Stable Ribonucleic Acid Synthesis during the Cell Division Cycle in Slowly Growing *Escherichia coli* B/r*

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**SUMMARY**

The rate of synthesis of stable nucleic acid was measured during the division cycle in slowly growing cultures of *Escherichia coli* B/r. The experimental patterns of synthesis were consistent with a discontinuous mode of chromosome replication and an exponentially increasing rate of stable RNA synthesis. The rates of tRNA and rRNA synthesis relative to the rate of total stable RNA synthesis were constant during the cell division cycle and were unaffected by replication of stable RNA cistrons.

In *vivo* transcription of ribosomal RNA cistrons requires the presence of a protein factor *ψ*, in addition to the other normal constituents of the RNA-polymerase transcriptional system (1). The activity of this transcriptional factor is inhibited by the nucleotide, guanosine tetraphosphate (2), which has been shown to accumulate during amino acid deprivation in stringent strains of *Escherichia coli* (3, 4). This suggests the possibility that the rate of stable RNA synthesis may not be dependent on the number of genes for stable RNA but may be limited by the activity of *ψ* which in turn, determines the fraction of RNA-polymerase molecules in the cell capable of transcribing stable RNA cistrons. In rapidly growing cultures of *E. coli*, the rate of stable nucleic acid synthesis increases continuously and uniformly during the cell cycle and is unaffected by replication of stable RNA cistrons (8). In slowly growing cultures, the pattern of stable nucleic acid synthesis is more complex. In this report I have analyzed this pattern of synthesis with respect to the relative contributions of DNA, tRNA, and rRNA as a function of cell age. In addition, possible mechanisms for the regulation of transcription of stable RNA cistrons are discussed.

**MATERIALS AND METHODS**

The organism used was *E. coli* B/r (ATCC 12407). Growth was in a minimal salts medium (9) supplemented with succinic acid (5% solution adjusted to pH 6.7 with tris(hydroxymethyl)-aminomethane) to a final concentration of 0.1% as the sole carbon source. This medium was inoculated with bacteria from a stationary phase succinate culture and incubated approximately 18 hours at 37°C with shaking prior to experiments. Radioactive labeling experiments were performed when the cell density reached 1 x 10^9 (Coulter counts). Radioisotopes, [2-^14C]uracil (56.3 mCi per mm) and [methyl-^3H]thymidine (23 Ci per mm), were obtained from New England Nuclear Corp.

The rates of uracil and thymidine incorporation during the division cycle were measured by pulse labeling cells in exponential phase of growth for 4 min and determining the amount of radioactivity incorporated into cells of different ages. This was accomplished by binding the cells to a GS Millipore membrane filter at the end of the labeling period and determining the radioactivity in the newborn cells eluted continuously from the membrane. These procedures have been described (8, 9). Briefly, the cells were bound to the membrane by filtration and washed with 100 ml of conditioned medium. The filter assembly was then eluted and elution was measured with a Coulter Counter model B. To obtain total incorporation, an aliquot from each sample of the effluent was immediately collected on a 24-mm HA Millipore membrane filter, washed with minimal medium, dried, and the radioactivity determined with Liquifluor (Nuclear Chicago Corp.) with a Nuclear Chicago Liquid scintillation system.

In order to determine the distribution of the pulse of [^14C]uracil between tRNA, DNA, and rRNA, a second aliquot from each sample of the effluent was incubated at 37°C with shaking for 50 to 150 min. Each sample was then mixed with 50 ml of exponential phase, unlabeled succinate-grown cells at a cell density of 2 to 3 x 10^9. Following centrifugation and disruption by passage through a French pressure cell, the lysates were extracted with phenol and the nucleic acid was purified by ethanol precipitation as previously described (8, 10). Each sample (approximately 0.5 mg of nucleic acid) was fractionated on a methylated albumin-Kieselgel column into tRNA, DNA, and 18 to 23 S rRNA by eluting with a linear NaCl gradient (0.35 to 1.06 M) in 0.05 M sodium phosphate buffer (pH 6.7). Five milliliter fractions were collected and the absorbance at 260 nm was determined with a Gilford 240 spectrophotometer. Following addition of unlabeled yeast RNA, the nucleic acid was precipitated with ethanol and the protein was removed by centrifugation.

