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Nucleotide Sequence of the Control Regions for the *glnA* and *glnL* Genes of *Salmonella typhimurium*

ROBERT HANAU,† RAJU K. KODURI,‡ NANCY HO,§ AND JEAN E. BRENCHLEY ‖ *

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

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We have partially characterized a DNA fragment encoding glutamine synthetase in *Salmonella typhimurium*. Restriction mapping and RNA polymerase binding studies identified two regions within the fragment which exhibit promoter activity when fused to *lacZ* in pMC1403, a plasmid used to detect transcriptional and translational control signals. DNA sequence analysis revealed that one region encodes amino acids corresponding to the amino terminus of the glutamine synthetase protein. The second region codes for the amino acids corresponding to the carboxy terminus of glutamine synthetase followed by a 330-nucleotide sequence containing an ideal Pribnow heptamer and a possible translation initiation signal. The location of this region is analogous to the position of the beginning of the *glnL* gene identified in *Escherichia coli*, and it is likely that the Pribnow heptamer is the RNA polymerase binding site for the *glnL* gene.

Studies of *Salmonella typhimurium* by Kustu et al. (13) and of *Escherichia coli* by Pahel and Tyler (23) have shown that the product of a regulatory locus distinct from, but closely linked to, the *glnA* (glutamine synthetase) gene is required for normal expression of *glnA* and other genes under nitrogen control. Subsequent investigations (16, 18) demonstrated that this regulatory locus is composed of two cistrons, *ntrB* and *ntrC* (also referred to as *glnL* and *glnG*, respectively, in studies of *E. coli* [5, 16, 22]), and that the products are involved in mediating both positive and negative control of *glnA*.

Recent genetic studies by Magasanik and coinvestigators (5, 22) and by Gutterman et al. (9) have demonstrated that *glnA*, *glnL*, and *glnG* comprise a single, complex operon. Their results indicate that (i) derepression of *glnA* leads to increased levels of *glnL* and *glnG* products due to transcription initiated at the *glnA* promoter, which proceeds in the direction of *glnL* and *glnG*; (ii) during growth conditions causing the repression of *glnA*, the regulatory products encoded by *glnL* and *glnG* are controlled by a second promoter located downstream from *glnA* at *glnL*.

We have previously reported the cloning of *glnA* from *S. typhimurium* into plasmid pBR322 and a preliminary biochemical characterization of the DNA (12). In this report, we extend our studies of the DNA from the *glnA* region of *S. typhimurium*. Our results support recent genetic findings and provide physical evidence for the existence of a promoter closely linked to but distal from the *glnA* gene.

MATERIALS AND METHODS

Chemical and DNA modifying enzymes. All chemicals were reagent grade and commercially available. Nitrocefin (Glaxo Research Ltd.) was a gift from H. Zalkin. The RNA polymerase holoenzymes, the large fragment of *E. coli* DNA polymerase I, T4 DNA ligase, and restriction endonuclease *PvuI* were purchased from New England BioLabs. Other restriction endonucleases and bacterial alkaline phosphatase (BAP · MATE) were purchased from Bethesda Research Laboratories. Polynucleotide kinase and calf intestine alkaline phosphatase were obtained from Boehringer Mannheim. Terminal transferase and nonradioactive deoxynucleoside triphosphates were purchased from P-L Biochemicals. [γ-^{32}P]ATP (~7,500 Ci/nmol) and α-^{32}P-labeled deoxynucleoside triphosphates (~3,000 Ci/nmol) were purchased from New England Nuclear. Cordycepin 5'-[α-^{32}P]triphosphate (~3,000 Ci/nmol) was purchased from Amersham Corp.

Plasmids and bacterial strains. The pBR322 (1) derivatives used in this study, pJB8 (12) and pMC1403 (3), have both been previously described. *E. coli* strain CU697, *rbs met ara* Δ(*pro-lac*) *thi* (obtained from H. E. Umbarger), was used as a recipient in transformations to detect the cloning of 5' control sequences from pJB8 into the *lac* fusion vector pMC1403. Hybrid

† Present address: Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907.
‡ Present address: Institute for Molecular Biology, University of California at Los Angeles, Los Angeles, CA 90024.
§ Present address: Laboratory of Renewable Resources Engineering, Purdue University, West Lafayette, IN 47907.
‖ Present address: Genex Corporation, 16020 Industrial Dr., Gaithersburg, MD 20877.

plasmids representing the desired fusions between DNA from pJB8 and *lac* in pMC1403 were subsequently used to transform strain JB1396, a Gln$^+$ Reg$^+$ *hsr ile leu met val trp* derivative of *S. typhimurium* LT-2.

