Three of Four GlnR Binding Sites Are Essential for GlnR-Mediated Activation of Transcription of the Amycolatopsis mediterranei nas Operon

Ying Wang, Jing-Zhi Wang, Zhi-Hui Shao, Hua Yuan, Yin-Hua Lu, Wei-Hong Jiang, Guo-Ping Zhao and Jin Wang


Published Ahead of Print 29 March 2013.

Updated information and services can be found at:
http://jb.asm.org/content/195/11/2595

These include:

**SUPPLEMENTAL MATERIAL**
Supplemental material

**REFERENCES**
This article cites 35 articles, 14 of which can be accessed free at:  http://jb.asm.org/content/195/11/2595#ref-list-1

**CONTENT ALERTS**
Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article),  more»

Information about commercial reprint orders:  http://journals.asm.org/site/misc/reprints.xhtml
To subscribe to another ASM Journal go to:  http://journals.asm.org/site/subscriptions/
Three of Four GlnR Binding Sites Are Essential for GlnR-Mediated Activation of Transcription of the *Amycolatopsis mediterranei nas* Operon

Ying Wang,*a,b Jing-Zhi Wang,*a Zhi-Hui Shao,*a Hua Yuan,*a Yin-Hua Lu,*a Wei-Hong Jiang,*a Guo-Ping Zhao,*a,b,c,d Jin Wang*a

CAS Key Laboratory of Synthetic Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China; State Key Laboratory of Genetic Engineering, Department of Microbiology and Microbial Engineering, School of Life Sciences, Fudan University, Shanghai, China; Shanghai-MOST Key Laboratory for Health and Disease Genomics, Chinese National Human Genome Center, Shanghai, China; Department of Microbiology and Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong SAR, China

In *Amycolatopsis mediterranei* U32, genes responsible for nitrate assimilation formed one operon, **nasACKBDEF**, whose transcription is induced by the addition of nitrate. Here, we characterized GlnR as a direct transcriptional activator for the **nas** operon. The GlnR-protected DNA sequences in the promoter region of the **nas** operon were characterized by DNase I footprinting assay, the previously deduced *Streptomyces coelicolor* double 22-bp GlnR binding consensus sequences comprising **a1**, **b1**, **a2**, and **b2** sites were identified, and the sites were then mutated individually to test their roles in both the binding of GlnR *in vitro* and the GlnR-mediated transcriptional activation *in vivo*. The results clearly showed that only three GlnR binding sites (**a1**, **b1**, and **b2** sites) were required by GlnR for its specific binding to the **nas** promoter region and efficient activation of the transcription of the **nas** operon in U32, while the **a2** site seemed unnecessary.

* Amycolatopsis mediterranei* is widely studied for its importance in producing the antitubercular rifamycins. It was found that nitrate, a significant nitrogen source for bacteria in the biosphere, markedly stimulated the yield of rifamycin SV by as much as 171% in *A. mediterranei* U32 fermentation, which simultaneously altered the expression of a series of genes related to both primary metabolism and secondary metabolism and thus was illustrated as the nitrate-stimulating effect (1). The assimilation of nitrate in bacteria requires nitrate reductase and nitrite reductase, which sequentially catalyze the reduction of nitrate to nitrite and then to ammonium (2). Although the assimilatory nitrate reductase was characterized from *A. mediterranei* decades ago, a **nas** operon consisting of **nasACKBDEF** genes encoding 7 enzymes/proteins responsible for nitrate assimilation and necessary for the nitrate-stimulating effect was just recently characterized (3). The transcription of the **nas** operon was activated by the addition of extracellular nitrate sources (3), while the activator for **nas** operon transcription remains unknown.

