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# Metal- and DNA-binding properties and mutational analysis of the transcription activating factor, B, of coliphage 186: A prokaryotic C4 zinc-finger protein

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## Abstract

Coliphage 186 B is a 72-amino acid protein belonging to the Ogr family of analogous transcription factors present in P2-like phage, which contain a Cys-X<sub>2</sub>-Cys-X<sub>22</sub>-Cys-X<sub>4</sub>-Cys presumptive zinc-finger motif. The molecular characterization of these proteins has been hampered by their insolubility, a difficulty overcome in the present study by obtaining B as a soluble cadmium-containing derivative (CdB). Atomic absorption spectroscopy showed the presence of one atom of cadmium per molecule of purified CdB. The UV absorption spectrum revealed a shoulder at 250 nm, characteristic of Cys-S-Cd(II) ligand-to-metal charge-transfer transitions, and the difference absorption coefficient after acidification ( $\Delta\epsilon_{248}$ , 24 mM<sup>-1</sup> cm<sup>-1</sup>) indicated the presence of a Cd(Cys-S)<sub>4</sub> center. Gel mobility shift analysis of CdB with a 186 late promoter demonstrated specific DNA-binding ( $K_{D,app}$  3–4  $\mu$ M) and the protein was shown to activate transcription in vitro from a promoter-reporter plasmid construct. The B DNA-binding site was mapped by gel shift and DNAase I cleavage protection experiments to an area between –70 and –43 relative to the transcription start site, coincident with the consensus sequence, GTTGT-N<sub>8</sub>-TNANCCA, from –66 to –47 of the 186 and P2 late promoters. Inactive B point mutants were obtained in the putative DNA-binding loop of the N-terminal zinc-finger motif and in a central region thought to interact with the *Escherichia coli* RNA polymerase  $\alpha$ -subunit. A truncated B mutant comprising the first 53 amino acids (B1–53) exhibited close to wild-type activity, showed a DNA-binding affinity similar to that of the full-length protein, and could be reconstituted with either Cd or Zn. Gel permeation analysis revealed that B1–53 was a majority dimeric species whereas wild-type B showed larger oligomers. 186 B therefore exhibits a potentially linear organization of functional regions comprising an N-terminal C4 zinc-finger DNA-binding region, a dispensable C-terminal region involved in protein self-association, and a central region that interacts with RNA polymerase.

**Keywords:** bacteriophage; cadmium substitution; Ogr; P2; zinc-finger

The transcription of genes in both eukaryotic and prokaryotic organisms is catalyzed by DNA-dependent RNA-polymerases. In addition, many genes require the influence of transactivating factors to initiate their transcription. These proteins most often bind to specific upstream DNA sequences in the promoter region of the

gene and interact with the RNA-polymerase holoenzyme. Zinc-finger proteins are a major class of transcription activators that are unlike other classes of DNA-binding proteins such as helix-turn-helix and base-helix-loop-helix classes in that they have no common structural element, except for the presence of a zinc-center with the general formula, Zn(Cys-S)<sub>4-n</sub>(His-N)<sub>n</sub>, where  $n = 0–2$ , and that they are subclassified as either C4, C3H, or C2H2 (Klug & Schwabe, 1995). Zinc-finger proteins are ubiquitous in eukaryotes but rarely found in prokaryotes (South & Summers, 1990) and, while they predominate as transcription factors in eukaryotes, no example of a prokaryotic transcription activator containing a zinc-finger structure has yet been characterized.

Although a great deal is known concerning the mode of recognition by zinc-finger domains of their target DNA sequences (Berg, 1995; Mandel-Gutfreund et al., 1995; Suzuki & Gerstein, 1995; Taylor et al., 1995; McBryant et al., 1996), the role of the coordinated zinc ion in the activation mechanism of the RNA-polymerase remains unclear. Recent studies have indicated that in both eukary-

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**Abbreviations:** SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; UV, ultra-violet; NMR, nuclear magnetic resonance; LMCT, ligand-to-metal charge-transfer; Tris, tris(hydroxymethyl) methylamine; PCR, polymerase chain reaction; IPTG, isopropyl- $\beta$ -D-thiogalactopyranoside; NNG, N-nitrosoguanidine.

otic and prokaryotic systems the sole function of transcription activating factors in initiation complex formation is to recruit the RNA-polymerase holoenzyme to the promoter (Barberis et al., 1995). However, the initiation of transcription requires conformational changes to be induced in the promoter DNA (Werner et al., 1996). It is possible that the zinc-finger motif may act as a kind of molecular spring where the coordinated zinc ion, by maintaining the protein in an entatic state, serves to store energy that can be utilized to induce the required twist or bend in the target DNA.

The ability of zinc-finger proteins to bind to DNA is generally dependent on the presence of the coordinated zinc ion. Thus, removal of the metal leads to loss of binding (O'Halloran, 1993). It has been suggested that this property may permit transcription activation by these proteins to be modulated by the cellular concentration of available zinc, which in turn can be regulated by the zinc-binding proteins, metallothioneins (MT) (Zeng et al., 1991a, 1991b; O'Halloran, 1993; Pountney et al., 1995a). Indeed, it has been demonstrated, both by *in vitro* and *in vivo* experiments, that metal-free MT is able to remove zinc from zinc-finger proteins, thereby abrogating their transcription activating competence (Zeng et al., 1991a, 1991b). To date, the only zinc-finger activator shown to respond directly to zinc through the zinc-finger domain is the MT transcription factor, MTF-1 (Radke et al., 1995).

Zinc-finger proteins also appear to be particularly sensitive to a variety of cellular reactants. Recent model studies on the zinc-binding site in alcohol dehydrogenase indicate that cysteine groups in zinc-finger-type sites are highly susceptible to oxidation and could represent a major target for damage linked to the pathophysiological effects of oxygen-derived free radicals (Crow et al., 1995). Indeed, the redox chemistry of cysteine in these proteins may in some cases play an important functional role in cellular regulation (Huang & Adamson, 1993; Civitareale et al., 1994). Moreover, similar zinc-bound cysteine thiolates in MT and in the *Escherichia coli* Ada protein have been shown to undergo alkylation reactions

with physiological alkylating agents (Myers et al., 1993; Yu et al., 1995; Sakashita et al., 1995). Wilker and Lippard (1995), using inorganic model complexes, have demonstrated that the rate at which thiolates are alkylated correlates to the net negative charge on the metal center, with thiolates in a  $[ZnS_4]^{2-}$  or C4-type site reacting more rapidly than those in C3H- or C2H2-type sites.

In order to study the relevant biological chemistry of zinc-finger proteins further it is desirable to have a model protein with a very simple molecular architecture. For this reason, we have commenced investigation of the 72-amino acid late-gene transcription activator B of the temperate coliphage 186 (Kalionis et al., 1986a, 1986b; Egan & Dodd, 1994). Coliphage 186 B is the sole phage function required for activation of the phage late genes (Dibbens & Egan, 1992; Dibbens et al., 1992) and belongs to a family of late-gene transactivating factors of 186-like phage, including P2 Ogr and P4  $\delta$ , which are capable of acting at each others promoters (Hocking & Egan, 1982a; Sauer et al., 1982). These proteins exhibit highly homologous primary structures (62% amino acid sequence identity between 186 B and P2 Ogr), including a conserved array of four cysteine residues with a Cys-X<sub>2</sub>-Cys-X<sub>22</sub>-Cys-X<sub>4</sub>-Cys motif expected to coordinate zinc in a zinc-finger structure (Table 1). Studies chiefly on P2 Ogr and P4  $\delta$  indicate that the proteins bind zinc (Lee & Christie, 1990), interact with the  $\alpha$  subunit of *E. coli* RNA-polymerase (Sunshine & Sauer, 1975; Fujiki et al., 1976; Halling et al., 1990; King et al., 1992; Ayers et al., 1994), have a region of the promoter DNA centered at -55 (Julien & Calendar, 1995), and contain a C-terminal region (ca. 20 amino acids) non-essential to protein function (Gebhardt et al., 1993). Further work has been hampered by the insolubility of the purified proteins other than as gene fusion products (Lee & Christie, 1990; Julien & Calendar, 1995). However, we have obtained the 186 B gene product as a soluble cadmium-containing derivative (CdB) and present the initial characterization of the protein in this paper.

