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The transition state transcription regulator *abrB* of *Bacillus subtilis* is a DNA binding protein

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The product of the *abrB* gene of *Bacillus subtilis* is an ambiactive repressor and activator of the transcription of genes expressed during the transition state between vegetative growth and the onset of stationary phase and sporulation. Purified AbrB protein binds specifically in a highly co-operative fashion to fragments of DNA containing the promoters it affects. DNase I footprints of the binding regions in these promoters revealed large protected areas of 50-120 nucleotides or more depending on the promoter. Methylation protection experiments gave protected guanine residues on only one face of the DNA helix. A consensus sequence could be deduced around these guanine residues that was not found around non-protected guanine residues in the footprint region. The results suggested that stationary phase functions and sporulation are repressed during active growth by AbrB and other transition state regulators by binding to the affected promoters in a concentration-dependent manner.
Key words: AbrB/*Bacillus subtilis*/footprinting/sporulation /transcription

Introduction

The earliest events in the initiation of the sporulation development cycle in *Bacillus subtilis* are controlled by the products of the *spoO* genes (Hoch, 1976). Mutations in these genes prevent the formation of the asymmetric septum characteristic of the onset of sporulation and appear to prevent the mutant from leaving the vegetative phase of growth. In addition to blocking sporulation at its earliest stage, *spoO* mutations of *B.subtilis* repress the expression of a wide variety of cellular processes usually associated with the end of exponential growth and the onset of sporulation (the transition stage) in this organism (Hoch, 1976). SpoO mutants are deficient in the production of several proteases, antibiotics and all known sporulation-associated products. Early efforts to dissect the pleiotropic effects of the *spoO* mutations led to the isolation of suppressor mutations that, while not compensating for the sporulation defect, were able to revert many of the other pleiotropic effects of these mutations (Guespin-Michel, 1971a,b; Ito *et al.*, 1971; Ito, 1973; Trowsdale *et al.*, 1979). The majority of these independently isolated suppressor mutations were subsequently shown to map at a single locus, termed *abrB* (Trowsdale *et al.*, 1979). Thus the *arbB* locus seemed to be the major locus responsible for regulating transition stage gene expression.

The *abrB* locus has been cloned and sequenced and has been shown to consist of a single gene which codes for a protein with an approximate mol. wt of 10 700 (Perego *et al.*, 1988). Mutations in this gene affect the transcription of a variety of genes, including *aprE* (subtilisin, Ferrari *et al.*, 1988), *tycA* (Maraheil *et al.*, 1987), *spoVG* (Zuber and Losick, 1987) and *spoOE* (M. Perego and J.A.Hoch, unpublished), and of itself (Perego *et al.*, 1988). Based upon these studies it has been suggested (Perego *et al.*, 1988) that the *arbB* protein functions as an ambiactive transcription regulator with both negative and positive effects on several genes that are expressed during the transition state between exponential growth and sporulation.

The concentration of *abrB* protein in the cell is controlled by the product of the *spoOA* gene (Perego *et al.*, 1988). Since the role of the *spoOA* protein is to initiate sporulation in response to the metabolic state of the cell, it appears that the *abrB* protein functions to repress a subset of genes whose transcription depends on signals received by the *spoOA* protein.

As an initial step towards an understanding of the molecular mechanism by which the *abrB* protein affects transcription and of how it interacts with the *spoO* gene products, we have purified the *abrB* protein from an *Escherichia coli* clone which overexpresses the *B.subtilis abrB* gene. In this paper we report upon this purification and studies which show that the *abrB* protein binds to the promoter DNA of its target genes.

Results

Purification of the AbrB protein

Initial experiments using crude extracts of induced (IPTG) versus uninduced JM109/pQAB3W cells indicated that the AbrB protein had DNA binding activity towards at least two of its target genes (*aprE* and *spoOE*). We therefore set out to purify sufficient amounts of the AbrB protein to characterize this binding activity. Our assay for following the course of AbrB purification was to examine the protein profile of the various fractions by PAGE (Figure 1). To confirm that the protein purified was actually AbrB, we subjected a portion of the protein to amino-terminal amino acid analysis. The sequence obtained was identical with the amino acid sequence deduced from DNA sequencing (Perego *et al.*, 1988) with one exception: the amino-terminal sequence of AbrB deduced from the DNA sequence was Met-Phe-Met-Lys-Ser . . ., whereas the results with the purified protein indicated that the protein began at the second methionine residue (i.e. Met-Lys-Ser . . .). While this seems

