NUCLEIC ACID SYNTHESIS IN *PSEUDOMONAS P*

(1) EFFECTS OF CELL CONCENTRATION

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The current hypothesis suggests that all types of RNA (soluble RNA, s-RNA; messenger RNA, m-RNA; and ribosomal RNA, r-RNA) are synthesized on DNA as a template. The regulation mechanisms of synthesis of each RNA have been studied in many laboratories. In our laboratory, nucleic acid synthesis of a strain of *Pseudomonas P* has been studied. The experiments to be described will show that when the cells are incubated at high concentration in a synthetic medium containing amino acids, r-RNA synthesis is almost completely inhibited, while syntheses of DNA, s-RNA and m-RNA continue. When the concentration of these cells is diluted to the normal concentration, specific synthesis of r-RNA is observed. This means that r-RNA synthesis can be specifically controlled by only modifying the cell concentration.

EXPERIMENTAL

Microorganism

Pseudomonas P (1) was grown in NB-P medium which contained 8 g of polypeptone in 1 l of distilled water. The pH was adjusted to 6.7. Cultivation was carried out at 30°C with shaking.

Media and Conditions for Incubation

"Conc. culture"

The logarithmically growing cells were harvested and washed once with Tris-MgCl₂ solution (0.05 M Tris buffer, pH 7.6, and 5 mM MgCl₂), and the cells were suspended in the incubation mixture, whose final composition was as follows: 100 mg wet cells (the final concentration was 25 mg/ml and this concentration was about 12 times as much as that of the logarithmically growing culture), 20 μ moles MgCl₂, 5 μ moles phosphate buffer, pH 6.7, 200 μ moles Tris buffer, pH 7.6, 0.2 mg L-tryptophan, 2 mg casamino acids and 1 μ mole each adenine, guanine, uracil and cytosine. The final volume was 4 ml. About 50–100 μ c carrier-free orthophosphate (³²P) was added prior to incubation. Incubation was carried out in a Monod tube at 30°C with shaking.

"Dil. culture"

The cells incubated in "conc. culture" were collected and washed once

with Tris-MgCl₂ solution containing 5 mM phosphate buffer. They were resuspended in NB-P medium at a final concentration of 2 mg wet cells per ml. This concentration of cells was about the same as that of the logarithmically growing culture.

Preparation of Nucleic Acids

The cells were collected after incubation and destructed by sonic oscillation in Tris-MgCl₂ solution. Nucleic acids were extracted by equal volumes of water-saturated phenol in the presence of 1% sodium dodecyl sulfate. Separation of nucleic acids into s-RNA, DNA and r-RNA was carried out by column chromatography on methylated albumin kieselguhr (MAK) prepared according to MANDELL and HERSHEY (2). All salt solutions used during chromatography contained 0.05 M phosphate buffer, pH 6.7, and elution procedure was accomplished with linear NaCl gradient ranging from 0.4 M to about 1.1 M.

Analysis of Ribosomal Fraction by Ultracentrifugation

The crude extract obtained by sonic oscillation was subjected to 13,000×*g* centrifugation for 20 min. to remove intact cells. The supernatant was centrifuged at 105,000×*g* for 120 min. and the pellet obtained was resuspended in Tris-MgCl₂ solution (the ribosomal fraction). After dialysis against 0.05 M Tris buffer, pH 7.6, containing 5 mM or 0.1 mM MgCl₂, 0.3 ml of this ribosomal fraction was put on 4.4 ml of linear sucrose gradient from 3 to 20% (W/W) dissolved in 0.05 M Tris buffer containing 5 mM or 0.1 mM MgCl₂ (3-4). Each was run at 35,000 rev./min. for 75 min. in a swinging bucket rotor, Spinco type SW-39, in a model-L. After centrifugation, successive 8-drop samples were removed through a syringe needle piercing the bottom of the tube. 3 ml of Tris-MgCl₂ solution was added to each fraction and O.D. at 260 mμ and the radioactivity determined.

Base Composition Analysis

r-RNA fractions purified by MAK column chromatography were pooled and after removal of salt by dialysis against distilled water, they were concentrated to about 5 ml at 40–50°C under reduced pressure. 40% trichloroacetic acid was added to a final concentration of 5%, and after the precipitate was washed once with alcohol-ether, it was subjected to digestion with 0.3 N KOH at 37°C for 18 hours. The resulting nucleotides were separated by two dimensional paper chromatography. The solvent systems were: isobutyric acid–1 N NH₄OH–0.1 M Na₂EDTA=100/60/1.6 (descending); and methanol–ethanol–conc. HCl–distilled water=50/25/6/19 (ascending). The U.V. absorption spots were cut and the radioactivity determined by a Packard Tri-carb scintillation counter.

