Amino Acid Regulation of Messenger Ribonucleic Acid Synthesis in T4-infected Escherichia coli

J. D. FRIESEN
Division of Biology, Kansas State University, Manhattan, Kansas 66502

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The effect of amino acid starvation on the synthesis of T4 messenger ribonucleic acid (mRNA) was studied by measuring the uptake of labeled precursors into Escherichia coli cells which were infected prior to amino acid starvation. The results indicated that the rate of uptake of labeled uracil and adenine into T4 mRNA was reduced by approximately 50% when amino acids were withheld from stringent K-12 strains, but not when they were withheld from a stringent strain B or a relaxed strain K-12. Chloramphenicol reversed the inhibition of precursor uptake resulting from amino acid starvation in the RC\textsuperscript{tr} K-12 strain. Pulse-labeling experiments reinforced these observations. Pulse-decay experiments in conjunction with actinomycin D indicated that the observed pattern of precursor uptake was probably not due to differences in mRNA decay rate. It is concluded that if amino acids exert any direct effect on the synthesis of T4 mRNA such an effect is much smaller than that observed for bulk RNA synthesis in the uninfected cell. These experiments also indicated that actual translation of the genetic message is not necessary for its transcription.

The fact that ribosomal ribonucleic acid (RNA) and transfer RNA are regulated by amino acids in RC\textsuperscript{tr} strains of Escherichia coli appears to be firmly established (7). Whether messenger RNA (mRNA) is so regulated is still a subject of some dispute (4, 10; J. Stubbs and B. Hall, personal communication; G. Edlin and P. Broda, personal communication). A class of mRNA which lends itself readily to study is the mRNA specific for T4 bacteriophage infected E. coli cells. Since all host RNA synthesis ceases after T4 infection (11, 12), it should be possible to measure phage messenger RNA synthesis directly by determining uptake of labeled RNA precursors into phage-infected cells.

The results of a series of such experiments, reported here, indicate that (i) the uptake of labeled precursors into T4 messenger RNA is reduced slightly, but reproducibly, by amino acid deprivation in RC\textsuperscript{tr} K-12 strains, but not at all in strain B RC\textsuperscript{tr} strains or strain K-12 RC\textsuperscript{tr1} mutants, (ii) phage mRNA synthesis can proceed in the complete absence of protein synthesis, and (iii) the rate of T4 mRNA decay depends upon conditions under which it is synthesized and under which it decays.

It is concluded that if amino acids exert any direct effect on the synthesis of T4 mRNA such an effect is much smaller than that observed for bulk RNA synthesis in the uninfected cell.

MATERIALS AND METHODS

Bacteria and phage. Two strains of E. coli K-12, CP 78 RC\textsuperscript{tr} and CP 79 RC\textsuperscript{tr1}, isogenic except for the RC locus (3), were used in most of these studies. Both of these strains require threonine, leucine, arginine, histidine, and vitamin B\textsubscript{12}. Many of the key experiments were repeated on another partially isogenic pair of K-12 strains, F 8 and F 74 (5), which require methionine and histidine. In addition, two strains of E. coli were used: strain F 40 (requiring methionine and thymine) is RC\textsuperscript{tr}; F 100 (requiring methionine, RC\textsuperscript{tr1}) was prepared from F 40 by mating this strain with Hfr Cavalli (requiring methionine and uracil) and selecting for thy\textsuperscript{r} recombinants, approximately 50% of which were RC\textsuperscript{tr1}.

Two amber mutants of T4 were used in these studies. Am 8.82 is unable to make lysozyme and behaves very similarly to T4 wild type with the exception that it does not lyse the nonpermissive host. Am N82 makes no deoxyribonucleic acid (DNA; 18) or late RNA; it makes only early RNA. The advantage of using this mutant is that it is unnecessary to correct H-uracil or H adenine incorporations for radioactivity incorporated into DNA. Both phages were kindly supplied by Werner Bode.

