Regulation of Ribosomal and Transfer Ribonucleic Acid Synthesis in Escherichia coli B/r*

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SUMMARY

The relative rates of tRNA, rRNA, and DNA synthesis were measured for steady state cultures of Escherichia coli B/r growing in succinate and glucose minimal media which support growth rates of 0.67 and 1.46 doublings per hour, respectively. Following a 2-fold increase in growth rate, the rate of tRNA synthesis increased approximately 2-fold relative to the rate of DNA synthesis and decreased 10 to 15% relative to the rate of rRNA synthesis. It is concluded that the synthesis of tRNA is regulated in a manner similar to the mechanism regulating rRNA synthesis. The 10 to 15% difference in the relative rates of synthesis is not the result of an increase in the relative frequency of ribosomal RNA cistrons at the higher growth rates, and therefore it seems that the mechanisms controlling rRNA and tRNA synthesis are at least partially independent.

In bacteria, changes in the amount of stable RNA are mainly due to an increase in the number of ribosomes per genome at faster growth rates (1, 2). In fact, Maelåe has indicated that the number of ribosomes per genome is proportional to $\mu$ (growth rate in doublings per hour) except at slow growth rates and that the tRNA content per genome is constant and independent of $\mu$ (3). This would suggest that the rate of rRNA synthesis is regulated while the rate of tRNA synthesis is unregulated and proportional to the number of transfer RNA genes present in the bacterium. In contrast, Rosset et al. (2) found that the tRNA : rRNA ratio remained essentially constant at growth rates above 0.4 doublings per hour. It is tenuous, however, to decide from the experimental data whether the ratio of tRNA : rRNA is in fact inversely proportional to $\mu$ as implied by Maelåe, or constant and independent of $\mu$ implying that the synthesis of both tRNA and rRNA are regulated. This uncertainty arises because the relative recoveries of rRNA and tRNA may vary with the growth rate and because many fractionation procedures involve enzymatic digestion of DNA resulting in contamination of tRNA by oligodeoxyribonucleotide products (2, 4). Recently Travers et al. (5) and co-workers (6, 7) tentatively identified a protein factor, $\psi_r$, and an unusual nucleotide, guanosine tetraphosphate, as possible elements in the system regulating stable RNA synthesis. Although the precise functions of $\psi_r$ and ppGpp in the regulation of RNA synthesis remain unclear, it appears that they may affect the rate of initiation of transcription of both ribosomal and transfer RNA genes.

In this paper, using a novel experimental approach, I distinguish between the following alternatives with respect to the regulation of stable RNA synthesis: (a) unregulated tRNA and regulated rRNA synthesis, (b) regulated synthesis of tRNA and rRNA by a common control mechanism, and (c) regulated synthesis of tRNA and rRNA by separate control mechanisms. The results indicate that both rRNA and tRNA are regulated, most probably by partially independent mechanisms.

MATERIALS AND METHODS

The bacterial strain used was E. coli D/r (ATCC No. 12407). All cultures were grown in minimal medium C (8) supplemented to a final concentration of 0.2% with either glucose or succinic acid (5% solution adjusted to pH 6.8 with tris(hydroxymethyl)-aminomethane) in a rotary shaker bath at 37°. Growth was monitored by measuring cell concentrations (Coulter counter, model B) and absorbance at 460 nm (Zeiss spectrophotometer). In all experiments the doubling times of exponential phase glucose or succinate cultures were between 42 to 44 min ($\mu = 1.46$) and 84 to 92 ($\mu = 0.67$) min respectively.

Exponential phase cultures were labeled with $5 \times 10^{-5} M [2-{^3}H]uracil$ (specific activity 56.3 mCi per mm) at a density of $4 \times 10^8$ cells per ml. After one cell doubling, nonradioactive uracil ($2 \times 10^{-4} M$) was added to the cultures. Under these conditions the radioactive uracil was rapidly exhausted in the medium, and virtually all the radioactivity was incorporated into stable nucleic acid during the first few minutes of the labeling period. Cultures were harvested by centrifugation ($7000 \times g$, 10 min, 6°) at a density of 2 to $4 \times 10^6$ cells per ml.

At various times following addition of nonradioactive uracil to the labeled cultures, the radioactivity in total nucleic acid (DNA and RNA) and in DNA was determined. Total nucleic acid was determined by precipitation of 1 ml of culture with an equal volume of 10% trichloroacetic acid. The DNA was determined following alkaline hydrolysis of 1 ml of culture with 1 ml of 1 M KOH (2 hours at 35°) and precipitation with 1 ml of 20% trichloroacetic acid. The insoluble precipitates were collected

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on nitrocellulose filters, washed with 5% trichloroacetic acid, dried, and counted as described.

