CHARACTERIZATION OF STRUCTURE, FUNCTION AND REGULATION OF THE *speB* GENE IN *ESCHERICHIA COLI*.

by

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Abstract

The speB gene of E. coli encodes agmatine ureohydrolase (AUH). AUH catalyses the hydrolysis of agmatine to urea and putrescine in a polyamine biosynthetic pathway. The plasmid pKA5, derived from an E. coli genomic library, was the source of a 2.97 kb restriction fragment containing the speB gene. Sequencing of this fragment revealed three intact open reading frames, ORF1 and ORF2 on one strand and ORF3 on the opposite strand, as well as a truncated open reading frame, ORF4, which terminated 92 kb upstream from ORF3. ORF2 and ORF3 were convergent, and overlapped by 85% of their sequence. ORF1 and ORF3 were separated by a sequence of two imperfect repeats containing four palindromes, three of which were overlapping. ORF3 represented the coding sequence of the speB gene. Two transcripts were detected from the speB gene: a shorter transcript, initiated 101 bp upstream from ORF3, and a polycistronic message, coding for ORF3 and ORF4. The short transcript was abundantly expressed when ORF4 sequences were deleted, but when ORF4 and its upstream sequences were present, the polycistronic message predominated and the amount of the monocistronic message was drastically reduced. The promoter producing the shorter transcript required only a -12 TATACT sequence for activity. Deletion of a 460 bp fragment comprising the 5'-region of ORF1 from a plasmid containing ORF1, ORF2 and speB reduced the activity of AUH by 83%. This fragment contained two divergently oriented promoters. The presence of ORF1 did not stimulate ß-galactosidase encoded by the speB promoter fused to lacZ. Agmatine induced transcription from speB but not from the ORF4 nor the ORF1 promoters. cAMP caused an 88% reduction

in the AUH activity of wild type *E. coli* K-12 but had no effect on the activity of plasmid encoded AUH. The activity of neither the *speB* nor the ORF4 promoters fused to *lacZ* or *phoA* were influenced by cAMP; in contrast, the *lacZ* promoter fused to *lacZ* or *phoA* was stimulated by cAMP. Thus, the role of cAMP and CRP on *speB* expression is indirect and limited to a single copy state.

I dedicate this work to my best friend,

,

with love and gratitude.

Acknowledgements

I am grateful to Dr. S. M. Boyle for allowing me to work in his laboratory, for many stimulating discussions and for letting me freely pursue my ideas. I also thank Dr. Boyle, , , , , Dr. S. Sriranganathan and Dr. Fang Lai for putting up with my enthusiasms, for picking me up when I was down and for making the laboratory a nice place to be. Furthermore, to Dr. Thomas W. Keenan, I express my strongest thanks for his creative support at a difficult time.

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General Introduction

Polyamines have been found in all studied organisms. Putrescine (1,4-diaminobutane) and spermidine (the aminopropyl derivative of putrescine) are the most ubignitous polyamine molecules found in both procaryotic and eucaryotic cells. Spermine ($NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$), which was the first known representative of these compounds, is present in most eucaryotic cells but is absent from E. coli and most other bacterial species (55). Unusual polyamines, composed of multiple units of diaminobutane and/or diaminopropane, as well as those containing tertiary or quaternary amino groups, have also been detected in microorganisms such as the thermophilic or acidophilic bacteria (36).

The observation in 1948 by Herbst and Snell that putrescine, spermidine or 1,3-diaminopropane is essential for growth of Haemophilus influenzae (23) sparked an interest in establishing the role of polyamines in life processes. The approach initially followed by many investigators was to isolate subcellular components of cells and examine their polyamine content (3, 51). Nearly all the studied macromolecular structures have been reported to be complexed with polyamines: DNA, tRNA, ribosomes and subcellular membranes. It became apparent that this approach did not necessarily reveal their in vivo location since at physiological pH polyamines are cations and have a high electrostatic affinity for all the negatively charged constituents of cells. During the process of isolation of the cellular components polyamines can readily be dislocated and bind to different structures. However, several of the reported results survived the scrutiny of this argument. For example, the presence

of large amounts of DNA associated polyamines in purified virus particles (not penetrable by polyamines) has shown that this association is physiologically significant; the bacteriophage T4 is packaged with enough putrescine and spermidine to neutralize about 50% of the negative charge in its DNA (4). Animal viruses (such as herpes virus, poxviruses, Influenza A and picorna viruses, among others) containing both DNA and RNA have also been found to contain large amounts of polyamines associated with their nucleic acids. The vaccinia virus, which upon infection causes rapid downregulation of synthesis of its host macromolecules, induces an ornithine decarboxylase (ODC, a putrescine biosynthetic enzyme) with characteristics differing from the enzyme present in noninfected host (55). This observation suggests that the novel ODC might be encoded by the virus, and thus underscores the importance of polyamines in the viral life cycle.

The above observations show that polyamines have a role in stabilization and packaging of nucleic acids *in vivo*. Furthermore, it is common knowledge among researchers handling nucleic acid preparations (28) that polyamines protect nucleic acids from denaturation, mechanical damage and enzymatic attack, and aid in isolation of intact macromolecular aggregates. For example, isolation of highly folded *E. coli* chromosome, still attached to fragments of membrane, was achieved when spermidine was added in the purification process (51). These effects of polyamines are believed to be the result of their cationic nature and ability to facilitate condensation between adjacent phosphate groups within the backbone of nucleic acids by preventing their electrostatic repulsion.

Because of the intimate contact that exists between polyamines and nucleic acids an effort has been placed on finding a role for polyamines in replication, transcription and translation. These studies have been largely facilitated by isolation of polyamine auxotrophic mutants of E. coli (20, 15) and by development of drugs inhibiting polyamine biosynthesis in both eucaryotic and procaryotic cells (45, 25). Seyfried and Morris measured the rate of incorporation of [^{3}H] thymidine into DNA of activated bovine lymphocytes as a function of the presence or absence in the culture media of the drugs MGBG, DGBG, TGBG or DFMO (45). MGBG (methylglyoxal bis[guanylhydrazone]) is an inhibitor of S-adenosylmethionine decarboxylase (SAM-decarboxylase), which catalyses the reactions leading from putrescine to spermidine (see Fig. 01) and spermine. MGBG also has cytotoxic effects on many cells which are not correlated with its action as an inhibitor of polyamine biosynthesis, and has been reported to inhibit replication in many eucaryotic cells. DGBG and TGBG (dimethylglyoxal bis [guanylhydrazone] and di-N',N''-methylglyoxal bis [guanylhydraxone] respectively) are also SAM-decarboxylase inhibitors but do not exhibit the broad cytotoxic activities of MGBG (45). DFMO (difluoromethylornithine) is a suicide inhibitor of ODC (25) and is unrelated to MGBG, DGBG or TGBG. All four drugs have shown the ability to inhibit DNA replication in activated lymphocytes and this activity was inversely correlated to the levels of polyamines (particularly spermidine and spermine) present in treated cells (45). A similar direct correlation between the level of spermidine and the rate of DNA replication have been observed in an E. coli mutant deficient in biosynthesis of putrescine and spermidine (17). In these cells the DNA replication is reduced by about 50% under conditions

of polyamine starvation. Addition of putrescine and spermidine to the growth media restored the replication rate to its optimal level within 20 minutes. However, when analogs of spermidine, containing C5-C8 substitutions in place of the C4 aliphatic chain, were added to the media, the longer chain analogs were unable to mimic the effect of spermidine on the replication rate. This indicated that the effect of the aliphatic chain length is specific for spermidine, and not mediated solely by its polycationic nature.

Polyamines have also been shown to effect transcription. In vitro studies on E. coli DNA dependent-RNA polymerase have shown that spermidine and spermine stimulated the polymerization catalyzed by this enzyme; an optimum effect was seen at 0.8 and 0.3 mM concentration of these polyamines respectively (1). The presence of optimal concentrations of spermidine did not change the profile of pH dependence of this reaction, indicating that the polyamine influences the reaction through interaction with nucleic acid rather than with the enzyme. The presence of 1 mM spermidine counteracted the inhibitory effect of exogenous RNA on the reaction. Thus, the polyamine must either prevent binding of RNA to the enzyme (and interfere with end product inhibition), or prevent hybridization of the added RNA to the DNA template (increasing the level of reinitiation). The first possibility seems more probable since the added RNA was either tRNA or rRNA, which would only hybridize to a small fraction of the template (native chromosomal E. coli DNA). In either case the polyamine action must have been exerted on the nucleic acid and seems to be rather nonspecific (although the possibility of spermidine preventing interaction between RNA and proteins might be worth a follow-up investigation).

Ribosomes were among the first macromolecular structures reported to contain large amounts of polyamines, although the stoichiometry of this association varied with different isolation procedures (3). In a mammalian cell-free translation system polyamines stimulated the rate of amino acid incorporation into polypeptides directed by Adenovirus 2 (Ad2) or 9S globin RNA 3-5 fold (6). Spermidine and spermine exhibited a larger stimulatory effect than putrescine. Addition of these polyamines also partially reduced the Mg^{2+} requirement of the *in vitro* translation process. The pattern of translation was also affected qualitatively by polyamines. In their presence the translation products of the Ad2 mRNA were enriched with high molecular weight proteins, resembling more the protein profile from Ad2 infected cells. This observation indicates that polyamines prevent premature termination in the eucaryotic *in vitro* translation process.

In bacterial cell-free translation, it was observed that extracts derived from polyamine auxotrophs of *E. coli* exhibited several fold higher translation rate when the bacteria were cultivated in the presence of polyamines, as compared to the rates observed in extracts from polyamine starved cells (15, 16). By reconstituting different fractions of the translation system derived from either polyamine starved or polyamine supplemented cells, Echandi and Algranati (15) found that the defective component of the polyamine starved translation extracts resides in the ribosomes rather than in the fraction containing other translation factors. In a follow-up study (16) the authors measured translation rates in extracts in which the ribosomes were reconstituted from the 30S and the 50S ribosomal subunits purified separately from putrescine

starved or supplemented polyamine auxotrophs. Reduction of the translation rate was observed only when the 30S ribosomal subunits were derived from polyamine starved cells. Furthermore, supplementation of the defective *in vitro* translation extracts with spermidine did not increase the translation rates. Sucrose gradient analysis of the ribosomes from starved or unstarved cells revealed an additional, lower density component in the 30S fraction from the polyamine starved cells, which might either represent a defective 30S particle or a precursor of the 30S subunit. The defective 30S particles from polyamine starved cells have a decreased affinity for association with the 50S subunit to form the 70S ribosome.

Igarashi et al. (24) studied the proteins from the 30S particles derived either from putrescine starved or supplemented *E. coli* polyamine auxotrophs on polyacrylamide gels. They observed that the polyamine starved cells produce much smaller amounts of the ribosomal protein S1. They repeated these experiments with another *E. coli* mutant, blocked at a different step of polyamine synthesis, and obtained the same results. They concluded that depletion of the S1 protein accounts for the defect in the 30S particles resulting in a lower translation rate in the polyamine starved cells. However, it is still unknown what mechanism causes the selective underproduction of the S1 protein.

Another indication that the 30S ribosomal subunit is the site of action of polyamines on translation came from the investigation by Goldemberg and Algranati (19) on the interaction between polyamines and streptomycin. Streptomycin is a cationic antibiotic that inhibits translation by binding to the 30S ribosomal subunit. Addition of increasing amounts of streptomycin to cultures of *E. coli* mutants unable to synthesize

polyamines resulted in a large decrease in protein synthesis only when the cells were supplemented with polyamines. When the cells were starved for polyamines the inhibitory effect of streptomycin on translation was much smaller. Another observation in this study was that depletion of polyamines resulted in production of smaller quantities of high molecular weight proteins. This could be the result of mis-reading or premature termination, followed by fast clearance of the defective proteins. Streptomycin, which is known for its negative effect on translation fidelity, produced the same effect, but only in the polyamine supplemented cells. Thus, the absence of polyamines confers on the cell the phenotype of streptomycin resistance.

The mechanisms of the polyamine mediated phenomena described above await full clarification. A unifying theory explaining all the pleiotropic effects of polyamines might never be found since it is obvious from the above examples that polyamines act on two levels: as ions that are able to nonspecifically neutralize negatively charged structures in the cell and substitute for other ions; or as small molecules capable of interacting specifically with proteins as is evident by their effect on translation. To distinguish between these two levels of polyamine action, it might be helpful to produce polyamine analogs with nonbasic ends (for example by substituting the primary amino groups with hydroxyl groups) and investigate their ability to mimick polyamine mediated actions in the polyamine auxotrophs.

Another way to establish the importance and possible roles of polyamines in cell physiology is through study of their synthesis. Most importantly, unraveling the patterns of regulation of polyamine synthesis

might lead to discovery of the processes for which their presence is essential.

Putrescine is an active diamine as well as an intermediate in the biosynthesis of both spermidine and spermine in all organisms. One pathway of putrescine synthesis, operating in both procaryotic and eucaryotic cells, is the decarboxylation of ornithine catalyzed by ornithine decarboxylase (ODC). The other pathway, found only in plants and in bacteria involves decarboxylation of arginine to agmatine, catalyzed by arginine decarboxylase (ADC); this is followed by hydrolysis of agmatine to putrescine, catalyzed by agmatine ureohydrolase (AUH) (53, 38, 39). The pathways involved in synthesis of putrescine and spermidine are illustrated in Fig. 01. In all studied organisms the biosynthesis of putrescine is closely connected to the metabolism of arginine. In eucaryotic cells and in some bacteria (13) ornithine is synthesized by hydrolysis of arginine in the reaction catalyzed by arginase. In bacteria ornithine is an intermediate in the pathway leading from glutamate to arginine (illustrated in Fig. 02). This pathway is strictly regulated by the availability of arginine through its feedback inhibition of step A (in E. coli among others) or step B. This pathway is the only source of arginine in E. coli which does not produce arginase (13).

The existence of the two putrescine biosynthetic pathways in E. coli K-12 and in E. coli B was established in 1966 by Morris and Pardee (29). They used in their experiments a strain of E. coli K-12 blocked in early steps of arginine biosynthesis and cultivated in media supplemented with either arginine or ornithine. They have established that the specific activities of the putrescine biosynthetic enzymes in these cells were

33, 13, and 83 nmol of product/min x mg protein for the ODC, ADC, and AUH respectively. About 40% of the cellular putrescine was formed by the ODC catalyzed reaction, while 60% was produced by the ADC-AUH pathway when surplus precursors were present. With these enzyme activities E. coli is able to produce polyamines in large excess of their steady state concentration, which the authors calculated to be about 100 nmol putrescine + spermidine per mg of protein. Thus, the cell has to produce about 1.7 nmol of putrescine per min per mg protein. It was found later that the pathway from ornithine is favored in unsupplemented or ornithine supplemented media, while the pathway from arginine is favored in arginine supplemented media (30, 50). Furthermore, when the internal and the external pools of arginine were differentially labelled, from ^{14}C-citrulline or ^{3}H-arginine respectively, it was observed that the putrescine was made preferentially from exogenous arginine, and that about 40-60% of putrescine was made before the exogenous arginine mixed with the endogenous arginine pool (50). A plausible explanation of this phenomenon was offered by Buch and Boyle, who found that the ADC is synthesized as a precursor (11) and resides in the cell envelope. As consequence of this compartmentalization the exogenous arginine is decarboxylated in the periplasmic space and enters the cytosol as agmatine.

The ODC and ADC enzymes have been purified and characterized. These enzymes are inhibited by putrescine and spermidine (5, 58). Moreover, Wright and Boyle have shown that the expression of ODC and ADC is negatively affected by adenosine-3'-5'-monophosphate (cAMP) (57, 58).

In E. coli cAMP is a mediator of carbon utilization. In catabolite repression the presence of glucose in growth media causes trasncriptional

repression of genes coding for enzymes that participate in degradation of sugars other than glucose. Glucose severely inhibits the activity of adenylate cyclase which catalyzes the synthesis of cAMP from ATP. When glucose is absent from the media, the level of cAMP in the cell increases (8). cAMP binds to the cAMP receptor protein (CRP) and the resulting cAMP-CRP complexes bind with high affinity to specific sequences of DNA residing at or near the promoters of the above mentioned genes (the lac, ara, and gal operons are among the best studied members of the catabolite repression regulon (9)). The exact mechanism of the interaction between the cAMP-CRP-promoter and the RNA polymerase is unknown, but the overall result of this binding is induction of catabolic genes. Most of the operons that are subject to catabolite repression are induced very strongly by the respective molecules whose catabolism they mediate (e.g. lactose). Thus, when the particular carbon source is present in the medium, and glucose is absent, these operons are fully active.

In recent years it has been found in several studies, that some bacterial genes are negatively regulated by cAMP-CRP (2, 31, 40). The most extensively studied case is that of the crp gene, coding for the CRP protein (2, 34, 35). The effect of cAMP-CRP on the crp gene is indirect: the cAMP-CRP induces transcription of a short RNA molecule complementary to the crp promoter sequences. The presence of this RNA molecule inhibits transcription of the crp gene, presumably by preventing the RNA polymerase from binding and initiating transcription from the crp promoter.

A systematic study of the AUH enzyme was initiated recently by Satischandran and Boyle. In several strains of E. coli K-12 encoding an active CRP protein AUH activity was reduced when cAMP was added to the

growth media. In isogenic crp strains the AUH activity was not affected by cAMP supplementation (43). The authors also found that the AUH activity increased approximately 2-2.5 fold when the cells were grown in the presence of agmatine. This increase was apparently independent of the cAMP mediated effect. The AUH enzyme was purified and its activity in vitro was found to be unaffected by either cAMP or putrescine (42).

The observation that AUH expression is stimulated by agmatine was supported by studies conducted in the laboratory of Halpern (21). However, their observations on the cAMP effect on expression of ODC, ADC, and AUH were different from the observations of Boyle's group. In the strains of E. coli K-12 used by Halpern's group, AUH behaves as member of the catabolic enzymes (i.e. high activity with succinate as the carbon source but drastically reduced activity with glucose as the carbone source). Furthermore, in their CRP^+ isogenic strains, the addition of cAMP to the media reversed the negative effect of glucose on the AUH activity. On the other hand, they found that neither ODC nor ADC activities in their strains were responsive to changes in carbon or nitrogen source (21).

Halpern's interest in the AUH enzyme is related to his studies on utilization of arginine as a sole nitrogen source in E. coli. The pathway of arginine degradation in E. coli is illustrated in Fig. 03. The ADC and AUH catalyzed reactions constitute the first two steps in this pathway. In the study of Shaibe et al. (47) glucose mediated catabolite repression of AUH could be relieved by use of a nitrogen source other than ammonia in the growth media. This phenomenon has been observed previously in E. coli K-12 grown on metabolites that function as either a carbon or nitrogen source (14).

The disagreement between the results of Halpern and Boyle with respect to regulation of AUH by cAMP might be a result of a mutation in the different strains involved; when Halpern tested one of Boyle's strains for ODC regulation by cAMP, he also observed a negative cAMP effect in this strain in agreement with observations of Wright and Boyle (21).

I started the study described in this dissertation with the intent to establish the mechanisms of action of the cAMP-CRP complex on the speB gene encoding AUH. The first step to achieve this goal was to localize and characterize the speB gene. I sequenced a fragment derived from the E. coli chromosome which conferred AUH activity on the host following its transformation. I localized the speB gene within this fragment and characterized the patterns of speB transcription. My results have shown that the speB expression is highly complex; it is regulated by expression of flanking genes (residing both upstream and downstream from speB). I found that agmatine is a transcriptional inducer of the speB gene while the inhibitory effect of cAMP is mediated through an unknown gene product.