Previously published procedures were used for large-scale isolation and purification of plasmid DNA (8). Restriction endonuclease digests were performed according to the recommendations provided by the commercial sources. DNA sequence was determined by the method of Maxam and Gilbert (17) from fragments labeled with ^{32}P at either the 5′ ends with polynucleotide kinase (4) or the 3′ ends by incubation with the large fragment of DNA polymerase (26) or terminal transferase (30). A total *SalI-SmaI* digest of pJB8 DNA was used for in vitro RNA polymerase binding studies (26), and the reactions were performed as described previously (12).

Plasmids pJB14, pJB15, and pJB16 were constructed by cloning *Hin*cII restriction fragments from pJB8 into the *Sma*I site of pMC1403. Approximately 3 pmol of pMC1403 DNA was digested with *Sma*I and treated with 200 U of bacterial alkaline phosphatase (BAP · MATE). The pMC1403 DNA was suspended in a 30-μl ligase reaction volume containing 400 U of T4 DNA ligase and 3 pmol of pJB8 DNA that had been digested with *Hin*cII. After incubation for 18 h at 20°C, an additional 400 U of ligase in 30 μl of buffer was added to the reaction, and the mixture was incubated at 20°C for an additional 20 h. Samples of the ligation mixture were used to transform strain CU697 according to the method described by Davis et al. (8). Ampicillin-resistant transformants were simultaneously screened for the acquisition of the Lac$^+$ phenotype by plating on LB (2) agar containing 50 μg of ampicillin and 4 μg of X-gal ml^{-1} (19). Of 272 transformed colonies, 6 were Lac$^+$ (blue color on X-gal-containing media), and the plasmid DNA from each of the 6 was analyzed by using a rapid isolation procedure for small amounts of plasmid DNA (11). Two classes of clones representing distinct gene fusions were identified, and these correspond to vectors pJB14 and pJB16. Vector pJB15 was derived from pJB14 by digesting 10 μg of pJB14 DNA with *Sma*I followed by incubation with 400 U of T4 DNA ligase in a 50-μl reaction volume for 24 h at 20°C. This mixture was used to transform CU697, and Amp^r Lac$^+$ derivatives were isolated as described above. Subsequently, plasmid DNA prepared from the pJB15 and pJB16 transformants of CU697 was used to transform JB1396.

Media and growth conditions. LB and minimal salts media have been described previously (2). Cells for physiology experiments were grown overnight at 37°C in minimal medium containing 20 mM glucose as the carbon source and either 20 mM $(NH_4)_2SO_4$ or 20 mM proline as the nitrogen source. Cells were then diluted into the same medium (cell density, ca. 10 Klett turbidity units) and grown at 37°C with vigorous shaking until they reached a density of about 100 Klett units. The cells were chilled on ice for 15 min and harvested by centrifugation at 12,000 × *g* for 10 min, washed in cold 0.85% NaCl, and resuspended in 1/50 volume of cold 25 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.3. Ultrasonic disrupted cell extracts were then prepared from the cell suspensions as described previously (2).

Enzyme assays. β-Galactosidase activity (24), glutamine synthetase activity (28), and protein (15) were determined by using previously published procedures. β-Lactamase (6) activity was determined by measuring the increase in optical density at 486 nm resulting from the hydrolysis of nitrocefin (21). The reactions were performed at 25°C in cuvettes containing 1 ml of 100 mM KH_2PO_4 (pH 7.0), 0.05 μmol of nitrocefin, and 2.5 to 10 μl of sonic extract (10 to 30 μg of protein). The molar extinction coefficient from the hydrolyzed form of nitrocefin was determined to be 1.41×10^7 mol^{-1}.

