing 0·1 M NaCl, 0·1 M Tris-HCl, pH 9·0,1 mM EDTA, 0·5 % sodium dodecyl sulphate, 4 µg/ml heparin and 0·1 % diethyl pyrocarbonate. RNA was isolated after deproteinisation with phenol/chloroform/isoamyl alcohol (50/50/1), by precipitation with ethanol from the aqueous phase. The RNA was washed with 2 M LiCl and stored in 10 mM Tris-HCl, pH 8·0 (containing 1 mM EDTA) at –20°C (Suzuki and Brown, 1972).

Radiolabelling experiments

In synchronous cultures: Carrier free [^{32}P]-phosphate and [^{14}C]-chlorella hydrolysate (sp. act. 20 mCi/matomC) as the case may be, were added to the cultures at the beginning of each phase, G_1, S, G_2 and M phases, respectively, and cells harvested at the end of the phase. Earlier experiments revealed that each phase was of 30 min duration (Somasundaram and Jayaraman, 1981a).

Under glucose repression/derepression conditions

In these experiments, the cells were exposed to [^{32}P] -phosphate for 1 h duration at various time intervals *viz*. 0–1 h, 1–2 h and so on.

In chase experiments, cells were labelled from 1-2 h and the radioactive label was chased by the addition of 0·01 M cold phosphate for a further period of 1 h. During the chase period additions were made as described under 'results'.

In vitro labelling

Synthesis of RNA by isolated mitochondria was followed according to the method of Newman and Martin (1982) with minor modifications.

In all cases, the RNA was precipitated with cold 10% trichloroacetic acid (TCA), followed by filtration through glass fibre filters (GF/A Whatman, 2·4 cm) and extensive washing with 5 % TCA and ethanol. The filters were dried and radioactivity determined using a toluene based scintillation fluid (Jeyaraj *et al*., 1982).

Gel electrophoresis of RNA

Electrophoresis of RNAs was carried out in 2 % acrylamide, 0·5 % agarose composite gels (Peacock and Dingman, 1968). Ethidium bromide stained gels were photographed under a short range ultraviolet source of 254 nm. The gels were dried, sliced into 3 mm bits and counted after hydrolysis (Jeyaraj *et al*., 1982). The S values were standardized by using yeast cytosolic rRNA and tRNA markers.

Results

RNA labelling in synchronous cultures

Figure 1 shows the pattern of synchrony, oxygen uptake, [^{14}C]-amino acid incorporation into mitochondrial proteins, and [^{32}P] -incorporation into mitochondrial RNA. It is clearly seen that mitochondrial RNA synthesis is most active in the S-phase, (table 1) whereas incorporation of amino acids into mitochondrial proteins and respiratory activities were maximal at G_2 phase (Somasundaram and Jayaraman, 1981a, b).

Analysis of labelled RNA species

The RNA samples labelled at various phases were analysed in agarose-acrylamide composite gels (figure 2). At G_1 phase, there is synthesis of very few species of RNA and the label is predominantly observed in a high molecular weight species (about 45S) in addition to a species slightly less than 14S. Although 14S rRNA is labelled, interestingly, 21S ribosomal RNA and 4S tRNAs are not labelled. The profile changes drastically during the S phase. In addition to the ribosomal RNAs and tRNAs, labeling is observed in the 'messenger region'. Further, in the high molecular weight region (35S-45S), 3 prominent peaks are seen. At the G_2 phase, the high molecular weight species have almost disappeared and labelling was prominent in the 'messenger region'. In the M phase, there was practically no labelling of any species.

