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Moderately thermostable phage Φ11 Cro repressor has novel DNA-binding capacity and physicochemical properties

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The temperate *Staphylococcus aureus* phage Φ11 harbors *cI* and *cro* repressor genes similar to those of lambdoid phages. Using extremely pure Φ11 Cro (the product of the Φ11 *cro* gene) we demonstrated that this protein possesses a single domain structure, forms dimers in solution at micromolar concentrations and maintains a largely α-helical structure even at 45°C. Φ11 Cro was sensitive to thermolysin at temperatures ranging from 55-75°C and began to aggregate at ~63°C, suggesting that the protein is moderately thermostable. Of the three homologous 15-bp operators (*O1*, *O2*, and *O3*) in the Φ11 *cI-cro* intergenic region, Φ11 Cro only binds efficiently to *O3*, which is located upstream of the *cI* gene. Our comparative analyses indicate that the DNA binding capacity, secondary structure and dimerization efficiency of thermostable Φ11 Cro are distinct from those of P22 Cro and λ Cro, the best characterized representatives of the two structurally different Cro families. [BMB reports 2009; 42(3): 160-165]

INTRODUCTION

The bacteriophage-encoded Cro proteins vary notably in structure, oligomerization ability and in thermal stability (1-6). While the Cro proteins of phages P22, 434, N15, and Xfaso 1 all bear similar α-helical folds, λ Cro and Pfl 6 Cro harbor homologous α-helix/β-sheet folds. These two Cro protein structural families share 25-40% identity at the primary sequence level. Of the Cro proteins adopting α-helical folds, all except N15 Cro exist primarily as monomers in solution. N15 Cro, λ Cro and Pfl 6 Cro all form dimers in solution but their dimerization efficiencies differ significantly. P22 Cro was shown to be more thermally stable than λ Cro. Interestingly, Cro proteins, which bind to operator DNAs as dimers, inhibit transcription from the operator-overlapped early promoters (7). To date however, Cro proteins from most temperate phages have yet to be investigated at the structural and functional level.

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Bacteriophage Φ11 uses *Staphylococcus aureus* as a host and, like lambdoid phages, harbors the *cI* and *cro* genes in divergent orientations (7, 8). The 239 amino acid product (CI) of the Φ11 *cI* gene shows moderate homology with λ repressor. Following partial purification, this protein was suggested to bind to the two 15-bp inverted repeats (with the axes of a partial twofold symmetry) or operator sites (*O1* and *O2*) in the Φ11 *cI-cro* intergenic region with variable affinity (9). Our preliminary analyses reveal that the 72 amino acid product (Cro) of Φ11 *cro* gene shares ~20-40% identity with experimentally characterized Cro or Cro-like proteins of phages Tuc2009 (10), Phig1e (11), A2 (12) and PhiSa3ms (13). In contrast, Cro proteins from phages 434 (14), P22 (15), and λ (7) showed moderate homology with the Cro/Cro-like proteins of the former phages including Φ11. After aligning the amino acid sequences from the above-mentioned Cro proteins, only one identical amino acid residue (Ala 20) and three highly conserved residues (Leu 5, Gln 16 and Phe 60) were detected in Φ11 Cro. Gln 16 and Ala 20 are part of the helix-turn-helix (HTH) DNA binding motif in Φ11 Cro. Interestingly, the organization of the predicted secondary structural elements in Φ11 Cro closely resembles that of Cro proteins of Tuc2009, PhiSa3ms, P22 and 434. The structure and the DNA binding capability of the Cro repressor in any temperate *S. aureus* phage have yet to be investigated experimentally.

Using extremely pure Cro repressor from the temperate *S. aureus* phage Φ11, we demonstrated that it neither binds to *O1* nor to *O2*, but instead binds to another homologous site (named *O3*) in the *cI-cro* intergenic region. The secondary structure and dimerization efficiency of Φ11 Cro were found to be intermediate to those of λ Cro and P22 Cro. In addition, Φ11 Cro shows moderate thermal stability.

