Autoregulation of the Plasmid Addiction Operon of Bacteriophage P1*

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The P1 plasmid addiction operon increases the apparent stability of a plasmid that carries it by killing plasmid-free (cured) segregants. The operon consists of a gene encoding an endotoxin responsible for death on mid-free (cured) segregants. The operon was stabilized by an autoregulatory circuit.

Bacteriophage P1, which lysogenizes Escherichia coli as an 100 kilobase pair, low copy plasmid (1), is spontaneously lost at a frequency of about 10⁻⁸ per generation (2). As is the case for other low copy number plasmids, this remarkable stability may be attributed to the combined effects of a partition system that ensures segregation of at least one plasmid to each daughter cell (reviewed in Refs. 3, 4) and an autoregulation system that kills cells cured of the plasmid (5).

There are two P1-encoded addiction proteins. A 126-amino acid toxin, Doc, causes death on curing; an unstable 73-amino acid antidote, Phd, prevents host death while the plasmid is retained. The corresponding genes form an operon in which phd, the antidote gene, precedes doc, the toxin gene (5). Although the antidote is less stable than the toxin, due to degradation by the host-encoded ClpXP protease, it is also synthesized at a higher rate than the toxin, so as to ensure toxin neutralization. Upon loss of the P1 plasmid, proteolytic degradation of the unrepliﬁed antidote unveils the toxic activity of Doc and causes post-segregational cell death (6).

Plasmid addiction elements functionally analogous to Phd/Doc include Cad/CadB of F, the PenP/PemK of R100 (identical to Kis/Kid of R1), and Pand/Pare of RK2 (and RP4). Although their toxin targets may differ and homology among the analogous proteins is weak, the structure of the operons and the details of autoregulation are strikingly similar (7). In this work, we examine the role of Phd and Doc in the autoregulation of the P1 addiction operon and discuss the possible similarity of antidote proteins to each other and to well-studied DNA-binding proteins.

EXPERIMENTAL PROCEDURES

Media—Growth medium was LB broth or LB agar supplemented as needed with antibiotics (10) ampicillin, 100 μg/ml chloramphenicol, 25 μg/ml kanamycin, 30 μg/ml, and spectinomycin, 40 μg/ml, except that drug-resistant lysogens of P1 were grown in media containing 125 μg/ml chloramphenicol or 15 μg/ml kanamycin.

Phage and Bacterial Strains—Standard methods were used for the isolation, growth, manipulation, and storage of a phage (8), P1 phage (9), and E. coli (30) (Table I).

Construction of lacZYA Transcriptional Fusion to the P1 Addiction Promoter—A plasmid, pG3, that contains the P1 addiction operon, including its promoter, was used as a template for a polymerase chain reaction with oligonucleotide primers HAL13 (5'-GGGAATTCGTGATAGCATTACCCGTA-3') and HAL15 (5'-GGGATCCGGATACGCTAAACACTCTGGTA-3'). The resulting DNA product was cloned into the primer-encoded EcoRI and BamHI restriction sites (underlined in primer sequences) and contains the first three codons of phd, the predicted promoter, and additional upstream sequences (nucleotides 5 to 374 (5)).

This DNA was digested with EcoRI and BamHI and cloned into the EcoRI-BamHI sites of the pRS415 transcriptional lacZAYA fusion vector (11) to produce pRDM064. The pRDM064 plasmid was isolated in strain BR6568, in which the P1 addiction operon, provided in trans from the compatible plasmid pG3, repressed expression of this potentially toxic, multicopy LacZAYA fusion. The fusion was transferred by homologous recombination from the pRDM064 plasmid to X5455, and integrated into the bacterial chromosome, as described previously (11).

Construction of Plasmids That Express phd and doc—A DNA fragment containing the coding sequences of phd (nucleotides 366–592 (5)) flanked by primer-encoded EcoRI and HindIII sites was produced by a polymerase chain reaction using pG3 template and oligonucleotide primers HAL20 (5'-GGGAATTCTATGCAATCCATTTACCTCGTA-3') and HAL22 (5'-GGGAAGCTTCTCATTATGCAGTT-3'). The DNA product was digested with EcoRI-HindIII and cloned into the EcoRI site of the pK233-2 Pₚₐₜ expression vector to generate pHAL20. Similarly, a DNA fragment containing the coding sequences of phd (nucleotides 366–592 (5)) flanked by primer-encoded EcoRI and HindIII sites was produced by another polymerase chain reaction using pG3 template and oligonucleotide primers HAL20 (described above) and HAL23 (5'-GGGAAGCTTCTCATTATGCAGTT-3'). The DNA product was digested with EcoRI and HindIII and cloned into the EcoRI and
Autoregulation of the P1 Plasmid Addiction Operon

