JOURNAL OF BACTERIOLOGY, Jan. 1980, p. 58-66
0021-9193/80/01-0058/09$02.00/0

Vol. 141, No. 1

In Vitro Synthesis of *Escherichia coli* Carbamoylphosphate Synthase: Evidence for Participation of the Arginine Repressor in Cumulative Repression

WILLY LISSENS,[1,3] RAYMOND CUNIN,[1,3] NORMAN KELKER,[4] NICOLAS GLANSDORFF,[1,3] AND ANDRÉ PIÉRARD[2,3]*

Erfelijkheidsleer en Mikrobiologie, Vrije Universiteit Brussel,[1] Laboratoire de Microbiologie, Faculté des Sciences, Université Libre de Bruxelles,[2] and Institut de Recherches du Centre d'Enseignement et de Recherches des Industries Alimentaires et Chimiques,[3] B-1070 Brussels, Belgium; and Department of Microbiology,[4] New York University School of Medicine, New York, New York 10016

A deoxyribonucleic acid-directed in vitro system for the synthesis of *Escherichia coli* carbamoylphosphate synthase has been developed, and its properties have been studied. The system uses the deoxyribonucleic acid of a lambda phage carrying the *car* genes (λd*carAB*) as template and mediates the synthesis of both subunits of the enzyme. This newly synthesized enzyme exhibits the properties of native carbamoylphosphate synthase. A study of the in vitro synthetic capacities of S-30 extracts from strains containing either a mutated or the wild-type allele of gene *argR* supports earlier suggestions, based on in vivo evidence, that the *argR* product is involved in cumulative repression of carbamoylphosphate synthase by arginine and the pyrimidines. Repression in vitro is as efficient as in vivo. In keeping with such observation it is shown that in vitro synthesis of carbamoylphosphate synthase is repressed by partially purified arginine repressor. Evidence was obtained which indicates that arginine repression of carbamoylphosphate synthase mainly operates at the level of transcription. This was based on the design of an in vitro transcription system for gene *carA*, the structural gene for the light subunit of carbamoylphosphate synthase. This system also allowed us to demonstrate that free arginine is the corepressor involved in carbamoylphosphate synthase repression. The present in vitro approaches, in addition to the information they have already provided, open new possibilities for further investigations on the mechanism of cumulative repression and, in particular, on the participation of pyrimidine end products in this regulatory mechanism.

The glutamine-dependent carbamoylphosphate synthase (EC 2.7.2.9) of *Escherichia coli* provides carbamoylphosphate for the arginine and pyrimidine biosynthetic pathways (26, 29). It consists of two different subunits coded by adjacent genes, *carA* and *carB* (21, 33). The light subunit (molecular weight 42,000), product of *carA*, bears the binding site for glutamine (physiological nitrogen donor). The heavy subunit (molecular weight 130,000), product of *carB*, catalyzes the formation of carbamoylphosphate from ammonia and carries the effector binding sites. It is responsible for the modulation of carbamoylphosphate synthase activity by the antagonistic effects of ornithine and by pyrimidine and purine nucleotides (3, 24).

The synthesis of carbamoylphosphate synthase is regulated through cumulative repression: partial repression occurs in the presence of arginine or a pyrimidine, and maximal repression (of the order of 95%) occurs in the presence of both end products (26, 29). The synthesis of the two subunits is coordinated under most conditions investigated (25). In keeping with this observation, genes *carA* and *carB* were shown to constitute an operon which is transcribed from *carA* to *carB* (7, 13; Gigot et al., manuscript in preparation; Crabeel et al., manuscript in preparation).

The *argR* mutations, which affect the repression of all eight arginine enzymes, impair the repressibility of carbamoylphosphate synthase as well (27). In addition, in strains carrying multiple copies of the *car* region, "escape" enzyme synthesis occurs in both the pyrimidine and the arginine pathways (14). These findings are taken as evidence that cumulative repression is mediated by the products of two regulatory genes respectively involved in the regulation of the two pathways. Progress in determining the mechanism of cumulative repression has been restrained by the absence of *car* operator mu-

tations (27) and by the poor understanding of the mechanism of enzyme repression in the pyrimidine pathway. Regulatory mutations that clearly affect a pyrimidine repressor are lacking, and difficulties have been met in the identification of the corepressor (25, 27). A similar situation exists in *Salmonella typhimurium*, which resembles *E. coli* with regard to the control of carbamoylphosphate synthesis (1, 2, 16, 18, 23, 31, 35).

With the aim of clarifying the mechanism of cumulative repression of carbamoylphosphate synthase, we have undertaken to develop a DNA-dependent in vitro system for the synthesis of the *E. coli* synthase. In this work, we describe the properties of this system and show how it can be used to demonstrate that the product of gene *argR* is directly involved in the in vitro repression of carbamoylphosphate synthase. The data presented in this paper have been the subject of preliminary reports (15, 20).

MATERIALS AND METHODS

Bacterial strains. The strains used for the preparation of S-30 extracts are derivatives of strain CSH73 obtained from Cold Spring Harbor Laboratory. Their derivation is indicated in Table 1.

Preparation of S-30 extracts. The S-30 extracts from strain CSH73 *carB8*, CSH73 *carA50*, and CSH73 *carB8 argR* were prepared according to Zubay et al. (37) as described by Miller (22).

