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Silencing of the *Escherichia coli bgl* promoter: effects of template supercoiling and cell extracts on promoter activity *in vitro*

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ABSTRACT

Regulation of the *Escherichia coli bgl* promoter involves the catabolite gene activator protein (CAP) and silencer elements that are located upstream and downstream of the promoter and its CAP binding site. The promoter is kept in a repressed state by the silencer elements and other normally active CAP-dependent or -independent promoters are repressed as well when flanked by these elements. To assess the mechanism of promoter repression, single round *in vitro* transcription was carried out with plasmids bearing either the wild-type *bgl* promoter or one of two derivatives that escape repression *in vivo* by different mechanisms: *C234* by improving the CAP binding site of the promoter and *Δ1* by a deletion within the upstream silencer sequence. Repression of the *bgl* promoter *in vitro* was shown to depend on template topology and the presence of cellular factors. With negatively supercoiled templates, all three promoters are transcribed to similar extents by purified *E.coli* RNA polymerase and no CAP dependence is apparent; with relaxed templates, transcription is CAP dependent, but the levels of transcription of the three promoters are comparable. Addition of crude cell extract to the simple transcription system leads to repression of all three promoter alleles in the absence of CAP. Repression of the mutant alleles but not of the wild-type promoter is completely relieved in the presence of the CAP–cAMP complex. The topology of the DNA template is also important in the differential regulation of these promoters. In the case of *C234*, repression by cell extract is completely relieved by CAP–cAMP on relaxed or negatively supercoiled templates, while complete derepression of *Δ1* by CAP–cAMP occurs on negatively supercoiled templates only. Repression by cell extract requires the presence of the histone-like protein H-NS. However, H-NS alone does not appear to be sufficient for specific silencing of the wild-type promoter, since repression of all three promoter alleles caused by purified H-NS protein is completely relieved by the CAP–cAMP complex. These data suggest that template topology, H-NS and other cellular factors are involved in the formation of a specific nucleoprotein structure in the *bgl* promoter–silencer region; the formation of this nucleoprotein structure keeps an otherwise active promoter in an inactive state.

INTRODUCTION

There is a paucity of information on the relationship between gene expression in bacteria and the structure and organization of the bacterial genome. Both histone-like proteins and DNA topology appear to influence the expression of a large number of genes. Two abundant histone-like proteins, HU and H-NS, for example, are known to affect numerous regulatory processes (reviewed in 1–4). Similarly, the effects of DNA supercoiling on gene expression in bacteria are well documented (for a recent review see 5). The effect of DNA supercoiling on transcription can be rather dynamic, as transcription itself may affect supercoiling of the template in the vicinity of the transcription complex (6).

The β-glucoside or *bgl* operon is one of the systems in *Escherichia coli* that are affected in their expression by DNA topology and by mutations in the *hns* gene encoding H-NS (7–9). The operon is cryptic in the wild-type (10) due to the very low expression level of its promoter (11,12). This low activity is brought about by upstream and downstream silencer elements of the promoter which possess the general capacity to inactivate promoters flanked by them (13). Initially, the *bgl* operon was discovered by identification of spontaneous mutants of *E.coli* capable of fermenting β-glucosidic sugars (10,14). Various types of mutations were found to activate the operon to different degrees (11,12,15,16) and these mutations share the common feature of abolishing the silencing of the *bgl* promoter by its flanking sequence elements (16). The majority of these mutations occur within a 223 bp region of the promoter itself. They include insertion of mobile elements IS1 or IS5 upstream and downstream of the promoter (15,16), point mutations within the binding site of the catabolite gene activator protein (CAP) that make the site closer to the consensus CAP site sequence (11,16) and deletions within the upstream silencer element (13,16). Interestingly, silencing is also relieved in cells carrying mutations in *hns*, and to a lesser extent in genes encoding the subunits of DNA gyrase (7–9). These findings are consistent with the notion that chromatin structure and the topological state of the promoter–silencer region might be crucial parameters in the inactivation of the promoter (13).

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Expression of the *bgl* operon in mutants that escape silencing is subject to catabolite control mediated by the CAP–cAMP complex (15) and is also regulated by aryl-β-glucosidic sugars (14). Metabolic control of operon expression does not take place at the promoter, however, and is instead mediated by transcriptional anti-termination at two terminators within the transcriptional unit itself (12,17,18). Anti-termination in response to availability of β-glucosidic sugars is modulated by reversible phosphorylation of the anti-terminator protein BglG by the β-glucoside-specific transport protein BglF (12,19–21).

