Mutational Analysis of the Bacterial Signal-Transducing Protein Kinase/Phosphatase Nitrogen Regulator II (NR\textsubscript{II} or NtrB)

MARIETTE R. ATKINSON AND ALEXANDER J. NINFA\textsuperscript{*}

Department of Biochemistry, Wayne State University School of Medicine, Detroit, Michigan 48201

Received 22 February 1993/Accepted 2 August 1993

The signal-transducing kinase/phosphatase nitrogen regulator II (NR\textsubscript{II}, or NtrB) is required for the efficient positive and negative regulation of \textit{glnA}, encoding glutamine synthetase, and the Ntr regulon in \textit{Escherichia coli}. The sensor of the system is a bifunctional uridylyltransferase–uridylyl-removing (UT-UR) enzyme encoded by \textit{glnD} (1, 3, 9, 11, 12, 31, 34). This enzyme is responsible for the modification and demodification of a signal transduction protein known as P\textsubscript{II}, the product of \textit{glnB}, in response to the nitrogen status of the cell (4, 7, 10, 34). Specifically, 2-ketoglutarate stimulates the uridylylation of P\textsubscript{II} by the UT activity, and glutamine stimulates the demodification of P\textsubscript{II}-UMP by the UR activity, so the ratio of 2-ketoglutarate to glutamine determines the extent of P\textsubscript{II} modification. This signalling of nitrogen status by the UT-UR enzyme via P\textsubscript{II} is responsible for the regulation of transcription from nitrogen-regulated promoters through the bifunctional kinase/phosphatase nitrogen regulator II (NR\textsubscript{II}), the product of \textit{glnL} (ntrB) (5). Under conditions of nitrogen limitation, NR\textsubscript{II} is autophosphorylated on residue His-139, and these phosphoryl groups are subsequently transferred to the transcription factor NR\textsubscript{I}, the product of \textit{glnG} (ntrC) (15, 16, 18, 25, 37). Phosphorylation of NR\textsubscript{I} converts NR\textsubscript{I} to the form able to activate transcription from nitrogen-regulated promoters (25). Under conditions of nitrogen excess, NR\textsubscript{II} and the unmodified form of P\textsubscript{II} act in concert to bring about the dephosphorylation of NR\textsubscript{II}–P and by so doing prevent the activation of transcription from nitrogen-regulated promoters (17, 18, 25). Nitrogen regulation of transcription is thus due to the control of the availability of P\textsubscript{II} for this interaction by the UT-UR enzyme (5). Several lines of evidence suggest that the NR\textsubscript{II}–P phosphatase activity resides in NR\textsubscript{II} and is elicited by P\textsubscript{II}. For example, physiology experiments with cells lacking P\textsubscript{II} suggest that some of the ability to negatively regulate \textit{glnA} expression is present, as opposed to the absence of this capacity in cells lacking NR\textsubscript{II} (2, 5, 8). In this report we present additional genetic evidence suggesting that the phosphatase activity is intrinsic to NR\textsubscript{II} and that the role of P\textsubscript{II} is entirely regulatory.

In addition to phosphotransfer from NR\textsubscript{II}, there is a second route by which NR\textsubscript{I} can become phosphorylated and activate transcription of \textit{glnA} and the Ntr regulon. Small phosphorylated metabolic intermediates such as acetyl phosphate and carbamyl phosphate can serve as direct substrates for NR\textsubscript{I} autophosphorylation, and the NR\textsubscript{I}–P so formed is able to activate transcription of \textit{glnA} (8). Experiments with intact cells have revealed that the most important phosphorylated metabolic intermediate in this process is acetyl phosphate, since cells lacking the capacity to synthesize acetyl phosphate are unable to activate \textit{glnA} expression in the absence of NR\textsubscript{II} (8). Alternatively, cells that lack NR\textsubscript{II} and retain the capacity to produce acetyl phosphate are unable to negatively regulate \textit{glnA} expression when the growth medium or genetic background causes an intracellular accumulation of acetyl phosphate. Thus, while NR\textsubscript{II} is not essential for the activation of \textit{glnA}, it is apparently essential for the efficient negative regulation of \textit{glnA} in response to nitrogen-excess growth conditions (6, 8, 21).

NR\textsubscript{I} and NR\textsubscript{II} are related to many other bacterial proteins that constitute two families of signal transducers known in the aggregate as the two-component systems (reviewed in references 23, 28, and 35). Analysis of the deduced amino acid sequences of the related histidine kinase (kinase/phosphatase) proteins has revealed that these proteins share a conserved domain, usually at the C-terminal end of the protein, and possess in addition unrelated domains. The kinase/phosphatase domain contains three highly conserved regions (23, 28, 35) (Fig. 1). Region 1, usually contained at the N-terminal end of the kinase/phosphatase domain, contains a conserved histidine residue that is the site of autophosphorylation (14, 24). The function of region 2, which contains two conserved asparagine residues, is unknown. Region 3 is a large glycinerich region that can be subdivided into three portions with

\textsuperscript{*} Corresponding author.
strong homology (regions 3.1, 3.3, and 3.5) separated by two variable spacers (regions 3.2 and 3.4) (Fig. 1). Region 3.5 is apparently involved in nucleotide binding, as shown in the accompanying paper (26).

