A COMPLEX OF VARIOUS ENZYMES CONCERNED WITH DNA SYNTHESIS IN YOSHIDA SARCOMA

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When the postmicrosomal supernatant from Yoshida sarcoma was filtered through a Sartorius membrane filter (0.1 μ pore size), incorporation of ^{14}C-thymidine into DNA was observed in the filter. The filter was found to contain over 80 % of the total DNA polymerase and thymidine kinase activities and 50 % of the total TMP kinase activity present in the postmicrosomal supernatant, while 100 % of the lactic dehydrogenase was found in the filtrate. When $MgCl_2$ was added to the postmicrosomal supernatant from Yoshida sarcoma, formation of the complex increased, maximal complex formation being observed with 1.0×10^{-2}M $MgCl_2$.

Most enzymes concerned with DNA synthesis are known to be present mainly in the postmicrosomal supernatant fraction[1-3]. Previously, we reported[4-6] that during fractionation of DNA synthesizing enzymes of postmicrosomal supernatant fractions from various tissues, the DNA polymerase, thymidine monophospho kinase (TMP kinase), thymidine kinase, uridine kinase and aspartate transcarbamylase were found in the pellet fraction of the postmicrosomal supernatant. The activities of DNA polymerase, TMP kinase and thymidine kinase were much higher in the pellet fractions of proliferating tissues, such as regenerating rat liver and Yoshida sarcoma than in those of non-proliferating, normal rat liver. The pellet fractions of regenerating rat liver and Yoshida sarcoma showed remarkable incorporation of various precursors (thymidine, TMP, deoxycytidine and dCMP) into DNA in the presence of a suitable DNA template, ATP and all four deoxynucleoside-5'-triphosphates required for DNA synthesis. These previous results suggested that enzymes concerned with DNA synthesis exist as a complex forming a functional unit. This paper reports

the existence of a complex of several enzymes concerned with DNA synthesis. This complex was isolated on a membrane filter and analyzed by Sephadex gel filtration.

MATERIALS AND METHODS

Ascites sarcoma cells. Yoshida ascites sarcoma of rats was used in this study. Ascites fluid was harvested 6 days after intraperitoneal inoculation of sarcoma cells. The sarcoma cells were suspended in 5 volumes of 0.01 M potassium phosphate buffer, pH 6.8 containing 0.14 M KCl and 1 mM EDTA and centrifuged at 150×g for 5 min. This washing procedure process was repeated three or four times and the cells were finally centrifuged at 1500×g for 10 min.

Enzyme and protein assays. DNA polymerase, TMP kinase, thymidine kinase and uridine kinase were assayed as described previously[6]. Lactic dehydrogenase was assayed as described by Wroblewski[7]. Protein was determined by the method of Lowry et al.[8].

RESULTS

Preparation of the complex of various enzymes concerned with DNA synthesis.

Packed ascites cells were homogenized in 1.5 volumes of 50 mM Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$ and 1 mM 2-mercaptoethanol using twenty strokes with a Teflon glass homogenizer. The homogenate was centrifuged at 105,000×g for 60 min. The resulting supernatant was diluted with ice cold 5 mM Tris-HCl buffer, pH 7.5, containing 5 mM $MgCl_2$ and then samples of 2.5 ml were filtered on a Sartorius membrane filter of 25 mm diameter (0.10 μ pore size). Sampling was completed within 3 min. The filter was transferred to 500 μl of incorporation mixture (20 μmole of glycine-NaOH buffer pH 8.0, 0.5 μmole of 2-mercapto-

Table I

INCORPORATION OF [^{14}C]-THYMIDINE INTO THE DNA FRACTION OBTAINED BY FILTRATION OF THE POSTMICROSOMAL SUPERNATANT FROM YOSHIDA SARCOMA AND THE EFFECTS OF DNase AND RNase UPON LABELING

	Incorporation of [^{14}C]-thymidine into DNA	
	cpm	%
Complete system	2237	100
+ DNase (100 μg)	285	13
+ RNase (100 μg)	1991	89

The standard assay system was used to measure incorporation of various precursors into DNA. After incubation for 60 min, mixtures were placed in a boiling water bath for 2 min and then incubated with the DNase I (100 μg) or RNase (100 μg) at 37° for 30 min. Then, they were treated with acid and radioactivity was counted as described in the text.

