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Silencing of *Escherichia coli bgl* promoter by flanking sequence elements

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Silencing of a transcriptional unit by flanking sequence elements has so far only been described for eukaryotic systems. Here, a similar system is described in bacteria. The *Escherichia coli bgl* operon (β-glucoside utilization) is normally cryptic due to very low promoter activity. However, low activity is not attributable to the quality of the promoter itself but is caused by its chromosomal context. The *bgl* promoter is perfectly active when tested outside of its normal context of a stretch of a few hundred base pairs. In addition, other promoters become inactivated when placed into the *bgl* region. Both the deletion of an upstream sequence element and the replacement of sequences located downstream result in promoter de-repression, demonstrating that silencing of promoters within this stretch of DNA *in vivo* is an active process brought about by the combined action of upstream and downstream chromosomal elements.

Key words: catabolite gene activator protein/chromatin structure/DNA topology/hns/transcription regulation

Introduction

In bacteria most of the transcriptional units seem to be generally accessible to the transcriptional machinery, i.e. to the binding of negatively or positively acting transcription factors and RNA polymerases. However, the amount of transcriptionally silent genetic information in the prokaryotic genome might be underestimated. A systematic analysis of the capacity of *Escherichia coli* to grow on certain sugars has, for example, shown that it contains at least four different systems for the utilization of aromatic β-glucosidic sugars which are all silent in the wild type (Prasad and Schaefler, 1974, Parker and Hall, 1990; Hall and Xu, 1992). Of these cryptic systems the *bgl* operon, originally discovered by Schaefler (1967), is the best characterized to date.

The *bgl* operon encodes all functions necessary for the regulated uptake and utilization of the β-glucosidic sugars salicin and arbutin (Prasad and Schaefler, 1974; Schnetz *et al.*, 1987), but it needs activation by spontaneous mutations that cause an enormous increase of activity of the normally very weak promoter (Reynolds *et al.*, 1986; Schnetz and Rak, 1988, 1992). The majority of these mutations are found in the vicinity of the promoter, within a target region of 223 bp (Schnetz and Rak, 1992). They include integration of mobile DNA elements upstream (Reynolds *et al.*, 1981; Schnetz and Rak, 1988) as well as downstream of the promoter (Schnetz and Rak, 1992), point mutations within the binding site of the catabolite gene activator protein (CAP) that make the site more similar to the consensus sequence (Schnetz and Rak, 1992), and deletions encompassing an upstream sequence element (Schnetz and Rak, 1992). These features are illustrated in Figure 1. The only point mutations that were isolated after chemical mutagenesis are located within the CAP binding site and are identical to the spontaneous mutations described above (Reynolds, *et al.*, 1986; Lopilato and Wright, 1990). The *bgl* operon is also active in host strains carrying mutations in the gyrase genes (DiNardo *et al.*, 1982) or in *hns* (Defez and de Felice, 1981; Higgins *et al.*, 1988), which encodes a highly abundant nucleoid associated protein H-NS (also termed H1). H-NS is a major component of the bacterial chromatin (Spassky *et al.*, 1984; Drlica and Rouvière-Yaniv, 1987).

It has been speculated that activity of the *bgl* promoter is inhibited by upstream sequences which might either provide a binding site for an unknown regulatory protein or have a specific DNA structure (Reynolds *et al.*, 1986; Lopilato and Wright, 1990). Analysis of activation by insertion of mobile element IS5 demonstrated that the mere insertion of small fragments of DNA (up to 150 bp) at various positions within this upstream region does not result in the drastic increase of promoter activity seen when the entire IS5 element of 1195 bp is integrated at identical positions. These results support the hypothesis that the region of the *bgl* promoter may be in an unfavorable topological or structural state, for which an upstream AT-rich sequence is partially responsible (Schnetz and Rak, 1992).