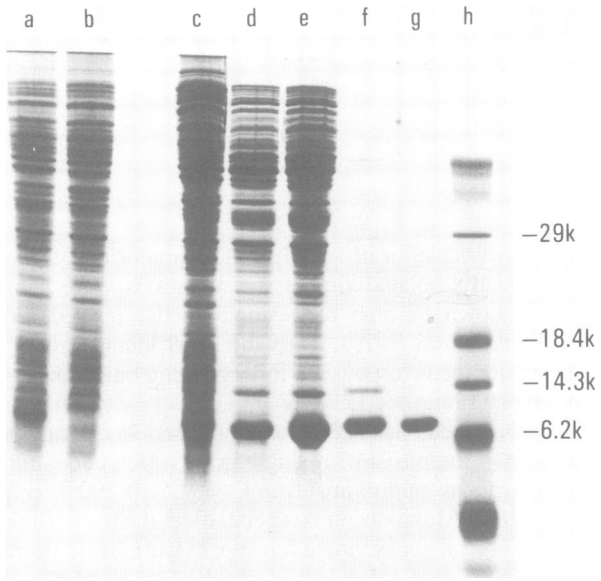

Fig. 1. Purification of the AbrB protein. (**a**) JM109/pQAB3W extract after induction with IPTG and (**b**) without IPTG; (**c**) crude extract; (**d**) 55–70% ammonium sulfate precipitate; (**e**) desalted sample after passage over P20 column; (**f**) DEAE 'shoulder' peak from column washing; (**g**) Heparin–agarose 50 mM KCl elution; (**h**) mol. wt standards with sizes indicated. See Materials and methods for details.

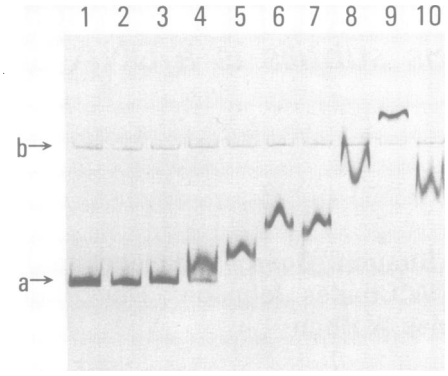

Fig. 2. Binding of the AbrB protein to a *Bam*HI–*Eco*RI fragment containing the promoter and upstream region of the subtilisin gene (*aprE*). The gel-retardation assay lane assignments: (**1**) no protein; (**2**) 1 μM AbrB protein; (**3**) 2 μM; (**4**) 3 μM; (**5**) 4 μM; (**6**) 6 μM; (**7**) 7.5 μM; (**8**) 10 μM; (**9**) 20 μM; (**10**) 10 μM *AbrB* protein and 2 μg sheared salmon sperm DNA. The band indicated by (a) is the 800-bp *Eco*RI–*Bam*HI fragment containing the *aprE* upstream region; the band indicated by (b) is the 7.5-kb linearized vector pJM783.

to indicate that the translation initiates at the second Met residue, we cannot rule out the possibilities of post-translational processing or that the protein produced in *E.coli* begins at the second Met, whereas in *B.subtilis* it begins at the first Met.

Binding of abrB protein to the DNA of its target genes

Several studies have implicated the *abrB* gene product in the transcription regulation of numerous genes whose products are expressed during the transition from exponential growth to sporulation. We were interested to learn if this effect was due to binding of AbrB to the promoter regions of its target genes. Using a gel-retardation assay we examined the DNA binding activity of the purified AbrB protein. The AbrB protein was found to bind to each of its target genes that we examined: *aprE* (Figure 2), *spoOE*, *abrB* and *hpr* (data not shown). The binding was specific: AbrB protein did not bind to plasmid DNA fragments that did not carry any of its target sequences, nor did it bind to a strong promoter from the *B.subtilis* phage φ29 (data not shown). In addition, an ~2000-fold excess of non-specific salmon sperm DNA was unable to compete with the target DNAs for AbrB binding (Figure 2, lane 10).

The binding (Figure 2) of the AbrB protein to the subtilisin, *aprE*, promoter is representative of the results obtained with the other target DNAs that we examined using the gel-retardation assay. The binding was highly co-operative, and seemed to occur at multiple, identical sites (see below). Interestingly, it appeared that the binding was quantized in some manner since for any given concentration of AbrB there was but a single discrete species of bound DNA with little smearing (see Figure 2). The reason for the wavy appearance of the AbrB bound DNA fragments as seen in Figure 2, lanes 4–10, is not known. Different conditions were tried in the assay itself (KCl concentration), the method of loading the gels (no dyes, glycerol versus sucrose), the voltage at which the gels were run and the composition of the running buffer (90 mM Tris-borate, pH 8.3, 1 mM EDTA; 45 mM Tris-borate, pH 8.3, 5×10^{-8} M EDTA) and in all cases the retarded fragments had this type of wavy appearance.

