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# MUTATIONALLY-CREATED TRANSCRIPTIONAL INITIATORS IN THE ARGININE CLUSTER OF *ESCHERICHIA COLI* K-12

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DAVID CHUNG-HONG CHEUNG

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MUTATIONALLY-CREATED TRANSCRIPTIONAL INITIATORS IN THE ARGININE CLUSTER OF *ESCHERICHIA COLI* K-12

APPROVED BY

Leon Unger

Robert Delaney

Joseph J. Zeretti

Martin J. Griffee

DISSERTATION COMMITTEE

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## CHAPTER I

## INTRODUCTION

### Promoter, Transcription Initiation Site

The promoter, as defined by Miller *et al.* (1), for a given operon, is the region in which transcription is initiated. Initiation includes all the processes involved in transcription up until the point at which the first RNA nucleotide is copied from DNA. This includes recognition and binding of RNA polymerase, and the opening up of the DNA duplex. Thus, a promoter governs the rate of transcription of a given operon. This rate can be altered by altering the promoter.

In the lactose system, there are three structural genes for the proteins β-galactosidase (gene *z*), galactoside permease (gene *y*) and thiogalactoside transacetylase (gene *a*) (2,3). The genes lie next to one another on the chromosome, in the order *z* - *y* - *a*, and are regulated by the repressor product of the *i* gene. In the absence of β-galactoside inducers of the system, the repressor interacts with the *lac* operator (*o*) to prevent synthesis of the three proteins. Jacob and Monod (3) have suggested that repression involves an inhibition of transcription of *lac* operon DNA into a messenger RNA (mRNA) copy. This

model for regulation has received strong support from experiments of Gilbert and Müller-Hill (4,5) who have isolated the repressor protein and have found that it binds specifically to lac operator double stranded DNA. The lac repressor is a tetramer of a molecular weight around 150,000 daltons. A horseshoe-shaped model of lac repressor covering 20-30 nucleotide pairs on the DNA has been postulated (6), and with normal DNA, repressor does not prevent formation of the complex with polymerase, although it does stop transcription (7). This agrees with the traditional idea that the action of repressor is to get in the way of the polymerase enzyme.

The initiation of mRNA synthesis was first hypothesized to occur at the operator, in the original Jacob and Monod theory (3). In this version of the model, the operator possessed a dual function: (1) interacting with the repressor, and (2) serving as an initiation point for transcription. Recognition of the promoter region awaited the isolation of four mutants by Scaife and Beckwith (8). These four promoter mutations have these important characteristics: (a) they are pleiotropically lac negative and function in a cis-dominant fashion; (b) they are not suppressible by any known nonsense or polarity suppressor; (c) they are $o^+$ (they do not manifest a defect in repressibility). Point mutations L8, L29 and L37, in the promoter, reduce the level of lac operon expression by 15-fold. L1, a deletion, results in a 50-fold reduction in lac operon expression (9).

The principle used in isolating these mutants was to screen UV-induced lac mutants for ones which were leaky (9). It was assumed that some mutants, by altering but not abolishing the promoter, would

lower the maximal levels of the lac enzymes. Using these mutations, Ippen et al. (10) were able to locate the promoter between laci and laco. L8 and L37 appear to be identical. L1 has been shown since to be a deletion extending into the i gene (11). L1 does not recombine with either of the two promoter point mutants (11). It was proposed that these mutations affected the process of initiation of transcription of the lac operon (12).

A further complication in the understanding of the lac promoter region arises from the fact that this promoter is recognized efficiently by RNA polymerase only in the presence of the catabolite gene activator protein (CAP), and adenosine 3'-5' cyclic monophosphate (13). The lac operon and other operons are said to be catabolite sensitive or subject to catabolite repression because the presence of glucose reduces their rate of expression. Glucose apparently brings about this result by decreasing the availability of adenosine 3'-5' cyclic monophosphate to CAP protein factor, which is absolutely dependent on this small molecule for transcription stimulating activity (13,14,15,16). It has been shown in vivo and by in vitro transcription systems that CAP protein and 3'-5' cyclic AMP are necessary in addition to RNA polymerase for high levels of expression of lac operon (7,12,17,18). That the promoter is the target site for catabolite repression is indicated by the work of Silverstone et al. (19) and Perlman et al. (20). These groups have shown that lac promoter mutations result in a loss of catabolite repression sensitivity. Furthermore, high level expression of lac that is insensitive to catabolite repression can result either from fusing the lac genes to a catabolite insensitive promoter (such as trp) (19),

or by generating second site, closely linked, revertants of promoter point mutations (21). Recently, Beckwith et al. (22) suggested that there are two distinct sites in the promoter by analysis of the promoter mutants of lac. One of these sites, between the region defined by deletion L1 and the lac operator, which normally promotes a low level (2%) of lac transcription, functions independently of CAP and cyclic AMP, possibly by interacting with RNA polymerase holoenzyme alone. The second site, the region covered by L1, is a site through which CAP protein and 3'-5' cyclic AMP stimulate lac transcription. They proposed that the function of the CAP and cyclic AMP complex is to bind to a site in the promoter, thus stimulating the initiation by RNA polymerase at the normally weak initiation site.

A similar delineation of the promoter and operator regions has been mapped for the trp operon in S. typhimurium (23). In other systems, such as his, the distinction is not so clear (24,25). The promoter and operator appear to be in reverse order in the arabinose operon (26).

## The Transcribing Enzyme and Its Initiation Factor Sigma

The steps involved in the synthesis of RNA by RNA polymerase in vitro occur in the following sequence (27,28,29): the binding of the enzyme to DNA at discrete sites, initiation of RNA chains involves strand selection and the exclusive formation of a purine ribonucleoside triphosphate at the 5' end, elongation of the RNA chains from the 5' to the 3' end with concomitant elimination of inorganic pyrophosphate until a termination site is reached at which newly synthesized single-stranded RNA chains are released from the DNA-enzyme complex. Recently, a termination protein, called rho (ρ) factor, has been discovered by Roberts

(30) in the E. coli crude extract, which causes the release of discrete RNA molecules in an in vitro reaction using bacteriophage λ DNA as template.

The DNA-dependent RNA polymerase of Escherichia coli is composed of at least four different polypeptide chains: $\alpha,\beta,\beta'$, and $\sigma$ (27, 28,29). On the basis of the known molecular weights and the relative contents of these components, the holoenzyme appears to have the structure $\alpha_2\beta\beta'\sigma$. The core enzyme with the structure $\alpha_2\beta\beta'$, devoid of the subunit $\sigma$, possesses all the enzyme activities associated with the polymerase for carrying out RNA synthesis, except that it cannot initiate RNA synthesis efficiently from the DNA duplex (27,28,29,31,32). Thus, the sigma subunit is essential for the proper initiation of transcription. The β subunit is the most important in the catalytic function of RNA polymerase, on the basis of the following investigations: (1) the β subunit may have the binding sites for the substrates (33); (2) the β subunit has the binding site for the antibiotic rifampin, a potent inhibitor of the initiation of RNA synthesis (33,34); (3) mutants resistant to another antibiotic, streptolydigin, which inhibits elongation by binding to the polymerase, have an altered β subunit (34,35).

Sigma factor determines the specificity of initiation in bacterial cells and is released from the DNA-enzyme complex shortly after initiation, leaving the $\alpha_2\beta\beta'$ core enzyme to elongate the RNA chain. Hinckle and Chamberlin (31,32) reported that core enzyme by itself associates nonspecifically with DNA in loose binding which is rapidly reversible. Interaction with sigma forms a more stable complex in which the enzyme is tightly bound and can no longer dissociate from the

DNA. This ensures preferential initiation of transcription from tight binding sites. They agreed that sigma opens the DNA duplex for the start of transcription. Initiation depends on temperature in a way which suggests that the first step in RNA synthesis is the action of sigma to melt the DNA to produce a region of local unwinding.

Granted that the sigma subunit may serve as a macromolecular allosteric effector to prime RNA polymerase for site selection, the question remains whether sigma itself bears structural information for recognition of the promoter sequence (36,37) or whether this information resides in the core polymerase but is expressed only when sigma is bound (34,38). The finding that sigma binds neither to DNA (31) nor to phosphocellulose (39) makes models for sigma function in which sigma binds directly to the DNA promoter sequence less attractive, although they are not ruled out. It had originally been assumed that sigma subunit must carry the information for promoter sequence recognition, since sigma was thought to be replaced by an equivalent phage protein after phage infection, leading to the initiation of RNA chains at new promoter sites (37,40,41). It has recently been shown that this mechanism is not used in the switch of promoter specificity by phage T7 (42). If this is so, then the mechanism by which a promoter site is altered in other phage and bacterial systems must function through alteration of the core polymerase or through the imposition of other polymerases or specificity-altering factors (38,43).

The universal and asymmetric distribution of pyrimidine-rich clusters (poly dC, dT regions) over the two strands of DNA isolated from a variety of bacteria, bacteriophages and higher organisms, prompted

Szybalski and coworkers (44) to put forward the hypothesis that pyrimidine clusters could be the specific points for RNA polymerase to initiate. This hypothesis is further substantiated by the fact, that in synthetic polymers like dG:dC, dA:dT and dTC:dAC, the pyrimidine-containing strands are preferentially transcribed by RNA polymerase (27,45). Moreover, X-ray diffraction studies have shown that the molecular conformation of the dG:dC or dA:dT polymers is different from that of the native DNA (27). Thus pyrimidine clusters might exhibit a higher affinity towards RNA polymerase.

## Internal Promoters, Naturally-Occurring and Mutationally-Created

### Tryptophan Operon

The tryptophan (trp) operon is presently understood to consist of five contiguous genes each specifying a different polypeptide chain. The genes are arranged in the same sequence relative to the metabolic pathway in E. coli and Salmonella typhimurium, although the letter designations of the genes differ. The order of the trp operon of S. typhimurium is promotor(P1)-operator(O)-trpA-trpB-trpE-trpD-trpC. The order of the promoter and operator sequence has been recently mapped by Callahan and Balbinder (46). The gene order in E. coli is PO-trpE-trpD-trpC-trpB-trpA. The order of the promoter and operator sequence is not entirely clear.

Investigations with the trp operon of S. typhimurium and E. coli reveal the existence of a relatively low-efficiency promoter element (P2) at or near the boundary between the second and the third genes of the operon in addition to the principal promoter (P1) in the vicinity

of the operator region (47,48).

The trp operon of S. typhimurium appears to be composed of two independent units, trpA-trpB and trpE-trpD-trpC, each possessing a promoter-like initiator element. However, the entire operon appears to function as a unit with respect to regulation by tryptophan, possessing a single operator region at the trpA end (47,49,50). Polarity mutations in the most operator-proximal gene (trpA) affect not only the function of the next gene (trpB), but also of the last three genes (trpE, trpD and trpC). However, the effect on trpB is more severe, and in even the strongest trpA polarity mutants, an appreciable basal level of activity for the distal three genes persists. Deletion mutations extending into the operon from the operator end (trpA side of the operon) and ending in trpA eliminate expression of trpB, but retain a low constitutive level of function of the distal three genes, trpE, trpD and trpC. Deletions ending in the second gene (trpB) yield essentially an identical result. However, expression of the three distal genes is completely lost in strains where the deletion extends past the boundary between second and the third genes (trpB-trpE), terminating in the third (trpE) or the fourth gene (trpD). Also the basal repressed level of the distal genes is decreased in strains with internal deletions eliminating the boundary between the second (trpB) and the third (trpE) genes.

These findings suggest that the apparent noncoordinate synthesis of the enzymes of the trp operon results from the presence of the second promoter-like initiator element (P2) at or near the trpB-trpE boundary. The level of constitutive synthesis of those enzymes specified by the distal three genes is 2-3% of that synthesized by a

partially constitutive, 5-methyltryptophan-resistant (repressor-deficient mutant) grown in the presence of tryptophan (47), and is about 40 to 70% of the activity of the fully repressed wild type strains (49).

A homologous, low-efficiency promoter-like element exists between *trpD* and *trpC* in the *trp* operon of *E. coli* (48). As in *S. typhimurium* polarity mutations in the two most operator-proximal genes (*trpE* and *trpD*) fail to reduce the low-level constitutive expression initiated by the internal promoter, whereas polarity mutations in the third and the fourth genes (*trpC* and *trpB*) produce appreciable negative pleiotropy on the expression of the most operator-distal gene of the operon. Comparison of the enzyme levels of a maximally repressed culture with those of fully derepressed *trpR*$^-$ constitutive cells indicates that constitutive *P2* function is approximately 2% of the maximal rate and is responsible for synthesis of 80% of the *trpC*, *trpB* and *trpA* polypeptides present in repressed cells (48,51). Recently, *P2* has been mapped within *trpD* near the operator distal end but on the operator proximal side of two *trpD* point mutants (51).

Since low-level constitutive expression of the three operator-distal genes is apparently not regulated by the *trp* repressor, the internal initiator (*P2*) seems to serve as a transcription initiator rather than as a special translational initiator (48,49).

A homologous internal initiator can be created by mutation within the *trp* operon of *S. typhimurium*. Mutants with deletions terminating inside *trpA* (as mentioned above) lack *trpB* expression because the promoter, the operator, and the operator-proximal segment of the first gene, *trpA*, are deleted. Phenotypically, *trpB*$^+$ derivatives have

been obtained by the induction of secondary mutations (called Ini) (50, 52). They were induced by mutagens such as 2-aminopurine, nitrosoguanidine, and diethylsulfate and occur within the remaining segment of the trpA gene. The resultant mutant strains are able to grow on anthranilate-supplemented medium, indicating that all the genes except trpA are functioning.

Further study of the genetic and physiological characteristics of these mutagen-induced initiator elements indicated that the Ini mutations could be created by a single DNA base-pair change (transition type) and were capable of generating a nucleotide sequence in the trpA gene which could initiate distal gene expression. There was also evidence that two distinct sites in the undeleted distal end of the trpA gene had the potential for becoming initiator elements as the result of a single base-pair change (50). In such $trpB^+$ strains, the restored trpB activity in all cases is between 0.4 and 0.7 times the fully repressed wild-type level. The activity was found to be constitutive with respect to trp regulation, being essentially the same in cultures grown under conditions of repression and derepression. The activity of the last three genes is increased to about the sum of the level restored by Ini mutation and that determined by P2. Since expression of Ini mutations is constitutive (independent of derepression of the operon), and since the mutations do not require the proximity of a translation-terminating mutation for function, they differ from the translational "restart" mutations described in the rII region of bacteriophage $T_4$ (53) and in the lac system of E. coli (54). It was concluded that Ini mutations are probably low-efficiency promoter elements serving as initiators of

transcription (52).

Wuesthoff and Bauerle (52) constructed recombinant Ini strains in which proximal deletions (supX) are replaced so that the tryptophan operon is complete and normal except for the Ini mutations. Such strains are prototrophs ($Anth^+$) and produced fully functional trpA enzyme. Under repressed conditions all five gene products in such reconstituted Ini strains show higher levels than the wild-type control. The increase in trpA expression is surprising. It was concluded that recombinants are able to translate the Ini mutations as an apparent missense sequence. The Ini mutations also function as initiators when in combination with strongly polar trpA nonsense and frameshift mutations which lie between the operator and the Ini site. It was concluded that Ini mutations probably create low-efficiency transcription-initiation promoters. A tryptophan operon, with a supX deletion of P1, the operator, and the operator-proximal part of trpA, and with an Ini mutation in the remaining part of trpA, would then make two types of mRNA molecules in about equal quantities. Transcription initiations at the Ini mutation would result in mRNA carrying the information for the trpB, E, D, and C genes, whereas the mRNA originating from initiations at P2 would code for the trpE, D, and C products.

Morse and Yanofsky (55) selected E. coli feeder colonies showing resistance to a combination of 6-methyltryptophan and 5-fluoroanthranilate in the presence of anthranilic acid. It was found that one has a mutation in the operator-distal half of the first structural gene trpE, which acts as an initiating element. Unlike the Ini mutations, this mutation ($trpE^R$) inactivates the trpE enzyme and simultaneously

causes a partial constitutive functioning of four operator-distal genes (*trpD*, *C*, *B*, and *A*) at 30-50% of the maximal level observed for *trpR*$^-$ constitutive mutants under maximal tryptophan-repressed conditions (compared to 4% for the wild type). The auxotrophy of the *trpE*$^R$ strain, due to the loss of *trpE* gene function, is not suppressed by nonsense suppressors. The *E*$^R$ mutant reverts spontaneously to prototrophy (frequency $2 \times 10^{-8}$), but reversion is not induced by 2-aminopurine, nitrosoguanidine, ethylmethan sulfonate or ICR. Reversion to prototrophy results in loss of the constitutivity of *E*$^R$ and the restoration of *trpE* gene expression. The mutation in the *trpE* gene apparently creates a transcription initiator, since the resultant constitutive expression of the more distal genes is not affected by tryptophan-mediated repression at the operator. Furthermore, the constitutive activity is unaffected by the polar effects of a nonsense mutation introduced into the operator-proximal portion of the *trpE* gene. Constitutivity is clearly the result of the initiation of transcription at or near the *E*$^R$ altered site, primarily for the distal four genes, as proved by hybridization studies of *trp* messenger RNA. They also noted that the operon with the initiation mutation can be derepressed to a significantly higher constitutive level by introducing a *trpR*$^-$ allele of the unlinked regulatory gene. This indicates that the transcription initiator mutation in the *trpE* gene does not prevent transcription initiated at the operator end (*Pl*) from proceeding down the operon to the more distal genes. The added increment of constitutive expression, however, is not as great as that produced by a wild-type strain carrying a *trpR*$^-$ allele, indicating some interference with the transcription initiated at *Pl*. It is possible

that the $E^R$ mutation involves the insertion of a foreign "promoter" internally into the trp operon and analogous to the insertions resulting in polar mutants of the lac and gal system (56,57,58,59,60).

A unique kind of initiator mutation, which may have a transcription-terminating component as well as an initiation component, has been reported by Callahan and Balbinder (61). They reported a mutation (trpA515) which maps in the "unusual" region at the boundary between trpA and trpB in S. typhimurium. The initiator mutation, which was selected in a strain bearing a strongly polar mutation trpA49 in the most operator-proximal part of trpA, causes a dependence on 5-methyltryptophan for utilization of anthranilate as a growth factor. This analogue is normally a potent growth inhibitor and corepressor of synthesis of the tryptophan biosynthetic enzymes. Given anthranilate, the double mutant grows better in the presence of 5-methyltryptophan than in its absence. When the trpA49 is removed by recombination, the presence of the initiator mutation alone causes trp auxotrophy and an absolute dependence on 5-methyltryptophan for growth with an anthranilate supplement.

In addition, trpA515 has the following unique enzymic properties: (1) trpA515 together with polar trpA49 has normal trpB function, a higher trpD expression (three times as much as the wild-type) under repressed conditions, and trpB and trpD expression is not affected by physiological and genetic derepression; that is to say, their expression is constitutive; (2) trpA515 alone, shows a similar low-level constitutive expression for trpB and trpD under repressed conditions. However, upon derepression, trpD is constitutive, but the trpB enzyme level is drastically reduced to 40%; (3) when trpA515 is combined with a supX

deletion which eliminates P1 and therefore eliminates all transcription at the operator end, the double mutant has low but significant constitutive trpB and trpD enzyme levels, which are not affected by the presence of tryptophan or 5-methyltryptophan, and does not require the latter in order to utilize anthranilate for growth. These mutants have similar characteristics to the double mutant trpA49 trpA515 except they can grow twice as fast without 5-methyltryptophan.

A possible explanation for this peculiar phenotype is that the mutation trpA515 creates a promoter for constitutive expression of the four operator-distal genes when the principal trp promoter (P1) is either inactive due to repression caused by tryphophan or 5-methyltryptophan or due to a supX deletion. However, when P1 is functioning under derepressed conditions, the mutation is read as a structural gene mutation in the "unusual" region and also acts as a terminator of transcriptions originating at P1, resulting in an extreme polarity effect for the four distal genes. The initiator mutation reverts spontaneously but is not induced to revert by mutagens. It responds negatively to tests for a nonsense mutation. It resembles insertion mutations with extreme polar effects (56,57,58,59,60).

## Histidine Operon

In the histidine operon of Salmonella typhimurium, the existence of internal promoters and internal initiator has been reported although the work is not as extensive as that for the tryptophan operon.

The histidine operon of S. typhimurium consists of promoter and operator regions followed by nine contiguous genes in the order of hisG-hisD-hisC-hisB-hisH-hisA-hisF-hisI-hisE. Two natural internal pro-

moters have been detected by Atkins and Loper (62) by using a highly sensitive intergeneric complementation test. They used a highly polar S. typhimurium his mutant with a deletion of the operator region as recipient for E. coli his episomes. It was concluded that there are two natural initiators internal in the histidine operon which they designated P2 and P3. P2 is in hisC or at the hisC-hisB boundary, and P3 is in hisF or at the hisF-hisI boundary.

St. Pierre (63) studied hisG203 deletion strains of S. typhimurium in which the second gene, hisD, is not functioning due to a deletion of the promoter, the operator, and the operator-proximal segment of the first gene, hisG. Secondary mutants could be selected for their ability to grow on histidinol, i.e., for restored expression of the intact hisD. Of 145 secondary mutants isolated, at least 91 contained point mutations mapping in the remaining distal portion of hisG. Seventy-nine of these secondary mutants were shown to be allelic and are located in region VI of hisG. The site of the secondary mutations is thus very critical for expression of the operon in hisG203. This site may, by base pair transition, become an initiator site. These initiation mutants are similar to the Ini mutants of the trp operon in the following ways: (1) both were isolated by base-transitional types of mutagens; (2) they all restore 50% of the gene-expression distal and adjacent to the initiator mutations (i.e., hisD, in this case); (3) like the Ini mutations, hisG activity is restored after hisG203 secondary mutations were transduced into G203$^{+}$. However, the mutations elicit a feedback hypersensitive and cold sensitive hisG enzyme.

However, the mechanism by which these mutations function

has not been fully elucidated. They may be either transcriptional or translational types of reinitiators.

A unique way to isolate internal initiation mutants of the his operon in S. typhimurium was demonstrated by McIntire and Loper (64). The method is based on the same principle of intergeneric complementation by which they detected internal promoters as described earlier. Spontaneous aminotriazole-resistant (AT) mutants were isolated by plating a diploid strain hisOGD223/F'hisB$^-$ on minimal medium in the presence of AT. Since AT is an inhibitor of one of the hisB enzymes, the diploid cannot grow. Two separate mutations conferring AT resistance cotransduce with the his P2 region, and do not impair function of hisC product. These mutants show an increase of hisB enzyme and a decrease of hisD expression. Therefore these mutants represent events for increased promotion at or near P2. The mode of action and mechanisms of these mutants have yet to be elucidated.

## The Regulation of Arginine Biosynthesis

In Escherichia coli, arginine biosynthesis proceeds from glutamic acid via an eight-step reaction sequence. The intermediates and the reactions are shown in Figure 1. The first half of the pathway proceeds via a series of acetylated intermediates and is unique in nature. Between ornithine and arginine the reaction sequence is the same as that originally shown in mammalian tissues (65). The common and systematic names of the enzymes in the arginine biosynthetic pathway are listed in Table 1.

The deacylation of acethylornithine to form ornithine is by two distinct mechanisms: a hydrolytic cleavage to release acetate, as

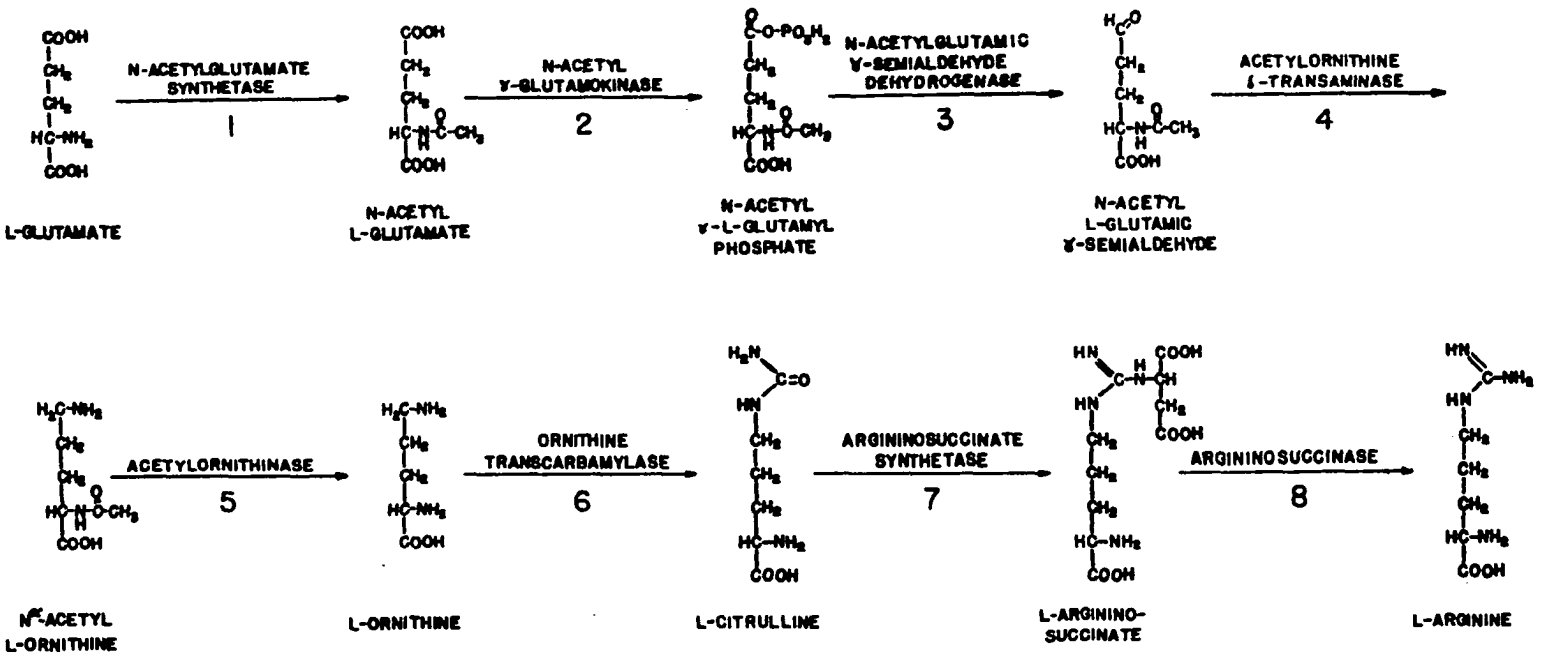



Figure 1. Arginine pathway in Escherichia coli.

TABLE 1

ENZYMES OF ARGININE BIOSYNTHESIS IN ESCHERICHIA COLI

| | Enzyme | |
|---|---|---|
| Step | Common name | Systematic name |
| 1 | N-Acetylglutamate synthetase | Acetyl-CoA:L-glutamate N-acetyltransferase (EC 2.3.1.1) |
| 2 | N-Acetyl-γ-glutamokinase | ATP:N-acetyl-L-glutamate 5-phosphotransferase |
| 3 | N-Acetylglutamic γ-semi-aldehyde dehydrogenase | N-Acetyl-L-glutamate γ-semi-aldehyde:NADP oxidoreductase (phosphorylating) |
| 4 | Acetylornithine δ-trans-aminase | α-N-Acetyl-L-ornithine:2-oxo-glutarate aminotransferase (EC 2.6.1.11) |
| 5 | Acetylornithinase | α-N-Acetyl-L-ornithine amido-hydrolase |
| 6 | Ornithine transcarbamylase | Carbamoylphosphate:L-ornithine carbamoyltransferase (EC 2.1.3.3) |
| 7 | Argininosuccinate synthetase | L-Citrulline:L-aspartate ligase (AMP) (EC 6.3.4.5) |
| 8 | Argininosuccinase | L-Argininosuccinate arginine-lyase (EC 4.3.2.1) |

demonstrated in E. coli (66), and an acetyl-transfer to glutamate, forming acetylglutamate as shown in Micrococcus glutamicus (67). The latter also constitutes a second mechanism accomplishing the first reaction of the biosynthetic sequence for arginine.