RESULTS AND DISCUSSION

Φ11 Cro is a single domain protein

Thirteen trypsin and eight chymotrypsin recognition sites were distributed throughout the primary structure of native Φ11 Cro (data not shown). These enzymes only cleaved those sites that were located in flexible regions or those that were exposed on the surface of the folded Cro molecule. To map such flexible region (s) in native Cro, we performed limited proteolysis of histidine-tagged Cro (named Cro here) with both trypsin and

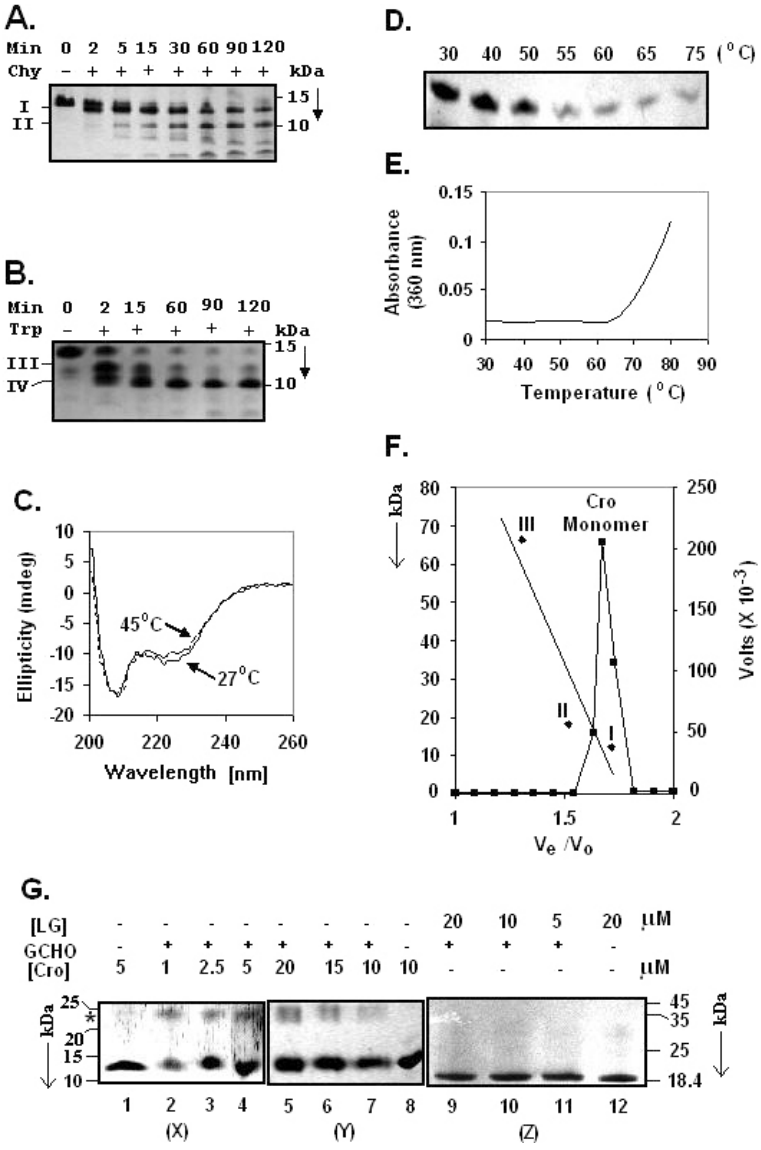

Fig. 1. Physicochemical characterization of Φ11 Cro. Chymotrypsin (A) and trypsin (B) digested Cro fragments were analyzed by Tris-tricine SDS-15% PAGE followed by silver staining. Fragments I and II were generated by chymotrypsin (Chy), whereas, fragments III and IV were generated by trypsin (Try). The molecular masses (in kDa) of marker protein bands were as shown at the right side of the gel. (C) Far UV CD-spectra of the Cro repressor were recorded at 25°C and 45°C according to Ganguly *et al*. (21). (D) SDS-15% PAGE analysis of thermolysin-cleaved Cro products. Proteolysis of Cro by thermolysin was performed essentially according to Hecht *et al*. (22). Briefly, each reaction mixture including 1 μg of Cro and 3.33 ng of thermolysin was incubated for 5 min at the indicated temperature followed by termination of the cleavage by the addition of EDTA. (E) Thermal aggregation of 5 μM Cro in Buffer C was performed according to Bandhu *et al*. (23). (F) Analytical gel filtration chromatography. Approximately 100 μl of 20 μM Cro in phosphate buffer was loaded onto a ~14 ml HPLC gel filtration column equilibrated in phosphate buffer and the absorbance of the eluted fractions, determined at 220 nm, were expressed as Volts. The column was calibrated with BSA (66 kDa, III), β-Lactoglobulin (18.4 kDa, II) and Cytochrome C (12.3 kDa, II). The molecular weights were plotted against V_e/V_o, where V_e and V_o denote the elution volume and void volume, respectively. The void volume of column, as determined by the elution of blue dextran, was 5.5 ml. (G) Glutaraldehyde (GCHO)-treated (+)/untreated(-) Cro/β-lactoglobulin (LG) solutions were analyzed by SDS-15% PAGE followed by either Coomassie blue (Y and Z) or silver (X) staining. Lanes 1-4, 5-8 and 9-12 contained ~0.65 μg, 2.5 μg and 1.8 μg protein, respectively. The concentrations of Cro and LG in the reactions were as indicated above the respective lanes. The molecular masses (in kDa) of marker protein bands are shown at both sides of the gel. An asterisk denotes dimeric Φ11 Cro.