Purified phage stocks were made according to the method of G. Kellenberger (personal communication).
A 1- or 2-liter culture of E. coli CR 63, grown with vigorous aeration to a level of 10^8 to 2 x 10^9 cells per ml in M9 medium (Na2HPO4, 0.7%; KH2PO4, 0.3%; NaCl, 0.03%; NH4Cl, 0.1%; CaCl2, 0.002%; MgSO4, 0.2%) plus 1% Casamino Acids and 0.1% glucose, was infected at a multiplicity of 0.1. At 3 hr after infection, the cells were harvested by centrifugation; the pelleted cells were lysed by adding chloroform and 100 μg of deoxyribonuclease and stirring for 5 min at room temperature. The lysate was then immediately layered on top of preformed CsCl gradients and centrifuged for 15 min in an SW 25.3 rotor at 60,000 x g. The opaque phage band was withdrawn by pipette and dialyzed against 0.01 M tris(hydroxymethyl)aminomethane (Tris), pH 7.5. The titer of the phage was typically of the order of 10^9 plaque-forming units (PFU)/ml.

Growth and infection of cells. Cells were grown at 37 °C in M9-glucose medium supplemented with the required amino acids at concentrations of 50 μg/ml (and thymine if necessary) plus 10^{-4} M Fe^{++}. When a density of 3 x 10^8 cells per ml was reached (optical density at 450 nm, 0.700), an input multiplicity of 6 (for strain B) or 20 (for strain K-12) phage was added. The extent of infection was monitored by measuring the fraction of cells surviving 10 min after infection. Experiments in which more than 1% of the cells were infected as judged by this criterion were rejected as unsuitable for analysis. Assuming a Poisson distribution, the actual multiplicity of infection was 4 to 5. Starvation was accomplished by a filtration method (4).

DNA and RNA extractions and formation of DNA-RNA hybrids. E. coli DNA was extracted according to the method of Thomas et al. (17), with the exception that 0.1 M ethylenediaminetetraacetate (Geygi Chemical Co., Ardsley, N.Y.) was present during lysis. Phage DNA was extracted by shaking purified phage stocks with phenol, followed by alcohol precipitation and dialysis against SSC (SSC = 0.15 M NaCl, 0.015 M trisodium citrate). Purification of RNA has been described (4). Annealing of RNA to DNA immobilized on nitrocellulose filters (6) has also been described previously (4); 30 μg of phage or host DNA was immobilized on each filter for the annealing reaction. Each reaction tube contained approximately 1 μg of total RNA (host plus phage).

Radioactive compounds and other chemicals. 3H-uracil (50 μCi/μg) and 3H-adenine (46.6 μCi/μg) were purchased from New England Nuclear Corp. (Boston, Mass.). Bovine pancreatic ribonuclease-A was purchased from Sigma Chemical Co. (St. Louis, Mo.), and deoxyribonuclease-1, electrophoretically purified, was from the Worthington Biochemical Corp. (Freehold, N.J.). Chloramphenicol was a gift of the Parke, Davis & Co. (Detroit, Mich.), and actinomycin was a gift of W. E. Scott, Hoffman-La Roche, Inc. (Nutley, N.J.).

RESULTS

Relative rates of uracil and leucine uptake in infected and uninfected cells. Figure 1a shows results of an experiment in which the rates of uracil uptake in infected and uninfected cultures of strains CP 78 RC^{str} and CP 79 RC^{rel} are compared. (All results have been normalized to an optical density at 450 nm of 0.600.) Uracil uptake in uninfected cells of both RC^{rel} and RC^{str} genotypes followed the expected pattern. The rate of uracil uptake into the RNA of fully supplemented phage-infected cells was approximately 15% that of the uninfected cells, and was greater than the uptake into amino acid-starved RC^{str} cells by a factor of about 4. The rate of uracil uptake by infected cells was almost identical in fully supplemented stringent and relaxed cells; the rate was very similar for am N82, which synthesizes only early mRNA, and for am 8.82, which synthesizes both early and late mRNA.

The rate of leucine incorporation into phage-infected cells was only about 5% that of uninfected cells (Fig. 1b); it was very nearly equal to the rate of leucine uptake by uninfected cells of stars of amino acid. Starvation of the infected cells for required amino acids led to a further reduction of leucine uptake by a factor which varied from four- to sixfold.

