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Genetic and Biochemical Analysis of Phosphatase Activity of *Escherichia coli* NRII (NtrB) and Its Regulation by the PII Signal Transduction Protein

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Mutant forms of *Escherichia coli* NRII (NtrB) were isolated that retained wild-type NRII kinase activity but were defective in the PII-activated phosphatase activity of NRII. Mutant strains were selected as mimicking the phenotype of a strain (strain BK) that lacks both of the related PII and GlnK signal transduction proteins and thus has no mechanism for activation of the NRII phosphatase activity. The selection and screening procedure resulted in the isolation of numerous mutants that phenotypically resembled strain BK to various extents. Mutations mapped to the *glnL (ntrB)* gene encoding NRII and were obtained in all three domains of NRII. Two distinct regions of the C-terminal, ATP-binding domain were identified by clusters of mutations. One cluster, including the Y302N mutation, altered a lid that sits over the ATP-binding site of NRII. The other cluster, including the S227R mutation, defined a small surface on the "back" or opposite side of this domain. The S227R and Y302N proteins were purified, along with the A129T (NRII2302) protein, which has reduced phosphatase activity due to a mutation in the central domain of NRII, and the L16R protein, which has a mutation in the N-terminal domain of NRII. The S227R, Y302N, and L16R proteins were specifically defective in the PII-activated phosphatase activity of NRII. Wild-type NRII, Y302N, A129T, and L16R proteins bound to PII, while the S227R protein was defective in binding PII. This suggests that the PII-binding site maps to the "back" of the C-terminal domain and that mutation of the ATP-lid, central domain, and N-terminal domain altered functions necessary for the phosphatase activity after PII binding.

The NRII-NRI (NtrB-NtrC) two-component signal transduction system regulates the transcription of genes encoding metabolic enzymes and permeases in response to carbon and nitrogen status in *Escherichia coli* and related bacteria (reviewed in reference 38). NRII is both a kinase and a phosphatase, catalyzing the phosphorylation and dephosphorylation of NRI (39). The phosphorylated form of NRI is a transcriptional activator of σ^{54}-dependent Ntr genes that acts by binding to enhancer sequences far from the promoters (39, 40). The enhancer-bound NRI~P interacts with the promoter-bound Eσ^{54} by means of a DNA loop (51) to activate the formation of the transcription open complex at these promoters (37, 45). Amplitude modulation of the NRI~P concentration results in the sequential activation and inactivation of genes, which differ in the responsiveness of their promoters to NRI~P (3).

The kinase and phosphatase activities of NRII are regulated by the PII signal transduction protein, which, on binding to NRII, inhibits the kinase activity of NRII and activates the NRII phosphatase activity (19, 20, 39). Signals of carbon and nitrogen status control the ability of PII to regulate NRII (23; reviewed in reference 38). PII is reversibly uridylylated by the *glnD* product, a bifunctional uridylyltransferase/uridylyl-removing enzyme, in response to the intracellular glutamine concentration (22). The uridylylated form of PII is unable to bind to NRII (4). In addition, the ability of PII to regulate NRII is controlled by the binding of 2-ketoglutarate to PII (22, 27, 31); binding of a single molecule of this allosteric effector to the trimeric PII favors the regulation of NRII by PII, while binding of additional effector molecules to PII blocks the ability of PII to regulate NRII (reviewed in reference 35).

The kinase activity of NRII, in which NRI becomes phosphorylated by NRII, occurs in two steps. In the first, dimeric NRII autophosphorylates in a bi-bi ping-pong reaction that proceeds by a *trans*-intramolecular mechanism in which ATP bound to the C-terminal domain of one NRII subunit phosphorylates the active-site histidine within the central domain of the opposing NRII subunit (42, 55). Figure 1A shows a model for the domain arrangement within the NRII dimer. In the second step, the N-terminal domain of NRI becomes phosphorylated by transfer of the phosphoryl group from the active-site histidine of NRII to an aspartate side chain of NRI (55). In the absence of NRI, the autophosphorylation of NRII is asymmetric in that unless the ADP generated in the reaction is enzymatically removed, the stoichiometry of NRII autophosphorylation approximates one per dimer (21). This asymmetry results from ~70-fold different equilibrium constants for the autophosphorylation of the "first" and "second" subunits of the NRII dimer (21). In the absence of NRI, the rate of the NRII autophosphorylation reaction is reduced by PII at saturating concentration but the final stoichiometry of NRII autophosphorylation at equilibrium is slightly increased by PII at saturating concentration (21).

The complex of PII and NRII brings about the rapid dephosphorylation of NRI~P (19, 25, 28, 39). It is not known whether this represents a distinct phosphatase activity of the

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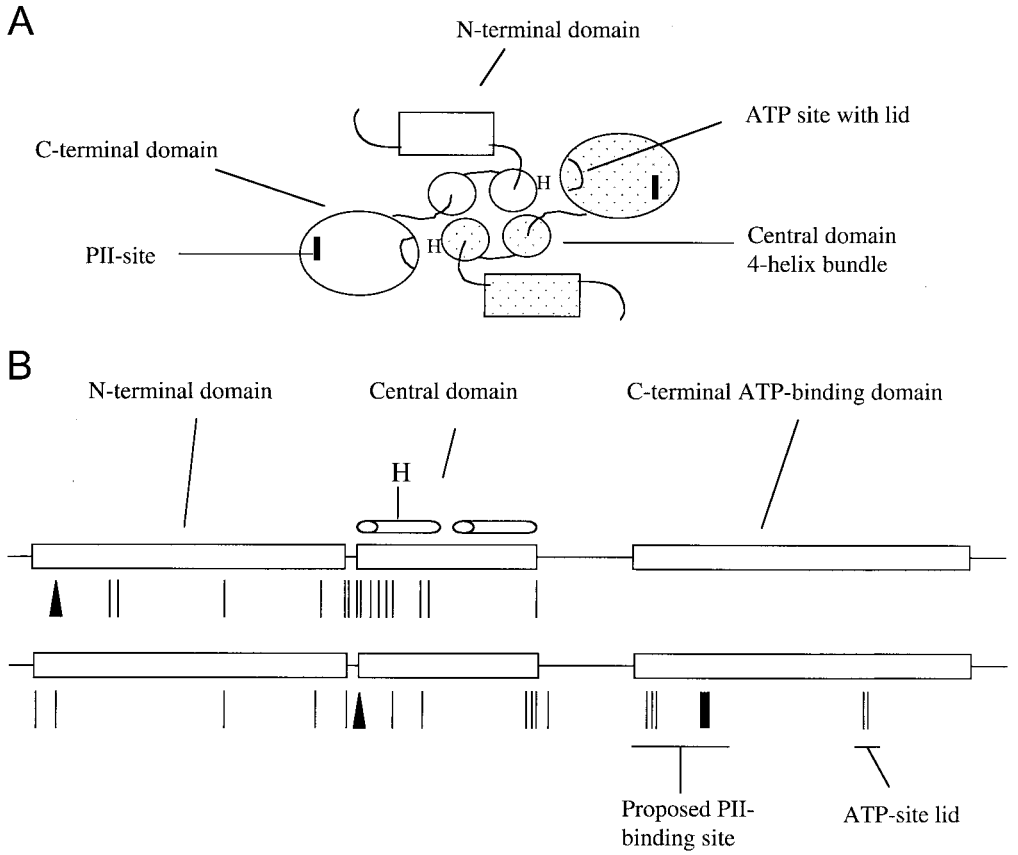

FIG. 1. Mutations affecting the phosphatase activity of NRII map to all domains of the protein. (A) Model for the domain organization of the NRII dimer based on structural information available for other two-component system transmitter proteins and biochemical studies of NRII. Each NRII subunit is composed of three domains: an unconserved N-terminal domain involved in intramolecular signal transduction, and central and C-terminal domains that together compose the conserved transmitter module. The central domain is involved in dimerization and probably forms half of a four-helix bundle containing His139, the site of autophosphorylation. The C-terminal domain is the ATP-binding kinase domain that directly interacts with PII. The dimer is shown viewed down the four-helix bundle. (B) Linear depiction of the domain structure of the 349-amino-acid NRII showing the distribution of mutations affecting the phosphatase activity. The top drawing shows the mutations obtained in the 1992 study (5), while the bottom drawing shows the mutations obtained in this study. The lines indicate amino acid substitutions, the bar indicates a deletion, and the triangles indicate insertions. The figure is roughly to scale.

complex or whether the complex acts to stimulate the intrinsic "autophosphatase" activity of NRI~P (28, 55). With this caveat in mind, we hereafter refer to this activity as the phosphatase activity. The phosphatase activity apparently does not directly require PII, in that the isolated central domain of NRII and a polypeptide that consisted of just the N-terminal and central domains of NRII displayed this activity at a very low level in the absence of PII and were not regulated by PII (19, 29). Also, phosphatase activity is displayed at a low level by a mutant form of full-length NRII, NRII (H139N), in which the active-site histidine within the central domain was converted to asparagine (6, 19, 25). The phosphatase activity of the H139N protein was strongly activated by PII (19). Since the H139N protein cannot become autophosphorylated, this indicates that activation of the NRII phosphatase activity by PII occurs even when the kinase activity is absent. In addition to its activation by PII, the phosphatase activity is activated by the binding of ATP or nonhydrolyzable ATP analogues to NRII.

Interestingly, polypeptides that consisted of just the central and C-terminal domains of NRII, that is, consisting entirely of the conserved "transmitter module" of NRII, were strongly defective for the phosphatase activity, even in the presence of PII (19). That is, the presence of the central domain is not sufficient for observation of phosphatase activity; thus, this domain must be able to adopt a conformation that permits the activity. Apparently, the N-terminal domain of NRII is required for this conformational state to occur when the C-terminal domain is present.

In addition to PII, *E. coli* contains a second PII-like protein, GlnK, that is expressed only in ammonium-starved cells (3, 7, 54). Purified GlnK, like PII, activates the phosphatase activity of NRII (8). The PII and GlnK proteins display 67% identity overall and are nearly identical in their T-loops. The T-loop is a loop at one end of the proteins (14, 16) that is implicated by cross-linking studies and mutational analysis of PII to be the site of interaction with NRII (24, 44). Thus, it is likely that PII and GlnK have a common binding site on NRII.

By analogy to other "transmitter" proteins, which contain N-terminal sensory domains (56), it was expected that PII would regulate NRII by interaction with its N-terminal do-

main. However, cross-linking studies indicated that PII interacts directly with the C-terminal ATP-binding domain of NRII (44). Also, PII regulates the ability of the isolated C-terminal domain of NRII to bring about the phosphorylation of the NRII central domain in an intermolecular reaction (19) and regulates the autophosphorylation of the isolated transmitter module of NRII (19). Thus, both enzymological and cross-linking studies were consistent with PII interaction with the ATP-binding C-terminal domain of NRII.

It was curious that no mutations affecting nitrogen regulation were ever identified that mapped within the C-terminal domain of NRII, although numerous mutations were identified that reduced the PII-dependent phosphatase activity (5). These were selected as allowing the use of a poor nitrogen source, arginine, as the sole nitrogen source by a strain that lacked the *glnD*-encoded uridylytransferase/uridylyl-removing enzyme. A *glnD* strain cannot use arginine as a nitrogen source because it cannot uridylylate PII, regardless of how low the intracellular glutamine concentration becomes. PII interacts with NRII and prevents the concentration of NRI~P from reaching the level necessary for activation of the *ast* operon, required for arginine utilization (49). Mutations that reduce the interaction of PII with NRII or that reduce the phosphatase activity of NRII without decreasing the kinase activity should then permit growth on arginine (5, 13). Many such suppressing mutations were obtained, and they mapped to many sites in the N-terminal domain of NRII, in the central domain of NRII, and in the linker joining these domains (5).

Recent studies of the regulation of nitrogen assimilation have clarified this issue. Cells lacking both PII and GlnK have a severe growth defect on minimal medium that results from overexpression of the *nac* gene, a part of the Ntr regulon (7, 11). The growth defect is most evident on nitrogen-rich minimal medium containing ammonium and less evident on nitrogen-limited medium containing arginine as the sole nitrogen source. The Nac protein is a LysR-type transcription factor that represses the *serA* promoter (11). The *serA* gene encodes phosphoglycerate dehydrogenase, the first step in the synthesis of serine, glycine, and C_1 units from glucose. Strains lacking both PII and GlnK, such as strain BK, have high levels of Nac accumulation and are starved for serine on ammonium-containing minimal medium (11). It was first observed that this phenotype was offset by inclusion of Casamino Acids (CAA) in the growth medium (7) and subsequently observed that serine or glycine were the components of CAA that rescued the poor growth phenotype (11). The phenotype is also offset by mutation of *nac*, *glnL (ntrB)* (encoding NRII), or *glnG (ntrC)* (encoding NRI) (7, 11). Thus, in retrospect, we could surmise that mutations that eliminate the binding of both PII and GlnK to NRII or otherwise completely inactivated the phosphatase activity of NRII, without limiting the kinase activity, would result in the unrestrained expression of Nac and poor growth in the absence of added serine or glycine on nitrogen-rich minimal medium. By inspection of old laboratory notebooks, we found that numerous such strains were obtained in previous experiments where suppressors were selected for the inability of the *glnD* mutant to grow on arginine as nitrogen source (M. R. Atkinson and A. J. Ninfa, unpublished data). In those studies, strains that grew poorly on nitrogen-rich glucose-ammonium-glutamine minimal medium were deliberately discarded (5). Here, we again selected suppressors of the inability of a *glnD* strain to use poor nitrogen sources and focused our attention on strains that mimic the phenotype of a strain lacking both PII and GlnK. We isolated many such mutant strains and observed that they contained mutations in all three domains of NRII. We purified NRII proteins containing representative mutations in each of the three domains and showed that the purified proteins retain kinase activity while lacking the phosphatase activity to varying degrees. Two distinct parts of the C-terminal ATP-binding domain were defined by clusters of mutations, and we show that one of these clusters probably corresponds to the PII-binding site of NRII.