RESULTS

Restriction endonuclease map of the *glnA* region. We previously described the cloning of the glutamine synthetase gene from *S. typhimurium* into pBR322 (12). One plasmid, pJB8, containing a 2.3-kilobase (kb) (*Hin*dIII to *Sal*I) insert that exhibited normal regulation of glutamine synthetase was used to construct a detailed restriction endonuclease map of the *glnA* region (Fig. 1). This map was used in subsequent studies to identify regions that would bind RNA polymerase and promote transcription of the *lac* genes in plasmid vector pMC1403 and for isolating specific fragments for DNA sequence analysis.

RNA polymerase binding studies. Previous work (12) suggested that RNA polymerase binding sites were present on two distinct *Hin*cII fragments contained within the 2.3-kb insert in pJB8 (see Fig. 1). To localize these binding sites more precisely, additional RNA polymerase binding studies were performed on pJB8 DNA that had been digested with *Sma*I and *Sal*I to generate smaller DNA fragments.

Figure 2, lane B, shows the pattern of pJB8 DNA retained by nitrocellulose filters after digestion with endonucleases *Sma*I and *Sal*I and incubation with RNA polymerase. For comparison, pJB8 DNA digested with *Sma*I and *Sal*I is shown in Fig. 2, lane A. Band I is a 3.8-kb fragment containing primarily pBR322 DNA. Bands II, III, and IV are the 1.4-, 0.6-, and 0.2-kb fragments from the *S. typhimurium* DNA insert (refer to Fig. 1). The relative efficiency of RNA polymerase binding was greater for the 0.6-kb fragment than for the 1.4-kb fragment. Binding of RNA polymerase to the 0.2-kb fragment was undetectable. The RNA polymerase binding to the 1.4-kb *Sma*I-*Sma*I fragment was expected and predicted on the basis of our previous results, which indicated the presence of an RNA polymerase binding site between *Hin*cII restriction endonuclease sites at 300 and 1,200 nucleotides (Fig. 1). The binding of RNA polymerase to the 0.6-kb *Sma*I-*Sal*I fragment verified the presence and more accurately describes the location of a second, potential control region within the pJB8 insert.

DNA sequence analysis of the region located between *Hin*cII endonuclease sites at 300 and 1,200 nucleotides. The nucleotide sequence was

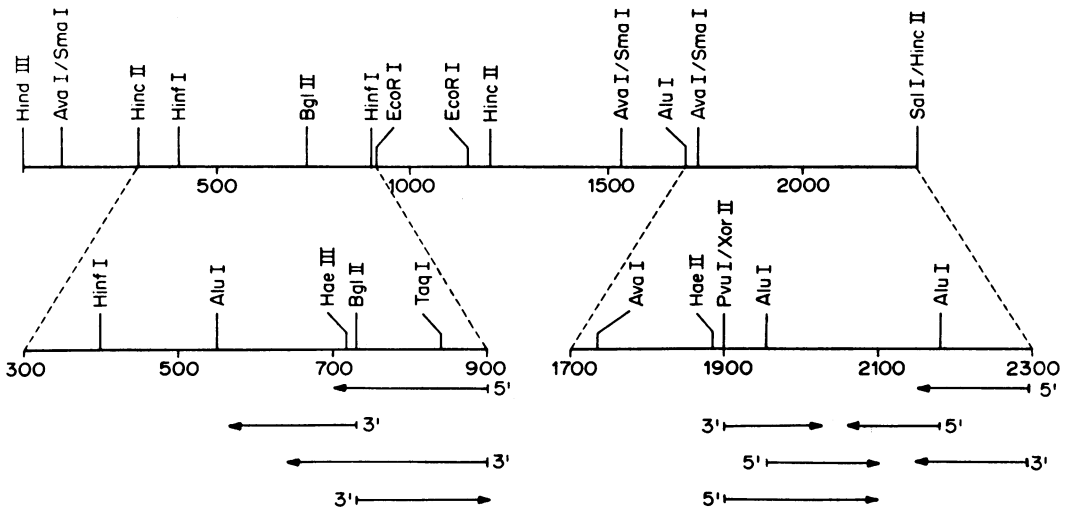

FIG. 1. Diagram of the restriction endonuclease sites in a 2,300-base pair *Hin*dIII to *Sal*I DNA fragment in pJB8 carrying the *glnA* region of *S. typhimurium*. The segments between 300 to 900 and 1,700 to 2,300 nucleotides are expanded to show more detail and sites for additional restriction endonucleases. Arrows correspond to regions that were sequenced. The tail of the arrow represents the ^{32}P-labeled end of the fragment, and the arrowhead represents the direction and extent to which each fragment was sequenced. The DNA strand labeled with ^{32}P is indicated by the 5′ and 3′ designation.

determined for several fragments located between the *Hinc*II endonuclease sites at 300 and 1,200 nucleotides (Fig. 1). A sequence corresponding to the amino-terminal amino acids of glutamine synthetase (13) was identified and is shown in Fig. 3. The results indicate that the translational initiation site for the peptide is located at nucleotide 780 of the pJB8 insert.