**Table 1.** Amino acid sequences of the late-gene transactivating factors of 186-like phage<sup>a</sup>

		1	10	20	30	40	50	60	70
186	B	MFH	CPKCHHA	AHARTSRYLTENTKERYHQ	CQNINCSCT	FMTMETIERFIVTPGAIDPAPP	HPTVGGQRPLWL		
P2	Ogr	MFH	CPLCQHA	AHARTSRYITDTT	KERYHQ	CQNVCNCSAT	FITCESVQRYIVKPGEV	HAVRPHPLPSGQQIMWM	
PSP3	Pag	MMH	CPLCQNA	AHARTSRYLSTETKERYHQ	CQNINCGCT	FITFETLSRFIVKPGTVDPAPP	HPIRNQQQLWL		
Nuc C		MMH	CPLCGHV	AHTRSSRYLSESTKERYHQCRNINCSCT	FATHESVARVIV	KPGDDIVPAQPHPPENQHKQSAAAL			
ϕR73	δ	MMR	CPFCRHS	AHTRTSRYVSDNV	KESYLOQCQNIYCSAT	FKTHESICAVIRSPVTEEK	PAPASTAPAVVRKVKG	CYSSPFNH	
P4 <sup>b</sup>	δI	MIY	CPSCGHV	AHTRAHFMDDG	TKIMIAQCRNIYCSAT	FEASESFFSDSKDGM	EYISGKQRYRDSLTSASC	GMKRPKRLMVT	
	δII	GYCC	RRCKGL	ALSRTSRRLSQEVT	ERFYVCTDPGCGLV	FKTLQTINRFIVRPVTPDEA	ERLHEKQELPPVRLKTQSY	SLRLE	
Consensus <sup>c</sup>		M--	CP-C-H-AH-RTSRY	---TKERY-QC-NI-CS-TF-T-E	---R-IV-PG-----				
Conserved		C	C	A	R	C	C	F	

<sup>a</sup>186 B, Kalionis et al., 1986a, 1986b; P2 Ogr, Birkeland & Lindquist, 1986; Christie et al., 1986; PSP3 Pag, R. Calendar, pers. comm.; NucC (from a cryptic prophage in *S. marcescens*), Christie et al., 1986; Jin et al., 1996; fR73 δ, Sun et al., 1991; P4 δ, Halling et al., 1990.

<sup>b</sup>The  $\delta$  protein of P4 contains two consecutive B-like sequences denoted  $\delta$ I and  $\delta$ II.

<sup>c</sup>Amino acids occurring in the same position in 5 or more sequences (dashes indicate no consensus amino acid). Conserved amino acids are shown in bold type.

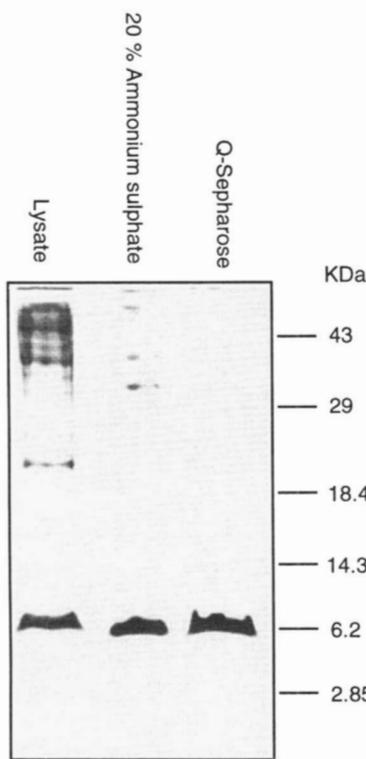
## Results

### Over-expression and purification

Similar to earlier studies on the P2 Ogr and P4  $\delta$  proteins, the 186 B gene product was obtained in high-yield from an *E. coli* plasmid-based expression system (5–10 mg protein/L culture) and was recovered as an insoluble aggregate. However, addition of 30  $\mu$ M CdSO<sub>4</sub> at the time of IPTG induction of B expression allowed soluble protein to be obtained and its molecular weight approximated the 8.3 kDa expected for B (Fig. 1). Subsequently, the protein was purified to homogeneity by precipitation with ammonium sulphate followed by anion exchange chromatography as described in Materials and methods (Fig. 1). The polypeptide molecular weight (8,281 Da) determined by electrospray ionization mass spectroscopy is consistent with that predicted from the amino acid sequence (Table 1).

### Metal-binding properties

The metal content of B obtained with the addition of CdSO<sub>4</sub> to the growth medium was determined using atomic absorption spectroscopy. The purified protein was found to contain 1.03 moles of cadmium per mole of protein. The UV absorption spectrum of the cadmium-containing protein (CdB) is illustrated in Figure 2A. In addition to the absorption maximum at 280 nm due to the aromatic amino acids, a broad shoulder at around 250 nm was observed. This feature is characteristic of cadmium-substituted metalloproteins where the cadmium(II) ion is coordinated by cysteine thiolate ligands, and was lost upon acidification of the protein to pH 2 (Fig. 2A), consistent with displacement of the metal ion by protons.



**Fig. 1.** Twelve percent SDS-PAGE of CdB at various stages of purification. Running buffer is Tris-Tricine, pH 8.

A difference spectrum, which represents only the metal-dependent absorption features, was generated by computer subtraction of the low pH spectrum from the high pH spectrum (Fig. 2B). Gaussian deconvolution of the difference spectrum revealed the presence of three individual transitions at 215, 231, and 248 nm, similar to those observed in a number of cadmium-substituted proteins known to contain cysteinate metal ligands (Henehan et al., 1993). These features are assigned to the cysteine thiolate-Cd(II) ligand-to-metal charge-transfer (LMCT) transitions also observed in simple cadmium-thiolate coordination compounds. The positions of the band maxima in the difference absorption spectrum of CdB correspond closely to those observed in the *Desulfovibrio gigas* rubredoxin Cd-derivative which contains a single Cd(Cys-S)<sub>4</sub> center (Henehan et al., 1993). Previous studies have shown that the intensity of the first (lowest energy) CysS-Cd(II) LMCT transition is proportional to the cysteine ligand-to-metal ratio with a per cysteine extinction coefficient,  $\Delta\epsilon_{\text{Cys}}$ , of about 6,000 M<sup>-1</sup> cm<sup>-1</sup> (Henehan et al., 1993). Therefore, the extinction coefficient ( $\Delta\epsilon_{248}$ , 24,000 M<sup>-1</sup> cm<sup>-1</sup>) obtained for the lowest energy transition of CdB indicates that the cadmium(II) ion is bound by four cysteine ligands (Fig. 2C). Comparison of the amino acid sequences of the B-like activator proteins (Table 1) revealed the presence of an array of four conserved cysteine residues in the N-terminus. It is most likely that the four cysteines involved in metal coordination are provided by this Cys-X<sub>2</sub>-Cys-X<sub>22</sub>-Cys-X<sub>4</sub>-Cys motif.

### In vitro transcription/translation assay

The ability of CdB to activate transcription at a 186 late promoter (pV) was assessed in vitro using a coupled transcription/translation system employing *E. coli* S30 extract. The biosynthesis of  $\beta$ -galactosidase, from the pV-LacZ reporter (pTMR1), was substantially enhanced in the presence of the protein. The activation observed was  $1,229 \pm 51$  nmol ONP/min/mg CdB, which is similar to that reported for the P4  $\delta$ -MalE fusion protein (287 nmol ONP/min/mg; Julien & Calendar, 1995) and one order of magnitude greater than that reported for the in vitro refolded P2 Ogr protein (20 nmol ONP/min/mg; Lee & Christie, 1990).