For shift-up experiments, a fresh stationary phase succinate culture was diluted 2000-fold into succinate medium containing [3H]uracil (10⁻⁸ M). When the density reached 10⁶ cells per ml, the bacteria were removed from the radioactive medium by centrifugation and resuspended in 2 volumes of conditioned succinate medium (8) containing nonradioactive uracil (2 x 10⁻¹⁵ M). After one cell doubling the culture was diluted 10-fold (to 10⁶ cells per ml) into previously warmed medium containing 0.2% glucose and uracil (2 x 10⁻¹⁵ M). The cultures were harvested when the density reached 3 x 10⁶ cells per ml. Growth in glucose media following the shift up represents a 30-fold increase in cell number and absorbance was equal to the exponential rate for over 3 generations before the cells were harvested.

Following centrifugation of approximately 40 ml of culture, the bacteria were resuspended in 10 ml of Tris-Mg buffer (0.05 M tris(hydromethyl)amino methane, 0.01 M MgCl₂, to pH 7.6 with HCl) and disrupted by passage through a French pressure cell (12,000 p.s.i.). The lysates were again centrifuged (15,000 x g, 10 min, 0°) in order to sediment unbroken cells and cellular debris. The supernatant solution was then extracted with an equal volume of redistilled phenol in the second peak. The phenol phase was re-extracted three more times with buffer and the aqueous fractions containing the nucleic acids were pooled. The nucleic acids were purified from phenol by three successive ethanol precipitations at -15°. Following the final precipitation, the nucleic acids were dissolved in 0.05 M sodium phosphate buffer, pH 6.7 (0.2 M in NaCl) and eluted with a linear NaCl gradient (0.2 to 1.5 M) in 0.05 M phosphate buffer, pH 6.7. Fractions (5 ml) were collected and the absorbance at 260 nm was determined in a Gilford 240 spectrophotometer. In most cases the nucleic acids from the entire fractions were precipitated with 1 ml of 30% trichloroacetic acid at 0° following addition of 50 µg of yeast RNA as carrier. In one glucose, one succinate, and one shift-up experiment, a 3 ml portion from each fraction was precipitated as described. A second 1-ml portion was added to an equal volume of 1 M KOH and incubated at 35° for 2 hours. The nucleic acid was then precipitated by addition of 1 ml of 20% trichloroacetic acid at 0°. All precipitates were collected on Millipore membrane filters (0.45 µm pore size), washed with 5% trichloroacetic acid, dried, and counted in 5 ml of Liquifluor (Nuclear Chicago Corporation) using a Nuclear Chicago liquid scintillation counter.

The hypochromicity of the nucleic acids was determined as follows: a methylated albumin Kieselguhr column (2 x 8 cm) was loaded with 3.0 mg of nucleic acid extracted from exponential phase glucose bacteria. The nucleic acids were eluted with a 100 ml of NaCl gradient and 2 ml of fractions were collected. The fractions within each peak were pooled and precipitated with 2 volumes of ethanol. The precipitates were then dissolved in 0.05 M sodium phosphate buffer (pH 6.7) to a final absorbance at 260 nm of 0.4 to 0.5. The melting profiles of the three nucleic acids were monitored at 260 nm in a Gilford recording spectrophotometer against a phosphate buffer blank over a temperature range of 25-90°. The temperature was increased at a linear rate of 0.5° per min. The melting profiles for

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>µ</th>
<th>Radioactivity*</th>
<th>Nucleotidesb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate . . .</td>
<td>0.67</td>
<td>2.25 ± 0.14</td>
<td>2.62</td>
</tr>
<tr>
<td>Glucose . . .</td>
<td>1.46</td>
<td>5.02 ± 0.20</td>
<td>5.77</td>
</tr>
<tr>
<td>Succinate:glucose . .</td>
<td>0.459</td>
<td>0.455</td>
<td>0.455</td>
</tr>
</tbody>
</table>

* Ratio and standard deviation of total acid-insoluble radioactivity (DNA + RNA) minus alkali-resistant, acid-insoluble radioactivity (DNA) to alkali-resistant acid-insoluble radioactivity (DNA).

To obtain the total nucleotides in DNA and RNA the radioactivity values were corrected for the differences in mole fraction of pyrimidine in DNA (50%) and RNA (43.5%), respectively.

The ratios of stable RNA nucleotides:DNA nucleotides in the [3H]uracil-labeled exponential phase succinate and glucose cultures were 2.62 and 5.77, respectively (Table I). These ratios were found to be proportional to µ (growth rate in doublings per hour). Furthermore, it was found that once the radioactivity was completely incorporated into stable nucleic acid, the ratio remained constant as long as the cultures remained in steady state growth, indicating that the turnover of stable nucleic acid was negligible.

