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Some properties of partially purified DNA-dependent RNA polymerases and changes of levels of their activities during development in *Dictyostelium discoideum*

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The nuclear DNA-dependent RNA polymerase (EC 2.7.7.6) of the cellular slime mold *D. discoideum* was separated into two enzyme fractions (I and II) by DEAE-Sephadex column chromatography. The activity of enzyme I which was eluted with 0.11 M $(NH_4)_2SO_4$ was insensitive to α-amanitin, whereas that of enzyme II eluted with 0.17 M $(NH_4)_2SO_4$ was completely inhibited by the same concentration of the drug. The $MnCl_2$ optimum of both the enzymes was at 2 mM. The optimal concentrations of $(NH_4)_2SO_4$ and $MgCl_2$ for enzyme I (25 mM and 5 mM, respectively) was apparently low relative to those for enzyme II (50 mM and 15 mM, respectively).

When the levels of these two enzyme activities were examined using the nuclear high salt extract in term of the difference of the α-amanitin sensitivity between the enzymes, the specific activity of enzyme I declined strikingly at the initial stage of the morphogenetic development, followed by a progressive decrease until the stage of fruiting body formation. On the other hand, the specific activity of enzyme II was relatively constant throughout development except for a significant increase in the cell-aggregation stage.

The cellular slime mold, *D. discoideum*, has an interesting life cycle. In the presence of food, cells grow as single amoebae, but when the food is exhausted, the amoebae stop growing and form multicelluar aggregates, which subsequently differentiate into fruiting bodies with spore and stalk cells (BONNER, 1967). These developmental events are accompanied with the changes of certain specific enzymes which depend on concomitant protein synthesis and prior RNA synthesis (NEWELL *et al.*, 1971; FIRTEL and BONNER, 1972). Furthermore, the DNA-RNA hybridization experiments with RNA synthesized at different stages present evidence that gene activity is being regulated at transcriptional level (FIRTEL, 1972).

The demonstration of multiple forms of DNA-dependent RNA polymerase (EC 2.7.7.6) in eukaryotes suggests that this enzyme may play an

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important role in the regulation of transcripition (ROEDER and RUTTER, 1969). In fact, this notion has been supported by evidence as to the distribution of the polymerase within cells; polymerase I found in nucleoli has been assumed to be responsible for the synthesis of rRNA, while polymerase II present in the nucleoplasm is thought to be involved in the synthesis of non-ribosomal RNA (ROEDER and RUTTER, 1970; BEEBEE and BUTTERROORTH, 1974). In addition, a certain amount of evidence has recently been accumulated as to the modulations of the activities of these two RNA polymerases during cell differentiation or after hormonal treatment (RUDICK and WEISMAN, 1973; SEBASTIAN *et al.*, 1974; GUILFOYLE *et al.*, 1975; YOUNG and WHITELEY, 1975).

The study in this communication is concerned with some properties of DNA-dependent RNA polymerase partially purified from isolated nuclei of *D. discoideum* amoebae and the change of the level of the polymerase activity during development. The results will show that nuclear RNA polymerase of this organism can be separated into two enzymes by DEAE-Sephadex chromatography; one is sensitive to α-amanitin and other insensitive, and also that the activity of the polymerase resistant to the agent significantly alters during development, while the enzyme activity sensitive to it is relatively constant.

Materials and Methods

Isotopes and chemicals: ^{14}C-Uridine 5'-triphosphate (59 mCi/mmol) was obtained from the Radiochemical Centre, Amersham. ATP, GTP, CTP, UTP, and rifampicin were purchased from Boehringer Mannheim, Mannheim; calf thymus DNA (grade I), bovine serum albumin, and pancreatic RNase [EC 2.7.7.16] from Sigma Chemical Company; DNase (RNase free) [EC 3.1.4.5] from Worthington Biochemical Corp.; α-amanitin from Calbiochem, San Diego; actinomycin D from Mann Research Lab., Inc., New York; cycloheximide from the Upjohn Company, Kalamazoo. All other chemicals were of analytical grade.

Organism: Amoeba cells of *D. discoideum*, strain NC-4 (haploid), were grown in association with *Escherichia coli* at 23°C according to the procedure of BONNER (1947) with a slight modification. This culture method gave a satisfactory result for synchrony of development. Cells were harvested in various stages of development, washed repeatedly by centrifugation with cold 14 mM phosphate buffer (pH 6.2) to thoroughly remove the bacterial cells.