The regulation of nitrate assimilation is diverse and may involve different enzymes and regulatory mechanisms in different microorganisms. In the Gram-negative *Klebsiella oxytoca*, the enzymes responsible for nitrate assimilation are encoded by the **nasFEDCB** operon (4–6), the expression of which is stringently controlled by both the NtrB/NtrC two-component system (TCS) (7) and the NtrC-regulated NasR transcription antiterminator (8–10). Instead of the Ntr system, global regulators in actinomycetes are responsible for the regulation of the nitrate assimilation as well as the whole nitrogen metabolism. In *Streptomyces coelicolor*, the **nasA** gene (encoding the nitrate reductase) and **nirB1B2** genes (encoding the nitrite reductase subunits), located separately on the chromosome, are all positively regulated by the global nitrogen regulator GlnR and a newly identified GlnR target, NnaR (11–14). In *Mycobacterium tuberculosis*, the causative agent of tuberculosi, GlnR is also characterized as an activator for the nitrite reductase-encoding genes (**nirBD**) (15), although the regulation for the nitrate reductase-encoding genes still remains unknown. Although genes responsible for nitrate assimilation form an intact **nasACKBDEF** operon in *A. mediterranei* (3), different from those with segregated operons dispersed in the chromosomes of *S. coelicolor* and *M. tuberculosis*, GlnR is still likely the governor for the regulation of nitrate assimilation in *A. mediterranei* because of its high similarities in both protein sequences (16) and regulatory functions (17) to those of *S. coelicolor* and *M. tuberculosis*.

So far, the GlnR binding consensus sequences have been widely studied in some model actinomycetes, which are comprised of double 22-bp GlnR binding boxes (“5-nt a site–n6-5-nt b site–n6”) in most cases in *S. coelicolor* (13) and in the promoter regions of **glnA**, **amtB**, and **amt1** in *Mycobacterium smegmatis* (16). However, the structure-functional aspects of the consensus sequences are complicated, e.g., they may consist of either triple 22-bp GlnR binding boxes as in the **amtB** promoter (18) or double a sites as in the promoters of **nasA** (14) and **SC05163** (19) in *S. coelicolor*. Besides, in *Streptomyces venezuelae*, the double a sites (GTnAC-n6-GTnAC) were found to be the most common GlnR binding consensus sequences, while in several cases, there was either only one 22-bp GlnR binding box (GTnAC-n6-GAnAC) or no identifiable GlnR binding consensus sequences being characterized (20). Therefore, although homology has been found among these GlnR proteins (16), the DNA sequences of their protected *cis* ele-
ments and the compositions of their binding sites are not all the same, which thus suggests that more work should be done to better understand the GlnR binding cis elements.

In this study, we proved that GlnR is a direct activator for the transcription of the nas operon in A. mediterranei U32. Based on the information on the GlnR binding cis elements in S. coelicolor, we in silico identified a similar double 22-bp GlnR binding box, consisting of a1, b1, a2, and b2 sites in the GlnR-protected sequences in the nas operon promoter in A. mediterranei U32. With sequential mutation analyses of the 4 deduced GlnR binding sites, we demonstrated that only 3 sites (a1, b1, and b2 sites) were required for GlnR’s specific binding of the promoter region of the nas operon in vitro and efficient activation of transcription of the nas operon in vivo. We also found that GlnR could form two distinct complexes with the nas promoter, and with the aid of DNase I footprinting analyses of the GlnR binding of the mutated nas promoter regions, the mechanism for GlnR binding of nas promoter is discussed.

MATERIALS AND METHODS

Bacterial strains, media, and primers. A. mediterranei strains were grown at 30°C in Bennet medium (21) supplemented with the appropriate nitrogen source (22). S. coelicolor strains were grown at 30°C in either the nitrogen-rich S medium (23) or the nitrogen-limited N-Evans medium (12) with either 5 mM nitrate or 20 mM glutamine as the sole nitrogen sources. When needed, apramycin (50 µg/ml), kanamycin (50 µg/ml), erythromycin (200 µg/ml), nalidixic acid (50 µg/ml), and ampicillin (100 µg/ml) were used. All the primers used in this study are listed in Table S1 in the supplemental material.

