DNA PRECURSORS AND THE ABSENCE OF THYMIDINE KINASE IN YEAST MITOCHONDRIA*

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1. Introduction

The in vitro incorporation of radioactive deoxyribonucleotides into m-DNA has been demonstrated with isolated mitochondria from many different organisms [1–9] including yeast [10,11]. Although deoxyribonucleosides and their mono-, di- and triphosphates were used as precursors for labelling m-DNA in higher eucaryotes, only dXTP was successfully used for labelling yeast m-DNA [10,11]. The absence of dTK in cell extracts of yeast and other microorganisms has been reported [12]. However, the bromodeoxyuridine resistant mammalian cells which also lack the cytoplasmic enzyme, still contain a mitochondrion-specific dTK [13,14]. We therefore asked whether yeast mitochondria contain a specific dTK and whether precursors other than dXTP can be used for the in vitro labelling of the m-DNA. Our results show that yeast mitochondria do not contain any detectable levels of dTK and that among thymine derivatives tested only dTDP or dTTP could be used as effective precursors for the in vitro labelling of m-DNA.

2. Materials and methods

[^{3}H]Adenine (11.2 Ci/mmole), [^{3}H]dT (2 Ci/mmole), [^{3}H]dTTP (20.9 Ci/mmole) and Aquasol were obtained from New England Nuclear; dT, dTMP, dTDP, dTTP, dATP, dCTP and dGTP from PL Biochemicals, PEP and PK were Sigma products, CsCl was the Harshaw Chemical Co. optical grade.

Mitochondria were prepared from the haploid strain of *Saccharomyces cerevisiae*, 273–10b. All operations described in this section were carried out at 3°C. Lactate-grown yeast cells (15 g), harvested in the log phase of growth, were washed twice with distilled water, once with TES, suspended in 10 ml TES and shaken for 15 sec with 50 g 0.5 mm glass beads on a Braun homogenizer (B. Braun, Melsungen, West Germany). After breakage, the pH was adjusted to 7.5 with 1 N-NaOH. Unbroken cells, nuclei and debris were removed by centrifugation at 2000 *g* for 20 min and the crude mitochondrial fraction collected by centrifugation at 17 500 rpm in a Spinco No. 30 rotor with one subsequent wash in TES. For further purification, the pellet was homogenized gently in 5 ml Urografin (methyl glucamine salt of *N,N'*-diacetyl-3,5-diamino-2,4,6,-triiodobenzoic acid) [15], density 1.25 g/cm^3, and overlaid in a centrifuge tube with lighter Urografin solution, density 1.15 g/cm^3. The sample was centrifuged in a Spinco SW 25.1 rotor for 2 hr at 25 000 rpm at 4°C. The purified mitochondrial fraction was collected from the top, while almost one half of the material (denser than 1.15 g/cm^3) remained at the interface between the Urografin solutions. The mitochondrial fraction was

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Abbreviations: dX – deoxyribonucleoside (X = A, C, G, T); DXMP – dXmonophosphate; dXDP – dXdiphosphate; dXTP – dXtriphosphate; dTK – thymidine kinase; m-DNA – mitochondrial DNA; TCA – trichloroacetic acid; PEP – phosphoenol pyruvate; PK – pyruvate kinase; TMS – (10 mM Tris–Cl, 10 mM $MgCl_2$, 0.25 M sorbitol, pH 7.5); TES – (10 mM Tris –Cl, 2 mM EDTA, 0.5 M sorbitol, pH 7.5)

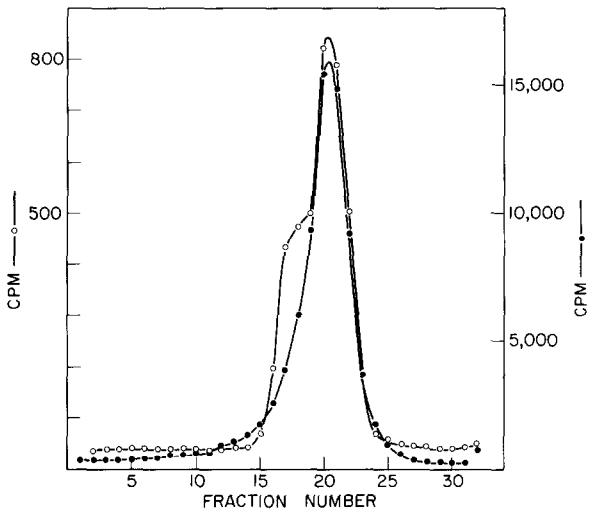

Fig. 1. CsCl-Distamycin A equilibrium density gradients of a) DNA extracted from crude mitochondrial fraction. Cells were labelled in vivo with [^{3}H] adenine and RNA was separated from DNA by apre-spin on a preparative CsCl gradient (o—o—o). b) DNA extracted from Urografin-purified mitochondria labelled in vitro with [^{3}H]dTTP (•—•—•).

