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Control of Deoxyribonucleic Acid and Ribonucleic Acid Synthesis in Pyrimidine-Limited *Escherichia coli*

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The effects of pyrimidine limitation on chromosome replication and the control of ribosomal and transfer ribonucleic acid syntheses were investigated. Chromosome replication was studied by autoradiography of ^{3}H-thymine pulse-labeled cells. Pyrimidine limitation did not affect the fraction of cells incorporating radioactive thymine during a short pulse, indicating that when growth is limited by the supply of pyrimidine, the time required for chromosome duplication increases in proportion to the time required for cell duplication. Control of ribosomal RNA and transfer RNA syntheses was examined by chromatographing cell extracts on methylated albumin kieselguhr columns. When growth was controlled by carbon-nitrogen limitation, the ratio of tRNA to total RNA remained roughly constant at growth rates above 0.5 doublings per hour. During pyrimidine limitation, however, the control of rRNA synthesis was apparently dissociated from the control of tRNA synthesis: the ratio of tRNA to total RNA increased as the growth rate decreased.

Steady-state pyrimidine limitation in cultures of *Escherichia coli* results in a decrease in the intracellular pyrimidine pools and an adjustment of the macromolecular composition (4). In this study we asked two rather unrelated questions concerning the effects of pyrimidine limitation on nucleic acid synthesis. The first concerned the relationship between chromosome replication and the cell cycle in normal and pyrimidine-limited cultures. The second question concerned the relative concentrations of ribosomal ribonucleic acid (rRNA) and transfer RNA (tRNA) in normal carbon-nitrogen-limited cultures (in which the growth rate was controlled by the carbon source or the addition of amino acids, vitamins, and other growth promoting substances to the medium) and in pyrimidine-limited cultures as a function of growth rate.

Control of the initiation and rate of chromosome replication as a function of growth rate in synchronized cultures of *E. coli* B/r has been well-documented by Helmstetter and Cooper (3, 6-8). In carbon-nitrogen-limited cells growing slower than 1 u (doublings per hour), initiation of chromosome replication seems to occur about the time of cell division, and replication requires a constant proportion of the cell cycle. A gap in deoxyribonucleic acid (DNA) synthesis is thought to occur during the final portion of the cell cycle. In cells growing faster than 1 u, chromosome replication requires only 40 min. This leaves a gap in DNA synthesis for cultures growing at a rate between 1.5 and 1 u. Cells growing faster than 1.5 u show no gap and possess chromosomes with multiple replicating points during some period of the cell cycle.

Pyrimidine limitation could affect chromosome replication in several ways. First, it might not alter the rate of DNA synthesis, and the chromosome replicating cycle would require only 40 min or the same amount of time required for chromosome duplication in normal cultures supplemented with uracil. Secondly, the time required for chromosome duplication could increase in proportion to the increase in the time required for cell duplication, occupying a constant proportion of the generation time, as in carbon-nitrogen-limited cells. Finally, pyrimidine limitation could result in an increase in the proportion of the cell cycle during which chromosome duplication occurs so that DNA synthesis occurs during most or all of the cell cycle. We have obtained evidence supporting the second hypothesis.

Rosset, Julien, and Monier (13) and more

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recently Forchhammer and Kjeldgaard (5) have studied the RNA composition in carbon-nitrogen-limited, steady-state cultures of *E. coli*. They have shown that the ratios of rRNA to DNA and tRNA to DNA decrease roughly in proportion to the total RNA to DNA ratio as the growth rate decreases. We have confirmed these results in carbon-nitrogen-limited cultures. However, in steady state pyrimidine-limited cultures, although the ratios of rRNA to DNA and total RNA to DNA decrease with decreasing growth rate, the ratio of tRNA to DNA does not decrease.

MATERIALS AND METHODS

Bacterial strains. Two strains used in these experiments have been described in the previous paper (4). Strain CYA 288 requires uracil or orotic acid (hereafter referred to simply as a uracil requirement), and PD101, a derivative of CYA 288, requires uracil and thymine. All cultures were grown and monitored as previously described (4). PD101 grew slightly more slowly than CYA 288.