Footprint analysis of the binding of AbrB proteins to aprE and spoOE promoters

To determine the location of AbrB binding to the target DNAs, footprinting assays were performed. When the non-template strand of *aprE* DNA was end labeled, AbrB protein protected from DNase I cleavage the sequences from −59 to +15 (relative to the start point of transcription, Figures 3 and 7A). When the template strand was labeled, the protected region was from −59 to ~ +25 (Figure 7A, data not shown). In both cases the protection was seen only at higher concentrations of AbrB protein; lower concentrations, while showing binding activity (see previous results with gel-retardation assays), did not appear to protect any sequences from DNaseI. In addition, the region protected exhibited an all or none protection pattern: there was no evidence of a single, higher affinity site serving as a nucleation point of AbrB binding follwed by enlargement of the protected region due to co-operative binding of further AbrB protein molecules. Any explanation for this phenomenon has to take into account the fact that in order to see a footprinting pattern of a given region most of the DNA molecules must have protein bound to them at that region. If a DNA molecule has multiple identical binding sites for a protein, with no site showing preferential binding of the initial protein regardless of whether or not subsequent binding is co-operative, then in the population it would appear that no one site was protected from DNase I until virtually all the sites were protected. It can be seen that binding of AbrB protein spanned the promoter region of *aprE*. Other studies (Ferrari *et al.*, 1988) have suggested that AbrB functions as a repressor of transcription of *aprE* and exerts its action between −40 and the start site of transcription. Our results are consistent with that hypothesis.

A study to determine which guanine residues were pro-

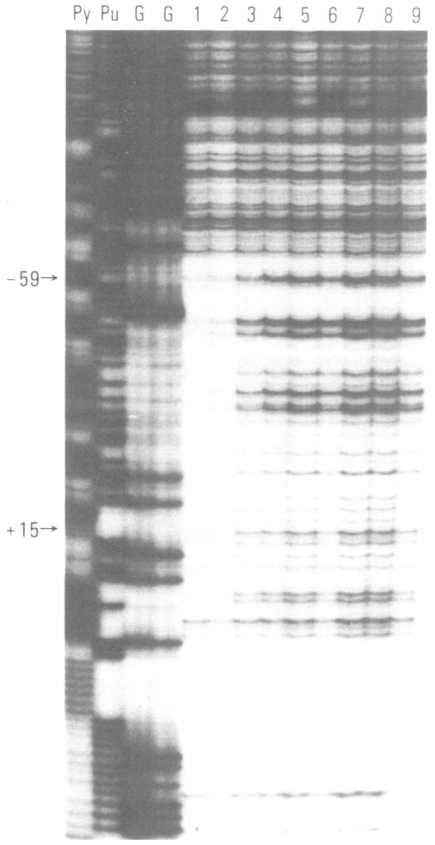

Fig. 3. DNase I protection experiments of the subtilisin promoter (*aprE*) by the AbrB protein. Shown are the results obtained when the non-template strand is labeled at its 3′ end. The Maxam–Gilbert C + T (Py), A + G (Pu) and G reaction lanes are shown for reference. **Lanes 8** and **9** contain no AbrB protein, while **lanes 1–7** contain 2, 1.5, 1, 0.5, 0.4, 0.2 and 0.1 μg AbrB protein respectively.

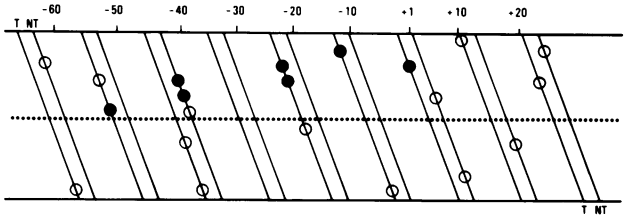

Fig. 4. Guanine residues in the *aprE* promoter region that are protected from methylation by binding of the AbrB protein. Shown is a planar projection of the DNA with 10.5 bp/turn of the helix. Protected guanines are indicated by closed circles, unprotected guanines by open circles. T, template strand; NT, non-template.

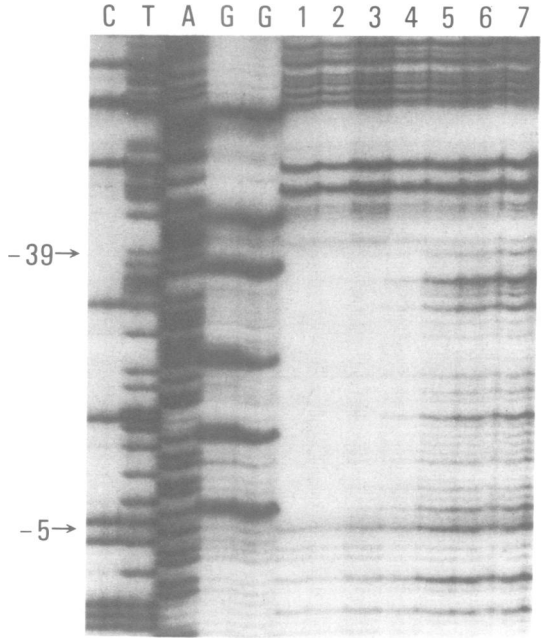

Fig. 5. DNase I protection experiments of the *spoOE* gene. Shown are the results obtained when the non-template strand is labeled at its 3′ end. The Maxam–Gilbert C, C + T (T), A + G (A) and G reactions are shown for reference. **Lane 7** contains no protein; **lanes 1–6** contain 3, 2, 1, 0.6, 0.3 and 0.1 μg AbrB protein respectively.

tected from chemical modification (methylation by DMS) by the binding of AbrB protein to the *aprE* promoter revealed that all protected guanines were on one face of the helix (Figure 4). While not all guanines on this face were protected, none of the guanines on the opposite face were protected (Figure 4). These data suggest that the AbrB protein binds predominantly to one face of the DNA helix.

