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# Genetic Regulation of Glycogen Biosynthesis in *Escherichia coli*: In Vitro Effects of Cyclic AMP and Guanosine 5′-Diphosphate 3′-Diphosphate and Analysis of In Vivo Transcripts

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**Glycogen accumulation in *Escherichia coli* is inversely related to the growth rate and occurs most actively when cells enter the stationary phase. The levels of the three biosynthetic enzymes undergo corresponding changes under these conditions, suggesting that genetic control of enzyme biosynthesis may account for at least part of the regulation (J. Preiss, Annu. Rev. Microbiol. 38:419–458, 1984). We have begun to explore the molecular basis of this control by identifying factors which affect the expression of the glycogen genes and by determining the 5′-flanking regions required to mediate the regulatory effects. The in vitro coupled transcription-translation of two of the biosynthetic genes, *glgC* (ADPglucose pyrophosphorylase) and *glgA* (glycogen synthase), was enhanced up to 26- and 10-fold, respectively, by cyclic AMP (cAMP) and cAMP receptor protein (CRP). Guanosine 5′-diphosphate 3′-diphosphate stimulated the expression of these genes 3.6- and 1.8-fold, respectively. The expression of *glgB* (glycogen branching enzyme) was affected weakly or negligibly by the above-mentioned compounds. Assays which measured the in vitro formation of the first dipeptide of *glgC* showed that a restriction fragment which contained 0.5 kilobases of DNA upstream from the initiation codon supported cAMP-CRP-activated expression. Sequence-specific binding of cAMP-CRP to a 243-base-pair restriction fragment from the region upstream from *glgC* was observed by virtue of the altered electrophoretic mobility of the bound DNA. S1 nuclease protection analysis identified 5′ termini of four in vivo transcripts within 0.5 kilobases of the *glgC* coding region. The relative concentrations of transcripts were higher in the early stationary phase than in the exponential phase. Two mutants which overproduced the biosynthesis enzymes accumulated elevated levels of specific transcripts. The 5′ termini of three of the transcripts were mapped to a high resolution. Their upstream sequences showed weak similarity to the *E. coli* consensus promoter. These results suggest complex transcriptional regulation of the glycogen biosynthesis genes involving multiple promoter sites and direct control of gene expression by at least two global regulatory systems.**

Glycogen represents the major form of stored carbon for *Escherichia coli* and many other procaryotes and provides a readily metabolized substrate for maintenance energy (42). The biosynthesis of glycogen requires three enzymes, ADPglucose pyrophosphorylase (EC 2.7.7.27), glycogen synthase (EC 2.4.1.21), and glycogen branching enzyme (EC 2.4.1.18), which are encoded by *glgC*, *glgA*, and *glgB*, respectively. The enzymes have been previously purified (9, 20, 27, 28). The genes have been cloned on a single 10.5-kilobase (kb) genomic fragment (41), and their nucleotide sequences have been determined (4, 5, 35). The genes are located at 75 min on the *E. coli* genome. The gene cluster also encodes the degradative enzyme glycogen phosphorylase. The gene for this enzyme has been alternatively designated *glgY* or *glgP* (47, 54). An open reading frame, *glgX*, which probably encodes a glucanase or glucosyl transferase, is located between *glgB* and *glgC* (47). The gene order is *glgY-glgA-glgC-glgX-glgB-asd*, and the genes are transcribed in the counterclockwise direction. The *asd* gene encodes aspartate semialdehyde dehydrogenase. It is not involved in glycogen metabolism but was used as a marker for the cloning of the *glg* genes. The nucleotide sequence of *asd* has also been determined (29).

The allosteric regulation of the first committed step of glycogen biosynthesis, catalyzed by ADPglucose pyrophosphorylase, has been extensively examined via biochemical analysis of wild-type and mutant enzymes (reviewed in reference 42). Glycogen biosynthesis is also controlled by variations in the levels of the biosynthetic enzymes according to growth rate and nutrient conditions (42). Two types of mutations which lead to elevated levels of the biosynthesis enzymes have been partially characterized. A mutation in *glgQ* increases the levels of all three enzymes 3- to 10-fold, is not closely linked to the structural genes in P1 transduction, and results in a substantially greater accumulation of enzymes encoded on multicopy plasmids (41–44). The mutation is therefore of the classical *trans*-acting form. A second mutation, *glgR*, is closely linked to the structural genes and results in enhanced levels of ADPglucose pyrophosphorylase and glycogen synthase but does not affect the level of glycogen branching enzyme (42–44).

The in vivo rate of glycogen biosynthesis has been shown to be affected by the cyclic AMP (cAMP)-cAMP receptor protein (CRP) system (17, 18, 37) and by the *relA* gene, which is required for the synthesis of guanosine 5′-diphosphate 3′-diphosphate (ppGpp) during the stringent response (10, 38, 51). The first evidence that cAMP may enhance the expression of *glgC* was obtained in an in vitro expression system which measures the formation of the first di- or tripeptide of specific gene products (51).

The expression of all three structural genes has now been examined in a coupled transcription-translation system, and the expression of *glgC* and *glgA* has also been measured

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with the dipeptide assay. A region which binds cAMP-CRP and therefore probably mediates the cAMP effects was detected upstream from *glgC*. The apparent initiation sites for transcripts in this region were mapped from wild-type strains and from *glgQ* and *glgR* mutants. Although qualitatively similar, the transcript patterns of the mutant strains showed quantitative differences, with respect to those of the wild type, which helped to further define the nature of the mutations.

## MATERIALS AND METHODS

**Biochemical reagents.** Tritiated amino acids and [$^{32}$P]ATP were obtained from New England Nuclear Corp., Boston, Mass., and [$^{35}$S]methionine was purchased from Amersham Corp., Arlington Heights, Ill. Purified isoacceptor tRNA species were purchased from Subriden RNA, Rolling Hills, Wash., except for $tRNA_f^{Met}$, which was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. RNA polymerase, micrococcal nuclease, and cAMP were from Sigma Chemical Co., St. Louis, Mo. ppGpp was obtained from Pharmacia, Inc., Piscataway, N.J. S1 nuclease was from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Biochemical reagents for the dipeptide assay were generously provided by H. Weissbach (Roche Institute of Molecular Biology, Nutley, N.J.). CRP was the kind gift of A. Revzin (Department of Biochemistry, Michigan State University, East Lansing). CRP was determined to be 95% pure and approximately 10% active in transcription assays and formed stable complexes with wild-type *lac* DNA at molar concentrations of 1:10 (DNA-CRP), as determined in the laboratory of A. Revzin. NtrA and NtrC610 were provided by S. Kustu and J. Keener (University of California, Berkeley). NtrC610 is a mutant form of the protein which is functional without phosphorylation (33). Other biochemical reagents were purchased from commercial sources and were of the highest quality available. Water was purified through a MilliQ system (Millipore Corp., Bedford, Mass.) before use.