chymotrypsin in separate reactions using standard techniques. Our data are presented in Fig. 1. Proteolysis of Cro by either trypsin (Fig. 1A) or chymotrypsin (Fig. 1B) yielded only one stable protein fragment (II or IV) of nearly 10 kDa. The data suggested that the majority of the enzymatic cleavage sites were buried in the interior of the folded Cro protein. The sequences of the first five N-terminal amino acid residues of fragments II and IV were determined to be NMQWN and GSEFN, respectively. This indicated that fragment II was generated by the cleavage of a peptide bond connecting amino acid residues 36 and 37, whereas, fragment IV was generated by digesting the peptide bond between residues 32 and 33. Because these cleavage sites were located in the histidine tagged region in Cro, our data suggested that native Cro is a single domain protein similar to that of P22 Cro, 434 Cro and λ Cro (7).

Secondary structure of Φ11 Cro

To find clues about the Cro secondary structure and to learn the effect of temperature on the conformation of Cro, CD spectra (200-260 nm) were recorded at different temperatures. Fig. 1C revealed that the spectrum of Cro at 25°C is very similar to that recorded at 45°C and that both spectra carry nearly identical negative elliptical peaks at both 208 and 222 nm. While the peaks obtained at 208 nm are nearly pointed, the peaks recorded at 222 nm are somewhat flattened. Although the presence of the histidine tag on Cro reduces its helical content

slightly as was evident from bioinformatic analysis of both native as well as histidine-tagged Cro (data not shown), the CD-spectrum of ϕ11 Cro appears to be somewhere between those of P22 Cro and λ Cro (6).

Moderate Φ11 Cro thermostability

The CD-spectrum of Φ11 Cro remained unaffected by increasing the incubation temperature from 25°C to 45°C (Fig. 1C), suggesting that Cro is thermostable. To see whether Φ11 Cro is truly thermostable, we studied both the thermolysin-mediated proteolysis and the aggregation of Φ11 Cro at different temperatures. Nearly 80% of Φ11 Cro was digested by thermolysin by raising the incubation temperature of proteolysis from 30°C to 55°C or even higher (Fig. 1D). Interestingly, the aggregation of Φ11 Cro began at ~63°C (Fig. 1E). Together, these data suggest that Φ11 Cro is a moderately thermostable protein and that this thermostability is greater than that observed for λ Cro, with a melting temperature close to 40°C (3).

