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Mutations in the *glnG* Gene of *Escherichia coli* That Result in Increased Activity of Nitrogen Regulator I

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Mutations in the *glnG* gene of *Escherichia coli* that result in increased activity of nitrogen regulator I (NR_I), the product of *glnG*, were obtained by two different selection procedures. The mutant proteins were purified and characterized. The concentrations of mutant proteins needed to activate transcription at the *glnAp2* promoter were three to four times lower than that of the wild-type NR_I. The rate of phosphorylation of these proteins and the stability of mutant NR_I phosphate were found to be similar to those of the wild-type NR_I. In one of the mutants, the site of the mutation was localized in the DNA region specifying the central domain of NR_I.

Nitrogen regulator I (NR_I) is a protein essential for the activation of transcription of genes of enteric bacteria in response to nitrogen deprivation of the cell (for a review, see reference 14). Promoters of these nitrogen-regulated (Ntr) genes share a characteristic nucleotide sequence recognized by core RNA polymerase coupled to a specific σ subunit, σ^{54} (1, 9, 11). The mechanism of the NR_I-mediated activation of transcription was elucidated through studies of the expression of the *glnALG* operon in *Escherichia coli*. This operon contains the structural genes for glutamine synthetase (*glnA*), NR_I (*glnG*), and NR_{II} (*glnL*). Three promoters have been identified within the *glnALG* operon (23, 27), and one of them, *glnAp2*, is responsible for the high level of expression of the operon under nitrogen limitation.

The *glnAp2* promoter is unique among Ntr promoters in that it is activated by NR_I at very low intracellular concentrations. It was found that this property results from the presence of high-affinity binding sites for NR_I located 100 and 130 base pairs upstream from the transcription start point (19, 24). When these binding sites were removed, the initiation of transcription from the *glnAp2* promoter required a high concentration of NR_I, similar to the concentration required to activate other Ntr promoters.

NR_I is only capable of activating the transcription of Ntr promoters after its conversion to NR_I phosphate. This phosphorylation is catalyzed by NR_{II}, the product of *glnL* (12, 18, 29); in the presence of P_{II}, the product of *glnB*, NR_{II} brings about the rapid dephosphorylation of NR_I phosphate (12, 18). The presence of P_{II} in cells growing with an excess of nitrogen is responsible for the lack of expression of Ntr genes. When these cells are subjected to nitrogen deprivation, the decrease in the intracellular concentration of glutamine and the increase in that of 2-ketoglutarate cause the enzyme uridylyl transferase, the product of *glnD*, to convert P_{II} to the innocuous P_{II}-UMP (4, 5). The removal of P_{II} enables NR_{II} to convert NR_I to NR_I phosphate, which in turn activates transcription at *glnAp2*. The resulting increase in the rate of transcription of the *glnALG* operon eventually results in the increase of the intracellular concentration of NR_I phosphate to a level that enables it to activate transcription at the other Ntr promoters. An increase in the availability of nitrogen in the growth medium results in an increase in the intracellular concentration of glutamine and a decrease in that of 2-ketoglutarate, which in turn causes the product of *glnD*, acting as a uridylyl-removing enzyme, to convert P_{II}-UMP to P_{II}, resulting in the dephosphorylation of NR_I phosphate and subsequent cessation of transcription initiation at Ntr promoters (5, 14).

While the genetic and physiological evidence for the involvement of P_{II} in the control of transcription at the *glnAp2* promoter (with NR_{II} acting as the mediator) is strong, the molecular mechanism of the putative P_{II}-NR_{II} interaction is so far unknown. Similarly, several points concerning activation of transcription by NR_I still require clarification. One of them is the dependence of NR_I activity upon phosphorylation of this protein by NR_{II}. With purified components one can obtain transcription from the *glnAp2* promoter only when NR_{II} is included in the system or when NR_I has been previously phosphorylated by NR_{II} (11; V. Weiss, A. J. Ninfa, and B. Magasanik, unpublished observation). However, NR_I-dependent transcription from the *glnAp2* promoter in vivo in *glnL*-deleted strains is still nitrogen regulated (3), and the only difference between $glnL^+$ and *glnL* deletion strains is that in the latter the response to the nitrogen limitation is much slower (7, 23). It is therefore possible that phosphorylation of NR_I is not absolutely necessary for its activity in vivo or that NR_I can be intracellularly phosphorylated by kinases other than NR_{II}.

We thought that to clarify at least some of the points concerning regulation of transcription by NR_I and NR_{II} it would be helpful to obtain and to characterize *glnG* mutations resulting in increased ability of the cells to activate transcription at *glnAp2* under conditions in which NR_I of the wild type is unable to do so. We have obtained such mutants. In this paper we present their characterization and describe the properties of the mutant NR_I proteins.

MATERIALS AND METHODS

Bacteria, phages, and plasmids. The bacterial strains, phages and plasmids used are listed in Table 1.

