JOURNAL OF BACTERIOLOGY, July 1968, p. 111-116

Vol. 96, No. 1
Printed in U.S.A.

Ribonucleic Acid Synthesis in T2-infected *Escherichia coli* During "Stringent" Control

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Received for publication 18 April 1968

The leucine auxotroph *Escherichia coli* 2961 exhibited stringent control of net ribonucleic acid (RNA) synthesis during amino acid starvation. After leucine was exhausted from the medium, the rate of uracil incorporation into RNA rapidly decreased to 2 to 4% of the prestarvation value. Infection of the starved cells with T2 phage stimulated uracil incorporation to a level equivalent to that of unstarved, T2-infected cells. The RNA synthesized during leucine starvation of the T2-infected cells consisted of T2 and *E. coli* messenger RNA, but stable ribosomal RNA (23*S* and 16*S*) did not appear to be synthesized. It is concluded that one or more T2-specific proteins are required to shut off host messenger RNA synthesis. Furthermore, transcription of *E. coli* and T2 deoxyribonucleic acid is not necessarily coupled to the translation of messenger RNA during stringent control of net RNA synthesis.

Infection of *Escherichia coli* by T-even bacteriophages (T2, T4, and T6) interrupts the synthesis of the host's ribosomal, transfer, and messenger ribonucleic acid (mRNA) (16, 20, 25). When protein synthesis is inhibited by the addition of chloramphenicol (CAP) prior to infection, all three kinds of RNA continue being synthesized (8, 17). On the basis of these results, it has been postulated that the T-even phages code for one or more proteins that repress host RNA formation. The activity of the hypothetical "repressor protein" is exerted not only on cellular RNA synthesis, but also on that of phage λ mRNA (8).

A plausible alternative to this interpretation must be considered. Amino acid starvation of "stringently controlled" strains of *E. coli* is known to cause an abrupt cessation of net RNA synthesis (14, 22). A genetic site for the control of RNA synthesis by amino acids has been determined (22). The addition of CAP releases RNA formation from the stringent control mechanism (9), the nature of which is still poorly understood (15). One can consider the hypothesis that the T-even phages shut off host RNA synthesis by a mechanism simulating stringent control. If this is true, then the failure of the T-even phages to block *E. coli* RNA synthesis in the presence of CAP can be explained without invoking phage-specific repressor protein(s). For instance, the attachment of the phage to the cell surface or the injection of phage deoxyribonucleic acid (DNA) into the cell might be sufficient to establish stringent control over host RNA synthesis, but the addition of CAP could release this inhibition.

To resolve this question, it was necessary to determine whether T-even phage could block RNA synthesis in *E. coli* under conditions that prevented protein synthesis, but did not release the cell's stringent control over its RNA. Amino acid starvation was the condition employed. The results of this investigation also bear on the question of whether transcription of DNA is necessarily coupled with the translation of mRNA into protein. If transcription is dependent on a coupled translation process, as suggested by some authors (2, 21), then neither T2 nor *E. coli* mRNA would be synthesized during amino acid starvation of T2-infected *E. coli*. However, if the two processes are not necessarily coupled, then amino acid starvation should not affect the rate of mRNA synthesis.

MATERIALS AND METHODS

Bacteria and media. E. coli 2961 pro⁻thre⁻leu⁻B_1⁻ (derived from K-12) is the leucine auxotroph described in this study. This strain exhibits stringent control of net RNA synthesis during amino acid starvation (11). Bacteria were grown with forced aeration at 37 C in M9-minimal medium (7) supplemented with 50 μg of the required amino acids per ml and 1.0 μg of vitamin B_1 per ml. Viable cell and phage assays were determined by standard plating techniques (7).

Culture conditions. An overnight culture supplemented with 50 μg of leucine per ml was diluted

1:50 into media containing 5 μg of leucine per ml. The culture was grown into log phase and was stored at 5 C prior to the onset of leucine exhaustion. Experimental cultures were started by diluting such log-phase cultures 1:5 into supplemented M9-minimal medium containing 5 μg of leucine per ml. Under these conditions, the culture increased exponentially to an optical density of 0.3 to 0.35, as measured at 660 mμ in the Zeiss spectrophotometer, and then abruptly ceased growing due to the exhaustion of leucine from the medium. The time at which the optical density readings first failed to increase was used to determine the approximate time of amino acid exhaustion; the cultures were subsequently starved for about 40 min. To show that the bacteria had exhibited the stringent effect, 1-ml samples were pulse-labeled with ^{3}H-uracil for 1 min both before and after leucine exhaustion. In these experiments, the rate of uracil incorporation decreased abruptly to 2 to 4% of the unstarved rate.

