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Genetics

A new *glnA*-linked regulatory gene for glutamine synthetase in *Escherichia coli*

(nitrogen metabolism/autogenous regulation/phage Mu/enzyme synthesis/*glnF*)

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ABSTRACT Mutations in the *glnA* region of the *Escherichia coli* chromosome due to Mu prophage insertion result in two phenotypic classes. One class is Gln^- and does not synthesize glutamine synthetase [L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2] under any growth condition. The other class produces a low level of glutamine synthetase under all growth conditions and is uncoupled from the regulatory effects of mutations in the *glnF* and *glnD* genes. Complementation analysis demonstrates that these two classes of insertions are in different cistrons. From these data we suggest that a regulatory gene, *glnG*, tightly linked to *glnA*, mediates both activation and repression of glutamine synthetase synthesis. An analysis of the evidence accumulated to date makes it unlikely that *glnG* is the only gene in the *glnA* region involved in the complex system of nitrogen regulation.

In the enteric bacteria, glutamine (Gln) synthetase [L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2] is not only responsible for the biosynthesis of glutamine, but also for the assimilation of ammonia into glutamate through a coupled reaction with glutamate synthase (L-glutamate:$NADP^+$ oxidoreductase, EC 1.4.1.13) (1, 2). In addition, numerous studies have led to the hypothesis that the Gln synthetase protein, independent of its enzymatic activity, serves as a positive control element for the synthesis of enzymes necessary for the utilization of various nitrogenous compounds (3).

Gln synthetase consists of 12 identical 50,000-dalton polypeptides, coded by the *glnA* gene. The biosynthetic activity of this protein is altered by a complex enzyme system that reversibly attaches an AMP moiety to each subunit of the Gln synthetase enzyme (1). The enzymatic activity of Gln synthetase is inversely proportional to the number of covalently bound AMP groups. The amount of Gln synthetase, which can vary over a 50- to 100-fold range (4), is regulated at the level of transcription (5).

A model for the nature of this regulation has emerged from analysis of a number of mutations linked to the *glnA* gene. One class of these mutations results in the loss of Gln synthetase activity (Gln^- phenotype), another in the high-level constitutive formation of the enzyme (GlnC phenotype), and the third in the low-level constitutive formation of the enzyme (3). Among this last class of mutants are many that suppress the Gln^- phenotype, which results from a mutation in the unlinked gene *glnF* (GlnR phenotype) (6). On the basis of three-point crosses which were performed with many *glnA*-linked mutations in *Klebsiella aerogenes* and complementation analysis, it was proposed that mutations resulting in the Gln^-, GlnC, or GlnR phenotypes are in the *glnA* gene, which is subject to autogenous regulation by Gln synthetase (3).

In this communication we report the isolation of two classes of mutants in which phage Mu has been inserted in the *glnA* region of the chromosome of *Escherichia coli*. The first class fails to synthesize Gln synthetase under any conditions. The second class is Gln^+, but produces a low level of the synthetase regardless of the availability of nitrogen or the presence of the *glnF* gene product. Analysis of these *glnA*-linked Mu insertions indicates that they are in a cistron that is distinct from *glnA*, which we have designated *glnG*. These studies clearly show that the *glnG* gene product is essential for the regulation of nitrogen metabolism and re-open the question of whether Gln synthetase plays any direct role in the regulation of its own synthesis. (Preliminary reports of these studies were given at the 1978 International Congress of Microbiology and at the 1979 ICN–UCLA Symposia on Molecular and Cellular Biology.)

MATERIALS AND METHODS

Growth media, enzyme assays, and transduction procedures have been described (7). Episome transfers were according to Miller (8).

Bacterial strains were constructed in this laboratory. All strains in the ET6000 series are derivatives of MC4100 (9) and are *rhaD* Δ (*lac*) U169 *thi strA*. All strains in the ET1000 series are derivatives of ET1101 (7) and are *rhaD metB*JKL136::tn5 *thi-1 strA9*. All merodiploid strains were made *rec*A56 by cotransduction with *srl*1300::tn10 and were constructed by using F′133 (from CGSC4265). In all of the complementation experiments, a portion of the culture was plated and colonies were tested both for transfer of the appropriate episomal markers and for episome segregation yielding the appropriate chromosomal markers.