**TABLE I**

| Description | Source |
|-------------|--------|
| Plasmids    |        |
| pbG2ts      |        |
| pG3         |        |
| pHAL0       |        |
| pKK223-3    |        |
| plad4       |        |
| pRD032      |        |
| pHAL20      |        |
| pRD064      |        |
| prS415      |        |
| pUC19       |        |
| Phages      |        |
| λRD12       |        |
| λRS45       |        |
| P1Cm        |        |
| P1kM        |        |
| Escherichia coli |        |
| BR4749      |        |
| BR6545      |        |

| Source |  |
|--------|--------|
|        | 40     |
|        | 5      |
|        | pU12 of (5) |
|        | 41     |
|        | This study |
|        | 11     |
|        | 11     |
|        | Lab collection |
|        | Lab collection |
|        | 43     |
|        | 44     |

a Sp, Ap, Km, Cm, and Str, resistance to spectinomycin, ampicillin, kanamycin, chloramphenicol, and streptomycin, respectively.

HindII sites of the pKK223-3 Plac expression vector to generate pRD032. The Plac expression constructs were isolated in the presence of lacI in order to repress maximally the cloned genes. The structures of pHAL20 and pRD063 were verified by digestion with restriction enzymes and by sequencing with the primers described by Miller (10) on toluene-permeabilized cells of a lysogen of MC1061 or derivatives of this lysogen. The λ lacl glycosylase, a transcriptional fusion of the P1 addiction promoter (Pr92) to lacZYA. Cells containing phd were grown in LB broth containing antibiotic a polymerase chain reaction to generate a labeled DNA fragment. Using this technique, a 189-bp fragment (nucleotides 252–440), in which the P1 addiction promoter was centrally located, was obtained with oligonucleotide primers ROY47 (5′-GATACTCGGGAGTGAC-3′) and ROY48 (5′-CTTCTCCAGCGCTTCAACATTGTT-3′). The radiolabeled DNA fragments were purified on 6% polyacrylamide gels in Tris-borate/EDTA buffer and eluted from gel slices in Maxam and Gilbert buffer (13). For mobility shift experiments and DNase I protection experiments, radiolabeled probe was used at a final concentration of approximately 0.1 to 3.0 μM, respectively.

Additionally, one primer was radiolabeled with T4 polynucleotide kinase and [γ-32P]ATP and then used with a second unlabeled primer in a polymerase chain reaction to generate a labeled DNA fragment. Using this technique, a 189-bp fragment (nucleotides 252–440), in which the P1 addiction promoter was centrally located, was used to detect the molar concentration of Phd. Samples of this preparation were used in DNA mobility shift and DNase I protection experiments. A small portion of this Phd preparation containing about 20 μg of protein was resuspended in 6 M guanidine hydrochloride and further purified over a high performance liquid chromatography C18 reverse phase column. High performance liquid chromatography-purified Phd was collected in a single fraction and subjected to amino-terminal sequence analysis on a 477A protein sequencer (Applied Biosystems). The 23-amino acid amino-terminal sequence MQSNFTARGNLSEVLNNVEAG determined by analysis matched the predicted amino-terminal sequence of Phd (5).

DNA Mobility Shift Assays—DNA mobility shift assays were performed as described previously (14, 15). Cell extracts or purified Phd was incubated with approximately 0.1 μM radiolabeled DNA for 20 min at room temperature in a solution containing 20 μM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 50 μM bovine serum albumin, 100 μM KCl, 31 μM calf thymus DNA, and 6% glycerol, electrophoresed on a 6% polyacrylamide gel in Tris/borate/EDTA buffer, which was then dried and autoradiographed.