AbrB is known to be involved in the expression of the *spoOE* gene. Transcription of *spoOE* is normally expressed only at the initial stage of sporulation (t_0); however, in cells possessing *abrB* mutations the expression of *spoOE* is constitutive during vegetative growth (M.Perego and J.A.Hoch, unpublished). This pattern differs from the *aprE* gene which is still subject to temporal control in the absence of the *abrB* gene product (Ferrari *et al.*, 1988). Thus the AbrB protein may function as the sole repressor of *spoOE* transcription. Footprinting (Figure 5) revealed that AbrB binds to a region of *spoOE* that included the promoter (Figure 7B). Binding of AbrB protein could prevent access of RNA polymerase to the promoter, thus accounting for the repressive effect during vegetative growth. Some signal, possibly *spoOA* dependent, has been postulated to inactivate the AbrB protein or lower its concentration at t_0 to allow expression of *spoOE* (Zuber and Losick, 1987; Perego *et al.*, 1988).

Binding of abrB protein to its own promoter

A previous study revealed that the amount of *abrB* mRNA was significantly elevated in *abrB* mutants (Perego *et al.*, 1988). This finding, along with that result that the AbrB protein binds to a DNA fragment containing the *abrB* promoter, indicates that the *abrB* gene may be subject to some form of autoregulation. Figure 6 illustrates a footprint experiment using AbrB protein and *abrB* DNA. A portion of the DNA to which AbrB protein bound (Figure 7C) included the −35 region of the P2 promoter and the entire (−35, −10, +1) P1 promoter (Perego *et al.*, 1988). Unlike the binding to *aprE* DNA, binding of the AbrB protein to its own gene seemed to occur in at least two distinct steps, the first involving a higher affinity site than the second. As can be seen in Figure 6, AbrB protein first protected the region from −43 to −14 (relative to P2) with very little, if any, protection of other regions seen. However, as the concentration of the protein was raised a further protected region extending upstream to at least −122 became apparent. This region was very large (minimum 80 bp) and, considered alone, the all-or-none nature of the appearance of the footprint in this large region resembled that seen for AbrB binding to *aprE* (minimum of 74 bp protected). Using the gel-retardation assay of AbrB binding, the results with *aprE* DNA and *abrB* DNA were essentially indistinguishable. However, the apparent presence of a higher affinity site from −43 to −14

in the *abrB* DNA implies that the binding characteristics of AbrB protein toward *aprE* and *abrB* were qualitatively different.

Discussion

Genetic studies suggested that the AbrB protein was an 'ambiactive' transcription regulator (capable of both negative and positive effects) controlling a variety of genes whose products are normally produced during the transition phase between vegetative growth and sporulation (Perego *et al.*, 1988). The studies reported here show that the AbrB protein binds specifically to the promoter regions of the genes that it controls. Thus AbrB acts directly as a negative regulator of the transcription of these promoters. We have previously suggested that AbrB is only one of a family of such ambiactive regulators that serve to prevent the onset of stationary phase and sporulation in actively growing cells (Perego *et al.*, 1988). The AbrB protein seems to be the sole regulator of the sporulation gene *spoOE* and the sporulation-associated antibiotic synthesis gene *tycA* (Maraheil *et al.*, 1987). It also represses genes with no apparent role in sporulation, e.g. *aprE*, consistent with its postulated role as a general regulator of stationary phase transcription. AbrB is also a positive regulator of the *hpr* locus which codes for a negative regulator with many of the characteristics of AbrB (Perego and Hoch, 1988). These regulators, and at least one other that is proposed to exist (Perego *et al.*, 1988), are in turn controlled by the *spoOA* gene product which is the active regulator of a sensing mechanism designed to decide a cell's fate between active growth and stationary phase. This mechanism amplifies its signals by using transition state regulators such as AbrB and Hpr as the direct repressors of many genes. The results of the present study help to elucidate the mechanism of this repression.

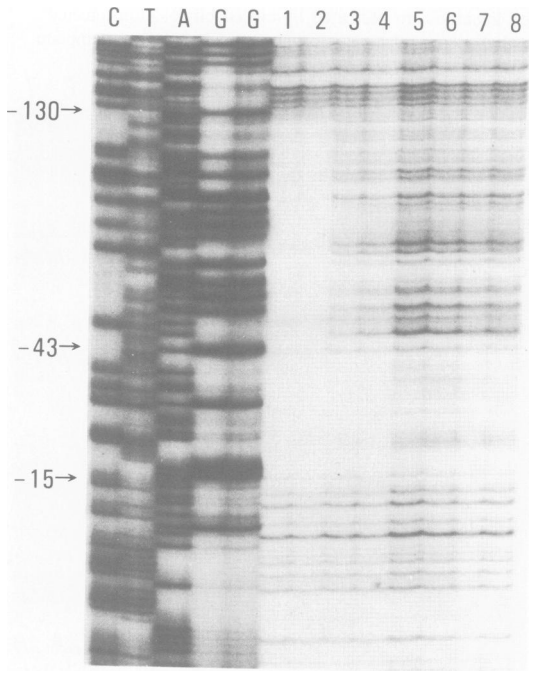

Fig. 6. DNase I protection experiments of the *abrB* gene. Shown are the results obtained when the template strand is labeled at its 5′ end. The Maxam–Gilbert sequencing reactions are shown for reference. **Lanes 7** and **8** contain no protein; **lanes 1–6** contain 3, 2, 1, 0.6, 0.3 and 0.1 μg AbrB protein respectively.

