JOURNAL OF BACTERIOLOGY, Dec. 1992, p. 7902–7909
0021-9193/92/247902-08$02.00/0

Vol. 174, No. 24

Identification and Characterization of Novel Low-Temperature-Inducible Promoters of *Escherichia coli*

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Received 17 April 1992/Accepted 13 October 1992

Escherichia coli **promoters that are more active at low temperature (15 to 20°C) than at 37°C were identified by using the transposon Tn*5-lac* to generate promoter fusions expressing β-galactosidase (β-Gal). Tn*5-lac* insertions that resulted in low-temperature-regulated β-Gal expression were isolated by selecting kanamycin-resistant mutants capable of growth on lactose minimal medium at 15°C but which grew poorly at 37°C on this medium. Seven independent mutants were selected for further studies. In one such strain, designated WQ11, a temperature shift from 37°C to either 20 or 15°C resulted in a 15- to 24-fold induction of β-Gal expression. Extended growth at 20 or 15°C resulted in 36- to 42-fold-higher β-Gal expression over that of cells grown at 37°C. Treatment of WQ11 with streptomycin, reported to induce a response similar to heat shock, failed to induce β-Gal expression. In contrast, treatment with either chloramphenicol or tetracycline, which mimics a cold shock response, resulted in a fourfold induction of β-Gal expression in strain WQ11. Hfr genetic mapping studies complemented by physical mapping indicated that in at least three mutants (WQ3, WQ6, and WQ11), Tn*5-lac* insertions mapped at unique sites where no known cold shock genes have been reported. The Tn*5-lac* insertions of these mutants mapped to 81, 12, and 34 min on the *E. coli* chromosome, respectively. The cold-inducible promoters from two of the mutants (WQ3 and WQ11) were cloned and sequenced, and their temperature regulation was examined. Comparison of the nucleotide sequences of these two promoters with the regulatory elements of other known cold shock genes identified the sequence CCAAT as a putative conserved motif.**

Many eukaryotic and prokaryotic proteins become insoluble when overexpressed in *Escherichia coli*. They typically accumulate in the cytoplasm in the form of inclusion bodies. The protein in these inclusion bodies can be solubilized and released by using strong chaotropic agents such as urea and guanidium hydrochloride. This process renders the task of isolating soluble, active protein difficult (21, 32). Several recent reports have indicated the utility of low-temperature culture conditions in improving intracellular solubility or secretion efficiency of heterologous proteins expressed in *E. coli*. In the case of human interferons α2 and γ, most of the protein was active and soluble when cultures were grown at temperatures lower than 30°C, whereas protein from cultures grown at 37°C was insoluble (33). A similar effect of temperature on protein solubility has been reported for the P22 tail spike protein, diphtheria toxin, ricin A chain, basic fibroblast growth factor, pro-subtilisin, and lipoxygenase L-2 (32, 34). Thus, it seems that a general, simple method for producing heterologous proteins in a soluble form in *E. coli* could be the use of a lower growth temperature for expression. Unfortunately, the expression vectors most commonly used to control recombinant protein production in *E. coli* are not readily amenable to low-temperature expression. For example, the lambda p_L-based systems are incompatible with low-temperature expression since they typically require temperature upshift for gene expression (36, 37). In addition to the potentially detrimental effect of high temperature on protein folding, this temperature upshift also results in induction of the heat shock proteins, several of which are proteases (4, 16, 19, 39). Some other commonly used promoters (e.g., *tac*) seem to show reduced efficiency at low temperature (13). Accordingly, an inducible system adapted for use at reduced temperatures would be of great interest.

Low-temperature-inducible promoters appear to occur naturally in *E. coli*. Indeed, a number of *E. coli* proteins show an increased rate of synthesis (in some cases 100-fold or greater) upon a temperature shift from 37°C to 10 to 20°C (12). Moreover, evidence points out that the cold shock proteins are coregulated, suggesting that a cold shock regulon may exist. In studies by VanBogelen and Neidhardt (41) using antibiotics that target ribosomes, it was found that effects of these antibiotics can mimic a temperature shift. One group of antibiotics, including kanamycin and streptomycin, induced heat shock proteins, whereas a second group, including chloramphenicol, erythromycin, and tetracycline, induced cold shock proteins. These findings suggest that stress proteins are induced in response to a change in the translational capacity of the cell and lend credence to the suggestion that a true cold shock regulon exists (41). The gene encoding one of these cold shock proteins, *cspA*, which maps at 79 min on the *E. coli* chromosome, has been cloned. Although this gene is tightly controlled and expressed at high levels when induced at low temperature, it is not clear at what level *cspA* regulation occurs. Expression of *cspA* is sensitive to small temperature shifts and transitory in nature (7). There are 13 other known proteins whose synthesis is cold shock inducible; seven of them have been identified (12, 41). These include the products of *nusA*, *infA*, and *infB* of the *nus-inf* operon (69 min) (10, 11, 29), *pnp* of the S15 operon (69 min) (27), *recA* (58 min) (9, 31), and *aceE,F* (3 min) (2, 12). More recently, the *hns* gene (27 min) (8, 24) encoding the 15.4-kDa nucleoid protein H-NS was also shown to belong to the putative cold shock regulon of *E. coli*