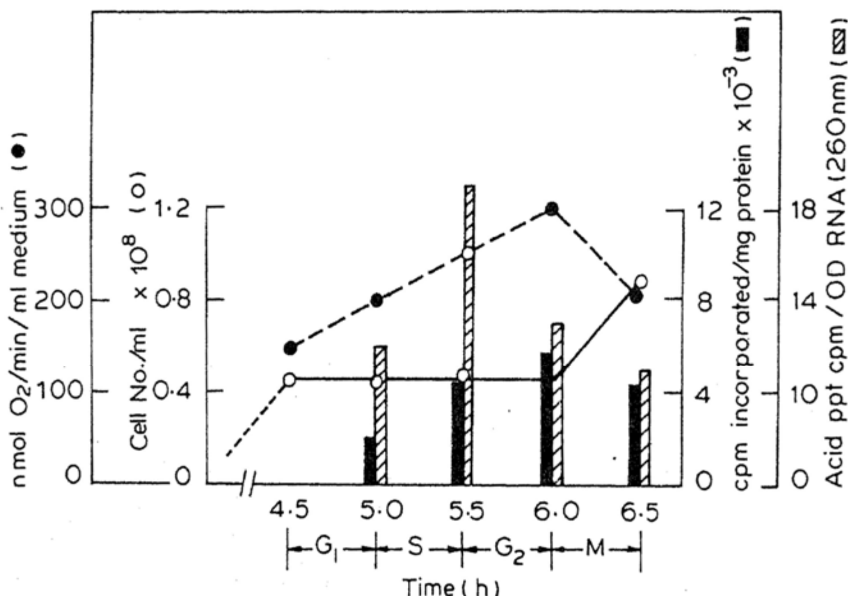

Figure 1. Pattern of synchrony, oxygen uptake, mitochondrial protein and RNA synthesis in synchronous cultures of yeast.

Synchronous cultures of yeast grown to two cell cycle divisions were labelled with either [^{14}C]-chlorella hydrolysate (2 μCi/ml medium) or [^{32}P]-phosphate (1 mCi/100 ml medium) Oxygen uptake,(●) cell growth (O), incorporation of [^{14}C]-chlorella hydrolysate into mitochondrial proteins (▨) and [^{32}P]-phosphate into RNA were monitored (■)at intervals of 30 min.

Table 1. In vivo labelling of RNA with [^{32}P]-phosphate in synchronous cultures of yeast.

Labelled RNA from synchronous cultures of yeast grown in the presence of [^{32}P]-phospate (1 mCi/100 ml medium) was extracted and the acid recipitable radioactivity determined. During nail dixic acid treatment, the antibiotic (50 μg/ml) was added alone with the label

Stages of growth	Acid precipitable radioactivity (cpm/mg RNA)
G_1	$3{\cdot}0 \times 10^5$
S	$4{\cdot}7 \times 10^5$
G_2	$3{\cdot}4 \times 10^5$
M	$2{\cdot}7 \times 10^5$

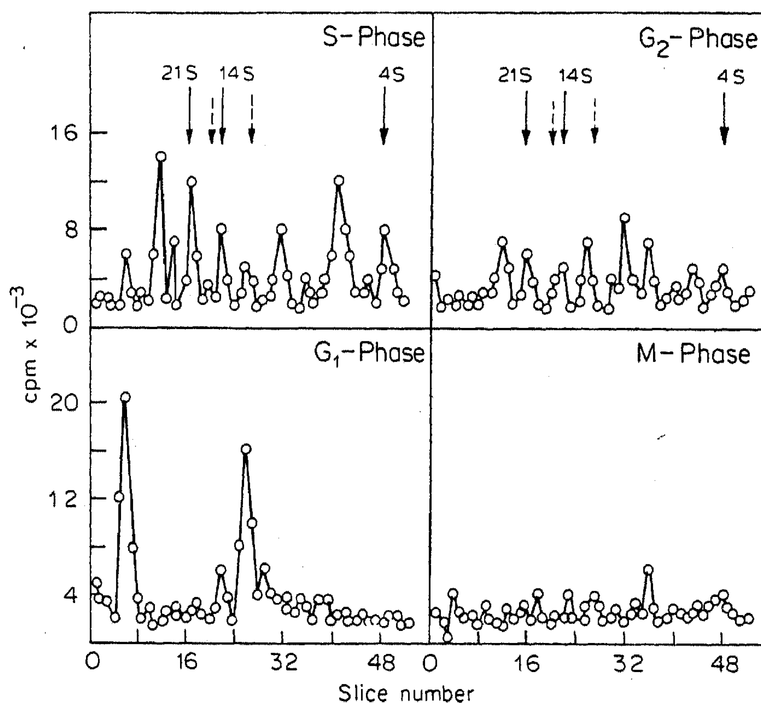

Figure 2. Analysis of labelled RNA species in synchronous cultures of yeast.

Labelled mitochondrial RNA ($1A_{260\,nm}$/slot) was analysed on a 2% acrylamide–0·5% agarose composite gel. At the end of the run the gel was sliced into 0·3 cm bits and processed for measuring radioactivity.