Methylation protection experiments using the AbrB protein and the subtilisin promoter showed that the protected G residues were on one face of the DNA helix (Figure 4). We showed earlier that the protein sequence of AbrB shared homology to other DNA binding proteins having the classical helix–turn–helix motif characteristic of DNA binding proteins (Perego *et al.*, 1988). The helix–turn–helix is presumed to interact with nucleotides in the major groove of the DNA thus stabilizing interactions which are base pair specific (reviewed in Pabo and Sauer, 1984). If the AbrB protein bound to DNA by polymerizing (head to tail) onto

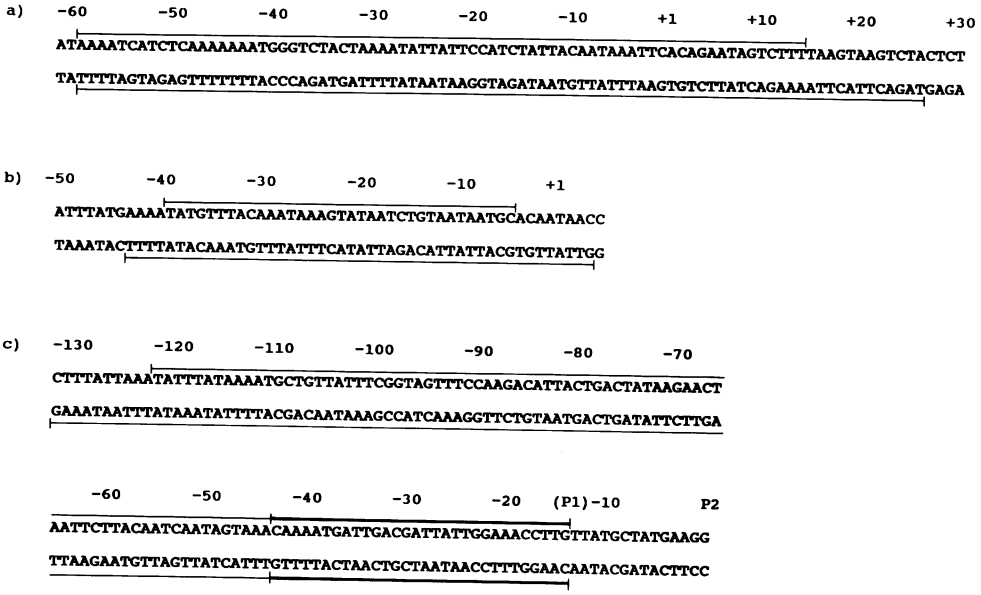

Fig. 7. Sequences protected by the AbrB protein. (**A**) *aprE* gene; (**B**) *spoOE* gene; (**C**) *abrB* gene. Areas protected from DNase attack due to the binding of the AbrB protein are indicated by the lines. Results obtained when each strand is labeled separately as shown. In (C) the heavy line indicates the region protected by lower concentrations of AbrB while the lighter line indicates those additionally protected by higher concentrations (see Figure 6 and text). The location of +1 in *spoOE* was determined in another study (M.Perego and J.A.Hoch, unpublished).

a nucleation complex which bound to a specific sequence (analogous to the gene 32 protein of T4; Alberts and Frey, 1970; McPheeters *et al.*, 1988) we would expect the AbrB protein to wind around the helix following the major groove of the DNA. Since we did not observe a methylation pattern consistent with this hypothesis, we would thus predict that the protein has some specific recognition sequence in the footprint areas which is repeated with a 10-bp periodicity.

