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Regulation of Early mRNA Synthesis After Bacteriophage T4 Infection of *Escherichia coli*

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Regulation of T4-specific mRNA synthesis was studied during leucine starvation of a leucine-requiring stringent *Escherichia coli* B strain. This was done by imposing starvation prior to T4 infection and then letting RNA synthesis proceed for different time periods. Rifampin or streptolydigin was added to stop further RNA synthesis, and protein synthesis was restored by addition of leucine. Samples were withdrawn at different times, and the enzyme-forming capacities for an immediate early and a delayed early enzyme were determined. It was found that, during conditions which elicit the stringent response in uninfected bacteria, immediate early mRNA is not stringently regulated. This conclusion contradicts the earlier conclusion of others, obtained by measuring incorporation of radioactive uracil; this is explained by the observation of Edlin and Neuhard (1967), confirmed and extended by us to the T4-infected cell, that the incorporation of uracil into RNA of a stringent strain is virtually blocked by amino acid starvation, whereas that of adenine continues at 30 to 50% of the rate seen in the presence of the required amino acid.

Salser et al. demonstrated by hybridization techniques that there is a sequential appearance of different classes of mRNA after infection of *Escherichia coli* cells by bacteriophage T4 (27). The mRNA formed in the presence of chloramphenicol is defined as the immediate early mRNA. This mRNA does not require protein synthesis to be initiated, in contrast to the delayed early mRNA, which is synthesized in the absence of chloramphenicol after about 2 min, and the late mRNA, which is formed after about 5 min at 30 C.

Lazzarini and Dahlberg (15) have shown that synthesis of both stable RNA and mRNA is restricted during amino acid starvation in uninfected cells, but in a noncoordinate manner, so that RNA synthesis is much enriched in mRNA during starvation. After T4 infection mainly T4 mRNA is synthesized (23), and DNA-dependent RNA polymerase undergoes several modifications (28). An unusual nucleotide, ppGpp, has been implicated in the regulation of RNA synthesis (3). This nucleotide is also synthesized after T4 infection during amino acid starvation of stringent cells (7).

The aim of the present work was to study the regulation of T4 mRNA in cells starved for amino acids. Earlier studies were performed with the aid of radioactive uracil (30). However, we have demonstrated that uracil incorporation is severely restricted under such conditions, which is consistent with the results from other laboratories (8, 12). Therefore, we have measured the accumulation of T4-specific mRNA formed during amino acid starvation by determining the enzyme-forming capacities after rifampin addition.

Our work shows that immediate early mRNA is not stringently regulated during conditions which elicit the stringent response in uninfected cells. We imposed amino acid starvation prior to T4 infection and let RNA synthesis proceed. After different time periods rifampin or streptolydigin was added to stop further RNA synthesis, and protein synthesis was restored by addition of amino acids. The formation of immediate and delayed early enzymes was measured.

MATERIALS AND METHODS

Media. For the growth of *E. coli* B40, Tris-mineral salts medium described by Fast and Sköld was used (9). This medium was supplemented with glucose (5 g/liter), $FeCl_3$ (10^{-5} M), and arginine (50 μg/ml). The experiments in which rifampin and streptolydigin were used were performed with *E. coli* AA446, which is highly permeable to these drugs. Cells of this strain will, however, lyse very easily. All experiments with AA446 were performed in M9 mineral salts medium (6). This medium was supplemented with glucose (5 g/liter), $FeCl_3$ (10^{-5} M), and leucine (50 μg/ml).