DNA sequence analysis of the region located between endonuclease sites *Sma*I and *Sal*I at 1,730 and 2,300 nucleotides. The assignment of the carboxy terminus of the *glnA* gene was confirmed by DNA sequence analysis of fragments obtained after digestion of the 0.6-kb *Sma*I-*Sal*I fragment with *Alu*I (refer to Fig. 1). Additional sequence information characterizing this region was obtained from fragments isolated after digestion of pJB8 DNA with restriction endonucleases *Pvu*I and *Sal*I. A portion of the sequence from the *Pvu*I site at 1,900 towards the *Sal*I site (Fig. 1) codes for the 14 carboxyl-terminal amino acids of glutamine synthetase (Fig. 4) (13; R. Heinrikson, personal communication). This region is followed by a sequence containing a consensus Pribnow heptamer (27) and additional sequences which represent a possible ribosome binding site (GGAG) (29) and a possible methionine initiation codon. These findings strongly suggest that this portion of the sequence corresponds to a control region for a gene(s) located downstream from *glnA*, presumably *glnL* and *glnG* (16).

Construction of *gln-lac* gene fusions. Our sequence analysis places the amino terminus and carboxy terminus of *glnA* at 780 and 1,965 nucleotides, respectively, on the restriction map (Fig. 1). These findings together with the results of the RNA polymerase binding studies and the presence of a Pribnow heptamer in the region after the *glnA* structural gene indicate that control regions may be present on each of the two *Hinc*II fragments contained within the pJB8 insert.

To determine whether DNA within either of the *Hinc*II fragments possesses the ability to regulate gene expression, both of these fragments were fused to *lacZ* by subcloning into the plasmid vector pMC1403 as described above (Materials and Methods). The position and orientation of the pJB8 DNA in the subclones were verified by restriction analysis. The *lac* fusion in pJB15 contains a portion of pJB8 DNA which corresponds to a region located between 1,730 and 2,300 nucleotides on the restriction map in Fig. 1 (Fig. 5A). This fusion brings the fragment containing the Pribnow heptamer into continuity with *lacZ*. The pJB8 DNA contained within the pJB16 fusion corresponds to the *Hinc*II fragment located between nucleotides 300 and 1,200 (Fig. 1), which contains at least a portion of the *glnA* control region.

Regulation of β-galatosidase in pJB15 and pJB16. Strain JB1396 was transformed with pJB15 and pJB16, the transformants were grown in glucose minimal medium with either 20 mM $(NH_4)_2SO_4$ or 20 mM proline as the nitrogen source, and the glutamine synthetase and β-galactosidase activities were measured. The glu-

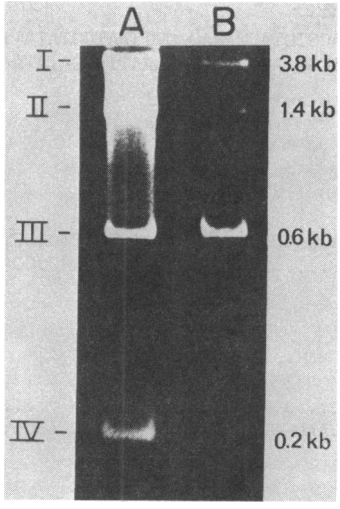

FIG. 2. Agarose gel analysis of RNA polymerase binding to DNA fragments from pJB8. The samples in lanes A and B each contained 5 μg of pJB8 DNA digested with *Sma*I and *Sal*I. Before electrophoresis, the sample in lane B was incubated with 5 μg of RNA polymerase and filtered through a nitrocellulose filter. Bands I, II, and III represent DNA fragments retained by the filter after RNA polymerase binding. Band IV corresponds to the 0.2-kb *Sma*I-*Sma*I fragment within the pJB8 insert and was not retained by the filter.