RESULTS

(³²P)-Nucleic Acids Synthesized in "Conc. Culture"

MAK column chromatography patterns of nucleic acids synthesized in

Fig. 1-a.

Fig. 1-b.

Fig. 1-c.

Fig. 1-d.

Fig. 1. MAK-column chromatography of nucleic acids prepared from "conc. culture"

The cells were resuspended to a final concentration of 25 mg wet cells/ml ("conc. culture") in ^{32}P i containing medium, (a) for 25 min., (b) for 50 min., (c) for 75 min., and (d) for 100 min. respectively. Nucleic acid were extracted by SDS-phenol method and separated by MAK-column chromatography. Approx. 100 samples of 4.5 ml each were collected. The absorbancy at 260 $m\mu$ (—) was measured and the radioactivity of each 0.5 ml (.....) was counted.

Fig. 2. The time course of the specific activity of nucleic acids separated by MAK-column chromatography. (In "conc. culture".)

From the cells of "conc. culture", nucleic acids were extracted at intervals and separated by MAK-column chromatography as shown in Fig. 1. The specific activity of each fraction was calculated.

"conc. culture" are shown in Fig. 1. As these experiments were made under the same conditions using the same medium, the specific activity of each pattern can be compared directly. In each instance, the O.D. profiles show s-RNA, DNA and two components of r-RNA (5).

A characteristic feature in these patterns is that incorporation of ^{32}P into r-RNA fractions is strongly inhibited, while DNA and s-RNA syntheses continue; this is clearly observed in Fig. 2, in which the time course of the specific activity of each fraction calculated from Fig. 1 is shown.

Another characteristic feature in Fig. 1 is that O.D. and count patterns of only r-RNA fractions show a discrepancy (6-7) which is not observed in s-RNA and DNA fractions.

The base composition analysis of ^{32}P -RNA in r-RNA fraction obtained from "conc. culture 25 min." (Fig. 1-a) was carried out. The result shown in Table 1 indicates that the base composition of this ^{32}P -RNA is similar to that of DNA, not to that of r-RNA of this strain.

Table 1. Base composition of ^{32}P -RNA Obtained in r-RNA Fractions of MAK-Column Chromatography.

Culture	Base Compositions (Moles per cent)					
	A	G	U	C	GC%	Pu/Py
"conc. culture 25 min."	20.5	31.4	16.0	32.1	63.5	1.08
"dil. culture 5 min."	23.7	29.8	20.0	26.4	56.2	1.15
"dil. culture 15 min."	24.3	32.1	20.6	23.0	55.1	1.30
"dil. culture 30 min."	26.0	32.2	24.4	17.4	49.6	1.39
r-RNA of <i>Ps. P</i>	24.6	33.0	23.2	19.2	52.2	1.36
DNA of <i>Ps. P</i>	19.5	30.5	19.5	30.5	61.0	1.00

The cells were collected from "conc. culture" or "dil. culture" as indicated. Nucleic acids were extracted and separated by MAK-column chromatography. r-RNA fractions were pooled and hydrolyzed with alkali. The nucleotides in the resulting hydrolysate were separated by two dimensional paper chromatography. The U.V. absorption spots were cut and their radioactivities were counted. For purpose of comparison, r-RNA base composition data are included in addition to DNA base composition data of this strain.

Characterization of ^{32}P -RNA of "conc. culture 25 min." was also carried out by ultracentrifugal analysis. As shown in Fig. 3, most of the ^{32}P -RNA existed on ribosomes only when the concentration of MgCl_2 was 5 mM. When it was decreased to 0.1 mM, the ^{32}P -RNA was released from the ribosomes (4, 8).

From these data concerning RNA synthesized in "conc. culture 25 min.", it was concluded that most of the ^{32}P -RNA found in the r-RNA fraction from MAK column chromatography has a feature characteristic of m-RNA and that true r-RNA synthesis is almost completely inhibited under this

Fig. 3. Sucrose density gradient analysis of ribosomal fraction obtained from "conc. culture 25 min."

Ribosomes were obtained from the cells of "conc. culture 25 min." and dialyzed against 0.05 M Tris-buffer (pH 7.6) containing: (a) 5 mM MgCl₂, (b) 0.1 mM MgCl₂. Centrifugation was carried out at 35,000 rev/min. for 75 min. in 3–20% sucrose gradient containing the buffer used in dialysis. (—) O.D. 260 mμ, (.....) cpm.