Isolation of nuclei: The preparation of nuclei was carried out using

some modifications of the method of SOLL and SUSSMAN (1973). The washed amoebae were suspended in ice-cold 5% (w/v) sucrose containing 5 mM $MgCl_2$ and 0.2% (v/v) Triton X-100 and then broken with a loosely fitting glass-teflon homogenizer. To the homogenate was immediately added an equal volume of 22% sucrose containing 5 mM $MgCl_2$ and 0.1% Triton X-100, and the mixture was left for 5 min in an ice bath. Nuclei were collected by centrifugation ($1000 \times g$, 10 min), followed by extensive washing with 13.5% sucrose containing 5 mM $MgCl_2$ and 0.15% Triton X-100 to remove contaminated cytoplasmic material. The nuclei so obtained were washed further three times with 13.5% sucrose containing 5 mM $MgCl_2$ and 10 mM dithiothreitol to eliminate the detergent and stocked at -80°C until use. All manipulations were done at 4°C.

Solubilization of RNA polymerase: RNA polymerase was solubilized by sonication of nuclei in a medium of high ionic strength according to the method of ROEDER and RUTTER (1969) with minor modifications. The entire procedure was carried out at 0°–4°C. Frozen nuclei were lysed in approximately 5 volumes of buffer A (50 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 10 mM dithiothreitol, 25% (v/v) glycerin), followed by the addition of 4 M $(NH_4)_2SO_4$ (adjusted to pH 7.9 with ammonium hydroxide) to make 0.3 M at the final concentration and then sonicated 4 times for 15-second period with 1-min interval at a maximum power using the Umeda Sonor 150 until the viscosity of the nuclear lysate disappeared. Immediately after sonication, the nuclear lysate was diluted with 2 vol. of buffer B which is identical to buffer A except that the concentration of dithiothreitol is 0.5 mM and reaggregated chromatin was removed by centrifugation at 45,000 rpm for 2 hr in a Spinco type 65 rotor. The resulting supernatant was termed the nuclear high salt extract.

When the extractability of RNA polymerase was tested with the nuclear preparation from cells harvested at two different stages of development (vegetative growth and culmination stages), more than 94% of total polymerase activity detected in sonicated nuclear lysates was recovered in nuclear high salt extracts obtained after high-speed centrifugation. This indicates that the method empolyed here gave a nearly satisfactory result for the solubilization of this enzyme.

DEAE-Sephadex column chromatography: The nuclear high salt extract obtained in the above was diluted with buffer B so that the final concentration of $(NH_4)_2SO_4$ may become 0.05 M and then directly applied to a DEAE-Sephadex A-25 column equilibrated previously with buffer B containing 0.05 M $(NH_4)_2SO_4$. The column was washed with two column vol-

umes of buffer B-50 mM $(NH_4)_2SO_4$ solution and eluted with a 0.05–0.4 M linear $(NH_4)_2SO_4$ gradient in buffer B at 4°C.

RNA polymerase assay: RNA polymerase activity was measured by the incorporation of ^{14}C-UMP from ^{14}C-UTP into acid-insoluble material. The standard reaction mixture (0.15 m*l*) contained; 50 mM Tris-HCl, pH 7.9, 2 mM $MnCl_2$, 5 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM each of ATP, GTP, and CTP, 0.05 mM UTP, 0.001 mM ^{14}C-UTP, 20 μg of calf thymus DNA, 150 μg of bovine serum albumin (omitted in the assay with nuclear high salt extracts), and 0.05 m*l* of the enzyme fraction. The concentrations of inorganic salts were altered depending on the experiments. The reaction mixture