In an attempt to understand the mechanism underlying the silencing of the *bgl* promoter, we first compared the wild-type promoter activity to that of two mutant derivatives, *C234* and *Δ1*, that are active *in vivo*, using a single round transcription system containing purified *E.coli* RNA polymerase. *C234* carries an improved CAP binding site and *Δ1* a deletion within the upstream silencer element. In such a simple transcription system, the wild-type *bgl* promoter was found to show the same high activity as its mutant derivatives. Moreover, on negatively supercoiled templates all three derivatives are independent of CAP. Addition of crude cell extract was found to repress all three promoter alleles. Repression of the mutant alleles, but not that of the wild-type promoter, is, however, fully relieved by the CAP–cAMP complex. In the case of the deletion derivative *Δ1*, complete relief of repression occurs only with negatively supercoiled templates; on the other hand, with the derivative *C234* carrying an improved CAP binding site complete derepression occurs with both relaxed and negatively supercoiled templates. The histone-like protein H-NS appears to be one of the factors necessary for specific repression: cell extract derived from a strain deficient for H-NS lacks the capacity to cause promoter repression and purified H-NS protein alone is able to repress all three promoters in the absence of CAP–cAMP. However, H-NS protein alone is insufficient for specific inactivation of the wild-type promoter and repression by H-NS is relieved for all three promoters by CAP–cAMP. These results indicate that DNA supercoiling and interactions between cellular factors and the *bgl* promoter–silencer region are important parameters in determining the activity of the promoter. Template topology and the binding of cellular factors may mutually influence each other in the formation of a specific nucleoprotein structure, within which a promoter is kept in a silenced state.

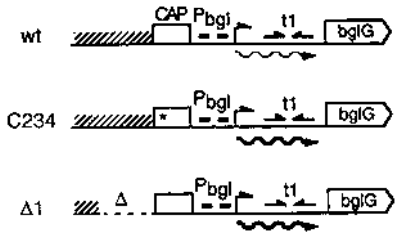

Figure 1. Relevant structures of templates used in *in vitro* transcription assays. The –35 and –10 regions of the *bgl* promoter are indicated by small rectangles. The site of transcription initiation is indicated by a bent arrow. Inverted arrows represent terminator *bgl-t1* in the leader of the *bgl* operon. *bglG* is the first gene of the operon. The CAP binding site is indicated by an open rectangle. The hatched area upstream of the CAP site represents the upstream silencer element. Wavy lines indicate transcripts which initiate at the *bgl* promoter and terminate in the leader of the operon at terminator *bgl-t1*. Plasmid pFDY433 (wt) contains the wild-type *bgl* promoter fragment. Plasmid pFDY433-C234 (C234) carries mutant derivative *C234* with an improved CAP binding site (16) and plasmid pFDY434 (Δ) carries allele *Δ1* containing a deletion within the upstream silencer element (13).

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli K12 strains S112 [= MC4100 Δ(*bglR-bglB*)::*tet*] and S114 [= PD32 Δ(*bglR-bglB*)::*tet*] were obtained by transduction of strains MC4100 [*araD139* Δ(*argF*$^-$*lac*)*U169 rpsL150 relA1 deoC1 ptsF25 rbsR flb5301*; 22] and PD32 (= MC4100 *hns*::*Ap*^{R}; 23), respectively, with phage T4GT7 (24) grown on R1360 [relevant genotype Δ(*bglR-bglB*)::*tet*; 16]. Plasmid pFDY433 was constructed by cloning a 1640 bp *Mlu*I–*Dra*I fragment of plasmid pFDX733 (18), which contains the *bgl* promoter region (from –449 to +1191 relative to the transcription start), into the polylinker of pUC12 (25). Plasmids pFDY433-C234 and pFDY434 were constructed similarly by cloning respective fragments isolated from plasmids pFDX733-C234 (16) and pFDY79 (13) into pUC12. Plasmid pFDY433-C234 carries an allele of the *bgl* promoter which is activated by a point mutation within the CAP binding site and in plasmid pFDY434 the *bgl* promoter is activated by a deletion within the upstream silencer element spanning bp –131 to –77 (allele Δ1, see Fig. 1). Plasmid pFDY400 carries the *hns* gene under control of *tacOP*. For construction of plasmid pFDY400, chromosomal DNA of strain W3110 [*IN*(*rrnD-rrnE*)*1*; CGSC no. 4474; 26] was amplified by polymerase chain reaction using oligonucleotide primers 5′-GGGAATTCATATGAGCGAAGCACTTAAAATTCTG and 5′-CGGGATCCTTTAAATTGTCTTAAACCGGAC. The resulting fragment spanning the *hns* coding region (27) was cut with *Nde*I and *Dra*I and cloned into expression vector pFDY45 (20) between its *Nde*I and *Pst*I sites (the latter terminus made blunt with T4 DNA polymerase). Sequencing of the insert revealed two silent deviations from the published sequence of the *hns* gene (27; EMBL accession no. X07688). At position 969 we detected a T instead of a C, resulting in a GTT codon instead of a GTC codon for valine, and at position 1032 we found an A instead of a G, resulting in AAA instead of AAC for lysine.