Chemicals. [5-^{3}H]dCMP ammonium salt (>3,000

mCi/nmol), L-[2-^{14}C]histidine (62 mCi/nmol), [8-^{14}C]adenine (59 mCi/mmol), [2-^{14}C]uracil (60 mCi/mmol), and [2-^{3}H]adenine (18 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, Great Britain. [5-^{3}H]uracil, 29 Ci/mmol, was purchased from New England Nuclear Corp., Chemicals, Dreieichenhain, Germany. [^{14}C]dHMP and unlabeled dHMP were synthesized according to Wiberg et al. (32). dCMP sodium salt, and *d*,l-tetrahydrofolic acid were purchased from Sigma, St. Louis, Mo. Rifampin sodium salt was obtained as a gift from Gruppo Lepetit, Milano, Italy. Streptolydigin (free acid) was a gift from Upjohn, Kalamazoo, Mich., and chloramphenicol was obtained as a gift from Parke, Davis and Co., Hounslow, Great Britain.

Bacteria and phages. The following strains of *E. coli* B were used. AA446, a rifampin- and streptolydigin-sensitive strain, is a derivative of AS19 (29) (AS19, *leu*$^-$, *rel*$^+$). This strain was a kind gift from E. Lund, Århus, Denmark (18). B40 (*arg*$^-$, *rel*$^+$) (10) was used. Suppressor strain CR63 was used for T4 amber mutant plating, and *E. coli* B was used for wild-type T4 plating.

T4 *amE*114 (gene 56$^-$, dCTPase$^-$ [EC 3.6.1.12]) was used under nonpermissive conditions, when enzyme-forming capacities were followed for a long time, in order to prevent lysis of the cells. This particular mutant was chosen because it is not defective in the structural genes of dHMP kinase (EC 2.7.4.4.) or dCMP HMase (EC 2.1.2.8.), the gene products of which were measured. T4 wild type was used in the other experiments.

Infected cells. Bacteria were grown and aerated at 37 C to about 5×10^8 cells/ml, unless otherwise indicated, in mineral salts medium. The cells were harvested by filtration or centrifugation and concentrated to 5×10^9 cells/ml. The experiments with AA446 were performed at 27 C; aeration was terminated at the time of infection and kept off for the remainder of the infection to minimize lysis of cells. By using small culture volumes, a large surface per volume was obtained, which gives good aeration. The experiments with B40 were performed at 30 C, and the aeration was also continued after infection.

At infection (time zero) four to eight phages per bacterium were added to the concentrated bacterial culture to obtain good adsorption conditions. After 2 or 3 min the infected cells were diluted 10-fold into previously actively aerated and prewarmed medium.

Phage adsorption and bacterial survival were determined routinely at 5 min. Adsorption was always higher than 95%, and bacterial survival was less than 1%.

Amino acid starvation was established by filtration of the culture, and the cells were washed on the filter three times with 3 ml of medium lacking amino acid and, finally, resuspended in medium lacking amino acid.

Determination of protein and RNA synthesis: cumulative incorporations. The rates of protein and RNA synthesis were followed by cumulative incorporations of [^{14}C]histidine and [^{14}C]uracil or [^{14}C]adenine, respectively. Samples of 0.10 ml were withdrawn at indicated times and pipetted onto filter paper disks (Whatman no. 3) and precipitated in ice-cold 5% trichloroacetic acid for at least 30 min at 0 C. Then the filters were dried, and the radioactivity was measured. When protein synthesis was determined by [^{14}C]histidine incorporation, the filter disks were boiled for 30 min before drying.

Pulse labeling (Fig. 4). The incorporation of [^{3}H]uracil and [^{3}H]adenine was measured as follows. Samples of 50 μl were pipetted into 2 ml of 0.3 N ice-cold NaOH. One-half of each sample was neutralized with 0.3 N HCl, and trichloroacetic acid was added to 5%. The remainder of each sample was treated in a 45 C water bath for 90 min; it was then cooled and neutralized with 0.3 N HCl and trichloroacetic acid was added. The precipitates were collected on membrane filters (type HA, 0.45-μm pore size, Millipore Corp.), which were dried and counted in the liquid scintillation counter. The radioactivity in the NaOH-hydrolyzed samples was subtracted from the total radioactivity in the corresponding samples to give incorporation in RNA.

Extracts for enzyme assays. For the determination of β-galactosidase (EC 3.2.1.23.) 1.0-ml samples were withdrawn at indicated times to 1 ml of toluene at 0 C, and the samples were vigorously shaken for 15 s and left on ice for at least 30 min (19).

