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Regulation of *Escherichia coli* Aspartate Transcarbamylase Synthesis by Guanosine Tetraphosphate and Pyrimidine Ribonucleoside Triphosphates

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The effects of guanosine tetraphosphate (ppGpp) and pyrimidine ribonucleoside triphosphates on *Escherichia coli* aspartate transcarbamylase (ATCase) synthesis were examined. To determine the effect of ppGpp, a stringent (*relA*$^+$) and relaxed (*relA*) isogenic pair of *E. coli* K-12 strains was starved for isoleucine, and the residual rate of synthesis of this enzyme was measured. It was necessary to starve the strains for uracil before the isoleucine limitation to maintain similar, low levels of UTP, the putative pyrimidine effector of ATCase synthesis. The isoleucine starvation of the stringent strain caused an immediate 10-fold increase in the intracellular concentration of ppGpp, which was coincident with the cessation of the synthesis of the enzyme. The elevated level of ppGpp then decayed until it reached an intracellular concentration similar to that found in unstarved cells. Enzyme synthesis resumed at this time. In the relaxed strain, the intracellular concentration of ppGpp did not increase upon isoleucine starvation and synthesis of the enzyme was not repressed. These experiments strongly indicated that ppGpp acts as a negative effector of ATCase synthesis. The repression of ATCase synthesis by ppGpp was demonstrated directly by using a *Salmonella typhimurium* (*relA*) in vitro coupled transcription-translation system with a lambda specialized transducing phage carrying the *E. coli* K-12 operon encoding the subunits of this enzyme (*pyrBI*) as a source of DNA. This in vitro system was also used to measure the effects of UTP and CTP on ATCase synthesis. Increasing the concentration of UTP in the in vitro reaction mixture resulted in strong repression of this synthesis, whereas increasing the CTP concentration did not affect synthesis significantly. Possible mechanisms for the regulation of *pyr* gene expression, including attenuation control, are discussed.

The biosynthesis of UMP in *Escherichia coli* and *Salmonella typhimurium* is catalyzed by six enzymes encoded by six unlinked genes and operons (4, 26, 29). The expression of these genes and operons is noncoordinate and subject to complex regulation. From experiments with mutant strains that permit the independent manipulation of the uridine and cytidine nucleotide pools, the expression of *pyrBI* (designated *pyrB* in *S. typhimurium*), *pyrE*, and *pyrF* appears to be repressed by a uridine nucleotide, whereas *pyrC* and *pyrD* expression appears to be repressed primarily by a cytidine nucleotide (17, 30, 32). The expression of *carAB* (designated *pyrA* in *S. typhimurium*), which is essential for both pyrimidine and arginine biosynthesis, is subject to cumulative repression by a pyrimidine nucleotide and arginine (1, 30). The exact identities of the pyrimidine regulatory nucleotides are unknown, although they must be phosphorylated to at least the diphosphate level to cause repression (32). The regulatory mechanisms in which these nucleotides function are also unknown.

In this study, guanosine tetraphosphate (ppGpp) is identified as another small-molecule effector of *pyr* gene expression. ppGpp accumulates in *E. coli*, *S. typhimurium*, and other bacteria during amino acid limitation and apparently functions as a pleiotropic regulatory molecule that redirects metabolism in response to this physiological stress (7, 9, 34). High levels of ppGpp apparently repress the synthesis of stable RNA, ribosomal proteins, and other macromolecular components of the protein synthesizing machinery (25) while stimulating the expression of amino acid biosynthetic operons and protein degradation (34, 35). These and other apparently ppGpp-mediated responses that occur upon amino acid limitation are collectively called the stringent response (13). The synthesis of high

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levels of ppGpp requires the *relA* gene product, which is an enzyme associated with the ribosome and stimulated by the binding of an uncharged tRNA (14). Mutants deficient in this enzymatic activity are called relaxed, because they do not accumulate high levels of ppGpp upon amino acid limitation and do not exhibit the stringent response (7). Because the primary function of the pyrimidine biosynthetic enzymes is to provide precursors for stable RNA synthesis, it seemed a likely possibility that ppGpp might also serve as an effector of *pyr* gene expression. Experiments presented in this paper show that the high levels of ppGpp that accumulate in an amino acid-starved stringent (*relA*$^+$) strain of *E. coli* K-12 are apparently sufficient to completely repress the synthesis of the pyrimidine biosynthetic enzyme aspartate transcarbamylase (ATCase; EC 2.1.3.2), which is composed of catalytic and regulatory subunits encoded by the *pyrBI* operon (29). Repression of the synthesis of this enzyme by ppGpp is demonstrated directly with an *S. typhimurium* (*relA*) in vitro coupled transcription-translation system with a lambda specialized transducing phage containing the *E. coli* K-12 *pyrBI* operon as a source of DNA. This in vitro system also provided an opportunity to measure the effects of pyrimidine ribonucleoside triphosphates on ATCase synthesis under more defined conditions than previously possible. The data suggest that UTP is a potent negative effector of ATCase synthesis but that CTP does not effect this synthesis significantly. In light of these results, possible mechanisms for the regulation of *pyr* gene expression are discussed.

