DNA SYNTHESIS BY A MEMBRANE-DNA COMPLEX FROM RAT LIVER MITOCHONDRIA

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A membrane-DNA complex isolated from Sarkosyl lysates of inner membrane-matrix preparations from rat liver mitochondria has been shown to carry out the synthesis of mitochondrial DNA *in vitro*. The incorporation of (^{3}H)dTTP is completely dependent upon Mg^{2+}, the endogenous mitochondrial DNA template, and four deoxynucleoside triphosphates. A number of inhibitors of DNA synthesis completely suppress the incorporation by the membrane-DNA complex. The incorporation of (^{3}H)dTTP takes place into the same types of replicative intermediates of mtDNA which are labeled when incorporation is allowed to take place in intact mitochondria *in vitro* or *in vivo*.

The M-band technique developed by Tremblay *et al*. (1) for the isolation of DNA associated with cellular membrane fractions has been used to demonstrate an attachment of (a) bacterial DNA to the cell membrane (2); (b) bacterial plasmid (3,4) and phage DNA (5-7) to the host cell membrane; (c) eucaryotic chromosomal DNA to the nuclear membrane (8,9); and (d) adenovirus and SV40 virus DNA (10,11) to the host nuclear membrane. In most of the systems studied the membrane-DNA complexes appear to contain the replicating portion of DNA but not the bulk of the non-replicating DNA. The isolation of a membrane-DNA complex by this method depends on the interaction of membrane phospholipid components with crystals of the detergent sodium N-lauroyl sarkosinate formed in the presence of magnesium ions (1,12). The presence of DNA in the M-band fraction results from its attachment originally to the membrane, since nucleoproteins or nucleic acids alone do not react with the Mg-Sarkosyl crystals (1,12).

We have reported the partial characterization of a membrane-DNA complex

from Sarkosyl lysates of inner membrane-matrix preparations from normal, 22-hr regenerating and fetal rat liver mitochondria (13). The percentage of total mtDNA[1] of the lysate present in the M-band was 4-fold higher in the complexes from 22-hr regenerating and fetal rat liver mitochondria than in normal mitochondria, whereas the percentage of membrane protein from all three sources was the same. The increased attachment of mtDNA to the inner membrane of mitochondria from actively proliferating tissues and the variation in the amount of DNA attached at different periods of time during the regenerative response suggested a possible functional significance for the attachment of mtDNA to the membrane during replication (13).

We report here on the ability of membrane-DNA complex from rat liver mitochondria to synthesize mtDNA in vitro when supplied with deoxynucleoside triphosphates and magnesium ions.

METHODS

Rat liver mitochondria and inner membrane-matrix fractions were prepared as previously described from this laboratory (13). The isolation and characterization of the membrane-DNA complex has been reported in detail (13) and will only briefly be described here. Sarkosyl NL-30 (10 µl) was layered on a discontinuous sucrose gradient containing 13.5 ml of 15% sucrose-TMK buffer (10 mM Tris-HCl, pH 7.4, 10 mM magnesium acetate, 0.10 M KCl) overlayered on 13.5 ml of 40% sucrose-TMK buffer. Three milliliters (15 mg protein) of inner membrane fraction were suspended in 8.5% sucrose-TMK buffer, layered over the Sarkosyl, and gently mixed with the detergent to lyse the membranes with minimum shearing of DNA. The gradient was immediately centrifuged at 13,000 rpm for 15 min in a Spinco SW 25.1 rotor. The Mg^{2+}-Sarkosinate containing the membrane-DNA complex was visible as a sharp white band at the interface of the 15% and 40% sucrose. Unless otherwise indicated, the complex was obtained by removing the first 12.5 ml of the gradient dropwise from the bottom of the tube and then collecting the complex as a single 3-ml fraction. The complexes from multiple gradients were pooled and exhaustively dialyzed for 21 hours against 20 mM Tris pH 8.5, 20% glycerol to remove Sarkosyl, which is inhibitory to DNA polymerases. Membrane-DNA complex prepared from normal rat liver mitochondria in this manner has been shown to be comprised of a small fragment of the inner mitochondrial membrane (14% of the total mitochondrial inner membrane-matrix protein) to which is attached 20% of the total DNA of the organelle (13).

