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Highly efficient DNA synthesis in isolated mitochondria from rat liver

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ABSTRACT

We have developed a highly efficient DNA-synthesizing system with isolated intact rat liver mitochondria. The ATP requirements for this *in organello* DNA synthesis are provided by endogenous synthesis in the presence of exogenous ADP and an oxidizable substrate. In this system, mitochondrial DNA synthesis strikingly proceeds at a constant rate for about 5 h at 37°C. Gel electrophoresis, hybridization and restriction enzyme analyses show that intact mitochondria synthesize nucleic acids with a size of 16.5 kb, that correspond to mitochondrial DNA, and that both DNA strands are replicated. This *in organello* DNA synthesis requires the supply of dNTPs and decreases at high ADP concentration in the incubation medium.

INTRODUCTION

Mammalian mitochondrial DNA (mtDNA) replication is a unidirectional and highly asymmetric process that requires two different origins (for a review see 1). The process begins by the initiation of the daughter heavy-strand (H-strand) synthesis on the displacement-loop (D-loop) region of the genome. When two-thirds of the H-strand has been synthesized, the origin of the light-strand (L-strand) is exposed as a single-stranded template molecule allowing the initiation of the light-strand synthesis.

Most of the knowledge about the mechanism of replication of mtDNA comes from studies of cultured cells. The verification of the model of replication in mammalian differentiated cells is of great interest since it will permit the investigation of the regulation of mitochondrial nucleic acid synthesis in different organs and tissues. However, this study is hampered by the difficulty of experimental manipulations. The mechanism of mtDNA replication can be approached by the construction of systems of DNA synthesis *in vitro*. In this way, earlier studies have shown initiation of the light (L)- and heavy (H)-strands in *in vitro* replication systems (2,3) and in permeabilized mitochondria from cultured cells (4), respectively, and replication of the complete H-strand in mitochondrial lysates of *Xenopus laevis* (5)

To investigate further the molecular mechanisms of mtDNA replication, under conditions that allow easy experimental manipulations, we have developed an *in organello* system for studying DNA synthesis utilizing isolated intact rat liver mitochondria. We have strikingly found that isolated mitochondria are able to synthesize DNA efficiently for at least 5 h. Furthermore, the results obtained strongly suggest that in this system both strands of the mtDNA are replicated.

MATERIALS AND METHODS

Isolation of rat liver mitochondria

The liver of one adult male Wistar rat (200 g) killed by decapitation was rapidly removed, chilled in homogenization medium (0.32 M sucrose, 1 mM K-EDTA, 10 mM Tris–HCl, pH 7.4) and cut in small pieces with scissors. All further operations were carried out at 2–4°C using sterile solutions and glassware. The minced tissue was resuspended in the same medium (approximately 4 ml/g of tissue) and homogenized in a loose-fitting Potter–Elvejhem homogenizer by using 4 up-and-down strokes. The homogenate was spun at 1,000 *g* for 5 min. The supernatant was aliquoted in Eppendorf tubes and centrifuged at full speed (13,000 *g*) for 2 min in a microfuge. The mitochondrial pellets were washed twice in homogenization buffer and pelleted. Finally, the mitochondrial pellet was resuspended in the appropriate incubation buffer (see below) and spun for 1 min. The number of washes employed in this preparation eliminate contaminating cytoplasmic RNA (estimated by the absence of 28S and 18S cytoplasmic rRNA in the electrophoretic patterns). The evaluation of the purity and integrity of the mitochondrial fraction was tested directly by electron microscopy (not shown). The mitochondrial protein concentration was determined by Waddel's method (6).

In organello labeling, isolation and analysis of mitochondrial DNA

Samples of the mitochondrial fraction (1 mg of mitochondrial protein) were resuspended in 0.5 ml of incubation buffer which contained 25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM K_2HPO_4, 0.05 mM EDTA, 5 mM $MgCl_2$, 1 mM ADP, 10 mM glutamate, 2.5 mM malate, 10 mM Tris–HCl, pH 7.4, 1 mg of fatty acid-free bovine serum albumin (BSA) per ml, 50 μM each of dATP, dGTP, and dTTP and 20 μCi of

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[α-^{32}P]-dCTP (800 Ci/mmol) in Eppendorf tubes. Unless otherwise specified, incubation was at 37°C for 5 h in a rotary shaker. After the incubation, the mitochondrial samples were pelleted at 13,000 *g* for 1 min and washed twice with 10% glycerol, 10 mM Tris−HCl, pH 6.8, 0.15 mM $MgCl_2$. Mitochondria lysis and nucleic acid extraction were carried out as described before (7). Nucleic acids were analyzed directly or after digestion with DNase-free RNase and the restriction enzymes *Bam*HI or *Eco*RI, by electrophoresis in 1% agarose slab gels. After the run, for analytical purposes, the gels were first stained with ethidium bromide, photographed under UV light and then dried and exposed for autoradiography at −70°C.