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RESULTS

In E. coli, there is a stringent control mechanism regulating the rate of uracil uptake and conversion to UMP (11, 12). During any time interval, the total increase in the cellular content of uracil is approximately the sum of (a) the incremental increase in the nucleotide pool size due to exponential growth, (b) the difference between the amount of mRNA synthesis and mRNA breakdown, and (c) the amount of DNA and stable RNA synthesis. In E. coli, at least 95% of the intracellular pyrimidine is found in stable nucleic acid while mRNA and the nucleotide pool account for the remaining 5% (13). Since 95% of the exogenous uracil entering the nucleotide pools compensates for incorporation of pyrimidine into stable RNA and DNA, the total uptake of uracil during a brief time interval represents a reasonable estimate of the rate of stable nucleic acid synthesis (see Reference 11 and "Appendix").

Total Stable Nucleic Acid Synthesis—The rate of stable nucleic acid synthesis (including DNA) during the division cycle of slowly growing cells was determined by pulse labeling exponential cultures growing in succinate minimal medium with uracil and employing the membrane elution technique to measure the incorporation of radioactivity into cells of different ages in the culture (Fig. 1). In the experiments illustrated in Curves A and C, radioactive uracil was added directly to the exponential phase cultures; in the experiment illustrated in Curve B, the cells were initially incubated with unlabeled uracil before the addition of the radioactive uracil pulse. The first samples of the effluent from the membrane-bound culture contained daughters of the cells which were about to divide in the exponential phase population at the time of the exposure to [14C]uracil. Subsequent samples during the first generation of elution contained progeny of progressively younger cells in the original population. This pattern repeats in the following generation, with the radioactivity per effluent cell at any age decreasing by a factor of 2 from one generation to the next. The pattern of stable nucleic acid synthesis during the division cycle is given from right to left in each generation of elution. In all cases the patterns were the same, the rate increased rapidly at the beginning of the division cycle (between ages 0 and 0.5 generation) and reached a maximum near the middle of the cycle. Following the maximum, a depression in the rate is evident (between ages of 0.6 and 0.9 generation) before the rate once again began to increase near the end of the cell cycle (ages 0.9 to 1.0 generation).

In slowly growing succinate cells, chromosome replication is confined to the first two-thirds of the cell division cycle (9, 14) and the ratio of DNA:RNA is relatively large. The temporal relationship between DNA and stable nucleic acid synthesis was determined by pulse labeling simultaneously with [3H]thymidine and [14C]uracil to measure the rates of DNA and stable nucleic acid synthesis, respectively (Fig. 2). The rapid increase in the rate of thymidine incorporation, corresponding to initiation of chromosome replication, occurs at, or slightly before, cell division while the gap in DNA synthesis (i.e. the period of decreased thymidine incorporation) occurs between cell age 0.6 to 0.9 generation. The uracil incorporation curve is identical with the three experiments illustrated in Fig. 1. From this experiment it is evident that the rate of stable nucleic acid synthesis (including DNA) increases rapidly during the period of DNA synthesis and the rate decreases slightly following completion of chromosome replication.

The relative contribution of chromosome replication to the rate of stable nucleic acid synthesis can be calculated from the
the time of initiation of chromosome replication and is based upon
the actual rate of thymidine incorporation observed in Fig. 2.
From these curves it is evident that the fluctuations observed in
the rate of stable nucleic acid synthesis during the cell division
cycle in succinate cells results at least partially, if not completely,
the discontinuous mode of chromosome replication.

Synthesis of tRNA, rRNA, and DNA—The relative rates of
tRNA, rRNA, and DNA synthesis as a function of cell age were
determined by measuring the proportion of the pulse of [3H]-
uracil ultimately incorporated into each of the three species of
nucleic acid. The total radioactivity per cell in 15 successive
effluent samples collected over a period of more than one gener-
ation is illustrated in Curve C (Fig. 1). A portion of each effluent
sample was mixed with exponential phase carrier cells and the
nucleic acid was extracted and chromatographically separated
into tRNA, DNA, and 16 to 23 S rRNA. The chromatographic
profiles of samples of the effluent containing cells of age 0.60,
0.08, and 0.70 generation at the time of the pulse label are illus-
trated in Fig. 3. The absorbance profiles in the samples repre-
sent the amount of the various species of nucleic acids in exponen-
tial phase cultures while the radioactivity profiles reflect the rates
of synthesis of tRNA, DNA, and rRNA in cells of a uniform
age.