In the work described in this report, we characterized the phenotypes resulting from alteration of eight of the most highly conserved residues within the kinase/phosphatase domain of NR1. Our results indicate that the negative and positive regulatory functions could be genetically separated and that these highly conserved residues and, indeed, the C-terminal 59 amino acids of NR1 were not required for the negative regulatory function.

**TABLE 1. Bacterial strains used in this study**

| Strain   | Genotype                          | Construction or source               |
|----------|-----------------------------------|-------------------------------------|
| JM109    | endA1 recA1 gyrA96 thi hsdR17 relA1 supE44 Δ(lac-proAB) [F' traD36 proAB lacF'ZDM15] | Promega                             |
| BW744    | recA1 creB510 thi Δlac-169 recA*  | B. Wanner                           |
| BW9301   | recA1 creB510 thi Δlac-169 recA*  | B. Wanner                           |
| BW10724  | recA1 Δlac-169 creB510 thi ΔrecA* | B. Wanner                           |
| YMC10    | hsr him Δlac-169 hutC_klebsiella  | B. Magasanik                        |
| YMC10S   | as YMC10, srfC::Tn10              | YMC10 × P1 BW744                    |
| YMC10R   | as YMC10, recA1                   | YMC10S × P1 BW9301                  |
| RB9132   | as YMC10, glnL2001                | B. Magasanik                        |
| RB9132S  | as RB9132, srfC300::Tn10          | RB9132S × P1 BW744                  |
| RB9132R  | as RB9132, recA1                  | RB9132R × P1 BW9301                 |
| TH16     | as YMC10, glnA::Tn5               | B. Magasanik                        |
| RB9060   | as YMC10, ΔglnB                    | B. Magasanik                        |
| BA       | as RB9060, glnA::Tn5              | RB9060 × P1 TH16                    |
| BL       | as RB9060, glnL2001               | BA × P1 RB9132                      |
| BLS      | as BL × P1 BW744                  | BL × P1 BW9301                      |
| BLR      | as BL, recA1                      | B. Wanner                           |
| BW18500  | lacX74 glnL2001 Δ(pta ackA hisQ hisP) zej-223::Tn10 | BW18500 × P1 |
| BW18500R | as BW18500, recA::cat             | BW18500 × P1                        |
| WS6005   | as RB9132, Δ(pta ackA hisQ hisP)  | Laboratory collection (8)           |
| WS6005R  | as WS6005, recA::cat              | WS6005 × P1                         |

**MATERIALS AND METHODS**

**Bacteriological techniques.** Media, preparation of plasmid DNA, preparation of competent cells, transformation of cells with DNA, preparation of P1vir phage lysates, P1-mediated transduction, and long-term storage of strains were as described previously (2, 22, 32). The bacterial strains used in this work are described in Table 1. The plasmids used in physiology experiments (see Table 2) were all similar to the previously described glnL+ plasmid pglN62 (2, 36), which is based on pBR322. The experiment whose results are shown in Table 4 used these and plasmids derived from pACYC184. Both types of plasmids contained a BamHI-HindIII DNA fragment that extends from the middle of the glnA gene to the beginning of the glnG gene. DNA ligations were performed in low-melting-point agarose by using gel-purified bands as described previously (22). Point mutations were constructed by oligonucleotide-driven mutagenesis using the Altered Sites mutagenesis kit (Promega) according to the manufacturer’s directions. The DNA sequence of each mutagenized allele was determined completely on one DNA strand, and the sequence containing the mutation and flanking nucleotides was determined on both strands by dideoxy sequencing of double-stranded plasmid DNA, as described previously (13). Sequencing was performed with a Sequenase kit (U.S. Biochemicals) and the set of primers described previously (2). The mutagenic oligonucleotides used in this study are as follows (the site of alteration is underlined): GCGGGCGCTGGCAATAGAGATTAAAT (codon 139, CAT→AAT); GGTGGCCAGCGCGCTGGGCTTA (codon 313, GGG→GCG); GTGCCGCGCGCTGGCAATTTAGATTTAAAAATCCCG (codon 139, CAT→GTT); GGCAACC GGCGTTGCTATATCCTGATGTCT (codon 315, GCC→GCG); AACGGCGGGCCGATTCCCCTGATCAT (codon 291, GGC→GAC); GAAAGAACGCGGGCGATCCCG (codon 289, GGG→GCG); GATGGTGGAAATAACCGGGGCG (codon 287, GAT→AAT); CTGAAATTAGTGCGCATGCGTACG (codon 248, AAT→GAT); CTGGCACTACGAT TAAAATCCCG (codon 140, GAG→CAG); and CGCGACATGCAGTTAATCCCG (codon 140, GAG→GCG).
**Bal 31 mutagenesis.** Plasmid pgln62 was cleaved at the unique NotI site within the glnL gene, and gel purified DNA was treated successively with Bal 31, DNA polymerase I Klenow fragment and deoxynucleoside triphosphates, and T4 DNA ligase as described previously (22), except that all steps were performed within the molten gel slice. The mutagenized DNA was then transformed into strain RB9132R (glnL2001 recA1; Table 1), and transformants were screened for the ability to negatively regulate GS on pyruvate-ammonia-glutamine medium by the microassay technique, as described previously (2), and for the loss of the NotI restriction site. Of the 63 transformants analyzed, 3 had lost the NotI restriction site but retained the ability to negatively regulate glnA on pyruvate-ammonia-glutamine medium in the RB9132R background; sequencing indicated that these transformants were a deletion of codon 307 (d307), a deletion of codons 307, 308, 309, and 310 (d307–310), and a large deletion that fortuitously resulted in a termination codon at codon 291, with all of the preceding sequence being wild type except for a silent mutation in the third position of codon 290 (tcr291). The remaining 60 isolates had lost both the NotI site and the ability to negatively regulate glnA and were not examined further.