DNase I Footprinting—Probing with DNase I was essentially as described previously (16). Purified Phd and radiolabeled DNA were incubated together for 20 min at room temperature in a solution containing 20 μM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 50 μg/ml bovine serum albumin, 100 μM KCl, and 31 μM calf thymus DNA. The formation of complexes was then probed by adding divalent

The abbreviations used are: Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; bp, base pair.
Table II

| Plasmids                        | Relevant plasmid genotype¹ | β-Galactosidase-specific activity in Miller units² | Relative repression³ |
|--------------------------------|----------------------------|-------------------------------------------------|----------------------|
| None                           | Control                    | 5200 (1)                                        | 1                    |
| P1Km                           | Pr92-Phd doc, low copy     | 135                                             | 9                    |
| P1Cm                           | Pr92-Phd doc, low copy     | 140                                             | 9                    |
| pGBK2ts                        | Control vector, modest copy| 3500 (1)                                        | 1                    |
| pG3                            | Pr92-Phd doc, modest copy  | 9                                                | 390                  |
| plad ¹, pKK223-3                | lad ⁴, control vector      | 4400 (1)                                        | 2000                 |
| plad ¹, pRD3032                | lad ⁴, P₉₅-Phd doc         | 2.2                                             | 9                    |
| plad ¹, pHAL20                 | lad ⁴, P₉₅-Phd             | 500                                             | 9                    |

¹ β-Galactosidase assays were performed in a λRD12 lysogen of MC1061, or derivatives of this lysogen, bearing the indicated plasmids. The λ prophage in each strain bears a transcriptional fusion of the P1 addiction promoter to lacZYA.

² β-Galactosidase-specific activity (Miller units) was averaged from four samples taken from cells growing exponentially in antibiotic-supplemented LB broth at 37°C. The standard deviation was less than 10% of the mean, except in the case of the strain containing pG3, in which case the standard deviation was 5 Miller units (55% of the mean).

³ Relative repression is β-galactosidase-specific activity of the appropriate (unpressed) control strain divided by the β-galactosidase-specific activity of the experimental strain.

RESULTS

Transcriptional Regulation of the P1 Addiction Operon—The promoter of the P1 addiction operon was fused to lacZYA, and a single copy of the fusion was integrated into the chromosome. This transcriptional fusion produced about 5200 Miller units of β-galactosidase in the absence of the P1 addiction proteins (Table II). A P1 prophage carrying the addiction operon repressed expression of the fusion approximately 40-fold, indicating that significant levels of repression are achieved in the natural context of the P1 addiction operon. A plasmid, pG3, which carries the P1 addiction operon and is maintained at a 500- fold higher copy number than a P1 prophage (1, 19) repressed expression of the fusion approximately 400-fold, or roughly 10-fold more than was observed with the low copy P1 prophage.

In order to test whether both proteins were involved in repression, yet avoid the complications of autoregulation, we constructed plasmids in which Phd or Phd and Doc were provided from a repressed P₉₅ promoter. Phd and Doc, supplied from such a construct, repressed expression of the transcriptional fusion to the addiction promoter more than 1000-fold (Table II). Phd alone, supplied from the same repressed P₉₅ promoter, repressed the addiction promoter by about 10-fold. These results indicate that, although Phd is sufficient for repression, Doc dramatically enhances repression.

Binding of Phd and Doc to the P1 Addiction Promoter—In order to characterize the mechanism of repression, crude cell extracts of cultures expressing either no P1 genes, phd alone, or phd and doc, were assayed for binding activity to a 403-bp DNA fragment containing the addiction promoter. Control extracts lacking P1 products did not interact with the labeled promoter fragment (Fig. 1A, middle lanes). DNA retardation was observed with extracts containing Phd (Fig. 1A, right lanes), consistent with the finding that Phd gives modest repression in vivo. Extract containing both Phd and Doc (Fig. 1A, left lanes) retarded the promoter fragment more than extracts containing only Phd. Furthermore, the Phd- and Doc-induced transition from unretarded to fully retarded DNA did not appear to involve a stable intermediate species, such as that produced by Phd alone. The absence of appreciable DNA-Phd complexes in extracts that presumably contain Phd in excess of Doc suggests that the DNA-Phd-Doc complex is more stable than the DNA-Phd complex. Stabilization of the repressive complex may be the mechanism by which Doc enhances repression.

Purification and Activity of Phd—Phd was overexpressed and purified by cation exchange chromatography, ammonium sulfate precipitation, and gel filtration chromatography, as described under “Experimental Procedures.” Purified Phd was estimated to be approximately 90% pure by silver staining (Fig. 2), and total amino acid analysis was consistent with the predicted composition of Phd. Purified Phd had DNA binding activity as detected by gel mobility shifts (Fig. 1B).