Protein was determined by the biuret (14) or Lowry methods (15) with crystalline bovine serum albumin as the reference. DNA was determined by the diphenylamine method (16) after washing the samples with trichloroacetic acid to remove sucrose. DNA was isolated by SDS and pronase digestion (17).

RESULTS AND DISCUSSION

Fig. 1 shows a sucrose gradient profile of a typical membrane-DNA complex

1. Abbreviations used: mtDNA, mitochondrial DNA; SDS, sodium dodecyl sulfate.

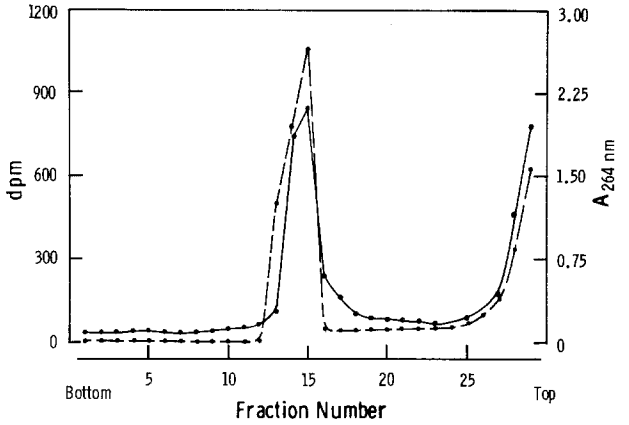

Fig. 1. Isolation of a membrane-DNA complex from normal rat liver mitochondrial inner membrane-matrix preparations. Inner membranes were incubated for 40 min at 37° in vitro in a reaction mixture containing per ml: Tris·HCl, pH 7.4 (50 μmoles); $MgCl_2$ (10 μmoles); pyruvate (7.5 μmoles); KCl (150 μmoles); three unlabeled deoxynucleoside triphosphates (15 nmoles each); (^{3}H)dTTP (10 μCi/15 nmoles); inner membranes (2 mg protein). The final volume was 5 ml. The inner membranes were isolated from the incubation medium by centrifugation at 12,500 g for 10 min and suspended in 8.5% sucrose in 10 mM Tris·HCl, pH 7.4, 10 mM $MgAc_2$, 0.1 M KCl buffer at a concentration of 3.3 mg membrane protein per ml. Aliquots of 3 ml were layered with Sarkosyl on discontinuous sucrose gradients and the membrane-DNA complex was isolated as described in Methods. Fractions (1 ml) of the gradient were collected dropwise from the bottom of the tube. The fractions were warmed to 37° to dissolve the Mg^{2+}-Sarkosyl crystals, and after appropriate dilution were read at A264 nm. The acid insoluble material in each fraction was precipitated in 10% TCA, and the precipitates were collected on filter paper discs, washed and counted as described in the legend for Table I. --------, $A_{264\ nm}$; •————•, dpm.

from an inner membrane-matrix preparation previously allowed to synthesize DNA in the presence of (^{3}H)dTTP. The profiles for membrane and labeled DNA show coincident peaks at the position of the Mg^{2+}-Sarkosyl crystals. This membrane-DNA complex incorporates (^{3}H)dTTP into mtDNA when incubated with (^{3}H)dTTP, 3 unlabeled deoxynucleoside triphosphates and Mg^{2+} in vitro (Table 1). Proof that the (^{3}H)dTTP is incorporated into mtDNA is presented in a later section. The incorporation is almost completely dependent on the presence of endogenous DNA of the complex as template, since the addition of DNase I to the incubation mixture inhibited 96%. RNase treatment had no effect. Treatment of the membrane-DNA complex with pronase (nuclease-free) also virtually abolished all incorporation. In addition to its obvious effect on the enzyme proteins of the membrane, nuclease-free pronase has previously

Table I

The Effect of Various Compounds on the Incorporation of (^{3}H)dTTP by a Membrane-DNA Complex from Rat Liver Mitochondria In Vitro

