Specific Binding of the E2 Subunit of Pyruvate Dehydrogenase to the Upstream Region of Bacillus thuringiensis Protoxin Genes*

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During sporulation, Bacillus thuringiensis produces inclusions comprised of different amounts of several related protoxins, each with a unique specificity profile for insect larvae. A major class of these genes designated cry1 have virtually identical dual overlapping promoters, but the upstream sequences differ. A gel retardation assay was used to purify a potential regulatory protein which bound with different affinities to these sequences in three cry1 genes. It was identified as the E2 subunit of pyruvate dehydrogenase. There was specific competition for binding by homologous gene sequences but not by pUC nor Bacillus subtilis DNA; calf thymus DNA competed at higher concentrations. The B. thuringiensis gene encoding E2 was cloned, and the purified glutathione-
S-transferase-E2 fusion protein footprinted to a consensus binding sequence within an inverted repeat and to a potential bend region, both sites 200–300 base pairs upstream of the promoters. Mutations of these sites in the cry1A gene resulted in decreased binding of the E2 protein and altered kinetics of expression of a fusion of this regulatory region with the lacZ gene. Recruitment of the E2 subunit as a transcription factor could couple the change in post exponential catabolism to the initiation of protoxin synthesis.

Most Bacillus thuringiensis subspecies contain multiple, plasmid-encoded protoxin genes that are very actively transcribed primarily during sporulation (1, 2). There is extensive synthesis of the related protoxins which are often packaged into the same inclusion. Each of these protoxins has a somewhat different specificity (7), and there may be synergism among some (8, 9).

Many but not all of these genes contain very similar overlapping promoters (2, 3) recognized in the mother cell during sporulation by qK and qK forms of RNA polymerase (2, 4). Dual promoters ensure transcription of these particular genes (cry1) throughout much of sporulation1 (5), but they are differentially transcribed (6). Control of expression of the cry genes is necessary not only to ensure a balance of transcription with mothercell spore genes which utilize the same forms of RNA polymerase but for regulating the relative amounts of the various protoxins and their assembly into an inclusion (2).

Given the similarity of the overlapping promoter regions for three of these cry1 genes, the sequences upstream for −1 kbp were examined and found to differ substantially (10).3, 4 These regions appear to be important for regulation because expression of cry1-lacZ fusion plasmids in B. thuringiensis was enhanced by their presence.5 Employing a gel retardation assay, a novel DNA binding protein was identified and purified, and its gene was cloned. The binding sites in the upstream regions of two of these cry genes were determined by footprinting. The effects of mutations in these sites indicated that this protein is likely to have a role in regulating the expression of this class of protoxin genes.

EXPERIMENTAL PROCEDURES

Cell Growth—B. thuringiensis subsp. kurstaki HD1, strain 80-21 (11), subsp. aizawai HD133, and a plasmid-cured (with mitomycin C) acrystalliferous derivative of B. thuringiensis subsp. kurstaki HD1 designated Mit96 were grown in G-Tris medium at 30 °C (12) in a New Brunswick incubator shaker. This medium contains 0.2% glucose as the principal carbon source, which was replaced with 0.1% potassium gluconate for the β-galactosidase assays (see below). Bacillus subtilis JH642 was grown in nutrient sporulation medium at 37 °C. Growth was monitored by A600 in a Perkin Elmer Model 35 spectrophotometer and sporulation in the phase microscope.

Isolation of Regions Upstream of the Promoters—A region of 280 bp upstream of the cry1A gene was prepared by PCR using oligonucleotides 5'-AATAGGATCCTTCCTATATTTACTTTGCCG-3', containing an EcoRI site.

The region upstream of the promoters of the cry1C gene was isolated as a 656-bp BglII/HindIII fragment from a 7-kbp EcoRI fragment containing this gene, including 2.5 kbp upstream of the promoters (13). This fragment was isolated from low melting agarose with GELase (Epicentre Technologies) and cloned into the HindIII and BamHI sites of pUC18. For gel retardation, this clone was digested with EcoRI and HindIII, and the 656-bp fragment was reisolated as described above.