Oligomeric state of Φ11 Cro

The Cro repressor encoded by Φ11 can dimerize spontaneously in solution as observed for λ Cro (3, 7) or can remain a monomer similar to that of P22 Cro (15). To understand the status of Cro in solution, we carried out both gel filtration chromatography and glutaraldehyde crosslinking of Cro at various concentrations. As shown in Fig. 1F, passage of a 20 μM solution of Cro through a gel filtration column produced a single peak that corresponded to monomeric Cro (~12.4 kDa) when compared to the elution profiles of protein standards. Similar analysis conducted with a 30 μM Cro solution also produced a single monomeric Cro-specific peak (data not shown), indicating that at micromolar concentrations Φ11 Cro exists as monomer in solution. Contrary to the results described above, glutaraldehyde-mediated crosslinking showed that Φ11 Cro predominantly forms a dimer at 1-20 μM concentrations. This dimerization appears to be specific because β-lactoglobulin did not form an oligomer under identical conditions (Fig. 1G). As observed from the CD-spectrum of Φ11 Cro (Fig. 1C), the dimerization efficiency of Φ11 Cro also appears intermediate to monomeric P22 Cro and dimeric λ Cro (3, 6). Previously, a mutant λ Cro harboring an Ala33Trp (as in P22 Cro) change, showed reduced dimerization efficiency and an increased thermal stability (3). Ala33 in λ Cro and Trp30 of P22 Cro were found to align with Leu32 of Φ11 Cro (data not shown), indicating that Leu32 may be responsible for the dimerization as well as the moderate thermal stability of Φ11 Cro.

Φ11 Cro binds to a single site in the Φ11 *cI-cro* intergenic region

To identify the possible Cro binding site (s) in the Φ11 *cI-cro* intergenic region, we performed DNase I footprinting analysis using Cro and radioactively labeled *O* DNA (Fig. 2A). The footprints of both the top and bottom strands of *O* DNA indicate that one extended region in *O* DNA was resistant to DNase I digestion in the presence of saturating amounts of Cro. More precisely, Cro protected the -128 to -47 and -127 to -54 regions of the top and bottom strand, respectively (Fig. 2B). The entire protected region harbors two putative 15 bp operator sites (*O3* and *O2*), which are separated by 31 bp. Interestingly, the *O1* site that showed binding affinity for Φ11 CI was not protected by Cro. In contrast, the *O3* site that did not interact with Φ11 CI (our unpublished data) was protected by Cro. The DNase I hypersensitive sites on both strands of the *cI-cro* intergenic region (designated by open circles; Fig. 2B) indicate that *O* DNA undergoes a conformational change of upon Cro binding.

To see whether Cro truly binds to both *O2* and *O3*, we carried out a gel shift assay using ^{32}P labeled *O* DNA and varied the Cro concentration. As shown in the Fig. 2C, one prominent shifted complex (designated I) gradually formed with increasing amounts of Cro. Additional gel shift assays using synthetic ^{32}P labeled DNAs (harboring either *O1*, *O2*, or *O3*) and varying the Cro concentration revealed that only O3 DNA bound appreciably to Cro (Fig. 2D). The Cro/*O3* DNA binding plot indicated that at Cro concentrations producing 50% *O3* DNA saturation, the apparent equilibrium dissociation constant was nearly 475 nM (Fig. 2E). We did not use Cro concentrations higher than 900 nM in any of our gel shift assays because non-operator *cspC* DNA showed weak binding to Cro at concentrations higher than 1,000 nM (data not shown).

Multiple Cro binding sites were shown to be present in the *cI-cro* intergenic regions in P22, 434, A2, Φg1e, Tuc2009 and λ (7, 10, 15, 16) phage. In contrast, the *cI-cro* intergenic region of *S. aureus* phage, similar to that of Φ11 or ΦSa3ms, bore only a single site supporting Cro binding (as mentioned above and in 13), indicating that the mechanism of Φ11 Cro or ΦSa3ms Cro action might be distinct from that observed for other lambdoid phages.

MATERIALS AND METHODS

Bacterial and phage strains and their growth conditions

S. aureus RN4220 and *E. coli* cells were routinely grown in Trypticase soy broth/agar (17) and Luria broth/agar (18), respectively. The growth media were supplemented with the appropriate antibiotics as needed. Phage ϕ11 and its growth conditions were as described previously (17).