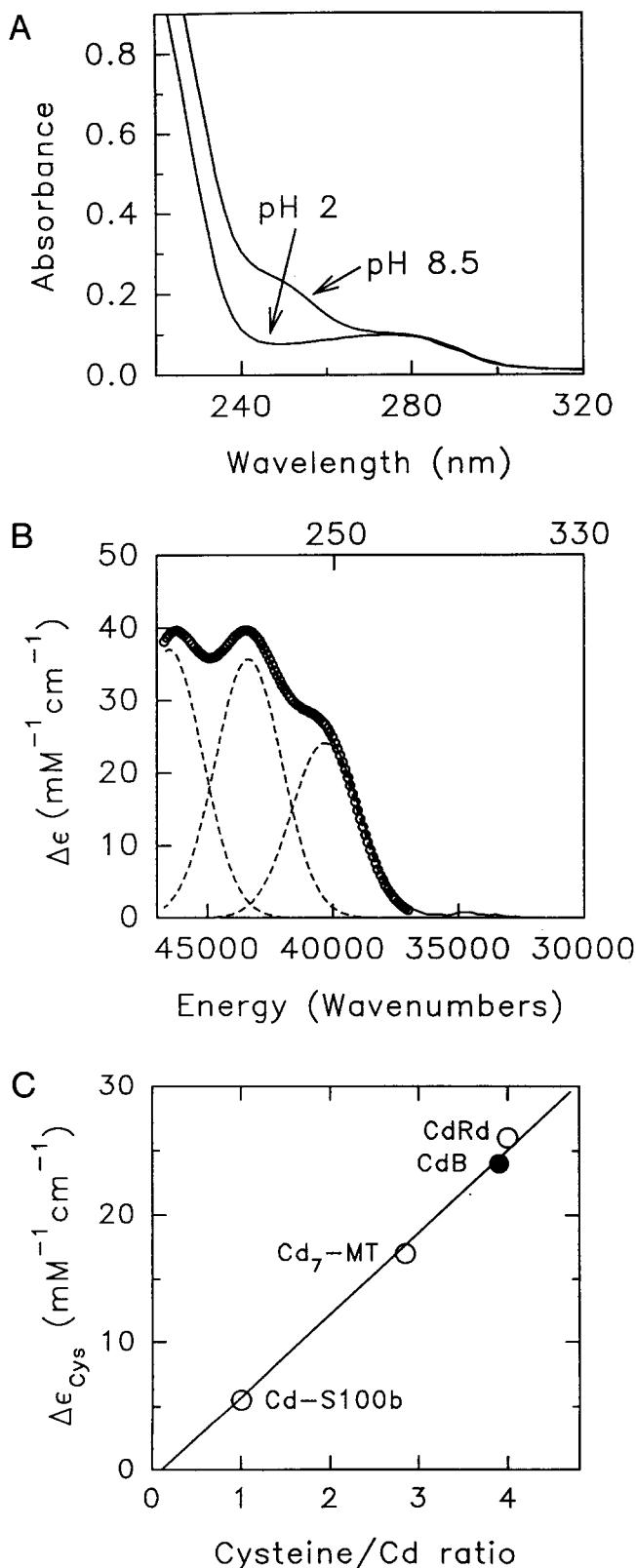
### Gel mobility shift analysis

The DNA-binding competence of CdB was determined by a gel mobility shift assay using a dsDNA fragment (600 bp) containing the 186 late promoter, pV (−263 to +229 relative to the transcription start site; Dibbens & Egan, 1992). A single retarded species was seen, which increased with increasing protein concentration (Fig. 3A). As the protein concentration was greatly in excess of that of the target DNA, an estimate of the apparent dissociation constant of the DNA:protein complex could be obtained from the concentration of protein required for 50% saturation of the DNA. This resulted in an apparent dissociation constant,  $K_{\text{D,app}}$ , for CdB of approximately 4  $\mu$ M (Fig. 3A). Under identical conditions no binding was observed with a similar-sized dsDNA fragment containing the 186 early promoters pR and pL (Fig. 3B), confirming that CdB interacts specifically with the 186 late-promoter.

Comparison of the DNA sequences of the late promoters of 186 and P2 revealed the presence of the consensus sequence GTTGT-N<sub>8</sub>-TNANCCA (see Table 2). Gel shift data obtained using a 41-base-pair dsDNA fragment from −76 to −35 of pV (Fig. 3C) showed a similar binding affinity for CdB to that of the longer DNA fragment (see above), thus indicating that the protein binding site is contained within this region of the promoter.

**DNAase I cleavage protection**

The region of the 186 late promoter, pV, to which B binds was determined by DNAase I cleavage protection experiments using



**Table 2.** Upstream region of the late promoters of phage 186 and phage P2<sup>a</sup>

		-75	-65	-55	-45 <sup>b</sup>
	:	:	:	:	:
186	pV	TAAGGTGCTGT <b>TG</b> TGTGGCCGGTTGTCCAGTCGTCA			
186	pJ	CATCCATTAGT <b>TG</b> TGCCAGACATCATCCAGCCCTGA			
186	p32	CAAATAAATAT <b>TG</b> TGTGAACCACCACCGGAGGCACG			
186	p12	CCAATGACG <b>TG</b> GACAACGCCACACAACAGCAC			
P2	pO	TAAGGTGCTGT <b>TG</b> TGTCACTGATAAGCCATCCGGGA			
P2	pF	TTACCGGTGGT <b>TG</b> TGCTGTCGATTAGCCAACCGGGAA			
P2	pV	TCCTGAATG <b>TG</b> TCTGGTAGTTCTACAAATGAATC			
P2	pP	TCAGTCCC <b>GG</b> AT <b>TG</b> GCTTATCACTGACACAACAGCAC			
Consensus <sup>c</sup>		--a-----gt <b>TG</b> t-----t-a-ccA-----			

<sup>a</sup>186 pV and p12, Kalionis et al., 1986a, 1986b; 186 pJ, Xue & Egan, 1995; 186 p32, Portelli, 1995; P2 pO, pF, pV, and pP, Christie & Calendar, 1983, 1985.

<sup>b</sup>Sequence position relative to the transcription start site.

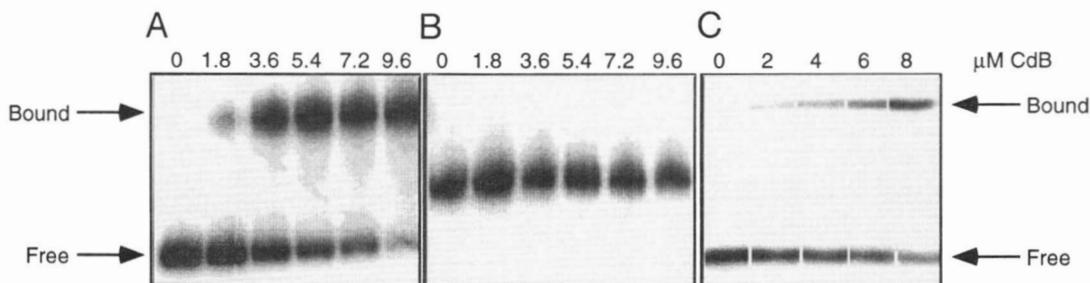
<sup>c</sup>Bases occurring at the same position in six sequences are shown in lower case, base occurring in seven sequences is shown in normal capital, conserved bases are shown in bold capital.

purified CdB. Fragments of dsDNA containing pV were <sup>32</sup>P-labeled at the 5' end of either the top or the bottom strand and subjected to treatment with DNAase I in the absence or presence of CdB. The pattern of cleavage obtained for the top strand is illustrated in Figure 4A and that of the bottom strand is shown in graphic representation in Figure 4B. Cleavage protections were observed from -70 to -43, and the degree of protection increased with increasing protein concentration. The concentration of CdB required to observe protection from DNAase I cleavage is in the same range as that used in the gel mobility shift assays described above. Approximately 50% protection was observed in the presence of 3 μM CdB, consistent with the protein binding site being occupied in half the DNA molecules at this concentration (Fig. 4B). In addition to the cleavage protections, several cleavage enhancements were also observed. Enhancements were seen on the top strand at -55 and -47, at each end of the protected region, and on the bottom strand at -72 and -73. A lack of cleavage protection was also observed at -67 on the top strand. All the DNAase I cleavage protections and enhancements observed are summarized in Figure 4C.

