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Autogenous Regulation of the *Bacillus subtilis glnRA* Operon

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Purified *Bacillus subtilis* GlnR was shown to bind with high affinity to a specific region that overlaps with the *glnRA* promoter site. The GlnR binding site includes four copies of a repeated sequence that may be the recognition site for the protein. GlnR inhibited transcription from the *glnRA* promoter in vitro.

Glutamine synthetase (GS; L-glutamate:ammonia ligase [ADP-forming], EC 6.3.1.2) is the major enzyme responsible for assimilation of ammonium ions into organic compounds in *Bacillus subtilis* (18). Its product, glutamine, is the donor of nitrogen atoms for synthesis of other amino acids, purines, pyrimidines, and vitamins (18). Regulation of GS synthesis and function is critical for cellular efficiency because GS activity provides a central building block and consumes ATP.

The concentration of GS protein in *B. subtilis* is regulated at the transcriptional level in response to the nitrogen source available in the medium (6, 7, 21). The gene encoding GS, *glnA*, lies in a dicistronic operon in which it is preceded by *glnR* (25), a gene whose product is required for negative regulation of the operon (20). Initial in vitro experiments with partially purified GlnR indicated that this protein binds to a DNA fragment that includes the promoter region of the operon (20). Later experiments showed that purified GlnR can retard the mobility of a fragment corresponding to positions −104 to +83 relative to the transcription start point (22). Furthermore, the region located between positions −104 and −35 was shown to be required for negative regulation in vivo (22). This region includes a dyad symmetry element between positions −40 and −60 within which a point mutation abolishes repression (22). The dyad element is a site of apparent GlnR-DNA interaction in vivo (10). The organization and regulation of the *B. subtilis glnRA* operon seem to be conserved in *Bacillus cereus* (15, 16) and *Staphylococcus aureus* (9).

In the present work, GlnR was purified to apparent homogeneity and shown to interfere with transcription by binding to four tandem segments of DNA that overlap the *glnRA* promoter site. The sites of binding correlate well with the results of mutagenesis experiments that define the *glnRA* operator site (22) and with in vivo determinations of GlnR-DNA contact points (10).

Purification of GlnR. Purification of GlnR was facilitated by high-level expression of the *glnR* gene from a phage T7 late promoter in *Escherichia coli*. After heat shock and rifampin treatment (to suppress transcription dependent on host RNA polymerase), almost all protein synthesized was GlnR (2). Induction of strains containing pT7-12 (carrying *glnR1*, an AUG-to-AAG start codon mutant version of *glnR*) or the vector pT7cat6 alone resulted in no significant accumulation of any GlnR-like polypeptide (2). GlnR was found in the insoluble fraction of overexpressing cells. Extraction of this fraction with low-salt buffers removed most other proteins (Fig. 1, lane 3). Further extraction of the insoluble material with 1 M NaCl solubilized up to 80% of the GlnR protein and yielded preparations that were 50 to 75% GlnR (Fig. 1, lane 4). High-salt extracts showed the same reactivity in DNase protection and gel shift experiments as did highly purified GlnR (see below). Further purification required that the protein concentration of GlnR-containing preparations be kept below 800 μg/ml, particularly when the salt concentration was decreased, to avoid aggregation of GlnR. The final preparation, after heparin-Sepharose chromatography and gel filtration, was more than 99% GlnR, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1, lane 7). The

