DNA Biosynthesis by Isolated Mitochondria Stimulation by Cytoplasmic Factors from Neoplastic and Regenerating Tissues

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SUMMARY

The incorporation of radioactively labeled dTTP or dATP by rat liver mitochondria in vitro is stimulated by factors present in the postmicrosomal fraction of rat and mouse tumors and in regenerating and fetal rat liver, but not in normal liver. The observed stimulation is completely abolished by ethidium bromide, a specific inhibitor of mitochondrial DNA polymerase in mammalian systems. The stimulatory activity is nondialyzable, heat labile, insensitive to DNase and RNase, and precipitated by 30 to 50% ammonium sulfate. Fractionation of the crude rat hepatoma supernatant on a DNA-cellulose column has led to the separation of two active peaks containing negatively charged proteins which bind specifically to DNA and which are eluted by 0.15 and 0.30 M NaCl, respectively. These eluates contain all of the stimulatory activity of unfractionated hepatoma supernatant.

INTRODUCTION

Rat liver mitochondria contain a genetic system consisting, in part, of 1 or more copies of a low molecular weight, circular M-DNA and a DNA polymerase which has been purified and characterized. The mitochondrial enzyme can be distinguished from its nuclear counterpart by chromatographic behavior, stimulation by concentrations of monovalent salts which are inhibitory to the nuclear enzyme, and specific inhibition by dyes such as ethidium bromide and acriflavine. However, the organelle is only semiautonomous at best, for many of the most important mitochondrial enzymes, including cytochromes c and α + α3, DNA and RNA polymerases, and at least some of the proteins of the mitochondrial ribosomes are known to be coded by nuclear genes and synthesized on cytoribosomes.

Nuclear-mitochondrial interaction via the cytoplasm would appear, therefore, to be of considerable importance in the regulation of M-DNA replication and in the biogenesis of the organelle.

In this communication, we present evidence that soluble factors in the postmicrososomal supernatant of fetal and regenerating rat liver, as well as those from solid rat and mouse tumors, stimulate the incorporation of radioactively labeled deoxyribonucleoside triphosphates into DNA by rat liver mitochondria in vitro.

MATERIALS AND METHODS

Male Wistar rats (150 to 170 g) were fasted overnight before decapitation. Partial hepatectomies were performed by removing the median and left lateral lobes, and the animals were sacrificed 22 hr posthepatectomy. Rat tumor (Walker R256 mammary carcinoma) and mouse 6C3HED Gardner lymphosarcoma were carried as solid tumors by s.c. transplantation.

Mitochondria were prepared and washed 5 times, as is routinely done in our laboratory. The purity of these mitochondria with respect to the lack of contamination by other subcellular components and bacteria has been described in detail.

Preparation of Supernatant Fraction. Postmicrosomal SF was prepared from fetal and regenerating rat liver and rodent tumors by homogenization in 3 volumes of 0.34 M sucrose-2 mM Tris buffer, pH 7.4, and then was centrifuged for 15 hr at 105,000 × g, to ensure the complete removal of membranous material. The supernatant was collected by aspiration and stored at −70°C; at this temperature, activity is retained for approximately 1 month. Protein was determined by the method of Lowry et al.

Chemical Compounds. Phosphoenolpyruvic acid, pyruvate kinase, and the unlabeled dNTP’s were obtained from the Sigma Chemical Co., St. Louis, Mo.

Standard Incubation Protocol. The reaction mixture contained (per ml) the following components: Tris buffer, pH 7.4 (50 μmoles); MgCl₂ (10 μmoles); 2-mercaptoethanol (5 μmoles); phosphoenolpyruvate (5 μmoles); pyruvate kinase (10 μg); KCl (125 μmoles); 3 unlabeled dNTP’s (30 μmoles each); and 15 μmoles of 1 of the following radioactively labeled dNTP’s (all of which were obtained from New England Nuclear, Boston, Mass.), dTTP-3H (333 mCi/mmole), dATP-3H (333 mCi/mmole), or dTTP-14C (45.4 mCi/mmole); and 2 mg of the appropriate swollen mitochondria.

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2 To whom correspondence should be addressed.
3 Special Fellow of the Leukemia Society of America.
4 The abbreviations used are: M-DNA, mitochondrial DNA; SF, 105,000 × g supernatant fluid; dNTP’s, deoxyribonucleoside triphosphates.