Examination of the protected areas of these promoters revealed no obvious candidates for consensus binding sequences. It may be that AbrB recognizes a three-dimensional structure of DNA that may be assumed by many different base sequences. Such a hypothesis has been advanced to explain the properties of the type II DNA-binding protein TF1 encoded by bacteriophage SPO1 (Greene *et al.*, 1986). Although both proteins are able to protect large areas in DNase I footprinting experiments, the mechanisms of binding as revealed by these and gel-retardation assays appear to be significantly different (Greene and Geiduschek, 1985; Greene *et al.*, 1986a,b; Sayre and Geiduschek, 1988). Additionally, AbrB does not share amino acid sequence homology with TF1 and other prokaryotic type II DNA-binding proteins such as *E.coli* HU (Greene *et al.*, 1984); nor have we observed non-specific binding of AbrB to DNA as is the case with TF1 (Johnson and Geiduschek, 1972, 1977; Greene *et al.*, 19876a). Despite these differences, both AbrB and TF1 may bind via recognition of a subtle three-dimensional DNA structure assumed by varying base sequences. At present it is impossible to say what this structure may be or what type of base sequences contribute to its formation. However, upon examination of the sequences surrounding the guanines protected from methylation we noted that in nine out of ten cases there was consensus in at least seven positions to the 8-bp sequences: TGNPu (A or T) NNA. In contrast, the sequences around the non-protected guanines occurring within the footprint regions show only 16% (5/31) with seven or eight matches to this consensus. In addition, examination of the sequences of the promoter regions of two genes to which AbrB does not bind, *spoOA* (Ferrari *et al.*, 1985) and the ϕ20 PE[3] promoter (Garvey *et al.*, 1985) revealed that 7/8 matches around guanine residues occur at a frequency of only 24% (9/27). A *t*-test of the differences between the sample means indicates that the homology of the sequences around the protected guanines is not due to chance. Based upon this analysis, it is tempting to speculate that this sequence may be important, but not necessarily sufficient, for the formation of the DNA structure that AbrB recognizes.

An unusual feature of the protein binding we have shown is the lack of linearity of binding of the DNA with AbrB input, suggesting that the initial binding of the protein is co-operative. At present we cannot determine whether the apparent co-operativity results from the sequential binding of monomers to the DNA, or whether the protein must form a multimer in solution before binding. Thus the binding of any one AbrB molecule to its target is weak, and binding may be observed only when the concentration of AbrB protein is sufficiently high that the protein will also bind to the adjacent target sites. This implies that AbrB binding to promoters will be very concentration dependent, a feature which we believe relates to the *in vivo* role of AbrB and which is consistent with the *in vitro* data we have presented here.

It has been shown elsewhere that the concentration of AbrB in the cell is under at least two control mechanisms (Perego *et al.*, 1988): repression by the SpoOA protein and a form of autoregulation. The initial binding site of the AbrB protein is on the downstream member (p2) of a pair of tandom, partially overlapping promoters responsible for transcription of the *abrB* gene (Figure 7C). We have shown previously that the downstream p2 promoter is also repressed by the *spoOA* gene product. It is the increase in the transcription of the AbrB p2 promoter in *spoOA* strains which accounts for a significant number of phenotypes usually associated with an *spoOA* mutation. In an *spoOA* mutant, the upsteam promoter (p1) did not change in level of transcription. A mutation in the structural gene of AbrB (*abrB4*) resulted in a dramatic increase in transcription from both p1 and p2. Thus the binding of AbrB affects both p1 and p2 transcription, as would be predicted by the extensive footprint we have shown in this paper. Since the level of AbrB is critical to its function, one might predict that mutations in either p1 or p2 would result in the *abrB* phenotype and, indeed, such mutations have been recovered (Perego *et al.*, 1988). While it may seem unusual for the AbrB protein to have two promoters it could be that the positive effects of the protein require a low level of the protein in the cell at all times. Thus p1 may be considered a constitutive promoter, although still sensitive to autoregulation, while p2 is inducible, although it is also sensitive to autoregulation.

When we initially found that the *abrB* gene was preceded by two promoters, we were somewhat perplexed as to how the *spoOA* gene product, which appears to control only one of the promoters, would lead to such a wide variety of phenotypes by affecting transcription from only the downstream promoter. The footprint and gel-retardation data we have included in this paper demonstrate a potential mechanism. It is clear that the binding of AbrB *in vitro* is highly co-operative, thus it is a change in the intracellular concentration, from moderate to high levels which will act to repress genes such as *spoOE*. Furthermore, it is clear that AbrB is required for activation of several genes (see below) as well as repression of others. *In vivo*, genes such as *spoOE* are relieved of repression by AbrB under the same conditions which the cell uses for AbrB activation. Thus the reduction of the AbrB concentration which leads to expression of *spoOE* (and related genes) should be accompanied by reduction, but not elimination, of the intracellular concentration of AbrB, as it is required for activation of other genes at the same time. The complex controls we have described employing AbrB thus seem to be correlated well with the *in vivo* expression of genes during the transition state.

AbrB has been implicated in the positive control of the *hpr* locus (Perego and Hoch, 1988; M.Perego and J.A.Hoch, unpublished). We carried out gel-retardation assays with a DNA fragment containing the *hpr* promoter. These experiments showed that the AbrB protein bound to the DNA and retarded the fragment. However, we were unable to obtain any footprint with *hpr* to determine where the binding region lay. Since the conditions for the binding and footprint reactions were entirely identical, and the same *hpr* fragments were used for gel retardation and the attempt to determine binding sites by DNase I footprinting, it appears that the mechanism of binding to the *hpr* DNA is in some way different from the binding to the other fragments we have studied. This difference is intriguing and may be related to the fact that the AbrB binding to *hpr* results in activation

of the promoter. Thus the binding may take a form which is intrinsically different than when the protein acts to repress transcription activity and the activated form bound to positively controlled promoter DNA may not be stable to DNase treatment. Further experiments to examine the location of the binding site by pre-modification of the DNA are in progress.