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(18). Interestingly, a DNA fragment from the proximal region of *hns* conferred a cold shock response to the expression of chloramphenicol acetyltransferase in vivo. On the basis of the experimental evidence, these authors have proposed that CS7.4, the product of *cspA*, is a transcriptional activator of the *hns* gene (18). If, in at least certain cases, this low-temperature regulation occurs at the transcriptional level, it may be possible to isolate tightly controlled promoters that are specifically activated when cultures are shifted to low temperature. Therefore, it is reasonable to believe that naturally occurring cold-inducible promoters in *E. coli* could be exploited to develop more effective and practical systems for low-temperature expression of heterologous proteins.

In an effort to evaluate the possibility of using cold-inducible promoters in plasmid-based vectors for heterologous gene expression, we undertook to identify, isolate, and characterize *E. coli* promoters that are more active at low temperature (15 to 20°C) than at 37°C. To this end, we utilized the P1::Tn*5-lac* delivery system to generate random Tn*5-lac* insertions in the *E. coli* chromosome. Tn*5-lac* insertions that resulted in low-temperature β-galactosidase (β-Gal) expression were isolated, two of the promoters were mapped, cloned, and sequenced, and their cold shock enhancement of β-Gal activity was studied. Our findings are reported in this article.

(A preliminary report of this work has been presented previously [26].)

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* MC4100 [F⁻ Δ(*argF-lac*)U169 *araD139 rpsL150 relA1 flbB5301 rbsR deoC ptsF25*] or its *recA56* derivative strain SE5000 was used throughout this study (kindly provided by T. J. Silhavy, Princeton University) (38). For most culture manipulations, bacteria were grown overnight aerobically at 37°C in LB medium (22). When necessary, medium was supplemented with 50 μg of kanamycin or ampicillin per ml. In cold shock experiments, a 1% inoculum from a starter culture was made into M9 minimal medium containing 0.2% glucose (22) without antibiotic addition. Growth was monitored by measuring the A_{600}. Temperature shifts were performed by transferring a portion of the culture into a water bath shaker, kept in a 4°C cold room and set at the appropriate temperature (15 or 20°C). Culture samples were collected at 1-h intervals. For continuous growth conditions, cultures were kept at the appropriate temperature after the initial inoculation, and the mid-log growth phase was reached in 2 days.

Genetic techniques. Standard procedures were used for the preparation of P1 phage lysates and P1 genetic transduction (38). Chromosomal *lacZ* transcriptional fusions were isolated by infecting SE5000 with P1::Tn*5-lac* (17). Agar plates were placed in a Precision 815 low-temperature incubator, and kanamycin-resistant colonies capable of growth on lactose minimal medium at 15°C after 4 to 5 days were selected. Mutants were isolated from separate transposition events.

β-Gal assay. Quantitative β-Gal assays were performed as described by Miller (22). Bacterial cells were lysed with sodium dodecyl sulfate and chloroform. β-Gal activity (in Miller units) was calculated as follows: $[(A_{420} - 1.75 \times A_{550}) \times 1{,}000]/[t \times v \times A_{600}]$, where t is the time in minutes and v is the volume of the culture used in the assay. *E. coli* SE5000, the parent strain of cold shock promoter transductants, does not show β-Gal activity at either 37 or 15°C.

Hfr and physical mapping. The mutations resulting from Tn*5-lac* insertions were transferred from SE5000 to MC4100 by P1 transduction; the cold-inducible phenotype in these transductants was confirmed, and these strains were then used as recipients in the Hfr mapping experiments. Hfr strains with Tn*10* insertions were obtained from Barbara Bachmann (*E. coli* Genetic Stock Center, Yale University, New Haven, Conn.). Rapid Hfr mapping experiments were done as described by B. L. Wanner (42). Exconjugants resistant to streptomycin (50 μg/ml) and tetracycline (20 μg/ml) were selected on LB medium. Colonies were toothpicked and scored for lactose phenotype loss on either MacConkey lactose indicator medium or LB-X-Gal medium supplemented with tetracycline (25 μg/ml) (X-Gal is 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Rapid physical mapping of cloned promoters was achieved by using the miniset collection of Kohara recombinant lambda phage library (14, 23). The membranes carrying the miniset were purchased from Takara Biochemical, Inc. (Berkeley, Calif.). Hybridization conditions used were those suggested by the manufacturer (40).