Infection with T2. Cultures were infected with T2 at an input multiplicity (MOI) of 15. The high MOI was necessary since T2 adsorbs poorly to this strain. Within 10 min at 37 C, there was less than 3% survival, as determined by the colony count method.

Determination of the rate of uracil incorporation. At various times, 1-ml portions of the culture were labeled for 1 min at 37 C with ^{3}H-uracil (~2 c/mmole, obtained from the New England Nuclear Corp., Boston, Mass.). The pulse was terminated by the addition of 1 ml of cold 20% trichloroacetic acid. The cells were collected by filtration onto membranes (Millipore Corp., Bedford, Mass.), and were washed with 30 ml of cold 5% trichloroacetic acid and then 20 ml of water at room temperature. After drying for 30 min at 100 C, the membranes were counted in a Nuclear-Chicago liquid scintillation spectrometer using toluene-liquifluor mix (New England Nuclear Corp.).

E. coli and T2 DNA. E. coli DNA was isolated from strain B/5 by a modification of the Marmur procedure (10) as previously described (7). We are grateful to W. S. Hayward for providing this DNA. T2 phage were grown on *E. coli* B/5 and were purified by two cycles of low- and high-speed centrifugation. Prior to the second centrifugation, the phage were treated with deoxyribonuclease (10 μg/ml) for 1 hr at 37 C. T2 DNA was purified by phenol extraction and dialysis, as described for λ DNA (7).

RNA isolation and hybrid analysis. Cultures were pulse-labeled and RNA was isolated as previously described (7). DNA-RNA hybrids were formed during a 4-hr incubation at 60 C and were assayed by the nitrocellulose filter method of Nygaard and Hall (18), as modified by Green (7).

Sedimentation analysis of RNA. RNA (0.2 ml) was layered on a 4.6-ml sucrose gradient [5 to 20% sucrose in tris(hydroxymethyl)aminomethane (Tris)-KCl (0.01 M Tris, *p*H 7.4, 0.05 M KCl)] and spun for 5 hr at 37,000 rev/min in the Spinco SW39 rotor. Fractions (two drops each) were collected from the bottom of the centrifuge tube and were diluted with Tris-KCl. The optical density at 260 mμ was determined with a Zeiss spectrophotometer. The samples were then precipitated with cold 10% trichloroacetic acid, washed with 15 ml of 5% trichloroacetic acid, dried, and counted in the liquid scintillation spectrometer.

RESULTS

Effect of T2 on the rate of uracil incorporation into RNA during amino acid starvation. E. coli 2961 had previously been shown to exhibit stringent control over RNA synthesis (11). After confirming this, we tested the effect of T2 phage infection on the rate of uracil incorporation during amino acid starvation. The rate was measured by pulse-labeling for 1 min with ^{3}H-uracil at various times before and after infection. The results are shown in Fig. 1.

Within 10 min after the cells exhausted their supply of leucine, ^{3}H-uracil incorporation into RNA decreased to 1.6% of the prestarved rate and remained at this level in the untreated control. (The following section will show that the ^{3}H-labeled acid precipitable material is in fact RNA.) The addition of leucine to the starved, uninfected cells rapidly restored their ability to incorporate ^{3}H-uracil. Of main interest here is the observation that T2 infection, in the presence or absence of leucine, caused ^{3}H-uracil to be incorporated into RNA at a rate 12-fold greater than in the starved, uninfected control. The addi-