MATERIALS AND METHODS

Bacteriological techniques. Mutant bacterial strains isolated in this work and plasmids used in this work are listed in Table 1. Luria broth (LB) and W-salts based minimal media were as described previously (5). Transformation with plasmid DNA, preparation of phage P1 vir lysates, P1 generalized transduction, and storage of strains were done by standard techniques or as described previously (32, 50).

DNA sequencing of chromosomal *glnL* alleles. The *glnL* gene was PCR amplified directly from bacterial colonies by using *Taq* polymerase, with the PCR primers described previously (5). The PCR products were purified from Tris-acetate–EDTA (TAE) agarose gels by using the Qiaex II gel extraction kit (Qiagen) as specified by the manufacturer and directly sequenced. Sequencing was performed by the University of Michigan DNA Sequencing Core Facility, using sequencing primers described previously (5, 6). In all cases, a clear single-strand sequence of the entire *glnL* coding region was obtained.

Strain constructions. For construction of strains bearing the mutant alleles listed in Table 1 in a wild-type *glnD* background, phage P1vir lysates were prepared on the Dϕ (or D[5]) suppressor strains and the *glnALG* region was crossed into strain YMC21Kϕ (Δ*glnALG glnKp-lacZYA*). Strain YMC21Kϕ is a glutamine auxotroph due to the lack of the *glnA*-encoded glutamine synthetase. Transductants were selected for glutamine prototrophy on glucose-ammonia-tryptophan minimal medium supplemented with CAA (CAA at the concentrations used does not supply enough glutamine to rescue the glutamine auxotrophy of YMC21Kϕ). Transductants were purified twice on the same medium and then screened for the constitutive phenotype on glucose-ammonia-glutamine-tryptophan minimal medium supplemented with CAA and containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). We refer to these as L*ϕ strains. Similarly, strains bearing the mutant *glnL* alleles in a *nac* background were constructed by crossing the *glnALG* region of the original suppressor strains into strain YMC21NKϕ (Δ*glnALG nac*::Cam^r *glnKp-lacZYA*), with transductions performed as above. We refer to these as L*Nϕ strains.

β-Galactosidase assays. Bacterial strains containing the *glnK* promoter-*lacZYA* fusion (7) were grown as indicated in the footnotes to the tables. Cells were disrupted by treatment with sodium dodecyl sulfate (SDS) and chloroform and directly assayed for β-galactosidase activity by the method of Miller (50), results are expressed in Miller units.

Construction of overexpression plasmids encoding NRII proteins. The *glnL* alleles of interest were PCR amplified from chromosomal DNA by using *Pfu* polymerase (Stratagene) (46). The primers were engineered to add an *Nde*I restriction site overlapping the ATG start codon and an *Eco*RI restriction site downstream of the termination codon. The upstream primer was 5′-CCCGCA GTCATATGGCAACAGGCACGCAGCCC-3′, and the downstream primer was as described previously (26). The PCR products were digested with *Nde*I and *Eco*RI and ligated into similarly digested pJLA503 (48). All constructs were sequenced over the entire *glnL* coding region to ensure that only the desired mutation was present. For construction of the pSJ4/*glnL* (L16R) plasmid expressing N-terminally histidine-tagged NRII (L16R), the *Nde*I-*Eco*RI *glnL* fragment from the appropriate pJLA503-based construct was swapped into similarly digested pSJ4. During the course of our studies, we observed that our wild-type overexpression plasmid contained a mutation, I141V, and that our expression plasmid for the A129T protein contained an additional mutation, I221V. For construction of a plasmid overexpressing a "true" wild-type NRII, a *Sal*I-*Sal*I fragment from pLOP (this fragment contains wild-type sequence) was used to replace the *Sal*I-*Sal*I fragment of pAP076 (containing the Y302N mutation). The resulting plasmid (pAP129 [Table 1]) was sequenced over the entire coding region to ensure that it encoded wild-type NRII. Similarly, for construction of a

TABLE 1. *E. coli* strains and plasmids used in this study

Strain or plasmid		Relevant genotype	Construction, reference, or source
Number	Name		
Strains[a]			
	YMC10ϕ	Wild type *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	7
	Dϕ	*glnD99*::Tn*10* *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	7
	N_2	*nac*::Cam^r	11
	Nϕ	*nac*::Cam^r *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	11
	BK_cϕ	Δ*glnB* Δ*mdl-glnK*::Cam^r *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	7
	BK_gϕ	Δ*glnB* ΩGm^{r}Δ *glnK1* *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	11
	BK_gNϕ	Δ*glnB* ΩGm^{r}Δ *glnK1 nac*::Cam^r *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	11
	Lϕ	Δ*glnL*2001 *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	7
	TEϕ	*recD1903*::Tn*10* *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	7
	YMC15	*glnL*2302 (A129T)	17
	RB9132	Δ*glnL*2001	13
	YMC21	Δ*glnALG*	17
	YMC21Kϕ	Δ*glnALG trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	YMC21 × TEϕ P1vir
AP1076	YMC21NKϕ	Δ*glnALG nac*::cam^r *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	YMC21Kϕ × N_2 P1vir
	BL21(DE3)	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	Novagen
WS1004		*glnD99*::Tn*10 glnL1004* (A138V)	5
WS1007		*glnD99*::Tn*10 glnL1007* (L122P)	5
WS1015		*glnD99*::Tn*10 glnL1015* (E120K)	5
WS4021		*glnD99*::Tn*10 glnL4021* (L154R)	5
WS4042		*glnD99*::Tn*10 glnL4042* (A35V)	5
WS5044		*glnD99*::Tn*10 glnL5044* (R115C)	5
AP1004	DL*(P188R)ϕ	*glnD99*::Tn*10 glnL* (P188R) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1005	DL*(Δ267-270)ϕ	*glnD99*::Tn*10 glnL* (Δ267-270) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1009	DL*(I12F)ϕ	*glnD99*::Tn*10 glnL* (I12F) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1010	DL*(S227N)ϕ	*glnD99*::Tn*10 glnL* (S227N) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1011	DL*(S227N)ϕ	*glnD99*::Tn*10 glnL* (S227N) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1012	DL*(D225V)ϕ	*glnD99*::Tn*10 glnL* (D225V) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1013	DL*(G150S)ϕ	*glnD99*::Tn*10 glnL* (G150S) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1015	DL*(D225V)ϕ	*glnD99*::Tn*10 glnL* (D225V) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1016	DL*(D225V)ϕ	*glnD99*::Tn*10 glnL* (D225V) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1017	DL*(G192D)ϕ	*glnD99*::Tn*10 glnL* (G192D) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1019	DL*(L16R)ϕ	*glnD99*::Tn*10 glnL* (L16R) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1020	DL*(S227R)ϕ	*glnD99*::Tn*10 glnL* (S227R) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1022	DL*(E107K)ϕ	*glnD99*::Tn*10 glnL* (E107K) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1023	DL*(INS/DUP)ϕ	*glnD99*::Tn*10 glnL* (INS/DUP) (resulting in a *glnL* composed of codons 1–120, followed by CAT, followed by codons 119–349) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1024	DL*(S227I)ϕ	*glnD99*::Tn*10 glnL* (S227I) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1025	DL*(Y302N)ϕ	*glnD99*::Tn*10 glnL* (Y302N) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1026	DL*(L228R)ϕ	*glnD99*::Tn*10 glnL* (L228R) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1027	DL*(L185P)ϕ	*glnD99*::Tn*10 glnL* (L185P) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1028	DL*(A138V)ϕ	*glnD99*::Tn*10 glnL* (A138V) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1030	DL*(P303L)ϕ	*glnD99*::Tn*10 glnL* (P303L) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1031	DL*(R116G)ϕ	*glnD99*::Tn*10 glnL* (R116G) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1033	DL*(S227N)ϕ	*glnD99*::Tn*10 glnL* (S227N) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1034	DL*(L83P)ϕ	*glnD99*::Tn*10 glnL* (L83P) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1035	DL*(D225V)ϕ	*glnD99*::Tn*10 glnL* (D225V) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1037	DL*(G187R)ϕ	*glnD99*::Tn*10 glnL* (G187R) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1038	DL*(R116C)ϕ	*glnD99*::Tn*10 glnL* (R116C) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
AP1043	DL*(P303T)ϕ	*glnD99*::Tn*10 glnL* (P303T) *trpDC700*::*putPA1303*[Kan^r Cam^s *glnKp-lac*]	Dϕ→Gargtrp$^+$
Plasmids			
	pBUC10	Amp^r, *glnB*$^+$ in pUC18	24
	pgln62	Amp^r, *glnL*$^+$ in pBR322	17
	pJLA503	Amp^r, temperature-inducible expression vector	48
	pSJ4	Kan^r, IPTG-inducible expression vector	Zhaohui Xu
	pLOP	Amp^r, overexpression of NRII (I141V)	42
	pLOP15	Amp^r, overexpression of NRII (A129T/I221V)	42
pAP073	pJLA503/*glnL* (S227R)	Amp^r, overexpression of NRII (S227R)	*glnL* of AP1020 PCR cloned *Nde*I-*Eco*RI into pJLA503
pAP076	pJLA503/*glnL* (Y302N)	Amp^r, overexpression of NRII (Y302N)	Same as AP073, except *glnL* of AP1025
pAP096	pJLA503/*glnL* (L16R)	Amp^r, overexpression of NRII (L16R)	Same as AP073, except *glnL* of AP1019
pAP121	pSJ4/*glnL* (L16R)	Kan^r, overexpression of histidine-tagged NRII (L16R)	*Nde*I-*Eco*RI fragment of pAP096 swapped into pSJ4
pAP129	true pLOP	Amp^r, overexpression of wild-type NRII	*Sal*I-*Sal*I fragment from pLOP replaced *Sal*I-*Sal*I fragment of pAP076
pAP131	true pLOP15	Amp^r, overexpression of NRII (A129T)	*Sal*I-*Sal*I fragment from pLOP replaced *Sal*I-*Sal*I fragment of pLOP15

[a] For clarity, only the parental strains are listed here. A complete strain list is available on request from the authors.

plasmid overexpressing a "true" NRII (A129T), the *Sal*I-*Sal*I fragment from pLOP (containing wild-type sequence) was used to replace the *Sal*I-*Sal*I fragment of pLOP15 (containing the extraneous I221V mutation). The resulting plasmid (pAP131 [Table 1]) was sequenced over the entire coding region to ensure that it encoded NRII (A129T) with no additional mutations.

Purified proteins. The preparations of PII, PII (E44C/C73S), and the N-terminal domain of NRI (NRI-N) were described previously (26, 44). NRII, NRII (I141V), NRII (S227R), NRII (Y302N), NRII (A129T/I221V), and NRII (A129T) were purified by a modified version of the method described previously (26, 41), as follows. Strain RB9132 (Δ*glnL*) (13) was transformed with the appropriate expression plasmid, and transformants were selected for ampicillin resistance. Cultures (4 liters) were grown at 30°C in LB medium containing ampicillin, with induction carried out by shifting the temperature to 44°C as described previously (26). Overexpression was sufficient in all cases to allow the course of the purification to be monitored by SDS-polyacrylamide gel electrophoresis. Cells were harvested by centrifugation, and the cell paste was stored at −80°C. The purification was essentially identical for the wild-type and mutant proteins, except for the differences noted for NRII (S227R). All steps of the purification were carried out at 4°C. The cell pellet was resuspended in buffer A [buffer C for NRII (S227R)], and cells were disrupted by sonication. Streptomycin sulfate (Sigma) was added to 1.5% (wt/vol) with stirring for 20 min, and the extract was clarified by centrifugation. Solid ammonium sulfate was added to the supernatant to 0.18 g/ml to precipitate NRII. The ammonium sulfate precipitate was collected by centrifugation, resuspended into a minimal amount of buffer C, and diluted to 50 ml with buffer B. This solution was loaded onto a ~70-ml ethyl agarose (Sigma) column equilibrated in buffer B. The column was washed with buffer B and eluted with a linear gradient of buffer B to buffer C. NRII eluted in a broad peak centered at ~0.05 g of ammonium sulfate per ml. Peak fractions containing NRII were pooled, and the NRII was precipitated with ammonium sulfate at 40% saturation. The ammonium sulfate pellet was resuspended in a minimal volume of buffer A [buffer C for NRII (S227R)] and loaded onto a ~500-ml Bio-Gel A 0.5 M (Bio-Rad) gel filtration column equilibrated in buffer D. For NRII (S227R), the column was equilibrated in 50 mM Tris-HCl (pH 7.5)–10% (vol/vol) glycerol–50 mM KCl–1 mM dithiothreitol (DTT). NRII eluted in a single peak corresponding to the dimeric protein. At this point, the preparation was ~95% pure as judged by visualization of Coomassie brilliant blue R-250 stained gels. NRII (S227R) peak fractions were pooled, dialyzed to storage buffer, and stored at −80°C. For the remaining proteins, a final ion-exchange chromatography step served mainly to concentrate the protein. Peak NRII fractions from the gel filtration column were pooled and dialyzed against buffer A. The dialyzed solution was loaded onto a ~15-ml DE52 (Whatman) column equilibrated in buffer A. After extensive washing, the NRII was eluted with a linear gradient of buffer A to buffer E. NRII eluted between ~140 and ~250 mM KCl depending on the mutant. The peak NRII fractions were pooled, dialyzed against storage buffer, and stored at −80°C. Typical yields from 4 liters of starting material were 50 to 100 mg of pure protein, although the NRII (S227R) yield (~20 mg) was lower due to losses over the course of the purification. Protein concentrations were determined by the method of Bradford (12) and are stated in terms of the monomer for NRI-N, the dimer for NRII, or the trimer for PII.