List of Abbreviations

1. Ad 2 - adenovirus 2
2. ADC - arginine decarboxylase
3. AUH - agmatine ureohydrolase
4. ATP - adenosine triphosphate
5. BME - ß-mercaptoethanol
6. cAMP - cyclic adenosine monophosphate
7. CRP - cAMP receptor protein
8. dATP - deoxyadenosine triphosphate
9. dCTP - deoxycytosine triphosphate
10. DFMO - difluoromethylornithine
11. DGBG - dimethylgloyoxal bis [guanylhydrazone]
12. dGTP - deoxyguanosine triphosphate
13. DNA - deoxyribonucleic acid
14. dNTP - deoxynucleoside triphosphate
15. E. coli - Escherichia coli
16. EDTA - ethyldiaminotetracetate
17. HEPES - N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
18. kb - kilobases
19. LB - Luria broth
20. MGBG - methylglyoxal bis [guanylhydrazone]
21. ODC - ornithine decarboxylase
22. RNA - ribonucleic acid
23. rRNA - ribosomal RNA
24. SAM - S-adenosylmethionine

25. SDS - sodium dodecyl sulfate

26. SST - standard saline citrate

27. STE - sodium-Tris-EDTA

28. TB - Terrific broth

29. TE - Tris-EDTA

30. TGBG - di-N'-N"-methylglyoxal bis [guanylhydrazone]

31. tRNA - transfer RNA

32. TTP - deoxythymidine triphosphate

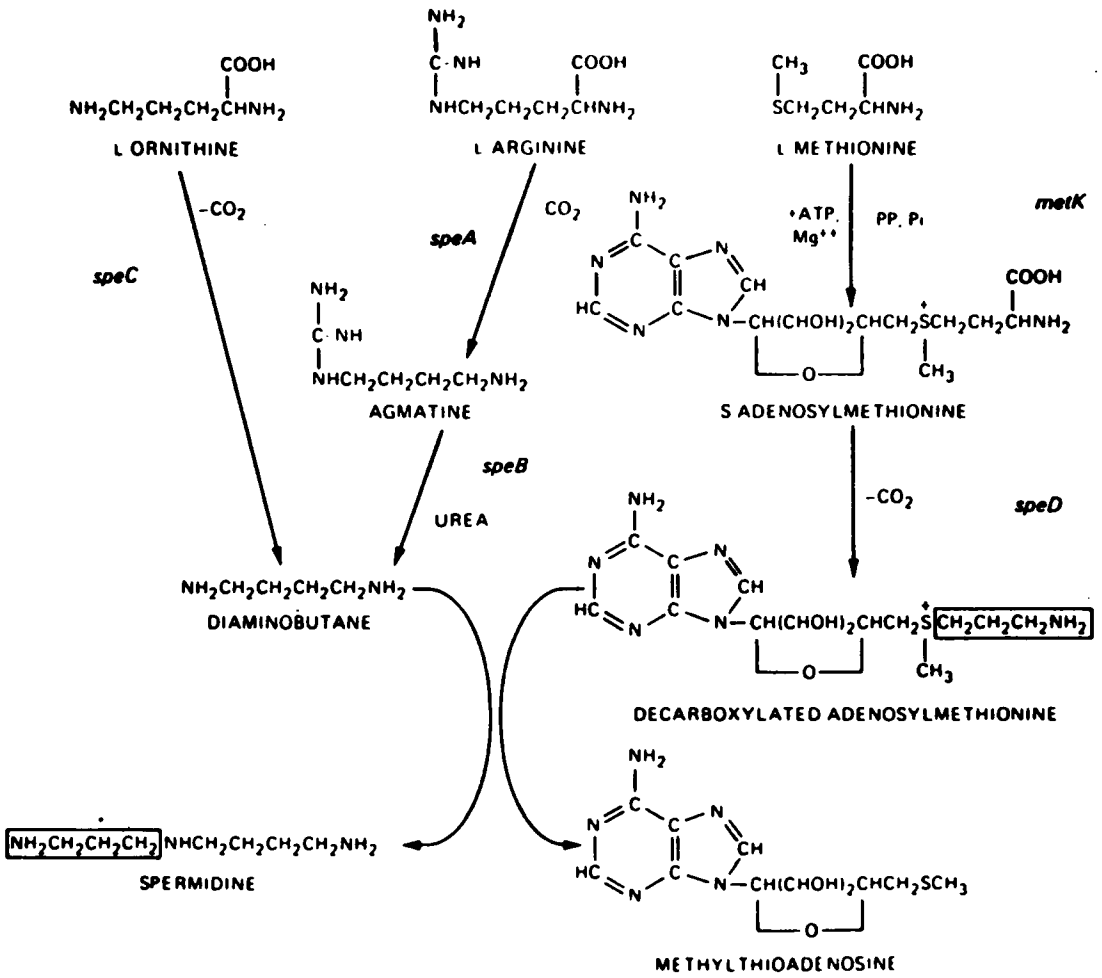


Fig. 01. Pathways of putrescine and spermidine synthesis in E. coli.

(This figure is derived from reference 53.)

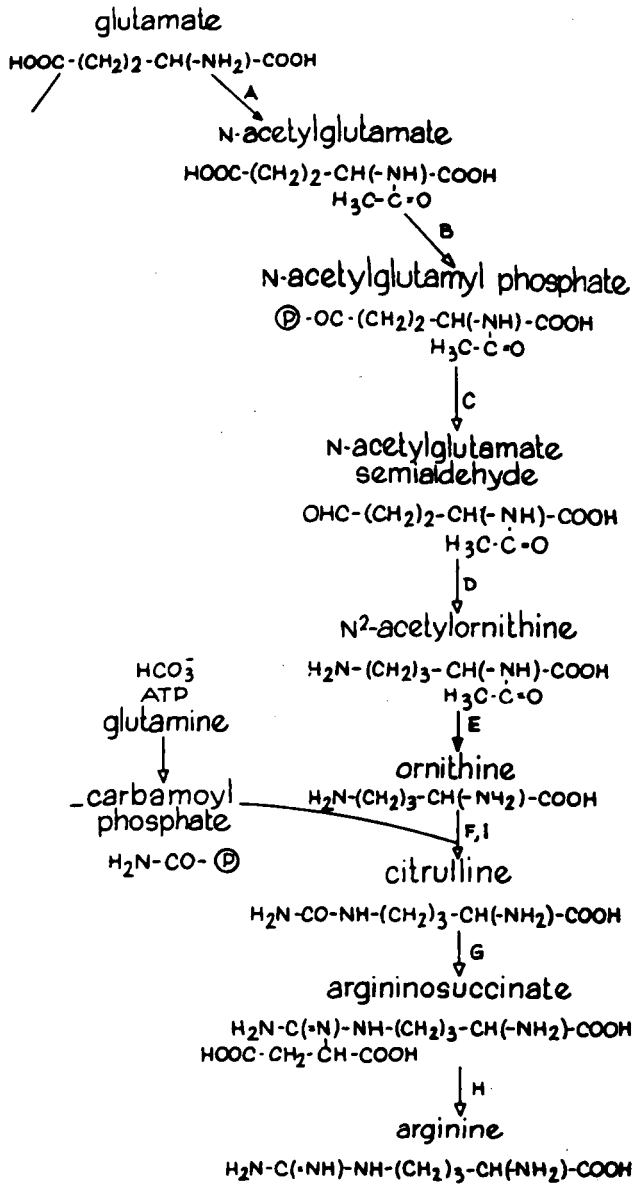


Fig. 02. Pathway of arginine synthesis in E. coli. (This figure is derived from reference 13.)

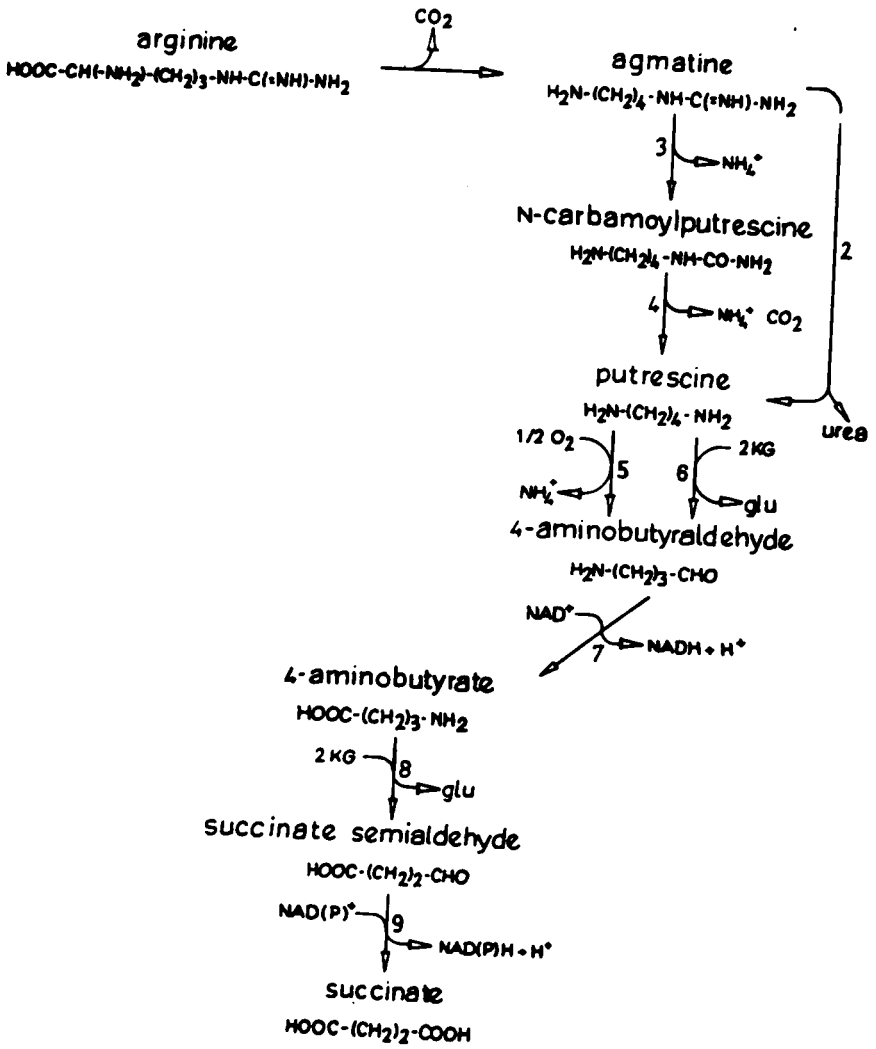


Fig. 03. Pathyway of arginine degradation in E. coli. (This figure is derived from reference 13.)

Part I

Analysis and sequence of the *speB* gene encoding agmatine ureohydrolase, a putrescine biosynthetic enzyme in *Escherichia coli*.

Abstract

The speB gene of E. coli encodes the enzyme agmatine ureohydrolase (AUH). AUH catalyses the hydrolysis of agmatine to urea and putrescine in one of the two polyamine biosynthetic pathways in E. coli. The plasmid pKA5, derived from an E. coli genomic library, was the source of a 2.97 kb restriction fragment containing the speB gene. Sequencing of this fragment revealed the presence of three intact open reading frames, ORF1 and ORF2 on one strand and ORF3 on the opposite strand, as well as a truncated open reading frame, ORF4, which terminates 92 kb upstream from ORF3. ORF2 and ORF3 are convergent, and overlap by approximately 85% of their sequence. ORF1 and ORF3 are separated by a sequence of two, 82 and 72 bp long, imperfect repeats (Rep1 and Rep2). Four palindromes, three of which overlap, are located within the repeats. ORF3 contained the coding sequence of the speB gene as it was the only ORF which expressed AUH activity. Two transcripts were detected from the speB gene: a shorter transcript, initiated 101 bp upstream from ORF3, and a longer polycistronic message, coding for both ORF3 and ORF4. The short transcript was abundantly expressed when the ORF4 sequences were deleted, but when the ORF4 and its upstream sequences were present the polycistronic message predominated, and the amount of the monocistronic message was drastically reduced. The promoter from which the shorter transcript was produced contained a -12 TATACT sequence but did not require any additional upstream sequences in order to permit expression of speB.

Acknowledgements

We thank Dr. Timothy Larson for E. coli CB806 and the plasmid pCB267; Dr. John Johnson for contributing ideas useful towards the development of our RNA purification method and S1-nuclease assay; Bob Moore and Dr. Dennis Dean for helpful discussions and Lisa Barroso for performing the primer extension assay on the 5'-end of the speB transcript. This work was supported by a grant (DMB-8508917) from the National Science Foundation (to S.M.B.) and by a graduate fellowship from the Department of Biochemistry and Nutrition (to M.B.W.S.).

INTRODUCTION

In Escherichia coli putrescine is synthesized either by decarboxylation of ornithine or by decarboxylation of arginine to agmatine followed by hydrolysis of agmatine to putrescine and urea (53). The last two reactions are catalyzed by the enzymes arginine decarboxylase (ADC) and agmatine ureohydrolase (AUH) respectively. The AUH protein has previously been purified from E. coli transformed with the plasmid pKA5; the enzymatic properties of AUH have been characterized (42). The subunit size of AUH, as deduced from its mobility on a sodium dodecyl sulfate polyacrylamide gel, is 38 Kd. The expression of AUH activity is antagonistically regulated by cAMP and agmatine. cAMP in the presence of the cAMP receptor protein (CRP) represses the expression of the speB gene, while agmatine induces it. These two modulators appear to act independently from each other (43); the mechanism of this differential regulation is unknown. The speB gene coding for AUH is located at approximately minute 63.5 on the E. coli chromosome. Although a large chromosomal fragment corresponding to this region, including the speB gene, is present in the pKA5 plasmid, the exact location of speB was not previously established. Here we report the nucleotide sequence of the speB structural gene as well as identification of the promoter responsible for transcription of one of two mRNAs encoding AUH. We also report mapping of the mRNA resulting from transcription initiated at this promoter.

Materials and Methods

Bacterial hosts, media and growth conditions.

E. coli CB806 (ΔlacZ, galK, phoA8, rpsL, thi, recA56) was used for all experiments involving the promoter vector pCB267 and its derivatives. E. coli DH5α (F^-, endA1, hsdR17, supE44, thi-1, recA1, gyrA96, relA1, phi80 Δ lacZ(d)M15), purchased from Bethesda Research Laboratories (BRL), was used as a host for all other plasmids.

The bacteria were grown in either LB (28) or TB (54) broth. An aliquot of cells from a frozen (-80°C) stock was inoculated into 3 ml of media containing 100 μg/ml ampicillin and grown overnight in a 37°C shaking waterbath. 0.5 ml of the overnight culture was inoculated into 50 ml of fresh media and grown to a density of about 90 Klett units. This culture was then used for either RNA purification or for preparation of cell extracts.

Vectors

Plasmid pBR322 was purchased from Bethesda Research Laboratories, Inc., plasmid pGEM®-3Z was purchased from Promega Corp. The promoter cloning vector pCB267 (43) was obtained from Dr. T. Larson the Department of Biochemistry and Nutrition (VPI & SU).

Purification of plasmid DNA.

50 ml of an overnight bacterial culture was centrifuged for 20 minutes at 4°C at 4000 x g. The cell pellet was washed with, and then resuspended in 5 ml of ice-cold STE (100 mM NaCl; 20 mM Tris-Cl, pH 7.5; 10 mM EDTA). 5 ml phenol/ chloroform (1:1) was added to the cell suspension and incubated for 20 minutes on a rotary shaker. The emulsified suspension

was centrifuged for 12 min at room temperature at 1200 x g; 2.5 volumes of ethanol was added to the aqueous phase, and the nucleic acid was precipitated at -20°C for one hour. The precipitate was collected by centrifugation at 13,400 x g for one hour; the pellet was redissolved in TE (10mM Tris-Cl, pH 8.0, 1 mM EDTA) and the plasmid DNA purified by CsCl banding (28).

Purification of total cellular RNA

The bacterial culture was treated as in the DNA purification procedure (see above) up to the first ethanol precipitation of the nucleic acids; except that 0.375 µl of ß-mercaptoethanol (BME) per ml of solution was added at each step. After addition of the phenol/chloroform the suspension was shaken for 30 seconds on a vortex mixer. The nucleic acid pellet was dissolved in 4 ml standard saline citrate (1 x SSC) (0.15 M NaCl, 0.015 M sodium citrate) - 1 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and 1.5 µl of BME. 2.5 ml of 7.5 M ammonium acetate was added, and the RNA was precipitated for 0.5 - 1 hour at 0°C. After centrifugation for 1 hour at 4°C at 13,400 x g, the RNA pellet was redissolved in 0.2 ml 1xSSC - 1mM HEPES. Sodium dodecyl sulfate (SDS) was added to 0.5% and the RNA was stored at -20°C.

AUH assay.

50 ml cultures were grown to a density of about 90 Klett units and pelleted by centrifugation for 20 minutes at 4°C at 4000 x g. The pellet was washed once with 5 ml, and then resuspended in 1 ml of ice-cold AUH reaction buffer (100 mM HEPES, 5 mM $MgCl_2$). The cells were disrupted by sonication: 5 x 1 minute exposures at 40% full scale using a microtip (Fisher, model 300 sonifier), spaced with 1 minute cooling on ice. Cell

debris was removed by two successive 10 minute centrifugations at 12,000 x g at 4°C. The supernatant constituted the cell extract used for assaying the AUH activity. The extracts were preincubated for 3 minutes at 37°C; the reaction was initiated by the addition of agmatine sulfate to 25 mM. At 1 minute intervals (over 5 minutes) 20 µl aliquots of the reaction mixture were transferred to 0.5 ml solution of Urease Buffer Reagent (Sigma Chemical Co., No. 640-5) at 0°C; the AUH reaction was stopped by this treatment. The urea content in these aliquots was measured with the Sigma Diagnostics® Urea Nitrogen Determination procedure No. 640.

Phosphatase A assays

The phosphatase A assays were performed as described by Schneider and Beck (44).

Generation of recombinant clones

Plasmids containing the DNA fragment of interest were hydrolysed with the appropriate restriction enzyme (purchased from Bethesda Research Laboratories, Boehringer Mannheim Corp. or Promega Corp.) as recommended by the manufacturer. The resulting DNA fragments were separated on a low gelling temperature agarose gel (NuSieve® GTG or SeaPlaque® purchased from FMC Bioproducts). The fragment of interest was identified and excised from the gel. Ligation of the fragment to a digested and dephosphorylated (28) vector, as well as the transformation were performed as described (22, 49).

Generation of the recombinant plasmids pKB1, pKB5, pKB2, pKB2S, pKB2H, pKG1 and pKG2.

The inserts of the deletion plasmids pKB1, pKB5, pKB2, pKB2S and pKB2H are shown schematically in Fig. 1B. The plasmid pKA5 (Fig. 1A) was digested with the restriction enzyme BamHI. Two of the four fragments were isolated (a unique BamHI site, not shown in Fig. 1A, resides in the pBR322 vector 375 basepairs beyond the right EcoRI insert/vector junction); the largest fragment, containing most of the pBR322 vector as well as the leftmost EcoRI - BamHI fragment of the pKA5 insert, was religated to form the plasmid pKB1. The adjacent 1.45 kb BamHI fragment was inserted into the BamHI site of the pKB1 plasmid to produce the plasmid pKB2. The plasmid pKB2 was cleaved with EcoRI and SmaI, the staggered ends were filled in with Klenow fragment of DNA polymerase I and religated to form the plasmid pKB2S. The pKB2H plasmid was constructed by cleavage of pKB2 with HindIII and religation. The insert of the plasmid pKB2S was subcloned into the vector pGEM®-3Z by a two step procedure in which the SmaI-BamHI fragment was first inserted into the SmaI/BamHI site of the vector, forming the plasmid pKG1, followed by insertion of the adjacent BamHI fragment into the pKG1 BamHI site in the same orientation as in the pKA5 and in the pKB2; this plasmid was called pKG2.

Generation of unidirectional deletions in the pKG2 plasmid.

The plasmid pKG2 was cleaved with the enzymes SphI and XbaI, or with the enzymes SacI and AvaI. These two pair of enzyme recognition sites are flanking either end of the insert in pKG2. Unidirectional deletions were then made in the pKG2 insert in both directions by the

ExoIII/ExoVII method (37). The derivative plasmids are shown in Fig. 2A.

Plasmid pKG3

Plasmid pKA5 was digested with PstI and the 2.95kb fragment was isolated and ligated into the PstI site of the pGEM®-3Z vector. In Fig. 2A only the AvaI-PstI fragment of the plasmid pKG3 is shown.

Plasmid pKG4

Plasmid pKG3 was cleaved with EcoRV and PstI. The 4.2 kb fragment was ligated into the pGEM®-3Z vector cleaved with SmaI and PstI (both EcoRV and SmaI produce blunt, mutually compatible, ends).