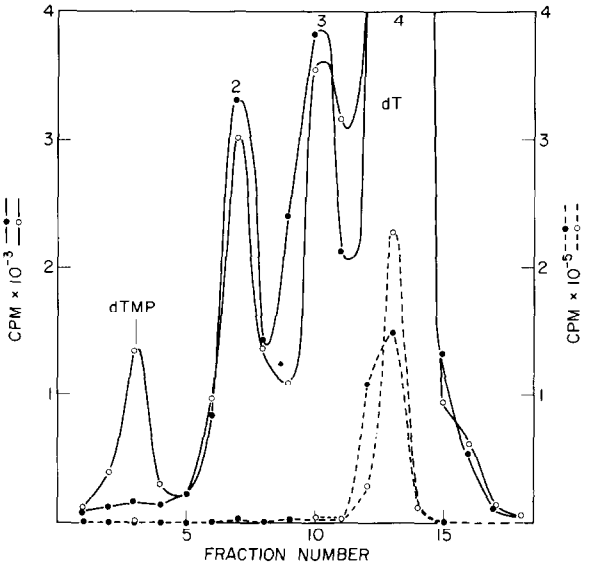

Fig. 2. Thin layer chomatrography of supernatants from dTK assay. Marker spots of dT and dTMP coincide with peaks 4 and 1, respectively. Supernatant of: (o—o—o), rat liver mitochondria, (•—•—•), yeast mitochondria. Origin at fraction 1, solvent front at fraction 23.

washed twice with TES and once with TMS. For the m-DNA analysis on CsCl-Distamycin A (fig. 1), Urografin-purified mitochondria (40 mg protein) were used for incorporation (conditions as described below with PEP and PK). Mitochondria were washed twice with TES, lysed, and the DNA was extracted and dialysed as previously described (16). The DNA solution (2.00 g) was mixed with 1.91 g CsCl and 0.125 mg Distamycin A (Calbiochem), the solution overlayed with mineral oil and the sample centrifuged for 60 hr at 35 000 rpm, 18°C in a SW 50.1 rotor. Fractions equal to 75 μl were collected and diluted with 0.8 ml H_2O and mixed with 10 ml Aquasol for ^{3}H counting (efficiency 30%). To mark the positions of nuclear and m-DNA, we extracted DNA from crude mitochondria prelabelled in vivo with [^{3}H]-adenine [16], removed the labelled RNA by one CsCl isopycnic centrifugation, and analysed the DNA on a CsCl-Distamycin A gradient identical to the one described above.

For dTK assay (fig. 2), mitochondria (0.5 mg protein) of either rat liver [17] or yeast were solubilized in 50 μl volume with 1% Triton, mixed with 50 μl of (10 mM ATP, 50 mM PEP, 80 μg/ml PK, 0.2 mM dithiothreitol, 25 μCi[^{3}H]dT, 10 mM Tris–Cl, 10 mM $MgCl_2$, pH 7.5) and incubated 15 min at 37°C. After that time, the mixture was cooled to 0°C, 50 μl of 30 mM dT and 50 μl of 20% TCA were added. After 30 min at 0°C, the precipitate was separated by centrifugation, and the supernatant neutralized with 20 μl 3 N ammonia. Ten μl of the supernatant was placed on silica gel TLC plate and the plate was chromatographed for 1 hr in isopropanol:NH_3 (3:2 by volume). The chromatogram (80 × 10 mm) was divided into 2.5 × 10 mm fractions; silica gel from each fraction was transferred into counting vials, shaken with 0.8 ml H_2O and then mixed with 10 ml Aquasol for ^{3}H counting. Marker spots of dT and dTMP were detected in UV light. For each point on the curves shown in figs. 3 and 4, mitochondrial protein (2.5 mg) was incubated 20 min at 37°C in 1.5 ml TMS, 33 μM dATP, dCTP, dGTP with 5 μCi[^{3}H]-dTTP. In experiment shown in fig. 3, 0.67 mg/ml PEP and 0.067 mg/ml PK were also present. After incorporation, an excess of cold dTTP was added, and the mitochondria were washed twice with TES, then homogenized into 3 ml of 0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris–Cl, pH 8.0 and precipitated with 3 ml

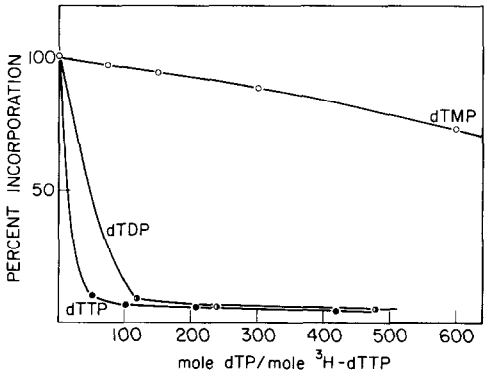

Fig. 3. The effect of thymidine phosphates on incorporation of [^{3}H] dTTP into m-DNA. Incorporation 20 min at 37° C in the presence of PEP and PK, the maximum cpm value (100% incorporation) 20 000 cpm/2.5 mg protein (○ dTMP, ◑ dTDP, ●, dTTP)'

20% TCA (at 0°C). The precipitate was washed 2 × with 5% TCA, dissolved in 1.5 ml 0.33 N NaOH (overnight), mixed with 13 ml Aquasol and the radioactivity was measured on a Beckman LS-250 scintillation counter.

