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Effects of Insertions and Deletions in *glnG* (*ntrC*) of *Escherichia coli* on Nitrogen Regulator I-Dependent DNA Binding and Transcriptional Activation

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Phosphorylated nitrogen regulator I (NR_I, also called NTRC), encoded by *glnG* (*ntrC*), stimulates transcription in *Escherichia coli* and other enteric bacteria from sites analogous to eukaryotic enhancers. We isolated 30 mutants, obtained without phenotypic selection, that have either a small insertion or deletion within *glnG*. Mutants were classified by the ability of NR_I to repress the $glnAp_1$ and *glnL* promoters and to activate two versions of the nitrogen-regulated $glnAp_2$ promoter; each activity was measured in cells grown with three concentrations of NR_I. The results were interpreted within the framework of the three-domain hypothesis of NR_I structure. NR_I is thought to contain a phosphorylated regulatory domain that controls binding of ATP, a central domain that hydrolyzes ATP and interacts with RNA polymerase, and a DNA-binding region of unknown extent. Our results suggest that the 70 amino acids from residue 400 to the carboxyl terminus constitute a DNA-binding domain that includes residues for specific contacts and dimerization. Our results also suggest that (i) transcription from $glnAp_2$ without specific NR_I-binding sites requires binding to sites with some similarity to the specific sites, and (ii) if an NR_I variant can stimulate transcription, then increasing the concentration of NR_I diminishes *glnA* expression for all mutants but one.

The *glnALG* operon in enteric bacteria is essential for growth in a nitrogen-limited environment. The genes of *glnALG* code for glutamine synthetase, nitrogen regulator II (NR_{II}; also called NTRB), and nitrogen regulator I (NR_I; also called NTRC), respectively (reviewed in references 24 and 35). NR_I-phosphate, together with RNA polymerase associated with σ^{54}, stimulates transcription from the $glnAp_2$ promoter (15, 17, 21, 28, 29). NR_{II} controls both the phosphorylation of NR_I and the dephosphorylation of NR_I-phosphate (19, 28). The activity of NR_{II} is determined by two regulatory proteins, P_{II} and the bifunctional uridylyltransferase/uridylyl-removing enzyme. During nitrogen-limited growth, NR_{II} phosphorylates NR_I. However, when nitrogen is abundant in the environment, NR_{II}, in conjunction with P_{II}, participates in the dephosphorylation of NR_I-phosphate.

NR_I binds to specific sequences upstream from the start site of transcription regardless of the degree of covalent phosphorylation—i.e., even when NR_I is in its transcriptionally inactive conformation (15, 29, 42). However, phosphorylation does affect binding by enhancing cooperative interactions between adjacent molecules bound to DNA (50). Neither NR_I nor NR_I-phosphate facilitates the binding of RNA polymerase near the start site of transcription (9, 15, 29, 42). NR_I-binding sites, like eukaryotic enhancers, can stimulate transcription from sites that are more than 1,400 bases upstream or downstream from the start site of transcription (29, 34). These sites can activate even when the RNA polymerase binding site is on a separate but topologically linked molecule (48). Several lines of evidence show that NR_I-phosphate from these sites contacts RNA polymerase with looping out of the intervening DNA (36, 47, 48). This contact catalyzes the isomerization of closed promoter complexes to open complexes (29, 31). Isomerization also requires the binding and hydrolysis of ATP by NR_I-phosphate (49).

This description of NR_I-dependent activation is undoubtedly a simplification. The hydrolysis of ATP and transcription from $glnAp_2$ may require an interaction between two dimers of NR_I-phosphate (49, 50). In addition, any model of activation must also consider two unusual features of regulation. Unlike other bacterial activators, NR_I-phosphate can stimulate transcription from $glnAp_2$ after deletion of its specific binding sites (20, 34, 43). We refer to such a promoter as the minimal $glnAp_2$ promoter. Although the mechanism of this activation is not entirely understood, it appears to be important. Some *glnG* mutants fail to grow with either arginine or proline as a nitrogen source, and NR_I from these mutants cannot stimulate transcription from the minimal promoter. In these mutant strains, transcription from the $glnAp_2$ promoter with the NR_I-binding sites was normal (43). These observations suggest that promoters similar to the minimal $glnAp_2$ promoter are physiologically important. Another unusual feature of NR_I-dependent regulation of *glnA* is the inhibitory effect of high levels of NR_I-phosphate. This inhibition is a major determinant in establishing the steady-state level of glutamine synthetase for wild-type cells (45).

Comparison with proteins homologous to NR_I, together with biochemical studies of NR_I and genetic analysis of *glnG*, suggests that NR_I contains three functional domains: a phosphorylatable amino-terminal regulatory domain (residues 1 to 120), a central domain (residues 140 to 380) that binds and hydrolyzes ATP and contacts RNA polymerase, and a putative DNA-binding helix-turn-helix structure near the carboxy terminus (residues 444 to 463) (6, 10–13, 16, 19, 38, 46, 49). Primary goals of this work were to determine the effect of disruptions in each region of NR_I and to further delineate the DNA-binding domain. Another goal was to

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analyze unusual aspects of regulation from $glnAp_2$ by correlating partial loss of function with different activities of NR_I. To these ends, we have isolated and characterized 30 different mutants, obtained without phenotypic selection, that contain either small insertions or deletions within *glnG*.

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MATERIALS AND METHODS

Strains. LR1 (*glnG10*::Tn*5* *lacI*^{q} *lacL8*) is a derivative of *Escherichia coli* K-12 strain W3110 (43). The *glnA* promoter region is intact in LR1 and all of its derivatives. Therefore, glutamine synthetase activity from nitrogen-limited cells is a measure of transcription from the wild-type $glnAp_2$ promoter (with all of the NR_I-binding sites) on the chromosome. Reporter strains, used to monitor separate aspects of $glnAp_2$ transcription, were derived from LR1 and contain derivatives of phage λ132 (43, 45). Phage λgln105 contains a fusion of the minimal $glnAp_2$ promoter—i.e., $glnAp_2$ without apparent NR_I-binding sites—to *lacZ*. Phage λgln107 contains a fusion of the NR_I-repressible *glnL* promoter to *lacZ*.