MATERIALS AND METHODS

Chemicals and other materials. Protosol, Omnifluor, and the radiochemicals L-[*U*-^{14}C]leucine (291 mCi/mmol), carrier-free $^{32}P_i$ in 0.02 N HCl, and L-[^{35}S]methionine (623 Ci/mmol) were obtained from New England Nuclear Corp. Pyruvate kinase was purchased from Boehringer Mannheim Corp. Polyethyleneimine cellulose plates (20 by 20 cm) were supplied by Brinkmann Instruments, Inc. (catalog no. PEI CEL 300). The plates were washed by ascending chromatography for 5 cm in 10% NaCl and then in deionized water. ppGpp was prepared as described (8). Bulk tRNA was isolated from wild-type *S. typhimurium* strain TA265 (6). All other chemicals were reagent grade and commercially available.

Bacterial strains. *E. coli* K-12 strains CP78-2 (*car-94 arg his leu thi*) and CP79-2 (*relA car-94 arg his leu thi*) are isogenic except for the *relA* locus. CP78-2 and CP79-2 were constructed by transducing strains CP78 (*arg his leu thi thr*) and CP79 (*relA arg his leu thi thr*), respectively, to *thr*$^+$ with bacteriophage P1 grown on strain AT2473 (*thi-1 relA1 car-94*). Strains CP78 and CP79 were provided by M. J. Fournier, and strain AT2473 was obtained from the Coli Genetic Stock Center (CGSC 4512). *E. coli* K-12 strain AD_{11}m5 [HfrH B_1 Δ(*proA/B-argF-lac*) Δ*argI* (λ*d valS argI pyrB b515 b519 S7* Δ(*att-cI*)) (λ *cI857 S7*)] carried the lambda phages used as sources of template DNA in in vitro reactions and was generously provided by Akihiko Kikuchi. The construction and characterization of the λ*d valS argI pyrB* specialized transducing phage (also designated yk14m5) has been previously described (19). This phage contains the entire *pyrBI* operon (19, 29). *S. typhimurium* strain TA705 [Δ *(hisOGDCBH)2253 hisT1504 relA1*] was used to prepare in vitro protein synthesizing extracts (S-30 extract).

Media and culture methods. The morpholinopropanesulfonic acid minimal medium described by Neidhardt (23) with 0.5 mM phosphate and 0.4% glucose was used to grow *E. coli* K-12 strains CP78-2 and CP79-2. Required amino acids were added at 100 μg/ml, and thiamine was present at 5 μg/ml. Uracil concentrations were as indicated below. Cultures were grown with shaking at 37°C. Other media and culture methods used were as desribed in the indicated references.

Preparation of cell extracts for ATCase assays. Forty-milliliter samples were withdrawn from 1-liter cultures at indicated times, and extracts were prepared as previously described (11).

ATCase assay. Enzymatic activity was measured as previously described (11).

Protein determination. Protein samples were precipitated in 10% trichloroacetic acid and assayed by the method of Lowry et al. (22), using crystalline bovine serum albumin as the standard.

Incorporation of [^{14}C]leucine into protein. Where indicated in the text, [^{14}C]leucine was added to 25-ml cultures to a final concentration of 1 μCi/ml. One-milliliter samples of labeled culture were taken at indicated times and added to tubes containing 0.2 ml of ice-cold 50% trichloroacetic acid. After at least 15 min on ice, the samples were heated for 10 min at 90°C to discharge aminoacyl-tRNAs. The samples were cooled on ice, and denatured protein was collected by filtration on 0.45-μm membrane filters (25 mm; Millipore Corp.). The filters were washed three times with 5 ml of ice-cold 10% trichloroacetic acid and three times with 3 ml of ice-cold 70% ethanol. The filters were dried and counted in 10 ml of PPO-POPOP-toluene scintillation fluid (4.2 g of 2,5-diphenyloxazole [PPO] and 0.063 g of 1,4-bis(5-phenyloxazolyl)benzene [POPOP] per liter of toluene). A 0-min sample was used as a blank to correct for entrapment of [^{14}C]leucine.

Nucleotide pools. Nucleotide pools were determined after growing 10-ml cultures for at least two generations in the presence of $^{32}P_i$ (specific activity in the medium, 40 μCi/μmol). At indicated times, 100-μl samples of labeled culture were added to 10 μl of ice-cold 11 N formic acid, mixed, and kept on ice for 30 min. Samples were either used immediately or stored at −20°C for up to several days without detectable hydrolysis of nucleotides. Insoluble material was removed by centrifugation for 4 min at 4°C in a Beckman microfuge. Twenty microliters of 0.4 M EDTA (pH 6.5) and 80 μl of a solution containing 1.25 mM each of the eight ribo- and deoxyribonucleoside triphosphates (pH 5) were added to 100 μl of the supernatant. A 50- or 100-μl portion was spotted on washed polyethyleneimine cellulose plates and chromatographed as previ-

ously described (24). Radioactive spots corresponding to ^{32}P-labeled ribonucleoside triphosphates and ppGpp were located by autoradiography, cut out, and counted in PPO-POPOP-toluene.

Template DNA. After heat induction of the *E. coli* double lysogen $AD_{11}m5$, the lambda phages were purified by CsCl density gradient centrifugation (18). Phage DNA was extracted as previously described (40). The bacterial genes contained in λ*d valS argI pyrB* (yk14m5) DNA originated from *E. coli* K-12 (19).

