# SELECTIVE INHIBITION OF THE *in vivo* TRANSCRIPTION OF MITOCHONDRIAL DNA BY ETHIDIUM BROMIDE AND BY ACRIFLAVIN

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SUMMARY : Acriflavin ($10^{-5}$M) as well as ethidium bromide ($3 \times 10^{-5}$M) selectively inhibited the *in vivo* transcription of mitochondrial DNA in normal yeast.

Ethidium bromide and some acridine dyes are known as potent mutagenic agents which act very specifically on some cytoplasmic genomes (Werbitzki, 1910; Ephrussi *et al*, 1949; Slonimski *et al*, 1968). These intercalating dyes induce cytoplasmic respiratory deficient mutations (*petites colonies* mutation) in yeast at concentration much lower than those required to induce chromosomal mutations. The mutation efficiency can be near 100 % under appropriate conditions at non lethal doses (Slonimski *et al*, 1968, Marcovich, 1951).

Among acridines, only some derivatives (e.g. euflavin, a component of the commercial acriflavin) are specific mutagens on yeast (Marcovich, 1951; Slonimski, 1953). An interesting feature of the action of these acridines is the fact that they specifically inhibit respiratory adaptation : when respiratory adaptation occurs in the presence of acriflavin, the yeast closely mimics the cytochromic phenotype of the petite colonie mutants, although it is genotypically still normal and capable of giving rise to wild type cells. Thus acriflavin appears to interfere with a especific reaction linking hereditary and non-hereditary determinism of cytochrome oxidase formation" (Slonimski, 1953). In the light of the present experiments, such a specific reaction can be localized at the transcription of mitochondrial DNA.

Some recent papers have reported that ethidium bromide inhibited specifically the *in vivo* transcription of mitochondrial DNA in Hela cells (Zylber *et al*, 1969; Attardi *et al*, 1969). We will describe below some experiments which show that acriflavin (ACF) as well as

ethidium bromide (EB) inhibits highly selectively the *in vivo* transcription of mitochondrial DNA in yeast.

## MATERIAL AND METHODS

(1) A respiratory sufficient strain of *Saccharomyces cerevisiae* was used (D243-2B-$R_1$, a $ad_1$).

(2) Labelling of RNA during respiratory adaptation : yeast was grown anaérobically in a glucose (5 %) - yeast extract (1 %) medium up to stationary phase. The cells were washed in the cold and suspended in a medium containing glucose (5 %) and Tris-maleate buffer (0.05 M, pH 6.5), with or without inhibitor. The suspension was aerated at 28° for respiratory adaptation. At the end of the first hour, radioactive phosphate (carrier-free, 10 mC per 200 ml suspension) was added. After 4 hours incubation, cells were washed and used for the preparation of $^{32}P$-labelled RNA. Technical details for respiratory adaptation have been fully described by Fukuhara (1967). It should be noted that in the present experiments, a pH 6.5 buffer was used for respiratory adaptation because both ACF and EB have almost no effect in the acidic media normally used for respiratory adaptation.

(3) Preparation of $^{32}P$-labelled RNA : the details of extraction and purification of RNA labelled during respiratory adaptation have been described previously (Fukuhara, 1968). The procedure can be summarized as follows : the labelled cells were extensively broken in a Braun shaker, the total homogenate was mixed with Sarkosyl (1 %) and one volume of phenol. After 3 hrs agitation, the mixture was centrifuged and the aqueous layer was mixed with 2 volumes of cold ethanol. The precipitated RNA was collected by centrifugation, dissolved in 0.1M NaCl (in 0.05M sodium phosphate buffer, pH 6.8). RNA was then adsorbed into a methylated albumin-Kieselguhr column. From the column, the 4S-5S class of RNA was eluted with 0.5M NaCl, then the 18S-28S ribosomal RNA class was eluted with 1.2M NaCl (see Fukuhara *et al*, 1969). Each RNA fraction was passed through a small column of Nitrocel S (Serva, Heidelberg) to eliminate residual radioactive impurities. RNA solutions were dialyzed against 0.3M NaCl-0.03M trisodium citrate.