The first enzyme in the pathway is N-acetylglutamate synthetase (EC 2.3.1.1), specified by gene argA, which converts L-glutamate to N-acetyl-L-glutamate, with acetyl-CoA as the acetyl donor (68). In 1963, Vyas and Maas (69) demonstrated by an in vivo method of acetylation of glutamic acid that the enzyme was subject to feedback inhibition by arginine. However, the activity could not be demonstrated in cell-free extracts. Recently, Haas and Leisinger were able to work out an in vitro assay of N-acetylglutamate synthetase both in Psuedomonas aeruginosa (70) and in E. coli K-12 (personal communication from T. Leisinger).

N-acetyl-γ-glutamokinase, the second enzyme in the pathway, is specified by argB and converts N-acetylglutamate to N-acetyl-γ-glutamyl phosphate (71).

N-acetylglutamic-γ-semialdehyde dehydrogenase, encoded in argC, catalyzes the NADPH-dependent formation of N-acetyl-glutamic-γ-semialdehyde from N-acetyl-γ-glutamyl phosphate (71).

Acetylornithine δ-transaminase (EC 2.6.1.11), specified by argD, converts N-acetylglutamic-γ-semialdehyde to N-acetylornithine with glutamate serving as the amino donor. The enzyme was shown to be dependent on glutamate and pyridoxal-5-phosphate for maximal activity and yields α-ketoglutarate as a by-product (72).

Acetylornithinase, the fifth enzyme of the arginine pathway, encoded in argE, catalyzes the hydrolytic cleavage of acetylornithine to

ornithine. Its activity is dependent upon the cobaltous ion and glutathione, and produces one mole of acetate for each mole of acetylornithine cleaved (66). An *in vitro* synthesis of N-acetyl-L-ornithine in *E. coli* K-12 has been recently demonstrated by Urm and coworkers (73).

Ornithine transcarbamylase (EC 2.1.3.3), enzyme 6, is encoded by *argF* and *argI* in *E. coli* K-12. This enzyme (OTC) requires carbamyl phosphate (74). Either of the two genes is able to produce a functional ornithine transcarbamylase (75). Recently it was shown (76) that when both are active in the same cell, the two gene products interact to form a family of four hybrid trimeric isoenzymes. In *E. coli* B and W, only the *argI* product is found.

Argininosuccinate synthetase (EC 6.3.4.5) is specified by *argG*. This enzyme has not been studied in *E. coli* except for some preliminary repression-derepression studies. It converts citrulline to argininosuccinate in the presence of ATP and aspartate (77).

Argininosuccinase, specified by *argH*, converts argininosuccinate to arginine with the release of fumarate (78).

The location on the *E. coli* chromosome of the eight genes which code for the biosynthetic enzymes, other genes associated with the arginine system, and selected reference markers, are shown in Figure 2 (79,80). The genes coding for the eight biosynthetic enzymes are located in six regions on the *E. coli* chromosome, with *argE*, *argC*, *argB*, and *argH* being clustered at minute 78.7 (79). The other structural genes are widely dispersed. The *argM* present in *E. coli* W, is responsible for production of an inducible transaminase (81,82,83); this gene appears to be another structural gene for a second acetylornithine

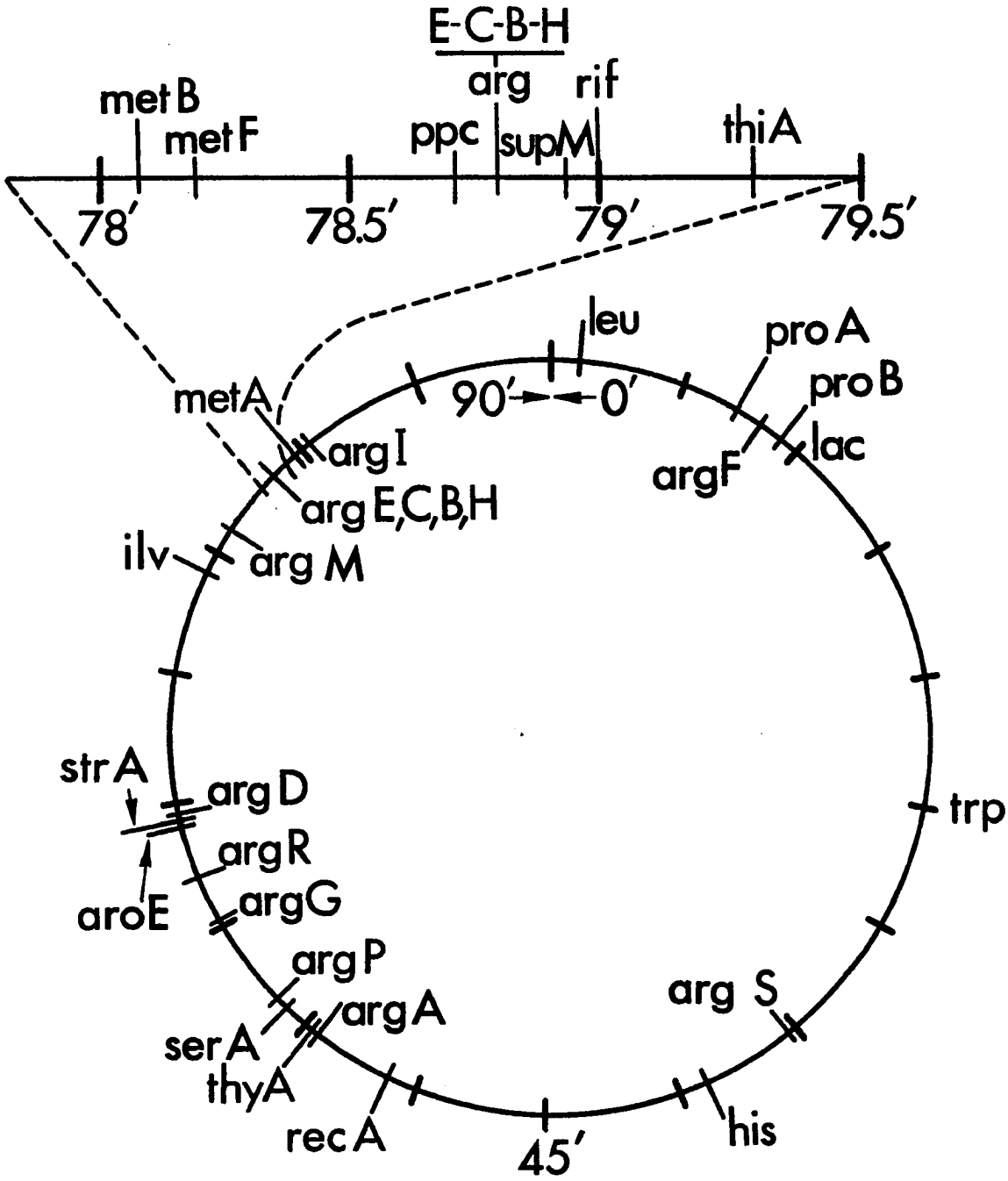



Figure 2. Linkage map showing genes of the arginine system in E. coli and reference markers.

δ-transaminase (personal communication from E. Jones). Ornithine transcarbamylase is encoded by two structural genes argF and argI (75). Both genes must be non-functional in order for a K-12 strain to be OTC-less. The arginine permease specified by argP, is responsible for the uptake of arginine, lysine and ornithine as well as canavanine (85,86), hence, argP mutants are also phenotypically canavanine-resistant. The other two genes which, when mutated, give rise to the phenotype of canavanine-resistance are the argR gene and the argS gene (87). The argS marker codes for arginyl-tRNA synthetase (88). A regulatory gene locus argR, controls the expression of all nine structural genes (90,91,92,93,94,95, 96). The arginine system has been termed a "regulon" (92).

The regulation of arginine synthesis in E. coli has been studied in three strains: W, K-12 and B. Though scattered, the genes in K-12 and W are controlled in parallel through repression by the end-product arginine (90,91,92,97), whereas in E. coli B, at least partial induction by arginine occurs (93,94,98). However, a repressible mutant was isolated from the nonrepressible strain B (93,94,98). The difference between strain K-12 and B now appears to reside in the nature of the argR alleles: strains carrying K-12 regulatory gene are repressible, those with the strain B gene are inducible (91,94,95). Indeed, in an elegant series of experiments involving merodiploids containing an amber nonsense mutation in the gene argR and various amber suppressor genes, Jacoby and Gorini (95) demonstrated that substitution of one amino acid by another in the argR gene protein product can change the type of regulation from that observed in E. coli B to that in E. coli K-12.

It was reported that in transient $argR^+/argR^-$ zygotes, $argR^+$

(or repressibility) is dominant over *argR*$^-$ (non-repressibility) (91). In permanent merodiploids for the *argR* region, arginine is able to repress synthesis of OTC, showing again that *argR*$^+$ is trans-dominant to *argR*$^-$ (92). Strains with amber and temperature-sensitive mutations in the *argR* locus (95) provide evidence for a protein repressor. Udaka (99), in 1970, reported the isolation from *E. coli* K-12 of a protein fraction, presumably containing the arginine repressor, whose characterization is still incomplete.

That arginine or a derivative can act as the corepressor for enzymes in the arginine biosynthetic pathway was first observed in 1953 (100) when it was discovered that arginine added to growing cultures of strain W of *E. coli* represses the formation of acetylornithinase. Since then it has been well documented that in the arginine biosynthetic pathway, repression of all eight biosynthetic enzymes in *E. coli* depends upon the level of intracellular arginine and upon the *argR* gene (90,93). It appears reasonable that the corepressor is arginine or a derivative and the aporepressor is specified by the *argR* gene. However, the mechanism by which they generate a repression signal is still under investigation.

Direct evidence suggesting a transcriptional control of arginine has been obtained recently. The fraction of total RNA hybridizing with the DNA of a $\phi$80 bacteriophage transducing the *argECBH* cluster is about 0.02% in *argR*$^+$ strains grown in the presence of excess arginine and increases to 0.4% under conditions of physiological (101,102) as well as genetic (*argR*$^-$mutants) derepression (102,103); it reaches the intermediate value of 0.15% in a wild-type *argR*$^+$ strain grown in the absence of arginine (101,102,104). These data are consistent with the view that

arginine signals repression by inhibiting the initiation of transcription of arg-mRNA and that the process is mediated in some way by the argR gene product. However, the possibility of a translational control signal operating simultaneously at a different level during repression, as suggested by Lavalle (105) cannot be ruled out.

By indirect and direct evidence, Vogel's group suggested that there are both transcriptional and translational aspects of arginine repression. McLellan and Vogel (106) explored the translational aspect of repression. They studied the accumulation of messenger RNA for arginine enzymes by starving auxotrophs for arginine, inhibiting further transcription with rifampin and measuring enzyme formation in the presence of either an excess, or a restricted, supply of arginine. For the $argR^+$ strain, little mRNA was found without starvation; for $argR^-$, a considerable amount of mRNA was demonstrated even without starvation. It was found that in the presence of excess arginine, there is substantial translation in an $argR^-$ strain, but relatively little translation in an $argR^+$ strain, apparently due to an accelerated degradation of mRNA under repressive conditions. Moreover, the formation and decay of mRNA were investigated directly by hybridization techniques (107). It was concluded that arginine exerts both translational as well as transcriptional repression on the arginine biosynthetic enzymes.

Further evidence of translational control was derived from studies with ribosomal inhibitors (108,109,110). Translational repression by arginine was inferred from the findings that tetracycline or streptomycin, during partial growth inhibition, will lower the differential rates of formation of the arginine enzymes when the conditions

are physiologically or genetically derepressed, but not when they are repressive.

Faanes and Rodgers showed that the arginine analogue, L-canavanine, represses the accumulation of translatable mRNA for three arginine enzymes in E. coli (111). However, the added canavanine has little or no effect on reducing the level of hybridizable argECBH messenger RNA under conditions in which canavanine prevents formation of translatable message. It apparently acts as a translational signal of repression.

In Neurospora crassa, arginyl-tRNA synthetase may play a role in the regulation of arginine biosynthesis. Nazario (112) has isolated arg10 (argininosuccinase) mutants which maintain high levels of ornithine carbamyltransferase even when grown in the presence of excess arginine. It was discovered that arginyl-tRNA synthetase is inhibited by the accumulated argininosuccinate in the cells, and this inhibition leads to a marked reduction in the percentage of charged arginyl-tRNA in the cells. Furthermore, inhibition of arginyl-tRNA synthetase activity by ornithine, citrulline, and argininosuccinate have been reported for Escherichia coli (113). Conversely, Hirshfield et al. (114) reported the repression of arginine biosynthetic enzymes by arginine is unaltered in canavanine-resistant mutants of E. coli possessing defective arginyl-tRNA synthetases. In addition, Celis and Maas (115) have reported that charging of arginyl-tRNA (five isoaccepting species) is not correlated with repression of arginine biosynthesis.

Faanes and Rodgers (111) have recently reported that canavanine repression of translatable messenger RNA for three arginine enzymes does

not occur in argS mutants unless canavanyl-tRNA is allowed to accumulate in such mutants. After 20 min growth with canavanine, E. coli strains containing a defective arginyl-tRNA synthetase (argS mutants) shows only 9% of $tRNA^{arg}$ which is protected from periodate oxidation, whereas an $argS^{+}$ strain has 42% charged $tRNA^{arg}$. However, they failed to detect a specific arginyl-tRNA species that might be involved in repression by canavanine. The data suggest that canavanine repression of the arginine pathway occurs only when high levels of canavanyl-tRNA are present, and thus support the notion that arginyl-tRNA synthetase plays a role in generating a repression signal.

Most recently, Williams (116) was able to show a role for arginyl-tRNA synthetase in the generation of the repression signal. Instead of isolating canavanine-resistant mutants derived from arginine bradytrophs in arginine-free enriched medium as performed by Hirshfield et al. (114), his canavanine-resistant mutants were derived from arginine prototrophs grown in minimal medium (116). These mutants were screened for nonrepressible synthesis of arginine; all such mutants (nonrepressible) are arginyl-tRNA synthetase mutants possessing about 30-70% of the normal synthetase activity. The mutant enzymes exhibit turnover in vivo and are less stable in vitro than those of the wild type at both $4^{\circ}C$ and $40^{\circ}C$; they also possess different affinities for both arginine and canavanine. Furthermore, in one case it was shown that (1) the mutant possesses unaltered uptake of arginine, and (2) that the mutant possesses diminished ability to incorporate canavanine into proteins and to attach canavanine to tRNA. Results of genetic experiments suggested that the mutants differ from the wild-type strain at only one

locus, that is to say that non-repressibility and reduced arginyl-tRNA synthetase activity are the results of a single mutation. Results also suggest that this mutation lies in the region of the chromosome designated for arginyl-tRNA (argS). It appears that arginyl-tRNA synthetase may be involved in some way in repression by arginine of its own biosynthetic enzymes. Furthermore, Williams and Williams (117) were able to show that one of the arginyl-tRNA synthetase mutants has reduced in vivo aminoacylation of two of the five isoaccepting species of $tRNA^{arg}$ and complete absence of aminoacylation of one of the five isoaccepting species. These data suggest that a finite level of aminoacylation of $tRNA^{arg}$ (all or one specific species) is essential for the generation of the repression signal. Based on the above evidence, it was proposed that arginyl-tRNA or arginyl-tRNA complexed with the synthetase is the corepressor or serves as the physiologically significant unit from which the corepressor is derived.

An interesting feature of the arginine system is the tight cluster formed by argE, C, B and H. These genes were found to be arranged in that order by three-point transduction tests and deletion mapping (118,119). Although the four loci appear to be adjacent, their expression is not strictly coordinated. Baumberg et al. (120) reported a two fold difference in the repressibility coefficients of argE and argH expression. Glansdorff and Sand (121) reached independently the same conclusion and concluded, in addition, that the expressions of argC, B and H present a strong degree of coordination, the repressibility coefficient of the corresponding enzymes being about 50. On the other hand, the repressibility coefficient for the synthesis of the enzyme

specified by argE is about 18.

The observation of polar effects of certain nonsense and deletion (presumably frameshift) mutations in argC and argB on the expression of B and H, and of H respectively (119,122), and of an argC mutation, probably frameshift, on the expression of B and H (89), are in agreement that nonsense and frameshift mutations exist in argC which exert a polar effect on argB and argH; nonsense and frameshift mutations in argB exert a polar effect only on argH. Nonsense and frameshift mutations in argH are nonpolar. These data together with the coordination of argB, C and H expression suggest that argCBH belongs to the same unit of expression and are polarized clockwise in the order C, B and H. Nonsense and frameshift mutation argE (119,123) are nonpolar, suggesting in agreement with the lack of coordination of argE with argC, B and H activities, that argE belongs to a different unit of expression.

Recently, both Glansdorff (124) and Jacoby (122) have reached independently the same conclusion that argE and argCBH form two operons transcribed in opposite directions (divergently) from an internal promoter-operator complex between argE and argC (Figure 3). Their results are discussed below.

Early in 1969, Glansdorff's group (119) had observed a peculiarity in cluster-gene expression of strain argEC-1, which has a deletion covering all of argE and the operator-proximal portion of argC. This strain has no trace of argE, argC and argB expression, but shows a low, constitutive, residual expression of argH, which is no longer repressible by arginine (124). They also showed that the argEC-1 mutation is trans-recessive. The properties of the argEC-1 deletion imply that argB

and H are controlled by an operator situated to the left of argC. Moreover, the same constitutive low level of argH expression is shown in another deletion mutant, P4XEC(B), in which the genes ppc, argE, argC, and probably a small portion of argB have been eliminated. These results suggest that the low residual level of argH observed is due to an internal initiator situated somewhere within argB or at the argB-H junction.

It was also observed that in a mutant carrying an argCB deletion (sup-102) which greatly lowers the rate of expression of argE but falls short of known argE markers, argH expression under repressed conditions is increased three and a half fold as compared to that of the normal wild-type strain (119,124). From sup-102 several derivatives were isolated in which the expression of argE is partly restored (124). In about a third of these strains, both argE and argH are expressed almost constitutively, and the mutations responsible appear to be cis-dominant and to map to the right of argE, probably between argE and argC. In two mutants with constitutive argE expression, argH appears to be deleted; since argE is constitutive in the two mutant strains, the deletion that removed argH might affect its operator. Since these two classes of mutations which reactivate argE expression constitutively are located on the right side of argE, it was suggested that an internal promoter-operator complex is situated between argE and argC.

Independently, and with a direct approach to the problem, Jacoby (122) succeeded in isolating operator mutations specific for genes in the arginine cluster. The technique employed is similar to that used for selecting operator mutations for argI (95,125), and relies on the observation that streptomycin-induced suppression of an arginine non-

sense mutation requires relief of repression such as imposed by the non-end product repressible *argR*$^{B}$ allele. Such operator mutations have been found. These $O^{c}$ mutations map between *argE* and *argC*, are cis-dominant, and cause partial constitutivity for *argE* as well as for *argCBH* (122). These results confirm Glansdorff's conclusions that the *argECBH* cluster comprises two operons transcribed divergently from an internal promoter-operator complex (Figure 3). The promoter-operator complex has not been genetically defined as yet. By preliminary hybridization studies, it was demonstrated that *argE* is transcribed from the heavy chain of the double stranded DNA, whereas *argCBH* is transcribed from the light chain (personal communication from R. Cunin).

The purpose of the work presented here is to gain more basic information as to the functional organization of the clustered arginine genes. We have isolated several mutants following nitrous acid mutagenesis. These mutants lose *argB* expression and concomitantly show a high *argH* expression under maximal arginine-repressed conditions. We have characterized them as to their locations in the arginine cluster both by three-point transduction tests and deletion mapping We have also studied their physiologic function when they are combined with either nonsense mutations or with promoter-operator deletions.

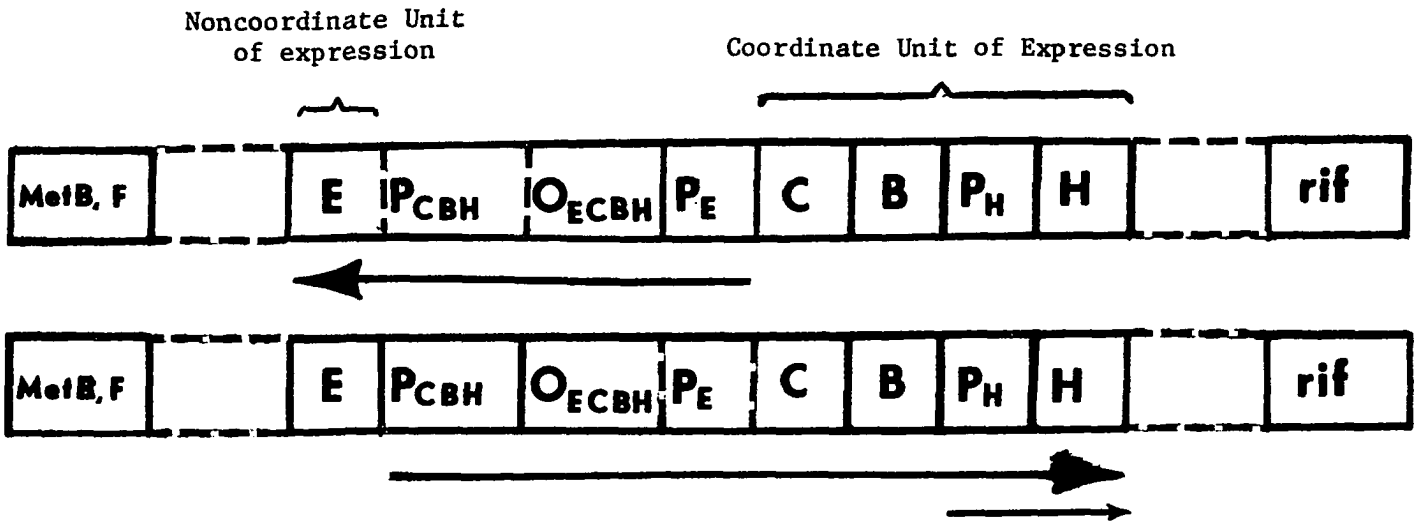



Figure 3. Proposed model for divergent transcription of arginine cluster in E. coli. Arrows indicate the direction of transcription.

| | |
|---|---|
| argE: | Acetylornithinase |
| argC: | Acetylglutamic-γ-semialdehyde dehydrogenase |
| argB: | Acetyl-γ-glutamokinase |
| argH: | Argininosuccinase |
| $arg O_{ECBH}$: | Operator for argECBH cluster |
| $P_E$: | Promoter for argE |
| $P_{CBH}$: | Promoter for argCBH |
| $P_H$: | Secondary promoter for argH |

# CHAPTER II

# MATERIALS AND METHODS

## Bacterial and Phage Strains

All strains of Escherichia coli and Salmonella typhimurium not arising as a result of genetic manipulation during the course of this study are listed in Table 2. Strains in use were maintained either on L agar slants, or in the case of merodiploids, on minimal medium agar slants supplemented so as to select for retention of the episome. All cultures were grown at 37°C and stored at 4°C.

Bacteriophage P1kc, a generalized transducing phage for E. coli, K-12 and B, is a clear-plaque mutant of P1.

## Chemicals

The following chemicals and enzymes were purchased from the Calbiochem Company: argininosuccinate (barium salt), glutathione (reduced), α-ketoglutarate, adenosine 5'-triphosphate, pyridoxal phosphate, pyruvate kinase, L-canavanine sulfate, Clelands reagent, L-arginine (arg), L-ornithine (orn), L-citrulline (cit), L-tyrosine, L-tryptophan (trp) and L-phenylalanine. 2-Aminopurine (AP), N-α-acetyl-L-ornithine (aco), N-acetyl-L-glutamic acid, phosphoenolpyruvate, p-aminobenzoic acid, p-hydroxybenzoic acid, thymine, nicotinamide adenine dinucleotide phosphate (NADP) were products of Sigma Chemical Company. Kanamycin

TABLE 2

LIST OF STRAINS

| Strain[a] | Other Designation | Mating Type | Genotype and Comments[b] | Source and References |
|---|---|---|---|---|
| EcK2 | K12,W.T. | $F^-$ | prototroph | - - - |
| EcK20 | KLF5/AB2463 | F' | F' *argE*$^+$/*argE*$^-$ *thi thr leu proA his str recA-13* $\lambda^-$ *lac gal ara xyl mtl* | K. B. Low (126) |
| EcK101 | Ra-2 | Hfr | $\lambda^-$; ←*metB argECBH rif* --- | K. B. Low (126, 127) |
| EcK111 | P4X6 | Hfr | *metB-1*;←*proB proA leu* - - - | D. Ezekiel (128) |
| EcK112 | P4X6R1 | Hfr | *metB-1 rif*;←*proB proA leu* | D. Ezekiel (128) |
| EcK164 | KL16-99 | Hfr | *thi* $\lambda^-$ *recA1*;←*thy recA* - - - *serA* | K. B. Low (126) |
| EcK172 | HCB6-21 | Hfr P4X | *metB* Δ(*argCB-1*);←*proB proA leu* - - | N. Glansdorff (119,124) |
| EcK173 | MN42 | Hfr P4X | *metB* Δ(*ppc-argECBH*) | N. Glansdorff (142) |
| EcK174 | P4XN1601 P4XEC(B) | Hfr P4X | *metB* Δ(*ppc-argECB*) | N. Glansdorff (119, 124) |
| EcK175 | Sup-102 | Hfr P4X | *metB* Δ[*argE*(?)-*argCB*] | N. Glansdorff (119, 124) |

TABLE 2 continued

| Strain[a] | Other Designation | Mating Type | Genotype and Comments[b] | Source and References |
|---|---|---|---|---|
| EcK176 | HCB7-18 | Hfr P4X | metB Δ(argEC-1) | N. Glansdorff (119, 124) |
| EcB177 | MG126 | F⁻ | aroE argR15 his strA40 $argC_{UGA}$ | G. Jacoby (122) |
| EcK180 | MG427 | F⁻ | Δ(ppc-argECBH) | G. Jacoby (122) |
| EcK183 | MG535 | F⁻ | thy $str^r$ $argO_{ECBH}$ $argR^+$ | G. Jacoby (122) |
| EcK185 | 30SOMA4 | Hfr P4X | argB-1 thi | N. Glansdorff (118, 119) |
| EcK186 | 12dTP67 | Hfr P4X | metB argB-5 | N. Glansdorff (119) |
| EcK187 | MN8A | Hfr P4X | metB argB-2 | N. Glansdorff (119) |
| EcK188 | 30SOMA2 | Hfr P4X | argH-3 thi | N. Glansdorff (118) |
| EcK189 | C600R9 | F⁻ | Δ(ppc-argECBH) supE | N. Glansdorff (119) |
| EcK190 | 342G1 | F⁻ | argH-2 thr leu thi his pro | N. Glansdorff (115) |
| EcK191 | P4XSB145 | Hfr P4X | metB argH-1 | N. Glansdorff (119) |
| EcK198 | RP31 | F⁻ | thi thyA argG metB his aroE str $\phi80^{sens}$ | W. Maas |

TABLE 2 continued

| Strain[a] | Other Designation | Mating Type | Genotype and Comments[b] | Source and References |
|---|---|---|---|---|
| EcW444 | 45A25 | $F^-$ | argG | H. J. Vogel |
| EcK496 | - - | $F^-$ | argH496 | D. Stroman |
| EcK1581 | - - | Hfr P4X6 | metB-1 argB | D. Stroman |
| EcK1583 | - - | Hfr P4X6 | metB-1 argB | D. Stroman |
| EcK1585 | - - | Hfr P4X6 | metB-1 argB | D. Stroman |
| EcK1586 | - - | Hfr P4X6 | metB-1 argB | D. Stroman |
| EcK1593 | - - | Hfr P4X6 | metB-1 argB[c] | D. Stroman |
| EcK1598 | - - | Hfr P4X6 | metB-1 argB | D. Stroman |
| EcK1637 | - - | Hfr P4X6 | metB-1 argB | D. Stroman |
| EcK1668 | - - | Hfr P4X6 | metB-1 argB | D. Stroman |
| EcK1734 | - - | Hfr P4X6 | metB-1 argB | D. Stroman |
| EcK1808 | - - | Hfr P4X6 | argB1511 | D. Stroman |
| EcK1809 | - - | Hfr P4X6 | argB1511 rif | D. Stroman |
| EcK1810 | - - | Hfr P4X6 | argC1418 | D. Stroman |
| EcK1811 | - - | Hfr P4X6 | argE492 rif | D. Stroman |
| EcK1817 | - - | Hfr P4X6 | argE492 | D. Stroman |
| EcK1818 | - - | Hfr P4X6 | argE492 rif | D. Stroman |
| EcK1820 | - - | Hfr P4X6 | argH496 | D. Stroman |

TABLE 2 continued

| Strain[a] | Other Designation | Mating Type | Genotype and Comments[b] | Source and References |
|---|---|---|---|---|
| EcK1982 | - - | Hfr P4X6 | *metB argH* | D. Stroman |
| EcK2007 | - - | Hfr P4X6 | *metB* Δ(*argBH*) | D. Stroman |
| EcK2009 | - - | Hfr P4X6 | *metB argH* | D. Stroman |
| EcK2041 | - - | Hfr P4X6 | *metB argB*[c] | D. Stroman |
| EcK2058 | - - | Hfr P4X6 | *metB argB* | D. Stroman |
| EcK2082 | - - | Hfr P4X6 | *metB argB* | D. Stroman |
| EcK2084 | - - | Hfr P4X6 | *metB argE* | D. Stroman |
| EcK2402 | - - | $F^-$ | *argR10* | E. Schneider |
| Sal27 | SL4040 | F' | F'*gal*$^+$ *attλ sup812*(ochre)/*gal*$^-$ *met trp fla str rfb* | J. J. Ferretti (153) |
| Sal428 | - - | $F^-$ | *hisG428* (ochre mutation) | J. J. Ferretti |
| Sal2100 | - - | $F^-$ | *hisG2100* (frameshift mutation) | J. J. Ferretti |
| Sal3018 | - - | $F^-$ | *hisD3018* (frameshift mutation) | J. J. Ferretti |

[a] EcK, *E.coli* K-12;
EcW, *E.coli* W;
EcB, *E.coli* B;
Sal, *Salmonella typhimurium*, LT-2.

