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Regulation of the *bgl* operon of *Escherichia coli* by transcriptional antitermination

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The *bgl* operon of *Escherichia coli* encodes all functions necessary for the regulated uptake and utilization of aryl β-glucosides. The operon is unusual, however, in that it is cryptic in wild-type strains, requiring activation by mutational events. The vast majority of these mutations are due to transposition of insertion elements into the promoter region of the operon. In this report we show that integration of IS5 into the vicinity of the *bgl* promoter (*P0*) enhances its activity by >60-fold thereby activating the operon. In the activated state the operon is subject to induction by substrate. Recent studies have shown that induction of the *bgl* operon by substrate involves antitermination within the leader of the operon. We now show that substrate-dependent regulation involves specific termination/antitermination of transcription at two signal structures flanking the first gene of the operon, *bglG*. Antitermination is mediated by the product of gene *bglG*. In the absence of substrate this antitermination is prevented by the action of the product of gene *bglF* (the second gene of the operon), which encodes the β-glucoside-specific transport protein (enzymeII^{Bgl} of the phosphoenolpyruvate-dependent phosphotransferase system, PTS) resulting in repression of the operon. The *bgl* promoter (*P0*) is not subject to substrate-dependent regulation. The *bgl* operon has two additional promoters (*P1* and *P2*) located within the terminators, which could also participate in regulation.

Key words: cryptic gene/gene regulation/insertion element/PTS/transcriptional termination

Introduction

Wild-type strains of *Escherichia coli* are unable to ferment β-glucosides. Spontaneous mutants arise, however, which can grow on certain β-glucosides such as salicin or arbutin revealing the presence of a cryptic operon known as *bgl* (Schaefler, 1967). This operon contains at least four genes, the first three of which (*bglG*, *bglF* and *bglB*) encode all functions necessary for regulated uptake and degradation of aryl-β-glucosides once the operon is decryptified (Schnetz *et al.*, 1987; Prasad and Schaefler, 1974; designations for the genes of the *bgl* operon used in this article conform to the revised nomenclature introduced by B.Bachmann in the forthcoming edition of the *E.coli* linkage map: B.Bachmann, personal communication). In a previous publication we reported the nucleotide sequence of the operon and assigned gene functions to open reading frames (Schnetz *et al.*, 1987). The 5′ region of the operon contains a 130 bp leader followed by gene *bglG* which encodes a positively acting regulatory protein. Distal to this gene is an extended intercistronic region 136 bp in length. The second gene of the operon, *bglF*, encodes the β-glucoside permease (enzymeII^{Bgl} of the phosphoenolpyruvate-dependent phosphotransferase system, PTS). A phospho-β-glucoside hydrolase is encoded by the third gene, *bglB*. Distal to *bglB* is a fourth gene of unknown function tentatively designated *bglX*.

About 98% of the Bgl$^+$ mutations are due to transposition of the mobile insertion sequences IS1 or IS5 into the proximal region of the operon (Reynolds *et al.*, 1981; Schnetz *et al.*, 1987; Ronecker and Rak, 1987) resulting in an elevated level of *bgl*-specific mRNA (Reynolds *et al.*, 1986). We present evidence that at least two promoters (*P0* and *P1*) are located upstream of *bglG* and that activation of the operon by insertion of IS5 is due to activation of the proximal promoter (*P0*). In an initial publication (Schnetz *et al.*, 1987) we formulated a detailed model for the regulation of the *bgl* operon in which we postulated transcriptional antitermination at the two terminators bracketing the first gene of the operon, *bglG*. Our model suggested that, in the presence of substrate, the product of gene *bglG* could function as a specific antitermination factor acting at both of these signals. In this report we present experimental results in support of this model. In the absence of a functional transport protein (enzymeII^{Bgl}) antitermination mediated by the *bglG* gene product is constitutive showing that enzymeII^{Bgl}, an integral membrane protein, also acts as a negative regulator of the operon (Mahadevan *et al.*, 1987 and data presented in this communication). Our model integrates this finding.

Results

Activation of promoter P0

Using *in vitro* techniques Reynolds *et al.* (1986) accurately mapped a promoter within the control region of the *bgl* operon. S1 mapping of *in vivo* RNA indicated that this promoter, henceforth called *P0*, is also active *in vivo*. The same transcriptional start site is utilized in both the wild-type operon and in an operon activated by insertion of an IS5 77 bp upstream of the start site (mutant *bglR-S7*::*IS5* in Schnetz *et al.*, 1987; unpublished results). To determine the activity of promoter *P0* in the wild-type operon and in the Bgl$^+$ mutant operon we measured the relative amount of *in vivo* RNA initiated at *P0* under equilibrium conditions. An end-labelled synthetic deoxyoligonucleotide complementary to nucleotides 1 – 26 of the mRNA transcribed from *P0* was used to probe RNA bound to nitrocellulose filters. Prior to the isolation of RNA, transformants were grown in synthetic medium with and without methyl β-D-glucoside (MBG) as *bgl*-specific inducer.

Figure 1 gives a schematic representation of the

experimental strategy (left panel) together with the resulting autoradiograms and the values obtained by densitometric scanning. No hybridization was detected with RNA isolated from the untransformed strain (not shown). Plasmid pFDX733 (containing the wild-type operon) directed the synthesis of significant amounts of *bgl*-specific transcript. Twice as much RNA was present when the bacteria were grown in the presence of inducer. The level of *bgl*-specific transcript in cells harbouring the mutant (Bgl^+) plasmid pFDX733-S7 are considerably higher: ~30-fold higher in the uninduced state, 130-fold higher in the induced state (Figure 1), i.e. induction in this case resulted in a 4-fold increase in the amount of *bgl* transcript. In order to test whether the apparent 2- to 4-fold increase in *bgl*-specific transcript in the presence of inducer is due to a stimulation of promoter *P0* or to a modulation in stability of the RNA within the probed region, we performed an identical experiment with a plasmid isogenic to pFDX733 but with the synthetic *tac* operator−promoter substituted for promoter *P0* (plasmid pFDX763 in Figure 1). As can be seen from the results in Figure 1, presence of *bgl*-specific inducer again led to a 4-fold increase in RNA indicating that the target for the inducer effect is not the promoter. Comparison of the results obtained for pFDX733-S7 and pFDX763 suggests that *bgl* promoter *P0*, when activated by insertion of IS5, is about three times as active as the formidable *tac* promoter.