For the determination of phage-specific enzymes, samples of 2 ml were withdrawn at indicated times to chilled test tubes on dry ice and acetone. These samples were thawed and disrupted by sonication in an MSE sonicator three times at 20 s each. Cell debris was removed by centrifugation at 25,000 $\times$ *g* for 10 min. The supernatant was frozen and stored at -20 C.

Enzyme assays. (i) β-Galactosidase. β-Galactosidase was assayed according to Miller (19). One unit in our experiments is defined as the increase in optical density at 420 nm $\times$ 10^3/min at 37 C.

(ii) dCMP HMase. dCMP HMase (EC 2.1.2.8.) was assayed using a modification by Wiberg et al. (33) of the procedure of Yeh and Greenberg (34); this procedure determines the release of the ^{3}H atom (as tritiated H_2O) from [5-^{3}H]dCMP. The background activity with uninfected bacteria was subtracted. Activity was measured as nanomoles of dHMP formed per hour and 1.5×10^8 cells at 37 C.

(iii) dHMP kinase. (dHMP kinase (EC 2.7.4.4.) was determined with [^{14}C]dHMP as a substrate according to Wiberg et al. (32).) Activity was measured as nanomoles of dHDP formed per hour and 1.5×10^8 cells at 37 C.

RESULTS

Synthesis of the early mRNA in the presence of chloramphenicol. To confirm the findings that dCMP HMase mRNA belongs to the immediate early class of mRNA and that dHMP kinase belongs to the delayed early class of mRNA (13, 17, 26), we followed mRNA synthesis in the presence of 20 μg of chloramphenicol per ml. At this concentration of chloramphenicol protein synthesis is lowered by 95% in 10 s (25). The drug was added 2 min prior to

T4 infection, and RNA synthesis was allowed to proceed for different time periods. Then rifampin (20 μg/ml) was added, and protein synthesis was restored by removal of chloramphenicol. Rifampin (20 μg/ml) shuts off RNA initiation within 30 s (11). By this method only T4 mRNA synthesized in the absence of protein synthesis could be translated into functional enzymes.

The activities of dCMP HMase and dHMP kinase are shown in Fig. 1A and B, respectively. Addition of rifampin prior to T4 infection blocked all synthesis of the enzymes. When rifampin was added at 1 and 5 min, respectively, after infection, however, increasing amounts of dCMP HMase were formed, whereas no significant amounts of dHMP kinase could be detected.

Synthesis of early mRNA during leucine starvation. Leucine starvation was imposed by filtration 5 min prior to T4 infection. Rifampin (20 μg/ml) or streptolydigin (100 μg/ml) was added at 9 min after infection, and protein synthesis was restored by addition of leucine (50 μg/ml) at 10 min.

From the results presented in Fig. 2A and B we conclude that dCMP HMase mRNA (immediate early) is synthesized during leucine starvation and that dHMP kinase mRNA (delayed early) is not. To rule out the possibility that dCMP HMase mRNA is only initiated but not elongated during leucine starvation, we made the same experiment with streptolydigin instead of rifampin. Rifampin blocks only the initiation of new RNA chains (31). Streptolydigin inhibits RNA chain elongation (4), and similar results were found if streptolydigin was used instead of rifampin (Fig. 2C).

Thus, dCMP HMase mRNA is both initiated and elongated during leucine starvation.

Efficiency of amino acid starvation. It could be argued that the method used by us does not really impose leucine starvation. To rule out this possibility, we measured the effect of leucine starvation on amino acid incorporation and on β-galactosidase formation. Fig. 3A shows the cumulative incorporation of [^{14}C]histidine by uninfected bacteria. Leucine starvation, imposed by this method, completely halts histidine incorporation for at least 20 min. Later in this experiment the cells are spontaneously supplied with leucine from lysed cells.