Purification of histidine-tagged NRII (L16R). Strain BL21(DE3) (Novagen) was transformed with the pSJ4/*glnL* (L16R) plasmid with selection for kanamycin resistance. A 4-liter culture was grown at 37°C in LB medium containing kanamycin. Mid-log-phase cells were induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 0.4 mM (final concentration) with growth for 4 h. Overexpression was sufficient to allow the course of the purification to be monitored by SDS-polyacrylamide gel electrophoresis. Cells were harvested by centrifugation and stored as a paste at −80°C. All steps of the purification were carried out at 4°C. For NRII (L16R), buffers A, D, and E were the same as listed below, except that they lacked DTT. The cell pellet was resuspended in buffer A and sonicated to break open the cells. The resulting extract was clarified by centrifugation, and the supernatant was loaded directly on a ~20-ml Ni-nitrilotriacetic acid-agarose (Quiagen) column equilibrated in buffer A. After extensive washing with buffer D, the column was eluted with a linear gradient of buffer D to buffer F. Histidine-tagged NRII (L16R) eluted in a broad peak centered at ~160 mM imidazole. Peak fractions were pooled and dialyzed against buffer A. The dialyzed solution was loaded onto a ~12-ml DE52 (Whatman) ion-exchange column equilibrated in buffer A. After extensive washing, the column was eluted with a linear gradient of buffer A to buffer E. Histidine-tagged NRII (L16R) eluted in a sharp peak centered at ~200 mM KCl. At this point, the preparation was ~95% pure. The protein concentration was determined by the method of Bradford (12). Peak fractions containing ~100 mg of pure protein were pooled, dialyzed against storage buffer, and stored at −80°C.

Proteolytic removal of the histidine tag from histidine-tagged NRII (L16R). A 9-mg sample of purified histidine-tagged NRII (L16R) was incubated with 2,000 U of rTEV protease (Invitrogen Life Technologies) overnight at 4°C in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 5% (vol/vol) glycerol, 0.5 mM EDTA, and 1 mM DTT. Analysis of the cleavage reaction product by SDS-polyacrylamide gel electrophoresis indicated that ~98% of the tagged NRII (L16R) was digested. The cleavage reaction product was dialyzed to 50 mM Tris-HCl (pH 7.5)–5% (vol/vol) glycerol–25 mM KCl and passed over a 2-ml Ni-nitrilotriacetic acid-agarose (Qiagen) column equilibrated in the same buffer to remove the residual tagged NRII (L16R), the cleaved tag, and the histidine-tagged protease. The flowthrough fraction containing purified cleaved NRII (L16R) was concentrated using a 10-kDa-molecular-mass cutoff Ultrafree-15 centrifugal filter device (Millipore), dialyzed to storage buffer, and stored at −80°C.

Buffers for protein purifications were as follows. Buffer A consisted of 50 mM Tris-HCl (pH 7.5), 10% (vol/vol) glycerol, 25 mM KCl, and 1 mM DTT; buffer B consisted of 50 mM Tris-HCl (pH 7.5), 10% (vol/vol) glycerol, 1 mM DTT, and 0.10 g of ammonium sulfate per ml; buffer C consisted of 50 mM Tris-HCl (pH 7.5), 10% (vol/vol) glycerol, and 1 mM DTT; buffer D consisted of 50 mM Tris-HCl (pH 7.5), 10% (vol/vol) glycerol, 200 mM KCl, and 1 mM DTT; buffer E consisted of 50 mM Tris-HCl (pH 7.5), 10% (vol/vol) glycerol, 500 mM KCl, and 1 mM DTT; and buffer F consisted of 50 mM Tris-HCl (pH 7.5), 10% (vol/vol) glycerol, 200 mM KCl, and 250 mM imidazole. Storage buffer was 50 mM Tris-HCl (pH 7.5)–50% (vol/vol) glycerol–100 mM KCl–1 mM DTT for NRII, NRII (I141V), NRII (Y302N), NRII (A129T/I221V), and NRII (A129T). NRII (S227R) was stored in 50 mM Tris-HCl (pH 7.5)–50% (vol/vol) glycerol–25 mM KCl–1 mM DTT. Histidine-tagged NRII (L16R) and cleaved NRII (L16R) were stored in 50 mM Tris-HCl (pH 7.5)–50% (vol/vol) glycerol–25 mM KCl.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis, nondenaturing (native) polyacrylamide gel electrophoresis, and urea-polyacrylamide gel electrophoresis were carried out as described previously (21, 36).

Autophosphorylation assays. The reactions for the autophosphorylation assays were similar to those described previously (21, 55). Briefly, the reactions were carried out on ice and the mixtures contained 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mg of bovine serum albumin (BSA) per ml, 50 μM 2-ketoglutarate, 0.5 mM [γ-^{32}P]ATP (or as indicated), 2 μM NRII or mutants as indicated, and 12 μM PII as indicated. For quantitative assessment, aliquots of the reaction mixtures were removed at various times, spotted on nitrocellulose filters, and washed in 0.1 M Na_2CO_3 (pH ≈11). The filters were dried briefly and counted in liquid scintillation fluid. For qualitative assessment by nondenaturing and urea-polyacrylamide gel electrophoresis, the reaction mixtures contained unlabeled ATP, and BSA and 2-ketoglutarate were omitted. Reactions were stopped by addition of 50 mM EDTA, and the mixtures were made 5% (vol/vol) glycerol to facilitate loading and analyzed on 10% nondenaturing polyacrylamide gels; alternatively, the reactions were stopped by addition of 4 M urea and the mixtures were analyzed on 10% polyacrylamide–6 M urea gels (21). The gels were stained with Coomassie brilliant blue R-250.

Combined kinase and phosphatase assay. The combined kinase and phosphatase assay was performed essentially as described previously (39). Briefly, the purified N-terminal domain of NRI (NRI-N, 30 μM) was incubated with NRII or mutant forms of NRII (0.3 μM) at 25°C in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.3 mg of BSA per ml, 50 μM 2-ketoglutarate, and 0.5 mM [γ-^{32}P]ATP. After 25 min, the reaction mixture was split into tubes containing either reaction buffer or various amounts of PII as indicated. Aliquots of the reaction mixtures were removed at various times and spotted onto nitrocellulose filters, which were washed extensively in 5% trichloroacetic acid. Filters were dried and counted in liquid scintillation fluid.

Cross-linking reactions. PII (E44C/C73S) was labeled with the photoactivatable, heterobifunctional cross-linking reagent *N*-(4-azido-2,3,5,6-tetrafluorobenzyl)-3-maleimidopropionamide (TFPAM-3) (Molecular Probes) as described previously (44). Cross-linking reactions were carried out as described previously (44). Briefly, reaction mixtures contained 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 50 μM 2-ketoglutarate, 0.5 mM ATP, 4.5 μM NRII (or mutant form of NRII), and 9 μM cross-linker-labeled PII. The reactions were carried out on ice, and UV exposure was for 20 min. Reactions were stopped by addition of SDS gel-loading buffer, and the mixtures were heated to 95°C for 5 min and analyzed on SDS–15% polyacrylamide gels; alternatively, the reactions were stopped by addition of 120 mM glycine (pH 9.0) and the mixtures were examined on 10% nondenaturing polyacrylamide gels. Gels were stained with Coomassie brilliant blue R-250.

Gel filtration assay for PII binding. The indicated NRII protein (12 μM) and PII (48 μM) were mixed on ice in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM $MgCl_2$, 50 μM free 2-ketoglutarate, and 0.5 mM

TABLE 2. Summary of sequenced *glnL* alleles

Strain	Codon	DNA change	Amino acid change[a]	BK phenotype[b]
AP1009	12	ATC→TTC	I→F	No
AP1019	16	CTG→CGG	L→R	Yes
AP1034	83	CTG→CCG	L→P	No
AP1022	107	GAG→AAG	E→K	Yes
AP1031	116	CGC→GGC	R→G	No
AP1038	116	CGC→TGC	R→C	No
AP1023	121	Insertion/duplication[c]		ND[d]
AP1028	138	GCA→GTA	A→V	No
AP1013	150	GGC→AGC	G→S	No
AP1027	185	CTG→CCG	L→P	No
AP1037	187	GGG→AGG	G→R	No
AP1004	188	CCG→CGC	P→R	No
AP1017	192	GGT→GAT	G→D	No
AP1012, AP1015, AP1016, AP1035	225	GAT→GTT	D→V	Yes
AP1010, AP1011, AP1033	227	AGC→AAC	S→N	No
AP1020	227	AGC→AGA	S→R	Yes
AP1024	227	AGC→ATC	S→I	Yes
AP1026	228	CTA→CGA	L→R	Yes
AP1005	266	ACC→ACG	None	
	267–270	Deletion	Δ267–270	No
AP1025	299	ACG→ACA	None	
	302	TAC→AAC	Y→N	Yes
AP1030	303	CCG→CTG	P→L	No
AP1043	303	CCG→ACG	P→T	Yes

[a] In the standard single-letter symbols.
[b] Indicated by poor growth similar to that of strain BK after 42 h of growth on GNGlnTrp medium at 37°C.
[c] Resulting in a *glnL* consisting of codons 1 to 120, followed by an insertion of CAT encoding histidine, followed by codons 119 to 349.
[d] ND, not determined.

free ATP. The free concentration refers to the amount of 2-ketoglutarate and ATP above that required for binding of one 2-ketoglutarate molecule and three ATP molecules per PII trimer and two ATP molecules per NRII dimer. After a 10- to 15-min incubation period, the reaction mixture (0.5 ml) was loaded onto a ~190-ml-bed-volume Sephadex G-100 (Pharmacia) column equilibrated in 50 mM Tris-HCl (pH 7.5)–100 mM KCl–1 mM $MgCl_2$–50 μM 2-ketoglutarate–0.5 mM ATP. The column was eluted with 50 mM Tris-HCl (pH 7.5)–100 mM KCl at 4°C. Fractions were collected and analyzed on SDS–15% polyacrylamide gels with Coomassie brilliant blue R-250 staining.

RESULTS

Isolation of spontaneous suppressors of the *glnD99*::Tn*10* mutation, revisited. To isolate severely phosphatase-deficient (constitutive) NRII mutants, some of which should be deficient for PII binding, we selected spontaneous suppressor mutations that permit a *glnD* mutant to grow on a poor nitrogen source (arginine) and focused on the mutants that grew poorly on nitrogen-rich glucose-ammonia-glutamine minimal medium. To qualitatively assess Ntr gene expression in cells grown on solid medium, we used a strain bearing the *glnD99*::Tn*10* mutation as well as a fusion of the nitrogen-regulated *glnK* promoter to *lacZYA* placed in single copy in the *trp* locus (strain Dϕ [7]). The *glnKp-lacZYA* fusion allowed us to assess the Ntr phenotype of strains on plates containing the β-galactosidase activity indicator X-Gal; the presence of this fusion within the *trp* operon results in a tryptophan auxotrophy that could be compensated for by inclusion of a low concentration of tryptophan in the medium. A *glnKp-lacZYA* fusion was chosen because activation of the *glnK* promoter requires a high intracellular concentration of NRI~P, permitting us to observe differences in NRI~P when it is near the high end of its physiological range (3). A total of 207 independent, spontaneous Ntr^+ pseudorevertants of strain Dϕ were isolated on defined glucose-arginine-tryptophan minimal medium at 37°C. After purification, the mutants were screened for the desired constitutive phenotype as detailed below.

Phenotypic screen for constitutive Ntr expression. The Dϕ suppressors were streaked onto solid nitrogen-rich glucose-ammonia-glutamine-tryptophan minimal medium supplemented with or without CAA and containing X-Gal to determine which of them exhibited constitutive expression of the *glnK* promoter and poor growth in the absence of CAA. Forty-four of the Dϕ suppressors exhibited the desired phenotype (data not shown). A range of growth properties were observed that varied from a subtle growth defect to very poor growth similar to that of strain BK.

Complementation test to determine mutations in *glnL*. To determine which of the constitutive Dϕ suppressors were a result of mutations in the *glnL* gene, we prepared competent cells of the 44 strains showing a range of growth defects on nitrogen-rich minimal medium and transformed them with plasmids encoding either wild-type PII (pBUC10 [24]) or wild-type NRII (pgln62 [17]). Transformants were selected for ampicillin resistance on LB plus glutamine medium and colony purified on the same medium. The purified transformants were streaked for single colonies on glucose-ammonia-glutamine-tryptophan minimal medium supplemented with CAA or not supplemented and containing ampicillin and X-Gal. The constitutive Dϕ suppressors could be roughly divided into four classes based on their phenotype in the presence of the PII- and NRII-encoding plasmids. Class I mutants exhibited good growth with or without CAA in the presence of pgln62 and restored nitrogen regulation of the *glnK* promoter (white colonies). These mutants exhibited poor growth with or without CAA in the presence of pBUC10 and remained constitutive for

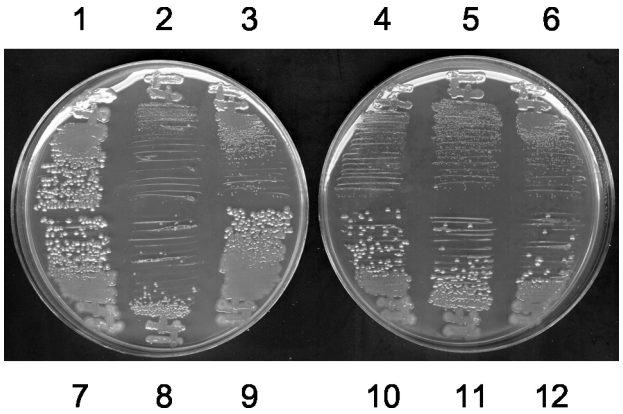

Fig. 2. Growth phenotypes of *glnL** alleles. Strains containing representative *glnL** alleles in a wild-type or *nac* background were streaked for single colonies on glucose-ammonia-glutamine-tryptophan minimal medium. Growth was carried out for 42 h at 37°C. The strains were as follows, with relevant genotypes in brackets: 1, YMC10ϕ [wild type]; 2, BK_gϕ [Δ*glnB*ΩGm^{r}Δ*glnK1*]; 3, L*(L16R)ϕ [*glnL* (L16R)]; 4, L*(L154R)ϕ [*glnL* (L154R)]; 5, L*(S227R)ϕ [*glnL* (S227R)]; 6, L*(Y302N)ϕ [*glnL* (Y302N)]; 7, Nϕ [*nac*::Cam^r]; 8, BK_gNϕ [Δ*glnB*ΩGm^{r}Δ*glnK1 nac*::Cam^r]; 9, L*(L16R)Nϕ [*glnL* (L16R) *nac*::Cam^r]; 10, L*(L154R)Nϕ [*glnL* (L154R) *nac*::Cam^r]; 11, L*(S227R)Nϕ [*glnL* (S227R) *nac*::Cam^r]; 12, L*(Y302N)Nϕ [*glnL* (Y302N) *nac*::Cam^r].

glnK promoter expression (blue colonies). It should be noted that the pBUC10 plasmid conferred somewhat poor growth on many strains, and therefore we concentrated mainly on the color of the colonies in the presence of this plasmid. Class II mutants exhibited good growth with or without CAA in the presence of pgln62 and gave a mixture of blue and white colonies. These mutants remained constitutive for *glnK* promoter expression in the presence of pBUC10 (blue colonies). Class III mutants exhibited good growth with or without CAA in the presence of pgln62 and restored nitrogen regulation of the *glnK* promoter (white colonies). These mutants also exhibited restored nitrogen regulation of the *glnK* promoter in the presence of pBUC10 (white colonies). Class IV mutants remained constitutive for *glnK* promoter expression in the presence of both pgln62 and pBUC10 (blue colonies). Some of the mutants were difficult to define to one specific class, and some overlap between classes existed. Approximately 30 of the constitutive Dϕ suppressors fell into classes I and II (data not shown). The mixtures of blue and white colonies for the class II strains were assumed to be due to the antagonism between a mutant *glnL* allele on the chromosome and the wild-type allele on the plasmid, which affect each other's expression.