Autoradiography. Cultures of PD101 were grown in the M63-B_1 medium containing glycerol, thymine, Difco Casamino Acids, and either uracil (20 μg/ml) or orotic acid (10 μg/ml). When the exponentially growing cultures reached an optical density (OD) of 0.5, they were diluted 1:1 into preconditioned labeling media. Radioactive labeling media were preconditioned by growing strain PD101 to an OD of 0.25. The cells were removed by centrifugation and the supernatant solution was removed and immediately used for labeling. The media were preconditioned to avoid a lag in growth and to remove radioactive impurities which might have been preferentially incorporated into macromolecular material during the pulse-labeling period (F. Forro, *personal communication*). The incorporation of either uracil-6-^3H (220 mc/mmole, New England Nuclear Corp.) or thymine-methyl-^{3}H (2,500 mc/mmole, Schwarz Bioresearch Corp.) was allowed to proceed for one-tenth of a generation. Incorporation was terminated by addition of formaldehyde to a final concentration of 4% and an excess of either cold uracil (500 μg/ml) or thymine (125 μg/ml) at 0 C. After 15 min of storage at 0 C, the cells were centrifuged and washed four times in ice-cold 0.1 M potassium phosphate buffer (*p*H 7.0) containing 1% formaldehyde. The cells could then be stored several weeks in the phosphate 1% formaldehyde solution.

After a final centrifugation to remove the formaldehyde, the cells were diluted from the phosphate buffer into 0.5% tryptone and spotted on clean "subbed" (2) glass microscope slides by using a 1-ml pipette. The spotted slides were placed on a hot plate at 45 to 50 C to dry. After cooling, the slides were placed in Carnoy's solution (3 parts absolute ethyl alcohol, 1 part glacial acetic acid) for 20 min and then 2 min each in aqueous solutions of 95, 85, 70, 50, 30, and 0% ethyl alcohol, respectively. The purpose of the acid-alcohol and graded-alcohol baths was to facilitate removal of intracellular thymine or uracil pools.

A modification of the autoradiographic processing procedures described by Caro has been used (2). The emulsion was prepared by dissolving 25 g of Ilford Nuclear Research Emulsion L4 in 20 ml of distilled water at 45 C to get a homogenous milky solution. With a uniform motion, the slides were dipped into the emulsion. They were allowed to dry in a vertical position in a light-proof box equipped with an air-circulating system. After several hours, the slides were removed from the box and placed in plastic slide boxes containing cotton-plugged vials of Drierite (anhydrous $CaSO_4$) as desiccant. The slide boxes were then placed in a light-proof metal desiccator (also containing Drierite) for exposure.

After the exposure period, the slides were processed in the following way: 5 min in Kodak D19 developer, 5 sec in distilled water, 4 min in Kodak F-24 nonhardening fixer, and finally five rinses in distilled water. All solutions were kept at 22 C during development. The slides were dried and stored in a dust-free area.

For examination, a large no. 1 glass coverslip was mounted with a drop of glycerol. The slide was examined with Zeiss phase-contrast and bright-field oil immersion objectives. After examination, the coverslips were removed, and the slides were rinsed in distilled water, dried, and stored for future examination.

RNA composition. Strain CYA288 was used in all experiments involving RNA composition as a function of growth rate. The pyrimidine-limited cultures were grown in the M63-B_1 medium. Either glucose, glycerol, or rhamnose was added as the carbon source; uracil was added as the pyrimidine source; and, in some cases, Difco Casamino Acids were also added.