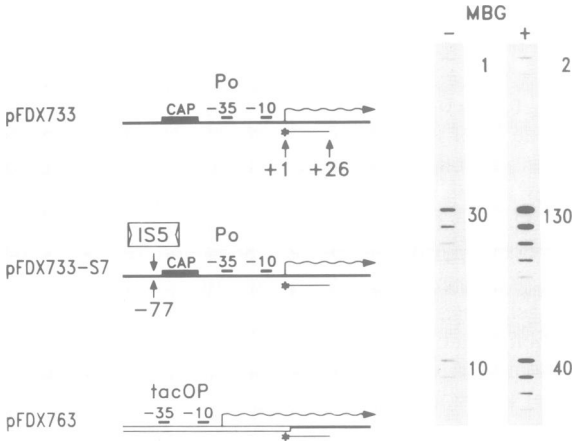

Fig. 1. Quantitative determination of RNA initiated from promoter *P0*. The relevant plasmid structures are diagrammed on the left: CAP denotes the cyclic AMP gene activator protein binding site detected upstream of *bgl* promoter *P0*. The −10 and −35 motifs of *P0* are indicated. The wavy line represents transcription (starting at position +1). An asterisk symbolizes the labelled 3′ terminus of the synthetic oligonucleotide used as a probe in the hybridization experiments. Plasmid pFDX733 carries the wild-type operon; plasmid pFDX733-S7 carries an insertionally activated *bgl* operon; plasmid pFDX763 is a derivative of plasmid pFDX733 in which the *bgl* promoter *P0* region is replaced by the *tac* promoter−operator (*tacOP*). In this plasmid the first 3 bp of the transcribed region of the *bgl* operon have also been substituted. Right panel: Autoradiograms of slot-blots of RNA isolated from transformants of the plasmids pFDX733, pFDX733-S7 and pFDX763 following hybridization with the radioactively labelled oligonucleotide. To the right of the autoradiograms the relative amounts of RNA estimated from the intensity of the autoradiographic signals are given. The intensity of the signal correlated with the wild-type *bgl* operon in the absence of inducer (pFDX733, −MBG) is taken as 1. Signal intensity was measured by densitometric scanning, and the resulting values were corrected for plasmid copy-number (copy-number varied <2-fold).

Inducer-specific antitermination

As a test of the hypothesis (Schnetz *et al.*, 1987) that the potential hairpin-forming sequences found in the leader and in the intercistronic region of genes *bglG* and *bglF*, (see Figure 2) function as conditional transcriptional terminators, we used the S1 technique to map 3′ RNA ends within these two regions. In the case of the hypothetical terminator in the leader, the experimental strategy and the results are given in Figure 3A and B, respectively. RNA was isolated from strain SL5235 transformed with plasmid pFDX733 (carrying the wild-type *bgl* operon) or with plasmid pFDX733-S7 (carrying the insertionally activated operon). Prior to isolation, cells were grown with or without MBG as inducer. The DNA fragment used to detect *bgl* transcripts was 3′ labelled (nucleotide +6 with respect to the transcription start). No hybridization was found with RNA isolated from cells containing plasmid pFDX733 (Figure 3B, lanes 1 and

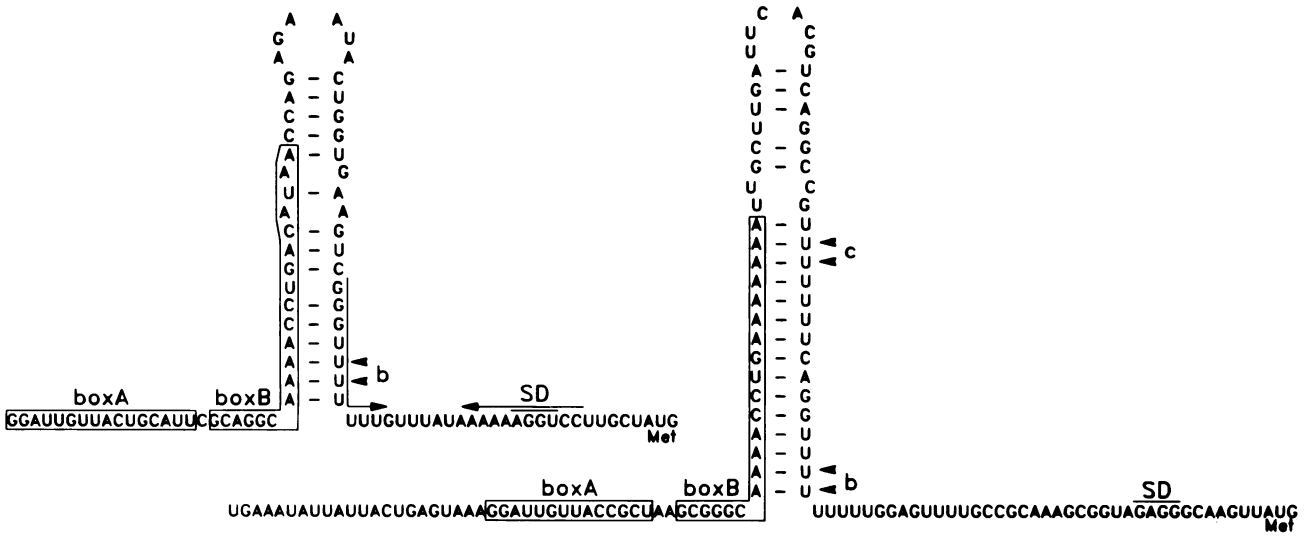

Fig. 2. Sequences of the leader and intercistronic regions showing terminators *t1* and *t2* (drawn as hairpins to suggest possible secondary structures of the RNA). Regions of homology (boxA and boxB) are boxed. Arrowheads labelled 'b' and 'c' designate the 3′ RNA termini detected in S1 experiments (see text). An alternative secondary structure which could interfere with the folding of *t1* and result in the sequestering of the translation start signal of *bglG* (SD) is marked by inverted arrows.

