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How To Achieve Constitutive Expression of a Gene within an Inducible Operon: the Example of the *nagC* Gene of *Escherichia coli*

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The *nagC* gene, encoding the NagC repressor/activator of the *nag* regulon, is part of the *nagBACD* operon. When the promoter-proximal *nagB* and *nagA* genes are induced 20- to 40-fold, the *nagC* gene is induced only two- to threefold. In addition to being transcribed as part of the polycistronic *nagBACD* mRNA, *nagC* is also expressed from two promoters located within the upstream *nagA* gene. These promoters are comparable in strength to the induced *nagB* promoter, resulting in a high basal level of the *nagC* mRNA. This means that when the *nagBA* genes are induced, there is a much smaller effect on the amount of *nagC* mRNA. The *nagC* gene is subject to low-level translation so that the amount of NagC protein is kept low despite the relatively high transcription levels.

Three sets of genes have been identified as being controlled by the NagC protein and thus are members of the *nag* regulon of *Escherichia coli*. They are the divergent *nagE-BACD* operons, the *glmUS* operon, and the *manXYZ* operon. All three sets of genes are implicated in the metabolism of the amino sugars *N*-acetylglucosamine (GlcNAc) and glucosamine (GlcN). The divergently expressed *nagE-BA* operons encode proteins necessary for the transport of GlcNAc and utilization of GlcNAc and GlcN as carbon sources (26, 36, 41, 43). They are negatively controlled by the NagC protein, which binds to the two operators overlapping the divergent *nagE-B* promoters, forming a DNA repression loop (33, 34). Two binding sites for the NagC protein also exist upstream of the *manXYZ* operon (33), which encodes a second transporter for GlcNAc and is the major transporter for GlcN as well as for other hexose sugars (5, 15). These genes are, however, only weakly repressed by NagC under normal cellular conditions (27, 41). The amino sugars are essential constituents of the peptidoglycan and lipopolysaccharide of gram-negative bacteria, and the NagC protein is involved in the regulation of their biosynthesis. The *glmUS* operon encodes proteins which synthesize GlcN-6-P and UDP-GlcNAc; the latter is the first dedicated precursor of the cell wall components (20, 21). The *glmUS* operon is positively controlled by NagC, which acts as an essential activator for one promoter and a weak repressor for a second, upstream promoter. Two NagC binding sites have been located in the *glmUS* upstream region, and both are necessary for activation (32).

The location of the gene for the NagC regulatory protein itself, within the inducible *nagBACD* operon (28, 30, 41), is somewhat unusual. Most transcriptional repressors and activators are expressed as isolated genes, possibly located near the loci that they control (e.g., *araC* and *lacI*) but transcribed from their own, usually low-level, promoters. However, Northern (RNA) blot analysis showed that *nagC* is expressed as part of the long *nagBACD* operon (28). These earlier studies also demonstrated that the majority of the transcripts induced from the *nagB* promoter (*nagBp*) have their 3′ ends after *nagB* or after *nagA*. The 3′ ends were mapped to sites within the coding sequence of the downstream gene, i.e., within *nagA* and *nagC*, and coincided with regions predicted to form stable secondary structures in the RNA. The putative RNA secondary structure within *nagC* is potentially very stable (ΔG = Ca. −40 kcal [−1674 kJ]/mol) and consists of two stem-loop structures held together by a third base-paired stem. The difference in *nagC*-specific mRNA levels between glucose (noninduced) and GlcNAc (induced) is much less than that observed for the *nagB* and *nagA* genes, and whereas the expression of *nagB* and *nagA* genes is strongly induced by growth on GlcNAc, very little induction of the expression of *nagC* was detected. The work described here is a first attempt to understand this phenomenon. Two promoters are localized within the *nagA* gene, and it is shown that they are responsible for the majority of *nagC* expression.

MATERIALS AND METHODS

Bacterial strains. *E. coli* IBPC5321 (*thi-1 argG6 argE3 his-4 xyl-5 rpslL ΔlacX74*), its *nagC*::Cm derivative IBPC529C (30), and the Δ*nagE-BACD*::Tc derivative IBPC590 (31) were used. The letter R after a strain name indicates the *recA1 srl*::Tn*10* derivative of the strain.

Construction of *nag-lacZ* fusions. In protein (or translational) fusions, β-galactosidase is expressed from a hybrid *nagC′-′lacZ* fusion protein, using *nag* transcriptional and translational signals. In operon (or transcriptional) fusions, a wild-type *lacZ* protein is expressed from its own translational initiation signals, but using the *nag* transcriptional signals.

Constructions of the *nagB′-′lacZ* and *nagD′-′lacZ* protein fusions have been described previously (29, 31). Protein fusions of *nagA* and *nagC* with *lacZ* were constructed by inserting the *Eco*RI-*Xmn*I and *Eco*RI-*Bal*I fragments (Fig. 1), respectively, into pMC1403 (3) digested with *Eco*RI and *Sma*I. The ligation of a *Bal*I site and *Sma*I site should have produced a run of six GC base pairs. This was apparently an unfavorable construction. Sequencing showed that at least two isolates had lost one GC base pair, and in another clone, a spontaneous deletion of 526 bp resulted in a fusion site 6 bp downstream of the *Stu*I site within the region predicted to form a stable secondary structure (Fig. 1) (28). This deleted fusion will subsequently be referred to as the N-terminal proximal fusion, whereas the fusion at the *Bal*I site (missing 1 bp) is called the distal fusion. Both types of constructs retained the *Bam*HI site at the *lacZ* junction. The *Bam*HI 5′ protruding ends were either filled in with Klenow enzyme or removed by S1 nuclease to produce the in-phase *nagC′-′lacZ* proximal and distal protein fusions. The *Eco*RI-*Bam*HI fragments from both the proximal and distal fusions were inserted into pRS415 (39) to produce operon (transcriptional) fusions.