**Bacterial strains and plasmids.** The strains used in these studies included wild-type *E. coli* K-12 3000 and *E. coli* B, *E. coli* B AC70RI (*glgQ*) (42), *E. coli* B SG3 (*glgR*) (24), and *E. coli* K-12 G6MD3 [Hfr *his thi* Str$^s$ Δ(*malA-asd*)] (49).

Plasmid pOP12 contains the glycogen biosynthesis gene cluster in pBR322, as well as an unrelated gene, *asd*, which was used as a marker for positive selection in obtaining the original clone (41). Plasmid pOP245 was constructed to contain *glgA* in pBR322 and has been previously described (35). Plasmid pPR1 was constructed by subcloning a 584-base-pair (bp) *Hin*cII-*Kpn*I restriction fragment, which extends from 0.5 kb upstream from the *glgC* coding region to 92 bp inside of the coding region, into the corresponding sites in the polylinker region of pUC19. Plasmid pPR2 was constructed by subcloning a 3.4-kb *Hpa*I fragment which was from pOP12 and which contained *glgC* and *glgA* into the *Hin*cII site of pUC19. Plasmid pJES40, which contains a *glnA'-'lacZ* fusion gene (32), was provided by S. Kustu and J. Keener. Plasmid DNA was prepared and subcloning steps were accomplished essentially as described previously (47).

**Preparation of DNA fragments.** Restriction fragments from pOP12 or pPR1 were prepared as previously described (47) or were purified by fast protein liquid chromatography with a MonoQ anion-exchange column (53). The fragments were eluted from the column with a linear gradient of NaCl in 10 mM Tris hydrochloride (pH 8.0) and analyzed by agarose gel electrophoresis with ethidium bromide-UV detection (39). The DNA was precipitated with ethanol, collected by centrifugation, washed once with 70% ethanol, dried in vacuo, redissolved in TE buffer (10 mM Tris hydrochloride, 1 mM EDTA, pH 8.0), and quantified by measuring the $A_{260}$.

**Coupled transcription-translation.** Assays which measured coupled transcription-translation utilized S-30 extracts of *E. coli* B. The extracts were prepared and assays were conducted as described by Chen and Zubay (14), with modifications introduced in the laboratory of H. Weissbach. Extracts were treated with micrococcal nuclease (25 U/ml) at 37°C for 30 min in the presence of 1 mM $CaCl_2$ to degrade endogenous nucleic acids. The nuclease was inactivated with ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) (4 mM) before the extracts were used. Reaction mixtures contained the following components and additional ones as indicated: S-30 extract (5.7 mg of protein per ml, assayed as described by Smith et al. [50]), 2 mM dithiothreitol, 30 mM phosphoenolpyruvate, 35 mM ammonium acetate, 2.9 mM ATP, 0.7 mM each CTP, UTP, and GTP, 65 mM potassium acetate, 0.8 mM spermidine hydrochloride, 3.6% polyethylene glycol 8000, 50 mM Tris acetate (pH 8.0), 10 mM dimethylglutaric acid (pH 6.0), 0.0286 mM methionine, 0.125 mM each of the other 19 amino acids, 1.29 mg of *E. coli* tRNA per ml, 28.6 μg of pyruvate kinase per ml, 30 μM $N^{5,10}$-methenyltetrahydrofolate, 14 μg of *E. coli* RNA polymerase per ml, magnesium acetate (approximately 15 mM, optimized for each extract), and [$^{35}$S]methionine (1,030 Ci/mmol; 0.43 or 0.86 mCi/ml). Reactions were carried out at 37°C for 1 h in Microfuge tubes containing 35 μl of reaction mixture. Reactions were started immediately after the addition of plasmid DNA (0.10 pmol or as otherwise indicated) by transferring the tubes from an ice bath to 37°C and were terminated by the addition of 35 μl of sodium dodecyl sulfate sample buffer (0.125 M Tris hydrochloride [pH 6.8], 4% sodium dodecyl sulfate, 20% glycerol, 10% 2-mercaptoethanol).

Proteins in the reaction mixtures were denatured at 100°C for 90 s, and 10- or 15-μl aliquots of the reaction mixtures were subjected to one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (36) with a Protean II slab gel system (Bio-Rad Laboratories, Richmond, Calif.). The gels were stained with Coomassie brilliant blue R-250 (0.23 g/200 ml in acetic acid-ethanol-water [1:5:4]) and destained with acetic acid-ethanol-water (1:2.5:6.5). The gels were equilibrated with water, treated with sodium salicylate, and dried in vacuo prior to fluorography (13). For quantification of individual proteins, a small amount (1 to 2 μg) of each of the purified, denatured glycogen biosynthesis enzymes was added directly to the sample wells which contained the S-30 assay mixtures prior to electrophoresis. After fluorography, the dried gels were rehydrated and the individual bands of protein were excised. The gel slices were dissolved in 30% $H_2O_2$ (22), and the radioactivity was quantified by liquid scintillation spectrometry.

**DNA-directed dipeptide synthesis.** The methods and conditions used for the analysis of gene expression based upon in vitro synthesis of the amino-terminal dipeptides of specific gene products were essentially as described previously for the expression of *glgC* (52). The components cAMP, CRP, NtrA, and NtrC610 were added at 5 μM, 2 μg per reaction, 80 μg per reaction, and 800 μg per reaction, respectively. DNA was added at 0.10 pmol per reaction. The amino-terminal dipeptides of ADPglucose synthetase, glycogen synthease, and β-lactamase are fMet-Val, fMet-Gln, and fMet-Ser, respectively. Purified isoacceptor tRNA species $tRNA_2^{Val}$, $tRNA_{3b}^{Ser}$, and $tRNA_{NUG}^{Gln}$ were activated to a

specific radioactivity of approximately 4,000 cpm/pmol with the $^3$H-amino acids. The dipeptides were isolated with Dowex 50 $H^+$ (8) and quantified by liquid scintillation spectrometry. The results were calculated as the amount of product formed (in picomoles) in 1 h per 35-μl reaction volume and are the averages of duplicate reactions.