2). RNA isolated under the same conditions from transformants carrying plasmid pFDX733-S7 yielded a signal at the predicted termination site within the oligo(U) stretch 3′ of the stem−loop structure (marked in Figure 2). RNA isolated from parallel cultures grown with (lane 4) and without (lane 3) inducer gave signals at the same position (marked 'b'). Only the RNA isolated from cells grown in the presence of inducer (compare lanes 3 and 4) yielded a strong signal corresponding to the full-length hybrid ('a' in Figure 3B). Thus, a terminator (*t1*) maps in the leader of the *bgl* operon, the action of which is partially overcome when inducer is present in the growth medium.

The same approach was used to investigate the second presumptive terminator, *t2*. RNA was isolated from strain SL5235 harbouring plasmid pFDX771, which carries the *bglG−bglF* intercistronic region downstream of the *tac* operator−promoter (*tacOP*) and the *laci* gene (encoding the *lac* repressor and thus permitting regulation of the *tacOP*).

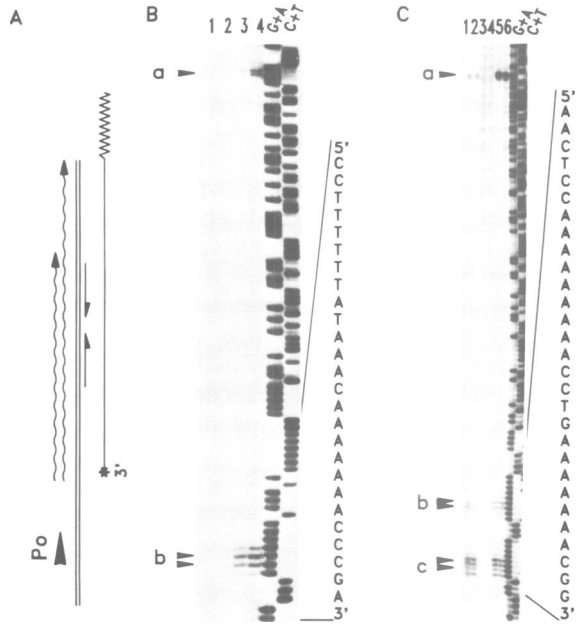

Fig. 3. Mapping of 3′ RNA termini at terminators *t1* and *t2*. Hybridization was done using aliquots of the RNA preparations used for slot-blots. DNA−RNA hybrids were treated with the single strand-specific endonuclease S1. Products of the S1 treatment were separated on a 6% (denaturing) gel next to a sequencing ladder (G + A and C + T reactions; Maxam and Gilbert, 1980) for the DNA strand used as probe. (**A**) Strategy for mapping *t1*. The fragment used as probe carries a heterologous 5′ tail of vector sequences symbolized by a zig-zag line. For explanation of other symbols see Figure 1. The position of the 3′ label is marked with an asterisk. (**B**) Autoradiogram. **Lanes 1** and **2**: RNA isolated from cells harbouring plasmid pFDX733 with the wild-type *bgl* operon. **Lanes 3** and **4**: RNA isolated from cells transformed with plasmid pFDX733-S7 carrying the insertionally activated operon. **Lanes 1** and **3**: −MBG; **lanes 2** and **4**: +MBG; 'a': full length hybrid; 'b': termination signal within the oligo(U) stretch at the end of the stem−loop structure of terminator *t1*. (**C**) Mapping of terminator *t2*. Strategy was in principle as for *t1*. **Lanes 1−4**: RNA isolated from transformants of plasmid pFDX771; **lanes 1** and **2**: +IPTG, **lanes 3** and **4**: −IPTG; **lanes 1** and **3**: −MBG; **lanes 2** and **4**: +MBG. **Lanes 5** and **6**: RNA isolated from double transformants containing plasmids pFDX771 and pFDX510 (expressing *bglG*). Cells were grown in the presence of IPTG. **Lane 5**: −MBG, **lane 6**: +MBG. Signals corresponding to full length homology to the probe DNA ('a'), to transcript terminated at the expected site ('b') and to transcript terminated at an oligo(U) stretch within the stem of the putative terminator ('c') are marked (see also Figure 2).

The results are given in Figure 3C. In the absence of isopropyl β-D-thiogalactoside (IPTG, inducer for *tacOP*) no signals at all were detectable (lane 3: −MBG, lane 4: +MBG). In the presence of IPTG, i.e. with the *tacOP* fully induced (lanes 1 and 2) three signals were visible (marked 'a', 'b' and 'c' in Figure 3C). Signal 'a' corresponds to the full-length hybrid, signal 'b' to a transcript terminated at the predicted site and signal 'c' to the oligo(U) stretch within the stem−loop structure of the presumptive terminator *t2*. The positions of the latter two signals are also marked in Figure 2. Addition of MBG to the medium (lane 2) had no significant effect on the resulting S1 signals. When RNA isolated from double transformants harbouring, in addition to plasmid pFDX777, plasmid pFDX510 (with a copy of gene *bglG* expressed from the *gal* promoter) was investigated, the same three signals were detectable (lanes 5 and 6). In this case, however, the signal corresponding to the full-length hybrid ('a' in Figure 3C), i.e. to non-terminated RNA, was much stronger than the signals corresponding to terminated RNA ('b' and 'c', lanes 5 and 6). The relative intensity of the different signals again was unaltered when cells were grown in the presence (lane 6) or absence (lane 5) of MBG. We conclude that the potential stem−loop structure in the intercistronic region is active as a terminator and that the product of gene *bglG* alleviates termination at this site. Moreover, antitermination under these conditions, i.e. in the absence of *bglF* gene product, is independent of the presence of a *bgl*-specific inducer.