A second series of fusions, called the sandwich fusions, was made by inserting a set of fragments internal to the *nagAC* genes (Fig. 2) into the *Bam*HI site of

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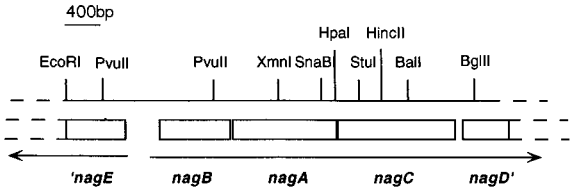


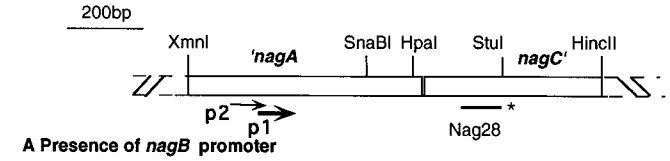
	Glc	GlcNAc	Ind	nagC	Ind
nagB'-'lacZ	57	1240	22	1350	27
nagBA'-'lacZ	17	330	19	620	36
nagBAC'-'lacZ	13	23	1.8	31	2.8
nagBACD'-'lacZ	55	55	1.0	54	1.0
nagD'-'lacZ	43	44	1.0	NT	

FIG. 1. Induction of the genes of the *nagBACD* operon. The relevant restriction sites in the *nagE-BACD* region of the chromosome are shown. The dark lines indicate the fragments cloned into pMC1403 to produce the different translational fusions. Activities of the fusions are measured from single-copy λ lysogens in the wild-type strain, IBPC5321, growing in minimal medium with glucose or GlcNAc as the carbon source. The β-galactosidase values are the mean of two independent cultures. Induction (Ind) is calculated as the fold increase in β-galactosidase expression during growth in the presence of GlcNAc compared with glucose or in the *nagC* strain (IBPC529C) compared with the wild type. NT, not tested.

pRS/EB (35). This plasmid carries the 200-bp intergenic *nagE-nagB* region, as an *Eco*RI-*Bam*HI fragment, in pRS415 (39), producing a *nagBp-lacZ* transcriptional fusion. The blunt-ended restriction fragments from the *nagA-C* region were ligated with a linker and then digested with *Bam*HI, which converts the blunt end to a *Bam*HI site. The same fragments, with *Bam*HI linkers, were also inserted into pRS415 alone to test for inherent promoter activity. A *nagBp'nagAC'-'lacZ* translational sandwich fusion was made by replacing the *Bam*HI *lacZ* junction from pRS415 with the corresponding region of the protein fusion vector pNM481 (23).

All fusions were transferred to λRS45 by in vivo recombination as described previously (39), and the resulting phages were used to form lysogens with IBPC5321R, IBPC529C, or IBPC590. Monolysogens were identified, and β-galactosidase activities were measured on four aliquots at two different optical densities of cultures growing exponentially in minimal MOPS [3-(*N*-morpholino)propanesulfonic acid] medium (24) supplemented with 50 μg each of arginine and histidine per ml and 0.2% glucose or GlcNAc at 30°C as described previously (22, 29). At least two and normally four to six cultures were tested for each lysogen, and standard errors between cultures were less than 10%.

Mutagenesis. The *nag-29* and *nag-30* mutations were made by oligonucleotide-directed mutagenesis, using the method of Kunkel as described by Sambrook et al. (37), on the *Xmn*I-*Stu*I fragment cloned into pT7T318U (Pharmacia) as a template. Both mutations change 4 bp and create *Stu*I and *Sal*I restriction sites,



A Presence of *nagB* promoter	Glc	GlcNAc	Ind
nagBp →	70	2050	29
→	28	847	31
→	685	1330	1.9
→	178	371	2.1
→	420	957	2.3
→	118	3130	27
→	74	2120	29
B Absence of *nagB* promoter			
	723	776	1.1
	167	142	0.9
	436	467	1.1
	2.9	N.T.	
	5.1	4.9	1.0

FIG. 2. Activity of *nagCp*s in the presence and absence of *nagBp*. The relevant restriction sites in the *nagA-C* region are indicated. The relative positions of $nagCp_1$ and $nagCp_2$ are shown, as is the location of the Nag28 oligonucleotide; the asterisk indicates the 5′ end of the oligonucleotide. The Nag28 oligonucleotide lies within the region capable of forming a stable secondary structure in the RNA. (A) Restriction fragments from the *nagA-C* region of the chromosome, as indicated by the horizontal lines, were inserted into the *Bam*HI site of pRS415/EB after the addition of *Bam*HI linkers. pRS415/EB carries the 200-bp intergenic *nagE-B* region as an *Eco*RI-to-*Bam*HI fragment shown by the short line with an arrowhead. This creates the sandwich fusions in which *nagBp* transcribes into the *nagA-C* region DNA inserted before *lacZ*. (B) The fragments shown in panel A were inserted into the *Bam*HI site of pRS415. The values are β-galactosidase activities of the different fusions carried by single-copy λ lysogens in IBPC5321R grown in minimal medium with glucose or GlcNAc as the carbon source. The induction factor (Ind) is the ratio of the expression during growth on GlcNAc divided by that on Glc. The β-galactosidase values are the means of two to four independent cultures. N.T., not tested.

which were confirmed by sequencing. Two independently mutated fragments were reinserted into the *lacZ* fusion constructs.