Conditions for in vitro protein synthesis. Reaction conditions and components were similar to those previously described (3, 31, 34). Each reaction mixture (50 μl) contained: Tris-acetate, 50 mM (pH 8.0); potassium acetate, 58 mM; ammonium acetate, 27 mM; magnesium acetate, 10 mM; calcium acetate, 4 mM; dithiothreitol, 1.3 mM; folinate, 20 μg/ml; *S. typhimurium* bulk tRNA, 0.3 mg/ml; 19 amino acids (minus methionine), 0.2 mM each; [^{35}S]methionine, 0.025 mM (18 μCi); ATP, 2 mM; GTP, CTP, and UTP, 0.5 mM unless indicated differently; phosphoenolpyruvate, 20 mM; *p*-toluenesulfonyl fluoride, 30 μg/ml; polyethylene glycol 6000, 35 mg/ml; template DNA, 0.2 mg/ml; and S-30 extract, 3.5 mg of protein/ml. Pyruvate kinase was included in the S-30 extract and is essential for the regeneration of nucleoside triphosphates during the in vitro reaction (concentration in the reaction mixture was 0.65 μg/ml). Reactions were initiated by the addition of S-30 extract, and the reaction mixtures were incubated at 37°C with rapid shaking for 70 min. Reactions were stopped by the addition of 50 μl of 2× Laemmli sample buffer (20) and placed in a boiling water bath for 2 min.

Quantitation of individual proteins synthesized in vitro. Proteins synthesized in vitro were precipitated with 1 ml of acetone for at least 15 min. The samples were centrifuged and the pellets rinsed with 1 ml of 90% acetone. The pellets were dissolved in 200 μl of Laemmli sample buffer with heating. Samples (50 μl) were run on a 12% sodium dodecyl sulfate-polyacrylamide slab gel (2) to separate [^{35}S]methionine-labeled proteins. The gel was stained with Coomassie blue and dried. Labeled proteins were located by autoradiography, and then each sample lane was cut transversely into 1-mm slices. The radioactivity in each slice was measured by dissolving the gel in 10 ml of 3% Protosol and 0.4% Omnifluor in toluene and counting in a scintillation spectrometer.

HPLC. The concentrations of ribonucleoside mono-, di-, and triphosphates in in vitro reaction mixtures were measured by high-performance liquid chromatography (HPLC). To a 50-μl in vitro reaction mixture was added 50 μl of 2 N formic acid. After 30 min on ice, precipitated material was removed by centrifugation, and the pH of the supernatant was adjusted to 5.5 with 1 N NaOH. Nucleotides in a 20-μl sample of this supernatant were separated on a Partisil SAX anion exchange column (4.6 by 250 mm) purchased from Altex. A Waters HPLC system was used that consisted of two M6000A solvent delivery systems, a model 660 solvent programmer, a U6K injector, and a model 440 dual-wavelength absorbance detector. Nucleotides were eluted with a 45-ml, nonlinear 10 mM KH_2PO_4 (pH 5.5) to 600 mM KH_2PO_4 (pH 5.5) gradient (gradient no. 7 on programmer) with a flow rate of 1 ml/min. Nucleotide standards were used to calibrate the system.

RESULTS

Effect of ppGpp on ATCase synthesis in vivo. Initial experiments designed to measure the effect of ppGpp on ATCase synthesis involved starving stringent (*relA*$^+$) and relaxed (*relA*) *E. coli* K-12 cultures for isoleucine and then measuring the differential rate of residual synthesis of this enzyme (data not shown). Isoleucine starvation was induced by the addition of 400 μg of valine per ml of culture medium (12), which permits protein synthesis to continue at approximately 10% of the unstarved rate in both strains. Although these experiments demonstrated that ATCase synthesis was preferentially inhibited in *relA*$^+$ cells starved for isoleucine, the interpretation of the results was complicated because the intracellular concentration of UTP, a putative negative effector of the synthesis of this enzyme (17, 32), changed in parallel with that of ppGpp (5).

To assess the effect of ppGpp on ATCase synthesis by using *relA*$^+$ and *relA* strains, it was necessary to select physiological conditions that maintained similar intracellular concentrations of UTP in both strains during amino acid starvation. The conditions chosen were to sequentially starve pyrimidine-auxotrophic *relA*$^+$ and *relA* strains, first for uracil and then for isoleucine by valine addition. Figure 1 shows the changes in nucleotide pools in sequentially starved CP78-2 (*relA*$^+$ *car-94*) and CP79-2 (*relA car-94*) cells. Uracil starvation was imposed by allowing the cultures to exhaust a growth-limiting supplement of uracil. Uracil starvation caused the UTP pools in both strains to decrease from an estimated 1 mM to between 0.05 and 0.1 mM. These low levels of UTP are sufficient to derepress ATCase synthesis nearly 100-fold (data not shown). Approximately 20 min after the depletion of the uracil supplement, valine was added to the cultures. The UTP levels remained low in both strains. In the *relA*$^+$ cells, there was an immediate 10-fold increase in the intracellular concentration of ppGpp. This concentration was estimated to be approximately 0.6 mM. The ppGpp pool then decreased slowly for several hours until it reached levels found in unstarved cells. In the *relA* cells, the ppGpp level did not increase after valine addition, but actually decreased slightly. The changes in the CTP, GTP, and ATP levels in both strains were qualitatively similar and did not correlate with changes in ATCase synthesis (see below).