Experiments presented in this work were aimed at the characterization of elements involved in keeping the *bgl* promoter inactive in wild type cells. Specifically, I addressed the question of whether low activity of the *bgl* promoter is characteristic of the promoter sequence itself or dictated by its sequence context. For these studies I made use of the well characterized *lac* system. I used the *lac* promoter and its CAP independent mutant derivative *lacUV5* to replace the *bgl* promoter. In addition, activity of the *bgl* promoter within the *lac* promoter sequence context was tested. The data revealed that low promoter activity of the *bgl* locus is not promoter specific but is brought about by the *bgl* sequence context. Specifically, I found that sequence elements located upstream and downstream act together to silence promoters within the *bgl* control region.

Results

An upstream sequence element is necessary for promoter inactivation

In order to analyze the potential role of upstream sequences in inactivation of the *bgl* promoter, I determined β-

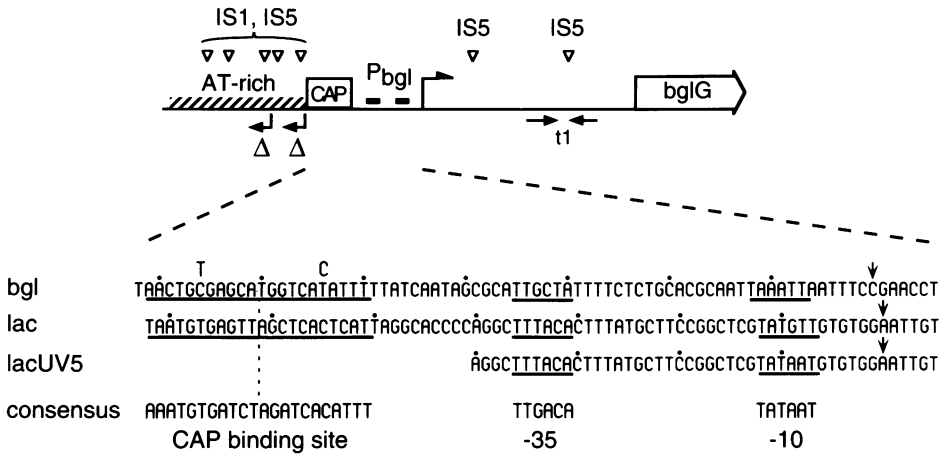


Fig. 1. Schematic representation of the *bgl* promoter region and comparison of its sequence with that of the *lac* promoter. Upper, the position of the *bgl* promoter (P_{bgl}) is marked by two filled boxes representing the –35 and –10 sequence motifs, respectively. Inverted arrows denote a transcriptional terminator (t1) within the leader of the *bgl* operon, at which the product of the first gene of the operon (*bglG*) alleviates termination (Mahadevan and Wright, 1987; Schnetz and Rak, 1988). Also indicated is the binding site for the catabolite gene activator protein (CAP) and an upstream AT-rich region. Spontaneous transposition of insertion sequences IS1 and IS5 into the vicinity of P_{bgl} leads to a drastic increase of activity of P_{bgl} (Reynolds *et al.*, 1986; Schnetz and Rak, 1988, 1992; Lopilato and Wright, 1990). Vertical open arrowheads mark integration sites of such transposition events. The promoter is also activated by deletions originating within the AT-rich region and extending to the left (rectangular arrows marked with Δ) and by point mutations improving the CAP binding site (as indicated in the sequence given in the lower part) (Lopilato and Wright, 1990; Schnetz and Rak, 1992). Lower, sequence comparison of the *bgl* and *lac* promoter regions with the consensus sequences for promoters (Lisser and Margalit, 1993) and CAP binding sites (Berg and von Hippel, 1988). Binding sites for the CAP protein as well as the –35 and –10 promoter sequence motifs are marked. The transcriptional start sites are indicated by vertical arrows.