Antibiotic-mediated β-Gal induction. The protocol described by VanBogelen and Neidhardt was used in conducting the antibiotic-mediated induction experiments (41). Briefly, *E. coli* WQ11 was grown at 37°C in 0.4% glucose–amino acids–MOPS medium (MOPS is morpholinepropanesulfonic acid). When the culture was in the exponential phase of growth, the antibiotic chloramphenicol, tetracycline, or streptomycin was added at various concentrations. Treatment of bacterial cells with antibiotics at a low concentration for 75 min mimics cold shock from 37 to 20°C or heat shock from 28 to 49°C, while treatment with antibiotics at a high concentration for 45 min mimics cold shock from 37 to 10°C or heat shock from 28 to 49°C.

Nucleic acid manipulations. For various nucleic acid manipulation techniques, standard procedures, described by Sambrook et al. (30) or Ausubel et al. (1), were used. The *Eco*RI fragment of the *lacZ* gene from pCB267 (35) served as a probe for Southern and Northern (RNA) blot analyses. This probe does not hybridize to either DNA or RNA from MC4100.

Cloning and sequencing. pHC79 cosmid (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) digested with *Eco*RI was ligated with chromosomal DNA obtained from *E. coli* WQ mutants digested with the same enzyme and then packaged in vitro by using GigapackII Gold extract (Stratagene, La Jolla, Calif.). *E. coli* SE5000 was then phage infected, and ampicillin-resistant blue transformants were selected on LB-X-Gal plates. Subclones were generated in pBR322 or the promoterless probe plasmid vector pQF50 (6). Double-stranded plasmid DNA sequencing was accomplished by using the Sequenase kit version 2.0 (U.S. Biochemicals, Cleveland, Ohio) as previously described (15). A 22-mer primer extending from the left end of Tn*5* into the transcriptional fusion site was used to carry out the sequencing protocol.

RESULTS

Isolation and identification of cold-inducible Tn*5-lac* insertions. Cold-inducible transcriptional fusions to β-Gal were isolated in *E. coli* SE5000 by utilizing P1::Tn*5-lac*. Tn*5-lac* is a transposable promoter probe which, when inserted in the correct orientation downstream from a promoter, creates a transcriptional unit producing a polycistronic *trpA-lacZYA* mRNA (17). Stop codons in all three reading frames prevent

TABLE 1. Level of β-Gal expression in *E. coli* WQ11 at various temperatures

Growth phase and temp[a]	β-Gal expression (Miller units)[b]	Fold induction
Exponential phase		
37°C	4.8	1
37 to 20°C shift	117	24
37 to 15°C shift	71	15
20°C	175	36
15°C	200	42
Stationary phase		
37°C	12.4	1
37 to 20°C shift	97	8
37 to 15°C shift	157	13
20°C	183	15
15°C	206	17

[a] Where temperature shifts are shown, β-Gal assays were performed 4 h after the shift as described in Materials and Methods.
[b] Values are averages of two independent experiments in duplicate. Similar treatment of the parent strain *E. coli* SE5000 yielded no β-Gal units.

TABLE 2. Cold induction of β-Gal expression in *E. coli* Tn*5-lac* insertion strains

Mutant	Time (h) at 15°C	β-Gal expression (Miller units)[a]	Fold induction[b]
WQ1	0	89 ± 51	1
	3	255 ± 28	3
WQ3	0	43 ± 10	1
	3	309 ± 156	7
WQ5	0	125 ± 85	1
	3	600 ± 185	5
WQ6	0	81 ± 10	1
	3	431 ± 106	5
WQ11	0	7.8 ± 0.5	1
	3	95 ± 9	12

[a] β-Gal values are averages of three independent experiments in duplicate ± standard deviations.
[b] Fold induction with respect to values for cells growing exponentially at 37°C.

translation in the wrong reading frame of *trp-lac* fragments. Thus, observed enhancement of β-Gal expression can almost certainly be attributed to transcriptional rather than translational activity. Randomly generated Tn*5-lac* chromosomal insertions that resulted in low-temperature-regulated β-Gal expression were identified by selecting for kanamycin-resistant mutants that were capable of growth on lactose minimal medium at 15°C but which grew poorly at 37°C on this medium. By using this system, several colonies were isolated at a frequency of 0.01% and confirmed by β-Gal cold induction in liquid culture. A total of seven mutants were selected for further studies.

