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Governor of the *glnAp2* promoter of *Escherichia coli*

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Summary

Low-affinity sites for the activator NRI~P (NtrC~P) that map between the enhancer and the *glnAp2* promoter were responsible for limiting promoter activity at high concentrations of NRI~P in intact cells and in an *in vitro* transcription system consisting of purified bacterial components. That is, the low-affinity sites constitute a 'governor', limiting the maximum promoter activity. As the governor sites are themselves far from the promoter, they apparently act either by preventing the formation of the activation DNA loop that brings the enhancer-bound activator and the promoter-bound polymerase into proximity or by preventing a productive interaction between the enhancer-bound activator and polymerase. The combination of potent enhancer and governor sites at the *glnAp2* promoter provides for efficient activation of the promoter when the activator concentration is low, while limiting the maximum level of promoter activity when the activator concentration is high.

Introduction

The σ^{54}-dependent promoters of bacteria are unique in their requirement for distant activator sites that function as enhancer elements (Reitzer and Magasanik, 1986; Ninfa *et al*., 1987; reviewed by Kustu *et al*., 1989; Atkinson and Ninfa, 1994). These enhancer elements bind the activator, which stimulates the rate of open complex formation at the promoter (Popham *et al*., 1989). The enhancer-bound activator interacts with the promoter-bound polymerase by means of a DNA loop that brings the activator and polymerase into proximity (Su *et al*., 1990). In the absence of activator, polymerase binds the promoter and forms a stable closed complex in which the DNA strands are not melted (Ninfa *et al*., 1987; Sasse-Dwight and Gralla, 1988; Popham *et al*., 1989). Interaction of the activator with the polymerase in the closed complex results in the isomerization of the closed complex to the transcriptionally active open complex (Popham *et al*., 1989). After formation of the open complex, activator appears to have no additional role in transcriptional activation, such as activation of promoter clearance or transcription elongation (Ninfa *et al*., 1989). Recent studies suggest that reinitiation follows the same kinetic pathway as the first initiation event, that is no subassembly of the transcriptional apparatus remains at the promoter after initiation (Bondarenko *et al*., 2002).

Several lines of evidence suggest that the role of the enhancer element is to increase the local concentration of the activator in the vicinity of the closed complex of polymerase and promoter. Studies of the nitrogen-regulated *glnAp2* promoter, which expresses the nitrogen assimilation enzyme glutamine synthetase (GS), indicated that activation from this promoter was only dependent on the enhancer when the concentration of the activator in the reaction mixtures was low; at high activator concentrations, the enhancer was not required for promoter activity (Ninfa *et al*., 1987). DNA binding by the activator is apparently not required for transcriptional activation, as a mutant form of the activator that is unable to bind to DNA is able to initiate transcription *in vitro* when provided in sufficient concentration (Porter *et al*., 1993). Kustu and colleagues showed that enhancer-dependent activation could occur when the enhancer and promoter were located on separate, concatenated circular DNA molecules (Wedel *et al*., 1990). This experiment suggests that activator interacts directly with the polymerase in the closed complex and appears to exclude 'tracking' mechanisms or mechanisms that require the activator to send a signal through the DNA, such as by altering its topography.

Nitrogen-regulated Ntr promoters of *Escherichia coli* are activated by the phosphorylated form of the *glnG* (*ntrC*) protein NRI~P (NtrC~P) (Ninfa and Magasanik, 1986), which binds to enhancer sequences found upstream from the regulated promoters. At the *glnAp2* promoter, there are two adjacent high-affinity NRI binding sites (sites 1 and 2) centred at position −110 relative to the site of transcription initiation (Hirschman *et al*., 1985) that serve as the enhancer (Reitzer and Magasanik, 1986; Ninfa *et al*., 1987). In experiments with purified components, these sites were able to stimulate transcriptional

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activation when moved to new locations far upstream or downstream from the promoter (Ninfa *et al*., 1987). Other nitrogen-regulated promoters also contain a pair of NRI binding sites upstream from the promoter. In the case of the nitrogen-regulated *glnK* and *nac* promoters, one of the two sites is degenerate in sequence, and these enhancers do not bind NRI as avidly as the *glnAp2* enhancer (Feng *et al*., 1995a; Atkinson *et al*., 2002). Phosphorylation of NRI results in the oligomerization of NRI and highly co-operative binding to adjacent NRI binding sites (Weiss *et al*., 1992). The oligomerized NRI~P bound to the enhancer has high ATPase activity that is somehow involved in melting the DNA strands to form the open complex (Popham *et al*., 1989; Weiss *et al*., 1991).

The phosphorylation and dephosphorylation of NRI is regulated by a complex signal transduction system in response to changes in the intracellular nitrogen status (reviewed by Ninfa *et al*., 2000). In addition, the intracellular concentration of NRI is regulated in part by activation of the *glnAp2* promoter of the *glnALG* operon (Pahel *et al*., 1982; Reitzer and Magasanik, 1985; Atkinson *et al*., 2002). The effect of these regulatory mechanisms is that the intracellular concentration of NRI~P is increased in nitrogen-starved cells. This amplitude modulation of the NRI~P concentration results in the sequential activation of Ntr promoters as cells become starved. That is, promoters with potent enhancers, such as the *glnAp2* promoter and the *glnHp2* promoter, are activated first, followed by the activation of the *glnK* and *nac* promoters only when the NRI~P concentration reaches the level required for interaction with their enhancers. Studies with purified components have indicated that activation of *glnHp2* required a slightly higher concentration of NRI~P than did activation of *glnAp2* (Carmona and Magasanik, 1996), and activation of *nacp* and *glnKp* required a much higher concentration of NRI~P than did activation of *glnAp2* (Feng *et al*., 1995a; Atkinson *et al*., 2002). Also, studies with intact cells have shown that *glnAp2* is activated before *glnKp* and *nacp* as cells become nitrogen starved (Atkinson *et al*., 2002). Recently, we examined the expression profile of *glnK*, *nac* and *glnA* promoter fusions to *lacZYA* in mutant cells unable to reduce the phosphorylation state of NRI in response to signals of nitrogen status (Blauwkamp and Ninfa, 2002a). We were surprised to observe that, in such cells, the expression of the *glnKp–lacZYA* and *nacp–lacZYA* fusions proceeded unchecked as the cells became nitrogen starved, whereas the expression of a *glnAp–lacZYA* fusion did not. This result indicated that a mechanism for limiting the expression of the *glnAp–lacZYA* fusion was operating, but did not address the nature of this mechanism.