Plasmid pB15N

The plasmid pB15N was constructed by deletion of the SmaI-NruI fragment from the clone B15.

Plasmid pBB15N

In all the sequencing deletion clones designed "B", a HindIII restriction site, derived from the pGEM®-3Z vector, is flanking the junction between the deleted end of the insert and the vector. Construction of the plasmid pBB15N is illustrated schematicaly in Fig. 3. In the first step, the HindIII-NruI fragment, containing the ORF3, was cloned into pBR322, cleaved with HindIII and NruI; this resulted in a large deletion of the tetR gene within the vector. The resulting intermediate plasmid was deleted for the EcoRI-HindIII fragment to remove the upstream portion of the tetR promoter.

Construction of plasmids pCO3B15P and pCO3B32P.

Plasmids pCO3B15P and pCO3B32P contain the deletion proximal fragments of the plasmids B15 and B32 respectively. The insert in the plasmid B15 is shown in Fig. 2A. In the plasmid B32 an additional 36 basepairs are deleted. In both plasmids a HindIII site is located in the vector immediately adjacent to the right insert/ vector border. The exact location of this junction is shown in Fig. 5A. The plasmids B15 and B32 were digested with HindIII and BamHI. The approximately 850 basepair fragments were separately ligated into HindIII-BamHI sites of the vector pCB267 (43), upstream from the promoterless phoA gene.

Sequencing.

The sequencing was performed by the Sanger dideoxy (41) method using the Sequenase™ protocol and reagents purchased from the United States Biochemical Corporation. Fig. 2A shows the extent of sequence data derived from each of the deletion plasmids.

Primer extension and the S1-nuclease assays.

The primer extension and the S1-nuclease assays were performed as described (56). For the 5'-mapping of the speB transcript, a 20 nucleotide long synthetic oligonucleotide complementary to the 5'-region of the speB gene, was used as a primer. The primer was synthesized by the Department of Microbiology and Immunology, Virginia Commonwealth University. The exact location of the region to which the primer is complementary, is shown in Fig. 5. For use in the S1-nuclease assay the primer was extended on the B15 template; the primer was annealed to the B15 template as described in the Sequenase™ protocol and the primer/template mixture was treated as in the labelling reaction except for following modifications:

a mixture of 0.5 mM dCTP, dGTP and TTP was substituted for the sequenase labelling mixture; 5 µl of ^{35}S-dATP (1250Ci/mmol; 12.5 mCi/ml; 10.4 mmol/ml), purchased from E.I.Du Pont de Nemours and Co., was used per reaction; the reaction mixture was incubated at 37°C for 10 minutes, followed by addition of 2 µl of 0.5 mM solution of all four dNTP's and additional 5 minutes incubation at 37°C.

For use in the S1-nuclease mapping of the 3'-end of the speB transcript, the T7 universal primer of the GemSeq K/RT™ sequencing system, purchased from Promega Biotech Corporation, was annealed to the BglII digested pB15N template and extended as described above.

Northern hybridization

Total cellular RNA from the strains DH5α [pKA5] and DH5α [pBB15N] was electrophoresed in duplicate on an agarose/formaldehyde gel. The electrophoresis, Northern transfer and hybridizations were performed as described by Selden (46). The speA specific probe was complementary to nucleotides 605-462 of the speB gene (Fig. 6), and the ORF4 specific probe was complementary to sequences 289-545 upstream from the 3'-end of ORF4. The probes were constructed by extension of the T7 universal primer (as in the S1-nuclease mapping, described above) using the PstI digested plasmids A53 and A42 (shown in Fig. 2A) as templates, respectively.

RESULTS

1. Location of the sequences necessary for AUH expression.

Plasmid pKA5 contains a 7.5 kb insert, derived from an E. coli genomic library (12), cloned into the EcoRI site of the plasmid pBR322. This insert was shown to contain the genes speB, speA, and metK, encoding agmatine ureohydrolase, arginine decarboxylase and methionine adenosyltransferase respectively (10). We updated the restriction map of the pKA5 plasmid (Fig. 1A). In order to localize the restriction fragment that contains the intact speB gene, we constructed a series of deletions and assayed the deletion clones for the AUH activity (Fig. 1B). The clones bearing pKB1 and pKB5 plasmids were negative for AUH activity. When the inserts from these plasmids were reconstituted in the original order in the plasmid pKB2, AUH activity was restored indicating that the internal BamHI site is interrupting the speB gene. Further deletions in the rightward direction from the EcoRI site revealed that the 460 bp SmaI - HindIII fragment is necessary for speB expression. Previous results indicated that removal of sequences to the left or to the right of the unique BalI site also inactivates the speB gene [C. Satishchandran; PhD Thesis (1985)]. Therefore we concluded that the shortest available restriction fragment that contains an intact speB gene is the one present in the plasmid pKB2S.

2. Nucleotide sequence of the pKB2S insert.

We subcloned the insert of the plasmid pKB2S into the vector pGEM®-3Z, creating the plasmid pKG2. A chance existed that during the two step cloning procedure we may have left behind a small, undetected BamHI

fragment. To insure this was not the case we later subcloned the fragment PstI - EcoRV from the pKA5 plasmid into the pGEM®-3Z vector (plasmid pKG4) and sequenced it through the intact BamHI site thus validating the reconstitution. Fig. 2A shows the strategy used for sequencing the 2.97 kb insert of the pKG2 plasmid. Sequence analysis (Fig. 2B) revealed the presence of three complete open reading frames (ORF1, ORF2, and ORF3) and the 3' end of another open reading frame (ORF4) terminating 92 nucleotides upstream from ORF3. ORF1 and ORF2 have the same orientation and are separated by 31 nucleotides. ORF2 and ORF3 are divergently oriented and overlap by 969 nucleotides. A sequence of two 82 bp long imperfect tandem repeats (Rep 1 and Rep 2), with 70 bp homology, extend between the diverging 3' ends of ORF1 and ORF3, and overlap the 5' end of ORF2. The two repeats are 31 bp apart. The region of repeats is very G-C rich and contains 4 palindromic sequences, three of which are overlapping as shown in Fig. 7. A 37 nucleotide long palindromic structure is located at the 5' end of ORF3. It has the potential of forming a loop with 15 nucleotide long stem, including one mismatch and a seven nucleotide long "bubble". The palindrome overlaps the start of ORF3 by 15 nucleotides and is terminated in the ORF3 direction by five T's.

3. Identification of the speB gene.

To establish which of the three ORF's encodes AUH, we assayed three of the deletion clones for AUH activity (Fig. 2A). Only clone A14, containing ORF2 and ORF3, overexpressed AUH activity (Fig. 2C). Since neither clone B31, containing ORF1, nor clone B33, containing ORF1 and ORF2, exhibited increased AUH activity, ORF3 must contain the coding sequence of the speB gene. We also performed an S1-analysis on the mRNA purified

from the A14 clone using a primer complementary to the sequences upstream from the ORF3 (data not shown). This experiment revealed that most of the speB transcripts are initiated at a site 92 bp beyond the insert/-vector junction. This nucleotide is the transcription start point for the α-peptide of ß-galactosidase in pGEM®-3Z. Thus the speB gene in the A14 clone is transcribed from the lacZ promoter of the vector. Thirty-one basepairs downstream from the start of ORF3 is a Shine-Dalgarno consensus sequence followed 7 basepairs downstream by a methionine codon (Fig. 5A and Fig. 7). Both these sites are deleted in the clone B33 (see Fig. 5A), which does not express AUH activity. Together with the observation that the speB expression in the A14 clone is directed from the lacZ promoter of the vector, this constitutes a strong indication that the above mentioned methionine codon must be the initiation codon for speB translation.

4. Mapping of the 5' end of the speB transcript and the location of the speB promoter.

We used primer extension and the S1-nuclease methods to identify the transcription start of the speB gene. With the primer extension method (Fig. 4A) a major band appears one nucleotide prior to the start of the 37 nucleotide long palindromic sequence (shown in Fig. 5 and 7). The S1-nuclease assays, performed on the total cellular RNA's derived from clones bearing the plasmids A14 (not shown), pKA5 or pBB15N (Fig. 4 A, B and C), all reveal strong bands at positions corresponding to the one nucleotide mismatch in the stem. These results strongly indicate a presence of a cruciform structure within the DNA/RNA hybrid prior to the S1-nuclease digestion. Upstream from this sequence two bands are visible in the

lane representing RNA derived from the pKA5 bearing clone; a strong band at position 113 bp, and a very weak band at position 102 upstream from the start of ORF3. The position 113 bp upstream from ORF3 marks the insert/vector junction of the B15 plasmid (see Fig. 5 for the exact limit of the B15 deletion clone) which was the template for the extension of the primer used in this S1-analysis. Thus, the sequences of the primer that extend beyond this point are complementary to the template but not to the upstream pKA5 sequences. Therefore a band at this position signifies a mRNA species that is initiated upstream from this point. When the S1-analysis was repeated using a primer extended on a template in which homology to the transcript ends at position 160 bp upstream from the start of ORF3 (data not shown), two bands still appear on the autoradiogram; the lower band is at the same position as before, but the higher band is at position 160 bp upstream of ORF3. Thus the longer speB transcript must be initiated more then 160 bp upstream from ORF3. The faint lower band, visible in this experiment signifies a transcript starting 102 bases upstream from ORF3. Twelve bases upstream from the nucleotide equivalent to this band is a TATACT sequence which is strongly homologous to the E. coli -10 TATAAT promoter consensus sequence. Since this sequence marks the end of the B15 insert (see Fig. 5A), we used this deletion to map the location of the promoter initiating the shorter speB transcript. We isolated the speB gene fragment, present in the deletion plasmid B15, by subcloning it into the pBR322 vector (Fig. 3). The strain harboring the resulting plasmid pBB15N exhibited elevated AUH activity (Fig. 5C). When mRNA derived from this clone was used in the S1-analysis (Fig. 4C), a band appeared at the same location as the lower

band from the analysis of the pKA5 RNA (Fig. 4B), while the upper band disappeared. These results confirmed the presence of two speB transcripts and suggested that the shorter transcript is initiated from a promoter that does not require any sequences beyond position -12 for activation. In the construction of the pBB15N plasmid we removed the entire sequence containing the tetR promoter of the vector to avoid interference with the speB expression. Still a small possibility existed that presence of some unidentified sequences upstream from the vector/insert junction might have resulted in a coincidental complementation of the "truncated" speB promoter in the insert. To exclude this possibility we constructed the plasmid pCO3B15P. This plasmid contains the upstream fragment of the speB gene identical to the one present in pBB15N, joined in a transcriptional fusion to the promoterless phoA gene within the pCB267 vector. As seen in Fig. 5B, the expression of the phoA gene was activated in this construct. When additional 36 bases were deleted, as in the plasmid pCO3B32P which represents a derivative of the deletion clone B32, the promoter activity was drastically reduced.

5. Establishing the origin of the longer speB transcript.

To establish if the longer speB transcript is coding both for AUH and for ORF4, the RNA from pKA5 and from pBB15N bearing strains were hybridized to a probe specific for either speB or ORF4 (Fig. 6). The autoradiograph revealed that a speB probe hybridized only to a 3.2 kb band in the RNA from pKA5 and to one 1.5 kb band in the RNA from pBB15N. The sizes of these transcripts conform to the molecular weights for the polycistronic and monocistronic messages expected from our RNA mapping and ORF length analyses. This confirms the observation from the S1-analyses

(Fig. 4B and 4C) that the shorter transcript is produced by a strain bearing pKA5 in trace amounts as compared to the amount of the longer transcript; in contrast the strain harbouring the pBB15N produces comparatively high quantities of the shorter transcript. On the membrane hybridized to the ORF4 probe the band representing the long transcript is visible in the pKA5 transformed cells while the shorter transcript is no longer detected in the pBB15N transformed cells. These results indicate strongly that the longer transcript is a polycistronic message encoding both the speB and the ORF4 sequences. Additionally, since no other RNA crossreacts with the ORF4 probe, this longer transcript must be the major product of the genes represented by ORF4 and speB.

6. Mapping of the 3' end of the speB transcript.

The 3'-end of the speB transcript was mapped by S1-analysis of the total cellular RNA derived from the clone pBB15N. The primer was homologous to the sequences flanking and overlapping the 3'-end of ORF3 (nucleotide 1304 to nucleotide 939, as depicted in Fig. 7). Four bands of equal intensity appear on the autoradiogram (Fig. 4D); they are positioned at a distance of 254-257 nucleotides from the 3'-end of the primer. This corresponds to the position 1193-1196 of the speB transcript (Fig. 7). This position marks the sequences directly downstream from the last of the three overlapping palindromes present within the direct repeat region (Fig. 7 and Fig. 2B). Thus this palindrome is the transcription termination signal in the transcription of speB mRNA.

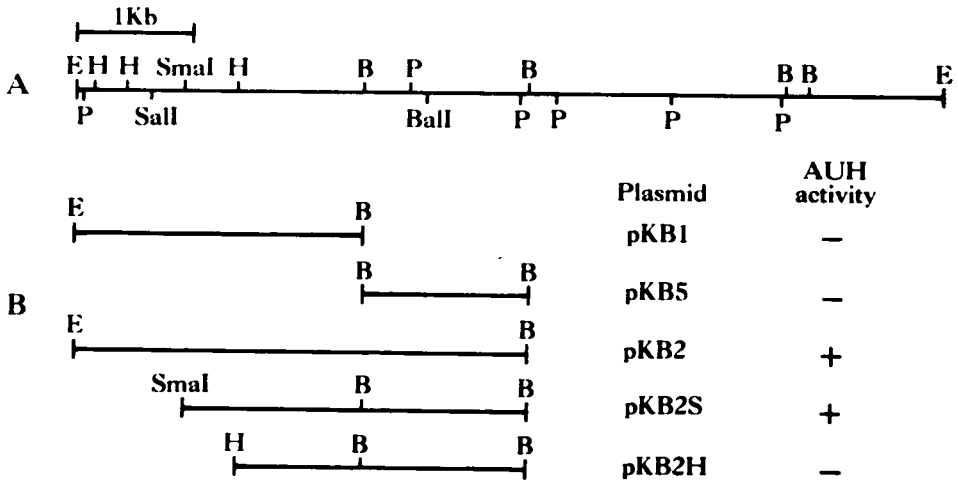
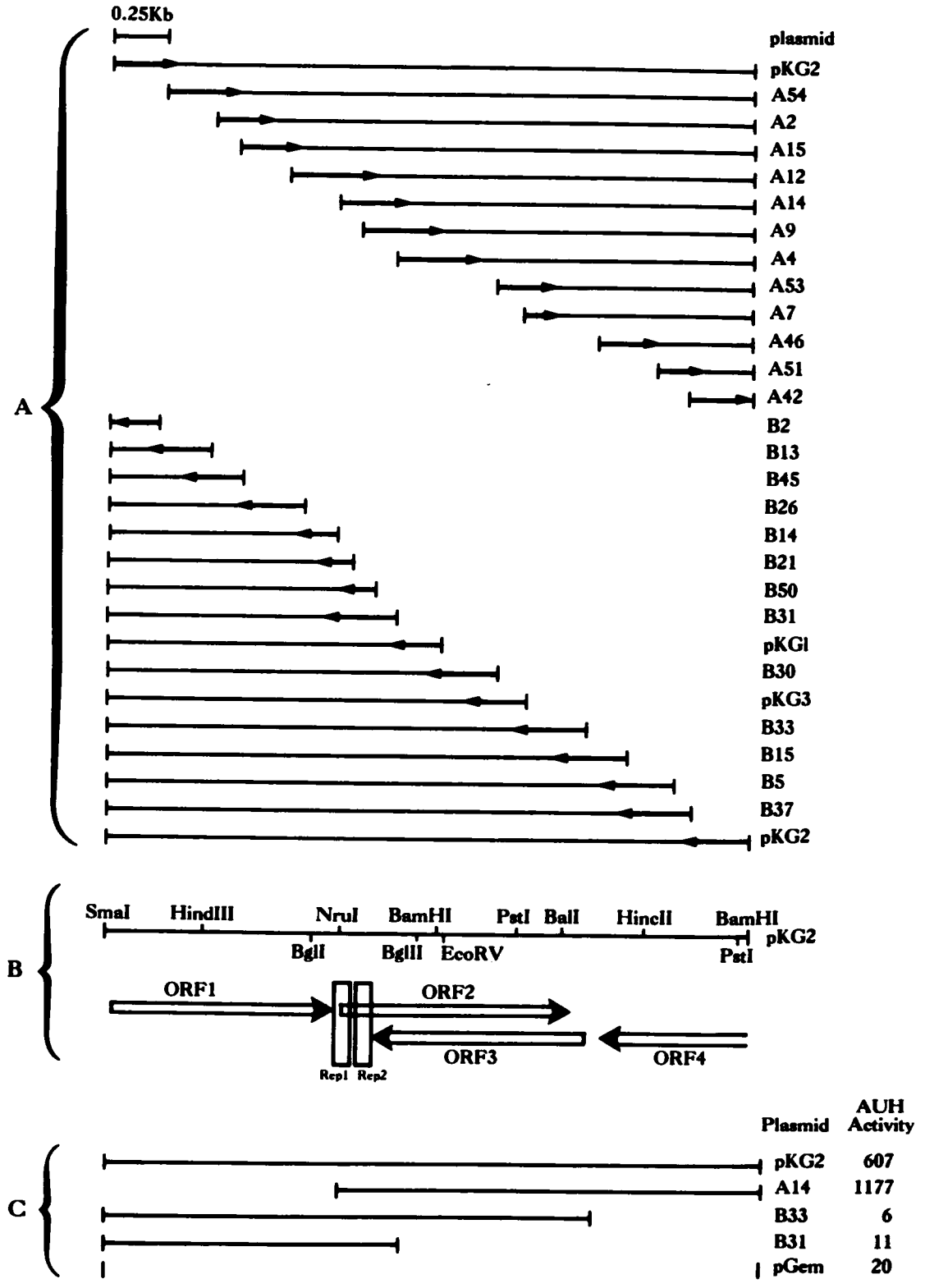


Fig. 1

Mapping of the location of the speB gene. A. Restriction map of the E. coli chromosome fragment present in the plasmid pKA5. Symbols: E- EcoRI;H - HindIII;P -PstI; B- BamHI. B. Subclones of the pKA5 insert used to determine the location of the speB gene. The ability of the plasmids to express the AUH activity was determined by enzyme assays; the vector is pBR322.

Fig.2

Sequencing strategy of the pKG2 plasmid, sequence analysis and identification of the speB gene. A. The deletion clones used for sequencing of the pKG2 insert.The short vertical lines represent the junction with the vector (pGEM-3Z). Arrows represent the extent and direction of sequencing. B. Top; restriction sites deduced from the sequence data; Bottom: location of the open reading frames (ORFs) as deduced from the sequencing data. Repl and Rep2 boxes represent the region of long tandem repeats. C. The ability of the deletion plasmids from A to express AUH activity in E. coli DH5α. The specific activity is expressed in nmoles of urea/min/mg protein.


A
0.25Kb
plasmid
pKG2
A54
A2
A15
A12
A14
A9
A4
A53
A7
A46
A51
A42
B2
B13
B45
B26
B14
B21
B50
B31
pKGl
B30
pKG3
B33
B15
B5
B37
pKG2
B
SmaI
HindIII
NruI
BamHI
PstI
BalI
HincII
BamHI
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BglI
BglII
EcoRV
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ORF1
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B33 6
B31 11
pGem 20

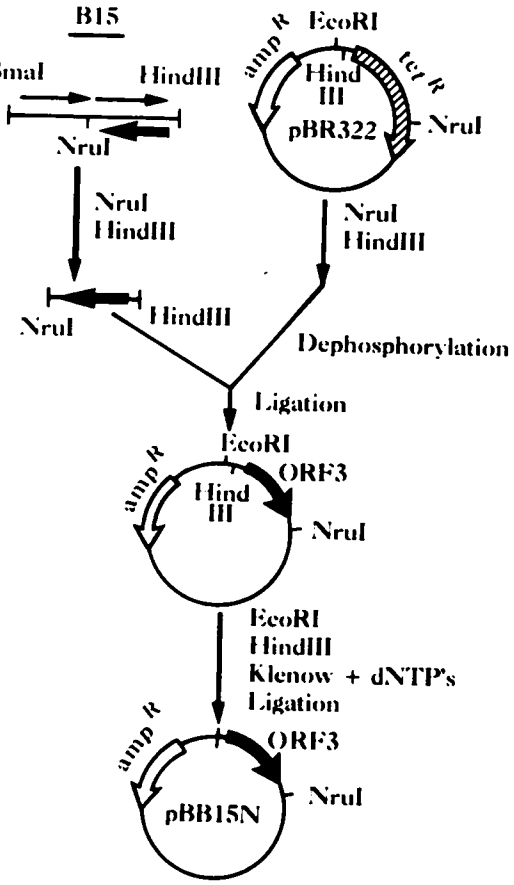


Fig. 3

Subcloning of speB gene into pBR322. The solid arrow represents ORF3. The procedures are described in Materials and Methods.

