

Cooperative Interaction of CI Protein Regulates Lysogeny of *Lactobacillus casei* by Bacteriophage A2

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The temperate bacteriophage A2 forms stable lysogens in *Lactobacillus casei*. The A2-encoded *ci* product (CI), which is responsible for maintaining the A2 prophage in the lysogenic state, has been purified. The CI protein, which is a monomer of 25.3 kDa in solution, specifically binds to a 153-bp DNA fragment that contains two divergent promoters, P_L and P_R. These promoters mediate transcription from *ci* and a putative *cro*, respectively. Three similar, although not identical, 20-bp inverted repeated DNA segments (operator sites O₁, O₂, and O₃) were found in this segment. CI selectively interacts with O₁, which is placed downstream from the transcription start point of the *cro* gene, and with O₂ and O₃, which overlap with the –35 region of the two promoters. Using a heterologous RNA polymerase, we have determined the transcription start points of P_L and P_R. CI exerts a negative effect on the in vitro transcription of P_R by repositioning the RNA polymerase in a concentration-dependent manner. CI, when bound to O₁ and O₂, enhances the positioning of the RNA polymerase with the P_L promoter. Our data indicate that the CI protein regulates the lytic and lysogenic pathways of the A2 phage.

Lysogenic phages regulate their developmental fate by encoding a product (termed CI in the case of phage λ) that acts as a transcriptional regulator. CI binds to two operator regions on the phage genome, designated O_R and O_L, which are each further divided into three closely spaced binding sites (16 to 18 bp) termed operators O_{R1/L1} to O_{R3/L3} (reviewed in reference 27). Interspersed with the operators are two major promoters, P_R and P_L. In the P_R region, the binding of a CI dimer to its highest-affinity binding site (O_{R1}) turns off the P_R promoter, repressing the genes that mediate the lytic development of the phage. Furthermore, this interaction stabilizes the association of a second CI dimer to the O_{R2} site. This activates the P_{RM} promoter, which is divergent from P_R, thereby activating the transcription of genes responsible for the maintenance of lysogeny (13, 17, 19, 21, 27). When the repressor concentration becomes high enough to cause occupancy of the O_{R3} site, the transcription of *ci* is prevented. This leads to a reduction of repressor concentration which, in turn, provokes its unwinding from O_{R3} and the resumption of *ci* expression. The repressor behaves then as an autogenous regulator of its own synthesis that functions positively at low concentrations and negatively at high concentrations (reviewed in reference 27). In other words, the commitment between lytic and lysogenic development is thus critically dependent on the ability of the repressor to discriminate between these different operators.

Very little is known about the commitment between lytic and lysogenic development from bacteriophages from gram-positive bacteria. Analysis of the genomes of different lactococcal phages reveals that *rlt* (24), BK5-T (4), and Tuc2009 (32) code for a product that shows significant homology with the CI repressor of lambdoid phages. Unlike them, the lactococcal phages seem to have only one early region. In the case of phage

rlt, this may be subdivided into two regions (O₁ and O₂) with dyad symmetry (21 bp) separated by a 2-bp spacer, into which the –35 consensus regions of the two divergent promoters are embedded (24). In the case of phage BK5-T, there are three regions (O₁ to O₃) of dyad symmetry (18 bp) separated by 10- and 24-bp spacers. The putative O₁ and O₃ sites lie within the –35 and –10 consensus regions, whereas O₂ is placed between the two divergent promoters (4). In the case of the temperate bacteriophage A2, which infects *Lactobacillus casei* and *Lactobacillus paracasei* strains (16), the CI protein shows significant identity with the CI protein of lambdoid phages. The A2 *ci* gene encodes a 224-amino-acid polypeptide with a predicted molecular mass of 25,277 Da. It appears to be the central player in the maintenance of the lysogenic state because (i) it is synthesized in lysogenic cultures, presumably blocking the lytic development of superinfecting A2, and (ii) *L. casei* derivatives containing a chromosomal copy of *ci* are completely resistant to phage infection. In front of *ci* there are three regions (O₁ to O₃) of 20 bp, displaying partial symmetry, that are separated by 26- and 32-bp spacer stretches, respectively (20) (Fig. 1).