Low-temperature-inducible β-Gal expression in strain WQ11. One of the first isolates which showed low-temperature-inducible β-Gal expression was designated WQ11. In this strain, a temperature shift from 37 to either 15 or 20°C resulted in induction of β-Gal expression with either exponential- or stationary-phase cells (Table 1). The magnitude of the induction levels upon temperature downshift varied from 8- to 24-fold, depending upon temperature and growth conditions. This induction level was maximum between 2 and 4 h after temperature downshift. In mid-log-phase cultures, continuous growth at either 20 or 15°C resulted in a 36- to 42-fold-higher β-Gal expression, respectively, than that in cells grown at 37°C. If cultures were assayed in the stationary phase, cells continuously grown at low temperature typically had expression levels similar to that of cells subjected to a temperature downshift, i.e., 15- to 17-fold induction. Generally, the best β-Gal induction by transient or continuous exposure to low temperature was observed when cells grown exponentially were used.

Low-temperature-inducible β-Gal expression in other Tn*5-lac* insertion mutants. The other cold-inducible Tn*5-lac* insertion mutants identified were designated WQ1 through WQ6. β-Gal expression studies on these strains are shown in Tables 2 and 3. Mutants WQ2 and WQ4 were not analyzed in these studies since they were found to contain multiple Tn*5-lac* insertions (see below). Upon a temperature downshift from 37 to 15°C, β-Gal induction ranged from 3- to 12-fold (Table 2) in the various mutants. Exponential-phase cells obtained from continuous growth at 15°C displayed β-Gal levels that were roughly two- to threefold greater than those seen in a downshift from 37 to 15°C, whereas stationary-phase cells showed levels roughly equivalent to those observed in a temperature downshift (Table 3), except in the case of WQ6 where the levels in exponential- and stationary-phase cells were similar. In all of the isolated Tn*5-lac* mutants, β-Gal was expressed at 37°C, albeit to a lower level than during cold shock. The leakiness of these promoters could result from a weak or unstable transcriptional repressor or from a nonobligatory cold-inducible transcriptional activator (see Discussion). Incidentally, growing these mutants at 42°C or heat shocking them from 37 to 42°C did not induce β-Gal expression but instead resulted in a slightly decreased β-Gal expression when compared with levels at 37°C (data not shown).

Induction of β-Gal in strain WQ11 by selected ribosomally active antibiotics. VanBogelen and Neidhardt (41) have proposed that ribosomes can act as sensors of heat or cold shock in *E. coli*, on the basis of their observation that antibiotics which target the ribosomes can mimic either heat shock or cold shock by inducing the full complement of the respective stress proteins. Treatment of *E. coli* WQ11 with streptomycin, an antibiotic reported to induce a response similar to heat shock, failed to induce β-Gal expression. On the other hand, treatment of WQ11 with either chloramphenicol or tetracycline, shown to mimic a cold shock response, resulted in a fourfold induction of β-Gal expression in this

TABLE 3. β-Gal expression after extended growth of *E. coli* Tn*5-lac* insertion strains at 15°C

Mutant	Exponential phase		Stationary phase	
	β-Gal expression (Miller units)[a]	Fold induction	β-Gal expression (Miller units)[a]	Fold induction
WQ1	707	8	655	3
WQ3	845	20	503	6
WQ5	1,849	15	1,006	6
WQ6	580	7	579	5

[a] β-Gal values are averages of three independent experiments in duplicate.
[b] Fold induction with respect to values from cells in exponential or stationary phase.

TABLE 4. Effect of antibiotic addition on β-Gal expression in WQ11 at 37°C

Antibiotic (concn)[a]	β-Gal expression (Miller units)[b]	Fold induction
None	9.7	1
Chloramphenicol (10 μg/ml)	35.0	4
Tetracycline (10 μg/ml)	34.4	4
Streptomycin (10 μg/ml)	6.8	1
Chloramphenicol (2.5 μg/ml)	33.2	4
Tetracycline (0.5 μg/ml)	34.6	4
Streptomycin (4 μg/ml)	9.8	1

[a] Treatment with antibiotics at a high concentration for 45 min mimics cold shock from 37 to 10°C or heat shock from 28 to 49°C, while treatment with antibiotics at a low concentration for 75 min mimics cold shock from 37 to 20°C or heat shock from 28 to 49°C.

[b] β-Gal values are averages of two independent experiments in duplicate.

strain (Table 4). This is two- to threefold lower than the induction observed upon temperature downshift (Table 1). Similar results were found with *E. coli* WQ3 (data not shown). These results further substantiate the classification of the putative promoters identified by transposon mutagenesis as cold shock promoters.