Since the data shown in Fig. 1 indicate that the level of uracil incorporation in infected cells is very low, it is obvious that uptake of uracil can be used to measure phage mRNA synthesis only if care is taken to assure that an insignificant fraction of the cell population remains uninfected. Accordingly, only those experiments in which greater than 99% of the cells were infected (as judged by cell killing) were admitted for analysis. The efficacy of the amino acid starvation was tested in each of the experiments reported below by comparing the rates at which the fully supplemented and amino acid-starved infectious centers took up labeled leucine. A 20-min period of amino acid starvation was inserted just prior to the addition of label to help deplete amino acid pools in the infected cells.

Effect of amino acid starvation on phage mRNA synthesis. Figure 2 shows uracil uptake into several strains of E. coli infected with T4 am N82. All data have been adjusted to an optical density at 450 nm of 0.600. In the RC^{str} K-12 strain CP 78 (Fig. 2a), there was a modest, but consistently observed, reduction of approximately 50% in the rate of uracil uptake by amino acid-starved infected cells. This was reversed by the addition of chloramphenicol. In the isogenic RC^{rel} strain CP 79 (Fig. 2b), removal of amino acids had virtually no effect on uracil incorporation. Growing the cells in medium containing unlabeled uracil prior to infection did not change the incorporation patterns (unpublished data). Similar results were observed with another (parti-
ally) isogenic pair of K-12 strains, F 74 and F 8, starved of methionine and histidine (unpublished data).

In strain B RCstr (Fig. 2c), amino acid starvation barely suppressed uracil uptake although addition of chloramphenicol appeared to stimulate it. In strain B RCrel (Fig. 2d), amino acid starvation, if anything, stimulated uracil uptake. It should be noted that the rate of uptake of uracil into infectious centers of the B strains was only about one-third that of the K-12 strains.

In the control (fully supplemented) cultures of all strains studied here, uptake of uracil was approximately linear with time during the 25 min of the experiment. In these experiments, there was no indication of a decreasing rate of uptake, thought to be due to equilibration of the uracil pool in phage-infected cells (16). The reason for our failure to observe this phenomenon is not clear, but may have resulted from the filtration and resuspension of infectious centers, which was an essential part of the experimental procedure.

The K-12 strains were studied further by labeling am N82-infected cells with adenine (Fig. 3a...
and b) and am 8.82-infected cells with uracil, subtracting the DNA component in the latter case (Fig. 3c and d). In all cases, starvation of the phage-infected strain CP 78 RCstr led to approximately 40 to 50% reduction in the rate of isotope incorporation, and this was specifically reversed by addition of chloramphenicol at the time of starvation. In the RCrel strain (CP 79), amino acid starvation or addition of chloramphenicol was without significant effect on precursor uptake.

As evidence that total isotope uptake is indeed an accurate reflection of incorporation into T4-specific RNA, an experiment similar to that shown in Fig. 1a was performed in which RNA

![Graph of uptake of 3H-uracil into stringent and relaxed strains of T4 am N82-infected E. coli.](http://jb.asm.org/

**Fig. 2.** Uptake of 3H-uracil into stringent and relaxed strains of T4 am N82-infected E. coli. Cultures of E. coli (as indicated below) growing exponentially at a titer of 2.5 × 10^8 cells/ml were infected with T4 am N82 as described in Materials and Methods. At 10 min after infection, the cultures were shifted by the filtration method to medium lacking the required amino acids indicated below. Following 20 min of starvation, the cultures were divided into five portions (this is time zero on the graph). Three contained 3H-uracil (1 μg/ml; 10 μc/ml): of these, one contained all amino acids required by the strain, a second lacked the amino acids indicated below, and a third lacked these same amino acids but contained 250 μg of chloramphenicol per ml. The remaining two portions of the cultures contained 3H-leucine (2 μg/ml; 2 μc/ml): one of these contained all amino acids required by the strain, and the other lacked all amino acids. The data for the leucine incorporations are not shown, but were included in each experiment to control the efficacy of amino acid starvation. Samples were prepared for counting as described in the legend to Fig. 1. All data are the means of three experiments. Symbols: ○, plus all required amino acids; ●, minus required amino acids as indicated below; △, minus required amino acids as indicated below plus chloramphenicol. (a) CP 78 RCstr starved of threonine, histidine, and arginine. (b) CP 79 RCrel starved of threonine, histidine, and arginine. (c) Strain B RCstr (F40) starved of methionine. (d) Strain B RCrel (F100) starved of methionine.
FIG. 3a and b. $^1$H-adenine uptake by T4 am N82-infected E. coli. These experiments were performed exactly as described in the legend to Fig. 2, except that labeling was done with $^1$H-adenine (1 µg/ml; 10 µc/ml). (a) Strain CP 78 RCstr starved of threonine, histidine, and arginine. (b) Strain CP 79 RCrel starved of threonine, histidine, and arginine.