**Mobility shift assay for cAMP-CRP binding.** The interaction of cAMP-CRP with restriction fragments was detected by virtue of the decreased electrophoretic mobility of bound versus free DNA (21, 23). The reaction mixtures contained restriction fragments (20 nM), cAMP (200 μM), and CRP (1 μM) in a buffer solution consisting of 20 mM Tris hydrochloride (pH 8.0), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 100 mM KCl in a 25-μl final volume. The reaction mixtures were gently mixed and incubated for 15 min at 37°C to allow binding. Loading dye (2.5 μl containing 25% Ficoll, 0.5% bromophenol blue, and 0.5% xylene cyanol) was added to the reaction mixtures, which were mixed and immediately loaded onto vertical slab gels (5% polyacrylamide; 17 by 14 by 0.075 cm). The gels and the upper buffer chamber contained 200 μM cAMP, and the entire system used TBE running buffer (0.09 M Tris base, 0.09 M boric acid, 2.5 mM EDTA). After electrophoresis (30 mA for 50 min), the gels were treated with ethidium bromide and DNA was detected by fluorescence under UV illumination (39).

**Analysis of in vivo transcripts.** The identification of 5′ termini of transcripts encoding ADPglucose pyrophosphorylase was carried out by S1 nuclease protection analysis (7, 19). Restriction fragments with 5′ extended termini were treated with calf intestinal alkaline phosphatase (39) and labeled to a high specific radioactivity with $^{32}$P in the polynucleotide kinase reaction (40). Labeled fragments were cut at a secondary restriction site, and the uniquely labeled fragments were isolated by polyacrylamide gel electrophoresis (39) and used as probes.

Cultures for RNA isolation were grown in Kornberg medium (1.1% $K_2HPO_4$, 0.85% $KH_2PO_4$, 0.6% yeast extract, 0.5% glucose) at 37°C with gyratory shaking at 200 rpm. Growth was monitored by measuring the $A_{600}$. Cells were collected by centrifugation and rapidly frozen in solid $CO_2$-ethanol. RNA was isolated as described by Aiba et al. (2), quantified by measuring the $A_{260}$, and stored at −80°C in an ethanolic suspension before use. The labeled DNA was added directly to thawed aliquots of RNA, and the mixtures were chilled to −80°C. The nucleic acid-containing precipitates were obtained by centrifugation, and the supernatant solutions were examined with a Geiger-Muller counter to ensure that the radioactivity remained in the precipitate. The precipitated nucleic acids were washed with 70% ethanol, dried in vacuo, carefully solubilized in hybridization buffer (39), and denatured at 85°C for 15 min. The samples were rapidly transferred to 53°C, and hybridization reactions were carried out at 53°C overnight. Nuclease digestions were initiated by the addition of S1 nuclease (25 U/50 μg of RNA) in ice-cold S1 buffer (39), and the mixtures were incubated at 37°C for 30 min. The reactions were stopped, and the protected fragments were obtained as described previously (39). Protected fragments were dissolved in loading buffer (100% formamide containing 0.1% bromophenol blue and 0.1% xylene cyanol), heated at 90°C for 5 min, and analyzed on 5 or 6% polyacrylamide sequencing gels (8 M urea in 0.5× TBE; 0.04 by 60 cm) with size markers prepared from probe DNA which had been subjected to the G and G+A sequencing reactions (40). The running voltage was adjusted manually during electrophoresis to maintain a temperature of 50 to 60°C. The gels were dried in vacuo and subjected to autoradiography with an intensifying screen (Cronex Lightning-Plus; Du Pont Co., Wilmington, Del.). Quantification of fragments was accomplished by densitometric analysis of the autoradiograms with a GS300 transmittance/reflectance scanning laser densitometer (Hoefer Scientific Instruments).

Experimental controls were used to ensure that probe DNA was free from nicks (electrophoretic analysis of intact probes on denaturing gels) and to confirm that the protected fragments were dependent upon *glgC* expression (RNA from the deletion strain, G6MD3, was hybridized to probes). The rRNA species present in each of the RNA preparations were examined by formaldehyde agarose gel electrophoresis (39) to assess the general quality of each preparation of RNA, and the quantity of $^{32}$P was monitored during the experimental procedures to ensure that probe DNA was hybridized in excess (25- to 50-fold) relative to RNA.

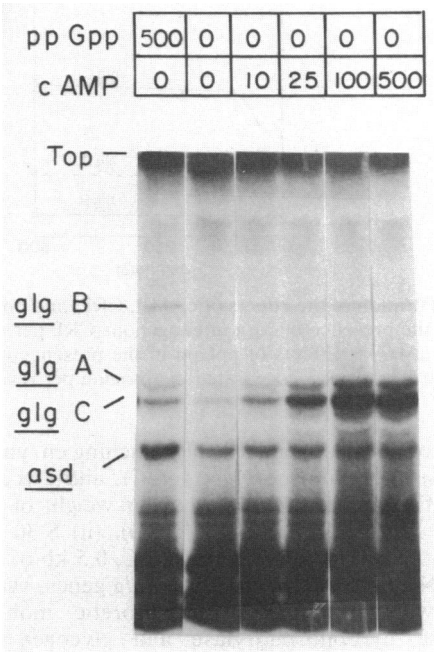



FIG. 1. Effects of ppGpp and cAMP on the in vitro synthesis of [$^{35}$S]methionine-labeled proteins directed by pOP12. Reactions contained 1 μg of pOP12 and 1 μg of exogenous CRP in a 35-μl final volume and were conducted as described in Materials and Methods. The positions of unlabeled standards of ADPglucose pyrophosphorylase (*glgC*), glycogen synthase (*glgA*), and glycogen branching enzyme (*glgB*) were determined by Coomassie blue staining, and the position of the gene product of *asd* was estimated from the known molecular weight of the protein (29). The concentrations of cAMP and ppGpp are micromolar.