Generation of DNA topoisomers

Supercoiled samples containing <1% nicked molecules of pFDY433, pFDY433-C234 and pFDY434 were prepared by three consecutive equilibrium centrifugations in CsCl containing excess ethidium bromide (28). Each plasmid was then treated with vaccinia virus topoisomerase in the absence and presence of varying amounts of ethidium bromide to prepare samples that were negatively supercoiled to different extents. In a typical reaction, 25 µg DNA were incubated with 12 U vaccinia topoisomerase in 100 mM NaCl, 40 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 50 µg/ml bovine serum albumin for 1 h at 37°C in the presence of different quantities of ethidium bromide. The reaction was stopped by extraction with phenol, followed by one phenol/chloroform and two chloroform extractions. DNA was precipitated with ethanol and resuspended in 100 µl TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). Specific linking differences of the samples were measured from the distributions of topoisomers resolved by agarose gel electrophoresis in the presence of chloroquine.

Preparation of cell extracts

Two liter cultures containing 100 mM potassium phosphate, pH 6.2, 10 g/l yeast extract (Difco), 10 g/l glucose, 1 mg/ml thiamine (29) were inoculated with cells diluted from an overnight culture and were grown with vigorous shaking at 37°C. After 2.5 h growth (OD_{600} increased to ~0.8), cultures were placed on ice and immediately chilled by the addition of 0.5 l frozen 100 mM potassium phosphate, pH 6.2. Cells in each 2 l culture were pelleted by centrifugation for 20 min at 4200 r.p.m., washed in 50 ml 30 mM Tris-acetate, pH 7.8, 7 mM magnesium acetate, 150 mM potassium glutamate and 10% glycerol and resuspended in 2 ml of the same buffer containing 1 mM each of phenylmethylsulfonyl fluoride (PMSF) and dithiothreitol (DTT). Cell suspensions were sonicated and the resulting lysates centrifuged for 1 h at 100 000 *g* to remove debris. Subsequently, lysates were treated with staphylococcal nuclease (1 U/µl lysate) in the presence of 1 mM $CaCl_2$ for 1 h at 37°C. The reaction was stopped by the addition of EGTA (2 mM final concentration). Cell lysates were further purified over a Sephadex G25 column (NAP column; Pharmacia) pre-equilibrated with 150 mM potassium glutamate, 30 mM Tris-acetate, pH 7.8, 7 mM magnesium acetate, 1 mM DTT, 1 mM PMSF, 10% glycerol and 2 mM EGTA. Aliquots of cell lysates were rapidly frozen in liquid nitrogen and stored at –80°C. Protein concentrations were determined by the BioRad Protein Assay according to the protocol supplied by the manufacturer.

Overexpression and purification of H-NS

Strain BMH71-18 [Δ(*lac-pro*) *thi supE/F′ lacI*^{q} *lacZ MΔ15*; 30] transformed with plasmid pFDY400 was grown in 2 l of 2YT medium (31) containing 50 µg/ml ampicillin. At an OD_{600} of 0.7 cells were induced with isopropyl-β-D-thiogalactoside (1 mM final concentration), grown for an additional 30 min, harvested on ice, pelleted by centrifugation (20 min, 4200 r.p.m.), washed in 100 mM NH_4Cl, 20 mM Tris–HCl, pH 7.2, 1 mM EDTA, 10% glycerol and frozen. Purification was performed essentially as described (32). Briefly, cells were thawed, PMSF (1 mM final concentration) and 2-mercaptoethanol (7 mM final concentration) were added and the cells were disrupted by two passages through a French press cell (15000 p.s.i.). Lysates were cleared by centrifugation (40 000 r.p.m., 1 h) and loaded onto a Phosphocellulose (P11; Whatman) column equilibrated in 100 mM NH_4Cl, 20 mM Tris–HCl, pH 7.2, 1 mM EDTA, 7 mM 2-mercaptoethanol, 10% glycerol. Proteins were eluted using an NH_4Cl gradient. Fractions containing H-NS protein were pooled and loaded onto a Q Sepharose column equilibrated in 100 mM NaCl, 20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 7 mM 2-mercaptoethanol, 10% glycerol and proteins were eluted by a NaCl gradient in the same buffer. Purified H-NS protein was concentrated and stored at –80°C in 200 mM NaCl, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 5 mM 2-mercaptoethanol, 40% glycerol.