(4) DNA-RNA hybridization : the nitrocellulose membrane technique of Gillepsie and Spiegelman (1965) was used. Mitochondrial DNA and nuclear DNA were highly purified by hydroxyapatite chromatography. Mutual contamination between the two DNA was not detectable. The DNA was denatured by alkali, and immobilized on 25 mm nitrocellulose filter (MF50). All DNAs used were labelled with $^{3}H$-adenine. Hybridization of immobilized

DNA with $^{32}P$ labelled RNA was carried out in a final volume of 2 ml containing 0.3M NaCl and 0.03M trisodium citrate and various concentration of $^{32}P$ RNA. Each incubation bottle contained a mitochondrial DNA-filter (5 $\mu$g DNA), a nuclear DNA-filter (5 $\mu$g DNA) and a blank filter (no DNA). After 20 hrs incubation at 60°, the filters were washed in the NaCl-sodium citrate solution, treated with pancreatic ribonuclease (10 $\mu$g/ml, 60 minutes at room temperature) and assayed for radioactivity ($^{3}H$ for DNA and $^{32}P$ for RNA). Technical details for hybridization have been described by Fukuhara, Faurès and Genin (1969).

Acriflavin was purchased from British Drug House, London, and ethidium bromide from Boots Pure Drug Co., Nottingham. All radioactive materials were the products of the Commissariat à l'Energie Atomique.

## RESULTS AND DISCUSSION

The respiratory adaptation system without cell growth was chosen for the study of the transcription of mitochondrial DNA. The RNA extracted from normally adapting cells contains the RNA classes which hybridize with mitochondrial DNA as well as the classes which hybridize with nuclear DNA. When a inhibitor blocks specifically the transcription of mitochondrial DNA, the total RNA preparation will fail to form *in vitro* hybrids with mitochondrial DNA but not with nuclear DNA. Figure 1 illustrates the case observed with EB. The RNA extracted from EB-treated cells showed a low level of hybridization with mitochondrial DNA as compared to the normal RNA (Fig. 1A). Such a difference was not found in the hybridization with nuclear DNA (Fig. 1B). Clearly only the transcription of mitochondrial DNA had been inhibited by EB. This is in good agreement with the fact that, under these conditions, the incorporation of $^{14}C$-uracil into total cell RNA was only slightly inhibited by EB, since the major part of cellular RNA synthesis is due to the transcription of nuclear DNA. In the experiment presented in Figure 1, the inhibition of transcription of mitochondrial DNA did not appear complete at the dye concentration applied ($3 \times 10^{-5}$M). In fact, the rate of $QO_2$ increase, taken as a measure of mitochondrial protein synthesis, was repressed by about 80 % by the drug under the same conditions. It has not been examined whether the residual transcription is due to a delay of the action of EB on yeast cells or the inhibition remained incomplete during the whole period of adaptation. At that concentration of EB, we observed a massive formation of petite colony mutants as revealed by plating the cell suspension on glycerol-agar at the end of respiratory

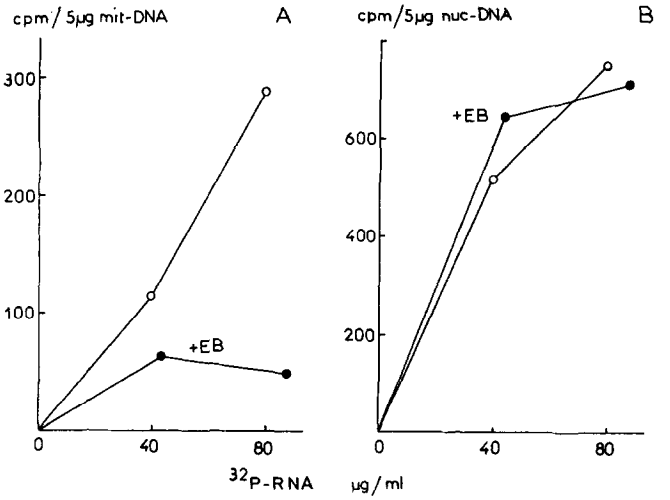


Figure 1 : Hybridization of mitochondrial and nuclear DNA with the RNA synthetized in the presence of ethidium bromide.

