Analysis of abrB Mutations, Mutant Proteins, and Why abrB Does Not Utilize a Perfect Consensus in the −35 Region of Its σ^A Promoter*

(Received for publication, September 18, 1995, and in revised form, November 10, 1995)

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The Bacillus subtilis global regulator AbrB is a DNA-binding protein composed of six identical monomers of 96 amino acids that shows specificity to the promoter regions of its target genes including its own. We have sequenced thirteen previously uncharacterized abrB mutations. Four mutant AbrB proteins were purified, and their DNA-binding properties and multimeric structures were examined. AbrB23 (R25S) had no appreciable DNA binding activity but retained a hexameric structure, indicating that Arg25 is important in DNA interactions. Three other mutant proteins, AbrB1 (C56Y), AbrB19 (Gln83 to a termination codon), and AbrB100 (L69P), showed decreased DNA binding and altered multimeric interactions. Analysis of the expression and AbrB binding affinities of mutant abrB promoters demonstrated that a consensus −35 region is incompatible with proper autoregulation of the abrB gene.

AbrB is a DNA-binding transcriptional regulator of numerous genes that commence expression at the end of vegetative growth and the onset of stationary phase and sporulation (1, 2). Its primary function is to prevent inappropriate expression of nutrient limitation-induced functions at times when they are not needed. The regulatory role of AbrB is not limited to events associated with growth cessation because it also modulates expression of some genes during slow exponential growth in suboptimal environments. In at least two cases, histidine utilization (3) and ribose transport (4), this involves antagonism of the catabolite repression response caused by slowly metabolizable sugars.

AbrB does not share significant amino acid homology with other known classes of DNA-binding proteins (5, 6). It is a hexamer of identical small subunits (96 amino acids), and it appears that the integrity of the hexameric structure is critical for DNA binding (7). DNasel footprinting on over 20 chromosomal targets has revealed defined protection regions ranging in size from 24 to over 100 contiguous base pairs (3, 4, 8–11).

Examination of these regions has failed to identify a simple DNA consensus sequence that can be unequivocally assigned as a recognition determinant (11). It is believed that AbrB recognizes a specific three-dimensional DNA structure that can be assumed by a finite subset of differing base sequences (1, 8, 11, 12). Using in vitro selection of random oligonucleotides, we have isolated seemingly optimal AbrB-binding sites that possess two to four occurrences of a 5-bp motif (TGGNA) separated by defined spacings (12). However, multiple regularly spaced examples of this motif are rare in chromosomal binding sites. It is thought that such optimal sites are infrequently used in vivo because they are incompatible with promoter structures and with the mechanism responsible for rapid relief of repressive effects due to AbrB-DNA interactions.

Mutations in the promoter and coding regions of abrB have been isolated, and a few of them have been sequenced and characterized (7, 13–19). In this communication, we report the sequence of additional abrB mutations and the functional analysis of mutant AbrB proteins and promoters. Three amino acid residues critical for either DNA binding or multimeric associations were identified. The differential biochemical properties of four mutant proteins suggest that the AbrB monomer might be divided into separable DNA-binding and multimerization domains.

Three mutations in the −35 region of the major abrB promoter (13) were analyzed with respect to in vivo promoter strength and their being subject to autoregulation (7). Our results resolve a previously noted (13) paradox: one class of mutations producing an AbrB phenotype actually results in a perfect consensus to the −35 region sequence utilized by σ^A RNA polymerase. We demonstrate that in the absence of regulation, this promoter is indeed more actively transcribed, but that the mutation responsible concomitantly strengthens AbrB binding affinity, increasing the degree of vegetative autoregulation in vivo, thus accounting for the observed AbrB phenotype. Significantly, this mutation produces a sequence that more closely resembles an optimal binding site selected in vitro. A promoter down mutation that diminishes AbrB binding affinity shows less similarity to the optimal in vitro site. Both of these binding site/promoter mutations affect AbrB interaction only with the higher affinity portion (−14 to −43) of the total binding region (−14 to −125).