Cell growth and enzyme assays. The minimal medium (W salts) used for growth of cells, growth and harvesting of cells for enzymatic assays, and assays for glutamine synthetase and β-galactosidase have been described previously (25, 39). The minimal medium for nitrogen-limited growth contained W salts, 0.4% glucose, and 0.2% glutamine; the minimal medium for carbon-limited growth contained W salts, 0.4% glycerol, 0.2% $(NH_4)_2SO_4$, and 0.2% glutamine. When applicable, β-galactosidase and glutamine synthetase were assayed from the same cell extract. Enzyme activities are expressed as units (micromoles per minute) per milligram of protein.

Plasmid constructions. The plasmids generated for this study are listed in Table 1 and are derivatives of pBLS1 (43), except pSP55, which is derived from pgln55 (see below). All plasmids contain a fusion of the *lacUV5* promoter to *glnG*. To construct the 26 mutants listed in the first part of Table 1, we employed a form of linker mutagenesis that generates in-frame insertions within NR_I (4). The linkers were single-stranded hexameric oligonucleotides containing a sequence complementary to either a 2- or 4-base restriction site overhang. Linker insertion created a new restriction site from the combination of linker sequences and adjacent nucleotides at the insertion site. Insertion of the linkers resulted in the addition of two codons, except when indicated otherwise.

The mutant plasmids were generated as follows. Plasmid pBLS1 was digested with either *Hin*PI, *Hpa*II, or *Taq*I. These sites were chosen because there are 27 *Hin*PI, 12 *Hpa*II, and 7 *Taq*I sites within *glnG*. An empirically determined concentration of ethidium bromide was included in the digestion mixture to limit digestion to a single site (30). Linearized DNA was purified after electrophoresis in a low-melting-point agarose gel. Digestion with *Hin*PI, *Hpa*II, or *Taq*I results in a 5′ GC overhang. The plasmid DNA was then ligated to linkers, either CGGGCC or CGTCGA; the CG of the linker hybridizes to the GC overhang left by the restriction enzyme. Ligation of the linker to these sites created a unique restriction site, either *Apa*I or *Sal*I (Table 1). To remove excess linkers, we treated the ligated DNA mixture with either *Apa*I or *Sal*I. A gene coding for resistance to kanamycin, obtained from plasmid pUC4kapa (Pharmacia) by digestion with either *Apa*I or *Sal*I, was ligated to linker-containing DNA. Resistance to kanamycin was used to select the mutants. The marker gene was subsequently removed, and the plasmid was religated. Usually, the site of the insertion could be unambiguously located after digestion with a combination of restriction enzymes that cut the plasmids frequently. Ambiguities are noted in Table 1; when there are ambiguities, knowledge of the exact location of the insertions would not strengthen or detract from the conclusions of this work. We tested for two or more inserts by analyzing for a restriction site that would not be found in plasmids with a single insert. For example, tandem CGGGCC linkers would have created an *Sma*I site (CCCGGG).

Different methods were used to construct the seven mutants listed in the bottom part of Table 1. Plasmid pBLS7 is a deletion derivative of pBLS1. Plasmid pBLS1 is essentially pBR322, except that the *tet* gene between *Eco*RI and *Pvu*II is replaced with a fusion of the *lacUV5* promoter to a promoterless *glnG*. The region between the *lac* promoter and *glnG* codes for the ribosome-binding site and the first six codons of *glnA*. This is followed by the last 42 codons of *glnL*, followed by the entire *glnG* gene. NR_I from cells with pBLS1 is the same size as NR_I from wild-type cells (44). A deletion between the *Asp* 718 and *Eco*RV sites of pBLS1 deletes the first 139 codons of *glnG* and creates a fusion of *glnA* to *glnG*. The sequence of this fusion protein before residue 140 of NR_I is M-S-A-E-H-V-K-L-V-P.

Plasmid pCB12 contains a deletion of residues 143 to 398 of NR_I and was constructed from pSP57 and pSP68. Both parental plasmids contain a unique *Sal*I site at the insertion site. The *Pst*I-*Sal*I fragment of pSP68 containing the amino-terminal end of *glnG* and the carboxy-terminal half of *bla* was ligated to the *Pst*I-*Sal*I fragment of pSP57 that contained the carboxy-terminal end of *glnG* and the amino-terminal region of *bla*. The resulting plasmid, pCB12, has an intact *bla* gene and an *Sal*I site in place of residues 143 to 398 of NR_I. Plasmid pCB30 contains a deletion of residues 23 to 398 of NR_I. The construction of pCB30 was identical to that of pCB12, except that the starting plasmids were pSP66 and pSP57.

Plasmids pSP4, pSP5, and pSP8 were constructed from pBLS1 with linkers inserted into one of three *Mlu*I sites within *glnG*. Linearized full-length DNA, which had been cut once with *Mlu*I, was blunt-ended, isolated, and ligated to an 8-bp *Hin*dIII linker. NR_I from pSP4 acquired 4 amino acids (RKLA) between residues 348 and 349 of *glnG*. Both pSP5 and pSP8 have insertions that contain stop codons that result in deletion of a few residues near the extreme carboxyl terminus of NR_I. They were constructed like pSP4, except that the *Hin*dIII linker was inserted into the *Mlu*I site close to the end of *glnG* (at codon 460, which is left intact by this procedure). The plasmids were then digested with *Hin*dIII and blunted by the Klenow fragment; codons 461 and 462 of *glnG* were restored by this step. Plasmid pSP8 was created by adding a 12-bp *Nhe*I linker (CTAGCTAGCTAG) to the DNA with the filled-in *Hin*dIII linker. NR_I from pSP8 is a 462-amino-acid polypeptide with a 6-amino-acid deletion from the carboxyl terminus. The construction of pSP5 was similar to that of pSP8 except that before insertion of the *Nhe*I linker, a second *Hin*dIII linker was added, cut with *Hin*dIII, and filled in. The introduction of the second *Hin*dIII linker restored codon 463. NR_I from pSP5 contains 464 residues; the first 463 are identical to wild-type NR_I (468 residues), but residue 464 is leucine instead of the glutamate present in wild-type NR_I.