tamine synthetase levels increased approximately 20-fold in cultures of JB1396 grown in glucose-proline medium as well as in comparable cultures of JB1396 transformed with PJB15 and pJB16 (Table 1). However, the β-galactosidase activities showed little variation when cultures of JB1396 transformed with pJB16 were grown in glucose-NH_4^+ and glucose-proline media (Table 1). In contrast to the 20-fold increase in glutamine synthetase, cultures of JB1396 transformed with pJB16 showed only a slight increase in β-galactosidase (cf. lines 5 and 6) when proline replaced NH_4^+ as the nitrogen source.

In addition to measuring glutamine synthetase and β-galactosidase activities, we examined the levels of β-lactamase (6) in cultures of JB1396 transformed with pJB15 and pJB16. For pBR322 and its derivatives, the level of β-lactamase in cultures of transformants is directly proportional to plasmid copy number (20, 31, 32). β-Lactamase levels were 17-fold lower when proline replaced NH_4^+ as the nitrogen source in cultures of JB1396 transformed with pJB16 (Table 1). Similarly, when cultures of JB1396 transformed with pJB15 were grown in glucose-NH_4^+ and glucose-proline media, the levels of β-lactamase were sevenfold lower in the glucose-proline cultures (Table 1). These results indicate that the glucose-proline cultures of JB1396 transformants contained 7- and 17-fold fewer copies of pJB15 and pJB16, respectively, than similar cultures grown in glucose-NH_4^+.

We used the levels of β-lactamase as an indication of relative gene copy responsible for directing the synthesis of β-galactosidase. Table 1 shows the β-galactosidase levels measured in glucose-NH_4^+ and glucose-proline cultures of JB1396 transformed with pJB15 and pJB16 which have been normalized with regard to plasmid copy number. The specific activity of β-galactosidase per copy of pJB16 is about 20-fold higher in glucose-proline medium than in glucose-NH_4^+ medium, and this increase is comparable to that observed for glutamine synthetase in these cultures. The specific activity of β-galactosidase per copy of pJB15 is similar in both media, although slightly higher in glucose-NH_4^+ than in glucose-proline. These results are expected if pJB16 and pJB15 represent *lac* fusions of the *glnA* and *glnL* control regions, respectively, and are similar to the results described by Pahel et al. (22) in studies of *E. coli*.

DISCUSSION

Two RNA polymerase binding sites are present in the pJB8 insert. One site is located be-

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                HaeIII    725  BglII              750
5' TTACGGCGACACGGCCAGCAGAATTGAAGATCTCGTTACCACGACGACCGATCATGACCA
   AATGCCGCTGTGCCGGTCGTCTTAACTTCTAGAGCAATGGTGCTGCTGGCTAGTACTGGT

          775                  800
ATCCGGGAGAGTACAAGTATGTCCGCTGAACACGTTTTGACGATGCTGAACGAGCACGAA '3
TAGGCCCTCTCATGTTCATACAGGCGACTTGTGCAAAACTGCTACGACTTGCTCGTGCTT
          MetSerAlaGluHisValLeuThrMetLeuAsnGluHisGlu
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FIG. 3. Nucleotide sequence of a segment of the *glnA* gene encoding the N-terminal amino acids of glutamine synthetase. The sequence was determined from fragments isolated from *Bgl*II and *Eco*RI digests of pJB8 DNA and corresponds to a segment located between nucleotides at about 700 and 820 on the restriction map (Fig. 1). The inferred amino acid sequence for the first 14 amino acids is indicated. The amino acids which are underlined represent those which have been previously identified (13). The positions of the *Hae*III and *Bgl*II restriction endonuclease sites are indicated for reference.