RNA was extracted by the phenol-detergent procedure from cells labelled with $^{32}P$ phosphate during respiratory adaptation, in the presence and in the absence of ethidium bromide ($3 \times 10^{-5}$M).(See Methods). RNA was purified on a methylated albumin-Kieselguhr column as described in Methods, and the 1.2M NaCl eluate (containing mainly ribosomal RNA classes,mitochondrial and cytoplasmic) was used for hybridization. The abscissa represents increasing concentrations of $^{32}P$-RNA and the ordinate stands for the $^{32}P$ counts (ribonuclease resistant DNA-RNA hybrids) fixed to membrane-bound DNA at each concentration of RNA. For hybridization conditions, see Materials and Methods. A represents the hybridization with mitochondrial DNA and B the hybridization with nuclear DNA. Specific radioactivities of the $^{32}P$-RNA were 2980 c/m/µg for normal RNA and 3150 c/m for the RNA of ethidium bromide treated cells. $^{32}P$ counts found on blank filters were between 7 and 42 c/m (increasing with the RNA concentration), which were substracted from experimental values.

adaptation (Table 1). During the 4 hrs respiratory adaptation, the total cell number increased less than 10 %. Since 40 % of the cell population was transformed into petites colonies during this time the mutation must have occurred in the mother cell population as described by Slonimski et al (1968).

The inhibition of respiratory adaptation and of transcription of mitochondrial DNA, as well as the highly mutagenic action of EB could all be due to the association of the dye with mitochondrial DNA bases. It is however of interest to ask the question whether or not the selective inhibition of mitochondrial DNA transcription is in some way involved in the mechanism of cytoplasmic mutation. In order to check this possibility, we examined the effect of another mutagenic agent, ACF,

Table 1 : Effect of ethidium bromide and acriflavin on respiratory adaptation and their mutagenic action.

At time 0 and time 4 hrs of respiratory adaptation, aliquots of the suspension were taken and the respiration was measured with an oxygen electrode (Gilson) using ethanol as substrate. Other aliquots were plated on agar plates containing 0.1 % glucose, 2 % glycerol and 1 % yeast extract Difco. The numbers of "grande" and "petite" colonies were determined after 4 days incubation.

| Addition | Rate of $Q_{O_2}$ increase ($\mu l\ O_2/mg$ dry $wt/hr^2$) | Inhibition % | Plating after 4 hrs adaptation, % petites colonies |
|---|---|---|---|
| - | 4.5 | | 1.4 |
| EB $3\times10^{-5}M$ | 1.0 | 77 | 40 |
| ACF $10^{-5}M$ | 1.2 | 72 | 2.7 |

which, in contrast to EB, does not induce mutation when cells do not undergo multiplication or budding (Ephrussi and Hottinguer, 1951). In the case of respiratory adaptation under non growing conditions, the relatively weak mutagenic effect of ACF is attributed to the small proportion of budding cells. Slonimski (1953) has observed previously that the inhibition of respiratory adaptation by ACF preceeded the induction of mutation, as if the former was the cause of the latter.

Figure 2 shows that ACF inhibits mitochondrial DNA transcription in a way very similar to EB. In Figure 2 A and B, one can see that the synthesis of low molecular weight RNA (mainly 4S size) transcribed from mitochondrial DNA was also inhibited. Figure 2 C and D show the inhibition of the synthesis of mitochondrial ribosomal RNA classes as in the case of Figure 1 for EB. (In answer to the communication of our results, Dr. D. Luck let us know that Drs. P. Lizardi and D. Wood at Rockefeller Institute have obtained very similar results on Neurospora). Under these conditions, the respiratory adaptation was inhibited by about 70 % as in the case of ED. The rate of total RNA synthesis, measured by $^{14}C$-uracil incorporation in vivo, was little affected by ACF.

Therefore, we have shown that during short-time respiratory adaptation, two intercalating dyes very strongly inhibited specific transcription of mitochondrial DNA and respiratory adaptation. Nevertheless, one of these dyes (EB) was highly mutagenic whereas the other (ACF) was not. Thus we have eliminated the possibility that the specific inhibition of mitochondrial transcription is necessarily followed by mutation.