The cry1D (14) was cloned as a 3.8 kbp KpnI fragment from B. thuringiensis subsp. aizawai HD133, strain 5 (11), into the Escherichia coli B. thuringiensis shuttle vector pH73101 (15). A 2.2-kbp NdeI fragment embracing most of the coding region was deleted from this plasmid, creating p1D from which the cry1D upstream region including the promoters was isolated as a 1.6 kbp KpnI-NdeI fragment.

The E2 binding site containing a potential bend region was mutated from 5'-CTCAATTTGTGATATGAAAAGGAAATG to 5'-CTCAGTCTCTTCTATGAGAACGACAATG (bold letters indicate changes including the creation of an MspHI site for screening) employing oligonucleotide 5'-CCTCTTTCCTGTTGTCAAATAGCAACTGAG. The inverted repeat (IR; see Fig. 4) was mutated from 5'-CCCTGAAATCTTTGTTGAAATG to 5'-CCCTGAAATCTTTGTTGAAATG with the introduction of a PsI site for screening. The cry1Ab upstream region as a 310-bp HindIII fragment (see Fig. 4) was cloned into pGEM 11(++) for production of single-stranded template in E. coli C326 (duT, ung-). Mutagenesis followed the procedure of Kunkel (16), and plasmids produced in E. coli DH5α were screened initially for the

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2 The abbreviations used are: kbp, kilobase pairs; IR, inverted repeat; bp, base pairs; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PDH, pyruvate dehydrogenase; GST, glutathione S-transferase.

3 M. Geiser, personal communication.

4 A. Aronson, unpublished results.
with the heated, 50–60% ammonium sulfate fraction from strain Mit9. Retardation was observed when protein and 32P-labeled DNA were mixed in 20 mM Tris, 20 mM NaCl, 10 mM MgCl2, pH 7.5, and incubated with 10 units of T4 polynucleotide kinase and 300 μCi of [β-32P]ATP (150 mCi/mmol) per μg of DNA for 90 min at 37 °C in a total volume of 25 μl. To remove the label from one end, the 32P-PDNA was digested with BanHI for footprinting the transcribed strand and with EcoRI for the nontranscribed strand. The labeled DNA was purified by excision from a low-melting agarose gel and digestion with GELase.

Gene Retardation Assays—Retardation assays (19) were performed in 3.5% native polyacrylamide gels (acylamide:bisacylamide ratio of 19:1) as per Fried and Crothers (20) and Garner and Revzin (21). After investigating several buffers, it was found that the best retardation was observed when protein and 32P-labeled DNA were mixed in 20 μl of 0.5 × TBE and incubated at 16 °C for 20 min. Five μl of bromphenol blue-xylene cyanol in 5 μl sucrose was added to the samples, which were loaded onto vertical gels prepared in 0.5 × TBE. One-mg fractions were collected, concentrated 4-fold by lyophilization, and dialyzed against 0.5 × TBE. Fractions were assayed for binding activity as described below.

Purification was also performed by excising and eluting the retarded DNA band from polyacrylamide gels. Elution was performed in a Little Blue Tank elutrap system (ISC0) using 0.05 × TBE in the sample wells and 0.5 × TBE in the electrophoresis chamber. The DNA-protein complex concentrated in this way was dissociated by addition of KCl to 0.4 M for 8 h at 27 °C. The DNA was then digested for 1 h at 37 °C with 20 units of DNase I, and the protein was fractionated in 10% SDS-PAGE (16). The gel was transferred to polyvinylidene difluoride and stained (18). Protein bands were excised, and the sequence of the first 25 amino acids was determined in an automated sequenator (Purdue Center for Macromolecular Structure).