Media, growth conditions, and enzyme assays. The minimal and L broth (LB) media used have been described previ-

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TABLE 1. Bacterial strains, phages, and plasmids used in this study

Strain, phage, or plasmid	Relevant genotype	Source or reference(s)
YMC21	Δ(*glnA-glnG*)*2000* Δ*lacU169*	3
YMC17	*glnG*::Tn*5*	2
YMC26	*glnD99*::Tn*10*	T. Hunt
TH52	*glnG*::Tn*5 lacI*^{q}	T. Hunt
λgln101	*glnA'-'lacZ*	2
pgln31	*glnG*$^+$	2; this paper
pgln110	*glnL*$^+$	20, 27
pTH8	Wild-type *glnAp2* promoter	11
pAN6	*glnAp2* promoter with NR_I-binding sites deleted	19
pUC17	lac_p	28
pUC19	tac_p	28
pgln1128	*glnG1128*	This paper
pAN218	lac_p *glnG1128*	This paper
pgln316-19	tac_p *glnG316*	This paper
pgln401 to pgln408	*glnG* recombinants	This paper

ously (21). Nitrogen-limited medium (Ggln) contained 0.4% D-glucose and 0.2% L-glutamine. Nitrogen-excess medium was LB supplemented with 0.2% glutamine (LBgln). The Ntr$^-$ phenotype was scored on glucose minimal medium containing 0.2% L-arginine hydrochloride or 0.2% L-proline as a sole nitrogen source. Cultures for enzyme assays were grown at 30°C. Cell harvest and enzyme assays were as described previously (21).

Hydroxylamine mutagenesis. Hydroxylamine mutagenesis of DNA was carried out as described previously (10, 27).

Purified proteins. Core RNA polymerase and σ^{54} were purified as described previously (11). NR_{II} was purified by A. Ninfa and V. Weiss as described by Ninfa et al. (20). NR_I was purified as described previously (11, 22, 28). NR_I316 and NR_I1128 were purified in the same way from the TH52 strain bearing *glnG316* and *glnG1128* cloned in plasmids pUC19 and pUC18, respectively. Wild-type and mutant NR_I proteins were judged to be at least 90% pure as determined by visual inspection of a Coomassie blue-stained polyacrylamide gels. CheA and CheY proteins were kindly provided by J. Stock.

Transcription assay. Preparation of DNA templates and the transcription assays (see Fig. 2) were performed as described by Ninfa et al. (19). In the transcription assays illustrated in Fig. 1 and 4 a single change was made: only ATP was present in the reaction mixture during formation of the open complex, and GTP and CTP were added together with radioactive UTP and heparin to allow the formation of full-length transcripts. Electrophoresis of transcription products in the urea-acrylamide gels and autoradiography were performed as described previously (11).

Phosphorylation of NR_I. Phosphorylation of NR_I and its separation from NR_{II} were carried out as described by Weiss and Magasanik (29).

DNA sequencing. Restriction fragments *Eco*RV-*Bal*I, *Bal*I-*Pvu*II, and *Bal*I-*Sca*I from pgln31 and pgln316 were gel purified, cloned in the Bluescript plasmid (Stratagene), and sequenced by following the protocol provided by the same manufacturer.

Plasmid construction. Standard restriction analysis and molecular cloning procedures were followed (15).

Plasmid pgln31 is a derivative of pgln25 (2) and was obtained by adding *Eco*RI linkers to the *Hin*cII-*Cla*I fragment of pgln25, removing the *Sal*I-*Cla*I portion by *Sal*I digestion, and cloning the resulting *Eco*RI-*Sal*I fragment into *Eco*RI-*Sal*I sites of pBR322. The cloned fragment contains the 3' portion (500 base pairs) of the *glnL* gene and the complete *glnG* gene.

Plasmid pgln1128 was obtained by cloning the 12-kilobase *Hin*dIII fragment containing the *glnALG* operon from the original *glnG1128* isolate into pBR322. The recombinant plasmids were transformed into strain YMC21, and transformants were selected for ampicillin resistance and glutamine prototrophy. DNA from the selected plasmids was cleaved with *Hin*dIII and *Sal*I, and the fragment containing *glnG1128* was subcloned into similarly cleaved pBR322, giving rise to pgln1128. Plasmids pAN218 and pgln316-19 were obtained by inserting the *glnG*-containing fragments from pgln1128 and pgln316 into pUC18 and pUC19, respectively (28).

Plasmids pgln401 and pgln402 were made by digestion of pgln31 and pgln316 with *Eco*RI and *Sca*I, gel purification of the two resulting fragments from each plasmid, and reciprocal ligation of fragments derived from pgln31 and pgln316 (see Fig. 5).

Plasmids pgln403 and pgln404 were made in an analogous way by exchanging the *Bal*I-*Eco*RI fragments derived from pgln31 and pgln316.