TABLE 1. Plasmid list

Mutant plasmid constructed by:	Amino acids added[a] or deleted[b]	Insertion site in *glnG*	Oligonucleotide inserted
Linker mutagenesis of *glnG*			
pSP6	36::G-P	*Hin*PI	CGGGCC[c]
pSP7	185/186::G-P[d]		
pSP15	448::G-P		
pSP17	406::G-P		
pSP19	185::R-A		
pSP20	252::R-A		
pSP23	252::(R-A)*n*[e]		
pSP27	193::G-P		
pSP37	185/186::G-P[d]		
pSP40	310::R-A		
pSP59	412::S-T	*Hin*PI	CGTCGA[f]
pSP60	406::S-T		
pSP61	36::V-D		
pSP62	64::S-T		
pSP63	116/117[g]::R-R/V-D		
pSP64	71::R-R		
pSP66	22::S-T		
pSP67	185/186/189/193[g]::X-X		
pSP68	142::S-T		
pSP56	341::S-T	*Hpa*II	CGTCGA[f]
pSP56A	341::(S-T)*n*[e]		
pSP57	398::S-T		
pSP70	9::V-D	*Taq*I	CGTCGA[f]
pSP71	Δ238–283; 237-V-D-284[b]		
pSP72	283::V-D		
pSP73	237::V-D		
Other methods[h]			
pBLS7[i]	Δ1–139	NA[j]	NA
pCB12	Δ143–398;142-S-T-399[b]	NA	NA
pCB30	Δ23–398;22-S-T-399[b]	NA	NA
pSP4	348::R-K-L-A	*Mlu*I	CGCGCAAGCTTG
pSP5	Δ465–468;464E→L[b]		CGCGCAAGCTCAAGCTCTAGCTAGCTAGCTAG[k]
pSP8	Δ463–468[b]		CGCGCAAGCTCTAGCTAGCTAGCTAG[k]
pSP55	128::Q-A-S-L	*Fnu*DII	CAAGCTAGCTTG

[a] The residue before the insertion and the residues inserted are indicated. For example, for pSP6, glycine and proline have been inserted between codons 36 and 37 of NR_I.
[b] The deleted codons (inclusive) and the residues that replace the deleted amino acids are indicated.
[c] This linker creates an *Apa*I site.
[d] The possible site of insertion in pSP7 and pSP37 is either codon 185 or 186. These two plasmids are different because cells with either plasmid have a different phenotype (Table 3). In either case, G-P would be inserted. Knowing the exact site of insertion would not change our conclusions.
[e] The insertion in this plasmid contains multiple linkers.
[f] This linker creates an *Sal*I site.
[g] The insertion sites in these plasmids could not be localized with certainty because there are two or more *Hin*PI sites within these regions.
[h] Described in Materials and Methods.
[i] A more complete description of this plasmid is provided in Materials and Methods.
[j] NA, not applicable.
[k] The underlined sequence is the stop codon.

Plasmid pgln55 has an 8-bp *Hin*dIII linker (CAAGCTTG) inserted in an *Fnu*DII site within *glnG*. This plasmid was digested with *Hin*dIII, and the single-stranded overhangs were filled in. The ligation product was pSP55. The insertion in pSP55 results in a 4-amino-acid insertion (QASL) between residues 128 and 129 of NR_I.

Immunological detection of NR_I. The experimental procedures described by Sambrook et al. (40) were followed with some modifications. The transfer buffer contained 25 mM Tris base, 192 mM glycine, and 20% methanol. The protein concentration of the cell extract was determined as described previously (23). Polyclonal anti-NR_I antiserum (rabbit), anti-rabbit immunoglobulin G-biotin conjugate, and ExtrAvidin-alkaline phosphatase conjugate (Sigma) were used for immunodetection. The buffer for immunoassay contained 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 0.05% (vol/vol) Tween 20. The chromogenic method for detection of alkaline phosphatase has been described (40). The buffer for the chromogenic reaction contained 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 50 mM $MgCl_2$.

TABLE 2. Activities of wild-type NR_I in LR1/pBLS1[a]

Measured parameter (assay method)	Plasmid and genotype	Effect of NR_I concentration on NR_I-dependent activities (mU/mg of protein)[b]		
		Low	Intermediate	High
Activity from the wild-type $glnAp_2$ promoter[c] (glutamine synthetase)	pBLS1(*lacUV5p-glnG*$^+$)	5,150 (267)	1,890 (204)	911 (56)
	pBR322(*glnG*)	151 (12)	150 (11)	154 (14)
Activity from the minimal $glnAp_2$ promoter[d] (β-galactosidase)	pBLS1(*lacUV5p-glnG*$^+$)	479 (42)	1,494 (63)	1,820 (128)
	pBR322(*glnG*)	138 (14)	152 (15)	244 (30)
Repression: *glnLp* assay[e] (β-galactosidase)	pBLS1(*lacUV5p-glnG*$^+$)	147 (53)	188 (56)	245 (17)
	pBR322(*glnG*)	802 (14)	854 (20)	891 (69)
Repression: $glnAp_1$ assay[f] (glutamine synthetase)	pBLS1(*lacUV5p-glnG*$^+$)	72 (6)	53 (11)	100 (23)
	pBR322(*glnG*)	217 (13)	198 (23)	217 (15)

[a] The results are the average of 16 independent assays for wild-type $glnAp_2$ promoter activity, 7 assays for minimal $glnAp_2$ promoter activity, and 4 DNA-binding assays. Numbers in parentheses are the standard errors of the mean.

[b] The expression of NR_I was induced by three different concentrations of IPTG, which result in low, intermediate, and high intracellular concentrations of NR_I relative to levels found in wild-type cells. These concentrations are discussed in Results.

[c] Cells were grown in nitrogen-limited medium.

[d] Cells lysogenic for λgln105 were grown in nitrogen-limited medium. Phage λgln105 contains a minimal $glnAp_2$ promoter (without NR_I-binding sites) fused to *lacZ*.

[e] Cells lysogenic for λgln107 were grown in nitrogen-limited medium. λgln107 contains the NR_I-repressible *glnL* promoter fused to *lacZ*.

[f] Cells were grown in carbon-limited medium; under this condition, *glnA* is expressed from the NR_I-repressible $glnAp_1$ promoter.

RESULTS

Strategy. We generated mutations within *glnG* by insertion of small oligonucleotides (Materials and Methods) because the effects of such mutations can be relatively mild (4, 5). We mutagenized *glnG* in pBLS1, which contains a fusion of the *lacUV5* promoter to *glnG*. We screened for mutagenized plasmids in LR1, in which the transposon Tn*5* disrupts the chromosomal *glnG*. The use of plasmid-borne *glnG* simplified mutagenesis and mutant screening and avoided the complications of autogenous regulation. An additional advantage of this overall strategy is that mutants could be isolated without phenotypic selection.