The labeled nucleic acids from these cultures were extracted, purified and separated using methylated albumin Kieselguhr column chromatography. Fig. 1, a and b, illustrates the typical chromatographic profiles obtained from succinate and glucose bacteria, respectively. The radioactivity and absorbance profiles are qualitatively similar and the peaks correspond to tRNA, DNA and 16 to 23 S rRNA. The 5 S RNA was a normal structural component of the 50 S ribosome (2) and its rate of synthesis is proportional to the rates of synthesis of 16 S and 23 S rRNA, probably because all three species are cotranscribed (10). In the chromatographic separation employed here, the 5 S RNA elutes as a shoulder of the tRNA peak. Measurements of the proportion of 5 S RNA in the tRNA peak (11) and calculation (2) of the proportion based upon the respective molecular weights of 23 S and 5 S RNA indicate that approximately 15% of the RNA in the tRNA peak is 5 S RNA. The pyrimidine content of 5 S RNA is similar to tRNA (12, 13) and the relative rate of synthesis of material in the tRNA and rRNA peaks vary by only 10 to 15% between the two growth rates studied here. Therefore, correction for 5 S RNA is negligible, amounting to only a 1 to 2% increase in the already existing 10 to 15% difference in the relative rates of tRNA and rRNA synthesis following the shift-up. This slight correction has been ignored.
Ribosomal and Transfer RNA Synthesis

Fig. 1. Chromatographic analysis of nucleic acids from exponential phase bacteria growing in succinate medium (A) and glucose medium (B). The nucleic acids were labeled, extracted, and chromatographically separated as described in "Materials and Methods." The four peaks present in the radioactivity (X---X) and absorbance 260 nm (O-O) profiles represent tRNA, DNA, and 16 S to 23 S rRNA in order of elution. The peak referred to as tRNA contains about 15% 5 S RNA (see Footnote 1) while rRNA refers to the material in the 16 S to 23 S peaks.

Table II

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>Specific activity ratioa</th>
<th>Correctedb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uncorrected</td>
<td>Corrected</td>
</tr>
<tr>
<td>tRNA:DNA</td>
<td>tRNA:rRNA</td>
<td>tRNA:DNA</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.925 (0.046)</td>
<td>1.30 (0.03)</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.801 (0.044)</td>
<td>1.34 (0.04)</td>
</tr>
<tr>
<td>Nutritional shift-up</td>
<td>0.490 (0.007)</td>
<td>1.495 (0.03)</td>
</tr>
</tbody>
</table>

a These values represent the means and ranges (difference between the maximum and minimum values) of three separate determinations for each of the three growth conditions. Included are the values calculated from the data illustrated in Figs. 1 and 2.

b Correction factors 1.08 and 0.847 for the tRNA:DNA and tRNA:rRNA ratios were determined from the extinction coefficients of the mononucleotides, base compositions (13), and the hyperchromicity of the tRNA, DNA, and rRNA. The hyperchromicities used for the corrections were measured following ethanol precipitation of the pooled nucleic acids in each peak by monitoring A_{260} as a function of temperature. Values of 1.22, 1.32, and 1.25 were obtained for tRNA, DNA, and rRNA, respectively. Hyperchromicity values for tRNA and rRNA determined by alkaline hydrolysis were 1.22 and 1.24, respectively.

The specific activity (radioactivity per absorbance at 260 nm) of the nucleic acid in each peak was calculated and the ratios of specific activity of tRNA:DNA and tRNA:rRNA were determined (Table II). The values represent the mean and the range of three separate MAK chromatography profile experiments for each growth condition. These ratios are completely independent of any differential recovery of the nucleic acid during extraction and purification. The ratios are expected to be approximately equal to 1.0 following correction for (a) the mole fraction of pyrimidine, (b) the extinction coefficients of the mononucleotides, and (c) the hyperchromicity. The slight variations observed result from uncertainty in the values used to correct the specific activity ratios in addition to experimental error.