[b] ←——————→ point of origin;
Δ ( ), designates deletion.

c *argX* mutants.

sulfate (USP) and neomycin sulfate (USP) are from Bristol Laboratories and Charles Pfizer & Co., Inc., respectively. Rifampin (rif) and streptomycin sulfate (USP) are from Schwartz/Mann. Diethylsulfate (DES) and ethyl methanesulfonate (EMS) were purchased from Fisher Scientific Company and Eastman Organic Chemical, respectively. N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was obtained from Aldrich Chemical Co., Inc. o-Aminobenzaldehyde was a product of K and K Chemical Co. and was redistilled before use. Arginase was purchased from Worthington Biochemical Corporation. ICR-191 was a gift from Dr. Hugh J. Creech.

All other reagents and solvents were obtained commercially in the highest grade available.

## Media and Reagents

All media were purchased from Difco Laboratories except N-Z Case, which was purchased from Sheffield Chemicals. N-Z Case is a vitamin-free enzymatic digest of casein.

Minimal Medium A (MMA). MMA (129) was prepared ten times concentrated (10X), stored over chloroform (2 ml/l), and diluted with distilled water just prior to sterilization by autoclaving. The 10X MMA contained in g/l: $K_2HPO_4$, 70.0; $KH_2PO_4$, 30.0; sodium citrate.$2H_2O$, 5.0; $MgSO_4.7H_2O$, 1.0; $(NH_4)_2SO_4$, 10.0. Glucose, added aseptically after autoclaving from a sterile stock solution of 25%, was used as a carbon and energy source in a final concentration of 0.5%. Glucose was omitted when MMA was used to wash and suspend cells.

Minimal Medium E (MME). MME (66) was prepared fifty times concentrated (50X), stored over chloroform (2 ml/l), and diluted with distilled water prior to sterilization by autoclaving. The 50X MME

contained in g/l: $MgSO_4.7H_2O$, 10.0; citric acid.$H_2O$, 100.0; $K_2HPO_4$, 500.0; $NaNH_4HPO_4$, 175.0. Glucose (0.5%, final concentration) was added separately after sterilization as above. Liquid stock cultures were prepared as MME + 0.2% glucose + 0.2% N-Z Case.

Arginine-free Medium (AF). AF medium was prepared by rehydrating Difco arginine assay medium with MMA (26.0 g/l MMA) and adding glucose (0.25%, final concentration) after autoclaving. The arginine assay medium was sterilized by filtration and added aseptically to MMA. AF + Can refers to arginine-free medium supplemented with L-canavanine (100 µg/ml, final concentration).

Peptone-beef Extract (PBE). PBE (130) contained 1% peptone and 0.3% beef-extract dissolved in distilled water, and was adjusted with NaOH to a pH of 7.6.

Nutrient Broth (NB). NB is rehydrated Difco nutrient broth (8.0 g/l of distilled water). NBG refers to NB supplemented with glucose (0.5%, final concentration).

Brain-heart Infusion Broth (BHIB). BHIB is rehydrated Difco brain-heart infusion broth (37.0 g/l of distilled water).

L Broth. L broth (131) contains in g/l: tryptone, 10.0; yeast extract, 5.0; NaCl, 10.0. The pH of L broth was adjusted to 7.0 with NaOH. Glucose was added to a final concentration of 0.1%.

LC broth. LC broth (132) is L broth to which $CaCl_2$ (final concentration, $2.5 \times 10^{-3}$ M) has been added.

Saline refers to 0.85% NaCl. It was used routinely to dilute bacterial cells.

Phage dilution buffer (133) contains in g/l: $Na_2HPO_4$, 7.0;

$KH_2PO_4$, 3.0; $NH_4Cl$, 1.0; $MgSO_4.7H_2O$, 0.25; NaCl, 5.0; gelatin, 0.02.

Unless otherwise noted, amino acids were added to minimal media in a final concentration of 100 µg/ml to supply the auxotrophic requirements of a particular strain. Solid media contained 16.0 g of agar per liter of media and soft agar had 7.5 g per liter of media.

"Aro mixture" (personal communication from W. Maas) contained: 0.01% p-aminobenzoic acid, 0.01% p-hydroxybenzoic acid, 0.5% tyrosine, 0.5% tryptophan, 0.5% phenylalanine. It was sterilized by autoclaving and added to medium aseptically to give a 1:100 dilution. It was added to L broth when necessary to fulfill the requirements of aroE auxotrophs.

Thymine was added to MMA to a concentration of 100 µg/ml. It was reduced to 50 µg/ml when added to L broth.

Rifampin (rif) was dissolved in 95% ethyl alcohol to a concentration of 100 mg/10 ml, and added to solid or liquid medium prior to the addition of glucose.

## Growth Conditions of Bacterial Cultures

Bacteria from stock slants were inoculated directly into broth. These liquid cultures were incubated at 37°C. Liquid cultures of E. coli, W, were grown without aeration, whereas those of K-12 and B were grown with aeration in a water bath rotatory shaker (Fermentation Design Inc.) at a constant speed of 250 rpm. Growth in liquid media was followed turbidimetrically in a Klett-Summerson colorimeter fitted with a No. 66 filter. Log-phase cells were routinely achieved by adding an equal volume of the same liquid medium to an overnight culture and incubating for 2 hours.

## Viable Cell Count

A standard curve relating viable cell concentration to colorimeter reading was constructed for logarithmic phase K-12 cells growing in L broth (Figure 4). Samples were withdrawn periodically, diluted with buffer, and 0.1 ml aliquots of appropriate dilutions were spread in triplicate onto the surface of NBG agar plates. After 24 hours incubation, the number of colonies per plate was counted.

## Reversion Studies

### Spontaneous Reversions

The bacteria were grown overnight in 3.0 ml L broth. Fresh L broth (3.0 ml) was added and the cultures were reincubated for two hours. The turbidity was measured and the cells were centrifuged, washed with 2.0 ml saline, and resuspended in saline to a density of 2.0 X $10^9$ cells/ml. Aliquots of 0.1 ml were spread onto solid MMA + methionine (met) + 1.25% (v/v) nutrient broth but without the requirements for which prototrophy was being sought. Plates were spread in triplicate. The number of colonies arising after five days of incubation at 37°C is a direct indication of the spontaneous reversion frequency.

### Mutagen-induced Reversions

The cells were prepared exactly as for spontaneous reversion. After the cells were spread, 50 µl of NTG (2 mg/ml), 10 µl of EMS (undiluted), 5 µl of DES (undiluted), 50 µl of 2-AP (2 mg/ml), or 10 µl of ICR-191 (1 mg/ml) (applied in subdued light) were applied onto the agar surface of each plate. Plates were incubated for 5 days and scored for colony growth peripheral to a clear zone of inhibition. Controls were

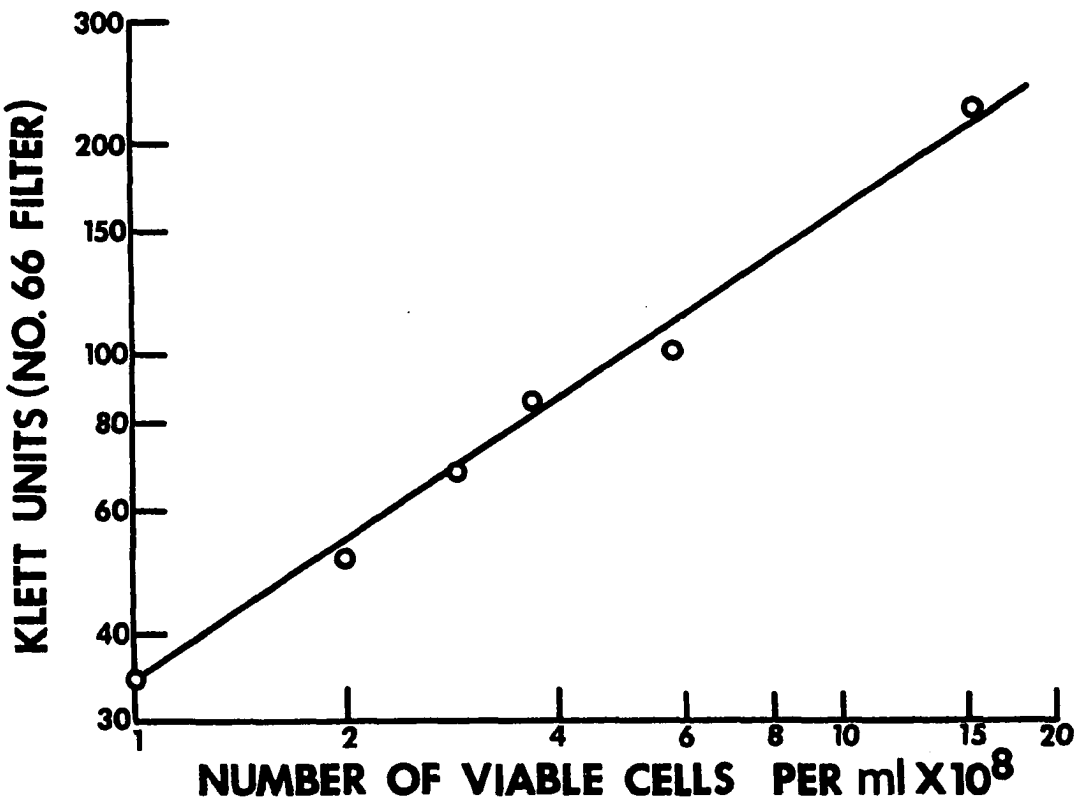



Figure 4. Standard curve relating number of viable cells of E. coli K-12 to Klett units.

performed with previously characterized mutants as well as with media from which mutagens had been omitted.

### Phenotypic Curing

The cells were prepared exactly as for spontaneous reversion. After the cells were spread, aliquots of the following antibiotics were placed onto the surface of the agar: 0.2 ml of kanamycin sulfate (3.3 mg/ml); 0.1 ml of neomycin sulfate (15 mg/ml); and 0.1 ml of streptomycin sulfate (15 mg/ml). The plates were incubated for 5 days and scored for colony growth peripheral to a zone of inhibition. Controls were performed as described above.

### Preparation of Cell-Free Extracts

Bacteria from stock slants were inoculated into 3.0 ml of MME + 0.2% glucose + 0.2% N-Z Case (liquid stock culture), and grown overnight. Cells were inoculated into side-arm flasks containing 50 ml of the designated medium and cultivated overnight to a cell density corresponding to 40-70 Kletts units (mid-log phase). An appropriate volume of cells was transferred into 150 or 300 ml of the same medium to give a turbidity of approximately 8 Klett units, and the flasks were reincubated. When the cell density reached 40-50 Klett units, the cultures were chilled and collected by centrifugation at $4^{\circ}C$ in a Sorvall RC2-B centrifuge (GSA rotor) at 10,000 rpm for thirty minutes. The cells were washed with MMA, resuspended in 4.0 ml of 0.1 M phosphate buffer, pH 7.4, containing $1 \times 10^{-4}$ M Cleland's reagent (extract buffer), and sonified for 60 seconds in a 10 ml beaker with a Branson sonifier (Model S125) at a power setting of 4.0. Cell debris and unbroken cells were removed by

centrifugation at 4°C in a Sorvall RC2-B centrifuge (SS-1 rotor) at 15,000 rpm for 10 min. Portions of the cell-free extract were dispensed into two tubes and kept frozen until assayed.

## Enzymes Assays and Protein Determination

### Lowry Protein Determination

The procedure employed was a modification (134) of the Lowry method. Bovine serum albumin was used as a standard.

### N-Acetyl-γ-Glutamokinase Assay

The assay is essentially that of Vogel and McLellan (135) as improved by Stroman (80). The reaction mixture contained: 60 μmoles of $KPO_4$ buffer (pH 7.4), 1.2 μmoles of ATP, 9.9 μmoles of phosphoenolpyruvate, 3.0 E.U. of pyruvate kinase, 20 μmoles of N-acetyl-L-glutamate, 3.0 μmoles of $MgCl_2$, 4.0 μmoles of KF, and enzyme extract, in a total volume of 0.6 ml. The reaction was started by the addition of N-acetyl-L-glutamate and was stopped by addition of 0.5 ml of 0.1% 2,4-dinitrophenylhydrazine in 4N HCl. The reaction yields as by-product, ADP, which in the presence of pyruvate kinase and phosphoenolpyruvate, leads to the formation of pyruvate and subsequent regeneration of ATP. The pyruvate produced was determined colorimetically, as its 2,4-dinitrophenylhydrazone, in a Klett-Summerson colorimeter fitted with a No. 54 filter. One unit of N-acetyl-γ-glutamokinase activity is defined as that amount of enzyme which catalyzes the formation of 0.1 μmole of pyruvate in 15 minutes at 37°C.

N-Acetylglutamic-γ-Semialdehyde Dehydrogenase Assay

The assay is a modification of the procedure of Vogel and McLellan (136) as described by Stroman (80). The reaction mixture contained: 3.6 μmoles N-acetylglutamic-γ-semialdehyde, 100 μmoles glycine-NaOH buffer (pH 9.8), 40 μmoles of $K_2HPO_4$, 0.6 μmoles of NADP, and enzyme extract, in a final volume of 1.5 ml. The reaction was started by addition of NADP, and was measured as the increase in absorbance at 340 mμ due to the reduction of NADP which occurs when N-acetylglutamic-γ-semialdehyde is converted enzymatically to N-acetylglutamyl phosphate (the reverse reaction).

One unit of N-acetylglutamic-γ-semialdehyde dehydrogenase activity is defined as that amount of enzyme which causes an increase in absorbance of 0.010 per minute at 37°C.

N-Acetylornithine δ-Transaminase Assay

This enzyme was assayed by the procedure of Vogel and Jones (137). The reaction mixture contained: 50 μmoles of $KPO_4$ buffer (pH 8.0), 9.4 μmoles of pyridoxal phosphate, 1.7 μmoles of α-ketoglutarate (pH 6.0-6.5), 1.4 μmoles of acetylornithine, and enzyme extract, in a final volume of 0.5 ml. The reaction was started by the addition of extract, stopped by the addition of 0.3 ml of 6N HCl, and boiled for 30 min (to hydrolyze the N-acetylglutamic-γ-semialdehyde to glutamic-γ-semialdehyde).

This assay depends on the acid hydrolysis to glutamic-γ-semialdehyde of the enzymatically produced N-acetylglutamic-γ- semialdehyde. Glutamic-γ-semialdehyde cyclizes spontaneously to $\Delta^1$-pyrroline-5-carboxylic acid which in turn reacts with o-aminobenzaldehyde to yield

a yellow dihydroquinazolium compound. One unit of acetylornithine δ-transaminase activity is defined as that amount of enzyme which yields an absorbance of 0.100 at 440 mμ per 15 minutes at 37°C. The value of 0.100 corresponds to 0.86 μmoles of Δ'-pyrroline-5-carboxylic acid per hydrolyzed reaction mixture.

### Acetylornithinase Assay

The method of Vogel and McLellan (138) was used to assay acetylornithinase activity. The assay is based on the determination with ninhydrin of the ornithine produced. The reaction mixture contained: 40 μmoles of $KPO_4$ buffer (pH 7.0), 0.1 μmole of $CoCl_2.6H_2O$, 0.4 μmole of glutathione, 3.0 μmoles of acetylornithine, and enzyme extract, in a final volume of 0.5 ml. Color change was read in a Klett-Summerson colorimeter fitted with a No. 42 filter. The reaction was started by the addition of substrate (aco), and stopped by the addition of 1.5 ml of ninhydrin reagent and boiled for 10 min.

One unit of acetylornithinase activity is defined as that amount of enzyme which catalyzes the formation of 0.10 μmole of ornithine in 10 min at 37°C.

### Argininosuccinase Assay

This enzyme was determined by a modification of the method of Ratner et al. (139). Instead of determining the urea released, this assay utilizes ninhydrin to measure the ornithine formed. The ornithine is formed by added arginase from the arginine produced by argininosuccinase. The reaction mixture contained: 10 μmoles of $KPO_4$ buffer (pH 7.5), 4.0 E.U. arginase, 1.5 μmoles of argininosuccinate as the

potassium salt, and enzyme extract, in a total volume of 0.5 ml. The reaction was started by the addition of substrate (argininosuccinate), stopped by the addition of 1.5 ml of ninhydrin reagent, and boiled for 10 min.

One unit of argininosuccinase activity is defined as that amount of enzyme which catalyzes the formation of 0.10 µmole of ornithine in 15 minutes at 37°C. This assay is able to detect as little as 0.006 µmole of ornithine produced, but is hardly reproducible at this level. The lower limit of the assay which gives reproducible results is about 0.01 µmole of ornithine, with an upper limit of at least 0.4 µmole of ornithine produced.

Argininosuccinase activity has been shown to be proportional to incubation time for at least 180 min (Figure 5). Argininosuccinase activity is linear with protein concentration at least up to 1 mg protein per ml of extract (Figure 6). It has also been shown by Stroman (80) that argininosuccinase activity is proportional to protein concentration; however, the linear relationship shows a break at about 1.2 mg protein. Therefore, all extracts were diluted to below 1.2 mg protein per ml of extract before assay.

## Bacteriophage Techniques

### Lysate Preparation

Lysates of P1*kc* were prepared by the soft-agar overlay technique. The bacteria used as donors were grown overnight in 3.0 ml LC broth. Fresh LC broth (3.0 ml) was added and the cultures incubated for two hours. To 2.5 ml of melted LC soft agar, were added $10^6$ P1*kc* phage and

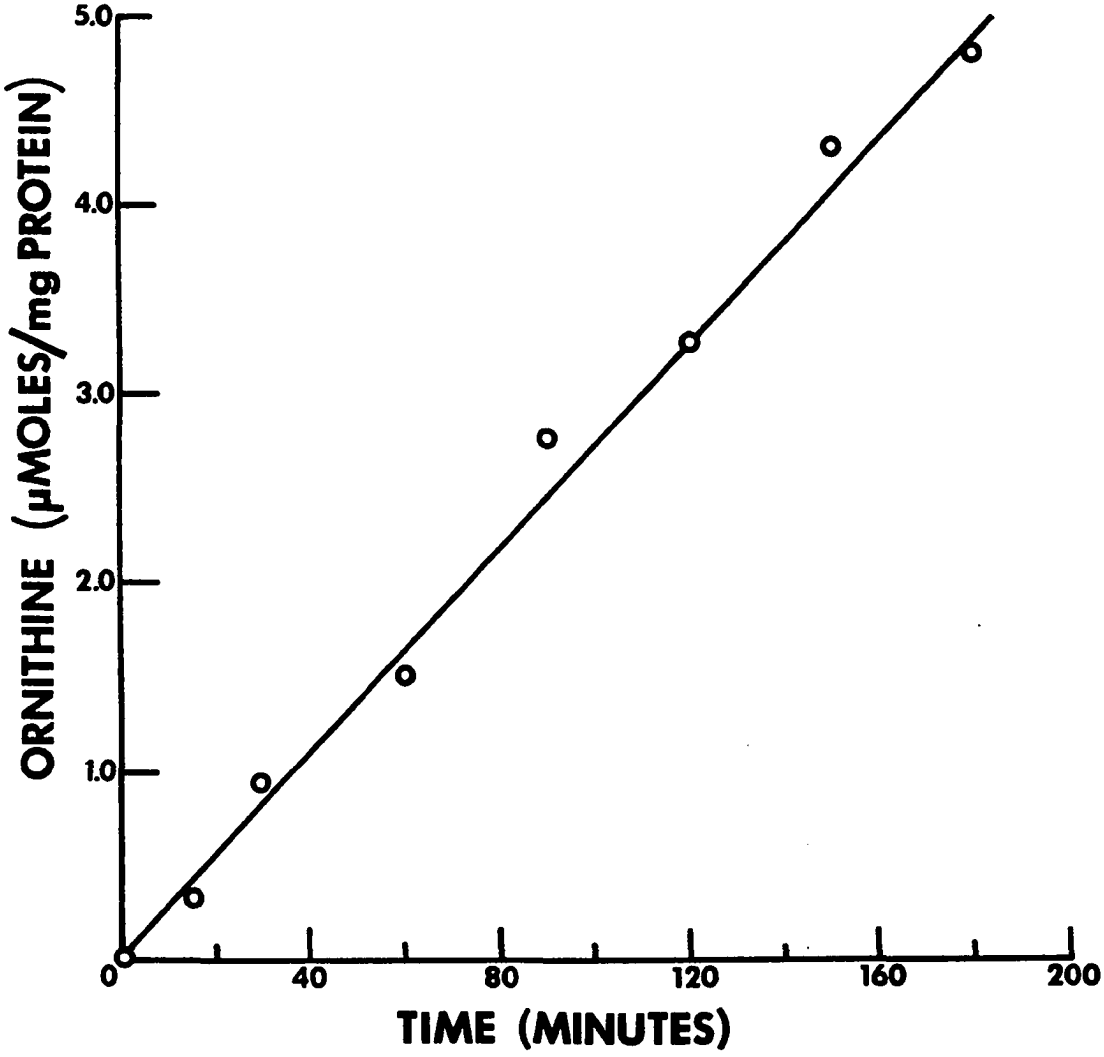



Figure 5. Proportionality of argininosuccinase to time.

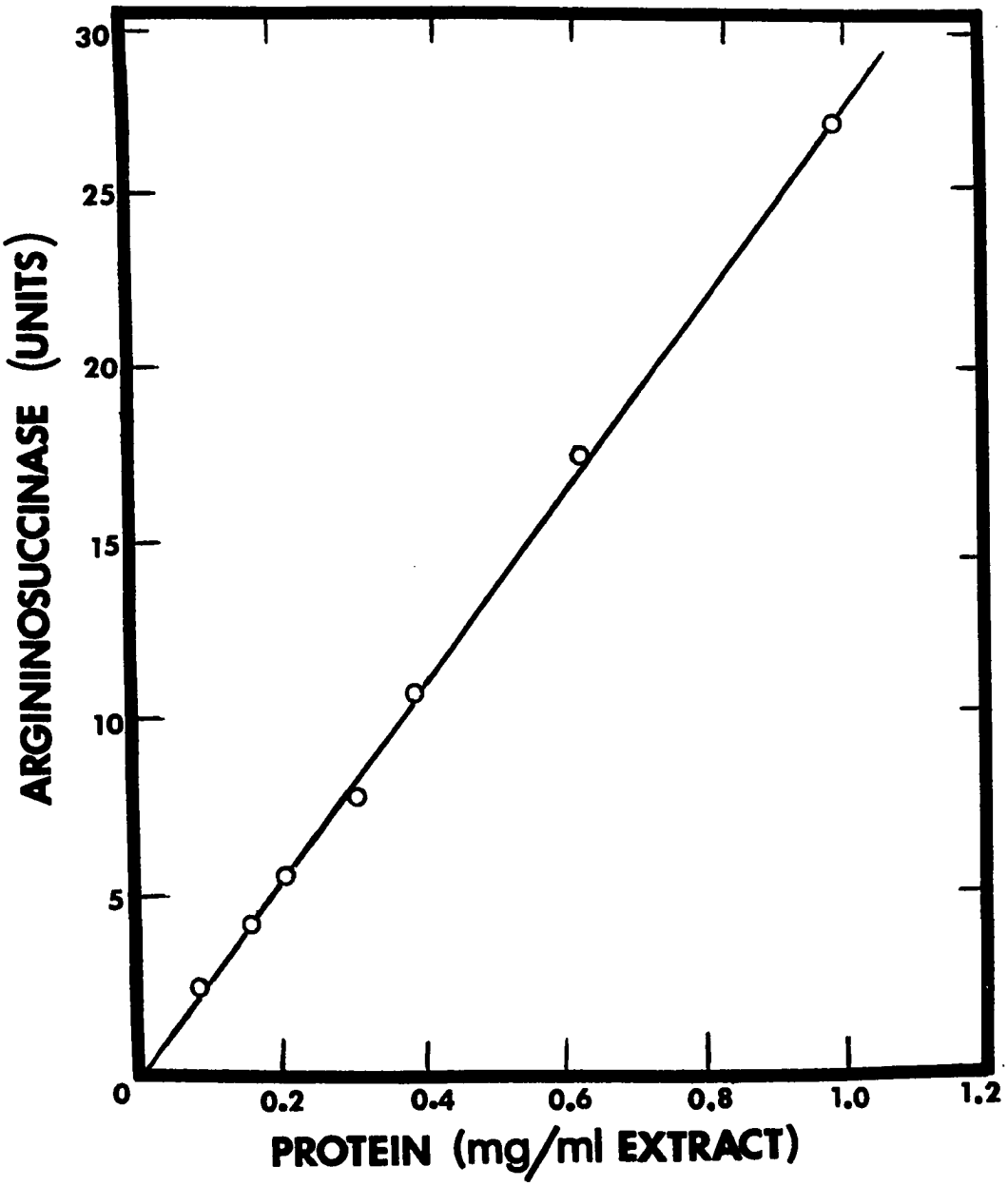



Figure 6. Activity of argininosuccinase as a function of protein concentration.

