A CYTOPLASMIC MEMBRANE-ASSOCIATED DNA FROM RAPIDLY PROLIFERATING TISSUE *

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Summary. A membrane fraction obtained from the post-microsomal supernatant of Novikoff hepatoma and rat tissues has been shown to contain nucleic acid which can stimulate DNA synthesis. Based on the insensitivity to specific RNases and the sensitivity to DNase, the stimulatory nucleic acid is presumed to be DNA. The occurence of this cytoplasmic membrane-associated DNA is related to the proliferative state of the tissue.

Since the work of Bollum and Potter (1), the presence of high levels of DNA polymerase in the post-microsomal supernatant has been verified in a wide variety of eukaryotic cells (2-6). The ambiguous sub-cellular distribution of this enzyme - that is, distinct from the location of its template - may not only be a consequence of cellular fractionation but may have physiological significance which remains unexplained. Recent studies of Baril *et al* (7,8) have shown that a membrane fraction obtained from the post-microsomal supernatant of Novikoff hepatoma and regenerating rat liver contains high levels of a high molecular weight (6-8 S) DNA polymerase.

It has been further demonstrated (9) that this cytoplasmic membrane fraction is the *predominant* cellular site for several deoxyribonucleotide synthesizing enzymes[ribonucleotide reductase, thymidine kinase, thymidylate synthetase (9), and dCMP deaminase (10)], as well as for the high molecular weight DNA polymerase

We now report that this cytoplasmic membrane fraction from rapidly proliferating tissues (Novikoff hepatoma, regenerating rat liver, and normal rat spleen) contains DNA which can function as template and/or initiator in

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the synthesis of polydeoxyribonucleotides. In contrast, the nucleic acid derived from the corresponding normal rat liver cytoplasmic membrane fraction is virtually incapable of stimulating in vitro DNA synthesis.

Materials and Methods

The cytoplasmic membrane fraction is prepared as previously described (8). After overnight centrifugation (75,000 x g) of the post-microsomal supernatant, the resulting pellet is resuspended by Dounce homogenization in the lower 1/5 of the supernatant. This suspension is placed atop a discontinuous sucrose gradient (0.8 - 2.0 M) as described by Murray et al (11). Upon centrifugation of this gradient, the cytoplasmic membrane fraction bands at approximately 1.3 M sucrose.

Nucleic acid extracts are prepared as follows: fractions are diluted 1:10 in 10% NaCl and heated 10 min at 90°. After centrifugation (1,000 x g for 20 min), four volumes of chilled (-20°) ethanol are mixed with the supernatant. Nucleic acid sedimented by centrifugation (15,000 x g for 30 min) is resuspended in 10 mM NaCl.

DNA synthesis is measured by the incorporation of dTMP-H^3 (Schwartz-Mann) into acid-insoluble product by a modification of the filter paper disc method of Bollum (12).

Results

Several reports (13-16) have indicated the possible involvement of RNA in the biosynthesis of DNA by means of oligoribonucleotides which may serve to initiate long-chain DNA synthesis. The cytoplasmic membrane fraction, previously shown to contain a low but rather constant amount of RNA, has been tested for its ability to stimulate DNA synthesis. Nucleic acid from this cytoplasmic membrane fraction has in fact demonstrated competence as template and/or initiator for DNA synthesis (Table 1). The preponderance of RNA in the cytoplasmic membrane nucleic acid has suggested RNA as the active nucleic acid. In order to test this hypothesis, the

TABLE I

NUCLEASE PRE-TREATMENT OF CYTOPLASMIC MEMBRANE NUCLEIC ACIDS:

EFFECT UPON ABILITY TO STIMULATE DNA SYNTHESIS

CYTOPLASMIC MEMBRANE-ASSOCIATED NUCLEIC ACID	NUCLEASE TREATMENT	dTMP INCORPORATION (CMP)
No Addition	-	495
Novikoff Hepatoma	-	12,350
Novikoff Hepatoma	RNase	10,775
Novikoff Hepatoma	DNase	551
Normal Rat Spleen	-	1250
Normal Rat Spleen	RNase	1670
Normal Rat Spleen	DNase	530
12-Hour Regenerating Liver	-	1740
12-Hour Regenerating Liver	RNase	1010
12-Hour Regenerating Liver	DNase	580

All assays are carried out in a two-step incubation. 0.1-0.3 A_{260} units of nucleic acid extract are incubated with and without nuclease for 30 min at 37° in 180 µl 0.02 M glycine buffer pH 7.0. RNase-treated tubes contain 10 µg pancreatic RNase A(pre-heated to destroy DNase) while DNase-treated tubes contain 5 µg DNase and 0.8 µmoles $MgCl_2$.

