*Proc. Nat. Acad. Sci. USA*
Vol. 70, No. 1, pp. 111–114, January 1973

# Autonomy of Mitochondria in *Saccharomyces cerevisiae* in Their Production of Messenger RNA

**(RNA synthesis/polysomes/inhibition/ethidium bromide/mitochondrial genome)**

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*Communicated by Marcus Rhoades, October 26, 1972*

**ABSTRACT** **In *ts*$^{-}$136, a temperature-sensitive mutant of *Saccharomyces cerevisiae*, nuclear and mitochondrial RNA production can be inhibited selectively by exposure to 36° and ethidium bromide, respectively. Using the programming of mitochondrial polysomes, as measured by their ability to form nascent polypeptide chains, as an assay for functional messenger RNA, we have determined its response to temperature shifts and ethidium bromide. Only ethidium bromide produced a measurable effect; in contrast the cell-sap system responded exclusively to temperature shifts. We conclude that transcription of mitochondrial DNA is sufficient and that import of messenger RNA transcribed from nuclear chromosomes makes no measurable contribution to intramitochondrial protein synthesis.**

Mitochondria, ubiquitous organelles of eukaryotic cells, contain their own semi-autonomous system of gene expression. The current view is that only the primary structure of the constituent stable RNA species [ribosomal RNAs (rRNAs), some or all transfer RNAs (tRNAs)] but not of its multitudinous polypeptides (ribosomal proteins and initiation, elongation, and termination factors, etc.) is encoded in mitochondrial (mt)DNA (1). A major uncertainty remains: to what extent is mtDNA competent to code for mitochondrial mRNA, or, in other words, which, if any, of the polypeptides synthesized by the mitochondrial machinery are actual products of mitochondrial genes? Some investigators believe that most, or perhaps all, of mitochondrial mRNA is an import of nuclear origin (2, 3), while others have provided evidence for mitochondrial transcripts with at least some of the properties expected of a mRNA (4), and even for the export of such species into the cell sap (5).

A critical inquiry into this problem requires the following: (*i*) a means for effectively shutting down nuclear and mtRNA selectively, and (*ii*) some way for assessing the presence and activity of mRNA as well as its response to (*i*). These conditions can be met, particularly in *Saccharomyces cerevisiae*, since it has been shown that: (*i*) ethidium bromide inhibits transcription and appears to be restricted to mitochondrial events in its specificity (1, 6–8); a temperature-sensitive mutant (*ts*$^{-}$136) is available that shuts down production of all nuclear RNA including mRNA (9, 10), and (*ii*) the ability to program polyribosomes, as measured by their capacity to support initiation and propagation of nascent polypeptide chains, provides a sensitive operational assay for mRNA in both the cell-sap and the mitochondrial systems (9–11).

In this communication, we show that cells and spheroplasts of *ts*$^{-}$136 can be used to demonstrate that all functional mitochondrial mRNA is produced inside the organelle and that effective mitochondrial protein synthesis does not depend on the import of mRNA.

## METHODS

*Strains and Culture Conditions.* Strains (obtained from L. H. Hartwell, University of Washington, Seattle) used in these studies were: (*1*) *S. cerevisiae* A364A, haploid, P $\rho^{+}$ (*a ade*$_1$ *ade*$_2$ *ura*$_1$ *tyr*$_1$ *his*$_1$ *lys*$_2$ *gal*$_1$); (*2*) *S. cerevisiae ts*$^{-}$136, derived from A364A by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. They were grown to midexponential phase at 23° in medium YM-1L that contained (g/liter): yeast extract, 5; peptone, 10; yeast nitrogen base (filter-sterilized), 6.7; adenine sulfate, 0.01; uracil, 0.01; succinic acid, 10; NaOH, 6; Na-D,L-lactate, 30, final pH 6.8. The labeling medium was YM-5L, which is the same as YM-1L except that the peptone and yeast extract are reduced 5-fold.