Southern and Northern analyses. To verify that the kanamycin-resistant transductants resulted from transposition of Tn*5-lac* into the *E. coli* chromosome and that the selected mutants did not harbor multiple Tn*5-lac* insertions, a Southern blot analysis was carried out on all mutants. Isolated chromosomal DNA digested with *Eco*RI and probed with a labelled *lacZ* fragment resulted in a single hybridizable band, as expected, since there is one *Eco*RI site in Tn*5-lac* (data not shown). In two of the transductants, WQ2 and WQ4, several hybridizable fragments were observed, indicative of multiple Tn*5-lac* insertions, and, thus, these mutants were not further characterized. Among the other five transductants, no two transductants displayed the same-size hybridizable fragment, indicating that the Tn*5-lac* transposition was likely to have occurred in five different regions on the *E. coli* chromosome.

The higher levels of β-Gal activity exhibited by the mutants upon temperature downshift to 15°C could be attributed to increased transcription of the Tn*5-lac* cassette or to enhanced translation of β-Gal protein. In an effort to distinguish between these possibilities, we performed a Northern blot analysis, the results of which are shown in Fig. 1. In *E. coli* mutants WQ3 and WQ11, an elevated level of a 4.0-kb message, the expected size for the *trpA-lacZ* fusion, was observed at 15°C as compared with that at 37°C (Fig. 1, lanes 3 to 6). Since the total expected size of the *trpA-lacZYA* mRNA transcript is about 6.0 kb, it appears that there was significant degradation, presumably from the 3′-end of this polycistronic message. One possible explanation is that the *pnp* gene product, which is a cold shock protein with 3′→5′ RNase activity, contributes to message degradation in these strains. Also, IPTG (isopropyl-β-D-thiogalactopyranoside) induction of wild-type *E. coli* at 15°C resulted in extensive β-Gal message degradation (not shown). No signals on the blot were detected from the total RNA isolated from a mutant with a Tn*5-lac* insertion which does not express β-Gal (not shown) or from a wild-type *E. coli* without IPTG induction (Fig. 1, lanes 1 and 2).

Cold shock promoter mapping experiments. Prior to cloning and sequencing the cold shock promoter regions from the various mutants, a rapid Hfr mapping technique was used to

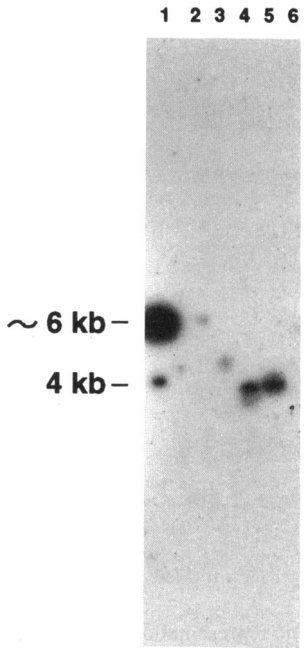

FIG. 1. Temperature downshift induction of *trpA-lacZ* fusion mRNA in selected WQ strains. A Northern blot analysis of total RNA from various *E. coli* strains using the *Eco*RI *lacZ* fragment from pCB267 as a probe is shown. Cells were grown in LB medium at 37°C (lanes 1, 2, 3, and 6) or cold shocked at 15°C for 3 h (lanes 4 and 5). Lanes: 1 and 2, *E. coli* wild type plus (lane 1) and minus (lane 2) IPTG; 3 to 6, cold shock promoter transductants WQ3 (lanes 3 and 4) and WQ11 (lanes 5 and 6).

narrow the map location of these Tn*5-lac* insertions on the *E. coli* chromosome. To date, 8 of the 14 known cold-inducible genes have been identified and mapped (7, 12, 18) (Table 5). On the basis of our Hfr mating results, at least two insertion mutants, WQ11 and WQ6, mapped to a region on the *E. coli* chromosome where no known cold-shock-inducible genes have been shown to reside (28 to 44 min and 12 min, respectively). Mutants WQ1 and WQ3 mapped to 75 to 90 min, while mutant WQ5 mapped to 43 to 61 min.

In an effort to further pinpoint the chromosomal location of the cold shock promoter regions, we carried out rapid

TABLE 5. Chromosomal location of cold shock genes in *E. coli*

Mutant or gene	Genetic mapping (min)	Kohara map (min)	Reference
Mutant			
WQ1	75–90	ND[a]	This work
WQ3	75–90	81	This work
WQ5	43–61	ND	This work
WQ6	12	ND	This work
WQ11	28–44	34	This work
Gene			
cspA	ND	79	7
hns	ND	27	8, 18
nus-inf operon	69	ND	2, 12
S15 operon	69	ND	2, 12
recA	58	ND	2, 12
aceE,F	3	ND	2, 12

[a] ND, not determined.