DNA Labeling—DNA was treated with calf intestinal alkaline phosphatase (CIP; 2 units per mg DNA) at 37 °C for 45 min, extracted with phenol and precipitated with ethanol. The DNA was then dissolved in 10 mM Tris, 20 mM β-mercaptoethanol, 10 mM MgCl2, pH 7.5, and incubated with 10 units of T4 polynucleotide kinase and 300 μCi of [γ-32P]ATP (150 mCi/mmol) per μg of DNA for 90 min at 37 °C in a total volume of 25 μl. To remove the label from one end, the 32P-PDNA was digested with BanHI for footprinting the transcribed strand and with EcoRI for the nontranscribed strand. The labeled DNA was purified by excision from a low-melting agarose gel and digestion with GELase.

Cloning the pdhC Gene from B. thuringiensis—The pdhC gene encoding the E2 subunit of pyruvate dehydrogenase (PDH) from B. thuringiensis was cloned as a fusion with the glutathione S-transferase gene in the pGEX-KG expression vector (22). PCR oligonucleotides: 5′-TAGGAGGTCGGGATCCGTGGCATTTGAATT-3′ containing a HindIII site in the insert and then sequenced to confirm the changes. The mutagenized HindIII fragment was isolated from the pGEM clone as described above, and the fragment inserted into the single HindIII site in a lacZ fusion vector containing the cryIA promoter region (see below). The orientation was established on the basis of the sizes of analogous and heterologous DNAs (A and B) or by different sources of the binding protein (C). A, lane 1, 100 ng of 32P-cryIA fragment; lane 2, plus 8 μg of total protein from a crude extract of strain Mit9; lane 3, 4 μg of the 50–60% ammonium sulfate fraction from Mit9; lane 4, as in lane 3 plus 200 ng of pUC18; and lane 5, as in lane 3 plus 200 ng of the cryIC fragment. B, lane 1, 2 μg 32P-cryIA fragment; lane 2, plus 2 μg of the Mit9 heparin-agarose fraction containing 1.5 μg of E2 antigen, lanes 3 and 4, as in lane 2 plus 40 or 100 ng of sonicated calf thymus DNA, respectively; lanes 5 and 6, as in lane 2 plus 10 or 20 ng of cryIA fragment, respectively. Panel C, lane 1, 2 ng 32P-cryIA fragment; lanes 2 and 4, as in lane 1 plus either 2.5 or 5 μl of the 50–60% ammonium sulfate fraction from Mit9 containing 2 or 4 μg of E2 antigen, respectively; lanes 3 and 5, as in lane 1 plus 3 or 6 μl of the 50–60% ammonium sulfate fraction from B. subtilis containing 2 or 4 μg of E2 antigen, respectively.

FIG. 2. Gel retardation assays of the competition by homologous and heterologous DNAs (A and B) or by different sources of the binding protein (C). A, lane 1, 100 ng of 32P-cryIA fragment; lane 2, plus 8 μg of total protein from a crude extract of strain Mit9; lane 3, 4 μg of the 50–60% ammonium sulfate fraction from Mit9; lane 4, as in lane 3 plus 200 ng of pUC18; and lane 5, as in lane 3 plus 200 ng of the cryIC fragment. B, lane 1, 2 μg 32P-cryIA fragment; lane 2, plus 2 μg of the Mit9 heparin-agarose fraction containing 1.5 μg of E2 antigen, lanes 3 and 4, as in lane 2 plus 40 or 100 ng of sonicated calf thymus DNA, respectively; lanes 5 and 6, as in lane 2 plus 10 or 20 ng of cryIA fragment, respectively. Panel C, lane 1, 2 ng 32P-cryIA fragment; lanes 2 and 4, as in lane 1 plus either 2.5 or 5 μl of the 50–60% ammonium sulfate fraction from Mit9 containing 2 or 4 μg of E2 antigen, respectively; lanes 3 and 5, as in lane 1 plus 3 or 6 μl of the 50–60% ammonium sulfate fraction from B. subtilis containing 2 or 4 μg of E2 antigen, respectively.

presence of an additional MspHI or PstI site in the insert and then sequenced to confirm the changes. The mutagenized HindIII fragment was isolated from the pGEM clone as described above, and the fragment inserted into the single HindIII site in a lacZ fusion vector containing the cryIA promoter region (see below). The orientation was established on the basis of the sizes of HpaI restriction fragments.