Preparation of DNA. Template DNA for the synthesis of carbamoylphosphate synthase was prepared from induced lysates of the double lysogenic strain Jef8 (*metB thrA carB8*) (λd*carAB*37.9)$^+$ (λ199)$^+$ described previously (14). Phage propagation and purification in CsCl gradients have been described previously (14). The DNA of the λd*carAB* phage was obtained by phenol extraction followed by overnight dialysis against 10 mM Tris-acetate (pH 8). The DNA concentration was determined by reading the optical density at 260 nm.

The DNA for the synthesis of β-galactosidase was prepared from induced lysates of doubly lysogenic strain CSH44 [F$^-$ *tonA* Δ*lac thi* (λ c1857 *St68 h80*)$^+$ (λ c1857 *St68 h80* d*lac*)$^+$] as described by Miller (22).

Template DNA for the in vitro transcription of gene *carA* was prepared from strains C600-pGW1 and C600-pGW2. These strains contain plasmids which have been constructed in vitro by joining a restriction fragment comprising gene *carA* to the colicinogenic factor pMB9. The construction of these plasmids will appear elsewhere (Crabeel et al., manuscript in preparation). Their DNA was prepared by a modification of the method of Clewell and Helinski (5).

Conditions for in vitro synthesis and assay of carbamoylphosphate synthase. Unless otherwise indicated, the synthesis mixture for carbamoylphosphate synthase contained in 0.1 ml: 4.7 μmol of Tris-acetate (pH 8.0); 3.1 μmol of ammonium acetate; 0.16 μmol of dithiothreitol; 0.062 μmol each of CTP, GTP, and UTP; 0.25 μmol of ATP; 3.1 μg of folinic acid; 11.5 μg of *E. coli* tRNA; 1.9 μmol of phosphoenolpyruvate; 0.022 μmol each of the 20 amino acids; and optimal concentrations of magnesium acetate, potassium acetate, and calcium acetate as discussed in Results. Unless otherwise indicated, the DNA concentration was 5 μg per 0.1 ml of reaction mixture. These ingredients were incubated with gentle shaking for 3 min at 37°C before the addition of 0.6 mg of S-30 extract protein. The synthesis mixtures were shaken in a 37°C

TABLE 1. *Bacterial strains and their derivation*

Strain	Genotype	Derivation
CSH73	HfrH Δ*lac* Δ(*ara-leu*) *thi*	Cold Spring Harbor Laboratory
CSH73b	HfrH Δ*lac* Δ(*ara-leu*) *thi carB8*	From a *thr* derivative of CSH73 by transduction with strain Jef8 Thr$^+$ (Hfr *metB carB8*) (21)
CSH73 *carB8*	HfrH Δ*lac* Δ(*ara-leu*) *thi carB8* Δ(*ppc-argECBH*)	From strain CSH73B by transduction with strain MN42 [Hfr *metB* Δ(*ppc-argECBH*)] (11), followed by penicillin selection for recombinants using succinate (and not glucose) as a carbon source and displaying a requirement for arginine and uracil that is not satisfied by citrulline and uracil
CSH73 *carA50*	HfrH Δ*lac* Δ(*ara-leu*) *thi carA50* Δ(*ppc-argECBH*)	From strain CSH73 *carB8* by transduction with strain P4XSB25 (*thrA metB*) and selection of a Car$^+$ Thr$^-$ recombinant and, in a second step, transduction with strain RC50 (F$^-$ *thi carA50*) and screening for a Thr$^+$ Car$^-$ recombinant
CSH73 *carB8 argR11*	HfrH Δ*lac* Δ(*ara-leu*) *thi carB8* Δ(*ppc-argECBH*) *argR11*	From strain CSH73 *carB8* after penicillin selection of a *mdh* derivative (6), followed by transduction with strain JC411ER11 K (F$^-$ *thi metB argR11*) carrying a nonsense *argR* mutation and screening for a Mdh$^+$ *argR* recombinant The *argR11* and *mdh* markers were 75% cotransducible

water bath for an appropriate period of time (usually 60 min).

After this incubation, carbamoylphosphate synthase was assayed by coupling it with ornithine carbamoyltransferase. The previously described assay (21, 27) has, however, been modified to increase its sensitivity. The assay mixture, containing 10 μmol each of ATP, magnesium chloride, and L-glutamine, 6 μmol of ornithine, 50 μmol of potassium phosphate (pH 7.5), 250 U of partially purified *E. coli* ornithine carbamoyltransferase, and 5 μmol of $NaH^{14}CO_3$ (0.6 mCi/mmol) in 1 ml, was added, and the incubation was continued for 150 min at 37°C. The reaction was then stopped by addition of 1 ml of 0.25 M trichloroacetic acid. The precipitated proteins were removed by centrifugation, and the excess of $NaH^{14}CO_3$ was eliminated by bubbling air for 5 min. A 1-ml sample was transferred to a polyethylene scintillation vial containing 10 ml of scintillation cocktail (4 parts of Triton X-100 to 6 parts of a solution containing 0.5% 2,5-diphenyloxazole and 10% naphthalene in reagent-grade dioxane). The samples were counted in a model LS-100 Beckman liquid scintillation spectrometer with an efficiency of 55%.

A blank obtained by omitting the DNA from the synthesis mixture (usually of the order of 300 cpm) was routinely subtracted from the results. In earlier experiments, considerable $H^{14}CO_3^-$ incorporation occurred even when DNA was omitted from the synthesis mixture. This interfering activity, which was increased 30-fold by acetyl coenzyme A, was due to phosphoenolpyruvate carboxylase acting on the phosphoenolpyruvate present in the synthesis mixture. Such blank values were considerably reduced once we used strains harboring a deletion of the *ppc* gene for the preparation of S-30 extracts. Under optimal conditions, the number of counts per minute corresponding to the activity of the newly synthesized enzyme was from 4,000 to 5,000 over the blank values.