Footprinting of the *glnAp2* promoter has revealed that, in addition to the high-affinity NRI binding sites that serve as the enhancer, the *glnA* control region contains three additional sites (sites 3, 4 and 5, centred at –90, –65 and –45 bp from the transcription start site) that are occupied only at very high concentrations of NRI~P (Hirschman *et al*., 1985; Ninfa *et al*., 1987). Experiments with purified components indicated that activation of *glnAp2* was reduced at very high NRI~P concentration (Feng *et al*., 1995b). Also, experiments with intact cells indicated that overexpression of NRI caused a reduction in transcription from *glnAp2* under nitrogen-limiting conditions. For example, hyperexpression of NRI from the multicopy plasmid pgln53 resulted in reduced expression of the *glnA* gene encoding glutamine synthetase (Chen *et al*., 1982). In another study, hyperexpression of NRI from a multicopy plasmid under the control of the *lac* promoter resulted in reduced expression of *glnA* (Shiau *et al*., 1992). This study used a *glnA* promoter reporter construct that contained a 700 bp insertion between the enhancer and promoter. It was observed that, in such a context, overexpression of NRI resulted in reduced *glnA* expression in intact cells regardless of the presence or absence of sites 3, 4 and 5 (Shiau *et al*., 1992). These results led to the conclusion that neither sites 3, 4 and 5 nor regulation of the flexibility of the DNA were responsible for the reduction in *glnA* expression at high activator concentration (Shiau *et al*., 1992).

Here, we examined the role of the low-affinity NRI binding sites in limiting the activity of the *glnA* control region when NRI was expressed under physiological conditions. Specifically, we examined the effect of scrambling the sequences of the low-affinity sites 3 and 4 (using the nomenclature of Hirschman *et al*., 1985) on the *glnAp2* expression profile. Our results indicated that, under physiological conditions, these sites act as a governor responsible for setting the upper boundary of expression from the promoter when the intracellular NRI~P concentration was high. However, under non-physiological conditions, such as when NRI was hyperexpressed from a multicopy plasmid, limitation of the *glnAp2* promoter was only partially dependent on sites 3 and 4. Thus, under the latter conditions, multiple mechanisms for restraining expression of the wild-type promoter were indicated. We also examined the role of sites 3 and 4 in activation of the *glnAp2* promoter in an *in vitro* transcription system, and found that these sites limited promoter activation at high concentrations of NRI~P.

Results

Sequences downstream from the promoter were not required to govern glnAp–lacZYA *fusions*

We described previously a fusion of the *glnA* promoter region to promoterless *lacZYA* which, when located in single copy within a chromosomal 'landing pad',

expressed β-galactosidase co-ordinately with the expression of GS from the natural wild-type *glnA* gene [Table 1 (Apϕ2), Fig. 1]. Studies of this fusion showed that the *glnA* promoter was expressed before similar fusions to the *glnK* and *nac* promoters as cells became starved (Atkinson *et al*., 2002) but that, unlike the *glnKp* and *nacp* fusions, it did not display 'runaway' expression in starved cells that cannot limit the extent of NRI phosphorylation (Blauwkamp and Ninfa, 2002a).

The *glnAp–lacZYA* fusion described above contains a portion of the *glnA* transcribed leader sequence and a portion of the *glnA* structural gene, followed by a synthetic stop codon and the *lac* leader mRNA and *lac* structural genes. Thus, the properties of the fusion may not have been entirely caused by the properties of the promoter. We therefore constructed another *glnAp–lacZYA* fusion (Apϕ), in which the first transcribed nucleotide from *glnAp2* is from the *lac* leader (*Experimental procedures*). Expression of both fusions was observed to parallel the expression of glutamine synthetase in various genetic backgrounds (Table 1). More importantly, hyperexpression of NRI from pgln53 resulted in a reduction in the level of expression of both fusions (Table 2). We interpret this as indicating that regulation of the fusions by high NRI~P did not require sequences downstream from the promoter.

Low-affinity NRI~P binding sites 3 and 4 of the glnA *control region were responsible for limiting promoter expression under physiological conditions*

To study the effects of sites 3 and 4, we engineered promoters in which the sequence of each site was multiply mutated to a sequence having no similarity to an NRI binding site, separately and together (Fig. 1). DNase I footprinting studies indicated that these mutations eliminated detectable binding of NRI~P to the altered sites 3 and 4, even at very high concentrations of NRI~P (data not shown). Specifically, we observed that, at 150 nM NRI, in the presence of excess NRII and ATP to phosphorylate the NRI, weak footprinting of sites 3 and 4 was observed with a wild-type template and that, at 300 nM NRI (with excess NRII and ATP), sites 3 and 4 were completely footprinted when a wild-type template was examined. Under the same conditions, the region containing scrambled sites 3 and 4 on a DNA template in which both sites were altered showed no detectable footprinting when NRI was at 150 nm, 300 nM or 600 nM along with excess NRII and ATP (data not shown).

The mutant promoters were used to form fusions analogous to the largest *glnAp–lacZYA* fusion (i.e. Apϕ2) and incorporated into the *E. coli* chromosome in single copy within the *trp* operon. In each case, limitation of the promoters by hyperexpression of NRI from pgln53 was defective, indicating a role for sites 3 and 4 in this process (Table 2). Indeed, the level of expression from these promoters was elevated relative to the wild type, even in the absence of NRI hyperexpression. This suggested that some governing of *glnAp2* expression by sites 3 and 4 occurs in nitrogen-limited wild-type cells at physiological levels of NRI~P. Reduction in *glnAp2* expression upon hyperexpression of NRI from pgln53 was not completely eliminated by mutations to sites 3 and 4 in these experiments (Table 2).