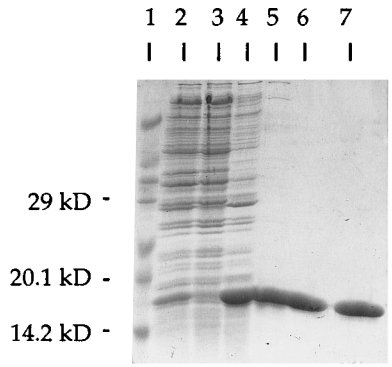

FIG. 1. Purification of GlnR. The figure shows a Coomassie blue-stained SDS–15% polyacrylamide gel of samples at various stages of purification. In lane 1, molecular mass markers (with masses in kilodaltons [kD] indicated on the left) were run. A 1-liter culture of *E. coli* K38 carrying plasmids pGP1-2 (27) and pT7-54 (20) was induced to produce GlnR protein as described previously (20). After being harvested, the cells were resuspended at 0°C in 50 mM Tris-HCl (pH 8.0)–0.5 mM EDTA–5 mM $MgCl_2$–2 mM $CaCl_2$ (TEMC) and broken by three passages through a French pressure cell at 10,000 lb/in^2 (lane 2). After centrifugation at 17,500 × *g* for 15 min, the pellet was extracted with low-salt buffer (TEMC containing 5% [vol/vol] glycerol and 400 μg of phenylmethylsulfonyl fluoride [PMSF] per ml) (lane 3). Several extractions of the pellet with 50 mM Tris (pH 8.0)–0.5 mM EDTA–1 M NaCl–400 μg of PMSF per ml followed. The high-salt extract used in some experiments was the third extract of the pellet, which appeared to be >66% GlnR as judged by SDS-PAGE. This extract was adjusted to 50% glycerol and stored at −20°C. For complete purification of GlnR, the high-salt extracts were combined (lane 4), diluted to 200 mM NaCl, and applied to a column of heparin-Sepharose CL-4B. Elution was done with a buffer containing 50 mM Tris (pH 8.0), 0.5 mM EDTA, 5% glycerol, 5 mM $MgCl_2$, and a linear gradient of 0.1 to 0.65 M KCl. The peak fractions (lane 5) were subjected to gel filtration chromatography on Bio-Gel P-30 in 50 mM Tris (pH 8.0)–0.5 mM EDTA–5% glycerol–0.8 M KCl. The peak fractions from the gel filtration were combined, concentrated, and dialyzed against 50 mM Tris (pH 8.0)–0.5 mM EDTA–0.8 M KCl–50% (vol/vol) glycerol. Any precipitate that formed during dialysis was removed by centrifugation. The concentration of protein in the final preparation (lanes 6 and 7) was 600 μg/ml by the Bradford assay and 711 μg/ml by A_{205}.

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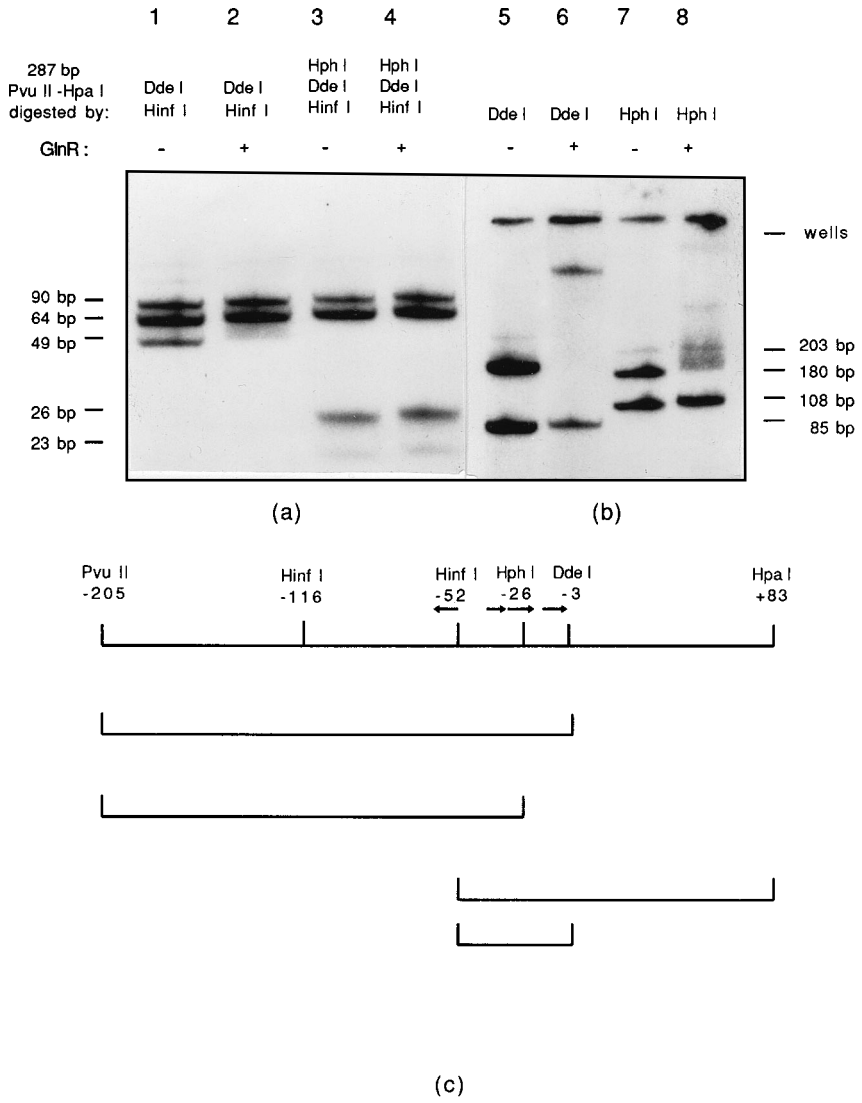