In vitro transcription assay

Transcription assays were performed at 37°C in transcription buffer (TB; 30 mM Tris-acetate, pH 7.8, 7 mM magnesium acetate, 150 mM potassium glutamate, 1 mM DTT, 100 µg/ml BSA, 1 mg/ml tRNA, 10% glycerol). DNA (250 ng or ~0.09 pmol in 4 µl TB) was mixed with 10 µl TB containing, when indicated, cAMP (100 µM), CAP protein (11.8 ng, ~0.5 pmol), H-NS (8 pmol) or cell extract (25 µg total protein). Simultaneously, 3 µl TB containing 0.2 U RNA polymerase (~1–2 pmol; Boehringer-Mannheim) were added. After 20 min incubation to allow binding of proteins to DNA, single round transcriptions were started by the addition of 3 µl of a nucleotide/heparin mixture [final concentrations of 200 µM each of ATP, CTP and GTP, 2 µM [^{32}P]UTP (40 Ci/mmol final activity)]. The same results were obtained when DNA was preincubated with CAP/cAMP or cell extract for 10 min prior to the addition of RNA polymerase (not shown). When single round transcription was started 1 min after addition of RNA polymerase, DNA (in 4 µl TB) was preincubated for 10 min with 10 µl TB containing CAP (11.8 ng, ~0.5 pmol) and cAMP (100 µM) as indicated, before 3 µl TB containing 0.2 U RNA polymerase were added. The reaction was started 1 min later by the addition of 3 µl nucleotide/heparin mixture as above. Samples were incubated for an additional 10–15 min and 12 µl of a stop buffer (90% formamide, 0.05% bromphenol blue, 0.05% xylene cyanol) were added to each reaction mixture. Five microliters of each quenched sample were loaded on a 6% sequencing gel [6% acrylamide:bisacrylamide (19:1), 7 M urea, 72 mM Tris-borate, pH 8.3, 1.6 mM EDTA]. Gels were dried onto Whatman 3MM paper and autoradiographed (Reflection autoradiography film; Dupont) or exposed to a Fuji imaging plate for quantitation of radioactivity with a Fuji BioImage Analyzer.

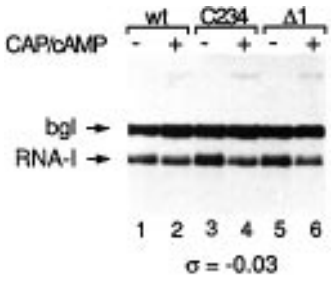

Figure 2. *In vitro* transcription activity of the wild-type *bgl* promoter and alleles *C234* and *Δ1*. DNA templates having a specific linking difference of –0.03 were assayed in single round *in vitro* transcription (Materials and Methods). Lanes 1 and 2, wild-type *bgl* promoter; lanes 3 and 4, allele *C234*; lanes 5 and 6, allele *Δ1*. Without CAP/cAMP, lanes 1, 3 and 5; with CAP/cAMP, lanes 2, 4 and 6.

RESULTS

In a simple *in vitro* transcription system the wild-type *bgl* promoter is active and does not require CAP–cAMP

In vitro activity of the wild-type *bgl* promoter was compared to that of two mutant alleles that escape silencing *in vivo* by different mechanisms: allele *C234* carries a point mutation that improves the CAP binding site (16) and allele *Δ1* carries a deletion within the upstream silencer region (13; see Fig. 1 for relevant structures). Single round transcription assays were carried out and relative activities of the promoters were determined by quantitation of the radioactive transcripts after gel electrophoresis. As shown in Figure 2, in a simple *in vitro* transcription system containing *E.coli* RNA polymerase and supercoiled DNA templates with a specific linking difference σ of –0.03, the wild-type promoter and its mutant derivatives were equally active in the absence of CAP and none of the three promoters was significantly stimulated by CAP–cAMP. Because the *bgl* promoter activity is strictly CAP dependent *in vivo*, the lack of stimulation by CAP *in vitro* was