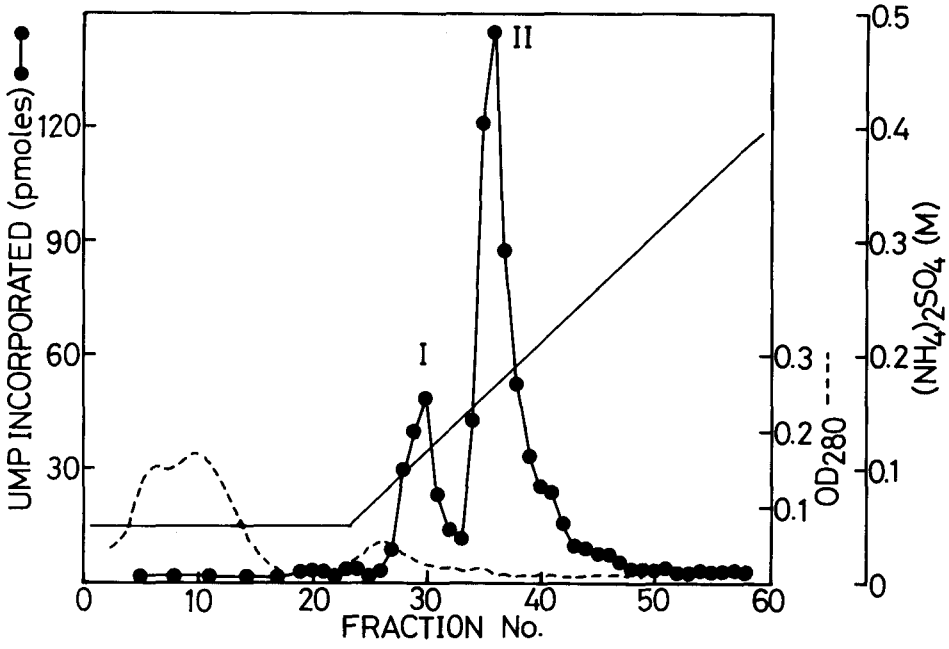

Fig. 1. DEAE-Sephadex column chromatography of nuclear RNA polymerase from *D. discoideum* amoebae at interphase of morphogenesis.

The nuclear high salt extract was prepared from isolated nuclei of interphase amoebae and then applied to DEAE-Sephadex A-25 column (1 cm×20 cm) and eluted with a linear gradient of 0.05-0.4 M $(NH_4)_2SO_4$ in buffer B. Three m*l* fractions were collected and aliquots (0.05 m*l*) of the fractions were used for the determination of enzyme activity. The concentration of $(NH_4)_2SO_4$ in the reaction mixture was not adjusted to become constant. The molarity of $(NH_4)_2SO_4$ in each fraction was monitored by refractive index. For details of the chromatography and assay conditions for the enzyme activity, see *Materials and Methods*. ——, $(NH_4)_2SO_4$ concentration (M); ●——●, enzyme activity; – – –, absorbance at 280 nm.

was incubated for 40 min at 23°C and the enzyme reaction was terminated by the addition of 2.2 m*l* of ice-cold 10% trichloroacetic acid containing 0.04 M $Na_4P_2O_7$. Trichloroacetic acid-precipitated material was collected on Whatman glass fiber filters (GF/C), extensively washed with 80 m*l* of cold 5% trichloroacetic acid containing 0.04 M $Na_4P_2O_7$, and finally with 2 m*l* of 75% ethanol. After drying, the filters were placed in 5 m*l* of toluene-based scintillation fluid and the radioactivity was measured in the Beckman liquid scintillation spectrometer.

DNA determination: The DNA fraction in the nuclear lysate was obtained by the method of Schmidt-Tannhauser-Schneider (VOLKIN and COHN, 1954) and its amount determined by the modified method of BURTON (1956).

Results

Figure 1 shows the pattern of DNA-dependent RNA polymerase activity after DEAE-Sephadex column chromatography of the nuclear high salt extract from cells at interphase (between vegetative growth and morphogenesis). Two distinct peaks of the polymerase activity were observed, which could be easily separated from the bulk of the A_{280}-absorbing material. The polymerase which was eluted at 0.11 M in $(NH_4)_2SO_4$ gradients was termed enzyme I and that at 0.17 M enzyme II. As shown in Fig. 2, the activities of enzymes I and II are almost linearly increased during 60-min period of

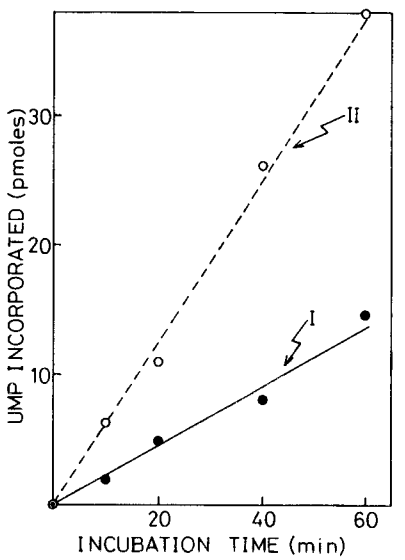

Fig. 2. Time course of RNA polymerase activity.