## RESULTS

**In vitro expression of glycogen biosynthesis genes.** The purpose of this study was to examine the effects of potential regulatory factors on the in vitro expression of each of the three biosynthesis genes. Several proteins encoded by pOP12 were synthesized in the S-30 system (Fig. 1). The synthesis of the four products identified in Fig. 1 was absolutely dependent upon the presence of pOP12 and did not occur in reactions which had no added DNA or contained pBR322 (see also Fig. 3, lanes A and B; other data not shown). Identification of these products was based upon the following. (i) The mobilities of the products were compared

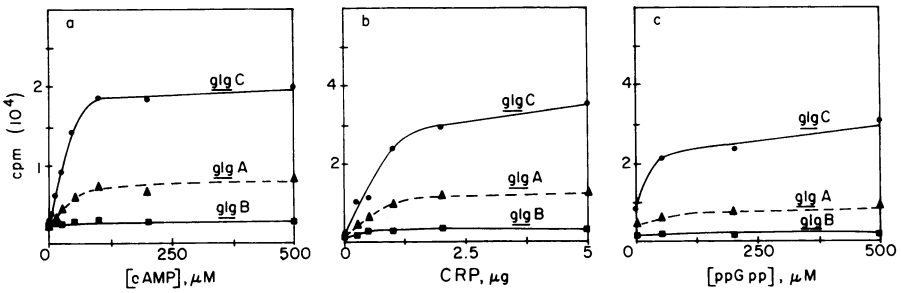



FIG. 2. Titration of the effects of cAMP, CRP, and ppGpp on the synthesis of glycogen biosynthesis enzymes in S-30 extracts. (a) Effects of cAMP in the presence of 2 μg of exogenous CRP per reaction. (b) Effects of increasing CRP levels in the presence of saturating levels of cAMP (100 μM). (c) Effects of ppGpp in the presence of cAMP (100 μM) and CRP (1 μg per reaction). Approximately $8.4 \times 10^3$ cpm is equivalent to 1 pmol of incorporated methionine per reaction per h.

with those of purified standards of branching enzyme (*glgB*), ADPglucose pyrophosphorylase (*glgC*), and glycogen synthase (*glgA*) and the known molecular weight of aspartate semialdehyde dehydrogenase (*asd*; 29). (ii) S-30 reactions directed by pPR2, which contains *glgC*, 0.5 kb of upstream flanking DNA, and *glgA* but no other *glg* genes, synthesized products with the same electrophoretic mobilities as ADPglucose pyrophosphorylase and glycogen synthase (data not shown). (iii) S-30 reactions directed by pOP245, which was constructed by subcloning a 1.7-kb *PvuI* fragment which contained *glgA* only into pBR322 (35), synthesized only the product which migrated with glycogen synthase (data not shown). The addition of cAMP and CRP increased the pOP12-directed synthesis of ADPglucose pyrophosphorylase (*glgC*) and glycogen synthase (*glgA*) but not that of branching enzyme (*glgB*) or aspartate semialdehyde dehydrogenase (*asd*). The synthesis of the products of *glgC*, *glgA*, and *asd* was slightly enhanced by ppGpp.

Quantification of incorporated [$^{35}$S]methionine (Fig. 2) allowed the estimation of saturation levels for cAMP, CRP, and ppGpp (100 μM, 2 μg/35 μl, and 150 to 200 μM, respectively). The data shown in Fig. 1, Fig. 2, and Table 1 were obtained from three independent experiments. These and other experiments reproducibly demonstrated the following. (i) cAMP and CRP enhanced *glgC* expression more strongly than *glgA* expression (approximately threefold) and had weak or negligible effects on *glgB*. (ii) ppGpp enhanced *glgC* expression less effectively than did cAMP and CRP and affected *glgC* expression to a greater extent than *glgA* expression. The effect of ppGpp on *glgB* expression was either weak (the maximal effect observed in Table 1 was 1.3-fold) or nonexistent. The maximal stimulation of expression by cAMP and CRP observed in the experiment shown in Fig. 2 was 26-fold for *glgC* and 10-fold for *glgA*; ppGpp increased the expression 3.6- and 1.8-fold, respectively. Table 1 shows the effects of various combinations of the three regulatory compounds. ppGpp activated the synthesis of *glgC* independently of cAMP and CRP; however, its effects were more pronounced in the presence of these factors. The observation that cAMP is able to cause some stimulation in the absence of added CRP is probably the result of the presence of endogenous CRP from the S-30 extract.

TABLE 1. Effects of cAMP, CRP, and ppGpp on the synthesis of glycogen biosynthesis enzymes in S-30 extracts

| Presence (+) or absence (−) of: | | | Expression[a] of: | | |
|---|---|---|---|---|---|
| cAMP (100 μM) | CRP (28.6 μg/ml) | ppGpp (250 μM) | *glgC* | *glgA* | *glgB* |
| − | − | − | 1.0 | 1.0 | 1.0 |
| + | − | − | 2.5 | 1.3 | 1.0 |
| + | + | − | 6.3 | 2.2 | 1.0 |
| − | − | + | 1.6 | 1.1 | 1.1 |
| + | − | + | 6.0 | 2.4 | 1.0 |
| + | + | + | 18.8 | 4.8 | 1.3 |

[a] Results are expressed as the relative incorporation (in counts per minute) of [$^{35}$S]methionine for each gene product. The directly determined counts per minute for the basal reactions, i.e., those containing no activators, were 719, 950, and 1,036 for ADPglucose pyrophosphorylase (*glgC*), glycogen synthase (*glgA*), and branching enzyme (*glgB*), respectively. Under the conditions for these assays, approximately $2.8 \times 10^3$ cpm was equivalent to 1 pmol of methionine incorporated per 35-μl reaction per h.

Since the levels of glycogen biosynthetic enzymes increase severalfold because of nitrogen depletion (see reference 42 for a review), it was reasonable to test whether the genes are under the control of the nitrogen starvation regulatory system. The *ntrC* and *ntrA* genes encode a specific DNA-binding protein and an alternate sigma factor for RNA polymerase, respectively, which directly regulate genes in this system (33). The effects of NtrA and NtrC on the expression of genes in the S-30 assay are shown in Fig. 3. Although these factors resulted in significantly enhanced expression from a *glnA'-'lacZ* gene fusion (lane H), they did not increase the synthesis of the glycogen biosynthesis proteins (lanes D and F).