Muc62 lysates were prepared and sensitive cells were infected as described by Casadaban (9). Cells of ET6001 were grown to 4×10^8/ml in LB medium, centrifuged, resuspended in 1 vol of absorption medium (5 mM $CaCl_2$/1 mm $MgCl_2$/0.85% NaCl), and infected with Muc62 at a multiplicity of 0.8. After absorption for 30 min at 30°C (15% survival), the infected cells were diluted into glucose minimal medium with glutamine as sole nitrogen source and grown for two generations. The cells were then centrifuged, washed, and resuspended in glucose minimal medium with arginine as sole nitrogen source. Penicillin was added (6000 units/ml final concentration) after one-half generation of growth. Two generations after penicillin addition penicillinase was added and cells were spread on glucose minimal agar screening plates containing excess arginine (0.2%) and limiting glutamine (0.01%) as nitrogen sources. This treatment previously yielded spontaneous mutants in *glnA*, *glnF*, and *gltB* among the small colonies on the selective plates (7).

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Abbreviations: Tc^R, tetracycline resistance; $NaDodSO_4$, sodium dodecyl sulfate.

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Spontaneous *glnF* revertants of strain ET1130 were isolated at a frequency of 10^{-6} by plating onto glucose/ammonia minimal plates without glutamine.

RESULTS

Isolation of Mu Insertion Strains with Nitrogen-Regulation-Negative (Reg^-) or Glutamine-Requiring (Gln^-) Phenotype. Cells of strain ET6001 that failed to use arginine as the sole nitrogen source were isolated after infection with Muc62 phage and one cycle of penicillin treatment. In six separate selections from 1 to 10% of the colonies were small on the screening plates containing arginine as major nitrogen source and growth-limiting amounts of glutamine. Among these small colonies, between 0.5 and 5% either required glutamine for growth (Gln^- phenotype) or were Gln^+ but failed to use arginine as sole nitrogen source (Reg^- phenotype). Four independent Gln^- and six independent Reg^- Mu insertion strains were isolated in this manner.

Mu Insertion Strains with Gln^- Phenotype. Two strains, ET6037 (*gln-11*::Mu) and ET6044 (*gln-12*::Mu), carrying Mu insertions resulting in the Gln^- phenotype were chosen for further study. They have no detectable Gln synthetase transferase activity under any growth condition tested (see Table 2), do not revert to Gln^+ (less than 10^{-10}) at 30°C, and fail to make a protein that migrates with the same mobility as the Gln synthetase polypeptide on sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gels (Fig. 1). These strains, which contain the *hut* operons from *K. aerogenes* (hut_K), failed to increase the level of histidase during nitrogen-limited growth (Table 1).

The *gln-11*::Mu and *gln-12*::Mu mutations were mapped by growing P1 on the Gln^+ strain ET1214 containing a tetracycline resistance element (*zig2*::tn10) that is 80% linked to the *glnA* locus (10). This lysate was used to transduce strains ET6037 and ET6044 to tetracycline resistance (Tc^R). In each case 75% (80/106) of the Tc^R transductants became Gln^+. All of these Tc^R Gln^+ transductants now grew at 44°C and failed to release Mu phage, indicating that the Gln^- phenotype was caused by the Mu prophage insertion. This result was confirmed by growing P1 on a Tc^R Gln^- transductant obtained from either strain ET6007 (*gln-11*::Mu) or strain ET6044 (*gln-12*::Mu) and using this lysate to transduce a wild-type strain, ET6002, to Tc^R. Gln^- colonies appeared among these Tc^R transductants at a reduced frequency of 10% (5/50). This lower cotransduction frequency is presumably due to incorporation of the Mu DNA into the phage P1 particle, decreasing the probability of concomitantly packaging the adjacent bacterial genome, and to zygotic induction. All of the Tc^R Gln^- transductants of ET6002 were now unable to grow at 44°C, indicating the presence of the Muc62 prophage. The Mu insertion in ET6007 is not complemented by any of six independent *glnA*-linked point mutations that result in the Gln^- phenotype. Therefore, the Muc62 insertions in ET6007 and ET6044 are designated *glnA11*::Mu and *glnA12*::Mu.