Plasmids pgln407 and pgln408 were obtained by digestion of pgln31 and pgln316 with *Eco*RI followed by partial digestion with *Eco*RV and by an exchange of the *Eco*RV-*Eco*RI fragment between the two plasmids.

RESULTS

Isolation of *glnG* mutants. We were interested in obtaining mutations in *glnG* that would result in NR_I capable of strong activation of expression at *glnAp2*, possibly by creating an NR_I active conformation irrespective of the presence of NR_{II} and of P_{II}. Two approaches to select such mutants were chosen.

In the first case we mutagenized in vitro the pgln31 plasmid containing the *glnG* gene. After hydroxylamine treatment the *Sal*I-*Eco*RI fragment containing *glnG* was cut out, purified by gel electrophoresis, and ligated to the large *Eco*RI-*Sal*I fragment of pBR322. The ligation mixture was then used to transform strain YMC21(λgln101), and the cells were plated on LBgln-Xgal medium. Strain YMC21(λgln101) is deleted for the *glnALG* operon and contains the *lacZ* gene fused to the *glnAp2* promoter in a single copy on the λ prophage (2). It forms light blue colonies on the LBgln medium when transformed with plasmid pgln31, which carries a promoterless *glnG* gene and therefore contains wild-type NR_I at a low intracellular concentration. Transformation of YMC21(λgln101) with plasmids bearing mutagenized *glnG* DNA yielded several dark blue colonies, which appeared with a frequency of $\sim 5 \times 10^{-4}$. β-Galactosidase assays done on strains derived from these colonies confirmed that the level of the enzyme in cells grown under nitrogen excess was higher than that in the same strain bearing the wild-type *glnG* allele. Two strains containing mutant *glnG* alleles on plasmids pgln316 and pgln317 were chosen for more detailed study.

The second series of *glnG* mutants was isolated by a different selection strategy. We attempted to obtain *glnG* mutations which suppressed the phenotype resulting from the *glnD* mutation. These mutations would in effect allow the cell to bypass the negative regulatory signal coming through the UTase-P_{II}-NR_{II} signal transduction pathway. Since previous experience had indicated that the loss of the *glnL* product, NR_{II}, results in such a suppression, we isolated

TABLE 2. Effect of *glnG* on expression of *glnA*

E. coli strain and growth medium	*glnA* expression with plasmid[a]:				
	pBR	pgln31	pgln316	pgln317	pgln1128
YMC21 (Δ*glnALG* λgln101)					
Ggln	9	100	335	222	146
LBgln	8	5	64	77	38
YMC17 (*glnG*::Tn*5*)					
Ggln	3	100	79	78	98
LBgln	4	2	28	38	24
YMC26 (*glnD*::Tn*10*)					
LBgln	3	3	16	15	17

[a] The levels of β-galactosidase in cells of strain YMC21 and of glutamine synthetase in cells of strains YMC17 and YMC26 were measured. The results are given as percentages of the level of β-galactosidase in strain YMC21 (pgln31) grown on Ggln (4,360 nmol of product formed per min per mg of protein) or of the level of glutamine synthetase in strain YMC17(pgln31) grown on Ggln (1,150 nmol of product formed per min per mg of protein).

spontaneous *glnD* suppressors in a strain that contained a multicopy plasmid, pgln110, which was responsible for the overproduction of NR_{II}. The suppressor mutants were selected for their abilities to grow in a medium containing arginine as the sole nitrogen source (Ntr^+ phenotype). We observed that overproduction of NR_{II} itself resulted in the Ntr^- phenotype even in the absence of the *glnD* mutation, possibly by sequestration of NR_I. Our selection scheme for obtaining a *glnG* mutant in the *glnD*(pgln110) genetic background thus required not only that the mutant form of NR_I be unresponsive to the negative signal coming through the signal-transducing pathway but also that this NR_I be insensitive to the overproduction of NR_{II}.

Starting with the *glnD*(pgln110) strain, we isolated by direct selection strains that were able to utilize arginine as a sole nitrogen source. Several isolates were obtained that had high glutamine synthetase activities when grown on the nitrogen-excess medium (LBgln). In order to focus on suppressor mutations that map in the *glnALG* region, phage P1vir was then grown on each of these isolates and used to transduce a strain containing a deletion of *glnALG* to glutamine prototrophy (5). These transductants were then screened for elevated glutamine synthetase levels after growth on LBgln. Several transductants which had this property were identified, and one of them bearing the *glnG1128* allele was chosen for further study.

In order to preserve and to study the *glnG1128* allele, we cloned the *glnALG* region from the original isolate into pBR322 and then subcloned the fragment containing *glnG* into the same vector, giving rise to pgln1128.