The synthetic activities were expressed as units (nanomole of carbamoylphosphate formed per hour) of carbamoylphosphate synthase activity formed per hour per milliliter of synthetic mixture.

Conditions for in vitro β-galactosidase synthesis and assay. The in vitro synthesis of β-galactosidase was performed as described above for carbamoylphosphate synthase except for the addition of 1.25 μmol of cyclic AMP to 100 μl of reaction mixture and for the replacement of λd*carAB* DNA by λd*lac* DNA. The DNA concentration was 5 μg per 0.1 ml of reaction mixture. After 1 h of synthesis, 1 ml of β-galactosidase assay buffer (0.1 M sodium phosphate, pH 7.3, 0.14 M β-mercaptoethanol, and 0.35 ml of *o*-nitrophenyl-β-D-galactoside per ml) was added and incubated at 30°C for an appropriate period of time (between 10 and 20 h). The reaction was stopped by the addition of 1 drop of glacial acetic acid, and the precipitate was removed by centrifugation. The supernatant was mixed with an equal volume of 1 M Na_2CO_3 and read against a distilled-water blank at 420 nm. The efficiency of in vitro synthesis of β-galactosidase was comparable to that reported by Zubay et al. (38).

Separation of complementary phage DNA strands. Separation of λd*carAB* and λ199 DNA strands was performed according to the method of Szybalski et al. (32). Using polyuridylic acid-polyguanylic acid, the sense strand for carbamoylphosphate synthase, which is also the leftwards-transcribed strand of λ*carAB*, appeared as the light strand in the gradients (Crabeel et al., manuscript in preparation).

In vitro transcription of the *carA* gene of *E. coli*. In vitro transcription of the *carA* gene was performed as described by Cunin et al. (9) for the *arg-ECBH* cluster. The reaction mixture (200 μl) contained 25 mM Tris-hydrochloride (pH 7.9), 8 mM $MgCl_2$, 0.13 M KCl, 0.1 mM dithiothreitol, 0.2 mM GTP, CTP, and ATP, 0.1 mM [^{3}H]UTP (specific radioactivity, 1,000 to 2,000 cpm/pmol), and 1.4 μg (about 20 molecules per DNA molecule) of purified RNA polymerase (Boehringer). It was preincubated for 4 min at 37°C, and the reaction was started by the addition of 10 μg of *carA* template DNA per ml. When present, L-arginine and partially purified arginine repressor were added to a final concentration of 0.5 mM and 125 μg of protein per ml of synthesis mixture, respectively. The reaction was stopped after 15 min by a further 10 min of incubation in the presence of RNase-free DNase (30 μg/ml), followed by chilling in the presence of 0.2% sodium dodecyl sulfate. *carA*-specific mRNA was measured by hybridization (8) with the *l*-strand of the DNA from λd*carAB*37.9. The *l*-strand of λ199 was used to determine the *carA* nonspecific DNA-RNA hybridization.

Materials. Arginine repressor was partially purified as described by Kelkei et al. (17). Amino acids, nucleotides, and phosphoenolpyruvate were from Sigma Chemical Co, St. Louis, Mo. RNA polymerase and tRNA were purchased from Boehringer, Mannheim, Germany; dithiothreitol was obtained from K and K Laboratories Inc., Plainview, N.Y. $Na_2{}^{14}CO_3$ and [^{3}H]UTP were supplied by the Radiochemical Centre, Amersham, England

RESULTS

In vitro synthesis of carbamoylphosphate synthase. In vitro synthesis of carbamoylphosphate synthase was performed using S-30 extracts of two different strains having no detectable overall carbamoylphosphate-synthesizing activity. The first strain, CSH73 *carB8*, contains a large deletion in gene *carB* but synthesizes normal amounts of *carA* product, the small subunit of the enzyme. The second strain, CSH73 *carA50*, bears a mutation in *carA* that results in a complete absence of synthesis of *car* mRNA as well as of both subunits of carbamoylphosphate synthase (Gigot et al., manuscript in preparation). The S-30 extracts of the two strains were found to be equally efficient (Table 2) when used for the in vitro synthesis of carbamoylphosphate synthase in a system directed by the DNA from the phage λd*carAB* (14). This indicates true de novo synthesis of both subunits of the enzyme. The activities measured at zero time of incubation or in the absence of template DNA were not significantly different from the values obtained with buffer in

TABLE 2. *In vitro synthesis of carbamoylphosphate synthase*[a]

Composition of the synthesizing mixture	Carbamoylphosphate synthase activity (U/h per ml of synthesizing mixture)	
	CSH73 *carA50*	CSH73 *carB8*
Complete	157.3	144.0
Complete (glutamine omitted from the assay mixture)[b]	2.6	2.1
Complete minus DNA	1.4	1.6
Containing S-30 extract plus DNA plus buffer	0	0

[a] Synthesis was conducted for 90 min with S-30 extracts in the presence of 40 μg of λd*carAB* template DNA.

[b] The concentration of glutamine in the assay was 10 mM; the concentration of glutamine due to carry-over from the synthesis was 0.022 mM.