The polymerases (enzymes I and II) were obtained by DEAE-Sephadex chromaography of the nuclear high salt extract from interphase amoebae. The activities of enzymes I and II were assayed at 0.037 M and 0.057 M $(NH_4)_2SO_4$, respectively. Other experimental conditions were the same as described in *Materials and Methods*. ●——●, enzyme I; ○——○, enzyme II.

incubation under standard conditions, indicating that the enzyme preparations so obtained do not include a significant amount of proteinase and nuclease.

Properties of RNA polymerases: Some porperties of the above two enzymes were examined with the partially purified enzyme preparations obtained by DEAE-Sephadex chromatography. As indicated in Table I, both the enzymes require DNA as a template and other three ribonucleoside 5'-triphosphates for the incorporation of ^{14}C-UTP into acid-insoluble fraction. DNase and pancreatic RNase significantly inhibited the incorporation of ^{14}C-UTP mediated by the enzymes under standard assay conditions. α-Amanitin which is known to specifically suppress the activity of eukaryotic RNA polymerase participating in synthesis of non-ribosomal RNA completely prohibited the activity of enzyme II but exhibited no inhibitory effect on the activity of enzyme I. The activity of both the enzymes was not influenced by rifampicin, a specific inhibitor of bacterial RNA polymerase. Actinomycin D caused inhibition of both the enzyme activities, while cycloheximide scarecely affected them.

The enzyme preparations desalted by dialysis were used in the experiment of metal ion (Mn^{2+} and Mg^{2+}) dependence. It is shown in Figs. 3 A and

TABLE 1 Properties of RNA polymerases of *D. discoideum*.

The polymerases (enzymes I and II) were prepared from isolated nuclei of interphase amoebae as described in the legend to Fig. 1 and assayed under standard conditions.

Conditions	Enzyme I		Enzyme II	
	UMP incorporation (pmoles/0.15 m*l* of reaction mixture*)	%	UMP incorporation (pmoles/0.15 m*l* of reaction mixture*)	%
Complete	7.0	100	31.6	100
-DNA	0	0	0	0
-ATP	0.2	2.8	1.6	5.0
-GTP	0	0	5.7	18.0
-CTP	0	0	3.2	10.1
-$MgCl_2$, -$MnCl_2$	0.2	2.8	2.5	7.9
+DNase (15 μg)	1.3	18.5	0	0
+RNase (10 μg)	1.6	22.8	10.5	33.2
+α-Amanitin (10 μg)	9.6	137.1	0	0
+Actinomycin D (5 μg)	2.0	28.5	1.0	3.1
+Rifampicin (10 μg)	7.1	101.4	33.8	106.9
+Cycloheximide (100 μg)	6.0	85.7	27.9	88.2

* The same enzyme preparations were used in these tests.