Sequencing of *glnL* alleles. The *glnL* gene of the class I and II mutants was sequenced as described in Materials and Methods. Three of the 30 class I and II mutants contained the wild-type *glnL* allele (data not shown). These strains may contain *glnG* mutations that encode NRI proteins less responsive to NRII; they were not pursued further. The constitutive Dϕ suppressors bearing mutations in the *glnL* gene, hereafter referred to as *glnL** alleles, are summarized in Table 2. In total, 27 independent mutants were obtained in *glnL* that were distributed over the entire gene. Twenty-five of the mutations involved nucleotide changes resulting in single amino acid alterations in the protein, one involved a deletion, and one involved an insertion and duplication. The amino acid substitutions involved 17 different codons, with two of the mutations, D225V and S227N, being obtained multiple times. For three codons, 116, 227, and 303, multiple alterations to different amino acids were obtained. Two of the mutants, Δ267–270 and Y302N, also contained an additional silent mutation that does not result in an amino acid change in the protein. With the exception of two mutations, L83P and A138V, the current set of mutations was different from those previously isolated (5). Nine unique mutations were obtained in the C-terminal ATP-binding domain of NRII. Five of these clustered in a patch of amino acid residues from positions 225 to 228, one was a

TABLE 3. Effect of *glnL** alleles on the expression of the nitrogen-regulated *glnK* promoter under nitrogen-rich conditions in a wild-type *glnD* background

Strain[d]	Relevant genotype[b]	β-galactosidase activity[a] on GNGIn Trp + 5x CAA[c]
YMC10ϕ	Wild type	0 (1)
BK_cϕ	Δ*glnB* Δ*mdl-glnK*::Cam^r	2,100 (1)
L*(I12F)ϕ	*glnL* (I12F)	130
L*(L16R)ϕ	*glnL* (L16R)	1,470
L*(L83P)ϕ	*glnL* (L83P)	910
L*(E107K)ϕ	*glnL* (E107K)	1,980
L*(R116G)ϕ	*glnL* (R116G)	600
L*(R116C)ϕ	*glnL* (R116C)	200
L*(L185P)ϕ	*glnL* (L185P)	1,080
L*(G187R)ϕ	*glnL* (G187R)	900
L*(P188R)ϕ	*glnL* (P188R)	190
YMC10ϕ	Wild type	0 (1)
BK_gϕ	Δ*glnB* ΩGm^r Δ*glnK1*	1,790 (1)
Lϕ	Δ*glnL2001*	140
L*(A35V)ϕ	*glnL* (A35V)	110
L*(R115C)ϕ	*glnL* (R115C)	100
L*(E120K)ϕ	*glnL* (E120K)	300
L*(L122P)ϕ	*glnL* (L122P)	2,220
L*(A129T)ϕ	*glnL* (A129T)	540
L*(A138V)ϕ	*glnL* (A138V)	470
L*(G150S)ϕ	*glnL* (G150S)	50
L*(L154R)ϕ	*glnL* (L154R)	1,680
L*(G192D)ϕ	*glnL* (G192D)	470
YMC10ϕ	Wild type	0
BK_gϕ	Δ*glnB* ΩGm^r Δ*glnK1*	1,650
BK_gNϕ	Δ*glnB* ΩGm^r Δ*glnK1 nac*::Cam^r	1,680
L*(D225V)ϕ	*glnL* (D225V)	1,990
L*(S227N)ϕ	*glnL* (S227N)	1,240
L*(S227R)ϕ	*glnL* (S227R)	1,880
L*(S227I)ϕ	*glnL* (S227I)	1,950
L*(L228R)ϕ	*glnL* (L228R)	2,010
L*(Δ267–270)ϕ	*glnL* (Δ267–270)	840
L*(Y302N)ϕ	*glnL* (Y302N)	1,350
L*(P303L)ϕ	*glnL* (P303L)	720
L*(P303T)ϕ	*glnL* (P303T)	1,660

[a] β-Galactosidase activities are given in Miller units and are the average for duplicate cultures except where indicated by the number of cultures in parentheses. Results from duplicate cultures differed by <10%. Cultures were grown overnight in the indicated medium, diluted to an optical density at 600 nm of 0.02 and grown at 30°C to an optical density of 0.5.

[b] In addition to the genotype shown, all strains contain *trpDC700*::*putPA1303* [Kan^r *glnKp-lac*], which is a fusion of the *lacZYA* operon to the nitrogen-regulated *glnK* promoter.

[c] The medium used was GNGln Trp + 5x CAA (glucose-ammonia-glutamine-tryptophan plus Casamino Acids) and contained kanamycin. Glucose was present at 0.4% (wt/vol), ammonia and glutamine were present at 0.2% (wt/vol), tryptophan was present at 0.04 mg/ml, and 5× casein hydrolysate was present at 0.5% (wt/vol).

[d] The breaks in the table indicate experiments performed on separate days.

deletion of amino acids 267 to 270, and the other three involved the adjacent amino acids 302 and 303. A comparison of the positions of mutations isolated in the present study and in the 1992 study (5) is shown in Fig. 1.

Growth properties of the *glnL alleles.** Examination of the growth phenotype of the *glnL** alleles is hampered when X-Gal-containing medium is used, due to the intrinsic growth inhibition caused by X-Gal itself. To more accurately determine the growth phenotype of the *glnL** alleles, we examined the growth of the strains listed in Table 2 on glucose-ammonia-glutamine-tryptophan minimal medium lacking X-Gal. The results are summarized in Table 2 and indicate that mutations causing a severe growth phenotype were not limited to the C-terminal kinase domain of NRII.

As a first step in the analysis of the *glnL** alleles, they were transduced into a genetic background that contained a wild-type *glnD* locus and the *glnKp-lacZYA* fusion. We refer to the products of these backcrosses as L*ϕ strains. In addition, we transduced the *glnL** alleles isolated in 1992 that had the constitutive phenotype (5) into the same background to allow a comparison with the present mutations. The strain constructions were performed as described in Materials and Methods.

Figure 2 shows the poor-growth phenotype of representative L*ϕ strains on glucose-ammonia-glutamine-tryptophan minimal medium. All the *glnL** alleles that exhibited the poor-growth phenotype in the *glnD99*::Tn*10* background also exhibited the phenotype in a wild-type *glnD* background (Fig. 2 and data not shown). Interestingly, two of the *glnL** alleles from the 1992 study, L122P and L154R (L154R was mistakenly labeled as L154P in reference 5), exhibited the poor growth phenotype when transduced into a clean background whereas the original *glnD99*::Tn*10* suppressor strains bearing these *glnL** alleles did not exhibit this poor-growth phenotype (Fig 2 and data not shown). Sequencing of the *glnL* gene of the newly constructed strains revealed that these strains did in fact contain the L122P and L154R mutations (data not shown). Presumably, the original *glnD99*::Tn*10* suppressor strains containing these alleles picked up suppressor mutations in a gene other than *glnL,* such as *nac* (see below), which alleviated the poor-growth phenotype.

Previous studies indicated that the severe poor-growth phenotype of strain BK was due to high levels of NRI~P that resulted in overexpression of the *nac* gene encoding the transcription factor Nac (11) and that a null mutation in *nac* alleviated the poor-growth phenotype. We transduced the *glnL** alleles into a genetic background containing a null mutation in *nac* as described in Materials and Methods. We refer to these as L*Nϕ strains. The growth phenotype of the L*Nϕ strains was examined on glucose-ammonia-glutamine-tryptophan minimal medium as above. In every case where a *glnL** allele exhibited the poor-growth phenotype, the poor growth was alleviated by a null mutation in *nac* (Fig. 2 and data not shown).

Effect of the *glnL alleles on expression of the nitrogen-regulated *glnK* promoter in intact cells.** We examined expression of the *glnK* promoter in the L*ϕ strains in physiology experiments as described in Materials and Methods. The experiments were carried out in nitrogen rich glucose-ammonia-glutamine-tryptophan minimal medium supplemented with 5× CAA and containing kanamycin. The CAA ensured that the strains exhibiting the growth defect on minimal medium would grow. The results are summarized in Table 3. YMC10ϕ (wild type) exhibited normal nitrogen regulation of the *glnK* promoter, while BKϕ exhibited unregulated high expression of the *glnK* promoter, as expected. The *nac* mutation had no effect on *glnK* promoter expression, as indicated by strain BKNϕ. We also examined *glnK* promoter expression in a strain bearing a deletion of *glnL* (Lϕ). In this strain, the low level of *glnK* promoter expression is due to phosphorylation of NRI by the metabolic intermediate acetyl- phosphate (18). The L*ϕ strains all exhibited unregulated expression of the *glnK* promoter to various extents, varying from levels similar to those observed in Lϕ to those observed in BKϕ. The L*ϕ strains exhibiting the highest levels of *glnK* promoter expression contained *glnL** alleles that confer the growth phenotype. These results suggest that the *glnL** alleles encode NRII proteins that lack phosphatase activity, have elevated kinase activity, or both. Presumably the *glnL** alleles exhibiting low levels of *glnK* expression similar to strain Lϕ encode NRII proteins that are only slightly defective in phosphatase activity or have only slightly elevated kinase activity, although this experiment does not rule out the possibility that these mutants encode completely inactive proteins.

We examined ammonia regulation in strains bearing the *glnL** alleles by assaying *glnK* promoter expression in the L*Nϕ strains containing a mutation in *nac* along with the *glnL** alleles and the *glnKp-lacZYA* fusion. The *nac* mutation allowed us to grow the strains in the absence of CAA; thus, we were able to compare *glnK* expression on minimal medium with or without ammonia. Table 4 shows the results obtained with representative strains. YMC10ϕ exhibited normal regulation by ammonia, and BKNϕ exhibited high expression of *glnK* regardless of the nitrogen status, as expected. The *nac* mutation did not have any effect on *glnK* expression. We observed that strain BKNϕ retained some regulation of *glnK* expression by ammonia. Since this strain lacks PII and GlnK and cannot negatively regulate NRI~P, the regulation observed probably stems from phosphorylation of NRI by acetyl phosphate. The L*Nϕ strains exhibited high expression of *glnK* regardless of the nitrogen status of the medium. The results obtained in the absence of ammonia, where PII and GlnK should be fully uridylylated, suggest that the *glnL** alleles encode NRII proteins that are active kinases. The results obtained in both media, taken together, suggest that the *glnL** alleles encode NRII proteins that lack the phosphatase activity to various extents. Lesions in NRII could lead to defective phosphatase activity for various reasons including loss of catalytic residues, loss of residues involved in conformational changes associated with PII activation of the phosphatase activity, and loss of residues required for PII binding.