System	(^{3}H)dTMP Incorporated
	% of Control
Complete System[a]	100
+ Pancreatic DNase I, 200 μg	4
+ Pancreatic RNase, 200 μg	98
+ Nuclease-free pronase, 200 μg	10
- 3 Unlabeled dNTPs	3
- Mg^{2+}	<1
+ Ethidium bromide, 5 μg	28
+ Actinomycin D, 100 μg	<1
+ 2-Mercaptoethanol, 5 mM	30
+ Sarkosyl (0.1%)	6

a) The complete system consisted of the following components per ml: Tris·HCl, pH 8.4 (37°) (100 μmoles); $MgCl_2$ (30 μmoles); 3 unlabeled dNTPs (270 nmoles each); (^{3}H)dTTP, (10 μCi/210 pmoles); membrane-DNA complex containing 250 μg protein and 3 μg mtDNA. The various compounds were added in the amounts listed in the Table. Incubations were carried out in duplicate with shaking for 60 min at 37°. The complete system contained 1330 dpm/3 μg of mtDNA template (5320 dpm/mg membrane protein). The reaction was stopped by the addition of Sarkosyl to a final concentration of 0.1% and trichloroacetic acid to 10%. The labeled membrane-DNA complex was collected and washed by the filter disc method previously described from our laboratory (23). The discs were placed in scintillation vials and digested with 0.5 ml Protosol for 15 min, after which they were counted in an Ominfluor counting solution. Acid-insoluble radioactivity was determined in an Intertechnic SL-30 scintillation system. The background was 18 cpm. Counting efficiency was determined with an external standard by the channels ratio method. Efficiency for (^{3}H) was 45 - 50%. Incorporation represents the average of duplicate samples which agreed within 5% or less.

been shown to release acid insoluble DNA from the mitochondrial membrane-DNA complex (13).

Synthesis of DNA by the membrane-DNA complex is completely dependent on the presence of all 4 deoxynucleoside triphosphates and magnesium ion (Table I). Optimal concentration for deoxynucleoside triphosphates was found to be 2.7×10^{-4}M, and for Mg^{2+}, 30 mM. Incorporation of (^{3}H)dTTP in the presence of 15 mM Mg^{2+} was only 75% of the maximal incorporation, and Mg^{2+} at 45 mM inhibited the incorporation 80%. The Mg^{2+} concentration required by

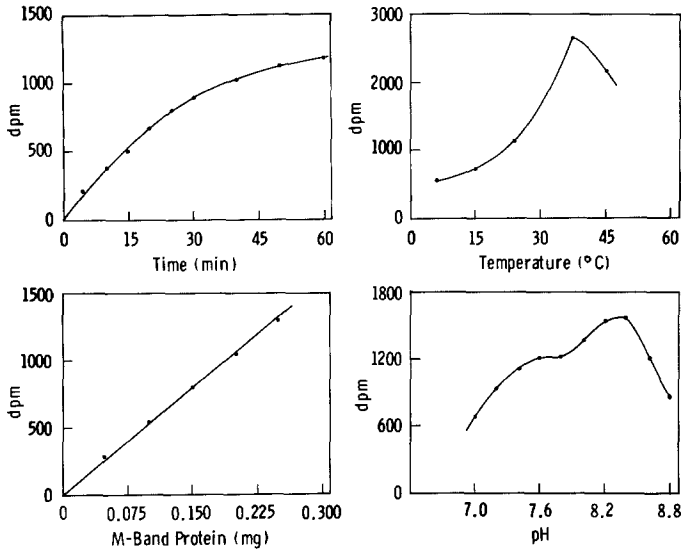

Fig. 2. The effect of time, temperature, pH and concentration of membrane-protein on the incorporation of (^{3}H)dTTP into the cold acid insoluble fraction by the membrane-DNA complex. The incubation conditions were as described in the legend to Table I. The reaction was stopped at the appropriate time and the samples washed and counted as described in the legend to Table I. The pH curve was carried out using 100 mM Tris·HCl and the appropriate pH was adjusted at 37°.

the membrane-DNA complex for optimal activity is rather high in comparison with the 8-10 mM Mg^{2+} required by the purified mitochondrial DNA polymerases (18,19). The high Mg^{2+} requirement may reflect the fact that the membrane-DNA complex represents a multi-enzyme complex for the synthesis of DNA and that each of the enzymes requires Mg^{2+}. Alternatively the high Mg^{2+} concentration may suppress a mitochondrial alkaline DNase present in the membrane-DNA complex, thereby manifesting an increase in labeled product. Evidence for such an enzyme in rat liver mitochondria which is inhibited by 20 mM Mg^{2+} has been reported (19).