A more physiological assay for regulation of the *glnA* promoter region is to examine the expression profile of fusions as cells deplete a limiting supply of ammonia and become nitrogen starved. Previous studies showed that governing of *glnAp2* was most obvious in cells lacking the GlnK signal transduction protein. These mutant cells are unable to activate the dephosphorylation of NRI~P once starved, and display unlimited expression of *glnKp–lacZYA* and *nacp–lacZYA* upon starvation (Blauwkamp and

Table 1. Glutamine synthetase and β-galactosidase expression in adapted cultures.

Strain	Glutamine synthetase[a] (n[b])		β-Galactosidase[c]	
	Ggtrp[d]	GNgtrp	Ggtrp	GNgtrp
YMC10Apϕ[e] (WT)	1690 (4.1)	180 (8.2)	2330	110
BApϕ (*ΔglnB2306*)	1310 (3.1)	1350 (11.3)	2770	1820
KcApϕ (*Δmdl-glnK::cam*[r])	1620 (3.4)	210 (9.4)	2080	200
YMC10Apϕ2 (WT)	1240 (3.8)	193 (8.9)	2290	170
BApϕ2 (*ΔglnB2306*)	1630 (3.7)	1620 (11.4)	3780	2380
KcApϕ2 (*Δmdl-glnK::cam*[r])	1700 (3.6)	240 (9.5)	3020	230

a. GS transferase activity. Cultures were grown overnight in the indicated medium, diluted to an OD_{600} of ≈0.02 and grown at 30°C to an OD_{600} of ≈0.5.
b. Adenylylation state expressed as average number of adenylylated subunits per GS dodecamer.
c. β-galactosidase in Miller units. Cells were permeabilized using chloroform and SDS.
d. Media used were: Ggtrp, glucose–glutamine–tryptophan; GNgtrp, glucose–ammonia–glutamine–tryptophan. In all cases, tryptophan was present at 0.004% (w/v), glucose 0.4% (w/v) and the nitrogen sources at 0.2% (w/v).
e. Strains designated Apϕ contain the *glnA* promoter fused to *lacZ* at the transcription +1. Strains designated Apϕ2 contain the *glnA* promoter fused to *lacZ* at +165.

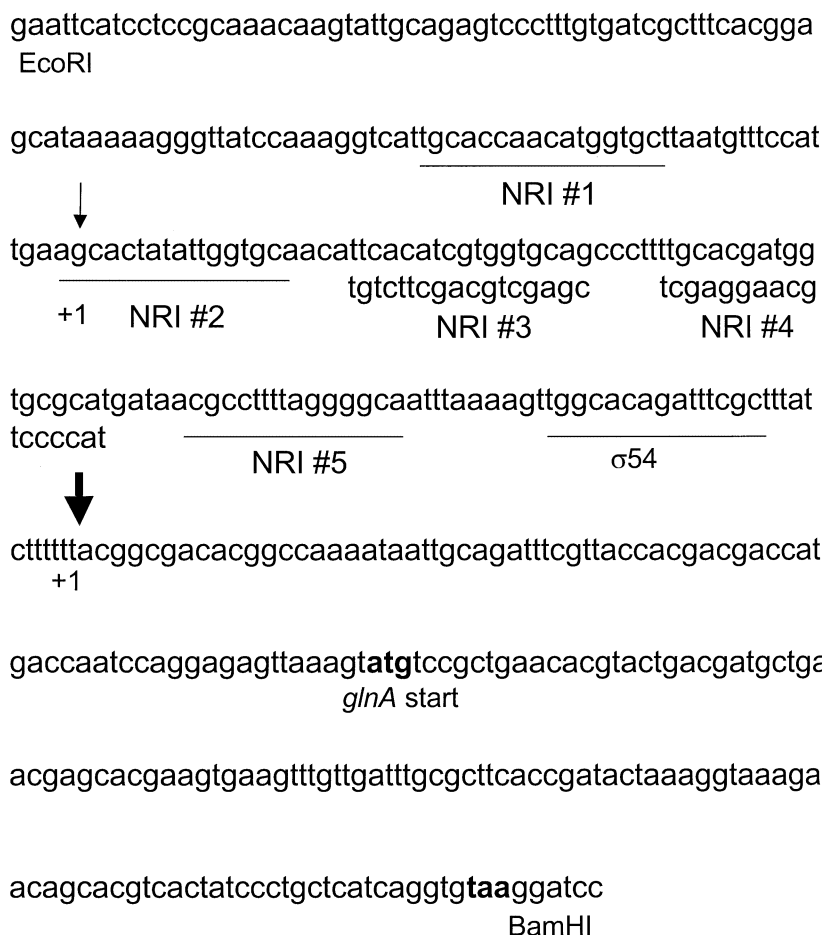

Fig. 1. Features of the wild-type and mutant *glnA* promoters. The DNA sequence of the *glnA* promoter fused to *lacZ* is shown including the PCR-generated restriction sites. The NRI and σ^{54} binding sites are underlined. The σ^{70} transcription start site is indicated with a small arrow. The σ^{54} transcription start site is indicated with a large arrow. Mutant DNA sequences introduced to NRI binding sites 3 and 4 are below the wild-type sequence. The *glnA* start codon and the stop codon introduced by PCR are shown in bold type.

Ninfa, 2002a). However, *glnAp–lacZYA* expression was limited in cells lacking GlnK as the cells became starved (Blauwkamp and Ninfa, 2002a; Fig. 2A). In contrast, fusions in which site 3, site 4 or both sites 3 and 4 had been mutated displayed an obvious defect in limiting *glnA* expression in starved cells lacking GlnK (Fig. 2A). Cells in which both sites 3 and 4 were mutated seemed to be more severely affected than cells lacking either site 3 or 4 alone (Fig. 2A). These results show that sites 3 and 4 play a role in limiting *glnA* expression under physiological conditions. Interestingly, the presence or absence of sites 3 and 4 had no apparent effect on the level of fusion expression during growth on ammonia or on the kinetics of induction upon depletion of the limiting ammonia in cells lacking GlnK (Fig. 2A).