Synthesis of riboprobes and hybridization

To prepare single-stranded hybridization probes, we used a fragment of rat mtDNA containing part (855 bp) of the COI gene, that was cloned in the plasmid vector Bluescribe (8) which has the T3 and T7 RNA polymerase promoters next to the polylinker cloning sites. To obtain transcripts from both strands of the fragment, 1 μg of recombinant plasmid was linearized upstream or downstream of the cloned fragment and then incubated alternatively with 20 units of T7 or T3 RNA polymerases in reaction mixtures containing 0.5 mM of each of the four NTPs, 8 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl, 10 mM dithiothreitol, 20 units RNasin, 40 mM Tris−HCl, pH 8.0. The incubation was for 1 h at 37°C. At the end of the reaction, 30 units of RNasin, 20 μg/ml of RNase-free DNase and 20 μg of tRNA carrier were added. After incubation for 10 min at 37°C, the samples were phenol extracted and ethanol precipitated in the presence of 2 M ammonium acetate, for several times. The RNA pellet was resuspended in T.E. (10 mM Tris−HCl, pH 7.5, 1 mM EDTA). The correct synthesis of RNAs with a size corresponding to the insert was checked by electrophoresis in 1% agarose gels and ethidium bromide staining.

Cold RNA (riboprobes), synthesized as indicated above, were blotted directly and fixed onto nitrocellulose filters and hybridized with newly synthesized labeled mtDNA previously treated with DNase-free RNase and *Bam*HI. Prehybridization, hybridization and washing of the filters were performed as already reported (9).

RESULTS

DNA synthesis in isolated intact mitochondria

We have previously shown that isolated intact rat liver mitochondria when incubated in an appropriate medium transcribe mtDNA in a way that closely resembles the *in vivo* process (10,11). Furthermore, RNA synthesis occurred efficiently for more than 3 h and among its products we could detect the presence of 7S RNA considered to be the primer of the synthesis of the H-strand of the mtDNA (10,12,13, and manuscript in preparation). These facts prompted us to test if intact mitochondria could replicate their DNA under the same conditions of incubation. Therefore, mitochondria were purified from rat liver and incubated in a basic medium in the presence of 1 mM ADP, glutamate, malate, phosphate and [α-^{32}P]-dCTP at 37°C for 5 h. The labeled nucleic acids were extracted and analyzed by electrophoresis in a 1% agarose slab gel (Figure 1A, lane b). The radioactivity was distributed in three different areas of the gel. The first band was located in the upper part of the gel, close to the origin of the lane. The second band migrated to a region corresponding to a size of 16.5 kb. Finally there were two bands at the bottom of the gel with a size of 200−400 bp. When the samples were heated at 95°C for 10 min before loading of the gel, the band in the upper part of the gel moved to a position equivalent to approximately 25 kb (Figure 1A, lane a). These data suggest that the band migrating to a position corresponding to around 16.5 kb represets open-circular mtDNA, while the slow moving bands represent concatenated or circular oligomers (14). All the components separated by electrophoresis in these experiments were completely digested when treated with DNase (Figure 1B, lane d) indicating the DNA nature of all the bands. In agreement with these results, RNase digestion before electrophoretic analysis did not alter the mobility of the bands (Figure 1B, lane e).

When the DNA that was synthesized *in organello* was digested with restriction endonucleases (*Bam*HI and *Eco*RI), the restriction pattern of the labeled products was that expected for rat mtDNA (Figure 1B, lanes b and e) and identical to that obtained by ethidium bromide staining of purified rat mtDNA treated with the same enzymes (not shown). As shown, both the bands of