Mu Insertion Strains with Gln^+ Reg^- Phenotype. Three of the six Gln^+ Reg^- strains, ET6008 (*gln-1*::Mu), ET6049 (*gln-2*::Mu), and ET6052 (*gln-4*::Mu), were chosen for further study. These strains did not use arginine, proline, ornithine, γ-aminobutyrate, or serine as sole source of nitrogen in glucose minimal medium, but were able to use glutamine or aspartate. In contrast to the wild-type strain, the mutant strains ET6080 and ET6082 did not contain derepressed levels of histidase when grown under nitrogen-limiting conditions. However, normal derepression of histidase synthesis by the cyclic AMP/cyclic AMP binding protein system was observed when succinate was the carbon source, indicating that the Reg^- strains do not produce an inhibitor of histidase synthesis. (Table 1).

Although this growth phenotype was similar to that of *gltB* mutant strains lacking glutamate synthase (7), cells of strains ET6008, ET6049, and ET6052 contained the same level of glutamate synthase as the parent strain whether grown under nitrogen excess or limiting conditions (data not shown). However, these strains differ from the parent in that cells grown in glucose minimal medium with either excess nitrogen or a growth-rate-limiting nitrogen source always contained the same low level of Gln synthetase activity (Table 1). The adenylylation state of this low level of enzyme varied with the growth condition in parallel with that of the parent ET6002. The increased Gln synthetase level seen under carbon-limiting conditions in the Reg^- strains will be discussed in a subsequent publication. It appeared that the Gln synthetase polypeptide from the Gln^+ Reg^- mutant strains migrated on $NaDodSO_4$/polyacrylamide gels with the same mobility as the wild-type polypeptide (data not shown).

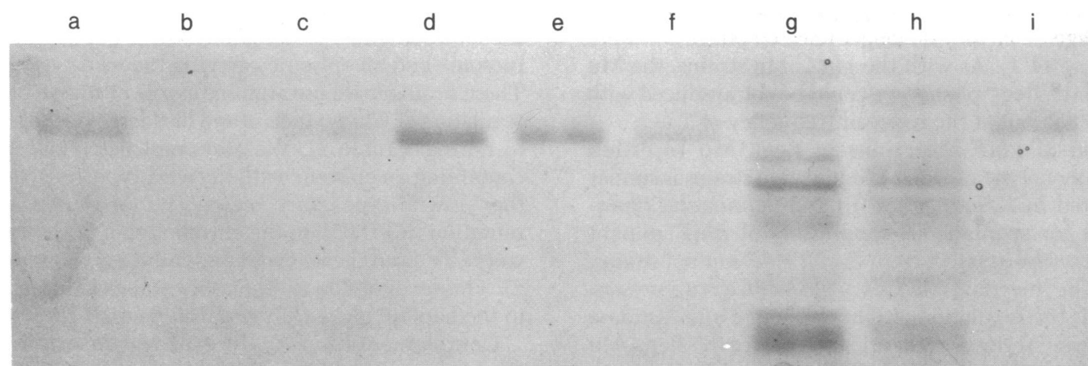

FIG. 1. Analysis of protein preparations on $NaDodSO_4$/polyacrylamide gels. Cells were grown on glucose minimal medium with aspartate as the sole source of nitrogen, except for the Gln^- strain, which was grown in the presence of glutamine. Gln synthetase preparations from cells grown in this laboratory were prepared by the polyethylene glycol method of S. Streicher and B. Tyler (unpublished data). Gln synthetase standards of the *E. coli* enzyme (provided by D. Eisenberg) were prepared by the method of Woolfolk *et al.* (16). Gln synthetase standards of the *K. aerogenes* enzyme (provided by S. Streicher) were prepared by Zn^{2+} precipitation. All lanes (except b) were loaded with the same number of units of Gln synthetase transferase activity. Lane a, standard Gln synthetase from *E. coli* ($M_r \approx 50{,}000$, highly adenylylated). Lane b, polyethylene glycol preparation from a *glnA*::Mu mutant. Lane c, polyethylene glycol preparation from wild-type cells (highly adenylylated Gln synthetase). Lane d, polyethylene glycol preparation from merodiploid cells that are *glnG*::Mu $glnA^+$/$glnG^+$ *glnA*::Mu. Lane e, standard Gln synthetase from *E. coli* ($M_r \approx 50{,}000$, primarily nonadenylylated). Lane f, crude extract of merodiploid cells that are *glnG*::Mu $glnA^+$/$glnG^+$ *glnA*::Mu. Lane g, crude extract of wild-type cells. Lane h, crude extract of *glnA*::Mu cells. Lane i, standard Gln synthetase from *E. coli* ($M_r \approx 50{,}000$, primarily adenylylated).

