PRESENCE OF Ca^{2+}, Mg^{2+}-DEPENDENT ENDONUCLEASE STIMULATING FACTOR IN RAT LIVER AND TESTIS NUCLEI.

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SUMMARY: A factor was extracted from isolated rat liver and testis nuclei which stimulated rat liver and bull semen Ca^{2+}, Mg^{2+}-dependent endonuclease activity and Micrococcal Ca^{2+}-dependent nuclease. The factor did not influence pancreatic deoxyribonuclease I; whereas it inhibited the Mg^{2+}-dependent acid endonuclease of rat liver. It retained its stimulating capacity after boiling for 60 min. Its action was lost on incubation with pronase. It enhanced the ability of bull semen Ca^{2+}, Mg^{2+}-dependent endonuclease to activate the template of rat liver residual chromatin for DNA synthesis.

INTRODUCTION

Isolated rat liver nuclei and chromatin possessed a Ca^{2+}, Mg^{2+}-dependent endonuclease (1-3). This enzyme was partially purified by Ishida et al. (2). In a previous study we showed that the Ca^{2+}, Mg^{2+}-dependent endonuclease can activate the template of isolated rat liver nuclei and chromatin for DNA synthesis in vitro (3).

In the present paper evidence will be presented to show that a factor exists in rat liver and testis nuclei which is capable of stimulating the Ca^{2+}, Mg^{2+}-dependent endonuclease of rat liver in vitro.

MATERIALS AND METHODS

Pancreatic deoxyribonuclease I and nuclease from Staphylococcus aureus were purchased from Worthington Corp., Wilmington, N.J. Pronase was purchased from Calbiochem, Los Angeles, Calif. Bull semen was purchased from Eastern Artificial Insemination Cooperative, Inc., Ithaca, N.Y. [Methyl-^{3}H]-thymidine (51.5 Ci/mmole) was purchased from New England Nuclear Corp., Boston, Mass.

Preparation of [^{3}H]DNA gel: Culture of E. coli K 38 was obtained through the generosity of Dr. P.H. Model. Deoxyadenosine (100 µg/ml) and [^{3}H]thymidine (1 mCi/20 ml) were added and the cultures incubated for 2 h at 37°C. [^{3}H]DNA was isolated according to Smith (4). [^{3}H]DNA gel was prepared as described by Melgar and Goldthwait (5). The radioactivity of the gel suspension was adjusted to 20,000 cts/min/100 µl/0.5 µg DNA.

Assay for endonuclease activity: The assay system for Ca^{2+}, Mg^{2+}-dependent

endonuclease contained 100 µl of [^{3}H]DNA gel, 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM 2-mercaptoethanol, and appropriate amounts of enzyme preparation in a total vol of 200 µl. The Mg^{2+}-dependent acid endonuclease was assayed in a system which contained 50 mM phosphate buffer (pH 6.12), 50 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 100 µl of [^{3}H]DNA gel and appropriate amounts of enzyme in a total vol of 200 µl. Reaction mixture was incubated at 35°C for 5 min. The reaction was stopped by placing the tubes in ice and by the addition of 200 µl of 20 mM EDTA (pH 8.0). The mixture was centrifuged at 1000g for 2 min. Radioactivity in 200 µl of the supernatant was measured in a Packard Liquid Scintillation Spectrometer with the use of Aquasol as scintillator. One unit of enzymic activity is defined as the release of 4000 cts/min of radioactive material from [^{3}H]DNA gel into the supernatant fraction on incubation for 30 min under the prescribed assay conditions.

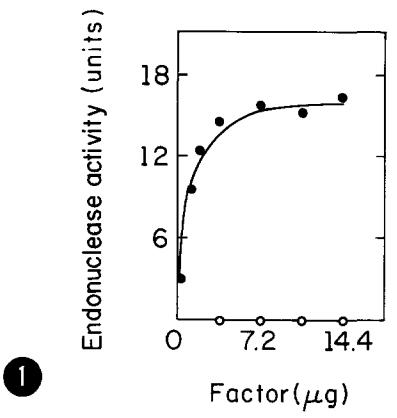
The Ca^{2+}, Mg^{2+}-dependent endonuclease activity was assayed by an alternative method using [^{3}H]DNA (2).