S1 analysis on *nag-lacZ* fusion templates. Total RNA was made from cultures of the lysogens exponentially growing in minimal MOPS medium (24) with 50 μg each of arginine and histidine per ml and with 0.2% glucose or GlcNAc as the carbon source. The cultures were cooled on ice, and the bacteria were harvested by centrifugation and washed with 10 mM Tris-HCl (pH 8.0)–10 mM $MgSO_4$–1 mM EDTA. After being resuspended in 0.15 M sucrose–20 mM sodium acetate (pH 5.0)–1% sodium dodecyl sulfate, the cells were immediately lysed by addition of an equal volume of water-saturated phenol and mixing at 65°C for 15 min. The hot phenol extraction was repeated once, chloroform extraction was performed, and the nucleic acids were recovered by ethanol precipitation and stored frozen in sterile water. The S1 probes were made by PCR using the plasmids carrying the different sandwich fusions as templates. The primers and templates are indicated in the figure legends. The downstream primer was 5′ end labelled with [γ-^{32}P]ATP and polynucleotide kinase. The radioactively labelled, amplified fragments were purified from an agarose gel. After denaturation of the probe and RNA (10 min at 75°C), hybridization was carried out in 80% formamide–40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer (pH 6.4)–0.4 M NaCl–1 mM EDTA (final volume of 50 μl) overnight and generally at 54°C. The hybridized mixture was transferred to 0.4 ml of ice-cold S1 buffer (30 mM sodium acetate [pH 4.6], 0.25 M NaCl, 1 mM ZnCl, 5% glycerol, 100 U of S1 nuclease [Boehringer Mannheim]) and incubated at 37°C for 30 min. The S1-resistant fragments were analyzed on 8% denaturing polyacrylamide gels.

In vitro transcription (IVT). The templates were made by PCR on pRS415-derived plasmids carrying the wild-type or mutant *Xmn*I-*Stu*I fragments in *lacZ* fusions, with or without the *Eco*RI-to-*Bam*HI *nagBp* fragment. The oligonucleotides used as primers were Nag28 (Fig. 2) and RBP22, which is homologous to the *bla* gene of pRS415. The amplified fragments were digested with *Eco*RI to remove DNA corresponding to the pRS415 vector prior to purification from an agarose gel. The templates (about 50 ng) were preincubated with 0.5 U of *E. coli* RNA polymerase (Boehringer Mannheim) in 40 mM Tris-HCl buffer (pH 8.0)–10 mM $MgCl_2$–100 mM KCl–5 mM dithiothreitol–500 μg of bovine serum albumin per ml at 37°C for 15 min. Polymerization was initiated by the addition of 25 μM UTP containing [α-^{32}P]UTP (0.5 μCi) and 100 μM each GTP, ATP, and CTP. The reaction was stopped after 15 min by extraction with phenol. The transcription products were recovered by ethanol precipitation and analyzed on a 6% denaturing acrylamide gel.

RESULTS

The *nagC* gene is not induced under conditions which induce the upstream *nagBA* genes. The expression of the divergent *nagE* and *nagB* genes has been extensively studied by fusions with *lacZ*. Both genes are induced in parallel by growth on GlcNAc (the inducing condition) or by the presence of a *nagC* mutation, which results in complete derepression due to the absence of the repressor (29, 30, 34). To examine the expression of the downstream genes *nagA*, *nagC*, and *nagD*, longer DNA fragments, starting within the *nagE* gene and ending within the *nagA*, *nagC*, or *nagD* gene, were used to construct *nagBA′-′lacZ*, *nagBAC′-′lacZ*, and *nagBACD′-′lacZ* protein fusions (Fig. 1). The levels of expression of these fusions (carried by single-copy lambda lysogens) under noninducing (glucose) and inducing (GlcNAc) growth conditions, in the presence of a wild-type *nagC* gene, are given in Fig. 1. While the fusions with *nagB* and *nagA* genes are induced about 20-fold by growth on GlcNAc, the *nagBAC′-′lacZ* fusion is induced only 2-fold. The effect of a *nagC* null mutation on expression of the *nagBAC′-′lacZ* fusion was similar, less than a threefold induction (Fig. 1). No induction of the *nagD* gene is detected, but this is not unexpected since most of its expression is due to monocistronic *nagD* transcripts. Northern analysis detected discrete mRNA species covering the *nagD* gene, and S1 mapping located 5′ mRNA ends consistent with consensus promoter −35 and −10 sequences immediately upstream of *nagD*. This finding implies that the bulk of *nagD* expression is from a promoter located in the intergenic *nagC-nagD* region (28). Protein fusions carrying just this intergenic region give a level of *nagD′-′lacZ* β-galactosidase activity only slightly lower than that of the entire *nagBACD′-′lacZ* fusion (Fig. 1). It should be remembered that as no phenotype or function has yet been associated with the NagD protein, it is not sure that this gene belongs to the *nag* regulon.

TABLE 1. Comparison of translational and transcriptional *nagC-lacZ* fusions

Fusion[a]	Translational fusions			Transcriptional fusions		
	β-Galactosidase activity (Miller units)[b]		Induction[c]	β-Galactosidase activity (Miller units)		Induction
	Glc	GlcNAc		Glc	GlcNAc	
nagBAC-lacZ (proximal)	15	28	1.8	940	2,300	2.4
nagBAC-lacZ (distal)	11	23	2.0	460	900	2.0
nagBp′nagAC-lacZ (sandwich)	13	26	2.0	685	1,330	1.9

[a] The *nagC-lacZ* junctions of the proximal fusion and the sandwich fusion are near and at the *Stu*I site, respectively, and that of the distal fusion is at the *Bal*I site (Fig. 1). The sandwich fusion contains the 838-bp *Xmn*I-to-*Stu*I fragment downstream of *nagBp*.

[b] Mean of results from two or four independent cultures.

[c] Calculated as described in the legend to Fig. 1.

The *nagC* gene is subject to poor translational initiation. Several lines of evidence suggested that the *nagC* gene is poorly translated (see Discussion). Two sets of fusions, translational (protein) and transcriptional (operon), were constructed at two points within the *nagC* gene: proximal to the translational start site, near the *Stu*I restriction site, and distal, at the *Bal*I site (Fig. 1). β-Galactosidase activity from the proximal protein fusion (i.e., of the hybrid *nagC′-′lacZ* protein) is 60- to 80-fold lower than that from the operon fusion at the same point, where *lacZ* is expressed from its own translational initiation signals (Table 1). This observation is consistent with the idea that translation of *nagC* is inefficient and limits the expression level of the gene. Despite the large difference in β-galactosidase activities, the induction of the operon fusions, like that of the protein fusions, is only twofold during growth on GlcNAc (Table 1). Moreover, the levels of the β-galactosidase activities of the *lacZ* fusions at the proximal and distal positions within *nagC* differ by no more than a factor of 2; i.e., the amount of translatable mRNA has been reduced by about 50% at a point more than halfway through the protein. Thus, some polarity exists, but it does not differentially affect the level of induction of the translational and transcriptional fusions. Since the same results are observed with both the transcriptional fusions and the translational fusions (Table 1), most of the experiments described below used transcriptional fusions.