**GS assays.** In all experiments, we used the transferase assay in the presence of Mn, as described previously (2). Since both adenylated and nonadenylated GS are active in this assay, the results reflect the total intracellular concentration of GS. Cells were grown exactly as described previously (2) and were harvested when the culture density reached an optical density at 600 nm of 0.5 (see Table 2). The qualitative GS microassay was performed as described previously (21). Total protein was determined by the method of Lowry et al. (19). All data are the averages of the results from at least two separate cultures grown from separate colonies.

**Immunoblotting.** An “amplified alkaline phosphatase” kit from BioRad was used for immunoblotting according to the instructions of the manufacturer.

**RESULTS**

**Experimental system for the structure-function analysis of NR\textsubscript{h}.** We constructed the mutations shown in Fig. 2A and B as described in Materials and Methods. In each case, the mutant allele was inserted into the multicopy cloning vector pBR322 such that the wild-type glnL promoter regulates the various glnL alleles. These constructions created mutant analogs of the previously described glnL+ plasmid pgln62 (36). We then examined the expression of GS (glnA product) in cells of various genetic background containing these plasmids. We also examined the intracellular concentration of NR\textsubscript{h} programmed by these plasmids in cells containing a large internal deletion within the chromosomal glnL gene (glnL2001), using the immunoblotting technique.

In wild-type cells, two promoters provide for the expression of glnL, encoding NR\textsubscript{h}. When grown on nitrogen-limiting medium, the intracellular concentrations of NR\textsubscript{h}, NR\textsubscript{a}, and GS are each increased as a result of the activation of the chromosomal glnALG operon from the nitrogen-regulated glnAp\textsubscript{2} promoter (29). This promoter is activated by NR\textsubscript{a}–P (25, 29). When grown on nitrogen-excess medium, the glnAp\textsubscript{2} promoter is not active (29, 30); expression of NR\textsubscript{a} and NR\textsubscript{h} then depends on the glnL promoter (36), which is repressed by NR\textsubscript{a}–P (5, 29).

We used the immunoblotting technique to directly examine the intracellular concentration of NR\textsubscript{h} in wild-type cells grown in glucose-glutamine (Ggln, nitrogen-limiting) and glucose-ammonia glutamine (GNgln, nitrogen-excess) media and ob-
served that in 5 µg of total cell protein, NR$_{II}$ was barely detectable when the cells had been grown on GN$_{glN}$ medium but was clearly detectable when the cells had been grown on Gln medium (Fig. 3). As shown, there is at least a 10-fold regulation of the intracellular concentration of NR$_{II}$ by nitrogen in wild-type cells. Exactly the opposite regulation was seen when NR$_{II}$ was programmed from only the multicopy pgln62 plasmid, which contains the glnL promoter but lacks glnAp$_2$ (Fig. 3). In this case, cells grown on nitrogen-excess medium had a high intracellular concentration of NR$_{II}$ (similar to the level seen in nitrogen-starved wild-type cells), but cells grown on nitrogen-limiting medium, which should result in an elevated intracellular concentration of NR$_{II}$, had little NR$_{II}$ (Fig. 3). As shown, the regulation in this case was also approximately 10-fold (Fig. 3). Thus, cells of the glnL2001 strain containing pgln62 have about a 10-fold-higher NR$_{II}$ concentration than wild-type cells when grown on nitrogen-excess medium and about a 10-fold-lower NR$_{II}$ concentration than wild-type cells when grown on nitrogen excess medium.

Surprisingly, these variations in the intracellular concentration of NR$_{II}$ had only a minor effect on the regulation of GS, with the most prominent difference being the more efficient repression of GS in the glnL2001 strain containing pgln62 (Table 2). We also examined the expression of GS in wild-type cells containing pgln62; as shown, in this background the presence of the plasmid had essentially no effect (Table 2).

Because the pgln62 plasmid restored nitrogen regulation to the glnL2001 strain, we used analogous plasmids containing mutations to discern the effects of the mutations on the positive and negative regulatory functions of NR$_{II}$. In order to ascertain whether various mutant alleles resulted in a dominant negative phenotype, we introduced each allele (on a plasmid) into strain YMCl0R, which has an intact Ntr signal transduction system and the capacity to synthesize acetyl phosphate but is recombination deficient because of the recA1 allele. To see whether various mutations affected the negative regulatory function of NR$_{II}$, we introduced the plasmids into strain RB9132R, which is able to synthesize acetyl phosphate.