Fig. 4

Mapping of the 3'- and the 5'-end of the speB transcript. All sequencing reactions and the 5'-end mapping experiments (A, B, C) were performed using the primer depicted in Fig. 5A. Panel A. Primer extension mapping of the 5'-end of the speB transcript, Line 1: RNA derived from clone A14, Line 2-5:sequencing reactions on the A14 template. Panel B. S1-nuclease assay on RNA derived from clone pKA5. Lines 1-4:sequencing reactions on B15 template; Lines 5-8: the S1-nuclease assays on (5)250 µg RNA; (6)200 µg RNA;(7) 150 µg RNA;(8)100 µg RNA. Hybridizations were performed at 39°C. Panel C.S1-nuclease assay on RNA derived from the pBB15N clone. Lanes 1-4 as in panel B; Line 5: the S1-nuclease assay on 200 µg of RNA. Hybridization was performed at 45°C. Panel D.S1-nuclease mapping of the 3'end of the speB transcript. Lanes 1-4, sequencing reactions on plasmid B32; Lane 5:S1-assay on 200 mg of pBB15N RNA. The hybridization was performed at 50°C.

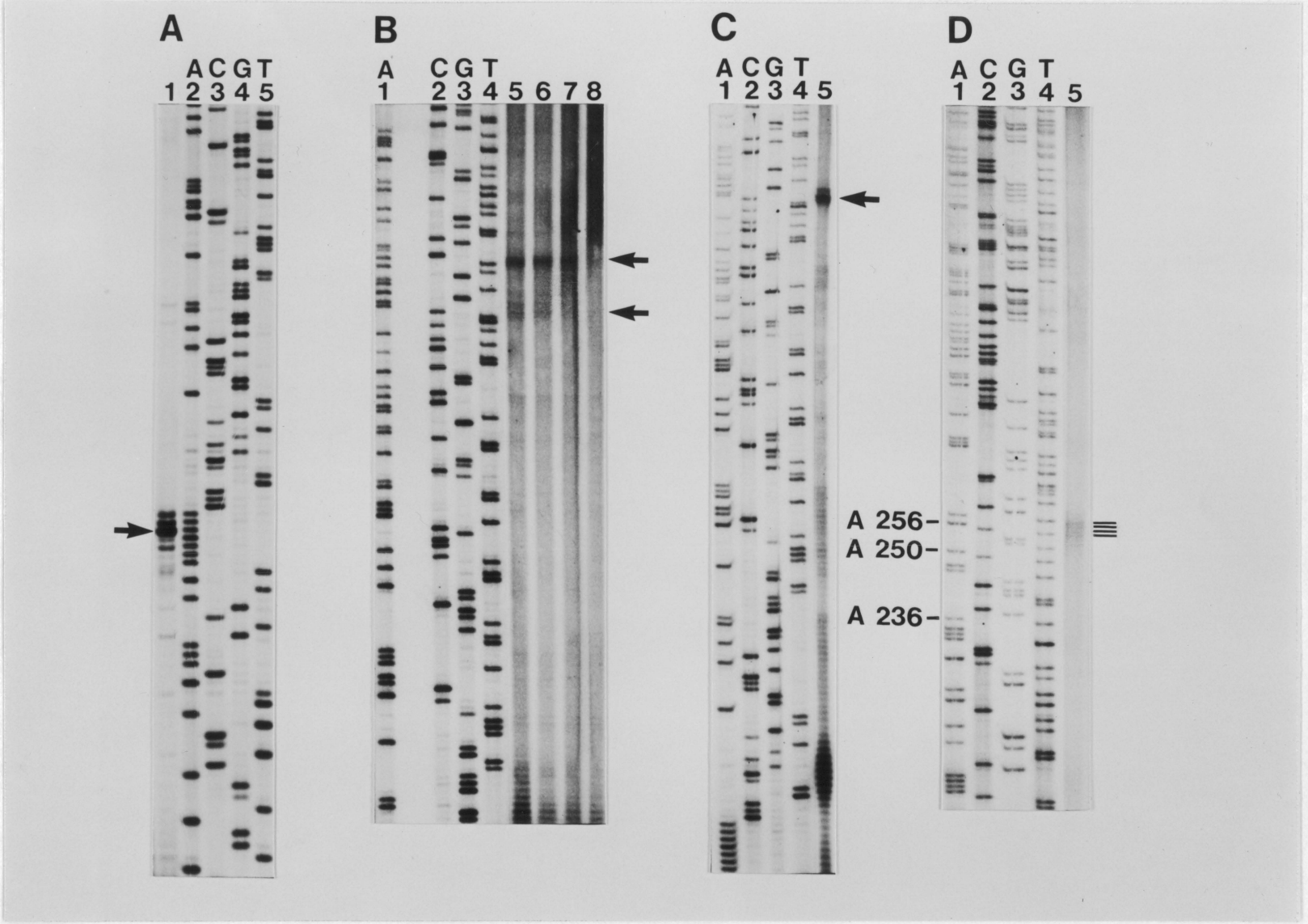
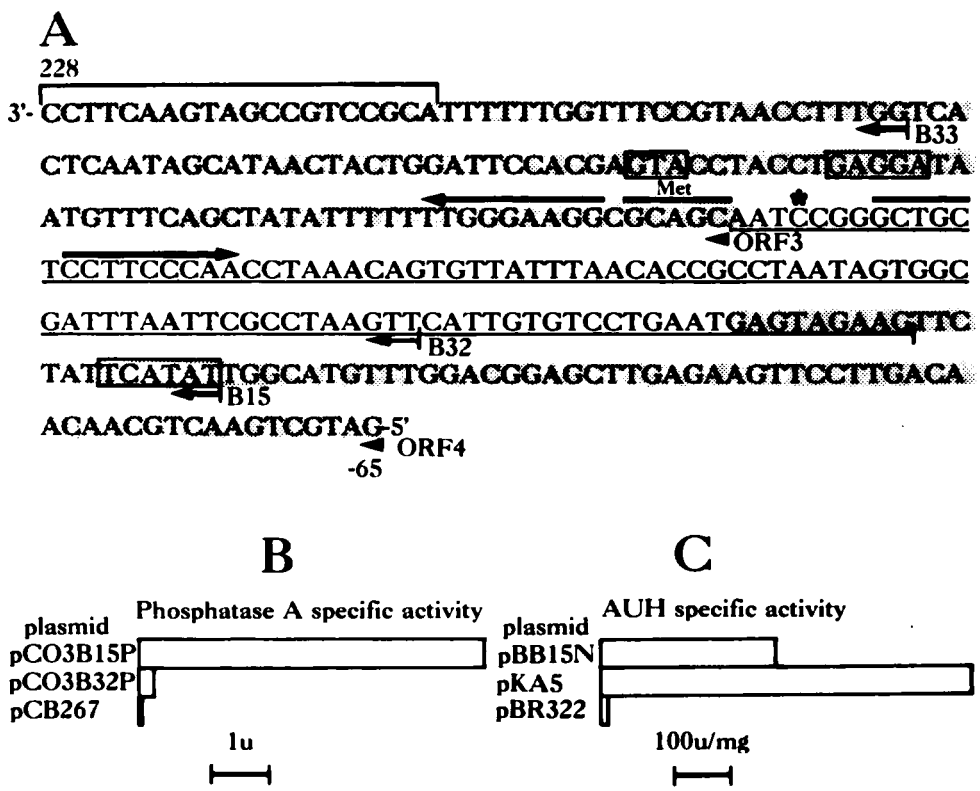

A
A C G T
1 2 3 4 5
B
A C G T
1 2 3 4 5 6 7 8
C
A C G T
1 2 3 4 5
A 256-
A 250-
A 236-
D
A C G T
1 2 3 4 5

Fig. 5

Localization of the speB promoter by deletion analysis. A. The upstream sequences of the coding strand of the speB gene; the orientation is as in Fig. 2B. The bracket over the first twenty nucleotides indicates the region of complementarity to the oligonucleotide primer used in the 5'-mapping experiments. Small arrows with clone names to the right indicate the end of the inserts in the named deletion plasmids. The methionine initiation codon as well as the sequences with homology to the ribosome binding site and to the -10 promoter consensus are boxed. The star indicates the center of symmetry in the palindromic sequence that is marked by an arrow over the participating nucleotides. The nucleotide sequences that are part of ORF3 or ORF4 are shadowed. The leader sequence that precedes ORF3 is underlined. B. The ability of the DNA fragments derived from the indicated deletion clones to promote transcription of the promoterless phoA gene. C. The ability of indicated deletion clones to express AUH activity.
Phosphatase A specific activity 1U = $OD_{410} \times 10^3$/vol of culture (ml) x time(min) x cell density (Klett units). AUH activity 1U = 1 nmole urea/min/mg protein.


A
228
3'- CCTTCAAGTAGCCGTCCGCATTTTTTGGTTTCCGTAACCTTTGGTCA
B33
CTCAATAGCATAACTACTGGATTCCACGAGTACCTACCTGAGGATA
Met
ATGTTTCAGCTATATTTTTTTGGGAAGGCGCAGCAATCCGGGCTGC
ORF3
TCCTTCCCAACCTAAACAGTGTTATTTAACACCGCCTAATAGTGGC
GATTTAATTCGCCTAAGTTCATTGTGTCCTGAATGAGTAGAAGTTC
B32
TATTCATATTGGCATGTTTGGACGGAGCTTGAGAAGTTCCTTGACA
B15
ACAACGTCAAGTCGTAG-5'
ORF4
-65
B
Phosphatase A specific activity
plasmid
pCO3B15P
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pCB267
1u
C
AUH specific activity
plasmid
pBB15N
pKA5
pBR322
100u/mg

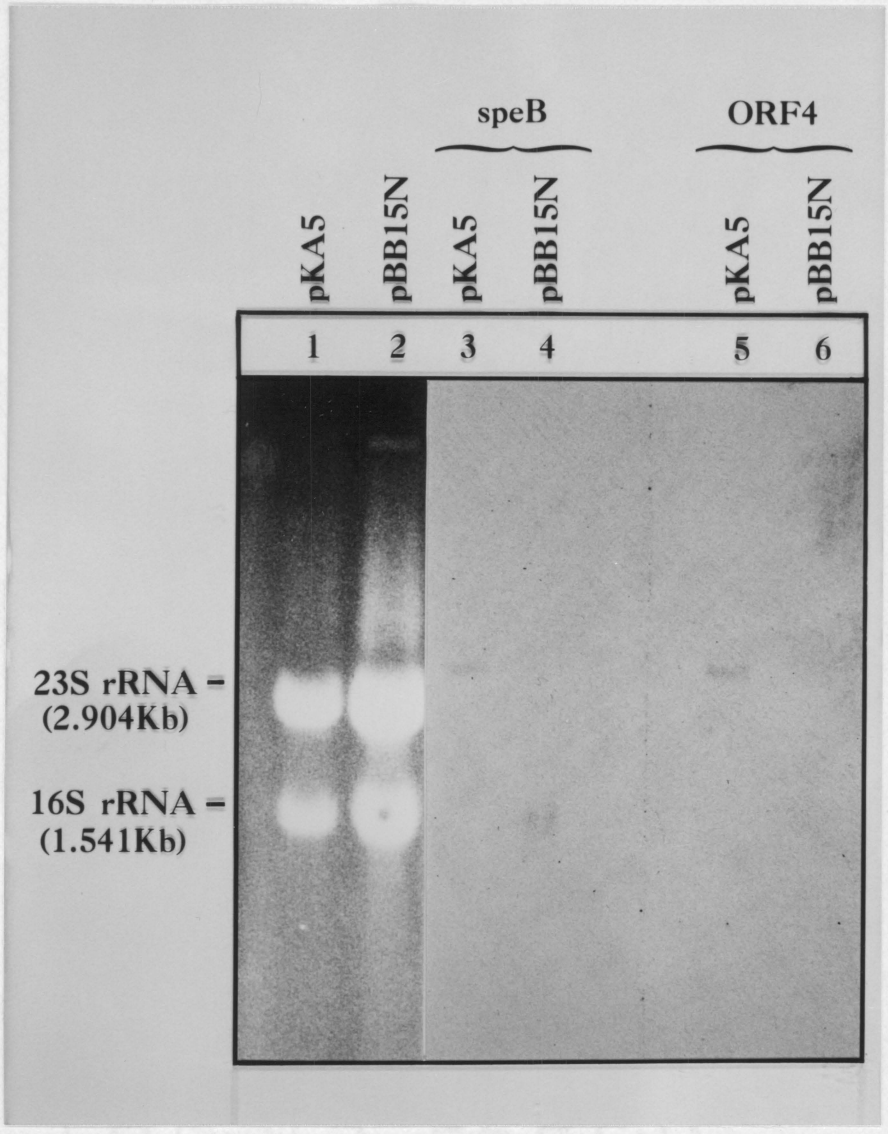
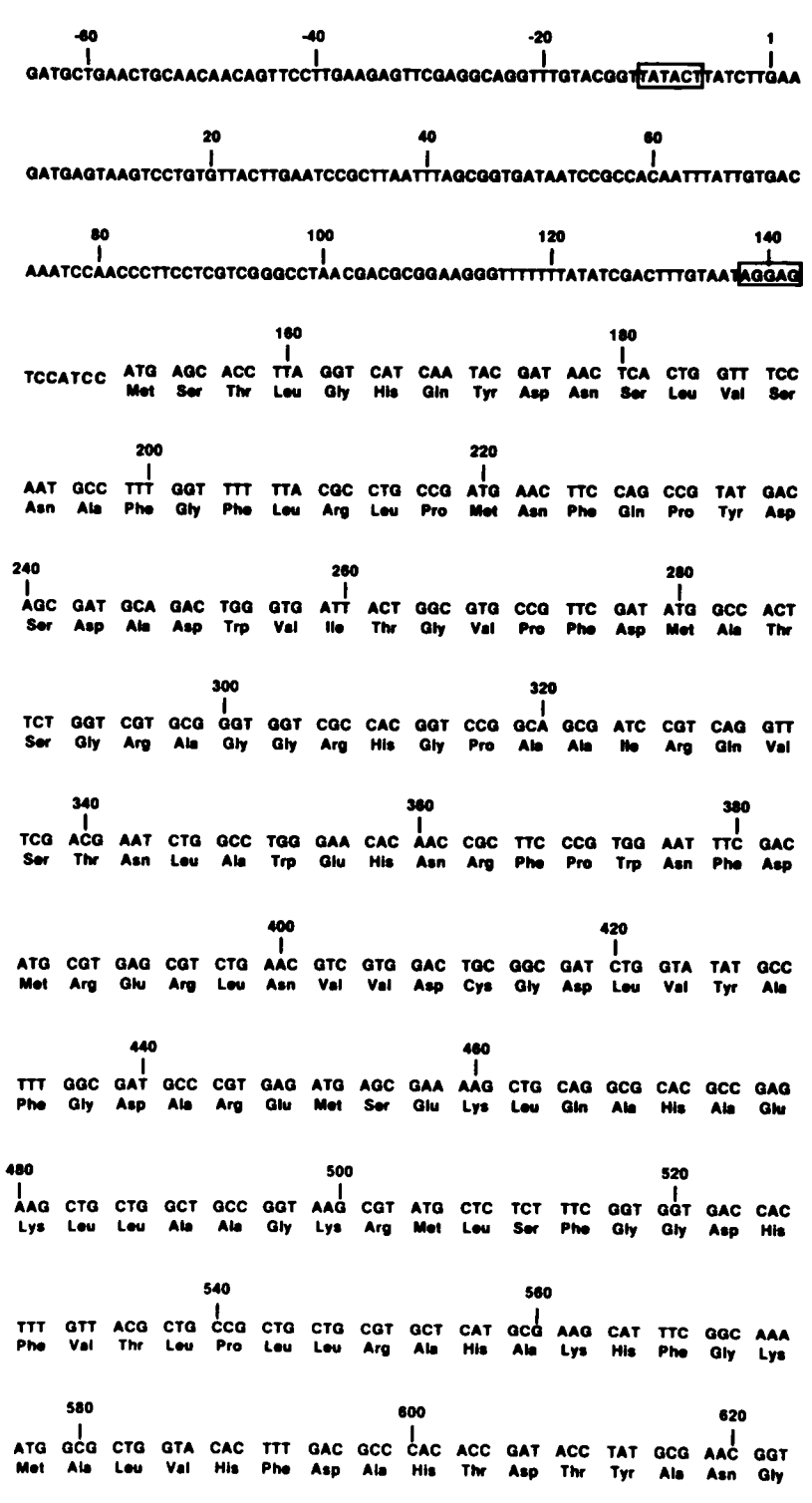


Fig. 6

Northern hybridization of RNA derived from E. coli DH5α bearing the plasmid pKA5 or pBB15N. Lanes 1 and 2: ethidium bromide stained gel before transfer. Lanes 3-6: autoradiogram of the hybridized membranes. The plasmid is indicated above the lane and the probe is indicated above the bracket.

Fig. 7

Nucleotide and amino acid sequence of the speB gene. The amino acid sequence of the speB gene product, starting with the initiating methionine codon, as deduced from the sequence, are given below the codons. The promoter consensus sequence and the ribosome binding site are boxed. Start of transcription is assigned #1. The homologous nucleotides within the long tandem repeat sequence are underlined. The palindromic sequences are indicated by arrows with stars at the center of symmetry. The thick lined arrow indicates the palindrome which terminates the speB transcript.