*Isolation of inactive B mutants*

B gene product expressed from the pEC434 plasmid is lethal to *E. coli* carrying a 186 lysogen (Dibbens & Egan, 1992). The lethality

**Fig. 2.** Optical properties of CdB. **A:** UV absorption spectra of CdB at pH 8.5 and pH 2. **B:** Difference spectrum of spectrum at pH 2 subtracted from spectrum at pH 8.5, representing the metal-dependent absorption features (see Text). Dashed lines are composite Gaussian bands. The sum of the composite bands is shown as open circles. **C:** Correlation between the extinction coefficient,  $\epsilon_{\text{Cys}}$ , of the lowest energy Cd(II)-CysS ligand-to-metal charge-transfer transition and the cysteine/Cd ratio (taken from Henehan et al., 1993). Hollow circles are reference proteins (CdRd, cadmium rubredoxin; Cd-S100b, cadmium S100-b protein; Cd<sub>7</sub>-MT, cadmium metallothionein) and CdB is shown as a solid circle.



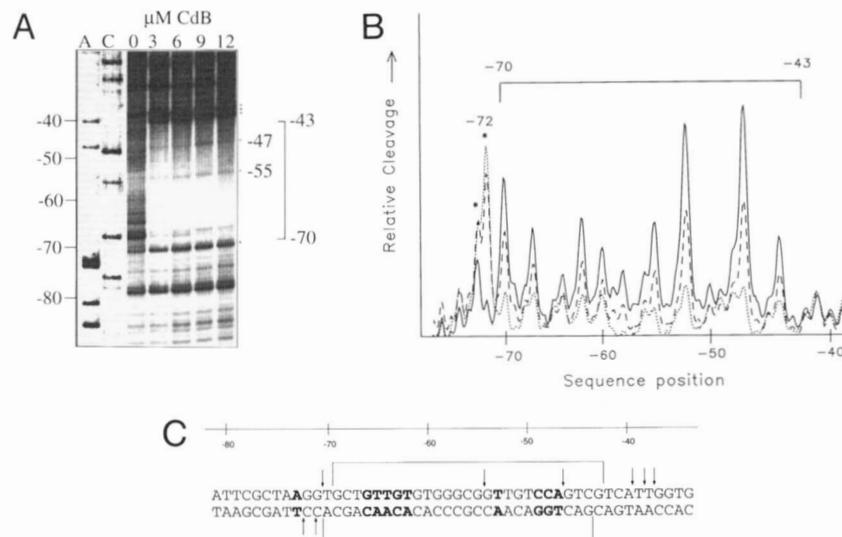
**Fig. 3.** Gel mobility shift assays of CdB vs. 186 promoters. **A:** The 186 late-promoter, pV (+229 to -263 relative to the transcription start site). **B:** The 186 early promoters pR/pL. **C:** A fragment of pV from -76 to -35. The concentration of CdB added is indicated above each lane.

phenotype was associated with the 10–22% *Bam*H1 fragment of 186 containing the *VUTSR* operon (Finnegan & Egan, 1979), since cells transformed with a plasmid bearing the 10–22% fragment (pRPT1) were found to be sensitive to B expression. This system was used to screen for inactive B mutants obtained after random mutagenesis of the B gene. Colonies appearing on antibiotic plates after transformation of nitrosoguanidine-mutagenized cells carrying plasmid pEC434 with plasmid pRPT1 were expected to express inactive B mutants. This was confirmed by the inability of an amber mutant of 186 carrying a defective B gene (186c<sup>l</sup>tsp *Bam*57; Hocking & Egan, 1982b) to form phage plaques on a lawn of these cells (efficiency of plating  $<10^{-8}$ ), whereas unmutagenized pEC434 cells were able to complement 186 *Bam* phage for plaque formation. Subsequent DNA sequencing of 14 pEC434 derivatives carrying inactive B genes revealed single mutagenic base changes in each. Among the 14 mutant genes, 7 different missense point mutations and 2 nonsense point mutations were identified (Fig. 5).

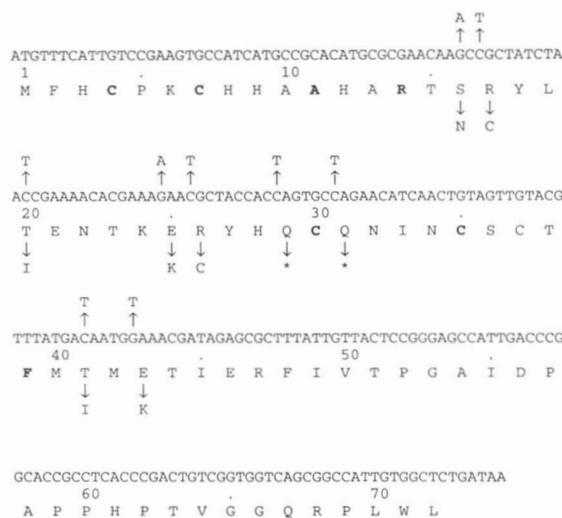
Five of the missense mutations resulted in amino acid substitutions (S16N, R17C, T20I, E25K, and R26C) in the putative DNA binding loop between the two pairs of cysteine metal ligands in the N-terminus, with the remaining two mutations, T41I and E43K, occurring in the central, serine/threonine-rich region of the polypeptide thought to be involved in contacting the RNA polymerase (see Discussion and Table 1).

#### Construction and characterization of an active truncated B mutant

It has been reported that a plasmid-encoded mutant of the P2 Ogr gene product terminating after the first 51 amino acids is able to complement a P2 mutant lacking an active Ogr gene in a phage burst assay (Gebhardt et al., 1993). We constructed a similar, truncated B mutant by engineering a stop codon in place of the codon for P52 in the B gene (Table 1), but found that the truncated gene



**Fig. 4.** DNAase I cleavage protection analysis of CdB on pV. **A:** Experiment with top DNA strand radiolabeled, i.e., that shown in Table 2, showing A and C chain termination reactions using the same primer on pV DNA (left). The concentration of CdB present in each reaction is indicated at the top of each lane. **B:** Experiment with bottom DNA strand radiolabeled in graphical representation, showing rectangular integrations of gel lanes for reactions containing 0 (solid line), 3 (dashed line) and 6 (dotted line)  $\mu$ M CdB. Regions of cleavage protection are indicated by square brackets and enhanced cleavage products are indicated by asterisks. Sequence positions are indicated relative to the transcription start site. **C:** Summary of the observed DNAase I cleavage protections (brackets) and enhancements (arrows) observed on each DNA strand of pV. The consensus sequence from the P2 and 186 late promoters is indicated in bold print.



**Fig. 5.** Inactive B mutants isolated by NNG mutagenesis. The DNA base changes observed are indicated above the wild-type DNA sequence (top row) and the resultant amino acid changes are indicated below the corresponding protein sequence (bottom row). Standard single letter codes are used for bases and amino acid residues and nonsense mutations are indicated by asterisks. Conserved amino acids are shown in bold type.