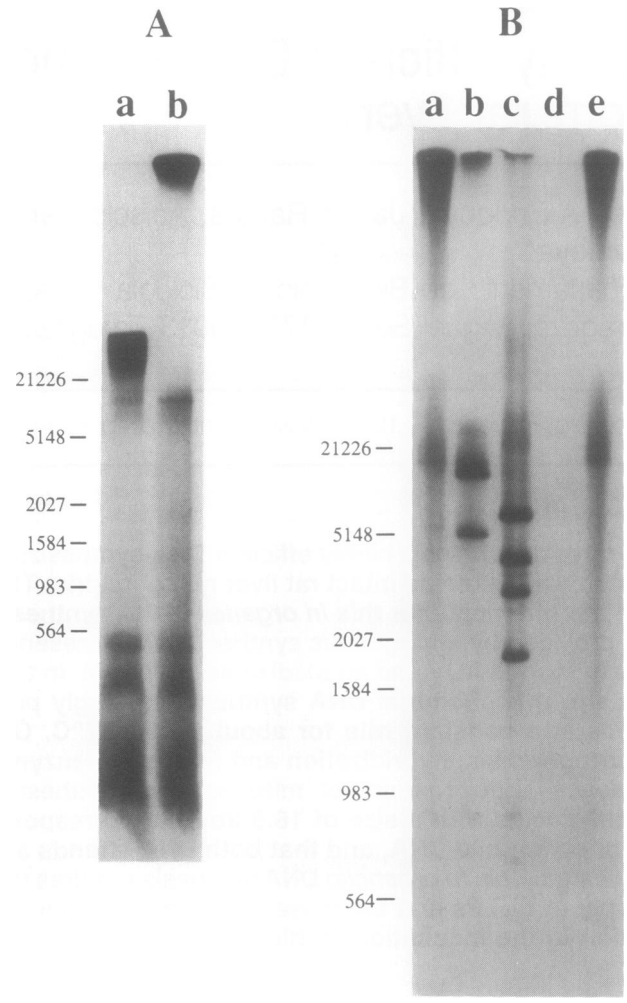

Figure 1. DNA synthesis *in organello*. Autoradiograms after electrophoresis through agarose slab gels of mtDNA synthesized in intact isolated mitochondria incubated under the standard conditions described in Materials and Methods. (**A**) Newly synthesized DNA before (lane b) and after treatment at 95°C for 10 min (lane a). (**B**) Newly synthesized DNA (lane a) treated with *Bam*HI (lane b), *Eco*RI (lane c), RNase-free DNase (lane d) and DNase-free RNase (lane e). Numbers on the left of each panel indicate size of DNA markers in bp.

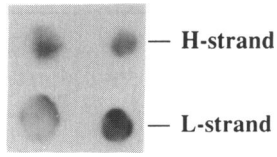

Figure 2. Analysis of the strand polarity of newly synthesized mtDNA by dot-blot hybridization. Cold riboprobes corresponding to each of the two DNA strands of a fragment of rat mtDNA containing part of the COI gene, cloned in Bluescribe, were synthesized as indicated in Material and Methods, fixed onto nitrocellulose filters and hybridized with labeled mtDNA synthesized *in organello* . H- and L-strand indicate the strand from which the riboprobe present in the nitrocellulose membrane was synthesized. Blots from two different experiments are shown. No hybridization was observed when yeast tRNA was used as a control (not shown).

the upper part of the gel and that of 16.5 kb were digested by the restriction enzymes originating the expected fragments, indicating therefore that both bands were composed of mtDNA.

In order to check if both strands of the mtDNA were synthesized *in organello*, the newly synthesized labeled mtDNA was treated with RNase and *Bam*HI. After phenol extraction and ethanol precipitation, the mtDNA fragments were denatured and blot-hybridized to specific mitochondrial cold riboprobes corresponding to each of the two strands that were fixed to the hybridization membrane. As shown in Figure 2, mtDNA hybridized with both H- and L-strand derived riboprobe.

Effect of incubation conditions on DNA labeling in isolated mitochondria

The synthesis of full-length daughter strands requires approximately 1 h and the entire cycle is completed in approximately 2 h (1). Therefore, it was important to study the effect of various times of incubation on the synthesis of mtDNA in isolated organelles. The results strikingly showed that, at 37°C, the incorporation of [^{32}P]-dCMP into mtDNA was linear for 5 h and then plateaued (Figure 3A). As shown in the electrophoretic analysis of intact mtDNA and after digestion with *Bam*HI (Figure 3B), this incorporation was due to synthesis of mtDNA. These analyses also show that after short periods of time of incubation the band migrating at approximately 16.5 kb was proportionally more labeled than the upper band. In addition, after digestion with *Bam*HI one can see that only one band, that containing the origin of H-strand was labeled (the upper band represents undigested DNA). However, after 1 h of labeling the two mtDNA bands originated by *Bam*HI digestion were visible. We have interpreted these results as a progressive synthesis of DNA starting at the D-loop region.