TABLE 3. *Nitrogen substrate dependence and regulatory properties of in vitro-synthesized E. coli carbamoylphosphate synthase*

Composition of the assay mixture	Carbamoylphosphate synthase activity (U/h per ml of synthesizing mixture[a])	
	Expt 1	Expt 2
Complete (with 10 mM glutamine)	266.2	82.5
Glutamine[b] omitted	4.9	
Glutamine omitted,[b] plus 100 mM NH_4Cl	35.9	
Complete[c] plus 1.8 mM UMP[d]		50.1
Complete plus 3.6 mM UMP		49.2
Complete plus 5.4 mM UMP		41.9
Complete plus 10.8 mM UMP		38.0

[a] Synthesis was conducted for 60 min in the presence of 50 μg of λd*carAB* template DNA for experiment 1 or of 40 μg for experiment 2. S-30 extract was from strain CSH73 *carB8 argR11* in experiment 1 and from CSH73 *carB8* for experiment 2.

[b] As in the experiment of Table 2, the concentration of glutamine in the assay mixture due to carry-over from the synthesis mixture was 0.022 mM.

[c] Complete, the assay mixture always contained 6 mM ornithine.

[d] The values obtained for UMP concentrations below 1 mM were poorly reproducible due to UMP disappearance catalyzed by the S-30 extract enzymes.

place of the synthesis mixture. The synthesis of carbamoylphosphate synthase was dependent on and proportional to the concentration of added DNA up to about 50 μg/ml of synthesis mixture.

The substrate dependence and the regulatory properties of the in vitro-synthesized enzyme were similar to those of native carbamoylphosphate synthase. The activity of the enzyme synthesized in the presence of S-30 extracts from both strains mentioned above was dependent on the presence of glutamine (Table 2) and was 10-fold lower when this nitrogen substrate was replaced by ammonium chloride (Table 3). This activity, like that of the native enzyme (24, 26), was partially sensitive to UMP in the presence of ornithine (Table 3). The inhibition of UMP in the presence of 6 mM ornithine was no greater than 60% even for UMP concentrations exceeding 10 mM.

Optimization of this in vitro-synthesizing system with regard to the concentrations of Mg^{2+}, Ca^{2+}, and K^+ ions was repeated for each new S-30 preparation. The optimal concentrations varied in the range of from 11 to 12 mM for Mg^{2+}, 4.5 to 9 mM for Ca^{2+}, and 38 to 45 mM for K^+. Assuming that the in vitro-synthesized enzyme has the same turnover number as the native enzyme (350,000 units per mg of protein for the assay conditions used), the system permitted the synthesis of 100 U (nmol of carbamoylphosphate formed per h) of carbamoylphosphate synthase per h per ml of synthesis mixture. This corresponds to about 1.25×10^{12} enzyme molecules per h per ml of mixture and to about 1.3 molecules per h per *carAB* gene cluster.

Time course of in vitro synthesis of carbamoylphosphate synthase. The synthesis of carbamoylphosphate synthase was not proportional to time during the first 30 min of synthesis. After this period, synthesis proceeded at a constant rate for about 1 h (Fig. 1A). Further incubation led to an arrest of net enzyme synthesis, followed by a decrease of the amount of carbamoylphosphate synthase present. The shape of the curve corresponding to the early period of synthesis could, however, be significantly modified (for a typical curve, see Fig. 1B) if the samples were incubated for prolonged periods in the presence of chloramphenicol before being assayed. This result may reflect inefficient assembly of the enzyme at low concentrations of subunits and suggests that subunit aggregation is the limiting step during in vitro synthesis of carbamoylphosphate synthase. Similar effects have been observed with other enzymes (4, 10, 30).

Arginine repression of the in vitro synthesis of carbamoylphosphate synthase. The DNA-directed system described above has been found suitable to study in vitro the repression of carbamoylphosphate synthase. This was shown first by comparing the ability to mediate in vitro synthesis of S-30 extracts from isogenic

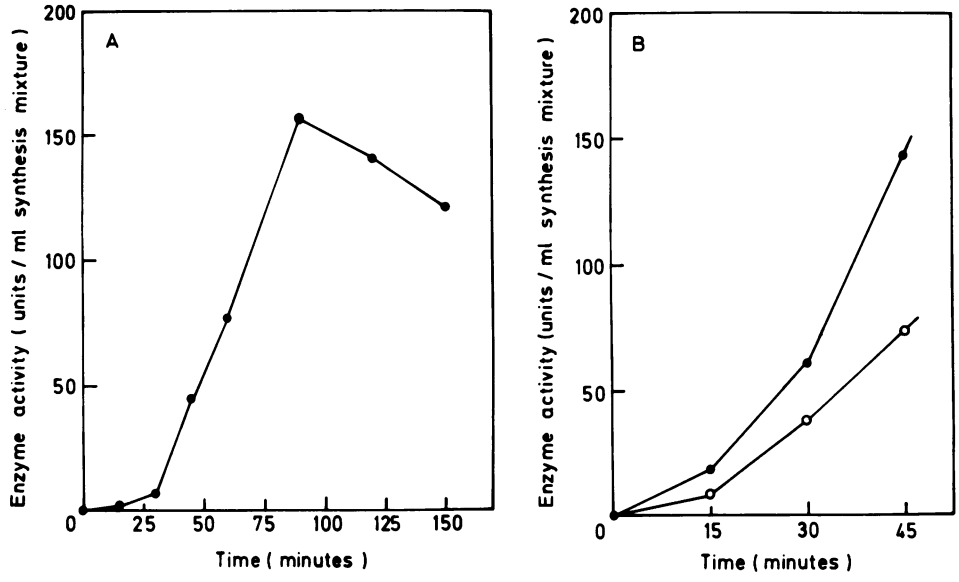

FIG. 1. *Time course for the synthesis of carbamoylphosphate synthase. (A) Kinetics of in vitro synthesis of the enzyme over a period of 150 min. (B) Effect of a maturation period on the early stage of synthesis. S-30 extracts from strain CSH73 carA50 were used. Protein synthesis was arrested at the indicated times by addition of chloramphenicol (20 μl of a 1.2-mg/ml solution). Enzyme activity was determined either immediately* (○) *or after incubation for 6 h at 4°C* (●).