Assay for DNA synthesis: The procedure was described in a previous report (6).

Determination of protein and DNA: Protein was determined by the method of Kihara and Kuno (7) and DNA was measured by the diphenylamine reaction (8).

Isolation of nuclei and chromatin: Nuclei were isolated from adult male testis and liver and chromatin prepared as described in a previous report (6). The pellet of nuclei was washed twice with Medium A which contained 0.25 M sucrose, 5 mM Tris-HCl (pH 8.0), 3 mM $CaCl_2$, 1 mM EDTA and suspended in a medium containing 30% glycerol, 1 mM EDTA, 2 mM 2-mercaptoethanol, 50 mM Tris-HCl (pH 8.0).

Isolation of Mg^{2+}, Ca^{2+}-dependent endonuclease: The nuclei preparation containing 200 mg of DNA was collected by centrifugation at 10,000g for 15 min and suspended in a medium which contained 5% glycerol, 25 mM EDTA, 50 mM Tris-HCl (pH 7.4), 0.1 M NaCl. The suspension was centrifuged and the sediment was resuspended in a medium containing 5% glycerol, 0.35 M NaCl, 50 mM Tris-HCl (pH 7.4), 2 mM EDTA. The mixture was centrifuged at 10,000g for 15 min. To the supernatant solid ammonium sulfate was added. The material precipitated between 50-85% saturation with ammonium sulfate was collected and resuspended in Medium B which contained 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 20% glycerol and the suspension was dialyzed against Medium B overnight. The retentate was centrifuged and subjected to chromatography on a carboxymethyl cellulose column equilibrated with Medium B. The column was eluted stepwise by the addition of Medium B containing 0.2 and 0.3 M NaCl. The enzyme was eluted with the latter medium. Fractions possessing enzymic activity were pooled and dialyzed against Medium B overnight. The retentate was centrifuged and used as the endonuclease preparation.



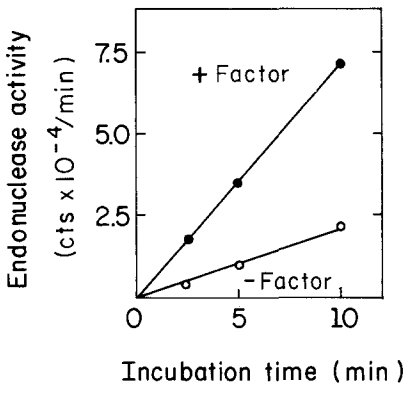


Fig. 1. Effect of stimulating factor on Ca^{2+}, Mg^{2+}-dependent endonuclease activity of rat liver. The assay system contained 70 ng of enzyme protein. Factor only (○); enzyme plus factor (●).

Fig. 2. Influence of stimulatory factor on the kinetics of Ca^{2+}, Mg^{2+}-dependent endonuclease activity. The assay system contained [^{3}H]DNA gel (400,000 cts/min, 4.5 μg DNA, 0.35 μg of enzyme protein and 7.2 μg of stimulatory factor. With stimulating factor (●); without factor (○).

Isolation of stimulating factor: Isolated rat liver or testis nuclei containing 28 mg of DNA were suspended in 20 ml of a medium which contained 25 mM EDTA, 0.35 M NaCl (pH 8.0). The suspension was homogenized and centrifuged at 10,000g for 15 min. To the supernatant acetone was added. The precipitate formed between 30-60% by vol was collected by centrifugation. The pellet was suspended in 5 ml of 20 mM Tris-HCl (pH 8.0). The suspension was boiled for 10 min, and centrifuged at 10,000g for 15 min. The supernatant was dialyzed against 4 liters of 20 mM Tris-HCl (pH 8.0) overnight.

Results and discussion: Rat liver Ca^{2+}, Mg^{2+}-dependent endonuclease activity increased with increasing amounts of nuclear factor added to the reaction mixture (Fig. 1). Maximum stimulation of the enzymic activity occurred when 7.2 μg of factor protein was added. Endonuclease activity assayed with [^{3}H]-DNA used in place of DNA gel (2) was stimulated by the addition of the factor to the reaction mixture (Fig. 2).