**EXPERIMENTAL PROCEDURES**

Bacterial Strains—Bacillus subtilis strains carrying abrB mutations were obtained from J. A. Hoch (19). Strains with the abrB alleles designated abs24 and tdB24 (14, 15) were obtained from the Bacillus Genetic Stock Center. Escherichia coli DH5α and DM1 were purchased from Life Technologies, Inc. BL21 was from Invitrogen, Inc. All B. subtilis strains containing spo0A alleles, abrB alleles, and abrB-lacZ

**Abbreviations used are:** bp, base pairs; PCR, polymerase chain reaction; TEMED, N,N,N',N'-tetramethylethylenediamine; Q83ter, mutation of Gin residues to a termination codon.

*This research was supported in part by Grant GM46700 from the National Institutes of Health. This is publication 9549-MEM from the National Institutes of Health. This is publication 9549-MEM from the National Institutes of Health.

† Supported by summer internship Grant 503RR03010 awarded to the Scripps Research Institute by the National Institutes of Health.

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1 M. A. Strauch, unpublished results.
fusions were derivatives of J H642 (trpC2 pheA1).

Sequence Determination of abrB Mutations—Chromosomal DNA from abrB strains was prepared by the procedure of Marmur (20). Segments of the abrB gene were amplified using standard PCR techniques. Primers and their position of annealing relative to the P2 start point of transcription (13) were as follows: ABPC4, CTATGGATCCTAGCTCC (nt 1–20); ABPC2, GCTATGGTGAATTTTTGT (nt 221 to 230); ABPC5, AGGCTAGCTCCGGAG (nt 49 to 65); ABPC8, CATATAAAGTTTCTCCCC (nt 77 to 96); and ABPC6, GTACCAAAACCTGTTGACCC (nt 452 to 473). For reference, the coding region of abrB begins at +69 and ends at +356 (13). The ABPC4, ABPC2, and ABPC5 oligonucleotides contained an additional 8 bp on their 5’ ends that efficiently amplified an EcoRI site. ABPC5 and ABPC6 had 5’ extensions specifying BamHI sites. Previous phenotypic analysis and complementation studies (13, 19) had defined the approximate location (i.e. promoter versus coding region) of the abrB mutations. Putative promoter mutations were amplified using ABPC4 (or ABPC2) and ABPC8 primers. Coding region mutations were amplified via ABPC5 and ABPC6 primers. Fragments were ligated into EcorI-BamHI-digested pUC19 and clones obtained as transformants of E. coli DH5α cells. Plasmid DNA was prepared using the anion exchange columns of Qiagen, Inc., and double-stranded sequencing was performed using the sequencing system (U. S. Biochemical, Inc.). At least three independent clones were sequenced for each fragment in order to identify and eliminate any possible PCR-induced mutations.

Protein Purification—Wild-type ABRB was purified as described previously (8). EcoRI–HindII fragments from pUC19 clones containing the coding regions of mutant abrB genes were inserted into the expression vector pKQV4 (8). The mutant proteins were overexpressed and purified using the following modifications of the procedures developed for wild-type ABRB. For AbrB1, E. coli DH5α was the host strain; a 0–30% (NH₄)₂SO₄ fraction contained the protein, and it was obtained in purified form (>95% purity) as a flow-through fraction (10 ml KCl) from a DEAE-triacylsar column fractionation. For AbrB19, E. coli DM1 was the host; the 40–50% (NH₄)₂SO₄ fraction contained the protein; and purified (95%) protein was eluted from a DEAE-triacylsar column using a 2.5–50 mM KCl gradient. For AbrB100, E. coli DH5α was the host; a 40–50% (NH₄)₂SO₄ fraction contained the protein, and it was collected in purified (>95%) form in a flow-through (10 ml KCl) fraction from DEAES-triacylsar. For AbrB23, E. coli B21 was the host; cells were grown at 37°C to an A₆₀₀ of 0.6, shifted to 30°C and induced (1 mM isopropylthio-β-galactoside) for 4 h; the protein was collected in a 40–50% (NH₄)₂SO₄ fraction and purified by DEAE-triacylsar and heparin agarose (2.5–50 mM KCl gradient) column fractionations.