When the OD of an exponentially growing culture reached 0.5, a 50-ml portion of the culture was centrifuged, and the cells were suspended in 20 ml of 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 7.6) and 0.01 M $MgCl_2$ (TM buffer). The cells were again centrifuged, resuspended in 10 ml of TM buffer, and broken by passage through a French pressure cell at 7,000 lb/in^2. The unbroken cells and cell debris were removed by centrifugation at 15,000 $\times$ *g* for 10 min. RNA was extracted according to the procedures of Rosset, Julien, and Monier (13). The DNA was not removed, however, since it did not interfere with the chromatographic fractionation of RNA. Approximately 10% of the total RNA (determined by orcinol assay) was lost with the unbroken cells and cell debris following passage through the French pressure cell. Approximately 90% of the remaining RNA was recovered after the extraction and ethyl alcohol precipitations. The recovery of RNA from pyrimidine-limited and carbon-nitrogen-limited cultures was independent of growth rate.

For chromatography, the nucleic acid was dissolved in 8 to 10 ml of 0.05 M sodium phosphate buffer (*p*H 6.7) and 0.2 M NaCl. The entire sample (0.5 to 0.75 mg of RNA) was placed on a methylated serum albumin kieselguhr (MAK) column (1 by 12.5 cm; reference 12) at room temperature for fractionation of the nucleic acids. The column was washed

with 10 ml of 0.2 M NaCl and 10 ml of 0.4 M NaCl. All NaCl solutions were in 0.05 M sodium phosphate buffer (*p*H 6.7). Fractions (5 ml) were collected, and the OD of each fraction was determined at 260 nm in a Gilford spectrophotometer.

Electrophoresis of RNA. Nucleic acid extracts from cultures labeled with either ^{14}C-uracil (0.016 μc/ml) or ^{14}C-orotic (0.033 μc/ml) were prepared as described above. Approximately 200 μg of RNA were applied to a gel (1.2 by 10 cm) containing 7.5% acrylamide and 0.2% bisacrylamide. Electrophoresis was carried out for 7 hr at a current setting of 7.5 ma per tube. The gel buffer was 0.04 M Tris, 0.02 M sodium acetate, and 0.002 M ethylenediamenetetraacetate (*p*H 7.8). The gels were stained overnight by using 1% lanthanum acetate and 2% acridine orange in 15% acetic acid. After destaining in 15% acetic acid, the RNA bands were cut out, dried, and dissolved in 0.1 to 0.2 ml of 40% hydrogen peroxide at 45 C. One milliliter of NCS reagent (Amersham-Searle) was added to each sample, followed by 10 ml of Bray's solution (1). The vials were counted, and the counts were corrected for quenching by the method of channels ratio.

RESULTS

DNA replication. We have used autoradiography of ^{3}H-thymine-pulsed cells to compare the proportion of cells in normal and pyrimidine-limited populations that are in the gap between rounds of DNA replication. Portions from a normal (supplemented with 20 μg of uracil per ml; u = 1.03) and pyrimidine-limited, exponential culture (supplemented with 10 μg of orotic acid per ml; u = 0.43) were pulse-labeled with ^{3}H-thymine for one-tenth of a generation. The cells were autoradiographed, and the fractions of unlabeled cells after different periods of exposure were estimated (Table 1). Both ^{3}H-thymine-pulsed samples gave an average of about 0.25 grains per cell per day of exposure. The proportion of cells not associated with developed photographic grains remained equal to about 8 or 9% in the thymine-pulsed cultures as the exposure period was increased. A portion from the normal culture was also pulsed with ^{3}H-uracil for one-tenth of a generation to determine the fraction of metabolically inactive cells. The uracil pulse produced an average of 0.5 grains per cell per day of exposure. The proportion of unlabeled cells from the uracil-pulsed culture remained at approximately 1.5%. The 6 to 7% of the population incorporating uracil but not thymine probably represents metabolically active cells with all chromosomes in the gap between rounds of DNA replication during at least most, and probably all, of the labeling period. Thus pyrimidine limitation appears to have had no effect on the proportion of cells in the culture that are in the gap between rounds of DNA replication.