The factor retained its stimulatory capability after boiling for 60 min. However, its stimulating activity was destroyed on treatment with pronase (Table I). In addition to stimulating Ca^{2+}, Mg^{2+}-dependent endonuclease of rat liver and bull semen the factor enhanced Micrococcal nuclease activity (Table II). On the other hand, it did not stimulate pancreatic deoxyribonuclease I. Moreover, it inhibited the activity of Mg^{2+}-dependent acid endo-

TABLE I

Effect of Stimulatory Factor on Ca^{2+}, Mg^{2+}-dependent Endonuclease Activity.

Ingredients added	Activity (units/30 min/reaction mixture)
Control	3.12
+ factor	16.32
+ pronase-treated factor	3.39
+ boiled pronase	3.79

Each ingredient was dissolved in buffer. The mixtures were incubated at 37° for 30 min and boiled for 10 min. To the mixture was added the partially purified Ca^{2+}, Mg^{2+}-dependent endonuclease and [^{3}H]DNA gel. The amounts of the stimulating factor, pronase and endonuclease used were 14.4, 90 and 0.07 µg of protein, respectively.

TABLE II

Effect of Rat Liver and Testis Stimulatory Factor on the Activities of Various Nucleases.

Enzyme	Protein ng	Nuclease activity (units/30 min/reaction mixture) control	with liver factor	with testis factor
DNAase I, pancreas	0.5	4.68	3.06	4.22
Nuclease, Micrococcus	0.2	1.68	7.62	10.80
Alkaline endonuclease, rat liver	70	3.12	16.32	16.10
Acid endonuclease, rat liver	880	8.46	2.04	2.10
Alkaline endonuclease bull semen	0.12	1.02	10.02	11.88

Pancreatic DNAase I and Micrococcal nuclease were assayed as described in Worthington Enzyme Methods (9), and rat liver alkaline nuclease and semen alkaline nuclease as described in the text. The standard assay systems contained 14.4 and 12.0 µg of stimulatory factor protein from rat liver and testis, respectively. The alkaline endonucleases purified 750-fold from bull semen and 37-fold from rat liver and the acid endonuclease purified 48-fold from rat liver were used.

nuclease of rat liver (Table II). Changing the order of addition of ingredients to the assay system did not alter the stimulatory capacity of the factor.

To be certain that the factor did not in some unknown manner influence

TABLE III

Effect of Stimulatory Factor on the Ability of Bull Semen Ca^{2+}, Mg^{2+}-dependent Endonuclease to Activate the Template of Rat Liver Residual Chromatin for DNA Synthesis.

	Incubation time (min)	Template activity [^{3}H]TMP incorporation (cts/min)	
		without factor	with factor
Residual chromatin	60	1321	311
+ Bull semen endonuclease	60	1604	6998

The reaction mixture was incubated at 25°C. The values presented were obtained by subtracting the control values (zero time) of the incorporation of [^{3}H]TMP from the 60 min values. The control values with and without added factors were 4049 and 3689 cts/min, respectively. The amounts of bull semen endonuclease and stimulating factor used were 0.24 ng and 14.4 μg of protein, respectively.

the endonuclease assay system, various amounts of the factor were added after completion of the assay. The endonuclease activity was not affected. The mode of action of the factor on endonuclease activity is not known. It was previously demonstrated that the Ca^{2+}, Mg^{2+}-dependent endonuclease activated the template of chromatin for DNA synthesis (3). The ability of the factor to potentiate the capacity of the endonuclease to activate the template of chromatin was investigated (Table III). In the presence of the factor, the endonuclease increased the template activity by over fourfold as compared to the control value (Table III). The present results suggest that the factor might regulate endonuclease activity and determine the number of nicks which can be formed. Although the factor has not been fully characterized it might be related to the w factor of *E. coli* or the superhelix relaxing protein (10,11). The purification and characterization of the factor and its mode of action on the endonuclease activity are under investigation.

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