Previously, the in vitro expression of *glgC* was examined with an assay which measures the synthesis of the first dipeptide of specific gene products (52). This assay has the particular advantage of using only purified components. An analysis of dipeptides formed with pOP12 as the genetic template confirmed previous results which showed that the synthesis of the amino-terminal dipeptide of *glgC* was enhanced by cAMP and CRP (7- to 10-fold in our experiments) and that neither cAMP nor CRP alone was sufficient. The expression of fMet-Gln, the amino-terminal dipeptide of *glgA* was enhanced 2.4- to 2.8-fold by cAMP and CRP. The effects on the expression of both dipeptides were dependent on plasmid pOP12. pBR322 had no effect, and the synthesis of fMet-Ser, the dipeptide of the *bla* gene, which is expressed from independent promoters on pOP12 and pBR322, also was not enhanced. Recently, the nucleotide sequences of two open reading frames encoded by pOP12 were reported (47). Therefore, the nucleotide sequence of all but about 0.7 kb of DNA upstream from the *asd* gene in this 15-kb plasmid is known. The deduced amino-terminal dipeptides of these two open reading frames are fMet-Thr (*glgX*) and fMet-Asn (*glgY*); that of the *asd* product is fMet-Lys. The possibility that either fMet-Val or fMet-Gln is the

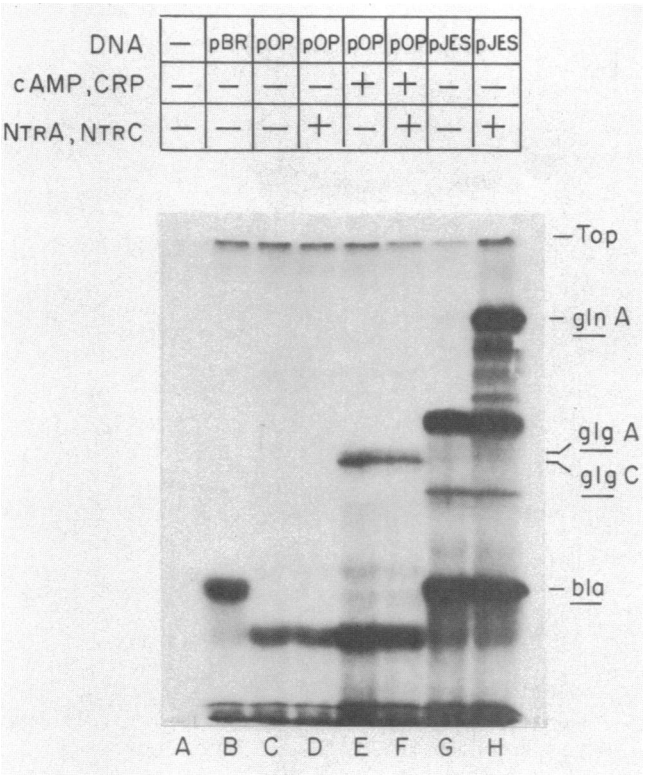



FIG. 3. Effect of NtrA and NtrC proteins on the synthesis of the glycogen biosynthesis enzymes in S-30 extracts. Plasmid DNA was present at 0.28, 1.0, and 5.0 μg per reaction for pBR322 (pBR), pOP12 (pOP), and pJES40 (pJES), respectively. The concentration of cAMP was 100 μM; CRP, NtrA, and NtrC610 were added at 1 μg, 80 ng, and 800 ng per reaction, respectively. The *glnAp2* promoter present in pJES40 is fused to *lacZ* and directs the synthesis of a β-galactosidase fusion protein (32).

product of a gene other than *glgC* or *glgA*, respectively, is therefore remote.

The dipeptide assay was also used to examine the expression of *glgC* and *glgA* from restriction fragments. Table 2 demonstrates that an *Hpa*I fragment which extends from 0.5 kb upstream from *glgC* through the *glgC* and *glgA* coding regions was capable of directing the synthesis of fMet-Val and fMet-Gln. The synthesis of fMet-Val directed by the *Hpa*I fragment was enhanced 2.5-fold by cAMP and CRP, versus the 7- to 10-fold effect with plasmid pOP12; however, cAMP and CRP no longer enhanced the synthesis of fMet-Gln from this fragment. Essentially the same results were obtained with two different preparations of the DNA fragment. This result shows that a promoter site which is capable of mediating the activation of *glgC* expression by cAMP-

TABLE 2. Formation of N-terminal dipeptides directed by an *Hpa*I restriction fragment of pOP12 which contains *glgC* and *glgA*

| System | pmol of the following dipeptide: | | |
|---|---|---|---|
| | fMet-Val (*glgC*) | fMet-Gln (*glgA*) | fMet-Ser (*bla*) |
| Complete | 0.49 | 0.17 | 0.09 |
| Without cAMP | | 0.17 | |
| Without CRP | | 0.15 | |
| Without cAMP and CRP | 0.20 | 0.16 | 0.00 |
| Without *Hpa*I | 0.00 | 0.00 | 0.00 |

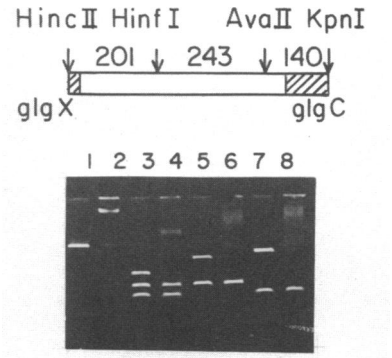
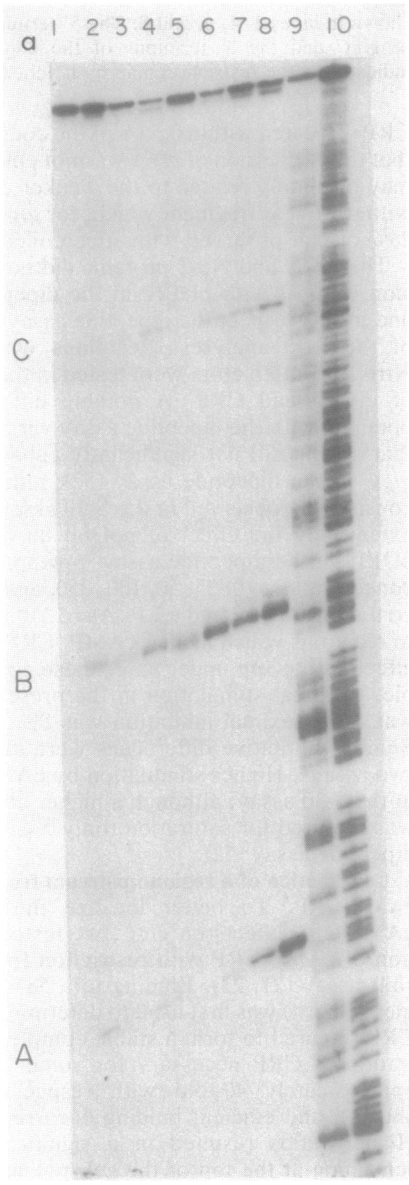