Purification of mutant NRII proteins. To understand the molecular basis for our in vivo observations, we purified representative mutant NRII proteins and characterized their properties in vitro. Plasmids causing the hyperexpression of NRII mutants were constructed as described in Materials and Methods. All the mutants that were cloned were overexpressed, but many of the mutant proteins were found to be insoluble when overexpressed at 44°C from the pJLA503 plasmid (data not shown). In particular, none of the N-terminal domain mutants that were cloned were produced in soluble form expressed from pJLA503. However, we obtained proteins

TABLE 4. Effect of *glnL** alleles on the expression of the nitrogen regulated *glnK* promoter under nitrogen-limiting and nitrogen-replete conditions in a *nac* background

Strain[d]	Relevant genotype[b]	β-Galactosidase activity[a] on:	
		GGlnTrp[c]	GNGlnTrp[c]
YMC10ϕ	Wild type	2,340 (1)	0 (1)
Nϕ	*nac*::Cam^r	1,900 (1)	0 (1)
BK_gNϕ	Δ*glnB* ΩGm^{r}Δ *glnK1 nac*::Cam^r	7,340 (1)	5,830 (1)
Lϕ	Δ*glnL 2001*	400 (1)	60 (1)
L*(I12F)Nϕ	*glnL* (I12F) *nac*::Cam^r	4,800	850
L*(L16R)Nϕ	*glnL* (L16R) *nac*::Cam^r	5,830	4,930
L*(R116C)Nϕ	*glnL* (R116C) *nac*::Cam^r	4,750	1,020
L*(A129T)Nϕ	*glnL* (A129T) *nac*::Cam^r	4,700	1,900
L*(L154R)Nϕ	*glnL* (L154R) *nac*::Cam^r	5,410	4,500
L*(G187R)Nϕ	*glnL* (G187R) *nac*::Cam^r	4,540	3,100
YMC10ϕ	Wild type	1,330	0
Nϕ	*nac*::Cam^r	1,390	0
BK_gNϕ	Δ*glnB* ΩGm^{r}Δ *glnK1 nac*::Cam^r	5,540	3,380
L*(D225V)Nϕ	*glnL* (D225V) *nac*::Cam^r	5,180	3,480
L*(S227N)Nϕ	*glnL* (S227N) *nac*::Cam^r	3,990	2,550
L*(S227R)Nϕ	*glnL* (S227R) *nac*::Cam^r	5,170	3,780
L*(S227I)Nϕ	*glnL* (S227I) *nac*::Cam^r	4,630	3,240
L*(L228R)Nϕ	*glnL* (L228R) *nac*::Cam^r	5,130	4,050
L*(Δ267–270)Nϕ	*glnL* (Δ267–270) *nac*::Cam^r	3,100	1,660
L*(Y302N)Nϕ	*glnL* (Y302N) *nac*::Cam^r	4,200	2,900
L*(P303L)Nϕ	*glnL* (P303L) *nac*::Cam^r	3,440	1,600
L*(P303T)Nϕ	*glnL* (P303T) *nac*::Cam^r	4,530	3,530

[a] β-Galactosidase activities are given in Miller units and are the average for duplicate cultures except where indicated by the number of cultures in parentheses. Results from duplicate cultures differed by <10%. Cultures were grown overnight in the indicated medium, diluted to an optical density at 600 nm of 0.02, and grown at 30°C to an optical density of 0.5.

[b] In addition to the genotype shown, all strains contain *trpDC700*::*putPA1303* [Kan^r *glnKp-lac*], which is a fusion of the *lacZYA* operon to the nitrogen regulated *glnK* promoter.

[c] Media used were G GlnTrp (glucose-glutamine-tryptophan) and GN Gln Trp (glucose-ammonia-glutamine-tryptophan) and contained kanamycin. Glucose was present at 0.4% (wt/vol), ammonia and glutamine were present at 0.2% (wt/vol), and tryptophan was present at 0.04 mg/ml.

[d] The break in the table indicates experiments performed on separate days.

with mutations in the C-terminal region of NRII that were overexpressed in soluble form, permitting purification. We chose to purify two proteins with mutations in the C-terminal kinase domain, NRII(S227R) and NRII(Y302N), both of which caused a severe growth phenotype in cells. The S227R mutation lies in a cluster of mutations obtained within the N-terminal part of the ATP-binding domain of NRII (residues 225 to 228), while the Y302N mutation lies within a cluster of mutations obtained in the C-terminal part of this domain (residues 302 to 303). In small-scale tests, these two mutant proteins seemed to be the most soluble of those mapping to the two clusters and exhibiting the severe growth phenotype in cells. In addition, we purified the previously studied constitutive NRII mutant NRII2302 (2, 25, 26, 34, 39, 43, 57), hereafter referred to as NRII (A129T). We chose this mutation because it was expressed in soluble form and because it was representative of a number of mutations that were obtained in the central domain of NRII spread throughout residues 111 to 154 (5; this study). The A129T protein does not bring about the poor growth phenotype in cells. The three mutants, NRII (S227R), NRII (Y302N), NRII (A129T), and the wild-type protein were purified to ~95% purity as described in Materials and Methods.

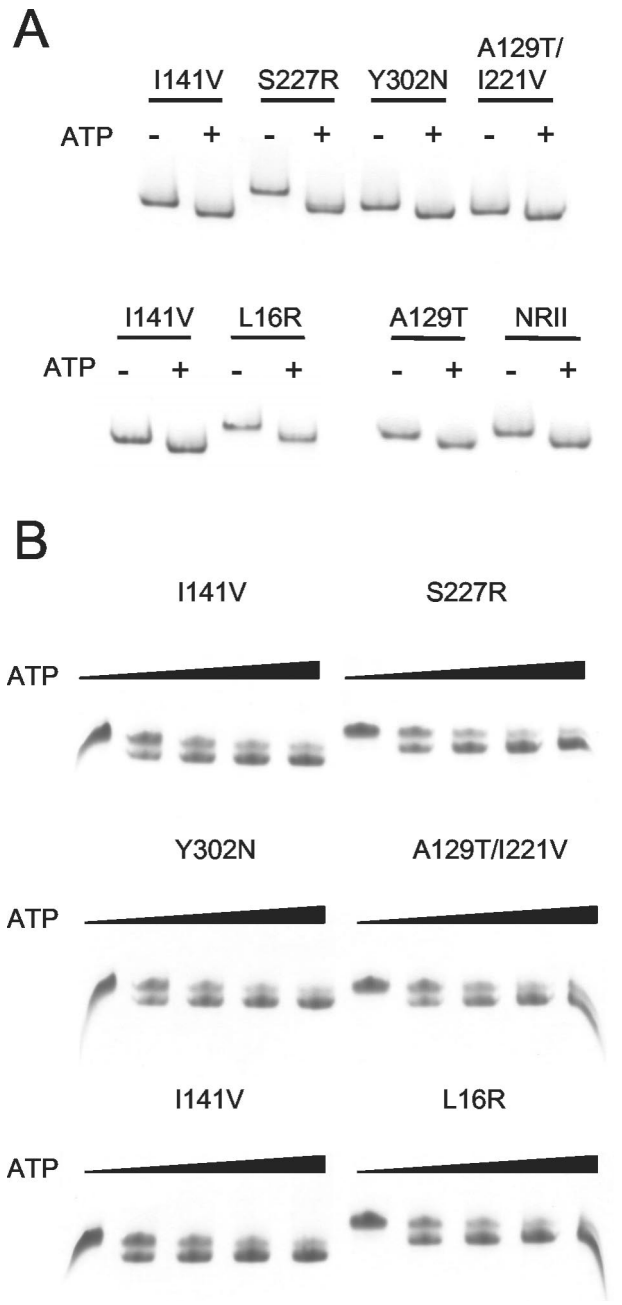

FIG. 3. Gel analysis of the autophosphorylation activities of NRII mutants. (A) Nondenaturing polyacrylamide gel electrophoresis of autophosphorylation reaction mixtures. NRII (2 μM) or mutant forms of NRII were incubated on ice for 20 min in reaction mixtures containing 0.5 mM ATP or as indicated, and autophosphorylation reactions were performed as described in Materials and Methods. Reactions were stopped by addition of 50 mM EDTA, and the mixtures were analyzed on 10% nondenaturing polyacrylamide gels stained with Coomassie brilliant blue R-250. The panel shows results from three separate gels. (B) Urea-polyacrylamide gel electrophoresis of autophosphorylation reactions. The indicated NRII proteins (2 μM) were incubated on ice for 30 min in reaction mixtures containing 0, 0.02, 0.1, 0.5, or 2 mM ATP (left to right), as described in Materials and Methods. Reactions were stopped by addition of 4 M urea, and the mixtures were analyzed on 10% polyacrylamide–6 M urea gels stained with Coomassie brilliant blue R-250. The panel shows results from three separate gels.

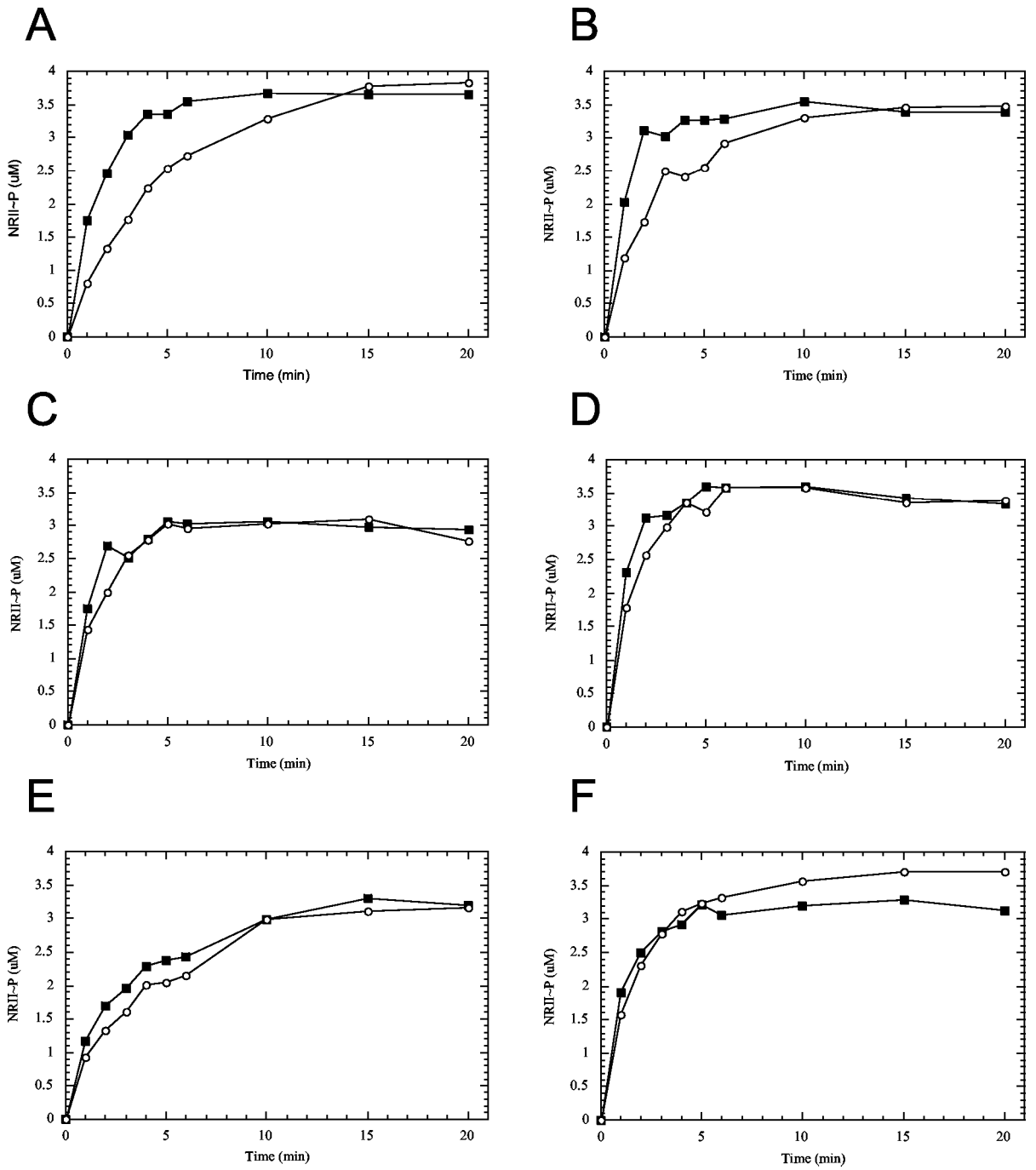

FIG. 4. Effect of PII on the autophosphorylation activities of NRII mutants. NRII (2 μM) or mutant forms of NRII were incubated on ice in reaction mixtures containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 0.5 mg of BSA per ml, 50 μM 2-ketoglutarate, and 0.5 mM [γ-^{32}P]ATP in the absence (■) or presence (○) of 12 μM PII. At various times, aliquots were spotted onto nitrocellulose filters and analyzed as described in Materials and Methods. (A) NRII; (B) NRII (I141V); (C) NRII (S227R); (D) NRII (Y302N); (E) NRII (L16R); (F) NRII (A129T).

To obtain an N-terminal domain mutant expressed in soluble form, we cloned the *glnL* (L16R) gene into the IPTG-inducible expression plasmid pSJ4, as described in Materials and Methods. The pSJ4 plasmid is a derivative of pET30a (Novagen) allowing the expression of N-terminally histidine-tagged proteins at 37°C. The plasmid encodes a fusion protein consisting of a 29-amino-acid tag at the N terminus of the protein, including a sequence of eight histidine residues, a spacer, and a cleavage site for rTEV protease (Invitrogen Life Technologies) followed by the protein of interest (Z. Xu, personal communication). Histidine-tagged NRII (L16R) expressed from the pSJ4 plasmid was soluble (data not shown). The tagged NRII (L16R) was purified to ~95% purity as described in Materials and Methods. For the in vitro experi-