Molecular biological, biochemical and biophysical techniques

Plasmid DNA isolation, digestion of DNA by restriction enzymes, ligation of DNA fragments, transformation, polymerase chain reaction (PCR), estimates of the amount of protein and DNA, native and SDS-PAGE, polyacrylamide gel staining, sequencing of PCR generated DNA using Φ11 DNA fragments as templates, analytical gel filtration chromatography, glutaraldehyde crosslinking, the recording of CD spectra, partial proteolysis, thermal aggregation and N-terminal end sequenc-

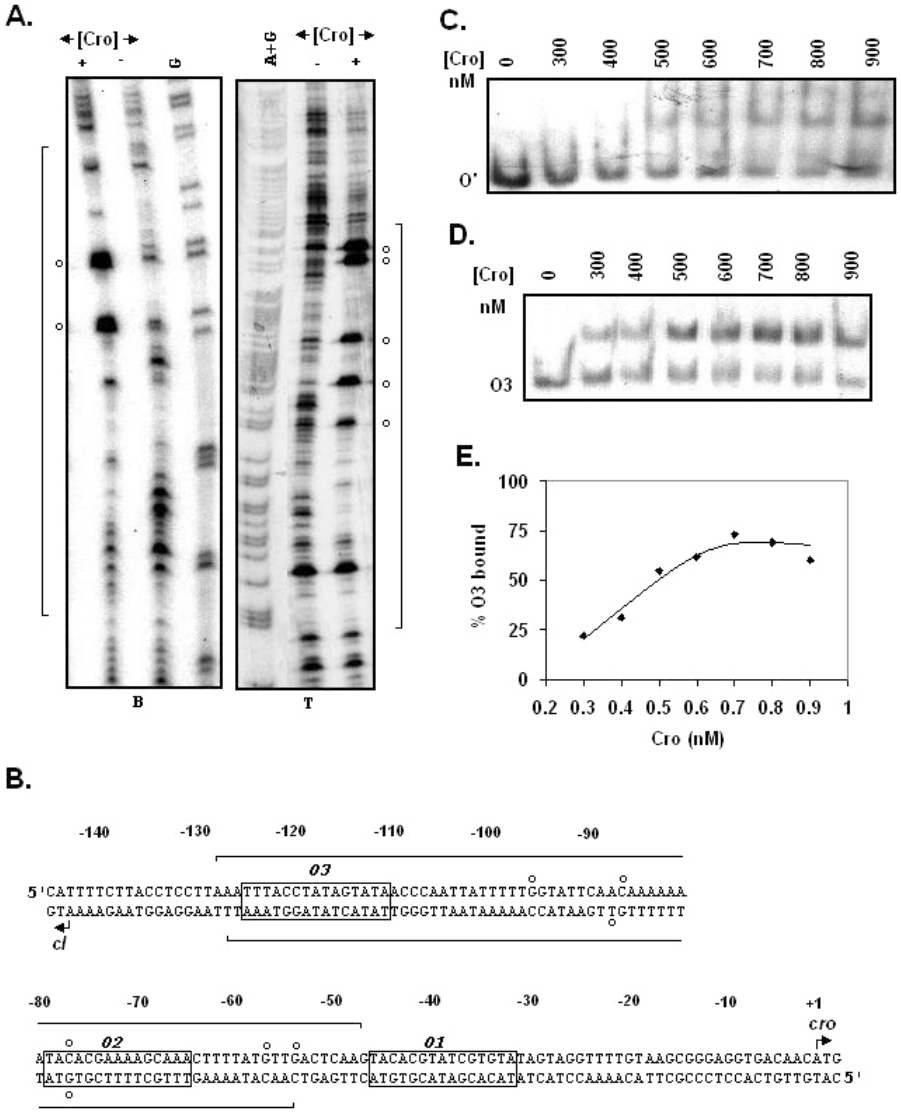

Fig. 2. Operator DNA-Φ11 Cro interaction. (A) Autoradiograms showing the DNase I footprints with the top (T) and bottom (B) strands of *O* DNA. The + and - indicated the DNase I-cleaved fragments of ^{32}P labeled *O* DNA, generated in the presence and absence of Cro, respectively. G and G+A are markers generated from the labeled *O* DNA as previously published (26). Angled lines and open circles denote the DNase-I resistant regions and the DNase I hypersensitive sites, respectively. (B) The DNA sequence of the Φ11 *cI-cro* intergenic region. Angled lines, circles and angled arrows indicate the DNase I-protected regions, the DNase I hypersensitive sites and the start codons of CI and Cro, respectively. The 15-bp inverted repeats (designated *O1*, *O2* and *O3*) are in the boxed in areas. The first base of the start codon of Cro was considered +1 and the whole DNA sequence was numbered according to that +1 designation. (C) Autoradiogram of the gel shift assay exhibiting the interaction of the said amounts of Cro to ~0.5 nM ^{32}P-labeled O' DNA. I denotes shifted complex. (D) Autoradigram of gel shift assay showing the binding of indicated concentrations of Cro to ~0.5 nM ^{32}P labeled *O3* DNA. (E) Equilibrium binding of Cro to *O3* DNA. The amounts of *O3* operator bound by Cro were estimated by scanning the autoradiogram shown in Panel D and plotting the densitometric data against the corresponding Cro concentrations (300-900 nM).

ing of proteolytic enzymes generating peptide fragments were performed using standard published procedures (9, 18-23). Genomic DNA from Φ11 phage particles was isolated according to Lee and Iandolo (17).

Cloning, overexpression and purification of Φ11 Cro