argR and *argR*$^+$ strains. S-30 extracts from an *argR* strain have consistently been found more effective, by a factor of about two, than extracts of strains carrying the wild-type *argR*$^+$ allele. This observation led us to perform an experiment in which the synthetic ability of mixtures of various proportions of S-30 extracts from *argR* and *argR*$^+$ strains was measured (Fig. 2). The experiment was designed in such a way that the total amount of S-30 protein was kept constant. Yet we observe that the synthetic capacities of *argR*$^+$ and *argR* S-30 extracts are not simply additive. For instance, if 20% *argR*$^+$ S-30 extract is mixed with 80% of *argR* extract, the resulting activity amounts to 67% of that of the *argR* S-30 extract, a lower value than the 89% expected if we assume that no repression occurs and that the activities are simply additive (see Fig. 2). We also observed that the addition of 2.5 mM arginine to the system results in a significant reduction of the synthetic capacity of pure *argR*$^+$ S-30 extract or of mixtures of argR$^+$ and *argR* extracts; in contrast, the synthetic activity of pure *argR* extract was little affected by arginine. Another amino acid, proline, was without effect on the synthesis of carbamoylphosphate synthase. Using the same incubation mixture and the same S-30 extracts but with *lac* DNA as template, the synthesis of β-galactosidase remained constant under conditions which widely affect the synthesis of carbamoylphosphate synthase (Fig. 2).

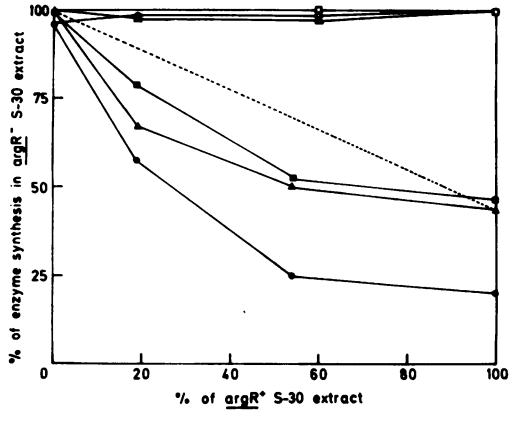

FIG. 2. *Repression of synthesis of carbamoylphosphate synthase by varied amounts of S-30 extracts from argR*$^+$ *and argR strains. Various proportions of S-30 extract from strains CSH73 carB8 and CSH73 carB8 argR11 were used in the presence of the appropriate template DNA for the synthesis of carbamoylphosphate synthase and of β-galactosidase as a control; the total amount of S-30 protein was kept constant. The synthesis of carbamoylphosphate synthase was conducted in the presence of the usual reaction mixture, which contains each amino acid at a concentration of 0.22 mM* (▲)*, with the same mixture plus 2.5 mM arginine* (●)*, and with the same mixture plus 2.5 mM proline* (■)*. The same conditions were used for β-galactosidase and are respectively indicated by* (△)*,* (○)*, and* (□)*. The dotted line represents the theoretical activity resulting from balanced addition of the activities of the two S-30 extracts.*

Participation of the arginine repressor to the in vitro repression of carbamoylphosphate synthase. The results reported in the previous section are consistent with the presence in excess in the *argR*$^+$ S-30 extract, and the absence in the *argR* extract, of an aporepressor which becomes active in the repression of carbamoylphosphate synthase after having bound arginine, a corepressor which is present in too low a concentration in the normal synthetic mixture to achieve maximal activation of the aporepressor. In confirmation of such findings, we have been able to show that partially purified arginine repressor (17) does inhibit the in vitro reaction. The data in Table 4 show that 70% inhibition of the synthesis was obtained upon the addition of the repressor alone to the synthesis mixture. Arginine alone at a 2.5 mM concentration had no effect, yet the simultaneous addition of arginine and the repressor caused a 91% inhibition. No supplementary inhibition was observed in the presence of proline. The same repressor preparation, however, caused a 35% inhibition of the in vitro synthesis of β-galactosidase, an inhibition that was not increased by arginine. The reduction of the synthesis of carbamoylphosphate synthase was thus not entirely due to repression but did partly result from a nonspecific inhibition of protein synthesis. The contribution of true repression by arginine repressor (plus arginine) in this effect is thus estimated to be about 85%.

Arginine repression of *car* genes transcription. In the previous sections we have shown that the arginine repressor is directly involved in the repression of carbamoylphosphate synthase in a system for coupled in vitro transcription and translation of the *car* genes. At this stage it was of interest to determine whether this control operates at the transcription level and if free arginine is the corepressor involved; we therefore developed an in vitro system for the transcription of one of the *car* genes, *carA*. This gene has been isolated following *Eco*RI restriction of the defective λd*carAB* bacteriophage and joined in vitro to the colicinogenic factor pMB9. The expression of the *carA* gene carried by such plasmids normally responds to cumulative repression by arginine and uracil, thus showing that it includes the control region of the *carAB* operon (7; Crabeel et al., manuscript in preparation). These plasmids have been used as a source of template DNA for the in vitro transcription of gene *carA*; *carA* mRNA has been estimated by hybridization with the "left" strand of phage λd*carAB*.

