DNA-NUCLEOTIDYL TRANSFERASE ACTIVITY IN SUPERNATANT AND MEMBRANE FRACTIONS FROM NORMAL AND REGENERATING RAT LIVER MITOCHONDRIA

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Received 2 June 1969

1. Introduction

Since the demonstration of DNA in mitochondria [1–3], there has been considerable study of its biochemical role. It is now relatively certain that mitochondrial DNA has the capacity to both transcribe [4–6] and translate [7,8]. It has also been shown that incorporation of precursors into mitochondrial DNA proceeds continuously and at a greater rate than that of the nuclear DNA incorporation. Nass [9] has reported that mitochondrial DNA incorporation proceeds at 18 times the rate of the nuclear incorporation in the 12 hr regenerating rat liver. He has also shown that incorporation of $^{32}P_i$ into mitochondrial DNA increases 8–10 fold in the 24 hr regenerating liver as compared to the non-regenerating (normal) liver. It seemed likely that enzymes of DNA synthesis were involved in this incorporation. We have previously reported the presence of a DNA-dependent DNA-nucleotidyl transferase which had been extracted and partially purified from normal and regenerating rat liver mitochondria [10]. We now report that system in more detail and give new data showing the following: (1) The specific activity of the sonicate extract from mitochondria of regenerating rat liver is 5–10 fold greater than that from the non-regenerating liver mitochondria; (2) The specific activity of the mitochondrial enzyme extracted from the very young, actively growing, rat liver is 2–3 times greater than that from the adult; (3) The transferase was found associated with the mitochondrial membranes, and a primer-enzyme-membrane complex is suggested. The similarity to bacterial DNA replication is discussed.

2. Methods

2.1. *Enzyme extraction and purification*

Mitochondria (from normal or regenerating rat liver) were suspended in 0.02 M tris-HCl, 0.01 M EDTA, 0.05 M mercaptoethanol (Buffer A, pH 7.5) and sonicated for one minute on a Biosonik Ultrasonicator (model BPI). KCl was added to a concentration of 0.15 M and the sonicate centrifuged at 40 000 RPM on the Spinco Model L Ultracentrifuge (Rotor #50) for 1 hr. The resulting supernatant was used as the enzyme source unless otherwise indicated. All procedures were carried out in the cold (4°C).

2.2. *DNA-nucleotidyl transferase assay*

The enzyme incubation and radioactivity determination were carried out according to the procedures of Keir [11,12]. The standard reaction mixture was as follows: 5 μmoles tris-HCl (pH 7.5); 15 μmoles KCl; 100 mμ moles EDTA; 2 μmoles $MgSO_4$; 1 μmole mercaptoethanol; 50 mμ moles each of d-ATP, d-CTP, d-GTP (Sigma); 50 mμmoles ^{3}H-TTP (Schwarz) (4.8 c/m mole, diluted with cold TTP to give 1×10^6 dpm per 50 mμmoles used in assay); DNA primer, 10–100 μg of calf thymus DNA (Sigma); mitochondrial supernatant enzyme, 100 to 300 μg protein. The components were incubated at 37°C, cooled to 0°C, and the DNA material precipitated by addition of 1.5 ml cold H_2O, 0.2 ml heat denatured herring sperm DNA, and 0.5 ml perchloric acid (6%). The entire suspension was filtered through a Millipore Filter Holder containing 2 filter discs (Fiberfilm T-20A-60 teflon-coated fiber filter paper, Bel-Art Products, Pequannock, N.J.)

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and the filter discs washed four times with cold 0.2 N perchloric acid, followed by 100% ethanol. The discs were then placed in vials containing 0.5 ml hyamine hydroxide and 10.0 ml scintillation fluid. The vials were heated at 57°C for 10 min, cooled and radioactivity determined in a Packard-Tri Carb liquid scintillation spectrophotometer (6% efficiency for ^3H).

2.3. *Preparation of mitochondrial membrane fraction*

In a number of preliminary experiments, the mitochondrial membranes were tested for DNA-nucleotidyl transferase activity. The membrane fraction was prepared according to the method of Wheeldon [8]. Washed mitochondria were suspended in a medium of 250 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, and 50 mM tris-HCl, pH 7.6 (1 g mitochondrial protein/10 ml medium). This suspension was homogenized with 10% sodium deoxycholate (10% volume of homogenate) and layered on a discontinuous sucrose gradient (1.5 M and 0.5 M). The gradient was spun at 40 000 RPM for 1 hr (Spinco Rotor #SW39). The membrane fraction (interface) was removed and suspended in Buffer B (Buffer A plus 0.15 M KCl). Protein in these fractions was determined by the method of Lowry et al. [13].