Similarly, uninfected cells cannot synthesize β-galactosidase during our conditions of leucine starvation (Fig. 3B). We conclude that leucine starvation is complete for at least 20 min. There is no uptake during this time of [^{14}C]adenine or [^{14}C]uracil (Fig. 3C and D). The adenine result shows that our conditions for starvation also impose the stringent response in uninfected cells; the uracil result is inconclusive on this point (see below).

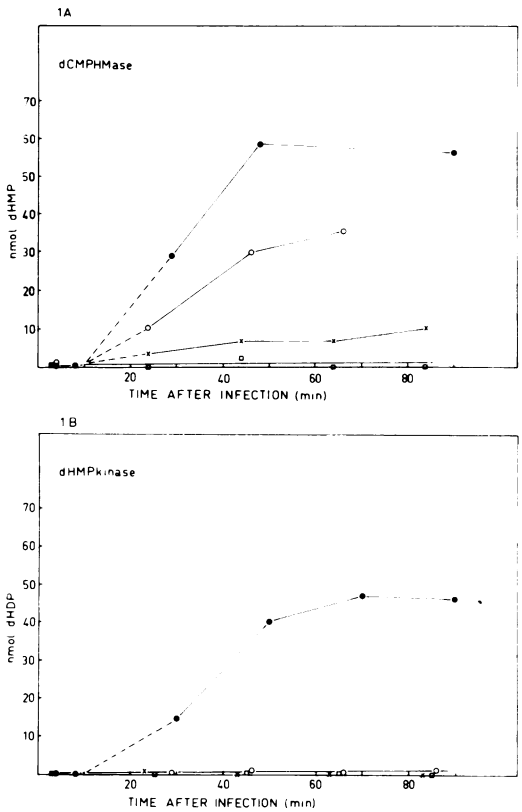

FIG. 1. *Early enzyme activities in E. coli AA446 infected with T4 amE114. Cells were grown in M9 mineral salts medium at 37 C. The cells were harvested by filtration and resuspended in M9 medium to a final concentration of 5 × 10⁸ cells/ml. Then the temperature was lowered to 27 C and the cells were infected (time zero). Two minutes after infection the cells were diluted 10-fold in the same medium. At different times extracts were prepared and assayed as described in Materials and Methods. (A) dCMP HMase activity (nanomoles of dHMP formed/h · 1.5 × 10⁸ cells at 37 C). (B) dHMP kinase activity (nanomoles of dHDP formed/h·1.5 × 10⁸ cells at 37 C). Symbols: (□) chloramphenicol −2 min, rifampin −1 min, −chloramphenicol +4 min; (×) chloramphenicol −2 min, rifampin +1 min, −chloramphenicol +4 min; (○) chloramphenicol −2 min, rifampin + 5 min, −chloramphenicol +6 min; (●) chloramphenicol −2 min, −chloramphenicol +10 min.*

Incorporation of [^{3}H]uracil and [^{3}H]adenine in RNA. Since earlier investigations in amino acid control of T4-specific mRNA formation relied upon uracil incorporation data, we made a comparison between [^{3}H]uracil and [^{3}H]adenine incorporation under starvation