Expression and purification of the recombinant A. mediterranei GlnR. The A. mediterranei U32 glnR gene was amplified with primers EXAMR_f and EXAMR_r using U32 chromosome DNA as a template. Primers EXAMR_f and EXAMR_r were designed to introduce EcoRI and HindIII sites at the 5’ and 3’ termini, respectively. The PCR product was digested with EcoRI and HindIII and then inserted into the corresponding sites of PET28a vector to form the expression plasmid pEXAMR. Expression and purification of the recombinant GlnR were performed according to the methods recommended by the manufacturer (Novagen, Darmstadt, Germany), and protein concentrations were determined using the Bradford method (24). Purified A. mediterranei GlnR was stored in the storage buffer (20 mM Tris-Cl [pH 8.0], 25 mM KCl).

Electrophoretic mobility shift assay (EMSA). The promoter regions of the nas operon were either PCR amplified employing primer pair nasP_f/nasP_r or annealed with equal molar amounts of synthetic oligonucleotides (see Table S1 in the supplemental material). Purified probes were labeled with [γ-32P]ATP using T4 polynucleotide kinase (T4 PNK) (NEB). The binding of His-tagged GlnR to labeled DNA was performed at room temperature in a total volume of 20 µl containing 10 mM Tris-HCl (pH 8.0), 25 mM KCl, 1.0 mM MgCl₂, and 0.1 mM dithiothreitol (DTT). To prevent nonspecific binding, sheared salmon sperm DNA was added to a final concentration of 100 ng/µl. After 20 min of incubation, the fragments were separated by a 5% nondenaturing polyacrylamide gel (unless specified) buffered with 0.5× Tris-buffered EDTA (TBE). Gels were scanned with an FLA-7000 phosphorimager (FujiFilm Corporation, Japan).

RNA extraction, reverse transcription-PCR (RT-PCR), and primer extension. For normal RNA extraction, A. mediterranei strains were grown in liquid Bennet medium for 48 h before being inoculated into fresh liquid Bennet medium supplemented with 0.8% KNO₃ or 0.8% (NH₄)₂SO₄ for further culture for 24 h. For time course analyses, Bennet medium-cultured strains were inoculated into fresh Bennet medium with 0.8% KNO₃ and cells were harvested at different time points. Total RNA was extracted using TRizol reagent (Invitrogen). RNA was treated with RNase-free DNase I (Promega) to prevent contamination of trace genomic DNA. Reverse transcription was performed with a random hexamer primer using 3 µg RNA in a total volume of 30 µl employing SuperScript III reverse transcriptase (Invitrogen). PCR was performed employing 20-ng reaction mixtures as the template to check the transcription of nasA and glnR genes and using the rpOB gene as the internal control. A negative control was made by following the same procedures except that the addition of reverse transcriptase was omitted. Two independent samples were used for analyses.

For primer extension assay, Bennet medium-cultured strain U32 was inoculated into Bennet medium with nitrate supplementation and a further 24-h incubation was performed before the cells were harvested. Total RNA was prepared as described above, and about 110 µg RNA was used to identify the transcription initiation site of the nas operon, using γ-32P-labeled primer U32nasA-FPE, which was complementary to the 16th to the 40th nucleotides of the nasA protein coding sequence (CDS) (25). The analysis of reverse transcripts was performed on a 6% PAGE gel with 7 M urea, followed by phosphorimager scanning.