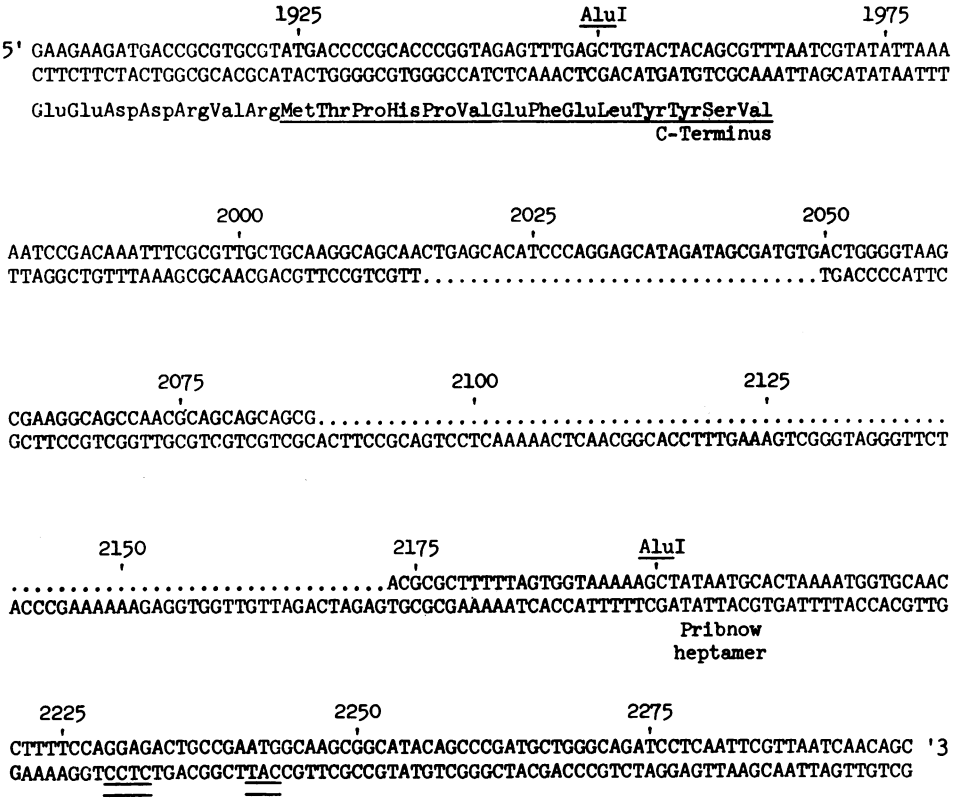

FIG. 4. Nucleotide sequence corresponding to the carboxy terminus of the *glnA* gene. The sequence was determined from fragments isolated from *Pvu*I, *Sal*I, and *Alu*I digests of pJB8 DNA. The dots represent portions of the particular strand which were not sequenced. The inferred amino acid sequence for the carboxy terminus is shown. The 14 amino acids which are underlined are those found at the carboxy terminus of the glutamine synthetase protein (13; R. Heinrikson, personal communication). The locations of the Pribnow heptamer as well as a possible ribosome binding site (GGAG) and a possible translation initiation codon (ATG) are indicated. The *Alu*I restriction sites and nucleotide numbers are shown relative to their position on the restriction map in Fig. 1.

tween *Hin*cII restriction sites at nucleotides 300 and 1,200. DNA sequence analysis of restriction fragments from this region shows that nucleotides encoding the amino-terminal amino acid of glutamine synthetase are located at nucleotide 780. Because this represents the start of the glutamine synthetase structural gene, it is reasonable to assume that the RNA polymerase binding occurs in a region located between nucleotides 300 and 780 and that this segment contains sequences which correspond to or represent a portion of the *glnA* promoter.

A second RNA polymerase binding site is located between the *Sma*I and *Sal*I restriction sites at nucleotides 1,730 and 2,300. DNA sequence analysis of this region shows that the carboxy-terminal portion of *glnA* is located at nucleotide 1,965. The mapping of the *glnA* coding sequences between nucleotides 780 and 1,965 is consistent with biochemical studies of the glutamine synthetase protein (7, 14) which predict the size of the structural gene to approximately 1,200 nucleotide pairs. Further analysis of the sequence from fragments isolated from the *Sma*I-*Sal*I region indicates the presence of a Pribnow heptamer, downstream from *glnA*, centered at about nucleotide 2,200. In addition, our results show that the Pribnow heptamer is followed by sequences which represent a possible ribosome binding site and a possible translation initiation codon.

The *Hin*cII and *Sma*I-*Sal*I restriction fragments were fused to *lacZ* by cloning into pMC1403. The eight amino-terminal codons of *lacZ* are absent in pMC1403 (3). β-Galactosidase activity resulting from the gene fusions is therefore dependent upon the presence of 5'-control sequences located within the insert. The results in Table 1 show that the fusions in both pJB15 and pJB16 are capable of directing the synthesis of hybrid β-galactosidase in cells transformed with these plasmids.