Plasmid pgln1128 and two plasmids obtained by the first of the two selection procedures described above (pgln316 and pgln317) were transformed into strains YMC21(λgln101), YMC17 (*glnG*::Tn*5*), and YMC26 (*glnD*::Tn*10*), and the levels of β-galactosidase and glutamine synthetase in the resulting transformants grown under nitrogen excess and nitrogen limitation were measured. The results given in Table 2 show that all three *glnG* mutations studied resulted in significant increases in β-galactosidase expression from the *glnAp2* promoter relative to that obtained in the strain containing the wild-type *glnG* allele. The difference was 10- to 15-fold in the case of cells grown under nitrogen excess and up to 3-fold for cells grown under nitrogen limitation. Similar differences, but only in strains grown in nitrogen-excess medium, were observed in the glutamine synthetase levels in the YMC17 strain transformed with the mutant and wild-type alleles. All three mutant *glnG* alleles allowed the utilization of arginine by the YMC26 (*glnD*::Tn*10*) strain and were responsible for an over 10-fold elevation of the glutamine synthetase level in this strain compared with the wild-type allele. This is not surprising in the case of

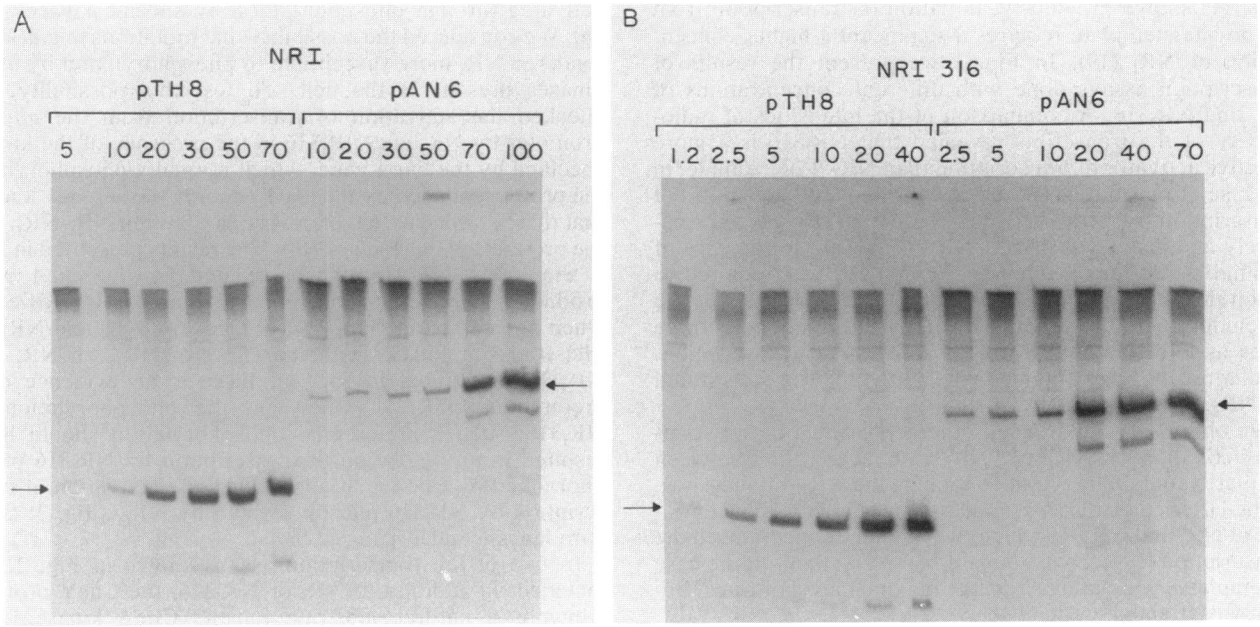

FIG. 1. Activation of transcription from the *glnAp2* promoter by NR_I (A) and NR_I316 (B). The DNA templates were either pTH8, containing the wild-type *glnAp2* promoter, or pAN6, in which the NR_I-binding sites had been deleted. Concentration of NR_I or NR_I316 (nanomolar) is given above each lane. Arrows indicate transcripts initiated at *glnAp2*.

glnG1128, which was selected as a suppressor of the *glnD* mutation, but it appears noteworthy that *glnG316* and *glnG317*, which were selected in a completely different way, also shared these properties.

Purification of the *glnG1128* and *glnG316* products. To verify that the mutations studied were actually in *glnG* and were not, for example, mutations in a small portion of the *glnL* gene present in the pgln1128 and pgln316 plasmids which could result in overproduction of the *glnG* product, as well as to study the mutant proteins in more detail, we sought to overproduce and purify the *glnG1128* and *glnG316* products.

In the case of pgln1128, we cloned the *glnG*-containing DNA fragment into the ptac12 plasmid downstream of the ptac12 promoter. The resulting plasmid, pAN202, was unstable and made cells nonviable even in the presence of the $lacI^q$ mutation. A slightly more stable plasmid, pAN218, was obtained by fusing *glnG1128* to a weaker *lac* promoter, pUC18. There were no problems with the stability of plasmid pgln316-19, which was obtained by the cloning of *glnG316* into pUC19.