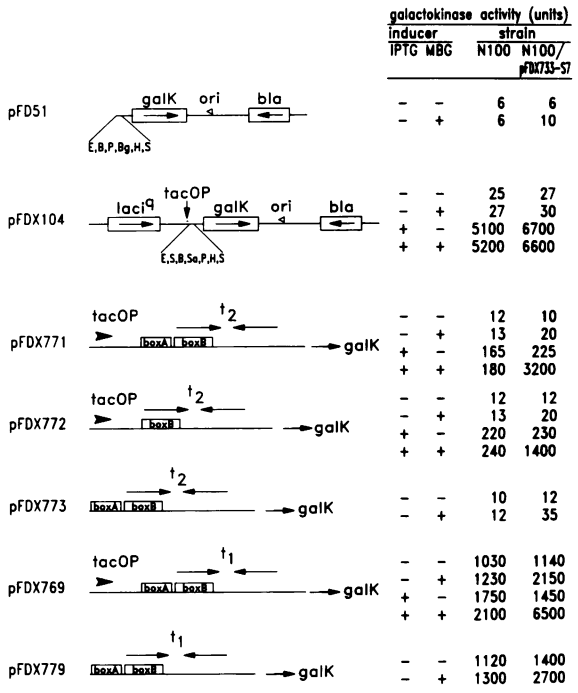

Fig. 4. Determination of the efficiency of termination/antitermination of terminators *t1* and *t2* and detection of additional promoter activity. The relevant plasmid structures are given on the left. The galactokinase activity in units (nmol galactose phosphorylated per min and OD_{546} of bacterial culture) synthesized under the direction of the various plasmids in the presence or absence of an insertionally activated *bgl* operon (pFDX733-S7) *in trans* both with and without the addition of MBG and IPTG is given in the columns at the right. pFD51: the empty promoter test vector; pFDX104: the empty terminator test vector; pFDX771: terminator *t2* in pFDX104; pFDX772: *t2*ΔboxA in pFDX104; pFDX773: *t2* in pFD51; pFDX769: *t1* in pFDX104; pFDX779: *t1* in pFD51.

Estimation of the efficiency of termination/antitermination and detection of two additional promoters, P1 and P2

To determine the efficiencies of termination and antitermination at the two *bgl* terminators *t1* and *t2*, the leader and intercistronic regions were cloned into terminator test vector pFDX104. To check these two regions for the presence of additional promoter activity, corresponding fragments were also cloned into promoter test vector pFD51. The resulting recombinant plasmids were transformed into strain N100 and into the same strain carrying Bgl^+ mutant plasmid pFDX733-S7. The transformants were grown in synthetic medium with or without MBG. In the case of plasmid pFDX104 and its derivatives, parallel cultures were grown with and without IPTG as inducer of the *tacOP*. Aliquots of these cultures were assayed to determine galactokinase activity. Figure 4 gives the relevant plasmid structures accompanied by the experimental results. Plasmid pFDX104 directs the synthesis of 25 units of galactokinase activity in the uninduced state and of ~5000 units upon induction with IPTG. Addition of MBG has no significant effect. The slight effect of the presence of plasmid pFDX733-S7 *in trans* may be due to a change in plasmid copy number. When terminator *t2* is inserted between *tacOP* and the *galK* reporter gene (pFDX771), the uninduced level of enzyme activity is lowered by ~50%. Induction of the *tac* promoter results in synthesis of only 165 units of galactokinase, i.e. the efficiency of terminator *t2* is ~97%. Addition of MBG has a drastic effect, but only in the presence of the insertionally activated *bgl* operon (pFDX733-S7): enzyme activity goes up from 180 to 3200 units, which is only ~50% lower than the activity determined for vector plasmid pFDX104. This increase can only be due to inducer-dependent transcriptional antitermination, since under the same conditions the identical *t2*-containing fragment in the absence of the *tac* promoter (pFDX733) directs the synthesis of only 35 units of galactokinase.

Both terminators, *t1* and *t2*, contain regions of homology proximal to and extending into the stem−loop structure (designated boxA and boxB; Schnetz *et al.*, 1987; see Figure 2). In an attempt to test whether the boxA motif is involved in termination/antitermination, plasmid pFDX772 was constructed. As can be seen from the data in Figure 4, the boxA motif is not essential for terminator function. Nevertheless, boxA clearly makes a significant contribution to the efficiency of antitermination (1400 versus 3200 units). Finally, the minute but significant amount of galactokinase activity correlated with plasmid pFDX773 indicates that a weak promoter, which we call *P2*, is located within terminator *t2*. Results obtained for parallel constructions with the DNA segment containing terminator *t1* are also given in Figure 4: when a fragment spanning the leader but lacking promoter *P0* was inserted into promoter test vector pFD51 the resulting clone directed the synthesis of >1100 units of galactokinase activity (pFDX779), demonstrating that an additional promoter of considerable strength is located within the leader. We designate this promoter *P1*. A 2-fold stimulation of enzyme synthesis was observed when the insertionally activated *bgl* operon (on plasmid pFDX733-S7) was present *in trans* and MBG was added to the medium. Essentially the same results were obtained for the parallel construction pFDX769 in the absence of IPTG (Figure 4).

In the absence of the activated *bgl* operon *in trans* induction of the *tac* promoter results in a 70% increase in enzyme activity (1750 versus 1030 units and 2100 versus 1230 units). Induction by IPTG resulted in only a 27% increase in the presence of the insertionally activated *bgl* operon but without MBG (1450 versus 1140 units). While the complexity of this system with two promoters and a terminator signal does not permit a cut-and-dried interpretation, the results obtained for the case of the activated operon *in trans* with both inducers (IPTG and MBG) present are conclusive: addition of MBG to the medium resulted in galactokinase values comparable to those found for the fully induced vector pFDX104 (6600 versus 6500 units). Obviously, terminator *t1* is efficiently overcome in the presence of a functional *bgl* operon and operon-specific inducer.