0.2 ml of donor cells, and the entire mixture was poured over an LC agar plate. The plate was incubated for 8-10 hours until confluent lysis of cells had occurred; then 4.0 ml of L broth were pipetted onto the surface of the soft agar and allowed to remain for 2-3 hours. The broth was collected, treated with chloroform to kill remaining bacteria, centrifuged to remove cell debris, and stored at $4^{o}C$ in a screw-capped tube with a drop of chloroform in the bottom. The stock lysate was titered, using the same procedure as described above, to determine the number of phage (plaque forming units- pfu) per ml. Only lysates with titers of more than $1 \times 10^{10}$ infective particles per ml were used for construction of strains or for mapping.

The recipient cells were grown overnight in 5.0 ml of LC broth. After addition of 5.0 ml of fresh LC broth, the culture was incubated for two hours. The turbidity of a 5.0 ml sample, withdrawn aseptically was determined in a Klett-Summerson colorimeter with a No. 66 filter. The remaining 5.0 ml of culture was centrifuged and resuspended in LC broth to give $1.0 \times 10^{9}$ cells per ml. These cells were mixed gently with phage at a multiplicity of infection (MOI) of 5 phage per cell, and incubated for 30 min without shaking. Infected cells were centrifuged, washed with 3.0 ml MMA and resuspended in 1.0 ml MMA. Appropriate dilutions were spread onto selective media. Controls were performed (1) by spreading uninfected cells to ascertain the occurrence of mutations which might mimic the desired transductants, and (2) by spreading lysate alone to determine whether unlysed donor cells or contaminants were present which might mimic transductants. Transductants formed good-sized colonies after 48 hours incubation at $37^{o}C$.

Dominant allele to recessive allele. If the donor marker is recessive to the recipient allele, then it is necessary to allow segregation of the two alleles in the "transductant" before placing the bacteria on selective media. For example, in the case of transducing a $rif^r$ allele into a recipient which contains a $rif^s$ allele, the infected cells were inoculated into MMA broth plus any auxotrophic requirements of the recipient (but in the absence of rif) and incubated in a shaking water bath at 37°C for 6 hours before plating on agar with rif.

On the other hand, if an $argR^+$ recipient was to be transduced to $argR^-$, the transductants were inoculated into liquid medium (AF + supplements) and incubated for 6-8 hours before being plated on AF + Can agar plates. Canavanine-resistant colonies were considered presumptive $argR^-$ mutants. If the recipient is auxotrophic for arginine and the block occurs before ornithine, ornithine (100 µg/ml) is added to AF + Can agar to fulfill the auxotrophic requirement. The addition of orn (100 µg/ml) to AF + Can agar media does not allow growth of the $argR^+$ cells.

The ability to excrete excess arginine, together with resistance to canavanine, are the two criteria for distinguishing an $argR^-$ genotype on solid media. Hence, it is necessary to check the ability of canavanine-resistant, presumptive $argR^-$ transductants to cross-feed an arginine auxotroph. The auxotrophic strain EcW444 (argG) was grown overnight in 3 ml of BH broth. An additional 3 ml of BH broth was added and, following a two hour incubation, the cells were centrifuged, washed once with, and resuspended in, 2.0 ml of saline. A 0.1 ml aliquot of a 1:200 saline dilution was spread as a lawn of cells on MMA + orn (200

μg/ml) + any growth requirements of presumptive argR⁻ transductants other than arginine.

The "argR⁻" strains were inoculated onto the plates as stabs with a straight-wire inoculating needle. Crossfeeding (detectable as a halo of growth around a stab) was apparent after 24 hours of incubation.

## Growth Response of Arginine Auxotrophs on Arginine Intermediates

All arginine auxotrophs isolated in the course of this study were characterized, after purification by streaking twice for isolated colonies, as to their ability to grow on MMA plus acetylornithine (argA, B, X, C, or D mutants), ornithine (argE mutants), citrulline (argF or I mutants), or arginine (argG or argH mutants).

## Deletion Mapping

The method used in constructing a deletion map was modified from that of Blume and Balbinder (140). A mutation is considered to be a deletion if it (1) is not revertible spontaneously or by mutagens and (2) is a multisite mutation (does not give prototrophic recombinants when crossed with several nonidentical point mutants).

Stock phage lysate was prepared routinely by propagation on strain MN42 (EcK173) which has a deletion of the arginine cluster. This lysate was recycled twice on donor cells prior to use in mapping.

### Spot Plate Test

Recipient cells were grown overnight in 3.0 ml LC broth. To this culture, 3.0 ml of fresh LC broth were added. Following incubation for two hours to reach log phase, the cells (0.1 ml) were spread as a

lawn onto MMA + auxotrophic requirements of recipients with the exception of arginine. A drop of phage which had been propagated twice on the donor was spotted onto the recipient lawn. In this way, 16 donors could be tested on the same plate. The donor phage were prepared by making a 1:10 dilution of the lysate in phage dilution buffer and irradiating with UV light to give 10% survival of phage (Figure 7). This degree of irradiation increases the frequency of transduction and hence the sensitivity of the spot plate test. Phage were irradiated with a 15 watt germicidal UV lamp (GE15T8) positioned 40 cm above the open petri dish. The number of transductants arising after 48 hours of incubation was scored. Any cross which yielded 6 or fewer transductants was repeated using the half plate test. Controls were run with uninfected cells and with lysates.

### Half Plate Test

The recipient lawn and the donor phage were prepared as above. This test, however, consisted of spreading 0.1 ml of irradiated phage over half a plate, thus increasing by 10-20 fold the number of phage-infected bacteria. Consequently, the sensitivity of detection of *arg*$^+$ transductants was enhanced 10-20 fold. Any cross which had 10 or fewer transductants was repeated using the whole plate test.

### Whole Plate Test

This was done as a standard transduction as described above.

## Construction of Double Mutants

Construction of all strains was by P1*kc* transduction.

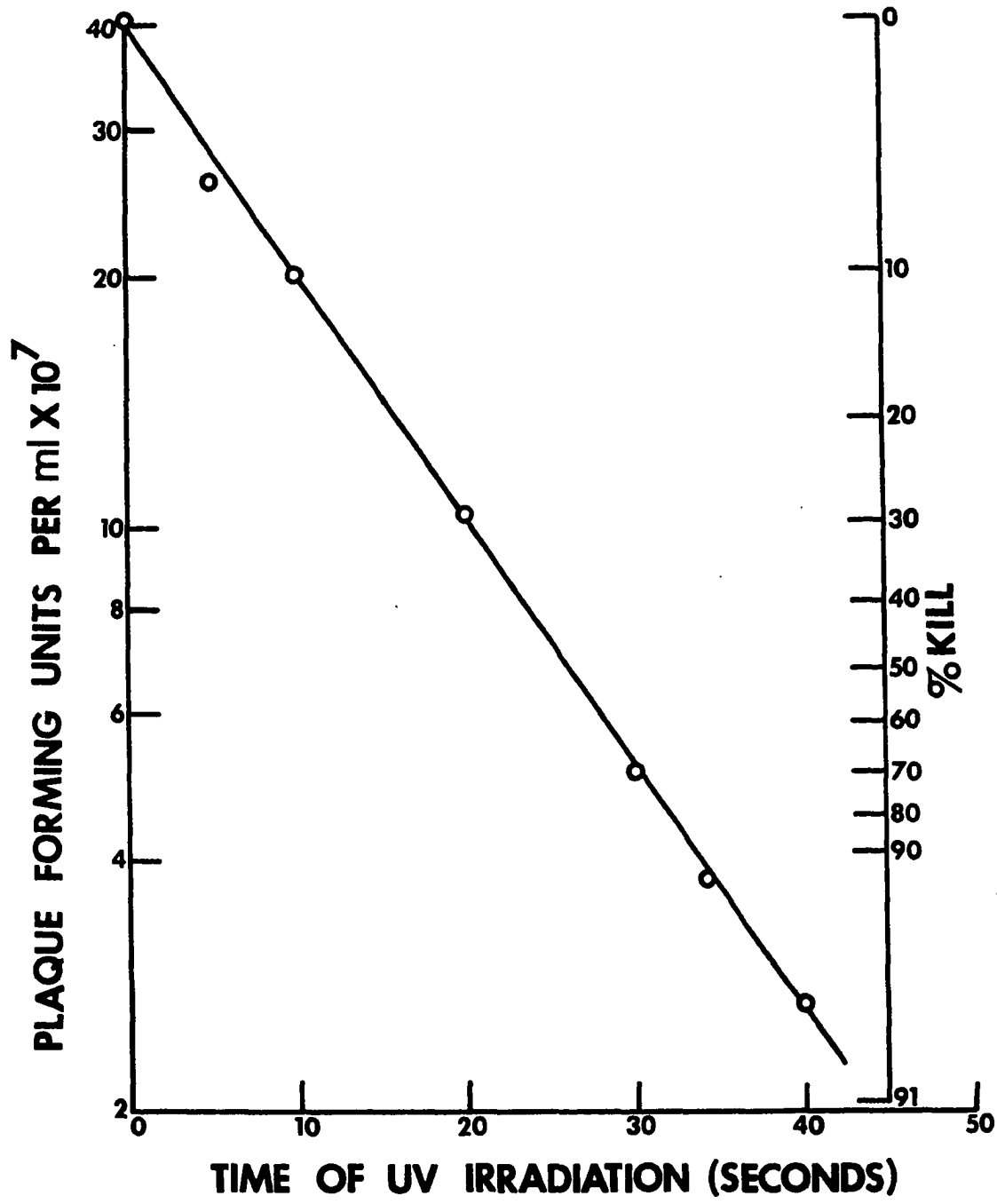



Figure 7. Survival of bacteriophage P1kc as a function of UV dosage.

Construction of *argE argX* Double Mutant

Strain EcK2041 (*metB argX2041*) was first made rifampin-resistant (Rif-R) by using EcK112 (*metB* $rif^r$) as a donor. Transductants were selected for Rif-R (plated on NBG + rif agar) and scored for the $Aco^-$ and $Met^-$ phenotypes.

To construct the double mutant (*argE argX*), strain EcK2041a (*metB argX2041* $rif^r$) served as the donor and EcK2084 (*metB argE* $rif^s$) as the recipient. Transductants were plated on MMA + aco + rif + met + limiting arginine (4 µg/ml) to select for $arg^-$ $rif^r$. The presumptive double mutants (*argE argX*) appear as tiny colonies since they cannot synthesize arginine from acetylornithine and the exogenous supply of arginine is growth-limiting. Therefore, the tiny Rif-R colonies were scored for an $Orn^-$ phenotype (i.e., ability to grow on ornithine but not on acetylornithine) which reflects the presence of the *argE* mutation in the transductants. The presence of the *argX* mutation in the presumptive *metB argE argX* $rif^r$ strains was confirmed genetically by spot-transduction tests with the original *metB argX2041* mutant (EcK2041) serving as donor, and the presumptive *metB argE argX* $rif^r$ strains were used as recipients. The donor lysate was prepared by UV irradiation of a 1:10 dilution in phage dilution buffer as described above. Aliquots of 0.2 ml of diluted lysate were spread on MM + met. Presumptive double mutants were suspended in a drop of saline (containing $2.5 \times 10^{-3}$ M $CaCl_2$) and spotted with a sterile tooth pick onto the donor lawn. In this way, 16 presumptive double mutants could be tested on the same plate. Any of those strains which failed to yield prototrophic arginine recombinants by spot-transduction were purified twice before further

tests by whole plate transduction. Strains which showed an Orn$^-$ phenotype and which did not yield *arg*$^+$ transductants when crossed with an *argX* donor were taken to be *argE argX* double mutants. The presence of the altered *argX* site was further confirmed by recovery of *argX* among the transductants resulting from an out-cross with a wild-type (*arg*$^+$ *rif*$^s$) donor (EcK2). Progeny were plated on MMA + aco + rif. Recombinants which were *metB*$^+$ *argE*$^+$ were scored for *argX* (Aco$^-$). Finally, the presence of the *argE argX* mutations in the double mutant was further confirmed by enzyme assay.

By this procedure mutant EcK3613 (*metB argE argX2041 rif*$^r$) was constructed.

### Construction of *argX argH* Double Mutant

EcK496 (*argH*) was made Rif-R (plated on MMA + arg + rif). The resulting *argH rif*$^r$ markers (EcK496-5) were cotransduced into recipient *argX2041* (*metB argX rif*$^s$). Selection was for Rif-R and *argH* on MMA + met + aco + rif + limiting arginine (4 µg/ml). Recombinants carrying *argH* would be expected to give small colonies due to the limiting supply of arginine. Progeny which were Rif-R and Arg$^-$ were subjected to spot and whole plate transduction tests with *argX2041* as donor as described above. Any mutants which failed to yield *arg*$^+$ were considered presumptive *argX argH* double mutants. The presence of *argX* in the presumptive double mutant was demonstrated by outcross of *argH* utilizing *arg*$^+$ *rif*$^s$ wild-type strain (EcK2) as the donor. Transductants were plated on MMA + aco + met. Any *argH*$^+$ recombinants were screened for *argX* (Aco$^-$). Loss of the *Arg*$^-$ phenotype, together with the appearance of the Aco$^-$ phenotype, was taken as confirmation of the presence of the *argX* marker in the

double mutant. The presence of the argH mutation in the double mutant was further confirmed by enzyme assay.

Strain EcK3615 (metB argX2041 argH rif$^r$) was constructed by this procedure.

Construction of argEC-1 argX Double Mutant

Strain EcK1593 (metB argX1593) was made Rif-R by using EcK112 (metB rif$^r$) as a donor (selected on NBG + rif). The resulting strain is EcK1593-6 (metB argX1593 rif$^r$). The Rif-R derivative of argX2041 (EcK2041a) was constructed as described above. The argX rif$^r$ markers of both strains (EcK1593-6, and EcK2041a) were cotransduced into EcK176 (metB argEC-1). Recombinants were selected on MMA + met + orn + rif. On this selective media, strains carrying argEC-1, but not argX, grow slowly and form very tiny colonies after 48 hours of incubation; strains bearing argX, but not argEC-1, grow more rapidly and form large colonies. The argEC-1 argX double mutants would be expected to yield medium sized colonies. Therefore, medium-sized colonies were picked among rif$^r$ recombinants and screened for an Orn$^-$ (argEC-1) phenotype. All Orn$^-$ transductants were further tested for the presence of the argX mutation by spot and whole plate transductions with argX2041 and argX1593 as the donors, respectively. All those which failed to yield arg$^+$ transductants were assayed to prove they were double mutants.

Strains EcK3725 (metB argEC-1 argX2041 rif$^r$) and EcK3764 (metB argEC-1 argX1593 rif$^r$) were constructed by this procedure.

The rif$^r$ marker was introduced into EcK176 (metB argEC-1) by using EcK112 (metB rif$^r$) as donor. Transductants were plated on L agar + rif. The strain EcK3726 (metB argEC-1 rif$^r$) thus isolated served as

a control in enzyme assays.

Introduction of Polar *argC*$_{UGA}$ into P4X6 (EcK111)

EcK2084 (*metB argE*) was made *rif*$^r$ (EcK2084-1) by using EcK112 (*metB rif*$^r$) as the donor (plated on MMA + orn + met + rif). The polar *argC*$_{UGA}$ marker was introduced into 2084-1 (*metB*$^-$ *argE rif*$^r$) with EcB177 (*metB*$^+$ *argC*$_{UGA}$ *rif*$^s$ *aroE argR15 his strA40*) as the donor. The cells were plated on MMA + aco + rif to select for *metB*$^+$ *rif*$^r$ recombinants which were screened in turn for the Aco$^-$ phenotype. The Aco$^-$ phenotype should reflect the presence of the *argC* mutation. The presence of the *argC* mutation was confirmed by enzyme assay. Strain EcK3653 (*argC*$_{UGA}$ *rif*$^r$) was constructed by this procedure.

Construction of Polar *argC*$_{UGA}$ and *argX* Double Mutant

The polar *argC*$_{UGA}$ mutation was transduced into EcK3613 (*metB*$^-$ *argE*$^-$ *argX rif*$^r$) with EcB177 (*metB*$^+$ *argC*$_{UGA}$ *rif*$^s$ *aroE argR15 his strA40*) as the donor. Transductants were plated on MMA + aco + rif to select for *metB*$^+$ *rif*$^r$ progeny which were scored for Aco$^-$. Transductants that showed an Aco$^-$ phenotype were further tested by spot transduction with an *argX2041* lysate. Recombinants that failed to yield *arg*$^+$ were purified twice, and then subjected to the whole plate transduction test with both *argX2041* and EcB177 as donors. Transductants which failed to yield any *arg*$^+$ with either donor were assumed to have incorporated both the *argX* and *argC* mutant sites. The presumptive double mutants were assayed to confirm the presence of both the *argC* and *argX* mutations. Strain EcK3648 (*argC*$_{UGA}$ *argX2041 rif*$^r$) was constructed in this manner.

### Transfer of $\underline{argO}_{ECBH}$ into P4X6 (EcK111)

The $\underline{argE}^+$ $\underline{argO}_{ECBH}$ loci were cotransduced into EcK2084-1 ($\underline{metB}^-$ $\underline{argE}^-$ $\underline{argO}^+$ $\underline{rif}^r$); the donor was EcK183 ($\underline{metB}^+$ $\underline{argE}^+$ $\underline{argO}^c$ $\underline{rif}^s$ $\underline{thy}$). Prototrophic ($\underline{metB}^+$ $\underline{argE}^+$) recombinants were selected on MMA + rif. Since the $\underline{argO}^c$ locus is cotransduced with the $\underline{argE}$ gene at a very high frequency, it was expected that almost all of the $\underline{argE}^+$ progeny would also be carrying the $\underline{argO}^c$ marker. The presence of the $\underline{argO}^c$ mutation was confirmed by enzyme assay. Strain EcK3766 ($\underline{argO}_{ECBH}$ $\underline{rif}^r$) was constructed by this procedure.

### Construction of $\underline{argO}_{ECBH}$ $\underline{argX}$ Double Mutant

The prototrophic strain EcK3766 ($\underline{argO}_{ECBH}$ $\underline{rif}^r$) constructed above was used as a donor to construct an $\underline{argO}_{ECBH}$ $\underline{argX}$ double mutant. The recipient was EcK3613 ($\underline{metB}^-$ $\underline{argE}^-$ $\underline{argX2041}$ $\underline{rif}^r$). Cells were plated on MMA + aco + rif to select for $\underline{metB}^+$ $\underline{argE}^+$ recombinants. The $\underline{argE}^+$ progeny were scored for the $Aco^-$ ($\underline{argX}$) phenotype. Such $Aco^-$ recombinants were presumed to have the $\underline{argX}$ locus intact. The presence of both the $\underline{argO}_{ECBH}$ and $\underline{argX}$ loci was confirmed by enzyme assay. Strain EcK3666 ($\underline{argO}_{ECBH}$ $\underline{argX2041}$ $\underline{rif}^r$) was constructed by this method.

## Construction of Merodiploids

### Introduction of Markers into Hfr Genome

EcK101 (Hfr Ra-2), which transfers the arginine cluster early, is ideal for use as a donor in construction of merodiploids bearing an F' factor containing the $\underline{argECBH}$ genes.

EcK101, which is prototrophic, was made $\underline{rif}^r$ and $\underline{argH}^-$ by using EcK496-5 ($\underline{argH}$ $\underline{rif}^r$) as a donor. Transductants were selected for

*rif*$^r$ on L agar + rif and scored for the *arg*$^-$ (*argH*) phenotype. Strain EcK3769 (Hfr Ra-2, *argH rif*$^r$) was constructed by this procedure.

In a separate cross, the *rif*$^r$ marker was introduced into EcK101, with EcK112 (*metB rif*$^r$) serving as donor. Selection was for *rif*$^r$ on L agar + rif. Strain EcK3768 (Hfr Ra-2, *rif*$^r$) was constructed in this manner.

## Introduction of Markers into F$^-$ Genome

EcK198 (*argG*$^-$ *metB thi his aroE thyA str*$^r$) was made *arg*$^+$ by transduction using EcK111 (*argG*$^+$ *metB*) as donor. The resulting *arg*$^+$ strain, EcK3728 served as recipient in a cross with a double *arg*$^-$ mutant, EcK3615 (*metB argX2041 argH rif*$^r$). Rif-R recombinants were selected on MMA agar and scored for *arg*$^-$ (*argH*). The *arg*$^-$ progeny were also tested by whole plate transduction with *argX* as a donor to confirm that *argX* had been cotransduced with *argH*. The resulting strain (EcK3731) has the following genotype: F$^-$, *metB argX2041 argH rif thi his aroE thyA str*$^r$.

Another strain was constructed as above, but with the use of a different donor, EcK2041a (*metB argX2041 rif*$^r$), to introduce only *rif argX* into the same recipient (EcK3728). The resulting strain, EcK3737, has the following genotype: F$^-$, *metB argX2041 argH*$^+$ *rif thi his aroE thyA str*$^r$.

## Construction of *recA* Derivatives of F$^-$ Strains

Since *recA* is closely linked to *thyA*, *thy*$^+$ recombinants may be examined for the Rec$^-$ character.

The two *thyA* F$^-$ strains, EcK3731 and EcK3737, constructed

above were mated with EcK164 (Hfr, thyA$^+$ recA1) according to B. Low (126). Matings were carried out by growing donor and recipient strains in L Broth + thymine + aro mixture at 37°C to a concentration of about 2 x $10^8$ cells/ml, and then mixing them in a ratio of 1:10. The cultures were shaken gently for aeration in a water bath for 30 min. Aliquots were pipetted into chilled saline buffer with streptomycin (final concentration, 100 µg/ml), mixed vigorously on a Vortex, and plated onto selective media with streptomycin. Recombinant (thy$^+$ str$^r$) colonies were scored for the Rec$^-$ phenotype by testing their UV sensitivity as described by Clark and Margulies (141). Cells were streaked on L agar plates together with known recA$^-$ (EcK164) and recA$^+$ (EcK3731 and EcK3737) controls, and were irradiated in the dark for 25 seconds with a General Electric GE15T8 15-watt germicidal lamp positioned at a distance of 25 cm above the petri dish. Plates were incubated overnight in the dark. Strains which are Rec$^-$ are more sensitive to UV than those which are Rec$^+$. Diminished growth, after UV irradiation, compared to that of the rec$^+$ controls, was taken to reflect the presence of the recA1 marker. Strains EcK3744 (F$^-$, metB argX2041 argH$^+$ rif$^r$ thi his aroE recA1 str$^r$) and EcK3735 (F$^-$, metB argX2041 argH$^-$ rif$^r$ thi his aroE recA1 str$^r$) were derived by this procedure.

## Construction of Merodiploids

Merodiploids were constructed by the ingenious method of Low (126), who discovered that "recombinants" for an early marker introduced by an Hfr into a recA F$^-$ strain are actually partial diploids.

The procedure for the isolation of the F' involves mixing the donor and recipient in a ratio of 1:5 and mating for 60 minutes in a

water bath as described above.

EcK3769 (Hfr, *metB*$^+$ *argH*$^-$ *rif*$^r$) was crossed with the F$^-$ strain, EcK3744 (*metB*$^-$ *argX2041* *argH*$^+$ *rif*$^r$ *thi* *his* *aroE* *recA1* *str*$^r$), by conjugation. In addition, EcK3768 (Hfr, *metB*$^+$ *rif*$^r$) was mated with F$^-$ strain EcK3735 (*metB*$^-$ *argX2041* *argH*$^-$ *rif*$^r$ *thi* *his* *aroE* *recA1* *str*$^r$). Selection was for *met*$^+$ *arg*$^+$ progeny by plating on MMA + str + supplements (with the omission of arginine and methionine).

The diploid state of recombinants (*metB*$^+$ *arg*$^+$) obtained was verified by the ability of the merodiploids to transfer their episomes to F$^-$ strains, and by the high frequency of segregation in L broth of markers carried on the F-merogenote.

Presumptive merodiploids with *argH*$^+$ *rif*$^r$ markers on the episome were tested by transferring the episome to EcK180 (which has a deletion of *ppc* and the entire *arg* cluster) using the cross-streak technique on MMA agar plates. In addition, presumptive merodiploids with *argH*$^-$ *rif*$^r$ markers on the episome, were tested by transferring the episome to an F$^-$ derivative of EcK2041 (*metB* *argX2041*) using the cross-streak technique on MMA agar plates. A positive control was performed with a known merodiploid (KLF5).

The diploids were further tested for their ability to segregate the episome in L broth. In order to increase the frequency of their segregation, both diploids (strains 3742 and 3750) were incubated overnight with vigorously shaking in a water bath. An aliquot (0.1 ml) of the culture was re-inoculated into fresh L broth and recycled overnight. Appropriate dilutions were made and plated on selective agar plates. Of 296 colonies of EcK3742 tested, 100 were both Arg$^-$ Met$^-$.

For EcK3750, 8 of 108 colonies tested were $Aco^-$ $Met^-$.

The merodiploids EcK3750 (F' *metB*$^+$ *argX*$^+$ *argH*$^-$ *rif*$^r$/*metB*$^-$ *argX2041* *argH*$^+$ *rif*$^r$ *thi* *his* *aroE* *recA1* *str*$^r$) and EcK3742 (F' *metB*$^+$ *argX*$^+$ *argH*$^+$ *rif*$^r$/*metB*$^-$ *argX2041* *argH*$^-$ *rif*$^r$ *thi* *his* *aroE* *recA1* *str*$^r$) were constructed by this method.

# CHAPTER III

# RESULTS

## Characteristics of argX Mutants

During the course of studies on the arginine cluster in Escherichia coli K-12, David Stroman, a former graduate student in this laboratory, had isolated several mutants following nitrous acid mutagenesis. Preliminary studies revealed that these mutants are arginine auxotrophs which show a concomitant inability to repress normally the argH enzyme, argininosuccinase.

### Growth Requirements of argX Mutants

Two selected argX mutants, argX1593 and argX2041, together with selected argB mutants which were subsequently shown to map in close proximity to the argX mutations, were characterized by their growth responses on intermediates of the arginine pathway (Table 3). These mutants are unable to grow on MMA, MMA + Glut, and AF medium. They are able to grow on MMA + Aco, MMA + Orn, MMA + Cit, MMA + Arg, and AF + Arg. Thus, they are acetylornithine-requiring auxotrophs. An $Aco^-$ phenotype is expected of an argA, B, C, or D mutant. The block is tight since the mutants show no leaky growth even after five days of incubation on MMA, MMA + Glut, and AF medium. The nutritional requirement is specific for arginine or its appropriate precursor since none of the

TABLE 3

GROWTH RESPONSES OF *argX* MUTANTS

AND SELECTED *argB* MUTANTS

| Strain | Supplement [a,b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | MMA | Glut | Aco | Orn | Cit | Arg | AF | AF + Arg |
| P4X6 | + | + | + | + | + | + | + | + |
| *argB1585* | - | - | + | + | + | + | - | + |
| *argB1586* | - | - | + | + | + | + | - | + |
| *argB1637* | - | - | + | + | + | + | - | + |
| *argX1593* | - | - | + | + | + | + | - | + |
| *argX2041* | - | - | + | + | + | + | - | + |

[a] Abbreviations: MMA, minimal medium A; Glut, L-glutamate; Aco, acetylornithine; Orn, ornithine; Cit, citrulline; Arg, arginine; AF, arginine-free assay medium.