![FIG. 3. Immunoblot analysis of NR$_{II}$. Polyclonal rabbit anti-NR$_{II}$ was used with an “amplified alkaline phosphatase” kit (BioRad). Cells were grown exactly as for the GS assay. Each gel lane contained 5 µg of total cell protein or the indicated amount of purified NR$_{II}$, STDs, prestained marked proteins. The polyclonal antibody used cross-reacts with a cellular protein that is not nitrogen regulated; this band serves as a useful internal control and is also shown.](https://jb.asm.org/)

**TABLE 2. Phenotypes resulting from the introduction of wild-type and mutant glnL plasmids into various strains**

| Plasmid$^a$ | recA Gln | glnL recA Gln | glnL glnB recA Gln | pta ackA Gln | Gln | Gln | Gln | PNgln | PNgln |
|-------------|-----------|----------------|-------------------|-------------|-----|-----|-----|-------|-------|
| None        | 2,008     | 305            | 232               | 931         | 383 | 2,000 | 1,096 | 465   | 1,975 |
| glnL*       | 1,764     | 213            | 360               | 1,616       | 112 | 228  | 2,460 | 1,033 | 1,095 |
| H139N       | 123       | 234            | 366               | 75          | 103 | 238  | 52    | 146   | 53    |
| H139V       | 2,043     | 219            | 356               | 80          | 102 | 2,064 | 209   | 74    | 1,887 |
| E140Q       | 196       | 205            | 340               | 75          | 117 | 136  | 34    | 72    | 177   |
| E140A       | 120       | 235            | 281               | 84          | 172 | 203  | 38    | 59    | 152   |
| N248D       | 1,914     | 219            | 354               | 1,170       | 133 | 186  | 1,347 | 936   | 1,698 |
| D287N       | 1,717     | 234            | 263               | 1,955       | 435 | 337  | 2,381 | 1,395 | 1,697 |
| G299A       | 1,872     | 239            | 306               | 1,682       | 133 | 172  | 1,875 | 1,258 | 1,584 |
| ter291      | 1,110     | 128            | 234               | 62          | 90  | 247  | 18    | 65    | 202   |
| G291A       | 2,020     | 242            | 355               | 1,871       | 142 | 225  | 2,264 | 1,339 | 1,389 |
| dG307       | 1,750     | 199            | 314               | 1,696       | 164 | 285  | 1,771 | 1,200 | 2,539 |
| d307–310    | 1,618     | 168            | 283               | 581         | 97  | 251  | 1,375 | 779   | 1,265 |
| G313A       | 1,725     | 173            | 325               | 804         | 91  | 223  | 1,150 | 666   | 1,130 |
| G315A       | 1,702     | 173            | 292               | 37          | 105 | 143  | 24    | 74    | 291   |

$^a$ All plasmids are analogous to pgln62 (glnL*) and contain the indicated mutations, as described in the text. ter indicates a termination codon at the indicated codon, and d indicates deletion of the indicated codon or codons.

$^b$ Recipient strains were YMC10R (recA), RB9132R (glnL recA), BLR (recA glnL glnB), and WSM605 (pta ackA recA glnL). Complete phenotypes of these strains are provided in Table 1. Gln medium contains glucose (0.4%, wt/vol) as the carbon source and glutamine (0.2%, wt/vol) as the sole nitrogen source and is nitrogen limiting. GN$_{glN}$ medium contains in addition ammonium sulfate (0.2%, wt/vol) and is nitrogen excess. PNgln medium contains pyruvate (0.4%, wt/vol) as the sole carbon source and ammonium sulfate and glutamine as nitrogen sources (each 0.2%, wt/vol) and is nitrogen excess.
is recA1, and contains the chromosomal glnL2001 mutation. Previous results had shown that in this strain background, growth on pyruvate, causing high intracellular acetyl phosphate levels, resulted in elevated GS levels even in the presence of ammonia (8) (Table 2). The ability of plasmid-encoded altered NR_{II} proteins to negatively regulate GS on pyruvate-ammonia medium in this strain background was then determined. To see if the negative regulatory activity of altered NR_{II} proteins depended on the P_{II} protein, as it does in wild-type cells, we also examined the expression of GS in strain BLR, which is able to synthesize acetyl phosphate and is recA1 glnL2001 ΔglnB (glnB encodes the P_{II} protein). To most clearly assess the positive regulatory function of plasmid-borne mutant glnL alleles, we introduced the plasmids into strain WS6005, which is recA1 glnL2001 and lacks the capacity to produce acetyl phosphate because of the deletion of pta and ackA (8). This strain is unable to activate glutamine synthetase under any conditions because it lacks the capacity to form NR_{II}−P (8), and we examined whether the introduction of plasmids encoding altered NR_{II} proteins restored the ability to activate GS on nitrogen-limiting medium. Finally, we further examined the positive regulatory function of NR_{II} by examining whether plasmids encoding altered NR_{II} proteins could complement strain RB9132R for growth on the poor nitrogen source arginine. Previous results have indicated that the activation of GS requires only a modest intracellular concentration of NR_{II}−P but that growth on arginine as the sole nitrogen source requires a high intracellular concentration of NR_{II}−P (27). Thus, this complementation assay is a more stringent assay of the positive regulatory function of NR_{II} than the measurement of GS expression (27).

The H139N, E140Q, and E140A mutations resulted in altered NR_{II} proteins that are transdominant negative regulators of GS synthesis. When the 13 mutant plasmids were introduced into the YMCl0R background (wild type except recA1), most had essentially no effect on nitrogen regulation (Table 2). The H-139→N (H139N), E140Q, and E140A constructs were remarkable in that they largely eliminated GS synthesis in nitrogen-limiting medium in the wild-type background; that is, they were transdominant negative regulators. The ter291 construct behaved as if it was a leaky or weak transdominant negative regulator.