Regulatory role of terminators t1 and t2

From the above data it is clear that termination/antitermination at both terminators plays a key role in regulation of the *bgl* operon. In order to determine to what extent each of the two signal structures participates in regulation, we compared inducibility of the active operon with inducibility of the active operon deleted for one or the other of the terminator structures. Strain JF201 (Δ *bgl*) was transformed with recombinant plasmids. Parallel cultures in synthetic medium were grown with and without the *bgl*-specific inducer MBG and, where necessary, with and without IPTG. Subsequently, the amounts of phospho-β-glucosidase synthesized under the direction of the various plasmids was determined. Figure 5 gives abbreviated diagrams of the various plasmids and the results of the enzyme assays. Plasmid pFDX733 (containing the wild-type, i.e. non-activated operon) directed the synthesis of very low levels of enzyme activity. Nevertheless production of phospho-β-glucosidase was inducible. Plasmid pFDX733-H3 contains

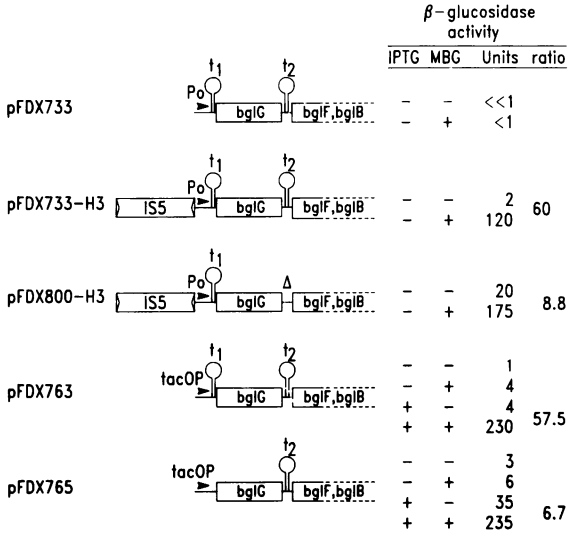

Fig. 5. Contribution of *t1* and *t2* to the regulation of the *bgl* operon. β-Glucosidase activity (specific activity in units) synthesized under the direction of plasmids carrying the wild-type *bgl* operon and various mutants thereof (diagrammed on the left) was determined. The ratio of induction by MBG is given in the rightmost column. pFDX733: wild-type operon; pFDX733-H3: *bgl* operon activated by spontaneous integration of IS5; pFDX800-H3: pFDX733-H3 deleted for terminator *t2*; pFDX763: pFDX733 with *tacOP* substituted for *bgl* promoter *P0*; pFDX765: pFDX763 deleted for terminator *t1*. Plasmids with the *tacOP* were accompanied by plasmid pFDX333 carrying the *lac* repressor gene.

the *bgl* operon activated by integration of an IS5. This plasmid showed an induction factor of 60 (Figure 5). When terminator *t2* was deleted (plasmid pFDX800-H3) the uninduced level of enzyme synthesis was raised 10-fold whereas the induced level was raised by only ~50%. The corresponding induction rate was about nine. To permit investigation of the role of terminator *t1* in *bgl* regulation plasmid pFDX763, in which the *tac* promoter is substituted for *P0*, was constructed. Induction by MBG gave nearly a 60-fold increase in activity (in the presence of IPTG). Deletion of terminator *t1* (pFDX765) led to nearly a 9-fold increase of the basal level. The induction factor was about seven. It can therefore be concluded that the two terminator regions contribute about equally to the regulatability of the *bgl* operon.

Discussion

A model for the regulation of the *bgl* operon is presented in Figure 6. Both IS5 (Reynolds *et al.*, 1982) and IS1 (Ronecker and Rak, 1987; Schnetz *et al.*, 1987) integrate in both orientations into various sites upstream of the cryptic *bgl* operon resulting in its activation. IS1 does not contain any promoter sufficiently active to account for turn-on of the adjacent *bgl* operon (Saedler *et al.*, 1972). IS5 directs low but significant transcription of adjacent regions in only one orientation (Rak *et al.*, 1982; Rak and von Reutern, 1984; our unpublished results). In neither case, therefore, can transcription originating within the IS element explain activation of the *bgl* operon. Recent studies have shown that activation of *bgl* in Bgl^+ mutants is due to an increase in *bgl*-specific mRNA initiated at a unique promoter. This promoter (*P0*) has been accurately mapped *in vitro* (Reynolds *et al.*, 1986). The same transcription start appears to be used *in vivo* (data not shown).