DNase I footprinting assay. The DNase I footprinting experiments were carried out either with γ-32P-labeled probes, according to the method of Leblanc and Moss (26), or with 6-carboxyfluorescein (FAM)-labeled probes (18). In employing the γ-32P-labeled probe method, primer U32nasA-FPR was first end labeled with [γ-32P]ATP using T4 PNK, and then a 205-bp DNA fragment was amplified by primer U32nasA-FFP and end-labeled primer U32nasA-FPR. Purification of the probe was performed using the Wizard SV Gel and PCR Clean-Up system (Promega). The appropriate probe (about 150,000 cpm) was first incubated at room temperature with different amounts of purified His₆-GlnR and 0.2 µg sheared salmon sperm DNA in a total volume of 40 µl in the same buffer as that used in EMSA. After 30 min of incubation, 0.3 units DNase I (Promega) was added, quickly mixed, and incubated at 25°C for 1 min, followed by precipitation of the digested probes. The sequencing ladders were prepared with the same primer, U32nasA-FPR, using the fmol DNA cycle sequencing system (Promega). The digestion products together with the ladders were analyzed on 6% polyacrylamide-7 M urea sequencing gels, followed by phosphorimager scanning.

To analyze the GlnR-protected regions in the mutated promoter regions of the nas operon, FAM-labeled probes were adopted and two rounds of PCR amplification were employed. First, primers U32nasA-FPR/FAM and U32nasA-FFP were used to amplify the promoter regions, which were then used as the templates for preparation of the probes with FAM-labeled M13F and U32nasA-FFP. The DNase I footprinting procedures and conditions were the same as those that we described before (18).

Construction of the in vivo reporter system. The promoter region of the nas operon was amplified with primers RSnas1 and RSnas10, and the product was then cloned to the HinCII site of pM9 (pBluescript II KS digested with NotI and HindIII, Klenow blunted, and self-ligated; kindly provided by Jun Ma and Zilong Zhang), producing pMZnasP. The direction of the insertion was verified to ensure that the promoter direction was from T7 to T3, and the inserted fragment was sequenced. The ermE gene was amplified with primer pair RSERF_F and RSERF_R, which was introduced to the HinCII site of pM9. The ermE gene was then excised from the plasmid with EcoRV and XhoI and inserted into the same sites of pMZnasP, producing pMZnasPer. Finally, the apramycin resistance gene (apr) was cut from pBcam (apramycin resistance cassette cloned into pBCKs, provided by Xiaoming Ding) with SmaI and introduced into the Ecl136II site of pMZnasPer to produce pMZnasP, employing ermE as the reporter gene. Mixed primers composed of RSnas1 (0.5 µM), diluted RSnas2 (0.01 µM), diluted RSnas3 (0.01 µM), and RSnas10 (0.5 µM) were used for amplification to synthesize the a1 site-mutated nas promoter. Similarly, mixed primers composed of RSnas1 (0.5 µM), diluted RSnas4 (0.01 µM), diluted RSnas3 (0.01 µM), and RSnas10 (0.5 µM) were used for synthesis of the b1 site-mutated promoter; mixed primers composed of RSnas1 (0.5 µM), diluted RSnas7...
RESULTS

GlnR activates the transcription of the nas operon in A. mediterranei U32. The transcription of the nas operon in U32 was remarkably elevated when medium was supplemented with nitrate (Fig. 1) (3), while in the glnR null mutant Rk (27), no transcriptional activation of the nas operon could be detected under the same condition (Fig. 1A). To exclude the possibility that GlnR affects the stability of the mRNA of the nas operon, we performed further time course analyses of the transcription of the nas operon. With the increase of incubation time, the nas transcription gradually increased in the wild type, while no increase of nas transcription could be detected in the glnR null mutant (Fig. 1B). Therefore, one may conclude that GlnR activates the transcription of the nas operon in U32.

Characterization of the promoter of the nas operon in A. mediterranei U32. A primer extension assay identified the transcript of the nas operon, which initiated from an adenine (A) and was located 27 nucleotides (nt) upstream of the nasA translational start codon (Fig. 2A). Preceding the transcription initiation site was the typical –10 box (TAGCGT) usually identified in the Streptomyces vegetative promoters (22, 27) (Fig. 2D).