FIG. 2. Gel mobility shift assays. The 287-bp *Pvu*II-*Hpa*I fragment from pHJS54 (21) was gel purified and further digested with *Hph*I, *Dde*I, or *Hin*fI. *Dde*I and *Hin*fI digests were end labeled by being filled in with [α-^{32}P]dATP and DNA polymerase I Klenow fragment (New England Biolabs, Inc.). (The *Pvu*II-*Hin*fI and *Dde*I-*Hpa*I fragments comigrated at ~90 bp.) *Hph*I digests were labeled with [γ-^{32}P]ATP by an exchange reaction of T4 polynucleotide kinase (New England Biolabs, Inc.). GlnR was mixed with 50 to 150 ng of DNA fragments in a binding buffer containing 10 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 5% glycerol, 50 mM sodium acetate, 0.05% Nonidet P-40, and calf thymus DNA (800 μg/ml). After incubation at room temperature for 15 min, the samples were mixed with dye-loading buffer and applied to gels of 12% acrylamide–bis-acrylamide (30:1) in Tris-borate-EDTA (TBE) buffer. Voltage was applied during the loading process and continued at room temperature for 30 min or less. Molecular size indicators refer to unshifted DNA fragments. (a) Lanes: 1 and 2, *Hin*fI-*Dde*I double digest without and with GlnR, respectively; 3 and 4, *Hin*fI-*Dde*I-*Hph*I triple digest without and with GlnR, respectively. (b) Lanes: 5 and 6, *Dde*I-cut DNA without and with GlnR, respectively; 7 and 8, *Hph*I-cut DNA without and with GlnR, respectively. (c) Summary of the above experiments and others (data not shown) with fragments shifted by GlnR-containing extracts. The fragments shown are the ones that were shifted. Arrows represent the locations of the repeated sequence ACATCANAT (see the text).

apparent mass of GlnR, as estimated by gel filtration, was 25 to 31 kDa, suggesting that the bulk of the GlnR protein (monomer with a size of 15,834 Da) was dimeric.

Gel mobility shift assays. We had previously shown that a high-salt extract of GlnR-overproducing *E. coli* retards a *glnRA* promoter-containing restriction fragment in gel mobility assays (20). To narrow the site of binding, the 287-bp *Hpa*I-to-*Pvu*II *glnRA* promoter-containing restriction fragment (corresponding to positions −205 to +83) was purified and further digested with *Hin*fI, *Hph*I, and *Dde*I, either alone or in combination. These smaller fragments were end labelled and used in gel retardation assays with partially purified GlnR (i.e., the high-salt extract shown in Fig. 1, lane 4). As shown in Fig. 2, an amount of GlnR that completely retarded the 203-bp *Pvu*II-*Dde*I fragment spanning the −205 to −3 region of the promoter (coordinates defined relative to the transcription start point) gave only partial shifting of the 180-bp *Pvu*II-*Hph*I fragment spanning positions −205 to −26. Note also that the mobilities of the shifted complexes were different, suggesting that complexes have different conformations. In experiments for which the results are not shown, fivefold-more protein was required to retard fully the 180-bp fragment or the 49-bp *Hin*fI-*Dde*I fragment spanning positions −52 to −3. When DNA triply digested with *Hph*I, *Dde*I, and *Hin*fI was used, no fragments were retarded even at high GlnR concentrations, even though the 23-bp *Hph*I-*Dde*I and 26-bp *Hin*fI-*Hph*I fragments represent overlapping regions of fragments that are retarded. These results are consistent with the findings of