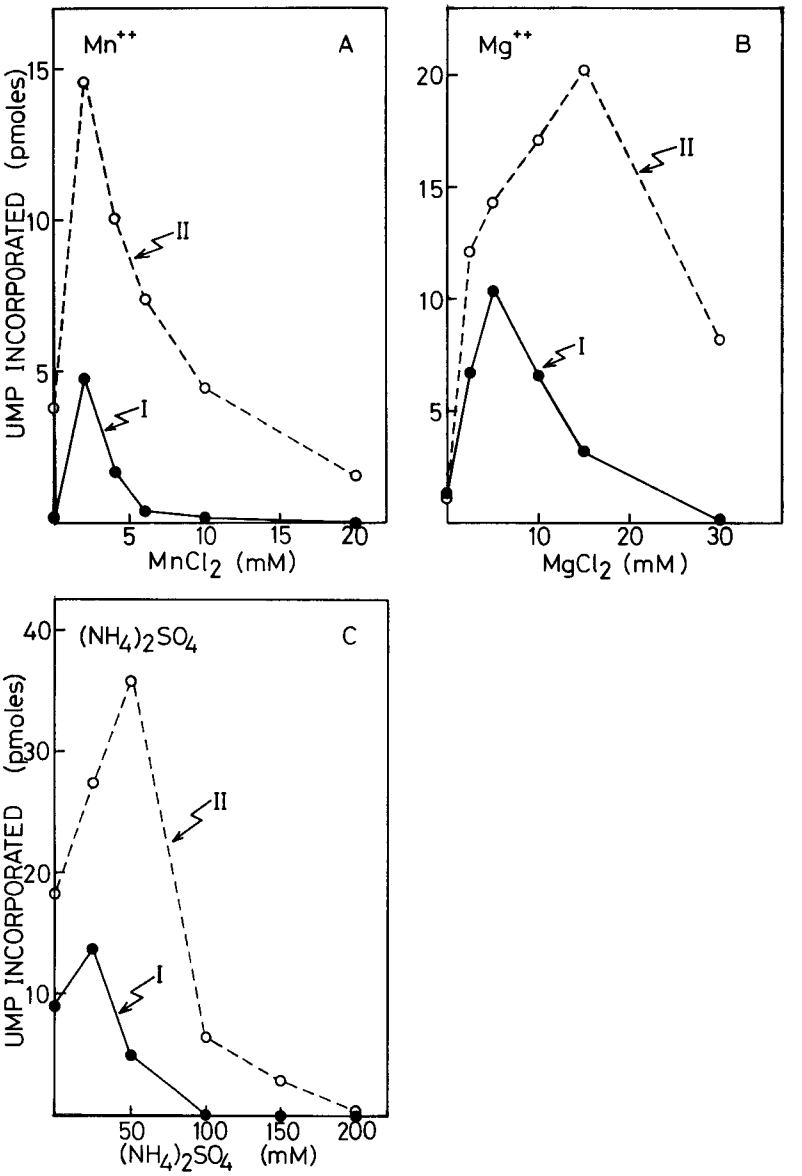

Fig. 3. Effects of divalent metal ions and ionic strength upon RNA polymerase activity.

The polymerases (enzymes I and II) were obtained, as in Fig. 1, from isolated nuclei of interphase amoebae. In order to remove $MgCl_2$ and $(NH_4)_2SO_4$, the enzyme preparations were dialyzed for 10 hr at 4°C against buffer B lacking $MgCl_2$. (A), $MnCl_2$; $MgCl_2$ and $(NH_4)_2SO_4$ were not contained in the reaction mixture. (B), $MgCl_2$; $MnCl_2$ and $(NH_4)_2SO_4$ were not contained in the reaction mixture. (C), $(NH_4)_2SO_4$; the reaction mixture contained 2 mM $MnCl_2$ and 5 mM $MgCl_2$. Other experimental conditions were the same as described in *Materials and Methods*. ●——●, enzyme I; ○---○, enzyme II.

3 B that an optimal concentration of $MnCl_2$ was 2 mM for both the enzymes and that of $MgCl_2$ was 5 mM for enzyme I and 15 mM for enzyme II. Figure 3 C shows the ionic strength dependence for the activity of both the enzymes. The maximum activity of enzymes I and II was observed at 25 mM and 50 mM of $(NH_4)_2SO_4$ respectively.

Thus, the above results suggest that enzymes I and II obtained here are undoubtedly two types of DNA-dependent RNA polymerase present commonly in nuclei of eukaryotic cells.

Alterations of levels of RNA polymerase activity during development: In order to see whether or not RNA polymerase activity varies during development of this organism, the activity levels of enzymes I and II were examined with the nuclear high salt extracts of cells in several developmental stages. As described in the above, since enzyme I was entirely insensitive to the inhibitory action of α-amanitin but enzyme II sensitive to it, the activity level of enzyme I in the nuclear high salt extract is to be represented by the activity resistant to α-amanitin (33 μg/m*l*) and that of enzyme II can therefore be estimated by subtracting the drug-resistant activity from the activity measured in the absence of the drug. The experimental results thus obtained are summarized in Table 2, in which the levels of the enzyme activity are normalized to the value per definite amount (mg) of *D. dis-*

TABLE 2. Alterations in the levels of RNA polymerase activity in nuclear high salt extracts during development.

The nuclear high salt extract was prepared at four different stages of development as described in *Materials and Methods*. The polymerase activity was assayed under standard conditions but including 50 mM $(NH_4)_2SO_4$ at the final concentration. The level of enzyme I was represented by the activity resistant to α-amanitin (33 μg/m*l*) and that of enzyme II by the activity abolished by the drug. Each value is the average in two or three separate experiments.