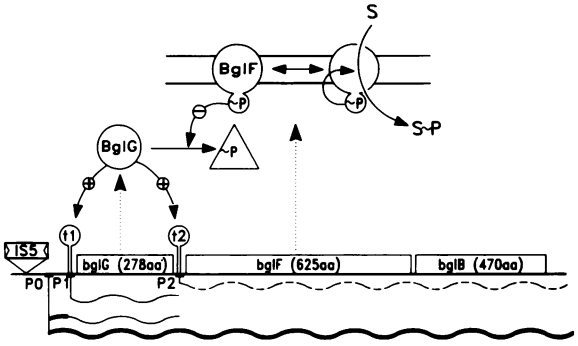

Fig. 6. A model for the complex regulation of the *bgl* operon. Promoter *P0* maintains a constant level of transcription that is not modulated by substrate. In the uninduced state transcripts terminate at terminators *t1* and *t2*, whereas in the induced state termination is relieved by the antiterminator protein (*bglG* gene product). Promoters *P1* and *P2* ensure basal levels of synthesis of the positively acting regulatory protein BglG and of the transport protein BglF (note its additional role in negative regulation of the operon) thereby maintaining inducibility by substrate. *P0* may also contribute to a basal expression of gene *bglG*. Activity of *P0* is susceptible to modulation by the catabolite gene repression system providing for a catabolite-regulated component of the basal *bglG* expression. In the wild-type operon the level of transcription initiated by *P0* is insufficient to express the operon. Mutational events (e.g. integration of insertion element IS5 into the vicinity of *P0*) enhance the activity of *P0* providing for higher levels of transcription. Terminators *t1* and *t2* are specifically recognized and inactivated by protein BglG (an antiterminator protein). Protein 'BglF' is the enzymeII^{Bgl} of the phosphoenolpyruvate-dependent phosphotransferase system (PTS) (see Saier *et al.*, 1988; Saier, 1985; Postma and Lengeler, 1985). In the PTS, a phosphoryl group is transferred from phosphoenolpyruvate to enzyme I and from enzyme I to protein HPr. HPr then relays the phosphoryl group to the individual, sugar-specific transport systems, in this case enzymeII^{Bgl}. Our data indicate that phosphate group translocation proceeds via phosphorylation of histidine residue 547 of enzymeII^{Bgl}. The phosphate group is then translocated to histidine residue 306 and finally to the sugar as it crosses the membrane. (Saier *et al.*, 1988; K.Schnetz, G.A.Daniels, M.H.Saier and B.Rak, unpublished). We postulate that in the absence of a phosphorylatable substrate the phosphate group is transferred to the antiterminator protein BglG (drawn as a circle) thereby curtailing its antiterminator activity (triangle).

In order to determine the molecular basis of insertional activation of the *bgl* operon we analysed the mRNA initiated from the *bgl* promoter *P0*. The results show conclusively that *P0* is enhanced ~60-fold by an IS5 present in its vicinity (mutant *bglR-S7*:: *IS5*; Figure 1). The same result was obtained when a Bgl^+ mutant with IS5 integrated in the opposite orientation was analysed (mutant *bglR-H3* :: *IS5*, data not shown). The signals responsible for the activation of promoter *P0* are located entirely within IS5 and are active from positions upstream as well as downstream from promoter *P0* in an orientation-independent manner (K.Schnetz and B.Rak, in preparation).

In the induced state promoter *P0* is responsible for expression of the operon. Substrate-specific induction of the operon, however, does not involve modulation of the activity of *P0* (Figures 1 and 5), but a substrate-dependent transcriptional antitermination mechanism. Two terminators (*t1* and *t2*) which flank *bglG*, the first gene of the operon, are the targets for this specific antitermination (Figure 6). Gene *bglG* product is responsible for antitermination at both terminators.

Data obtained from S1 experiments (Figure 3) together with enzyme measurements on promoter and terminator test constructions (Figure 4) showed that efficient antitermination occurs at both terminators, and revealed the presence of two additional promoters *P1* and *P2* (Figure 6). Terminator *t2* (distal to gene *bglG*) has an efficiency of >97%, and termination is relieved by the product of gene *bglG*, the *bgl*-specific antiterminator protein. In the case of this terminator the efficiency of antitermination was 50% (Figure 4). Deletion of the boxA sequence motif proximal to terminator *t2* (see Figure 2) did not influence the efficiency of the terminator. Efficiency of antitermination, however, dropped to ~22% indicating that this sequence motif, which is conserved at both terminators, quantitatively participates in the antitermination reaction.

Antitermination at *t1* has also been postulated by Mahadevan and Wright (1987). These authors also came to the conclusion that the product of *bglG* is an antiterminator. The results of the expression studies on the 5′ region including terminator *t1* must, however, be evaluated more cautiously (Figure 4, see also Mahadevan and Wright, 1987; Mahadevan *et al.*, 1987). The activity of promoter *P1* is quite high. It is not clear exactly how promoter *P1* interacts with the activated promoter *P0*. *P1* may also interact with regulatory components of the system. The results presented in Figure 1 show that the half-life of mRNA initiated at promoter *P0* is longer in the induced state, an effect which is probably due to difference in the length of the 3′ terminus. This adds another level of complexity. It is, nevertheless, clear from the data given in Figure 4 that, upon induction of *bgl*, efficient antitermination occurs at terminator *t1* and

t2. Moreover, the data for the *bgl* operon deleted for one or the other of the terminators (Figure 5) suggest that each of the terminator segments contributes about equally to the regulatability of the operon.

The capability of the product of gene *bglG* to mediate antitermination at terminator *t2* is independent of induction (Figure 3C). However, when gene *bglF* (β-glucoside permease; enzymeII^{Bgl} of the PTS; Schnetz *et al.*, 1987; Fox and Wilson, 1968; Postma and Lengeler, 1985; Saier, 1985) is also expressed, relief of termination becomes dependent on the presence of inducer (Figure 4). Similar data were obtained for terminator *t1* (data not shown). Thus enzymeII^{Bgl}, an integral membrane protein with phosphotransferase activity (see Figure 6) shows a phenotype typical of a negative regulatory protein. To explain this phenotype, direct negative interference between enzymeII^{Bgl} and the antitermination protein has been postulated (Mahadevan and Wright, 1987). Our own experimental evidence, however, (to be published elsewhere) indicates that, in the absence of substrate, enzymeII^{Bgl} exerts it negative regulatory effect by phosphorylating the product of *bglG* thereby curtailing its antiterminating activity (Figure 6).

The weak promoter *P2* located within or just downstream of the terminator *t2* (Figure 4) may serve to ensure the synthesis of enough enzymeII^{Bgl} in the uninduced state to antagonize antitermination by the *bglG* protein, thereby keeping up a basal level of transport and negative regulatory function (Figure 6). The role of promoter *P1*, which is located within terminator *t1* (Schnetz *et al.*, 1987 and Figure 4), could be the maintenance of a concentration of *bglG* gene product high enough to ensure inducibility (Figure 6).