*Labeling of Spheroplasts.* Cells were converted into spheroplasts as described (11), suspended in the original volume of YM-5L plus 1 M sorbitol, and incubated with the appropriate precursors ([$^{3}$H]uracil, 40 Ci/mmol; [$^{3}$H]leucine, 36 Ci/mmol, and [$^{14}$C]formate, 59 Ci/mol, all from New England Nuclear). Reaction was stopped by addition of crushed ice, 4 mg/ml of chloramphenicol, and 100 μg/ml of cycloheximide, and centrifugation at 3° (10 min at 4000 rpm in a Sorval RC-1). The pellet was suspended in one-twentieth of the original volume of 0.5 M sorbitol-NMT [10 mM $MgCl_2$–100 mM $NH_4Cl$–10 mM Tris·HCl (pH 7.4)] and blended for 15 sec.

*Isolation of Mitochondria and Cell Sap.* Lysed spheroplasts were centrifuged 3 times at 600 × *g* for 10 min. The pellets were discarded and the supernatant was centrifuged at 20,000 × *g* for 20 min. The pellet, resuspended in 0.01 of the original volume of 0.5 M sorbitol-NMT, constitutes the mitochondrial fraction; the supernatant is the cell-sap fraction.

*Isolation of Ribosomes, Samples for Counting, etc.* are described in ref. 11 and in the figure legends.

## RESULTS

### Experimental design

The experiments to be described have been designed to answer the following question: Is transcription by either of the two systems *sufficient* for mitochondrial mRNA function or are both *necessary?* This question is asked in two ways. After the specificity of the two blocks of RNA synthesis (exposure to 36° for nuclear RNA synthesis, and to ethidium bromide for mtRNA synthesis) is first established by measuring their effect on the kinetics of incorporation of uracil into mitochondrial and cell-sap RNA of the ts mutant, is a shift to this nonpermissive temperature (nuclear block) sufficient, necessary, or ineffective in the absence of a subsequent or simultaneous exposure to ethidium bromide (mitochondrial