The N-terminal His-tag fused GlnR (His6-GlnR) of A. mediterranei U32 was produced in Escherichia coli BL21(DE3) and purified by nickel-nitritotriacetic acid (Ni-NTA) affinity chromatography. With purified His6-GlnR and the promoter region of the nas operon, electromobility shift assay (EMSA) results indicated...
that GlnR was able to specifically bind to the nas promoter in vitro (Fig. 2B). A DNase I footprinting assay, employing γ-32P-labeled probes was further employed to precisely characterize the GlnR-protected DNA sequences. Along with the increase of GlnR, a GlnR-protected region of 49 nt was clearly identified within the nas promoter, extending from nucleotide 91 to 42 relative to the translational start site of the nasA gene in the coding strand (Fig. 2C). Almost the same GlnR-protected region was obtained when the DNase I footprinting assay was performed employing the FAM-labeled probes (see Fig. 4A).

Because the A. mediterranei GlnR (AME_GlnR) is not only structurally homologous to that of S. coelicolor (SCO_GlnR) (27) but also able to complement the glutamine auxotrophic phenotype of the S. coelicolor glnR null mutant in trans (17), we propose that the two GlnRs share much homogeneity in their binding DNA consensus sequences. The AME_GlnR-protected sequences in the nas promoter were compared with the typical double 22-bp SCO_GlnR binding motif (13), and a similar double 22-bp AME_GlnR binding motif, consisting of a1, b1, a2, and b2 sites, was identified in silico (Fig. 2D).

Only three out of the four GlnR binding sites are required for GlnR-mediated transcriptional regulation of the nas operon. Transition mutations of individual nucleotides within each site were constructed to further characterize the four GlnR binding sites in the nas promoter. The transcription of both rpoB and nasA was used as the internal control. Abbreviations: WT, wild-type promoter; a1M, promoter with mutation in a1 site; b1M, promoter with mutation in b1 site; a2M, promoter with mutation in a2 site; b2M, promoter with mutation in b2 site. Am, Bennet medium with 0.8% ammonium; Ni, Bennet medium with 0.8% KNO3.
without any changes in the binding patterns of GlnR/DNA observed, which suggested that the a2 site was not required by GlnR for binding to the nas promoter in vitro.

The mutated promoters described above together with the wild-type promoter were further fused to a reporter gene (ermE, erythromycin resistance gene) and integrated into the chromosome of A. mediterranei U32 upstream of the promoter of the nas operon via single crossover recombination (see Materials and Methods). Transcriptional analyses of ermE with RT-PCR were conducted to measure the activities of the mutated promoters. The transcript of the nasA gene from the native nas operon, with its native promoter, was used as an internal control. The RT-PCR results showed that the transcription of nasA was remarkably enhanced under nitrogen-limited conditions and remained at the same level among all the tested strains, but the transcription of ermE was activated only under the wild-type promoter of the nas operon and the a2 site-mutated promoter (Fig. 3C and D). Mutations in either the a1 site, the b1 site, or the b2 site resulted in the failure of activation of ermE transcription, which therefore demonstrated that all of the three sites were required for GlnR-medi- cated in vivo transcriptional regulation.

Two distinct steps are found during the process of GlnR binding of the nas promoter. When the wild-type nas promoter was employed for EMSA (Fig. 3B), the amount of complex II increased along with the increase of GlnR concentration while the amount of complex I did not change significantly. The findings thus suggested that complex I might be the intermediate complex, with partial binding of GlnR, while complex II was likely the final complex, with GlnR fully bound.

Except for the mutations in the a2 site, all other mutations demonstrated changes of band shift patterns (Fig. 3B). Specifically, when the a1 site was mutated, only complex II was formed, which indicated that the a1 site was required for the formation of complex I. As the complex II formed under this condition was extremely weak, one could also infer that the formation of complex I facilitated the formation of complex II. When the b1 site was mutated, no complexes could be detected, demonstrating that the b1 site was the most pivotal and necessary for the formation of both complexes. Mutation in the b2 site resulted in failure of complex II formation, but with simultaneous increase of complex I. The results not only showed that the b2 site was necessary for the formation of complex II but also indicated that complex II was formed on the basis of complex I. Therefore, once the formation of complex I was blocked, the accumulation of complex I could be observed. In summary, a1 and b1 sites were required by GlnR to form complex I, while b1 and b2 sites contributed to the formation of complex II with GlnR.