Purification of the Binding Protein—Strain Mit9 was grown in 2 liters of G-Tris medium until about 70% of the cells contained phase bright endospores. B. subtilis JH642 was grown in 500 ml of nutrient sporulation medium until >50% contained bright endospores (the maximum is about 70% versus >90% for Mit9). Cells were harvested by centrifugation and washed with 20 ml of 100 mM KCl, 5 mM EDTA, pH 8.0. The pellets were suspended in 1/5 volume of buffer A (50 mM Tris, pH 7.4, 1 mM EDTA, 100 μg/ml phenylmethylsulfonyl fluoride), washed twice with this buffer, resuspended in 1/50 the original volume of buffer A plus 1 mM dithiothreitol, and lysed in a French press at 9000 p.s.i. The lysate was centrifuged at 4500 × g for 10 min at 4 °C. The supernatant was withdrawn and centrifuged in an Eppendorf microcentrifuge for 10 min at 4 °C. This supernatant was then heated to 45 °C for 10 min to inactive DNases while leaving binding activity unaltered.

The heated extract was fractionated by the addition of solid (NH4)2SO4 to final concentrations of 25, 40, 50, 60, 80, and 100% of saturation. The protein precipitate after each step was collected by centrifugation at 8000 × g for 20 min at 4 °C, dissolved in 0.5 × TBE (0.04 M Tris, 0.04 mM sodium borate, 2 mM EDTA, pH 8.0), and dialyzed overnight at 4 °C against 2 changes of 2 liters each of this buffer. Each fraction was then tested for binding activity as described below.
BamHI site and 5'-ATAGGGGAATCTCGAGCTACCAACATTTA-3' containing a XhoI site were based on the sequences of the B. subtilis pdhC gene (23) and used to clone a 1350-bp region of DNA from B. thuringiensis into E. coli. It was sequenced and had a deduced open reading frame encoding a polypeptide of the expected size.

A fusion protein consisting of B. thuringiensis PDH-E2 fused to glutathione S-transferase was produced after transformation of pCB117 into E. coli TG1 and induction with isopropyl-1-thio-galactopyranoside (22). The fusion protein that was purified by elution from a glutathione-agarose column (24) reacted with anti-PDH-E2 antibody from Staphylococcus aureus, kindly provided by Dr. H. Hemila (23). The glutathione S-transferase could not be removed by thrombin digestion without disrupting the B. thuringiensis PDH-E2 protein, which contains an internal thrombin cleavage site.

To obtain a less extensive E2 fusion protein, this gene was also cloned into the His6 expression vector, pQE30 (Qiagen). The clone was grown in LB-ampicillin (25 \( \mu \)g/ml) and expression was induced by addition of 1 mM isopropyl-1-thio-galactopyranoside for 3 h. Cells were lysed as per the Qiagen manual. Although there was substantial His\(_6\)-E2 in the soluble fraction, the E2 protein solubilized from the pellet with 6 M urea in 0.05 M Na\(_2\)HPO\(_4\), 0.3 M NaCl, pH 8.0 was most active in gel retardation. The latter was fractionated on a Ni\(^{2+}\)-agarose column using a step gradient of 0.1–0.3 M imidazole in the above buffer. After dialysis for 18 h at 4 °C against 4000 volumes of 0.5 M TBE, fractions were assayed for gel retardation activity, and the most active fraction was stored at −80 °C.