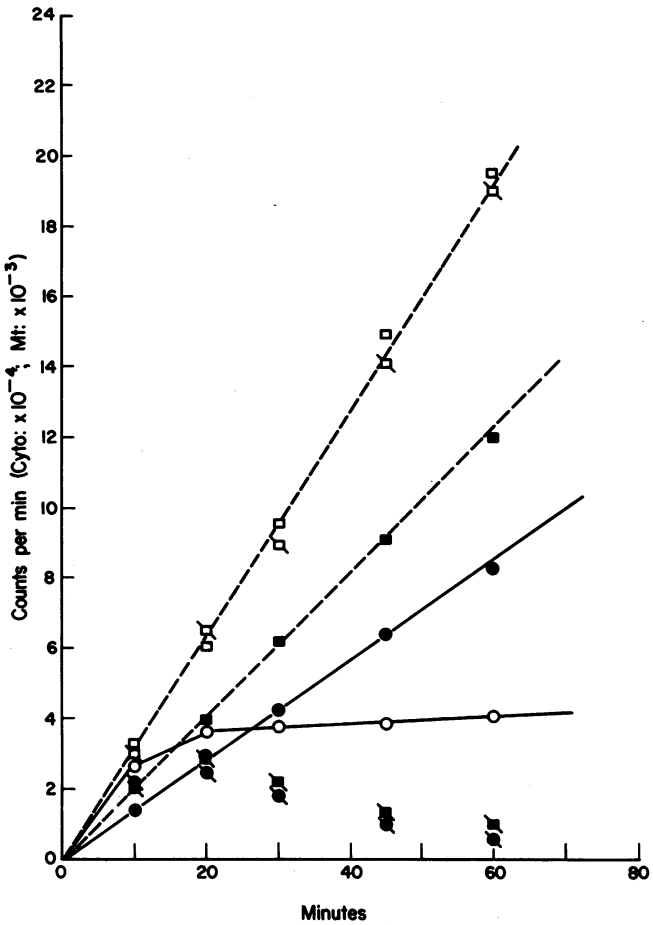


FIG. 1. Incorporation of [³H]uracil at 36°. A364A (wild type) and *ts*⁻136 were grown (50 ml each) in YM-1L at 23° until $A_{600}$ = 0.5. The cells were harvested and spheroplasts were made. The spheroplasts were suspended in YM-5L–1 M sorbitol and transferred to a 36° incubator; after 20 min [³H]uracil (5 μCi/ml) was added. 12-ml samples were taken from each flask at the times indicated, iced, and harvested. The spheroplasts were lysed and mitochondrial (*Mt*)(1 ml) and cell-sap (*Cyto*) (5 ml) fractions were isolated. 0.2-ml aliquots were put on filter paper discs. The discs were put in 10% cold trichloroacetic acid for 2 hr, washed twice with 10% cold trichloracetic acid, suspended in EtOEt–EtOH (2:1), washed twice with this mixture and once with EtOEt, and dried. Their radioactivity was then measured (11). The values shown are total cpm determined on replicate samples. (□, ■) cell-sap and mitochondrial fractions, respectively, from A364A; (○, ●) cell-sap and mitochondrial fractions, respectively, from *ts*⁻136. *Crossed symbols* (⊠, ⊠, ⊘, ⊘) represent data obtained when 10 μg/ml of ethidium bromide was added to the spheroplast suspension at the time of the shift to 36°.

block) to eliminate mRNA function? After all functional mRNA is first eliminated as a result of the double block, is a shift down (selective release of nuclear block) sufficient to re-establish a functional system?*

* The converse, i.e., release of the mitochondrial block by removal of ethidium bromide is not feasible because, in addition to the transcriptional block, exposure to this agent also produces irreversible effects on mtDNA; experiments with acriflavine, which circumvent this difficulty, are in progress.

**Blocks exerted by shift-up and ethidium bromide are specific**

The data shown in Fig. 1 demonstrate that in *ts*⁻136 a shift-up to 36°, a restrictive temperature for nuclear RNA synthesis in this strain (9), blocks incorporation of uracil into RNA outside, but is without effect on this process inside, the mitochondria. Conversely, exposure to ethidium bromide for 10 min completely blocks incorporation of this precursor into mtRNA at the restrictive temperature, or at 23°, the permissive temperature—as well as into mtRNA of the wild type at either temperature—without affecting cell-sap RNA.

**Functional mitochondrial mRNA is unaffected by a shift-up but blocked by ethidium bromide**

A shift-up of *ts*⁻136 to 36°, followed by incubation at this temperature for 60 min, is sufficient to eliminate all mRNA function in the cell sap, as measured by the disappearance of polysomal structures (9, 10), and the inability of these structures to catalyze polypeptide chain initiation or elongation (Table 1). As judged by any of these criteria cell-sap functions are, however, inert to exposure to ethidium bromide at either 36° or 23° (compare Fig. 3*A* and *C*) (as they are in the wild type). Conversely, functional mRNA-containing ribonucleoprotein particles, isolated from highly purified mitochondria, are unaffected by the elevated temperature, as measured by