Classification of mutants. Three parameters were assayed from the mutants: measures of transcription from two types of $glnAp_2$ promoters (wild-type and the minimal promoter) and an indirect measure of DNA binding by assessment of NR_I-dependent repression of transcription from $glnAp_1$. Each activity was measured from cells at three concentrations of NR_I. We manipulated the intracellular concentration of NR_I by varying the concentration of isopropyl-β-D-thiogalactopyranoside (IPTG). We refer to the levels of NR_I that result from the three concentrations of IPTG as low, intermediate, and high (45). A low concentration of NR_I (in LR1/pBLS1 grown without IPTG) is a concentration lower than that found in nitrogen-limited wild-type cells; a high level of NR_I (cells grown with 1 mM IPTG) is a level higher than that in wild-type cells. An intermediate concentration of IPTG is defined as the concentration that induces the level of glutamine synthetase found in nitrogen-limited wild-type cells. The level of glutamine synthetase is very sensitive to changes in the level of NR_I (45). Therefore, we assumed that the concentration of NR_I in LR1/pBLS1 that results in a wild-type level of glutamine synthetase is the same concentration of NR_I found in nitrogen-limited wild-type cells. This assumption was verified by immunodetection of NR_I (44).

Table 2 shows the levels of these three activities measured from LR1/pBLS1 (*glnG*$^+$); Table 3 summarizes these activities for 30 mutants. Glutamine synthetase activity, a measure of transcription from the wild-type $glnAp_2$ promoter on the chromosome, varied inversely with the concentration of NR_I, as observed previously (45). High concentrations of NR_I inhibit transcription from $glnAp_2$ by a mechanism that is not fully understood. Transcription from the minimal $glnAp_2$ promoter (without specific NR_I-binding sites) on the chromosome was assessed by measuring β-galactosidase activity from λgln105 lysogens. Its activity rose with increasing NR_I (43). These results were expected and have been analyzed elsewhere (43, 45).

Specific binding to DNA in vivo was generally assessed by one of two different methods, although sometimes both were used. For the first method, cells were grown in carbon-limited medium and then glutamine synthetase activity was determined. With this growth medium, *glnA* transcription initiates from the NR_I-repressible $glnAp_1$ promoter (33). Therefore, in wild-type cells, as well as in plasmid-bearing LR1 cells, *glnA* expression (glutamine synthetase activity) is a measure of binding of unphosphorylated NR_I to the two high-affinity sites that overlap $glnAp_1$. It is not known whether repression requires one or two molecules of NR_I; however, repression probably involves cooperative binding to DNA. The second method measures binding of NR_I-phosphate to DNA. λgln107 lysogens contain the NR_I-repressible *glnL* promoter fused to *lacZ* (45). Measurement of β-galactosidase activity in these lysogens allows in vivo assessment of the binding of NR_I-phosphate to a single high-affinity site when cells are nitrogen limited. Wild-type NR_I at all concentrations repressed the *glnL* and $glnAp_1$ promoters equally well, suggesting that either assay provides a qualitative assessment of binding to specific sites (Table 2). However, in one mutant, our assays suggested that a variant NR_I repressed $glnAp_1$ but not *glnLp*. Because this result suggests that the $glnAp_1$ assay may be more sensitive than

TABLE 3. NR_I-dependent activities in mutants as a function of concentration of NR_I

Group	Mutant plasmid	Lesion	Effect of concentration of NR_I on:						
			DNA binding[a]			Wild-type $glnAp_2$ activity[b,c]		Minimal $glnAp_2$ promoter activity[b,d]	
			Low	Intermediate	High	Low	Intermediate	Intermediate	High
1	pSP17	406::G-P	−	−	−	−	−	−	−
	pSP60[e]	406::S-T	−	−	−	−	−	−	−
	pSP15[e]	448::G-P	−	−	−	−	−	−	−
	pSP8	Δ463–468	−	−	−	−	−	−	−
2	pSP6[e]	36::G-P	−	−	+	−	−	−	−
	pSP55[e]	128::Q-A-S-L	−	−	+	−	−	−	−
	pSP7[e]	185/186::G-P	−	−	+	−	−	−	−
	pSP71	Δ238–283	−	−	+	−	−	−	−
3	pSP59	412::S-T	−	+[f]	+[f]	−	−	−	−
	pSP5	Δ465–468	−	+	+	+	+++	−	+
4	pSP70	9::V-D	+	+	+	−	−	−	−
	pSP68	142::S-T	+	+	+	−	−	−	−
	pSP73	237::V-D	+	+	+	−	−	−	−
	pSP20	252::R-A	+	+	+	−	−	−	−
	pSP23	252::(R-A)*n*	+	+	+	−	−	−	−
	pSP56A	341::(S-T)*n*	+	+	+	−	−	−	−
	pSP4	348::R-K-L-A	+	+	+	−	−	−	−
5	pSP40	310::R-A	+	+	+	++	+	−	−
	pSP56	341::S-T	+	+	+	+	−	−	−
6	pSP63	116/117	+	+	+	+	−	−	+
	pSP72	283::V-D	+	+	+	+	−	+	+
7	pSP66	22::S-T	+	+	+	+++	+++	+	+
	pSP61	36::V-D	+	+	+	+++	+++	+	+
	pSP62	64::S-T	+	+	+	++	++	+	+
	pSP64	71::R-R	+	+	+	++	++	++	+
8	pSP19	185::R-A	+	+	+	+++	+++	++	+
	pSP67	185/186/189/193::X-X	+	+	+	+++	+++	−	++
	pSP37	193::G-P	+	+	+	+++	+++	++	+++
	pSP27	398::S-T	+	+	+	+++	+++	+	+++
	pSP57	185/186::G-P	+	+	+	+++	+++	+	++

[a] +, greater than 50% binding; −, less than 50% binding (also see Fig. 2). For all mutants, binding to specific sites was assessed by measuring glutamine synthetase expressed from the NR_I-repressible $glnAp_1$ promoter, which appears to be the more sensitive of the two assays for DNA binding. In addition, for about half of the mutants, binding was determined by *glnLp* assay (discussed in Results, but also see Table 2). Both methods gave the same results, except for pSP59 (see footnote *e*).