-60 -40 -20 1
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20 40 60
GATGAGTAAGTCCTGTGTTACTTGAATCCGCTTAATTTAGCGGTGATAATCCGCCACAATTTATTGTGAC
80 100 120 140
AAATCCAACCCTTCCTCGTCGGGCCTAACGACGCGGAAGGGTTTTTTTATATCGACTTTGTAATAGGAG
160 180
TCCATCC ATG AGC ACC TTA GGT CAT CAA TAC GAT AAC TCA CTG GTT TCC
Met Ser Thr Leu Gly His Gln Tyr Asp Asn Ser Leu Val Ser
200 220
AAT GCC TTT GGT TTT TTA CGC CTG CCG ATG AAC TTC CAG CCG TAT GAC
Asn Ala Phe Gly Phe Leu Arg Leu Pro Met Asn Phe Gln Pro Tyr Asp
240 260 280
AGC GAT GCA GAC TGG GTG ATT ACT GGC GTG CCG TTC GAT ATG GCC ACT
Ser Asp Ala Asp Trp Val Ile Thr Gly Val Pro Phe Asp Met Ala Thr
300 320
TCT GGT CGT GCG GGT GGT CGC CAC GGT CCG GCA GCG ATC CGT CAG GTT
Ser Gly Arg Ala Gly Gly Arg His Gly Pro Ala Ala Ile Arg Gln Val
340 360 380
TCG ACG AAT CTG GCC TGG GAA CAC AAC CGC TTC CCG TGG AAT TTC GAC
Ser Thr Asn Leu Ala Trp Glu His Asn Arg Phe Pro Trp Asn Phe Asp
400 420
ATG CGT GAG CGT CTG AAC GTC GTG GAC TGC GGC GAT CTG GTA TAT GCC
Met Arg Glu Arg Leu Asn Val Val Asp Cys Gly Asp Leu Val Tyr Ala
440 460
TTT GGC GAT GCC CGT GAG ATG AGC GAA AAG CTG CAG GCG CAC GCC GAG
Phe Gly Asp Ala Arg Glu Met Ser Glu Lys Leu Gln Ala His Ala Glu
480 500 520
AAG CTG CTG GCT GCC GGT AAG CGT ATG CTC TCT TTC GGT GGT GAC CAC
Lys Leu Leu Ala Ala Gly Lys Arg Met Leu Ser Phe Gly Gly Asp His
540 560
TTT GTT ACG CTG CCG CTG CTG CGT GCT CAT GCG AAG CAT TTC GGC AAA
Phe Val Thr Leu Pro Leu Leu Arg Ala His Ala Lys His Phe Gly Lys
580 600 620
ATG GCG CTG GTA CAC TTT GAC GCC CAC ACC GAT ACC TAT GCG AAC GGT
Met Ala Leu Val His Phe Asp Ala His Thr Asp Thr Tyr Ala Asn Gly

Fig. 7

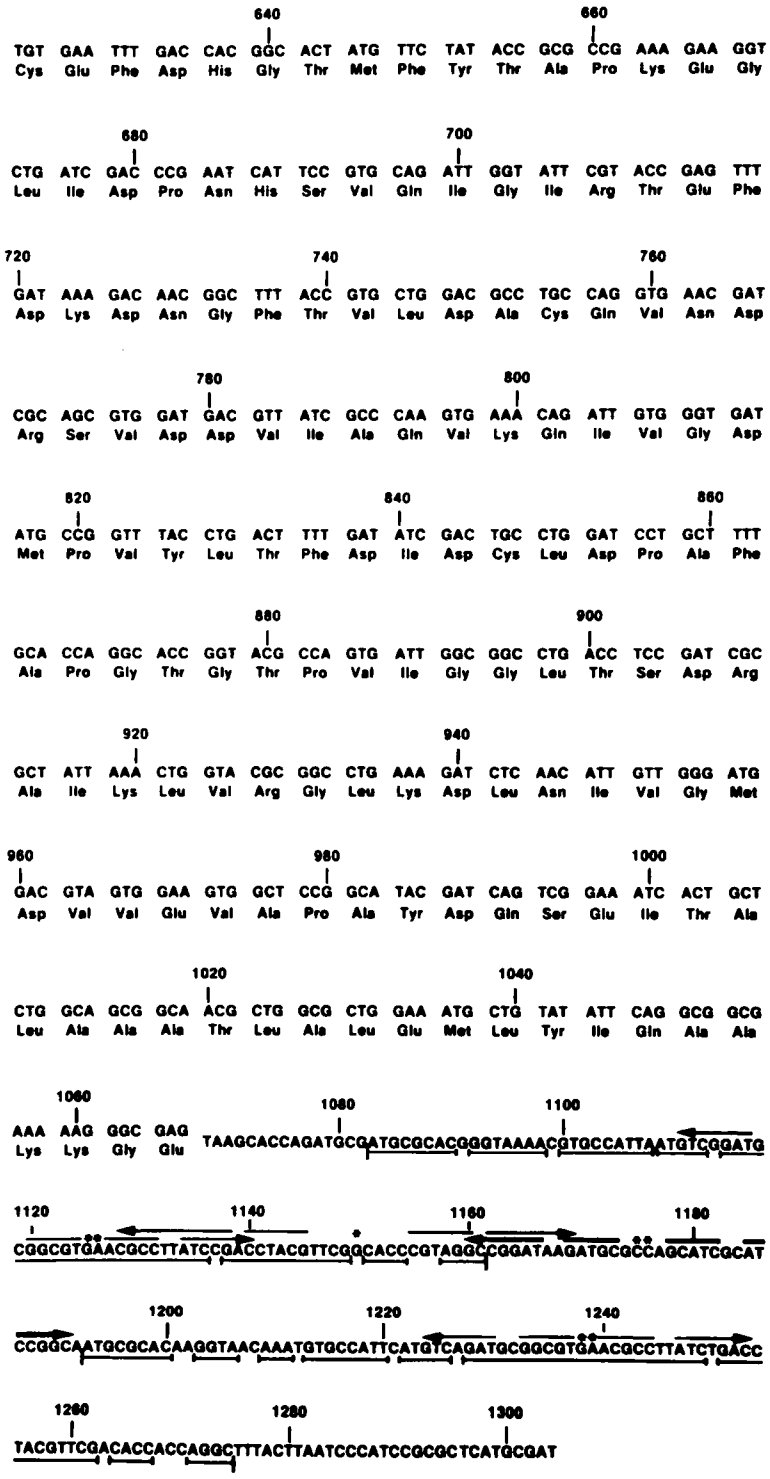


Figure 7 (continued)

Discussion

Within the 7.5 kb insert of the pKA5 plasmid we have identified and sequenced the DNA necessary for the expression of AUH activity. Sequence analysis revealed the presence of three open reading frames, ORF1, ORF2 and ORF3. ORF1 and ORF2 are arranged in tandem and are separated by 31 nucleotides, while ORF3 is on the opposite strand and overlaps ORF2 by 864 nucleotides. Among deletion clones harboring the individual ORF's, only the clone with ORF3 overexpresses AUH activity. Thus ORF3 represents the coding region of the speB gene. The molecular mass of AUH, deduced from its sequence, is 33,409 daltons. The molecular mass previously established from the mobility of the purified enzyme on a SDS polyacrylamide gel is 38 kd (42). We do not know the reason for the 4.6 kd discrepancy.

Two imperfect (86% identity) tandem repeats, 82 and 72 nucleotides long, are located between the 3'-ends of the converging ORF1 and ORF3 sequences (Fig. 2B). Four palindromic sequences are present within this region. Three of them are overlapping (Fig. 7). We have shown that the speB transcript is terminated at the end of the third overlapping palindrome distal from the speB gene.

S1-mapping of the 5'-end of the speB transcript revealed that two species of mRNA are involved in synthesis of the AUH protein. The start and the end point of the shorter transcript, as well as the location of the promoter, from which it is initiated were mapped. An unusual feature of this promoter is that while it contains a sequence, TATACT at position-12, which differs by only one nucleotide from the TATAAT -10 consensus for the σ^{70} recognised promoters, there were no upstream sequences resembling

the -35 consensus (TTGACA); nor were they necessary for the initiation of transcription from this promoter (Fig. 5).

The presence of a longer transcript containing the speB message was evident from the results of the S1-mapping experiments, illustrated in Fig. 4B and 4C. Since the promoter of the speB gene overlaps the sequence of ORF4 (which represents the speA gene encoding arginine decarboxylase, Robert Moore, personal communication, VPI & SU), this longer transcript could originate either from a second promoter within the speA gene or from the speA promoter itself. The results of the Northern hybridization experiment (Fig. 6) confirmed the latter hypothesis. Thus AUH in the E. coli is encoded by one open reading frame, but synthesized from two transcripts: one monocistronic, directed from the speB promoter, and one polycistronic, directed from the (speA) ORF4 promoter. From the relative intensities of the bands representing these two transcripts in the S1-analyses and in the Northern hybridization experiment, the transcription of the monocistronic speB message appears repressed when the polycistronic transcript is produced. The cellular environment in the strain DH5α [pKA5], in which the speB is expressed primarily as a polycistronic message differs only from the one in the strain DH5α [pBB15N], in which the monocistronic message is primarily expressed, by the presence of increased amounts of proteins (or RNA) encoded by the pKA5 insert. Consequently, the switch between polycistronic and monocistronic must be mediated by the DNA flanking speB (which includes any product of the speA promoter) rather than by an intervention of a gene product encoded elsewhere on the chromosome.

In the course of our analysis of the 5'-end of the speB transcript we have discovered that a 37 basepair long, G-C rich palindromic sequence present at the start of ORF3, forms a loop in both the RNA and the DNA. This structure has a 15 basepair long stem (including one bubble due to one basepair mismatch) and a 7 base long single stranded loop. This cruciform structure formed within the RNA:DNA heteroduplex survives at the highest stringency prehybridization conditions used in our S1-analyses. It seems likely, therefore, that it might also form in vivo within RNA, DNA or both. The palindrome ends with a track of seven T's in the speA and ORF4 coding direction, resembling a rho-independent terminator structure. However, it does not stop the transcription from the speB promoter initiated 79 bases upstream, nor does it prevent the read through from speA into speB resulting in the polycistronic transcript. Nevertheless, it is possible that this structure might be involved in regulation of expression of the speB gene.

Part II

The influence of cyclic AMP, agmatine and flanking genes on expression of the *speB* gene encoding agmatine ureohydrolase in *Escherichia coli*.

Abstract

The speB gene of E. coli encodes the putrescine biosynthetic enzyme, agmatine ureohydrolase (AUH). The speB gene is transcribed either from its own promoter or as a polycistronic message initiating from the promoter of the speA gene, encoding arginine decarboxylase (ADC). Two open reading frames are present on the complementary strand of speB: ORF1 and ORF2 respectively; ORF2 overlaps the speB coding region. Deletion of a 460 bp fragment comprising the 5'-region of ORF1 from a plasmid containing ORF1, ORF2 and speB reduced the activity of AUH by 83%. The 460 bp fragment contains two promoters: one in the direction of ORF1 and one strong divergent promoter. ORF2 does not have its own promoter but there is very low level of ORF2 transcription initiated from the ORF1 promoter. The presence of ORF1 does not stimulate ß-galactosidase expressed from the speB promoter fused to lacZ. Agmatine induced transcription from speB but not from the speA promoter. Agmatine did not affect the transcription of ORF1, nor the effect of ORF1 on the activity of the speB promoter. cAMP caused an 88% reduction in the AUH activity of wild type E. coli K-12 but had no effect on the activity of plasmid encoded AUH. The activities of ß-galactosidase or alkaline phosphatase expressed from speB or speA promoters fused to lacZ or phoA genes were not influenced by cAMP; in contrast, the activity of ß-galactosidase expressed by lacZ promoter fused to lacZ or phoA was stimulated by cAMP. Thus, the effect of cAMP and CRP on speB expression is indirect and limited to a single copy state.

Acknowledgements

We thank Bob Moore for helpful discussions and the promoter of the speA gene. This work was supported by a grant (DMB-8508917) from the National Science Foundation (to S.M.B.) and a graduate fellowship from the Department of Biochemistry and Nutrition (to M.B.W.S.).

Introduction

In E. coli putrescine can be synthesized from arginine by decarboxylation of arginine to agmatine, catalyzed by arginine decarboxylase (ADC), followed by hydrolysis of agmatine to putrescine, catalyzed by agmatine ureohydrolase (AUH) (51). ADC and AUH are encoded by the genes speA and speB respectively which reside around minute 63.5 on the E. coli chromosome (7). Genetic studies on regulation of the speB expression by cAMP and agmatine were performed before this gene was fully characterized. Halpern and Metzer (21) reported that cAMP supplementation of the strain CS101B of E. coli K-12 induced the expression of AUH as measured by activity assays in crude extracts. In contrast Satishchandran and Boyle (43) showed that cAMP supplementation of other E. coli K-12 strains inhibited the expression of the AUH in crude extracts. The inhibition of AUH by cAMP required the cAMP receptor protein (CRP) since crp strains did not exhibit AUH repression when supplemented with cAMP. They concluded that cAMP was either directly or indirectly involved in the transcriptional regulation of the speB gene (43). E. coli transformed with the plasmid pKA5, which contains a 7.5 kb E. coli chromosomal fragment encoding both the speA and the speB genes (10), as well as several other strains of E. coli K-12 show increased AUH activity following agmatine supplementation of the growth media (43).

We have recently sequenced the speB gene and characterized its pattern of expression (Part I). The speB gene is encoded by one open reading frame (ORF); however it can be transcribed as either a monocistronic or a polycistronic message. The speB promoter, initiating transcription

of the monocistronic message is unusual in that it does not require any sequences beyond the Pribnow box for its activity. The polycistronic message is initiated from the speA gene (R. Moore, personal communication, VPI & SU), residing immediately upstream from speB in the E. coli chromosome.

In our initial search for the speB gene we identified a 2.97 kb DNA fragment which, when inserted into a plasmid (pKB2S), resulted in over-production of AUH activity. Deletion of a 460 bp fragment from one end of this insert caused a 83% drop in AUH activity. After we established the location of the speB gene within the 2.97 kb fragment, it became obvious that the 460 bp deletion did not map within the speB gene. Rather this deletion included the 5'-border of ORF1, the first of two open reading frames (ORF1 and ORF2) located on the strand complimentary to the speB coding strand.

These observations revealed that speB expression is more complex than anticipated and have prompted us to investigate the nature of the relationship between ORF1, ORF2 and speB. Furthermore, in view of the revealed interactions between speB and speA, and between speB and ORF1 or ORF2, we attempted to establish which of these genes were the primary target for the regulatory actions of agmatine and cAMP-CRP. The principle results of this investigation are: agmatine is a transcriptional inducer of the speB gene; cAMP-CRP does not effect either speB or speA promoter activity directly, but acts through an unidentified mediator. ORF1 encodes a trans-acting factor that influences speB at a post-transcriptional level.

Materials and Methods

Bacterial hosts, media and growth conditions.

In all experiments involving the vector pCB267 and its derivatives, the bacterial host was E. coli CB608 (lacZ, galK, phoA8, rpsL, thi, recA56) (T. Larson, Department of Biochemistry and Nutrition, VPI & SU). E. coli DH5α (F^-, endA1, hsdR17, supE44, thi-1, recA1, gyrA96, relA1, phi80, lacZ (Δ)M15) (Bethesda Research Laboratories, Inc.) was used as a host for other plasmids. Wild type E. coli K-12 strain HY1 was obtained from Dr. Yamasaki, Carleton University, Ottawa, Canada.

The bacteria were grown as described (Part I). MOPS minimal media (32) with 0.2% glucose was used for all experiments in which the enzyme activities of strains supplemented with agmatine or cAMP were measured. For growth of E. coli DH5α and its derivatives, 25μg/ml of all 19 L-amino acids (except arginine) and 1 mM thiamine was added to the minimal media. For all lacZ promoter assays, 1mM IPTG (isopropylthiogalactoside) was added to the media. cAMP or agmatine sulfate (Sigma Chemical Co.) was used at 5 mM and 10mM respectively.

Vectors

Plasmid pBR322 was purchased from Bethesda Research Laboratories, Inc., plasmid pCB267 (43) was obtained from T. Larson. Plasmid pGem®-3Z was purchased from Promega Corp.

Purification of plasmid DNA and generation of recombinant clones.

All procedures were as described previously (Part I).

AUH assay.

AUH assays were performed as described previously (Part I). Preparation of the wild type E. coli K-12 extract was from a 200 ml culture; the final volume of the crude extract was 1.0 ml.

Phosphatase A and ß-galactosidase assays.

These assays were performed as described in (43).

Generation of the recombinant plasmids pKB2, pKB2S, pKB2H, pBB15N and pCO3B15N.

Generation of these plasmids was described previously (Part I).

Generation of recombinant plasmids pCO1L, pCO201L, pCO2L, pCO12P, pCO12L, pCO3P and pCO3PO1.

The inserts in all these plasmids are DNA fragments derived from pKA5. The original location of each fragment in pKA5 is depicted in Fig. 5. Generation of the plasmid pCO3PO1 is shown schematically in Fig. 3.

The plasmid pCO1L was generated by ligation of the SmaI-HindIII fragment of pKA5 into the SmaI and HindIII digested vector pCB267.

Plasmid pCO2L was generated by digesting the deletion plasmid B31 (Part I Fig. 2) with HindIII and ligation of the resulting 0.88 kb fragment into HindIII digested pCB267. The right HindIII restriction site in this insert is derived from the vector of the source plasmid B31 and not from pKA5.

The plasmids pCO12L and pCO12P were generated by ligation of the 0.95 kb HindIII fragment of pKA5 (Fig. 5) into HindIII digested pCB267 in both orientations.

Plasmid pCO3P was generated by ligation of the BamHI fragment of pKA5 (shown in Fig. 5) into BamHI digested pCB267.

The names of these plasmids reflect the orientation of their inserts with respect to the orientation of either the phoA or the lacZ promoterless structural genes: "O" in the name designates the ORF in the insert, while "L" or "P" designs the structural gene (lacZ or phoA respectively), which is codirectional with the ORF. Thus "O1L" in the name translates into "ORF1 is in the same orientation as the lacZ structural gene"; "O3P" in the name translates into "ORF3 (the coding sequence of speB) is in the same orientation as phoA".

The plasmid pCO3PO1 was generated by substitution of the 1.93 kb SacI-BglII fragment of the plasmid pCO3P with the SmaI-BglII fragment derived from pKA5 (Fig. 3 and Fig. 5).

Generation of the plasmid pCO4P.

The insert in this plasmid is a BamHI fragment of a deletion clone, obtained from R. Moore (from this laboratory). This fragment contains the promoter of ORF4 (speA) and the ORF4 sequences down to the BamHI site which maps within it (Fig. 5). This fragment was ligated into the BamHI site of the pCB267 vector. The ORF4 promoter in this plasmid activates the phoA gene of the vector.

Generation of the plasmids pCLL and pCLP.

The 0.39 kb PvuII fragment of the plasmid pGEM®-3Z contains the promoter of the lacZ gene with intact operator and CRP binding site, as well as a 120 bp fragment of the structural lacZ sequence. This fragment was ligated into the SmaI site of the pCB267 in both orientations, creating the plasmids pCLL and pCLP.

DNA sequencing.

The strategies and procedures used in sequencing of the insert of the plasmid pKB2S (Fig. 1) were described in Part I.

Results

Influence of neighboring sequences on expression of the speB gene.

Our initial search for the speB gene within the insert of the pKA5 plasmid showed that removal of a 460 bp SmaI-HindIII fragment (about 750 bp downstream from the speB transcription terminus) drastically reduced AUH expression (Fig. 2A). This fragment includes the upstream sequences and the 5'-end of the ORF1 (See Fig. 1 for the nucleotide and amino acid sequence of the pKA5 derived fragment of chromosomal DNA present in the plasmid pKB2S). Our initial hypothesis was that ORF1 or ORF2 might encode a protein necessary for activation of the speB expression. To test this hypothesis we searched for the presence of a functional promoter upstream from ORF1. We constructed a transcriptional fusion between the 460 bp SmaI-HindIII fragment and the promoterless lacZ gene in the vector pCB267. Strains bearing this construct produced blue colonies on an X-gal indicator plate; when assayed for ß-galactosidase transformants exhibited a 29 fold increase over background (Fig. 2B). Plasmids pCO2L and pCO201L were constructed to establish the presence of an independent promoter for ORF2 or a read-through transcription from ORF1 into ORF2, respectively. The results (shown in Fig. 2B) indicated that ORF2 is not transcribed from its own promoter and that a transcriptional terminator must be present upstream from the BglII site (Fig. 2B), preventing a read-through from the ORF1 promoter into the ORF2. To establish if the ORF1 is coding for a transcriptional regulator of the speB expression we inserted the SmaI-BglII fragment of pKA5 (Fig. 3), containing the entire ORF1 and flanking sequences, into the speB promoter: phoA transcriptional

fusion plasmid, pCO3P, creating the plasmid pCO3PO1 (Fig. 3). If the ORF1 gene product activates transcription from the speB promoter, the phosphatase A activity measured in the pCO3PO1 bearing strain should be significantly higher than in the strain bearing the plasmid pCO3P. The phosphatase A activities in these two strains are shown in Fig. 4. No significant difference in the speB promoter activity (i.e., speB::phoA) was found between these two strains grown either in complex or in minimal media. Thus the lack of response of the speB promoter with respect to the ORF1 gene product is not due to an unknown effector missing in the defined media.

Influence of agmatine and cAMP on the promoter activity of speB and neighboring promoters.

The activities of the reporter enzymes (phosphatase A or ß-galactosidase) were assayed in strains bearing the promoter fusion plasmids following their growth in defined media supplemented with 5 mM cAMP or 10 mM agmatine sulfate (Fig. 5). All three strains bearing the speB promoter (plasmids pCO3B15P, pCO3P and pCO3PO1) exhibited over a 100% induction with agmatine. The plasmid pCO3B15P which contained only the minimal upstream sequence essential for the speB promoter activity, and was deleted for all sequences upstream from position -11 (Part I), was fully induced with agmatine. Thus the sequences recognized by agmatine must be located within or downstream from the speB promoter.