[b] Present at a concentration of 100 µg/ml.

components of AF medium supports growth.

## Linkage of *argX* Mutants to *rif* and *metB*

As mentioned in Chapter I, the arginine cluster is composed of *argE-C-B-H* and in the order as written. In crosses by P1*kc* transduction, David Stroman (80) determined that the *rif* locus lies outside of the arginine cluster to the right (clockwise) of *argH*, and that it has an average cotransduction frequency to the arginine cluster of approximately 0.55. Additionally, it was found that the linkage between *rif* and *metB* is approximately 0.10. Since *metB* lies to the left (counterclockwise) of *argE-C-B-H* and *rif*, it is reasonable to visualize that the arginine cluster is situated between *metB* and *rif*, with *rif* on the right side of *argE-C-B-H* and *metB* on the left of the arginine cluster (Figure 2).

The linkage by transduction of the *argX* ($Aco^-$) mutations to *rif* and *metB* was measured in order to determine whether the mutations were in one of the clustered genes or in one of those scattered genes (*argA*, *argD*) which would be expected to give an $Aco^-$ phenotype. The *argX* mutants, EcK2041a (*metB*$^-$ *argX2041* *rif*$^r$) and EcK1593-6 (*metB*$^-$ *argX1593* *rif*$^r$), served as donors and a *metB*$^+$ *argX*$^+$ *rif*$^s$ strain (K-12, wild-type) as recipient. Transductants were selected for *rif*$^r$ and scored for *Aco*$^-$ (*argX*) and *Met*$^-$ (*metB*). Results are shown in Table 4. Mutations *argX2041* and *argX1593* show a linkage to rif of 0.50 and 0.42, respectively. The cotransducibility of *metB* to *rif* is approximately 0.10. These results are consistent with the linkage studies performed by Stroman (80) and with the conclusion that the *argX* ($Aco^-$) mutations appear to occur within the genes (*argB* or *argC*) of the arginine cluster which would give an $Aco^-$ phenotype. It remained to be determined by

TABLE 4

LINKAGE OF *rif* TO *argX* AND *metB*

| Donor | Recipient | Selected Marker | Recombinants | |
|---|---|---|---|---|
| | | | *argX*$^-$ | *metB*$^-$ *argX*$^-$ |
| *metB*$^-$ *argX2041* *rif*$^r$ | *metB*$^+$ *argX*$^+$ *rif*$^s$ | *rif*$^r$ | $\frac{161}{320} = 0.50$ | $\frac{32}{320} = 0.10$ |
| *metB*$^-$ *argX1593* *rif*$^r$ | *metB*$^+$ *argX*$^+$ *rif*$^s$ | *rif*$^r$ | $\frac{134}{320} = 0.42$ | $\frac{36}{320} = 0.11$ |

direct enzyme assay whether these strains were *argB* or *argC* mutants.

## Reduced Repressibility of Nonisogenic *argX* Mutants

The nonisogenic *argX* mutants, EcK2041 and EcK1593, were assayed for the cluster enzymes E (acetylornithinase), C (N-acetylglutamic-γ-semialdehyde dehydrogenase), B (N-acetyl-γ-glutamokinase) and H (argininosuccinase), and for a noncluster enzyme, D (acetylornithine δ-transaminase). An *argR*$^-$ derivative of *argX2041* was constructed as a canavanine-resistant, arginine-excreter as described in Chapter II. The specific activities are shown in Tables 5 and 6. Wild type (P4X6) enzyme activities are shown in parenthesis. In order to discuss the regulation of the various enzymes in such a way as to eliminate differences in the way some authors measure and define units of activity and to normalize and compare the range of repression-derepression behaviors of different enzymes, it is useful to express the results in terms of three ratios: *ratio A* equals the specific activity of the *argR*$^-$ strain grown in arginine-supplemented media divided by the specific activity of the *argR*$^+$ strain grown in arginine-supplemented media, and represents the total repression-derepression range; *ratio B* equals the specific activity of the *argR*$^-$ strain grown without exogenous arginine divided by that of the *argR*$^+$ strain grown without arginine and represents the fold increase above the partially derepressed rate of synthesis reflecting the intracellular, steady-state level of endogenously synthesized arginine; *ratio C* equals the specific activity of the *argR*$^+$ strain grown without arginine divided by the specific activity of the *argR*$^+$ strain grown with arginine and represents the fold decrease below the partially derepressed rate of synthesis.

TABLE 5

ALTERED REPRESSIBILITY OF ARGININOSUCCINASE (*argH*)

IN THE NONISOGENIC *argX2041* MUTANT

| Strain | Supplement | Specific Activity (units/mg protein)[a] | | | | |
|---|---|---|---|---|---|---|
| | | *argE* | *argC* | *argB* | *argH* | *argD* |
| 2041 $R^+$ | orn | 27.3 (27.7) | 4.0 (3.5) | N.M.[b] | 1.1 (1.0) | 2.8 (2.7) |
| 2041 $R^+$ | arg | 14.5 (12.9) | 1.9 (1.7) | N.M. | 0.67 (0.17) | 0.7 (0.7) |
| 2041 $R^-$ | orn | 227 (251) | 64.7 (70.1) | Low | 9.3 (8.8) | 8.1 (9.3) |
| 2041 $R^-$ | arg | 229 (245) | 67.4 (71.7) | Low | 9.4 (8.2) | 8.7 (9.9) |

| Ratio[c] | *argE* | *argC* | *argB* | *argH* | *argD* |
|---|---|---|---|---|---|
| A | 15.8 (18.9) | 35.5 (32.6) | ___ | 14.0 (55.3) | 12.4 (12.4) |
| B | 8.3 (9.1) | 16.2 (20.0) | ___ | 8.5 (8.8) | 2.9 (3.4) |
| C | 1.9 (2.1) | 2.1 (2.1) | ___ | 1.6 (5.9) | 4.0 (3.9) |

[a] The numbers in parenthesis are the specific activities for the wild type strain (P4X6).

[b] Not measurable.

[c] The numbers in parenthesis are the ratios for the wild type strain (P4X6).

TABLE 6

ALTERED REPRESSIBILITY OF ARGININOSUCCINASE (*argH*)

IN THE NONISOGENIC *argX1593* MUTANT

| Strain | Supplement | Specific Activity (units/mg protein)[a] | | | | |
|---|---|---|---|---|---|---|
| | | *argE* | *argC* | *argB* | *argH* | *argD* |
| 1593 $R^+$ | orn | 30.4 (27.7) | 3.9 (3.5) | N.M.[b] | 1.1 (1.0) | 2.6 (2.7) |
| 1593 $R^+$ | arg | 15.0 (12.9) | 1.8 (1.7) | N.M. | 0.45 (0.17) | 0.6 (0.7) |

| Ratio[c] | *argE* | *argC* | *argB* | *argH* | *argD* |
|---|---|---|---|---|---|
| C | 2.0 (2.1) | 2.2 (2.1) | ___ | 2.5 (5.9) | 4.3 (3.9) |

[a] The numbers in parenthesis are the specific activities for the wild type strain (P4X6).
[b] Not measurable.
[c] The numbers in parenthesis are the ratios for the wild type strain (P4X6).

The argR$^+$ derivatives of both argX2041 and argX1593 show a high argininosuccinase (argH) activity under fully repressive conditions with arginine. In argX2041, the specific activity for argH is 0.67 (Table 5), compared with the wild-type value of 0.17, and represents an almost four-fold increase in argH expression. In argX1593, the specific activity for argH is 0.45 (Table 6). This value represents approximately a two and a half-fold increase above the corresponding wild-type activity of 0.17. Hence, argininosuccinase is not repressible normally by arginine in argX mutants carrying the argR$^+$ allele.

When the argX strains are grown in the presence of ornithine to effect partial derepression, the increase in argH expression is not observed (Tables 5 and 6). Moreover, the argX mutation has no detectable effect on argH expression in a genetically derepressed argR$^-$ derivative of argX2041, when grown in the presence of either arginine or ornithine (Table 5). Kinase (argB) activity is not measurable in either of the argX mutants under partially derepressed or fully repressed conditions. However, a low enzyme B activity was detected in the argR$^-$ derivative of argX2041, which represents approximately 1-2 per cent of the corresponding level for the wild-type argR$^-$ strain. The other arginine enzymes specified by cluster genes (argE and argC) and the noncluster enzyme (argD) behave normally both in the argR$^+$ and the argR$^-$ strains.

These studies show that these two argX mutants are pleiotropic. They are deficient in argB enzyme (N-acetyl-γ-glutamokinase) and show a concomitant inability to normally repress argH expression. Moreover, the argX mutations enhance gene expression in a clockwise (argH), but

not counterclockwise (*argC*, *argE*) direction (Figure 3). The effect of *argX* on *argH* expression is detectable only under fully repressed conditions.

### Reduced Repressibility of Isogenic *argX* Mutants

Subsequent mapping of the *argX2041* mutation shows that it is confined to the *argB* gene and is located to the right of *argC1810* (see below) (see Figure 9). In order to construct isogenic strains carrying the *argX* mutation, it is ideal to transfer only the *argX* region to a suitable recipient. A rifampin-resistant derivative (EcK2041a) of *argX2041* was used as a donor in P1*kc* transduction. A *metB*$^+$ *argC* *rif*$^S$ mutant (EcK1810) was used as a recipient. The rationale for using an *argC* strain as recipient was to ensure that only the piece of genetic material extending from *rif* to *argX*, but not beyond *argC*, was transduced into the recipient. Rifampin-resistant recombinants were selected on nutrient agar so that the *argX* mutation might be cotransduced with *rif*. The *rif*$^r$ progeny were scored for the Aco$^-$ phenotype which reflects the presence of either the *argC* or *argX* gene, or both. The *rif*$^r$ Aco$^-$ strains were subjected to spot transduction tests using EcK1810 (*argC*) and EcK2041 (*argX*) as donors. Strains (*rif*$^r$ Aco$^-$) which failed to give prototrophic recombinants with both the *argC* and *argX* lysates were considered to be presumptive *argC* *argX* double mutants. The presumptive double mutants were purified twice and subjected to whole plate transduction tests to reaffirm the presence of the *argC* and *argX* mutations. Strain EcK3506 (*argC1810* *argX2041* *rif*) was constructed in this manner. Enzyme activities found for the isogenic strain 3506 and its *argR*$^-$ derivative (EcK3493 $R^-_{10}$) are shown in Table 7.

TABLE 7

ALTERED REPRESSIBILITY OF ARGININOSUCCINASE (argH)
IN AN ISOGENIC argX2041 STRAIN

| Strain[a] | Supplement | Specific Activity (units/mg protein)[b] | | | | |
|---|---|---|---|---|---|---|
| | | argE | argC | argB | argH | argD |
| 3506 $R^+$ | orn | 23.6 (27.3) | N.M.[c] | N.M. | 0.9 (1.1) | 2.3 (2.8) |
| 3506 $R^+$ | arg | 13.7 (14.5) | N.M. | N.M. | 0.55 (0.67) | 0.6 (0.7) |
| 3493 $R^-_{10}$ | orn | 193 (227) | N.M. | Low | 9.2 (9.3) | 8.0 (8.1) |
| 3493 $R^-_{10}$ | arg | 197 (229) | N.M. | Low | 9.2 (9.4) | 8.8 (8.7) |

| Ratio[d] | argE | argC | argB | argH | argD |
|---|---|---|---|---|---|
| A | 14.4 (15.8) | ____ | ____ | 16.7 (14.0) | 14.7 (12.4) |
| B | 8.2 (8.3) | ____ | ____ | 10.2 (8.5) | 3.5 (2.9) |
| C | 1.7 (1.9) | ____ | ____ | 1.6 (1.6) | 3.8 (4.0) |

[a] All strains carry argC and rifampin-resistant (rif) genes.
[b] The numbers in parenthesis are the specific activities for the nonisogenic argX2041 mutant.
[c] Not measurable.
[d] The numbers in parenthesis are the ratios for the nonisogenic argX2041 mutant.

The corresponding values for the nonisogenic argX2041 mutant are listed in parenthesis for comparison purposes. The isogenic strain exhibits a high level of argH activity under repressed conditions similar to that of the nonisogenic strain. It has a value of 0.55 compared to 0.67 for the nonisogenic strain. Activities of argE and argD in the isogenic $argR^+$ and $argR^-$ strains are essentially similar to those of the nonisogenic $argR^+$ and $argR^-$ strains.

## Lack of Effect of argX on argH Expression in an $argO_{ECBH}$ argX2041 Double Mutant

The argX mutations affect argH expression only under fully arginine-repressed conditions. They do not affect argH expression either during partial physiologic derepression (supplemented with ornithine) (Tables 5 and 6) or during full genetic derepression (in an $argR^-$ derivative) (Tables 5 and 7). Therefore, it was necessary to ascertain whether an argX mutation has any effect during partial genetic derepression (in an $argO^c$ mutant). The arginine cluster has its own, specific operator site which has been determined independently by Jacoby (122) and Glansdorff (124) to be located between argE-argC. Jacoby (122) devised an ingenious method for the isolation of an $argO_{ECBH}$ mutant. This $argO_{ECBH}$ mutation causes a partial derepression of the clustered genes without any effect on nonclustered genes.

Jacoby's $argO_{ECBH}$ mutation was put into P4X6 and also into argX2041. Intermediate steps involved utilization of an argE strain, and construction of an argE argX2041 double mutant. Rationale and details for construction of the argE argX double mutant EcK3613 were described in Chapter II. Since the argE enzyme (acetylornithinase)

occurs after the argX (argB) enzyme (kinase) in the arginine biosynthetic pathway, the presence of an argE ($Orn^-$) mutation will "mask" the phenotype of an argX ($Aco^-$) mutation. In order to ensure the presence of both the argX and argE markers, it was necessary to recover argX ($Aco^-$) from the double mutant. As shown in Table 8, wild-type strain (K-12) was used as donor in P1kc transduction, with the presumptive argE argX strain as recipient. Recombinants on selective medium (deprived of ornithine and methionine but with added acetylornithine) were screened for $Aco^-$ mutants. Of 150 $Orn^+$ transductants screened, 5 were actually also $Aco^-$. A control was performed using an argE strain (2084-1) as recipient, and no $Aco^-$ recombinants were recovered.

The $argO_{ECBH}$ and $argO_{ECBH}$ argX mutants were assayed together with the control strains argX2041, argE, argX argE, and P4X6 (wild-type). The results are presented in Table 9. It was found that there is no significant difference in enzyme H activity between strains $argO_{ECBH}$ (3.7) and $argO_{ECBH}$ argX (3.2) under repressed conditions. It is, therefore, concluded that the argX mutation does not affect argH expression in a partially genetically derepressed $argO_{ECBH}$ mutant.

## Nature of the argX Mutations

The argX mutations were classified by the criteria of Whitfield, Martin and Ames (143). These correlate the spontaneous revertibility, and the response of each mutant to chemical mutagens, to several aminoglycoside antibiotics (phenotypic curing), and to suppression by known suppressors. Mutants with previously characterized mutations were tested in parallel with the argX strains. The results are shown in Table 10.

Spontaneous reversion rate. Mutants argX2041 and argX1593

TABLE 8

RECOVERY OF *argX* (Aco$^-$) FROM PRESUMPTIVE DOUBLE MUTANTS

BY OUT-CROSS WITH *E. COLI* K-12 WILD TYPE

| Donor[a] | Recipient[b] | Selected Marker | Unselected Marker (*argX*$^-$) |
|---|---|---|---|
| *metB*$^+$ *argE*$^+$ *argX*$^+$ | *metB*$^-$ *argE*$^-$ *argX*$^-$ (3613) | *metB*$^+$ *argE*$^+$ | $\frac{5}{150} = 0.033$ |
| | *metB*$^-$ *argE*$^-$ *argX*$^+$ (2084-1) | *metB*$^+$ *argE*$^+$ | $\frac{0}{150} = 0$ |
| *metB*$^+$ *argE*$^+$ *argX*$^+$ | *metB*$^-$ *argX*$^-$ *argH*$^-$ (3615) | *argH*$^+$ | $\frac{15}{200} = 0.075$ |
| | *metB*$^+$ *argX*$^+$ *argH*$^-$ (496-5) | *argH*$^+$ | $\frac{0}{200} = 0$ |

[a] *E. coli* K-12 (Wild type).

[b] All strains carry the *rif* gene.

TABLE 9

ABSENCE OF *argX* EFFECT ON *argH* EXPRESSION IN A PARTIALLY DEREPRESSED $argO_{ECBH}$ MUTANT

| Strain | Genotype[a,b] | Specific Activity (units/mg protein)[c] | | |
|---|---|---|---|---|
| | | *argE* | *argH* | *argD* |
| P4X6 (Control) | Wild type | 12.9 | 0.17 | 0.7 |
| 2084-1 (Control) | *argE* | N.M.[d] | 0.18 | 0.6 |
| 3613 (Control) | *argE argX2041* | N.M. | 0.58 | 0.7 |
| 2041 (Control) | *argX2041* | 16.7 | 0.58 | 0.6 |
| 3766 | $argO_{ECBH}$ | 136.5 | 3.7 | 0.5 |
| 3666 | $argO_{ECBH}$ *argX2041* | 127.4 | 3.2 | 0.6 |

[a] All strains are *rif* except for P4X6.

[b] All strains are *metB* except for strains 3766 and 3666.

[c] After growth in the presence of excess arginine (100 µg/ml).

[d] Not measurable.

TABLE 10

PROPERTIES OF argX MUTANTS

| Mutant | Origin | Type of Mutation | Revertibility | | | | | | Phenotypic Curing | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Spontaneous Frequency | NTG | AP | EMS | DES | ICR-191 | Sm | Km | Nm |
| argX2041 | $HNO_2$ | ____ | $5.1 \times 10^{-9}$ | - | - | - | - | - | - | - | - |
| argX1593 | $HNO_2$ | ____ | $1.8 \times 10^{-9}$ | - | - | - | - | - | - | - | - |
| argC3653 | AP | UGA | ____ | H R | + | H R | H R | - | ++ | - | - |
| hisG428 | ___ | UAA | ____ | H R | + | H R | H R | - | - | ++ | + |
| hisG2100 | ___ | F. S. | ____ | - | - | - | - | + | - | - | - |
| hisD3018 | ___ | F. S. | _____ | - | - | - | - | + | ___ | ___ | ___ |

Abbreviations: NTG, N-methyl-N'-nitro-N-nitrosoguanidine; AP, 2-aminopurine; EMS, ethyl methanesulfonate; DES, diethylsulfate; Km, kanamycin sulfate; Sm, streptomycin sulfate; Nm, neomycin sulfate; H R, high reversion frequency: F.S., frameshift mutation .

were plated on MMA + met + 1.25% nutrient broth as described in Chapter II. Viable cell counts were made by plating appropriate dilutions, in triplicate, onto L agar. The spontaneous reversion frequency was calculated by dividing the number of revertants by the total number of colonies plated. The results (Table 10) show that both argX2041 and argX1593 have very low spontaneous reversion frequencies of approximately $10^{-9}$.

Reversions by chemical mutagens. The argX mutants were tested with NTG, EMS, DES, AP and ICR-191. NTG, EMS and DES are alkylating agents and are believed to cause base pair substitution by transition and transversion (143). In rare events, NTG and DES also cause single base deletions (144,145). AP is a base analogue which causes base pair substitutions of only the transition type (143). All nonsense and missense mutants are reverted by NTG, and all nonsense and 67% of missense mutants are reverted by DES. About 61% of the nonsense mutants, but only 43% of the missense mutants, respond to AP. ICR-191 is an acridine half-mustard which is believed to induce primarily frameshift (deletion or addition of one or more bases) mutations and it has been shown to be 69% effective in inducing frameshift reversions (143).

The data presented in Table 10 show that argX mutants are not revertible by any of the base substitution or frameshift mutagens, whereas the known nonsense mutations (argC3653 and hisG428) are reverted by all the mutagens except ICR-191. Known frameshift mutations (hisG2100 and hisD3018) are reverted only by ICR-191.

Phenotypic curing. It is known that aminoglycoside antibiotics, such as streptomycin, kanamycin, and neomycin, lead to phenotypic sup-

pression by causing misreading of messenger ribonucleic acid (143). Phenotypic curing is manifested in spot tests by the appearance of a solid ring of growth peripheral to the zone of inhibition caused by the antibiotic. All amber and ochre mutants show phenotypic curing, whereas frameshift mutants give negative results, and only 33% of the missense mutants respond to at least one of the antibiotics (143). As shown in Table 10, the argX mutants fail to respond to all three antibiotics, whereas known nonsense mutants are suppressed by one or two of the antibiotics.

Suppression by known nonsense suppressors. Two suppressor-bearing strains were available which were suitable for testing the suppression patterns of the argX mutants. One of the strains, EcK189, carries a supE (amber suppressor) on the chromosome and also a deletion of the entire arginine cluster. Sal27 is an F' strain which carries an ochre suppressor on the episome. Both strains are unable to grow on MMA + met. P1kc was propagated on argX2041 and argX1593. An aliquot of each lysate was streaked across a line of early log phase cells of EcK189 (supE) on MMA + met agar plates. In addition, log-phase cells of the F' donor (ochre suppressor) were cross-streaked against argX1593 and argX2041 log-phase cells on MMA + met agar plates. The appearance of growth at or past the line of recipient cells was taken as a positive test for suppression of argX. Both argX strains failed to be suppressed by either the amber or ochre suppressor.

Although revertible spontaneously, argX1593 and argX2041 are not revertible by mutagens known to revert nonsense and missense mutants nor by the one frameshift mutagen tested. Furthermore, they are neither

suppressible by antibiotics nor by the two known nonsense suppressors used. Apparently, the argX mutations are neither of the nonsense nor missense type. Since ICR-191 reverts only 69% of frameshift mutants (143), and since both argX mutants were derived by means of nitrous acid mutagenesis which is effective in inducing deletion mutations in E. coli (146), and in view of their ability to revert spontaneously, the possibility cannot be excluded that the argX mutants may be of the short (frameshift) deletion type.

Effect of Reversion to $argX^+$ on argH Expression

The argX2041 mutant ($Aco^-$ phenotype) was made prototrophic ($Aco^+$ phenotype) by spontaneous reversion and by transduction to $argX^+$, using P4X6 (wild-type) as a donor. EcK3754 and 3755 were isolated as revertants, and EcK3751 and 3753 were constructed by transduction. These prototrophic strains were assayed for the argE (acetylornithinase) and argH (argininosuccinase) enzymes after growth under repressive conditions. The results are given in Table 11. Revertants 3754 and 3755 show an $Aco^+$ ($argX^+$) phenotype and a concomitant loss of the enhanced expression of argH.

The findings are consistent with the view that the pleiotropy of the argX mutants is due to a single mutational event. Two types of spontaneous revertants were observed. In one type (3755) argininosuccinase activity is reduced to the normal wild-type level (0.14). In the second type (3754) expression of argH is decreased to approximately 30% (0.05) of that in the wild-type).

The $argX^+$ prototrophs (EcK3751 and 3753) constructed by transduction show normal wild-type argH activity.

TABLE 11

RESTORED REPRESSIBILITY OF SPONTANEOUS REVERTANTS AND PROTOTROPHIC TRANSDUCTANTS OF argX2041

| Strain | Origin[a] | Pertinent Genotype[b] | Doubling Time (min)[c] | Specific Activity (units/mg protein)[c] | |
|---|---|---|---|---|---|
| | | | | argE | argH |
| P4X6 | —— | W.T. | 48 | 12.9 | 0.17 |
| 2041 | $HNO_2$ | argX$^-$ | 49 | 14.5 | 0.67 |
| 3754 | S.R. | argX$^+$ | 45 | 12.5 | <0.05 |
| 3755 | S.R. | argX$^+$ | 47 | 15.0 | 0.14 |
| 3751 | Tran. | argX$^+$ | 46 | 13.6 | 0.19 |
| 3753 | Tran. | argX$^+$ | 43 | 13.2 | 0.19 |

[a] S.R., spontaneous revertants; Tran., P1kc transductants.

[b] All strains carry the metB gene

[c] After growth in the presence of arginine (100 µg/ml).

The argX2041 site on the argB structural gene may well be very specific and critical for the expression of argH. On the one hand, the site can be created by mutation to promote argH expression. On the other hand, spontaneous reversion can inactivate the argX site and lead to diminished argH expression. Although the reversions have not been mapped, there is presumably a critical site in the argB gene, the modification of which may lead alternatively to enhanced (argX2041) or reduced (3754) expression of argH function.

## Genetic Analysis of argX Mutations

A fine-structure map of the argX mutations in the arginine cluster was constructed. The approach involved: construction of a deletion map, that is an ordering of arginine cluster markers by a series of overlapping deletion mutations; location of the cluster point mutations within the appropriate deletions; ordering of point mutations which lie in the same region of the deletion map with respect to one another by three-point crosses with an outside marker. This outside marker should have a high cotransduction frequency with the clustered arginine genes in order to be useful.

### Three-Point Crosses

In three-point crosses, double mutants with closely linked markers were crossed with single mutants for the determination of the order of the mutations by donor-switching as described by Glansdorff (118). Selection was for prototrophic transductants in the respective crosses. The design of the three-point crosses is illustrated in Figure 8. The rif locus was used as an outside linked marker in all the three-

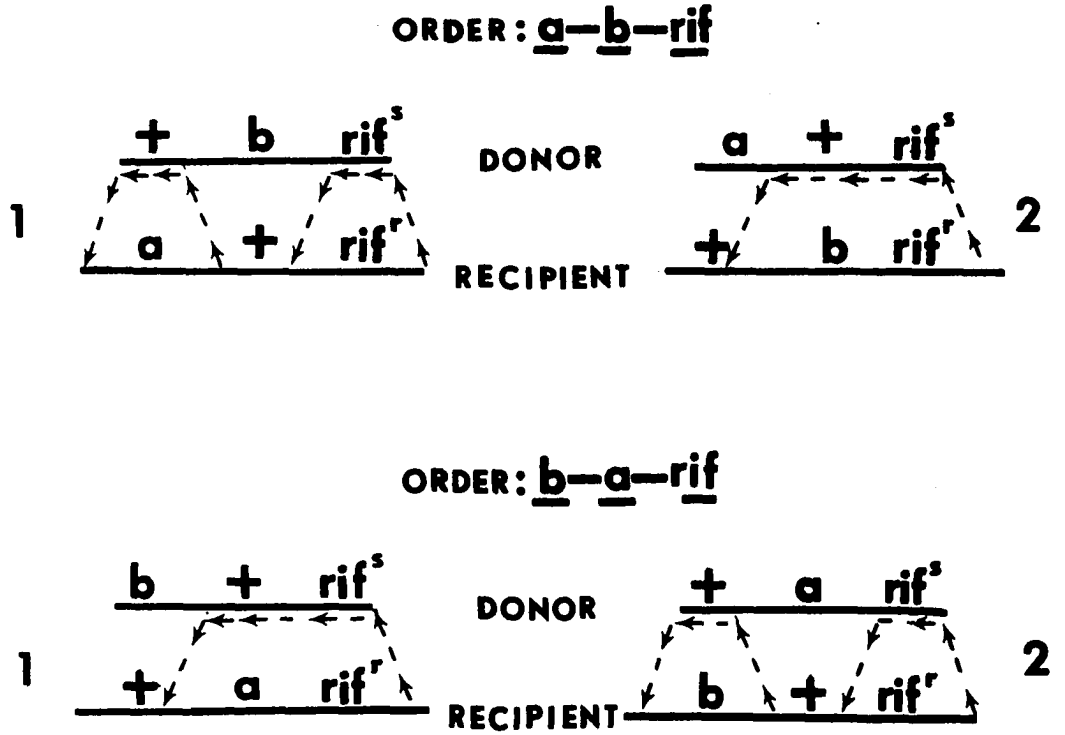



Figure 8. The mapping of sites by three-point crosses.