TABLE 4. *Repression of in vitro synthesis of carbamoylphosphate synthase by partially purified arginine repressor*

Addition to synthesis mixture[a]	Enzyme synthesis			
	Carbamoylphosphate synthase		β-Galactosidase	
	U/h per ml of synthesis mixture	% Maximal synthetic activity	U/h per ml of synthesis mixture	% Maximal synthetic activity
None	82.5	100	1.08	100
Arginine (2.5 mM)	81.9	99.3		
Proline (2.5 mM)	82.4	99.9		
Arginine repressor[b]	25.4	30.8	0.71	65.7
Arginine (2.5 mM) + arginine repressor	7.1	8.6	0.73	67.6
Proline (2.5 mM) + arginine repressor	26.4	32.0	0.69	63.9

[a] Synthesis was conducted for 60 min in the presence of 40 μg of λd*carAB* DNA. S-30 extract was from strain CSH73 *carB8 argR11*.

[b] The amount of arginine repressor used corresponded to 190 μg of protein per ml of synthesis mixture.

The results of several independent experiments using two different plasmids, PGW1 and PGW2, are shown in Table 5. In the previous section, we observed that the partially purified repressor preparation was responsible for a nonspecific arginine-independent inhibition of protein synthesis. A similar effect also occurred at the transcription level, since a 37% inhibition resulted from the presence of the repressor preparation in the absence of any added arginine. Under these conditions, the addition of arginine consistently resulted in a supplementary inhibition, which may thus be regarded as repression of *carA* transcription. On the average, a 63% repression was obtained.

DISCUSSION

An in vitro system for the synthesis of *E. coli* carbamoylphosphate synthase by DNA-directed transcription and coupled translation has been developed. The efficiency of the system (1.3 molecules of enzyme synthesized per h per molecule of *carAB* template at 37°C) is comparable to that reported by Zubay (36) for the in vitro system of β-galactosidase.

The in vivo rate of synthesis of carbamoylphosphate synthase is about 5,000 molecules per h per *car* gene in totally derepressed cells; it is thus considerably lower than the rate of 42,000 molecules per h per *lac* gene that can be deduced from the data of Kennel and Riezman (19) regarding the synthesis of β-galactosidase by fully induced cells. The in vitro synthesis of carbamoylphosphate synthase therefore appears relatively efficient.

Using this cell-free system, we have obtained in vitro evidence for a previously suggested (27) participation of *argR* product in cumulative

TABLE 5. *Repression of carA transcription by partially purified arginine repressor*

DNA template	Arginine repressor[a]	L-Arginine (0.5 mM)	Total RNA synthesis	Hybridization input (cpm)[b]	Hybridized on *l*-λd*carAB* DNA (cpm)	Hybridization (%)	% Repression
PGW2	−	−	452	27,938	5,078	18.2	58
	+	−	158	13,594	1,563	11.5	
	+	+	124	19,089	906	4.8	
PGW2	+	−	156	12,971	749	5.7	79
	+	+	135	8,520	96	1.2	
PGW1	+	−	119	12,384	394	3.2	50
	+	+	81	8,004	125	1.6	
PGW2	+	−	167	20,276	1,804	8.9	63
	+	+	123	14,917	492	3.3	

[a] The amount of arginine repressor used corresponded to 125 μg of protein per ml of synthesis mixture.
[b] The mRNA yield was different in each experiment, so the hybridization input was also different in each case. cpm, Counts per minute.

repression of carbamoylphosphate synthase. The comparison of the synthetic activities of S-30 extracts from strains carrying either the *argR*$^+$ or the *argR* alleles yielded a repression efficiency of 80%. This value is in fair agreement with the 70 to 80% repression estimated by comparing the specific carbamoylphosphate synthase activities of an *argR* strain grown in minimal medium and of a wild-type strain grown in minimal medium plus arginine (27, 28).

The participation of *argR* product to cumulative repression was confirmed by demonstrating that in vitro transcription of gene *carA* as well as the in vitro synthesis of the enzyme were repressed by partially purified arginine repressor. These observations have important implications. First, since it is known that the repressor preparations are devoid of aminoacyl tRNA synthetase (17), they demonstrate, as was already established for the other arginine enzymes (9), that free arginine is the corepressor involved in the repression of carbamoylphosphate synthase. In addition, they show that arginine repression is primarily mediated at the transcriptional level. These results are thus consistent with the conclusion of another study (28) that was more peculiarly concerned with the question of translational control in the synthesis of carbamoylphosphate synthase.

Calculation of the number of arginine repressor molecules per *E. coli* cell has so far been performed on the basis of in vitro repression of two enzymes of the arginine pathway, namely, acetylornithinase (34) and ornithine carbamoyltransferase (4); the estimates were, respectively, 206 and 67. Such values have essentially been obtained by assuming, in analogy with what has been observed for the tryptophan system (38), that in an *argR*$^+$ S-30 extract with a saturating arginine concentration, all repressor molecules are engaged in repression. The value of Urm et al. (34) may however be questioned, insofar as only one repressor molecule could possibly bind per *argECBH* control region (11); the alternative estimate would be 103 arginine repressors per cell. We have performed similar calculations for the values given in Fig. 2 for carbamoylphosphate synthase, making the additional assumption that maximal repression represents 80% of the synthetic activity of an *argR* S-30 extract. Under such conditions, half-maximal repression (actually 40% of the activity displayed by an *argR* S-30 extract) will correspond to a mixture containing 18% of *argR*$^+$ S-30 extract. Assuming the mass of protein per cell to be 10^{-13} g, calculation of the number of arginine repressor molecules per *E. coli* cell yielded a value of 40, which is of the same order but nevertheless lower than the previous estimates (67 and 103).