The specific activity (radioactivity per absorbance) of the nu-
ucleic acid in each peak was calculated and compared to the
specific activity of the total RNA (tRNA and 16 S to 23 S rRNA
peaks) in the same sample (Table I). The ratio of specific activ-
ity of tRNA, or rRNA, or DNA to that of the total RNA reflects

Although unstable RNA (3- to 4-min half-life) represents
approximately 50% of the instantaneous rate of total RNA synthe-
sis, virtually all the radioactive uracil is incorporated into stable
nucleic acid within 18 min or 20% of the doubling time following
the termination of the pulse label (Reference 13 and unpublished
experiments).
termination of chromosome replication plus the expansion of the deoxyribonucleotide pools, and incorporation due to repair synthesis. In addition, when labeling with uracil, a lag in incorporation into stable nucleic acid results from the synthesis of unstable RNA. This lag along with the high rate of thymidine incorporation during this period are sufficient to account for the high specific activity ratio of DNA to total RNA observed from cell age 0.6 to 0.9 generation.

DISCUSSION

In rapidly growing cultures of E. coli B/r (1.3 to 2.4 doublings per hour) the rate of stable nucleic acid synthesis increases continuously and uniformly during the cell division cycle (8). In such cultures the DNA:RNA ratio is relatively small and DNA is synthesized throughout the cycle. Initiations and terminations of chromosome replication contribute relatively little to the rate of total nucleic acid synthesis, and the rate of stable RNA synthesis increases exponentially during the cell cycle (8).

In the experiments with slowly growing cultures (0.65 to 0.75 doubling per hour) reported here, the rate of stable nucleic acid synthesis fluctuates during the division cycle (Fig. 1). Conclusions concerning the mode of synthesis of stable RNA are complicated by the relatively large contribution of DNA to total nucleic acid synthesis during the period of chromosome replication. Comparison of the experimental curves with the results predicted by a theoretical model indicates that the periodicity of chromosome replication could account for the fluctuations observed in the patterns of synthesis of total stable nucleic acid. Therefore, the rate of stable RNA synthesis most probably increases exponentially during the cell cycle as in rapidly growing cells. The rates of tRNA and rRNA synthesis in both rapidly (9) and slowly growing cells are both proportional to the rate of total stable RNA synthesis. If the rate of synthesis were, in fact, limited by the dosage of stable RNA cistrons, the ratio of tRNA synthesis to total stable RNA synthesis should have de-

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\(^a\) Proportion of the total RNA (tRNA and rRNA) eluting in the tRNA peak.

\(^b\) Ratio of specific activities of tRNA, rRNA, and DNA to total RNA in each sample.

\(^c\) Standard deviation expressed as percentage of the mean.

Fig. 5. The differential rate of uracil uptake was determined with the expression \((U_s - U_i)/k_M(t_f - t_i)\) where \(t\) is the time after addition of uracil, \(U\) is the uracil incorporated between 0 and \(t\) min, \(k\) is the first order growth rate constant, and \(M\) is the mass of the culture at the midpoint of the time interval. When an exponential phase succinate culture reached a density of \(10^8\), 4 \(\times\) \(10^{-4}\) \(\text{M}\) uracil and \(10^{-6}\) \(\text{M}\) [\(^{14}\text{C}\)]uracil were added. At regular time intervals 1-ml portions were removed and either collected directly on Millipore filters and washed with 5- to 6-ml portions of minimal medium (uptake, \(\bullet\)) or precipitated with 1 ml of 10% trichloroacetic acid for 30 min at 0°C before collecting on a filter (trichloroacetic acid insoluble, \(\chi\)). All washes contained \(10^{-4}\) \(\text{M}\) uracil. The growth rate (88 min doubling time) was determined by monitoring the absorbance 460 nm and cell number (Coulter counts).
increased abruptly following replication of the ribosomal RNA cistron clusters (15-18). Therefore, it appears that stable RNA synthesis is regulated either at the level of initiation of transcription or by the degradation of newly synthesized RNA, and this regulation thus nullifies any effect of gene replication which might otherwise influence the rate of stable RNA synthesis.