Effect of mutations on the P_{II}−dependent negative regulatory function of NR_{II}. As noted above, GS is elevated in cells lacking NR_{II} (RB9132R) when they are grown on pyruvate-ammonia-glutamine medium (8). Introduction of the wild-type glnL allele on pglm62 greatly reduced this GS activity in the RB9132R background, which contains P_{II}, but not in the BLR background, which lacks P_{II} (Table 2). This reflects the importance of P_{II} in the negative regulatory function of NR_{II} (Table 2). (Note, however, that there was a modest negative regulation of GS synthesis by the wild-type glnL allele even in the BLR strain, which lacks P_{II}, indicating that negative regulation by NR_{II} is not entirely dependent on P_{II}.) Each of the mutant glnL plasmids except the H139V construct was able to bring about the negative regulation of GS in the RB9132R background. (The H139V construct seemed to have a very weak P_{II}−independent negative regulatory activity that can be discerned only by comparing the expression of GS on Gln and GNgn media with that of the plasmidless controls.) Since the H139N construct was able to negatively regulate GS, we can state that none of the sites altered in this work are essential for the negative regulation of GS. In the case of the H139N, E140Q, E140A, ter291, and G315A mutations, negative regulation did not require P_{II} (Table 2).

Effect of mutations on the positive regulatory activity of NR_{II}. As shown in Table 2, introduction of wild-type NR_{II} (programmed by pglm62) into strain BLR (glnL2001 glnB recA1) resulted in elevated GS levels on the nitrogen-rich GNgn medium. This inappropriate activation of GS expression is apparently due to the positive regulatory activity of NR_{II} acting in the absence of P_{II}, as observed previously in similar experiments (5). We examined the expression of GS in the BLR strain background containing the set of mutant plasmids (Table 2). As shown, the N248D, D287N, G289A, G291A, and dG307 constructs were similar to the wild-type construct in causing the elevated expression of GS on GNgn medium in the strain BLR background. Thus, these mutations do not eliminate the positive regulatory function of NR_{II}. The d307−310 and G313A constructs caused a more modest elevation of GS levels on GNgn medium in this strain background; thus, these constructs are partially deficient in the positive regulatory function of NR_{II}. The H139N, H139V, E140Q, E140A, ter291, and G315A constructs in the strain BLR background resulted in a level of GS lower than that in the plasmidless control in all media tested. These constructs are therefore defective in the positive regulation of GS.

To more clearly assess the positive regulatory function, we examined the effect of introducing the set of mutant plasmids into strain WS6005, which is glnL2001 and cannot produce acetyl phosphate. Cells lacking both NR_{II} and the capacity to synthesize acetyl phosphate are unable to activate expression of glnA, and introduction of pglm62 into this background restored the ability to activate GS upon nitrogen starvation (8) (Table 2). As shown, the N248D, D287N, G289A, G291A, dG307, d307−310, and G313A constructs restored or partially restored the ability to activate GS. These mutations have effects ranging from quite modest (G291A) to clearly observable (d307−310) but do not eliminate the positive regulatory function of NR_{II} (Table 2). In contrast, the H139N, H139V, E140Q, E140A, ter291, and G315A mutations eliminated the positive regulatory function of NR_{II}. Either these mutant alleles lack the positive regulatory function entirely or this function is obscured by the negative regulatory function. The results obtained with this strain background mirror the results with the BLR strain background discussed above.

The most rigorous test for the positive regulatory function of NR_{II} is the complementation of the glnL2001 recA1 strain (RB9132) for growth on the poor nitrogen source arginine. When this test was performed with pglm62 and the set of mutant plasmids, it was observed that pglm62 and the G289A and G291A constructs clearly complemented the chromosomal glnL2001 mutation and that the dG307 construct gave poor but discernible complementation (Table 3). None of the other plasmid constructs were able to complement the glnL2001 mutant for growth on arginine as the sole nitrogen source. Thus, the N248D, D287N, G313A, and d307−310 constructs are clearly defective in the positive regulatory function of NR_{II}. It should be noted that for the N248D and D287N constructs, this defect is not readily apparent from the GS data (compare Tables 2 and 3).

Yet another way to assess the effect of mutations on the positive regulatory function of NR_{II} is to assess the effect of mutations on the repression of the glnL promoter by NR_{II}. Previous work has indicated that this promoter is repressed by NR_{II}−P (5, 36, 37); thus, the extent of repression is a measure of the intracellular NR_{II}−P. We examined this repression by directly measuring the relative intracellular concentration of NR_{II} in RB9132R cells (recA1 glnL2001) containing plasmids, using the immunoblot technique (Fig. 3). As shown, those mutations that did not eliminate the positive regulatory function of NR_{II} (d307, D287N, N248D, G289A, G291A, and
G313A) also did not eliminate the repression of NR_{11} synthesis in nitrogen-limiting Gln medium, whereas those mutations that eliminated the positive regulatory function (H139N, H139V, E140A, E140Q, and G315A) also eliminated this repression of NR_{11} synthesis (Fig. 3). The product of the ter291 allele could not be reproducibly observed in immunoblotting experiments; in several experiments it was observed as a very faint band, but in the experiment shown in Fig. 3, it was not observed. Thus, with the exception of those for the ter291 protein, the results of the GS assays and the immunoblotting assays are consistent.