This paper deals with the molecular characterization of the CI repressor protein of bacteriophage A2 and how it acts to control gene expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *Escherichia coli* XL-1 Blue (5) and DH10B (GIBCO BRL) were used as recipients for plasmid constructions. *E. coli* BL21(DE3)/pLys was used in expression studies of the genes cloned into plasmid pET11a (31). Plasmids pLys (31) and pUC18 (35) have been previously described.

E. coli was grown and maintained on Luria medium (29). Antibiotic resistance transformants were selected with 100 μg of ampicillin per ml and/or 25 μg of chloramphenicol per ml as appropriate.

DNA manipulations. Plasmid DNA was obtained by the alkaline lysis method (3) and purified through a CsCl gradient (29). Analytical and preparative gel electrophoresis of plasmid DNA and restriction fragments was carried out in 0.8% (wt/vol) agarose–Tris-borate horizontal slab gels or 8% (wt/vol) polyacrylamide–Tris-borate gels. The purification of DNA fragments was carried out by electroelution (29).

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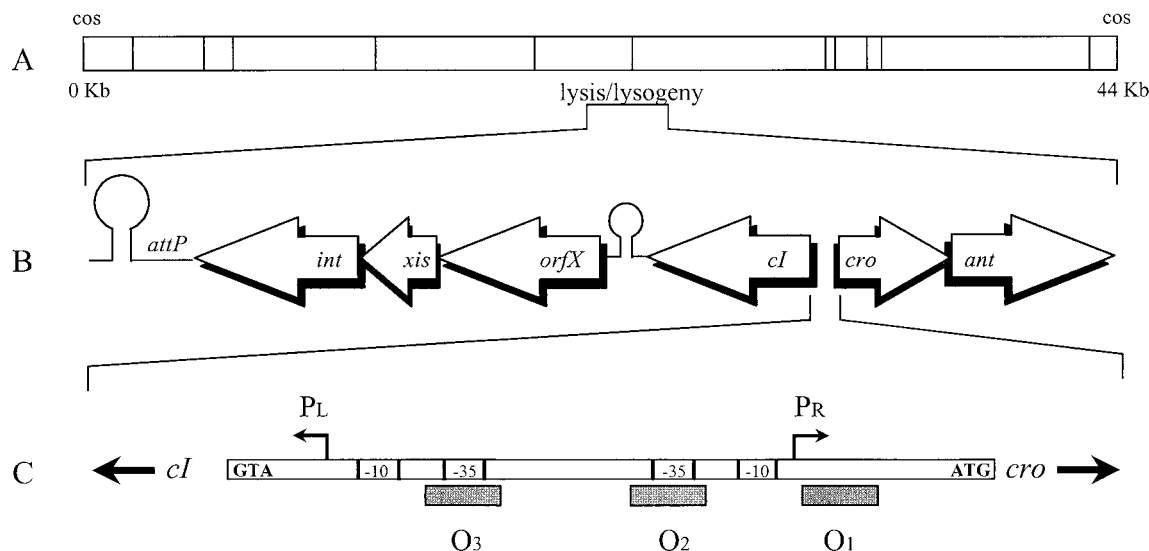


FIG. 1. Schematic representation of the A2 genome showing the location and organization of the development control region. (A) *EcoRI* physical map of the A2 genome. (B) Organization of its central region. The arrows refer to sequences functionally identified or similar to *cI* (repressor), *orfX* (unknown function), *xis* (putative excisionase), *int* (integrase), *cro* (putative homologue of λ *cro*), *ant* (putative antirepressor), and *attP* (site of attachment to the bacterial chromosome). (C) Scheme of the genetic switch region, located in the intergenic stretch between *cI* and *cro*. The transcription start sites of the P_L and P_R promoters and the relative positions of the -10 and -35 hexamers with respect to the three operator sequences (O_1 to O_3) are indicated.

The 153-bp DNA segment containing the intergenic region between *cI* and putative *cro* genes was obtained by PCR amplification with the primers 5'-TG GATTGCCTCCTTCGTTTC-3' and 5'-ACCTCAAGAATGATTTTAACAC CG-3'. The amplified DNA fragment was purified and then cloned into *HincII*-cleaved pUC18 to generate pUO183. A 183-bp *BamHI-HindIII* segment containing the 153-bp intergenic region was purified by electroelution and end labeled by filling in the staggered ends with T7 DNA polymerase in the presence of [α - 32 P]dCTP (3,000 Ci/mmol; Amersham) and cold dTTP, dATP, and dGTP.