RNA composition. The nucleic acids isolated from steady state cultures were fractionated on MAK columns. Figure 1 illustrates the separation into peak A containing tRNA and 5*S* RNA and the double peak B containing mainly 16 and 23*S* ribosomal RNA. The center peak contains DNA almost exclusively (12, 13). The amount of DNA recovered in this peak, however, varies quite drastically from one preparation to the next, whereas the RNA recoveries are fairly constant. The amounts of RNA in peaks A and B were determined by summing the OD values at 260 nm of the fractions in each peak (Fig. 1, bars). The ratios of the RNA in peak A to the total RNA in peak A plus peak B were determined in pyrimidine-limited and in carbon-nitrogen-limited, steady-state cultures. The proportion of the total RNA found in peak A for carbon-nitrogen-limited cultures increases only slightly as the growth rate decreases from 2.0 to 0.5 u (Fig. 2). At growth rates below 0.5 u, the ratio increases more rapidly. In pyrimidine-limited cultures, however, the ratio of RNA in peak A to total RNA increases rapidly as the growth rate decreases from 1.1 to 0.4 u. The response of the mechanism controlling RNA synthesis is clearly different during pyrimidine limitation as compared to carbon-nitrogen limitation over the same range of growth rates.

Two types of RNA have been shown to occur

TABLE 1. *Fractions of unlabeled cells after radioactive pulse*

Exposure period	^{3}H-thymine pulse: normal culture		^{3}H-thymine pulse: pyrimidine-limited culture		^{3}H-uracil pulse: normal culture	
	Frequency	Per cent	Frequency	Per cent	Frequency	Per cent
days						
28	111/1257	8.8 ± 1.6[a]	78/897	8.7 ± 1.8	12/1089	1.8 ± 0.6
49	80/966	8.3 ± 1.9	73/831	8.8 ± 1.9	29/2419	1.2 ± 0.4
70	52/564	9.2 ± 2.4	57/566	10.0 ± 2.5	15/916	1.64 ± 0.8
144	163/2081	7.8 ± 1.2	165/1926	8.5 ± 1.2	39/2358	1.65 ± 0.4

[a] Ninety-five per cent confidence interval for the estimate of the number of unlabeled cells in the pulse-labeled sample.

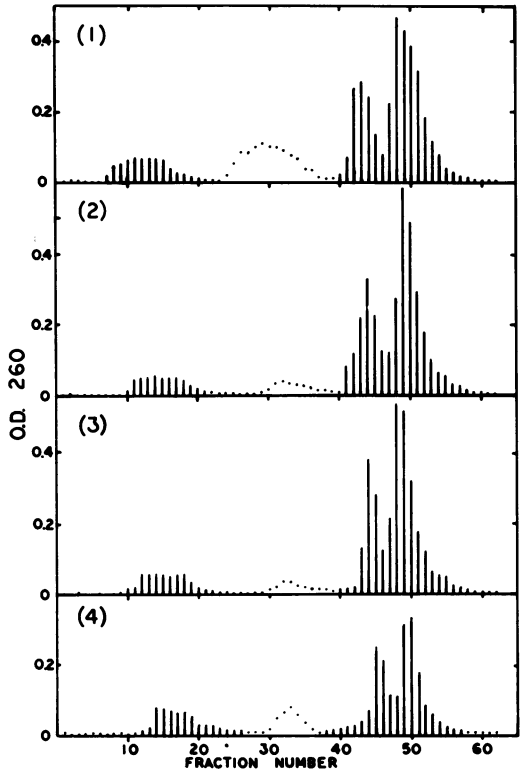

FIG. 1. *MAK column chromatography of nucleic acid extracts. The first peak, containing mainly tRNA, is called peak A (bars) and the last double peak containing mainly rRNA is called peak B (bars). The center peak contains mainly DNA (points). (1) Extract from glycerol-uracil culture (u = 0.6) with a ratio of the area under A to the total area under A and B equal to 14.7%. (2) Extract from glycerol-orotic acid (100 μg/ml)-Casamino Acids culture (u = 1.1) with a ratio of 12.2%. (3) Extract from glycerol-uracil-Casamino Acids culture (u = 1.15) with a ratio of 13.9%. (4) Extract from glycerol-orotic acid (20 μg/ml)-Casamino Acids culture (u = 0.5) and a ratio of 23.1%.*