Figure 2 shows the effect of valine addition on ATCase synthesis in uracil-starved CP78-2 and CP79-2. The cells were grown and starved as described in the legend to Fig. 1. In the *relA*$^+$ cells, derepressed enzyme synthesis was inhibited immediately after valine addition and the appearance of high levels of ppGpp (Fig. 1). This

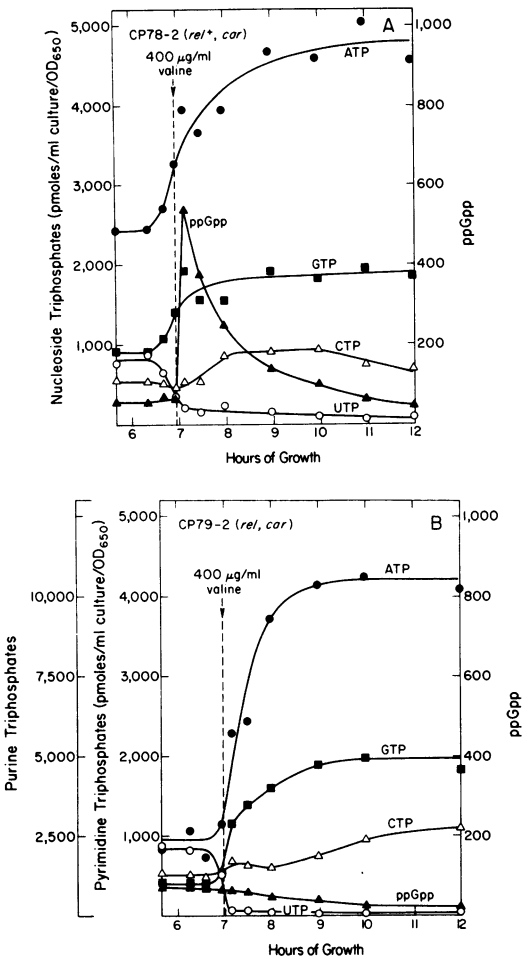

FIG. 1. Changes in nucleotide pools in CP78-2 (*relA*$^+$ *car-94*) and CP79-2 (*relA car-94*) sequentially starved for uracil and isoleucine. Cultures of CP78-2 (A) and CP79-2 (B) were grown in minimal medium supplemented with 5 μg of uracil per ml. This supplement was exhausted after approximately 6.5 h of growth (optical density at 650 nm [OD_{650}] ≃ 0.5). Approximately 20 min after the depletion of uracil, valine (400 μg/ml) was added (arrow) to induce isoleucine starvation. Samples taken during growth and starvation were assayed for acid-soluble nucleotides. Symbols: ●, ATP; ■, GTP; △, CTP; ○, UTP; and ▲, ppGpp.

inhibition lasted 3 h, after which time enzyme synthesis resumed. This resumption of enzyme synthesis was confirmed in several similar experiments and always was coincident with the return of ppGpp levels to those found in unstarved cells (Fig. 1). In the *relA* cells, the addition of valine did not inhibit the synthesis of ATCase, which parallels that of total protein (see below). The possibility that ppGpp or another factor present in extracts of *relA*$^+$ cells was inhibiting ATCase activity during enzyme assays (Fig. 2) was eliminated by mixing extracts of *relA*$^+$ and *relA* cells and demonstrating additive enzymatic activity.

To demonstrate that the differential response of ATCase synthesis in CP78-2 and CP79-2 (Fig. 2) was not due to differences in the rate of total protein synthesis, this rate was measured in cells grown under conditions identical to those above (Fig. 3). Total protein synthesis was similar in both strains for at least 1 h after valine addition. Protein synthesis in the *relA*$^+$ cells proceeded at approximately the same rate for 5 h after valine addition. The resumption of ATCase synthesis in *relA*$^+$ cells (Fig. 2) was not accompanied by an increase in total protein synthesis. In the *relA* cells, total protein synthesis stopped approximately 2 h after valine addition. This may have been due to the accumulation of aberrant proteins that are synthesized in amino acid-starved *relA* cells (28).

In the experiment shown in Fig. 1A, the elevated level of ppGpp induced by valine addition decayed at a rate much faster than that in

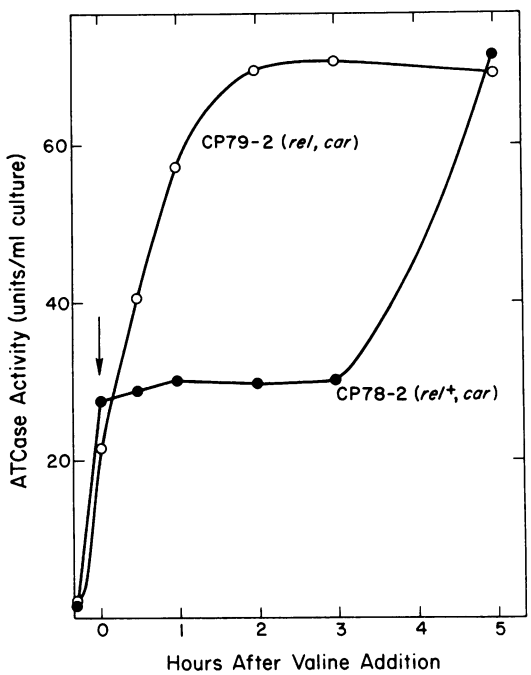

FIG. 2. Effect of valine addition on ATCase synthesis in CP78-2 (*relA*$^+$ *car-94*) and CP79-2 (*relA car-94*) previously starved for uracil. Cultures of CP78-2 (●) and CP79-2 (○) were grown and starved as described in the legend to Fig. 1. At approximately 20 min before valine addition at an optical density at 650 nm of ≃0.5, both cultures had exhausted the uracil supplement and began to derepress ATCase synthesis. Samples were taken as indicated and assayed for ATCase activity.