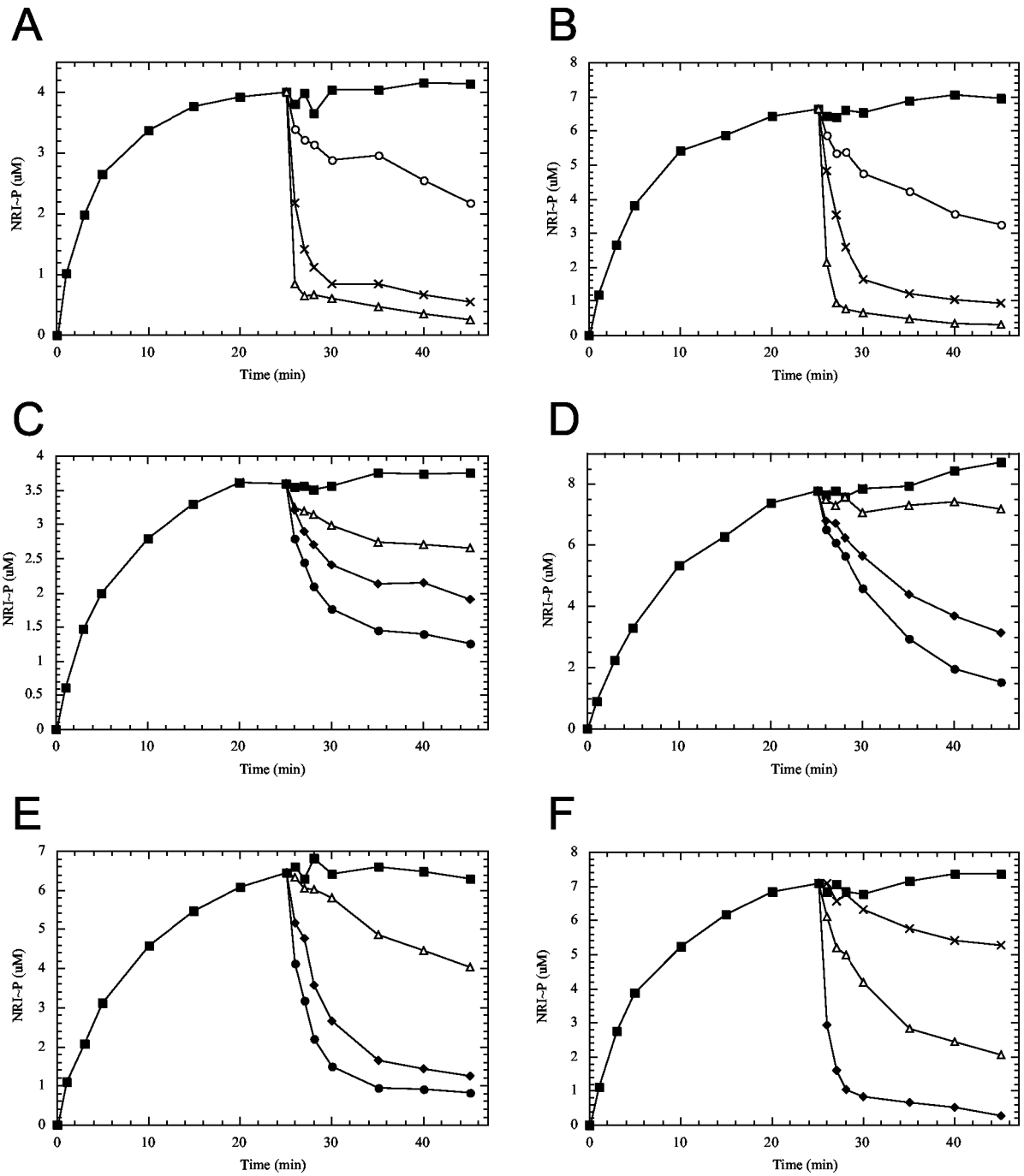

FIG. 5. Kinase and phosphatase activities of NRII mutants. Reaction mixtures containing 30 μM NRI-N, 0.3 μM NRII (or mutant NRII), 50 μM 2-ketoglutarate, and 0.5 mM [γ-^{32}P]ATP were incubated at 25°C. After 25 min, the reaction mixtures were split into tubes containing buffer (■), 0.075 μM PII (○), 0.15 μM PII (×), 0.30 μM PII (△), 1.0 μM PII (◆), or 3.0 μM PII (●). At the indicated times, aliquots were spotted onto nitrocellulose filters and analyzed as described in Materials and Methods. (A) NRII; (B) NRII (I141V); (C) NRII (S227R); (D) NRII (Y302N); (E) NRII (L16R); (F) NRII (A129T).

ments described below, we used NRII (L16R) that had the tag removed by protease digestion with rTEV protease as described in Materials and Methods. The cleaved NRII (L16R), hereafter referred to as NRII (L16R), contains an additional three amino acids at the N terminus of the protein (Gly-Ser-His).

After much of the work described below in this paper was completed, we discovered that the overexpression plasmids encoding wild-type NRII and NRII (A129T), designated pLOP and pLOP15, respectively (Table 1), contained extraneous mutations. DNA sequencing of the plasmids revealed that pLOP encodes NRII (I141V), containing a mutation in the central domain of NRII, near the active-site histidine, and pLOP15 encodes the double-mutant NRII (A129T/I221V), containing

an extra mutation in the C-terminal ATP- and PII-binding domain. These mutations probably arose during PCR amplification of the *glnL* gene with *Taq* polymerase. We constructed plasmids encoding the "true" wild-type NRII and a "true" NRII (A129T) as described in Materials and Methods. We overexpressed the correct versions of the proteins and purified them as above to ~95% purity. Results are presented below for both the pLOP- and pLOP15-encoded proteins containing extraneous mutations and the correct proteins, and they are clearly labeled with the identity of the protein. Effects of the extraneous mutations are described where appropriate below.

The NRII mutants exhibit normal autophosphorylation. The autophosphorylation activity of NRII can be assessed qualitatively by nondenaturing (native) polyacrylamide gel electrophoresis (21). On these gels, the phosphorylated NRII dimers migrate faster than do the unphosphorylated NRII dimers, allowing assessment of the fraction of NRII that is active. NRII (I141V), NRII (S227R), NRII (Y302N), NRII (A129T/I221V), NRII (L16R), wild-type NRII, and NRII (A129T) all exhibited normal autophosphorylation (Fig. 3A), since qualitatively all of the NRII became phosphorylated in reaction mixtures that contained excess ATP. At least qualitatively, the extraneous I141V and I221V mutations had no effect on the autophosphorylation activity. Previous results indicated that the phosphorylated species on nondenaturing gels corresponds to the hemiphosphorylated dimer, consisting of NRII dimers in which one subunit is phosphorylated (21). A simple qualitative test for the asymmetry of autophosphorylation involves analysis of phosphorylated NRII on urea-polyacrylamide gels. These gels allow separation of the unphosphorylated and phosphorylated subunits of NRII (21). All of the NRII proteins examined here exhibited normal asymmetry of autophosphorylation as assayed by the urea-polyacrylamide gel technique (Fig. 3B).

PII does not significantly affect the autophosphorylation activities of the NRII mutants. The autophosphorylation activity of the NRII proteins was quantitatively assessed in reaction mixtures containing [γ-^{32}P]ATP, as indicated in Materials and Methods. Previous results indicated that the PII protein inhibits the rate of NRII autophosphorylation while slightly increasing the stoichiometry of phosphorylation (21). In the absence of PII, wild-type NRII, NRII (I141V), NRII (S227R), NRII (Y302N), and NRII (A129T) all exhibited similar rates and levels of autophosphorylation while NRII (L16R) exhibited a slightly diminished rate of autophosphorylation (Fig. 4). In the presence of excess PII (12 μM), only the wild-type protein and NRII (I141V) clearly displayed a diminished rate of autophosphorylation, while the other mutant proteins were less affected by the presence of PII. Similar experiments with the NRII (A129T/I221V) protein indicated that it was the same as the A129T protein (data not shown). Thus, the I141V and I221V mutations seemed to have no effect on NRII autophosphorylation.

The NRII mutants are active kinases but lack phosphatase activity to various extents. We examined the purified NRII mutants for their ability to serve as NRI kinases and for their ability to bring about the dephosphorylation of NRI~P in the presence of the PII protein. We used a truncated version of NRI consisting of the amino-terminal receiver domain (amino acids 1 to 118, NRI-N) as the substrate for phosphorylation and dephosphorylation in an assay described previously (26, 39) and performed as described in Materials and Methods. Previous studies indicated that NRI-N was sufficient to serve as a substrate for NRII and was capable of being dephosphorylated in the presence of NRII and PII (20, 26, 28). Figure 5A shows typical results obtained with wild-type NRII, where under our experimental conditions, approximately 13% of the NRI-N became phosphorylated after 25 min and subsequent addition of PII to 0.3 μM (trimer) was capable of bringing about the rapid dephosphorylation of NRI-N~P. By comparison, under the same conditions, NRII (I141V) brought about phosphorylation of ~22% of the NRI-N substrate (Fig. 5B); that is, the I141V mutation apparently increased the ability of NRII to act as an NRI-N kinase. As observed with wild-type NRII, NRII (I141V) was a potent NRI-N~P phosphatase on addition of PII (Fig. 5B). In contrast, NRII (S227R) displayed wild-type kinase activity but was a very poor phosphatase in the presence of an equimolar ratio of PII (Fig. 5C). The phosphatase activity of NRII (S227R) was increased at elevated concentrations of PII (Fig. 5C). NRII (Y302N) exhibited elevated kinase activity, resulting in the phosphorylation of ~25% of the NRI-N under our experimental conditions, and was a very poor phosphatase in the presence of PII (Fig. 5D). As with NRII (S227R), NRII (Y302N) phosphatase activity was increased at elevated PII concentrations (Fig. 5D). NRII (L16R) also exhibited elevated kinase activity (~22% of NRI-N phosphorylated) and poor phosphatase activity, albeit slightly better than that of NRII (S227R) and NRII (Y302N) (Fig. 5E).

We reexamined the phosphatase activity of the intensively studied NRII mutant, NRII (A129T). Previous results indicated that this mutant did not display obvious phosphatase activity in the presence of PII (25, 26, 39). In two of those studies, the NRII (A129T/I221V) protein was studied (25, 26). Since those studies were carried out in the absence of 2-ketoglutarate, an activator of PII when present at low concentration (23, 27, 31), the deficiency in phosphatase activity of NRII (A129T) was probably overstated. We observed that NRII (A129T) exhibited elevated kinase activity, as reported previously (39), and was partially defective as a phosphatase (Fig. 5F). When assayed at elevated PII concentrations (1 μM), this protein could bring about the nearly complete dephosphorylation of NRI-N~P (Fig. 5F). Thus, NRII (A129T) retained more phosphatase activity than did NRII (S227R), NRII (Y302N), and NRII (L16R). These results are consistent with the in vivo results obtained above, which indicated that in intact cells NRII (A129T) was more efficient at bringing about the negative regulation of the *glnK* promoter than were NRII (S227R), NRII (Y302N), and NRII (L16R) (Tables 3 and 4). Results obtained with the NRII (A129T/I221V) protein were identical to those obtained with NRII (A129T) indicating that the extraneous I221V mutation had no apparent effect (data not shown).

PII-binding ability of the NRII mutants. The results above indicated that the purified NRII (S227R), NRII (Y302N), NRII (L16R), and NRII (A129T) proteins were defective in phosphatase activity. To determine whether the lack of phosphatase activity was due to a decreased ability to interact with PII, we assayed PII binding in a cross-linking assay described previously (44). NRII (I141V), NRII (Y302N), NRII (A129T/

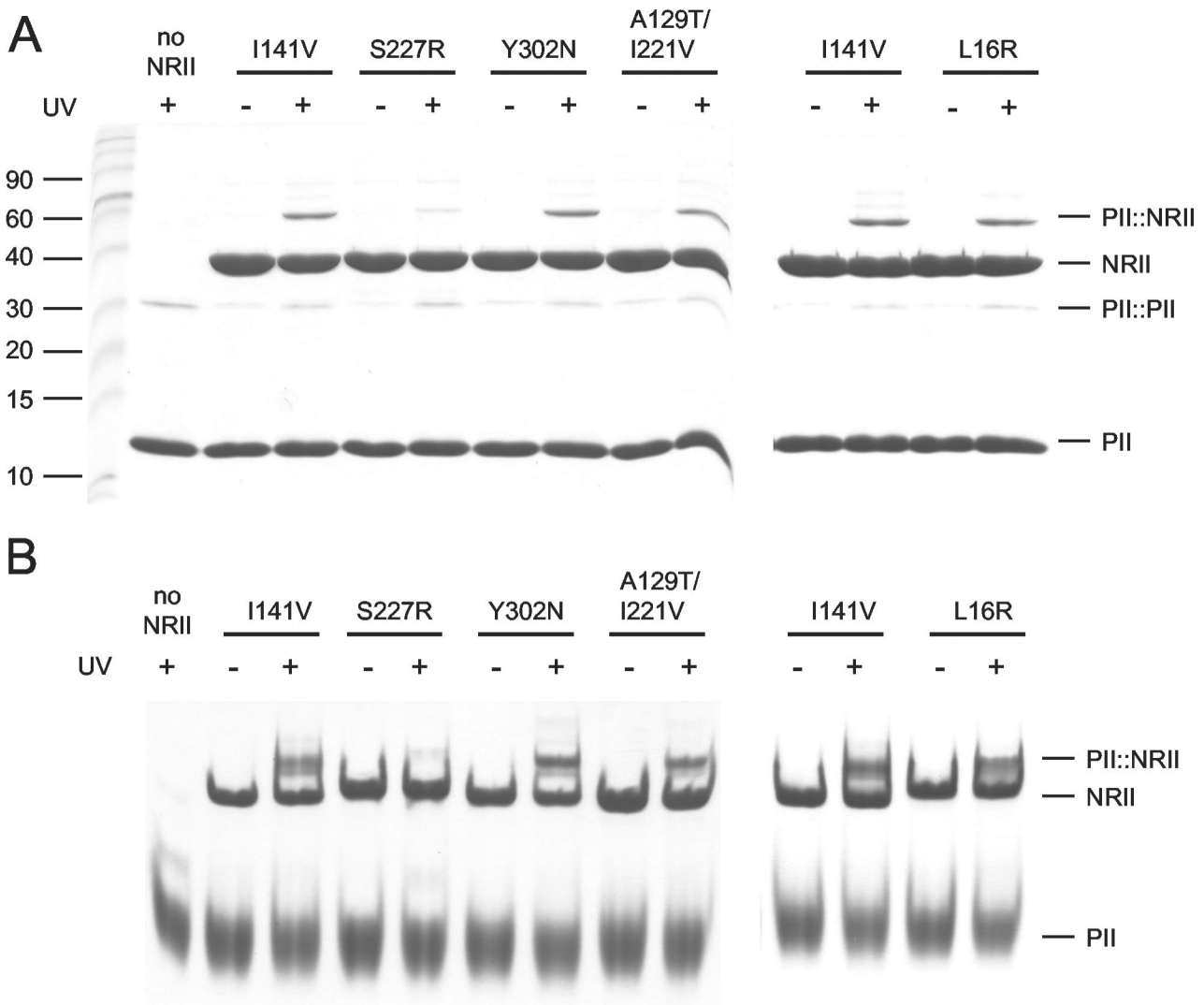

FIG. 6. Cross-linking of PII to NRII mutants. PII (E44C/C73S)-TFPAM-3 (9 μM) was incubated with the indicated NRII protein (4.5 μM) in reaction mixtures containing 50 μM 2-ketoglutarate and 0.5 mM ATP as described in Materials and Methods. Cross-linking was initiated by exposure to UV light for 20 min as indicated. (A) Reactions were stopped by addition of SDS gel-loading buffer, and the mixtures were analyzed on SDS–15% polyacrylamide gels. Molecular mass markers are indicated in kilodaltons. (B) Alternatively, reactions were stopped by addition of 120 mM glycine (pH 9.0) and the mixtures were analyzed on 10% nondenaturing polyacrylamide gels. For both panels, the gels were stained with Coomassie brilliant blue R-250.