Materials and methods

Construction of pQAB3W

A *Kpn*I−*Pst*I fragment containing the *lacI*^{q} gene from pMJR1560 (Amersham) was made blunt ended via the 3′ → 5′ exonucleolytic activity of the Klenow fragment of DNA Polymerase I (Bethesda Research Laboratories). This fragment was inserted into the *Pvu*II site of pKK223-3 (Pharmacia) to produce pKQV4. A 420-bp *Dra*I−*Hin*dIII fragment from pJM5153 (Perego *et al.*, 1988) containing the intact *abrB* gene was ligated into *Sma*I−*Hin*dIII-digested pKQV4 to form pQAB3W. This construction placed the *abrB* gene in the proper orientation downstream from the *tac* promoter of pKQV4.

pQAB3W was transformed into *E.coli* JM109 (Yanisch-Perron *et al.*, 1985) made competent by the procedure of Cohen (1973) with selection for Ap^R. Selected transformants were screened for the production of *abrB* protein by the following method. Cells were grown at 37°C in LB broth containing 50 μg/ml ampicillin to an OD_{600} of 0.6. The culture was divided in two, one half receiving 1 mM IPTG, the other half none. Incubation was continued for 2 h at 37°C. One milliliter of each culture was pelleted by centrifugation and resuspended in lysis buffer (0.125 M Tris, pH 6.8, 10% glycerol, 1% sodium lauryl sulfate, 140 mM β-mercaptoethanol). After boiling for 5 min the samples were electrophoresed on a polyacrylamide gel (5% stacking, 15% separating) using the buffer system of Laemmli (1970). The gels were stained with Coomassie Blue and visually inspected. All JM109/pQAB3W Ap^R transformants tested showed overproduction of a protein band of ~10 000 daltons (deduced mol. wt of *abrB* protein) in the presence of IPTG, and the absence of the band in the absence of IPTG (Figure 1, lanes A and B). Thus the expression of the *abrB* gene from the *tac* promoter of pQAB3W is very tightly controlled by the presence on each plasmid of the *lacI*^{q} gene but is fully inducible by 1 mM IPTG.

Purification of the abrB protein

Six liters of LB broth containing 50 μg/ml ampicillin were inoculated with JM109/pQAB3W and grown at 37°C to an OD_{600} of 0.6. IPTG was added to 1 mM and incubation continued for 2 h. The cells were pelleted by centrifugation, washed twice (10 mM Tris, pH 8.3, 1 mM EDTA, 100 mM NaCl, 50 μg/ml phenylmethylsulfonyl fluoride) and resuspended in Buffer A (10 mM Tris, pH 8.3 at 4°C, 1 mM EDTA, 10 mM $MgCl_2$, 10 mM KCl, 10 mM β-mercaptoethanol, 50 μg/ml PMSF). Lysozyme was added to a final concentration of 300 μg/ml and the suspension incubated at 37°C for 15 min. All subsequent steps were performed at 4°C. The cells were sonicated using a Heat systems-Ultrasonics Model W-220F sonicator with microtip at maximum output for five cycles of 1-min bursts/1 min rests. After centrifugation at 27 000 *g* for 15 min the supernatant was removed and used as the crude extract. The crude extract was diluted with Buffer A to give a final protein concentration of 15 mg/ml. An aliquot of 10% streptomycin sulfate was added dropwise to a final concentration of 1% with gentle stirring. After 30 min the suspension was centrifuged at 35 000 *g* for 15 min. Solid ammonium sulfate, $(NH_4)_2SO_4$, was added slowly to the supernatant to give a final concentration of 55%. After gentle stirring for 15 min the suspension was allowed to stand for an additional 15 min without stirring before centrifugation at 35 000 *g* for 15 min. Solid $(NH_4)_2SO_4$ was added slowly to the supernatant to give a final concentration of 70% and the suspension treated as above. The resulting protein pellet [55−70% $(NH_4)_2SO_4$ cut] was resuspended in the minimum volume necessary of Buffer A and applied to a 14 cm × 2 cm desalting column of P20 (Biorad) equilibrated and developed with Buffer A (we had previously determined empirically that the native AbrB protein was excluded by P20). Approximately 50 mg of the desalted protein sample was applied to a 7 cm × 1 cm DEAE trisacryl M (IBF-Biotechnics, France) column equilibrated with Buffer A. The column was washed with 3 vol of Buffer A and the majority of the AbrB protein was found to elute off the column as a shoulder peak near the end of the wash (see Results). The AbrB-containing fractions were pooled and applied to a 7 cm × 1 cm Heparin−agarose column (Sigma, 760 mg/ml). The column was washed with 3 vol Buffer A and the AbrB protein was eluted with 3 vol of Buffer A and 50 mM KCl. The AbrB-containing fractions were pooled, concentrated via partial lyophilization and dialyzed overnight at 4°C versus 100 vol of Buffer A. Glycerol was added to 30% final concentration and the preparation stored at −20°C. Throughout this procedure the prescence of the AbrB protein was monitored by protein fractionation on polyacrylamide gels as described above. Figure 1 shows the purification of the AbrB protein through various steps in this procedure.

