REGULATION OF argF mRNA SYNTHESIS, PERFORMED IN VITRO.

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Received March 12,1975

SUMMARY: The specific synthesis of argF mRNA directed by the argF gene carried on the specialized transducing bacteriophage λh80C$_1$857dargF, performed in vitro, is described with the use of an S180 extract from a strain carrying argR$^-$. Synthesis of argF mRNA is biphasic at approximately 7 minutes. The regulation of argF mRNA synthesis by the specific arginine holorepressor present in an S180 extract prepared from a strain carrying the argR$^+$ allele is described.

INTRODUCTION

The genes involved in arginine biosynthesis in E. coli K 12 are scattered around the chromosome constituting a regulon with 7 operators (1,2,3,4) controlling 9 genes. The results of in vivo studies (1,3,4) have shown that the concentration of all the enzymes in the arginine pathway are repressed when wild cells are grown in the presence of arginine and derepressed in the absence of exogenous arginine. A number of mutations have been isolated (4,5) which map in the argR region and lead to constitutive expression of all enzymes in the pathway. In order to ascertain the precise physico-chemical mechanism of regulation of this important regulon it is necessary to develop in vitro studies of mRNA synthesis and the regulation thereof. This work describes the first experiments which demonstrate the synthesis of argF mRNA and the specific regulation of argF mRNA synthesis by extracts containing arginine holorepressor.

MATERIALS AND METHODS

Chemicals

Dithiothreitol, UTP, ATP, CTP and GTP were purchased from Calbiochem. [^{3}H] CTP (22.6 Ci/mM) and [^{3}H] uridine (40.4 Ci/mM) were purchased from New England Nuclear. Deoxyribonuclease (RNase free), bovine pancreatic ribonuclease and T$_1$ ribonuclease were purchased from Worthington. Yeast RNA was purchased from Sigma. Cesium chloride was purchased from Columbia Organic Chemicals. Glucose, yeast extract and Bacto tryptone were purchased from Difco.

Bacteria and Bacteriophage

Strains utilized in this work are listed with their genotype and origin in Table 1. The procedures for growth of bacteria and bacteriophages have been described (6). Phage lysates were clarified by centrifugation at 8000

Table I

Bacteria and Bacteriophage

Strain	Genotype	Origin
EJ112	F^-thi (lac pro argF)$^{\nabla}$ argI str^R	our collection
EJ113	F^-thi (lac pro argF)$^{\nabla}$ argI argR15 str^R $spec^R$	our collection
$\lambda h80C_1857dargF$		our collection
λC_1857S7		N. E. Kelker
$\phi 80_v$		our collection

rpm for 15 minutes and phage was sedimented for 12-16 hours in the presence of 10% polyethylene glycol 6000 (Arthur Thomas) and 0.5 M NaCl as described by Yamamoto, Alberts, Benzinger, Hawthorne and Preiber (11). The sediment was collected by centrifugation at 5000 rpm for 5 minutes and the pellet carefully resuspended in 1/50 of the original lysate volume of Tl buffer (Tl buffer is: 6 x 10^{-4}M $MgSO_4$, 5 x 10^{-4}M $CaCl_2$, 6 x 10^{-3}M Tris HCl pH 7.3, 0.1% w/v gelatin). The concentrated phage was sedimented for 2 hours at 30,000 rpm into a cushion comprising 2 ml of 1.4 density CsCl above 2 ml of 1.6 density CsCl using a 50.2 Ti rotor in the Beckman ultracentrifuge. Phage were further purified by repeated centrifugation in a cesium chloride density gradient using 1.4, 1.5, 1.6 block gradients for a period of 4 to 6 hours; 1.4, 1.6 block gradients for 12-16 hours or running to equilibrium in 1.5 density CsCl using either the 50.2 Ti, 50 Ti or 75 Ti angle rotors. Following centrifugation, gradients were harvested by upward displacement by fluorinert FC 40 (Minnesota Mining and Manufacturing Co., St. Paul, Minn.) with an ISCO gradient fractionator coupled to a Chromatronix dual wavelength absorbance monitor with an 0.5 mm path length flowcell.

Isolation of DNA

Template DNA was isolated as described by Zubay et al. (6). DNA utilized for hybridization studies was further purified by following the third phenol extraction by extraction with chloroform: isoamyl alcohol (24:1 v/v), the aqueous phase was adjusted to 25 μg/ml in Pronase and incubated for 2 hours at 37°C. Two additional phenol extractions were performed followed by a final chloroform: isoamyl alcohol extraction. The aqueous layer was dialyzed exhaustively against 10 mM EDTA, pH 8.0 and stored at 4°C over a drop of chloroform.