isoleucine-starved cells not subjected to a previous uracil starvation (data not shown). This more rapid decay could be due to a gradual reduction in the rate of total mRNA synthesis, similar to that observed in cells starved only for uracil (21). Because the availability of mRNA is essential for the maintenance of high levels of ppGpp (14), a reduction in the rate of mRNA synthesis should result in reduced ppGpp levels. Eventually, the availability of mRNA could become limiting for protein synthesis, which would permit the level of charged isoleucyl-tRNA to increase. The resumption of ATCase synthesis that occurs in *relA*$^+$ cells 3 h after valine addition (Fig. 2) cannot be due to such an increase. Sufficient charged isoleucyl-tRNA is available immediately after valine addition to support total protein synthesis at a rate of 10% of that in unstarved cells (data not shown). This rate of total protein synthesis is sufficient to support derepressed ATCase synthesis as shown in *relA* cells (Fig. 2 and 3). It is conceivable that in *relA*$^+$ cells, only proteins containing few or no isoleucine residues are synthesized during the first 3 h after valine addition. This is clearly not the case because protein synthesis during this time has been shown to include essentially all of the proteins that were synthesized before the isoleucine limitation (28).

Effect of ppGpp on ATCase synthesis in vitro. The apparent repression of ATCase synthesis by ppGpp observed in *relA*$^+$ cells was examined directly by using an in vitro DNA-dependent coupled transcription-translation system. This system consisted of an S-30 extract prepared from an *S. typhimurium relA* mutant and defective in in vitro ppGpp synthesis (34), DNA from a lambda specialized transducing phage carrying the *E. coli pyrBI* operon, and other components necessary for transcription and translation. The concentrations of ppGpp and UTP added to the reaction mixtures were chosen to simulate the conditions in starved *relA*$^+$ cells (Fig. 1A). The effect of ppGpp on the synthesis of the ATCase catalytic subunit, which is encoded by the first structural gene (*pyrB*) of the *pyrBI* operon (29), is shown in Fig. 4. Synthesis was severely repressed by ppGpp, with 15-fold repression at 1 mM ppGpp. The regulatory subunit of ATCase (molecular weight, 17,000), which is encoded by *pyrI* (29), was also synthesized in these experiments. The regulatory subunit band was obscured in one-dimensional gels by similarly sized lambda proteins and was detected on two-dimensional O'Farrell gels (27). Comparison of the levels of the regulatory subunit at 0 and 0.5 mM ppGpp indicated that the synthesis of this subunit was also repressed by ppGpp (data not shown). Attempts to uncouple in vitro transcription and translation (34) were unsuccessful because of extremely low levels of ATCase synthesis.

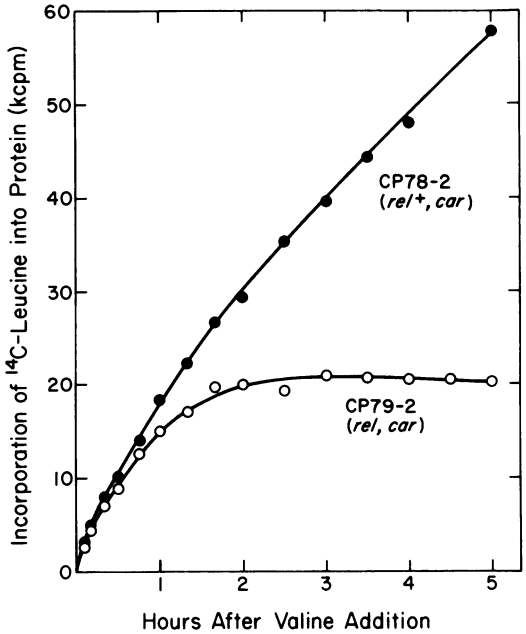

FIG. 3. Effect of valine addition on protein synthesis in CP78-2 (*relA*$^+$ *car-94*) and CP79-2 (*relA car-94*) previously starved for uracil. Cultures of CP78-2 (●) and CP79-2 (○) were grown and starved as described in the legend to Fig. 1. At the time of valine addition, [^{14}C]leucine was added to the culture. The incorporation of [^{14}C]leucine into protein was measured as described in the text. Similar rates of protein synthesis were obtained when protein concentrations were determined colorimetrically (22).

The effect of ppGpp on the synthesis of proteins in vitro was highly specific. The lambda phage used as a DNA template carried the *E. coli* genes *valS* and *argI* in addition to *pyrBI*. The gene products of *valS* and *argI* are valyl-tRNA synthetase and ornithine transcarbamylase, respectively. The synthesis of valyl-tRNA synthetase was repressed by ppGpp, but substantially less than that observed for ATCase synthesis (Fig. 4). At 1 mM ppGpp, the synthesis of valyl-tRNA synthetase was repressed approximately threefold. The synthesis of ornithine transcarbamylase was stimulated by ppGpp (Fig. 4). There was substantial stimulation even at low concentrations of ppGpp, and at 1 mM ppGpp the stimulation was approximately sevenfold. The synthesis of lambda DNA-encoded proteins, the lower-molecular-weight bands in Fig. 4A, appeared to be uniformly stimulated by ppGpp. This stimulation was estimated from densitometer tracings of the autoradiogram in Fig. 4A to be approximately twofold at 1 mM ppGpp for the major lambda proteins.