Determination of Molecular Weights—Native polyacrylamide gel electrophoresis was performed using a modification of the procedures described in Hedrick and Smith (21) and the Sigma Technical Bulletin MKR-137. A series of slab gels (0.8 × 150 × 200 mm) containing polyacrylamide concentrations of 5, 6, 7, 8, 9, and 10% (separating gel) were used. All gels utilized a 4–16% acrylamide stacking gel. The electrophoresis was performed using a modification of the procedures described in Hedrick and Smith (21) and the Sigma Technical Bulletin MKR-137. The electrophoretic mobilities of the proteins were determined for each. Lines were fitted using linear regression. The percentage of gel electroforessed into the native polyacrylamide gel determinations underestimate the molecular weights of the mutant proteins and the wild type by native polyacrylamide gel electrophoresis (Table I). Two of the mutants, as well as wild type, appeared as two bands: a major (80%) and a minor form. The major band of wild type exhibited an approximate molecular weight of 54,000 in this assay. AbrB23 showed a similar banding pattern and molecular weight as wild type. AbrB1 and AbrB100 formed a single band in these native gels, and their molecular weights were approximately half that of the major species of wild type. The major form of AbrB19 exhibited a molecular weight similar to that of AbrB1 and AbrB100. These results indicated that the alternations C56Y, L69P, and Q83ter weakened or destroyed subunit interactions, whereas R255 did not.

We feel that the molecular weight values obtained from our native polyacrylamide gel determinations underestimate the true sizes of the proteins. A previous study (7) using gel filtration chromatography indicated that wild-type ABRB was a hexamer with a molecular weight of 63,000. The discrepancy between the two types of determinations is probably related to the electric charge of the proteins.

DNA Binding Assays—Gel retardation and DNase I footprinting assays were performed as described previously (8). Plasmid pMAS3011 (22), which contains the 38-bp AbrB-binding site from the spo0E gene in the polylinker region of pSE280 (23), was the source of the control spo0E site. The 76-bp NheI–Hpal fragment of pMAS3011, end-labeled using [32P]dATP and Klenow enzyme, was used in gel retardations for determination of the EcoRI–HindII fragment (labeled EcoRI) of pMAS3011 was used. EcoRI–HindII fragments (labeled at EcoRI) of plasmids (pUC19 derivatives) containing in vitro selected optimal AbrB-binding sites (12), and EcoRI–BamHI fragments (labeled at BamHI) of the clones containing wild-type and mutant abrB promoters were also used in DNase I footprinting experiments as described under “Results.”

β-Galactosidase Assays—Mutant abrB promoters (EcorI–BamHI fragments) were transferred from the pUC19 clones (used for sequencing) into the lacZ fusion vector pDH32 (24). The fusions were inserted in B. subtilis strains as single-copy integrants at the amyE locus using standard transformation procedures (25). J H21600 (26) was used as the source of a wild-type ABRB–lacZ fusion. β-Galactosidase assays were performed as described (26).
proteins might still possess some degree of binding affinity to other sites, we employed a pool of oligonucleotides having a central portion of 44-bp randomized positions as the substrate in gel retardation assays of binding. Wild-type AbrB is capable of binding a fraction of the random oligonucleotides, and, in fact, this methodology was the basis of selecting optimal in vitro AbrB binding sequences (12). However, even at concentrations at least 5-fold higher than those required to see wild-type AbrB binding, AbrB23(R25S) gave no evidence of significant binding to any substrates in the pool (Fig. 2). A small portion of the pool was bound only by higher concentrations of AbrB1 (C56Y), whereas AbrB19(Q83ter) and AbrB100(L69P) were capable of bindingsome of the substrates present, albeit at reduced affinity relative to wild type (Fig. 2).