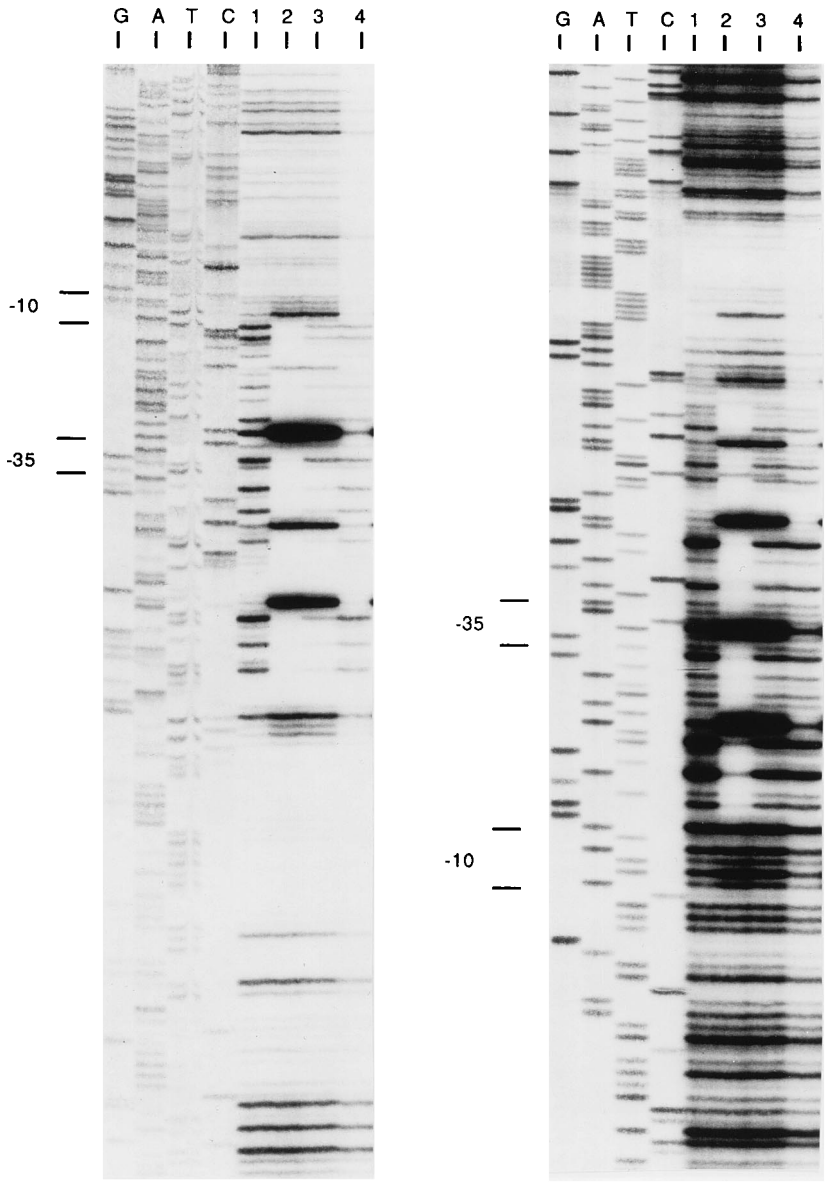

FIG. 3. Protection of *glnRA* promoter region from DNase I by purified GlnR. A 324-bp *glnRA* promoter-containing *Taq*I fragment (corresponding to positions −216 to +108) was gel purified from a digest of pSB20 (a plasmid in which the *Taq*I fragment was cloned at the *Acc*I site of pUC8; 2) that had been dephosphorylated by treatment with calf intestinal alkaline phosphatase (Boehringer Mannheim). The fragment was labeled at both ends with T4 polynucleotide kinase and [γ-^{32}P]ATP. Samples were cut with *Hpa*I for bottom-strand analysis or with *Pvu*II for top-strand analysis. These treatments removed 24- or 10-bp labeled fragments, respectively, from one or the other end of the DNA. After deproteination, ~0.1 pmol of labeled DNA was added to GlnR in 45 μl of binding buffer (Fig. 2) containing 2 μg of calf thymus DNA per ml and 1.5 mM $MgCl_2$. After incubation for 30 min at room temperature, 0.15 U of RQ1 DNase (Promega) was added, and the incubation was continued for 1 min at room temperature. The reaction was stopped by the addition of EDTA–sodium acetate–tRNA carrier–ethanol. Samples were centrifuged, dried, and resuspended in formamide-dye mix, boiled, and loaded onto a 6% acrylamide sequencing gel. Sequencing reactions were run in parallel on the gel to determine the locations of protected regions. The two oligonucleotide primers used in sequencing reactions had the same 5′ ends as the labeled *Taq*I 5′ ends except for the terminal phosphate. Lanes: G, A, T, and C, sequencing ladders; 1 and 4, no GlnR; 2, 6 nM GlnR monomer; 3, 3 nM GlnR. DNA sequencing was done by the dideoxy chain termination method of Sanger et al. (17), with double-stranded DNA as the template and synthetic oligonucleotides as the primers for modified T7 DNA polymerase (Sequenase; U.S. Biochemicals).