We could also observe an effect of sites 3 and 4 on limiting promoter expression in wild-type cells (Fig. 2B). All the fusions displayed a similar level of *glnA* expression during growth on ammonia, which was increased upon exhaustion of the ammonia (Fig. 2B). After overnight starvation, the fusions with mutations in sites 3, 4 or both 3 and 4 were expressed at a higher level than the wild type (Fig. 2B), but at a considerably lower level than in cells lacking GlnK (Fig. 2A).

As the mutations to sites 3 and 4 resulted in an increase in the expression of the *lacZYA* fusions, the possibility existed that the mutations had created new promoters. To examine this, we studied the effects of a mutation that deletes *glnL* and *glnG*, eliminating NRII and NRI, on the expression of the fusions (*Experimental procedures*). Deletion of *glnL* and *glnG* reduced the expression of the

Table 2. Effect of hyperexpression of NRI from a multicopy plasmid on β-galactosidase expression from *glnA* promoter fusions.

Strain	Fusion	NRI sites present	β-galactosidase[a] pBR322	β-galactosidase[a] pgln53
YMC10 (WT)	glnApϕ	1, 2, 3, 4, 5	2330	430
	glnApϕ2	1, 2, 3, 4, 5	2910	540
	glnApϕ5	1, 2, 4, 5	3620	1005
	glnApϕ6	1, 2, 3, 5	3660	1410
	glnApϕ7	1, 2, 5	4480	2190

a. Miller units. Cells were permeabilized using chloroform and SDS. Cultures were grown overnight in glucose–glutamine–tryptophan medium, diluted to an OD_{600} of ≈0.02 in the same medium and grown at 30°C to an OD_{600} of ≈0.7. Results are the average of three experiments.

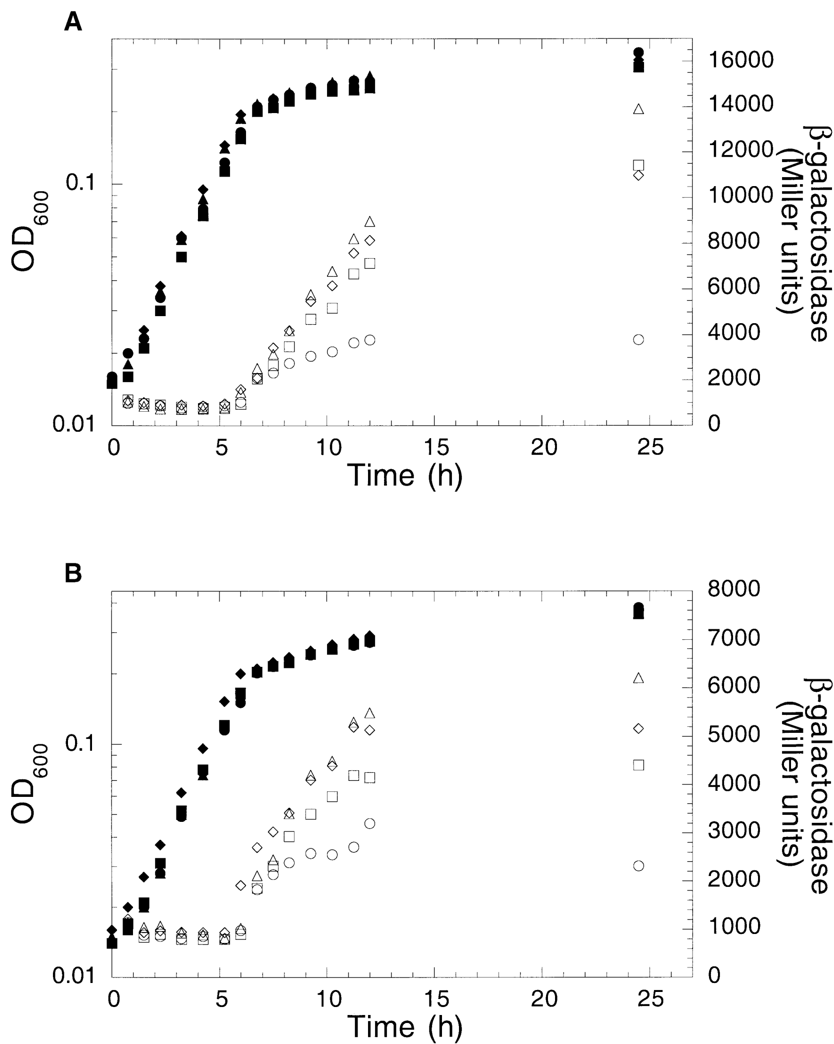

Fig. 2. Induction of *glnAp2* and *glnAp2** upon shift to nitrogen-starved growth. Cells were incubated 30°C overnight in defined minimal W-salts medium containing 0.4% (w/v) glucose, 0.2% (w/v) ammonium sulphate and 0.004% (w/v) tryptophan. The cells were then washed and diluted into fresh media as above, only containing 0.005% (w/v) ammonium sulphate. Samples were assayed for expression of the reporter β-galactosidase using the Miller assay on SDS–chloroform-permeabilized cells. Solid symbols, OD_{600}; open symbols, β-galactosidase.
A. KgApΦ2 [wild-type *glnAp–lacZ* fusion (filled circles)], KgApΦ5 [NRI site 3 mutant *glnAp–lacZ* fusion (filled squares)], KgApΦ6 [NRI site 4 mutant *glnAp–lacZ* fusion (filled diamonds)], KgApΦ7 [NRI sites 3 and 4 mutant *glnAp–lacZ* fusion (filled triangles)].
B. YMC10ApΦ2 [wild-type *glnAp–lacZ* fusion (filled circles)], YMC10ApΦ5 [NRI site 3 mutant *glnAp–lacZ* fusion (filled squares)], YMC10ApΦ6 [NRI site 4 mutant *glnAp–lacZ* fusion (filled diamonds)], YMC10ApΦ7 [NRI sites 3 and 4 mutant *glnAp–lacZ* fusion (filled triangles)].