The question may consequently be asked as to whether the repressor molecules are less efficient (have a lower affinity) towards the *car* operon than towards the other *arg* operators or whether the arginine repressor, before binding to the *car* operator, has to interact with another molecule that could possibly be pyrimidine specific.

A detailed understanding of the mechanism of cumulative repression will without doubt depend on the identification of the pyrimidine regulatory element which might be involved in cumulative repression. Preliminary observations in this laboratory indicate that in vitro repression of the synthesis of carbamoylphosphate synthase by pyrimidine nucleotides may be achieved and is stimulated by a preparation obtained after fractionating a crude cell-free extract. It may thus become possible to isolate the

regulatory molecule responsible for this effect and check its activity on in vitro synthesis of other pyrimidine enzymes such as aspartate carbamoyltransferase, which has recently been synthesized in a cell-free system programmed with the DNA from a λ transducing phage (12). Such developments open the way to the study of regulation in the pyrimidine pathway, a study which has long been hampered by the difficulty of isolating pyrimidine regulatory mutants.

ACKNOWLEDGMENTS

We thank P. Halleux for her help in an early stage of this work.

This investigation was supported by a grant from the Fonds de la Recherche Fondamentale Collective-Fonds voor Collektief Fundamenteel Onderzoek. W.L. holds a fellowship from the Instituut voor Aanmoediging van het Wetenschappelijk Onderzoek in Nijverheid en Landbouw.

LITERATURE CITED

1. **Abd-El-Al, A., and J. L. Ingraham.** 1969. Control of carbamylphosphate synthesis in *Salmonella typhimurium*. J. Biol. Chem. **244**:4033–4038.
2. **Abdelal, A. T. H., and J. L. Ingraham.** 1975. Carbamylphosphate synthetase from *Salmonella typhimurium*. Regulation, subunit composition and function of the subunits. J. Biol. Chem. **250**:4410–4417.
3. **Anderson, P. M., and A. Meister.** 1966. Control of carbamylphosphate synthase by purine and pyrimidine nucleotides. Biochemistry **5**:3164–3169.
4. **Cleary, M. L., R. T. Garvin, and E. James.** 1977. Synthesis of *Escherichia coli* K-12 isoenzymes of ornithine transcarbamylase, performed *in vitro*. Mol. Gen. Genet. **157**:155–165.
5. **Clewell, D. B., and D. R. Helinski.** 1969. Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. U.S.A. **62**:1159–1166.
6. **Courtright, J. B., and U. Henning.** 1970. Malate dehydrogenase mutants in *Escherichia coli* K-12. J. Bacteriol. **102**:722–728.
7. **Crabeel, M., D. Charlier, G. Weyens, A. Feller, N. Glansdorff, and A. Piérard.** 1978. Repressible expression of an *Escherichia coli carA* gene inserted into a colicinogenic factor. Arch. Int. Physiol. Biochim. **86**: 911–912.
8. **Cunin, R., A. Boyen, P. Pouwels, N. Glansdorff, and M. Crabeel.** 1975. Parameters of gene expression in the bipolar *argECBH* operon of *E. coli* K-12. The question of translational control. Mol. Gen. Genet. **140**:41–60.
9. **Cunin, R., N. Kelker, A. Boyen, H.-L. Yang, G. Zubay, N. Glansdorff, and W. K. Maas.** 1976. Involvement of arginine in *in vitro* repression of transcription of arginine genes C, B and H in *Escherichia coli* K-12. Biochem. Biophys. Res. Commun. **69**:377–382.
10. **Dohi, M., H. Inouye, A. Kikuchi, and L. Gorini.** 1978. *In vitro* synthesis of ornithine transcarbamylase. J. Biochem. (Tokyo) **84**:1389–1400.
11. **Elseviers, D., R. Cunin, N. Glansdorff, S. Baumberg, and E. Ashcroft.** 1972. Control region within the *argECBH* gene cluster of *Escherichia coli* K-12. Mol. Gen. Genet. **117**:349–366.
12. **Feller, A., W. Lissens, N. Glansdorff, and A. Piérard.** 1978. Synthesis *in vitro* of *Escherichia coli* aspartate carbamoyltransferase. Arch. Int. Physiol. Biochim. **86**: 941–942.
13. **Gigot, D., M. Crabeel, A. Feller, W. Lissens, A. Piérard, and N. Glansdorff.** 1978. Transcription polarity of the *carAB* gene cluster of *Escherichia coli*. Arch. Int. Physiol. Biochim. **86**:915–916.
14. **Glansdorff, N., C. Dambly, S. Palchaudhuri, M. Crabeel, A. Piérard, and P. Halleux.** 1976. Isolation and heteroduplex mapping of a λ transducing phage carrying the structural genes for *Escherichia coli* K-12 carbamoylphosphate synthase: regulation of enzyme synthesis in lysogens. J. Bacteriol. **127**:302–308.
15. **Halleux, P., M. Crabeel, N. Glansdorff, and A. Piérard.** 1975. Synthesis *in vitro* of *Escherichia coli* carbamoylphosphate synthase. Arch. Int. Physiol. Biochim. **83**:186–187.
16. **Justesen, J., and J. Neuhard.** 1975. *pyrR* identical to *pyrH* in *Salmonella typhimurium*: control of expression of the *pyr* genes. J. Bacteriol. **123**:851–854.
17. **Kelker, N., W. K. Maas, H.-Y. Lang, and G. Zubay.** 1976. *In vitro* synthesis and repression of argininosuccinase in *Escherichia coli* K-12. Partial purification of the arginine repressor. Mol. Gen. Genet. **144**:17–20.
18. **Kelln, R. A., J. J. Kinahan, F. K. Folterman, and G. A. O'Donovan.** 1975. Pyrimidine biosynthetic enzymes of *Salmonella typhimurium* repressed specifically by growth in the presence of cytidine. J. Bacteriol. **124**: 764–774.
19. **Kennel, D., and H. Riezman.** 1977. Transcription and translation initiation frequencies of the *Escherichia coli lac* operon. J. Mol. Biol. **114**:1–21.
20. **Lissens, W., R. Cunin, N. Glansdorff, and A. Piérard.** 1979. *In vitro* evidence for a participation of the arginine repressor in cumulative repression of *Escherichia coli* carbamoylphosphate synthase. Arch. Int. Physiol. Biochim., **87**: 630–631.
21. **Mergeay, M., D. Gigot, J. Beckmann, N. Glansdorff, and A. Piérard.** 1974. Physiology and genetics of carbamoylphosphate synthesis in *Escherichia coli* K-12. Mol. Gen. Genet. **133**:299–316.
22. **Miller, J. H.** 1972. Cell-free synthesis of β-galactosidase, p. 419–424. *In* J. H. Miller (ed.), Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **O'Donovan, G. A., and J. C. Gerhard.** 1972. Isolation and partial characterization of regulatory mutants of the pyrimidine pathway in *Salmonella typhimurium*. J. Bacteriol. **109**:1085–1096.
24. **Piérard, A.** 1966. Control of the activity of *Escherichia coli* carbamoylphosphate synthetase by antagonistic allosteric effectors. Science **154**:1572–1573.
25. **Piérard, A., N. Glansdorff, D. Gigot, M. Crabeel, P. Halleux, and L. Thiry.** 1976. Repression of *Escherichia coli* carbamoylphosphate synthase: relationship with enzyme synthesis in the arginine and pyrimidine pathways. J. Bacteriol. **127**:291–301.
26. **Piérard, A., N. Glansdorff, M. Mergeay, and J. M. Wiame.** 1965. Control of the biosynthesis of carbamoylphosphate in *Escherichia coli*. J. Mol. Biol. **14**: 23–36.
27. **Piérard, A., N. Glansdorff, and J. Yashphe.** 1972. Mutations affecting uridine monophosphate pyrophosphorylase or the *argR* gene in *Escherichia coli*. Effects of carbamoylphosphate and pyrimidine biosynthesis and on uracil uptake. Mol. Gen. Genet. **118**:235–245.
28. **Piérard, A., W. Lissens, P. Halleux, R. Cunin, and N. Glansdorff.** 1980. Role of transcriptional regulation and enzyme inactivation in the synthesis of *Escherichia coli* carbamoylphosphate synthase. J. Bacteriol. **141**: 382–385.
29. **Piérard, A., and J. M. Wiame.** 1964. Regulation and mutation affecting a glutamine-dependent formation of carbamylphosphate in *Escherichia coli*. Biochem. Biophys. Res. Commun. **15**:76–81.
30. **Pouwels, P. H., and J. Van Rotterdam.** 1972. *In vitro* synthesis of enzymes of the tryptophan operon of *Esch-*