[b] +++, greater than 67%; ++, 34 to 67%; +, 10 to 33%; −, less than 10%.

[c] Complete promoter activity was assessed by measuring glutamine synthetase from nitrogen-limited cells. Results with a high concentration of NR_I are not given because of the inhibition by NR_I at this concentration (see Table 2).

[d] Activity of the minimal $glnAp_2$ promoter (without the NR_I-binding sites) was assessed by measuring β-galactosidase from cells lysogenic for λgln105.

[e] NR_I encoded by these plasmids was unstable (see Fig. 1).

[f] Binding of NR_I from pSP59 is detectable by the $glnAp_1$ assay but not the *glnLp* assay.

the *glnLp* assay, all mutants were assessed by the $glnAp_1$ assay.

Based on these assays of transcriptional activation and repression, we divided the mutants into eight groups (Table 3). Four mutations resulted in complete loss of function: loss of repression and loss of activation of transcription (Table 3, group 1). Five mutants had phenotypes similar to that of LR1/pBLS1 (with wild-type *glnG*) (Table 3, group 8). Therefore, by using linker mutagenesis, we obtained 21 mutants with a partial loss of function.

Protein stability. Variant NR_Is that have a reasonably high level of activity in any of these assays are obviously stable, but variants that function poorly or not at all may be unstable. Therefore, we analyzed all variants that repressed less well than did wild-type NR_I (Table 3, groups 1 to 3) for stability by an immunodetection method. The steady-state levels of NR_I from pSP5, pSP8, pSP17, and pSP59 were detectable, although these variants were apparently less stable than wild-type NR_I (Fig. 1). NR_Is from pSP6, pSP7, pSP15, pSP55, and pSP60 were either barely detectable or undetectable, suggesting that these variants were unstable. Even though NR_I polypeptide was not detectable from cells carrying pSP6, pSP7, or pSP55, sufficient NR_I was induced by 1 mM IPTG to repress well.

NR_I-dependent repression. Figure 2 summarizes the results for repression of $glnAp_1$ for mutants with stable NR_I variants in relation to the location of the mutations. The locations of some mutations that diminish repression are consistent with the assignment of a DNA-binding helix-turn-helix motif to residues 444 through 463 (Fig. 2B). Six amino acids were

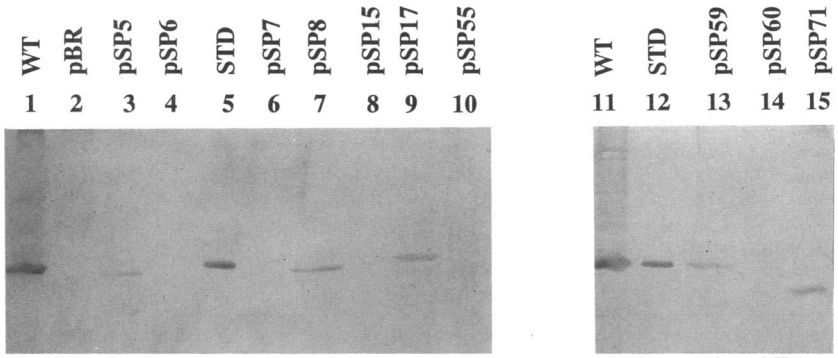

FIG. 1. Immunological assay of NR_I from mutants in which NR_I had a detectable DNA-binding defect. NR_I was detected in extracts from whole cells that contained the indicated plasmids. Cells were grown in nitrogen-limited medium with 1 mM IPTG to fully induce *glnG* expression. Plasmid pBLS1 contains wild-type (WT) *glnG* (lanes 1 and 11); cells with pBR322 (lane 2) do not contain NR_I. Forty micrograms of total protein was loaded into each lane, except lanes 5 and 12 (STD; standard). For lanes 5 and 12, 100 ng of purified NR_I was applied. NR_I from pSP71 is smaller than wild-type NR_I because pSP71 contains a deletion.

deleted from the carboxyl terminus of NR_I from pSP8, including 1 amino acid (residue 463) at the carboxyl end of the second helix. This mutant NR_I failed to repress (Table 3, group 1; Fig. 2A). On the other hand, only 4 amino acids from the carboxyl terminus were deleted in plasmid pSP5, which belongs to group 3 in Table 3. NR_I from this plasmid repressed $glnAp_1$ at an intermediate or high concentration (Table 4) and probably has an intact helix-turn-helix structure. (Since LR1/pSP5 has a number of anomalous properties, Table 4 shows more data for this mutant.)

Not only did alterations within the proposed helix-turn-helix structure affect repression, so did two insertions between residues 406 and 444. NR_I from plasmid pSP59 has a 2-amino-acid insertion between codons 412 and 413. This variant protein repressed transcription from $glnAp_1$ but only for cells grown with intermediate or high concentrations of IPTG (Table 3, group 3). However, this mutant NR_I could not repress transcription initiated from the *glnL* promoter, which contains only one high-affinity binding site (44). Plasmid pSP17 has an insertion that adds 2 amino acids between codons 406 and 407 (Table 3). The variant NR_I from this plasmid failed to repress even at a high concentration (Table 3, group 1).

Alterations between residues 1 and 398, which contain the regulatory and central activation domains, had no effect on repression if the variant NR_I was stable (Fig. 1 and 2A). Therefore, we examined repression by NR_I variants with large deletions of the regulatory domain, the activation domain, or both (Table 5). $NR_I(\Delta 143–398)$ from pCB12 repressed transcription from $glnAp_1$ completely and from *glnLp* as well as did wild-type NR_I. Purified $NR_I(\Delta 143–398)$ bound as well to *glnLp*-containing DNA in vitro as did wild-type NR_I (8). This purified variant was a dimer (8). $NR_I(\Delta 1–139)$ and $NR_I(\Delta 23–398)$ also repressed expression from *glnLp* and $glnAp_1$ in vivo, but not as well as did wild-type NR_I (Table 5). Preliminary results with purified $NR_I(\Delta 23–398)$ indicate that this variant also bound DNA, but wild-type NR_I binds with greater affinity. Preliminary evidence suggests that purified $NR_I(\Delta 23–398)$ is also dimeric (8).