Plasmid pET11a-*cI* was constructed as follows: primers 5'-GAGGTGAGAC ATATGAAAAC-3', which includes the starting point for the translation of *cI*, and 5'-CAGCTTTTAACGTGGATCCGGG-3', which corresponds to a sequence located downstream of it, were used to amplify the encompassing region. The primers were designed to introduce *NdeI* and *BamHI* restriction sites (underlined). The amplified DNA segment was purified, digested with the above enzymes, and ligated to *NdeI-BamHI*-cleaved pET11a, and the ligation mixture was transferred into *E. coli* BL21(DE3)/pLys.

Protein purification. The overexpression of CI was achieved by the addition of 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) (Boehringer Mannheim) to exponentially growing cultures of *E. coli* BL21(DE3)/pLys carrying pET11a-*cI*. After 30 min, rifampin was added (to a final concentration of 200 μ g/ml), and the cells were further incubated for 90 min. The cells were harvested, resuspended in 25 ml of buffer A (50 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, 1 mM dithiothreitol [DTT], 0.1% Tween 20) per liter of culture containing 1 M NaCl, and broken by passage through a French press. During the purification procedure all steps were performed at 4°C. Crude extracts (total protein concentration of about 20 mg/ml) were centrifuged (12,000 \times g for 30 min), the supernatant was discarded, and the pellet was washed twice with 25 ml of buffer A. The resulting pellet was thoroughly resuspended in the same volume of buffer A containing 1 M NaCl, until complete dissolution was obtained, and was then diluted four times in buffer A to achieve a final concentration of 250 mM NaCl. This sample was loaded onto a Q-Sepharose column (Pharmacia) equilibrated with the same buffer without DTT. The column was washed with 10 column volumes of buffer and eluted with a gradient of increasing salt concentrations (250 mM to 1 M NaCl). The fractions containing pure CI (as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) were pooled, diluted in glycerol (50% final concentration), and stored at -20°C . The protein was measured by using the Bio-Rad protein assay.

The CI protein was analyzed by matrix-assisted laser desorption-ionization-time of flight (MALDI/TOF) mass spectrometry with dihydroxybenzoic acid as the matrix on the HP G2025A MALDI/TOF system (Hewlett-Packard). The residual fractions were analyzed by peptide sequence analysis with the HP G1005A protein sequencing system (Hewlett-Packard).

Filter binding assays. The formation of DNA-protein complexes was measured as described previously (11). In short, the standard reaction was performed for 30 min at 30°C, and the mixture contained 475 pg (81 pM) of the 32 P-labeled 183-bp DNA segment (see above) and 126 ng (100 nM) of CI in a total volume of 50 μ l of buffer B (50 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, 0.01% Tween 20, 100 mM NaCl, 5% glycerol), unless stated otherwise. Buffer B (1 ml) was added to stop the reaction, and the mixture was filtered and extensively washed with

buffer B. Filters were dried, and the radioactivity bound to them was determined by scintillation counting. The DNA retained on the filter was corrected for the retention of radioactively labeled DNA in the absence of protein (1 to 3%). The specific activity of the labeled DNA was measured as trichloroacetic acid-precipitable material. All reactions were performed in duplicate.

Electrophoretic mobility shift assay (EMSA). The end-labeled 183-bp DNA fragment (240 pg or 81 pM) was incubated with increasing amounts of CI protein in 25 μ l of buffer B at 30°C for 30 min, in the presence of poly(dI)-poly(dC) as an unspecific competitor (at a final concentration of 4 ng/ μ l). The reaction mixture was loaded onto a 5% (wt/vol) nondenaturing polyacrylamide gel. Similar reaction conditions were used with the *Bacillus subtilis* vegetative RNA polymerase (σ^A -RNAP) and in the competition experiments between σ^A -RNAP and CI, except that the reaction mixture was loaded onto a 4.5% (wt/vol) nondenaturing polyacrylamide gel. Gels were run at 100 V in TAE buffer (40 mM Tris-acetate, 1 mM EDTA [pH 8]) at room temperature. Gels were vacuum dried and analyzed by autoradiography. Quantification of the repressor-operator complexes separated in these gels was performed by using an Instant Imager (Packard). The degree of DNA-protein hybrid formation was quantified by dividing the amount of complex formed by the amount of residual free DNA.