Neither the activities of speB, ORF4 nor ORF1 promoters were affected by cAMP. Promoter activities in clones pCO2O1L and pCO2L exhibited a small but significant stimulation by agmatine. Overall, this effect seems to be negligible, since even with agmatine, the level of expression

promoted by this fragment is only 2.7 times higher then background (see the absolute promoter strength column in Fig. 5). The presence of plasmid encoded ORF1 does not enhance the agmatine induction in the strain hosting pCO3PO1; nor is the ORF1 promoter itself (pCO12, pCO12L and pCO12P) affected by either agmatine or cAMP.

The SmaI-HindIII fragment containing the ORF1 promoter also exhibits very strong promoter activity (3.6 times the strength of the lacZ promoter as seen on Fig. 5) in the orientation opposite to ORF1. Both promoters are entirely contained in the SmaI-HindIII fragment since the strains bearing the adjacent fragment present in the plasmid pCO2L produce white colonies on phoA indicator plates. The designers of the pCB267 plasmid have warned against use of this vector for promoter control experiments involving very strong promoters fused to the phoA structural gene (43). Strong expression of phoA slows the growth of the host bacteria which could influence the copy number of the plasmid. We have indeed experienced this problem with the plasmid pCO12L, in which the strong promoter is fused to the phoA gene, and, to a lesser extent with the plasmid pCO12P. Without effectors growth of the strain CB806 [pCO12L] is 10% slower than growth of the strain CB806 [pCO12P], in which the strong promoter is fused to the lacZ gene. With cAMP or agmatine the growth rate of strain CB806 [pCO12L] was decreased by 13% or increased by 29% respectively, as compared to the growth rate with no effectors added to the media. This observation indicated that the strong promoter divergent to ORF1 might be induced by cAMP and repressed by agmatine. The effects of the cAMP and agmatine on this promoter in the plasmid pCO12P (Fig. 5) have been inconsistent; however the results give some indication of a contrasting

cAMP and agmatine effect. The general conclusion from these experiments is that there is a possibility that this promoter is influenced by cAMP and agmatine. However, the studies of these effects have to be conducted in a low copy number vector or in a single copy state. We do not know if the strong promoter is expressing a gene since the putative open reading frame is outside the sequenced area. The fact that this promoter activity can be measured in the plasmids pCO12P and pCO12L indicates that no transcription termination signal is encountered within the 500 bp stretch downstream from the sequenced promoter area.

Fusions of the lacZ promoter with either the phoA or the lacZ indicator genes (Fig. 5) were inducible by cAMP. This indicated that the lack of response of the measured promoters to cAMP were not due to mutations in any of the genes required for transcriptional regulation by cAMP.

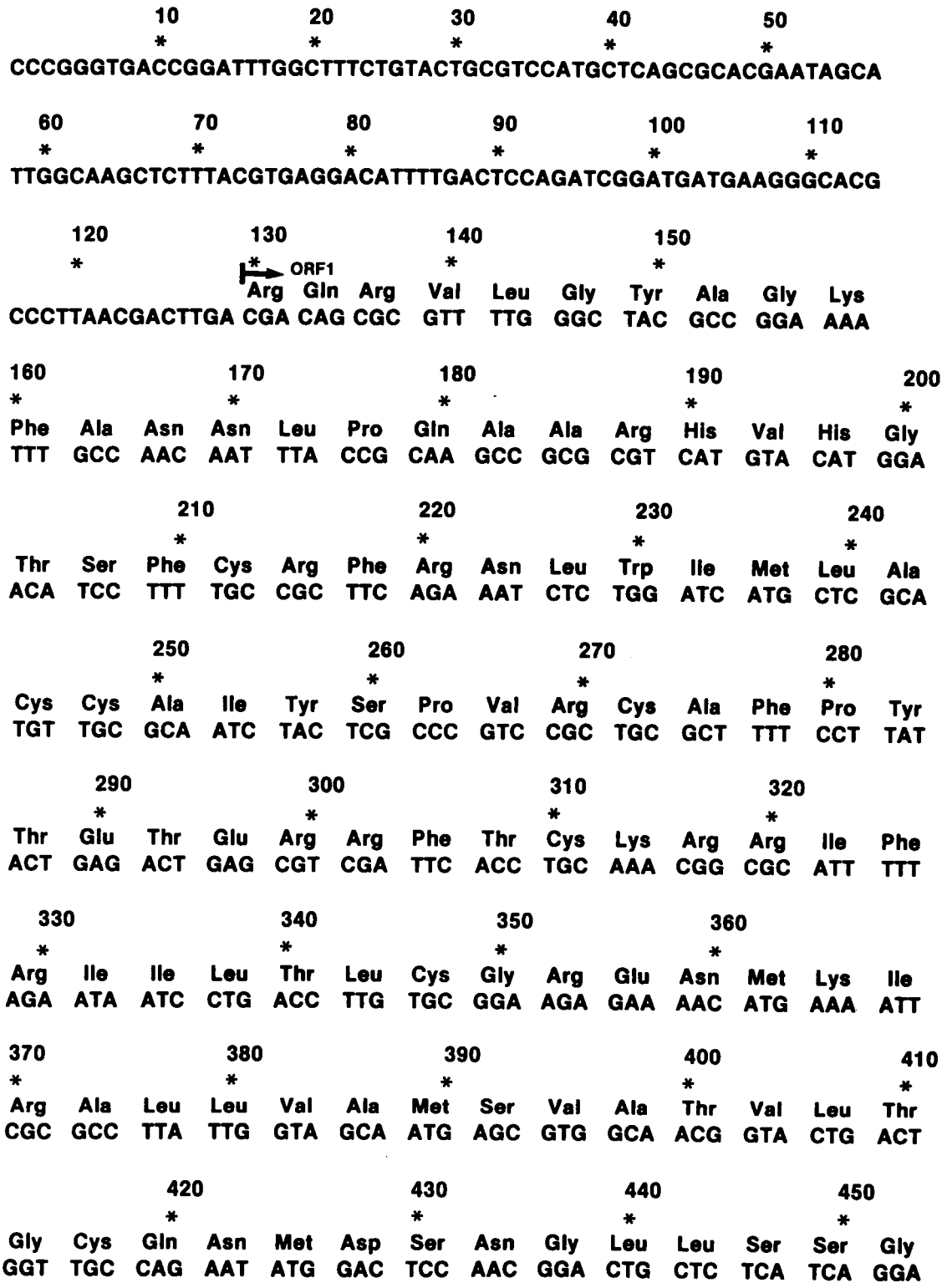
AUH activities were measured in E. coli DH5α [pKA5], DH5α [pBB15N] and in wild type E. coli K-12 grown with or without agmatine (Fig. 6). The cAMP did not cause any increase in expression of the plasmid encoded AUH but it reduced drastically the activity of the chromosomally encoded AUH in wild type E. coli K-12. In contrast, AUH activity encoded by plasmid pBB15N was increased 100% by agmatine. To test the cAMP responsiveness of the E. coli DH5α we chose the strain DH5α [pGem-3Z]. The chromosomal copy of the lacZ operon in E. coli DH5α is deleted for the codons 11-41 of the lacZ gene. It produces an inactive ß-galactosidase, whose activity can be restored by complementation with the α-peptide of ß-galactosidase encoded by the plasmid pGem-3Z. As shown in Fig. 6 the

ß-galactosidase level increased in this control strain by 575% as result of cAMP supplementation.

Fig. 1

Nucleotide sequence of the 2.971 kb insert encoding speB and flanking regions in the plasmid pKB2S.

The noncoding speB strand and flanking regions are shown. The amino acid sequences of ORF1 (130-1116) and ORF2 (1151-2183) are given above the nucleotide sequence; the amino acid sequence of AUH is given below the sequence. The sequence of ORF4 is indicated by triplet codons. Long arrows underneath the sequences indicate the palindromes present within the long tandem repeats. The thicker arrow represents the speB terminator and speB promoter and a Shine-Dalgarno sequences are boxed.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120 130 140 150
ORF1
Arg Gln Arg Val Leu Gly Tyr Ala Gly Lys
CCCTTAACGACTTGA CGA CAG CGC GTT TTG GGC TAC GCC GGA AAA
160 170 180 190 200
Phe Ala Asn Asn Leu Pro Gln Ala Ala Arg His Val His Gly
TTT GCC AAC AAT TTA CCG CAA GCC GCG CGT CAT GTA CAT GGA
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Thr Ser Phe Cys Arg Phe Arg Asn Leu Trp Ile Met Leu Ala
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Cys Cys Ala Ile Tyr Ser Pro Val Arg Cys Ala Phe Pro Tyr
TGT TGC GCA ATC TAC TCG CCC GTC CGC TGC GCT TTT CCT TAT
290 300 310 320
Thr Glu Thr Glu Arg Arg Phe Thr Cys Lys Arg Arg Ile Phe
ACT GAG ACT GAG CGT CGA TTC ACC TGC AAA CGG CGC ATT TTT
330 340 350 360
Arg Ile Ile Leu Thr Leu Cys Gly Arg Glu Asn Met Lys Ile
AGA ATA ATC CTG ACC TTG TGC GGA AGA GAA AAC ATG AAA ATT
370 380 390 400 410
Arg Ala Leu Leu Val Ala Met Ser Val Ala Thr Val Leu Thr
CGC GCC TTA TTG GTA GCA ATG AGC GTG GCA ACG GTA CTG ACT
420 430 440 450
Gly Cys Gln Asn Met Asp Ser Asn Gly Leu Leu Ser Ser Gly
GGT TGC CAG AAT ATG GAC TCC AAC GGA CTG CTC TCA TCA GGA

Figure 1

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                460                 470                 480                 490
                 *                   *                   *                   *
Ala  Glu  Ala  Phe  Gln  Ala  Tyr  Ser  Leu  Ser  Asp  Ala  Gln  Val
GCG  GAA  GCT  TTT  CAG  GCT  TAC  AGT  TTG  AGT  GAT  GCG  CAG  GTG

          500                 510                 520                 530
           *                   *                   *                   *
Lys  Thr  Leu  Ser  Asp  Gln  Ala  Cys  Gln  Glu  Met  Asp  Ser  Lys
AAA  ACC  CTG  AGC  GAT  CAG  GCA  TGT  CAG  GAG  ATG  GAC  AGC  AAG

     540                 550                 560                 570
      *                   *                   *                   *
Ala  Thr  Ile  Ala  Pro  Ala  Asn  Ser  Glu  Tyr  Ala  Lys  Arg  Leu
GCG  ACG  ATT  GCG  CCA  GCC  AAT  AGC  GAA  TAC  GCT  AAA  CGT  CTG

580                 590                 600                 610                 620
 *                   *                   *                   *                   *
Thr  Thr  Ile  Ala  Asn  Ala  Leu  Gly  Asn  Asn  Ile  Asn  Gly  Gln
ACA  ACT  ATT  GCC  AAT  GCG  CTA  GGC  AAC  AAT  ATC  AAC  GGT  CAG

               630                 640                 650                 660
                *                   *                   *                   *
Pro  Val  Asn  Tyr  Lys  Val  Tyr  Met  Ala  Lys  Asp  Val  Asn  Ala
CCG  GTA  AAT  TAC  AAA  GTG  TAT  ATG  GCG  AAG  GAT  GTG  AAC  GCC

          670                 680                 690                 700
           *                   *                   *                   *
Phe  Ala  Met  Ala  Asn  Gly  Cys  Ile  Arg  Val  Tyr  Ser  Gly  Leu
TTT  GCA  ATG  GCT  AAC  GGC  TGT  ATC  CGC  GTC  TAT  AGC  GGG  CTG

     710                 720                 730                 740
      *                   *                   *                   *
Met  Asp  Met  Met  Thr  Asp  Asn  Glu  Val  Glu  Ala  Val  Ile  Gly
ATG  GAT  ATG  ATG  ACG  GAT  AAC  GAA  GTC  GAA  GCG  GTG  ATC  GGT

750                 760                 770                 780
 *                   *                   *                   *
His  Glu  Met  Gly  His  Val  Ala  Leu  Gly  His  Val  Lys  lys  Gly
CAC  GAA  ATG  GGG  CAC  GTG  GCG  TTA  GGC  CAT  GTG  AAA  AAA  GGA

790                 800                 810                 820                 830
 *                   *                   *                   *                   *
Met  Gln  Val  Ala  Leu  Gly  Thr  Asn  Ala  Val  Arg  Val  Ala  Ala
ATG  CAG  GTG  GCA  CTT  GGT  ACA  AAT  GCC  GTG  CGA  GTA  GCT  GCG

               840                 850                 860                 870
                *                   *                   *                   *
Ala  Ser  Ala  Gly  Gly  Ile  Val  Gly  Ser  Leu  Ser  Gln  Ser  Gln
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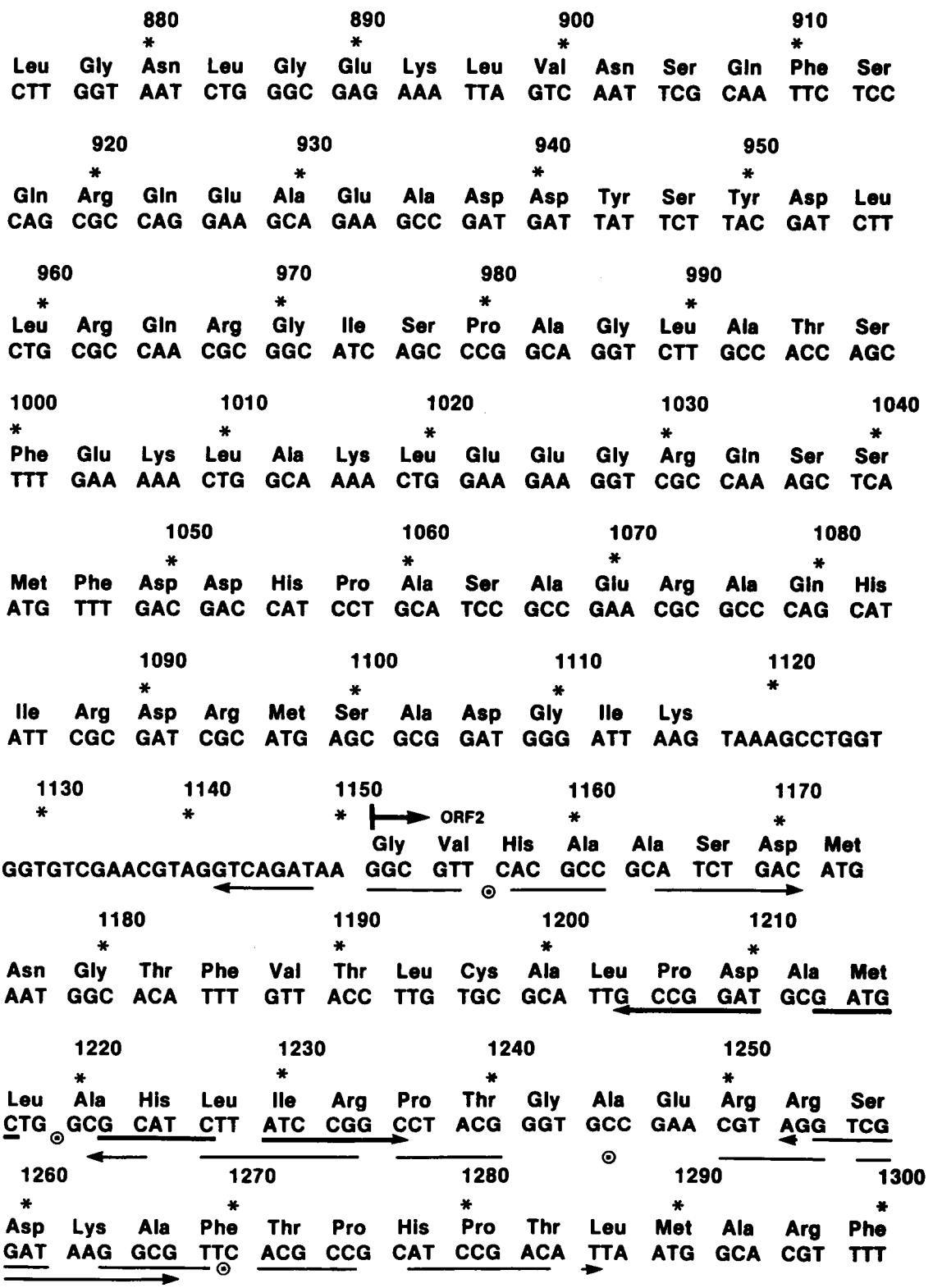
Figure 1 (continued)



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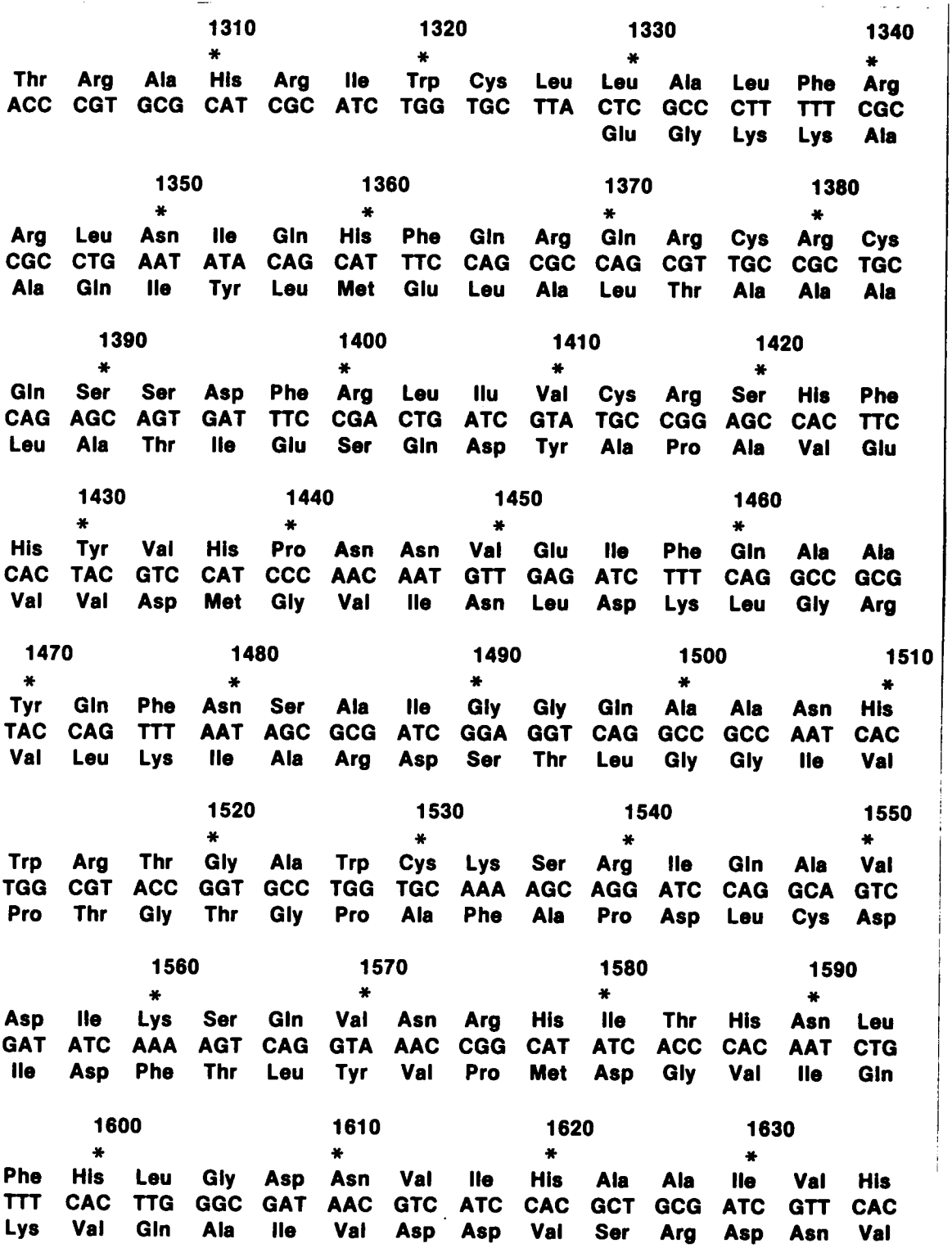

Figure 1 (continued)