To establish the relationships between the relative rates of tRNA, rRNA, and DNA synthesis, an exponential phase succinate culture was labeled with [14C]uracil, grown for one generation in conditioned succinate medium containing nonradioactive uracil, and then shifted to glucose medium and grown for several further generations until the culture reached steady state. The distribution of the radioactivity between DNA and RNA was not affected by the shift-up and remained equal to the succinate value of approximately 2.3. However, chromatographic analysis of the purified nucleic acids (Fig. 2) shows that the specific radioactivities of tRNA, rRNA, and DNA, and thus also the ratios of these specific radioactivities have changed; the tRNA:DNA specific activity ratio has decreased to 0.46 and the tRNA:rRNA ratio has increased to 1.26. The tRNA:DNA ratio represents approximately a 2-fold decrease when compared with the steady state glucose and succinate ratios. This result clearly indicates that the rate of tRNA synthesis relative to the rate of DNA synthesis was about 2-fold higher in the steady state glucose as compared with the steady state succinate bacteria. Likewise, the rate of tRNA synthesis relative to the rate of rRNA synthesis was 10 to 15% lower in steady state glucose as compared with succinate cultures.

Since the bulk of stable RNA is ribosomal RNA and since the remaining small fraction represented by tRNA varies only 10 to 15% between glucose and succinate growth rates, these results corroborate the earlier findings that the number of ribosomes per genome, to a first approximation, is proportional to μ (1-3).
Following a nutritional shift-up from succinate to glucose medium, the rate of tRNA synthesis was found to increase approximately 2-fold relative to the rate of DNA synthesis and to decrease 10 to 15% relative to the rate of rRNA synthesis. These results lead to the conclusion that tRNA:DNA ratio is not a constant as postulated by Maaløe (see introductory section), and that tRNA synthesis is regulated. In addition, the small although significant difference in the ratio of the rates of tRNA to rRNA synthesis in succinate and glucose medium may result either from separate control mechanisms or secondary effects on the same primary control mechanism (for example, gene dosage).

Using the equation for the age distribution in an exponential phase population (14) and the model of Cooper and Helmstetter (15) for the regulation of chromosome replication in E. coli B/r, it is possible to calculate gene frequencies in exponential phase population (14) and the model of Cooper and Helmstetter (15) for the regulation of chromosome replication in E. coli B/r, it is possible to calculate gene frequencies in exponential phase culture growing at different growth rates. The origin to terminus ratios in glucose and succinate cultures were calculated to be 2.0 and 1.74, respectively, indicating that the maximum differences in the relative frequencies of two different loci would be only 13% between the two growth rates. In E. coli, the rRNA genes are clustered near the origin and the tRNA genes near the terminus of replication and if a unique fraction of the total RNA polymerase is active in transcription of stable RNA genes (both rRNA and tRNA) then, in fact, a 13% increase in the rate of rRNA synthesis relative to tRNA synthesis resulting entirely from a gene dosage effect would be anticipated following the shift-up. However, the following observation rules out the gene dosage effect: the model would predict that the distribution of active RNA polymerase between ribosomal RNA and transfer RNA genes would show a gene dosage effect during the cell division cycle. For bacteria growing in glucose medium where initiation of chromosome replication occurs immediately following completion of the previous round (approximate cell age 0.5 generations) the origin to terminus ratio is virtually constant during the entire cell cycle as is the ratio of the rates of synthesis of tRNA:rRNA (16). However, in succinate bacteria, the tRNA:rRNA synthesis ratio is also constant whereas the origin to terminus ratio decreases from 2.0 during the first two-thirds to 1.0 during the final one-third of the division cycle (17). Therefore, the 10 to 15% decrease in the rate of tRNA synthesis relative to rRNA synthesis following the shift-up is not due to a gene dosage effect. From these considerations and earlier observations (16, 17), it is concluded that the synthesis of tRNA is regulated by a mechanism similar to the mechanism regulating the synthesis of rRNA. The small differences observed may result either from a second order effect on the same primary control system or from separate primary control systems.

Although the precise mechanisms regulating stable RNA synthesis are still unclear, any pretranscriptional mechanism may resemble the $\Psi_{ppGpp}$-mediated system recently described (4-6). It should be noted that the experiments reported here measure only the rate of synthesis of rRNA and tRNA. The results do not preclude the possibility of a posttranscriptional control which results in the selective degradation of newly synthesized (i.e. still nascent) rRNA and/or tRNA molecules.

In summary, whether the mechanisms regulating tRNA and rRNA synthesis are pre- or posttranscriptional, or both, it is evident that these mechanisms nullify any effect of gene dosage and respond in a similar, although not identical, manner to changes in the steady state growth rate.

Note Added in Proof—Two recent reports (18, 19) indicate that approximately 10% of the in vitro transcription product from E. coli DNA and purified RNA polymerase (containing the $\sigma$ factor) is ribosomal RNA. Addition of $\Psi_{ppGpp}$ stimulates the synthesis of RNA but does not increase the proportion of the transcripts which are rRNA (18). These experiments indicate that $\Psi$ is not required for in vitro transcription of rRNA genes but do not rule out the possibility that rRNA genes are controlled in a positive manner in vivo (18).

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