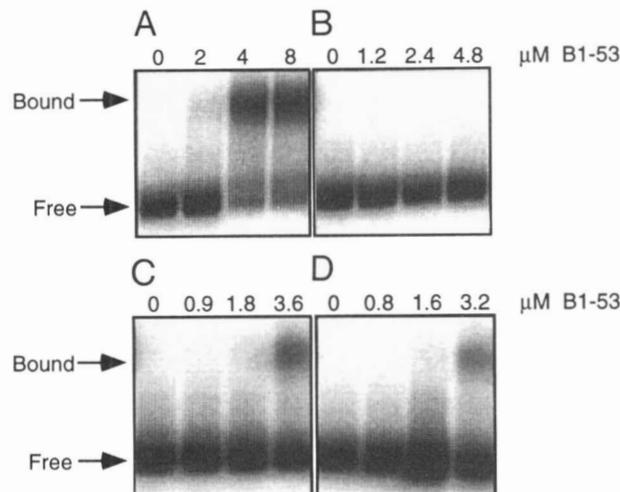
product expressed from the plasmid vector was unable to complement the 186 *Bam*57 mutant for plaque formation (Table 3). We next constructed truncated B genes coding for the first 52 and 53 amino acids. The 52 amino acid mutant was also found to be inactive but the 53 amino acid protein exhibited an activity close to that of the full-length protein (Table 3). The plasmid vector expressing the active truncated mutant (B1-53) was transformed into the same strain used to over-express the wild-type B and the truncated gene product was purified as the Cd-derivative (CdB1-53) with a yield of 10–12 mg/L of culture. Atomic absorption analysis of CdB1-53 showed a cadmium content identical to that of CdB and the UV absorption spectrum contained Cd-dependent features closely similar to CdB (data not shown). The molecular weight of B1-53 according to SDS-PAGE was close to that predicted from the amino acid sequence (6.3 kDa) and was confirmed by electrospray ionisation mass spectrometry (data not shown).

Purified CdB1-53 was tested for DNA-binding competence by gel shift analysis against a dsDNA fragment containing pV (+180 to -70) and was found to bind with a similar affinity to that of the wild-type protein (Fig. 6A). No binding was observed to a DNA fragment containing the 186 gene, *tum* (data not shown).

**Table 3.** Activity of truncated B mutants

Host	Efficiency of plating <sup>a</sup>
HMS174 (pKS1) - wild-type B	1
HMS174 (pDLP1) - P52*	<10 <sup>-3</sup>
HMS174 (pDLP2) - G53*	<10 <sup>-3</sup>
HMS174 (pDLP3) - A54*	0.92

<sup>a</sup>Efficiency of plating of 186*c/lsp* *Bam*57 on the non-suppressing host carrying the wild-type B plasmid was defined as 1. This efficiency of plating was 50% of that of *Bam*57 on a suppressing host (C600). Asterisks indicate stop codons.



**Fig. 6.** Gel mobility shift analysis of various forms of the truncated B protein (B1-53) vs. pV. **A:** CdB1-53. **B:** Metal-free B1-53. **C:** Cd-reconstituted B1-53. **D:** Zn-reconstituted B1-53. Protein concentrations are indicated above each gel lane.

#### Metal removal and reconstitution

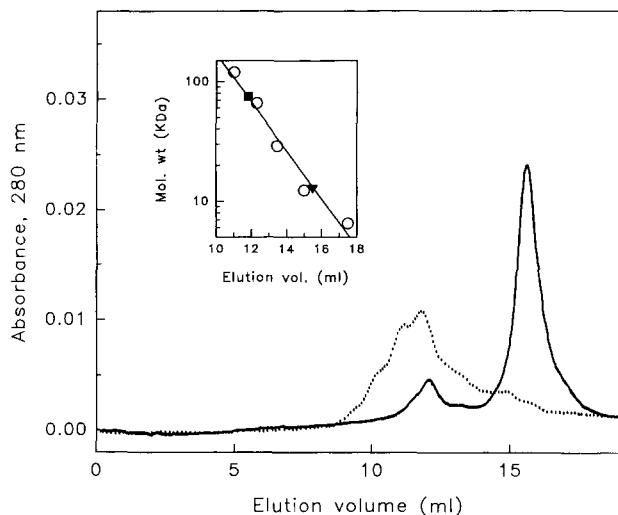
Removal of cadmium from wild-type CdB by desalting at low pH abolished DNA-binding, and attempts to reconstitute the metal-free protein by addition of Cd(II) or Zn(II) were unsuccessful (data not shown). DNA-binding was also abolished after cadmium was removed from CdB1-53 (Fig. 6B), but was restored by reconstitution with either Cd(II) or Zn(II) ion (Fig. 6C,D) in contrast to the full-length protein.

#### Gel permeation chromatography

Chromatographic analysis using a calibrated gel permeation column (Fig. 7) showed for the full-length protein (CdB) a broad feature centered at an apparent molecular weight of 75 kDa (dotted line and inset). Under identical conditions, the truncated derivative, CdB1-53, (see Fig. 7, solid line) showed the presence of a majority species with an apparent molecular weight of 13 kDa (see inset, Fig. 7) and a small amount of a high molecular weight species centered at approximately 70 kDa.

#### Discussion

The B gene product is the sole phage function required for activation of the 186 late-gene promoters (Dibbens & Egan, 1992), comprises 72 amino acids and exhibits high sequence homology to the analogous activators, Ogr,  $\delta$ , Pag,  $\delta$ , and NucC, from the phages P2, P4, PSP3,  $\phi$ R73, and a cryptic prophage identified in *S. marcescens*, respectively (Table 1). Each of these proteins contains a Cys-X<sub>2</sub>-Cys-X<sub>22</sub>-Cys-X<sub>4</sub>-Cys motif, two in the case of P4  $\delta$  which is a head to tail covalent dimer of the B-like sequence (see Table 1). This motif is proposed to be involved in zinc-binding in a novel zinc-finger-type DNA-binding domain (Lee & Christie, 1990). Previous <sup>65</sup>Zn blotting studies of Lee and Christie (1990) on the Ogr protein of P2 have indicated that these proteins are capable of binding zinc; however, no direct evidence was obtained to demonstrate that the protein contains a zinc-finger structure. These proteins are able to functionally substitute for each other



**Fig. 7.** Analytical gel permeation chromatography of CdB (dotted line) and CdB1-53 (solid line). The elution volumes of molecular weight standards are shown (inset) plotted vs. molecular weight (open circles). The elution volumes corresponding to the major CdB (solid square) and CdB1-53 (solid triangle) peaks are indicated corresponding to their apparent average molecular weights.

(Hocking & Egan, 1982a; Sauer et al., 1982), and so must recognize similar DNA sequences. Recent DNAase I cleavage protection experiments by Julien and Calendar (1995) on the  $\delta$  protein of P4 as a malE fusion have shown that this protein binds over a region of the P2 late promoters from -71 to -43. Further molecular studies of these proteins has been hampered by the insolubility of the gene products. However, we have overcome these difficulties by obtaining the B protein of phage 186 in a soluble Cd-containing form, CdB, which retains biological activity. The greater solubility of the Cd-derivative may be due to the approximate 10,000-fold higher affinity of cysteine thiolates for cadmium over zinc, resulting in a reduced lability of the metal ion or could be a consequence of the reduced charge density of the larger Cd(II) ion.

We have purified CdB from an *E. coli* plasmid-based expression system and have characterized the metal- and DNA-binding properties of the protein. We have shown by atomic absorption measurements that the protein binds one atom of Cd per protein molecule. Analysis of the UV absorption spectrum of CdB shows that the single coordinated Cd(II) ion is bound by four cysteine thiolate ligands. The amino acid sequence of the B protein contains only five cysteine residues, all of which are in the N-terminal portion of the polypeptide. Four of these cysteine residues form an array conserved in the amino acid sequences of all B-like activators with the Cys-X<sub>2</sub>-Cys-X<sub>22</sub>-Cys-X<sub>4</sub>-Cys motif (Table 1). These data therefore support the presence of a zinc-finger-like metal-binding domain with the structure, M<sup>II</sup>(Cys-S)<sub>4</sub>. Similar C4 zinc-finger domains have been found in a number of other DNA-binding proteins (Klug & Schwabe, 1995).