3. Results and discussion

Sonic extracts of mitochondria from normal and regenerating rat liver contain DNA-nucleotidyl transferase activity. Table 1 lists the effects of some additions or deletions to the complete enzyme system from both normal and regenerating rat liver. Maximal activity requires a DNA primer (native DNA is utilized nearly as well as thermally denatured DNA), Mg^{++}, and the presence of all three nonradioactive deoxynucleoside triphosphate. This last point is taken as evidence for a replicative type of transferase. DNA-ase incubated with the complete system destroyed all of the activity as did boiling the enzyme. Other workers have shown relatively similar requirements for incorporation of radioactive precursors into mitochondrial DNA material [14,15] or for purified mitochondrial DNA polymerase activity [16–19].

Table 1
DNA nucleotidyl transferase: requirements for normal and 24 hr regenerating rat liver mitochondrial enzyme.

Assay mixture	% Standard assay
Complete	100*
Complete minus DNA primer	30
Complete minus dATP, dCTP, dGTP	15
Complete native DNA as primer	80–90
Complete minus Mg^{++}	30
Complete (DNA-ase added)	<5
Complete (enzyme boiled)	<5

* 100% standard assay activity for the normal transferase was in the range of 0.8–1.5 units; for the 24 hr regenerating transferase the range was 6.3–16.9 units.

Fig. 1 is a concentration curve for the transferase extracted from liver mitochondria of older rats (285–320 g); young rats (100–120 g), and hepatectomized rats (24 hr post hepatectomy). In these assays denatured calf thymus DNA was used as a primer. The enzyme activity is highest in the 24 hr regenerating liver extract (12 units), and lowest in the normal (non-regenerating) extract from the older rats (1.2 units) (one unit is equivalent to 1 mμmole ^3H-TMP residue incorporated per mg mitochondrial protein per hour). This difference in enzyme activity parallels the data on $^{32}P_i$ incorporation into DNA [9]. Fig. 1 also shows a higher enzyme activity in the extract

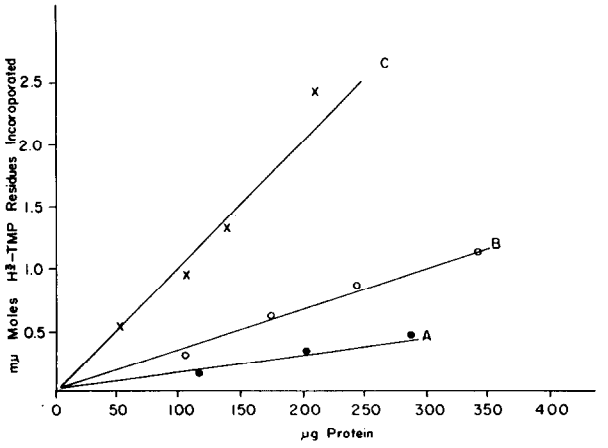

Fig. 1. DNA-nucleotidyl transferase: Concentration curve A, from liver mitochondria of old rats (285–320 g); B, from liver mitochondria of young rats (100–120 g); C, from liver mitochondria of hepatectomized rats (24 hr, post hepatectomy).

Table 2
DNA-nucleotidyl transferase of mitochondrial membrane fraction.

Experiment	mμmoles ^{3}H-TMP residues incorporated/hr/mg mitochondrial protein		
	Endogenous primer	Exogenous primer	
		denatured calf thymus DNA	native calf thymus DNA
1	6.3	3.2	3.0
2	6.0		2.8
3	4.76	2.4	

from young, actively growing rats (3.2 units) as compared to the old rats. This indicates that the more actively growing the tissue, the greater the rate of mitochondrial DNA synthesis, and this is reflected in the order of transferase units (i.e., regenerating: young:old).

The enzyme activity has been localized on the mitochondrial membranes. Table 2 (experiment 1) shows that a membrane fraction from normal liver mitochondria gave a specific activity of 6.3 units (endogenously primed). This activity is much higher than that of the comparable sonicate enzyme (i.e., 1.2 units). When exogenous DNA primer was added, it was inhibitory to the enzyme. These results are similar to those obtained with a bacterial DNA-DNA polymerase-membrane complex [20], and suggest that a membrane-DNA-DNA nucleotidyl transferase complex may be the replicating unit in both mitochondria and bacteria [20–22]. Preliminary analysis has qualitatively identified the presence of DNA in these membrane fractions. A similar mechanism of bacterial and mitochondrial DNA replication is another piece of evidence suggesting a bacterial-mitochondrial homology [3].

Acknowledgements

We thank Mrs. Virginia Morris for skilled technical assistance, and Dr. Maher Mansour for thoughtful discussion. S.R.S. thanks Professor H.M.Keir for help and advice on measuring DNA nucleotidyl transferase.

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