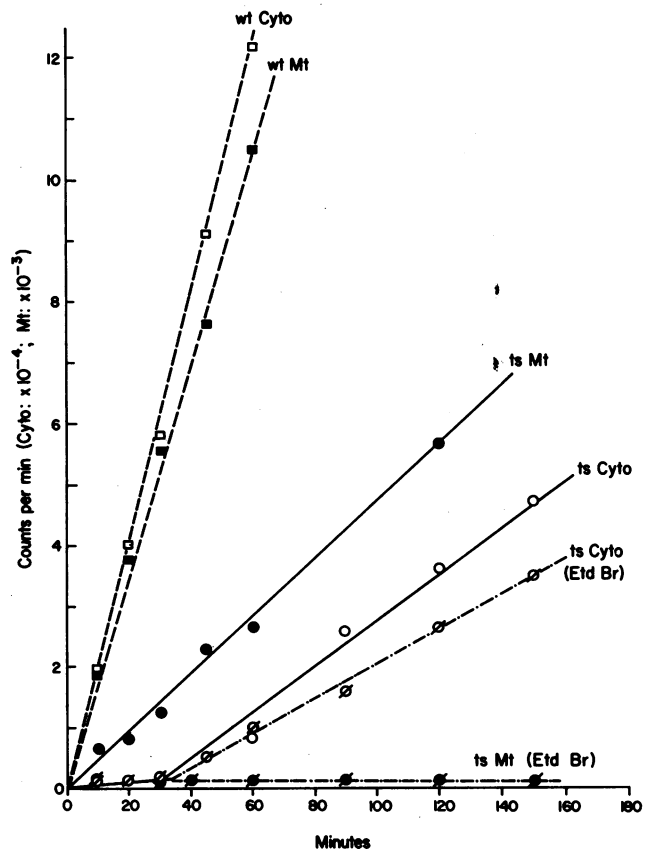


FIG. 2. [³H]Uracil incorporation after shift-down from 36° to 23°. Procedure as in Fig. 1, except that the spheroplasts were kept for 60 min at 36 and shifted down to 23°. Then 10 μg/ml of ethidium bromide was added to one sample and no drug to another. 20 min later [³H]uracil (5 μCi/ml) was added to the samples, and 10-ml aliquots were taken at the appropriate time. Symbols are as in Fig. 1.

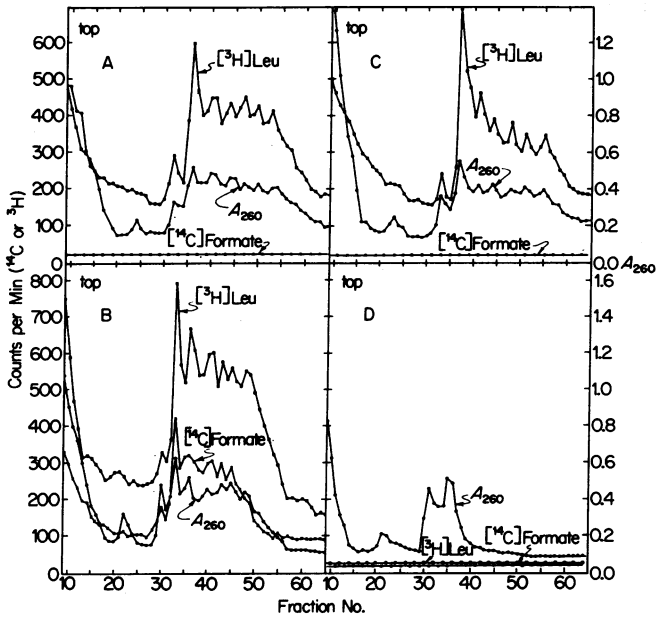


FIG. 3. Polypeptide synthesis on ribosomes. *ts*-136 cells were grown in YM-1L medium (200 ml each) to $A_{600}$ = 0.4 and then shifted to 36°; after 60 min at this temperature one-half of the cells received 10 μg/ml of ethidium bromide. Then both samples were incubated 60 min longer at 36° before they were shifted down to 23° and incubated for 30 min. The cells were harvested and spheroplasts were made at 23°. They were suspended in YM-5L (supplemented with 10 μg/ml of ethidium bromide for the cells treated with this agent at 36°). After 30 min, [$^3$H]leucine (5 μCi/ml) and [$^{14}$C]formate (2.5 μCi/ml) were added. The reaction was stopped after 10 min by addition of crushed ice plus 100 μg/ml of cycloheximide and 4 mg/ml of chloramphencol. The spheroplasts were sedimented and lysed by blending in 0.5 M sorbitol–NMT buffer. Mitochondrial and cell-sap fractions were isolated. Mitochondria were washed by sedimentation from 5 mM EDTA–100 mM $NH_4Cl$–10 mM Tris·HCl in 0.5 M sorbitol and lysed in 2 ml of 2% Triton X-100–NMT buffer. The lysates were layered on top of a 0.3–1.4 M sucrose in NMT buffer linear gradient and centrifuged for 4 hr in an SW27 rotor in an L2 Beckman centrifuge, at 26,000 rpm. The gradients were fractionated from the top, and $A_{260}$ and radioactivity were measured (11). *A* and *C*, cell sap; *B* and *D*, mitochondria; *A* and *B* in the absence of, *C* and *D* in the presence of ethidium bromide. Results similar to *D* were obtained when the cells incubated with ethidium bromide were analyzed just before the shift-down; analogous results for leucine were also found when 4 mg/ml of chloramphenicol was substituted for ethidium bromide. However, with this inhibitor the $A_{260}$ profile resembled that shown in *B*! Thus, the technique can distinguish between programmed functional, but inhibited, particles and nonfunctional ones.