Gel mobility shift studies with CdB using the 186 late-promoter, pV, show that the cadmium-containing protein binds specifically to the target DNA in vitro and that only bases within the region -76 to -35 relative to the transcription start site are required for efficient DNA-binding. DNAase I cleavage protection experiments map the B binding site to a region between -70 and -43. This is quite similar to the results obtained by Julien and Calendar (1995) with the P4  $\delta$ -MalE fusion gene product.

In addition, we observed DNAase I cleavage enhancements at -55, -47 and at each end of the protected region. Enhanced sensitivity to DNAase I cleavage at particular sequence positions is often observed in areas of DNA which are bent or twisted such that access to the minor groove is improved and is commonly seen in regions flanking protein binding sites (Ausubel, 1995). Hence, these data are consistent with at least two protein binding sites on the DNA centered at approximately -55. Thus, the pattern of cleavage protections and enhancements obtained is in good agreement with the consensus sequence, GTTGT-N<sub>8</sub>-TNANCCA, of the late promoters of 186 and P2 (Table 2), indicating that B binds directly adjacent to the RNA-polymerase  $\sigma$ -subunit contact sites at -35 and -10 (Lisser & Margalit, 1993).

The ability of B to bind to promoter DNA is clearly dependent on the presence of metal ion. Removal of cadmium abolished binding and, at least in the case of the C-terminal truncation mutant, B1-53, the DNA-binding activity could be restored by reconstitution with either Cd(II) or Zn(II).

Random mutagenesis studies on the B gene have identified two regions for mutations which cause inactivation of the gene product, a region between amino acids 16 and 26 within the N-terminal zinc-finger motif and between amino acids 41 and 43 within a serine/threonine-rich central region of the polypeptide. The inactivating mutations near the N-terminus occur between the two pairs of conserved cysteines, Cys 4-Cys 7 and Cys 30-Cys 35, of the zinc-finger motif (Table 1) and most likely interfere with DNA-binding. This region of the polypeptide contains a conserved arginine residue, Arg 14 (Table 1, bold), and a lysine, Lys 24 (Table 1), conserved in all of the B-like sequences except for the second B-like sequence of P4  $\delta$ . Lysine and arginine residues are frequently involved in specific base to side-chain hydrogen-bonding involving guanine and thymine bases (Mandel-Guttfreund et al., 1995; Taylor et al., 1995). Hence, it is strongly inferred that this region of the protein constitutes the DNA-binding loop.

There is a substantial amount of evidence to show that the C-terminal domain of the *E. coli* DNA-dependent RNA-polymerase  $\alpha$ -subunit interacts directly with specific transcriptional activators (Blatter et al., 1994; Busby & Ebright, 1994). Indeed, the first indication that the  $\alpha$ -subunit was involved in activator interactions was provided by the *E. coli* *rpoA109* mutation, which prevents lytic growth of phage P2 and P4 (Sunshine & Sauer, 1975; Fujiki et al., 1976). This mutation results in an amino acid substitution in the C-terminal region of  $\alpha$  (L290H) in a hydrophobic surface patch of the protein (Jeon et al., 1995) and is thought to sterically hinder interaction with the P2 and P4 late-activators, Ogr and  $\delta$ , respectively. The lytic development of 186 is not blocked in *rpoA109* (Sauer, 1979). However, after random mutagenesis of the *rpoA* gene, an *rpoA* mutant carrying the identical single base change as in *rpoA109* was obtained that significantly depressed (to 35% of wild type) the activation of 186 pV by B (T. Rathjen, pers. comm.). Thus, B interacts with the same part of RNA-polymerase as Ogr and  $\delta$ . Suppressor mutants have been obtained in P2 (Ogr1) and in P4 (Org1) which overcome the block to late activation imposed by *rpoA109*. These two suppressor mutants contain, respectively, the amino acid changes Y42C in Ogr (Christie et al., 1986) and T127A in the second B-like sequence of  $\delta$  (Halling et al., 1990), equivalent to position 44 in the B sequence. Thus, the T41I and E43K inactivating mutations in B are adjacent to the compensatory mutations in Ogr and  $\delta$  which allow those proteins to function with the L290H substitution in  $\alpha$ . These mutations are therefore expected to interfere with contact between B and the RNA-polymerase

$\alpha$ -subunit. It is worth noting that T41 (Table 1) occurs in each of the B-like sequences, except for the first B-like sequence of P4  $\delta$  ( $\delta$ I) and E43 (Table 1) occurs in each of the sequences, except for the second part of P4  $\delta$  ( $\delta$ II). Interestingly, Gebhardt and Lindquist (1991) have reported that the double Ogr mutant F39L, Y42C is unable to function, even though each single mutation has little effect. Further studies on the three-dimensional structure of B should enable a more detailed description to be made of the amino acid(s) involved in the interaction with RNA-polymerase.

Our truncation mutagenesis studies on B are in good agreement with those conducted by Gebhardt and co-workers (1993) on P2 Ogr, showing that about the last 20 amino acids of the protein are not essential for promoter activation. Unlike Ogr, we have found that the consensus residues P52 and G53 (Table 1) are required for B function. Furthermore, the 53-amino acid active, truncated B-mutant (B1–53) was found to bind to the promoter DNA with an affinity similar to that of the full-length protein. Although the function of the C-terminal region of the B-like activators is unclear, the amino acid sequence is rich in proline and other hydrophobic residues (Table 1), making it a good candidate for involvement in protein-protein interactions. This hypothesis is also consistent with the findings of Julien and Calendar (1995) in their studies on the P4  $\delta$  fusion with the maltose-binding protein, MalE. These authors report that the  $\delta$ -MalE fusion product by itself is inactive and that optimal activity is attained when both fused and unfused  $\delta$  gene products are present in approximately a molar 1:1 stoichiometry. The unfused  $\delta$  protein alone precipitates from solution. Hence, it seems likely that the active  $\delta$  species will be a polymer, being at least a heterodimer of fused and unfused proteins. Indeed, gel permeation chromatographic analysis of CdB shows the presence of multiple high-molecular weight oligomers with an average molecular weight of 75 kDa, most likely due to a majority octameric species. By comparison, the truncated protein, CdB1–53, is characterized by a major species of 13 kDa, corresponding to a dimer, and smaller amounts of higher-order oligomers. Sedimentation equilibrium experiments on CdB1–53 are consistent with a monomer/dimer equilibrium, with approximately 75% of the protein present as the dimeric species at the concentration used for the chromatography experiments (K.E. Shearwin, pers. comm.). This is in good agreement with the chromatographic data. Hence, these results indicate a reduced propensity of CdB1–53 to oligomerize compared with CdB, consistent with the C-terminal region of B being involved in protein self-association.

#### Linear organization of functional regions

B appears to exhibit a modular organization of functional regions within the polypeptide chain, comprising an N-terminal zinc-finger-type DNA-binding region (residues 4–35), a C-terminal region (residues 54–72) involved in the formation of B oligomers and a central region which interacts with RNA-polymerase. Interestingly, a similar arrangement of zinc-finger, RNA-polymerase contact and oligomerization regions has been reported recently in the much larger (591 amino acids) EBF transcription factor (Hagman et al., 1995). Figure 8 illustrates a hypothetical schematic structure of B, summarizing the current evidence. By virtue of its apparent molecular simplicity, B should represent a valuable model to study the biological chemistry of zinc-finger-type transcription activators and how they interact both with DNA and RNA-polymerase. Further studies will focus on developing a more sophisticated struc-

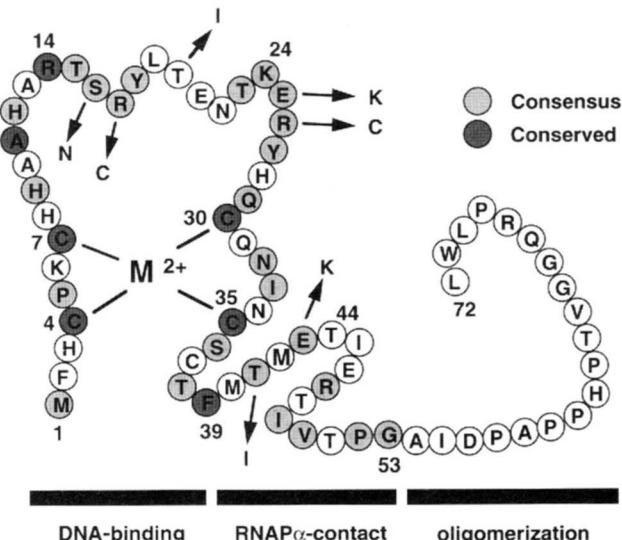


Fig. 8. Hypothetical schematic structure of 186 B. Circles represent amino acid residues denoted by the standard single-letter code.  $M^{2+}$  represents the coordinated divalent metal ion. Arrows indicate inactivating point mutations.

tural model of CdB1–53 using multidimensional NMR methods (Pountney et al., 1995b).