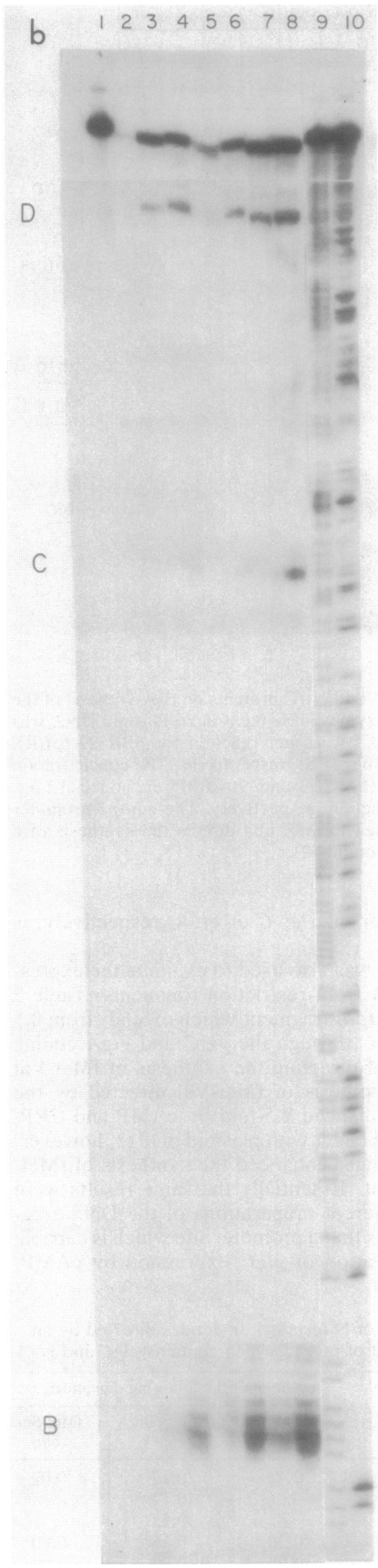

FIG. 4. Mobility shift assay for the detection of CRP-DNA complexes. A *Hinc*II-*Kpn*I fragment which contains approximately 0.5 kb of DNA upstream from *glgC* is shown at the top. Binding reactions contained this fragment either intact (lanes 1 and 2) or after further digestion with *Ava*II and *Hin*fI (lanes 3 and 4), *Hin*fI (lanes 5 and 6), or *Ava*II (lanes 7 and 8). Concentrations of the binding reaction components were as follows: restriction fragments, 20 nM; CRP, 1 μM; and cAMP, 200 μM. CRP was added to the reactions shown in lanes 2, 4, 6, and 8. The 5′ terminus of the coding region of *glgC* and the 3′ terminus of the coding region of *glgX* are indicated in the restriction map by hatched bars.

CRP is located within 0.5 kb of the coding region. The reason that the expression of *glgA* was not enhanced is not clear but may be simply related to the weaker cAMP-CRP activation with the linear fragment which, for *glgC*, was approximately 25% of that observed with supercoiled pOP12.

The NtrA and NtrC proteins did not enhance the expression of *glgC* with pOP12 in the dipeptide synthesis assay, and in this respect the dipeptide assay confirmed the results of the S-30 analyses. Reactions which contained NtrA, NtrC, or both factors were tested in the presence or absence of cAMP and CRP. A notable difference in the results obtained with the dipeptide assay versus the S-30 assay was that ppGpp did not significantly enhance the expression of *glgC* in the dipeptide assay (52), although its effects were consistently observed in the S-30 assay. We examined more extensively the effect of ppGpp on *glgC* expression from pOP12 in the dipeptide assay. ppGpp was added in various concentrations (0, 25, 50, 100, 250, and 500 μM) to reactions containing cAMP and CRP. Also, 250 μM ppGpp was added to reactions which lacked cAMP, CRP, or both factors. The effects of ppGpp under all of these conditions were negligible. Maximal stimulation in the presence of 50 μM ppGpp was 3%; maximal inhibition was 8% with 500 μM ppGpp. Some quantitative differences were also observed with the two assays. Higher stimulation by cAMP could be achieved in the S-30 assay, although a higher concentration, 100 μM, was required for saturation (only 5 μM was required in the dipeptide assay; 52).

**Localization of a region upstream from *glgC* which binds to cAMP-CRP.** To better localize the region required for cAMP-CRP effects on *glgC*, we tested the specific interaction of cAMP-CRP with restriction fragments in a mobility shift assay (21, 23). Binding to a 584-bp *Hinc*II-*Kpn*I fragment (Fig. 4) was first used to determine the concentration of CRP required to form a stable complex. The molar concentration of CRP necessary for some binding to occur was approximately 40-fold with respect to DNA (data not shown), and efficient binding occurred at a 50-fold excess. Higher ratios resulted in a significant amount of DNA remaining at the top of the gel, presumably because of the binding of multiple CRP proteins to each fragment. The


a
1 2 3 4 5 6 7 8 9 10
C
B
A


b
1 2 3 4 5 6 7 8 9 10
D
C
B

*Hin*cII-*Kpn*I fragment was hydrolyzed with restriction enzymes which cut at single sites in the fragment, and these secondary fragments were tested. It is clear that a 243-bp *Hin*fI-*Ava*II fragment contained the minimum region necessary for binding with respect to the fragments which were examined (Fig. 4). This fragment spans a region from −297 to −48 relative to the initiating AUG codon.

**Analysis of the 5′ termini of in vivo *glgC* transcripts.** Inspection of the nucleotide sequence in the region upstream from *glgC* revealed no obvious consensus promoter. Therefore, S1 nuclease protection analysis was used to localize the putative initiation sites for transcripts. Two different double-stranded probes labeled at the 5′ termini of the sense strands were used. Some of the parameters for this analysis, such as appropriate hybridization temperature and RNA and probe concentrations, were first established with small gels (0.075 by 20 cm) prior to analysis on 60-cm sequencing gels. Similar protection patterns were observed at both 47 and 53°C (data not shown).