Different previous studies have proposed that the presence of Mg^{2+} and/or Mn^{2+} ions in the incubation medium is required for the activity of the various enzymes associated with the replication of mtDNA (15). Therefore, the effect of various concentrations of $MgCl_2$ and $MnCl_2$ on the *in organello* labeling of mtDNA was also analyzed. As shown in Figure 4, increasing concentrations of $MgCl_2$ stimulated progressively the incorporation of [^{32}P]-dCMP into mtDNA to reach a maximum (corresponding to a 5-fold increase) at 5 mM; at higher concentrations the stimulatory effect decreased and then remained constant at 2-fold up to 15 mM. The use of increasing concentrations of $MnCl_2$ instead of $MgCl_2$ produced an increase of incorporation of label (measured as precipitated cpm) of more than 150-fold

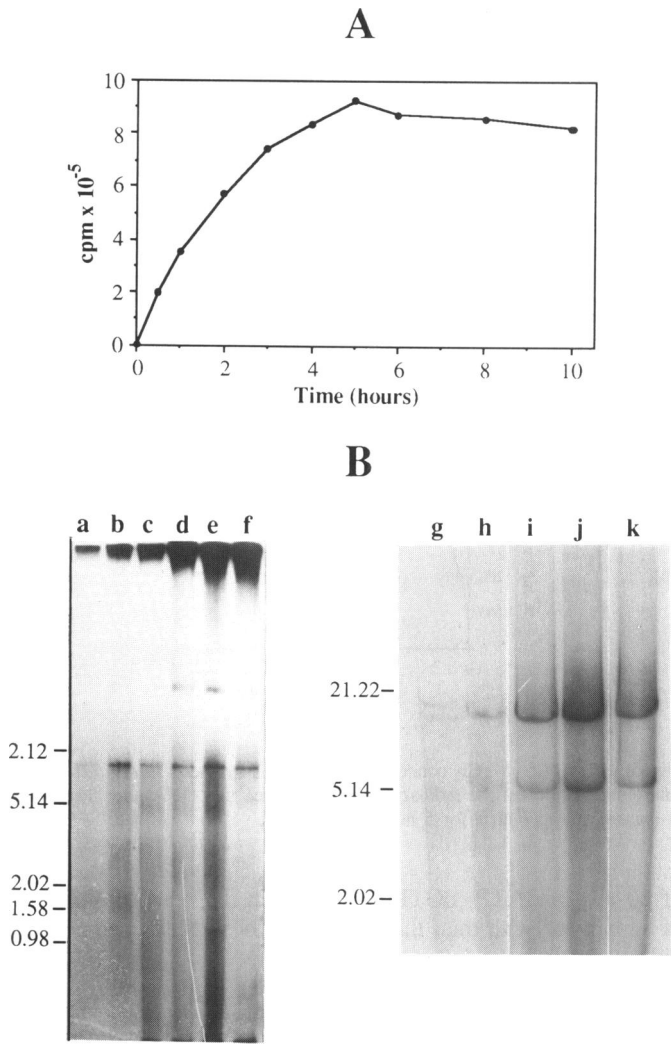

Figure 3. Time course of mtDNA synthesis *in organello*. (**A**) Radioactivity incorporated into mtDNA after different times of incubation at 37°C. (**B**) Autoradiogram after electrophoresis through agarose slab gels of intact mtDNA (lanes a–f) and mtDNA digested with *Bam*HI (lanes g–k) after 0.5 (lanes a and g), 1 (lanes b and h), 2 (lane c), 3 (lanes d and i), 5 (lanes e and j), and 6 h (lanes f and k) of incubation. Numbers on the left indicate size of DNA markers in kbp.

between 0 and 5 mM (not shown). However, the electrophoretic analysis of the newly synthesized products showed that this increase did not correspond to *in organello* synthesized mtDNA. Furthermore, the comparison of the autoradiograms of the mtDNA synthesized in the presence of $MgCl_2$ and $MnCl_2$ showed that the level of incorporation of the label into mtDNA was higher using Mg^{2+} salts (data not shown).