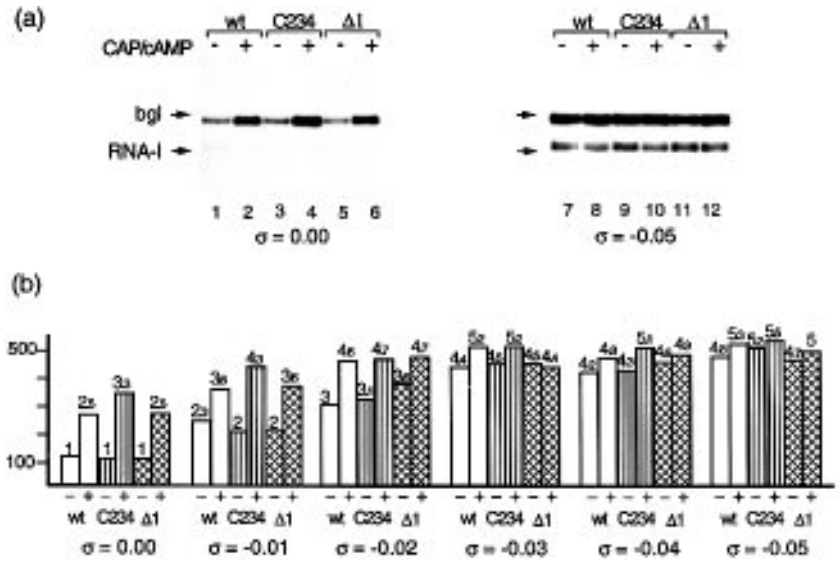

Figure 3. Influence of template supercoiling on regulation by CAP/cAMP of the wild-type *bgl* promoter and alleles *C234* and *Δ1*. Promoter activities were assayed *in vitro* using a series of DNA templates negatively supercoiled to different degrees (σ = 0.00, –0.01, –0.02, –0.03, –0.04 and –0.05). Assays were performed in the absence (–) and presence of CAP/cAMP (+). (**a**) Representative autoradiograms of gels containing electrophoretically separated transcripts. Lanes 1–6, covalently closed relaxed DNA templates (σ = 0.00); lanes 7–12, negatively supercoiled DNA (σ = –0.05). (**b**) Quantitation of radiolabeled transcripts in gels by exposure to Fuji imaging plates and subsequent analysis with a Fuji Bio Image Analyzer. Amounts of radioactivity incorporated into the transcript initiated at the *bgl* promoter are expressed in arbitrary units. Values are averages of at least two independent experiments. The templates used were: wt, pFDY433 carrying the wild-type *bgl* promoter; C234, pFDY433-C234 (improved CAP site); Δ, pFDY434 (deletion within upstream silencer element).

unexpected; transcription of negatively supercoiled template bearing the well-characterized CAP-dependent promoter *lac*, which was also examined in the aforementioned assays as a control, showed the expected CAP–cAMP dependence (data not shown).

In vitro activity of the *bgl* promoter is dependent on template supercoiling

In order to test whether in this *in vitro* system the lack of CAP dependence and the lack of differences in the activities of the three promoters might be caused by the degree of supercoiling of the DNA template employed, transcription assays were repeated with templates of varying degrees of supercoiling, ranging from σ = 0 to σ = –0.05. Representative gel autoradiograms of this series of experiments are depicted in Figure 3a and relative promoter activities obtained by quantitation of the radiolabeled RNA products are shown in Figure 3b. It can be seen from lanes 1, 3 and 5 in Figure 3a and the corresponding data in Figure 3b, that with a relaxed DNA template and in the absence of CAP all three promoters exhibited low activity; promoter activity gradually increases as the template becomes more negatively supercoiled, reaching a plateau around σ = –0.03. Thus in the absence of CAP–cAMP all three promoters are similarly sensitive to negative supercoiling. The presence of CAP–cAMP stimulates transcription of all three *bgl* promoters on templates of a low level of negative supercoiling: activation by the CAP–cAMP complex is 2.5- to 3-fold with relaxed DNA (σ = 0) and remains significant for DNA with σ = –0.02; above σ = –0.03 the stimulatory effect of CAP–cAMP becomes less apparent. Identical results were obtained when the concentration of RNA polymerase was decreased 4-fold, indicating that CAP independence at elevated negative superhelicity is not caused by a corresponding increase in promoter occupancy of RNA polymerase (data not shown). In these experiments DNA templates were preincubated with RNA polymerase and CAP protein for 20 min prior to the initiation of single round transcripions by the addition of nucleoside triphosphates and heparin. The possibility that different promoter alleles might differ in their kinetics of promoter occupancy, or that CAP–cAMP might alter the rates differently, appears to be unlikely. Identical results were obtained when RNA polymerase was preincubated for only 1 min prior to initiation of single round transcriptions (see Fig. 4 for representative results). With relaxed DNA, stimulation by CAP was 3.5- to 5-fold, while with negatively supercoiled DNA (σ = –0.04) no CAP dependence was apparant. The *bgl* promoter-bearing plasmids used in these experiments also carry the RNA-I promoter, the activity of which is known to be strongly dependent on template supercoiling (33). Transcription initiated at the RNA-I promoter showed the expected supercoiling dependence (marked in Fig. 3a).

The above results demonstrate that DNA supercoiling affects *bgl* promoter activity and may influence its regulation by CAP–cAMP. These data also suggest that the topological state of the template might be an important parameter in keeping the wild-type *bgl* promoter in an inactive state. However, in the simple transcription system employed the activity of the wild-type *bgl* promoter was always observed to be similar to that of the two mutant derivatives, irrespective of the degree of supercoiling of the template. This suggests that template topology cannot be the sole determinant of specific silencing of the wild-type *bgl* promoter and that binding of cellular factors to the silencer–promoter region might be involved in this process.