**Complementation between mutant glnL alleles.** Previous work with the related CheA kinase, part of the two-component regulatory system that controls chemotaxis, has indicated that certain cheA mutations can be complemented by other cheA mutations in recombination-deficient cells (33). Those experiments indicated that the positive regulatory function of CheA is due to separate, compartmentalized functions that can be provided by separate CheA monomers in intact cells. We examined whether the positive regulatory function of NR_{11} was similarly due to separate compartmentalized functions by examining whether different glnL mutations could complement one another in intact cells. We chose for this analysis the H139V mutation, which causes greatly deficient positive and negative regulatory activities of NR_{11}, and the d307–310 and G313A mutations, which modestly affect the positive regulatory function but essentially do not affect the negative regulatory function (Table 2). In order to perform the analysis, these alleles were subcloned into the vector pACYC184, which carries a tetracycline resistance determinant. The abilities of combinations of these alleles to complement were then examined by sequentially introducing pairs of plasmids into strain RB9132R (recA1 glnL2001) and examining GS expression. As shown in Table 4, the combination of G313A or d307–310 with H139V resulted in complementation of GS expression. Thus, as with CheA, the positive regulatory function of NR_{11} seems to be due to separate, compartmentalized functions.

**DISCUSSION**

We examined the effects of mutations at the highly conserved residues within the kinase/phosphatase domain of NR_{11}, a member of the histidine kinase family of the two-component regulatory systems. The main conclusion from our work is that, with the exception of the H139V mutation, none of the mutations analyzed had a significant effect on the negative regulatory function of NR_{11}. Since the alteration of histidine 139 to asparagine resulted in a protein capable of negative regulation (indeed, a transdominant negative regulator), it seems unlikely that histidine 139 is directly involved in the negative regulatory activity. Rather, the introduction of a valine at this position may distort the structure of the protein. Thus, we must conclude from our results that none of the highly conserved sites that we altered has a direct role in the negative regulatory function, that is, the phosphatase activity. Furthermore, since the ter291 allele is a negative regulator of glnA, the C-terminal 59 amino acids of NR_{11} are apparently not required for the negative regulatory function.

In previous work we isolated and characterized glnL mutations that suppress the Ntr defect resulting from a leaky glnD mutation that decreases the P_{11}-UT activity and thus results in the inability to convert P_{11} to the innocuous P_{11}-UMP under conditions of nitrogen limitation (2). That selection should have identified mutations affecting the negative regulatory function of NR_{11}, of 16 mutations that were characterized, 15 mapped either in the nonconserved N-terminal domain of NR_{11} or in a cluster flanking His-139 (2) (shown for comparison in Fig. 2C). None of the previously isolated mutations that appear to affect the negative regulatory function mapped to the highly conserved residues shared by the histidine kinase proteins of the two-component systems. In the current work we demonstrated that the highly conserved residues shared by the histidine kinases of the two-component systems are not directly involved in the negative regulatory function.

Previous genetic analysis of glnL had indicated that the negative and positive regulatory functions of NR_{11} can be genetically separated (20, 21), and our work extends this conclusion by identifying mutations that result in this separation. For example, His-139 is required for the positive regulatory function, but not for the negative regulatory function, of NR_{11}. In a number of cases, very highly conserved residues were altered with little effect on glnA regulation. For example, the highly conserved N-248, D-287, G-289, G-291, and G-313 residues were not essential for the positive or negative regulatory function of NR_{11}.

### Table 3: Complementation of the Ntr+ phenotype of a strain containing the glnL2001 mutation by various plasmid-borne glnL alleles

| Strain          | glnL plasmid | Growth on glucose-arginine |
|-----------------|--------------|----------------------------|
| YMC10R (recA1)  | None         | -                          |
| RB9132R (recA1 glnL2001) | glnL + | +                          |
|                 | G313A        | -                          |
|                 | H139N        | -                          |
|                 | H139V        | -                          |
|                 | E140Q        | -                          |
|                 | E140A        | -                          |
|                 | N248D        | -                          |
|                 | D287N        | -                          |
|                 | G289A        | +                          |
|                 | ter291       | -                          |
|                 | G291A        | +                          |
|                 | dG307        | +/-                        |
|                 | d307–310     | -                          |
|                 | G313A        | -                          |
|                 | G315A        | -                          |

* Growth was assessed visually after 40 h; cultures were grown in triplicate (three separate isolates) in liquid glucose-arginine medium.

### Table 4: Intragenic complementation by different glnL alleles

| Strain          | Plasmid(s) | GS (nM/min/mg) |
|-----------------|------------|---------------|
| YMC10R (wild type) | None       | 2.098/0.305   |
| RB9132R (recA1 glnL2001) | G313A-pBR322 | 804/91        |
|                 | H139V-pBR322 | 80/102        |
|                 | d307–310-pBR322 | 581/97       |
|                 | G313A-pACYC184| 1,017/62      |
|                 | H139V-pACYC184| 111/60        |
|                 | d307–310-pACYC184 | 1,030/66   |
|                 | H139V-pBR322 + G313A-pACYC184 | 2.066/155   |
|                 | H139V-pACYC184 | 1.828/146     |
|                 | d307–310-pACYC184 | 1.860/98    |
|                 | H139V-pACYC184 + d307–310-pBR322 | 1.717/146   |

* For further details, see the footnotes to Table 2.
loration of glnA. These alleles resulted in a subtle defect in positive regulation. Thus, we assume that such alleles of glnL result in altered proteins with slightly diminished kinase activity in vivo.