After the initial incubation all tubes are heated 5 min at 90° and, after cooling, 65 µl of DNA polymerase assay components are added: 1.25 µmoles $MgCl_2$, 3.125 µmoles glycine buffer pH 8.0, 75 mµmoles dithiothreitol, 78 mµmoles each of dATP, dGTP, and dCTP, 5 µl TTP-H^3(0.5 mCi/ml, 17.3 Ci/mmole) and 100 µg Novikoff cytoplasmic membrane protein. After incubation at 37° for 60 min, acid-insoluble counts are assayed as described in Methods.

nucleic acid extracts have been pre-incubated with and without specific nucleases prior to use in a DNA polymerase assay (Table 1). The incorporation of deoxynucleotide into acid-insoluble product is not affected by the action of pancreatic RNase A upon the membrane nucleic acid ex-

TABLE II

COMPARISON OF EFFICIENCIENCIES OF VARIOUS CYTOPLASMIC MEMBRANE-ASSOCIATED NUCLEIC ACIDS INSTIMULATION OF DNA SYNTHESIS

SOURCE OF MEMBRANE-ASSOCIATED NUCLEIC ACID	dTMP INCORPORATION CMP/A_{260} Units
Normal Rat Liver	310
Normal Rat Liver	350
Normal Rat Liver	240
12-Hour Regenerating Liver	1880
12-Hour Regenerating Liver	1920
12-Hour Regenerating Liver	2340
24-Hour Regenerating Liver	1330
36-Hour Regenerating Liver	210
36-Hour Regenerating Liver	450
Normal Rat Spleen	6700
Novikoff Hepatoma	5300
Novikoff Hepatoma	11,500
Novikoff Hepatoma	16,500

All assays are carried out in 250 µl of solution containing the following: 1.25 moles $MgCl_2$, 3.125 moles glycine buffer pH 8.0, 75 mµmoles dithiothreitol, 78 mµmoles each of dATP, dGTP and dCTP, 5 µl TTP-H^3 (0.5 mCi/ml, 17.3 Ci/mmole), 0.1-0.3 A_{260} units of nucleic acid extract and 100-150 µg Novikoff cytoplasmic membrane protein. Incubation is 60 min at 37°.

tracts while DNase pre-treatment abolishes incorporation. Also, stimulation of DNA synthesis by Novikoff cytoplasmic membrane nucleic acid is abolished by pre-treatment with snake venom phosphodiesterase, is insensitive to RNase T_1, and is not inhibited by RNase T_2. These data indicate that the nucleic acid from the cytoplasmic membrane fraction which stimulates DNA synthesis is DNA.

We have also observed that cytoplasmic membrane nucleic acids from various sources differ greatly in their ability to serve as template and/or initiator in DNA synthesis and that this ability is correlated with the rate of proliferation of the tissue (Table II). Rat liver cytoplasmic membrane extracts have been found to be consistently inactive while comparable Novikoff extracts have considerable activity. Intermediate activity has been observed with rat spleen membrane nucleic acid extracts and low but consistent activity has been found with 12 hour regenerating liver extracts. The declining activity of the 24- and 36-hour regenerating liver extracts is contrary to the generally observed correlation of membrane nucleic acid template and/or initiator competence with the rate of tissue proliferation.

A comparison of DNA synthesis utilizing "activated" calf thymus DNA and Novikoff cytoplasmic membrane nucleic acid has been made (Table III). Both reactions require all four deoxyribonucleoside triphosphates for maximal activity and have only marginal activity with one triphosphate. ATP does not significantly affect incorporation while the sulfhydrl inhibitor N-ethylmaleimide as well as Actinomycin D are quite inhibitory. These data suggest a close correspondence between the two templates.

Discussion

We have demonstrated that the cytoplasmic membrane fraction from Novikoff hepatoma, rat spleen and regenerating rat liver contains DNA which can serve as template and/or initiator for DNA synthesis. The cytoplasmic membrane fraction also contains several deoxyribonucleotide and DNA synthesizing enzymes which all show definite responses to cellular proliferation (17,18). Together with the predominance of these enzymes in the cytoplasmic membrane fraction (9,10), this data has suggested that a complex of deoxyribonucleotide and DNA synthesizing enzymes may exist in this fraction.

TABLE III

COMPARISON OF ACTIVATED CALF THYMUS DNA AND CYTOPLASMIC MEMBRANE NUCLEIC ACID IN DNA SYNTHESIS

	% CONTROL	
TREATMENT	NOVIKOFF CYTOPLASMIC MEMBRANE NUCLEIC ACID	ACTIVATED CALF THYMUS DNA
Complete	100	100
-dNTP	33	25
-2dNTP	23	15
-3dNTP	5	5
+ATP (2.0 mM)	110	89
+N-Ethylmaleimide (0.3 mM)	8	3
+Actinomycin D (40 mg/ml)	28	7

All assays are carried out as in TABLE II.

100% (activated calf thymus DNA) = 22,000 cpm

100% (Novikoff cytoplasmic membrane nucleic acid) = 2490 cpm

Previously, "cytoplasmic" DNAs have been described in association with microsomes (19), plasma membranes (20), and mitochondria (21). With the exception of the mitochondrial DNA, no physiological function has been determined for these DNAs. Hypotheses regarding the function of these DNAs include gene amplification and information transfer (19). Because of the problems inherent in cellular fractionation procedures and the associated potential for artifactural redistribution of cellular components, it would be unwise to suggest that the observed localization of cytoplasmic membrane DNA and DNA synthesizing enzymes reflects the "true" localization in the intact cell. What does seem to be of significance is

the possible association between this DNA and a group of DNA synthesizing enzymes uniquely responsive to the demands of cellular proliferation, as well as the striking difference in cytoplasmic membrane DNA content between rapidly and slowly proliferating tissues.

Further investigations will be required to clarify the nature of the post-microsomal membrane-associated DNA and its potential relationship to the deoxyribonucleotide and DNA synthesizing enzymes of this membrane fraction.

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