Protein concentrations were determined by the procedure of Lowry *et al.* (1951) and by the formula of Warburg and Christian (Layne, 1957). Where feasible, these values were quantitatively checked by comparison of stained bands on gels to stained bands of known amounts of the low mol. wt proteins found in the standards obtained from Bethesda Research Laboratories.

Amino-terminal amino acid analysis of the purified AbrB protein was performed at The Agouron Institute, San Diego, CA, using an Applied Biosystems Inc. gas-phase microsequencer.

Gel-retardation assay

DNA fragments containing the promoters and surrounding regions of various genes used in the assay of AbrB binding were the following: *aprE* (subtilisin), 800 bp *Eco*RI−*Bam*HI of pJM818 (Ferrari *et al.*, 1988); *abrB*, 800 bp *Bam*HI−*Hin*dIII of pJM5134 (Perego *et al.*, 1988). *spoOE* 360 bp *Eco*RI−*Pst*I of pJM7144 (Perego and Hoch, 1987); *hpr*, 330 bp *Pst*I−*Bam*HI of pJM2485 (M.Perego and J.A.Hoch, unpublished).

After digestion with the appropriate enzymes, the fragments were labeled (at both ends) with [α-^{35}S]dATP using the Klenow enzyme. The DNA was fractionated on 5% polyacrylamide gels and stained with ethidium bromide. The appropriate bands were excised, electroeluted and extracted once with phenol saturated with TE (10 mM Tris, pH 8, 1 mM Na_2 EDTA), once with phenol/$CHCl_3$ (1:1), once with 1-butanol and ethanol precipitated. The labeled DNA was resuspended in TE.

Binding of AbrB to the DNA fragments was performed at 37°C in 10 μl reaction volumes containing 0.04 M Hepes, pH 8, 0.1 M KCl, 0.01 M $MgCl_2$, 0.1 mg/ml bovine serum albumin, 0.1 mM dithiothreitol, 0.1 mM Na_2EDTA, 10% glycerol. Approximately 10 000 c.p.m. labeled DNA was used per reaction and the amount of AbrB protein was varied. The reactions (at 37°C) were initiated by the addition of the protein and allowed to proceed for 5 min. Loading dye (5 μl, 0.1% xylene cyanol, 0.1% bromophenol blue, 60% sucrose) was added and the samples immediately applied to a 5% polyacrylamide gel running at 7 V/cm at 37°C. After electrophoresis the gels were dried and subjected to autoradiography.

DNase I footprinting and methylation protection

The nature of the DNA fragments used in the footprinting experiments was essentially the same as described in the gel-retardation assays except that they were labeled at only one end using either [α-^{35}P]dATP (3000 Ci/mmol, Amersham) and the Klenow enzyme, or [γ-^{32}P]ATP (7000 Ci/mmol, ICN) and T4 polynucleotide kinase (United States Biochemical Corporation). For each fragment examined both the transcribed and the untranscribed strands were labeled in separate experiments. The binding reactions were performed as described for the gel retardation assays using 2.5 nmol or less labeled DNA. (In separate experiments using the same fragment, no difference in the footprint pattern with identical protein concentrations was observed using as little as 10 pmol or as much as 2.5 nmol DNA). After the 5-min binding reaction, 2 μl of 0.1 mg/ml DNase I was added. Five seconds later, 5 μl of stop solution (0.1 M Na_2EDTA, 0.5% SDS) were added and the reactions placed on ice until completion of the series. Then 1−2 μl of 2 mg/ml sheared salmon sperm DNA and 1 ml 95% ethanol were added and the reactions placed at −70°C for 1 h. The DNA was collected by centrifugation, dried and resuspended in 5 μl loading dye (0.1% xylene cyanol, 0.1% bromophenol blue, 10 mM Na_2EDTA, 95% deionized formamide) and 2.5 μl of each sample was loaded on the gels (see below). The same labeled DNA fragment used for footprinting was also subjected to chemical sequencing procedures (Maxam and Gilbert, 1980) to produce a ladder with which to compare the footprint patterns. Sequencing and footprinting samples were electrophoresed on 6% polacrylamide/6 M urea gels, dried and subjected to autoradiography.

The protoclol for determining which guanine residues were protected from methylation by dimethyl sulfate due to the binding of AbrB was as follows. AbrB protein was bound to the DNA fragment in 10 μl of the G reaction buffer supplied with the New England Nuclear Maxam and Gilbert sequencing kit. After 2 min at 37°C the reaction was transferred to 21°C and 290 μl additional G reaction buffer was added, and the G sequencing reactions were carried out as usual (Maxam and Gilbert, 1980). Identical amounts (in c.p.m.) of protected versus unprotected G reactions were electrophoresed alongside each other. A guanine was considered protected if its corresponding band was absent or visibly reduced (at least 50%) in intensity compared to the unprotected reaction.

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