	Specific activity* (nmoles of UMP incorporated/mg of DNA)			
	Vegetative growth	Interphase	Aggregation	Migration~ Culmination
Enzyme I	15.38	7.97	2.51	1.33
Enzyme II	7.32	7.92	9.78	7.06
Total	22.70	15.89	12.29	8.39
Ratio of enzyme I/II	2.10	1.01	0.26	0.19

* The specific enzyme activity is expressed as the amount of UMP incorporated per mg of *D. discoideum* nuclear DNA which is to be contained in 0.15 m*l* of the reaction mixture, assuming that all DNA present in sonicated nuclear lysates can be recovered in the nuclear high salt extract obtained after high-speed centrifugation.

coideum DNA present in sonicated nuclear lysates, because the extent of the contamination of cytoplasmic material into the nuclear preparation from which the high salt extract was obtained was not always identical in separate experiments. It is apparent that the specific activity of enzyme I is the highest at the vegetatively growing stage and thereafter decreases gradually with the progerss of development. On the other hand, enzyme II exhibits nearly constant activities throughout development, though a slight increase is seen in the aggregation stage. As a result, the ratio of the specific enzyme activity between enzymes I and II became progressively smaller as the development proceeds.

As mentioned above, almost all (more than 94%) of the polymerase activity present in sonicated nuclear lysates was capable of being recovered in the nuclear high salt extract and furthermore the specific activity of the enzymes could be expressed with the amount of DNA contained in sonicated nuclear lysates. The above data, therefore, will probably be considered as more reliable indication of the levels of the enzyme activity. Thus, it seems certain that the activity of RNA polymerase, particularly enzyme I, changes during development of this slime mold.

Discussion

The cell differentiation of *D. discoideum* amoebae has been shown to be accompanied with the changes of the cell metabolism including RNA synthesis (GREGG, 1966; SUSSMAN, 1966; WRIGHT, 1973; LONG and COE, 1974; MIZUKAMI and IWABUCHI, 1970; LODISH *et al.*, 1974). In the present study, we examined some properties of DNA-dependent RNA polymerase of this organism which was partially purified by DEAE-Sephadex chromatography and the alteration of the levels of the enzyme activity during development. The experimental results have demonstrated that *D. discoideum* NC-4 possesses two types of RNA polymerases; one is termed enzyme I which is insensitive to α-amanitin and the other is enzyme II sensitive to the drug. Therefore, it is highly probable that enzyme I resembles the nucleolar RNA polymerase which has been assumed to transcribe rRNA and enzyme II is similar to the nucleoplasmic RNA polymerase which is thought to be involved in synthesis of mRNA (RUTTER *et al.*, 1974).

In the analysis of the polymerase by DEAE-Sephadex chromatography, we did not detect the third peak of the polymerase activity reported by PONG and LOOMIS (1973) who obtained *D. discoideum* RNA polymerase from an axenic strain, A3. Whether the inconsistent results are due to the difference of the strains or to the difference of the methods used for preparation

of the enzyme has yet been obscure.

In the experiment where the levels of the activity of two RNA polymerases in the nuclear high salt extract were examined by utilizing the difference in their sensitivity to α-amanitin, we observed that the specific activity of enzyme I markedly varied throughout development, while that of enzyme II was relatively constant except for a slight but significant increase in the cell-aggregation stage. Similar phenomenon has recently been reported in intact nuclei of *D. discoideum* amoebae by SOLL and SUSSMAN (1973), who observed that the nuclei isolated from vegetative cells and from cells in morphogenetic phase display significant differences in the ability of α-amanitin-sensitive and -resistant UTP incorporations into RNA. We have obtained evidence that the alteration of the ratio of enzyme I to enzyme II in the specific activity is in good parallel with the change of the proportion of the synthesis rate between rRNA and messenger-like RNA (MIZUKAMI and IWABUCHI, 1970; YAGURA, T., YANAGISAWA, M. and IWABUCHI, M., manuscript in preparation).

To explain the changes of RNA polymerase activity during development, at least two possibilities will be offered. One is that there is the factor(s) which can influence the enzyme activity or the template activity of DNA. The second is the quantitative changes of the enzyme itself. Some experiments to elucidate these points are in progress with RNA polymerase preparations of enzymes I and II purified by DEAE-Sephadex chromatography. The experimental results so far obtained, which will be published elsewhere, appear to support the notion that the changes of the activity level of enzymes I and II during development are possibly due to the quantitative alterations of these enzymes.

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