Plasmids pAN218 and pgln315-19 were transformed into strain TH52 ($lacI^q$), and the mutant proteins were expressed and purified as described in Materials and Methods. We estimate the purity of NR_I1128 and NR_I316 to be greater than 90%, as judged from their appearance on the Coomassie blue-stained polyacrylamide gels (not shown).

Activation of transcription at *glnAp2* by purified NR_I1128 and NR_I316. We examined the ability of purified NR_I1128 and NR_I316 to activate the initiation of transcription in a defined transcription system. Previous results with the purified components of *E. coli* had indicated that four proteins (RNA polymerase, σ^{54}, NR_I, and NR_{II}) were required and sufficient to stimulate transcription from the *glnAp2* promoter (11). We used two different plasmids in the supercoiled form as templates in our transcription experiments. One, pTH8, contains a native *glnAp2* promoter with the full set of the NR_I-binding sites; the second, pAN6, lacks these sites. As shown previously, initiation of transcription from the no-sites template requires a significantly higher concentration of NR_I (19). In Fig. 1 we present the results of transcription assays done with different concentrations of NR_I and NR_I316. A comparison of the intensities of radioactivity of the transcripts revealed that NR_I316 is a more effective activator of transcription than NR_I. For example, in the case of plasmid pTH8 there was almost no activation of transcription by 5 nM NR_I, but 5 nM NR_I316 was approximately as effective as 20 nM NR_I. Similarly, in the case of plasmid pAN6, approximately 70 nM NR_I was required to match the activation of transcription by 20 nM NR_I316. We conclude, therefore, that NR_I316 is approximately three times as effective as NR_I as an activator of transcription. Similar results were obtained when NR_I1128 was substituted for NR_I316 (data not shown).

We observed a difference in the dependence of transcription activation on NR_I and NR_I316 concentration between templates containing or not containing the NR_I-binding sites. In the latter case, there seemed to be a threshold concentration of NR_I and NR_I316 which, when overstepped, raised the transcription level from almost none to maximal. In the case of templates containing sites, the maximal level of transcription was attained gradually.

Although NR_I316 has a significantly higher activity than NR_I, it still required the presence of NR_{II} for full activation of transcription from the *glnAp2* promoter. In the absence of NR_{II}, transcription was activated slightly by NR_I316 and not at all by NR_I (Fig. 2; compare lanes 2 and 5).

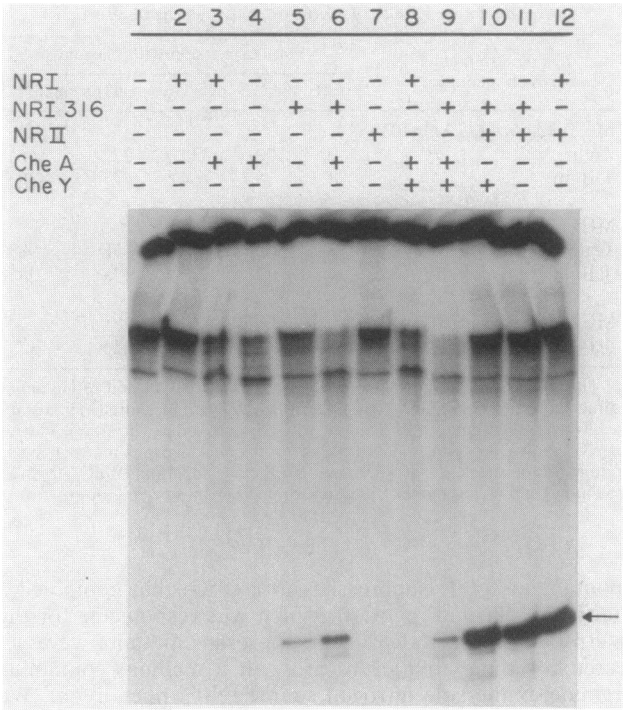

FIG. 2. Activation of transcription from the *glnAp2* promoter by NR_I and NR_I316 phosphorylated by the CheA kinase. The composition of transcription was identical for all assays with the exception of the proteins listed above each lane. pTH8 DNA was used as a template. Proteins were added at the following concentrations: NR_I and NR_I316, 2.5 μM; NR_{II}, 150 nM; CheA, 2.3 μM; CheY, 10 μM. Arrow indicates transcript initiated at *glnAp2*.

In *E. coli* and other enteric bacteria, several proteins are activated through phosphorylation by specific kinases (14, 26). We considered the possibility that mutations in *glnG* had rendered NR_I more susceptible to phosphorylation by other kinases present in the cell. To test this possibility, we checked the activation of transcription from the *glnAp2* promoter by NR_I and NR_I316 in the presence of the kinase specified by the *cheA* gene, which normally phosphorylates the protein encoded by the *cheY* gene. It was already known that there is some extent of cross talk between NR_I-NR_{II} and the products of *cheY-cheA* (17). The results presented in Fig. 2 show that NR_I316 phosphorylated by the *cheA* gene product is more active than similarly treated NR_I. However, when one compares the effect of CheA on NR_I and NR_I316 (illustrated in Fig. 2) with the effect of NR_{II} on NR_I and NR_I316 (illustrated in Fig. 1), there is no evidence of a preferential effect of CheA on the phosphorylation of NR_I316; such a preferential phosphorylation should have resulted in an activation of transcription by NR_I316 phosphorylated by CheA that approaches the activation of transcription by NR_I316 phosphorylated by NR_{II} (Fig. 2; compare lanes 6 and 11).