**DNase I Footprinting**—Fifty ng of purified, end-labeled DNA was incubated with varying amounts of the E2 protein and 5 µg of poly(dIdC) in 20 µl of 0.5 × TBE. The binding reaction was carried out for 20 min at 16 °C, after which 0.05 units of DNase I (Boehringer Mannheim) in 12.5 mM MgCl\(_2\) was added and the tubes incubated at 25 °C for 90 s. The reaction was stopped by the addition of 5 µl of 30 mM EDTA and extraction of the mixture with phenol-chloroform. The DNA was precipitated with 2 volumes of ethanol and dissolved in Sequenase stop buffer (United States Biochemical). After heating at 90 °C for 5 min, samples were loaded onto a 6% polyacrylamide gradient gel containing 8 M urea in 90 mM Tris, 89 mM borate, 2.5 mM EDTA, pH 8.3 (16) and electrophoresed at 1000 V and 75 mA. Gels were then dried and autoradiographed.

DNA sequence was obtained according to the standard method for double-stranded DNA sequence analysis (16) with the addition of Mn\(^{2+}\) to the reaction mix to allow sequence determination near the primer (25).

**β-Galactosidase Activity**—A cryIAb promoter-lacZ fusion was constructed in an E. coli/Bacillus shuttle vector. A HindIII-digested, PCR-cloned 310-bp upstream fragment from the cryIAb gene (containing the region from −218 to −528; see Fig. 5) was inserted in both orientations into a single HindIII site upstream of the protoxin promot-

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**Table 1**

Comparisons of the amino acid sequences of the N-terminal 25 residues of PDH E2 proteins from several Gram (+) bacteria with that from Bacillus thuringiensis

| Bacterial species | Sequence of E2 protein | % Identity |
|-------------------|------------------------|-----------|
| Bacillus thuringiensis | AFEFKLPDIGEGIHEGEIVKP | 80 |
| Bacillus subtilis | AFEFKLPDIGEGIHEGEIVKVF | 92 |
| Bacillus steareothermophilus | AFEFKLPDIGEGIHEGEIVK | 87 |
| Staphylococcus aureus | AFEFKLPDIGEGIHEGEIVKVF | 92 |
| Enterococcus faecalis | AYQFKLPDIGEGIHEGEIVKVF | 80 |

*Sources are given in Refs. 24 and 40. Letters in bold indicate differences (mostly conserved) from the B. thuringiensis sequence.*

**Fig. 4. Footprints of the transcribed (panel A) and nontranscribed (panel B) strands of the cryIAb upstream region DNA (50 ng).** The E2-GST fusion protein was added in amounts of 60 ng (lane 2A) and 150 ng (lane 3A) or 10, 50, and 200 ng in lanes 2B, 3B, and 4B, respectively. Arrowheads indicate DNase hypersensitive sites.
ers. This fragment containing the mutated bend or IR regions was also inserted into this vector, and the plasmids were electroporated (28) into strain 50–21. Cells were grown in G-Tris medium (12) containing 0.1% yeast extract and 0.1% potassium glutamate plus 5 μg/ml chloramphenicol. Differences in induction of β-galactosidase (but not growth) were more evident when potassium glutamate rather than glucose was the primary carbon source. Samples of 0.2 ml were taken at various times during growth and sporulation, sonicated for 10 s, and 30–50 μl were assayed for β-galactosidase activity (27) as modified by Giacomini et al. (28).

Characterization of the Binding Protein—


tation of the PDH E2 Subunit

Gene-specific Binding of the PDH E2 Subunit

Results

Binding Activity in Sporulating Cells—The 50–60% (NH₄)₂SO₄ fraction of a crude extract from sporulating but not from growing cells of B. thuringiensis strain Mit9 retarded a 280-bp fragment from the cry1A upstream region, a 656-bp fragment from cry1C, and an ~1.6-kbp fragment from cry1D (Fig. 1). In all cases, at least a 1,000-fold excess of poly(dC) was present. The further addition of a small excess of homologous unlabeled DNA competed for binding, whereas the same concentration of pUC18 did not (Fig. 2A). Even a 100-fold excess of pUC18 DNA (either linear or as 0.3 + 2.4-kb PvuII fragments) or sonicated B. subtilis DNA did not compete. There was some inhibition of retardation by a 50-fold excess of sonicated calf thymus DNA (Fig. 2B).