DNase I footprinting. The *EcoRI-SphI* or *HindIII-KpnI* DNA fragments obtained from pUO183 were end labeled at the *EcoRI* or *HindIII* sites with Klenow DNA polymerase and [α - 32 P]dATP (3,000 Ci/mmol; Amersham). End-labeled DNA (81 pM) was incubated for 30 min at 30°C in the absence or presence of CI and/or *B. subtilis* σ^A -RNAP in 20 μ l of buffer B plus poly(dI)-poly(dC) as an unspecific competitor (at a final concentration of 50 ng/ μ l), followed by the addition of freshly diluted DNase I (Boehringer Mannheim) (at a concentration to obtain, on average, one cut per molecule). The mixture was incubated at 30°C for 3 min, and the digestion was stopped by the addition of 1 μ l of 0.5 M EDTA, pH 8. The DNA was precipitated, redissolved in a loading buffer, electrophoresed on a 6% denaturing polyacrylamide gel, and autoradiographed. Chemical sequencing reactions (22) for purines, obtained by an express protocol (2), were run in parallel to determine the sizes of the DNA fragments generated.

In vitro runoff transcription assays. Plasmid pUO183 digested with *XmnI* was used as a template for in vitro transcription assays. The reaction mixtures contained, in 25 μ l of a solution consisting of 3 nM DNA, 0.2 mM each ATP, CTP, GTP, and UTP, 25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 90 mM ammonium sulfate, 2 mM DTT, 7.5 U of RNasin (Promega), 30 nM *B. subtilis* σ^A -RNAP, and different amounts of CI. After 30 min of incubation at 30°C, the reactions were stopped with 1 μ l of 0.5 M EDTA, pH 8. The RNAs were precipitated and then analyzed by primer extension as follows. The pellet was dissolved in 10 μ l of a solution containing 50 mM Tris-HCl (pH 7.5), 40 mM KCl, 7 mM magnesium acetate, 2 mM DTT, 200 μ M each dCTP, dGTP, and dTTP, 100 μ M [α - 32 P]dATP (3,000 Ci/mmol), 4 U of avian myeloblastosis virus reverse transcriptase (Promega), 10 U of RNasin, and 0.5 pmol of primers 5'-CGCCAGG TTTTCCAGTCACGA-3', for the P_R promoter, and 5'-GCGGATAACAA TTTACACAGG-3', for the P_L promoter. The reaction mixture was incubated at 42°C for 60 min, and the reaction was stopped by the addition of 0.5 μ l of 0.5 M EDTA, pH 8, and 100 μ l of TE buffer (10 mM Tris, 1 mM EDTA, pH 8). Unincorporated nucleotides were removed by passing the sample through a