To further understand the possible mechanism for GlnR binding to the nas promoter region, a DNase I footprinting assay was used to precisely characterize the GlnR-protected DNA sequences in the above-described mutated promoters (Fig. 4). Consistent with the EMSA results, when the a1 site was mutated, the GlnR-protected region extended from nucleotide −81 to −42, including the b1-a2-b2 sites only, and the protection was much weaker than that with the wild-type promoter. No protection by GlnR could be observed when the b1 site was mutated, which was consistent with the EMSA result. When the a2 site was mutated, the same GlnR-protected region as that of the wild-type promoter was obtained, once again demonstrating that the a2 site was dispensable for GlnR binding. The b2 site mutation, which caused the formation of complex I only with GlnR binding, resulted in the protection of a1-b1 sites by GlnR only, proving that complex I was formed by GlnR binding of a1-b1 sites.

During the process of GlnR binding of the nas promoter, two distinct steps can be found (see Fig. S1 in the supplemental material). First, GlnR binds to a1 and b1 sites to form complex I, which actually has been demonstrated via the DNase I footprinting assay employing the b2-site-mutated probe. On the basis of complex I formation, GlnR binds to the b2 site to produce complex II. However, due to the low resolution of EMSA and the fact that purified recombinant GlnR oligomerizes in solution, it is still unclear how many sites (two or three) are occupied by GlnR in complex II. Therefore, three different types of complex II are proposed for further demonstration, where in II(a) (see Fig. S1C), a dimer of GlnR binds to b1-b2 sites and the a1 site is released while all three necessary sites are occupied either by a trimer of GlnR in II(b) or by a tetramer of GlnR in II(c) (see Fig. S1D and E).

DISCUSSION

The GlnR binding cis elements are complex and diverse. The cis elements for GlnR binding and regulation have been the subject of research for years. The GlnR binding consensus sequences are complex and diverse for different promoter regions with respect to both the number of GlnR binding boxes and the distances between the boxes (12–14, 16, 18, 20, 28, 29). For example, Tiffert et al. (13) proposed a 22-bp GlnR binding consensus sequence comprised of an “a site” of gTnAc and a “b site” of GaAc with a fixed distance of 6 bases in between. However, further studies proved that the number of GlnR binding sites might vary from one to six in different GlnR target genes (18, 19). Here in this study, we demonstrated that GlnR was the direct regulator for the transcription of the nas operon in A. mediterranei U32 under nitrate-supplemented conditions. Only three GlnR binding sites (a1, b1, and b2 sites) rather than the four identified in silico were required for GlnR to bind to the DNA in vitro and to regulate the gene transcription in vivo, while the a2 site seemed unnecessary.