In two of the transcription assays shown in Fig. 2, we included (in addition to NR_I or NR_I316) the CheY protein, which is a natural substrate for the CheA kinase. This resulted in a decrease in the amount of transcript obtained from *glnAp2* (Fig. 2; compare lanes 3 and 6 with lanes 8 and 9), indicating that the CheY protein effectively competes

with NR_I for CheA and supporting the view that NR_I and NR_I316 are actually activated by the CheA kinase.

Phosphorylation and stability of mutant NR_I phosphate. The properties of NR_I316 and NR_I1128 could conceivably have been the reflection of greater stability of the corresponding phosphates than of NR_I phosphate. The wild-type NR_I phosphate is quite unstable, with a half-life of about 5 min at 37°C (12, 29). We examined the rate of phosphorylation of NR_I316 phosphate generated from NR_I316 and ATP in the reaction catalyzed by NR_{II} and compared this with the rate of formation and stability of wild-type NR_I phosphate. The stabilities of NR_I phosphate and NR_I316 phosphate were assayed after purification of these proteins from the phosphorylation mixture by a small-scale heparin-Sepharose batch affinity separation. Both the rates of phosphorylation of mutant and wild-type proteins and the stabilities of the corresponding phosphorylated proteins were exactly the same (Fig. 3). Similarly, no difference in stability was observed between phosphorylated NR_I and NR_I1128 (results not shown).

The phosphorylated ^{32}P-labeled NR_I316 separated from NR_{II} by heparin-Sepharose chromatography was then used

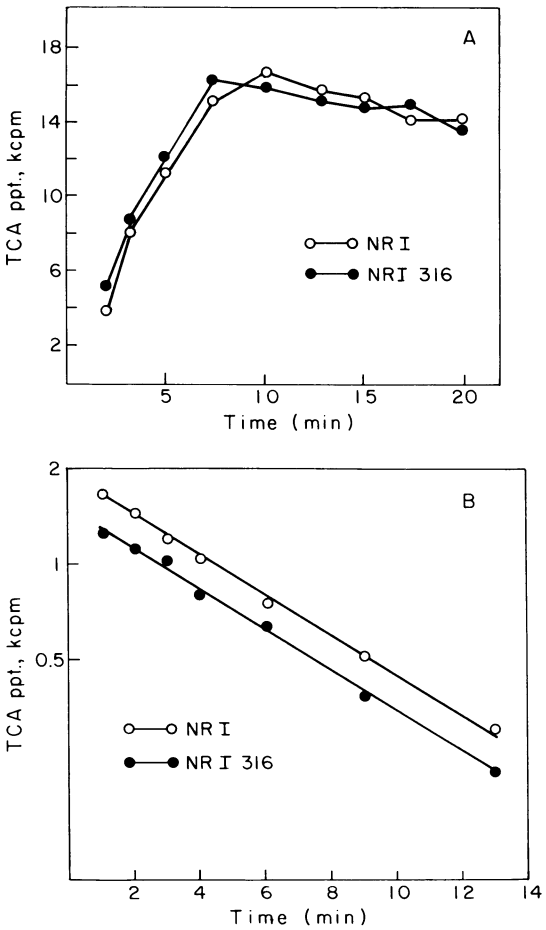

FIG. 3. Phosphorylation of NR_I and NR_I316 by NR_{II} (A) and stability of the phosphorylated proteins (B). (A) NR_I (3 μM) was incubated at 37°C in the presence of NR_{II} (2.8 μM) and [γ-^{32}P]ATP. Samples (10 μl) were withdrawn at the indicated times, spotted on glass filters, and analyzed as described previously (18). (B) NR_I and NR_I316 were phosphorylated by NR_{II}, separated from NR_{II}, and incubated at 37°C. At the indicated times, aliquots were spotted on glass filters and analyzed as described previously (18).