In summary, the four stable variants with lesions between residue 406 and the carboxyl terminus repressed transcription from $glnAp_1$ less well than did wild-type NR_I. In contrast, the 16 stable variants with insertions between the amino terminus and residue 398 repressed transcription as

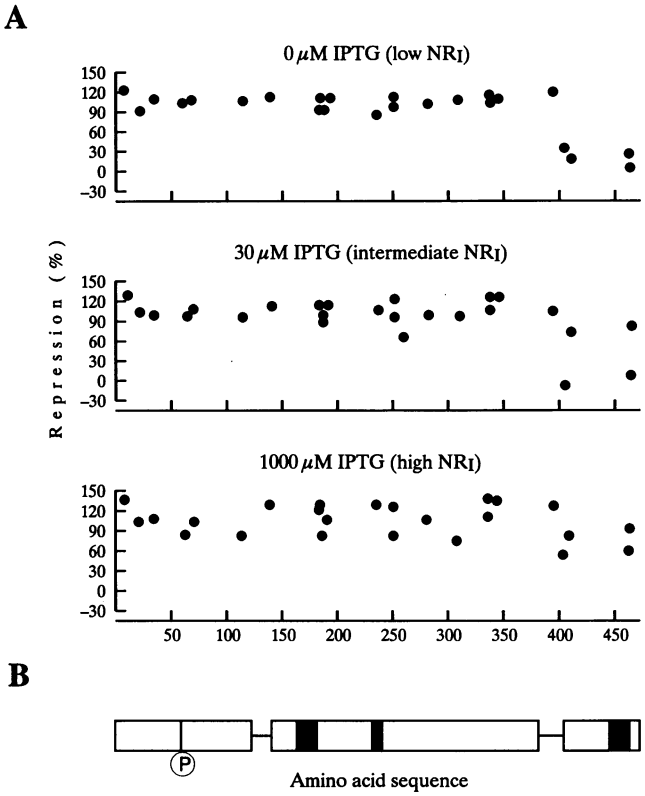

FIG. 2. Repression activity of NR_I variants versus the location of lesions. (A) Each point represents an NR_I variant with a lesion in the indicated location. Cells were grown with three concentrations of IPTG that resulted in low, intermediate, or high levels of NR_I. Repression was measured by assay of the glutamine synthetase expressed from the NR_I-repressible $glnAp_1$ promoter. The results are normalized to the activity of wild-type NR_I, which is defined as 100% (complete repression); 0% is the level observed in a strain without NR_I. Some results are expressed as negative values because the level of glutamine synthetase was slightly higher (i.e., no repression) than in cells without NR_I. (B) The three open boxes represent the domains of NR_I: a regulatory domain (residues 1 to 120), a central domain (residues 140 to 380), and a DNA-binding domain (residues 400 to 468). The P indicates the site of phosphorylation. The two solid boxes within the central domain represent regions with homology to other nucleotide-binding proteins. The solid box in the DNA-binding domain is the proposed helix-turn-helix structure.

TABLE 4. Activities of NR_I in LR1/pSP5[a]

Measured parameter (assay method)	Plasmid	Effect of NR_I concentration on NR_I-dependent activity (mU/mg of protein)[b]		
		Low	Intermediate	High
Stimulation of transcription from the wild-type *glnAp*$_2$ promoter[c] (glutamine synthetase)	pBLS1(*glnG*$^+$)	5,380 (1,004)	2,320 (218)	840 (60)
	pBR322(*glnG*)	187 (38)	207 (39)	214 (28)
	pSP5	698 (118)	5,400 (1,025)	3,760 (191)
Repression: *glnLp* assay[d] (β-galactosidase)	pBLS1(*glnG*$^+$)	138 (16)	165 (24)	234 (15)
	pBR322(*glnG*)	742 (42)	782 (24)	790 (23)
	pSP5	764 (57)	269 (32)	271 (20)

[a] Cells were grown in nitrogen-limited medium. Results are the average of four different assays for complete promoter activity and three assays for DNA binding. Numbers in parentheses are the standard errors of the mean.
[b] See footnote *b*, Table 2.
[c] See footnote *c*, Table 2.
[d] See footnote *e*, Table 2.

well as did wild-type NR_I. Complete deletion of the central domain had no effect on repression, while deletion of the regulatory domain diminished repression to some extent.

NR_I-phosphate-dependent activation of transcription. Figure 3 summarizes the results of assays of NR_I-dependent transcription from *glnAp*$_2$ versus the location of the mutation. NR_I from only three plasmids, pSP8, pSP17, pSP71, was stable but could not repress *glnAp*$_1$. These NR_Is presumably bind DNA poorly and also failed to stimulate transcription from either the wild-type or the minimal *glnAp*$_2$ promoter (Table 3, groups 1 and 2). Conversely, all mutant NR_Is that activated transcription from *glnAp*$_2$ also repressed well. These correlations imply that DNA binding is necessary, even for activation of the minimal *glnAp*$_2$ promoter, which has no apparent specific binding sites. Since our assays for DNA binding involve binding to specific high-affinity sites, our results suggest that activation from the minimal promoter requires binding to sites with some similarity to high-affinity NR_I-binding sites. There is only one apparent exception to these correlations. NR_I from pSP5 at a low concentration did not detectably repress *glnAp*$_1$ but could stimulate transcription from the wild-type *glnAp*$_2$ promoter, albeit to only 13% of the reference level (Table 4). Our repression assays measure the binding of either one NR_I-phosphate to a single high-affinity site or two molecules of unphosphorylated NR_I to adjacent sites. However, activation from the wild-type *glnAp*$_2$ promoter involves cooperative binding of two molecules of NR_I-phosphate to adjacent sites (50). Since our assays may fail to detect weak binding that is enhanced by cooperative interactions, we cannot conclude that this altered NR_I does not bind DNA when this

TABLE 5. Repression by NR_I variants with deletion of the regulatory domain, the central activation domain, or both[a]

Plasmid	Description	IPTG (μM)	Sp act (mU/mg of protein)[b] of:	
			β-Galactosidase[c]	Glutamine synthetase[d]
pBLS1	Wild-type NR_I	0	151 ± 20	4,730 ± 40
		10	165 ± 2	1,008 ± 9
		1,000	207 ± 4	610 ± 6
pBR322	No NR_I	0	661 ± 9	212 ± 4
		10	713 ± 11	200 ± 5
		1,000	700 ± 14	178 ± 2
pBLS7	Deletion of regulatory domain (Δ residue 1–139)	0	713 ± 18	188 ± 3
		10	319 ± 12	20 ± 2
		1,000	306 ± 9	17 ± 3
pCB12	Deletion of activation domain (Δ residue 143–398)	0	175 ± 5	4 ± 1
		10	221 ± 16	ND[e]
		1,000	244 ± 3	ND
pCB30	Deletion of regulatory activation domain (Δ residue 23–398)	0	675 ± 14	193 ± 3
		10	590 ± 13	171 ± 5
		1,000	245 ± 11	11 ± 3