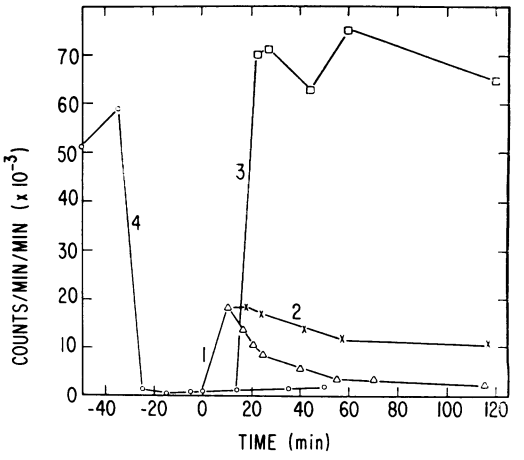

FIG. 1. *Stimulation of uracil incorporation by T2 infection of leucine-starved E. coli. E. coli 2961 was grown in limiting leucine medium. After starving for 35 min, the culture was divided and treated as follows: (1) T2 added at zero-time (t = 0), △; (2) T2 added at t = 0, leucine (50 μg/ml) at t = 12 min, ×; (3) leucine (50 μg/ml) added at t = 14 min, □; (4) no additions, ○. At t = 10 min, there was 1.3% survival of the infection. Samples (1 ml) of the cultures were taken at the designated times and were immediately pulse-labeled for 1 min with ^{3}H-uracil (1 μc/ml).*

tion of T2 thus restored the rate of uracil incorporation to that of nonstarved T2-infected cells. However, the T2 effect was transient. By 60 min after infection, the rate of incorporation of ^{3}H-uracil was only slightly greater than in the control. There was no phage production during the 60 min in the absence of leucine.

Formation of E. coli and T2 mRNA by amino acid-starved cells. The following experiment enabled us to determine whether amino acid starvation prevented T2 phage from shutting off host mRNA synthesis. Previous results had indicated that CAP prevents T2 from completely inhibiting *E. coli* mRNA synthesis (8).

E. coli 2961 was grown in limiting leucine and 1-ml samples were tested for ^{3}H-uracil incorporation before and after leucine depletion. In this experiment, the rate of uracil incorporation decreased to 3.9% of the prestarved rate. After starving for 40 min, one portion of the culture was supplemented with leucine (50 μg/ml) and T2 was added 5 min later; the remaining cells were infected in the absence of leucine. There was 1.5% survival 9 min after adding T2 to the leucine-starved culture. One-ml samples of the leucine-starved, T2-infected cells were tested for ^{3}H-uracil incorporation by 1-min pulses. At 5 and 10 min after T2 infection, ^{3}H-uracil incorporation was stimulated 5- and 17-fold, respectively, as compared to the starved, uninfected cells. The remainder of each culture was pulse-labeled with ^{3}H-uracil from 10 to 11 min after infection, and RNA was isolated for DNA-RNA hybrid analysis (*see* Materials and Methods). Less than 0.5% of the radioactivity in the RNA preparation remained acid precipitable after incubation with ribonuclease (5 μg/ml) or 0.2 N NaOH for 2 hr at 37C.

The results shown in Table 1 indicate that *E. coli* mRNA and T2 mRNA were synthesized in the leucine-starved, T2-infected cells, whereas only T2 mRNA was synthesized in the unstarved cells. The relative rate of T2 mRNA synthesis was the same in both cultures. The rate of *E. coli* mRNA synthesis in the starved, infected cells was approximately 10% of that in nonstarved, uninfected *E. coli*. Similar results were obtained when CAP was used to inhibit protein synthesis (8). It is therefore concluded that the rate of mRNA synthesis, as measured by DNA-RNA hybridization, is not affected by amino acid starvation of a "stringent" strain of *E. coli*. It is also apparent that protein synthesis is required in order for T2 phage to block completely the formation of host mRNA.

Kinetics of ^{3}H-uracil incorporation into the RNA of leucine-starved cells following T2 infection. The results shown in Fig. 1 might be interpreted to mean that T2 infection of leucine-starved cells stimulated the rate of RNA synthesis 12-fold, but by 60 min after infection RNA synthesis nearly stopped. This interpretation requires that the rate of RNA synthesis not be limited by the cell's ability to incorporate uracil into the RNA precursor pool during amino acid starvation. To test this supposition, leucine-starved cells were infected and the incorporation of ^{3}H-uracil was monitored over an extended period. The results are presented in Fig. 2.