Effect of UTP and CTP on ATCase synthesis in

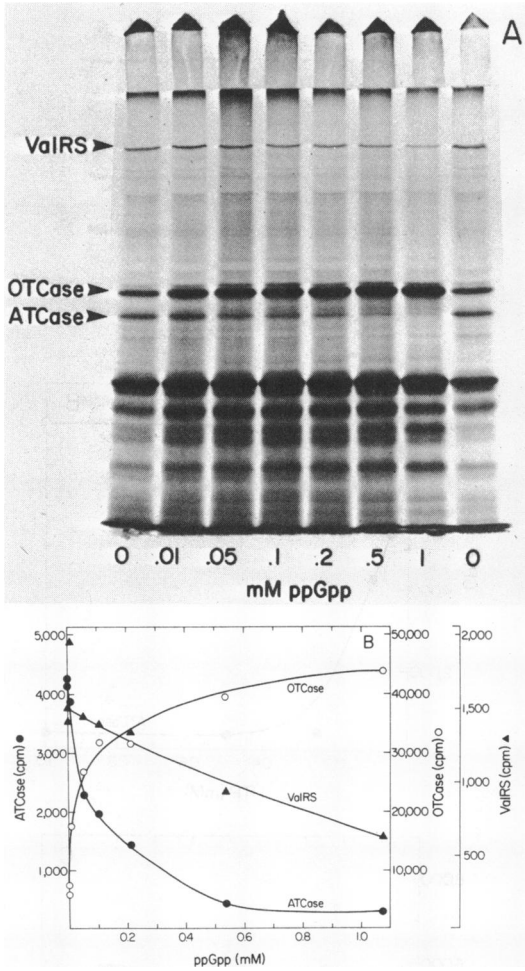

FIG. 4. Effect of ppGpp on the synthesis of ATCase and other proteins in vitro. (A) Autoradiogram of a sodium dodecyl sulfate-polyacrylamide gel used to separate [^{35}S]methionine-labeled proteins synthesized in vitro in the presence of various concentrations of ppGpp. The UTP concentration was 0.1 mM and the DNA template was λ*d valS argI pyrB* (yk14m5). The bands corresponding to the ATCase catalytic subunit (molecular weight, 33,000) and ornithine transcarbamylase (OTCase; molecular weight, 35,000) were identified by coelectrophoresis with purified proteins (provided by Ying Yang.) The valyl-tRNA synthetase band (ValRS; molecular weight, 110,000) was located by using molecular weight standards. None of these bands were present when in vitro protein synthesis was directed by λ *c*I857 S7 DNA (data not shown). (B) The level of each labeled *E. coli* protein was measured by counting the radioactivity in the appropriate band in the gel as described in the text. Background counts were determined from slices of the gel adjacent to each protein.

vitro. Previous in vivo studies indicated that ATCase synthesis was repressed by a uridine nucleotide, probably UTP or a metabolically related nucleotide (17, 30, 32). The *pyrBI*-directed in vitro system provided an opportunity to measure the effect of UTP on the synthesis of this enzyme under more defined conditions. As shown in Fig. 5A and B, increasing the concentration of UTP in the in vitro reaction mixture resulted in strong repression of the synthesis of the ATCase catalytic subunit (regulatory subunit synthesis was not measured). Maximum repression ranged from 20- to 55-fold in several experiments. The greatest repression was seen in experiments with the lowest background counts, which permitted more accurate measurement of low levels of the catalytic subunit. This repression was highly specific. Increasing the UTP concentration stimulated the synthesis of all other proteins (Fig. 5A). The synthesis of ornithine transcarbamylase and lambda proteins was stimulated up to 40% (Fig. 5B). The synthesis of valyl-tRNA synthetase was stimulated threefold. This increased stimulation may reflect increased sensitivity to premature transcription termination at low UTP concentrations of the long mRNA requried for the synthesis of valyl-tRNA synthetase. When no UTP was added to the in vitro reaction mixture, transcription was supported by uridine nucleotides presumably present in the S-30 extract, which could be rapidly phosphorylated to UTP by the pyruvate kinase-phosphoenolpyruvate nucleoside triphosphate-regenerating system included in the reaction mixture. The actual concentration of UTP in the in vitro reaction mixture under these conditions was approximately 0.05 mM as measured by HPLC (see above).

The effect of CTP on ATCase synthesis in vitro was also measured. As shown in Fig. 5A and C, increasing the concentration of CTP resulted in only slight, if any, repression of catalytic subunit synthesis. This is consistent with previous results from in vivo studies (17, 30, 32). Increasing the concentration of CTP in the in vitro reaction mixtures caused a slight stimulation (0 to 25%) of the synthesis of ornithine transcarbamylase and lambda proteins and a twofold increase in the synthesis of valyl-tRNA synthetase (Fig. 5A and C). When no CTP was added to the reaction mixture, the actual CTP concentration was estimated to be approximately 0.05 mM due to endogenous cytidine nucleotides.