Preparation of S180 Extracts

The strains EJ112 and EJ113 were utilized for the production of S30 extracts according to Zubay (6) except that the extract was adjusted to 1 mM

arginine immediately after passage through the French press and the incubation step was omitted. Supernatant solutions were prepared by centrifugation of S30 extracts for 2 hours at 44,000 rpm in the SW 50.1 rotor at 4°C; the upper two-thirds of the cellular extract were collected and utilized as S180 which was rapidly frozen and stored in 0.5 ml aliquots at -82°C.

Transcription of the argF Operon, in vitro

Standard reaction mixtures for in vitro transcription contained per ml: 23 mM Tris-HCl (pH 7.9), 15 mM $MgCl_2$, 100 mM KCl, 0.1 mM sodium EDTA, 0.1 mM dithiothreitol, 0.15 mM ATP, UTP, GTP, 0.075 mM [^{3}H] CTP (specific activity 26.2 Ci/mM), 50 µg λh80C$_1$857dargF DNA and 300 µl of S180 cell extract. The reaction mixture was first incubated without NTP's for 5 min. at 37°C; the reaction was initiated by the addition of NTP's and terminated by the addition of 2.2 ml of a cold solution (buffer Q) containing 100 mM Tris-HCl (pH 7.0), 3 mM magnesium acetate, 0.2 mg/ml tRNA and 25 µg/ml of deoxyribonuclease. After 15 min. at 4°C an aliquot (50 µl) was removed, 50 µl of BSA solution (5 mg/ml), 400 µl of 10 mM EDTA and 500 µl of 20% TCA were added. The mixture was permitted to stand at 4° for 30 minutes, collected on a glass fibre filter (Whatman GF/C) and washed with 10 ml of 5% TCA. The filter was dried and [^{3}H] labeled RNA determined by counting in Ready Solv IV in a Beckman LS230 scintillation spectrometer. [^{3}H] RNA was extracted with an equal volume of phenol saturated with 50 mM sodium acetate (pH 5.2) and 10 mM $MgCl_2$. RNA was precipitated by the addition of 2 volumes of 95% ethanol (precooled to -20°C) and standing at -20° for 16 hours; the precipitate was collected by centrifugation at 12,000 rpm for 15 minutes at 4°C in the Beckman J21B centrifuge. RNA was dissolved in 1 ml of 4 x SSC (SSC is 0.15 M NaCl + .015 M Na_3 citrate (pH 7.0)) and a 50 µl aliquot was removed for the determination of [^{3}H] RNA as described.

Hybridization Procedures

The quantity of argF mRNA synthesized in vitro was determined using the hybridization procedure of Gillespie and Spiegelman (8). Solutions of [^{3}H] labeled RNA were adjusted with 4 x SSC to yield a solution containing 2 x 10^5 cpm/ml. A quantity of RNA (2.0 x 10^4 cpm) was prehybridized for 24 hours at 67° to a mixture of denatured DNA isolated from φ80 and λC$_1$857S7 (3.0 µg and 4.0 µg respectively). Specific argF mRNA remained in solution and was determined by permitting 150 µl of supernatant solution to hybridize for 16 hours at 67° to 2 µg of denatured λh80C$_1$857dargF DNA immobilized on a nitrocellulose filter.

RESULTS AND DISCUSSION

DNA isolated from the specialized transducing bacteriophage λh80C$_1$857dargF was utilized as a template for the direction of mRNA synthesis utilizing an S180 cell extract as a source of RNA polymerase. The time course of synthesis of total mRNA was linear for 10 minutes as determined from the measurement of total [^{3}H] labeled RNA precipitated with cold 10% TCA. The time course of specific argF mRNA synthesis is presented in figure 1. This data was obtained by performing in vitro synthesis for specific periods of time, arresting the reaction by transferring an aliquot (100 µl) of the reaction mixture to 2.2 ml

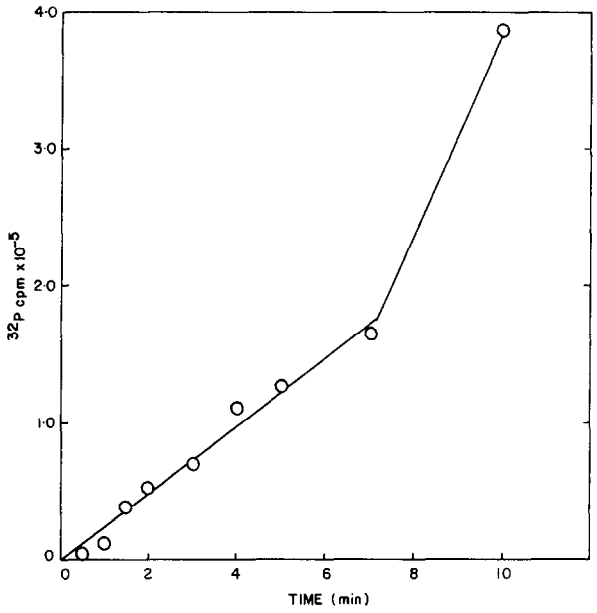

Figure 1. time course of argF mRNA transcription utilizing λh80C$_1$857dargF DNA template and an S180 extract from EJ113 (argR$^-$).