We did not undertake an analysis of the oligonucleotide sequences bound by the mutant proteins, so it was not known if they represented the same classes as those bound by wild type or if the binding activities of the mutant proteins were also altered in specificity. To examine if the altered DNA binding properties of the mutant proteins still retained specificity characteristics of the wild type, we performed DNase I footprinting experiments on three different substrates. No protection by AbrB1 or AbrB23 was seen using the spo0E site, but AbrB100 was observed to protect the same region as wild type, although much higher protein concentrations were required (Fig. 3). Only partial protections of the spo0E site were observed using the higher concentrations of AbrB19 (Fig. 3). The BS16 and C34 sites had been selected in vitro from pools of random sequence oligonucleotides (12) and gave 25- and 45-bp DNase I footprint regions using wild-type AbrB. AbrB23 did not bind to these sites, whereas AbrB1, AbrB19, and AbrB100 did show

5 The sequences of the DNase I protected regions are: BS16, TTCAACTGGGAAATATGCTCCTCCA, and C34, CAGATGGAAACA-TATGTAATCTAGGGAATATGTGGTGCCCTAA.
Fig. 3. DNaseI footprints of AbrB proteins on the spo0E binding site. Protein concentrations are 20 (lanes a), 4 (lanes b), and 2 μM (lanes c). Lanes R and Y are Maxam-Gilbert purine and pyrimidine sequencing reactions, respectively. The DNA target fragment is described under “Experimental Procedures” and was obtained from a clone of the 38-bp portion of the spo0E gene that corresponds to the AbrB-binding site. The sequence of this site is AATATGTTTACAAATAAGTATAATCTGTAATAATGCA. It can be read from the sequencing ladder adjacent to the AbrB protection regions by using its complement and reading from bottom to top.

Analysis of AbrB Proteins—Among the mutations giving an AbrB phenotype, a number were located in the –35 region of the major promoter (P2), occurring at three different positions (see above). To confirm that each of these (represented by abrB8, abrB11, and absB24) lowered expression of abrB, we constructed lacZ fusions (see “Experimental Procedures”) and examined them in vivo levels of transcription. Our initial assays were performed in a wild-type background as well as in one (spo0A abrB) that removes the known regulators of abrB transcription (7, 13). In the wild-type background, all three mutant promoters gave lower expression levels than the control, nonmutant promoter (data not shown). In the spo0A abrB strain, the abrB11 and absB24 promoters were still deficient, but, somewhat unexpectedly, the abrB8 promoter was stronger than the wild-type promoter (data not shown). This indicates that changing the abrB promoter to have a perfect –35 σ70 consensus (TTGACA as in abrB8) does produce a stronger promoter but only in the presence of Spo0A, AbrB, or both.

For a number of reasons, we suspected that the absence of AbrB was responsible: 1) Spo0A is a repressor of AbrB (28), and spo0A strains overexpress AbrB (13); 2) results had shown that the mutant promoter did not express well in spo0A mutants (13); and 3) the AbrB autoregulatory binding site encompasses the –35 region, whereas the Spo0A binding site does not (7, 8, 28). To test this hypothesis, we examined abrB8 transcription in a number of genetic backgrounds. The presence of AbrB repressed abrB8 transcription regardless of the nature of the spo0A allele (Fig. 4A). These results implied that the single base pair mutation of abrB8 increased both the intrinsic strength of the promoter and the affinity of AbrB binding to the autoregulatory site. Because abrB8 gives an AbrB-phenotype, the net result must be a lowering of the intracellular AbrB levels. The results of Fig. 4B confirm this and demonstrate that the lower AbrB levels transcribed from an abrB8 promoter are capable of repressing an abrB-lacZ fusion in vivo.

Final proof that the abrB8 mutation results in a stronger AbrB binding affinity was obtained by in vitro DNaseI footprinting assays (Fig. 5). AbrB was able to protect the –14 to –43 region of the abrB8 promoter at much lower protein concentrations than those needed to see protection of the corresponding region of the wild-type promoter. Binding to the upstream (weaker affinity) region from –44 to –125 was largely unaffected by abrB8. We note that the region in the vicinity of –81 of the abrB8 promoter is more susceptible than the wild-type promoter to DNaseI cleavage, both in the presence and the absence of bound AbrB. This relative hypersensitivity implies that the single base pair change of abrB8 at –29 can in some way affect the overall DNA structure as far away as –81. We do not know the nature of this alteration, but it does not appear to significantly affect overall AbrB binding to the upstream region.