Striking homologies have been noticed between the signal structures involved in the regulation of the *bgl* operon and regulatory sequences from *Bacillus subtilis* (Schnetz *et al.*, 1987). Furthermore, the amino acid sequence deduced for the *bglG* gene product is highly homologous to amino acid sequences deduced for possible counterparts in *B.subtilis* (Schnetz *et al.*, 1987; Steinmetz *et al.*, 1988). These homologies suggest that the regulatory system of the *bgl* operon is the prototype of a family of conserved regulatory mechanisms represented in both Gram-negative and Gram-positive bacteria. The compact regulatory unit nested within the *bgl* operon with the two composite signal structures closely flanking the antiterminator gene predisposes it as a module that could insert itself into various genetic entities and assume regulatory functions provided a potential antiterminator modulating system (such as the phosphotransferase activity of enzymeII^{Bgl}, see above) is available.

Materials and methods

Bacterial strains

N100 is *E.coli* K12 *pro galK recA* (Rak and von Reutern, 1984); JF201 is *E.coli* K12 Δ *lac (x74)* Δ *(pho-bgl) ara* B1$^-$ (Reynolds *et al.*, 1986); SL5235 is *Salmonella typhimurium* LT2 *metA metD trpD leu rpsL hsdL* (r^-m^+) *hsdSA*(r^-m^+) *hsdSB*(r^-m^+) (Schnetz *et al.*, 1987).

Plasmids

Plasmids were constructed using standard recombinant techniques (Maniatis *et al.*, 1982). Where necessary staggered ends were made blunt with DNA polymerase (Klenow large fragment) before ligation. Plasmid pFDX733 (Schnetz *et al.*, 1987) contains the *bgl* wild-type operon on plasmid vector pACYC177 (Chang and Cohen, 1978). The Bgl$^+$ mutant plasmids pFDX733-S7 (Schnetz *et al.*, 1987) and pFDX733-H3 are spontaneous insertion mutants of pFDX733 with a 'tagged' copy of IS5 (IS5-Sal, Schnetz *et al.*, 1987) in orientation II (Rak *et al.*, 1982) integrated at position −77 and with a wild-type IS5 in orientation I integrated at position −93 upstream of promoter *P0* respectively. (The transcription start site of promoter *P0* is position +1; Figure 1.) The integration sites were determined by DNA sequencing. The promoter test plasmid pFD51 has been described elsewhere (Rak and von Reutern, 1984, see Figure 4). Plasmid pFDX773 was generated by ligating a 220 bp *Ssp*I−*Dra*I fragment (from plasmid pFDX733) carrying the *bglG*−*bglF* intercistronic region into the singular *Sma*I site of plasmid pFD51. Plasmid pFDX779 was constructed by ligating a 119 bp *Bst*NI−*Eco*O109 fragment spanning the *bgl* leader (from position +6 to position +124) into the *Sma*I site of plasmid pFD51. Terminator test plasmid pFDX104 is essentially pFD105 (Alt-Mörbe *et al.*, 1986). The relevant structures of this plasmid are given in Figure 4. Plasmid pFDX771 contains a 220 bp *Ssp*I−*Dra*I fragment spanning the *bglG*−*bglF* intercistronic region substituted for the 41 bp *Sma*I fragment of pFDX104. Plasmid pFDX772 is identical to pFDX771 but lacks the boxA motif of terminator *t2* [32 bp from *Dde*I to *Ssp*I (Schnetz *et al.*, 1987)]. Plasmid pFDX769 contains the 119 bp *Bst*NI−*Eco*O109 fragment extending from position +6 to position +124 of the *bgl* leader substituted for the *Sma*I fragment of pFDX104. In plasmid pFDX763 the 455 bp segment between *Mlu*I and *Bst*NI of pFDX733 containing *bgl* promoter *P0* is replaced by an 800 bp *Pvu*II−*Sma*I fragment of pFDX104 with the *tac* operator promoter (Amann *et al.*, 1983). The related construction pFDX765 was derived from plasmid pFDX733 by replacing the 567 bp segment (between *Mlu*I and *Eco*O109) spanning promoter *P0* and terminator *t1* by the 800 bp *tacOP* fragment from pFDX104. Plasmid pFDX800-H3 is a derivative of pFDX733-H3 deleted for the 107 bp *Ssp*I−*Fnu*4 fragment containing the intercistronic region between *bglG* and *bglF*. In plasmid pFDX510, a derivative of pACYC177, the structural gene *bglG* is under the control of the *gal* promoter; distal to *bglG* is the lambda terminator *t0*. Plasmid pFDX333 carries the *lac* repressor gene cloned into pBR322 (E.Schwartz and B.Rak, unpublished). Details of the construction of the latter two plasmids will be published elsewhere.

Isolation of RNA

RNA was isolated by a 'hot phenol' method: strain SL5235 was transformed with various plasmids and the transformants were grown in synthetic medium containing glycerol as carbon source and all necessary supplements (Miller, 1972). For induction of the *bgl* operon and the *tac* promoter, MBG (5 mM) and IPTG (1 mM) respectively were added when the cultures reached an OD_{600} of 0.15. After an additional 2 h of growth 10 OD_{600} units of culture were pelleted, washed with Mg−saline (0.85% NaCl, 10 mM $MgSO_4$), resuspended in 0.9 ml buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris−HCl, pH 8.0) and frozen. After slow thawing at 0°C, 100 μl lysozyme (10 mg/ml) were added, followed 5 min later by the addition of 2 ml phenol, 800 μl Na-acetate, pH 5.2, 100 μl SDS (10%) and 50 μl macaloid (1 mg/ml). The sample was placed in an 80°C water bath and incubated for 6 min with continuous mixing using a loose-fitting glass pestle. The mixture was chilled on ice and spun to separate the phases. The aqueous phase was removed, phenolysed and extracted with chloroform/isoamyl alcohol (24:1). Nucleic acids were precipitated (overnight at −20°C) by the addition of 0.1 volume of 20% Na-acetate and 2 volumes of ethanol, washed once with 80% ethanol, dried, dissolved in buffer (10 mM Tris−HCl, pH 7.4, 5 mM $MgCl_2$) and incubated with 10 μg DNase (RNase-free) for 30 min at 37°C. Following DNase treatment the sample was extracted once with phenol and once with chloroform/isoamyl alcohol, ethanol-precipitated twice, dried and dissolved in H_2O.