FIG. 3c and d. 3H-uracil uptake by T4 am 8.82-infected E. coli. These experiments were performed exactly as described in the legend to Fig. 2. At each time interval, duplicate samples were pipetted into trichloroacetic acid and NaOH, as described in the legend to Fig. 1. (c) Strain CP 78 RCstr starved of threonine, histidine, and arginine. (d) Strain CP 79 RCrel starved of threonine, histidine, and arginine. All symbols are the same as in Fig. 2. All data are the mean values of three experiments.

was extracted from the phage-infected cells, purified, and annealed to T4 DNA and E. coli DNA. Incorporation into T4-specific RNA closely mirrored total trichloroacetic acid-precipitable incorporation (Fig. 4). In none of the 18 samples used in this experiment or other similar experiments was any significant amount of labeled RNA, above background, annealed to host DNA.

Decay of phage mRNA. It has been observed that in bacterial cells addition of chloramphenicol leads to a decreased rate of mRNA decay (4, 9). By analogy, it is possible that the observed chloramphenicol-induced enhancement of precursor uptake into T4-infected RCstr cells might be due to changes in the rate of decay of phage mRNA rather than in the rate of synthesis. This possibility was tested by a series of pulse-decay experiments (Fig. 5).

The data displayed in Fig. 5a do indeed indicate that the decay of mRNA synthesized in normally infected cells is slower when subsequently the infectious centers are placed in chloramphenicol ($t_{1/2} = 13$ min) than without the exposure to chloramphenicol ($t_{1/2} = 3.5$ to 4 min). On the other hand, Fig. 5a also shows that when synthesis of mRNA takes place in the presence of chloramphenicol the subsequent rate of decay in the presence of the antibiotic (5 min) is close to the normal rate. Similarly, when the pulse was administered to infectious centers during amino acid starvation and subsequent decay occurred during inhibited protein synthesis, the decay rate re-
Fig. 4. Hybridization of RNA extracted from T4 am N82-infected E. coli strain CP 78 RC<sup>ext</sup>. This experiment was carried out exactly as described in the legend to Fig. 2, except that 2-ml samples taken at intervals were frozen, and the RNA was purified and hybridized to T4 and E. coli DNA as described in Materials and Methods. The main body of the figure shows radioactivity annealed to T4 DNA. The insert shows total trichloroacetic acid-precipitable counts per minute. There was no radioactivity annealed to E. coli DNA in any of the samples. Symbols: O, plus threonine, leucine, arginine, and histidine; , minus threonine, leucine, arginine, and histidine; A, threonine, leucine, arginine, and histidine, plus chloramphenicol.

TABLE 1. Effect of amino acid starvation and chloramphenicol on the instantaneous rate of uracil uptake<sup>a</sup>

<table>
<thead>
<tr>
<th>Strain</th>
<th>Labeling conditions</th>
<th>Time of start of 1-min pulse (min)</th>
<th>Trichloroacetic acid-precipitable counts/minute</th>
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<td>CP 78 RC&lt;sup&gt;ext&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td>10</td>
<td>4,648</td>
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<sup>a</sup> The protocol for these experiments was essentially the same as described in the legend to Fig. 2. Following the 20-min starvation period (time zero in the experiment), the infected cultures (am N82) were divided into three parts; of these, one contained all amino acids required by the strain, a second lacked threonine (thr), leucine (leu), arginine (arg) and histidine (his), and a third lacked these amino acids but contained 250 µg of chloramphenicol per ml. At intervals thereafter, 1.0-ml samples from each of the three subcultures were added to a tube containing 3H-uracil (0.1 µg/ml; 4.5 µC/ml). Exactly 1 min later, 1.0 ml of ice-cold 10% trichloroacetic acid was added to stop incorporation. Time zero refers to the point at which the starved culture was distributed into the three subcultures.