The specificity of this binding was confirmed by footprinting (Figs. 4 and 6) as well as by the effectiveness of a B. thuringiensis protein fraction as compared with that from B. subtilis (Fig. 2C). There was greater retardation of the cry1A fragment by equivalent amounts of E2 in the 50–60% ammonium sulfate fraction from B. thuringiensis Mit9 than that from B. subtilis JH642 (Fig. 2C). A comparable preparation from E. coli did not retard.

Retention of 1 pmol of the cry1Ab fragment was complete when incubated with about 2 μg of the 50–60% ammonium sulfate fraction from B. thuringiensis. Complete retardation of the cry1C and cry1D DNAs required 10–20-fold more protein, indicating lower affinities and/or more binding sites. There were several degrees of retardation of these DNAs (arrows on the right panel of Fig. 1) in contrast to the cry1A DNA. The presence of multiple retarded complexes may be because of several binding sites with different affinities for the protein.

Characterization of the Binding Protein—The binding protein was isolated by electroelution of an excised, retarded band followed by dialysis and digestion with DNase I. This procedure resulted in recovery of a single protein of ~60 kDa (Fig. 3). The sequence of the first 25 residues of this band was 92% identical to that of dihydrolipoamide acetyltransferase, the E2 subunit of PDH from B. subtilis and Bacillus stearothermophilus (Table I). The E2 subunit is a 48-kDa protein containing lipoic acid (which decreases its mobility in SDS-PAGE).

Because sufficient quantities of E2 could not be renatured after elution from the retarded complex, the 50–60% (NH₄)₂SO₄ fraction was further purified by elution from a heparin-agarose column with a KCl gradient. The greatest binding activity (based on retardation of cry1A DNA) was in the 0.2–0.3 M KCl fraction that contained the E2 protein as >50% of the total based on staining and confirmed by immunoblotting with antisera against the B. subtilis PDH complex. The E1α and E1β subunits of PDH eluted at a lower salt concentration, and this fraction retarded poorly (41).

Footprinting of the Binding Sites—The purified glutathione S-transferase fusion protein protected three regions in the 280-bp cry1A DNA (Fig. 4, A and B and Fig. 5). The most distal of these and the inverted repeat share a common sequence. The third region was within a stretch of intrinsically bent DNA (29) with a 10-bp spacing between each of the protected regions. There were also several hypersensitive sites on both strands (arrows). A major protected site in the cry1C DNA was within an inverted repeat (convergent overlying arrows) based on footprinting results with both the transcribed and nontranscribed strands. Start sites of transcription from the BH (α5) and BTH (α8) promoters are indicated by arrows; the ribosome binding site is overlined. Lowercase letters indicate oligonucleotide primers used to construct a 310-bp HindIII PCR fragment of the upstream region. Letters in bold are the HindIII sites.

Function of the Upstream Region in cry1 Gene Transcription—The binding site within the bend region and the IR were mutated as described under “Experimental Procedures.” Both were found to have lower affinities for purified His₆-E2 protein than the wild type fragment (Fig. 7). The apparent Kd value for the wild type, assuming that it was the monomer of the His₆-E2 adduct that bound, was 4–6 nM. E2 is a multimer in the PDH complex (50), however, and it is likely that a multimeric form of this protein is required for binding to DNA. As mentioned above, only His₆-E2 extracted from the crude membrane fraction with 4–6 M urea in buffer was active. The extent of gel retardation by His₆-E2 purified from the E. coli clone was greater than that of E2 purified from B. thuringiensis (Figs. 1 and 7), implying different aggregation states perhaps because of concentration effects or the presence or absence of lipoamide.

Cells containing lacZ fusions with either the cry1A wild type
FIG. 6. Footprint of the nontranscribed strand of the cry1C upstream DNA (50 ng). Lane 1, no protein; lane 2, 1 µg of the heparin-agarose purified fraction; lanes 3–5, 60, 150, and 500 ng, respectively, of the E2-GST purified fusion protein. Protected sequences are indicated as are the DNase I hypersensitive sites (arrowheads). The major protected region is part of an inverted repeat that is very similar in sequence to the one in the cry1Ab sequence (Fig. 5).