#### Materials and methods

##### Chemicals and enzymes

Reagents used were analytical grade or better. Enzymes for DNA manipulation, synthetic oligonucleotides, and radiolabeled nucleotides were from Bresatec, Australia. Standards for atomic absorption measurement were supplied by Fluka, Switzerland. Column materials were from Pharmacia. High purity water was used throughout.

##### Spectroscopic methods

Atomic absorption measurements were made using a Varian SpectraAA spectrometer, equipped with computerized data analysis and deuterium baseline correction. Samples for atomic absorption measurements were diluted in 0.01 M HCl. Protein concentration of stock solutions was determined by Bradford reagent (BioRad), using bovine serum albumin as standard. UV absorption spectra were recorded with a Shimadzu U-160A spectrophotometer using 1 cm quartz cuvettes. Gaussian deconvolution of difference spectra was performed using the computer program SigmaPlot (Jandel). Electrospray ionization mass spectra were measured by Yoji Hayasaka at the The Australian Wine Research Institute on desalting polypeptides in either 0.01 M HCl or 0.1% trifluoracetic acid containing 30% v/v acetonitrile using a Perkin Elmer Psi-ex triple-quadrupole mass spectrometer.

##### Bacterial and bacteriophage strains

Strain E251 is a *str<sup>R</sup>* derivative of the non-permissive strain W3350 (Hocking & Egan, 1982b). HMS174 is a 186-sensitive strain expressing the T7 RNA-polymerase and C600 is a 186-sensitive, amber-suppressing strain (Miller, 1992). Strain MO 20-1 is a lacZ<sup>-</sup>

derivative of BL21 ( $\lambda$  DE3) which carries the *ams* temperature sensitive RNAase mutation linked to Tn10 (gift of Dr. M. Dreyfus, Paris; Lopez et al., 1994). 186 *c/tsp* and its amber mutant 186 *c/tsp* *Bam*57 are described by Hocking and Egan (1982c).

#### Plasmids

The B expression plasmid, pEC434, carrying the B gene with an *Nde*I restriction site engineered at the beginning of the coding region, is described by Dibbens and Egan (1992). Plasmid pKS1 was constructed by PCR amplification of the B gene coding sequence using pEC434 as template and the following oligonucleotide primers: primer 1: 5'-ATGAAACATATGCCACCCTTTA; primer 2: 5'-CGGGATCTTATCAGAGCCAC. The isolated product was then digested with *Nde*I and *Bam*HI (underlined) and ligated with the *Bam*HI-*Nde*I fragment of plasmid pET3a (Studier & Moffatt, 1986). Plasmids pDLP1, 2, and 3 were constructed using a similar approach to pKS1, substituting for primer 2 the following primers that introduce stop codons at amino acid positions 52, 53, and 54, respectively, before the engineered *Bam*HI site: pDLP1: 5'-CGGGATCCTTAAGTAACAATAAGCGCTCT; pDLP2: 5'-CGGGATCTTATCACGGAGTAACAATAAGCG; pDLP3: 5'-CGGGATCTTATCATCCGGAGTAACAATAAA GCG. Plasmid pLysS encoding T7 lysozyme was from Studier. Plasmid pTMR1, containing *lacZYA* transcribed from promoter, pV, was constructed by cloning the *Hind*III-*Eco*R1 fragment of plasmid pEC428 containing the *Alu*I-*Alu*I fragment (-263 to +229) of 186 (Dibbens & Egan, 1992) into plasmid pMRR9 (Neufing et al., 1996) in front of *lacZYA*. Plasmid pRPT1, which contains the lethal *VUTSR* operon of 186 transcribed from the 186 late promoter pV, was constructed by cloning the 3.6 kb *Bam*HI (10–22%) fragment of the 186 genome (Finnegan & Egan, 1979) into the *Bam*HI site of pACYC184, such that pV transcription opposes *tet*. All plasmid clones constructed using PCR were sequenced by dideoxy chain-termination sequencing using the Sequenase kit (USB) with the appropriate oligonucleotide primers and incorporation of [ $\alpha$ -<sup>35</sup>S]-dATP (Bresatec).

#### Preparation of DNA

Plasmid DNA for transformation of competent cells was prepared using the procedure of LeGouill et al. (1994). Plasmid DNA for sequencing and for in vitro transcription/translation assays was prepared by alkaline lysis and isopropanol precipitation followed by ultracentrifugation in 1 g/mL CsCl and then two rounds of ethanol precipitation according to Sambrook et al. (1989).

Linear DNA for constructing plasmid clones, gel mobility shift assays and DNAase I cleavage protection experiments was obtained by polymerase chain reaction (PCR) using Taq DNA-polymerase (Bresatec). Primers were radiolabeled by using ATP-dependent polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]-dATP (Bresatec). For cloning, PCR products and linearized vector DNA were purified by agarose gel electrophoresis and extraction of DNA fragments using glass milk (BresaClean, Bresatec). For gel mobility shift assays and DNAase I cleavage protection experiments, radiolabeled DNA was purified by polyacrylamide gel electrophoresis before use.

#### Overproduction and purification of B protein

The B gene product was obtained from MO 20-1 cells bearing the pKS1 and pLysS plasmids. Cells were cultured in Luria-Bertani

liquid medium (LB) containing the appropriate antibiotics. Typically, 50 mL aliquots taken from an overnight culture at 37 °C were inoculated into 500 mL of medium in 2 L flasks and incubated with shaking at 34 °C until an OD<sub>600</sub> of 1 was reached. The temperature was then shifted to 42 °C, the non-permissive temperature for the *ams* mutant, and 0.2 mM IPTG and 30  $\mu$ M CdSO<sub>4</sub> added. After incubation for a further 2–3 h, cells were harvested by centrifugation at 5,000  $\times$  g for 30 min at 4 °C (Sorval, GS3 rotor), washed with 50 mM Tris-HCl pH 8.5 and stored at -20 °C overnight. The frozen cells were thawed in 20 mL 50 mM Tris-HCl pH 8.5 and incubated at room temperature for 1 h to allow cell lysis. Cell debris was removed by centrifugation at 10,000  $\times$  g for 20 min at 4 °C and solid ammonium sulphate was added to the supernatant to 20% of saturation. After 20 min at 0 °C, the precipitated protein was collected by centrifugation at 15,000  $\times$  g for 20 min at 4 °C. The precipitate was then resuspended in 5 mL 20 mM Tris-HCl pH 8.5 and dialyzed vs. 1 L of the same buffer overnight at 4 °C (3,000 NMWCO membrane, Spectrapor). The dialysate was centrifuged at 10,000  $\times$  g for 10 min at 4 °C and the supernatant applied to a chromatography column (20 cm  $\times$  3 cm, BioRad) packed with either DEAE-Sephadex or Q-Sepharose Fast-Flow (Pharmacia). After washing with 2 column volumes of 20 mM Tris-HCl pH 8.5, the column was eluted with a linear gradient of 0–0.7 M NaCl/20 mM Tris-HCl pH 8.5 (total volume, 1.5 L) using a BioRad Econosystem liquid chromatography system. Column fractions containing B protein as judged by SDS-PAGE eluted at about 0.4 M NaCl (slightly later on Q-Sepharose) and were subsequently pooled and concentrated to between 100–400  $\mu$ M either by ultrafiltration (Filtron, 3,000 NMWCO membrane) or dialysis vs. solid polyethylene glycol 20,000 (Spectrapor, 3,000 NMWCO membrane), then dialyzed overnight vs. 1,000 volumes of 20 mM Tris-HCl pH 7.5. Aliquots were stored at -20 °C until required.