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RESULTS AND DISCUSSION

Incubation of swollen normal adult hepatic mitochondria with dTTP\(^{14}\)C or dATP\(^{3}\)H in vitro under assay conditions which allow DNA synthesis by isolated mitochondria results in very little or no incorporation into trichloroacetic acid-precipitable material over a period of 60 to 90 min (Chart 1). The level of incorporation observed in normal mitochondria varies, depending on such factors as the age of the animals and whether mitochondria are prepared from livers pooled from a number of rats or from a single animal, suggesting that DNA synthesis in normal rat liver mitochondria is controlled even when intact mitochondria are incubated in vitro. That the labeled dNTP is incorporated into M-DNA under these conditions has been thoroughly documented in this laboratory (6) and by Simpson's group (7, 11, 15). Furthermore, a highly purified and unique M-DNA polymerase can be prepared from these mitochondria (6, 11) that is capable of incorporating at a rapid rate. Some properties of the enzyme are presented in Table 1, providing proof that these rather inactive mitochondria are capable of synthesizing DNA. These data confirm those which have previously been published (6, 11) for mitochondrial DNA polymerase.

Under certain circumstances, mitochondria carry out considerable DNA synthesis. We have tested the incorporation of dTTP\(^{3}\)H by mitochondria from 22-hr regenerating rat liver and Walker carcinosarcoma. In a typical experiment, normal mitochondria incorporated 0.27 pmole/mg protein/30 min, whereas 22-hr regenerating mitochondria and Walker tumor mitochondria incorporated 0.90 and 0.97 pmole/mg protein/30 min, respectively. These results suggested that DNA synthesis is occurring in mitochondria isolated from actively proliferating tissues, possibly because the mitochondria were isolated from a cytoplasmic environment containing factors capable of stimulating M-DNA synthesis. Therefore, we tested
the ability of SF from actively proliferating tissues to stimulate the incorporation of labeled dNTP's by normal rat liver mitochondria.

The addition of SF from 22-hr regenerating or 19-day fetal rat liver causes a significant incorporation of the labeled dNTP into M-DNA over the entire period, but SF from normal rat liver does not affect the incorporation (Chart 1, a and c). Furthermore, SF from regenerating or fetal liver does not incorporate when incubated under nearly identical conditions, but in the absence of mitochondria.

SF from mouse 6C3HED lymphosarcoma also stimulates incorporation of dTTP-3H by adult mitochondria (Chart 1a). Under these conditions, liver SF from the same tumor-bearing mice did not stimulate incorporation, nor did mouse tumor SF incorporate, when incubated in the absence of mitochondria.

A qualitatively identical stimulation was observed with SF from Walker R256 carcinosarcoma of the rat and with SF from a fast-growing rat hepatoma (7777) obtained from Dr. Harold P. Morris.

The stimulation of incorporation in adult mitochondria as a function of SF concentration is presented in Chart 2. The response is linear up to 1000 µg/ml of regenerating SF protein and to about 800 µg/ml of mouse tumor SF protein. Stimulation by rat tumor SF was identical to that observed for regenerating SF. Inhibition occurs at higher concentrations of SF. Normal rat liver SF has no stimulatory effect on normal rat liver mitochondria at any concentration (Chart 2, inset).

The addition of ethidium bromide to the incubation concomitantly with SF abolished the stimulation at all concentrations of SF. Chart 2 presents the inhibition by the dye in 2 systems and at 2 concentrations of SF protein. Significant inhibition by ethidium bromide has been observed at concentrations as low as 1 µg/ml, suggesting that the incorporation is being carried out within the organelle by SF from actively proliferating tissues to stimulate the incorporation of labeled dNTP's by normal rat liver mitochondria.

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It is unlikely that SF acts by increasing the permeability of mitochondria to dNTP's, because permeability of the mitochondria is increased by pretreatment with 0.1 M phosphate buffer and because stimulation of incorporation is also observed with dATP, which enters the organelle by a specific transport mechanism. Furthermore, the activity of SF is not due merely to the presence of a DNA polymerase in the SF. SF incubated under identical conditions but in the absence of mitochondria shows no incorporation, but a small amount of incorporation does occur when an exogenous template is added to the reaction vessel. However, in the absence of exogenous DNA, the polymerase present in SF does not appear to enter mitochondria and use M-DNA as a template, nor does the M-DNA leave the organelle and serve as a template outside.

Evidence supporting this is as follows.

(a) The incorporation of dATP-3H by unswollen normal rat liver mitochondria is stimulated by rat hepatoma SF, and this incorporation is inhibited by actrylclloside, a specific inhibitor.

M-DNA polymerase. Meyer and Simpson (10) have shown that the synthesis of DNA by the mitochondria polymerase is 54 times more sensitive to the dye than is its nuclear counterpart.

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(a) The incorporation of dATP-3H by unswollen normal rat liver mitochondria is stimulated by rat hepatoma SF, and this incorporation is inhibited by actrylclloside, a specific inhibitor.
of ATP transport across the inner mitochondrial membrane (Table 2) which would not affect any polymerase activity outside of the organelle. (b) The addition of template-dependent, purified M-DNA polymerase (Fraction Mt-III; cf. Table 1) to the reaction vessel in the presence or absence of SF does not affect the incorporation by the intact mitochondria, suggesting that the mitochondrial enzyme itself is unable to penetrate the organelle and also that M-DNA is probably not leaking out.