Fig. 4. MAK-column chromatography of nucleic acids extracted from "dil. culture".

The "conc. culture 25 min." cells labelled with ³²Pi were collected and washed once with Tris-buffer. They were resuspended to a final concentration of 2 mg wet cells/ml ("dil. culture") and incubated at 30°C, (a) for 5 min., (b) for 15 min., and (c) for 30 min., respectively. Other procedures are as shown in Fig. 1. (—) O.D. 260 mμ, (.....) cpm.

incubation condition.

(³²P)-Nucleic Acids Extracted from "Dil. Culture"

1. After "conc. culture 25 min."

Changes of patterns of MAK column chromatography of nucleic acids from "conc. culture 25 min." to "dil. culture" are shown in Fig. 4 and the base compositions of each ³²P-RNA fraction are summarized in Table 1.

These results show that 15 min. incubation in "dil. culture" after "conc. culture 25 min." is sufficient to make nearly complete recovery of

Fig. 5. The time course of the specific activity of nucleic acids separated by MAK-column chromatography. (In "dil. culture" after "conc. culture 25 min.")

After "con. culture 25 min.", the cells were incubated in "dil. culture", and nucleic acids were extracted at intervals. Other procedures are as shown in Fig. 2.

normal r-RNA synthesis, judging from MAK column chromatography patterns and the base compositions. In Fig. 5, the time course of the specific activity of each fraction of MAK column chromatography is shown. s-RNA, DNA and r-RNA increased at about the same rates.

2. After "conc. culture 100 min."

After "conc. culture 100 min.", the cells were shifted to "dil. culture". Nucleic acids were extracted at intervals by SDS-phenol method and separated

by MAK column chromatography. The specific activity of each fraction was calculated and the time course is shown in Fig. 6.

It is very characteristic that only r-RNA synthesis occurred while DNA and s-RNA syntheses were inhibited, as compared with the results obtained in the system of "conc. culture 25 min—dil. culture", which are shown above.

Fig. 6. The time course of the specific activity of nucleic acids separated by MAK-column chromatography. (In "dil. culture" after "conc. culture 100 min.")

After "conc. culture 100 min.", the cells were incubated in "dil. culture" and other procedures as shown in Fig. 5.

DISCUSSION

It is widely known that the growth rate of bacteria and the rate of r-RNA synthesis have a close relationship. When bacteria are transferred into synthetic medium from nutrient broth (shift down), synthesis of r-RNA stops immediately, and when the cells are transferred again into growing medium (shift up), r-RNA synthesis is greatly increased. SPIEGELMAN (9) reported that use of shift down culture facilitated ready exhibition of m-RNA in the intact cells.

In our laboratory, the rate of r-RNA synthesis has been controlled by

modification of the concentration of the cells in the culture as described by MIZUNO (10).

From the patterns of MAK column chromatography, base composition analyses and the patterns of sucrose density gradient, it is concluded that in "conc. culture", r-RNA synthesis was almost completely repressed and that m-RNA synthesis could be detected. In "conc. culture", synthetic medium was used in order to learn the fate of ^{14}C -uracil incorporated into RNA, as described in the following report. It should be mentioned that even when N.B.-P medium (nutrient broth) was used in "conc. culture" instead of the synthetic medium, approximately the same phenomena were observed.

When the cells from "conc. culture" were resuspended to "dil. culture", r-RNA synthesis began to occur. It was remarkable that the rates of syntheses of s-RNA, DNA and r-RNA in "dil. culture" were very much influenced by the length of time during which the cells were incubated in "conc. culture". In the system of "conc. culture 25 min.—dil. culture", each nucleic acid was synthesized at about the same rate, but in the system of "conc. culture 100 min.—dil. culture", synthesis of r-RNA occurred but syntheses of DNA and s-RNA were completely inhibited. From these facts, it may be concluded that syntheses of r-RNA, s-RNA, m-RNA and DNA are controlled under different regulation mechanism.

SUMMARY

1. Logarithmically growing cells of *Pseudomonas* P were suspended to a final concentration of 25 mg wet cells/ml ("conc. culture"). Under these conditions, synthesis of r-RNA is almost completely inhibited and m-RNA is detected, while syntheses of s-RNA and DNA continue.

2. When these cells are resuspended to a final concentration of 2 mg wet cells/ml ("dil. culture"), synthesis of r-RNA begins. In "conc. culture 25 min.—dil. culture" system, DNA, s-RNA and r-RNA syntheses occur at about the same rate, but in "conc. culture 100 min.—dil. culture" system, the specific synthesis of r-RNA is observed.

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