After a short lag, ^{3}H-uracil was incorporated into the starved, infected cells at a nearly constant rate for at least 60 min. In contrast, when leucine was again added 5 min prior to infection, the incorporation of ^{3}H-uracil reached steady state within 25 min after infection. It is known that the rate of RNA synthesis and breakdown becomes equal under these conditions (1). In view of the data presented in Fig. 1, we propose that amino acid starvation eventually prevents exogenous uracil from entering an RNA precursor pool, whereas uracil incorporated soon after infection can be subsequently converted into RNA.

Sedimentation analysis and stability of the RNA synthesized during amino acid starvation. Since CAP is known to prevent T4 from shutting off the

TABLE 1. *Hybridization analysis of RNA synthesized after T2 infection*

Source of RNA	Specific activity (counts per min per μg)	Percentage of input RNA[a] hybridized with						Background[b] %
		E. coli DNA[c]			T2 DNA			
		70 μg	42 μg	21 μg	46 μg	23 μg	9 μg	
E. coli + T2 − leu	1,220	1.5	1.5	0.90	12.6	11.0	9.0	0.5
E. coli + T2 + leu	770	0	0	0.27	12.6	15.8	13.4	0.5

[a] The input counts/min ranged from 39,000 to 42,000 for the two RNA samples.

[b] The background for each RNA has been subtracted from the listed hybrid data.

[c] A maximum of 15% hybrid was obtained with this DNA and with pulse-labeled RNA from nonstarved, uninfected *E. coli*.

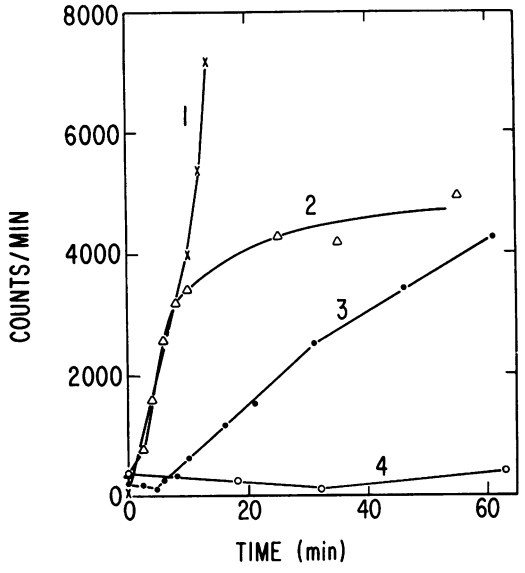

FIG. 2. *RNA synthesis in leucine-starved cells after T2 infection. Approximately 40 min after exhausting the supply of leucine, ^{3}H-uracil (0.5 μc per 5 μg/ml) was added to the culture (t = 0). The cells were distributed into four aeration tubes and were treated as follows: (1) leucine (50 μg/ml) added at t = 0 (×); (2) leucine (50 μg/ml) added at t = −5 min, T2 at t = 0 (△); (3) T2 added at t = 0 (●); (4) no additions (○). At the indicated times, 1-ml samples were acid-precipitated. There was 5% survival of infection at t = 5 min, 3% at t = 10 min.*

synthesis of *E. coli* ribosomal RNA (23*S* and 16*S*) and transfer RNA (4*S*) (17), it might be expected that leucine starvation would have the same effect. The following experiment was designed to test this possibility by sedimentation and DNA-RNA hybrid analysis.

A leucine-starved culture was prepared as in previous experiments. Pulse-labeled samples indicated that ^{3}H-uracil incorporation was reduced to 1.2% of the unstarved rate. Ten min after adding T2 phage, one portion of the culture was labeled with ^{3}H-uracil for 1 min and the remainder was labeled with ^{14}C-uracil for 20 min. Incorporation of label was linear throughout this period. After the indicated labeling period, the cultures were chilled in the presence of sodium azide (0.01 M) and combined, and RNA was extracted. Less than 1% of the ^{14}C and ^{3}H radioactivity was acid precipitable after ribonuclease treatment (5 μg/ml, 2 hr at 37 C).