Table II

ENZYME ACTIVITIES OBTAINED BY FILTRATION OF THE POSTMICROSOMAL SUPERNATANT FROM YOSHIDA SARCOMA

Fraction	Enzyme activity							
	DNA polymerase				TMP kinase		Thymidine kinase	
	Native*		Heat denatured**					
	pmole	%	pmole	%	nmole	%	nmole	%
Postmicrosomal supernatant	731	100	326	100	198	100	118	100
Filtrate	60	8	60	18	99	50	4	3
Fraction on filter (calculated)	671	92	266	82	99	50	115	97

Enzyme activities were assayed as described previously[6]. * Native calf thymus DNA used as primer ** Heat denatured calf thymus DNA used as primer

ethanol, 8 μmole of $MgCl_2$, 25 nmole each of dGTP, dATP and dCTP, 3 nmole of [^{14}C]thymidine (40 μCi/mole) 3.3 μmole of ATP, 65 μg of heat denatured calf thymus DNA) at 37° and incubated for 60 min in a vial for counting radioactivity. The reaction was stopped by adding 1 ml of cold 5 % trichloroacetic acid containing 1 % $Na_4P_2P_7$. The mixture was stood at 0° for 20

Table III

EFFECT OF $MgCl_2$ CONCENTRATION ON FORMATION OF THE COMPLEX OF VARIOUS ENZYMES CONCERNED WITH DNA SYNTHESIS

$MgCl_2$ concentration (mM)	Enzyme activity on membrane filter*			
	DNA polymerase**	TMP kinase	Thymidine kinase	Lactic dehydrogenase
	%	%	%	%
0	20.7	32.6	87.8	1.0
1.25	24.8	29.3	91.8	0.0
2.5	38.0	30.2	95.1	0.0
5.0	50.3	33.5	97.3	0.0
10.0	58.8	42.1	98.6	0.0
20.0	45.8	33.9	92.4	0.0

Packed ascites cells was homogenized in 1.5 volumes of 5 mM Tris-HCl (pH 7.5) containing 0.25 M sucrose and 1 mM EDTA. The homogenate was centrifuged at 105,000×g for 60 min. The resulting supernatant was diluted with ice-cold 5 mM Tris-HCl (pH 7.5) buffer containing various $MgCl_2$ concentrations and the samples of 2.5 ml were filtered using a Sartorius membrane filter of 25 mm diameter (0.10 μ pore size). Enzyme activities were assayed as described in "Materials and Methods".

* $\dfrac{\left[\text{Enzyme activity of postmicrosomal supernatant}\right] - \left[\text{Enzyme activity of filtrate}\right]}{\text{Enzyme activity of postmicrosomal supernatant}} \times 100$

** With heat denatured DNA as primer

min. Then the filters were washed three times with 15 ml of 5 % trichloroacetic acid, dried and counted in a liquid scintillation counter. As shown in Table I, the radioactivity on the filters showed marked incorporation of [^{14}C]thymidine into the acid insoluble fraction. Treatment of the acid insoluble fraction on the filters with DNase considerably decreased the radioactivity of the acid insoluble fraction, while treatment with RNase had no effect. To estimate the activities of enzymes concerned with DNA synthesis on this filter, the various enzyme activities in the postmicrosomal supernatant and in the filtrate were determined and from the results those on the filter were calculated. The results are shown in Table II. The filter was found to contain over 80 % of the total DNA polymerase and thymidine kinase activities and 50 % of the total TMP kinase activity present in the postmicrosomal supernatant. The present results support our earlier findings[6] that enzymes concerned with DNA synthesis exist in a complex as a functional unit.