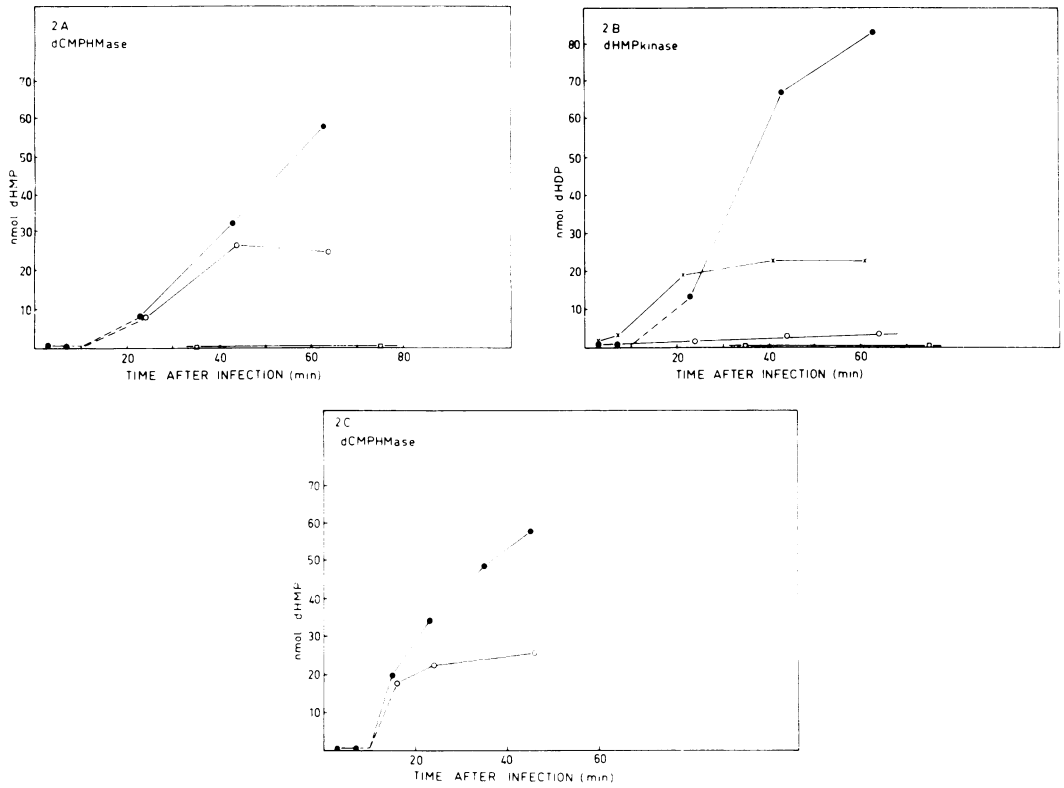

FIG. 2. *Early enzyme activities in E. coli AA446 infected with T4 amE114. Cells were grown, harvested, and infected as described in the legend to Fig. 1. Leucine was removed by resuspending the cells, after filtration, in a leucine-free medium. At different times extracts were prepared and assayed as described in Materials and Methods. (A) dCMP HMase activity. (B) dHMP kinase activity. Symbols: (□) resuspension in leucine-free medium −5 min, rifampin −1 min, +leucine −0 min; (○) resuspension in leucine-free medium −5 min, rifampin +9 min, +leucine +10 min; (●) resuspension in leucine-free medium −5 min, +leucine +10 min; (×) resuspension in medium containing leucine −5 min, rifampin +9 min. (C) dCMP HMase activity. Symbols: (○) resuspension in leucine-free medium −5 min, streptolydigin (100 μg/ml) +9 min, leucine +10 min; (●) resuspension in leucine-free medium −5 min, +leucine +10 min.*

conditions. Figure 4A shows that the incorporation of uracil is completely shut off when a culture of T4-infected *E. coli* B40 is starved for arginine. The incorporation of [^{3}H]adenine is reduced to about 50% compared to the control culture (Fig. 4B). The same results were obtained with T4-infected *E. coli* Cp78 starved for the whole set of required amino acids: arginine, histidine, leucine, and threonine.

DISCUSSION

We find that the early mRNA transcribed during treatment with chloramphenicol or during leucine starvation can code for the synthesis of dCMP HMase. Lembach and Buchanan found that this mRNA is also formed in the presence of 5-methyltryptophan (17). Since it is formed in the absence of protein synthesis, it belongs to the immediate early class by definition (26). This statement is also supported by the finding of Jayaraman (13), who has shown that dCMP HMase mRNA is synthesized in vitro in the presence of the terminator protein "rho" (13).

The demonstration that dCMP HMase mRNA is formed under conditions of efficient leucine starvation also (Fig. 3) implies that a T4-specific early messenger is synthesized independently of the *rel* gene of the host.