DNA within the *nagA* gene is responsible for the lack of induction. Both Northern and S1 experiments (28) showed that a large proportion of the induced *nagBA* transcripts had their 3′ ends within the *nagC* structural gene, localized to a region predicted to fold into a stable RNA secondary structure: two hairpin loops held together by a third base-paired stem. To try to attribute the lack of induction of the *nagC* gene to a particular DNA element and in particular to see if it was related to this structured RNA region, various restriction fragments from the *nagA-C* region were inserted downstream of a functional *nagB* promoter in a *lacZ* operon fusion. We have previously shown that a 200-bp *Eco*RI-to-*Bam*HI fragment, spanning the *nagE-B* operators and promoters, contains all the DNA necessary for NagC-specific regulation of the divergent *nagE-B* promoters (34). This fragment inserted into pRS415 (39) produces a *nagBp-lacZ* transcriptional fusion. Different restriction fragments from the *nagA-C* region, equipped with suitable *Bam*HI linkers, were inserted at the *Bam*HI site mark-

ing the *nagB-lacZ* junction downstream of *nagBp*. These constructions, in which an internal *nagAC* fragment is inserted downstream of the *nagB* promoter, will be referred to as sandwich fusions.

The presence of a 509-bp fragment (*Hpa*I-*Hinc*II; Fig. 1) from very near the 3′ end of *nagA* and carrying the 5′ half of the *nagC* gene including all the RNA secondary structure region reduced the level of expression from *nagBp* about 3-fold (from 70 to 28 U) but had no effect on the induction by growth on GlcNAc, which stayed at 30-fold (Fig. 2A). On the other hand, an 838-bp DNA fragment carrying the 3′ half of the *nagA* gene and terminating within the DNA of the putative mRNA secondary structure within *nagC* (*Xmn*I-*Stu*I; Fig. 1) enhanced the basal (glucose) expression level considerably (from 70 to 685 U) but reduced the induction by GlcNAc to twofold. Cutting this latter fragment into two, at either the *Sna*BI site or the *Hpa*I site (Fig. 1), and testing the two halves separately showed that the high basal level and lack of induction are both associated with the *Xmn*I-*Sna*BI region (Fig. 2A) and suggested the presence of a promoter in this region. Thus, the same restriction fragments were tested for promoter activity in the absence of the *nagE-B* promoter fragment (Fig. 2B). The three fragments which showed a high basal level in the sandwich constructions all show an intrinsic promoter activity which is independent of the growth medium, glucose or Glc NAc. The other restriction fragments derived from DNA downstream of the *Sna*BI site, which have no effect on the induction from *nagB*, have almost no promoter activity (Fig. 2B).

Localization of an internal promoter for *nagC*. The results described above are consistent with a promoter activity within the *nagA* gene. This promoter was mapped on the DNA inserts of the sandwich fusions. Total RNA was prepared from lysogens carrying the different sandwich fusions under induced conditions and from the fusion without *nagBp* during growth on glucose. The different mRNA preparations were used for S1 experiments using DNA probes specific for each sandwich fusion construct. In all cases, the RNA from the fusions with the induced *nagBp* contained a long mRNA whose length was compatible with a 5′ end corresponding to the induced *nagBp* (Fig. 3, bands B). With mRNA from either the induced or basally expressed fusions, a second strong band, labeled C1 in Fig. 3, was detected for the sandwich fusions containing the *Xmn*I-*Stu*I (lanes 2 to 5), *Xmn*I-*Hpa*I (lanes 8 to 11), and *Xmn*I-*Sna*BI (lanes 14 to 17) fragments but not for the *Sna*BI-*Stu*I fragment (lanes 20 to 23). The position of the promoter giving rise to this strong band in the *Xmn*I-*Sna*BI fragment can be deduced from each of the S1 experiments in Fig. 3A to C and predicts a consistent position, 475 bp upstream of the *Stu*I site. Inspection of the DNA sequence in this region suggested that the promoter sequence was TTCATT-16 bp-TATACT (underlined nucleotides correspond to those in agreement with the commonly accepted consensus for promoters [13, 14]). A promoter in this region was confirmed by IVT (see below). On the longest S1 template, *Xmn*I-*Stu*I, additional 5′ RNA ends were detected (D1, D2, and D3 in Fig. 3). The D1 and D2 protected fragments correspond to 5′ ends near the 3′ end of the *nagA* gene where no promoter activity was detected. The D3 end maps to the 5′ edge of the region predicted to fold into a stable secondary structure in *nagC*. 5′ ends in the same positions as D1 and D2 are detected by S1 mapping of chromosomally derived *nagAC* transcripts (27), and these 5′ extremities are likely to be the result of an RNA processing of the longer primary transcripts.