Although the GlnR binding consensus sequences of the a site and the b site were obtained on the basis of a dozen studies in S. coelicolor (12–14, 18), the DNA sequences of the GlnR binding sites were not conserved among the GlnR target genes, e.g., the a sites previously defined (see Fig. 2A in reference 13) and the a1 site in this study, which thus raises the question of whether the bioinformatically predicted GlnR binding site is accurate. Driven by this question and considering the fact that the a2 site seems dispensable, we propose a new GlnR binding motif in the promoter of the nas operon (see Fig. S2 in the supplemental material), where the a1 site is inverted and the b2 site is moved one base left and more conservative GlnR binding sites to form complex I, which has so far been proved true. Although the b1 site and the b2 site are separated by 16 bp, their spatial distance can be decreased through DNA bending after the binding of GlnR to the b1 and b2 sites, a mechanism of regulation which has been proven to be widely used by prokaryotes (for a review, see reference 30). Notably, the newly proposed b2 site is exactly the same as the a1 site in the glnA promoter of A. mediterranei U32 (see Fig. S2) (27). Of course, the proposed model still needs further examination, i.e., to determine whether GlnR is able to bind a pair of inverted sites.
Characterization of the GlnR-protected cis elements in the wild-type and mutated promoter regions of the nas operon in *A. mediterranei* U32 by DNase I footprinting assay using FAM-labeled probes. Probes in panels A to E were wild-type, a1M, b1M, a2M, and b2M promoters, respectively. Different amounts of GlnR were used for each assay, as labeled in the figure. (A) GlnR-protected DNA sequences in the wild-type *nas* promoter region were almost the same as those revealed by a traditional 32P-labeled probe (Fig. 2C). (B to E) The GlnR-protected regions are marked with dashed boxes, and the region protected in the wild-type *nas* promoter is indicated by a red underline. The mutated sites are labeled on the sequencing map by a blue underline. The sequences shown below the figure are self-explanatory. We also noticed a hypersensitive site in the promoter region, which is not shown in Fig. 2C. A possible explanation for this phenomenon is that the probe used here is shorter than that used in Fig. 2C (compare with Fig. 2D). When the a2 site was mutated, the DNase I digestion pattern was different from that of the wild type and the signal of the hypersensitive site was much weakened (D).
Complex II is required for GlnR-mediated transcriptional activation of the nas operon. As a direct transcriptional regulator for the nas operon in *A. mediterranei*, GlnR first binds to *a1* and *b1* sites, forming complex I. The formation of complex II is required for activation of the transcription of the *nas* operon, as the mutation of the *b2* site impedes transcriptional activation by GlnR. We also noticed that the formation of complex I apparently facilitates the formation of complex II, and without the formation of complex I, AME_GlnR binds to *b1* and *b2* sites of *nasP* DNA with an extremely low efficiency (Fig. 3B), which is probably the cause of the failure in *in vivo* regulation. On the other hand, although complex II can still be obtained when the *a1* site is mutated, which also has the same migration rate as that formed by GlnR binding to the wild-type promoter, it is still uncertain that the two complex IIs are the same in their composition because of the low resolution of EMSA. In addition, the purified recombinant GlnR oligomers in solution and thus may differ from the native GlnR in binding of the *nas* promoter to some extent. Therefore, based on present data, three possible models are proposed subject to further verification (see Fig. S1 in the supplemental material) and further *in vivo* studies, i.e., with careful mutation of the three sites to either strengthen or weaken the formation of complex I, and direct characterization of the oligomerization status of GlnR *in vivo* in response to different extracellular nitrogen sources may help us better understand the mechanism for GlnR binding and transcriptional regulation.

It is also worth noting that the LysR-type transcriptional regulators (LTTR) (e.g., LysR [31, 32], CatR [33], and CbbR [34]) can also form two protein-DNA complexes. With the availability of the crystal structure of LTTR (ChnR [35]), the binding processes have been uncovered [36]. First, a dimer of LTTR binds to the ribosome binding site (RBS) sequence to produce complex II (the intermediate complex), then another dimer of LTTR binds to the ABS (activation binding site) sequence to form complex I (the final complex), and the two dimers form a tetramer of LysR at the same time. Similarly, the formation of the final complex is dependent on the formation of the intermediate complex in LTTR binding. However, as GlnR belongs to the OmpR family, the oligomerization of GlnR can be different from that of LTTR after binding to DNA. Therefore, mechanical comparison with LTTR may not shed light on the mechanism for GlnR binding and regulation, and the crystal structure of GlnR, particularly crystal structures of GlnR-DNA complexes, may possibly bring direct clues.

ACKNOWLEDGMENTS

The work was supported by the National Natural Science Foundation of China (30830002 and 31121001); the National Basic Research Program of China (2012CB721102); the Natural Science Foundation of Shanghai, China (11ZR1442900); and the National Postdoctoral Science Foundation of China (2012M520820).

REFERENCES