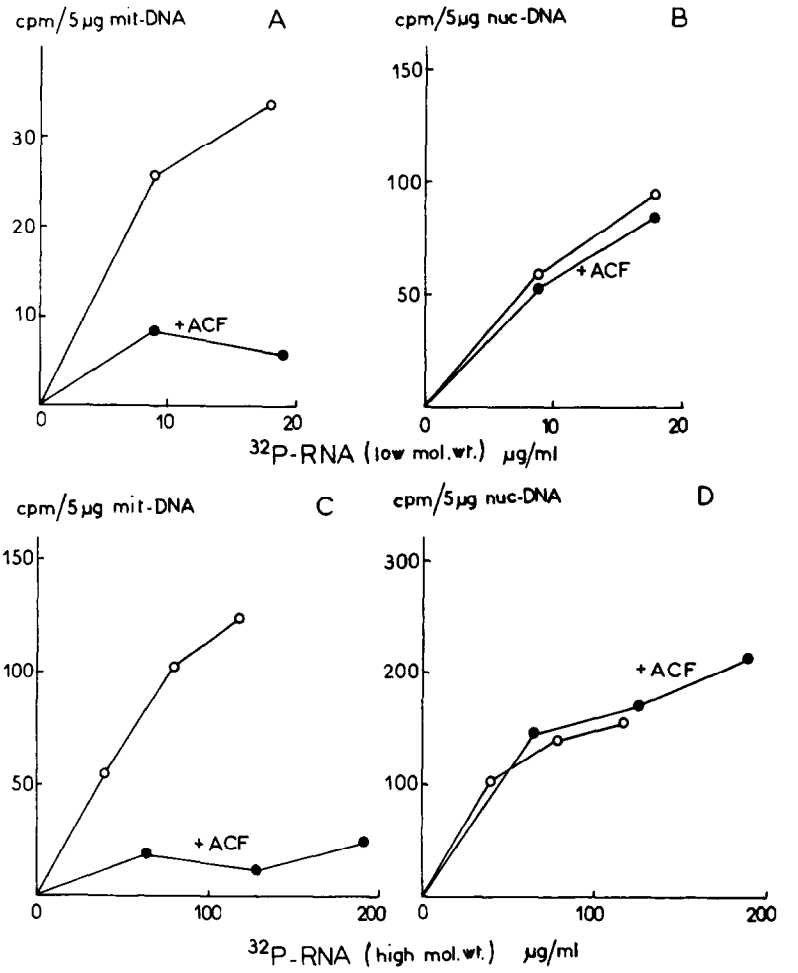


Figure 2 : Hybridization of mitochondrial and nuclear DNA with the RNA synthetized in the presence of acriflavin.

The experimental conditions were identical to those of Figure 1 except that ethidium bromide was replaced by acriflavin ($10^{-5}$M). In A and B, $^{32}$P RNA used was the 0.5M NaCl eluate from the methylated albumin-Kieselguhr column (see Methods), representing mainly the 4S class RNA (cytoplasmic and mitochondrial). In C and D, the 1.2M NaCl eluate was used, which contains mainly ribosomal RNA classes (cytoplasmic and mitochondrial). A and C show the hybridization with mitochondrial DNA, and B and D show the hybridization with nuclear DNA. $^{32}$P counts found on blank filters were approximately 10 counts per minutes which were substracted from experimental values. Specific radioactivities of the RNA were 1630 c/m/µg for the normal RNA and 2240 c/m/µg for the RNA of acriflavin treated cells.

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The effect of EB and of ACF on the formation of the yeast mitochondrial system can be understood as follows : the intercalation of

the dyes into mitochondrial DNA will produce altered DNA copies in DNA replication, on the one hand, and will block the transcription of mitochondrial DNA, on the other. The second effect is enough to explain the very specific inhibition of respiratory adaptation by acridine dyes.

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REFERENCES

ATTARDI G., ATTARDI B., STORRIE B., MATTOCIA L., LEDERMAN M. and ALONI Y.,(1969) in the International Symposium on "Autonomy and Biogenesis of Mitochondria and Chloroplasts",Canberra, Dec.8-13,1969

EPHRUSSI B., HOTTINGUER H. and CHIMENES A.M.,(1949),Ann. Inst. Pasteur, 76, 351

EPHRUSSI B. and HOTTINGUER H., (1951), Cold Spring Harbour Symp.,16,75.

FUKUHARA H., (1967), Biochem. Biophys. Acta, 134, 143

FUKUHARA H., (1968) in Biochemical Aspects of the Biogenesis of Mitochondria (E.C. Slater, J.M. Tager, S. Papa and E. Quagliariello), p. 303, Adriatica Editrice, Bari

FUKUHARA H., FAURES M. and GENIN C.,(1969), Mol.Gen. Genetics,104, 264.

GILLESPIE D. and SPIEGELMAN S., (1965), J. Mol. Biol., 12, 829

MARCOVICH H., (1951), Ann. Inst. Pasteur, 81, 452

SLONIMSKI P., (1953), in Adaptation in Microorganisms, Cambridge, University Press, London.

SLONIMSKI P.P., PERRODIN G. and CROFT J., (1968), Biochem. Biophys. Res. Comm., 30, 232

WERBITZKI F.W., (1910), Zbl. Bakt. Pt.1, Orig. 53, 303

ZYLBER E., VESCO C. and PENNAN S., (1969), J. Mol. Biol., 44, 195