erichia coli. Proc. Natl. Acad. Sci. U.S.A. **69**:1786–1790.
31. **Schwartz, M., and J. Neuhard.** 1975. Control of expression of the *pyr* genes in *Salmonella typhimurium*: effects of variation in uridine and cytidine nucleotides pools. J. Bacteriol. **121**:814–822.
32. **Szybalsky, W., H. Kubinski, Z. Hradecna, and W. C. Summers.** 1971. Analytical and preparative separation of the complementary DNA strands. Methods Enzymol. **210**:384–413.
33. **Trotta, P., M. E. Burt, R. H. Haschenmeyer, and A. Meister.** 1971. Reversible dissociation of carbamoylphosphate synthase into a regulated synthesis subunit and subunit required for glutamine utilization. Proc. Natl. Acad. Sci. U.S.A. **66**:1027–1035.
34. **Urm, E., H.-Y. Lang, G. Zubay, N. Kelker, and W. K. Maas.** 1973. *In vitro* repression of N-α-acetyl-L-ornithinase synthesis in *Escherichia coli*. Mol. Gen. Genet. **121**:1–7.
35. **Williams, J. C., and G. A. O'Donovan.** 1973. Repression of enzyme synthesis of the pyrimidine pathway in *Salmonella typhimurium*. J. Bacteriol. **115**:1071–1076.
36. **Zubay, G.** 1973. *In vitro* synthesis of protein in microbial systems. Annu. Rev. Genet. **7**:267–287.
37. **Zubay, G., D. Chambers, and L. Cheong.** 1970. Cell-free studies in the regulation of the *lac* operon, p. 375–391. *In* J. Beckwith and D. Zipser (ed.), The lactose operon. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Zubay, G., D. E. Morse, W. J. Schrenk, and J. H. M. Miller.** 1972. Detection and isolation of the repressor protein for the tryptophan operon of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **69**:1100–1103.