Broken lines represent cross-overs necessary to get $\underline{a}^+ \underline{b}^+ \underline{rif}^s$ recombinants. 1 and 2 are a pair of reciprocal crosses. If order is $\underline{a}$ - $\underline{b}$ - $\underline{rif}$, frequency of 2 > 1. If order is $\underline{b}$ - $\underline{a}$ - $\underline{rif}$, frequency of 1 > 2.

$\underline{a}$, $\underline{b}$ = $\underline{argE}$, $\underline{C}$, $\underline{B}$, $\underline{X}$, or $\underline{H}$;

$\underline{rif}^r$ = rifampin-resistance.

point crosses. Double mutants with *rif*$^r$ and an *arg* marker served as recipient, whereas a single mutant with a different *arg* marker was used as a donor. Transductants were spread on MMA + met agar plates. The *arg*$^+$ recombinants were picked, suspended in a drop of sterile saline, and tested for *rif*$^s$ by streaking on NBG + rif with sterile tooth picks. As shown in Figure 8, *a* and *b* are designated as two *arg* markers in the cluster. If the order is *a* - *b* - *rif*, and *b* is the donor marker, the formation of *a*$^+$ *b*$^+$ *rif*$^s$ recombinants requires a quadruple cross-over (Cross 1); if *a* is the donor marker, the same class may arise from a double cross-over. The value of the ratio (*a*$^+$ *b*$^+$ *rif*$^s$) / (*a*$^+$ *b*$^+$) is thus expected to be lower if *b* (Cross 1) rather than *a* (Cross 2) is contributed by the donor. Opposite predictions could be made if the order is *b* - *a* - *rif*$^s$. That is, the value of the ratio in Cross 2 will be lower than that in Cross 1 (Figure 8).

The results of the three-point crosses are given in Table 12. Reciprocal crosses were performed to determine the order of the two *argX* mutations and several *argB* mutations with respect to reference *argH*, *argB*, *argC*, or *argE* markers. Strains *argH496*, *argB1808*, *argC1810*, and *argE1817* were used as the reference mutations. Four pairs of crosses were performed for each *argX* or *argB* mutant. It was found that both the *argX1593* and *argX2041* mutations map between *argC* and *argH*. However, they are different loci since *argX2041* lies to the right of *argB1808* and *argX1593* to the left of *argB1808*. The following mutations were found to be located between *argC* and *argH*: *argB1581*, *argB1585*, *argB1586*, *argB1637*, *argB1668*, and *argB2058*. Mutations *argB1581*, *argB1668*, and *argB2058* map to the left of *argB1808*. On the other hand,

TABLE 12

ORDER OF *argX* AND *argB* WITH RESPECT TO *arg* MUTATIONS

AS DETERMINED BY THREE-POINT CROSSES

| Cross No. | Strains[a] (Recipient X Donor) | Recombinants (*arg*$^+$ *rif*$^s$/*arg*$^+$) | | Order |
|---|---|---|---|---|
| | | Ratio | % | |
| 1. | *rif*$^r$ *argX2041* X *argH* | 17/112 | 15.2 | *argX2041 argH rif*$^r$ |
| 2. | *rif*$^r$ *argH* X *argX2041* | 28/112 | 25.0 | |
| 1. | *rif*$^r$ *argX2041* X *argB* | 67/204 | 32.8 | *argB argX2041 rif*$^r$ |
| 2. | *rif*$^r$ *argB* X *argX2041* | 29/144 | 20.1 | |
| 1. | *rif*$^r$ *argX2041* X *argC* | 49/106 | 46.2 | *argC argX2041 rif*$^r$ |
| 2. | *rif*$^r$ *argC* X *argX2041* | 12/112 | 10.7 | |
| 1. | *rif*$^r$ *argX2041* X *argE* | 32/112 | 28.6 | *argE argX2041 rif*$^r$ |
| 2 | *rif*$^r$ *argE* X *argX2041* | 15/112 | 13.4 | |
| 1. | *rif*$^r$ *argX1593* X *argH* | 17/111 | 15.3 | *argX1593 argH rif*$^r$ |
| 2. | *rif*$^r$ *argH* X *argX1593* | 31/111 | 27.9 | |
| 1. | *rif*$^r$ *argX1593* X *argB* | 11/141 | 7.8 | *argX1593 argB rif*$^r$ |
| 2. | *rif*$^r$ *argB* X *argX1593* | 25/161 | 15.5 | |

TABLE 12 Continued

| Cross No. | Strains[a] (Recipient X Donor) | Recombinants ($arg^+rif^s/arg^+$) Ratio | % | Order |
|---|---|---|---|---|
| 1. | $rif^r$argX1593 X argC | 62/111 | 55.9 | argC argX1593 $rif^r$ |
| 2. | $rif^r$argC X argX1593 | 9/111 | 8.1 | |
| 1. | $rif^r$argX1593 X argE | 65/111 | 58.6 | argE argX1593 $rif^r$ |
| 2. | $rif^r$argE X argX1593 | 14/111 | 12.6 | |
| 1. | $rif^r$argB1581 X argH | 39/206 | 18.9 | argB1581 argH $rif^r$ |
| 2. | $rif^r$argH X argB1581 | 66/192 | 34.8 | |
| 1. | $rif^r$argB1581 X argB | 28/112 | 25.0 | argB1581 argB $rif^r$ |
| 2. | $rif^r$argB X argB1581 | 47/112 | 42.0 | |
| 1. | $rif^r$argB1581 X argC | 48/112 | 42.9 | argC argB1581 $rif^r$ |
| 2. | $rif^r$argC X argB1581 | 17/111 | 15.3 | |
| 1. | $rif^r$argB1581 X argE | 49/112 | 43.8 | argE argB1581 $rif^r$ |
| 2. | $rif^r$argE X argB1581 | 12/112 | 10.7 | |
| 1. | $rif^r$argB1585 X argH | 40/224 | 17.9 | argB1585 argH $rif^r$ |
| 2. | $rif^r$argH X argB1585 | 57/187 | 30.5 | |

TABLE 12 Continued

| Cross No. | Strains[a] (Recipient X Donor) | Recombinants ($arg^+rif^s/arg^+$) | | Order |
|---|---|---|---|---|
| | | Ratio | % | |
| 1 | *rif*$^r$*argB1585* X *argB* | 47/168 | 28.0 | *argB argB1585* *rif*$^r$ |
| 2 | *rif*$^r$*argB* X *argB1585* | 32/187 | 17.1 | |
| 1. | *rif*$^r$*argB1585* X *argC* | 48/112 | 42.8 | *argC argB1585* *rif*$^r$ |
| 2. | *rif*$^r$*argC* X *argB1585* | 12/112 | 10.7 | |
| 1. | *rif*$^r$*argB1585* X *argE* | 46/112 | 41.1 | *argE argB1585* *rif*$^r$ |
| 2. | *rif*$^r$*argE* X *argB1585* | 10/112 | 8.9 | |
| 1. | *rif*$^r$*argB1586* X *argH* | 8/112 | 7.1 | *argB1586 argH* *rif*$^r$ |
| 2. | *rif*$^r$*argH* X *argB1586* | 33/112 | 29.5 | |
| 1. | *rif*$^r$*argB1586* X *argB* | 60/200 | 30.0 | *argB argB1586* *rif*$^r$ |
| 2. | *rif*$^r$*argB* X *argB1586* | 37/161 | 23.0 | |
| 1. | *rif*$^r$*argB1586* X *argC* | 40/112 | 35.7 | *argC argB1586* *rif*$^r$ |
| 2. | *rif*$^r$*argC* X *argB1586* | 14/112 | 12.5 | |
| 1. | *rif*$^r$*argB1586* X *argE* | 34/112 | 30.4 | *argE argB1586* *rif*$^r$ |
| 2. | *rif*$^r$*argE* X *argB1586* | 15/112 | 13.4 | |

TABLE 12 Continued

| Cross No. | Strains[a] (Recipient X Donor) | Recombinants (*arg*$^+$*rif*$^s$/*arg*$^+$) | | Order |
|---|---|---|---|---|
| | | Ratio | % | |
| 1. | *rif*$^r$*argB1598* X *argH* | 7/79 | 8.86 | *argB1598 argH rif*$^r$ |
| 2. | *rif*$^r$*argH* X *argB1598* | 32/93 | 33.0 | |
| 1. | *rif*$^r$*argB1598* X *argB* | 0 | ____ | ———— |
| 2. | *rif*$^r$*argB* X *argB1598* | 0 | ____ | |
| 1. | *rif*$^r$*argB1598* X *argE* | 24/78 | 30.8 | *argE argB1598 rif*$^r$ |
| 2. | *rif*$^r$*argE* X *argB1598* | 5/96 | 5.2 | |
| 1. | *rif*$^r$*argB1637* X *argH* | 29/183 | 15.8 | *argB1637 argH rif*$^r$ |
| 2. | *rif*$^r$*argH* X *argB1637* | 46/207 | 22.2 | |
| 1. | *rif*$^r$*argB1637* X *argB* | 33/44 | 75.0 | *argB argB1637 rif*$^r$ |
| 2. | *rif*$^r$*argB* X *argB1637* | 16/52 | 30.8 | |
| 1. | *rif*$^r$*argB1637* X *argC* | 30/104 | 31.7 | *argC argB1637 rif*$^r$ |
| 2. | *rif*$^r$*argC* X *argB1637* | 15/112 | 13.7 | |
| 1. | *rif*$^r$*argB1637* X *argE* | 37/110 | 33.6 | *argE argB1637 rif*$^r$ |
| 2. | *rif*$^r$*argE* X *argB1637* | 12/104 | 11.5 | |

TABLE 12 Continued

| Cross No. | Strains[a] (Recipient X Donor) | Recombinants ($arg^+rif^s/arg^+$) Ratio | % | Order |
|---|---|---|---|---|
| 1. | *rif*$^r$ *argB1668* X *argH* | 41/208 | 19.7 | *argB1668 argH rif*$^r$ |
| 2. | *rif*$^r$ *argH* X *argB1668* | 59/208 | 28.4 | |
| 1. | *rif*$^r$ *argB1668* X *argB* | 44/192 | 22.9 | *argB1668 argB rif*$^r$ |
| 2. | *rif*$^r$ *argB* X *argB1668* | 50/144 | 34.7 | |
| 1. | *rif*$^r$ *argB1668* X *argC* | 47/112 | 42.0 | *argC argB1668 rif*$^r$ |
| 2. | *rif*$^r$ *argC* X *argB1668* | 16/112 | 14.3 | |
| 1. | *rif*$^r$ *argB1668* X *argE* | 32/112 | 28.5 | *argE argB1668 rif*$^r$ |
| 2. | *rif*$^r$ *argE* X *argB1668* | 8/112 | 7.2 | |
| 1. | *rif*$^r$ *argB2058* X *argH* | 29/112 | 25.9 | *argB2058 argH rif*$^r$ |
| 2. | *rif*$^r$ *argH* X *argB2058* | 45/112 | 40.2 | |
| 1. | *rif*$^r$ *argB2058* X *argB* | 28/128 | 21.9 | *argB2058 argB rif*$^r$ |
| 2. | *rif*$^r$ *argB* X *argB2058* | 56/143 | 39.2 | |
| 1. | *rif*$^r$ *argB2058* X *argC* | 24/72 | 33.3 | *argC argB2058 rif*$^r$ |
| 2. | *rif*$^r$ *argC* X *argB2058* | 24/108 | 22.2 | |

TABLE 12 Continued

| Cross No. | Strains[a] (recipient X Donor) | Recombinants ($arg^+rif^s/arg^+$) Ratio | % | Order |
|---|---|---|---|---|
| 1. | *rif*$^r$*argB2058* X *argE* | 43/112 | 38.4 | *argE argB2058 rif*$^r$ |
| 2. | *rif*$^r$*argE* X *argB2058* | 9/112 | 8.0 | |
| 1. | *rif*$^r$*argX2041* X *argB1637* | 13/111 | 11.7 | *argB1637 argX2041 rif*$^r$ |
| 2. | *rif*$^r$*argB1637* X *argX2041* | 1/111 | 0.9 | |
| 1. | *rif*$^r$*argX2041* X *argB1585* | 7/111 | 6.3 | *argX2041 argB1585 rif*$^r$ |
| 2. | *rif*$^r$*argB1585* X *argX2041* | 28/111 | 25.2 | |

[a] Reference Markers:

*argH* = *argH496*;

*argB* = *argB1808*;

*argC* = *argC1810*;

*argE* = *argE1817*.

argB1585, argB1586, and argB1637 map to the right of argB1808. One argB mutant, argB1598, fails to give any $arg^+$ recombinants with argB1808, and is presumed to map in the same locus as argB1808. Two argB mutants were mapped with respect to argX2041. Mutation argB1637 lies to the left of argX2041, whereas argB1585 is located to the right of argX2041.

## Deletion Mapping

In order to construct a deletion map, a large number of overlapping deletion mutants, particularly in the argB gene, are needed. Thus far, efforts to isolate a substantial number of cluster deletions have not been highly fruitful (80,118,119). Nevertheless, a number of useful cluster deletions are available. Multisite mutant (2007) has an extensive deletion of part of argH and most of argB, and has been particularly useful in mapping the argX strains. Other deletion mutants were given to us generously by N. Glansdorff. They are as follows: MN42 carries a deletion for almost the entire arginine cluster (the distal portion of argH is still intact); argCB-1 and sup-102 are deletions which cover argC and extend distally to include most of the argB gene; argEC(B) has argEC and part of argB deleted; argEC-1 carries a deletion of argE and the most proximal portion of argC. Some point mutants or small deletion strains, such as 185 (argB-1), 186 (argB-5), 187 (argB-2), 188 (argH-3), 190 (argH-2), and 191 (argH-1) were also provided by N. Glansdorff. These strains have been mapped rather extensively either by three-point crosses or by deletion mapping (118,119) as to their relative positions in the argB or argH genes. With these mutants and their known map-positions, together with other argB mutants from our own culture

collection, it was possible to construct a fine structure map of the argX mutants as to their loci in the argB gene.

The results are shown in Tables 13-15. Procedures and methods for scoring prototrophic recombinants are described in Chapter II. Table 13 represents the results of reciprocal crosses of the argX mutants with arg point and deletion mutants. Recipients are listed in the horizontal column and donors in the vertical column. As is indicated, argX2041 and argX1593 fail to give prototrophic recombinants with deletion mutants MN42, 2007, argCB-1, and sup-102, but give wild-type recombinants with the argEC(B) and argEC-1 strains. Further confirmation that argX2041 and argX1593 are not in the same locus was derived from the observation that they give prototrophic transductants when crossed with each other, but not with themselves (Tables 13 and 15). It is interesting to know that argX2041 crossed with 187 (argB-2) yields no recombinants (Table 13 and 15). Hence, argX2041 and 187 are thought to share at least a part of the same locus in the argB gene. Mutation 187 (argB-2) has been mapped by Glansdorff and found to be located in the distal portion of the argB gene and is included within both the argCB-1 and sup-102 deletions (119, 124).

As shown in Table 14, ten argB mutants as donors were crossed with six deletion mutants. It was found that all the argB mutations tested are included within deletions MN42, argCB-1 and sup-102. All except 1581, 1583, 1734, 2058, and 2082 map within 2007. However, 1581, 1583, 1734, 2058 and 2082 give no prototrophic recombinants when crossed with argEC(B). These argB mutations apparently lie within argEC(B) which must extend into the proximal portion of the argB gene, but not into 2007.

TABLE 13

RECIPROCAL CROSSES BETWEEN *argX* AND *arg* POINT AND DELETION MUTANTS

| D \ R | MN42 | 2007 | sup-102 (175) | CB-1 (172) | EC(B) (174) | EC-1 (176) | 185 | 186 | 187 | 2041* | 1593* | 1810 | 190 | 188 | 191 | 496 | 2009 | 1982 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MN42 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | + | + |
| 2007 | - | - | - | - | - | + | - | - | - | - | - | + | - | - | + | + | + | + |
| sup-102 | - | - | - | - | - | - | - | - | - | - | - | - | + | + | + | + | + | + |
| CB-1 | - | - | - | - | - | + | - | - | - | - | - | - | + | + | + | + | + | + |
| EC(B) | - | - | - | - | - | - | + | + | + | + | + | - | + | + | + | + | + | + |
| EC-1 | - | + | - | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + |
| 185 | - | - | - | - | + | + | - | + | + | + | + | + | + | + | + | + | + | + |
| 186 | - | - | - | - | + | + | + | - | + | + | + | + | + | + | + | + | + | + |
| 187 | - | - | - | - | + | + | + | + | - | - | + | + | + | + | + | + | + | + |
| 2041* | - | - | - | - | + | + | + | + | - | - | + | + | + | + | + | + | + | + |
| 1593* | - | - | - | - | + | + | + | + | + | + | - | + | + | + | + | + | + | + |
| 1810 | - | + | - | - | - | + | + | + | + | + | + | - | + | + | + | + | + | + |
| 190 | - | - | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + | + |
| 188 | - | - | + | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + |
| 191 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | + | + | + |
| 496 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | + | + |
| 2009 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | + |
| 1982 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - |

* *argX* mutants.

TABLE 14

CROSSES BETWEEN argB AND LARGE DELETION MUTANTS

| D \ R | MN42 | 2007 | CB-1 (172) | sup-102 (175) | EC(B) (174) | EC-1 (176) |
|---|---|---|---|---|---|---|
| 1581 | - | + | - | - | - | + |
| 1583 | - | + | - | - | - | + |
| 1585 | - | - | - | - | + | + |
| 1586 | - | - | - | - | + | + |
| 1598 | - | - | - | - | + | + |
| 1637 | - | - | - | - | + | + |
| 1668 | - | - | - | - | + | + |
| 1734 | - | + | - | - | - | + |
| 1808 | - | - | - | - | + | + |
| 2058 | - | + | - | - | - | + |
| 2082 | - | + | - | - | - | + |

TABLE 15

RECIPROCAL CROSSES AMONG *argB* AND *argX* MUTANTS

| D \ R | 185 | 186 | 187 | 1581 | 1583 | 1585 | 1586 | 1593* | 1598 | 1637 | 1668 | 1808 | 2041* | 2058 | 2082 | 1734 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 185 | - | + | + | + | + | + | + | + | - | + | + | - | + | + | + | + |
| 186 | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 187 | + | + | - | + | + | L | - | + | + | L | + | + | - | + | + | + |
| 1581 | + | + | + | - | - | + | + | + | + | + | + | + | + | - | - | - |
| 1583 | + | + | + | - | - | + | + | + | + | + | + | + | + | - | - | - |
| 1585 | + | + | L | + | + | - | + | + | + | + | + | + | L | + | + | + |
| 1586 | + | + | - | + | + | + | - | + | + | + | + | + | - | + | + | + |
| 1593* | + | + | + | + | + | + | + | - | + | + | - | + | + | + | + | + |
| 1598 | - | + | + | + | + | + | + | + | - | + | + | - | + | + | + | + |
| 1637 | + | L | L | + | + | + | + | + | + | - | + | + | L | + | + | + |
| 1668 | + | + | + | + | + | + | + | - | + | + | - | + | + | + | + | + |
| 1808 | - | + | + | + | + | + | + | + | - | + | + | - | + | + | + | + |
| 2041* | + | + | - | + | + | L | - | + | + | L | + | + | - | + | + | + |
| 2058 | + | + | + | - | - | + | + | + | + | + | + | + | + | - | - | - |
| 2082 | + | + | + | - | - | + | + | + | + | + | + | + | + | - | - | - |
| 1734 | + | + | + | - | - | + | + | + | + | + | + | + | + | - | - | - |

L, low number of recombinants (under 20 colonies).

* *argX* mutants

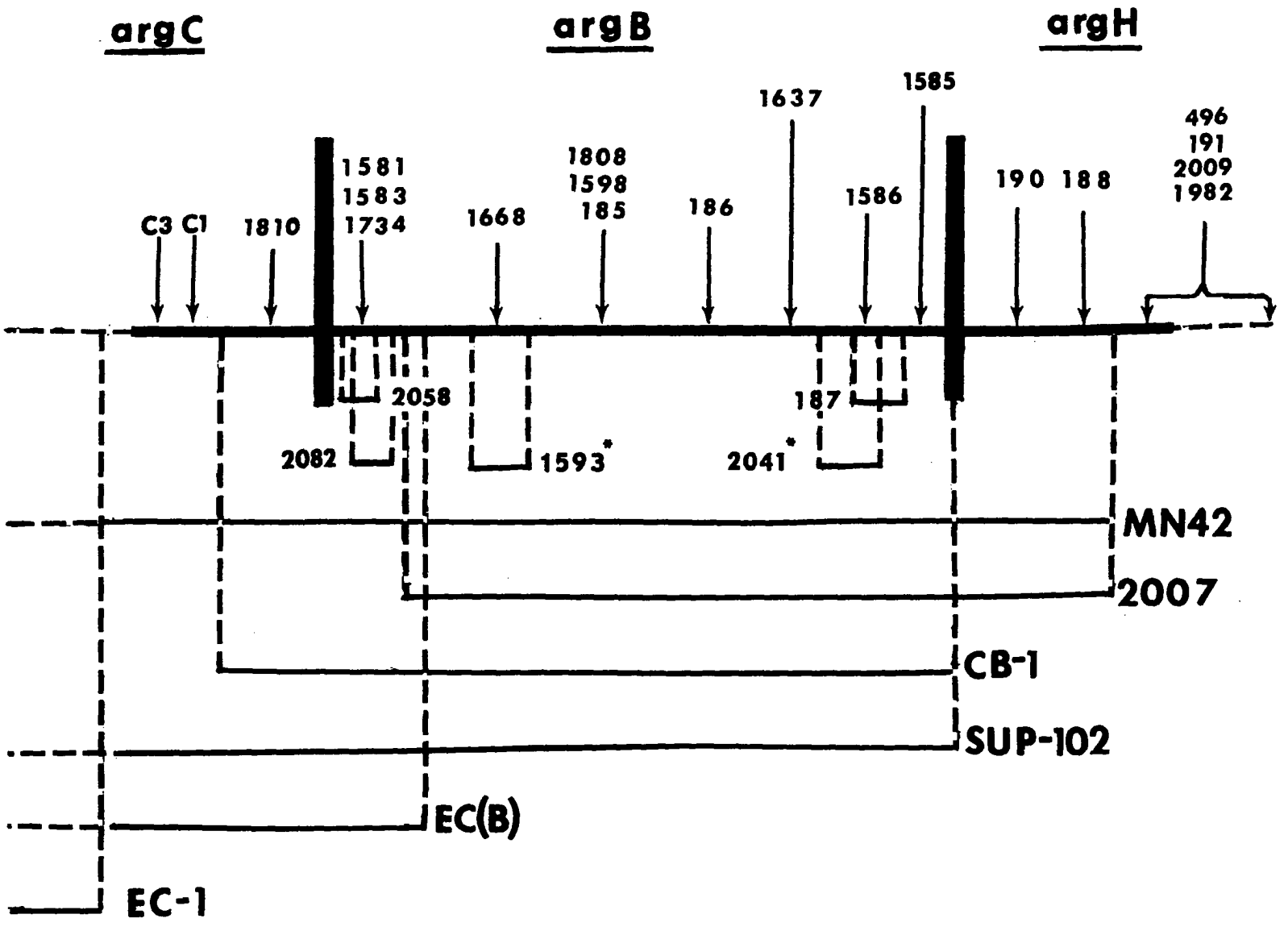
Moreover, it would appear that 2007 and argEC(B) overlap somewhere in the proximal argB gene, since no recombinants result from reciprocal crosses between 2007 and argEC(B) (Table 13).

As shown in Table 15, reciprocal crosses were performed among argB and argX mutants. Reciprocal crosses between argX2041, 187 (argB-2) and 1586 yield no prototrophic recombinants and, therefore, presumably occupy the same locus. Scarce transductants were found when argX2041 was crossed with either 1585 or 1637. Therefore, strains 1585 and 1637 probably map in close proximity to argX2041. It was previously determined that 1585 was to the right of argX2041 and 1637 to the left of it (Table 12). Since strain argX1593 fails to yield wild-type recombinants when crossed with strain 1668 (Table 15), they are presumed to share the same locus. Strain argB1808, which was the reference mutation used in the three-point crosses (Table 12), was found to map in the same locus as that of 185 (argB-1) and 1598 (Table 15). Strain 185 (argB-1) was formerly mapped by Glansdorff and shown to be in the proximal half of the argB gene (118,119).

The above data obtained by deletion mapping and by three-point crosses, together with information on the relative positions of some of Glansdorff's point mutations, were used to construct a "fine structure" map which is shown in Figure 9. Mutation argX1593 is placed in the proximal portion of argB, whereas argX2041 maps either at the extreme distal end of argB or at the argB-H junction.

Figure 9. Location of promoter mutations in argB. The numbers above the "chromosome" line indicate the approximate sites of arg point mutations. The lines below indicate the approximate regions of the chromosome which are missing in the various deletion strains. Termini of the deletions are approximate. The positions of C1 and C2, as assigned by N. Glansdorff (119,124,142), are included for reference purposes.

* argX mutations.


argC
argB
argH
C3
C1
1810
1581
1583
1734
1668
1808
1598
185
186
1637
1585
1586
190
188
496
191
2009
1982
2058
2082
1593*
187
2041*
MN42
2007
CB-1
SUP-102
EC(B)
EC-1

## Enzyme Activities of argB Mutants Which Map at or near argX2041

Several mutants which either map at the same locus as argX2041, or in close proximity to it, were tested for their growth responses (Table 3) and enzyme activities (Table 16).

It is noteworthy that although auxotroph argB1585 maps distal to argX2041, it has normal argininosuccinase (argH) when grown under arginine repression (Table 16). Therefore, the argB1585 point mutation must lie in the structural gene of argB, at or near the argB-H border, and does not affect a critical region of the argH gene. Strains argX2041, 187 (argB-2) and argB1586 appear to map at the same site (Table 15). Whereas the argX2041 mutant shows a marked increase in argininosuccinase (argH) activity under repressed conditions, the 187 mutant exhibits a substantial reduction in argininosuccinase activity, to approximately 6% of that of the wild-type. Strain 187 thus resembles the argX2041 spontaneous revertant EcK3754 (Table 11) in the sense of having a low residual expression of argH. The revertant has 30% of the wild-type argH expression (activity). Hence, the argX2041 site in the argB structural gene, appears to be critical for argH expression, since two different mutations at this site alternatively "turn off" or "promote" argH expression. On the other hand, strain argB1586 shows normal argH expression.