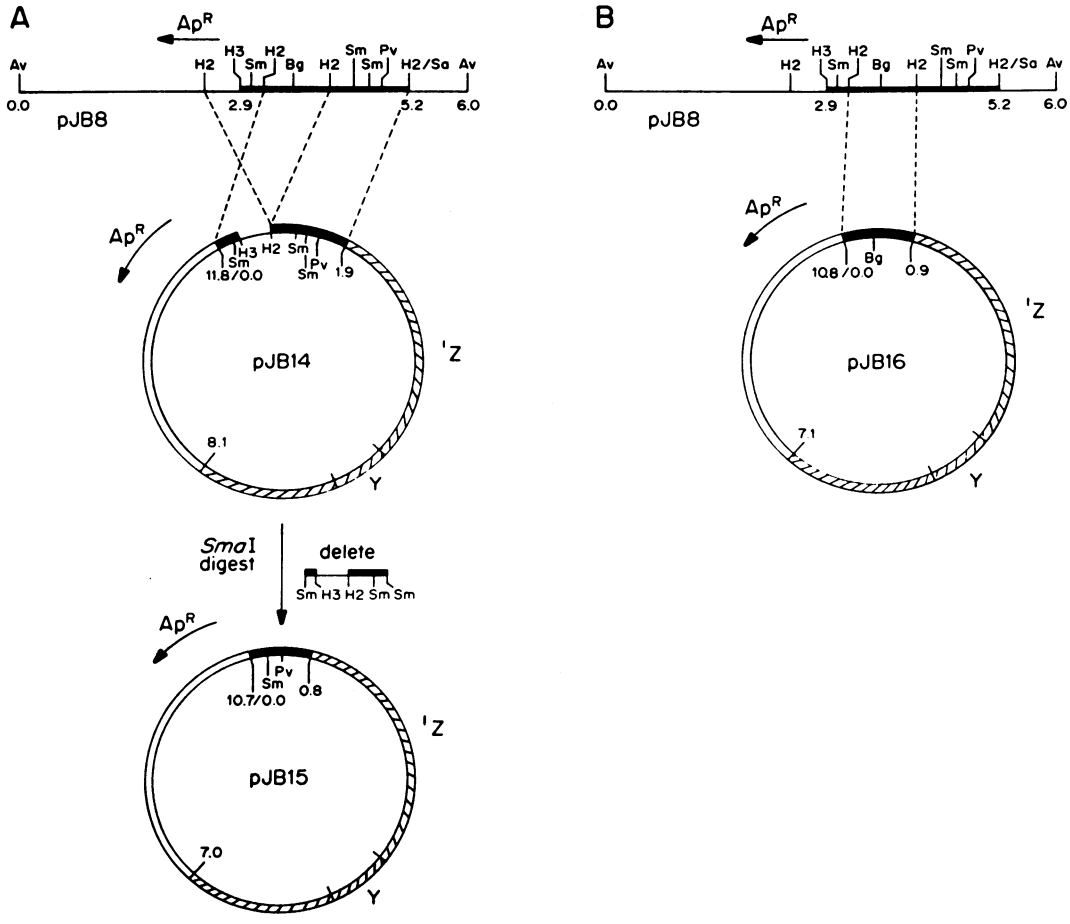

FIG. 5. Plasmids containing *gln-lac* gene fusions. Segments of the insert DNA from pJB8 were cloned into plasmid vector pMC1403 (3). pJB8 DNA (represented by the linear map) digested with *Hin*cII was ligated with pMC1403 DNA that had been digested with *Sma*I as described in the text. Two classes of fusions represented by clones pJB14 and pJB16 were isolated. Restriction mapping verified the position and orientation of the pJB8 *Hin*cII fragments which had been fused to *lacZ*. A portion of the insert in pJB14 was removed after digestion with *Sma*I and ligation to generate pJB15. The first 200 nucleotides of the insert in pJB15 correspond to an inversion of the *Sma*I-*Hin*cII fragment from the pJB8 insert (between nucleotides 100 and 300 on the restriction map, Fig. 1), which is followed by a 600-nucleotide segment from pJB8 containing the carboxy-terminal portion of *glnA* and the adjacent, downstream region. The insert in pJB16 corresponds to a 900-base pair *Hin*cII fragment, a portion of which encodes the amino-terminal amino acids of glutamine synthetase. Solid lines represent pJB8 DNA. Open lines correspond to pMC1403 DNA. Hatch marks denote pMC1403 DNA derived from the *lac* genes (3). Relevant restriction sites are indicated and have the following designations: Av, *Ava*I; Bg, *Bgl*II; H2, *Hin*cII; H3, *Hin*dIII; Pv, *Pvu*I; Sa, *Sal*I; Sm, *Sma*I. Kilobase coordinates for relevant sites and the location of the Ap^r (ampicillin resistance) determinant gene of the plasmids are shown.