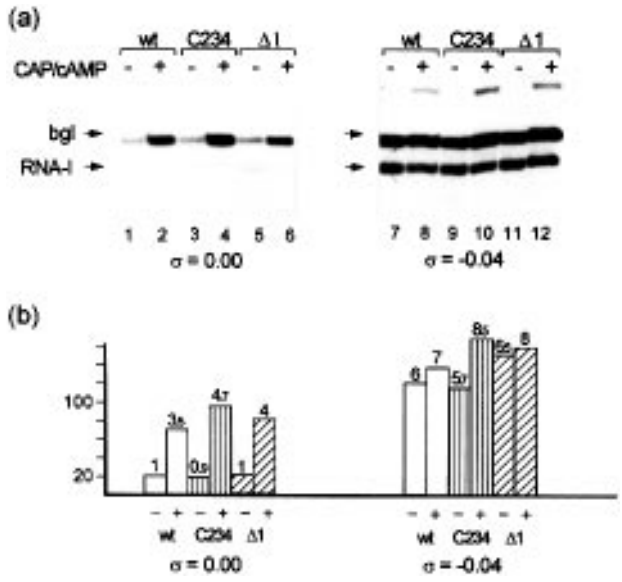

Figure 4. Influence of time of RNA polymerase promoter complex formation on promoter activity. Assays were performed in the absence (–) and presence (+) of CAP/cAMP. Single round transcription was started 1 min after the addition of RNA polymerase. (**a**) Autoradiogram of representative gels. Lanes 1–6, relaxed DNA (σ = 0); lanes 7–12, negatively supercoiled DNA (σ = –0.04). (**b**) Quantitation of the gels as described in the legend to Figure 3. Values are averages out of at least two independent experiments. Other symbols are the same as those displayed in Figure 3.

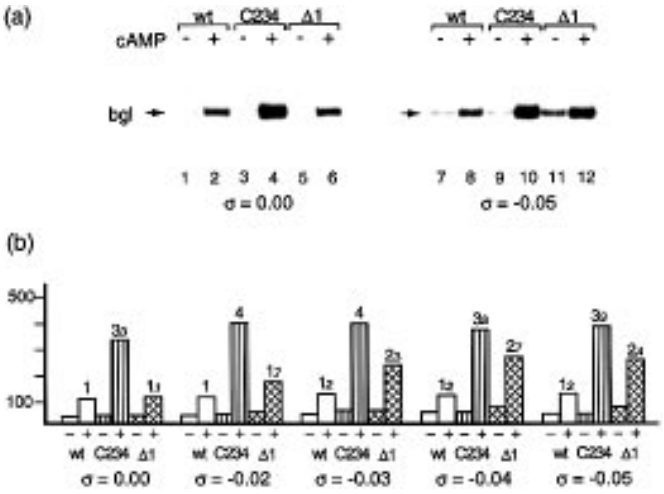

Figure 5. Differential promoter repression and restoration of CAP-dependent activation of mutant alleles of the *bgl* promoter by cell extract. Assays were performed as described in the legend to Figure 3, but in the presence of cell extract (1.25 μg/μl protein) and in the absence (–) or presence (+) of cAMP. (**a**) Autoradiogram of representative gels. Lanes 1–6, relaxed DNA (σ = 0); lanes 7–12, negatively supercoiled DNA (σ = –0.05). (**b**) Quantitation of the gels as described in the legend to Figure 3. Values are averages of at least three independent experiments. Other symbols are the same as those displayed in Figure 3.

Addition of cell extract to the purified transcription system leads to differential repression of the wild-type *bgl* promoter and restores regulation by CAP–cAMP

To test the hypothesis that cellular factors are involved in the specific silencing of the wild-type *bgl* promoter and in regulation by CAP–cAMP, crude cell extract was included in the assays. Figure 5b shows the resulting quantitative data of promoter activity, again accompanied by representative autoradiograms, shown in Figure 5a. The lack of topoisomerase or nuclease activity in the cell extract used, which if present would complicate the interpretation of the results, was confirmed by control experiments in which DNA templates were recovered after the transcription assays in the presence of cell extract; agarose gel electrophoresis of the samples, in the presence of chloroquine to resolve the topoisomers, showed that no significant change in template topology had occurred during incubation (results not shown).

Data in Figure 5b reveal that in the absence of CAP–cAMP the addition of cell extract to the transcription system causes severe repression of the activity of all three promoters. More importantly, increasing the extent of negative supercoiling of the template, which would stimulate promoter activity in the absence of cell extract, can no longer relieve repression in the presence of it. Furthermore, a rather different picture emerged when cAMP was added to the transcription system containing cell extract to activate CAP, which was present in saturating amounts in the cell extract (data not shown). In contrast to the simple *in vitro* system containing no cell extract, activated CAP was found to stimulate the wild-type promoter as well as its mutant derivatives over the entire range of template superhelicity employed. Addition of cell extract thus renders the *bgl* promoter CAP–cAMP dependent not only on relaxed templates, but also on negatively supercoiled DNA. Furthermore, whereas in the simple transcription system the wild-type promoter and the mutant derivatives behave identically, in the presence of cell extract the activities of the three promoters are stimulated to different extents in response to CAP–cAMP. On supercoiled DNA and in the presence of CAP–cAMP, the activity of the wild-type promoter is 4- and 2.5-fold lower than that of the mutant alleles *C234* and *Δ1*, respectively (lanes 7 to 12 in Fig. 5a and corresponding data in Fig. 5b). Activity of the wild-type *bgl* promoter remains low even in the presence of CAP–cAMP. The inclusion of cell extract in the transcription system thus appears to mimic the specific silencing of the wild-type *bgl* promoter and not its mutated alleles *C234* and *Δ1 in vivo*.