To clone the Φ11 *cro* gene, a 252 bp Φ11 DNA fragment [PCR amplified using genomic Φ11 DNA as template and primers PCR4 and PCR5 (Table 1)] was inserted into a pGEMT-Easy vector (Promega, U.S.A.) according to the manufacturer's protocol. One of the recombinant pGEM-T-Easy vectors carrying Φ11 *cro* with no mutation (as confirmed by DNA sequence analysis) was selected and designated pSAU1176. The Φ11 DNA insert, pSAU1176, was subcloned into an *E. coli* pET28a (Novagen, U.S.A.) expression vector to construct pSAU1259. The subcloning included 37 extra amino acid residues including six histidine residues at the N-terminal end of the Cro. After transforming pSAU1259 into competent *E. coli* BL21 (DE3) cells (Novagen, U.S.A.), a healthy transformant was selected, purified and stocked at -70°C.

To induce the expression of Cro, 0.5 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) was added to an *E. coli* (pSAU1259) cell culture in log phase. The culture was then grown for another 3 h at 32°C. The induced cells were harvested, washed with 0.9% NaCl and resuspended in a 1/10th volume of lysis Buffer A [20 mM Tris-chloride buffer (pH 8.0), 300 mM NaCl, 5% glycerol, 10 mM imidazole and 10 μg/ml PMSF (phenylmethane sulfonylfluoride)]. After disrupting the

Table 1. Primers used in the study

Name	Sequence (5'-3')	Purpose
PCR4	AAGCTTGCATTTGTTATGTCTCC	Synthesis of Cro ORF
PCR5	GAATTCAACATGCAATGG AATTTAATAAAG	Synthesis of Cro ORF
pHC1	GGATCCTAAATCTTCTTGAGTAC	Synthesis of O′ DNA
PCR11	GACTCAAGTACACGTATCGTGTATAGTAGGTTTA	Synthesis of *O1*DNA
PCR21	AAACCTACTATACACGATACGTGTACTTGAGTCA	Synthesis of *O1* DNA
IIa	ATTCAACAAAAAAATACACGAAAAGCAAACTTTTATGTTGACTCAAGTA	Synthesis of *O2* DNA
IIb	TACTTGAGTCAACATAAAAGTTTGCTTTTCGTGTATTTTTTTGTTGAAT	Synthesis of *O2* DNA
PCI51	GAATTCTCGCTAATTCTTTTTTATC	Synthesis of *O3* and O′ DNAs
IIId	TTTTTTTGTTGAATACCAAAAATAATTGGGTTATACTATAG	Synthesis of *O3* DNA
CSP4	CATGCCATGGATGAATAACGGTACAG	Synthesis of *S. aureus* cspC DNA
CSP6	CTCGAGCATTTTAACTACGTTTG	Synthesis of *S. aureus* cspC DNA

Note- Most DNA fragments synthesized here were also used in other study (unpublished data).

cells, Cro was purified from the crude extract by Ni-NTA column chromatography according to the method described for Φ11 CI (9).