wild-type fusion *glnApϕ*2 from 2910 Miller units of β-galactosidase to a basal level of 90 Miller units. Similarly, the fusions *glnApϕ*5–*glnApϕ*7 displayed basal levels of expression of 110, 100 and 120 Miller units, respectively, in cells deleted for *glnL* and *glnG*. This result indicates that, in all cases, fusion expression was dependent on NRII and NRI, implying that it resulted from expression from *glnAp2*. In another set of experiments, we examined the effect of the *glnD99::*Tn*10* mutation on reporter expression. Previous results indicated that, under the conditions in our experiments, this mutation reduces the expression of glutamine synthetase by about two-thirds (Bueno *et al*., 1985). We observed that, for all the fusions studied here, the *glnD99::*Tn*10* mutation resulted in a similar decrease in fusion expression (data not shown). These experiments are also consistent with the idea that expression of the fusions results in all cases from the activity of the *glnAp2* promoter.

Sites 3 and 4 play a role in the reduction of transcription from glnAp2 *at high activator concentration in an* in vitro *transcription system*

To examine the effects of sites 3 and 4 *in vitro*, we constructed supercoiled plasmid vectors based on the plasmid pTE103 (Elliot and Geiduschek, 1984; *Experimental procedures*). These transcription templates contain a strong rho-independent transcriptional terminator sequence derived from bacteriophage T7 positioned downstream from the *glnAp2* promoter, such that a transcript of 329 nucleotides (nt) is formed. Unexpectedly, we observed that both the wild-type template, pglnApOG7, and a template with mutations in both site 3 and site 4, pglnApOG8, produced two *glnAp2* transcript bands in our *in vitro* system that seemed to result from initiation at sites a few nucleotides apart (Fig. 3; data not shown). As a control in our *in vitro* transcription assays, we included the

supercoiled plasmid template pLR100 (Ninfa *et al.*, 1987), which has the wild-type *glnAp2* promoter driving the expression of a 420 nt transcript.

In vitro transcription assays were performed as described previously (Ninfa *et al.*, 1987; Feng *et al.*, 1995a; Liu *et al.*, 2002), using a combination of pLR100 and either pglnApOG7 or pglnApOG8 in the reaction mixtures (*Experimental procedures*). In single-cycle transcription assays in which transcription complexes were formed for a fixed time, we observed that high concentrations of NRI~P resulted in a reduced yield of transcripts from the wild-type promoter and a less severe reduction in the number of transcripts from the template bearing mutations in sites 3 and 4 (pglnApOG8; data not shown). The difference in yield of transcripts from the wild-type and mutant templates at high activator concentration was magnified when a multiple-cycle transcription assay was used (Fig. 3). Previous studies showed that the multiple-cycle transcription assay results in about four cycles of transcription/template under the conditions used here (Liu *et al.*, 2002).

Discussion

Our work has shown that the combination of high-affinity activator binding sites (enhancer) and low-affinity activator binding sites (governor) at the *glnAp2* promoter was responsible for the expression characteristics of this promoter under physiological conditions in which the expression of NRI was from the single-copy wild-type gene. The

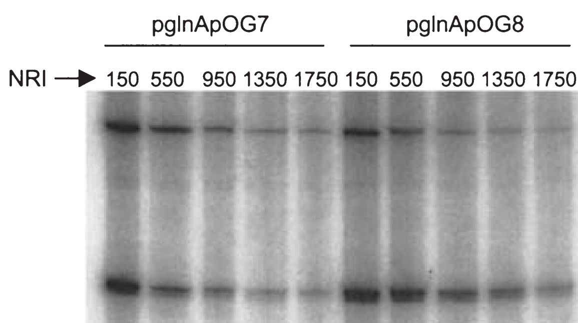

Fig. 3. Multiple-cycle transcription assay analysis of the role of sites 3 and 4 in activation of *glnAp2* at high activator concentration. Each transcription reaction mixture contained two templates, pLR100 (wild-type), which produces a 420 nt transcript (top band), and either pglnApOG7 or pglnApOG8, as indicated, which produce a doublet of about 329 nt (bottom band). NRI concentrations are in nM as indicated. Multiple-cycle transcription was initiated by the addition of [α-^{32}P]-UTP after preincubation of all other components at 37°C for 10 min. Transcription was for 15 min, after which reactions were stopped by the addition of EDTA to 0.05 M, NaCl to 0.5 M and tRNA to 100 μg ml^{-1}. Reactions were then extracted with phenol–chloroform, and RNA was precipitated with ETOH, resuspended in formamide and subjected to electrophoresis on a 6% polyacrylamide–7 M urea gel.

glnAp2 promoter was very sensitive to activation by NRI~P, yet the maximum level of expression was set by the governor sequences. In the absence of the governor sequences, the kinetics of activation appeared to be normal as cells became nitrogen starved and the intracellular concentration of NRI~P increased, but expression from the mutant promoters was dramatically elevated over that observed with the wild-type promoter in starved cells. Similar results were obtained in an *in vitro* transcription system, in which the yield of transcripts was higher from a template that lacked the governor sites than from the wild-type template at high activator concentrations.

Previous results have indicated that factors binding between the enhancer and the promoter may regulate expression from σ^{54}-dependent promoters. For example, binding of IHF at a site between the enhancer and the promoter is required for efficient activation of the *E. coli glnHp2* promoter (Claverie-Martin and Magasanik, 1991) and certain *nif* promoters of *Klebsiella pneumoniae* (Hoover *et al.*, 1990). Binding of IHF at these promoters is apparently required to facilitate the activation DNA loop and position the enhancer-bound activator and promoter-bound polymerase properly (Claverie-Martin and Magasanik, 1991). Similarly, the Nac protein of *Klebsiella aerogenes* represses transcription of the *nac* promoter by binding to a site located between the enhancer and the promoter (Feng *et al.*, 1995b). Repression by Nac appeared to result from bending of the DNA that adversely affected the formation of the activation DNA loop (Feng *et al.*, 1995b). Indeed, by altering the distance between the Nac site and the promoter, positions were identified at which the DNA bend induced by Nac stimulated transcription instead of repressing it (Feng *et al.*, 1995b). Together, the previous experiments with IHF and Nac show that topological regulation of the activation DNA loop is an important mechanism for the regulation of σ^{54}-dependent promoters.