We also performed footprinting experiments on the abrB11 and absB24 promoters (Fig. 5). The abrB11 mutation caused no visible change in AbrB binding extent or affinity. The absB24 mutation resulted in a complete loss of AbrB binding at –14 to –43, but the affinity of binding at –44 to –125 was not significantly altered, and, in fact, the DNaseI cleavage site at –81 was protected by AbrB.

DISCUSSION

We have sequenced a number of previously uncharacterized abrB mutations, including two, tolB24 and absB24, which are now proven to be abrB alleles. The majority of mutations located within the abrB coding region are highly disruptive (deletions, insertions, frameshifts, and nonsense). Only two different naturally occurring mutations that change a single amino acid residue were discovered. Perhaps many more point mutations changing single residues are phenotypically silent.

The AbrB23 protein (R25S) retains a hexameric structure but has completely lost specific DNA binding activity (Fig. 3). It is likely that it is also devoid of nonspecific DNA binding activity (see Fig. 2). This indicates that Arg25 is probably a critical residue involved in interaction of the hexamer with DNA, but the exact nature of the defect caused by its substitution with serine is unknown. Deducing the roles of the residues altered due to abrB1 (C56Y), abrB100 (L69P), and abrB19 (Q83ter) is more problematic. In each case, the mutant proteins exhibit altered subunit interactions, resulting in the predominant multimeric forms being smaller than wild type (Table I). As expected, all three are also defective in DNA binding properties but not to as severe an extent as AbrB23 (Figs. 2 and 3). However, these data do not allow a definite conclusion that the sole roles of Cys56, L69, or the carboxyl-terminal 14 amino acids are in formation of the multimer; some or all may also directly interact with DNA.

The C56Y, L69P, and Q83ter alterations do not completely destroy multimerization. The major proportion of the mutant proteins are in either trimer or tetramer forms with no evidence of significant dissociation into monomers. Although Cys56 is the sole cysteine residue in the monomer, it is unlikely that it participates in disulfide bonds between subunits. The results of the native gel determinations of molecular weights presented here indicate that AbrB1 is a tetramer, but a previous determination using gel filtration showed that a C56Y protein retained the hexameric configuration (7). We believe this discrepancy is related to the different methods used and that the Cys56 → Tyr change probably weakens subunit interactions with the effect being apparent only under the conditions of the native gel method (e.g. migrating in an electrical field). Additionally, using iodoacetamide and nonreducing gel methods (29, 30), we have found no evidence that disulfide bonds exist in wild-type AbrB.  

Recently, within the context of the B. subtilis genome sequencing project, two genes encoding proteins with high amino acid homology to AbrB have been discovered (31, 32). One...
encodes a 92-amino acid protein whose first 50 residues show 74% identity to residues 3–52 of AbrB (31). The other, designated spoVT, encodes a 178-amino acid protein whose first 51 residues show 68% identity to AbrB residues 3–52. Although the DNA binding properties of these proteins have not yet been reported, they both have been shown to affect transcription of genes associated with stationary phase and sporulation.7,8 Given that AbrB and these two proteins are regulators of gene expression during a differentiation process, it is tempting to speculate that the shared homology over the first 50 or so amino acids represents the Bacillus equivalent of the eucaryotic homeodomain (33, 34). Functional similarities between AbrB and homeodomain proteins have previously been noted (2).

A high proportion of the total number of abrB mutations examined occur in the promoter region. This probably reflects a bias in the original methods used to select abrB mutations as partial revertants of spo0A phenotypes. It is now known that spo0A abrB” cells grow faster than spo0A abrB− (3, 4). Because some promoter mutations could still express low levels of active AbrB (too low, however, to give an AbrB” phenotype for the characteristic used in the selection process), these might have grown faster and been more noticeable in the selection. Supporting this conclusion is the fact that for some AbrB phenotypes there is a detectable difference between promoter and coding mutations (3, 13, 19).