## Constitutivity of argH Expression Restored by argX in a Primary Promoter-Operator Deleted Mutant

It has been established that argX mutations enhance argH expression only under maximally repressed conditions, and not under partial

TABLE 16

ENZYME ACTIVITIES OF *argB* MUTANTS WHICH MAP AT OR NEAR *argX2041*

| Strain | Relative Specific Activity[a] | | | |
|---|---|---|---|---|
| | *argE* | *argC* | *argH* | *argD* |
| P4X6 (W.T.) | 100 | 100 | 100 | 100 |
| 2041 | 112 | 111 | *394* | 103 |
| 1585 | 91 | 116 | 95 | 87 |
| 1586 | 96 | 105 | 110 | 75 |
| 1637 | 96 | 147 | 105 | 105 |
| 187 (B-2) | 108 | N.D.[b] | *<6* | 113 |

[a] Cells were grown under fully repressive conditions (arginine, 100 µg/ml). Relative specific activities were calculated by designating as 100 per cent the specific activity of each enzyme in wild type (P4X6) grown under conditions of maximum-repression. Wild type specific enzyme activities (units/mg protein): enzyme E, 12.9; enzyme C, 1.7; enzyme H, 0.17; enzyme D, 0.7.

[b] Not determined.

physiologic (after growth in the presence of orn) or genetic ($\underline{argO}_{ECBH}$) derepression or full genetic ($\underline{argR}^-$) derepression. Therefore, its ability to function seems to correlate with that of the primary promoter ($P_{CBH}$) for the argC, B, and H genes. The primary promoter seems to be affected by arginine repression, whereas the argX mutations are apparently unaffected by arginine, and, in fact, they function only when the argX mutants are grown under repressive conditions. Therefore, efforts were made to separate the argX mutations from the primary promoter ($P_{CBH}$) in order to determine directly whether argX can function independently and whether the expression of argH, restored by argX, is affected by repression. From this study, it was also possible to infer whether the argX mutations act at the transcriptional or translational level.

Double mutants, argEC-1 argX2041 and argEC-1 argX1593, were constructed and assayed as described in Chapter II. Strain argEC-1 carries an operator-primary promoter ($P_{CBH}$) deletion in the arginine cluster (119,124). The results are shown in Table 17. Strain 3726, carrying the argEC-1 deletion, has a residual level of enzyme H activity of approximately 0.06 when grown under repressive conditions. This constitutive level of argininosuccinase activity is presumably due to a low-efficiency, unregulated internal promoter ($P_H$) situated somewhere between argB and argH (119,124). Under repressive conditions, strains 3725 (argEC-1 argX2041) and 3764 (argEC-1 argX1593) show an argininosuccinase specific activity of 0.43 and 0.33, respectively (Table 17). These values correspond to a 5.5 - 7 fold increase above the specific activity of 0.06 achieved by argEC-1 in the absence of the argX mutations. When strains 3764 and 3725 are cultivated under partially de-

TABLE 17

INSENSITIVITY TO REPRESSION OF *argH* EXPRESSION RESTORED BY *argX* IN A PROMOTER($P_{CBH}$)-OPERATOR DELETION MUTANT

| Strain | Genotype[a,b] | Supplement | Specific Activity (units/mg protein) | |
|---|---|---|---|---|
| | | | *argE* | *argH* |
| P4X6 | Wild type | orn | 27.7 | 1.0 |
| | | arg | 12.9 | 0.17 |
| 2041 | *argX2041* | orn | 27.3 | 1.1 |
| | | arg | 16.7 | 0.58 |
| 1593 | *argX1593* | orn | 30.4 | 1.1 |
| | | arg | 15.0 | 0.43 |
| 3726 | *argEC-1* | orn | ____ | 0.10 |
| | | arg | ____ | <0.06 |
| 3725 | *argEC-1 argX2041* | orn | ____ | 0.48 |
| | | arg | ____ | 0.43 |
| 3764 | *argEC-1 argX1593* | orn | ____ | 0.35 |
| | | arg | ____ | 0.33 |

[a] All strains carry the *rif* gene except for the wild type (P4X6).

[b] All strains carry the *metB* gene.

repressed conditions (grown with ornithine), they have essentially the same argH specific activity as under repressed conditions. It is apparent that the argX mutations can act as initiators in the absence of the primary promoter and restore argH expression. Moreover, the argX mutations no longer depend on the repressed state of the arginine cluster in order to function; they effect the same constitutive level of argininosuccinase under both repressed and partially derepressed conditions. Under repressed conditions, argX restores and actually enhances argininosuccinase to a level which is 1.9 - 2.5 fold above the wild-type activity of 0.17. However, under partially derepressed conditions, argX can only restore from 35% - 50% of the corresponding wild-type activity of 1.0.

The growth rates of these strains in the presence of ornithine or arginine are noteworthy (Table 18). The promoter-operator deleted strain, argEC-1, cultivated in the presence of arginine, has a doubling time of 49 minutes. However, when grown with ornithine, it has a longer doubling time of 120 minutes. This is apparently due to the limited amount of arginine synthesized because of the low level of argininosuccinase present. When strain argEC-1 was combined with either the argX1593 or argX2041 mutation, the doubling time of the resulting strains (after growth in orn) are accelerated from 120 minutes to an essentially normal doubling time of 57 minutes (compared to 51, 48, or 49 minutes for wild-type, argX2041, or argX1593, respectively). The resumed normal growth rate is presumably due to an elevated level of argininosuccinase promoted by the argX mutations. The growth curves of an argEC-1 argX2041 double mutant and an argEC-1 deletion strain, when supplemented with ornithine and arginine, are shown in Figure 10.

TABLE 18

DOUBLING TIMES OF PROMOTER($P_{CBH}$)-OPERATOR DELETION MUTANT WHEN CARRYING *argX* MUTATIONS

| Genotype | Supplement | Doubling Time (min) |
|---|---|---|
| P4X6 (W.T.) | Orn | 51 |
| | Arg | 48 |
| *argX2041* | Orn | 48 |
| | Arg | 49 |
| *argX1593* | Orn | 49 |
| | Arg | 52 |
| *argEC-1* | Orn | 120 |
| | Arg | 49 |
| *argEC-1 argX2041* | Orn | 57 |
| | Arg | 52 |
| *argEC-1 argX1593* | Orn | 57 |
| | Arg | 51 |

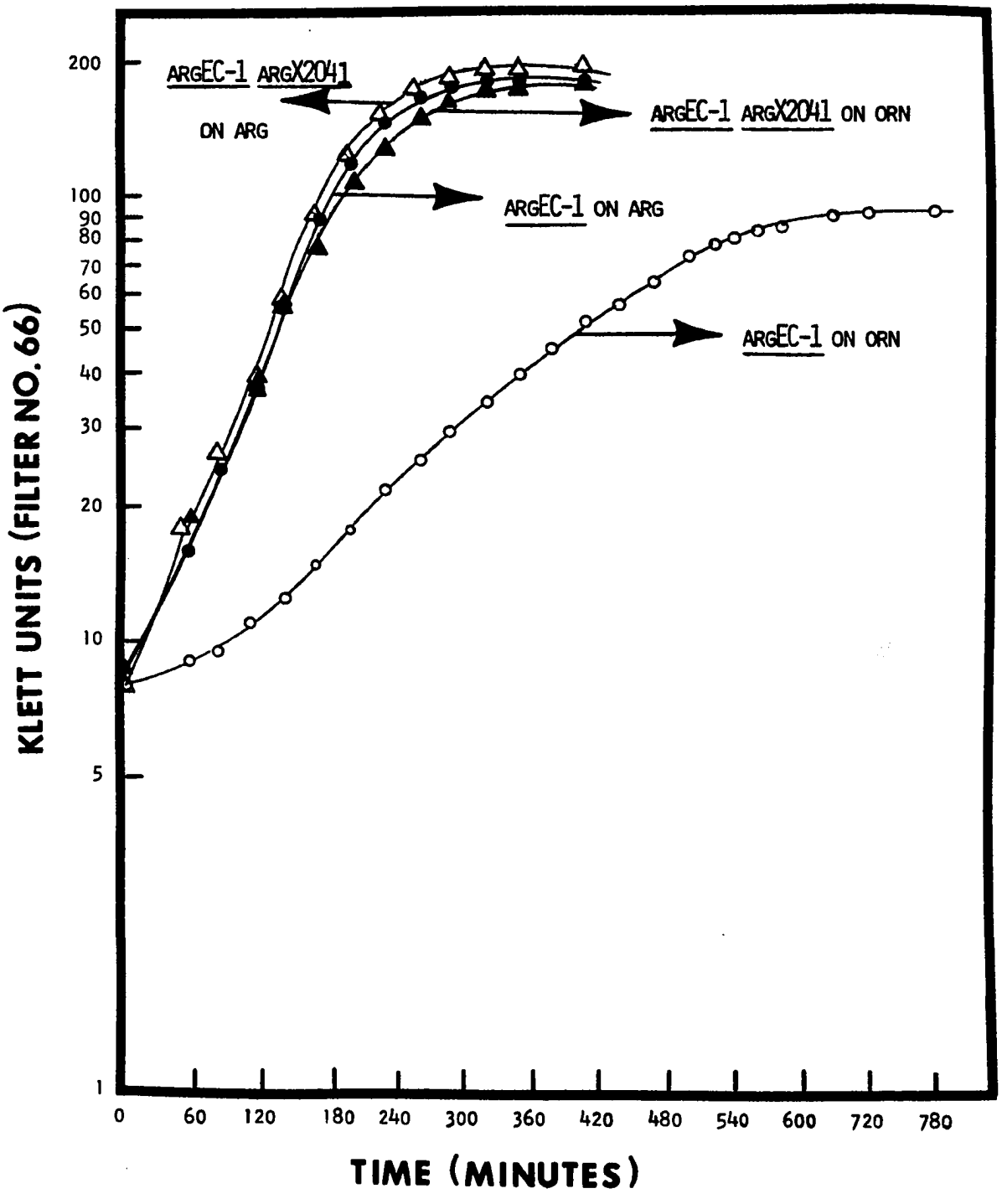



Figure 10. Growth curves of promoter($P_{CBH}$)-operator deletion mutant when carrying argX2041 mutation.

From the above data, it is reasonable to conclude that argX1593 and argX2041 are transcriptional types of initiators. Both argX mutants act to promote transcription when the normal initiation site ($P_{CBH}$) is deleted. It is unlikely that these argX mutants act at the translational level, because there would be hardly any mRNA for the arginine cluster in the argEC-1 strain due to the deletion of the primary promoter-operator region. However, there is a low level of transcription of argH, which is initiated at the internal promoter ($P_H$). This amount of mRNA certainly could not account for the enzyme H activity (1.9 - 2.5 fold increase above that of the wild-type when grown with arginine) in the argEC-1 argX mutants.

## Possible Involvement of An Operator in argX Mutant

Since the argX elements are not regulated by arginine, it was of interest to determine whether the argX mutation includes its own "operator" region. This "operator" might be able to interact with active repressor only at abnormally high concentrations of arginine, or might be sensitive to active repressor from another pathway. Strain argX2041 was cultivated in MMA with abnormally high arginine (200 µg/ml), or in enriched arginine-free assay medium with added arginine (100 µg/ml). Extracts were assayed for the argE and argH enzymes. The results, shown in Table 19, indicate that the argX strain has the same constitutive level of argininosuccinase activity either in enriched medium (arginine free assay medium containing arginine), or in MMA medium with a high concentration of arginine. It is concluded that the argX mutation does not include an operator region.

TABLE 19

AN OPERATOR NOT INCLUDED IN *argX2041* MUTATION

| Strain[a] | Medium | Arginine Supplement (μg/ml) | Specific Activity (units/mg protein) | |
|---|---|---|---|---|
| | | | *argE* | *argH* |
| P4X6 (W.T.) | Minimal | 100 or 200 | 16.2 | 0.17 |
| 2041 $R^+$ | Minimal | 100 | 14.9 | 0.67 |
| 2041 $R^+$ | Minimal | 200 | 15.6 | 0.64 |
| 2041 $R^+$ | Arginine-free assay | 100 | 14.5 | 0.71 |

[a] All carry the *metB* gene.

## Function of argX Mutation in Combination with Polar Nonsense Mutation in argC

Since an argX mutation is able to initiate and restore argH expression in a promoter-operator deletion mutant, it was of interest to determine whether the argX mutations might also be effective in reducing the polar effect exerted by a nonsense mutation (UGA) in argC on argH function. Mutant 3653 ($argC_{UGA}$ $rif^r$) shows a polar effect on argH expression. As shown in Table 20, when polar mutant 3653 is cultivated in arginine, argH expression is reduced to approximately 50% (0.08) of the corresponding wild-type specific activity of 0.17. However, when it is grown in the presence of ornithine, the enzyme H activity of 0.73 represents a loss of only 25% of the wild-type specific activity of 1.0.

The procedure for isolation of an argC argX double mutant was tedious, as described in Chapter II. An argE strain (2084-1) was used to construct an argC (3653) mutant, and an argE argX double mutant (3613) was used to construct an argC argX double mutant (3648). With these strains, it was possible to examine the effect of the argX mutation on operon expression after termination of translation has occurred at the operator-proximal argC gene. The growth curves and rates of the argC polar mutant and of the double mutant in ornithine and arginine are shown in Figure 11. The results strongly suggest that the argX2041 mutation indeed relieves the polar effect of the nonsense mutation on argH expression since the growth rate of the double mutant in ornithine is increased to that of the arginine control. Assays of the cluster enzymes verified this conclusion (Table 20). Under conditions of arginine repression, it was found that function of the previously almost inactive

TABLE 20

RELIEF BY *argX* OF POLAR EFFECT EXERTED BY A NONSENSE *argC* MUTATION ON *argH* EXPRESSION

| Strain | Genotype[a,b] | Supplement | Specific Activity (units/mg protein)[c] | | |
|---|---|---|---|---|---|
| | | | *argE* | *argC* | *argH* |
| P4X6 (Control) | W.T. | orn | 27.7 | 3.5 | 1.0 |
| | | arg | 12.9 | 1.7 | 0.17 |
| 2084-1 (Control) | *argE* | orn | N.M.[d] | 4.7 | 1.1 |
| | | arg | N.M. | 2.0 | 0.18 |
| 3613 (Control) | *argE argX2041* | arg | N.M. | 1.9 | 0.58 |
| 3653 | $argC_{UGA}$ | orn | 50.0 | <0.37 | 0.73 |
| | | arg | 16.7 | N.M. | <0.08 |
| 2041 | *argX2041* | orn | 27.3 | 4.5 | 1.1 |
| | | arg | 14.5 | 2.2 | 0.58 |
| 3648 | $argC_{UGA}$ *argX2041* | orn | 45.6 | <0.27 | 0.81 |
| | | arg | 12.3 | N.M. | 0.40 |

[a] All strains carry the *rif* gene except for P4X6.

[b] All strains carry the *metB* gene except for 3653 and 3648.

[c] After growth in the presence of excess arginine (100 µg/ml)

[d] Not measurable.

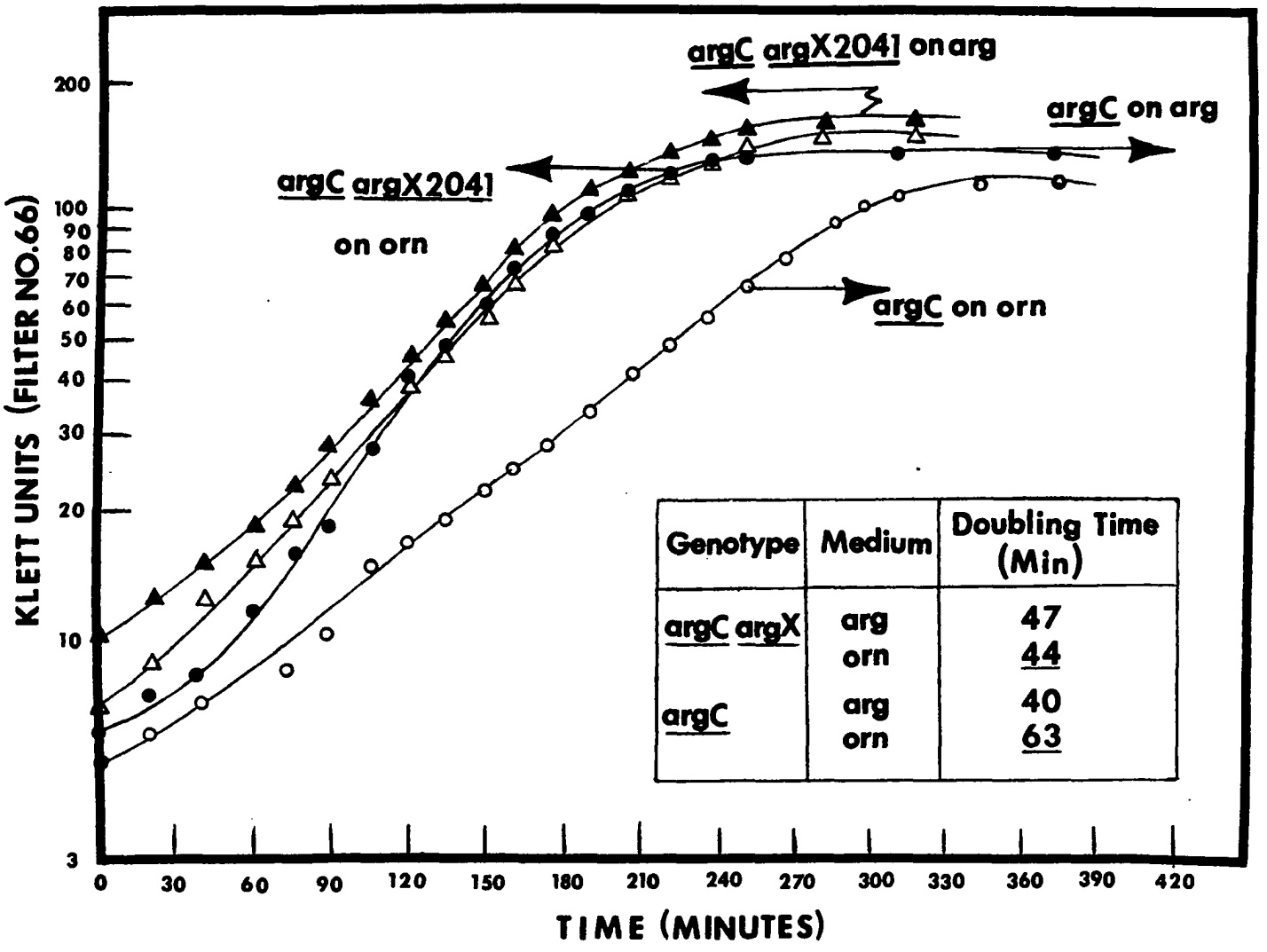



Figure 11. Growth curves of polar argC mutant when carrying argX2041 mutation.

argH gene is restored by the argX mutation in the polar argC strain. The specific argininosuccinase activity is 0.08 for the argC strain as compared to 0.40 for the argC argX strain. Therefore, argH expression in the double mutant is elevated to 235% above that in repressed wild-type strain. However, no such dramatic release of the polar effect is exerted under conditions of partial derepression, and in fact, there is essentially no significant relief of the polar effect. This finding is further evidence that argX mutations are unable to promote transcription when the primary promoter ($P_{CBH}$) is functioning even if only 75% efficiency.

In summary, the evidence indicates that the argX mutations act as efficient internal promoters in combination with polar mutations, as well as with promoter-operator deletion mutations.

## Effect of the argX Element on the Expression of argH in Merodiploids

Before construction of the diploids, an $F^-$ strain, EcK3615 (argX2041 $argH^-$), was isolated as described in Chapter II. The presence of the argX mutation was confirmed by demonstrating its recoverability via transduction from the double mutant (Table 8). An $argH^-$ strain was used as a control.

The merodiploids, EcK3742 (F' $metB^+$ $argX^+$ $argH^+$ $rif^r$ / $metB^-$ argX2041 $argH^-$ $rif^r$ thi his aroE recA1 $str^r$) and EcK3750 (F' $metB^+$ $argX^+$ $argH^-$ $rif^r$ / $metB^-$ argX2041 $argH^+$ $rif^r$ thi his aroE recA1 $str^r$), were constructed as described in Chapter II.

The two diploids, together with the corresponding exogenote and endogenote parents, were grown in the presence of arginine, and

assayed for the level of acetylornithinase and argininosuccinase. The results are given in Table 21. The exogenote (*argX*$^+$) is unable to reduce synthesis of argininosuccinase from the endogenote (*argH*$^+$) as demonstrated in the F' strain 3750 (F' *argX*$^+$ *argH*$^-$ /*argX*$^-$ *argH*$^+$). Therefore, it is concluded that *argX* constitutivity is cis-dominant. Parallel studies in the diploid strain 3742 (F' *argX*$^+$ *argH*$^+$/*argX*$^-$ *argH*$^-$) show that *argX* is trans-recessive, since *argX*$^-$ in the endogenote does not elevate *argH* expression in the exogenote (*argH*$^+$).

## Absence of Duplication of argH Gene in argX2041 Mutant

It has been reported that mutants which utilize ornithine slowly due to low *argH* expression could be reactivated to achieve an increased growth rate (142,147). Some of these fast ornithine-utilizers carry duplications of the *argH* gene in the close vicinity of the arginine cluster, but not within it. In view of the above report, it was necessary to rule out the remote possibility that a duplication of the *argH* gene is responsible for the enhanced argininosuccinase activity in the *argX* mutants.

The tactics to approach this problem were as follows. It was assumed that *argX2041* did indeed carry more than one copy of the *argH* gene [*arg*(H$^+$)$_n$]. An *argH*$^-$ marker (EcK496-5) was introduced into the *argX*$^-$ *arg*(H$^+$)$_n$ recipient (EcK2041), thereby replacing the presumed multiple *arg*(H$^+$)$_n$ gene copies with a single copy of *argH*$^-$ (as described in Chapter II). Recovery of the *argX* mutation from the double mutant EcK3615 (*argX*$^-$ *argH*$^-$) was performed as described in a previous section in the present Chapter, and the results are given in Table 8. The

TABLE 21

EFFECT OF *argX* ON THE EXPRESSION OF *argH* IN MERODIPLOIDS

| Strain | Genotype[a] (exo-/endogenote) | Doubling Time (min)[b] | Specific Activity (units/mg protein)[b] | |
|---|---|---|---|---|
| | | | *argE* | *argH* |
| 3769 | Hfr *argX*$^+$ *argH*$^-$ | 48 | 13.9 | N.M.[c] |
| 3744 | F$^-$ *argX*$^-$ *argH*$^+$ | 80 | 13.4 | 0.59 |
| 3750 | F' *argX*$^+$ *argH*$^-$/*argX*$^-$ *argH*$^+$ | 94 | 28.2 | 0.56 |
| 3768 | Hfr *argX*$^+$ *argH*$^+$ | 47 | 13.6 | 0.17 |
| 3735 | F$^-$ *argX*$^-$ *argH*$^-$ | 79 | 15.8 | N.M.[c] |
| 3742 | F' *argX*$^+$ *argH*$^+$/*argX*$^-$ *argH*$^-$ | 97 | 30.5 | 0.19 |

[a] All strains carry the *rif* gene. All strains except for 3769 and 3768, also carry *recA1*.

[b] After growth in the presence of excess arginine (100 µg/ml).

[c] Not measurable.

double mutant, EcK3615 (*metB*$^-$ *argX*$^-$ *argH*$^-$ *rif*$^r$) served as recipient in a transduction in which a copy of the *argH*$^+$ gene was introduced from a wild-type strain (P4X6). Cells were plated on MMA + met + aco agar to select for *Arg*$^+$ (*argH*$^+$) recombinants which were in turn scored for the Aco$^-$ phenotype (*argX*$^-$). It is reasonable to expect that the resulting *argX*$^-$ strain has only a single copy [*arg*(H$^+$)$_1$] of the *argH* gene. Argininosuccinase (*argH*) and acetylornithinase (*argE*) assays were performed on extracts of EcK3758 (*metB*$^-$ *argX*$^-$ *argH*$^+$) and related strains grown in the presence of arginine to determine whether there is enhanced expression of *argH* in an *argX* strain which reasonably may be expected to carry only a single *argH*$^+$ gene.

The results are represented in Table 22. Strain 3758 [*argX*$^-$ *arg*(H$^+$)$_1$] has an enzyme H specific activity of 0.68, as compared to 0.67 for *argX2041* [*argX*$^-$ *arg*(H$^+$)$_n$]. Since these values are essentially identical, it is apparent that the *argX* mutant (*argX2041*) does not have a duplication of the *argH* gene. However, the possibility cannot be excluded that an insertion of a piece of foreign DNA into the region between *argB* and *argH* had occurred, thereby inactivating the *argB* enzyme and concomitantly enhancing *argH* expression.

## Effect of Rifampin on the Efficiency of *arg* and *argX* Promoters

RNA polymerase must interact with the promoter sites in the arginine cluster. Since rifampin interferes with *in vivo* transcription-initiation at promoter sites, this antibiotic was used as a probe to examine the relative efficiencies of the arginine promoters. Preliminary studies were made to test the effect of rifampin on the effi-

TABLE 22

DUPLICATION OF *argH* GENE NOT PRESENT IN *argX2041* MUTANT

| Strain | Genotype[b] | Specific Activity (units/mg protein)[c] | |
|---|---|---|---|
| | | *argE* | *argH* |
| P4X6 (W.T.) | *argX*$^+$ *argH*$^+$ | 16.7 | 0.18 |
| 2041 | *argX*$^-$ *arg*(H$^+$)$_n$ | 17.0 | 0.67 |
| 3615[a] | *argX*$^-$ *argH*$^-$ | 15.8 | N.M.[d] |
| 3758 | *argX*$^-$ *arg*(H$^+$)$_1$ | 16.2 | 0.68 |

[a] Carries the *rif* gene.

[b] All strains carry the *metB* gene.

[c] After growth in the presence of excess arginine(100 µg/ml).

[d] Not measurable.

ciency of transcription-initiation at the naturally-occurring primary ($P_{CBH}$) and secondary ($P_H$) promoters, and also at the mutationally-created argX type ($P_X$) of promoter. All strains used were rifampin-resistant ($rif^r$). Cultures were grown in ornithine and arginine, with and without rifampin. The levels of enzymes specified by argE and argH were determined. The results are given in Table 23. Under repressed as well as partially derepressed conditions, the wild-type strain (P4X6) grown with added rif has essentially the same enzyme H activity as when it is grown without added rif. Whereas P4X6 (grown under repressed conditions) shows the same specific enzyme E activity with and without added rif under repressive conditions, it has higher enzyme E activity with rif (39) than without rif (27.7) under partially derepressed conditions (after cultivation in the presence of ornithine). This corresponds to an increase of almost 50%.