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of Q buffer. A sample (50 µl) was removed and total [^{3}H] CTP incorporated into acid precipitable counts determined as described. The rest of the sample was extracted with phenol, ethanol precipitated and the resulting precipitate redissolved in 1 ml of 4 x SSC. An aliquot (2 x 10^4 cpm) of the resulting RNA solution was annealed with 7 µg of λC$_1$857S7 and φ80 denatured DNA immobilized on a nitrocellulose filter at 67° for 24 hours in 4 x SSC. Following prehybridization to remove λ and φ80 specific mRNA an aliquot of the mRNA was permitted to hybridize with denatured DNA isolated from λh80C$_1$857dargF immobilized on a nitrocellulose filter.

The time course of argF mRNA synthesis is linear for approximately 7 minutes at which time an increase in the rate of mRNA synthesis is observed. The augmented rate of argF mRNA synthesis is presumably caused by readthrough from a powerful upstream phage promotor as found for the tryptophan operon (9).

In experiments designed to demonstrate regulation of argF mRNA synthesis the reaction was permitted to proceed for 2.0 minutes in order to avoid any

complication from readthrough from a neighboring phage promoter. The results of a set of experiments showing *in vitro* regulation of *argF* mRNA synthesis are shown in figure 2. Specific *argF* mRNA synthesis was permitted to proceed

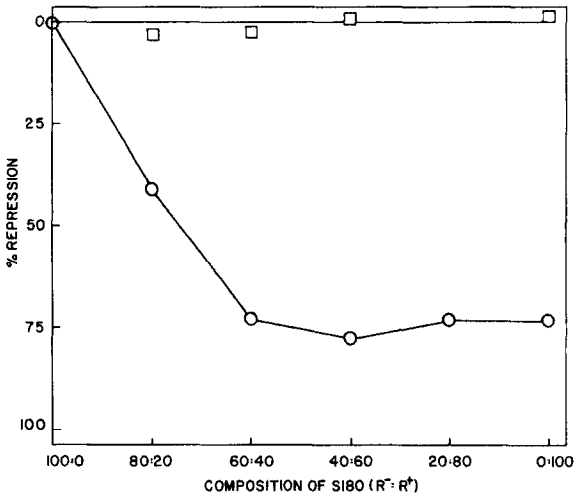

Figure 2. correlation of mRNA synthesis as a function of S180 composition. Specific *argF* mRNA synthesis O, Total mRNA synthesis □.

for precisely 2 minutes and the amount of *argF* mRNA synthesis determined as described. These determinations utilized varying proportions of S180 cell extracts isolated from strains carrying *argR*$^-$ and *argR*$^+$ alleles. It is apparent that specific *argF* mRNA is repressed dramatically by the incorporatior of 20% of an S180 extract obtained from a strain carrying a wild *argR* allele; the increase in concentration of S180 cell extract from the *argR*$^+$ strain to 40% resulted in repression of *argF* mRNA synthesis by about 70%. Utilization of increased concentrations of S180 cell extract from an *argR*$^+$ strain resulted in little additional repression of specific *argF* mRNA synthesis. Total mRNA synthesis was shown to be independent of the concentration of S180 cell extract from the *argR*$^+$ strain.

The fact that repression was not found to be complete is not unexpected as *in vitro* systems such as those used in this work have shown similar

repression figures (9, 10). This may be due to inherent leakiness of repression in the system and also to basal levels of transcription of mRNA from other bacterial genes carried on the specialized transducing bacteriophage. This latter possibility will be investigated by the use of competition experiments and also by the use of pure argF gene DNA as a probe.

The system described in this work is now being used as the basis of an *in vitro* assay for the argR gene product.

ACKNOWLEDGEMENTS: We are indebted to Dr. Luigi Gorini for introducing us to the arginine regulon; to Drs. Gottesman and de Crombrugghe for valuable discussions and to the American Cancer Society for financial support through grant VC131 to E.J. and through grant IN107 to D.S.

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