If the fraction of active RNA-polymerase molecules is limiting, the replication of stable RNA cistron clusters would not be expected to alter the rate of total stable RNA synthesis. Travers et al. (1) have recently characterized an activity from E. coli designated $\psi r$ which appears to stimulate the transcription in vitro of ribosomal RNA cistrons by the RNA-polymerase-\(\sigma\) factor complex. The $\psi r$ factor is also sensitive to the tetraphosphate of guanine (2), a compound which accumulates in stringent strains designated $\psi r$ which appears to stimulate the transcription in vitro of ribosomal RNA cistrons by the RNA-polymerase-\(\sigma\) factor complex. The $\psi r$ factor is also sensitive to the tetraphosphate of guanine (2), a compound which accumulates in stringent strains in E. coli following amino acid deprivation (3, 4).

These results (1-4) suggest that the rate of stable RNA synthesis is determined by the activity of $\psi r$ which would regulate the fraction of RNA-polymerase molecules in the cell capable of transcribing stable RNA cistrons. However, it might be anticipated that the distribution of active polymerase molecules transcribing tRNA and rRNA would be dependent upon the ratio of transfer to ribosomal RNA cistrons. Since the tRNA synthesized represents a small fraction of the total stable RNA synthesized, the rate of incorporation of a pulse label into tRNA would drop by approximately a factor of 2 following replication of the ribosomal RNA cistrons. In that case, since the rRNA cistrons are most likely clustered near the origin of chromosome replication (15-20) while the tRNA cistrons appear to be dispersed around the chromosome (21), the ratio of tRNA to total RNA specific activity would show a decrease approximately equivalent to the increase observed in the DNA to total RNA specific activity ratio occurring at the time of initiation of chromosome replication. In glucose-minimal medium the rate of DNA synthesis doubles midway through the division cycle and a clear and abrupt increase in the DNA specific activity ratio is evident. The DNA to total RNA ratio in succinate cells is also indicative of the pattern of chromosome replication and there is no reason to believe that similar changes in the rate of tRNA synthesis relative to the rate of total stable RNA synthesis would not be equally apparent. Since the tRNA specific activity ratio remains constant during the division cycle in both glucose (8) and succinate cells, it would appear that the transcription of the two classes of stable RNA cistrons is regulated independently. For example, there may exist two discrete classes of RNA-polymerase molecules capable of transcribing stable RNA cistrons, one transcribing only transfer RNA cistrons and the other only ribosomal RNA cistrons. Alternatively, the ribosomal cistrons may be dispersed, but rather clustered near the ribosomal RNA cistrons (7) so that the ratio of tRNA to rRNA genes remains virtually constant during the division cycle. In summary, the rates of rRNA and tRNA synthesis appear to be unaffected by replication of stable RNA cistrons and the fluctuations in the rate of stable nucleic acid synthesis during the division cycle in slowly growing cells most probably result from the discontinuous mode of chromosome replication.

Note Added in Proof—Although the precise function of $\psi r$ in the regulation of stable RNA synthesis remains uncertain (22), it is evident from the results reported here that cytoplasmic factors do limit the transcription (or net rate of synthesis) of rRNA and tRNA.

Acknowledgments—I wish to express my appreciation to Dr. Charles E. Helmantoller for his hospitality and helpful discussion and Dr. H. Weinfield for critically reading the manuscript.

APPENDIX

The differential rate of uracil uptake (increment of uracil uptake to increment of mass increase) when averaged over the first 4 min following addition of uracil to the growth medium is equal to the steady state rate, although the initial rate is clearly greater than the steady state rate and results in expansion of the nucleotide pools (Fig. 5). Between 3 and 4 min the rate decreases below the steady state rate, compensating for the initial expansion before approaching the true steady state rate by 3 to 4 min. In order to use the initial rate of uracil uptake as an estimate of the rate of stable nucleic acid synthesis, the pulse must be sufficiently long to cancel the effects of the initial pool expansion, or the culture must be initially incubated with nonradioactive uracil before the addition of the uracil pulse. Identical results are obtained with either of the two methods to estimate the rate of stable nucleic acid synthesis during the cell division cycle (Fig. 1).

REFERENCES

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