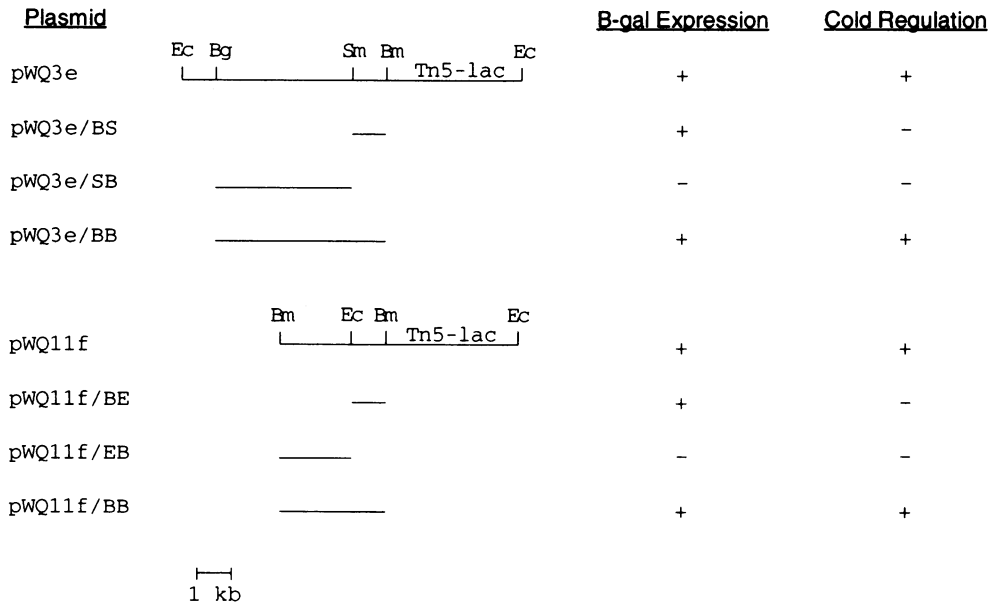

FIG. 2. Schematic representation of the cloning and subcloning of two cold shock promoter regions. Plasmids pWQ3e and pWQ11f are pBR322 derivatives made after initial pHC79 cosmid cloning. Subclones were generated in pQF50 (6). The resulting plasmids were examined for β-Gal expression and cold regulation. Restriction sites: Bm, *Bam*HI; Bg, *Bgl*II; Ec, *Eco*RI; Sm, *Sma*I. The units of β-Gal obtained with the various plasmids at 37 and 15°C were 19 and 340 for pWQ3e, 35 and 39 for pWQe/BS, 0 and 0 for pWQ3e/SB, 36 and 154 for pWQ3e/BB, 10 and 200 for pWQ11f, 28 and 33 for pWQ11f/BE, 0 and 0 for pWQ11f/EB, and 40 and 130 for pWQ11f/BB, respectively. These data are summarized as the presence (+) or absence (−) of β-Gal activity in the figure for simplicity.

physical mapping of two promoters we cloned (see below) by using the miniset collection of Kohara recombinant lambda phage library (14, 23, 40). Hybridization of the gene-mapping membrane with labelled probes encompassing the two cloned promoter sites yielded two dark spots on the autoradiogram because of overlapping of lambda phage clones in the ordered miniset of the Kohara library (data not shown). The position of the spots on the autoradiogram corresponds to the location of the promoters on the *E. coli* chromosome. Phages 302 and 303 hybridized to the promoter site from WQ11 (i.e., clones 2A9 and 5F9 or 34 min), and phages 560 and 561 hybridized to the promoter site from WQ3 (i.e., clones 2A1 and 7A1 or 81 min) (14, 23, 40).

Cloning and nucleotide sequence analysis. In an effort to complete the characterization of the cold shock promoters, they were first cloned, and then two of them, WQ3 and WQ11 (which exhibit the best cold inducibility), were subjected to nucleotide sequence determination. *Eco*RI-digested chromosomal DNA obtained from all mutants was ligated to *Eco*RI-digested cosmid vector pHC79. After in vitro packaging, *E. coli* cells were phage infected, and ampicillin-resistant blue transformants were selected on LB-X-Gal plates; representative clones were then evaluated for cold-inducible β-Gal expression. For *E. coli* WQ3 and WQ11, the clones were named pHC79-WQ3 and pHC79-WQ11, respectively. Subclones from these cosmid inserts were generated in pBR322 and designated pWQ3e and pWQ11f (Fig. 2). To identify the restriction fragment with the cold shock promoter activity, subclones from pWQ3e and pWQ11f were constructed by using the promoter probe high-copy-number vector pQF50 (6). The promoter activities of these two constructs were localized to 1.0-kb *Sma*I-*Bam*HI and *Eco*RI-*Bam*HI fragments, respectively. To our surprise, however, this subcloning resulted in a loss in cold shock regulation of β-Gal expression. Further investigation of this phenomenon revealed the requirement for sequences upstream from the 1-kb promoter region for maintaining cold shock regulation (Fig. 2). A 0.6-kb polymerase chain reaction fragment containing the promoter region from the *cspA* gene exhibited a similar loss of cold inducibility in our hands (data not shown).