<sup>b</sup> Backgrounds have been subtracted.

Seemed the normal rate (Fig. 5b). Similar results were obtained with both a K-12 strain and a B strain.

On the basis of the data in Fig. 5, it seems unlikely that the apparent differences in precursor uptake (Fig. 2a and 3) can be accounted for by differences in the decay rate of mRNA.

Pulse-labeling experiments. To gain further information on this point, short pulses (1 min) of 3H-uracil were fed to am N82-infected cells growing under a variety of conditions (Table 1). The amount of radioactivity incorporated during these short pulses ought to reflect the instantaneous rate of uracil uptake, relatively unclouded by complications arising from possibly differing decay rates. The data in Table 1 indicate that the instantaneous rate of uracil uptake was reduced by approximately 60% because of amino acid starvation.
of RCstr strain CP 78, and that the reduction was reversed by the addition of chloramphenicol. Amino acid starvation had no effect in strain CP 79 RCstr.

6-Azauracil experiments. The level of uracil uptake, and thus presumably the level of RNA synthesis, in T4-infected cells is much lower than in uninfected cells (Fig. 1a), and the data presented in Fig. 2a and 3a indicate that the effect of amino acid starvation in depressing the level of uracil uptake by infected RCstr cells is only about a twofold decrease. It is possible that, at the low level of RNA synthesis obtained during phage infection, the RNA synthetic system cannot be depressed during amino acid starvation by the same proportion as it is in the uninfected cell. In other words, the lower limit to the residual level of RNA synthesis during "stringency" might be an absolute, rather than a relative, one.

One way to shed some light on this was to find an alternate way to depress RNA synthesis in an uninfected RCstr culture and then, in addition, to starve such a culture of required amino acids. Strain F 74 RCstr was chosen for this experiment, and RNA synthesis was inhibited by the addition of 100 μg of 6-azauracil per ml. The addition of 6-azauracil inhibited the rate of RNA synthesis in the presence of amino acids by a factor of 3 to 4;

![Fig. 5. Decay of T4 mRNA. (a) A culture of strain CP 78 was infected with T4 am N82. At 10 min after infection, the culture was rapidly centrifuged (30,000 × g; 2 min), washed with 0.1× Tris, pH 8, centrifuged once again, and finally suspended in 0.1× Tris – 2 × 10⁻³ × EDTA, pH 8, for 2 min at 37°C (8). At this point, the culture was diluted 10-fold into warm complete growth medium and divided into two parts, one containing chloramphenicol and the other lacking it. Each culture was labeled for 4 min with ³H-uracil (0.5 μg/ml; 20 μc/ml) and then placed in tubes containing 40 μg of actinomycin D per ml plus 100 μg of unlabeled uracil. (In addition, a control culture was run in which the actinomycin D was added immediately before the addition of the label. These controls incorporated essentially zero counts/min above backgrounds in all the experiments reported.) At 1-min intervals, 0.1-ml samples from each culture were pipetted into 1.0 ml of cold 5% trichloroacetic acid. Symbols: ○, RNA labeled under normal conditions, decay under normal conditions; △, RNA labeled under normal conditions, decay in the presence of chloramphenicol; ▲, RNA labeled in the presence of chloramphenicol, decay in the presence of chloramphenicol. (b) An infected culture was starved of threonine, leucine, histidine, and arginine for 20 min and then washed with Tris and suspended in Tris-EDTA as described above. Subsequently, the culture was diluted 10-fold into warm growth medium lacking all four amino acids and labeled for 4 min with ³H-uracil during the amino acid starvation. Following this, actinomycin D and cold uracil were added to each culture and the decay was followed under 3 different conditions: ○, RNA labeled under amino acid starvation, decay under normal conditions; ●, RNA labeled under amino acid starvation, decay in the absence of amino acids; ▲, RNA labeled under amino acid starvation, decay in the presence of chloramphenicol.]