The affinity chromatography-purified Φ11 Cro migrated as expected on SDS-12% PAGE (data not shown), was recognized by an anti-his antibody (data not shown) and bound to Φ11- DNA *in vitro* (see above). The protein was about 97% pure as was evident from scans of the band intensity on the PAGE gel. The overall yield approached 0.5 mg Cro per liter of induced *E. coli* cell culture. Prior to our experiments, Cro in Buffer A was extensively dialyzed against Buffer B [10 mM Tris-Cl (pH 8.0), 200 mM NaCl, 1 mM EDTA, 5% glycerol and 100 μg/ml BSA], Buffer C (Buffer B minus BSA) or against a phosphate buffer [50 mM Na-phosphate buffer (pH 7.0), 200 mM NaCl, 1 mM EDTA, 5% glycerol]. The phosphate buffer did not affect the DNA binding activity of Cro (data not shown). The concentration of Cro was calculated using the molecular mass of monomeric Cro.

Synthesis of ^{32}P labeled DNA fragments

The 269 bp *Eco*RI-*Bam*HI DNA fragment (harboring *O1*, *O2* and *O3*) of pSAU1201 DNA (9) was renamed O DNA. O′ DNA (also carrying *O1*, *O2* and *O3*) was synthesized by PCR using pSAU1201 DNA and primers pHC1 and pCI15 (Table 1). Similarly, *O3* DNA was amplified using primers PCI15 and IIId (Table 1) and pSAU1201 DNA. The *S. aureus cspC* DNA was amplified using primers CSP4 and CSP6 (Table 1) and *S. aureus* Newman DNA (24). The DNA fragments described above were purified from agarose gels using a standard procedure. Oligos PCR11 & PCR21 and IIa & IIb (Table 1) were annealed to prepare *O1* and *O2* DNA, respectively. All DNA fragments were labeled with either [γ-^{32}P] ATP or [α-^{32}P] dATP using standard procedures (18).

To label the top strand of O DNA with ^{32}P, oligonucleotide pHC2 was end labeled with [γ-^{32}P] ATP prior to amplification of *O* DNA by *Taq* DNA polymerase using pSAU1201 DNA and pHC1 and labeled pHC2 oligonucleotides. The resulting DNA fragment was purified from agarose gels. Plasmid pSAU1201 was successively treated with *Eco*RI, Klenow polymerase and [α-^{32}P] dATP, and *Bam*HI. Bottom strand labeled *O* DNA was purified from agarose gels.

Gel shift assay

The gel shift assay was performed by a standard procedure (9) using 300-900 nM of Cro in Buffer B and ~0.5 nM of ^{32}P labeled DNA. Separate gel shift assays were carried out to investigate the binding of Cro to each of O′, *O1*, *O2*, *O3* and *cspC* DNAs. The *S. aureus cspC* DNA harboring no Φ11 operator site was found to bind Cro only weakly at concentrations of 1,100 nM and higher (data not shown).

DNase I footprinting assay

DNaseI footprinting was performed using a standard procedure with certain modifications as described below (25). Briefly, ~60 μM of a labeled DNA fragment (~5,000 cpm) was incubated with 500 μM of Cro in 50 μl Buffer C for 20 min on ice. The reaction mixture was made to 1 mM with $MgCl_2$ and treated with 0.15 units of DNase I for 4 min at room temperature. The addition of 90 μl of Stop solution [200 mM NaCl, 80 mM EDTA (pH 8.0), 1% SDS, 0.03% Glycogen] terminated the reaction. After cleaning the cleaved DNA fragments by phenol chloroform (1:1) extraction and ethanol precipitation, the cleaved DNA fragments were resuspended in sequencing gel buffer [(98% deionized formamide, 10 mM EDTA (pH 8.0), 0.025% bromophenol blue)]. Each labeled DNA was treated with DNase I in the absence of Cro and the recovered DNA fragments were used as controls. Finally, both experimental and control DNA fragments were analyzed by urea-6% PAGE along with G and/or A+G sequencing ladders [from identically labeled DNA fragments generated by standard procedures (26)].

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