Table 1. Gln synthetase and histidase regulation in haploid *E. coli* strains

Strain	Relevant genotype	Relevant phenotype*		Growth conditions†					
				N excess, C limiting		N excess, C excess		N limiting, C excess	
		Gln	Reg	GS‡	H'ase§	GS	H'ase	GS	H'ase
ET6002	Wild type	+	+	0.08	380	0.15	120	1.00	550
ET6037	*glnA11*::Mu	−	−	<0.005	405	<0.005	90	<0.005	110
ET6044	*glnA12*::Mu	−	−	<0.005	ND	<0.005	ND	<0.005	ND
ET6008	*gln-1*::Mu	+	−	0.16	ND	0.05	ND	0.06	ND
ET6080	*gln-2*::Mu	+	−	0.14	400	0.06	120	0.08	75
ET6082	*gln-4*::Mu	+	−	0.13	390	0.05	80	0.06	115
ET6095	*gln-100*	+	−	0.11	345	0.05	85	0.05	75
ET6096	*gln-101*	+	−	0.12	300	0.06	90	0.06	75
ET6059	*glnF208*::tn10	−	−	0.06	ND	≈0.01	ND	≈0.01	ND
ET6056	*gln-2*::Mu *glnF208*::tn10	+	−	0.14	ND	0.05	ND	0.06	ND
ET1298	*gln-1*::Mu *glnF100*	+	−	0.17	ND	0.06	ND	0.07	ND

* Gln and Reg phenotypes are described in the text. +, Wild-type phenotype; −, mutant phenotype.

† C, carbon; N, nitrogen. C excess is growth in the presence of 4 mg of D-glucose per ml; C limiting is growth in the presence of 4 mg of succinate per ml; N excess is growth in the presence of 2 mg of $(NH_4)_2SO_4$ and 2 mg of L-glutamine per ml; N limiting is growth in the presence of 2 mg of L-glutamine per ml.

‡ GS, Gln synthetase transferase activity expressed as nmol of product formed per min per mg of protein.

§ H'ase, histidase specific activity expressed as μmol of product formed per min per mg of protein. Histidase was not determined (ND) in strains without the *hutC202* mutation.

Since it has been proposed that autogenous regulation of the *glnA* gene is affected by the adenylylation state of Gln synthetase, we examined the phenotype of a *gln-1*::Mu strain (ET6126) containing the *glnD99*::tn10 mutation, which eliminates the uridylyltransferase protein of the adenylylation system. The level of Gln synthetase in this strain was identical to that in the *gln-1*::Mu strain without the *glnD* mutation (ET6008) although the enzyme was highly adenylylated, leading to the Gln$^-$ phenotype. In a control experiment, we found, as previously reported (11), that the *glnD* mutation alone leads to overadenylylation of Gln synthetase and to decreased production of the enzyme under all growth conditions.

The *gln-1*::Mu, *gln-2*::Mu, and *gln-4*::Mu mutations were shown to be in the *glnA* region by transducing the Gln$^+$ Reg$^-$ strains ET6008, ET6049, and ET6052 to Tc^R with P1 grown on ET1214 (*zig2*::tn10). Sixty-eight percent (53/68) of the total Tc^R transductants of these three strains now had the parental growth phenotype. All of these Reg$^+$ transductants had lost the Muc62 prophage, since they no longer released phage and were able to grow at 44°C. As with the *glnA*::Mu strains, the Mu prophage and the Reg$^-$ phenotype could be cotransduced with Tc^R into ET6002, but at the reduced frequency of 7%.

Suppression of *glnF* Mutations in Reg$^-$ Mu Insertion Strains. The phenotype of Reg$^-$ Mu insertion strains is similar to that reported in *K. aerogenes* (6) and *Salmonella typhimurium* (12) for spontaneous suppressors of *glnF* mutant strains. We have also isolated, from *E. coli glnF* mutant strains, spontaneous Gln$^+$ revertants that carry *glnA*-linked suppressors. We found that the regulation of Gln synthetase and histidase synthesis in these strains was identical to that in the Reg$^-$ Mu insertion strains (compare strains ET6095 and ET6096 with ET6080 and ET6082 in Table 1). Therefore, we tested the ability of these Reg$^-$ mutations to suppress *glnF* by transducing a wild-type strain (ET6001) and the Gln$^+$ Reg$^-$ mutant strains (ET6008, ET6049, and ET6052) with P1 grown on a strain (ET6059) that is devoid of the *glnF* gene product due to a tn10 insertion in *glnF*. Whereas 100% (25/25) of the Tc^R transductants of the parent ET6001 become Gln$^-$, none of the Tc^R transductants of the Gln$^+$ Reg$^-$ strains required glutamine. The *glnF*::tn10 could be rescued by P1 transduction from each of the Tc^R derivatives of the Gln$^+$ Reg$^-$ strains. Therefore, all three of these Reg$^-$ Mu insertions suppress the *glnF* mutation. In addition, the regulation of Gln synthetase in a Reg$^-$ Mu insertion strain is unaltered by the presence or absence of *glnF* gene product (Table 1).