Interestingly, in the presence of cell extract the two mutant derivatives reacted differently to DNA supercoiling. The derivative *C234* with an improved CAP binding site exhibits nearly identical low activity irrespective of template topology in the absence of cAMP and promoter activity on all templates is stimulated 8- to 10-fold by the addition of cAMP. Activity of *C234* thus appears to have been rendered independent of template superhelicity by components present in the cell extract. In the absence of cAMP the activity of the mutant derivative *Δ1* is likewise as low as that of the wild-type on relaxed or negatively supercoiled templates. However, whereas on relaxed DNA its activity in the presence of cAMP is indistinguishable from that of the wild-type promoter, with increasing degree of negative supercoiling of the template its activity steadily increases over that of the wild-type promoter, reaching a plateau of ~2.5-fold above that of the wild-type promoter at σ < –0.03. Thus, in contrast to allele *C234*, which is equally stimulated by the CAP–cAMP complex irrespective of the extent of supercoiling of the DNA template, the presence of

cell extract makes activation of the Δ*1* allele dependent on both the CAP–cAMP complex and template supercoiling.

H-NS is necessary but not sufficient for differential repression of the wild-type promoter

In vivo the *bgl* operon is activated by mutations within the *hns* gene (8,9). In order to test whether H-NS protein is a host factor necessary for differential repression of the wild-type *bgl* promoter the *in vitro* transcription assay was repeated with negatively supercoiled DNA templates (σ = –0.04) using cell extract prepared from a H-NS-negative strain. Quantitative data (Fig. 6a) and a representative gel (Fig. 6b) clearly show that this cell extract lacked the capacity for promoter repression: in cell extract deficient for H-NS protein the wild-type and the mutant promoters showed similar high, CAP–cAMP-independent activity, indicating that H-NS is a crucial factor in repression. In order to test whether H-NS is sufficient for specific repression of the wild-type promoter, the *in vitro* transcription was repeated with negatively supercoiled DNA (σ = –0.04) using purified H-NS protein instead of cell extract. A representative autoradiogram and quantitative data are shown in Figure 6c and d, respectively. In the absence of CAP–cAMP addition of H-NS protein caused complete repression of the wild-type promoter and also of allele *C234*, while allele Δ*1* was only partially repressed. Repression of all three promoters was relieved by CAP–cAMP to similar extents, indicating that H-NS is responsible for rendering the *bgl* promoter CAP dependent. Moreover, the data suggest that H-NS, albeit necessary, is not sufficient for specific repression. Thus additional host components are involved in specific repression of the wild-type promoter.

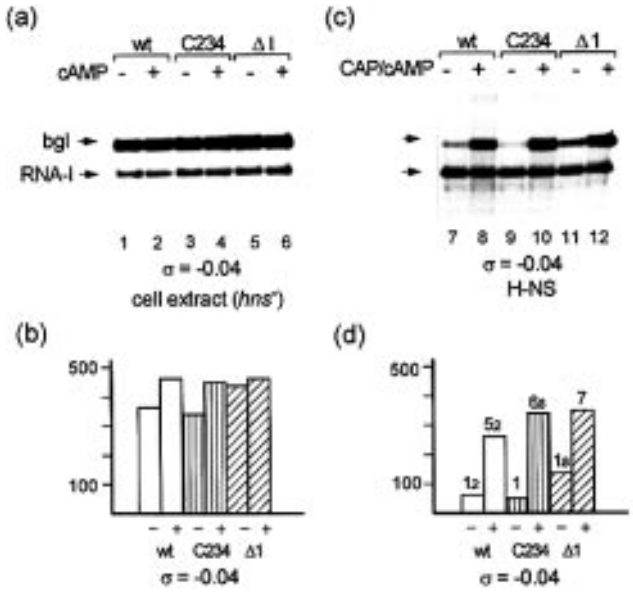

Figure 6. Activity of the wild-type promoter and alleles *C234* and Δ*1* in the presence of H-NS-deficient cell extract and restoration of CAP dependence by H-NS protein. Assays were performed with negatively supercoiled DNA (σ = –0.04) as described in the legend to Figure 3. (**a** and **c**) Autoradiogram of a representative gel. Lanes 1–6, in the presence of cell extract prepared from strain PD32 (*hns*⁻) (1.25 μg/μl protein) and in the absence (–) or presence (+) of cAMP; lanes 7–12, in the presence of H-NS protein (8 pmol) and the absence (–) or presence (+) of CAP/cAMP. (**b** and **d**) Quantitation of the gels as described in the legend to Figure 3. Values are averages out of at least two independent experiments. Other symbols are the same as those displayed in Figure 3.