There is a striking coincidence that the five mutations that appeared to eliminate the positive regulatory function entirely, H139N, G313A, E140Q, E140A, and ter291, also resulted in negative regulation of glnA in the absence of P$_{II}$. A current working hypothesis for the role of P$_{II}$ is that P$_{II}$ shifts NR$_{II}$ from a conformation in which it is a positive regulator to a conformation in which it is a negative regulator (5). If this is true, then these mutant NR$_{II}$ proteins appear to be able to adopt the conformation associated with negative regulation, even in the absence of P$_{II}$. One hypothesis for the mechanism of regulation by P$_{II}$ is that P$_{II}$ functions to shift the equilibrium between the two conformations of NR$_{II}$ to that associated with the phosphatase activity.

In the work reported in the accompanying paper, we purified and characterized the H139N, G313A, and ter291 proteins (17, 26). It was observed that the H139N and ter291 proteins are unable to be autocatalyzed, while the G313A protein is partially defective in autophosphorylation (26). Furthermore, it was observed that the H139N protein could bind ATP but that the G313A protein was partially defective in the binding of ATP (26). We have also observed that the H139N protein could bring about the dephosphorylation of NR$_{II}$ in the absence of P$_{II}$ (17). The results obtained in those studies thus serve to explain the phenotypes documented in this study.

Finally, in the accompanying report it was demonstrated that autophosphorylation of NR$_{II}$ occurs by a mechanism in which one subunit in the NR$_{II}$ dimer binds ATP and phosphorylates the other subunit (26). That result offers an explanation for the intragenic complementation between different glnL alleles documented in this report. Apparently, when H139V and either G313A or d307–310 are coproduced in cells, mixed dimers containing one of each type of subunit can be formed. The results with purified proteins suggest that it is within such mixed dimers that complementation of the autophosphorylation activity of NR$_{II}$ occurs (26).

ACKNOWLEDGMENTS

This work was supported by grant DMB6004048 from the NSF. We thank Boris Magasanik for stimulating discussions.

REFERENCES

1. Alder, S. P., D. Purich, and E. R. Stadtman. 1975. Cascade control of Escherichia coli glutamine synthetase. Properties of the P$_{II}$ regulatory protein and the uridylytransferase-uridylyl removing enzyme. J. Biol. Chem. 250:6264–6272.

2. Atkinson, M. R., and A. J. Ninfa. 1992. Characterization of Escherichia coli mutations affecting nitrogen regulation. J. Bacteriol. 174:4538–4548.

3. Bloom, F. R., M. C. Levin, F. Foor, and B. Tyler. 1978. Regulation of glutamine synthetase formation in Escherichia coli: characterization of mutants lacking the uridylytransferase. J. Bacteriol. 134:569–577.

4. Brown, M. S., A. Segal, and E. R. Stadtman. 1971. Modulation of glutamine synthetase adenylylation and deadenlylation is mediated by metabolic transformation of the P$_{II}$-regulatory protein. Proc. Natl. Acad. Sci. USA 68:2949–2953.

5. Bueno, R., G. Pahel, and B. Magasanik. 1985. Role of glnB and glnD gene products in the regulation of the glnALG operon of Escherichia coli. J. Bacteriol. 164:816–822.

6. Chen, Y.-M., K. Backman, and B. Magasanik. 1982. Characterization of a gene, glnL, the product of which is involved in the regulation of nitrogen utilization in Escherichia coli. J. Bacteriol. 150:214–220.

7. Engleman, E. G., and S. H. Francis. 1978. Cascade control of glutamine synthetase II. Metabolic regulation of the enzymes in the cascade. Arch. Biochem. Biophys. 191:602–612.

8. Feng, J., M. R. Atkinson, W. McCleary, J. B. Stock, B. L. Wanner, and A. J. Ninfa. 1992. Role of phosphorylated metabolic intermediates in the regulation of glutamine synthetase synthesis in Escherichia coli. J. Bacteriol. 174:6061–6070.

9. Foor, F. R., J. Cedergren, S. L. Streicher, S. G. Rhee, and B. Magasanik. 1978. Glutamine synthetase of Klebsiella aerogenes: properties of glnD mutants lacking uridylyltransferase. J. Bacteriol. 134:562–568.

10. Foor, F., Z. Reuveny, and B. Magasanik. 1980. Regulation of the synthesis of glutamine synthetase by the PII protein in Klebsiella aerogenes. Proc. Natl. Acad. Sci. USA 77:2636–2640.

11. Francis, S. H., and E. G. Engleman. 1978. Cascade control of glutamine synthetase. I. Studies on the uridylyltransferase and uridylyl removing enzyme(s) from E. coli. Arch. Biochem. Biophys. 191:590–601.

12. Garcia, E., and S. G. Rhee. 1983. Cascade control of Escherichia coli glutamine synthetase. Purification and properties of PII uridylytransferase and uridylyl-removing enzyme. J. Biol. Chem. 285:2246–2253.