The pWQ3e and pWQ11f were sequenced from the *Bam*HI transcriptional fusion site to about 500 bp upstream. The putative promoter region of these two clones had typical −10 and −35 elements (Fig. 3). When these promoter elements were aligned with similar sequences from known cold shock genes such as *cspA*, *nusA*, *hns*, and *pnp* (7, 10, 18, 27), a striking feature was the common motif CCAAT which has been proposed to be a binding site for a cold shock transcriptional activator (18) (see Discussion).

DISCUSSION

The microorganism *E. coli* is able to maintain balanced growth in rich medium over a wide temperature range, from 10 to 49°C, although the growth rate varies with temperature (12). When an *E. coli* culture growing at 37°C is shifted to temperatures below 20°C, this results in a lag period before exponential growth is resumed. At 10°C, the lag is 4 to 5 h long with a generation time of 24 h (12). During this lag time, the expression of at least 14 genes is increased between 2- and 10-fold. In the case of *cspA*, the gene encoding protein F10.6 (CS7.4), expression is either turned on de novo or increased dramatically, with a rate of synthesis more than 100-fold that seen at 37°C. To date, eight cold shock genes have been identified and characterized (see the introduction).

Tn*5-lac* has been used to study low-temperature developmental gene expression in *Myxococcus xanthus* (28). In the current study, we utilized Tn*5-lac* random mutagenesis in *E.*

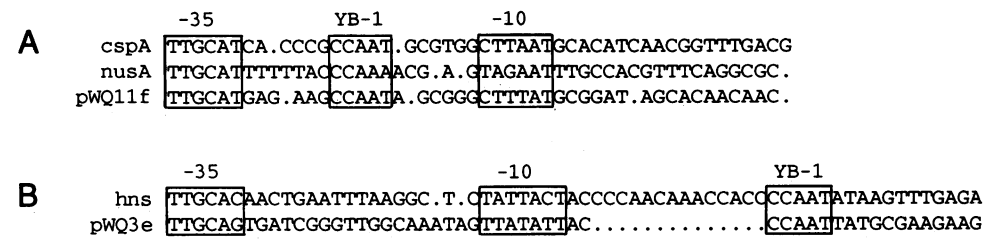

FIG. 3. Comparison of cold shock promoter sequences. Alignment of the −35 and −10 elements of WQ11 (A) and WQ3 (B) with other known cold shock promoters *cspA* (7), *nusA* (11), and *hns* (18). The −10 and −35 regions and the CCAAT common motif are boxed.

coli (17) to generate promoter fusions expressing β-Gal preferentially at low temperature. The purpose of this work was to identify and characterize undescribed cold shock genes and to study the regulation of the cold shock response. Ultimately, our intent is to investigate the potential use of cold-inducible promoters in a novel heterologous gene expression system in *E. coli* in an effort to alleviate the detrimental effect of high temperature on the folding and degradation of certain proteins.

In this work, we report the identification of five apparently novel promoters that behave as members of the putative cold shock regulon. This classification is evident by increased cellular levels of mRNA of the *trpA-lacZ* message upon temperature downshift (Northern analysis shown in Fig. 1) and increased β-Gal activity at 15°C compared with that at 37°C (Table 2). Another corroboration comes from the stimulation of β-Gal activity by antibiotics belonging to the C-group (cold shock) and not the H-group (heat shock) of antibiotics targeting the ribosomes (41) (Table 4). Upon subcloning two of the promoter fragments of WQ3 and WQ11, we found that, in both cases, the isolated proximal region of the transcriptional fusion is responsible for the observed promoter activity. However, somewhat surprisingly, these fragments did not show cold-shock-inducible β-Gal expression. A similar loss of inducibility by temperature downshift was also observed upon subcloning a 0.6-kb fragment containing the promoter region from another cold shock gene, *cspA* (data not shown). In contrast, La Teana et al. reported that a 110-bp fragment containing the *hns* cold shock promoter maintained the cold shock response upon subcloning (18).