Fig. 2a-d presents experiments to determine incorporation as a function of time, temperature, pH or concentration of M-band. At a pH optimum of 8.4 and at 37°, the incorporation of (^{3}H)dTTP into the DNA of the membrane-DNA complex is a linear function of the concentration of the membrane-DNA complex and of time over approximately a 20 min incubation period. Incorporation by the complex is inhibited above 45° C; exposure of the purified DNA polymerase

of rat liver mitochondria to 45^{o} for 20 min destroys 75% of the enzyme activity (Klunk, L. and Kalf, G., unpublished experiments).

The incorporation of (^{3}H)dTTP into the mtDNA of the complex is sensitive to several compounds known to inhibit DNA synthesis by both intact mitochondria and purified mitochondrial DNA polymerase *in vitro*. Complete inhibition was observed with Actinomycin D (Table I). Ethidium bromide, which at low concentrations has been shown to selectively inhibit the mitochondrial DNA polymerase (20,21), suppressed the incorporation by the complex 72% at 5 μg/ml. It is also significant that 2-mercaptoethanol inhibited incorporation of (^{3}H)dTTP by the membrane-DNA complex *in vitro*.

We have recently demonstrated that SH compounds, such as 2-mercaptoethanol, dithiothreitol, glutathione and cysteine, specifically inhibit the labeling of 39S and 27S circular forms of mtDNA with (^{3}H)dTTP by intact mitochondria *in vitro* (22). Furthermore, membrane-DNA complex prepared from inner membrane-matrix preparations pre-labeled in the presence of a SH compound contained greatly reduced label in the circular forms of DNA (22). Purified mitochondrial DNA polymerase is not inhibited by SH compounds but, in fact, is stimulated slightly by dithiothreitol (19,22). Taken together, these results suggest that the inhibitory effect of SH compounds on mtDNA synthesis is directed toward a membrane-DNA system.

The detrimental effect of low concentrations of Sarkosyl on the DNA polymerase activity of the membrane-DNA complex is also evident in Table I. The completeness of inhibition of incorporation by Sarkosyl emphasizes the need to use exhaustive dialysis to remove the detergent before attempting incorporation studies with the membrane-DNA complex. Conversely, we have also used Sarkosyl as a reagent for quickly and completely stopping the reaction (*cf.* legend to Table I).

The effect of exogenous DNA templates on the incorporation of (^{3}H)dTTP by the membrane-DNA complex is presented in Table II. Native calf-thymus DNA was capable of competing with the DNA of the complex for the polymerase; how-

Table II

The Effect of Exogenous DNA Templates on the Incorporation of (^{3}H)dTTP by the Membrane-DNA Complex

System	(^{3}H)dTMP Incorporated
	% of Control
Complete System[a]	
+ Calf-thymus DNA, 10 μg	80
100 μg	70
+ Activated calf-thymus DNA[b], 10 μg	263
100 μg	300
+ Poly(dA)·(dT_{10}), 12.5 μg	109
25.0 μg	127

a) The complete system was as described in the legend for Table I. Exogenous DNA was added according to the type and amount presented in the Table. Collection of the labeled membrane-DNA complex and determination of radioactivity was as described in the legend for Table I.

b) Activated calf-thymus DNA was prepared as described by Aposhian and Kornberg (27).

ever, an overall inhibition of incorporation was observed because the calf-thymus DNA, containing few if any free 3' OH ends, did not act as a suitable template for incorporation. Conversely, activated calf-thymus DNA could both compete effectively with the DNA of the complex for the polymerase and act as a template such that a 2- to 3-fold stimulation of incorporation was observed. Poly(dA)·(dT_{10}) was also tested because it has been observed previously in our laboratory that the activity of purified mitochondrial DNA polymerase is specifically stimulated with poly(dA)·(dT_{10}) 2-3-fold over that observed with activated calf thymus DNA (21). It appears, therefore, that poly(dA)·(dT_{10}) did not compete very well with the membrane-bound template for the polymerase or we would have expected a much higher level of incorporation.