glucosidase enzyme activity encoded by gene *bglB* of the *bgl* operon and directed by various mutant derivatives (Figure 2A, lines 1 – 6). These data were confirmed by S1 mapping experiments (Figure 2B, lanes 2 and 5), which show that the enzyme activities correlate with transcription initiated at the *bgl* promoter. The data given in line 2 show that the wild type operon directs synthesis of β-glucosidase activity at a level only slighty above background (lane 1). Insertion of mobile DNA-sequence IS5 into the upstream region of the promoter (line 3) and a point mutation within the CAP binding site (line 4) both result in a drastic increase of enzyme activity and thus of activity of the *bgl* promoter, confirming previous results obtained by direct quantitative determination of mRNA initiated at the *bgl* promoter (Schnetz and Rak, 1988, 1992). Deletion of an upstream sequence element which precisely removes the previously determined target region of integration of mobile elements upstream of the promoter (Schnetz and Rak, 1992) results in a similar increase of promoter activity (Figure 2A, line 5, also compare Figure 2B lanes 2 and 5). Larger deletions extending further upstream do not result in an additional increase of promoter activity (data not shown). The entire upstream sequence element responsible for promoter silencing encompasses ~100 bp (Figure 2 and my unpublished data). This silencer can be inverted (Figure 2A, line 6), and its distance to the promoter can be increased by at least 150 bp (Schnetz and Rak, 1992), without impairing its silencing activity.

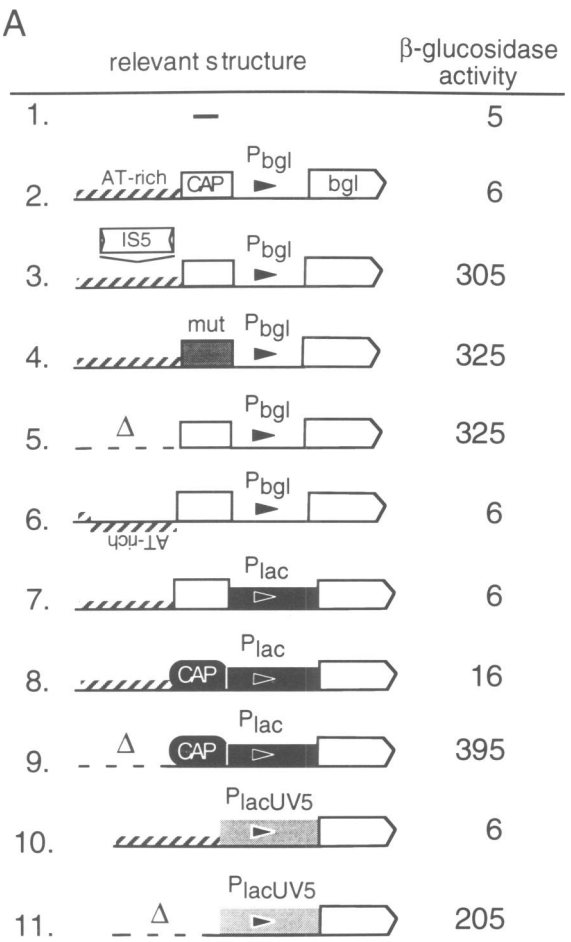
Other promoters become inactivated when placed into the bgl sequence context