13. Hattori, M., and Y. Sakaki. 1986. Dideoxy sequencing using denatured plasmid templates. Anal. Biochem. 152:232–238.

14. Hess, J. F., R. B. Bourret, and M. I. Simon. 1988. Histidine phosphorylation and phosphoryl group transfer in bacterial chemotaxis. Natl-Sci. (London) 336:139–143.

15. Hirschman, J., P.-K. Wong, K. Sei, J. Keener, and S. Kustu. 1985. Products of nitrogen regulatory genes ntrC and ntrE of enteric bacteria activate glnA transcription in vitro: evidence that the ntrA product is a sigma factor. Proc. Natl. Acad. Sci. USA 82:7525–7529.

16. Hunt, T. P., and B. Magasanik. 1985. Transcription of glnA by purified bacterial components: core RNA polymerase and the products of glnF, glnG, and glnL. Proc. Natl. Acad. Sci. USA 82:8453–8457.

17. Kamberov, E. S., M. R. Atkinson, and A. J. Ninfa. Unpublished data.

18. Keener, J., and S. Kustu. 1988. Protein kinase and phosphoprotein phosphatase activities of nitrogen regulatory proteins NTRB and NTRC of enteric bacteria: roles of conserved amino terminal domain of NTRC. Proc. Natl. Acad. Sci. USA 85:4976–4980.

19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.

20. MacNeil, T., D. MacNeil, and B. Tyler. 1982. Fine-structure deletion map and complementation analysis of the glnA-glnG region in Escherichia coli. J. Bacteriol. 150:1302–1313.

21. MacNeil, T., G. P. Roberts, D. MacNeil, and B. Tyler. 1982. The products of glnL and glnG are bifunctional regulatory proteins. Mol. Gen. Genet. 188:325–333.

22. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual, p. 230. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

23. Ninfa, A. J. 1991. Protein phosphorylation and the regulation of cellular processes by the homologous two-component regulatory systems of bacteria. Genet. Eng. 13:39–72.

24. Ninfa, A. J., and R. L. Bennett. 1991. Identification of the site of autophosphorylation of the bacterial protein kinase/phosphatase NR$_{II}$. J. Biol. Chem. 266:6888–6893.

25. Ninfa, A. J., and B. Magasanik. 1986. Covalent modification of the glnG product, NR$_{I}$ by the glnL product, NR$_{II}$, regulates the transcription of the glnALG operon in Escherichia coli. Proc. Natl. Acad. Sci. USA 83:5909–5913.

26. Ninfa, A. J., and B. Magasanik. 1982. Complex glnA-glnL-glnG operon of Escherichia coli. J. Bacteriol. 150:202–213.

27. Pahl, G., D. M. Rothstein, and B. Magasanik. 1982. Complex glnA-glnL-glnG operon of Escherichia coli. J. Bacteriol. 150:202–213.

28. Parkinson, J. S., and E. C. Kofoid. 1992. Communication modules in bacterial signaling proteins. Annu. Rev. Genet. 26:71–112.

29. Reitzer, L. J., and B. Magasanik. 1985. Expression of glnA in
Escherichia coli is regulated at tandem promoters. Proc. Natl. Acad. Sci. USA 82:1979–1983.

30. Reitzer, L. J., B. Mvosas, and B. Magasanik. 1989. Activation of glnA transcription by nitrogen regulator I (NRI)-phosphate in Escherichia coli: evidence for a long-range physical interaction between NRi-phosphate and RNA polymerase. J. Bacteriol. 171: 5512–5522.

31. Rhee, S. G., S. C. Park, and J. H. Koo. 1985. The role of adelylyltransferase and uridylyltransferase in the regulation of glutamine synthetase in Escherichia coli. Curr. Top. Cell Regul. 27:221–232.

32. Silhavy, T. J., M. L. Berman, and L. W. Enquist. 1984. Experiments with gene fusions, p. 107–111. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

33. Smith, R. A., and J. S. Parkinson. 1980. Overlapping genes at the CheA locus of Escherichia coli. Proc. Natl. Acad. Sci. USA 77:5370–5374.

34. Stadtman, E. R., E. Mura, P. B. Chock, and S. G. Rhee. 1980. The interconvertable enzyme cascade that regulates glutamine synthetase activity, p. 41–59 in J. Mora and R. Palacios (ed.), Glutamine: metabolism, enzymology, and regulation. Academic Press, Inc., New York.

35. Stock, J. B., A. J. Ninfa, and A. M. Stock. 1989. Protein phosphorylation and the regulation of adaptive responses in bacteria. Microbiol Rev. 53:451–490.

36. Ueno-Nishio, S., S. Mango, L. J. Reitzer, and B. Magasanik. 1984. Identification and regulation of the glnL operator-promoter of the complex glnALG operon of Escherichia coli. J. Bacteriol. 160:379–384.

37. Weiss, V., F. Claverie-Martin, and B. Magasanik. 1992. Phosphorylation of nitrogen regulator I of Escherichia coli induces strong cooperative binding to DNA essential for activation of transcription. Proc. Natl. Acad. Sci. USA 89:5088–5092.

38. Weiss, V., and B. Magasanik. 1988. Phosphorylation of nitrogen regulator I (NRI) of Escherichia coli. Proc. Natl. Acad. Sci. USA 85:8919–8923.