Quantification of transcripts

Ten micrograms of RNA and successive 1:2 dilutions thereof (brought to a total of 10 μg by adding yeast soluble RNA) were applied to sheets of nitrocellulose using a slot-blot apparatus (Schleicher and Schuell, Dassel/FRG). The synthetic deoxyoligonucleotide (26-mer) used for probing RNA blots was end-labelled with terminal deoxynucleotide transferase and [α^{32}P]dATP (3000 Ci/mM) (Deng and Wu, 1983) to give on the average 20 labelled residues per molecule. Hybridization (Church and Gilbert, 1984) was carried out overnight at 50°C. The relative copy number of the template plasmids was determined from alkaline lysates (Birnboim and Doly, 1979) by filter hybridization of the DNA (Church and Gilbert, 1984) using the same oligonucleotide as probe. All autoradiograms were evaluated by densitometric scanning.

S1 mapping of 3′ RNA termini

S1 mapping was done as described by Berk and Sharp (1977) using 50 μg of RNA and 100 000 c.p.m. of end-labelled fragment and a hybridization temperature of 55°C. The 'leader' fragment was labelled at the *Bst*NI site and the 'intercistronic' fragment at the *Dde*I site using DNA polymerase (Klenow large fragment). Hybrids were treated with 50 units of S1 nuclease for 30 min at 37°C.

Determination of enzyme activities

Galactokinase activity was determined as described previously (Rak *et al.*, 1982). Phospho-β-glucosidase activity was measured using a procedure adapted from the β-galactosidase assay (Miller, 1972): cells were grown in synthetic medium (see above). At an OD_{600} of 0.15, MBG (5 mM) and/or IPTG (1 mM) were added where indicated. After 2 h of growth the cells were harvested and resuspended in Z-buffer (100 mM Na-phosphate, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 50 μg/ml chloramphenicol). Two hundred microlitres of *p*-nitrophenyl β-D-glucoside (PNPG, 8 mg/ml in Z-buffer) were added to 1 ml of an appropriate dilution of the cell suspension in Z-buffer. After 30 min at 37°C the reaction was stopped by the addition of 0.6 ml 2 M $NaCO_3$. A drop of toluene was added, the suspension mixed for 30 s on a vortex mixer and cleared by centrifugation. The amount of product (nitrophenol) was determined by measuring the absorbance at 410 nm. One unit of phospho-β-glucosidase (specific activity) is defined as the amount of *p*-nitrophenol released per OD_{600} unit of cell suspension per min. To arrive at a convenient magnitude, units were calculated as follows:

$$1 \text{ unit} = A_{410} \times 1000/(v \times t \times OD_{600})$$

where v is the dilution, t the reaction time in min (30 min in this case) and OD_{600} the optical density of the culture (before dilution) measured at 600 nm.

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References

Alt-Mörbe,J., Rak,B. and Schroeder,J. (1986) *EMBO J.*, **5**, 1129–1135.
Amann,E., Brosius,J. and Ptashne,M. (1983) *Gene*, **25**, 167–178.
Berk,A.J. and Sharp,P.A. (1977) *Cell*, **12**, 721–732.
Birnboim,H.C. and Doly,J. (1979) *Nucleic Acids Res.*, **7**, 1513–1523.
Chang,A.C.Y. and Cohen,S.N. (1978) *J. Bacteriol.*, **134**, 1141–1156.
Church,G.M. and Gilbert,W. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1991–1995.
Deng,G.-R. and Wu,R. (1983) *Methods Enzymol.*, **100**, 96–116.
Fox,C.F. and Wilson,G. (1968) *Proc. Natl. Acad. Sci. USA*, **59**, 988–995.
Mahadevan,S. and Wright,A. (1987) *Cell*, **50**, 485–494.
Mahadevan,S., Reynolds,A.E. and Wright,A. (1987) *J. Bacteriol.*, **169**, 2570–2578.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Maxam,A.M. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499–560.
Miller,J. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Postma,P.W. and Lengeler,J.W. (1985) *Microbiol. Rev.*, **49**, 232–269.
Prasad,I. and Schaefler,S. (1974) *J. Bacteriol.*, **120**, 638–650.
Rak,B. and von Reutern,M. (1984) *EMBO J.*, **3**, 807–811.
Rak,B., Lusky,M. and Hable,M. (1982) *Nature*, **297**, 124–128.
Reynolds,A.E., Felton,J. and Wright,A. (1981) *Nature*, **293**, 625–629.
Reynolds,A.E., Mahadevan,S., LeGrice,S.F.J. and Wright,A. (1986) *J. Mol. Biol.*, **191**, 85–95.
Ronecker,H.-J. and Rak,B. (1987) *Gene*, **59**, 291–296.
Saedler,H., Besemer,J., Kemper,B., Rosenwirth,B. and Starlinger,P. (1972) *Mol. Gen. Genet.*, **115**, 258–265.
Saier,M.H. (1985) *Mechanism and Regulation of Carbohydrate Transport in Bacteria*. Academic Press, London.
Saier,M.H., Yamada,M., Erni,E., Suda,K., Lengeler,J., Ebner,R., Argos,P., Rak,B., Schnetz,K., Lee,C.A., Stewart,G.C., Breidt,F., Waygood,E.B., Peri,K.G. and Doolittle,R.F. (1988) *FASEB J.*, **2**, 199–208.
Schaefler,S. (1967) *J. Bacteriol.*, **93**, 254–263.
Schnetz,K., Toloczyki,C. and Rak,B. (1987) *J. Bacteriol.*, **169**, 2579–2590.
Steinmetz,M., Aymerich,S., Gonzy-Tréboul,G. and Le Coq,D. (1988) In Ganesan,A.T. and Hoch,J. (eds), *Genetics and Biotechnology of Bacilli*. Academic Press, in press.

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