The truncated B mutant (B1–53) was obtained from MO 20-1 cells bearing the pLysS and pDLP3 plasmids under the same growth conditions as the full-length protein. The protein was purified by precipitation with 40% saturation of ammonium sulphate after lysis in 20 mM Tris-HCl pH 9/30 mM  $\beta$ -mercaptoethanol. The pellet was washed 3–5 times with 20 mM Tris-HCl pH 6.8/10 mM  $\beta$ -mercaptoethanol, after which >95% pure B1–53 was resuspended in 10 mM Tris-HCl pH 9/10 mM  $\beta$ -mercaptoethanol to a final concentration of 0.5–1 mM. Residual traces of nucleic acid were removed if necessary by treatment with 0.1% polyethylene-imine. Protein was eluted from the pellet with 10 mM Tris-HCl pH 9/10 mM  $\beta$ -mercaptoethanol/0.4 M NaCl.

SDS-PAGE was run on protein samples using 12% polyacrylamide gels and the Tris-Tricine buffer system (Schagger & von Jagow, 1987). Protein molecular weight standards were: ovalbumin, 43,000; carbonic anhydrase, 29,000;  $\beta$ -lactoglobulin, 18,400; lysozyme, 14,300; bovine trypsin inhibitor, 6,200; Insulin ( $\alpha$  and  $\beta$  chains), 2,850 (Gibco BRL).

#### Metal removal and reconstitution

Metal-free protein was prepared by desalting at low pH. To a solution of protein (1–5 mg/mL), solid dithiothreitol was added to 100  $\mu$ M and incubated at room temperature for 30 min, after which the pH was adjusted to pH 1 with 4 M HCl. This was then applied to a column (30 cm  $\times$  3 cm) packed with Sephadex G-50 (Pharmacia), equilibrated with 10 mM HCl and eluted with 10 mM HCl at 4 °C. For reconstitution experiments, peak protein fractions (10–15  $\mu$ M) were purged with nitrogen gas, then adjusted to pH 9

with 3 M Tris base in the presence of 1.5 equiv. of either Cd(NO<sub>3</sub>)<sub>2</sub> or ZnCl<sub>2</sub> and centrifuged. Samples for gel shift analysis were diluted at least 1:1 and 10 mM  $\beta$ -mercaptoethanol was added. Samples of apo-protein were prepared identically to Zn- or Cd-reconstituted protein, but without the addition of metal.

#### Gel mobility shift assay

Assays were conducted essentially according to Ausubel (1995). Samples (10  $\mu$ L) containing 40 fmol of radiolabeled DNA, 0–20  $\mu$ M purified protein, 10% glycerol, 5 mM EDTA, 50 ng/ $\mu$ L sonicated salmon sperm DNA (Sigma), 30 mM NaCl, and 20 mM Tris-HCl pH 7.5 or pH 9 were incubated at 4 °C for 30 min, then electrophoresed at 500–700 volts (20 mA) on 5% polyacrylamide (10% in the case of the pV short fragment) containing 10% glycerol in 50 mM Tris-borate/1 mM EDTA, pH 8. The gel was then vacuum-dried onto Whatman paper at 70 °C and exposed to a Molecular Dynamics storage phosphor screen overnight, then read using a Molecular Dynamics phosphorimager.

#### In vitro transcription/translation assay

In vitro transcription/translation assays were performed using the Promega *E. coli* S30 extract kit for circular DNA essentially according to the manufacturers instructions. LacZ assays were performed according to Miller (1992). Each 25  $\mu$ L reaction contained 2  $\mu$ g of the plasmid pTMR1, carrying the pV-LacZYA construct and 0.9 or 3.6  $\mu$ g of purified CdB. Reactions were incubated at 37 °C for 1.5 h, then 475  $\mu$ L of  $\beta$ -galactosidase reaction buffer (Miller, 1992) was added and the samples incubated at 28 °C until yellow color developed, after which 0.25 mL of 1 M sodium carbonate was added and the absorption at 420 nm read 5 minutes later. CdB-dependent activation of LacZ transcription is reported in  $\beta$ -galactosidase units as nmol ONP produced ( $\epsilon_{420}$ , 4,500 M<sup>-1</sup> cm<sup>-1</sup>) in the 0.75 mL final volume per min per mg protein added.

#### DNAase I cleavage protection

DNAase I cleavage protection experiments were conducted essentially according to Neufing et al. (1996). The 50  $\mu$ L reaction volumes contained 20 mM Tris-HCl pH 8, 200 fmol <sup>32</sup>P-end labeled DNA and 0–12  $\mu$ M CdB. To this, 2  $\mu$ L of 0.05  $\mu$ g/mL DNAase I (Boehringer) was added and incubated at room temperature for 2 min, after which 50  $\mu$ L phenol was added to stop the reaction. The reactions were then mixed and centrifuged at 10,000  $\times$  g for 5 min and the supernatant removed. To this 107  $\mu$ L of 0.3 M sodium acetate pH 4.6/0.5% v/v glycogen/0.001% w/v SDS/98% ethanol was added and centrifuged at 15,000  $\times$  g at 4 °C for 15 min. The pellet was then washed with 70% ethanol, dried, and resuspended in 5  $\mu$ L of 30% v/v formamide loading buffer. The reactions were heated to 100 °C for 3 min and stored on ice immediately prior to electrophoresis on a 6% polyacrylamide/6 M urea gel. Marker lanes contained Sequenase (USB) dideoxy chain-termination sequencing reactions obtained with pTMR1 as template and using the appropriate <sup>32</sup>P-end labeled primer. Gels were then dried onto Whatman paper and either exposed to Kodak Biomax film or to a Molecular Dynamics storage phosphor screen.

#### Phage assays

Phage assays were performed according to Hocking and Egan (1982b). Cells were grown in bacto-tryptone (TB) broth to an

optical density of 0.6. Two hundred  $\mu$ L of culture was incubated with 100  $\mu$ L of phage stock for 15 min at room temperature, then mixed with 3 mL of 0.7% agar at 45 °C, poured onto TB plates and incubated overnight at 30 °C.

#### Random mutagenesis

E 251 cells containing the B expression plasmid pEC434 (Dibbens & Egan, 1992) in L broth (OD<sub>600</sub>, 0.4) were mutagenized using nitrosoguanidine (50  $\mu$ g/mL, 40 min, 37 °C, ≈50% killing) by the method of Miller (1992), subcultured 1:100 in L broth, incubated overnight at 37 °C, then transformed (Chung et al., 1989) with pV-VUTSR plasmid, pRPT1, and spread on antibiotic plates. Surviving colonies appearing overnight at 37 °C were tested for resistance to 186 cltsp Bam57.

#### Analytical gel permeation chromatography

Purified protein samples (100  $\mu$ L, 0.6 mg/mL) were applied to a column (Superose 12 HR 10/10, Pharmacia) equilibrated with 10 mM Tris-HCl pH 9/10 mM  $\beta$ -mercaptoethanol/0.1 M NaCl and eluted with the same buffer at a flow rate of 0.25 mL/min using a Pharmacia FPLC system. Eluant was monitored at 280 nm. Molecular weight calibration standards (yeast alcohol dehydrogenase, 120,000; bovine serum albumin, 66,000; carbonic anhydrase, 29,000; horse cytochrome C, 12,400 and aprotinin, 6,500) were obtained from Sigma.

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