Mutating *nagCp1* stimulates induction of *nagC* from *nagB*. A mutation, *nag-29*, which was expected to eliminate the *nagCp*

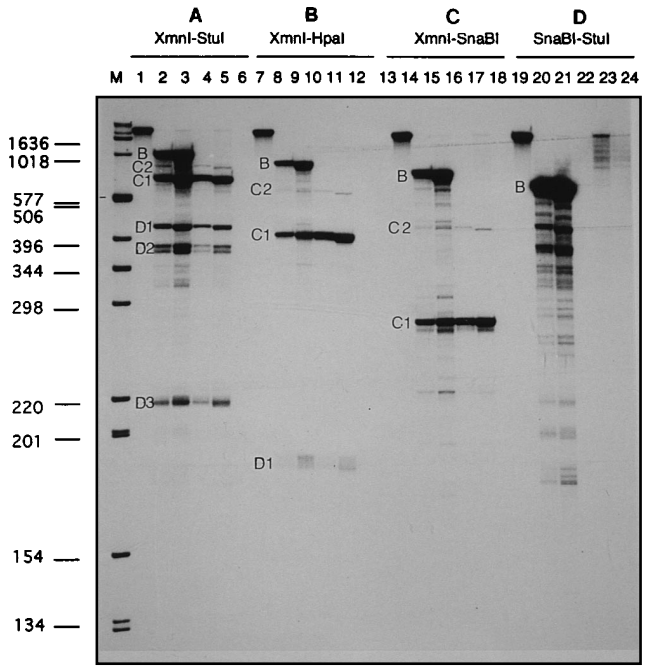


FIG. 3. S1 mapping of promoters on the sandwich *nagBp-′nagAC′-lacZ* fusions. Probes were made by PCR on plasmids carrying the sandwich fusions with the four internal *nagAC* fragments, as indicated above each panel, downstream of the *nagBp* promoter fragment. The primers used hybridized to DNA outside the *nag* DNA present in the four fusions; they were Lac22 (5′ end labelled), which hybridizes to the beginning of *lacZ*, and RBP22, which hybridizes to the *bla* gene of plasmid pRS415. Total RNA was extracted from exponentially growing cultures of lysogens carrying the *nagBp* sandwich fusions grown on GlcNAc or from the same fusions without the *nagBp* fragment grown on glucose. Each of the two types of RNAs were hybridized with their corresponding labelled DNA probes at 54°C overnight. The S1-resistant fragments were analyzed on an 8% denaturing acrylamide gel. The protected fragments B, C1, C2, D1, D2, and D3 are identified in the text. Lanes 2, 3, 8, 9, 14, 15, 20, and 21, mRNA from lysogens of the *nagBp* sandwich fusions grown on GlcNAc; lanes 4, 5, 10, 11, 16, 17, 22, and 23, mRNA from lysogens without *nagBp* grown on glucose; lanes 6, 12, 18, and 24, control hybridizations with 30 μg of tRNA. In lanes 2, 4, 8, 10, 14, 16, 20, and 22, 15 μg of total RNA was used per hybridization; in lanes 3, 5, 9, 11, 15, 17, 21, and 23, 30 μg of RNA was used. Lanes 1, 7, 13, and 19 show the probes. Lane M is a molecular weight marker; positions are indicated at the left in base pairs.

activity was constructed on the *Xmn*I-*Stu*I fragment by changing the −10 consensus from TATACT to AGGCCT, thus creating a *Stu*I site. This mutation was studied on fusions with and without *nagBp* in the presence and absence of GlcNAc (Fig. 4). In the absence of *nagBp*, the basal promoter activity was 57 U, showing that the majority of the promoter activity within the *Xmn*I-*Stu*I fragment had been eliminated. When the mutated fragment was cloned downstream of *nagBp*, the β-galactosidase activity increased a small amount, to about 90 U, during growth on glucose. Moreover when the upstream *nagBp* was induced by growth on GlcNAc, there was an 11-fold increase compared with growth on glucose.

To confirm that a promoter existed on the *Xmn*I-*Stu*I fragment and that it had been silenced by the *nag-29* mutation, IVT experiments were carried out on wild-type and mutated templates with or without *nagBp*. On the wild-type sandwich template, *nagBp Xmn*I-*Stu*I, three strong bands are detected (Fig. 5, lane 1), while on the same fragment carrying the *nag-29* mutation, only two of the three are detected (lane 3). The longest transcript, B, corresponds in size to that expected for a transcript from *nagBp*. The fainter band, E1, just heavier than the B transcript, presumably corresponds to a full-length (end-

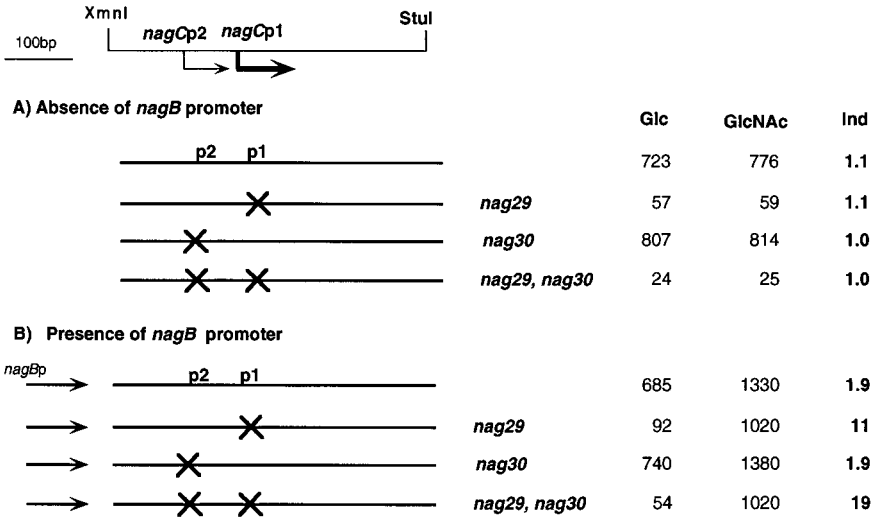


FIG. 4. Effects of mutations in $nagCp_1$ and $nagCp_2$ on *nagC-lacZ* expression. The effects of mutations *nag-29* and *nag-30* on the *Xmn*I-*Stu*I fragment in *lacZ* fusions carried by single-copy λ lysogens were determined by measuring β-galactosidase levels in the presence and absence of *nagBp* during growth on GlcNAc or glucose. The values are the means of two or three independent cultures. Ind (induction) is the ratio of the β-galactosidase-level during growth on GlcNAc to that during growth on glucose.

to-end) transcript on the template. The other two transcripts, C1 and C2, are expected to come from promoters present on the fragment, of which one, that giving rise to C1, is eliminated by the *nag-29* mutation. Transcription of the *Xmn*I-*Stu*I fragment, without *nagBp*, also detected the C1 and C2 transcripts in the absence of the *nag-29* mutation (lane 2) and only the C2 transcript in its presence (lane 4). The fainter band, E2, is the size of the template DNA and is presumably due to end-to-end transcription. The transcript, labelled C1, of about 450 nucleotides is of the size expected for the C1 band detected by S1 on the fusions. Its absence from transcripts made on templates carrying the *nag-29* mutation, with or without the upstream *nagBp* (lanes 3 and 4), confirms that it is due to a promoter which is inactivated by the *nag-29* mutation.