I221V), and NRII (L16R) were all cross-linked to PII in UV-dependent reactions, while NRII (S227R) exhibited severely diminished cross-linking (Fig. 6). In additional experiments, we observed that wild-type NRII behaved identically to NRII (I141V) (data not shown). These results suggest that the S227R mutation affects PII binding while the other mutations do not. The I141V and I221V mutations seemed to have no effect on PII binding.

As another assay for PII-NRII interaction, we used a chromatographic method in which the elution profile of a mixture of the proteins from a gel filtration column was examined (reviewed in reference 9). PII and NRII (I141V) coeluted from a gel filtration column when the column was preequilibrated in buffer containing the PII allosteric effectors ATP and 2-ketoglutarate at appropriate concentrations (Fig. 7A). Control reactions indicated that PII and NRII (I141V) did not coelute in the absence of ATP and 2-ketoglutarate, demonstrating specificity (data not shown). We took advantage of this result to examine the binding of mutant forms of NRII to wild-type PII. The reaction mixtures contained an excess of the PII protein, such that under suitable binding conditions PII eluted as two peaks corresponding to NRII-bound and free PII. PII coeluted from the gel filtration column with NRII (Y302N), NRII (A129T/I221V), and NRII (L16R), although the results indicated modestly reduced binding relative to the NRII (I141V) protein (Fig. 7C, D, and E, respectively). In contrast, PII failed to coelute with NRII (S227R), while NRII (S227R) eluted from the column later, at the position of free NRII, indicating that NRII (S227R) failed to bind PII in this assay (Fig. 7B). These results, taken together with the cross-linking assay results, strongly suggest that serine 227 of NRII is required for the interaction with PII and that NRII (S227R) is a poor

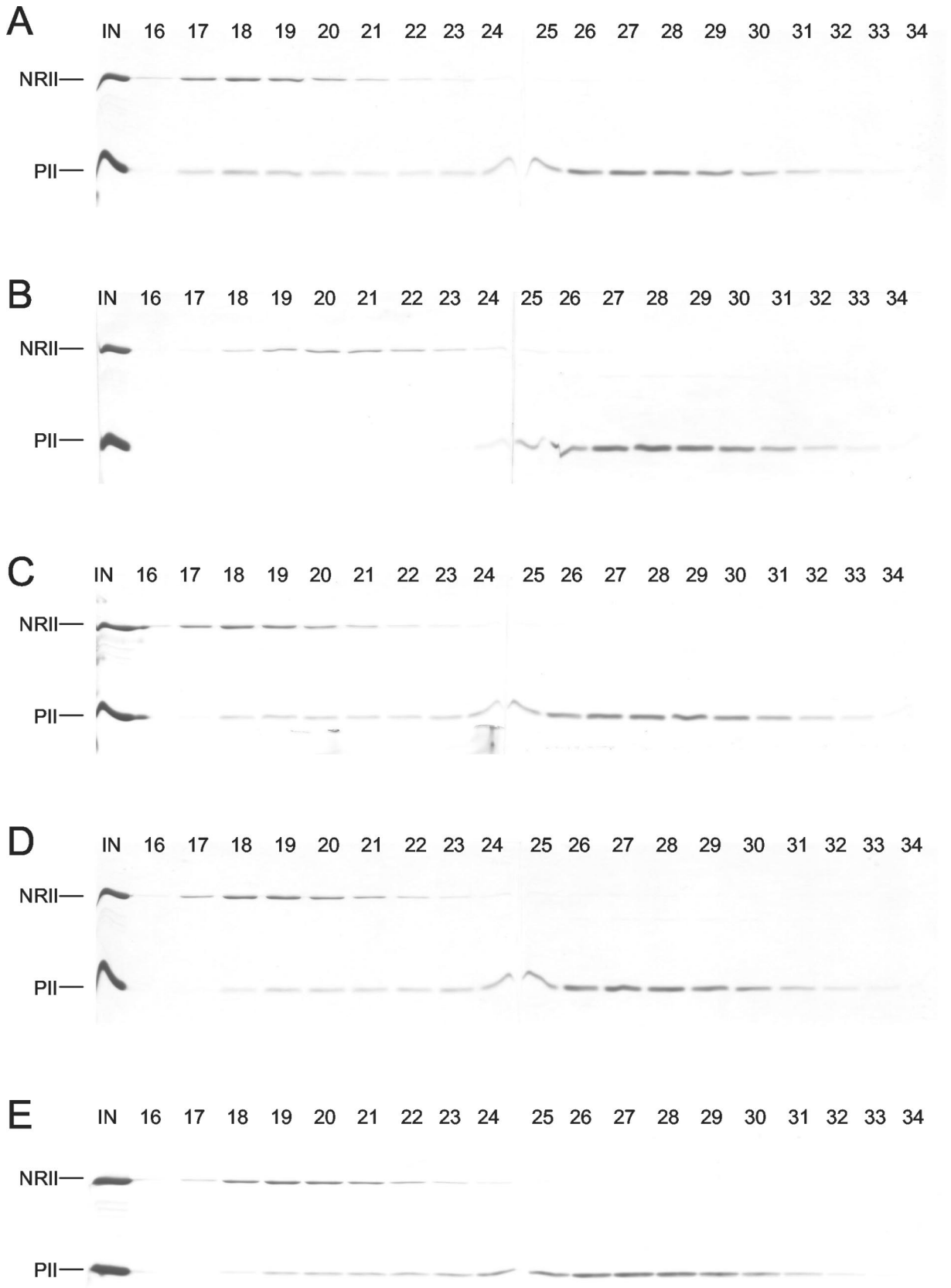

FIG. 7. Gel filtration assay for PII binding to NRII mutants. PII (48 μM) and the indicated NRII protein (12 μM) were mixed on ice in reaction mixtures containing 1 mM $MgCl_2$, 50 μM free 2-ketoglutarate, and 0.5 mM free ATP, as indicated in Materials and Methods. The bulk of the reaction mixture was loaded onto a Sephadex G-100 column equilibrated in buffer containing 1 mM $MgCl_2$, 50 μM 2-ketoglutarate, and 0.5 mM ATP and eluted at 4°C. Fractions were collected and analyzed on SDS–15% polyacrylamide gels stained with Coomassie brilliant blue R-250. Fraction numbers are indicated above the lanes, and IN indicates the column input. (A) NRII (I141V); (B) NRII (S227R); (C) NRII (Y302N); (D) NRII (A129T/I221V); (E) NRII (L16R).

phosphatase due to its inability to bind PII. Conversely, the results suggest that the Y302N, A129T/I221V, and L16R mutations do not directly affect PII binding and that these mutants are poor phosphatases for another reason.

DISCUSSION

Our results indicate that all three domains of NRII play a role in the PII-activated phosphatase activity and that two distinct surfaces of the C-terminal ATP-binding domain are involved in this activity. The approximate location of these surfaces on the C-terminal domain of NRII may be surmised by comparing the locations of the mutations to the known structure of the related EnvZ transmitter protein (Fig. 8). Since the structures of the ATP-binding domains of three different histidine kinases have been solved (EnvZ, PhoQ, and CheA [10, 33, 52]) and since these are all very similar, it is reasonable to expect that the corresponding domain from NRII has a similar structure. One of the mutation clusters (residues 302 to 303) maps to the lid over the ATP-binding site, while the other cluster (residues 225 to 228) maps to the "back" or opposite side of the domain (Fig. 8). A mutation in the latter cluster, S227R, was observed to severely diminish the binding of PII to NRII; therefore, this surface may correspond to the PII-binding site of NRII. Interestingly, the deletion mutation (Δ267–270) maps to a region adjacent to the 225 to 228 cluster (Fig. 8), suggesting that alteration of residues 267 to 270 may also affect PII binding, although we did not directly test the PII binding of this mutant. Comparison of our proposed PII-binding region of NRII to other transmitter proteins in GenBank indicates nonconservation of this part of the transmitter proteins, with only NRII proteins sharing sequences around positions 225 to 228. Our results with the purified A129T/I221V protein are consistent with those of a previous study showing that NRII (A129T) interacted with PII in a yeast two-hybrid assay (34).

Since all three domains of NRII are required for the PII-activated NRII phosphatase activity (19; this study), our results are not consistent with the idea of distinct and modular functions of the NRII domains. Rather, they are consistent with the idea that the three domains of NRII interact in a concerted fashion with little flexibility, as previously suggested by studies of the asymmetry of NRII autophosphorylation (21). In addition to mutations spread throughout the NRII domains, mutation clusters were obtained in regions of NRII that probably correspond to "hinge" regions between the domains (e.g., residues 185 to 192), again suggesting a concerted interaction between domains. Indeed, one would expect reduced PII binding to result from mutations that block conformational changes required for the phosphatase activity, and several of our mutations that did not appear to directly affect PII binding did result in a modest reduction in the binding of PII when assayed by the sensitive gel filtration method. We hypothesize that the A129T, Y302N, and L16R mutations affected the phosphatase activity at steps after PII binding.

From a physiological perspective, the PII-activated phosphatase activity of NRII is its most important activity, since cells can grow well on minimal medium in the absence of NRII kinase activity (13, 18) but display a severe growth defect in the absence of the NRII phosphatase activity (2, 7, 11). This growth defect is due to the hyperexpression of Nac that occurs in the absence of regulation of the concentration of NRI~P (11). Our results indicate that mutations in NRII eliminating its phosphatase activity resulted in the Nac-mediated growth

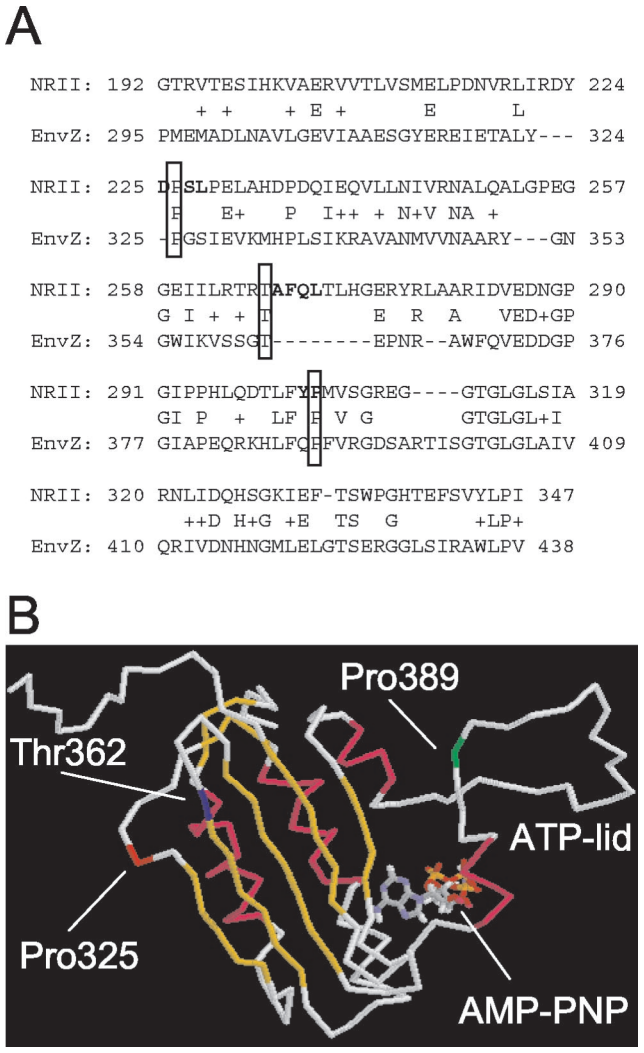

FIG. 8. Structure of the kinase domain of EnvZ modeled to show the approximate positions of the mutations obtained in NRII. (A) Amino acid sequence alignment of the kinase domains of NRII and EnvZ. The alignment was generated using a basic BLAST search of the *E. coli* genome with full-length NRII (accession number AAC76866) as the query (1). The residues shown in bold indicate the positions of the mutations obtained in the kinase domain of NRII. Shown boxed are conserved residues that were used as "anchor" points to represent positions of mutations obtained in NRII. Pro226 (NRII)/Pro325 (EnvZ) represents the cluster of mutations obtained at positions 225 to 228, Thr266 (NRII)/Thr362 (EnvZ) represents the deletion of residues 267 to 270, and Pro303 (NRII)/Pro389 (EnvZ) represents the cluster of mutations obtained at positions 302 to 303. (B) Nuclear magnetic resonance imaging structure of the kinase domain of EnvZ (52) shown as an alpha-carbon trace. Alpha-helices are shown in magenta, and beta-strands are shown in yellow. The AMP-PNP molecule is shown in stick representation with CPK coloring. The positions of Pro325 (representing the 225-to-228 cluster), Thr362 (representing the deletion), and Pro389 (representing the 302-to-303 cluster) are shown in red, blue, and green, respectively. The figure was generated using RasMol.

defect previously associated with the simultaneous absence of PII and GlnK. Thus, this growth defect is clearly shown to be due to loss of control of the NRI~P concentration. Since we used cells that contain PII and GlnK, the growth defect cannot be ascribed to the action of another PII/GlnK receptor protein.