To demonstrate that the incorporation of (^{3}H)dTTP was actually occurring on the membrane-DNA complex, "rebanding" experiments were performed. Membrane-DNA complex was labeled with (^{3}H)dTTP *in vitro*, the reaction was stopped by

the addition of Sarkosyl, and the membrane-DNA complex bound to the Mg^{2+}-Sarkosyl crystals was rebanded on a discontinuous sucrose gradient as before. The M-band was collected, and the radioactivity was determined as described in the legend to Fig. 1. Sixty-five per cent of the total radioactivity present in the membrane-DNA complex after incubation was retained by that complex after treatment with Sarkosyl and rebanding, indicating that the incorporation of (^{3}H)dTTP was indeed taking place on the membrane-DNA complex, using the membrane-bound DNA as template.

To prove that the incorporation of (^{3}H)dTTP by the membrane-DNA complex *in vitro* represents synthesis of mtDNA, the rebanded complex was treated with SDS and pronase and after concentration and dialysis, the solution was layered on a 5-20% linear sucrose gradient for analysis of labeled DNA.

The sucrose gradient profile from a typical experiment is presented in Fig. 3. Two peaks are seen containing labeled DNA with sedimentation values of 39S, 27S and a broad band of labeled DNA sedimenting between 5-25S with

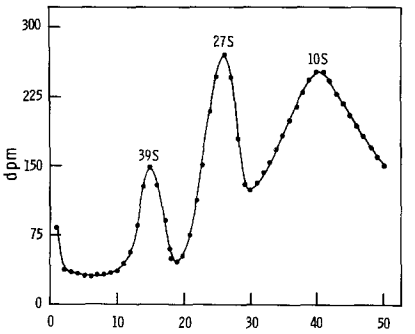

Fig. 3. Sedimentation velocity profiles of DNA from normal rat liver membrane-DNA complex labeled *in vitro* with (^{3}H)dTTP. Membrane-DNA complex was incubated in the reaction mixture as described in the legend to Table I, except that the usual 1 ml reaction mixture was increased 20-fold. After a 25 min incubation, the membrane-DNA complex was recovered by rebanding. The complex was treated with SDS at a final concentration of 2%, incubated for 2 hrs at 37° C with nuclease-free pronase at a concentration of 200 μg/ml and dialysed extensively at 4° C against buffer containing 10 mM Tris·HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl. The dialysate was concentrated to 0.2 ml in a collodion bag apparatus and layered onto a 4.8 ml linear sucrose gradient (5-20% in the above buffer), centrifuged in a Spinco SW 39 rotor at 38,000 rpm for 3.5 hrs at 4° C. Fractions (0.1 ml) were collected from the bottom of the tube directly onto filter discs, washed, and analyzed for radioactivity as described. Direction of sedimentation is from right to left. Identical results were obtained when the labeled DNA was isolated by the phenol method.

a peak value at 10S. These S values correspond to closed circles, open circles and replicating DNA fragments, respectively (23). This gradient profile is qualitatively similar to that observed for labeled DNA from intact mitochondria or inner membrane-matrix fractions incubated with (^{3}H)dTTP *in vitro* (17,23-25) and from mitochondria labeled *in vivo* (26). Treatment of the gradient fractions containing radioactivity with DNase I renders the label acid soluble (data not shown). Furthermore, no label was observed in closed circle forms of mtDNA isolated from a membrane-DNA complex incubated in the presence of 2-mercaptoethanol.

In view of the similarity in the labeling pattern of mtDNA between membrane-DNA complex labeled *in vitro* and whole mitochondria labeled *in vitro* and *in vivo*, it seems likely that *de novo* DNA synthesis is occurring on the membrane-DNA complex and therefore the complex may contain a replication complex.

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