This conclusion is further strengthened by the results presented in Fig. 4B, where it is shown by [^{3}H]adenine incorporation that early mRNA synthesis is not severely restricted during amino acid starvation.

Other workers (30) have used radioactive uracil to demonstrate that early mRNA is governed by the *rel* gene. Their results, however, are invalidated by the fact that uracil incorporation is blocked during amino acid starvation in stringent cells (Fig. 4), and thus mRNA synthesis goes undetected.

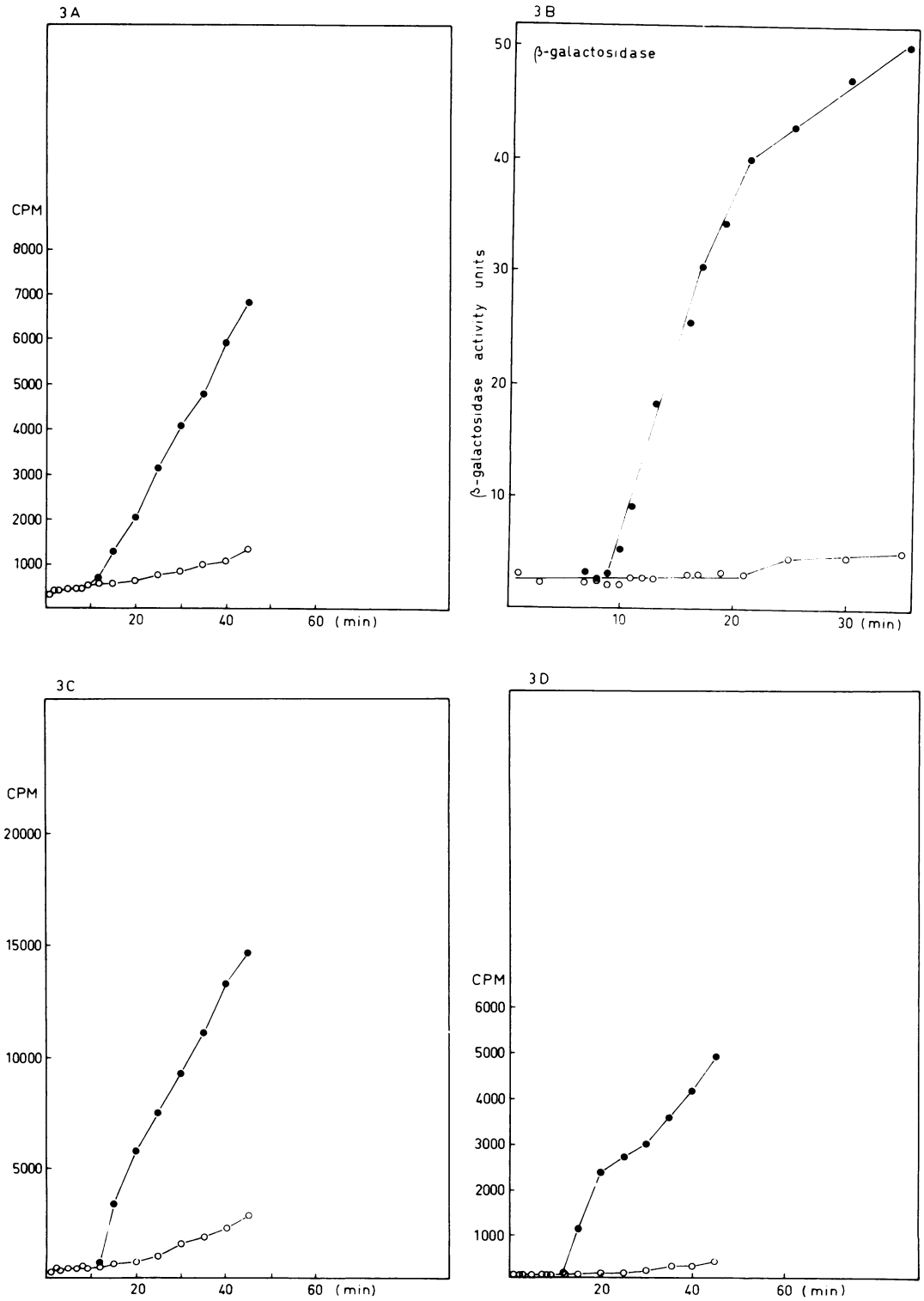

FIG. 3. *Efficiency of amino acid starvation in uninfected E. coli AA446. Cells were grown and leucine starvation was imposed as described in the legend to Fig. 2; then temperature was lowered to 27 C. (A) Protein synthesis: [^{14}C]histidine (0.2 μCi/ml and 2 μg/ml) at 0 min. Symbols:* (○) *resuspension in leucine-free medium −5 min;* (●) *addition of leucine +10 min. (B) Synthesis of β-galactosidase; resuspension in leucine-free medium at 0 min; induction by addition of isopropyl-β-D-galactopyranoside (to a final concentration of 0.5 × 10^{-3} M) +5 min. Symbols:* (○) *rifampin +14 min, +leucine +15 min;* (●) *addition of leucine +4 min, rifampin +14 min, +leucine +15 min. (C) Nucleic acid synthesis: [^{14}C]adenine (0.2 μCi/ml, 2 μg/ml) at 0 min. Symbols:* (○) *resuspension in leucine-free medium −5 min;* (●) *addition of leucine +10 min. (D) Nucleic acid synthesis: [^{14}C]uracil (0.2 μg/ml and 2 μCi/ml) at 0 min. Symbols:* (○) *resuspension in leucine-free medium −5 min;* (●) *addition of leucine +10 min.*