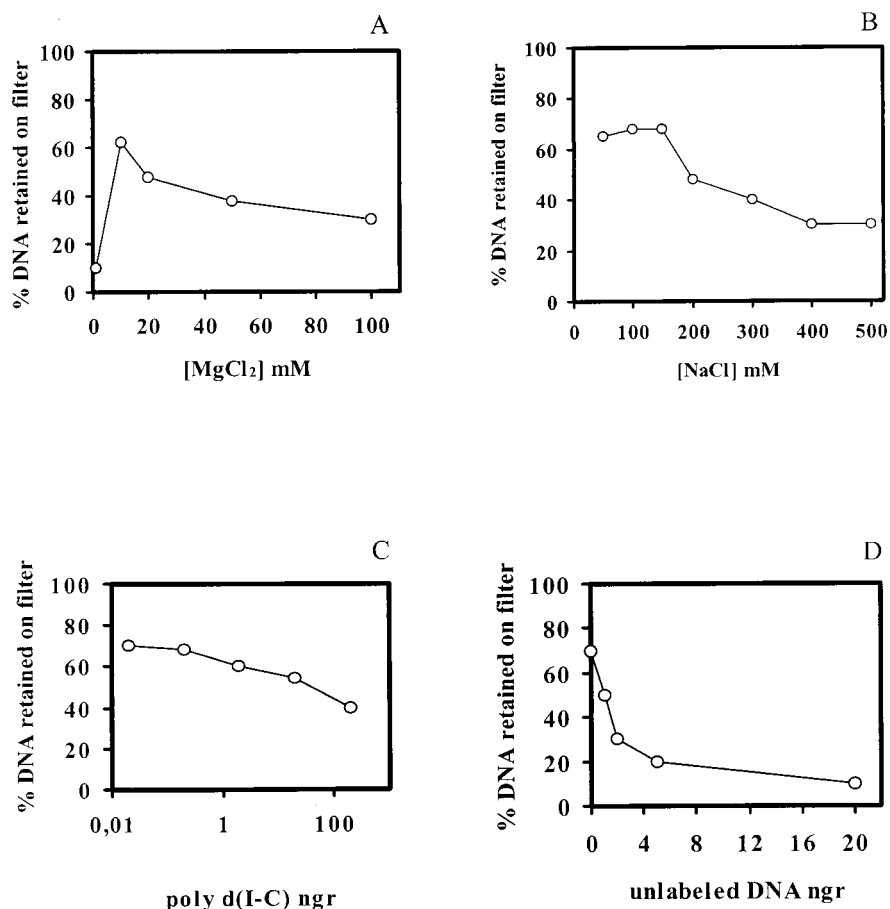


FIG. 2. Parameters involved in CI-DNA complex formation as detected with a filter binding assay. The CI binding parameter measured is indicated under each panel. Analytical binding reactions were performed in a 50- μ l volume with 81 pM 183-bp [32 P]DNA segment (475 pg) that comprises the *cl-cro* intergenic region and 100 nM CI (126 ng). The DNA retained on the filter was corrected for the retention of radiolabeled DNA in the absence of CI (about 2% of total DNA input).

Sephadex G-50 column. The resulting cDNAs obtained were precipitated, analyzed by denaturing PAGE (6%), and autoradiographed. Chemical sequencing reactions of purines (2, 22) were run in parallel to determine the sizes of the cDNAs obtained.

RESULTS

Purification of the bacteriophage A2 CI repressor. The CI protein was overexpressed as described in Materials and Methods. Under those conditions most of the CI, which accounted for about 2% of the total cell protein, was in the pellet of the crude extract centrifugations. This allowed the removal of soluble proteins and washing of the protein aggregates, resulting in the solubilization of most proteins coprecipitated with CI. The resulting pellet was solubilized in buffer A containing 1 M NaCl, and the solution was then diluted to lower the salt concentration to 0.25 M. The solubilized CI protein was further purified by a conventional chromatographic step (Q-Sepharose), rendering a product that was more than 95% pure, as judged by SDS-PAGE (data not shown).

Purified CI migrated as a 28,000-Da polypeptide under denaturing conditions, which is slightly more than expected from its size as deduced from the nucleotide sequence of *cI* (25,277 Da). The native molecular mass of the CI protein was obtained by MALDI/TOF mass spectrometry and turned out to be $25,235 \pm 13$ Da at nanomolar concentrations, which indicates that CI is a monomer in solution.

CI specifically binds to the A2 *cl-cro* intergenic DNA segment. Analysis of the genomic organization of the early region of phage A2 (1, 20) (Fig. 1) revealed that the putative rightward promoter (P_R) would transcribe the *cro* and *ant* open reading frames, while the leftward promoter (P_L) would govern the expression of the genes coding for CI and the site-specific recombination machinery. This organization allowed us to postulate that the decision between lytic and lysogenic development would be critically dependent on the ability of CI to act as the A2 repressor, probably by discriminating among possible operators located between P_L and P_R . In support of this hypothesis was the finding of a putative helix-turn-helix domain in the NH_2 -terminal half of CI that might mediate its DNA binding. To test whether the purified CI protein specifically binds to the early control region of the A2 genome, the 153-bp intergenic *cl-cro* region (obtained as a 183-bp DNA segment) was incubated with the CI protein and the mixture was subjected to filter binding assays.