The −35 region of the abrB promoter has one mismatch to the 5A consensus, but six identical independently isolated mutations presented a paradox: they resulted in a perfect −35 consensus. We have solved this paradox by showing that although the mutation to the perfect consensus does result in a stronger promoter, it concomitantly results in stronger negative autoregulation due to an increased affinity for AbrB binding in the region (Figs. 4 and 5).

Autoregulation of abrB had previously been correlated with...
AbrB binding to the –14 to –125 sequences of the promoter (7, 8). Within this large region, the area from –14 to –43 appeared to have higher binding affinity than the rest, as judged by DNase I footprinting assays (8). Our in vivo expression studies and in vitro footprinting assays of AbrB binding to the mutant abrB8 and absB24 promoters provide evidence that binding at the –14 to –43 sequences is the major component responsible for autoregulation. Although the abrB8 mutation increases the binding affinity of AbrB at –14 to –43, it does not have an effect on the binding affinity to the upstream region (Fig. 5). As discussed above, abrB8 is more strongly autoregulated than wild type. However, analysis of the absB24-IacZ fusion revealed only a 2–4-fold difference between wild-type and spo0A abrB backgrounds during vegetative growth (data not shown), suggesting that only autoregulation had been removed because this magnitude of difference is identical to that seen for the native promoter in wild-type versus spo0A−abrB+ cells (7). As seen in Fig. 5, AbrB can still bind to the –44 to –125 sequences of the absB24 promoter but does not bind at –14 to –43. Furthermore, AbrB binding affinity to the upstream (–44 to –125) region is independent of the presence (abrB8) or the absence (absB24) of AbrB bound at –14 to –43, indicating that no cooperativity exists between the regions. The role of the upstream binding region is unknown, but perhaps it functions to increase the localized concentration of AbrB immediately upstream of the medium-binding region.

In a previous study (12), we had selected optimal in vitro AbrB-binding sites that were characterized by regularly spaced repeated examples of a 5-bp motif (TGGNA; TNCCA on the opposite strand) and had shown that they could confer AbrB-mediated regulation in vivo. Nevertheless, examination of native AbrB-binding regions did not reveal a significant occurrence of these regularly spaced motifs. This seeming incongruity was explained by the hypothesis that optimal AbrB binding sites were probably incompatible with the function of most promoters and with the mechanism that leads to the rapid release of AbrB-mediated repression at the onset of stationary phase. Our footprinting results (Fig. 5) on the wild-type and mutant promoters support this hypothesis. The relative orientation of the in vitro selected motifs did not appear to be a critical factor for AbrB binding, and one class had examples of the type TNCCA-5bp-TGGNA. From –34 to –20 of the abrB promoter is a 15-bp sequence with partial homology to this arrangement of dual motifs. The downstream motif (–24 to –20) is a perfect match to TGGNA, but the aligned upstream motif only matches two of the defined positions in TNCCA (Fig. 6). However, the abrB8 mutation, which increases AbrB binding affinity (Fig. 5), results in three matches in the upstream motif; the absB24 mutation shows only one match and lowers AbrB binding affinity; the abrB11 mutation only changes the variable position (retaining the two wild-type matches) and has a binding affinity comparable with the unaltered promoter. Additionally, dual motifs found in the in vitro selected binding sites were usually spaced 5 bp apart (12). That this spacing (which places the motifs on the same face of the helix) is important for AbrB binding was evidenced by an in vitro generated variant of the –43 to –14 abrB region that possessed a deletion of two bases (Ts at –27 and –28) between the wild-type TGACG and TGGAA motifs and did not bind AbrB in DNase I and methylation protection assays.10

Acknowledgments—We are grateful to the Stein Endowment Fund for assistance in purchasing digoxigenides.

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9 In addition to the results in the cited reference, we have performed numerous control assays and consistently observed a 2–4-fold difference in abrB expression in wild type versus spo0A. The native promoter is further derepressed due to an abrB mutation (i.e., greater than 10–20-fold difference between wild-type and spo0A abrB backgrounds).

10 M. A. Strauch, unpublished results.