The simplest model to account for all the available data is that binding of NRI~P to the low-affinity (governor) sites adversely affects the formation of the activation DNA loop and, by so doing, reduces the probability of interaction between the enhancer-bound activator and the promoter-bound polymerase. For example, binding of the governor sites may limit the flexibility of the DNA, reducing DNA looping. Alternatively, the binding of NRI~P to sites 3 and 4 may somehow block the ability of the activator bound to the enhancer to interact with the promoter-bound polymerase. A unique aspect of the *glnA* system is that the same transcription factor, NRI~P, is required for activation and governing of the promoter. A consequence of this arrangement is that cells may activate *glnA* expression first upon becoming nitrogen limited, and this activation may be limited in severely starved cells co-ordinately with the activation of other Ntr

genes. Thus, amplitude modulation of the activator concentration may bring about complex patterns of gene activation and inactivation.

Interestingly, in our experiments in which NRI was hyperexpressed from a multicopy plasmid based upon pBR322, governing of *glnAp2* was defective but not eliminated by mutation of sites 3 and 4. Thus, it seems that, under these conditions, multiple mechanisms were responsible for limiting the promoter. In a previous study, in which NRI was expressed from the *lac* promoter on a very high-copy-number plasmid, it was concluded that promoter limitation resulted from a mechanism that did not depend on sites 3 and 4 (Shiau *et al*., 1992). Our results may be reconciled with the earlier results if the levels of NRI expressed from pgln53 in our experiments was lower than that obtained when it was expressed from a very high-copy-number plasmid bearing the *lac* promoter, as seems likely. Apparently, at very high activator concentration, the governor-independent mechanism dominates, and the contribution of sites 3 and 4 to limiting promoter activity becomes less evident.

The σ^{54}-dependent promoters of *E. coli* have several features that are reminiscent of eukaryotic promoters, such as the relative position independence of the enhancer sequences, regulation by covalent modification of the activator and regulation of promoter activity by factors that influence the topology of the DNA. These promoters form a gene cascade, in which amplitude modulation of the activator is used to effect a programme of gene expression (Atkinson *et al*., 2002). The use of governor sites to limit activation of the first gene in the cascade upon amplification of the activator may prove to be a common feature of such gene cascades.

Experimental procedures

Plasmids, site-directed mutagenesis and strain constructions

The plasmids, primers and strains used in this study are listed in Table 3. The plasmid pgln53 contains the *glnA* promoter fused to *glnG* (Chen *et al*., 1982). The plasmid pglnAplac2 containing the *glnA* promoter fused to *lacZ* at the transcriptional +1 was constructed by polymerase chain reaction (PCR) amplification of the *glnA* promoter using primers 5′-CCGGAATTCATCCTCCGCAAACAAGTATTGCAGAG and 5′-CGCGGATCCTAAAAAAGATAAAGCGAAATCTGTGCCAAC and cloned as an *Eco*RI–*Bam*HI fragment into pRS551 (Simons *et al*., 1987). To mutagenize the *glnA* promoter fused to *lacZ* at the transcriptional +165, it was first amplified by PCR using primers 5′-CCGGAATTCATCCTCCGCAAACAAGTATTGCAGAG and 5′-CGCGGATCCTTACACCTGATGAGCAGGGATAGTGAC and cloned as *Eco*RI–*Bam*HI into pUC18 (Yanisch-Perron *et al*., 1985), resulting in plasmid pglnAp1. Mutations were introduced to this plasmid using the Stratagene Quickchange mutagenesis system. The sequences of the mutations introduced are shown in Fig. 1. The *glnA* promoter mutant plasmids were pglnAp2 (NRI site 3), pglnAp3 (NRI site 4) and pglnAp5 (NRI sites 3 and 4). The mutant promoters were then fused to *lacZ* by cloning as *Eco*RI–*Bam*HI fragments into pRS551, resulting in pglnAplac5 (NRI site 3), pglnAplac6 (NRI site 4) and pglnAplac7 (NRI sites 3 and 4).

To place the *glnA* promoter–*lacZ* fusions in single copy within the *trp* operon of the *E. coli* chromosome, all plasmids (pglnAplac2, pglnAplac5, pglnAplac6 and pglnAplac7) were digested with *Pst*I, the linear DNA transformed into TE2680 (Elliot, 1992) by electroporation using a Bio-Rad gene pulser apparatus and recombinants selected for kanamycin resistance. The *glnA* promoter–*lacZ* fusions were moved into desired strains by P1*vir*-mediated transduction of YMC10 (Backman *et al*., 1981), RB9060 (Bueno *et al*., 1985), K_c (Atkinson *et al*., 2002) and K_3 (Blauwkamp and Ninfa, 2002a). The *glnA* promoter–*lacZ* fusions were amplified by PCR, and the DNA was sequenced at the University of Michigan DNA sequencing core to confirm that they were as designed.

Growth media, glutamine synthetase and β-galactosidase assays

Cells were grown at 30°C in defined W-salts media (Pahel *et al*., 1978) supplemented with carbon and nitrogen sources as indicated. The γ-glutamyl transferase activity of glutamine synthetase (GS) was measured as described previously (Rhee *et al*., 1985). Total GS was determined in reactions in which Mn^{2+} was the sole metal ion, and unadenylylated GS was determined in reactions in which Mg^{2+} was present in large excess over Mn^{2+}. In the presence of Mn^{2+}, both adenylylated and unadenylylated GS should be active, whereas only unadenylylated GS should be active when supplied with Mg^{2+}. Results were expressed as nmol of glutamylhydroximate formed min^{-1} mg^{-1} cell protein. β-Galactosidase levels were expressed in Miller units and were assayed with cells permeabilized using SDS and chloroform (Silhavy *et al*., 1984).