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however, removal of required amino acids from the 6-azauracil culture did not depress the rate of RNA synthesis to a level any lower than that reached in a control culture starved of the same amino acids (Fig. 6). The level to which "stringency" can depress the rate of RNA synthesis appears to have an absolute lower limit.

**DISCUSSION**

The interpretation of the present results hinges upon the validity of the assumption that the rate of precursor uptake into RNA is a true measure of the actual rate of phage mRNA synthesis. Several authors have presented evidence which indicates that the tendency in *E. coli* cells infected with T-even phages is for the uracil pool to appear to become compartmentalized. This is reflected in a gradual decrease in the rate of uptake of exogenous uracil (16) and the retention of precursor in the pool for long periods of time (1). The latter effect may occur in the present system, since otherwise it is difficult to explain the observation of a constant rate of accumulation of label into a class of molecules that is known to turn over rapidly [especially since the pulse experiments (Table 1) show that the instantaneous rate of uracil uptake does not change significantly after infection]. As a result of this, the fact that amino acid starvation appears to diminish uracil uptake in stringent strains of K-12, but not in stringent strains of B or relaxed strains of K-12, could be attributed to differences in pool behavior between the two strains.

Against this must be set the observations that the effects of amino acid starvation on precursor uptake in the K-12 strains (i) appear to be a function of the RC locus, since the strains of CP 78 and CP 79 are completely isogenic except for the RC locus (3), (ii) are specifically reversed by chloramphenicol, (iii) are very similar when either pyrimidine or purine is used as the labeled precursor, and (iv) are independent of the existence of a permanent "sink" for precursors, the phase DNA, which might influence the pool behavior.

Further, one could explain the failure to observe any depression of the rate of RNA synthesis upon amino acid deprivation in strain B RC<sup>+</sup> by noting that the level of phage mRNA synthesis in this strain is already threefold lower than in the K-12 strains (Fig. 2). Thus, if one can extrapolate the result for uninfected cells depicted in Fig. 6 to the phage-infected cell, one can speculate that the level of phage RNA synthesis in cells of strain B is incapable of being further depressed by amino acid starvation. It is also possible that the low levels of protein synthesis obtained after infection with *T*<sup>+</sup> bacteriophage might not permit efficient amino acid starvation (Fig. 1b).

With these reservations kept in mind, the tentative conclusion from the present data is that if *T*<sup>+</sup> mRNA is controlled at all by amino acids through the RC locus, it is controlled to a much smaller extent than is bulk RNA in the uninfected host.

There have been several previous studies of the effect of amino acid deprivation on RNA synthesis in T-even-infected cells. Sekiguchi and Cohen (13) concluded that amino acid starvation had very little effect on RNA synthesis in infected cells of strain 15. However, in their experiments, the cells were starved of essential amino acids before infection, a condition which leads to residual synthesis of host RNA (11, 12, 14), and this tends to obscure the applicability of Sekiguchi and Cohen's data (13) to the present problem. Similarly, Sharp and Green (14) concluded that T2
mRNA synthesis is not affected by amino acid starvation in a stringent strain of K-12. On the other hand, Edlin (2) concluded that amino acid starvation of a stringent strain inhibits the uptake of 5-fluorouracil; the effect was reversed by the addition of chloramphenicol. Whether or not this same pattern would be observed in a relaxed strain was not determined.

Like those of Sharp and Green (14), the present results indicate no obligatory coupling between mRNA transcription and translation (15) in the phage-infected cell, since there is clearly mRNA synthesis in the presence of a high concentration of chloramphenicol. This has also been observed in uninfected E. coli (4). It has not yet been determined whether in the case of T-even phage infection there is attachment of mRNA to the ribosome under conditions in which actual transcription is halted, as is the case in uninfected cells (5).

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LITERATURE CITED