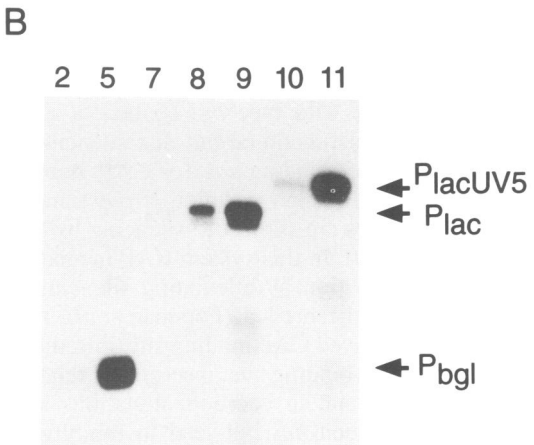
In lines 7–11 of Figure 2A, data obtained with derivatives of the *bgl* operon carrying various substitutions of the *bgl* control region by *lac* sequence elements are summarized. These measurements of β-glucosidase levels were confirmed by S1 mappings shown in Figure 2B (lanes 7–11), demonstrating that β-glucosidase activities indeed correlate with transcription initiated at the *lac* and *lacUV5* promoters. These results reveal that both the *lac* and *lacUV5* promoter become inactivated when placed into the *bgl* sequence context. Activity of the *lac* promoter (Figure 2A, line 7) is, like that of the *bgl* promoter (line 2), only slightly above background (line 1). Activity of the *lac* promoter remains low when it is fused to its own CAP binding site (line 8). However, when the upstream sequence element is deleted in this derivative (line 9), activity of the *lac* promoter increases as drastically as in the case of the *bgl* promoter carrying the same deletion (compare with line 5). Similar results were obtained when the CAP-independent *lacUV5* promoter was tested. Activity of the *lacUV5* promoter is likewise low within the *bgl* sequence context (line 10), and increases enormously when the upstream sequence element is deleted (line 11). Thus, a CAP-independent promoter also becomes inactivated in this context.

Downstream sequences are necessary for promoter silencing

When I replaced the downstream sequences by the *lacZ* gene in several of the constructs shown in Figure 2, I found that in addition to the upstream silencer element, downstream sequences are necessary for promoter silencing, i.e. the upstream silencer alone is not sufficient for inhibition of promoter activity. Figure 3A shows the relevant structures of these substitutions together with the resulting β-galactosidase enzyme activities. Figure 3B provides the corresponding S1 analyses as controls. As can be seen in line 1 of Figure 3A, enzyme activity is high when the *bgl* promoter, together with its intact upstream silencer sequences, is fused to the *lacZ* gene. This activity increases only slightly when the upstream silencing element is removed (line 2). Activity of the *lac*

promoter is likewise high in the presence of the upstream silencing element, not only when it brings along its own CAP site (line 4) but also when fused to the CAP site of the *bgl* promoter (line 3). This indicates that the wild type CAP site of the *bgl* promoter is functional and can perfectly substitute the CAP site of the *lac* promoter. Finally, the upstream silencer element alone has only a slight inhibitory effect on the *lac* promoter (line 5). Taken together, these data demonstrate that both upstream and downstream sequences are necessary for promoter silencing.





Discussion

Silencing of transcriptional units by the concerted actions of elements located upstream and downstream was initially described for the silent cassettes of the mating-type locus in yeast (Brand *et al.*, 1985; Laurenson and Rine, 1992). It was later found in telomeric and centromeric regions and has also been studied in X-chromosome inactivation (Gottschling *et al.*, 1990; Aparicio *et al.*, 1991; Lyon, 1991; Allshire *et al.*, 1994). Although the molecular details remain to be elucidated, it is generally believed that silencing in these systems (i.e. the formation of a transcriptionally inactive domain) is based on the spreading of a heterochromatin-like structure originating from inactivation centers [Rivier and Pillus (1994) and references therein].

In bacteria, several examples of silencing of gene expression were reported. Differential expression of chromosomes within the same cell was observed in studies of non-complementing diploids in *Bacillus subtilis* (Hotchkiss and Gabor, 1980). In *Caulobacter crescentus* cell differentiation the newly replicated chromosomes in the pre-divisional cell are unequal and are differentially active for some functions while silent for others (Gober *et al.*, 1991). Transcriptional silencing was also implicated in the regulation of genes in *E.coli* which are controlled by H-NS (Göransson *et al.*, 1990 and see below).

I presented evidence that the combined action of flanking sequence elements causes silencing of promoters in the *bgl* control region. The upstream silencing element encompasses ~100 bp. It can be inverted (Figure 2) and its distance to the promoter can be increased by at least 150 bp without impairing its activity (Schnetz and Rak, 1992).