DNase I footprinting and in vitro *transcription assays*

DNase I footprinting was conducted as described previously (Ninfa *et al*., 1987), except that the plasmid DNA used was prepared using a Concert midi-prep system (Marligen) instead of by density gradient centrifugation. DNase I was from Invitrogen Life Technologies, and optimal results were obtained using 0.1 unit of enzyme for 45 s at 22°C in reactions containing 5 nM linear, end-labelled DNA. The highest concentration of NRI used in our footprinting experiments was 600 nM, with NRII present at 40 nM and ATP present at 400 μM.

Single-cycle *in vitro* transcription assays were performed as described previously (Ninfa *et al*., 1987; Feng *et al*., 1995a), and multiple-cycle transcription assays were performed as described previously (Liu *et al*., 2002). Core RNA polymerase was from Epicentre and was used at 50 nM. σ^{54} and NRI were kind gifts from S. Monje and L. Field and were purified as described previously (Ninfa *et al*., 1987). σ^{54} was used at 50 nM, and NRI was used as indicated. NRII was a kind gift from A. Pioszak and was purified as described

Table 3. Strains, plasmids and primers used in this study.

Strain	Relevant genotype	Construction or source
YMC10	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$	Backman *et al*. (1981)
RB9060	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ ΔglnB2306	Bueno *et al*. (1985)
RB9040	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ Mu lysogen glnD99::Tn10	Bueno *et al*. (1985)
TH16	glnA::Tn5	Reitzer and Magasanik (1986)
SN24	endA1 thi-1 hsdR17 supE44 ΔlacU169 ΔglnLG lacI^q lacL8/λgln105	Schneider *et al*. (1991)
Kc	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ Δmdl-glnK::cam^r	Atkinson *et al*. (2002)
K$_3$	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ ΔglnK1 amtB$^+$	Blauwkamp and Ninfa (2002b)
YMC10A	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ glnA::Tn5	YMC10 × TH16 P1*vir*
YMC10LG	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ ΔglnLG	YMC10A × SN24 P1*vir*
TE2680	recD1903::Tn10 trpDC700::putPA1303 [kan^s cam^r lac]	Elliot (1992)
JM109	F′ traD36 proA + B + lacI^q Δ(lacZ)M15/Δ(lac-proAB)glnV44 e14 gyrA96 recA1 relA1 endA1 thi hsdR17	Yanisch-Perron *et al*. (1985)
XL1-Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′proAB lacI^{q}ZΔM15 Tn10]	Stratagene
MAAplac2	recD1903::Tn10 trpDC700::putPA130 [ϕ(glnAp-lacZ1) kan^r cam^s]	TE2680 × *Pst*I pglnAplac2
MAAplac3	recD1903::Tn10 trpDC700::putPA130 [ϕ(glnAp-lacZ2) kan^r cam^s]	Atkinson *et al*. (2002)
YMC10Apϕ	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ trpDC700::putPA130 [ϕ(glnAp-lacZ1) kan^r cam^s]	YMC10 × MAAplac2 P1*vir*
BApϕ	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ ΔglnB2306 trpDC700::putPA130 [ϕ(glnAp-lacZ1) kan^r cam^s]	RB9060 × MAAplac2 P1*vir*
KcApϕ	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ Δmdl-glnK::cam^r trpDC700::putPA130 [ϕ(glnAp-lacZ1) kan^r cam^s]	Kc × MAAplac2 P1*vir*
YMC10Apϕ2	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ trpDC700::putPA130 [ϕ(glnAp-lacZ2) kan^r cam^s]	Atkinson *et al*. (2002)
BApϕ2	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ ΔglnB2306 trpDC700::putPA130 [ϕ(glnAp-lacZ2) kan^r cam^s]	RB9060 × MAAplac3 P1*vir*
KcApϕ2	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ Δmdl-glnK::cam^r trpDC700::putPA130 [ϕ(glnAp-lacZ2) kan^r cam^s]	Kc × MAAplac3 P1*vir*
KgApϕ2	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ ΔglnK1 amtB$^+$ trpDC700::putPA130 [ϕ(glnAp-lacZ2) kan^r cam^s]	Blauwkamp and Ninfa (2002b)
DApϕ2	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ glnD99::Tn10 trpDC700::putPA130 [ϕ(glnAp-lacZ2) kan^r cam^s]	RB9040 × MAAplac3 P1*vir*
LGApϕ2	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ ΔglnLG trpDC700::putPA130 [ϕ(glnAp-lacZ2) kan^r cam^s]	YMC10LG × MAAplac3 P1*vir*
MAAplac5	recD1903::Tn10 trpDC700::putPA130 [ϕ(glnAp-lacZ5) kan^r cam^s]	TE2680 × *Pst*I pglnAplac5
MAAplac6	recD1903::Tn10 trpDC700::putPA130 [ϕ(glnAp-lacZ6) kan^r cam^s]	TE2680 × *Pst*I pglnAplac6
MAAplac7	recD1903::Tn10 trpDC700::putPA130 [ϕ(glnAp-lacZ7) kan^r cam^s]	TE2680 × *Pst*I pglnAplac7
YMC10Apϕ5	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ trpDC700::putPA130 [ϕ(glnAp-lacZ5) kan^r cam^s]	YMC10 × MAAplac5 P1*vir*
YMC10Apϕ6	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ trpDC700::putPA130 [ϕ(glnAp-lacZ6) kan^r cam^s]	YMC10 × MAAplac6 P1*vir*
YMC10Apϕ7	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ trpDC700::putPA130 [ϕ(glnAp-lacZ7) kan^r cam^s]	YMC10 × MAAplac7 P1*vir*
KgApϕ5	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ ΔglnK1 amtB$^+$ trpDC700::putPA130 [ϕ(glnAp-lacZ5) kan^r cam^s]	K$_3$ × MAAplac5 P1*vir*
KgApϕ6	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ ΔglnK1 amtB$^+$ trpDC700::putPA130 [ϕ(glnAp-lacZ6) kan^r cam^s]	K$_3$ × MAAplac6 P1*vir*
KgApf7	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ ΔglnK1 amtB$^+$ trpDC700::putPA130 [ϕ(glnAp-lacZ7) kan^r cam^s]	K$_3$ × MAAplac7 P1*vir*
DApϕ5	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ glnD99::Tn10 trpDC700::putPA130 [ϕ(glnAp-lacZ5) kan^r cam^s]	RB9040 × MAAplac5 P1vir
DApϕ6	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ glnD99::Tn10 trpDC700::putPA130 [ϕ(glnAp-lacZ6) kan^r cam^s]	RB9040 × MAAplac6 P1vir
DApϕ7	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ glnD99::Tn10 trpDC700::putPA130 [ϕ(glnAp-lacZ7) kan^r cam^s]	RB9040 × MAAplac7 P1vir
LGApϕ5	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ ΔglnLG trpDC700::putPA130 [ϕ(glnAp-lacZ5) kan^r cam^s]	YMC10LG × MAAplac5 P1vir
LGApϕ6	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ ΔglnLG trpDC700::putPA130 [ϕ(glnAp-lacZ6) kan^r cam^s]	YMC10LG × MAAplac6 P1vir
LGApϕ7	endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$ ΔglnLG trpDC700::putPA130 [ϕ(glnAp-lacZ7) kan^r cam^s]	YMC10LG × MAAplac7 P1vir