Studies using glucose repression/derepression conditions

Since working with synchronous cultures on a large scale was difficulty and also since the glucose repression/derepression system simulated the synchronous growth conditions with respect to mitochondriogenesis (Jayaraman *et al.,* 1975), we resorted to the latter system for further studies. The various changes that take place in this system as a function of time have been described in earlier communications (Jayaraman *et al.,* 1966, 1975).

[^{32}P]-Labelling of mitochondrial RNA at different time intervals was studied (table 2). Maximum incorporation into RNA was observed between 1 and 2 h. RNA species labelled at different times were analysed in the gel (figure 3). The following features were noticed.

(i) During the first 1 h, a few species mainly in the region between 4S and 14S are labelled. The 14S RNA is labelled while 21 S and 4S RNAs are not. This resembles the situation obtained in the G_1 phase cells, except that the high molecular weight RNA is not seen.
(ii) Between 1 and 2 h, there is a burst of transcriptional activity. The interesting point to note is the presence of atleast 4 radioactive peaks above 21S, ranging between 35S–45S. This resembles the pattern obtained in the S phase of synchronous cultures,
(iii) Between 2 and 3 h, the intensity of labelling of all species has decreased. Only one peak in the region above 21S was observed,
(iv) Between 3 and 5 h, there is very little synthesis of RNA.

Effect of second addition of glucose

In the next series of experiments, labelling was carried out in a similar way as shown in table 3, but at each time point a second addition of glucose was made to bring the final concentration to 1 %. There was more than 50 % inhibition in the period between 1–2 h, showing that glucose concentration is a factor in mitochondrial transcription (results not given).

Table 2. Changes in the total mitochondrial RNA and poly (A) RNA levels during repression/derepression.

Labelled RNA from different time hour mitochondria were extracted and the acid precipitable radioactivity and the radioactivity bound to poly(U) Millipore filters determined (see methods).

Time of growth	RNA yield A_{260}/50 ml culture	Acid precipitable cpm/ incorporated/mg RNA	cpm bound to poly (U) filter/mg RNA	% poly (U) RNA
10 min	1·12	$0·5 \times 10^5$	$0·02 \times 10^5$	3·7
1 h	1·80	$4·1 \times 10^5$	$0·21 \times 10^5$	5·8
2 h	2·40	$12·6 \times 10^5$	$1·80 \times 10^5$	14·9
3 h	3·52	$5·4 \times 10^5$	$0·57 \times 10^5$	10·6
4 h	3·60	$1·7 \times 10^5$	$0·13 \times 10^5$	7·5
5 h	3·72	$0·9 \times 10^5$	$0·03 \times 10^5$	3·8

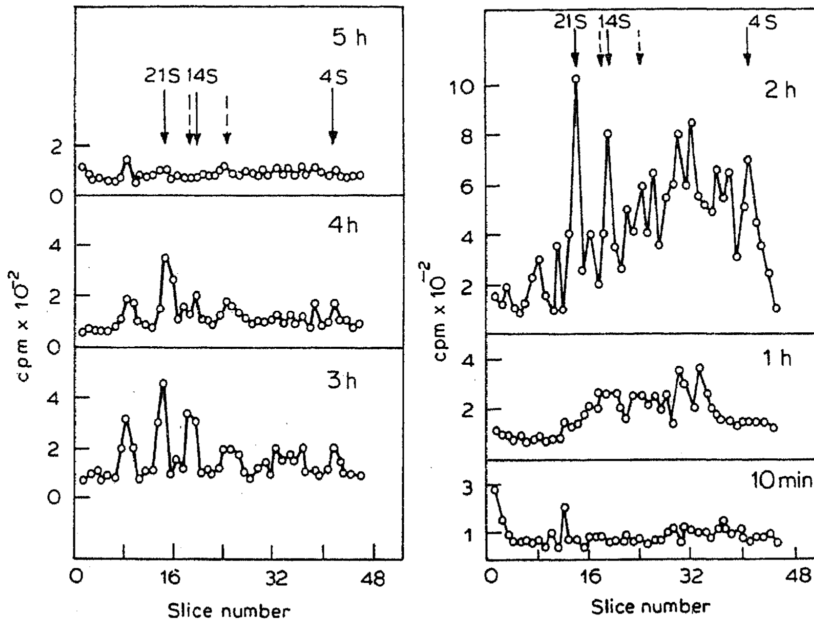

Figure 3. Analysis of labelled mitochondrial RNA in cells undergoing glucose repression/derepression.