Fig. 2. Silencing of the *bgl*, *lac* and *lacUV5* promoters within the *bgl* sequence context and definition of an upstream region necessary for silencing. (**A**) Transformants harboring the various plasmids were grown in synthetic medium containing methyl-β,D-glucoside as inducer of the *bgl* operon (Schnetz and Rak, 1988). Note that induction of this operon does not take place at the promoter but is mediated by transcriptional anti-termination at two terminators, *t1* (see Figure 1) and *t2*, which flank *bglG* (Mahadevan and Wright, 1987; Schnetz and Rak, 1988). Promoter activity was measured by determining β-glucosidase activity directed by gene *bglB* of the operon. Relevant plasmid structures are shown on the left. Filled arrowheads indicate promoters, Δ indicate deletions. *lac* segments are dark, *bgl* segments white. Other symbols are as in Figure 1. Enzyme activities are given on the right. Line 1, empty strain; 2, plasmid pFDX733 (wild type *bgl* operon); 3, pFDX733-S7 (upstream insertion of IS5); 4, pFDX733-C234 (mutant with improved CAP binding site); 5, pFDY79 (deletion of the upstream sequences); 6, pFDY289 (inversion of the upstream sequences); 7, pFDY154 (substitution by the *lac* promoter); 8, pFDY151 (substitution by the *lac* promoter and *lac* CAP site); 9, pFDY153 (as 8, with deletion of the upstream sequences); 10, pFDY241 (substitution of *bgl* promoter and CAP site by *lacUV5*) and 11, pFDY239 (as 10, with deletion of the upstream sequences). (**B**) S1 nuclease mapping of 5′ ends of RNA initiated at the *bgl*, *lac* and *lacUV5* promoters, respectively. RNA was isolated from the same cultures used for the β-glucosidase assays in (A). Numbers of lanes in the autoradiogram correspond to plasmid numbers in (A). S1 signals marked with arrows are at positions expected for transcription initiated at the *bgl*, *lac* and *lacUV5* promoters, respectively. Evaluation of the S1 signals by a PhosphorImager produced good quantitative correlations with enzyme activities shown in (A) (data not shown). Note that the primer used for generation of the probe primes from upstream of the termination site at terminator t_1 in the leader of the operon. The obtained signals are thus a direct quantitative measure for transcription initiated at the *bgl*, *lac* and *lacUV5* promoters, respectively.