Gln$^-$ and Reg$^-$ Mu Insertion Mutations Are Recessive. We constructed strains ET1803 and ET6801, with *glnA*::Mu on the chromosome and an episome carrying a wild-type *glnA* region. These strains grew normally on arginine as sole nitrogen source, and the synthesis of both Gln synthetase and histidase was normally regulated (Table 2). Thus, the *glnA*::Mu mutation was recessive to wild type.

Strains containing Mu insertions with the Reg$^-$ phenotype did not synthesize derepressed levels of Gln synthetase or histidase when grown under nitrogen limitation and also suppressed the Gln$^-$ phenotype of a *glnF* mutation. The following experiments demonstrated that *gln-1*::Mu and *gln-2*::Mu were recessive for both of these phenotypes. Strains ET1802 and ET6802 contained either *gln-1*::Mu or *gln-2*::Mu on the chromosome and an episome carrying the wild-type *glnA* region. These strains could use arginine as sole nitrogen source, and the regulation of Gln synthetase and histidase synthesis was restored to normal (Table 2). We also constructed two *glnF* strains containing an episome with the wild-type *glnA* region and either a wild-type *glnA* region (ET1804) or the *gln-1*::Mu mutation (ET1805) on the chromosome. Both of these strains were Gln$^-$ and the level of Gln synthetase was identical (Table 2). This level of Gln synthetase was lower than that observed in the haploid *gln-1*::Mu *glnF100* strain ET1298 (Table 1).

Complementation Analysis of Mu Insertion Mutations. We next considered the possibility that the insertions resulting in the Gln$^+$ Reg$^-$ phenotype may be in a gene distinct from *glnA*. If this were the case, then the two types of Mu insertion mutations should complement to restore regulation of Gln synthetase activity. Two insertions, *gln-1*::Mu and *gln-2*::Mu, causing the Gln$^+$ Reg$^-$ phenotype, did not complement for derepression of Gln synthetase when grown under nitrogen-limiting conditions (strain ET1807, Table 2). Strain ET1807 also did not use arginine as sole nitrogen source. In contrast, in merodiploid strains containing *glnA11*::Mu on the chromosome

Table 2. Gln synthetase and histidase regulation in strains merodiploid for *glnA* region*

	Relevant genotype		Relevant phenotype		Growth condition			
					N excess		N limiting	
Strain	Chromosome	Episome†	Gln	Reg	GS	H'ase	GS	H'ase
ET1803	*glnA11*::Mu	Wild type	+	+	0.26	ND	1.20	ND
ET6801	*glnA11*::Mu	Wild type	+	+	0.20	115	1.15	405
ET1802	*gln-1*::Mu	Wild type	+	+	0.24	ND	1.09	ND
ET6802	*gln-2*::Mu	Wild type	+	+	0.24	105	1.30 (1.90)‡	390
ET1804	*glnF100*	Wild type	−	−	0.02	ND	0.03	ND
ET1805	*gln-1*::Mu *glnF100*	Wild type	−	−	0.02	ND	0.02	ND
ET1807	*gln-1*::Mu	*gln-2*::Mu	+	−	0.14	ND	0.16	ND
ET6812	*glnA11*::Mu	*gln-1*::Mu	+	−	0.25	115	1.35 (6.01)‡	100
ET6813	*glnA12*::Mu	*gln-2*::Mu	+	−	0.23	110	1.31	95
ET1810	Δ[*glnA-rha*] VIII	*gln-1*::Mu	+	−	0.20	ND	1.48	ND
ET1806	*gln-1*::Mu	*glnA11*::Mu	+	−	0.19	ND	1.60 (4.10)‡	ND
ET6814	*gln-2*::Mu	*glnA11*::Mu	+	−	0.13	80	1.69	95

* Phenotypes, growth conditions, and enzyme activities as in Table 1 except that both growth conditions are C excess.