in peak A from MAK columns: tRNA and 5S RNA (13). The 5S RNA has been identified as a normal structural component of the 50S ribosome subunit, and its concentration reportedly varies in proportion to the ribosome content (13). To check this point with regard to pyrimidine-limited cultures, polyacrylamide gel electrophoresis has been used to further characterize peak A. Two distinct bands corresponding to 4 and 5S RNA have been observed after electrophoresis of either the total RNA extract or the RNA from peak A. (The 16 and 23S RNA species, when present in the sample, fail to penetrate the gel.) The ratios of radioactivity in the 5S band to the radioactivity in both the 4 and 5S bands have been determined for RNA extracts from normal ^{14}C-uracil-supplemented cultures and from pyrimidine-limited cultures supplemented with ^{14}C-orotic acid (Table 2). The ratio in uracil-grown cultures was found to be about 0.17, whereas, in pyrimidine-limited cultures, the ratio decreased from about 0.20 to about 0.10 as the growth rate decreased from 1.10 to 0.43 u. From the data given in Table 2 and Fig. 2, we have computed that the ratios of 5S RNA to rRNA are 0.029, 0.030, 0.030, and 0.031 for the four growth rates listed in Table 2, respectively. We conclude that the concentration of 5S RNA is proportional to the RNA content over the range of pyrimidine-limited growth rates we have used.

The results of diphenylamine, orcinol, and Folin assays to determine DNA, RNA, and protein contents of pyrimidine-limited cultures have been reported in the accompanying paper (4). The DNA to protein ratio was found to remain constant, whereas the RNA to protein ratio decreased as the growth rate decreased. The RNA to DNA, tRNA (peak A minus 5S RNA) to DNA, and the rRNA (peak B) to DNA ratios are illustrated in Fig. 3 as a function of pyrimidine-limited growth. As the growth rate decreases in pyrimidine-limited cultures, the amount of tRNA relative to DNA does not decrease, whereas the amount of rRNA relative to DNA decreases at least as rapidly as the total RNA to DNA ratio. In carbon-nitrogen-limited growth, on the other hand, the ratio of tRNA to DNA decreases proportionately with the decrease in the ratio of total RNA to DNA.

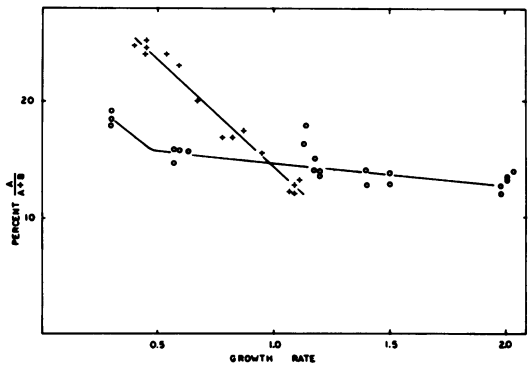

FIG. 2. *Ratios of nonribosomal RNA to total RNA. Pyrimidine-limited cultures (+) were supplemented with orotic acid (10 to 100 μg/ml). Carbon-nitrogen-limited cultures were supplemented with uracil and grown in Penassay broth, glucose-Casamino Acids, glycerol-Casamino Acids, glycerol-minimal, and rhamnose-minimal medium (in order of decreasing growth rates). The growth rate units are doublings per hour.*

TABLE 2. *Proportion of 5S RNA in peak A*

Pyrimidine supplement	Growth rate (u)	Proportion of 5S RNA[a]	No. of determinations
Uracil (20 μg/ml)	1.13	0.170 ± 0.014	5
Orotic acid (100 μg/ml)	1.10	0.202 ± 0.026	3
Orotic acid (30 μg/ml)	0.63	0.110 ± 0.012	3
Orotic acid (10 μg/ml)	0.43	0.095 ± 0.015	4

[a] Represents the radioactivity of the 5S RNA divided by the radioactivity of the 5S RNA plus the 4S RNA. Errors given encompass the ranges of all determinations.