[a] All plasmids were in LR1 (λgln107). Cells were grown in nitrogen-limited medium.
[b] All activities are the average of three determinations ± standard deviation.
[c] β-Galactosidase activity is a measure of transcription from the NR_I-repressible *glnLp*.
[d] Glutamine synthetase assesses *glnA* transcription. When glutamine synthetase exceeds the level found in cells with no NR_I, transcription initiated from *glnAp*$_2$. When the level of glutamine synthetase was lower than that in cells without NR_I, glutamine synthetase can be used to assess NR_I-dependent repression of *glnAp*$_1$.
[e] ND, not detectable (glutamine synthetase was less than 10 mU/mg of protein).

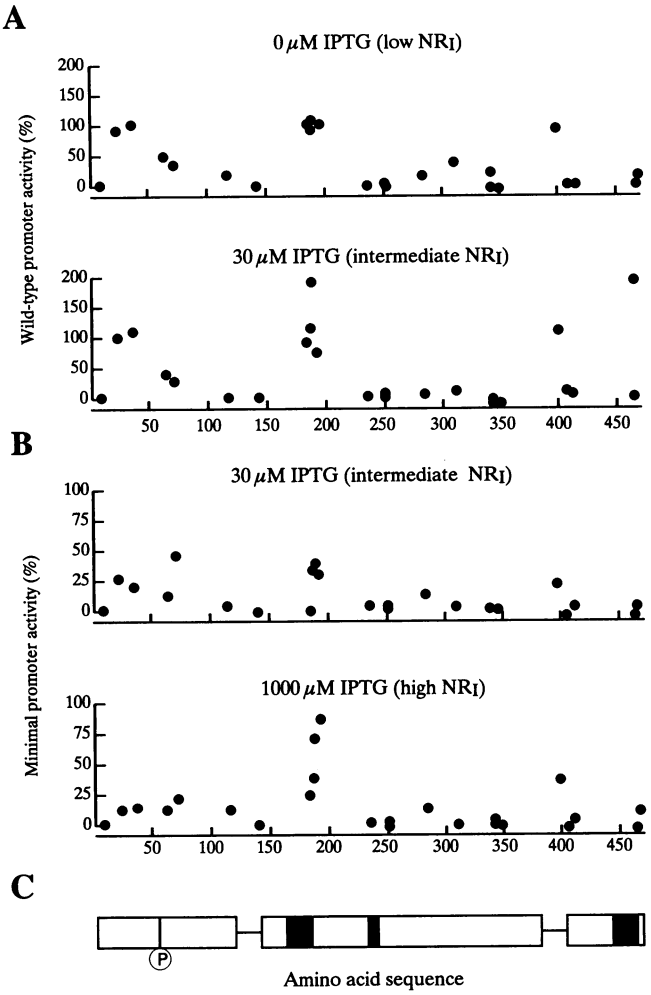

FIG. 3. Activation of the wild-type and minimal $glnAp_2$ promoters by NR_I variants versus location of the lesion. (A) Activation of transcription from the wild-type $glnAp_2$ promoter. (B) Activity from the minimal $glnAp_2$ promoter. In each case, the activity found in cells with wild-type NR_I is defined as 100%. The activity of the wild-type $glnAp_2$ promoter at a high concentration of NR_I is not shown because of the NR_I-dependent inhibition (Table 2). The activity of the minimal $glnAp_2$ promoter is not shown for cells with a low concentration of NR_I because there is little activation at this concentration (Table 2). (C) Proposed domain structure of NR_I (see Fig. 2 for details).

variant is present at a low concentration. Nonetheless, it does seem to bind at higher concentrations.

Central activation domain (residues 140 to 380). Almost all alterations in the central domain produced strains with essentially the same phenotype. The mutant NR_I repressed $glnAp_1$ normally (Table 3; Fig. 2) but generally did not stimulate or poorly stimulated transcription from the wild-type $glnAp_2$ promoter (Table 3, groups 4 to 6; Fig. 3A and B). NR_Is that failed to stimulate transcription from the wild-type $glnAp_2$ promoter also failed to activate expression from the minimal $glnAp_2$ promoter (Table 3; Fig. 3). However, some insertions within the central domain did not affect activation.

Amino-terminal regulatory domain (residues 1 to 120). Four of the six mutant NR_Is with alterations in the amino-terminal regulatory domain had only a minor defect in activity from the wild-type $glnAp_2$ promoter, but transcription from the minimal $glnAp_2$ promoter was severely impaired (Table 3, group 7; Fig. 3). These mutants grew slowly with arginine as a nitrogen source compared with an isogenic $glnG^+$ strain (44). These strains and previously isolated mutants in which the primary defect appears to be diminished phosphorylation of NR_I (43) have identical phenotypes. In contrast, two mutations in the regulatory domain (see pSP70 in group 4 and pSP63 in group 6, Table 3) virtually eliminated all transcription from $glnAp_2$, although NR_I from these mutants repressed $glnAp_1$ well (Table 3; Fig. 2 and 3).

NR_I-dependent inhibition of transcription from $glnAp_2$. Transcription from the wild-type $glnAp_2$ promoter is inhibited by a high concentration of NR_I (45). With only one exception, if NR_I at a low or intermediate concentration stimulated transcription from the wild-type $glnAp_2$ promoter, then a higher level of NR_I diminished expression (44). The mutation in pSP5 (the exception) decreased repression of $glnAp_1$ and therefore seemed to affect binding to DNA. At an intermediate concentration, this mutant NR_I bound DNA (i.e., repressed transcription from either $glnAp_1$ or $glnLp$) and activated transcription from the wild-type $glnAp_2$ promoter (Table 4). However, a further increase in the concentration of NR_I from pSP5 did not significantly diminish transcription from the wild-type $glnAp_2$ promoter (Table 4).