† The merodiploid strains contained the F′133 episome carrying either the wild-type *glnA* region or various Mu insertions cotransduced onto the episome (in a Rec$^+$ strain) via *zig2*::tn10.

‡ N limitation with 2 mg of L-aspartate per ml as sole nitrogen source.

and either *gln-1*::Mu (ET6812) or *gln-2*::Mu (ET6813) on the episome, Gln synthetase became fully derepressed in response to nitrogen-limited growth (Table 2). When the position of the two Mu insertions was reversed with respect to the chromosome and episome, as in strains ET1806 and ET6814, Gln synthetase also was able to fully derepress. However, none of these strains were able to use arginine as sole nitrogen source, and histidase synthesis did not increase in response to nitrogen-limited growth. Thus, there was complementation for Gln synthetase regulation, but the strains retained the Reg$^-$ phenotype.

The episome carrying *gln-1*::Mu was also mated into strain ET1257, which has a [*rha-glnA*] deletion generated by heat induction of a λ*cI*857 *xis* prophage illegitimately inserted into the *rha* gene cluster (10). The growth phenotype and Gln synthetase regulation of this merodiploid strain (ET1810) was identical to that of strains ET6812 and ET6813 (Table 2).

The merodiploid strains ET6812, ET6813, ET1806, and ET6814 had another unusual property. When these strains were grown with aspartate or limiting ammonia as the source of nitrogen, they produced 4.0–6.0 units of Gln synthetase activity per mg of protein (Table 2). This extraordinarily high level of enzyme activity is due to the production of a correspondingly large amount of the Gln synthetase polypeptide (Fig. 1). Lanes g (strain ET6002) and f (strain ET6812) were loaded with enough crude extract to give equal amounts of Gln synthetase activity. As can be seen in lane f, the Gln synthetase band in this crude extract appears identical to the purified preparations of the enzyme from wild-type cells in lanes e and i. It should also be noted that the Gln synthetase produced in this diploid had the same mobility as the wild-type Gln synthetase polypeptide.

Comparable results were obtained with the longer episome F′14, except in experiments in which the *glnA11*::Mu was on the episome over *gln-1*::Mu. In this case, the levels of Gln synthetase did not increase in response to nitrogen limitation. It is likely that this result was due to spontaneous shortening of the episome because we frequently observed the loss of the *rha* and *met* genes from this episome.

Together with the results presented in the previous sections, the complementation experiments show that the Mu insertions *gln-1*, *gln-2*, and *gln-4* define a new gene, which we designate *glnG*. Because the Gln$^-$ deletion, extending from *rha*, complemented the *glnG1*::Mu mutation, the *glnG* gene must be distal to *glnA* with respect to *rha*.

DISCUSSION

Mu prophage insertions have three properties relevant to the interpretation of the results presented in this paper. The first is that the mutant phenotype is due to premature termination of the transcript and, hence, the polypeptide product of the gene into which the Mu has been inserted (13). The second is that the Mu insertion is completely polar with respect to transcription of genes downstream in the same operon (14). The third property is that Mu insertions occur randomly both with respect to gross distribution in different areas of the chromosome (13) and with respect to the nucleotide sequences in a small defined area such as in the *lacZ* gene (15).

We considered three possibilities to explain the existence of Mu insertions near *glnA* that result in altered regulation of Gln synthetase synthesis but not in the total loss of Gln synthetase enzyme activity (Gln$^+$ Reg$^-$ phenotype). One is that the insertions are in the *glnA* gene. Another is that the insertion is near or in a control region for the *glnA* gene. The final possibility is that they are in a second gene, distinct from *glnA*, that is involved in the regulation of Gln synthetase levels.

If the Gln$^+$ Reg$^-$ strains contain Mu insertions in the *glnA* gene, they must result in the loss of some portion of the carboxy-terminal portion of the Gln synthetase polypeptide. The remaining part of the enzyme molecule would then be unable to autogenously regulate *glnA* transcription while retaining enzymatic activity and the ability to form dodecamers. The Gln synthetase protein produced by strains with Mu insertions resulting in the Gln$^+$ Reg$^-$ phenotype has the same mobility as the wild-type Gln synthetase protein on $NaDodSO_4$/polyacrylamide gels. We estimate, from the relative mobilities of the *K. aerogenes* and *E. coli* Gln synthetase polypeptides, that we could easily detect a difference of 2500 daltons in size. This is