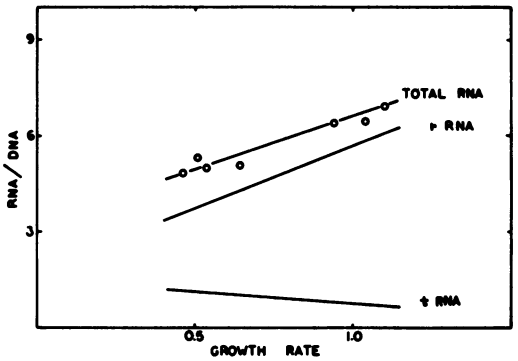

FIG. 3. *Ratios of RNA to DNA in pyrimidine-limited cultures. The amounts of tRNA and rRNA were determined by multiplying the total RNA* (○) *by the per cent RNA in peak A (minus the 5S RNA fraction) and peak B, respectively (obtained from Fig. 2), at the corresponding growth rates.*

DISCUSSION

By using the method of pulse-labeling and autoradiography, we have observed only about 8% unlabeled cells in both normal (1.04 u) and pyrimidine-limited (0.45 u) cultures after a pulse amounting to one-tenth doubling time (Table 1). This value is obviously an underestimate of the proportion of cells not synthesizing DNA, since cells initiating a new round of replication during the period of the pulse will be labeled. We estimate that if the gap in DNA synthesis occurs at the end of the cell cycle (just prior to cell division), a gap equal to 20% of the doubling time would give about 7 to 8% nonradioactive cells after a pulse of one-tenth doubling time. The purpose of these experiments, however, was not to determine the precise length of the gap in DNA synthesis but to compare normal and pyrimidine-limited cultures with respect to the proportions of cells not synthesizing DNA. We conclude that the proportions are the same in the two cultures, as are DNA contents per cell (4), and, therefore, that pyrimidine limitation increases the time required for chromosome duplication in proportion to the time required for cell duplication. The same conclusion has been reached for the case in which growth is limited by the source of carbon and energy (8).

When the growth rate in *E. coli* and *Salmonella typhimurium* is controlled by carbon-nitrogen or energy limitation or the addition of amino acids, vitamins, and other growth-stimulating compounds to the medium, the total RNA to protein ratio decreases as the growth rate decreases (9–11, 13). Initially, it was proposed that the decrease in RNA was entirely from a decrease in the concentration of ribosomes, and the amount of soluble or tRNA remained proportional to total protein or DNA (9, 10). More recently, however, it has been shown that the amounts of rRNA and tRNA decrease roughly in proportion to the decrease in total RNA (5, 13) down to doubling times of about 0.4 to 0.5 u, below which the ratio of tRNA to total RNA begins to increase (13). We have confirmed this general picture: our results with carbon-nitrogen-limited cultures show a very slight increase in the ratio of tRNA to total RNA as the growth rate decreases from 2.0 to 0.5 u (Fig. 2), and below 0.5 u the increase becomes more rapid.

In striking contrast to these results, however, we find that in pyrimidine-limited cultures, the ratio of tRNA to total RNA increases rapidly as the growth rate decreases from 1.1 to 0.4 u. The tRNA to DNA ratio does not decrease (but seems to increase) as the growth rate decreases, and the decrease in RNA concentration is largely from a decrease in the rRNA content of the culture. The concentration of 5S RNA decreases in parallel with the concentration of rRNA, but it amounts to only 3% that of rRNA. We are further attempting to characterize the RNA in peak A from MAK columns with respect to amino acid-accepting ability to determine whether the RNA composition changes as a result of pyrimidine limitation. We conclude that pyrimidine limitation leads to a disassociation of the control of tRNA and rRNA synthesis.

The amount of unstable RNA in log-phase cultures of *E. coli* has been estimated at 3% of

the total RNA (14). The ratio of messenger RNA to total RNA or rRNA in carbon-nitrogen-limited cultures as measured by stimulation of in vitro protein synthesis has been found to remain approximately constant, dropping off only slightly at slower growth rates (5). We have ignored the contribution of messenger RNA in our calculations of the RNA composition of cultures.

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