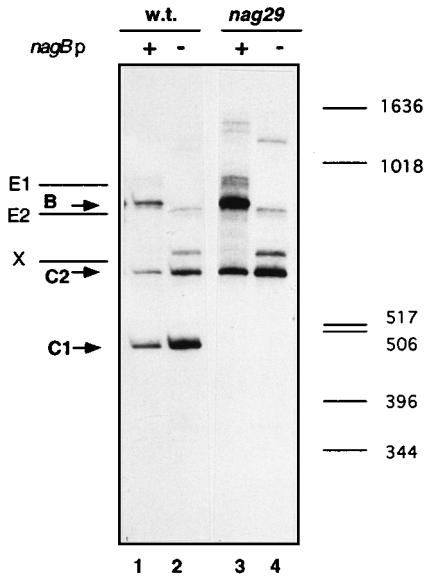


FIG. 5. IVT on wild-type and *nag-29* mutant fragments. The templates, made by PCR on the appropriate sandwich fusions, were the *Eco*RI-to-*Bam*HI *nagBp* joined to the wild-type *Xmn*I-*Stu*I fragment (lane 1), the wild-type (w.t.) *Xmn*I-*Stu*I fragment alone (lane 2), the *Eco*RI-to-*Bam*HI *nagBp* fragment joined to the *Xmn*I-*Stu*I fragment with the *nag-29* mutation (lane 3), and the *nag-29* mutant fragment alone (lane 4). The templates were preincubated with RNA polymerase, and transcription was initiated by the addition of nucleotide triphosphates containing [α-^{32}P]UTP. The transcription products were extracted with phenol, ethanol precipitated, and analyzed on a 6% denaturing acrylamide gel. The transcripts B, C1, and C2 are identified in the text. E1 and E2 are probably end-to-end transcripts on the templates. The origin of the band labelled X in lanes 2 and 4 is unknown. Positions of DNA molecular, weight markers are indicated at the right in base pairs.

Identification of a second promoter for *nagC*. The IVT experiment showed not just the two transcripts corresponding to the expected *nagB* and *nagC* promoters but also a strong intermediate band, labelled C2 (Fig. 5), synthesized on all templates with or without *nagBp*. The length of this transcript, about 600 nucleotides, predicted a 5′ end compatible with a faint band detected by S1 on the sandwich fusions, also labelled C2, on Fig. 3. By IVT, the two transcripts C1 and C2 have similar intensities, while by S1 mapping of in vivo-synthesized mRNA, band C2 is nearly 10-fold less intense. This difference could be due to the experimental conditions used, a linear template and a 15-min preincubation with RNA polymerase before addition of the nucleotides. It seemed possible that the C2 band could account for the 50 U of residual β-galactosidase activity observed with the fragment carrying the *nag-29* mutation. Its 5′ end was precisely located on the DNA sequence by S1 mapping of unlabelled in vitro-synthesized transcripts (data not shown) and predicted a possible promoter sequence as TCGACG-19 bp-TATTAT (underlined bases indicate those agreeing with the normally accepted consensus for −35 and −10 regions of promoters). A mutation, *nag-30*, was constructed to change the −10 sequence to GCTGAT, and its effects on fusions in the presence and absence of the *nag-29* mutation were studied (Fig. 4).

By itself, the *nag-30* mutation had no appreciable effect on the β-galactosidase activities from the *Xmn*I-*Stu*I fragment, since the fragment carrying it produced 740 to 810 U under uninduced conditions, irrespective of the presence of *nagBp*, and 1,380 U when the upstream *nagBp* was induced. However, in combination with *nag-29*, the basal level of expression decreased about twofold, to 24 U in the absence of *nagBp* and to 54 U in its presence. After activation of *nagBp* by growth on