DISCUSSION

Our goals were to probe the effects of disruption of each domain of NR_I and to analyze aspects of transcriptional activation. We have provided evidence for a larger DNA-binding domain than was previously suspected and have described the characteristic phenotypes of strains with mutations in each domain. Our results suggest that transcription from the minimal $glnAp_2$ promoter requires binding to DNA. We also report isolation of a mutant in which increasing NR_I did not diminish *glnA* expression.

DNA-binding domain. All lesions between residues 406 and 468 reduced repression of $glnAp_1$ and presumably affected binding to DNA. In contrast, 16 insertions between residues 1 and 398 that resulted in a stable variant (most variants were stable) had no effect on NR_I-dependent repression and presumably had little or no effect on DNA binding. Deletion of residues 143 to 398 had no effect on NR_I-dependent repression in vivo or DNA binding in vitro. Deletion of residues 1 to 139 or 22 to 398 diminished, but did not eliminate, NR_I-dependent repression or DNA binding. Preliminary results indicate that the purified $NR_I(\Delta 23–398)$ is dimeric. From these results, we suggest that residues 406 to 468 comprise a structural DNA-binding domain that contains residues for dimerization. Future biochemical experiments with the truncated versions of NR_I will resolve the following issues. Are all of the DNA-binding determinants in the proposed DNA-binding domain? What region of NR_I is involved in cooperative interactions?

Central activation domain. Two lines of evidence suggest that the central domain of NR_I binds and hydrolyzes ATP. First, it contains motifs found in many nucleotide binding proteins (14, 18, 37). One sequence motif between residues 167 and 173 (GXXGXGK) has been called a "phosphate gripper" (2). Structural evidence suggests that glycine-rich regions in other proteins form flexible loops that strongly bind the phosphates of nucleotides (22, 32). Alteration of this sequence in NR_I abolishes the ability to stimulate transcription (3). Second, mutations that abolish ATP hydrolysis alter amino acids in the central region, and NR_I from such

mutants fails to catalyze formation of open-promoter complexes (49).

The central domain of NR_I (from residues 140 to 380) is homologous to similar regions in other transcriptional activators, such as NifA of *Rhizobium meliloti*, the activator for genes of nitrogen fixation (16). A high level of a truncated derivative of NifA, containing only this central region, can activate transcription of the *nifH* promoter (16). This region of NR_I probably contains amino acids that contact σ^{54}-RNA polymerase. Except for insertions between the two nucleotide-binding motifs, insertions within the central domain eliminated transcription from both the wild-type and minimal $glnAp_2$ promoters (Table 3, mutants of groups 4 to 6; Fig. 3). All insertions outside the nucleotide-binding motifs of the central domain eliminated transcriptional activation. It is tempting to speculate that this essential region is involved in the interaction with RNA polymerase.

Amino-terminal regulatory domain. The first 120 amino acids of NR_I are homologous to a region found in the second component of all two-component regulatory systems. The site of NR_I phosphorylation, Asp-54 (27, 41), is within this domain. An analogous aspartate is phosphorylated in other response regulators, such as CheY (7). Since phosphorylation is required for transcriptional activation, we expected some mutations in this region to eliminate *glnA* transcription. All transcription from $glnAp_2$ was abolished by the insertion in pSP70 (Table 3; Fig. 3). Some insertions within the regulatory domain should diminish, but not eliminate, the extent of phosphorylation of NR_I. The phenotype of such mutants has been characterized previously (43). They cannot utilize arginine as a nitrogen source. NR_I from these mutants fails to stimulate transcription from the minimal $glnAp_2$ promoter but activates transcription from the wild-type $glnAp_2$ promoter to a normal level (43). In fact, four of the mutations in the regulatory domain produced strains with this previously characterized phenotype (Table 3, group 7; Fig. 3). Our results extend the previous findings and show that the regulatory domain contains the lesions that result in this phenotype.

Transcription from the minimal $glnAp_2$ promoter. Activators of σ^{54}-dependent promoters are unusual because they can stimulate transcription after deletion of the specific binding sites (20, 34). We have previously isolated mutants of NR_I that can stimulate transcription from the wild-type $glnAp_2$ promoter but not from the minimal promoter. Such mutants fail to utilize a number of nitrogen sources (43). This result suggests that the promoters for some Ntr genes are like the minimal $glnAp_2$ promoter and are necessary for growth in some media. One such gene may be *argT* of *Salmonella typhimurium*, which lacks identifiable NR_I-binding sites (1). We have previously shown that regulation of the minimal promoter differs significantly from that of the wild-type $glnAp_2$ promoter even though both have identical protein requirements (43). Nonetheless, insertions in the central domain eliminate transcription from both types of $glnAp_2$ promoters, suggesting that the mechanisms of activation are similar. In addition, we conclude that if NR_I could activate transcription from the minimal promoter, it could bind to specific NR_I-binding sites (Table 3; Fig. 2 and 3). This correlation suggests that specific DNA binding is necessary for activation of the minimal $glnAp_2$ promoter. In mutants where NR_I is less easily phosphorylated, there was little or no transcription from the minimal promoter, but normal transcription from the wild-type $glnAp_2$ promoter (43) (Table 3, group 7). Therefore, it is reasonable to speculate that phosphorylation may stabilize the binding to DNA necessary for activating transcription from the minimal $glnAp_2$ promoter. This speculation is consistent with the observation that NR_I detectably binds some sites only after phosphorylation (26).

Inhibition of transcription from the wild-type $glnAp_2$ promoter. An important mechanism in determining the steady-state level of glutamine synthetase in a wild-type strain is the inhibitory effect of a high concentration of NR_I on *glnA* expression (45) (Table 2). To account for this inhibition, we proposed that an interaction between two molecules of NR_I-phosphate bound to adjacent sites hinders contact between NR_I-phosphate and RNA polymerase (45). This hypothesis is too simple in light of emerging evidence that the ATPase activity of NR_I and transcriptional activation may require two dimers of NR_I (49, 50). Instead, to account for this inhibition, we propose that, depending on the number of phosphorylations or molecules of ATP or ADP bound, some complexes activate better than others. With one exception, inhibition by a high concentration of NR_I was observed in all mutants in which NR_I could activate transcription from the wild-type $glnAp_2$ promoter at a low or intermediate concentration. However, this inhibition was not observed in cells carrying plasmid pSP5 (Table 4). NR_I from pSP5 may be crucial in the investigation, with purified components, of the mechanism of this inhibition.

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