Priming of mtDNA replication at O_H could depend on short RNA primers that are synthesized from the L-strand transcription promoters (12,13). Therefore, it could be expected that the presence of both NTPs and dNTPs in the incubation medium were necessary for maximum incorporation of radioactivity into mtDNA. To study this effect, we added the four NTPs and/or the dNTPs except the labeled precursor. As shown in Table I, the addition of 50 μM of each of the four NTP did not change the incorporation of radioactivity into DNA. In contrast, DNA synthesis was strongly stimulated (2.5–3 times) by the presence

Table I. Requirements for *in organello* DNA synthesis

	[α^{32}P]-CMP incorporation (%)
Optimal	100
Omit dNTPs (dATP, dGTP, dTTP)	25
Omit dNTPs; Add NTPs	16
Add NTPs	106

DNA replication assays were carried out as described in Materials and Methods. Optimal conditions are 25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM K_2HPO_4, 0.05 mM EDTA, 5 mM $MgCl_2$, 1 mM ADP, 10 mM glutamate, 2.5 mM malate, 10 mM Tris–HCl, pH 7.4, 1 mg of fatty acid-free bovine serum albumin (BSA) per ml, 50 μM each of dATP, dGTP, and dTTP and 20 μCi of [α-^{32}P]-dCTP at 37°C for 5 h in a rotary shaker. The incubation mixture (0.5 ml) contains 1 mg of mitochondrial protein.

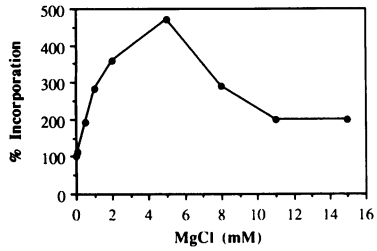

Figure 4. Effect of $MgCl_2$ concentration on DNA synthesis *in organello*. The data plotted represent the radioactivity incorporated into DNA after incubation of isolated mitochondria for 5 h at 37°C.

of 50 μM of dATP, dGTP and dTTP in the reaction mixture. In agreement with this, the addition of both NTPs and dNTPs did not change the latter value.

The effect of varying the mitochondrial concentration on *in organello* labeling of mtDNA is shown in Figure 5. The incorporation of label into mtDNA increased to reach a maximum at 2 mg of mitochondrial proteins per ml.

Figure 6A shows that *in organello* synthesis of mtDNA is dependent on the concentration of ADP in the incubation medium. Thus, increasing concentrations of ADP stimulated DNA labeling in isolated mitochondria with a maximum at 0.5–1 mM. However, at higher concentrations there was a progressive inhibition of the labeling. The mtDNA labeled in the presence of different ADP concentrations was digested with *Bam*HI and subsequently analyzed by gel electrophoresis (Figure 6B). As shown, in the absence of ADP only the upper band, corresponding to a fragment of mtDNA of 11,226 bp containing the origin of replication of the H-strand, was detected (lane a). At higher concentrations, the pattern of labeled material is identical to that of the *Bam*HI digests of rat mtDNA, although at 0.1 mM the lower band was still very faint.

DISCUSSION

The experiments described in this work show that isolated intact mitochondria from rat liver are able to support efficient mtDNA synthesis. Furthermore, the results obtained strongly suggest that in this system both strands of the mtDNA are replicated. Earlier studies with isolated intact mitochondria from chicken and rat liver did not exhibit an adequate rate of incorporation of DNA precursors into mtDNA and the analysis of the replication

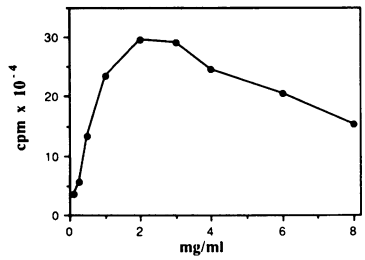

Figure 5. Effect of mitochondrial concentration on *in organello* synthesis of mtDNA. Radioactivity incorporated into mtDNA in assays utilizing various concentrations of mitochondria.

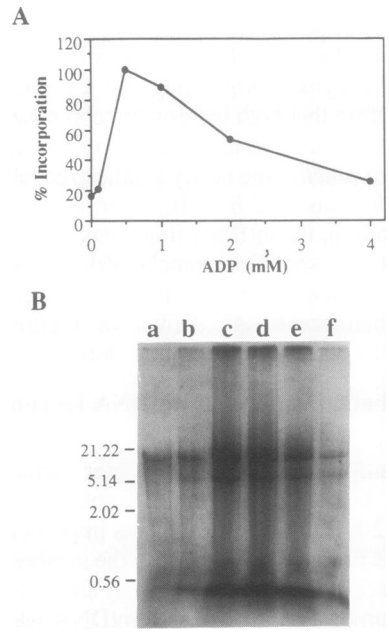

Figure 6. Effect of ADP concentrations on DNA synthesis *in organello*. (**A**) Radioactivity incorporated into mtDNA at different ADP concentrations. (**B**) Autoradiogram after electrophoresis through agarose slab gels of a *Bam*HI digestion of newly synthesized mtDNA labeled in the presence of 0, 0.1, 0.5, 1, 2, and 4 mM ADP (lanes a–f, respectively). Numbers on the left indicate size of DNA markers in kbp.

products was done by equilibrium centrifugation in CsCl (16–18).