To examine the effect of the growth phase on the relative abundance of transcripts, we isolated RNA from cells at the mid-exponential and early stationary phases. In addition, RNA was isolated from five different bacterial strains which vary in their capacities for glycogen biosynthesis. Figure 5a shows the results of mapping studies which used probe 1, a restriction fragment labeled at the *Bam*HI site (located 198 bp inside the coding region) and extending to the *Bgl*I site (427 bp upstream from the initiation codon) (see Fig. 6). Transcripts from all five strains were mapped simultaneously. Three distinct protected fragments, labeled A, B, and C, were observed, as was a fourth fragment which was not well resolved from the probe. RNA from deletion strain K-12 G6MD3 (lane 1) did not protect the three fragments, suggesting that the transcripts were dependent on the presence of the *glgC* gene and establishing the specificity of the analysis. Visual inspection of Fig. 5 shows that each transcript was found in greater relative abundance in the stationary phase than in the exponential phase in the strains which were compared (lanes 3, 5, and 7 versus lanes 4, 6, and 8). Obvious strain differences were also readily observed. *E. coli* B SG3, which accumulates elevated levels of ADPglucose synthetase and glycogen synthase because of the *glgR* mutation, exhibited higher levels of transcript B only, relative to wild-type *E. coli* B. *E. coli* B AC70RI, which accumulates high levels of all three biosynthesis enzymes, exhibited significantly higher amounts of all three transcripts. Especially striking was the abundance of transcript A from this strain. Analysis of the autoradiogram by densitometry allowed a more quantitative comparison of the relative amounts of the transcripts (Table 3). Probe 2 (Fig. 6) was also used to map the larger transcripts from each strain (Fig. 5b). In this case, the fourth protected fragment, D, was resolved from the probe. This gel also resolved fragment B into a group of seven to eight fragments which differed sequentially by one nucleotide. These forms may not necessarily suggest the presence of multiple initiation sites but are probably the result of the inherent heterogeneity of the S1 nuclease analysis (2). A summary of the experimental design and the results of the S1 nuclease analysis is shown in Fig. 6. The nucleotide sequences of the immediate 5′-flanking regions for three of the transcripts are compared with consensus sequences for *E. coli* promoters (26). A comparison of these proposed promoter sequences for *glgC* with promoters required for the expression of heat shock genes via $\sigma^{32}$ (25), promoters of chemotaxis and flagellar genes (6, 30), and promoters of nitrogen starvation genes which depend on $\sigma^{54}$ did not reveal any significant similarities.

TABLE 3. Scanning densitometry of $^{32}$P-labeled fragments protected by RNA from *glgQ* and *glgR* mutants

| Strain[a] | Growth phase | Relative amt[b] of transcript: | | |
|---|---|---|---|---|
| | | A | B | C |
| B (wild type) | Exponential | | 7 | 3 |
| | Stationary | 3 | 15 | 7 |
| SG3 (*glgR*) | Exponential | | 27 | 2 |
| | Stationary | 4 | 51 | 5 |
| AC70RI (*glgQ*) | Exponential | 41 | 48 | 12 |
| | Stationary | 100 | 64 | 14 |

[a] *E. coli* B is wild type with respect to glycogen biosynthesis. SG3 and AC70RI are mutants derived from *E. coli* B which have been shown to overproduce glycogen biosynthesis enzymes (see text).

[b] Data were collected from the autoradiogram in Fig. 5a. Arbitrary integration units were normalized with respect to the highest value (AC70RI, transcript A, stationary phase), designated 100.

## DISCUSSION

The variety of mechanisms which participate in the regulation of glycogen biosynthesis in *E. coli* attests to the importance of this process to the cell. The rate of glycogen accumulation varies with the nutrient composition of the growth medium and with the growth phase (42). Although the allosteric regulation of ADPglucose pyrophosphorylase activity is an important factor in controlling the rate of glycogen accumulation, this study establishes a significant role for the genetic control of the levels of biosynthetic enzymes in regulation.

Analysis of the in vivo rate of glycogen biosynthesis has indicated that *relA* mutants (*relA* is the gene responsible for the synthesis of ppGpp during the stringent response) are defective in glycogen accumulation (10, 38, 51). Likewise, mutations in the catabolite control system, including *crp* and *cya*, have been shown to decrease glycogen accumulation (17, 18, 37). These studies showed that *cya* but not *crp* mutants could be restored by the addition of cAMP to the culture medium. However, the mechanisms by which these two systems act was not ascertained. In fact, cAMP and CRP were proposed to be involved indirectly by affecting the synthesis of an unknown enzyme whose substrate or reac-

FIG. 5. S1 nuclease protection analysis of transcripts from the wild type and glycogen biosynthesis mutants. RNA was hybridized with a *Bam*HI-*Bgl*I probe (a) or an *Ava*II-*Hin*cII probe (b), and the reactions were treated with S1 nuclease. The protected fragments were subjected to electrophoresis on sequencing gels and detected by autoradiography (see Materials and Methods for details). In panel a, RNA was extracted from strains G6MD3 (lane 1), K-12 3000 (lane 2), B (lanes 3 and 4), SG3 (lanes 5 and 6), and AC70RI (lanes 7 and 8). RNA was extracted from cultures in the mid-exponential phase (lanes 3, 5, and 7) or the early stationary phase (lanes 1, 2, 4, 6, and 8). Lanes 9 and 10 contained the *Bam*HI-*Bgl*I probe subjected to the G and G+A reactions, respectively (40). In panel b, RNA was extracted from strains G6MD3 (lane 2), B (lanes 3 and 4), SG3 (lanes 5 and 6), and AC70RI (lanes 7 and 8). RNA was extracted from cultures in the mid-exponential phase (lanes 3, 5, and 7) or the early stationary phase (lanes 2, 4, 6, and 8). Lane 1 contained unreacted *Ava*II-*Hin*cI probe, and lanes 9 and 10 contained the *Ava*II-*Hin*cII probe subjected to the G+A and G reactions, respectively (40). A, B, C, and D represent protected fragments.

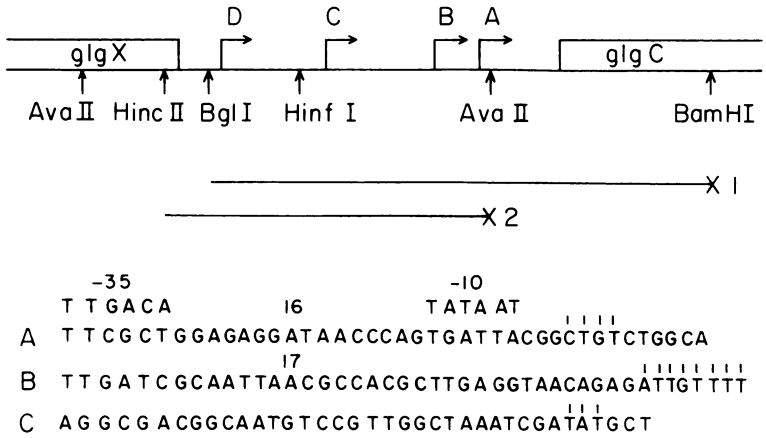



FIG. 6. Summary of S1 nuclease protection mapping analysis. The 5′ termini of transcripts identified as A, B, and C were mapped with the indicated probes (1 and 2) and located at approximately −60, −130, and −245, respectively, relative to the initiating AUG codon. The position shown for transcript D has not been determined at a high resolution. The $^{32}$P-labeled 5′ terminus of each probe is indicated by ×. The nucleotide sequences preceeding transcripts A, B, and C are shown along with the consensus sequences for *E. coli* promoters (26). The apparent initiation sites for the transcripts are shown as vertical lines above the sequence of the antisense (RNA-like) strand.