1/20th of the total size of the Gln synthetase monomer (50,000 daltons), and thus the Mu insertions leading to the Gln^+ Reg^- phenotype would be restricted to the carboxy-terminal 5% of the *glnA* gene. Because Mu prophage insertion is random, there should have been 20-fold more Mu insertions with the Gln^- phenotype than with the Reg^- phenotype if both types are in the *glnA* gene. We actually isolated Gln^- and Reg^- strains at approximately equal frequencies out of the same selection. Reconstruction experiments demonstrated that there was no preferential enrichment for either of the two types under our selection conditions. These results argue against both types of insertions being in the *glnA* gene.

If the Gln^+ Reg^- strains contain Mu insertions that have altered a control region for *glnA* transcription, then they should act in *cis* on Gln synthetase expression. However, because these insertions are recessive for both the inability to express elevated levels of Gln synthetase in $glnF^+$ strains and the ability to suppress the Gln^- phenotype of *glnF* mutant strains, this possibility is unlikely.

It is therefore likely that the Gln^+ Reg^- phenotype results from Mu insertion into a gene distinct from *glnA*. In agreement with this interpretation, we found that Gln synthetase regulation was restored in complementation tests between Mu insertions that cause the Gln^+ Reg^- phenotype (*gln-1*::Mu and *gln-2*::Mu) and Mu insertions or deletions that cause the Gln^- phenotype. We interpret this result to mean that the Gln^+ Reg^- Mu insertions are in a new gene, which we have designated *glnG*. Because Mu insertions are polar and the *glnA*::Mu and *glnG*::Mu insertions complement, they must be in separate transcriptional units. The *glnG* product mediates both activation and repression of *glnA* expression at the level of synthesis since the activity of Gln synthetase correlates with the amount of Gln synthetase polypeptide as determined by $NaDodSO_4$/polyacrylamide gel electrophoresis. Furthermore, the low level of expression of Gln synthetase observed in *glnG*::Mu mutant strains is uncoupled from the regulatory effect of a mutation in the adenylylation system although the biosynthetic activity of the enzyme is still subject to adenylylation system control.

Because there is a regulatory gene closely linked to *glnA*, the question remains whether all regulation previously attributed to Gln synthetase is in fact due to the *glnG* product. If this product alone were responsible for the regulation of *glnA* expression, one would expect complementation of a *glnA* mutant by a *glnG* mutant to restore normal regulation of Gln synthetase synthesis under all conditions. However, even though Gln synthetase regulation is restored in merodiploids containing *glnG*::Mu and *glnA*::Mu in a *trans* configuration, such strains synthesize an abnormally high level of the enzyme when grown under extreme nitrogen limitation (strains ET6812 and ET1806, Table 2). This very high level could be a physiological manifestation of the fact that these diploids retain the Reg^- phenotype and, therefore, might be more nitrogen starved than merodiploids containing a wild-type *glnA* region. However, strains ET6802, ET6812, and ET1806 have comparable growth rates on aspartate as sole nitrogen source. Furthermore, the phenotypes of *gltB* (7) and *glnG* mutant strains give no evidence for nitrogen regulation of aspartate use. Consequently, the possibility remains that Gln synthetase contributes to the regulation of its structural gene, *glnA*.

As far as the regulation of other systems such as *hut* is concerned, it is clear that this regulation is not entirely attributable to *glnG*. We show that insertion of Mu into *glnA* prevents the activation of histidase formation in response to nitrogen limitation. This insertion of Mu, in addition to inactivating *glnA*, due to polarity could have prevented the expression of a hypothetical gene located downstream in the same transcriptional unit. Thus, the failure to activate *hut* expression may reflect the lack of Gln synthetase or the product of a downstream gene or, simply, the inability of the mutant to produce glutamine. However, we have isolated *glnA* point mutants that produce Gln synthetase antigen without Gln synthetase enzymatic activity and that are tight glutamine auxotrophs (unpublished observations). As has been reported for *K. aerogenes* (4), in these point mutants histidase formation is activated even in the presence of ammonia, so that the failure of the *glnA*::Mu mutants to produce histidase at a high level cannot be due to their inability to produce glutamine. This suggests that either Gln synthetase itself or a downstream gene product is also involved in nitrogen regulation. This product cannot be from *glnG* because *glnA* and *glnG* are in different transcriptional units.

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