$A_{260}$, incorporation of leucine into nascent polypeptide chains or by chain initiation with formate (all in Fig. 3*B*). They also retain their ability to catalyze initiation of polypeptide chains, as measured by formation of fMet puromycin (11) or incorporation of leucine or formate into nascent chains (all shown in Table 1): all these activities, at either temperature, are eliminated by prior exposure to ethidium bromide. Similar results (not shown) are obtained with chloramphenicol, a specific inhibitor of mitochondrial translation, except that in this instance polysomal structures are retained.

TABLE 1. *Incorporation of leucine and formate into mitochondrial and cell-sap nascent chains (cpm)*

| Addition | Leucine | | Formate | | |
|---|---|---|---|---|---|
| | Mt* | Cell sap | Mt | Cell sap | fMet puromycin Mt |
| | wild type (A364A) | | | | |
| 36° | | | | | |
| None | 1970 | 9000 | 440 | 46 | 5790 |
| Chloramphenicol | 320 | — | 67 | — | 720 |
| Cycloheximide | 1830 | 240 | 460 | 42 | 5730 |
| Ethidium bromide | 510 | — | 130 | — | 1470 |
| 36 → 23° | | | | | |
| None | 1080 | 5050 | 240 | 22 | 5140† |
| | *ts*-136 | | | | |
| 36° | | | | | |
| None | 1810 | 315 | 460 | 45 | 4320 |
| Chloramphenicol | 325 | — | 65 | — | 540 |
| Cycloheximide | 1690 | 270 | 440 | 45 | 4330 |
| Ethidium bromide | 520 | — | 100 | — | 990 |
| 36 → 23° | | | | | |
| None | 1080 | 850 | 290 | 29 | 5240† |
| Cycloheximide | 1130 | 35 | 280 | 24 | |
| Chloramphenicol | 150 | — | 54 | — | |

* Mt, mitochondria.

† 23° without prior incubation at 36°.

All data are the means of duplicate or triplicate determinations of total cpm by spheroplasts. A *dash* indicates not measured. Spheroplasts (prepared at 23°!) were equilibrated for 10 min at 23° and then shifted to 36°; after 20 min aeration at this temperature they were divided into 50-ml aliquots. Appropriate drugs (4 mg/ml of chloramphenicol; 100 μg/ml of cycloheximide; 10 μg/ml of ethidium bromide) were added, and the samples were aerated for an additional 10 min (ethidium bromide, for 20 min). Precursors (5 μCi/ml of [$^3$H]leucine; 2.5 μCi/ml of [$^{14}$C]formate) were added, and incorporation was for 10 min. Spheroplasts were harvested, lysed, and ribosomes were isolated from the mitochondrial and cell-sap fractions (11; see also Fig. 3) by sedimentation for 120 min at 105,000 × *g*. Aliquots on filters were processed for counting. In the 36 → 23° experiment, prior incubation at 36° was for 60 min, followed by equilibration at 23° for 35 min, before addition of inhibitors. fMet puromycin was determined in a separate experiment, for measurement of chain initiation (11).

**Shift-down restores function only to cell-sap ribosomes**

When cells, previously incubated at 36° for 60–120 min, are restored to the permissive temperature of 23°, incorporation of uracil into cell-sap RNA resumes after a characteristic lag of 30 min. No such lag is observed for incorporation into mtRNA (Fig. 2). When these cells, previously completely blocked in *all* RNA synthesis by exposure to ethidium bromide at 36°, are restored to 23°, the rate of uracil-incorporation returns (after a similar lag) to that characteristic for this regime (Fig. 2). Coincident with this return the residual ribosomes in the cell sap (9) become reprogrammed into functional polysomal structures, whether or not ethidium bromide is present (Compare Fig. 3*A* and *C*, and Table 1). On the other hand, there is no evidence for import of any labeled RNA into mitochondria (Fig. 2). In another experiment, spheroplasts were exposed to a 30-min labeling period (with ethidium bromide added) 20

min after the shift-down and then to a chase of unlabeled uracil for an additional 60 min. 8600 cpm were incorporated into the cell sap, of which 1300 were retained after the chase (i.e., most was probably mRNA); no incorporation above background was observed in the mitochondrial fraction either before or after the chase. More conclusively, perhaps, in the presence of ethidium bromide polysomes decay and, in contrast to the cell-sap structures, the residual intramitochondrial ribosomes and subunits do not become reprogrammed for the support of polypeptide chain elongation or even initiation (compare Fig. 3*B* with Fig. 3*D*).