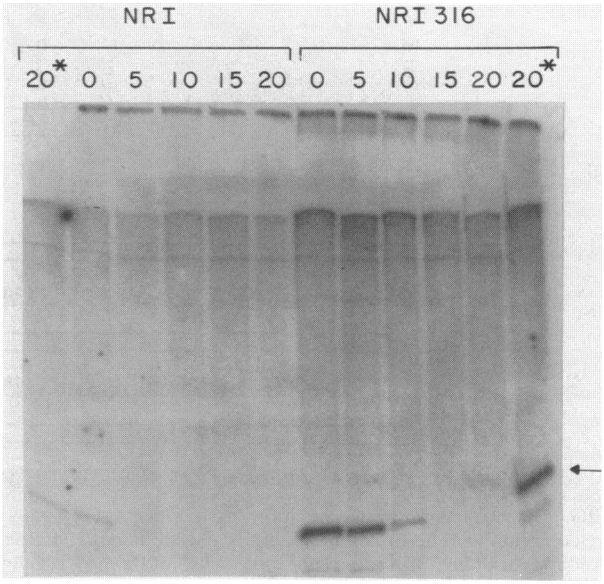

FIG. 4. Activation of transcription by NR_I phosphate and NR_I316 phosphate. NR_I and NR_I316 were phosphorylated by NR_{II}, separated from NR_{II}, preincubated at 37°C in the presence of $MgCl_2$ (10 mM), and used in the transcription assays. The time of preincubation (in minutes) is given above each lane. In two assays (*) NR_{II} was present at 150 nM. pTH8 DNA was used as a template. Arrow indicates transcripts initiated at glnAp2.

in the transcription assay along with ^{32}P-labeled phosphorylated NR_I obtained in the same way. The amounts of the two proteins were normalized for their radioactivity, which is an exact measure of the amount of phosphorylated protein in the preparation. Portions of NR_I316 phosphate and NR_I phosphate were incubated for a given length of time at 37°C and then added to the standard transcription assay system. The results shown in Fig. 4 indicate clearly that NR_I316 phosphate is much more effective in activation of transcription than NR_I phosphate and that both proteins lose the ability to activate transcription and phosphate at approximately the same rate (compare Fig. 3 and 4).

Localization of the mutation site within the *glnG* gene. We determined the site of the *glnG316* mutation by localizing it in a restriction fragment and then by DNA sequencing. The plasmids containing definite portions of the mutant and wild-type *glnG* genes were constructed and then transformed into the YMC21(λgln101) strain. Screening of transformants on LB-Xgal indicator plates allowed us to distinguish between mutant and wild-type phenotypes. The results shown in Fig. 5 permitted us to assign the site of mutation to the *Eco*RV-*Bal*I restriction fragment. The same result was obtained for the *glnG317* allele.

The DNA fragments spanned by the *Eco*RV-*Sca*I restriction sites (Fig. 4) from both pgln316 and pgln31 were then sequenced. The sequence for the wild-type gene differed from that published by Miranda-Rios et al. (16) for *E. coli glnG* by containing three additional bases, G, C, and G, after positions 425, 430, and 431. With these added bases the amino acid sequence of NR_I of *E. coli* becomes identical to that of *Klebsiella aerogenes* determined by Buickema et al. (6). The sequence of the mutant *glnG316* differed from the wild-type sequence by a single-base substitution changing the C in position 476 of the sequence described by Miranda-Rios et al. (16), which becomes position 479, to T. This

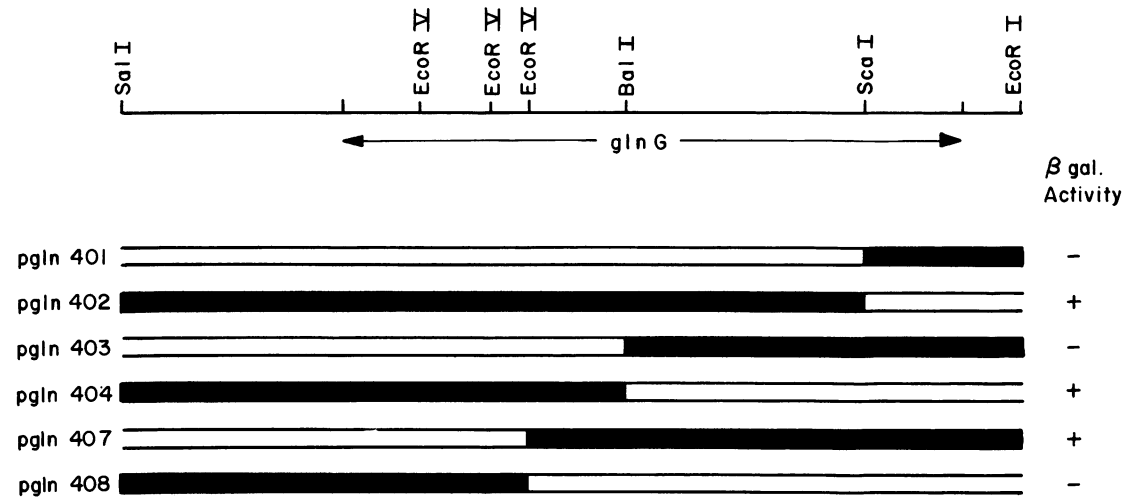

FIG. 5. Localization of the mutation site in *glnG316*. Plasmids containing fragment portions of DNA from pgln31 (□) and pgln316 (■) were constructed as described in Materials and Methods. These plasmids were transformed into YMC21(λgln101), and resulting colonies were scored for dark blue (+) versus light blue (−) color on the LB Gln Xgal medium, reflecting β-galactosidase activity. The fragment of *glnALG* DNA present in pgln31 and pgln316 is shown at the top of the figure. Restriction sites used in plasmid construction are indicated.

results in the replacement of the serine in position 160 by phenylalanine.