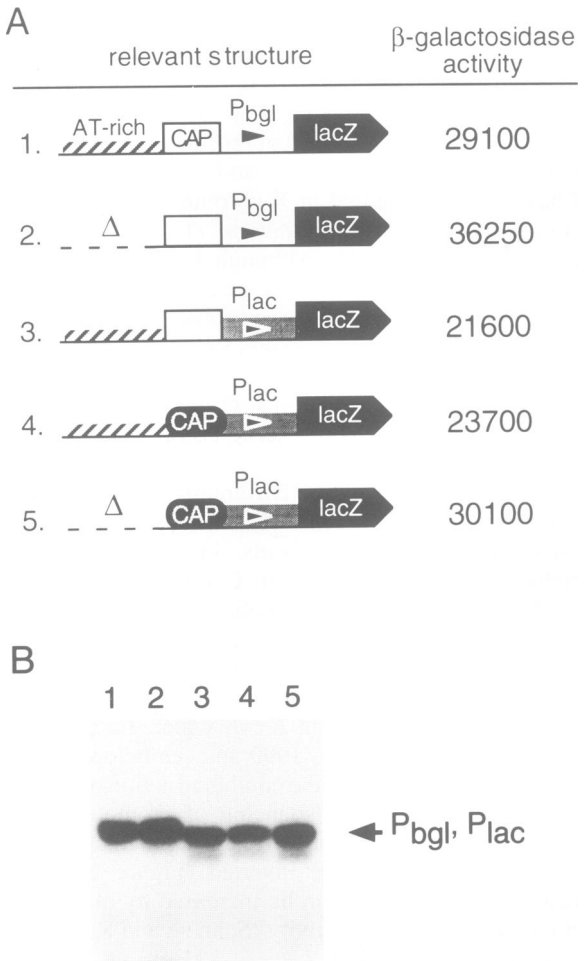


Fig. 3. Upstream and downstream sequences together are necessary for silencing. (**A**) Relevant plasmid structures (left) and associated β-galactosidase activities (right). Symbols are as in Figure 2. Line 1, pFDY378 (wild type *bgl* control region fused to *lacZ*); 2, pFDY445 (as 1, with deletion of the upstream sequences); 3, pFDY137 (as 1, with substitution of the *bgl* promoter by the *lac* promoter); 4, pFDY115 (as 3, but additional substitution of the *bgl* CAP site by the *lac* CAP site) and 5, pFDY117 (as 4, but deletion of the upstream sequences). (**B**) S1 mapping of 5′ ends of RNA. Numbers of lanes correspond to the plasmid numbers in (A). Intensity of S1 signals correlated with enzyme activities in (A) (PhosphorImager analysis; data not shown). Positions of S1 signals are as expected for RNA initiated at the respective promoters.

Activation (or rather inhibition of silencing) of the *bgl* promoter is caused by a wide variety of spontaneous mutations. Activation by the different types of mutation is likely to be based on different mechanisms. This is especially true for mutations that map *in trans*, such as mutations found in the gyrase (DiNardo *et al.*, 1982) and *hns* genes (Defez and de Felice, 1981; Higgins *et al.*, 1988). H-NS is one of the most abundant nucleoid-associated proteins in *E.coli* (Spassky *et al.*, 1984; Drlica and Rouvière-Yaniv, 1987), it binds to DNA non-specifically with a preference for AT-rich and curved sequences (Spassky *et al.*, 1984; Bracco *et al.*, 1989; Yamada *et al.*, 1991; Owen-Hughes *et al.*, 1992). Mutations in *hns* are pleiotropic and alter the expression of a number of genes [see Higgins *et al.* (1990) and references therein]. It has been suggested that H-NS might affect gene expression indirectly by influencing DNA supercoiling (Higgins *et al.*, 1988; Hulton *et al.*, 1990; Owen-Hughes *et al.*, 1992; Tupper *et al.*, 1994), or directly by acting as a transcriptional repressor (Göransson *et al.*, 1990; Forsman *et al.*, 1992; Dersch *et al.*, 1993; Ueguchi and Mizuno, 1993; Zuber *et al.*, 1994). In one case it appears to act by stabilizing a repressor–DNA complex (Falconi *et al.*, 1991). Activation of the *bgl* operon in gyrase or *hns* mutants point to both chromatin structure and DNA topology as being crucial parameters in the process of silencing by flanking sequence elements in this prokaryotic system.

The majority of spontaneous mutations activating the *bgl* promoter act *in cis* and are located upstream as well as downstream of the promoter (Figure 1; Schnetz and Rak, 1992). Based on the results presented here, activation by mobile elements should be attributable to their direct interference with one of the silencing elements or to a structural and topological change brought about in the vicinity of the mobile element. Previously we discussed a defective IS5 as a mobile enhancer of transcription (Schnetz and Rak, 1992). We found that the introduction of internal deletions into IS5 elements that are integrated within the *bgl* control region abolishes activation of the *bgl* promoter (Schnetz and Rak, 1992). In this case, the expression of an essential transposition function encoded by IS5 *in trans* partially restores promoter activation (Schnetz and Rak, 1992). This result suggests that insertion of small DNA segments alone do not necessarily interfere with the function of the silencing element, but that binding of a protein to such a segment can partially abolish silencer activity. However, larger DNA segments might alone be able to inactivate the silencer, since insertion of the entire IS5 sequence alone, and in the absence of any of its transposition functions, is sufficient for promoter activation (my unpublished data).