The corrected enzyme levels shown in Table 1 verify that the fusion in pJB16 (containing the *Hin*cII fragment located between nucleotides 300 and 1,200) contains control sequences which correspond to the *glnA* promoter. β-Lactamase activity was measured to determine relative plasmid copy number. After correcting for the lowered gene dose in glucose-proline cultures of the pJB16 transformants, regulation of the hybrid β-galactosidase was identical to that of glutamine synthetase.

The results in Table 1 also indicate that the response of the control sequence in the pJB15 fusion (containing the *Sma*I-*Sal*I fragment located between nucleotides 1,730 and 2,300) is quite different from that of the pJB16 fusion. The activity of hybrid β-galactosidase per copy of pJB15 was similar in glucose-proline and glucose-NH_4^+ cultures, although slightly elevated in the latter.

Studies by Magasanik's group (5, 22), MacNeil et al. (16), and Gutterman et al. (9) have

TABLE 1. Enzyme levels in pJB15 and pJB16 transformants of JB1396

Strain	N source[a]	Sp act (μmol min^{-1} mg of protein^{-1})			
		Glutamine synthetase	β-Galacto-sidase	β-Lactamase	β-Galacto-sidase[b]
JB1396	NH_4^+	0.07	<0.01	<1	
	Proline	1.39	<0.01	<1	
JB1396(pJB15)	NH_4^+	0.08	0.83	3,420	
	Proline	2.09	0.08	510	0.55
JB1396(pJB16)	NH_4^+	0.08	1.50	890	
	Proline	1.69	2.04	50	34.30

[a] Media and growth conditions are described in the text. $(NH_4)_2SO_4$ (20 mM) and proline (20 mM) were used as nitrogen sources as indicated.

[b] β-Galactosidase activities for glucose-proline cultures were corrected for differences in specific activity due to lowered plasmid copy number.

demonstrated that the order of the genes in the *glnA* region of *E. coli* is *glnA-glnL-glnG* and that they comprise a single transcriptional unit controlled by the *glnA* promoter. The results of Gutterman et al. (9) and Pahel et al. (22), which describe the regulation of *glnL* and *glnG*, indicate that these genes are regulated by a second promoter located at *glnL*. We propose that the *glnA*-distal control region contained in the pJB15 gene fusion is analogous to the proposed *glnL* promoter of *E. coli*.

Thus, we show that the 2.3-kb pJB8 insert which encodes *S. typhimurium* glutamine synthetase contains a promoter distinct from that which regulates *glnA*. Unlike *glnL*, we find no ideal Pribnow heptamer within the sequence immediately preceding the *glnA* coding region (Fig. 3) nor is one contained within the sequence which extends 165 nucleotides upstream from the *BglII* restriction site (unpublished data). Our sequence data also suggest that additional *glnA*-distal control regions exist. A sequence (CAGCCAACGCAGCAGCAG) which is located 101 nucleotides beyond the carboxy terminus of *glnA* could encode four glutamines if translated (Fig. 4). Pahel et al. (22) address the possibility that continuation of transcription distal to *glnA* may be regulated. If this intercistronic region is also involved in regulating *glnL* and *glnG*, it differs from previously described intercistronic regulation elements (10) because it precedes an apparent transcription initiation site at the Pribnow heptamer. We are characterizing this intercistronic region further to determine whether it is involved in a novel molecular mechanism which serves to better coordinate expression of the nitrogen regulatory genes *glnL* and *glnG* with the synthesis of glutamine synthetase.

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