Schreier et al. (22) that GlnR retards the mobility of a fragment corresponding to positions −104 to +83 and protects the promoter region from cleavage by *Hin*fI at position −52. The segments required for binding also correlate well with DNA sequences known to be required for regulation in vivo. That is, fragments extending from positions −63 or −52 to −8 responded at least partially to GlnR-dependent regulation but a fragment spanning positions −35 to −8 did not respond (22).

Binding in vitro was enhanced twofold by the addition of 0.05% Nonidet P-40 and was improved an additional 1.5- to 2-fold by the inclusion of 50 mM sodium acetate. The addition of NaCl or KCl (20 to 100 mM) or 20 mM $MgCl_2$ inhibited

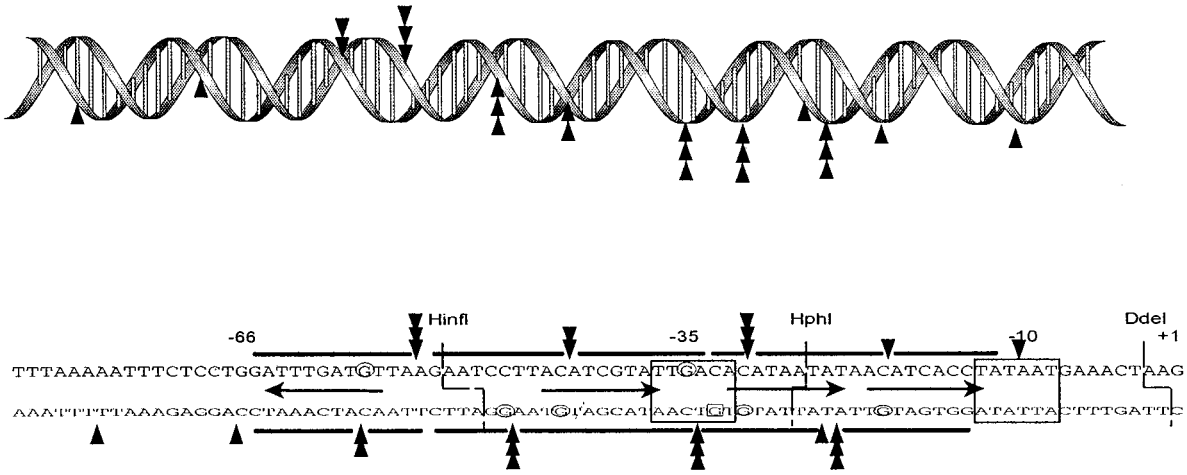

FIG. 4. Summary of protection by GlnR of *glnRA* promoter region DNA from DNase I and DMS. ▲, DNase I hypersensitive site; Ⓖ, DMS-protected G residue; 🄶, DMS-enhanced G residue; ——, DNase I-protected sites.

binding up to twofold. Under optimal conditions, a 2:1 molar ratio of GlnR to DNA was required for 50% shifting of DNA fragments, and the dissociation constant for GlnR (taken to be equivalent to the concentration needed to shift 50% of the DNA molecules containing the binding site when GlnR is in excess and the concentration of DNA is well below the dissociation constant) was estimated at 10^{-11} M. Ammonium ion, glutamate, glutamine, and ATP, separately or together, had no effect on binding in this study. Schreier (19) has found substantial inhibition of GlnR binding by 50 mM glutamate and other carboxylic acids. We have not seen this effect with glutamate but cannot rule out differences in reaction conditions or GlnR preparation.