Activity of the *bgl* promoter is also increased by point mutations within the binding site for CAP. Although the CAP binding site appears to match the consensus sequence quite well, in neither one of its half-sites is the most conserved TGTGA motif present (Figure 1; Berg and von Hippel, 1988). Both of the two known point mutations which cause activation of the *bgl* promoter restore this motif in either one of the half-sites (Figure 1). *In vitro* affinity of CAP to one of these mutant sites is indeed higher (Reynolds *et al.*, 1986). Results presented in Figure 3 show that the wild type *bgl* CAP binding site is perfectly functional within the *lac* context. Within the *bgl* context, activity of the *lac* promoter is low when fused to the *bgl* CAP binding site and only slightly increases when fused to its natural CAP binding site (Figure 2). These data suggest that within the wild type *bgl* context, a regular CAP binding site is not functional but that silencing can be overcome by a better binding site for CAP. A related phenomenon was found in the regulation of *pap* transcription. The *pap* promoters are subject to silencing by H-NS (Göransson *et al.*, 1990). In this system CAP mediates its positive regulatory function by alleviating silencing and thus functions as an anti-repressor (Forsman *et al.*, 1992). In the *bgl* case an improved CAP binding site, introduction of an additional protein binding site, insertion of relatively large DNA segments and, in addition, mutations in the gyrase genes and in gene *hns*, all lead to inhibition of promoter silencing. These superficially different alterations

might share a common mechanistic feature in that they alter a chromatin structure unfavorable for transcription, possibly formed by the binding of H-NS and other proteins, or that these alterations block the spreading of such a structure into the promoter region.

Up to this time, the organization of the bacterial chromosome is not known in much detail (Drlica and Rouvière-Yaniv, 1987; Pettijohn, 1988). The *bgl* control region might represent a microdomain in which a certain chromatin structure and topological state unfavorable for transcription is maintained. Transcriptionally silent domains such as the *bgl* control region might also reveal structures which may play a role in the overall organization of the bacterial chromosome.

Material and methods

Bacterial strains and plasmids

Escherichia coli K12 strain R1360 is *ara Δ(lac-pro) strA thi Δ(bglR-bglB)::tet* (Schnetz and Rak, 1992) and strain BMH71-18 (=R1404) is *Δ(lac-pro) thi supE/F' lacI^q lacZΔM15 proA$^+$ proB$^+$* (Kramer *et al.*, 1984).

Plasmid pFDX733 which carries the wild type *bgl* operon cloned into a pACYC177 backbone (Chang and Cohen, 1978), and plasmids pFDX733-S7 and pFDX733-C234 in which the *bgl* operon is activated by spontaneous mutations have been described (Schnetz *et al.*, 1987; Schnetz and Rak, 1988, 1992). Plasmid pFDY79 is a derivative of plasmid pFDX733. It carries a deletion from position –131 to –77 (coordinates are given relative to the transcription start sites indicated in Figure 1) and a T to G substitution at position –72 which generates an *Aff*II site at this position. Plasmid pFDY289 was derived from plasmid pFDY79. A 57 bp *Mse*I fragment spanning positions –130 to –74 was inserted in inverse orientation into pFDY79 which was linearized at the *Aff*II site at position –77. Ends were made blunt by Klenow polymerase. In plasmid pFDY154 the *bgl* promoter (position –39 to +6) in pFDX733 was replaced by a fragment carrying the *lac* promoter (position –40 to +36, followed 3′ by the linker sequence AATTCCC). Plasmids pFDY151 and pFDY153 were derived from pFDX733 and pFDY79, respectively. In these plasmids, the *bgl* promoter together with its CAP site (position –72 to +6) was substituted by the corresponding *lac* sequences (–72 to +36 and the linker AATTCCC). In plasmids pFDY241 and pFDY239, which are again derived from pFDX733 and pFDY79, the *bgl* CAP binding site and promoter (position –72 to +6) was replaced by a fragment carrying the *lacUV5* promoter (position –40 to +36, followed by a 16 bp linker). Plasmids pFDY378 and pFDY445 were derived from pFDX733 and pFDY79 by fusion of the *lacZ* gene downstream of the *bgl* promoter (the sequence at the fusion is TTAAATTTCCG:ATTGTG-AG, the –10 box corresponding to the *bgl* promoter is underlined; see also Figure 1). In these plasmids the *lac* operator is positioned downstream of the *bgl* promoter which can indeed be repressed by the *lac* repressor (not shown). In plasmids pFDY137, pFDY117 and pFDY115, the fragment encompassing the respective *lac* control regions together with *lacZ* are as in the *lac* operon. Upstream of the promoters these constructs are identical to those in plasmids pFDY154, pFDY151 and pFDY153, respectively.