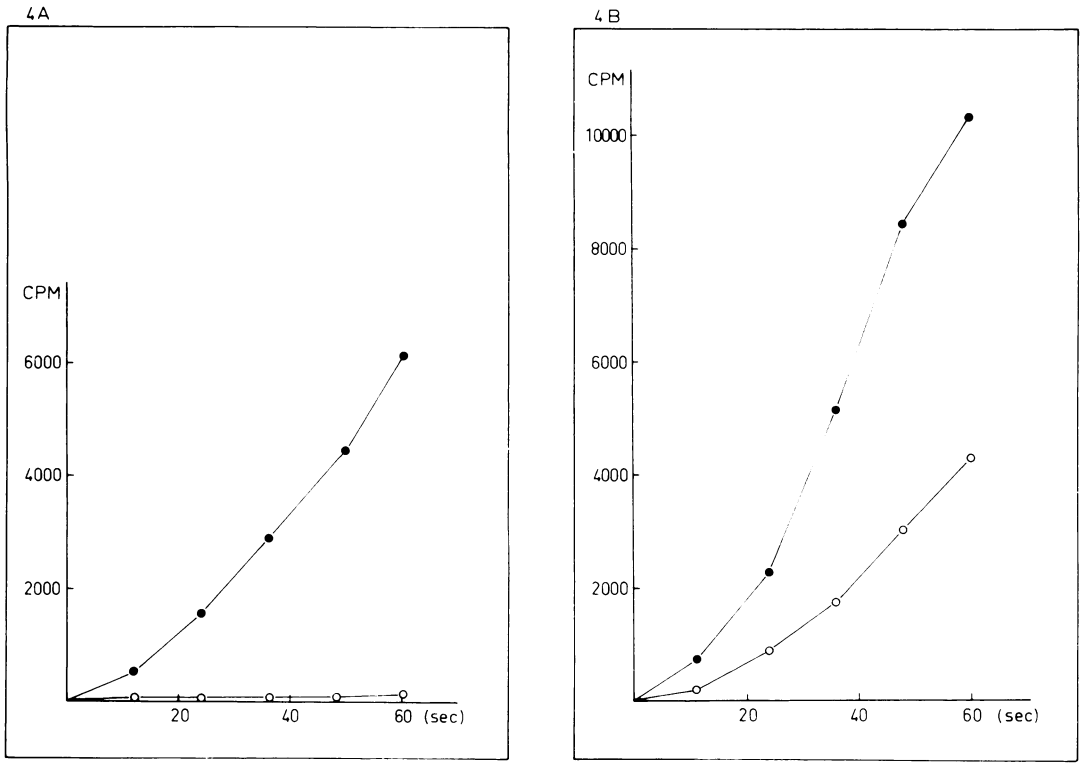

FIG. 4. *Incorporation into RNA of uracil and adenine in T4-infected E. coli B40. A culture of E. coli B40 (1.5 × 10⁸ cells/ml) was infected with T4am⁺ at 30 C. Six minutes after infection 5 ml was transferred to the bottom of a 250-ml Erlenmeyer flask on ice. The clock was stopped during the filtration procedure, and 3 ml was then filtered. After filtration the cells were transferred to a test tube and resuspended in fresh medium. The clock was started, and the culture was aerated for 5 min. Eleven minutes after infection (time zero) 500 μl was transferred to a test tube containing 10 μl of [³H]uracil (29 Ci/mmol) or [³H]adenine (18 Ci/mmol). Every 12 s 50-μl samples were withdrawn and treated as described in Materials and Methods. (A) Symbols: (●) incorporation of [³H]uracil, after filtration and resuspension in medium containing arginine; (○) incorporation of [³H]uracil, after filtration and resuspension in medium lacking arginine. (B) Symbols: (●) incorporation of [³H]adenine, after filtration and resuspension in medium containing arginine; (○) incorporation of [³H]adenine, after filtration and resuspension in medium lacking arginine.*

Restriction of uracil uptake concomitant with continued uptake of adenine has also been observed in stringent strains by Edlin and Neuhard (8). Hochstadt-Ozer and Cashel (12) showed that the concentration of ppGpp increases drastically upon amino acid starvation of a stringent strain and that the formation of UMP from uracil by membrane vesicles is inhibited by ppGpp. The synthesis of AMP from adenine is also affected, but the inhibition is milder.

That formation of early mRNA is not affected by amino acid starvation has also been demonstrated by Legault-Démare et al. (16) by use of hybridization techniques. By similar methods Olsnes and Hauge (24) found a four-fold reduction of the amount of mRNA under the same conditions. The lower amount of mRNA in their case is probably an underestimate due to degradation of unprotected mRNA during the extraction at 20 C.