Labelled mitochondrial RNA isolated from different time points in cells undergoing repression/derepression was analysed in an agarose-acrylamide composite gel, the gel was sliced and processed for measuring radioactivity.

Table 3. Chase of the 2 h labelled mitochondrial RNA in the presence of glucose and antibiotics.

Cells labeled from 1-2 h were chased for 1 h in the presence of excess of cold phosphate containing either 1% glucose, nalidixic acid (50 μg/ml), chloramphenicol (5 mg/ml) or cycloheximide (100 μg/ml).

Time of chase	Acid precipitable cpm incorporated/mg RNA
0 h chase	$11{\cdot}4 \times 10^5$
1 h chase:	
control	$9{\cdot}3 \times 10^5$
+ Glucose	$7{\cdot}7 \times 10^5$
+ Cycloheximide	$8{\cdot}6 \times 10^5$
+ Chloramphenicol	$7{\cdot}5 \times 10^5$
+ Nalidixic acid	$6{\cdot}1 \times 10^5$

Chase experiments

In order to assess the turnover of the RNAs synthesised between 1 and 2 h, a few chase experiments were carried out. Cells were labelled between 1 and 2 h and at that time 0·01 M cold phosphate was added to chase the label. After 1 h chase, cells were harvested and mitochondrial RNA isolated and analysed. The chase also was carried

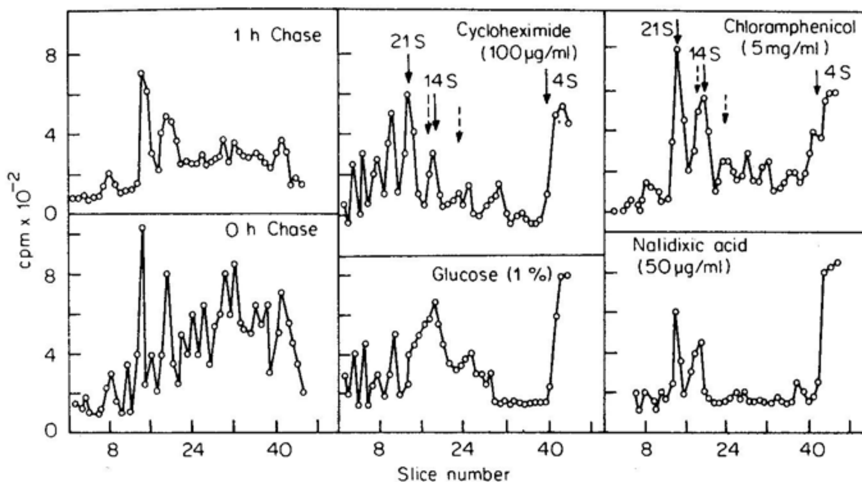

Figure 4. Analysis of labelled mitochondrial RNA undergoing turnover.
Cells undergoing repression/derepression were labelled from 1-2 h with [^{32}P] –phosphate and chased for the next 1 h in the presence of excess 0·01 M phosphate. The chase was also carried out in the presence of inhibitors as indicated. The mitochondrial RNA was extracted and analysed in an agarose-acrylamide composite gel.

out in the presence of glucose (1 % final concentration) and the protein synthetic inhibitors, cycloheximide and chloramphenicol. Results are given in table 3. The gel profiles of the RNA species after chase gave some interesting results (figure 4). After 1 h chase, the high molecular weight species (barring one peak) have disappeared and there is considerable reduction in 7S–11S region, whereas the 21S, 14S and 4S species are more stable.

Interesting changes are observed when the chase was carried out in the presence of glucose. There is an accumulation of species in the high molecular weight range (about 14S, infact) while the small molecular weight species have decreased label. A similar situation is obtained in the presence of cycloheximide. In the presence of chloramphenicol, labelling in the high molecular weight weight range declines, while labeled 4S-11S species persist.

RNA synthesis by isolated mitochondria

The results of experiments in which [^{32}P] -labelling of RNA by isolated mitochondria are given in table 4. Conforming to the pattern obtained in *in vivo* labelling experiments, mitochondria isolated from cells at 2 h of growth show maximal incorporation. The incorporation is insensitive to chloramphenicol and oligomycin but inorganic pyrophosphate and a mixture of nalidixic acid and actinomycin D cause drastic inhibition (table5).

Mitochondrial RNA polymerase activity

The RNA polymerase activity of mitochondria isolated from cells at different times were also analysed and the results given in table 6 again show the highest activity at 2 h.