FIG. 7. Gel retardation of 0.5 pmol of cry1A wild type (lanes 1–3), mutated IR (lanes 4–7), and mutated bend region (lanes 8–10) DNAs. Lanes 1, 4, and 8, no protein; lanes 2 and 3, 1.0 µg of His₆-E2; lanes 5 and 6, 2.0 µg of His₆-E2; lanes 7 and 10, 3.0 µg of His₆-E2.

or mutant (bend and IR) upstream regions were sampled during sporulation, and the kinetics of β-galactosidase synthesis was determined (Fig. 8). Addition of the upstream DNA resulted in an enhancement of β-galactosidase synthesis. Both the initial rate and the final activity were reduced in strains containing the mutated upstream regions.

DISCUSSION

Regulation of protoxin genes is of interest not only because of their insecticidal properties but from the perspective of how a cell recruits regulatory elements for a group of structural genes that have very likely become part of the genetic repertoire of this Bacillus relatively late in evolution. One aspect of the regulation is the presence of dual overlapping promoters, which ensures a constant rate of transcription of these cry genes during an extended period of sporulation. In addition, each of these genes is independently regulated, so factors other than the promoters must be involved. The sequences upstream of the promoters for the differentially regulated cry1A, cry1C, and cry1D genes differ substantially (although some features are shared; see below) so we began a search for DNA binding proteins.

Extracts of sporulating but not vegetative cells of B. thuringiensis subsp. kurstaki contained a protein that bound to regions of DNA upstream of the cry1Ab, cry1C, and cry1D gene promoters. The major binding protein in the heparin-purified fraction from B. thuringiensis was identified as the E2 subunit of PDH. The purified GST-E2 or His₆-E2 fusion proteins footprinted to specific sites in the cry1A and cry1C upstream regions. The presence of three close binding sites in the cry1A but not the cry1C sequence may account for the higher affinity for the E2 oligomer by the former (Fig. 1) and thus a basis for the differential regulation of these cry 1 genes.

The evidence for specific binding of this novel DNA binding protein is 1) the presence of a consensus binding sequence (5'–AAAGAT/gG/tAA) in two of the three sites in the cry1A sequence and within the inverted repeat in the cry1C sequence. There was also binding to an intrinsically bent region in the cry1A DNA. The binding site(s) in the cry1D DNA have not been mapped. 2) There was optimal competitive binding by the homologous DNA and very little or no competition by nonspecific DNAs such as poly(dI-dC), pUC18, or B. subtilis DNAs. Sonicated calf thymus did compete at higher concentrations (Fig. 2B), probably because of the complexity of sequences in this DNA including potential bend regions. 3) There was no binding to a digest of pUC18, which included a fragment similar in size (about 0.3 kbp) to that of the cry1A upstream fragment. 4) There was higher affinity binding by the E2 protein from B. thuringiensis as compared with that from B. subtilis. While the deduced sequences of the B. thuringiensis and B. subtilis E2 proteins are >80% identical, there is considerably less homology in and around the so-called hinge region. 4 This region of E2 links the lipoyl domain to the E1 and E3 binding sites (30), and it is a potential DNA binding region (37).
The stoichiometry and patterns of DNA retardation indicated extensive cooperativity in the binding, probably involving conformational changes to allow some form of the E2 protein to bind. The icoshedral core of PDH is comprised of 60 E2 subunits (30), so it is likely that some multimeric form is involved in binding as indicated by the requirement of a urea extraction in the purification protocol of His6-E2 from E. coli. The role of lipoamide, which is a component of E2 in the PDH complex, is not known. Whatever conformational changes of the DNA occurred could be reversed by treatment with protease K, demonstrating that the continued presence of E2 was essential. In many respects, the binding was similar to that of the Lrp consensus binding sequence in this promoter region is thus the cry IAb, cry IC, and cry ID genes.

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