tion product could alter the activity of ADPglucose pyrophosphorylase (37). The compound ppGpp has also been suggested to be a negative allosteric effector of ADPglucose pyrophosphorylase (16), although such a role for this compound is not consistent with the results obtained with *relA* mutants (10, 38, 51) or with the results of the present study. It is now apparent that both of these global regulatory systems act to enhance the expression of two of the biosynthesis genes, *glgC* and *glgA*. A preliminary report which showed that the in vitro synthesis of the first dipeptide and tripeptide of *glgC* was stimulated by cAMP and CRP was published previously (52).

The observation that cAMP and ppGpp appear to be able to independently affect the in vitro expression of *glgC* is similar to the results for the expression of *lacZ* (45). There may be a further similarity in the mechanisms of genetic control for these two systems, in that ppGpp enhances the expression of *lacZ* in S-30 extracts (45) but not in a defined transcription system (1), as observed for *glgC* (52; T. Romeo and J. Preiss, unpublished data). Although there is persuasive evidence that ppGpp interacts directly with RNA polymerase to directly alter the transcription of various genes (11 and references therein), it is conceivable that this nucleotide acts in a somewhat different fashion in systems such as *lac* and *glg*. A small protein which may help to mediate the effects of ppGpp on *lacZ* under certain conditions has been partially characterized (1).

The coordinate increase in the levels of ADPglucose pyrophosphorylase and glycogen synthase under a variety of conditions has been proposed as an indication that the glycogen biosynthetic genes for these enzymes constitute an operon (34, 42, 44). The gene for branching enzyme, *glgB*, may not be part of that operon, since the level of this enzyme does not increase coordinately under certain growth conditions (44). The observation that the in vitro synthesis of branching enzyme, unlike that of the other two enzymes, is not significantly enhanced by cAMP or ppGpp is consistent with this interpretation. In addition, the gene which encodes branching enzyme is separated from *glgC* and *glgA* by an intervening region which may encode glucan hydrolase or transferase (47). However, *glgB* may be part of a glycogen regulon, since the levels of branching enzyme as well as ADPglucose pyrophosphorylase and glycogen synthase are all enhanced in AC70RI (42), presumably because of the effects of the mutation upon a *trans*-acting regulatory factor. The fact that the initiation codon of *glgA* overlaps with the stop codon of *glgC* (35) suggests that the coordinate synthesis of their gene products may involve translational coupling (3, 15, 48).

Several types of evidence are consistent with the conclusion that the promoter(s) required to mediate the effects of cAMP and ppGpp on the expression of *glgC* and probably *glgA* is located immediately upsteam from the *glgC* coding region. (i) The in vitro synthesis of the first dipeptide of *glgC* is enhanced by cAMP and CRP when the gene is present on an *Hpa*I restriction fragment which contains 0.5 kb of DNA upstream from the *glgC* initiation codon and the *glgC* and *glgA* coding regions. (ii) cAMP-CRP selectively binds to a 243-bp restriction fragment from the region immediately upstream from *glgC*. (iii) In vivo transcripts apparently initiate in this region. (iv) A *lacZ* gene fusion which contains 0.5 kb of DNA upstream from *glgC* and 0.2 kb of the *glgC* coding region places *lacZ* expression under *glgC* control. The gene fusion shows cAMP and ppGpp regulation in vitro and in vivo (T. Romeo, J. Black, A. Gardiol, and J. Preiss, unpublished observation). Of course, these results do not exclude the possibility that in this system ppGpp may have effects on the early phase of translation.

The increase in the levels of *glgC* transcripts in the early stationary phase relative to the mid-exponential phase indicates that the accumulation of the glycogen biosynthesis enzymes in the stationary phase is the result of transcriptional control, although the results do not rule out contributions from other mechanisms. A similar conclusion is suggested by a study by Cattanéo et al. (12), who showed that the addition of actinomycin D to cultures at the beginning of the stationary phase inhibits glycogen accumulation. The present study also showed that the glycogen-overproducing mutants SG3 and AC70RI are affected at the transcriptional level. In the case of SG3, the observation that only the level of the transcript B is elevated suggests a specific location for the mutation upstream from this transcript. The AC70RI mutation acts in *trans* with respect to the glycogen biosynthesis enzyme levels and leads to apparent overproduction of all of the *glgC* transcripts. Transcript A levels are especially high in this strain, approximately 25-fold with respect

to those in strains B and SG3. Although AC70RI has been described as a derepressed strain (42), the basis of the phenotype unfortunately is unclear. The results of the present study indicate that the mutation affects transcriptional regulation but do not determine the nature of the factor which is altered. Since branching enzyme as well as ADPglucose pyrophosphorylase and glycogen synthase are overproduced in this strain, the mutation probably affects a factor other than cAMP-CRP or ppGpp. The fact that in vivo *lacZ* expression is similar in strains AC70RI and B is also consistent with this interpretation (T. Romeo and J. Preiss, unpublished observation). Obviously, a major effort will be directed toward the further characterization of mutant AC70RI and the regulatory factor(s) involved.

Several possible explanations could account for the poor similarity of the −10 and −35 regions of the identified transcripts with respect to the *E. coli* consensus promoter sequences (26). It may be a consequence of the fact that the expression of *glgC* is regulated by at least two activator systems, which could compensate for the weak promoters (46). Also, some or all of the transcripts may be initiated via an alternate sigma factor for RNA polymerase (31 and references therein). Although none of the promoter sequences for the known alternative sigma factors are similar to those of the *glgC* promoters, perhaps the required sigma factor is one which has not been identified. It is conceivable that one or more of the transcripts may have arisen by endonucleolytic processing. We are therefore cloning the putative promoters and will analyze the in vitro and in vivo expression from the isolated regions. Such an analysis should confirm the existence of the proposed promoters and allow the regulation of each to be examined independently.

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