## DISCUSSION

Our results show that the temperature-sensitive mutant, *ts*$^{-}$136, in conjunction with the inhibitor ethidium bromide, can be used to study the relative contribution and importance of nuclear and mitochondrial RNA synthesis. They also indicate that formation and decay of *functional* polysomes measured by incorporation of a radioactive precursor into nascent polypeptide chains on these particles provides a sensitive, qualitative assay for the presence of functional mRNA not only in the cell sap (9, 10), but in mitochondria (11) as well.

By means of our particular experimental design we have attempted to answer whether import of mRNA into mitochondria is either necessary or sufficient to program their ribosomes for active polypeptide synthesis; we find that such import is completely dispensable, while the converse holds for mRNA synthesized inside the particle in a process sensitive to ethidium bromide—presumably on mtDNA. One possible source of ambiguity in the interpretation of these results resides in the observation that ethidium bromide may inhibit mitochondrial translation directly, and not just as a consequence of its interference with transcription (1, 12). The results of the temperature-shift experiments could then be interpreted in terms of such a translational inhibition of mitochondrial polysomes programmed with a population of very long-lived mRNAs of nuclear origin. We consider this alternative unlikely for the following three reasons: (*i*) Since we have shown that ethidium bromide is a specific and potent inhibitor of mitochondrial transcription under precisely the conditions of this particular experiment, the most parsimonious interpretation would rely on this demonstrated fact. (*ii*) Since we have repeated the shift-down experiments after prior exposure to 36° for as long as 2 hr, the half-life of the hypothetical mRNA would have to be >2 hr, i.e., half a generation time under conditions where the same species in the cell-sap breaks down within 23 min (9). (*iii*) This "stable" mRNA would then have to decay rapidly under conditions of the postulated translation block by ethidium bromide (at either 36° or 23°); this decay is contrary to the effect of most translational inhibitors on polysomal stability (13), including that of cycloheximide on cell-sap polysomes (10), or of chloramphenicol on mitochondrial ones in these strains. Another possible source of ambiguity is that many of the experiments made use of spheroplasts rather than intact cells, and it might be argued that these structures are no longer capable of supporting certain events required for complete cellular function, specifically the mitochondrial import of nuclear mRNA. This *ad hoc* hypothesis is highly unlikely; yeast spheroplasts have been shown to be fully competent to biosynthesize nucleic acids and proteins in Hartwell's laboratory (9, 10) as well as in our own (11), including the elaboration of active mitochondria after release from catabolite repression. Finally, one might argue that ethidium bromide blocks not the synthesis of mitochondrial mRNA but precisely the transport into mitochondria of the nuclear variety: any substantial contribution of this mechanism is ruled out by the results of the shift-up experiments.

We, therefore, conclude that, at least in ascomycetes, transcripts of mtDNA are sufficient to account for the programming of the protein-synthesizing machinery of the organelle, and the latter need not depend on the import of mRNA transcribed from nuclear genes. Similar inferences have been drawn by Reijnders *et al.* from hybridization data of total stable mtRNA with nuclear DNA (14). Therefore, for these organisms at least, the only polypeptide products translated by the organelle are the ones encoded in the genome of the organelle.

We thank Prof. Leland H. Hartwell for supplying us with the strains required for this investigation and for his counsel concerning their use, and Dr. Fred Feldman for many stimulating discussions. This research was supported by Research Grant PHS R01 GM 12228 from the Institute of General Medical Sciences. HRM is a recipient of a Research Career Award K06 GM 05060 from this Institute. KD was supported by Consejo de Desarrollo Cientifico y Humanistico, Universidad Central de Venezuela. This is publication no. 2137 from the Chemical Laboratories, Indiana University.

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