Table 4. *In vitro* labelling of mitochondrial RNA.

Isolated mitochondria from cells undergoing repression/derepression at different time periods of growth were labelled with 100 μCi/ml of [^{32}P]-phosphate as described in 'methods' and the RNA extracted and analysed.

Time of growth	Acid precipitable cpm incorporated/mg RNA
0	$0{\cdot}53 \times 10^5$
1	$1{\cdot}57 \times 10^5$
2	$9{\cdot}50 \times 10^5$
3	$2{\cdot}60 \times 10^5$
4	$1{\cdot}80 \times 10^5$
5	$0{\cdot}90 \times 10^5$

Table 5. Effect of inhibitors on the *in vitro* labelling of mitochondrial RNA.

Isolated mitochondria from cells harvested at 2h were labeled *in vitro* with [^{32}P] in the presence of various inhibitors like nalidixic acid (50 μg/ml), + actionmycin D (5 μg/ml), chloramphenicol (5 mg/ml) and the incorporation determined.

Sample	Acid precipitable cpm incorporated/mg RNA	% inhibition
Control	$41{\cdot}7 \times 10^5$	
+Nalidixic acid and Actinomycin D	$5{\cdot}2 \times 10^5$	87·5
+Chloramphenicol	$31{\cdot}1 \times 10^5$	25·4
+Oligomycin	$40{\cdot}7 \times 10^5$	2·4
+Inorganic pyrophosphate	$3{\cdot}1 \times 10^5$	92·6

Table 6. Mitochondrial RNA polymerase activity as a function of repression/derepression

Mitochondria were isolated from cells grown to different time periods. The mitochondrial RNA polymerase was extracted and assayed using [^{3}H]-ATP, as described under 'methods' Nalidixic acid was used at a concentration of 50 μg/ml.

Time	Acid precipitable cpm incorporated/mg protein/h	+nalidixic acid	% inhibition
10 min	$1{\cdot}6 \times 10^5$	$7{\cdot}5 \times 10^4$	53·1
1 h	$2{\cdot}04 \times 10^5$	$6{\cdot}2 \times 10^4$	69·6
2 h	$2{\cdot}79 \times 10^5$	$1{\cdot}44 \times 10^4$	94·8
3 h	$1{\cdot}74 \times 10^5$	$1{\cdot}22 \times 10^4$	93·0
4 h	$1{\cdot}34 \times 10^5$	—	—
5 h	$1{\cdot}13 \times 10^5$	$9{\cdot}1 \times 10^4$	19·5

Table 7. Mitochondrial poly (A) polymerase activity during repression-derepression.

DEAE cellulose-purified mitochondrial poly(A) polymerase from different time hour mitochondria was analysed as described in 'methods' and the activity defined as p mol ATP incorporated/mg protein/h.

Time	$[^3H]$-ATP incorporated/mg protein/h cpm	p mol
0	$0·71 \times 10^5$	40·0
1	$1·50 \times 10^5$	76·5
2	$5·40 \times 10^5$	274·6
3	$2·60 \times 10^5$	130·6
4	$2·70 \times 10^5$	135·4
5	$2·20 \times 10^5$	111·2

Inclusion of nalidixic acid in the assay system at 50 μg/ml concentration shows more than 90 % inhibition in the case of 2 h and 3 h mitochondria, whereas it is less at other times. The enzyme activity is inhibited when cells are exposed to higher concentration of glucose before harvesting (results not given).

Poly A adenylation and poly A polymerase activity

There exists a controversy about the existence of poly A tails in yeast mitochondrial RNA (Groot et al., 1974; Aujane and Freeman, 1976). However in our strain, we have found that mitochondrial RNA isolated from cells harvested at 2 h, has 14·9 % poly A content and at 3 h it drops to 10·6 % (table 2). Poly (A) polymerase activities were also monitored (table 7) and once again maximal activity of 254 p mol [3H]-ATP incorporated/mg protein/h is obtained at 2 h.

Discussion

'Glucose repression' is a terminology used with reference to the expression of mitochondrial energy transducing functions. Baldacci and Zennaro (1982) have reported on the mitochondrial RNA metabolism of 'repressed' yeast cells (i.e. cells grown to mid exponential phase in 15 % glucose medium) and compared that with that of 'derepressed cells' (i.e. cells grown in the presence of glycerol).