Table 3. *cont.*

Plasmid	Relevant features and construction	Source
pRS551	*lacZ* fusion vector	Simons *et al*. (1987)
pUC18	Ampicillin-resistant cloning vector	Yanisch-Perron *et al*. (1985)
pgln53	*glnA* promoter fused to *glnG*	Chen *et al*. (1982)
pTE103	Transcription vector	Elliot and Geiduschek (1984)
pLR100	Wild-type *glnAp2* promoter in pTE103	Ninfa *et al*. (1987)
pglnAplac2	PCR-amplified *glnA* promoter with 3′ end at +1 cloned as *Eco*RI–*Bam*HI fragment into pRS551	
pglnAp1	PCR-amplified *glnA* promoter with 3′ end at +165 cloned as *Eco*RI–*Bam*HI into pUC18	
pglnAp2	NRI site 3 of pglnAp1 mutagenized	
pglnAp3	NRI site 4 of pglnAp1 mutagenized	
pglnAp4	Mutation of NRI site 4 added to pglnAp2	
pglnAplac5	*Eco*RI–*Bam*HI fragment from pglnAp2 containing mutation of NRI site 3 cloned into pRS551	
pglnAplac6	*Eco*RI–*Bam*HI fragment from pglnAp3 containing mutation of NRI site 4 cloned into pRS551	
pglnAplac7	*Eco*RI–*Bam*HI fragment from pglnAp4 containing mutation of NRI sites 3 and 4 cloned into pRS551	
pglnApOG7	*Eco*RI–*Bam*HI fragment amplified from pglnAp1 using primers glnApLRUS1 and glnApLRDS1 and cloned into pTE103	
pglnApOG8	*Eco*RI–*Bam*HI fragment amplified from pglnAp4 using primers glnApLRUS1 and glnApLRDS1 and cloned into pTE103	

Primer	Sequence and description
glnApU.S.2	CCGGAATTCATCCTCCGCAAACAAGTATTGCAGAG Upstream primer for cloning *glnA* promoter (*Eco*RI)
glnApD.S.3	CGCGGATCCTAAAAAAGATAAAGCGAAATCTGTGCCAAC Downstream primer for cloning glnA promoter at +1 (*Bam*HI)
glnApD.S.5	CGCGGATCCTTACACCTGATGAGCAGGGATAGTGAC Downstream primer for cloning glnA promoter at +165 (*Bam*HI)
mutNRI#3US1	CTATATTGGTGCAACATTGTCTTCGACGTCGAGCCCTTTTGCACG (mutagenesis primer for NRI site 3)
mutNRI#3DS1	CGTGCAAAAGGGCTCGACGTCGAAGACAATGTTGCACCAATATAG (mutagenesis primer for NRI site 3)
mutNRI#4US	GTGGTGCAGCCCTTTTCGAGGAACGTCCCCATGATAACGCCTTTTAG (mutagenesis primer for NRI site 4)
mutNRI#4DS	CTAAAAGGCGTTATCATGGGGACGTTCCTCGAAAAGGGCTGCACCAC (mutagenesis primer for NRI site 4)
mutNRI#4US2	GACGTCGAGCCCTTTTCGAGGAACGTCCCCATGATAACGCCTTTTAG (mutagenesis primer for NRI sites 3 and 4)
mutNRI#4DS2	CTAAAAGGCGTTATCATGGGGACGTTCCTCGAAAAGGGCTCGACGTC (mutagenesis primer for NRI sites 3 and 4)
S1224S	CGCCAGGGTTTTCCCAGTCACGAC (New England Biolabs M13/pUC sequencing primer)
pRS551rev1	GGCTGTGGGATTAACTGCGCGTCGCCG (pRS551 sequencing primer)
glnApLRUS	CGGGGTACCGGATCCAATTGTGAGCGCTCACAATTGCACCAACATGGTGCTTAATGTTTCC Upstream PCR primer to clone *glnA* promoter into pTE103 (*Kpn*I–*Bam*HI)
glnApLRDS	GGGAATTCAAGCTTAATTGTGAGCGCTCACAATTAAAAAAGATAAAGCGAAATCTGTGCC Downstream PCR primer to clone *glnA* promoter into pTE103 (*Kpn*I–*Bam*HI)

previously (Ninfa *et al*., 1986). In single-cycle assays, NRII was used at 25 nM, whereas in multiple-cycle assays, NRII was used at 100 nM.

The transcription template pLR100, containing wild-type *glnAp2* promoter, has been described previously (Ninfa *et al*., 1987). Templates pglnApOG7 and pglnApOG8, containing the wild-type promoter and promoter with NRI binding sites 3 and 4 mutated, respectively, were constructed by PCR amplification of either pglnAp1 or pglnAp4, using primers glnApLRUS and glnApLRDS (Table 3). The PCR products were cloned into pTE103as *Bam*HI–*Hin*dIII fragments.

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