DISCUSSION

In vivo the wild-type *bgl* promoter is kept in an inactive state by sequence elements flanking the promoter and its CAP binding site (13). Inactivation of the promoter by these silencer elements can be overcome by a variety of mutations that result in an increase in promoter activity *in vivo*, which may be as much as 80-fold (16; unpublished results). Experiments reported in this work show that the wild-type *bgl* promoter and its mutant derivatives are equally active in a simple *in vitro* transcription system containing *E.coli* RNA polymerase as the only protein. In each case promoter activity is lowest with a relaxed DNA template and negative supercoiling of the template increases promoter activity, reaching a plateau around a specific linking difference of about –0.03 (Fig. 3). These data indicate that activity of the wild-type *bgl* promoter or its mutant derivatives *C234* and Δ*1* is equally favored by negative supercoiling of the template. It is also clear from these data that the silencer elements by themselves are incapable of repressing the promoters. Recently, regulation of *bgl* promoter activity by DNA topology *in vitro* was reported (34). In this case a difference was found between the activity of the wild-type promoter and a mutant derivative which is activated *in vivo* by integration of insertion element IS1. However, these data are difficult to compare, since CAP–cAMP was not included in the assays and linking numbers of the individual DNA templates were unspecified (34).

Addition of crude cell extract to the simple transcription system results in repression of promoter activities irrespective of template supercoiling (Fig. 5). Again, the wild-type and the two mutant derivatives exhibited no significant difference in these experiments. It is plausible that this repression results from binding of cellular factors in the extract to the promoter, making the promoter less accessible to the RNA polymerase. The formation of protein–DNA structures in silencing genes has been suggested in a large number of cases. In bacteria, for example, the formation of multimeric H-NS complexes on DNA has been implicated in the inactivation of a number of genes (32,35,36) and the occlusion of RNA polymerase from a specific region of the chromosome by the formation of a nucleoprotein filament was suggested to account for gene silencing mediated by the F plasmid SopB protein (37). Interestingly, *in vivo* the *bgl* operon is known to be activated in cells carrying mutations in the *hns* gene (8,9). The *in vitro* data presented here confirm that H-NS is necessary for repression of the *bgl* promoter but insufficient for specific repression of the wild-type promoter (Fig. 6).

A further determinant in the regulation of *bgl* promoter activity is CAP, the cAMP-dependent catabolite gene activator protein. *In vivo*, regulation of *bgl* promoter activity by CAP is unusually tight (K.Schnetz, unpublished results). *In vitro*, as reported in this work, wild-type *bgl* promoter and its mutant derivatives respond differently to the CAP–cAMP complex in the presence of additional cellular factors. In the case of the wild-type promoter, repression imposed by cellular factors is only partially overcome by CAP–cAMP and low promoter activity was observed with relaxed or negatively supercoiled templates. In contrast, complete relief of repression of the mutant derivatives was observed (Fig. 5; compare with Fig. 3). Derepression by CAP has also been

implicated in the *pap* system, which, similar to the *bgl* operon, is also activated in *hns* mutants (38).

How could CAP relieve repression of the mutant derivatives more effectively than repression of the wild-type *bgl* promoter? In the case of the *C234* mutant, improvement in the binding of CAP may shift the balance between the binding of CAP, H-NS and other cellular factors in favor of the former, which may in turn affect the productive binding of RNA polymerase. In the case of the allele Δ*1*, it is plausible that repression by cellular factors can be overcome by CAP only when the template is in a negatively supercoiled state. The DNA segment deleted within the upstream silencer of Δ*1* probably encompasses sites that are involved in the binding of cellular factors and removal of such sequences may disfavor the formation of a chromatin structure necessary for maintaining the promoter in its repressed state. Binding of CAP to its less than optimal wild-type recognition site in the Δ*1* promoter might be sufficient to overcome repression only if the template is negatively supercoiled.

In the *in vitro* system the difference in the observed promoter activities between wild-type *bgl* promoter and its activated mutant derivatives is maximally 4-fold, while that seen *in vivo* is in the range of 8- to 80-fold (16; K.Schnetz, unpublished results). Nevertheless, the *in vitro* data constitute a significant step towards an understanding of the molecular mechanism underlying the cryptic state of the *bgl* promoter *in vivo*. The data are consistent with a model in which H-NS and other cellular factors bind to the *bgl* promoter–silencer region to form a characteristic nucleoprotein structure. The formation of this putative structure would render the promoter region less accessible to RNA polymerase and CAP and thus inhibit early steps of transcriptional initiation. It is likely that the formation of such a nucleoprotein structure is tightly coupled to local DNA topology, which is influenced by both the actions of the DNA topoisomerases and processes including transcription (6). The development of an *in vitro* system exhibiting preferential repression of the wild-type *bgl* promoter relative to its derivatives that are active *in vivo* provides an opportunity to assess which cellular proteins are likely to be involved in the formation of the putative nucleoprotein complex.

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