In the in vitro experiments described above, the concentrations of nucleoside mono-, di-, and triphosphates in the reaction mixtures were monitored at various times by HPLC. Nucleoside mono- and diphosphates present in S-30 extracts and nucleoside triphosphate solutions were nearly quantitatively phosphorylated to nucleoside triphosphates within the first minutes after initiating the reactions. The nucleoside triphosphate levels were then maintained nearly

constant for at least 30 min, at which time the bulk of transcription has been completed (34). At 60 min, the triphosphate levels had decayed to 30 to 70% of their maximum levels, with UTP levels being the most stable. At this time, considerable accumulation of nucleoside mono- and diphosphates had occurred. It is suggested from these measurements that it was indeed UTP or CTP that primarily affected ATCase synthesis in the experiments where the levels of these nucleotides varied.

DISCUSSION

The results presented in this study indicate that ppGpp acts as a negative effector of ATCase synthesis in *E. coli*. By using amino acid-starved stringent (*relA*$^+$) and relaxed (*relA*) strains a strong inverse correlation between the rate of synthesis of this enzyme and the intracellular concentration of ppGpp was shown. In these experiments, changes in the rate of total protein synthesis or in the intracellular concentration of UTP could not account for the observed changes in the rate of enzyme synthesis. These changes were unrelated to changes in the intracellular concentration of ATP or GTP, which apparently have some affect on *pyr* gene expression (15).

Repression of ATCase synthesis by ppGpp was demonstrated in vitro by using an *S. typhimurium* (*relA*) coupled transcription-translation system with template DNA from a lambda transducing phage containing the *E. coli pyrBI* operon. The *S. typhimurium* (*relA*) system was used because it appears to be much more sensitive to added ppGpp than in vitro systems with S-30 extracts prepared from relaxed strains of *E. coli* (31). Because the regulation of *pyrBI* expression is very similar, if not identical, in *E. coli* and *S. typhimurium* (30, 32), it is unlikely that nonphysiological responses would result from the use of this heterologous system. The effect of added ppGpp on in vitro protein synthesis in this system is highly specific and reflects the physiological role of this pleiotropic regulatory molecule. The synthesis of valyl-tRNA synthetase, a component of the protein synthesizing machinery, is repressed, whereas the synthesis of the arginine biosynthetic enzyme ornithine transcarbamylase is stimulated. Also, the effects of UTP and CTP on ATCase synthesis in vitro are consistent with results from in vivo studies (30, 32).

The regulation of ATCase synthesis in vitro caused by varying the concentration of UTP indicates that this nucleotide is the pyrimidine regulatory effector of *pyrBI* expression in vivo. The UTP concentrations used in vitro were similar to those found in *E. coli* and *S. typhimurium* cells grown under conditions that repress or derepress ATCase synthesis. The highest UTP

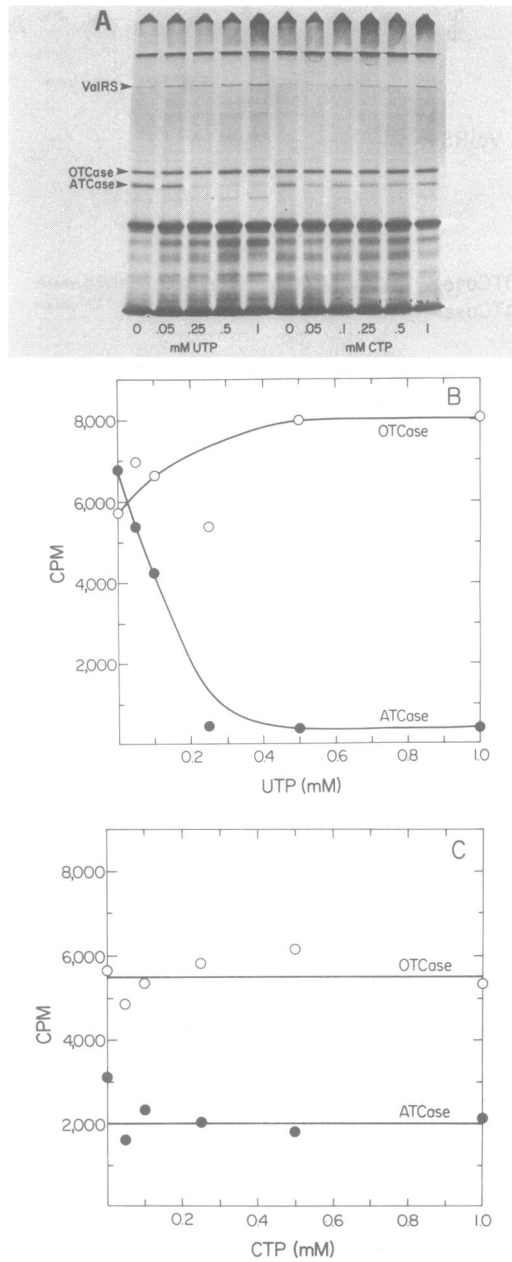

FIG. 5. Effect of various UTP or CTP concentrations on the synthesis of ATCase and other proteins in vitro. (A) Autoradiogram of a sodium dodecyl sulfate-polyacrylamide gel used to separate [^{35}S]methionine-labeled proteins synthesized in vitro with UTP or CTP concentrations varied as indicated. Protein bands were identified as in the legend to Fig. 4. When the concentration of UTP was varied, the CTP concentration was 0.5 mM; when CTP was varied, UTP was 0.1 mM. The DNA template was λ*d valS argI pyrB* (yk14m5). (B) and (C) The levels of the ATCase catalytic subunit and ornithine transcarbamylase (OTCase) were measured as described in the text and plotted as a function of the UTP or CTP concentration.

concentration used was 1 mM, which is approximately equal to the concentration found in cells grown in the presence of uracil. At 0.05 mM UTP, the lowest concentration used in vitro, enzyme synthesis was maximally derepressed 55-fold. This was comparable to the 100-fold derepression observed in uracil-starved cells in which the intracellular UTP concentration was approximately 0.05 mM. A striking similarity between the regulation of ATCase synthesis in vivo and in vitro is that substantial derepression occurs only when the UTP concentration is below an apparent threshold of approximately 0.2 mM (Fig. 5B and reference 32).