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	*			*			*				*		
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CTG	GCA	GGC	GTC	CAG	CAC	GGT	AAA	GCC	GTT	GTC	TTT	ATC	AAA
Gln	Cys	Ala	Asp	Leu	Val	Thr	Phe	Gly	Asn	Asp	Lys	Asp	Phe

	1680			1690			1700			1710			1720
	*			*			*			*			*
Leu	Gly	Thr	Asn	Thr	Asn	Leu	His	Gly	Met	Ile	Arg	Val	Asp
CTC	GGT	ACG	AAT	ACC	AAT	CTG	CAC	GGA	ATG	ATT	CGG	GTC	GAT
Glu	Thr	Arg	Ile	Gly	Ile	Gln	Val	Ser	His	Asn	Pro	Asp	Ile

			1730			1740			1750				1760
			*			*			*				*
Gln	Thr	Phe	Phe	Arg	Arg	Gly	Ile	Glu	His	Ser	Ala	Val	Val
CAG	ACC	TTC	TTT	CGG	CGC	GGT	ATA	GAA	CAT	AGT	GCC	GTG	GTC
Leu	Gly	Glu	Lys	Pro	Ala	Thr	Tyr	Phe	Met	Thr	Gly	His	Asp

		1770			1780				1790			1800	
		*			*				*			*	
Lys	Phe	Thr	Thr	Val	Arg	Ile	Gly	Ile	Gly	Val	Gly	Val	Lys
AAA	TTC	ACA	ACC	GTT	CGC	ATA	GGT	ATC	GGT	GTG	GGC	GTC	AAA
Phe	Glu	Cys	Gly	Asn	Ala	Tyr	Thr	Asp	Thr	His	Ala	Asp	Phe

	1810				1820			1830			1840		
	*				*			*			*		
Val	Tyr	Gln	Arg	His	Phe	Ala	Glu	Met	Leu	Arg	Met	Ser	Thr
GTG	TAC	CAG	CGC	CAT	TTT	GCC	GAA	ATG	CTT	CGC	ATG	AGC	ACG
His	Val	Leu	Ala	Met	Lys	Gly	Phe	His	Lys	Ala	His	Ala	Arg

	1850			1860			1870				1880		
	*			*			*				*		
Gln	Gln	Arg	Gln	Arg	Asn	Lys	Val	Val	Thr	Thr	Glu	Arg	Glu
CAG	CAG	CGG	CAG	CGT	AAC	AAA	GTG	GTC	ACC	ACC	GAA	AGA	GAG
Leu	Leu	Pro	Leu	Thr	Val	Phe	His	Asp	Gly	Gly	Phe	Ser	Leu

	1890			1900			1910			1920			1930
	*			*			*			*			*
His	Thr	Leu	Thr	Gly	Ser	Gln	Gln	Leu	Leu	Gly	Val	Arg	Leu
CAT	ACG	CTT	ACC	GGC	AGC	CAG	CAG	CTT	CTC	GGC	GTG	CGC	CTG
Met	Arg	Lys	Gly	Ala	Ala	Leu	Leu	Lys	Glu	Ala	His	Ala	Gln

			1940			1950			1960				1970
			*			*			*				*
Gln	Leu	Phe	Ala	His	Leu	Thr	Gly	Ile	Ala	Lys	Gly	Ile	Tyr
CAG	CTT	TTC	GCT	CAT	CTC	ACG	GGC	ATC	GCC	AAA	GGC	ATA	TAC
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Figure 1 (continued)

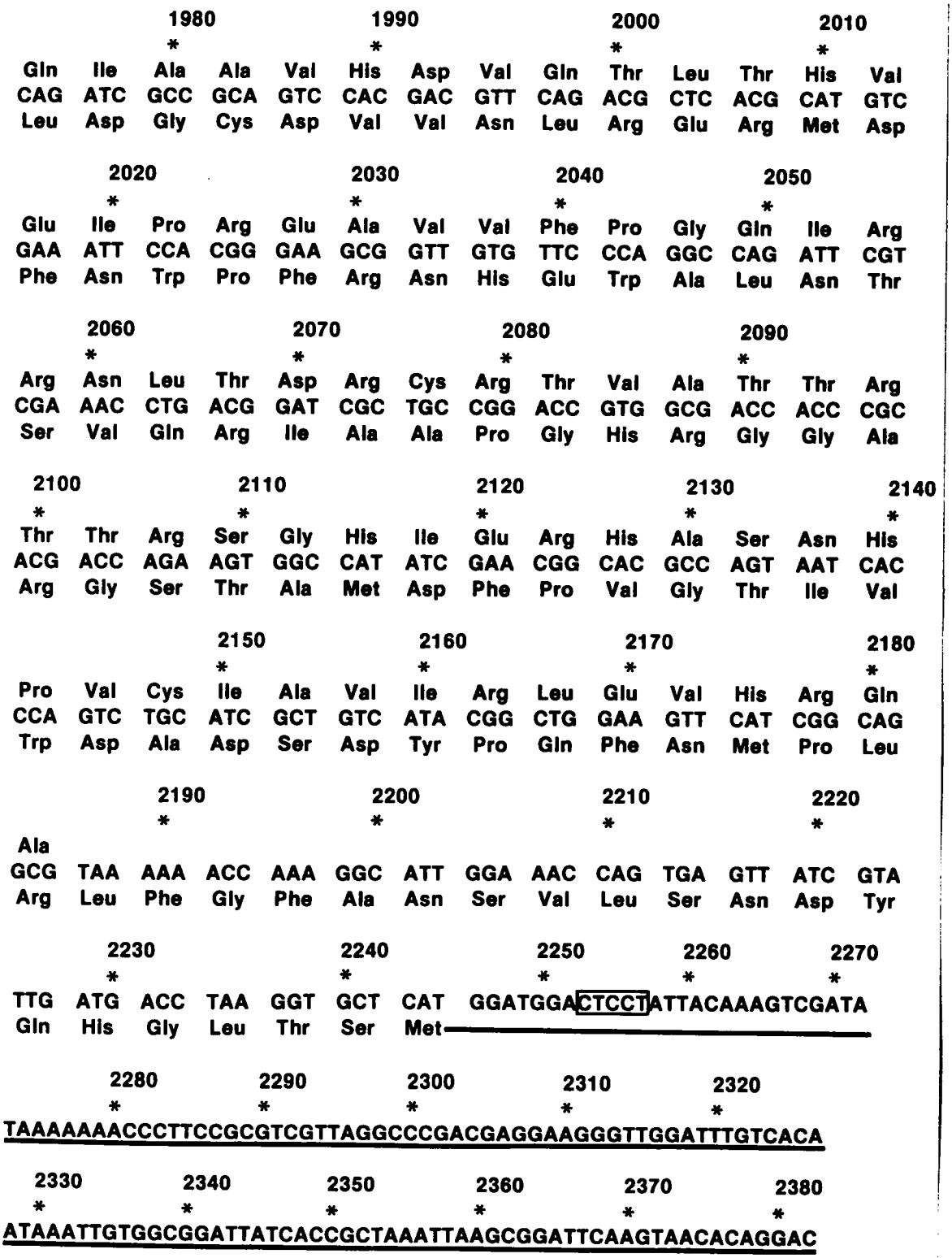


Figure 1 (continued)

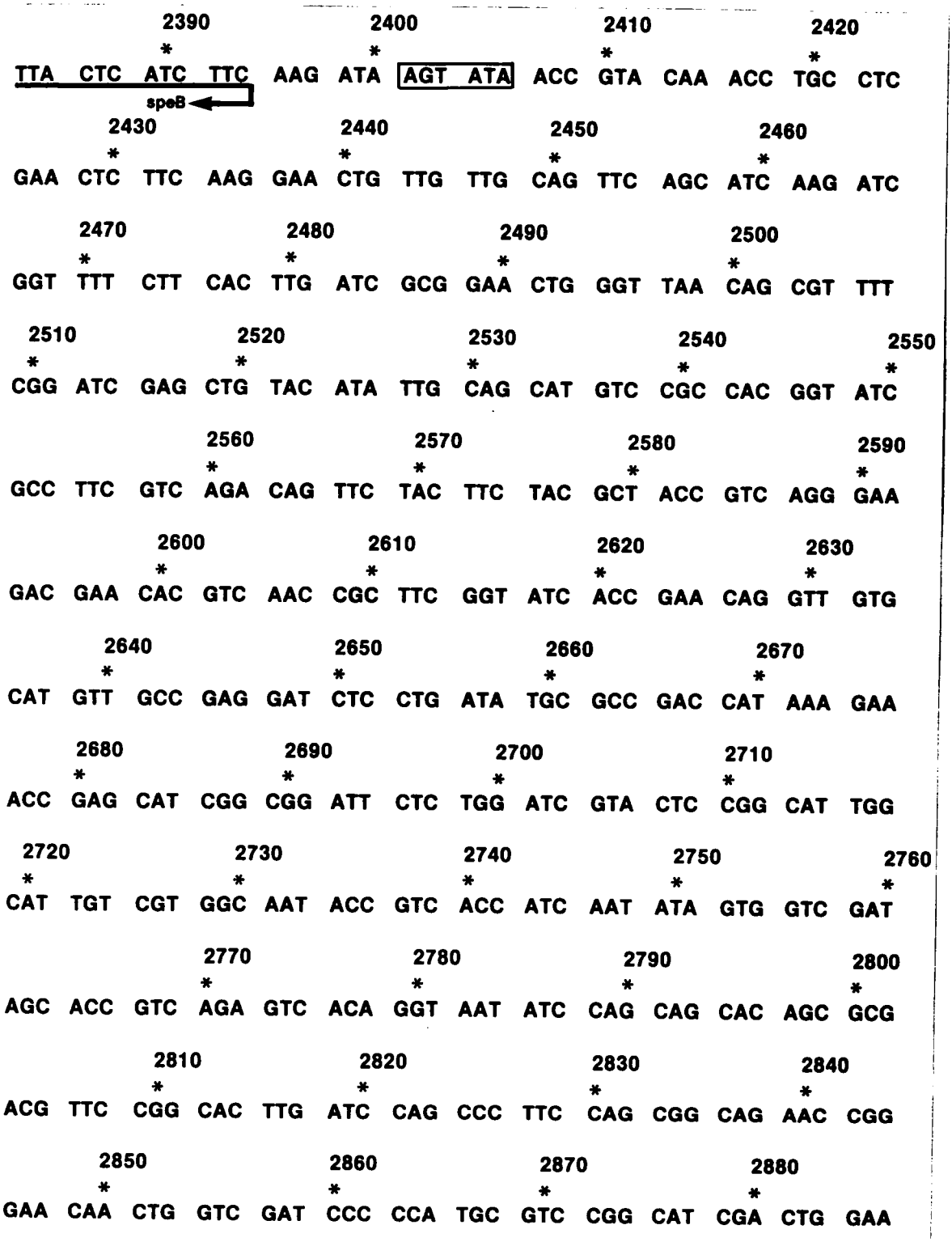


Figure 1 (continued)

```
      2890              2900              2910              2920
      *                 *                 *                 *
CAG  CGA  GAA  GTT  GAC  GTA  CAT  TTT  GTC  CGC  CAT  ACG  TTC  CTG

 2930              2940              2950              2960              2970
 *                 *                 *                 *                 *
CAG  CTC  GTC  GAT  AAT  CGG  ACG  ATG  AGC  ACG  GTT  TTG  CGG  ATC

C
```

Figure 1 (continued)

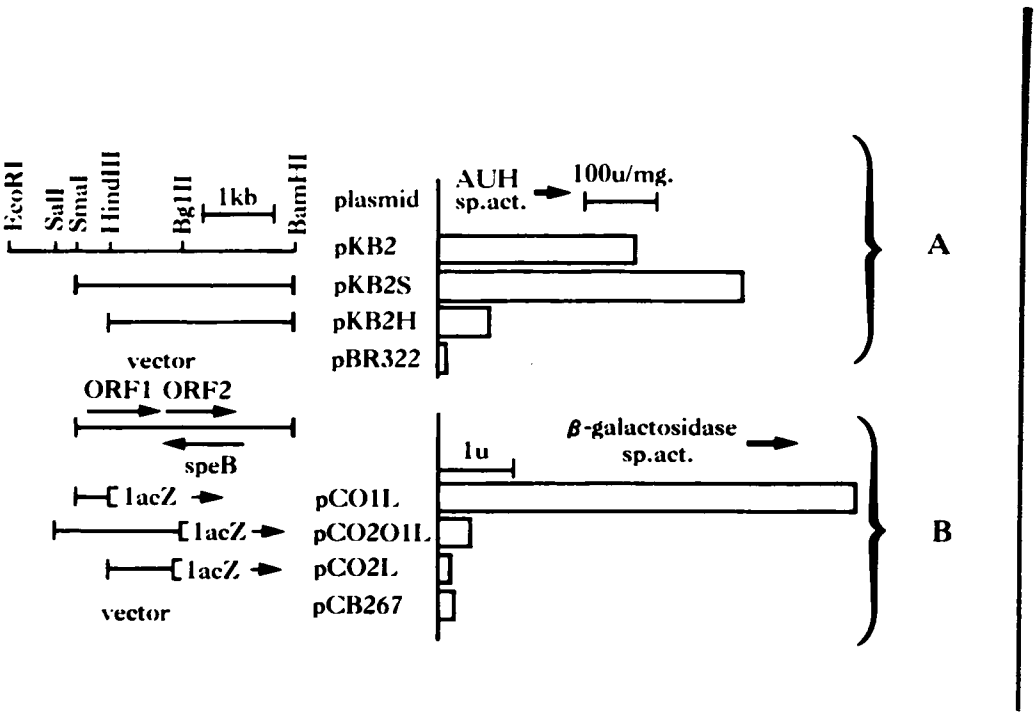


Fig. 2

Influence of deletions on speB expression and promoter activity of flanking regions.

The horizontal lines in the left portion specify the inserts in the named plasmids. Projection of these lines on the restriction map (top of A) or on the structural map (between A and B) specify their size and origin relative to open reading frames. A. the ability of strains containing the specified deletion plasmids to express the AUH activity. 1u of AUH activity = 1nmol urea/min. B. the ability of DNA fragments derived from pKB2 to activate transcription (in the direction indicated by the arrows) of the promoterless lacZ gene in the vector pCB267. All cultures were grown in complex media. 1u of ß-galactosidase activity = $OD_{410} \times 10^{-2}$/reaction time (min) x cell density (Klett units).

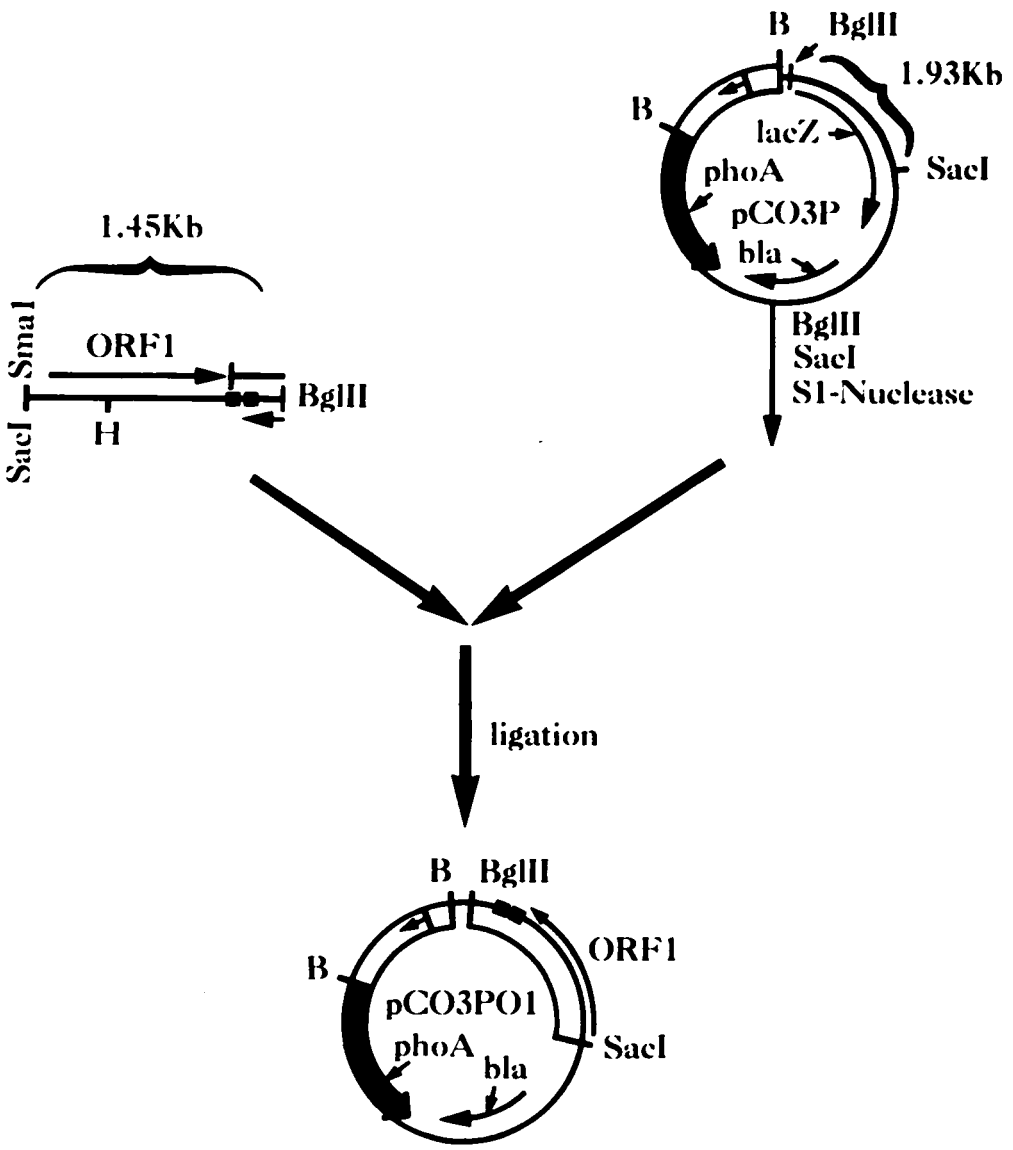


Fig. 3

Construction of the plasmid pCO3PO1.

The two black boxes downstream from ORF1 in the SacI-BglII insert depict the position of the two long tandem repeats (Fig. 1) containing the speB terminator and three other pallindromes.

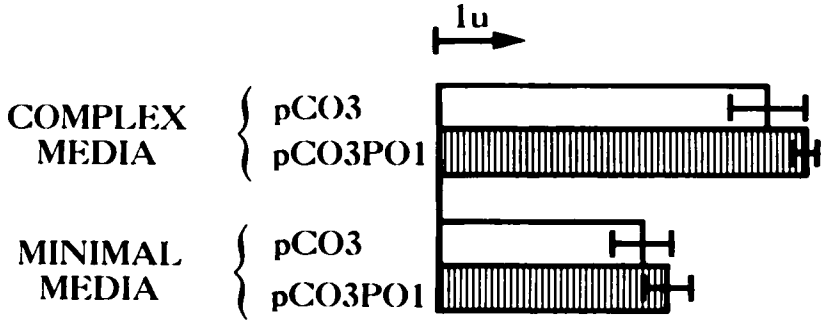


Fig. 4

Phosphatase A activity of the strains bearing the plasmid pCO3P or pCO3PO1 in complex or minimal media.

lu of activity = OD_{410} x 10^{-3}/reaction time (min) x cell density (Klett units/ml).

Fig. 5

Influence of cAMP or agmatine on the promoter activities encoded in DNA fragments derived from the pKA5 insert.

Top: structural map (with relevant restriction sites indicated) of the 7.5 kb pKA5 insert and the location of sequences included in the speB transcripts (wavy lines); E-EcoRI, B-BamHI, H-HindIII, N-NruI, S-SmaI.

Bottom: the horizontal lines specify the inserts in the named plasmids and the direction (arrows) in which promoter activity was measured. Projection of the lines on the pKA5 map specify the fragments original location. The "promoter strength" column lists the specific activities resulting from the indicator gene expression (phoA or lacZ) when no effectors were included in the growth media. lu is defined as in Fig. 4.

[a] - data from three or more ß-galactosidase assays.

[b] - data from three or more phosphatase A assays.

[c] - result of one phosphatase A assay.