The formation of late mRNA has been found to be independent of the *rel* gene by Donini and Edlin (5). Therefore, it now seems as if the synthesis of global mRNA after T4 infection is independent of the *rel* gene. Furthermore, this is consistent with the idea of noncoordinate control of mRNA synthesis in uninfected bacteria (15).

In contrast to dCMP HMase mRNA, mRNA that codes for dHMP kinase could not be detected in the presence of chloramphenicol or during leucine starvation, and consequently it belongs to the delayed early class.

There are at least two explanations for the fact that this class of mRNA requires protein synthesis for detection. (i) Initiation of delayed early mRNA requires protein synthesis. (ii) Delayed early mRNA, which probably is synthesized promoter distally according to Brody et

al. (2), is synthesized in the absence of protein synthesis but degraded before translation. Degradation of distal mRNA in the presence of chloramphenicol has been ascribed to endonuclease A in both uninfected (20, 21) and T4-infected cells (1), since a mutant *E. coli su*A, defective in this enzyme (14), allowed the whole polycistronic messenger to be conserved.

When protein synthesis is blocked by amino acid starvation of stringent cells, this enzyme seems to be inhibited, since both proximal and distal mRNA are conserved (22). Therefore, if delayed early mRNA is synthesized during amino acid starvation, it is pertinent to believe that it can be detected by our methods. Since we have not found any significant amounts of dHMP kinase messenger formed during leucine starvation, we suggest that delayed early mRNA has not been synthesized. This supports the idea that protein synthesis is required for the transition from immediate early to delayed early transcription, and thus explanation (i) (above) is favored.

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