DNase I footprinting. End-labeled DNA fragments containing the *glnRA* promoter region were mixed with purified GlnR and then exposed to DNase I under conditions in which the nuclease would cleave the average DNA molecule once. Figure 3 shows that in the presence of GlnR, a large segment of the *glnRA* promoter region was protected against cleavage by DNase I. This region extends from positions −64 to −13 on the top strand and from positions −63 to −15 on the bottom strand. Approximately 0.45 pmol of the GlnR monomer was required to give maximal protection from DNase I to about 0.1 pmol of DNA (Fig. 3) under optimal buffer conditions, consistent with a 4:1 binding stoichiometry.

The protected bases were arranged in four segments, each separated by a single site of hypersensitivity to DNase I (Fig. 4). Similar patterns of DNase I reactivity in the presence of DNA-binding proteins have been observed in several other systems (24 and references therein) and are attributed to bending of the DNA helix, causing a distortion that opens the minor groove and makes it more accessible to DNase I (4, 11). All four protected regions are associated with the sequence 5′-ACATCANAT-3′, centered at positions −59, −41, −28, and −18. (The version of the sequence at position −59 is inverted relative to the others.) The hypersensitive sites are found between the 5′ A and the adjoining C and, on the opposite strand, between the second and the third bases beyond the 3′ end on the sequence. This arrangement of hypersensitive phosphodiester bonds on opposite strands is probably due to DNase I cutting of both strands at a single site in the minor groove, as these bonds are expected to lie directly across the minor groove from each other (26).

The ability of purified GlnR to protect the promoter region against methylation by dimethyl sulfate (DMS) was also tested (data not shown). Again, GlnR either prevented or enhanced modification of the DNA at specific bases (Fig. 4). Protected G residues at position −57 on the top strand and at positions −20, −30, and −43 on the bottom strand are complementary to the C residues in the conserved fourfold-repeated sequence and correspond closely to those at which GlnR-dependent protection occurs in vivo (10).

Effect of GlnR on in vitro transcription. The 324-bp *Taq*I fragment corresponding to positions −216 to +108 was mixed with *B. subtilis* $E\sigma^A$ RNA polymerase and assayed for transcription in the presence or absence of heparin-Sepharose-purified GlnR. Figure 5 shows that synthesis of the *glnRA* transcript was greatly inhibited by GlnR protein. As determined by densitometry, transcription was inhibited by approximately 50% at 7 nM GlnR and by 85% at 14 nM GlnR. Utilization of the *glnR*-independent promoter, *veg* (lanes 5 to 8), and a vector promoter (lanes 1 to 4) was not affected by the amounts of GlnR tested.

Conclusions. The results presented here show that GlnR purified from *E. coli*, as expected from analysis of its predicted amino acid sequence (25) and the properties of mutant strains (20, 22), binds with high affinity to a region of DNA that overlaps with the *glnRA* promoter region (Fig. 4). By doing so, it presumably interferes with binding of RNA polymerase to the same DNA sequence, consistent with its ability to inhibit transcription in vitro. Maximal binding of GlnR requires a GlnR-to-DNA ratio of >4 and DNA sequences extending from position −10 to a point located between positions −52 and −63. The exact stoichiometry of binding cannot be determined, however, because the fraction of purified GlnR that is active is unknown.

The extended sequence needed for maximal binding in the gel shift assay correlates well with the pattern of protection and enhancement seen in experiments with DNase I and DMS. This pattern lends itself to the following interpretation: GlnR binds to four neighboring DNA domains (each of which includes a version of the consensus sequence 5′-ACATCANAT-3′) and, because of interactions among the protein molecules, causes the DNA helix to be distorted. Such distortion was suggested previously on the basis of permanganate modification studies (10). We have been unable to observe any order of