[d] - the bar graph represents data related to this value.

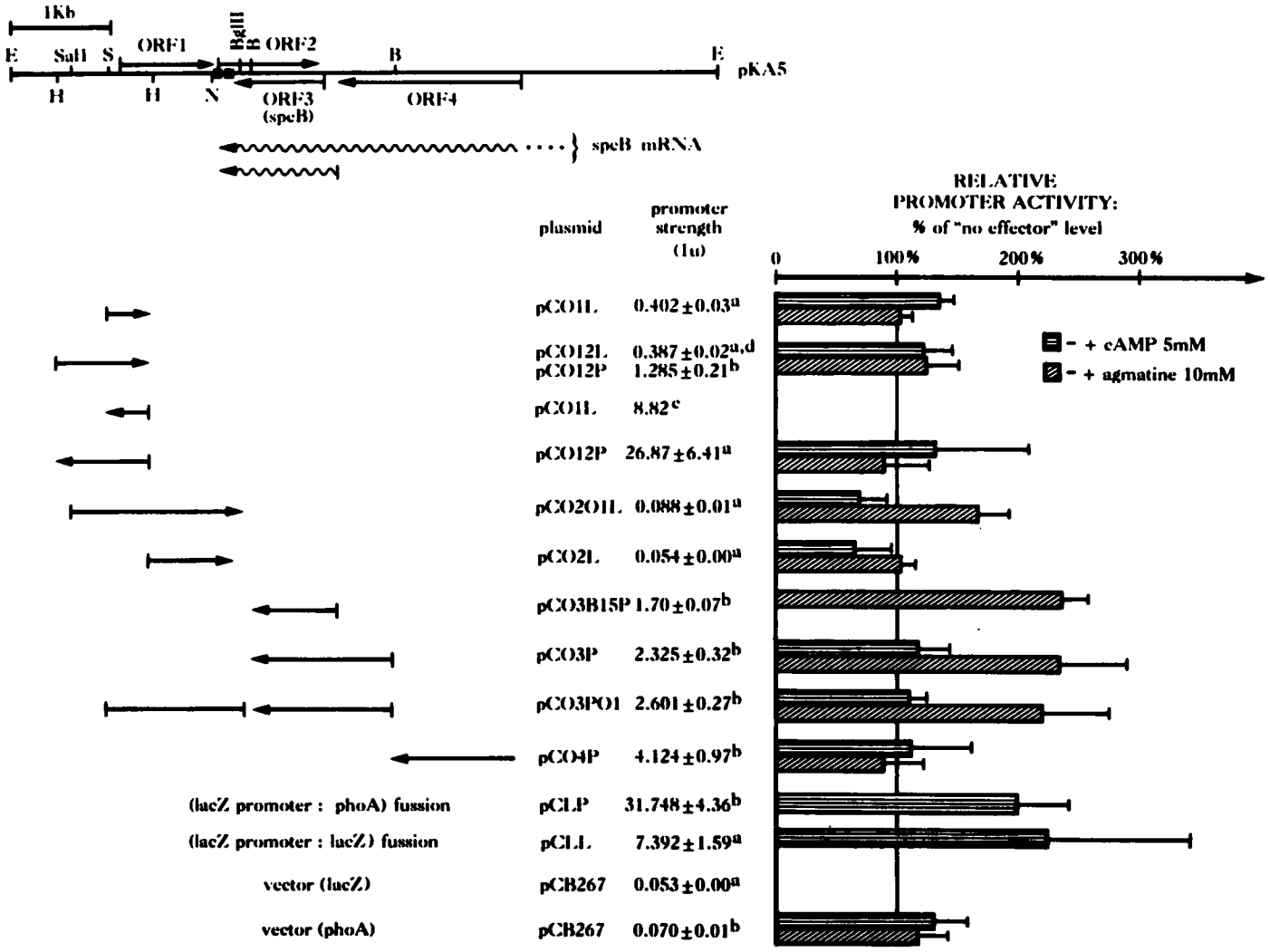
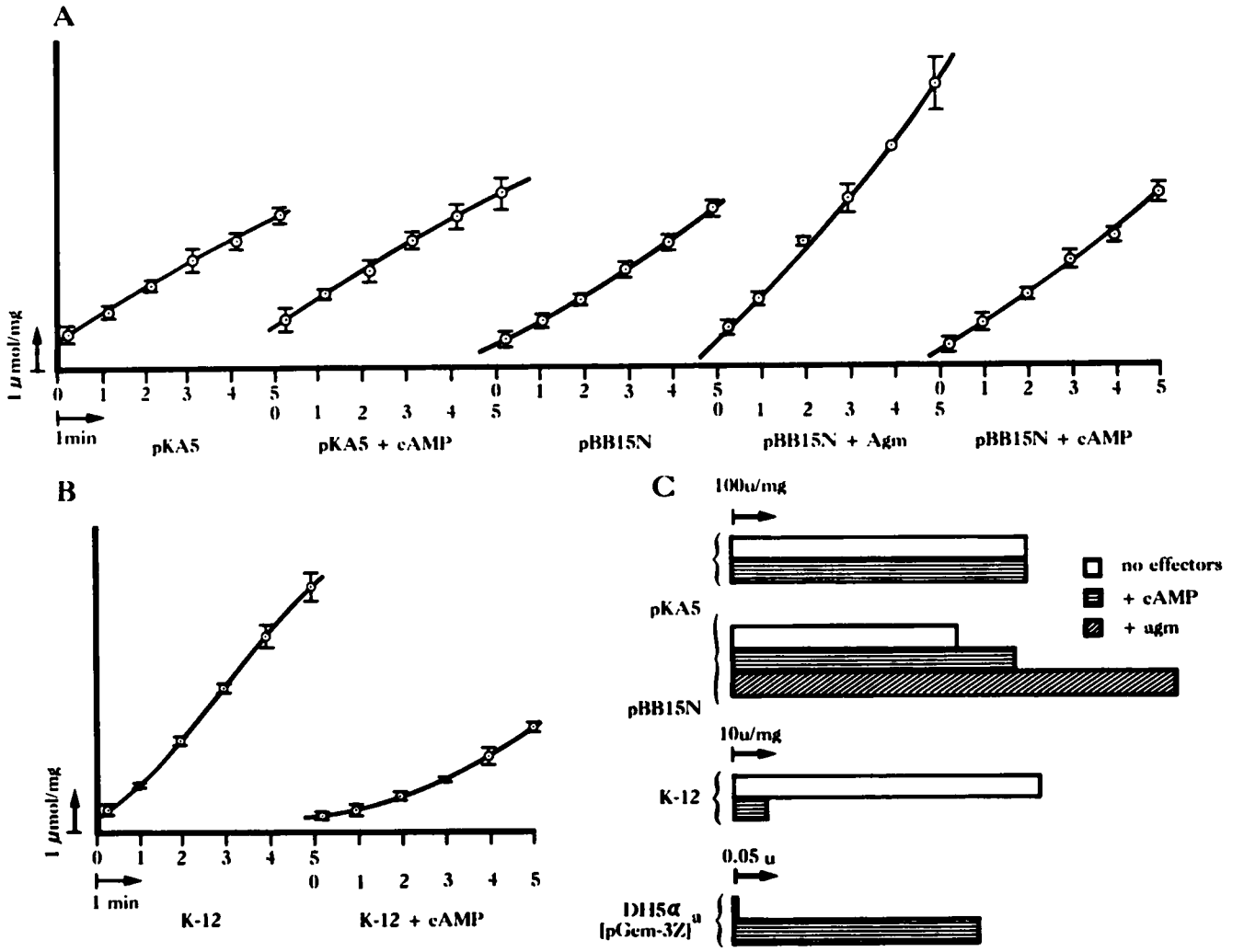

1Kb
E
SalI
S
ORF1
BglII
B
ORF2
B
E
pKA5
H
H
N
ORF3
(specB)
ORF4
specB mRNA
plasmid
promoter strength (1u)
RELATIVE PROMOTER ACTIVITY:
% of "no effector" level
0
100%
200%
300%
- + cAMP 5mM
- + agmatine 10mM
pCO1L 0.402±0.03a
pCO12L 0.387±0.02a,d
pCO12P 1.285±0.21b
pCO1L 8.82c
pCO12P 26.87±6.41a
pCO2O1L 0.088±0.01a
pCO2L 0.054±0.00a
pCO3B15P 1.70±0.07b
pCO3P 2.325±0.32b
pCO3PO1 2.601±0.27b
pCO4P 4.124±0.97b
(lacZ promoter : phoA) fussion
pCLP 31.748±4.36b
(lacZ promoter : lacZ) fussion
pCLL 7.392±1.59a
vector (lacZ)
pCB267 0.053±0.00a
vector (phoA)
pCB267 0.070±0.01b

Fig. 6

Influence of cAMP or agmatine supplementation of the growth media on AUH activity in strains bearing the pKA5 or the pBB15N plasmids, or in wild type E. coli K-12.

A and B: Progress of the AUH catalyzed reactions; C: Specific activities of AUH; values derived from the progress curves shown in A and B. Data are from three experiments. lu of AUH activity is defined as in Fig. 2A.

[a]B-galactosidase specific activity measured on cell extracts. lu = OD_{410}/min x mg protein.


A
1 μmol/mg
1min
pKA5
pKA5 + cAMP
pBB15N
pBB15N + Agm
pBB15N + cAMP
B
1 μmol/mg
1 min
K-12
K-12 + cAMP
C
100u/mg
no effectors
+ cAMP
+ agm
pKA5
pBB15N
10u/mg
K-12
0.05 u
DH5α [pGem-3Z]

Discussion

We have previously reported the presence of two open reading frames, ORF1 and ORF2, on the strand opposite the speB coding strand (Fig. 1). Deletion of a fragment containing the upstream sequences of ORF1 results in loss of plasmid encoded AUH activity. We found two divergent promoter activities within this fragment; the promoter responsible for transcription in the direction opposite to ORF1 is between 15 and 70 times stronger than the ORF1 promoter (depending on the growth media Fig. 5). No promoter activity was found in sequences immediately upstream from ORF2 and only trace read-through was observed from ORF1 promoter. In light of the above observations, ORF1 seems to represent a gene involved in regulation of speB expression. Since our results have excluded ORF1 encoding a transcriptional activator of the speB promoter (Fig. 4), its function must be connected to post-transcriptional regulation of the speB expression; the nature of which remains to be identified.

Satishchandran and Boyle (43) showed that cAMP in presence of cAMP receptor protein (CRP) inhibited the expression of AUH activity in various strains of E. coli K-12. They also demonstrated that agmatine stimulated expression of AUH encoded either on the chromosome or on pKA5 plasmid. Our observations showed that the speB is expressed from either its own promoter or from speA (arginine decarboxylase encoded by ORF4) (Part I). In addition, the evidence presented here showed that the sequences downstream from the speB coding region effect AUH expression. Thus it is of interest to learn which one of these promoters is the direct target of the regulatory action of the cAMP and agmatine. Our results (Fig. 5)

indicated that while agmatine induces the activity of the speB promoter (and thus activates the transcription of the monocistronic speB message), it does not affect either of the promoters of ORF4 or ORF1.

cAMP did not influence the expression of any of the mentioned promoters nor did it effect the AUH activity encoded by pKA5 or the pBB15N plasmids (Fig. 5 and Fig. 6). Our previous results have demonstrated (Part I) that speB is expressed mainly as a polycistronic transcript in the DH5α [pKA5], but as a monocistronic transcript in the DH5α [pBB15N]. Thus these results additionally confirm the insensitivity of the ORF4 and the speB promoters, respectively, towards the cAMP-CRP mediated control. However, when a chromosomally encoded AUH was assayed in the wild type E. coli K-12, a significant inhibition by cAMP was clearly noted. This phenomenon can be explained by making two assumptions: that the cAMP effect is indirect, mediated by a product of a cAMP-CRP regulated gene, and that the high copy number expression of the plasmid coded AUH prevents our detection of this effect. For this latter assumption to be true the regulatory protein must be encoded by a region of the chromosome not present in the pKA5 insert and either its expression in the cell or its affinity for the target (DNA, RNA or protein) must be low. Support for this hypothesis is provided by the observation of Satishchandran and Boyle (42), who showed that maximal induction by agmatine on the AUH expression was observed after approximately half of a generation (30 minutes); in contrast, maximum repression of AUH by cAMP was observed after 2.5 generations.

The progress curves of the AUH catalyzed reactions show post-transnational influences on the activity of the enzyme. In extracts from pKA5

the curves are flattened at very early timepoints. Wild type E. coli K-12 and DH5α [pBB15N] extracts show an activating influence which causes the curves to assume parabolic shapes. For each strain the presence of cAMP or agmatine in the growth media does not influence the shape of the curve but rather the initial velocity of the reactions. This indicates that agmatine and cAMP effect the initial concentration of the active enzyme, while the factors influencing the progress of the reactions are independent of these two effectors. Differences in the shapes of the reaction curves between the strains must reflect the relative proportions of the involved proteins as effected by their single or multiple copy expression. Thus the relative concentration of AUH versus the neighboring proteins, encoded by the pKA5 insert are approximately equal in the E. coli DH5α [pKA5] and in the wild type E. coli K-12; although in the E. coli DH5α [pKA5] the absolute concentrations of all these proteins are higher. In E. coli DH5α [pBB15N] the relative proportions of the AUH to the remaining pKA5 encoded proteins is higher than in the other two strains. The conclusion which emerges from this analysis is that the activating factor responsible for the upward tendency in the AUH progress curves representing the E. coli DH5α [pBB15N] and the wild type E. coli K-12 must either be an intrinsic property of the AUH enzyme (for example its activation by one of the reaction products), or another protein encoded elsewhere on the chromosome. However, the inhibitory factor which causes early inactivation of AUH in E. coli DH5α [pKA5] and in the wild type E. coli K-12 must be encoded by the pKA5 insert.

General Discussion

The results of the investigation reported here have answered several questions and generated new ones. In the first category is the question of linkage between expression of the speA and the speB genes. It was known since the original mapping of the putrescine biosynthetic genes (27) that speA maps very close to speB in the E. coli chromosome, and the possibility of these two genes being expressed from an operon was considered. Inactivation of speA or speB by insertion of the bacteriophage Mu did not produce any polar effect (52), which indicated that these two genes are expressed independently from each other. My results show that indeed speB can be fully expressed from its own promoter but when speA is expressed, speB is preferentially transcribed from the speA promoter.

The inducibility of the speB, but not the speA, promoter by agmatine provides an additional example of the strict linkage of putrescine biosynthesis to the availability of arginine. Several reports have indicated that the ADC and the AUH expression is induced by arginine (47, 30, 50). I did not measure the speB promoter activity as effected by arginine supplementation. However, Satishchandran and Boyle (43) observed that arginine does not increase AUH activity in a speA mutant. Together with my results these observations suggest that expression of polycistronic speA and speB is induced by arginine, while the expression of monocistronic speB is not. The total picture of the pathway selection for biosynthesis of putrescine emerges as follows: when the exogenous supply of arginine is low, ornithine is produced from glutamate by the

pathway illustrated in Fig. 02, and the ODC pathway is preferred; when the exogenous supply of arginine is high, the ODC pathway is inactivated by lack of ornithine (the synthesis of which is feedback inhibited by arginine), and the ADC/AUH pathway is induced; when exogenous agmatine is present, the synthesis of the AUH, but not of the ADC, is increased due to induction of the monocistronic speB.

We do not know if expression of the speB gene causes downregulation of expression from the speA operon. If such a relation exists it is not mediated by agmatine, since my results show that agmatine does not repress the speA promoter. Further clarification of the mechanisms responsible for coordination between the monocistronic and the polycistronic expression of AUH will be possible only after a thorough investigation of the regulation of the speA promoter activity.

Both the S1-analyses and the Northern hybridization experiment have indicated that the monocistronic expression of speB is repressed when the speA operon is transcribed. We do not know what molecule is mediating this repression. This effect could be mediated by either a nutrient molecule, the speA mRNA or by ADC itself. The first possibility can be tested by analysis of RNA produced by pKA5 bearing strains grown with arginine or agmatine supplementation. My RNA analysis was performed on E. coli grown in complex media and thus was not controlled enough to answer these questions. The two latter possibilities can be evaluated by assaying the speB promoter activity in a plasmid created by inserting the speA gene into the plasmid pCO3B15N in such a way that no readthrough from the speA to the speB is possible.

The simplified RNA purification procedure used in my experiments should facilitate a greater accuracy in the RNA analyses by shortening the preparation time elapsing between the harvest of cells and the denaturation of proteins. I have shown that treatment of E. coli with phenol/chloroform effectively disrupts the cell envelope and protects the released RNA from degradation. Results not shown in this dissertation have indicated that even the cooling and rinsing of cells is not necessary to purify RNA. Thus, aliquots of cultures can be transferred immediately to the vial containing equal volume of phenol/chloroform, releasing the RNA in native form. The ethanol precipitated nucleic acids from the supernatant can then be loaded directly on a denaturing gel and analysed by Northern hybridization. In my experience, the procedure is selective for RNA as no detectable DNA bands are visible in the Northern hybridization procedure. Designing a method which would facilitate the transfer of DNA together with RNA would provide the best possible internal standard to quantify the RNA signal.

One of the unanswered questions arising from my results is: what is the function of the palindrome residing between the speB promoter and the Shine-Dalgarno sequence of the speB gene? This sequence apparently does not function as a terminator (although it looks like one). I examined its similarity to other structures involved in post-transcriptional repression in E. coli (18). The RNA secondary structures known to be involved are located near the Shine-Dalgarno sequence or the initiation codon (and generally within the +13 to -20 bp of the initiation codon). The intergenic speA-speB palindrome starts 33 bp upstream from the initiation codon of the speB gene; thus the probability of it functioning

in translational inactivation of speB is rather small. However, a different group of palindromic sequences appearing at the 3'-end or within the intercistronic region of some operons have been described (33). The best known example of these sequences is the REP sequence of E. coli. 500-1000 copies of this highly conserved sequence of inverted repeats have been found in the E. coli chromosome. When transcribed in RNA the REP sequences form stable loops and in all studied cases their presence is connected to a selective increase of the half-life of the mRNA transcribed from the upstream gene. Newburry et al. (33) have demonstrated that the presence of the REP sequence in the intercistronic region of the malEFG operon between the malE and the malF gene accounts for the selective stability of the malE mRNA. The speB palindrome is not homologous to the REP sequence; however, palindromes other than REP, although not as well studied, have been reported downstream from preferentially stable RNAs expressed from operons (26). Our situation is different in that the whole polycistronic speAB message is preferentially expressed over the monocistronic speB message. At this time the role of the palindromic sequence in this or other regulatory phenomenon cannot be excluded.

The experiments described in Part II of this dissertation suggested that the ORF1 might be coding for a post-transcriptional inducer of the speB gene. Additionally, an inhibitory involvement of the putative gene expressed from the promoter divergent from ORF1 was indicated by the 30% increase of AUH activity in the strains bearing the pKB2S plasmid as compared to the AUH activity in the pKB2 bearing strain. Satishchandran and Boyle (42) found that a strongly acidic inhibitory protein was complexed

with the AUH protein through the first stages of their purification of the AUH from a pKA5 bearing strain. A complex of AUH with this inhibitor exhibits a pI value of 5.5 while the purified AUH protein has a pI of 8.3. Dissocation of this inhibitor at the DEAE-Sephacel chromatography step resulted in three fold activation of the recovered enzyme. A plausible explanation for such a strong effect is that the inhibitor is encoded on the same multicopy plasmid as the AUH. A tentative indication for presence of a pKA5 encoded inhibitor was derived from analysis of the progress curves from the AUH reactions (see discussion section in Part II). Since both the ORF1 and ORF2 codons indicate basic proteins, the possibility that the strong divergent promoter directs transcription of the acidic inhibitor protein must be considered. The EcoRI-SmaI fragment located 5' to ORF1 in pKA5 is currently being sequenced in our laboratory.

It will be germane to the regulation of speB to establish whether the genes neighboring speB are encoding a post-translational effector of the AUH. This could be tested by mixing extracts containing the effector (e.g. protein) in question with an extract of cells overproducing AUH (such as the extract of pBB15N bearing strain) and measuring the AUH activity as function of the changing concentrations of the putative effector.

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