The success of the method employed here may be due to the efficiency of the incubation medium used, an isotonic incubation buffer in which the energy requirements are provided by exogenous ADP in the presence of an oxidizable substrate (glutamate plus malate). This medium has previously been proved to be also very efficient for mtDNA transcription and RNA processing in isolated organelles (10, and manuscript in preparation) and to work better for mitochondria isolated from differentiated cells than other media described before (19,20). In fact, in this incubation medium, and with the smooth but continuous agitation provided by a rotary shaker, the mitochondria are maintained functional for very long periods of time allowing the replication to be linear up to 5 h. It is quite

probable that such a long incubation time allows the replication of both DNA strands to be completed.

Evidence that the *in organello* synthesis analyzed here was the result of DNA replication and not of DNA repair comes from the facts that the incorporation of label was linear with the time of incubation (5 h), and that the restriction analysis of DNA shows a progressive labeling of the fragments at different times of incubation and ADP concentrations. This, together with previous evidence in other systems (permeabilized mitochondria and mitochondrial lysates) (4,5), points out that DNA synthesis is due to true replication.

MtDNA synthesis in isolated organelles is dependent to a considerable extent on an external ADP source. Thus, in the absence of ADP the efficiency of mtDNA synthesis is very low, due to the low level of intramitochondrial ATP, resulting in a lack of elongation of the nascent chains. Therefore, only one fragment, that containing the origin of replication of the H-strand, is visible in the *Bam*HI restriction endonuclease digestion pattern. As the concentration of ADP increases, the intramitochondrial ATP concentration would be higher and the whole molecule could be replicated with the consequent appearance of the two *Bam*HI fragments.

The system described here is also dependent on the presence of dNTPs. The absence of three of them reduces the synthesis to 25%; this residual synthesis can be assigned to endogenous dNTP pool. This dependence was also found in the synthesis of mtDNA produced in mitochondrial lysates (5). However, the addition of the four NTPs does not modify the incorporation of label into mtDNA. This result contrasts with that previously obtained with permeabilized mitochondria where the synthesis of mtDNA was dependent on the presence of NTPs in the incubation medium (4). This is likely due to the high intramitochondrial NTP pool in intact isolated mitochondria. In fact, transcription in isolated mitochondria proceeds for long periods of time without the addition of exogenous ribonucleotides (10, and manuscript in preparation).

In contrast to previous work with mitochondrial lysates (5), our results show that the presence of magnesium ions in the incubation buffer is more efficient than manganese ions for the synthesis of mtDNA as measured by electrophoretic analysis. The high increase of radioactivity incorporated in isolated mitochondria in the presence of increasing concentrations of $MnCl_2$ (measured as precipitated cpm) could be due to precipitation of unincorporated nucleotides in the presence of Mn salts and was not accompanied by any specific increase of the DNA bands in the autoradiogram. Therefore, it can be concluded that the determination of precipitated cpm is not a reliable method to estimate the incorporation of labeled precursor into nucleic acids in *in organello* DNA synthesis and that, whenever is possible, electrophoretic analysis and subsequent quantitation of the autoradiogram should be carried out.

In this work we have not analyzed the existence of initiation events although it is likely that these are achieved in this system as it has been shown earlier in permeabilized mitochondria and in mitochondrial lysates (4,5,16). The synthesis of 7S RNA, primer of the synthesis of the H-strand of the mtDNA (12,13), also found in transcription systems with isolated organelles (10), and the long period of linear incorporation of radioactivity into mtDNA, which doubles the estimated time of replication (1), points in the same direction. The possible relation of the two smallest bands obtained in the electrophoretic pattern of the replication products with nascent DNA strands remains to be investigated.

The efficiency of mtDNA synthesis in isolated organelles, without the constraints imposed by the nucleo-cytoplasmic compartment, described in this paper make this system very valuable for future studies on the mechanisms of mtDNA replication and on the effect that biological variability may have in the regulation of DNA synthesis.

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