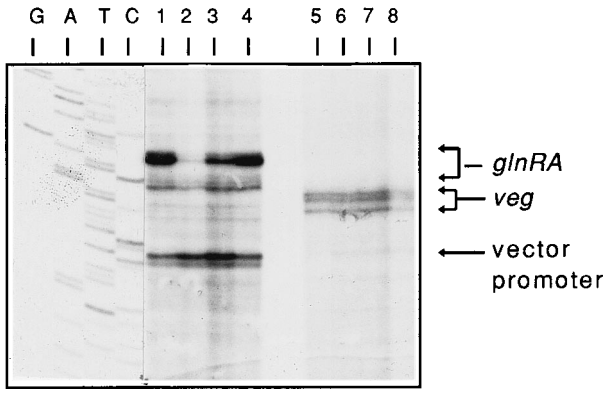

FIG. 5. Inhibition of in vitro transcription by GlnR. A *Taq*I digest of pSB20 (Fig. 3) was used as a template for the synthesis of a 108-nucleotide runoff transcript that initiated at the *glnRA* promoter. (A slightly shorter *glnRA* transcript and vector transcripts were also produced.) The *B. subtilis veg* promoter, cloned in pPH9 (13), was used as a control promoter. pPH9 was linearized by treatment with *Bam*HI. To measure transcription in vitro, heparin-Sepharose-purified GlnR was incubated for 15 min at room temperature with the DNA template in a buffer containing 40 mM Tris-HCl (pH 8.0), 100 mM sodium acetate, 10 mM magnesium acetate, 0.2 mM dithiothreitol, 0.1 mM EDTA, 5% glycerol, 0.05% Nonidet P-40, and 100 μg of bovine serum albumin per ml. Transcription was initiated by the addition of nucleotides and *B. subtilis* σ^A RNA polymerase (28) and continued for 10 min at 37°C. The final concentrations of the reactants were 5 nM RNA polymerase; 5 nM pSB20 (*glnRA* promoter) or 3.2 nM linearized pPH9 (*veg* promoter); 400 μM adenylyl (3′→5′) adenosine (ApA) (for *glnRA*) or adenylyl (3′→5′) guanosine (ApG) (for *veg*); 50 μM (each) GTP, ATP, and CTP; 15 μM UTP plus 2.25 μCi of [α-^{32}P]UTP (500 Ci/mmol); and 0.05% Nonidet P-40. Lanes: 1 to 4, *glnRA* template; 5 to 8, *veg* template; 1 and 5, no GlnR; 2 and 6, 20 ng of GlnR (14 nM dimer); 3 and 7, 10 ng of GlnR (7 nM dimer); 4 and 8, 5 ng of GlnR (3.5 nM dimer); G, A, T, and C, sequencing ladder of pSB20, used to estimate the lengths of transcripts.

occupancy of the four sites, suggesting a high degree of cooperativity of GlnR monomers or dimers during binding. The downstream sites are better placed as sites for repression (12), but they may not by themselves bind GlnR tightly enough to cause repression. We hypothesize that full repression requires binding of GlnR between positions −40 and −14, aided by interaction with GlnR molecules bound in the −64 to −40 region. The consensus sequences are conserved in *B. cereus*, in which *glnRA* regulation seems to be similar to that in *B. subtilis* (15).

GlnR alone is not sufficient to regulate the *glnRA* operon in vivo in response to the nitrogen source. Genetic experiments have shown that GS is also required for proper regulation of *glnRA* (3, 8, 21, 23, 29) and other nitrogen metabolism operons (1, 5, 14). The effects of *glnA* mutations do not appear to be due to any decrease in the intracellular glutamine pool (7). Nakano and Kimura (16) reported that GS stimulates binding of *B. cereus* GlnR to the *glnRA* promoter region in gel mobility shift assays. We confirmed that GS has a small stimulatory effect (two- to fourfold) on binding of *B. subtilis* GlnR to the *glnRA* promoter region (data not shown), but we have been unable to ascribe a mechanism to this stimulation or to assure its physiological relevance. The in vitro effect does not require GS enzymatic activity, binding of GS to DNA, or formation of a stable GS-GlnR-DNA complex (2). Moreover, GS does not alter the specific interaction of GlnR with the DNA sequence (2). If this effect reflects the in vivo role of GS in *glnRA* regulation, one might imagine that GS modulates GlnR function in response to nitrogen availability by determining whether GlnR is able to achieve its most active conformation.

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