As snake venom phosphodiesterase and DNase I have no effect on the incorporation of labeled dNTPs by intact mitochondria in the presence or absence of SF (Table 2), it is unlikely that nonspecific nuclease(s) present in SF can enter the organelle and cause the stimulation by "nicking" M-DNA.

Furthermore, SF does not appear to affect the activity of S'-endonuclease (12), present in intact or sonically disrupted mitochondria, nor does this enzyme abolish the stimulation by SF.

Recently, further purification of the factors from rat hepatoma (7777) SF has been accomplished by chromatography on DNA-cellulose columns according to the method of Alberts et al. (1). This method relies upon the fact that many of the proteins which function on DNA inside the cell bind tightly to DNA at physiological ionic strengths in vitro; at higher salt concentrations, these proteins are reversibly released from the DNA.

DNA-free hepatoma SF was prepared by incubation with pancreatic DNase I (20 μg/ml) for 15 min at 23° followed by dialysis against 2 changes of a buffer composed of 0.05 M NaCl, 10⁻³ M 2-mercaptoethanol, 10⁻³ M EDTA, and 0.02 M Tris-HCl, pH 8.1.

Treated hepatoma SF (5 ml) was loaded onto a native calf thymus DNA-cellulose column (1) at 2 ml/hr. The column (1.0-ml packed volume; 0.5 mm X 5 cm) had been prewashed for several hours at 4° with dialysis buffer containing 10% glycerol. After loading, the column was washed again with this buffer, and then the bound proteins were eluted by stepwise increases in buffer NaCl solution. Elution was carried out with the following concentrations of NaCl: 0.15, 0.30, 0.40, 0.50, 0.60, and 2.0 M. Fractions (0.5 ml) were collected at a flow rate of 2 ml/hr, and the fractions containing protein were located by spectrophotometric determination at 280 nm in a Zeiss PMQ II spectrophotometer.

### Table 4

<table>
<thead>
<tr>
<th>Additions</th>
<th>TMP-³H incorporated (pmole/mg protein)</th>
<th>Stimulation (-fold)</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>0.19</td>
<td>0</td>
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<tr>
<td>Unfractionated hepatoma SF</td>
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<td>2</td>
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<tr>
<td>(1000 μg)</td>
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<tr>
<td>Unfractionated hepatoma SF + ethidium bromide (4 μg)</td>
<td>0.02</td>
<td></td>
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<tr>
<td>NaCl eluates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15 M</td>
<td>0</td>
<td></td>
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<tr>
<td>(44 μg)</td>
<td>0.31</td>
<td>1.6</td>
</tr>
<tr>
<td>(88 μg)</td>
<td>0.50</td>
<td>2.6</td>
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<tr>
<td>(44 μg) + ethidium bromide (4 μg)</td>
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<tr>
<td>0.30 M</td>
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<tr>
<td>(44 μg)</td>
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<tr>
<td>(88 μg)</td>
<td>0.62</td>
<td>3.3</td>
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<td>(44 μg) + ethidium bromide (4 μg)</td>
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Preliminary examination of the factors on polyacrylamide disc gel electrophoresis has indicated that, at pH 8.0, they migrate rapidly to the anode and appear in the gel slightly behind the bovine serum albumin marker protein, suggesting that these proteins are highly negatively charged at the pH at which they bind to the DNA-cellulose.

The binding of these negatively charged proteins to DNA in the presence of greater than 0.1 M NaCl, where simple electrostatic interaction between non-DNA-related proteins and DNA should be largely prevented, suggests that the proteins are recognizing DNA as a specific substrate.

Alberts et al. (1) have found that the vast majority of specific DNA-binding proteins (i.e., T₄-induced "gene 32 protein") required for DNA replication, in T₄ phage-infected Escherichia coli) are negatively charged under the conditions used for chromatography.

It is apparent from the results reported here that the postmicrosomal SF from actively proliferating tissues, such as rodent tumors and fetal and regenerating rat liver, contains soluble protein factors which appear to bind specifically to DNA and which stimulate the incorporation of dTTP's into M-DNA by adult rat liver mitochondria incubated in vitro.
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under conditions that support DNA synthesis. That the incorporation is being carried out within the organelle by M-DNA polymerase is evidenced by the complete inhibition of the reaction by the mitochondria inhibitor, ethidium bromide. Nuclear-mitochondrial interaction via the cytoplasm thus appears to be of significance in the replication of M-DNA.

Work on the further purification of the factors from 22-hr regenerating rat liver and hepatoma SF and on a determination of their role in M-DNA replication is actively being pursued in our laboratory.

ACKNOWLEDGMENTS

We are indebted to Mr. M. E. Stekula of the Pathology Department of Jefferson Medical College for furnishing us with the Walker carcinosarcoma and the Gardner lymphomas used in this study.

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