We observed that to maintain cold shock regulation, a region positioned at least 1 kb upstream from the WQ3 and WQ11 promoters was required (it is located at or upstream of the *Sma*I restriction site in pWQ3e and at or upstream of the *Eco*RI site in pWQ11f). The reason or nature of this requirement is not obvious. One possible explanation is that DNA supercoiling and/or certain upstream sequences are necessary for the cold shock regulation. It is noteworthy that *E. coli* H-NS, a cold shock protein whose synthesis is increased three- to fivefold at 10°C (18), has been shown to be a bacterial DNA-binding protein with high affinity for curved DNA (44) and has been implicated in the condensation of the bacterial chromosome and organization of the nucleoid (8). It is possible that at low temperature H-NS or another protein alters the compaction of DNA, leading to lower transcriptional activity in the case of the smaller, but not the larger, promoter fragments from WQ3 and WQ11. In an attempt to investigate the effect of DNA structure on cold shock inducibility, we transformed a gyrase-defective mutant (*gyrA96* in JM108) of *E. coli* (gyrase is an enzyme that supercoils DNA) with pWQ3e and pWQ11f and with pWQ3e/BS and pWQ11f/BE. Upon temperature downshift from 37 to 15°C, we observed that the cold inducibility of β-Gal from pWQ3e and pWQ11f was lost. Conversely, pWQ3e/BS and pWQ11f/BE carrying the shorter promoter fragments did not acquire β-Gal cold inducibility (25). The loss of cold inducibility with pWQ3e and pWQ11f in the gyrase-deficient strain implies that DNA supercoiling plays a role in cold shock regulation. While this paper was under review, Jones et al. (11a) reported that the A subunit of DNA gyrase is a cold shock protein. This reinforces the idea that DNA supercoiling plays an important role in the cold shock response. It is also worth mentioning that one of the four promoters (P1) for the *topA* gene (topoisomerase, an enzyme that unwinds DNA) is a heat shock promoter. Although topoisomerase is not a heat shock protein per se, its levels are stable during temperature upshifts (20). Since both gyrase and topoisomerase activities have a direct influence on the superhelicity of the chromosome, there may be a role for them and/or other DNA-binding proteins in expression of genes during cold or heat shock, respectively. It is also possible that specific sequences located upstream from the short WQ3 and WQ11 promoter fragments are required for cold shock regulation, as suggested in preliminary DNA swapping experiments which indicated that the upstream region could not be substituted for by a nonspecific piece of DNA of similar size (25).

In addition to the putative −35 and −10 classical promoter elements present in the sequenced fragments from both WQ3 and WQ11 mutants, these promoter regions were also characterized by the presence of a CCAAT motif which is also found in other promoter sequences of cold shock genes (in *nusA*, the sequence seems to be CCAAA). As shown in Fig. 3, the CCAAT motif can be found either between the −35 and −10 regions (Fig. 3A) or shortly after the −10 region (Fig. 3B). Perhaps of significance, the same motifs are found in *cspA* and *nusA* at 350 and 15 bp upstream from the −35 region, respectively (7, 11). The presence of this sequence in several promoters of cold shock genes might not be coincidental and could play a role during activation of these genes. Recently, La Teana et al. (18) reported that cold shock activation of the *hns* gene is mediated by CS7.4, the product of *cspA*, and that an *hns* 110-bp promoter fragment to which CS7.4 binds comprises a CCAAT motif. This observation promotes the exciting possibility that the CCAAT motif found in other cold shock promoters is also recognized by the CS7.4 protein. Accordingly, CS7.4 could be a universal transcriptional activator-regulator of the cold shock regulon. Several lines of experimental evidence support this view. (i) *cspA* expression precedes and exceeds that of other cold shock genes upon temperature downshift (12). (ii) *cspA* expression is sensitive to small temperature changes and transitory in nature (7). (iii) *cspA* has been suggested to be

subjected to autoregulation (7). (iv) The regulatory region of *cspA* has two CCAAT motifs (7). Finally, (v) the most compelling argument that CS7.4 is a DNA-binding protein with binding specificity for a CCAAT motif was made by Wistow (43). Indeed, the amino acid sequence of CS7.4 was shown to be homologous to a region of the human DNA-binding proteins DbpA, DbpB, and YB-1. Of these eukaryotic transcriptional factors, YB-1 has been shown to bind to the CCAAT-containing Y-box of HLA class II genes and to display an absolute requirement for CCAAT (5).

Various functions have been ascribed to CS7.4. The most plausible of these is preparing cells to reinitiate protein synthesis since exposure to low temperature results in the accumulation of 70S ribosomes and a block in translation initiation (3, 12). Alternatively, CS7.4 could be an antifreeze protein which increases survival at low temperature (7). Clearly, the function of CS7.4 must await mutational analysis of the protein and its putative CCAAT-binding site. Future work should answer questions regarding the role of the CCAAT motif and other regulatory sequences in cold shock genes including *cspA* itself.

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