3. Results and discussion

The incorporation results reported here were obtained with mitochondria purified on a discontinuous Urografin gradient [15]. To ascertain that after this purification, precursor molecules are incorporated only into m-DNA and not into possibly contaminating nuclear DNA, we analyzed the labelled DNA on a CsCl-Distamycin A density gradient [18] and compared the profile with that of a DNA extract from crude mitochondria prelabelled in vivo. For better comparison, the two profiles are drawn to the same scale and superimposed in fig. 1 (compare also with fig. 3, ref. [18]). The nuclear DNA contamination is seen as a clear shoulder for DNA extracted from crude in vivo prelabelled mitochondria. The peak of the in vitro labelled DNA is somewhat skewed toward higher densities. This is due to fragmentation of m-DNA during incorporation [19] and separation of m-DNA pieces with density higher than 1.683. Heterogeneity of yeast m-DNA is well described in the literature [20,21]. A more detailed analysis of the in vitro labelled m-DNA will be published elsewhere [19].

Our early experiments showed that [^{3}H] dT was not incorporated into yeast m-DNA in vitro, in contrast to the known behavior of animal mitochondria. This fact in itself, while excluding significant incorporation into contaminating bacteria (which would prefer dT to dTTP), does not answer whether mitochondrial dTK is present or absent because the block could be farther along the pathway. We therefore assayed directly dTK in mitochondrial lysate, taking rat liver mitochondrial lysate as a control since rat liver mitochondria have low but detectable levels of mitochondrial dTK [6]. The dTK was assayed as described in Materials and methods and the results are shown in fig. 2. Peaks 1 and 4 are at positions of dTMP and dT markers, respectively. Peaks 2 and 3 represent unidentified decomposition products of dT which are always present even in fresh samples of [^{3}H] dT. The amount of these impurities increases on storage of [^{3}H] dT at a rate of about 1% a month. As seen, no dTMP is present in a supernatant from yeast mitochondria while it is clearly detectable in the rat liver mitochondrial supernatant. Only about 0.8% of the dT was converted to dTMP by rat liver mitochondria; a finding consistent with the low dTK activity previously observed [6]. It is therefore very likely that yeast mitochondria do not contain any specific dTK.

When the isolated mitochondria are allowed to incorporate [^{3}H] dTTP in the presence of PEP, PK

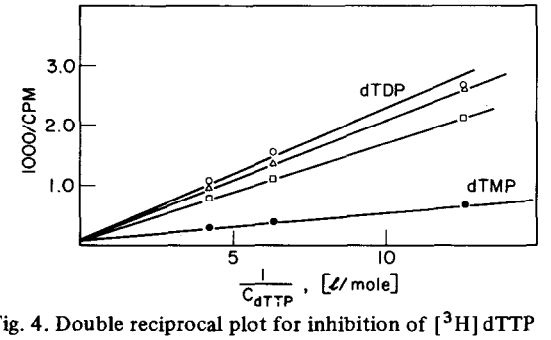

Fig. 4. Double reciprocal plot for inhibition of [^{3}H] dTTP incorporation into m-DNA by dTMP and dTDP in the absence of PEP and PK. Incorporation 20 min at 37°C. dTMP concentrations: (●) 11.9, 47.6, 95.2 μmole/1; dTDP concentrations: (□) 9.6;(△) 38.3; (○) 76.5 μmole/1.

and different concentrations of dTMP, dTDP and dTTP, only dTDP or dTTP significantly suppress the incorporation of the radioactive precursors (fig. 3). The large effect of dTDP and the relatively small effect of dTMP may reflect either competition or inhibition through a non-competitive mechanism. To distinguish between the two alternatives, we varied concentrations of both [^{3}H]dTTP and cold dTMP and dTDP in the absence of PEP and PK (fig. 4). No inhibition of incorporation occurred with dTMP since all nine points fall on a single straight line. The small difference in this respect from the experiment displayed in fig. 3 is probably due to absence of PEP and PK. The dTDP results in fig. 4 are consistent with a competitive mechanism because all lines have an intercept common with that of the dTMP line.

In contrast to mitochondria of higher eucaryotes, isolated mitochondria of yeast can use only dTDP or dTTP as effective precursors for the in vitro labelling of m-DNA. The inhibition of [^{3}H]dTTP incorporation by dTDP is competitive. The presence of a triphosphate group is a structural requirement for binding of a nucleotide to DNA polymerase of *E. coli* [22]. We think therefore that dTDP is phosphorylated to dTTP in our reaction, in agreement with the finding that yeast mitochondria contain nucleoside diphosphokinase [23]. Our results indicate that thymidylate kinase (phosphorylation of dTMP), while perhaps not completely absent in yeast mitochondria, is not very active under the in vitro conditions.

It has often been suggested that dTK is an important regulatory enzyme of DNA biosynthesis, inducible under conditions of rapid growth and DNA synthesis [24]. Its absence in yeast mitochondria as demonstrated here may manifest a significant difference in the regulation of m-DNA biosynthesis between yeast and higher eucaryotes.

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