DNase I Footprint of Pure Phd on the phd-doc Promoter—in order to identify the DNA sequences to which Phd bound, we tested the ability of Phd to protect the promoter from DNase I digestion. These footprinting experiments were performed with purified Phd and a 189-bp fragment of DNA in which the putative promoter was centrally located. DNase I footprints showed protection of a perfect 8–10-bp palindromic site at 2 and 10 nm Phd. At 50 nm Phd, a second imperfect palindromic site was also protected (Fig. 3A). Similar protection patterns were observed on both DNA strands. The centers of the perfect and imperfect palindromes are separated by 13 bp. The two palindromic sites span the region between the −10 region of the putative promoter and start codon of phd (Fig. 3B).

Phd Binding Sites—The footprinting experiments suggested that a single palindromic site would be sufficient for recognition by Phd. As expected, a 27-bp synthetic DNA fragment containing the perfect 10-bp palindromic, flanked by restriction site linkers, was retarded by Phd in the same manner as a 189-bp fragment containing the whole promoter region (Fig. 1B, 0–21 nm Phd). To facilitate resolution of the small 27-bp DNA fragment, we used a higher percentage of acrylamide and increased the voltage at the beginning of the run. Under these conditions, the Phd-DNA complex appeared as a doublet (Fig. 1B, 21 nm Phd), which we interpret to be two alternative forms
of one molecular complex. Between 21 and 105 nM Phd, the 189-bp fragment containing the complete promoter region underwent an additional shift (Fig. 1B). This second shift probably reflects the filling of the second site, as observed in footprinting experiments. Consistent with this interpretation, the 27-bp fragment of DNA, which contains only a single palindromic site, did not undergo the second shift.

**DISCUSSION**

**Physiology of Autoregulation—**The plasmid addiction operon of bacteriophage P1 contains two genes, phd and doc, encoding, respectively, an antidote and a toxin. A chromosomally integrated, transcriptional lacZ fusion to the putative promoter of the P1 addiction operon was repressed about 400-fold when the addiction operon was furnished in trans on a moderate copy plasmid (Table II, pG3), indicating that transcription of the operon is negatively regulated by one or more products of the operon. Since the lacZ fusion was repressed approximately 40-fold in P1 lysogens, it appears that transcriptional repression occurs under physiological conditions.

In principle, autoregulation could buffer the cellular expression of the plasmid addiction operon against fluctuations in plasmid copy number. Evidence that this might be the case for P1 comes from the observation that when the copy number of the addiction operon was increased (from that of P1 prophage to that of pG3), expression of the operon decreased. The suggestion that levels of addiction proteins remain largely unaffected by variations in plasmid copy number, short of plasmid elimination, is consistent with the proposed post-segregational function of the operon. Autoregulation ensures that should plasmid copy numbers dip to levels that incur the risk of plasmid loss, the addiction system will remain fully primed.

**Role of Phd in Autoregulation—**Expression of phd, without doc, was sufficient to repress expression of a lacZ fusion to the addition operon almost 10-fold (Table II). Consistent with this observation, purified Phd protected a region in the putative promoter encompassing the P1 addiction promoter was incubated with the indicated amount of crude cell extract, per 10-μl reaction, and analyzed on a 6% polyacrylamide gel. B, two radiolabeled DNA fragments, a 189-bp DNA fragment containing the promoter region and a 27-bp fragment containing a single 10-bp palindrome, were incubated with the indicated amount of purified Phd and analyzed on a 11% polyacrylamide gel. See "Experimental Procedures" for details.

**FIG. 1. DNA mobility shifts.** A, a 403-bp radiolabeled DNA fragment encompassing the P1 addiction promoter was incubated with the indicated amount of crude cell extract, per 10-μl reaction, and analyzed on a 6% polyacrylamide gel. B, two radiolabeled DNA fragments, a 189-bp DNA fragment containing the promoter region and a 27-bp fragment containing a single 10-bp palindrome, were incubated with the indicated amount of purified Phd and analyzed on a 11% polyacrylamide gel. See "Experimental Procedures" for details.
promoter from digestion with DNase I. The protected region, located between the −10 region of the putative promoter and the start codon of phd, encompasses a perfect and an imperfect palindromic (Fig. 3). These palindromic sites are 8–10 bp in length and are centered 13 bp apart. The palindromic nature of the DNA sites protected from DNase I suggests that Phd, like many DNA-binding proteins, might bind as a dimer. DNA retardation and DNase I footprinting experiments show that a single perfect palindrome is sufficient for recognition by Phd (Fig. 1B, Fig. 3).