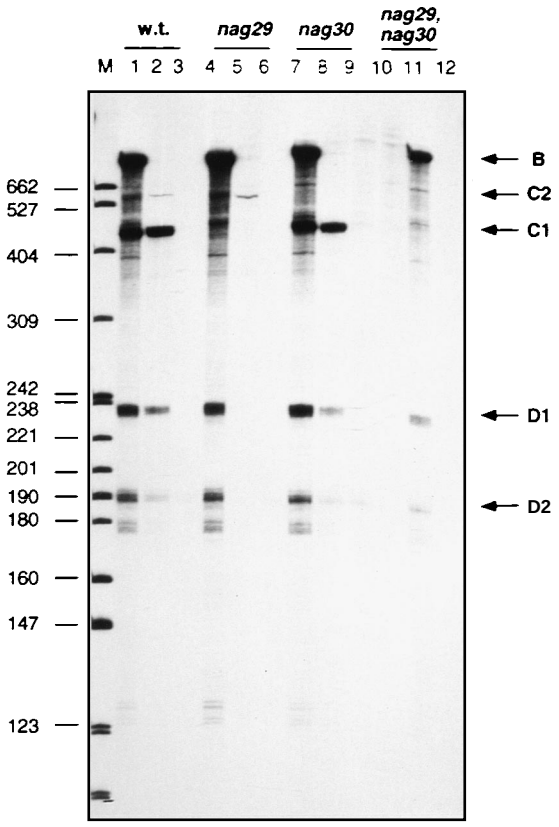


FIG. 6. S1 mapping of promoters on the *Xmn*I-*Stu*I *lacZ* fusions carrying the *nag-29* and/or *nag-30* mutations with or without *nagBp*. Probes were made by PCR on plasmids carrying the sandwich fusions with the *nagBp* fragment upstream of the *Xmn*I-*Stu*I fragment carrying the different mutations as indicated at the top. The oligonucleotides used as primers were 5′-end-labelled Nag28, homologous to the beginning *nagC* DNA just upstream of the *Stu*I site, and RBP22, homologous to the *blp* gene on plasmid pRS415. mRNA was made from lysogens in 1BPC590 Δ*nagEBACD*). The use of this strain eliminates chromosome-derived *nag* transcripts and derepresses the *nagB* promoter (no repressor). Total RNA (30 μg) from lysogens carrying the fusions with the *nagB* promoter fragment (lanes 1, 4, 7, and 11) or without the *nagB* promoter (lanes 2, 5, 8 and 10) was hybridized with the corresponding probes overnight at 54°C. In lanes 3, 6, 9, and 12, 30 μg of tRNA was used. The S1-resistant fragments were analyzed on an 8% denaturing acrylamide gel. The protected bands B, C1, C2, D1, and D2 are identified in the text. w.t., wild-type. Lane M is a molecular weight marker. Positions on the left are indicated in base pairs.

GlcNAc, there was a nearly 20-fold induction of expression, to 1,020 U (Fig. 4).

S1 protection experiments with mRNA prepared from lysogens carrying the single *nag-29* and *nag-30* mutations as well as the *nag-29 nag-30* double mutation and the wild type are shown in Fig. 6. Lanes 1, 4, 7, and 11 show mRNA from the sandwich fusions when *nagBp* has been induced, while lanes 2, 5, 8, and 10 have mRNA from lysogens with just the *Xmn*I-*Stu*I fragment. Band B is the induced *nagB* transcript. The *nag-29* mutation removes the strong transcript C1, while the *nag-30* mutation removes the weaker transcript, C2, as predicted. The protected fragments D1 and D2 correspond to the identically labelled bands in Fig. 3 and are probably the result of mRNA processing (see above).

No evidence for translational coupling between *nagA* and *nagC*. There are just eight nucleotides between the termination codon of *nagA* and the *nagC* initiation codon (CAATAAGAG AAAGTATGACA, the termination and initiation codons are underlined), and there is no good Shine-Dalgarno sequence. This arrangement could favor translational coupling whereby the same ribosome translates both *nagA* and *nagC*. This does not seem to be the case, since the expression of a translational *nagBp nagC′-′lacZ* sandwich fusion, which is missing *nagB* and the beginning of *nagA*, gives β-galactosidase activities almost identical to those of the full operon *nagBAC′-′lacZ* fusions under uninduced and induced conditions (Table 1). This result shows that the induced *nagBp* mRNA is translated equally for *nagC* whether *nagA* is expressed or not.

DISCUSSION

Promoters within the *nagA* gene are responsible for *nagC* expression. Two promoters, $nagCp_1$ and $nagCp_2$, defined by the mutations *nag-29* and *nag-30*, have been localized within the *nagA* gene. The promoters have been mapped by S1 analysis of RNA prepared from lysogens carrying *nagAC-lacZ* fusions (Fig. 3 and 6) and also on the mRNA expressed from the chromosomal *nag* genes (data not shown). Two criteria, expression of a promoterless *lacZ* gene in vivo and production of transcripts by RNA polymerase in vitro, show that the 5′ ends correspond to true promoters rather than mRNA processing sites. Moreover, the introduction of mutations into their −10 consensus sequences eliminates the transcripts in vitro and in vivo (Fig. 5 and 6).

However, the relative strengths of the two promoters measured by two techniques, *lacZ* fusions and IVT, are very different: they are of about equal intensity by IVT but 10-fold different in vivo by S1 analysis of the fusions, with the upstream one being the weaker. This is not simply due to promoter occlusion (1), since p_2 does not increase (significantly) when p_1 is mutated (Fig. 6). It is also worth noting that the strong transcription from the induced *nagBp* does not seem to diminish appreciably the transcription coming from the *nagCp*s on the fusions. This finding is in contrast to some other documented cases in which transcription from strong upstream promoters inhibits a downstream promoter (1, 9). Judging from the levels of β-galactosidase activity, $nagCp_1$ is a fairly strong promoter, giving about 700 Miller units of activity. This value can be compared with those for the basal *nagBp* (70 U), the induced *nagBp* (2,500 U), and the induced wild-type *lacZp* (1,000 U) (22) $nagCp_2$ seems to be responsible for only about 35 to 40 U in vivo. The fact that the IVT experiment suggests that the two promoters, $nagCp_1$ and $nagCp_2$ exhibit comparable activity could imply that $nagCp_2$ is subject to some additional transcriptional regulation in vivo. However, it cannot be excluded that the relative transcriptional intensities observed in vitro are artifacts of the experimental conditions used. The *Xmn*I-*Stu*I fragment, carrying both *nag-29* and *nag-30* mutations, still exhibits 22 to 25 U of β-galactosidase activity, which could mean that there is yet another, weak promoter within the *Xmn*I-*Stu*I fragment.

The data presented in Fig. 2 and 3 show that the major *nagCp* activity exists in the *Xmn*I-to-*Sna*BI fragment of the *nagA* gene. When present on three different DNA fragments the β-galactosidase activities measured are about 750, 150, and 450 Miller units. Since the fragment with the lowest β-galactosidase value is that with the intermediate length, this finding suggests that the absolute β-galactosidase activities reflect something other than promoter strength, such as the translatability or stability of the hybrid *nagA-lacZ* mRNA. An interdependence of transcription, translation, and mRNA degradation has been described previously (45).