The mechanism of the PII-activated NRII phosphatase activity is not addressed by our study; nevertheless, some speculations are possible. Previous studies showed that the isolated central domain of NRII, containing the active-site histidine residue involved in the phosphotransfer reaction, displayed very weak phosphatase activity (19, 29). This is consistent with the identification of mutations in the central domain of NRII that reduce the phosphatase activity (5; this study). Conceivably, the N-terminal and C-terminal domains of NRII are required to allow the binding of PII to shift the central domain into the conformation with potent phosphatase activity. PII influences the conformation of the central domain by binding to the "back" of the C-terminal ATP-binding domain. The lid over the ATP-binding site is also required for the phosphatase activity; this part of NRII may be necessary for transmission of the signal provided by PII binding to the back of the C-terminal domain or may form part of the "phosphatase active site". The "ATP site lid" must, by definition, map near to the active-site histidine in the intact dimer, since the transfer of the phosphoryl group from ATP to the active-site histidine in the central domain requires apposition of these segments of NRII. The N-terminal domain of NRII may act as an "anvil" to limit the flexibility of the central domain and allow it to be forced into the conformation with phosphatase activity. The L16R mutation may affect the ability of the central domain to be put into the phosphatase conformation.

Previous studies of other transmitter proteins have suggested that their N-terminal domains act as sensory domains and that in some cases transmembrane signaling results in control of the kinase and phosphatase activities of the cytoplasmic transmitter module by an extracellular N-terminal domain (15, 53). However, our studies show that PII controls NRII by binding to its C-terminal domain (44; this study). To reconcile these findings, we speculate that the family of transmitter proteins may be designed to integrate multiple signals, with sensation by both the C-terminal and N-terminal domains. For example, another (unknown) intracellular signal may regulate NRII activities by interaction with its N-terminal domain.

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REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Atkinson, M. R., T. A. Blauwkamp, and A. J. Ninfa.** 2002. Context-dependent functions of the PII and GlnK signal transduction proteins in *Escherichia coli*. J. Bacteriol. **184:**5364–5375.
3. **Atkinson, M. R., T. A. Blauwkamp, V. Bondarenko, V. Studitsky, and A. J. Ninfa.** 2002. Activation of the *glnA*, *glnK*, and *nac* promoters as *Escherichia coli* undergoes the transition from nitrogen-excess growth to nitrogen starvation. J. Bacteriol. **184:**5358–5363.
4. **Atkinson, M. R., E. S. Kamberov, R. L. Weiss, and A. J. Ninfa.** 1994. Reversible uridylylation of the *Escherichia coli* PII signal transduction protein regulates its ability to stimulate the dephosphorylation of the transcription factor nitrogen regulator I (NRI or NtrC). J. Biol. Chem. **269:**28288–28293.
5. **Atkinson, M. R., and A. J. Ninfa.** 1992. Characterization of mutations in the *glnL* gene of *Escherichia coli* affecting nitrogen regulation. J. Bacteriol. **174:**4538–4548.
6. **Atkinson, M. R., and A. J. Ninfa.** 1993. Mutational analysis of the bacterial signal-transducing protein kinase/phosphatase nitrogen regulator II (NRII or NtrB). J. Bacteriol. **175:**7016–7023.
7. **Atkinson, M. R., and A. J. Ninfa.** 1998. Role of the GlnK signal transduction protein in the regulation of nitrogen assimilation in *Escherichia coli*. Mol. Microbiol. **29:**431–447.
8. **Atkinson, M. R., and A. J. Ninfa.** 1999. Characterization of the GlnK protein of *Escherichia coli*. Mol. Microbiol. **32:**301–314.
9. **Beeckmans, S.** 1999. Chromatographic methods to study protein-protein interactions. Methods **19:**278–305.
10. **Bilwes, A. M., L. A. Alex, B. R. Crane, and M. I. Simon.** 1999. Structure of CheA, a signal-transducing histidine kinase. Cell **96:**131–141.
11. **Blauwkamp, T. A., and A. J. Ninfa.** 2002. Nac-mediated repression of the *serA* promoter of *Escherichia coli*. Mol. Microbiol. **45:**351–363.
12. **Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. **72:**248–254.
13. **Bueno, R., G. Pahel, and B. Magasanik.** 1985. Role of *glnB* and *glnD* gene products in regulation of the *glnALG* operon of *Escherichia coli*. J. Bacteriol. **164:**816–822.
14. **Carr, P. D., E. Cheah, P. M. Suffolk, S. G. Vasudevan, N. E. Dixon, and D. L. Ollis.** 1996. X-ray structure of the signal transduction protein PII from *Escherichia coli* at 1.9 Å. Acta Crystallogr. Sect. D **52:**93–104.
15. **Cavicchioli, R., R. C. Chiang, L. V. Kalman, and R. P. Gunsalus.** 1996. Role of the periplasmic domain of the *Escherichia coli* NarX sensor-transmitter protein in nitrate-dependent signal transduction and gene regulation. Mol. Microbiol. **21:**901–911.
16. **Cheah, E., P. D. Carr, P. M. Suffolk, S. G. Vasudevan, N. E. Dixon, and D. L. Ollis.** 1994. Structure of the *Escherichia coli* signal transducing protein PII. Structure **2:**981–990.
17. **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.
18. **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.
19. **Jiang, P., M. R. Atkinson, C. Srisawat, Q. Sun, and A. J. Ninfa.** 2000. Functional dissection of the dimerization and enzymatic activities of *Escherichia coli* nitrogen regulator II and their regulation by the PII protein. Biochemistry **39:**13433–13449.
20. **Jiang, P., and A. J. Ninfa.** 1999. Regulation of the autophosphorylation of *Escherichia coli* NRII by the PII signal transduction protein. J. Bacteriol. **181:**1906–1911.
21. **Jiang, P., and A. J. Ninfa.** 2000. Asymmetry in the autophosphorylation of the two-component system transmitter protein NRII (NtrB) of *Escherichia coli*. Biochemistry **39:**5058–5065.
22. **Jiang, P, J. Peliska, and A. J. Ninfa.** 1998. Enzymological characterization of the signal-transducing uridylyltransferase/uiridylyl-removing enzyme (E.C. 2.7.7.59) of *Escherichia coli* and its interaction with the PII protein. Biochemistry **37:**12782–12794.
23. **Jiang, P., J. Peliska, and A. J. Ninfa.** 1998. Reconstitution of the signal transduction bicyclic cascade responsible for the regulation of Ntr gene transcription in *Escherichia coli*. Biochemistry **37:**12795–12801.
24. **Jiang, P., P. Zucker, M. R. Atkinson, E. S. Kamberov, W. Tirasophon, P. Chandran, B. R. Schefke, and A. J. Ninfa.** 1997. Structure/function analysis of the PII signal transduction protein of *Escherichia coli:* genetic separation of interactions with protein receptors. J. Bacteriol. **179:**4342–4353.
25. **Kamberov, E. S., M. R. Atkinson, P. Chandran, and A. J. Ninfa.** 1994. Effect of mutations in *Escherichia coli glnL* (*ntrB*), encoding nitrogen regulator II (NRII or NtrB), on the phosphatase activity involved in bacterial nitrogen regulation. J. Biol. Chem. **269:**28294–28299.
26. **Kamberov, E. S., Atkinson, M. R., P. Chandran, J. Feng, and A. J. Ninfa.** 1994. Signal transduction components controlling bacterial nitrogen assimilation. Cell. Mol. Biol. Res. **40:**175–191.
27. **Kamberov, E. S., M. R. Atkinson, and A. J. Ninfa.** 1995. The *Escherichia coli* PII signal transduction protein is activated upon binding 2-ketoglutarate and ATP. J. Biol. Chem. **270:**17797–17807.
28. **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.
29. **Kramer, G., and V. Weiss.** 1999. Functional dissection of the transmitter

module of the histidine kinase NtrB in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **96:**604–609.
30. **Kustu, S., E. Santero, D. Pophan, and J. Keener.** 1989. Expression of σ^{54} (*ntrA*)-dependent genes is probably united in a common mechanism. Microbiol. Rev. **54:**367–376.
31. **Liu, J., and B. Magasanik.** 1995. Activation of the dephosphorylation of nitrogen regulator I-phosphate of *Escherichia coli*. J. Bacteriol. **177:**926–931.
32. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Marina, A., C. Mott, A. Auyzenberg, W. A. Hendrickson, and C. D. Waldburger.** 2001. Structural and mutational analysis of the PhoQ histidine kinase catalytic domain: insight into the reaction mechanism. J. Biol. Chem. **276:** 41182–41190.
34. **Martinez-Argudo, I., P. Salinas, R. Maldonado, and A. Contreras.** 2002. Domain Interactions on the *ntr* signal transduction pathway: two-hybrid analysis of mutant and truncated derivatives of histidine kinase NtrB. J. Bacteriol. **184:**200–206.
35. **Ninfa, A. J., and M. R. Atkinson.** 2000. PII signal transduction proteins. Trends Microbiol. **8:**172–179.
36. **Ninfa, A. J., and D. P. Ballou.** 1998. Fundamental laboratory approaches for biochemistry and biotechnology. Fitzgerald Science Press, Bethesda, Md.
37. **Ninfa, A. J., E. Brodsky, and B. Magasanik.** 1989. The role of NRI phosphate in the activation of transcription from the nitrogen-regulated promoter *glnA*p2. UCLA Symp. Mol. Cell. Biol. **95:**43–52.
38. **Ninfa, A. J., P. Jiang, M. R. Atkinson, and J. A. Peliska.** 2000. Integration of antagonistic signals in the regulation of bacterial nitrogen assimilation. Curr. Top. Cell. Regul. **36:**32–76.
39. **Ninfa, A. J., and B. Magasanik.** 1986. Covalent modification of the *glnG* product, NRI, by the *glnL* product, NRII, regulates the transcription of the *glnALG* operon in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **83:**5909–5913.
40. **Ninfa, A. J., L. J. Reitzer, and B. Magasanik.** 1987. Initiation of transcription at the bacterial *glnAp2* promoter by purified *Escherichia coli* components is facilitated by enhancers. Cell **50:**1039–1046.
41. **Ninfa, A. J., S. Ueno-Nishio, T. P. Hunt, B. Robustell, and B. Magasanik.** 1986. Purification of nitrogen regulator II, the product of the *glnL* gene of *Escherichia coli*. J. Bacteriol. **168:**1002–1004.
42. **Ninfa, E. G., M. R. Atkinson, E. S. Kamberov, and A. J. Ninfa.** 1993. Mechanism of autophosphorylation of *Escherichia coli* NRII: trans-phosphorylation between subunits. J. Bacteriol. **175:**7024–7032.
43. **Pahel, G., A. D. Zelentz, and B. M. Tyler.** 1978. *gltB* gene and regulation of nitrogen metabolism by glutamine synthetase in *Escherichia coli*. J. Bacteriol. **133:**139–148.
44. **Pioszak, A. A., P. Jiang, and A. J. Ninfa.** 2000. The *Escherichia coli* PII signal transduction protein regulates the activities of the two-component system transmitter protein NRII by direct interaction with the kinase domain of the transmitter module. Biochemistry **39:**13450–13461.
45. **Popham, D. L., D. Szeto, J. Keener, and S. Kustu.** 1989. Function of a bacterial activator protein that binds to transcriptional enhancers. Science **243:**629–635.
46. **Saiki, R. K.** 1990. Amplification of genomic DNA, p. 13–20 *In* M. A. Innis, D. H., Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, San Diego, Calif.
47. **Sanders, D. A., B. L. Gillece-Castro, A. L. Burlingame, and D. E. Koshland, Jr.** 1992. Phosphorylation site of NtrC, a protein phosphatase whose covalent intermediate activates transcription. J. Bacteriol. **174:**5117–5122.
48. **Schauder, B., H., Blocker, R., Frank, and E. G. J. McCarthy.** 1987. Inducible expression vectors incorporating the *Escherichia coli atpE* translation initiation region. Gene **52:**279–283.
49. **Schneider, B. L., A. K. Kiupakis, and L. J. Reitzer.** 1998. Arginine catabolism and the arginine succinyltransferase pathway in *Escherichia coli*. J. Bacteriol. **180:**4278–4286.
50. **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.
51. **Su, W., S. Porter, S. Kustu, and H. Echols.** 1990. DNA-looping and enhancer activity: association between DNA-bound NtrC activator and RNA polymerase at the *glnA* promoter. Proc. Natl. Acad. Sci. USA **87:**5504–5508.
52. **Tanaka T, S. K. Saha, C. Tomomori, R. Ishima, D. Liu, K. I. Tong, H. Park, R. Dutta, L. Qin, M. B. Swindells, T. Yamazaki, A. M. Ono, M. Kainosho, M. Inouye, and M. Ikura.** 1998. NMR structure of the histidine kinase domain of the *E. coli* osmosensor EnvZ. Nature **396:**88–92.
53. **Utsumi, R., R. E. Brisette, A. Rampersaud, S. A. Forst, K. Oosawa, and M. Inouye.** 1989. Activation of bacterial porin gene expression by a chimeric signal transducer in response to aspartate. Science **245:**1246–1249.
54. **van Heeswijk, W. C., S. Hoving, D. Molenaar, B. Stegeman, D. Kahn, and H. V. Westerhoff.** 1996. An alternative PII protein in the regulation of glutamine synthetase in *Escherichia coli*. Mol. Microbiol. **21:**133–146.
55. **Weiss, V., and B. Magasanik.** 1988. Phosphorylation of nitrogen regulator I (NRI) of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **85:**8919–8923.
56. **West, A. H., and A. M. Stock.** 2001. Histidine kinases and response regulator proteins in two-component signaling systems. Trends Biochem. Sci. **26:**369–376.
57. **Zimmer, D. P., E. Soupene, H. L. Lee, V. F. Wendisch, A. B. Khodursky, B. J. Peter, R. A. Bender, and S. Kustu.** 2000. Nitrogen regulatory protein C-controlled genes of *Escherichia coli*: scavenging as a defense against nitrogen limitation. Proc. Natl. Acad. Sci. USA **97:**14674–14679.