The systems we have used in these studies offer us the facility to follow the mitochondrial transcriptional pattern as a function of the stages of mitochondriogenesis. Using these systems, we have restricted ourselves to investigating the total transcriptional activity of the mitochondria at various stages, and looking at the newly synthesised RNA species in a qualitative way. In this limited context, we have designated any RNA species above 21S as putative 'precursors' and the species appearing between 4S and 14S as the 'messenger region'. The translatability of the RNAs, isolated at various times and conditions, has been studied in rabbit cell-free reticulocyte lysate system and the pattern of $[^{14}C]$ -amino acid incorporation into TCA

precipitable material in such experiments, follows closely the appearance and disappearance of species in the messenger region (Nithyakalyani Raghavan and J. Jayaraman, unpublished data).

In synchronous cultures, maximal mitochondrial transcription occurs during the S phase, followed by translation and expression of electron transport enzyme activities in the subsequent phase whereas G_1 and M phases are silent. It is interesting to note that a high molecular weight RNA species is labelled at this phase. When the cells enter the S phase, the mitochondrial transcriptional machinery is very active, synthesising several species from 45S–4S. In the G_1 phase, labelling is mainly found in the 'messenger region' apart from rRNAs and tRNAs.

A somewhat analogous situation is obtained in the glucose repression-derepression condition also. Maximal incorporation of [^{32}P] -phosphate into RNA occurs between 1 and 2 h of growth and the gel profile shows a remarkable similarity to the RNA obtained from S-phase cells. Between 2 and 3 h labelling is mainly in the 'messenger region' with considerable reduction in high molecular weight species representing the RNA processing period. Assay of transcription-associated enzyme activities, RNA polymerase and poly A polymerase, as well as in vitro incorporation of [^{32}P]-phosphate into RNA, all substantiate the fact that between 1–2 h [which will correspond to the mid-exponential phase cells grown in higher concentration of glucose by other workers (Hendler et al., 1975) and which is defined as 'repressed phase'] has the maximal mitochondrial transcriptional activity.

We deduce from these experiments that late G_1 or early S phase cultures in synchronous growth conditions, or after 1 h growth under the repression-derepression conditions, would be the time point to try and locate the nuclear trigger, proposed by Neupert's group (Neupert and Rucker, 1976; Neupert, 1977). The effect of cAMP and hemin, suggested as possible trigger molecules (Somasundaram *et al*., 1980; Chandrakumar and Padmanabhan, 1980) was tried in our system but found a small but overall increase in transcription (1·2 fold in the case of cAMP and 4 fold in the case of hemin, table 8), but the gel patterns remained the same (data not presented). Only hybridisation studies using mitochondrial DNA probes will answer the question as to whether any specific species are induced. Increasing the glucose concentration in the medium during the period of active growth-diminished transcription in accordance with the results of Baldacci and Zennaro (1982). Interestingly, the apparently nuclear-coded RNA polymerase activity was also inhibited.

Table 8. Effect of hemin and cyclic AMP addition on the synthesis of mitochondrial RNA.

Cells undergoing repression/derepression were labeled from 0-1 h with [^{32}P]-phospate (2 mCi/200 ml medium in the presence of either hemin (2.5 μg/ml) or cyclic AMP (5 mM)

Sample	Acid precipitable cpm incorporation/gm RNA	Stimulation
Control	$1{\cdot}4 \times 10^5$	
+ hemin	$5{\cdot}0 \times 10^5$	257%
+ cAMP	$1{\cdot}6 \times 10^5$	14·3%

The chase experiments gave some interesting data. In these, cells were labeled between 1 and 2 h and then label chased for a further 1 h. In the control cells, the high molecular weight species disappeared as also most species in the messenger region. Only the rRNAs and tRNAs retained the label to a large extent. But if excess glucose was present during the chase, there was a net accumulation of high molecular weight species. Baldacci and Zennaro (1982) have also observed the accumulation of a high molecular weight RNA species, faulty maturation of *cob* precursor RNA and a reduction of *oli* 1 and *oxi* 1 mRNAs in repressed cells.

This could indicate that RNA processing may be a crucial step in the manifestation of 'glucose effect' on mitochondriogenesis.

Chase experiments in the presence of cycloheximide showed accumulation of high molecular weight RNA species, testifying to the cytosolic origin of the processing enzymes. Chloramphenicol did not show such an effect.

Precise hybridisation studies using mitochondrial DNA probes, which are under way, will answer some of the questions raised.

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