Similarity of Phd to Other Antidote Proteins and to Other Known DNA-binding Proteins—Phd and other plasmid-encoded antidote proteins, such as CcdA, PemL, and ParD, exhibit certain functional and structural similarities to each other and to well-studied members of the β-sheet family of DNA-binding proteins (20, 21), such as MetJ (22) and Arc (23). MetJ is a small dimeric repressor that binds cooperatively to two or more adjacent, palindromic 8-bp sites (24). Similarly, Arc dimers bind cooperatively to two adjacent (but asymmetric) sites (25). In both cases, specific DNA contacts of the dimer are mediated by a short, two-strand, antiparallel β-sheet which is inserted into the major groove of the DNA site. The antiparallel β-sheet of the dimer is formed by the amino-terminal regions of the constituent monomers.

Predictions of secondary structure (26, 27) suggest that there is a short β-sheet region near the amino terminus of Phd, followed by two or three α-helical domains. Similarly, predictions of secondary structure of CcdA indicate the presence of one or two β-sheets at the amino terminus, followed by one or more α-helices (28). A truncated CcdA protein lacking the amino-terminal 31 amino acids loses autoregulatory activity (28) but retains antidote activity (29), indicating that the amino terminus of CcdA may be specifically involved in DNA binding. ParD of RP4 binds DNA and may also be structurally similar to members of the β-sheet family of DNA-binding proteins (30).

Four antidote proteins involved in autoregulation (Phd, CcdA, PemL, and ParD) and two β-sheet DNA-binding proteins (Arc and MetJ) were compared, in all pairwise combinations, using the method of Needleman and Wunsch (18). Alignment scores were standardized by subtracting the mean score from 100 alignments using randomized protein sequences of the same composition and dividing by the estimated standard deviation from the randomized trials. Only one pair of proteins, CcdA and PemL, is significantly similar, as previously noted (31). As a group, the antidote proteins are slightly similar to each other (p > 97%) and to β-sheet DNA-binding proteins (p > 96%). Alignment scores fail to reflect the structural similarity of Arc to MetJ. The involvement of the antidotes in transcriptional repression and their (weak) similarity to each other and to β-sheet DNA-binding proteins is consistent with the hypothesis that the antidote proteins can bind to DNA via β-sheet structures.

Role of Doc in Autoregulation—Although expression of Phd alone was sufficient to repress a lacZ fusion to the Phd addiction promoter, repression was enhanced more than 100-fold when Doc and Phd were co-expressed (Table II). We have not yet been able to determine whether the toxic Doc protein can repress the promoter in the absence of Phd. Extracts containing Phd and Doc caused a greater retardation of the electrophoretic mobility of DNA bearing the promoter than did extracts containing Phd without Doc. Most likely, Doc participates directly in the repression complex by binding Phd or DNA or both.

Transcriptional Regulation of Operons Analogous to the P1 Addiction Operon—A number of large plasmids carry operons encoding antidote/toxin pairs that enhance plasmid stability by killing or arresting the growth of plasmid-free segregants. Where studied, these operons have been shown to be under negative, transcriptional autoregulation. In the case of ParD/ParE of RK2, ParD binds DNA at micromolar concentrations (32), but it is not clear whether the toxin, ParE, affects repression (30, 33, 34). In the case of CcdA/CcdB of F (28, 35–37) and PemL/PemK of R100 (38, 39), both antidote and toxin are required for full transcriptional repression and DNA binding activity. At the concentrations tested, the antidote, CcdA, did not bind to its promoter region in the absence of CcdB but in vivo, CcdA, much like Phd, represses expression of its promoter 5-fold (35). In the case of PemL/PemK, repression and DNA binding are detected only in the presence of both toxin and antidote.

It seems possible, given the functional and structural similarity of these operons, that the ways in which they are auto-regulated may be similar. A simple, unifying hypothesis is that 1) the antidote specifically interacts with DNA (shown in two out of four cases), possibly via β-sheet structures, and 2) the toxin enhances repression (shown in three out of four cases). How the P1 toxin contributes to repression is currently under study.

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