Enzyme assays

For determination of phospho-β-glucosidase activity, transformants of strain R1360 were grown exponentially in synthetic M9 medium (Miller, 1972) containing 1% glycerol and 0.66% casamino acids and harvested after 2 h of induction with methyl-β,D-glucoside at an OD_{600} of ~0.8. Phospho-β-glucosidase activity was determined as described (Schnetz and Rak, 1988). For β-galactosidase assays transformants of strain R1404 (=BMH71-18) were grown exponentially in the same medium as that used for β-glucosidase assays. β-galactosidase activity was determined after 30 min of induction with 1 mM isopropyl-β, D-thiogalactoside at an OD_{600} of ~0.8 as described (Miller, 1972). Enzyme activities were determined at least five times, and deviations were <10% for the high activities and <20% for the low activities. Copy numbers of plasmids did not vary significantly.

S1 nuclease mapping

RNA isolation and S1 nuclease mapping was performed as described (Schnetz and Rak, 1992). Briefly, probes were generated with T7 DNA polymerase following the plasmid sequencing protocol (USB sequenase 2.0 kit) but omitting dideoxynucleotides in the chase reaction. RNA (100 μg) was mixed with labelled DNA (2×10^6 c.p.m., ~0.5 pMol) lyophilized and resuspended in 40 μl of 3 M NaTCA, 50 mM PIPES pH 7.0, 5 mM EDTA, incubated at 65°C for 5 min and at 45°C for at least 4 h as described by Murray (1986). For S1 nuclease digestion 20 μl of the hybridization mixture were added to 190 μl of S1 buffer (250 mM NaCl, 40 mM Na–Acetat pH 5.5, 1 mM $ZnCl_2$, 20 μg/ml denatured calf thymus DNA, 500 U/ml S1 nuclease) and incubated for 30 min at 37°C. The reaction was stopped by the addition of phenol, which was followed by extraction with chloroform and ethanol precipitation. Samples were resuspended in sequencing gel loading buffer and loaded onto a 5% long ranger (AT-Biochem) sequencing gel next to a sequencing ladder (not shown) as size standard.

Oligonucleotide 5′-AACCCGACTTCACCAGTATTC and plasmids pFDX733, pFDY151 and pFDY241, respectively, were used as templates to generate the probes used for mapping of transcripts initiated at the *bgl*, *lac* and *lacUV5* promoters when placed within the *bgl* region. This oligonucleotide is complementary to the non-coding strand and thus to mRNA from position +84 to +114 for transcripts initiated at the *bgl* promoter, from position +121 to +141 for transcripts initiated at the *lac* promoter and from position +130 to +150 for *lacUV5* promoter initiated transcripts, respectively. Oligonucleotide 5′-GTTTTCCCAGT-CACGAC and plasmids pFDY378 and pFDY115 were used for generation of the probes specific for transcripts initiated at the *bgl* and *lac* promoter within the *lac* context. This oligonucleotide is complementary to RNA from position +80 to + 96 initiated at the *bgl* promoter and from position +79 to +95 for RNA initiated at the *lac* promoter.

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