If stable *E. coli* RNA had been formed, the longer labeling period with ^{14}C-uracil would have enriched for this type of RNA, and the pulse-labeled ^{3}H-RNA would have been relatively enriched for unstable mRNA. The ^{14}C-RNA might then have been expected to sediment mainly as 23*S* and 16*S* ribosomal RNA. Furthermore, the ^{14}C-RNA would have hybridized much less efficiently than the ^{3}H-RNA with both *E. coli* and T2 DNA.

However, these results were not observed. Sedimentation analysis on a sucrose gradient (Fig. 3) indicated that the ^{14}C- and ^{3}H-RNA had nearly identical sedimentation profiles, ranging from 4*S* to 23*S* and characteristic of mRNA (19). The hybridization data presented in Table 2 confirm this result. The ratio of ^{14}C to ^{3}H in the hybrids with T2 DNA and *E. coli* DNA was nearly the same as the ^{14}C to ^{3}H ratio of the RNA preparation, i.e., 0.361. If the ^{14}C had entered only ribosomal and transfer RNA, the ^{14}C to ^{3}H ratio would equal zero with these conditions of hybridization. We conclude that stable *E. coli* RNA is not synthesized in T2-infected cells during amino acid starvation.

DISCUSSION

Two main conclusions can be drawn from these experiments. The first is that amino acid starvation prevents T2 phage from completely blocking *E. coli* mRNA synthesis. Secondly, amino acid starvation does not affect the rate of mRNA synthesis in a T2-infected "stringent" strain of *E. coli*.

With regard to the first conclusion, it now appears certain that the T-even phages direct the synthesis of one or more proteins that are required to completely inhibit the formation of *E. coli* mRNA. It should be noted, however, that even when protein synthesis is inhibited, the rate of *E. coli* mRNA synthesis after T2 infection is greatly reduced (8). It would thus appear that at least two distinct processes are involved in the regulation of host RNA synthesis by T-even phages. Terzi has recently come to the same conclusion through examination of the effect of T4 on *Shigella* RNA synthesis (24).

The second conclusion is based on the following line of reasoning. Leucine starvation did not affect the level of uracil incorporated into RNA during a 1-min pulse shortly after T2 infection (Fig. 1). Leucine starvation also did not affect the fraction of ^{3}H-uracil incorporated into T2 mRNA (Table 1). Thus, it is apparent that the rate of T2 mRNA synthesis is unaffected during stringent control of net RNA synthesis. Furthermore, the ratio of T2 mRNA to *E. coli* mRNA synthesized during leucine starvation (Tables 1 and 2) was the same as that found when CAP was used to block protein synthesis (8). Thus, it may be concluded that in T2-infected cells the rate of synthesis of *E. coli* mRNA, as well as of T2 mRNA, is unaffected by stringent control. This

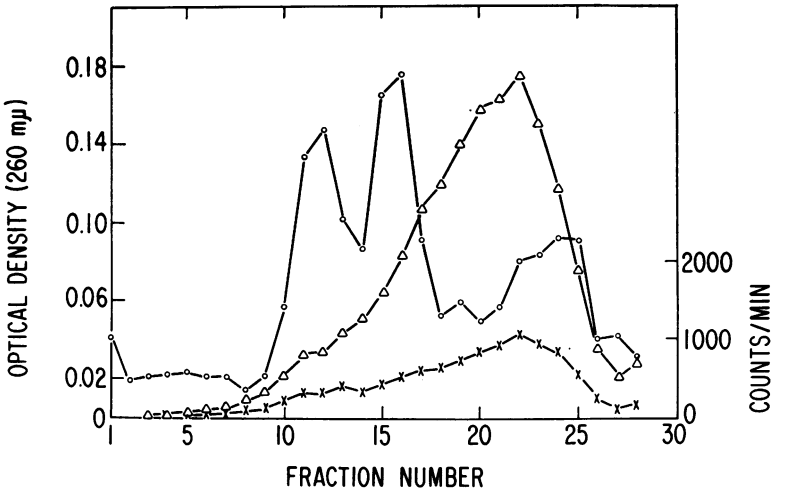

FIG. 3. *Sedimentation analysis of RNA synthesized during amino acid starvation. A culture of E. coli was starved for leucine for 40 min, then infected with T2 at t = 0. There was 0.5% survival at t = 9 min. One half of the culture was pulse-labeled with ^{3}H-uracil (1 μc/ml) from t = 10 to 11 min, the remainder with ^{14}C-uracil (0.5 μc per 2 μg/ml) from t = 10 to 30 min. RNA was extracted and sedimented through a 5 to 20% sucrose gradient for 5 hr at 37,000 rev/min. Fractions (two drops) were collected from the bottom of the tube, diluted, and assayed for ultraviolet absorbance as optical density at 260 mμ (○), acid precipitable ^{3}H (△) and ^{14}C (×) radioactivity.*