DISCUSSION

The *glnG* mutants described in this paper were obtained by two different selection strategies. The first strategy aimed for *glnG* mutants whose product, NR_I, would activate transcription from the *glnAp2* promoter more effectively than the wild-type protein. The second aimed for mutants producing NR_I insensitive to the negative signal transmitted through the UTase-P_{II}-NR_{II} pathway (14). It appears that whichever strategy was applied, the resulting mutants were phenotypically indistinguishable. All mutant alleles introduced into the cell on the promoterless plasmids caused a similar elevation of expression of genes transcribed from the *glnAp2* promoter, and all were found to suppress the phenotype resulting from the *glnD* mutation. The results of our experiments with purified NR_I316 and NR_I1128 confirmed the similarity of the mutations obtained by the two strategies.

The mutant proteins differed from wild-type NR_I by their increased ability to activate transcription at *glnAp2*. In contrast to wild-type NR_I, the mutant proteins could activate transcription in the absence of NR_{II}; however, the level of this activation was extremely low relative to that attainable by phosphorylated NR_I (Fig. 2; compare lane 2 with lane 5 and lane 5 with lanes 11 and 12). The more striking characteristic of the mutant proteins depended on their phosphorylation. In the presence of NR_{II}, they were as effective as NR_I when used at a three- to fourfold-lower concentration.

In our experiments, the activation of transcription by NR_I depended on the rate of its phosphorylation by NR_{II}, the rate of the spontaneous dephosphorylation of NR_I phosphate, the affinity of NR_I phosphate for its binding sites on the DNA template, and the rate of its interaction with the closed promoter-σ^{54}-RNA polymerase complex that resulted in the formation of the open complex. Our results showed that NR_I316 is phosphorylated by NR_{II} with the same kinetics as the wild-type protein and that it loses its phosphate at the same rate as wild-type NR_I phosphate. The fact that the greater ability of the mutant protein to activate transcription is equally apparent when templates with or without binding sites are used militates against the view that the mutant has increased affinity for the binding sites. Moreover, gel mobility retardation experiments (unpublished results) failed to reveal any difference in the ability of NR_I or the mutant form of NR_I to bind to DNA in the presence or absence of NR_{II} and ATP. It appears, therefore, that the mutation resulted in a form of NR_I phosphate with increased ability to catalyze the isomerization of the closed to the open promoter-σ^{54}-RNA polymerase complex. This view is in good accord with the location of the mutated codon in the central domain of NR_I.

NR_I belongs to a class of bacterial effector proteins with homology in their amino-terminal domains (13, 26). This domain contains the aspartate residue phosphorylated by NR_{II} (12, 29). A helix-turn-helix motif characteristic of DNA-binding proteins is located at the carboxy-terminal end of NR_I, and it has been shown that this portion of NR_I is responsible for the ability of NR_I to interact with its binding sites on the DNA template. The central domain of NR_I is homologous to the product of the *nifA* gene of *K. aerogenes* and to that of the *dctD* gene of *Rhizobium leguminosarum*, both of which are activators of transcription at σ^{54}-specific promoters, and therefore appears to be the domain of NR_I capable of interacting with the closed promoter-σ^{54}-RNA polymerase complex (8, 25).

It is not clear why only NR_I phosphate and not NR_I is capable of stimulating the conversion of the closed to the open promoter-RNA polymerase complex. The fact that the product of *nifA*, which stimulates a corresponding conversion at *nif* promoters, has no homology to NR_I in the amino-terminal domain that contains the phosphorylation site in NR_I (8) suggests that the phosphate attached to NR_I does not play an intrinsic role in the interaction of NR_I with the σ^{54}-RNA polymerase bound to the promoter. It is more likely that the attachment of the phosphate to NR_I results in a conformational change that allows the central domain of NR_I to make contact with the closed promoter complex. We may consider that even nonphosphorylated NR_I exists as an equilibrium mixture of an active and an inactive form and

that phosphorylation shifts the equilibrium in the direction of the active form. The mutations may have resulted in proteins that can more readily assume the active form. This assumption would explain both the slight ability of NR_I316 to activate transcription without being phosphorylated and the ability of phosphorylated NR_I316 to activate transcription more effectively than wild-type NR_I phosphate.

An increase in the ability of the mutant form of NR_I to activate the initiation of transcription at *glnAp2* would account reasonably for the phenotypes of the mutants whose *glnG* gene has been replaced by a mutated *glnG* gene. It is likely that in the case of the mutants neither growth in LBgln nor the lack of UTase can reduce the level of NR_I phosphate in the active conformation below that necessary for the activation of transcription at *glnAp2*.

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