What is the mechanism by which changes in the levels of UTP regulate *pyrBI* expression? A number of recent experiments indicate that the expression of *pyrBI* and probably other *pyr* genes is regulated by a mechanism similar in part to the coupled transcription-translation control mechanism of the histidine (16) and tryptophan (39) operons. In a *hisT* strain of *S. typhimurium*, in which the rate of translational elongation is slowed (D. Palmer and S. Artz, unpublished data), *pyrA* and *pyrB* expression is preferentially reduced. This repression is reversed by the addition of DNA-binding agents (e.g., proflavin, norharman, and netropsin), which presumably inhibit the rate of transcriptional elongation (R. J. Neill, Ph.D. thesis, University of California, Berkeley, 1979). In a *hisT*$^+$ strain of *S. typhimurium*, sublethal concentrations of streptolydigin, which is an inhibitor of transcriptional elongation (33, 38), preferentially stimulate *pyrB* expression (C. L. Turnbough, Jr., unpublished data). These results suggest that the expression of *pyrA* and *pyrB* is regulated by the relative rates of transcription and translation presumably of a leader DNA sequence immediately preceding the structural genes.

The promoter-regulatory region of the *E. coli* K-12 *pyrBI* operon has been sequenced, and DNA fragments containing this region have been transcribed in vitro (C. L. Turnbough, Jr., K. Hicks, and J. Donahue, Proc. Natl. Acad. Sci. U.S.A., in press). These experiments have identified a rho-independent termination site (10) 23 base pairs before the start of the *pyrB* structural gene. Transcription initiated at the *pyrBI* promoter or promoters (there appear to be two in vitro) is efficiently terminated at this site. Without termination, the leader transcript(s) can encode a 44-amino acid polypeptide which terminates three nucleotides before the start of the ATCase catalytic subunit. Within the polypeptide-encoding sequence of the leader transcript is a uridine-rich region that appears to be a uniquely strong pause site for RNA polymerase when low UTP concentrations limit the rate of transcription. This pause site is approximately 20 base pairs before the rho-independent terminator. These observations suggest the following mechanism for regulating *pyrBI* expression. Transcription is initiated at the *pyrBI* promoter(s). When UTP is scarce, the rate of transcription through the uridine-rich region of the leader transcript is slow enough to allow a ribosome to bind near the 5′ end of the transcript at the initiation codon for the leader polypeptide and translate up to the paused or slowly-transcribing RNA polymerase. When the rho-independent terminator is eventually transcribed, the formation of the terminator-encoded GC-rich hairpin necessary for termination is precluded or immediately disrupted by the adjacent translating ribosome. An adjacent translating ribosome can apparently disrupt the GC-rich hairpin necessary for transcriptional termination in the leader region of the histidine operon (16). The disruption of termination of the *pyrBI* leader transcript allows RNA polymerase to continue transcription into the structural genes. When UTP is abundant, RNA polymerase does not pause during the transcription of the leader region. This does not provide enough time for a ribosome to bind to the transcript and catch up to RNA polymerase before the formation of the GC-rich termination hairpin. In this case, transcription is terminated before the *pyr*B structural gene.

The expression of other *pyr* genes may also be regulated by such a coupled transcription-translation control mechanism. For the expression of *pyrE* and *pyrF*, which like *pyrBI* expression is regulated by a uridine nucleotide, the control mechanism could be entirely analogous to that described for *pyrBI*. For the expression of *carAB* (*pyrA*), *pyrC*, and *pyrD*, which is apparently sensitive to the levels of a cytidine nucleotide, the only required change in the mechanism would be that the pausing of RNA polymerase would be sensitive to the availability of CTP. Presumably, this sensitivity would be due to transcription of a cytidine rich-sequence in the leader transcript. In addition to a uridine- or cytidine-rich sequence, it is likely that other features in the leader transcript are required for RNA polymerase pausing.

ppGpp could cause repression of *pyr* gene expression by interacting with the proposed coupled transcription-translation regulatory mechanisms. It has been shown that ppGpp can inhibit the rate of translation (13, 28), which if it occurred preferentially would have a negative effect on *pyr* gene expression. ppGpp may also cause repression by an independent mechanism, such as the inhibition of transcriptional initiation at the *pyr* promoters (36). For *pyrBI*, preliminary experiments in vitro indicate that ppGpp preferentially inhibits the initiation of transcription at one of the two possible promoters (J. Donahue,

unpublished data). The DNA sequence of this promoter between the Pribnow box and the start of transcription is similar to the consensus sequence found in bacterial promoters subject to stringent control (37).

The regulation of *pyr* gene expression by ppGpp and pyrimidine ribonucleoside triphosphates described in this paper should provide the cell with substantial flexibility and sensitivity for the regulation of the pyrimidine biosynthetic pathway. The activity of the pathway can be adjusted with respect to the levels of its end products and also to the physiological state of the cell.

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