TABLE 2. *Hybridization of the $^{14}C^{3}H$-RNA synthesized during amino acid starvation*

Source of DNA	Percentage of input RNA[a] hybridized[b]		$^{14}C/^{3}H$ in hybrid
	^{14}C	^{3}H	
T2 (46 μg)	27.5	33.4	0.296
T2 (23 μg)	18.3	23.6	0.280
E. coli (70 μg)	3.1	3.2	0.352

[a] The $^{14}C^{3}H$-RNA had a ^{14}C to ^{3}H ratio of 0.361.
[b] The RNA input was 10,000 counts/min ^{3}H, 3,600 counts/min ^{14}C.

conclusion is strengthened by reports demonstrating that amino acid starvation does not prevent the synthesis of mRNA specific for certain enzymes (13, 20, 23), and also by the demonstration that normal amounts of mRNA, as assayed in an in vitro protein-synthesizing system, are present during amino acid starvation (4). Although these results imply that transcription and translation are not coupled, it is still possible that some initial step in protein synthesis, e.g., the binding of mRNA to ribosomes, may be required in order for transcription to occur.

The mechanism for the restriction of uracil incorporation into the RNA of "stringent" cells after amino acid starvation is as yet obscure. A recent report by Gallant and Cashel indicated that exogenous uridine triphosphate (UTP), but not uridine monophosphate (UMP) or uracil, could be incorporated into the RNA of plasmolyzed cells under stringent control (6). They suggested that amino acid starvation sets up a metabolic block in the conversion of UMP to UTP, thereby blocking RNA synthesis. However, the nucleotide pool has been found to decrease too slowly to account for the cell's rapid loss in ability to synthesize net RNA (3). Moreover, if UTP could not be formed during stringent control, mRNA synthesis would also stop. Other reports have also shown that this is not the case (4, 13, 20, 23). Whatever the mechanism for restricting uracil incorporation, it is apparent that T2 infection can reverse this block.

A comparison of Fig. 1 and 2 indicates that during amino acid starvation, RNA synthesis continues, but metabolic pools somehow regulate the incorporation of exogenous uracil into RNA. Whereas 1-min pulses showed a fourfold decrease in the rate of uracil incorporation between 10 and 60 min after T2 infection, there was only a 30% decrease in the rate of incorporation over the same period when uracil was added at the time of infection. In accordance with the model proposed by Morris and DeMoss (12), we suggest that T2 infection permits uracil to enter a pool that is established during stringent control by mRNA turnover. The results of Gallant and Cashel (6) can be accounted for by this proposal by assuming that nucleoside monophosphates, as well as

free bases, are excluded from the mRNA precursor pool during stringent control.

Of considerable interest is the finding that little, if any, stable 23*S* and 16*S* ribosomal RNA was synthesized after T2 infection of amino acid-starved cells (Fig. 3). Thus, in the case of infected cells, stringent control is apparently not exerted coordinately on the various classes of RNA, as suggested for uninfected cells (5). Nomura et al. (17) have shown that *E. coli* ribosomal and transfer RNA continue to be synthesized after T4 infection when CAP is used to block protein synthesis. However, since CAP is able to reverse the stringent control process (9), causing the accumulation of 23*S* and 16*S* ribosomal RNA during amino acid starvation, it is conceivable that T-even phages interrupt the synthesis of ribosomal and transfer RNA by a mechanism simulating stringent control. Finally, it should be made clear that the cell's failure to synthesize stable ribosomal RNA during stringent control does not preclude the possibility that this RNA is synthesized in an unstable form like mRNA.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant GM-14318 from the National Institute of General Medical Sciences. One of us (K. S.) was supported by Public Health Service research training grant GM-00702.

The authors are indebted to J. A. DeMoss for his critical review of the manuscript.

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