Effect of the internal *nagCp*s on transcription from *nagBp*. The presence of the *nagBp* upstream of the three fragments expressing *nagCp* activity has no effect on their β-galactosidase

expression levels during growth on glucose, but growth on GlcNAc produces a twofold enhancement of each value, giving 1,330, 370, and 960 U, respectively. The S1 experiment seems to confirm the twofold induction factor. Transcript B, derived from *nagBp*, is comparable in intensity to that from $nagCp_1$, C1 (Fig. 3A to C). Although the promoter strengths measured on different fusions vary with the DNA environment of the promoter-bearing fragment, the induction factor is consistent and appears to be more significant.

If the wild-type *Xmn*I-*Stu*I fragment is compared with those carrying the *nag-29* and *nag-30* mutations, which eliminate $nagCp_1$ and $nagCp_2$, a profound effect of the *nag-29* mutation is observed: it reduces the high constitutive promoter expression from this fragment and permits an 11-fold induction when the upstream *nagBp* is induced. The combination with the *nag-30* mutation, which removes a second promoter within the *nagA* gene, reduces the basal level again and permits an induction of 20-fold, which is nearer to, but still slightly less than, that found with the *nagB* promoter alone or in the presence of fragments from downstream of the *Sna*BI site.

Role of the secondary structure within the mRNA at the beginning of *nagC*. Previous S1 and Northern experiments showed that the majority of the transcripts derived from *nagB* have 3′ extremities either after *nagB* or after *nagA* at regions predicted to form a stable secondary structure within the RNA. The data in Fig. 3 show that the site after *nagA* is not correlated with the loss of induction of the *nagC* gene, since loss of induction is associated with DNA fragments from upstream, entirely within the *nagA* gene. There is evidence that the secondary structure, predicted to form in the RNA at the beginning of the *nagC* gene, is a site of stabilization of the upstream mRNAs against processive 3′ to 5′ exoribonucleases (27).

Effect of low *nagC* translation. As NagC is a regulatory protein, it is expected to be necessary only in small amounts inside the cell. The numbers of different transcription factors inside the bacterial cell have been estimated from a few copies to a few hundred copies depending on the regulatory protein and maybe on the number of potential sites it has to bind, e.g., *trpR* (16), *argR* (reviewed in reference 18), *deoR*, *cytR* (40), and *malT* (6). In agreement with this hypothesis, the basal level of NagC in wild-type cells is likely to be very low. When the *nagC* gene is placed downstream of the *lac* promoter on a pUC plasmid, IPTG induction allows the production of a crude bacterial extract containing overproduced NagC protein which has been successfully used for DNA binding experiments in vitro, although the overproduced protein is scarcely detectable on a gel (33). The use of a vector supplying, in addition to a strong promoter, a good Shine and Dalgarno sequence (38) increased the overproduction somewhat so that the overproduced protein was visible in a total bacterial extract and corresponded to a few percent of the total proteins (27). The experiments described here show that the promoters assigned to *nagC* expression are quite powerful, and it is likely that poor translation is limiting *nagC* expression. The same fragments cloned into protein and operon fusions give at least a 50-fold difference in β-galactosidase activities; i.e., the hybrid *nagC′-′lacZ* fusion protein is translated 50 times less efficiently than wild-type β-galactosidase. This is equally true for fragments carrying all of the *nagBAC* operon or the *Xmn*I-to-*Stu*I fragment with just the internal *nagCp*s (Table 1).

Model for the regulation of *nagC* expression. Two elements seem to be important to produce a constant, low level of the NagC protein within the bacterial cell. The primary element is the presence of the constitutive and relatively strong promoters within the *nagA* gene, specific for *nagC*. They effectively titrate out the transcripts coming from the upstream inducible *nagBp*. Second, to ensure that only a low level of NagC protein is synthesized, the *nagC* mRNA is poorly translated. The untranslated *nagC* mRNA produces some polarity and in addition is presumably subject to nuclease attack which is inhibited by the formation of a stable secondary structure near the beginning of *nagC*. This third element, the region of RNA capable of forming a stable secondary structure, is probably more important for the expression of *nagA* than for the expression of *nagC*. A subsidiary level of regulation, which cannot be excluded by the current data, is that the mRNAs derived from *nagBp* and *nagCp* are subject to different translational efficiencies.

The majority of transcription factors in *E. coli* are found in single-gene operons, although often in the vicinity of the genes that they control, e.g., *araC* and *lacI*. Many, but not all, are subject to a transcriptional autoregulatory control to keep their levels constant; examples are *crp*, (2, 11, 12, 25), *trp* (16), *cytR* (10), *fur* (7), *argR* (17), *purR* (19), and *galS* but not *galR* (42). The autoregulation observed is generally over a small range. There is a low level of autoregulation of the *nagC* gene in the sense that its expression increases almost twofold when *nagBp* is derepressed. This would facilitate the return to the repressed state as soon as the inducer is removed from the cell. In the cases of *trpR* (16) and *malT* (4), inefficient translation has also been cited as a mechanism to maintain low levels of regulatory proteins. It has been argued that limitation at the translational level should produce a more constant supply of a regulatory protein than limitation of the mRNA (4).

Besides *nagC*, a small number of regulatory genes have been located as part of complex operons; e.g., the mannitol repressor, *mtlR*, is downstream of the inducible genes for mannitol transport and catabolism, *mtlAD*, and it is apparently expressed at a low level (8), and the genes for positive and negative regulatory proteins have been located downstream of the *gut* operon (44). It will be interesting to see if these genes are expressed similarly to the *nagC* gene.

The experiments described here have demonstrated a novel strategy to maintain a low basal level of a protein in the cell, despite the presence of its gene within a highly inducible operon. Instead of relying on low-level transcription as is the case for several other transcription factors, *nagC* is expressed from quite powerful promoters, which serve to reduce the effective increase in concentration of the transcripts coming from upstream, and relies on a posttranscriptional mechanism, inefficient translation, to keep the level of NagC at the requisite low levels.

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