After growth in ornithine plus rif, argX2041 shows an enzyme H activity of 0.58, which is 40% lower than that after growth without added rif (1.0). Under repressed conditions with added rif, the argX mutant also has a correspondingly lower (approximately 40%) enzyme H activity of 0.38, as compared to 0.59 without added rif. It is noteworthy that the enzyme H activity of 0.38 shown by the argX mutant (grown under repressed conditions with added rif) is exactly equal to the enzyme H activity of strain argEC-1 argX2041 grown under the same conditions. Therefore, it is apparent that the primary promoter ($P_{CBH}$) does not function at all to initiate argH transcription in the argX mutant during growth in arginine with added rif. However, the function of $P_X$ (promoter created by argX mutation) is not affected by added rif

TABLE 23

EFFECT OF RIFAMPIN ON EFFICIENCY OF TRANSCRIPTION-INITIATION

AT ARGININE PROMOTERS

| Strain[a] | Promoter(s) Present | Supplement | Specific Activity (units/mg protein)[b] | | |
|---|---|---|---|---|---|
| | | | argE | argH | argD |
| P4X6 (W.T.) | $P_{CBH} + P_H$ | orn | 39.0 (27.7) | 1.0 (1.2) | 2.4 (2.6) |
| | | arg | 19.6 (15.0) | 0.19 (0.18) | 0.8 (0.6) |
| argX2041 | $P_{CBH} + P_X$ | orn | 24.4 (27.3) | 0.58 (1.0) | 2.3 (2.3) |
| | $+ P_H$ (?) | arg | 17.7 (16.7) | 0.38 (0.59) | 0.7 (0.9) |
| argEC-1 | $P_H$ | orn | ——— | 0.56 (0.11) | 6.8 (11.2) |
| | | arg | ——— | 0.08 (0.06) | 0.7 (1.0) |
| argEC-1 argX2041 | $P_X + P_H$ (?) | orn | ——— | 0.44 (0.48) | 6.9 (8.3) |
| | | arg | ——— | 0.38 (0.43) | 0.7 (0.6) |

[a] All strains carry the metB and rif genes.

[b] After growth in the presence of rifampin; values in parenthesis obtained after growth in the absence of rifampin.

since enzyme H activity does not change significantly when the argEC-1 argX2041 strain is grown in arginine either with (0.38) or without (0.43) rif. Hence, the lower enzyme H activity of the argX mutant when grown with rif is apparently due to the lower efficiency of the primary promoter ($P_{CBH}$) in the presence of the antibiotic.

When the argEC-1 strain is cultivated in ornithine plus rif, the argininosuccinase level is increased five fold above that of the same strain under the same conditions but without added rif. The specific activity is 0.56 as compared to 0.11. It would seem that rifampin acts either as an "inducer", or just binds to RNA polymerase to facilitate or direct transcription specifically at $P_H$ (low efficiency promoter between argB and argH) under partial derepression (with ornithine). However, under repressed conditions with rif, enzyme H is no longer "induced" and $P_H$ again has the same low efficiency (0.08) as it does without rif (0.06).

From the studies of the effect of rifampin, an unexpected property of the argX mutant was uncovered. The findings indicate that in the argX mutant, only $P_{CBH}$ and $P_X$ are still intact, whereas $P_H$ is either deleted by the presence of the argX mutation, or is completely nonfunctional. After growth in ornithine plus rif, strain argEC-1 (carrying $P_H$ as the only promoter) shows an enzyme H activity of 0.56 and strain argEC-1 argX2041 [$P_X + (P_H)$?] has an enzyme H activity of 0.44. If $P_H$ was present and functional in the argEC-1 argX strain, argH expression should be equal to the sum of $P_H$ (0.56) and $P_X$. In fact, argH expression (0.44) in argEC-1 argX2041 is somewhat less than that found for $P_H$ alone (argEC-1). Therefore, it is unlikely that $P_H$ is still

intact in the argX mutant. Additional support may be derived from comparing strains argX2041 and argEC-1 after growth with ornithine + rif. The enzyme H activity made available at $P_{CBH} + P_X + P_H(?)$ (0.58) is essentially the same as that contributed by $P_H$ alone (0.56).

The doubling times for strains P4X6, argX2041, argEC-1 and argEC-1 argX2041 are shown in Table 24. Although there is no marked difference in doubling times when these strains are grown with or without rifampin, there appear to be slightly shorter doubling times under repressed as well as partially derepressed conditions in the presence of rif.

The findings are in accord with the view that the rate of in vivo transcription-initiation of specific mRNA molecules at different promoter sites in rifampin-resistant strains is affected nonuniformly by rifampin, and that the effect of rifampin on initiation at $P_H$ appears to be influenced by arginine.

TABLE 24

EFFECT OF RIFAMPIN ON DOUBLING TIME OF *argX*, *argEC-1* AND *argEC-1 argX* STRAINS

| Strains[a] | Supplement | Doubling Time[b] (min) |
|---|---|---|
| P4X6 (W.T.) | Orn | 41 (47) |
| | Arg | 41 (51) |
| 2041 | Orn | 43 (48) |
| | Arg | 41 (49) |
| *argEC-1* | Orn | 114 (120) |
| | Arg | 41 (49) |
| *argEC-1 argX2041* | Orn | 50 (57) |
| | Arg | 45 (52) |

[a] All strains carry the *metB* and *rif* genes.

[b] After growth in the presence of rifampin; values in parenthesis obtained after growth in the absence of rifampin.

# CHAPTER IV

## DISCUSSION

Information for the synthesis of the enzymes which catalyze the terminal steps of arginine biosynthesis of Escherichia coli K-12 is encoded in nine structural genes, scattered in six regions of the chromosome. Five of the structural genes map at five different sites (Figure 2), whereas four structural genes are contiguous and form a cluster in the order argE-C-B-H (118). The unlinked genes are repressed by arginine in parallel, but not coordinately, as though each constitutes a distinct operon with its own operator site. It is reasonable to believe that the arginine genes constitute various functional units under negative transcriptional control by arginine through the mediation of an active protein repressor produced by the argR gene. The evidence in favor of this view for the arginine system is briefly discussed as follows:

(1) Evidence has been given which demonstrates the presence of cis-dominant operator regions for the argECBH cluster (122) and for argI (95,125).

(2) Evidence has been presented which suggests that arginine or a derivative acts as the corepressor for the system at the transcriptional level (101,102,103,104). RNA-DNA hybridization experiments support the view that arginine signals repression by inhibiting the initiation of transcription of argECBH messenger RNA via the involvement of the argR

product. However, there are indications that a translational control signal might operate simulataneously during arginine repression (105). The data also suggest that, in the presence of excess arginine, an accelerated degradation of mRNA occurs in the *argR*$^+$ strain (107).

(3) Based on studies of coordinacy (120,121), of polarity (89,119,122,123), and of *argO*$_{ECBH}$ mutants (122), it has been proposed that *argECBH* form two adjacent, but distinct, functional units. One unit is comprised of *argCBH* and is transcribed clockwise from the light strand, and the second unit consists of only *argE*, and is transcribed counterclockwise from the heavy strand (Figure 3). Whereas deletions covering the region between *argE* and *argC* result in a total nonfunction of *argE*, *C*, and *B*, they allow a constitutive level of *argH* to be maintained, presumably due to a low-efficiency internal promoter ($P_H$) at or near *argB* (119,124). It was therefore, hypothesized that the primary promoter-operator complex is located between *argE* and *argC*, and that a secondary promoter exists at or near the *argB-H* boundary. Although no direct measurements have been reported on the quantitative aspects of the *argECBH* mRNA, Krzyzek *et al*. (102) estimated that at least two discrete pieces of mRNA are involved. All the above results suggest that *argC*, *B*, and *H* are transcribed into a single polycistronic messenger RNA, and that *argE* is transcribed individually.

In the present work, two *argX* mutations have been described which are able to function as reinitiation elements in the arginine cluster of *E. coli* K-12. The mutants were isolated fortuitously from the wild type as acetylornithine-requiring auxotrophs following nitrous acid mutagenesis (80). They are deficient in *argB* expression and show a concomitant and

enzyme-specific inability to repress, normally, argininosuccinase. The expression of *argH* in *argR*$^+$ derivatives of the *argX* mutants is three- to four-fold higher than that in the wild type under arginine-repressed conditions (Tables 5, 6 and 7). The other arginine enzymes in the cluster (enzymes E and C) and the noncluster enzyme, D, behave normally. The *argX* mutations inactivate *argB* function, and concomitantly their intrinsic specific DNA sequences act as initiators to promote mRNA synthesis specifically for *argH* expression, in a clockwise manner, consistent with the divergent transcription model as proposed by Jacoby (122) and Glansdorff (124).

The *argX* mutations appear to be unique as initiators in that they function only under fully repressive conditions. They have no detectable effect on *argH* expression under either the partially derepressed condition (after growth with added ornithine or when combined with an $argO_{ECBH}$ mutation) (Tables 5, 6, 7 and 9), or the fully derepressed state resulting from the presence of an *argR*$^-$ allele (Tables 5, 6 and 7). Furthermore, *argX* shows no detectable effect under partial derepression when it is combined with a polar *argC* mutant even when the translation of mRNA transcribed from $P_{CBH}$ (primary promoter) is reduced by 25% (Table 20). It is possible that the effect of *argX* might not be detectable during full or partial genetic derepression, since it is masked by the high enzyme H activity (3.2-9.9) (Tables 5, 7 and 9) contributed by initiation at the primary promoter site ($P_{CBH}$). However, if the *argX* mutations were functional under partial derepression, the contribution by *argX* alone (enzyme H activity of 0.33-0.48) (Table 17) to *argH* expression in the *argC* mutant (enzyme H activity of 0.73) (Table 20) and in the R$^+$ derivatives (1.1-.1.2) (Tables 5, 6 and 7) would be proportionately large and, hence, readily detectable. Therefore, the

finding that the argX mutations do not enhance argH expression, either under partial physiologic derepression or in a polar argC strain grown in ornithine, is taken as strong evidence that argX mutations can actually function only under fully repressed conditions. The phenomenon, however, is not clear.

It seems that argX2041 and argX1593 have dual effects on the arginine cluster. Under maximal arginine repression, argX mutations create a promoter of transcription for the constitutive expression of the distal argH gene and are also read as structural gene mutations for the argB gene. However, under partially and fully derepressed conditions, the mutations are read only as structural gene mutations.

Callahan and Balbinder (61) have isolated a type of initiator mutation (trpA515) of the trp operon of S. typhimurium which may have a transcription-terminator component as well as an initiation component. This mutation (trpA515) is similar to the argX mutations in the sense that both function as initiators only under conditions of maximal repression. Upon derepression, the adjacent trpB expression of trpA515 is drastically reduced to 40%, whereas the other distal gene, trpD, has the same expression as under repressed conditions. The reason for this peculiar phenotype may be explained by the hypothesis that mutation trpA515 creates a promoter of transcription of the four operator-distal genes, whereas the primary trp promoter is inactive due either to repression by tryptophan or the presence of a supX deletion (deletion which covers the primary promoter-operator region and extends into the proximal part of the first structural gene). When the primary promoter is functioning normally in derepressed conditions, the mutation acts as a terminator of those transcriptions originating at

the primary promoter. The result is an extreme polarity effect for the four distal genes. In contrast, the argX mutations are probably read as nonpolar structural mutations in the argB gene, since the mutations do not affect the expression of the operator-distal argH gene under partially or fully derepressed conditions (Tables 5, 6, 7 and 9) (also see discussion in the latter part of this Chapter). Hence, argX mutations differ from trpA515 in this respect. However, an alternative explanation is that in argX mutants, $P_{CBH}$ is a highly efficient promoter for transcription. When $P_{CBH}$ is available for transcription (under partially and fully derepressed conditions), it may be recognized efficiently and preferred by RNA polymerase for initiation rather than $P_X$ (argX mutations).

Several internal promoter mutants of the trp and his operons of S. typhimurium (52,63) were isolated from strains carrying promoter-operator-structural gene deletions. Selection was for secondary mutations in the first operator-proximal structural gene which restore trp or his operon expression. With this in mind, argX1593 and argX2041 were introduced into the argEC-1 strain (with a deletion of the primary promoter-operator complex). Both argX mutations can function independently and are not affected by the absence of transcription at the proximal primary promoter ($P_{CBH}$) (Table 17). Thus, argX mutations act as initiators and restore argH function. The argH expression is constitutive with respect to arginine regulation, being essentially the same in cultures grown under conditions of repression and partial derepression of the arg cluster (Table 17). The finding that argX mutations can restore constitutive argH expression in the absence of the natural primary promoter is taken as strong evidence that they are internal promoters.

Further evidence that argX mutations are internal promoters is the finding that argX2041 is capable of relieving the polar effect exerted on argH by a translational terminator (nonsense mutation) in the argX-proximal argC gene (Table 20). Under repressive conditions, argH expression initiated at $P_X$ (argX2041) is at the same constitutive level in the polar argC mutant (enzyme H activity of 0.40) (Table 20) as it is in the primary promoter-operator deletion strain (enzyme H activity of 0.43) (Table 17). The argH expression initiated at $P_X$ in strains carrying these combined mutations is approximately two and a half-fold above that of the fully repressed wild type (0.17). Similarly, argH expression initiated at argX1593, when in combination with the promoter-operator deletion, shows a two-fold increase (enzyme H activity of 0.33) (Table 17). Therefore, the argX mutations can function more efficiently than the primary promoter ($P_{CBH}$) under repressed conditions.

The argX2041 mutation differs from the translational-restarters described in the rIIB region of bacteriophage T4 (53) and in the lac system (54) for the following reasons: (1) argX does not require the proximity of a translation-terminating nonsense mutation in order to function, whereas a translational-restarter requires a proximal translational-terminater in its close vicinity in order to express its function; (2) argX does not depend upon derepression of the cluster (Table 17), whereas the activity of the translational-restarter in the lac system is dependent on induction of the operon; (3) argX relieves the polar effect of a nonsense mutation and actually boosts argH expression two and a half-fold (efficiency of 250%) above that in the repressed wild type, even though the proximal translational-terminator is located within a relatively distant, different gene

(argC) (Table 20); on the other hand, a translational-restarter can apparently relieve only part or even most of the polar effect of a proximal translational-terminator which must be in its close vicinity within the same gene, but does not boost gene expression above 100% (repressed wild type) efficiency; (4) argX does not relieve the polar effect of a nonsense argC mutation on argH expression under partial derepression (after growth in ornithine) (Table 20): if argX were a translational-restarter, the same efficiency (250%) found under full repression should apply to the state of partial derepression.

The additivity of $P_{CBH}$ to the constitutive function of $P_X$ in argX mutants can be demonstrated by comparing the enzyme H activity in strains in which the argX mutations are combined with a $P_{CBH}$-deletion. The results are presented in Table 25. The argH expression transcribed at $P_{CBH}$ in the wild type (P4X6) was calculated by subtracting the value found for the $P_{CBH}$ deleted mutant from that for the wild-type strain under repressed conditions. The specific enzyme H activity transcribed at $P_{CBH}$ in argX mutants was calculated by subtracting the value of the $P_{CBH}$-deleted argX strain from the activity of argX mutants under repressed conditions. Since argH expression initiated at $P_{CBH}$ is essentially the same in the wild type (o.11) as it is in the argX1593 (0.10) and argX2041 (0.15), the argX mutations do not appear to have a polar effect on the translation of argH mRNA whose transcription is initiated at the proximal $P_{CBH}$ promoter under repressed conditions. Moreover, transcription at $P_X$ sites apparently does not interfere with the "downstream" transcription by RNA polymerase from $P_{CBH}$.

Cis- and trans-dominance tests were performed on argX2041 (Table

TABLE 25

ADDITIVE NATURE OF $P_{CBH}$(PRIMARY PROMOTER) AND $P_X$ UNDER REPRESSIVE CONDITIONS

| Strain | Promoter(s) presumed to function under repressive conditions* | argH |
|---|---|---|
| P4X6 (W.T.) | $P_{CBH} + P_H$ | 0.17 |
| argEC-1 | $P_H$ | <0.06 |
| argX1593 | $P_{CBH} + P_H + P_{X1593}$ | 0.43 |
| argX2041 | $P_{CBH} + P_H(?) + P_{X2041}$ | 0.58 |
| argEC-1 argX1593 | $P_H + P_{X1593}$ | 0.33 |
| argEC-1 argX2041 | $P_H(?) + P_{X2041}$ | 0.43 |

Calculations:

argH expression at $P_{CBH}$ in P4X6 = 0.17 - 0.06 = 0.11

argH expression at $P_{CBH}$ in argX1593 = 0.43 - 0.33 = 0.10

argH expression at $P_{CBH}$ in argX2041 = 0.58 - 0.43 = 0.15

*$P_{CBH}$: Natural primary promoter at argE-C border

$P_H$ : Natural secondary internal promoter near argB-H border

$P_X$ : Promoters created by either argX1593 or argX2041.

21). The results show that the argX effect on argH expression is cis-dominatn and trans-recessive and suggest that argX does not produce an altered polypeptide product which, in turn, causes an elevated argininosuccinase level. The findings are in accord with the view that the argX mutation is a transcriptional initiator. In addition, the elevated argininosuccinase level is probably not due to a duplication of the argH gene (Table 22). It remains a possibility that the argX mutations involve the insertion of a foreign "promoter" in the argB gene analogous to the insertions in the lac (57,58) and gal system (56,58,59). However, it is unlikely that argX mutations are insertions, since the known insertions cause extreme polarity effects on operon expression. Moreover, even if argX2041 is an insertion, it does not include an "operator region", since argH expression is at the same constitutive level when the argX strain is grown in enriched medium or in MMA with an abnormally high amount of arginine (Table 19).

Both argX1593 and argX2041 appear to be short frameshift deletions. They are not revertible by base-substitution mutagens or the frameshift mutagen ICR-191 (Table 10), and do not show phenotypic curing by aminoglycoside antibiotics. In addition, they are not suppressed by the UAA and UAG nonsense suppressors available in this laboratory. However, they do show a low spontaneous reversion frequency of approximately $10^{-9}$ (Table 10). It is of interest to know that although they may be frameshift mutants, the argX strains are not polar since the argH expression elevated by the argX mutations under fully repressive conditions is additive to that initiated at $P_{CBH}$ (Table 25). It is therefore concluded that both argX1593 and argX2041 are short "in phase" frameshift deletions.

Deletion analysis and three-point crosses reveal that argX mutations occur in two regions of the argB gene (Figure 9). The mutations map either in the proximal half of argB (e.g., argX1593), or in the vicinity of the argB-H border (e.g., argX2041).

There appears to be a natural secondary promoter ($P_H$) in the arginine cluster which is located in argB or at the argB-H border (119, 124). The basal level of enzyme H observed in polar point and deletion mutants [e.g., argB-5 (EcK186), argEC-1 and argEC(B)] is presumably due to this $P_H$ promoter. This is not ture, however, in strains harboring mutations which either destroy all known argB markers (e.g., argCB-1) or are located in the immediate vicinity of the argB-H junction [e.g., argB-2 (EcK187)]. Mutation argB-2 has been characterized as a short frameshift deletion (119). It has an extreme polar effect on argH function, reducing the basal level of enzyme H from 35% (specific activity 0.06) to approximately 6% of the repressed wild-type activity of 0.17 (Table 16). The basal constitutive level of argH expression is due to transcription at $P_H$ in the absence of $P_{CBH}$ and is presumably unaffected by polar mutations proximal to it. The only ways to further reduce the basal enzyme H level seem to be by inserting a polar or frameshift mutation distal to $P_H$ or simply by deleting $P_H$. The present author proposes, therefore, that argB-2 is either a frameshift deletion that destroys $P_H$, or is in the immediate vicinity of $P_H$, but distal to it.

It is surprising that one of the argX mutations (argX2041), as well as a point mutation (argB1586), map virtually at the same locus as argB-2 (Figure 9), and another argB point mutation (EcK1585) is located distal to them. Nevertheless, both 1586 and 1585 show normal enzyme H

activity (Table 16). It is very likely that $P_H$ is located within the structural gene of argB at the operator-distal end, adjacent to argH, and on the operator-proximal side of 1585. A similar internal promoter (P2) has been described by Jackson and Yanofsky (51) in the trp operon of E. coli. It was demonstrated to map within trpD at the operator-distal end. It was, therefore, proposed that the nucleotide sequence of P2 has two functions: (1) it codes for a sequence of amino acids for the carboxy-end of the trpD polypeptide; (2) it promotes low-efficiency transcription for the distal genes adjacent to trpD.

Since argX2041 maps in the same locus as argB-2, it must also be in the close vicinity of $P_H$. This is an unusual situation since $P_H$ (the natural internal low-efficiency promoter) and $P_X$ (the promoter created by mutation) may be situated side by side in the argB gene, unless $P_H$ is destroyed by the argX2041 mutation. If $P_H$ and $P_X$ do coexist, it would be of interest to study their functional integrity. However, studies of the effect of rifampin on the efficiency of arginine promoters (Table 23) reveal that it is likely that $P_H$ was destroyed by the same event that created argX2041. If $P_X$ and $P_H$ did coexist, it might be expected that enzyme H activity in the argEC-1 argX2041 strain grown in ornithine would be elevated by rifampin beyond the constitutive level to that of the sum of the transcriptions occurring at $P_X$ and $P_H$. This is not the case, as pointed out in Chapter III, since the expression of argH in the $P_{CBH}$-deleted mutant carrying the $P_X$ promoter is essentially the same as in the $P_{CBH}$-deleted strain not carrying the $P_X$ promoter. However, the possibility that interference exists between the two independent transcription processes at such close range cannot be eliminated.

Therefore, the specific base sequence of the low-efficiency $P_H$ may be changed into a more efficient promoter, i.e., argX2041, merely by deletion of several base-pairs. The number of bases deleted should be a multiple of 3 since argX2041 and argX1593 appear to be "in phase" frame-shift deletions, and since both mutants can be reverted spontaneously (Table 10), the number of deleted base-pairs should be small. The argX2041 prototrophic revertants (Table 11) show either a normally repressed argininosuccinase activity or a drastic reduction to below basal level as compared to the $P_{CBH}$-deleted strain (Table 17). This phenomenon is not understood since no studies have yet been made to characterize these prototrophs as to the nature and location of the "reversion", or to determine the enzyme B level. Furthermore, since only a few of these revertants were assayed for the argH enzyme, it is premature to draw any firm conclusions. It is possible to speculate, however, that "foreign" bases have been inserted into the deleted site in some of the revertants, and that the inserted bases may be translated as a missense sequence which can no longer serve as the transcription-initiator for argH expression.

Highly efficient transcription-initiators, possibly created by similar deletions, have been described by Morse and Yanofsky (55) in the trp operon of E. coli, and by Callahan and Balbinder (61) in S. typhimurium. Other low-efficiency transcription-initiation mutations created by only a single base-pair change in specific areas in the operator-proximal segment of the first structural gene were demonstrated in the trp operon of S. typhimurium (52) and in the his operon (63). The above results imply that as little as a single DNA base-pair change is capable of generating a nucleotide sequence which could serve to initiate gene expression. Although

the base sequence of a specific promoter element has not yet been elucidated, it may consist of a cluster of pyrimidine-rich codons, as proposed by Szybalski et al. (44). In addition, a primary promoter may be distinguishable from a low-efficiency internal promoter by an intrinsic specific base sequence or merely by its location in the operon. Since some of these initiation-mutations are also transcribed and translated into protein as missense mutations (e.g., "ini" mutations), there exists the hope that eventual determination of the amino acid change, as well as the sequence of the amino acids adjacent to the change, will disclose the nucleotide sequence of the promoters.

The significance of naturally occurring, low-efficiency internal promoters might be important with respect to evolutionary development of the gene organization in the prokaryote and eukaryote. It is well documented that a high degree of clustering of genes specifying biochemically related functions, especially in biosynthetic pathways, has been found for certain enterobacteria (148). The high frequency of gene clustering, however, does not appear to extend to eukaryotic organisms, such as Neurospora, or even to all bacteria (149). The clustering of genes, especially the existence of operons (contiguous, coordinately regulated units of functionally related genes) may provide a clue to the mechanisms by which metabolic pathways evolved. The extended operons, such as those for histidine and tryptophan found in E. coli and S. typhimurium, may be speculated to be the more primitive form of gene organization.

Horowitz (149) has proposed a theory to account for the evolutionary development of metabolic pathways. He proposed that the metabolic sequence evolved in a retrograde fashion following the sequential tandem duplication of genes, each step being followed by the occurrence of modi-

fying mutations in the newly duplicated gene, which conferred upon it the ability to specify an altered protein capable of metabolizing the newest intermediate in the growing pathway. The genes of a metabolic pathway, when found to be closely linked, were thus to have a common evolutionary origin. If the histidine and tryptophan operons are the more primitive form of gene organization, then the arginine system would be the more advanced form of gene organization found in enterobacteria. It is speculated that in the arginine system in E. coli K-12, the functionally related genes which are not highly clustered may also have arisen by tandem duplication but were dispersed later. The clustered arginine genes occur also in S. typhimurium (150) and in Proteus mirabilis (151). However, in Pseudomonas putida, the arginine genes are more widely scattered and show no close linkage among argE-C-B-H (152). The scattered-gene organization of psuedomonads therefore, more closely resembles the higher forms, the eukaryotes, rather than the classical prokaryotes of the family Enerobacteriaceae (152). It is not surprising that the argECBH cluster in E. coli is probably the vestige of an operon which once had even the scattered arginine genes linked together into one operon. We might speculate further that the high-efficiency primary promoter located between argE-C might once have been a low-efficiency internal promoter dominated by another promoter proximal to those genes in the system which used to be contiguous, but which are now scattered on the E. coli chromosome. The internal promoters which presently exist between argE-C and argB-H might be visualized as possible intermediary steps between the clustered and scattered types of gene organization. Glansdorff et al. (142,147) have suggested that mutants possessing specific duplicated genes, such as

*argH*, may be selected under conditions of severe polarity and that this represents a selective pressure for gene duplication.

# CHAPTER V

## SUMMARY

Two argX mutants have been described which are able to function as reinitiation elements for transcription in the arginine cluster of Escherichia coli K-12. These mutants, argX2041 and argX1593, were isolated as acetylornithine-requiring auxotrophs following nitrous acid mutagenesis. They are deficient in argB expression and show a concomitant and enzyme-specific inability to repress, normally, argininosuccinase (argH) synthesis. The specific activity of argininosuccinase in $argR^+$ derivatives of argX mutants is 3-4 fold higher than that in wild type after growth under arginine repression. The argX mutations have no detectable effect on argH expression under the partially derepressed condition (after growth in ornithine or when combined with an $argO_{ECBH}$ mutation), or the fully derepressed state of $argR^-$ derivatives. Moreover, an operator region does not appear to be included in the argX2041 mutation.

The argX mutations function independently and constitutively when in combination with a deletion of the primary cluster promoter ($P_{CBH}$) and also show an additive effect to $P_{CBH}$. This implies that argX affects transcriptional control. Additional evidence supporting the view that argX2041 is a transcriptional type of initiator is the finding that it relieves the polar effect exerted by a nonsense mutation in the proximal argC gene on argH expression.

The argX mutants are not revertible by base substitution mutagens or the frameshift mutagen ICR-191, and do not show phenotypic curing with aminoglycoside antibiotics. Furthermore, the argX mutations are not suppressed by two nonsense suppressors (UAG, UAA). However, they do show a low spontaneous reversion frequency of approximately $10^{-9}$. The findings suggest that argX mutations are short frameshift deletions. Although the argX mutations appear to be frameshift deletions, they are not polar since the argH expression initiated at $P_X$ (argX mutations) is additive to that initiated at $P_{CBH}$.

Mapping of the argX mutants by deletion analysis and by three point crosses revealed that argX mutations occur in two regions of the argB gene. They either map within argB (e.g., argX1593), or at the argB-H border (e.g., argX2041).

The effect of argX2041 on argH expression is cis-dominant and trans-recessive and is not due to duplication of the argH gene.

In the absence of arginine, rifampin lowers the efficiency of transcription at $P_{CBH}$, facilitates transcription at $P_H$ (the natural low-efficiency internal promoter), and has no effect on transcription at $P_X$ (argX2041). However, in the presence of arginine, rifampin seems to inhibit completely transcription at $P_{CBH}$, and has no effect on transcription at $P_H$ and $P_X$.

The results indicate that the argX mutations are non-polar, secondary, internal promoters, created by frameshift mutations, which act independently as transcription-initiators for the argH gene in a clockwise manner, and which are not under specific regulatory control.

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