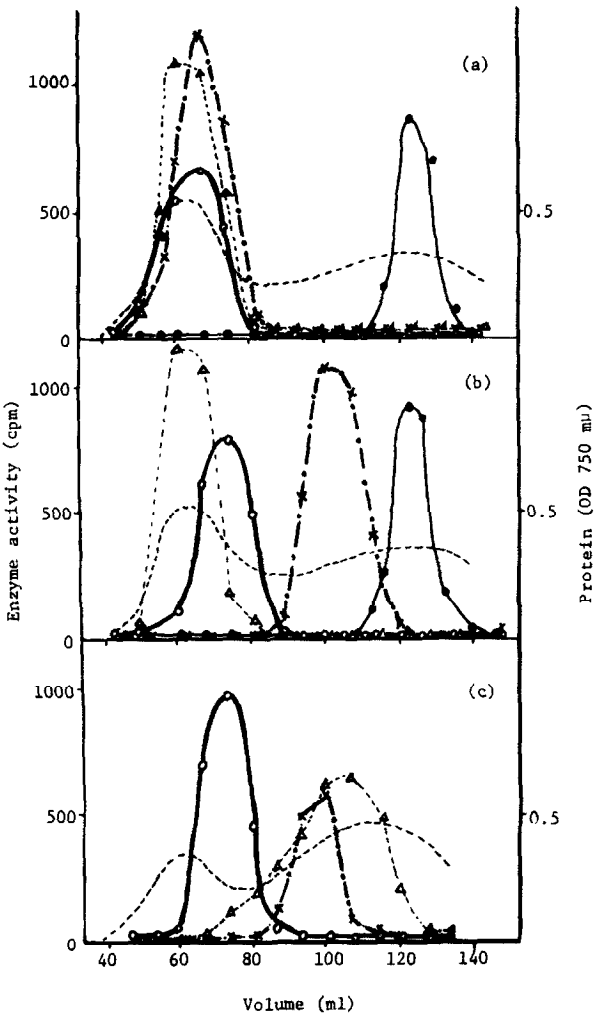

Fig. 1. Gel filtration of the postmicrosomal supernatant from Yoshida sarcoma on Sephadex G-200. The postmicrosomal supernatant was placed on a column of Sephadex G-200 (2.5×35 cm) and eluted with 5 mM Tris-HCl buffer (pH 7.5) containing 5 mM $MgCl_2$ (a) or 5 mM Tris-HCl buffer (pH 7.5) containing 0.5 M KCl (b). Conditions for (c) were as for (b) except that the postmicrosomal supernatant had been treated with bromelain (1/250 of the sample protein) at 25°C for 60 min. o—o DNA polymerase (heat denatured DNA used as primer), x—•—x Thymidine kinase, •—• TMP kinase, Δ····Δ Uridine kinase, ------ Protein

Effect of $MgCl_2$ formation of the complex of various enzymes concerned with DNA synthesis. Table III shows that addition of $MgCl_2$ to the postmicrosomal supernatant of Yoshida sarcoma resulted in increased association of DNA polymerase, TMP kinase and thymidine kinase with the complex. The optimal effect of $MgCl_2$ on incorporations of DNA polymerase, TMP kinase

and thymidine kinase into the complex was observed at a concentration of 1.0×10^{-2}M $MgCl_2$. Lactic dehydrogenase of the postmicrosomal supernatant from Yoshida sarcoma was not found associated with this complex.

Gel filtration pattern of the complex of various enzymes concerned with DNA synthesis from the postmicrosomal supernatant. Fig. 1a shows the elution patterns of the postmicrosomal supernatant from Yoshida sarcoma cells. The column was eluted with buffer of low ionic strength. DNA polymerase, thymidine kinase and uridine kinase appeared entirely in the void volume (indicated by the peak of Blue Dextran) but TMP kinase was eluted later. Gel filtration of the complex on Sephadex G-200 resulted in release of TMP kinase. On the other hand, the DNA polymerase and thymidine kinase associated with this complex were easily solubilized by treatment with a solution of high ionic strength, such as 0.5 M KCl (Fig. 1b). Uridine kinase was separated from the complex by treatment with bromelain (Fig. 1c).

DISCUSSION

In this work a complex of various enzymes concerned with DNA synthesis was separated by centrifugation and filtration on a Sartorius membrane. We found that formation of this complex was increased by addition of $MgCl_2$ (1.0×10^{-2}M). When the postmicrosomal supernatant fraction of Yoshida sarcoma cells was subjected to gel filtration on Sephadex G-200. DNA polymerase, thymidine kinase and uridine kinase were found in the void volume, while TMP kinase was eluated late. This suggests that the former three enzymes are combined tightly in the complex while TMP kinase is only loosely combined and so is easily released. The mode of attachment of TMP kinase to the complex is obscure. Its dissociation from the complex on Sephadex G-200 gel filtration might be due to removal of some unknown factor required for its association. Buffer of high ionic strength

(containing 0.5 M KCl) caused release of thymidine kinase, TMP kinase and DNA polymerase but not uridine kinase from the functional complex. The uridine kinase was separated from the complex by treatment with bromelain. The complex seemed to have a molecular weight of over 600,000, because, unlike the complex, 70 % blue dextran passed through Sartorius membrane filter (0.10 μ pore size), while the complex was eluted in the void volume from a Sephadex G-200 column.

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