The CI protein-DNA complex could be trapped on nitrocellulose filters, which allowed quantification of the affinity between its components. CI-DNA complex formation was strictly dependent on the presence of Mg^{2+} , with an optimum concentration at 10 mM (Fig. 2A). The extent of the reaction was partially reduced by concentrations of NaCl above 150 mM (Fig. 2B), while the presence of Tween 20 (0.1 to 1%) in the reaction mixture had no significant effect on complex forma-

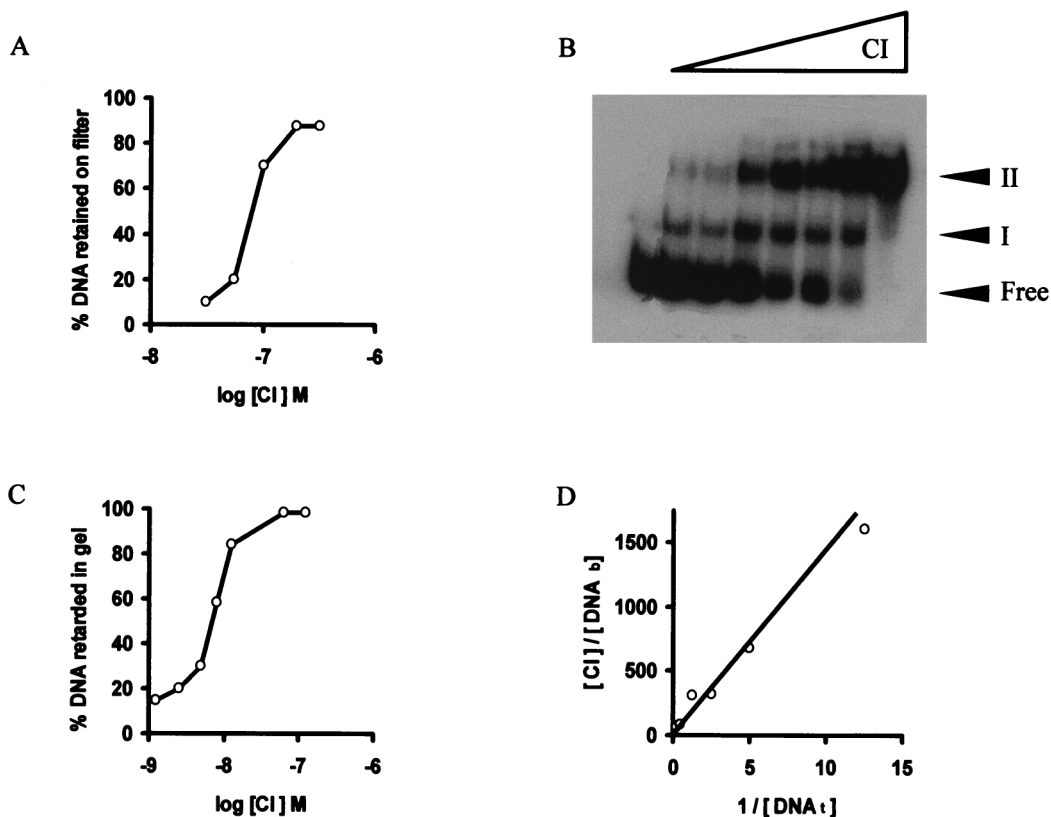


FIG. 3. Determination of the affinity between CI and the *cI-cro* intergenic DNA segment. The reaction mixtures were incubated for 30 min at 30°C. (A) Trapping of the ^{32}P -labeled 183-bp DNA segment (81 pM) on membrane filters in the presence of increasing concentrations of CI. (B) Gel shift assay of the complexes formed by increasing concentrations of CI (0, 1, 2, 4, 6, 8, 10, and 50 nM) and the DNA in the presence of a 400-fold excess of nonspecific [poly(dI)-poly(dC)] competitor DNA. The unbound probe (Free) and the protein-DNA complexes (I and II) are indicated. (C) Fraction of DNA bound by increasing concentrations of CI obtained from quantification of the phosphorimage of the autoradiograph shown in panel B. (D) Binding of CI (7 nM) to increasing concentrations of DNA. [CI] is the total protein concentration, $[DNA_b]$ is the concentration of substrate bound to CI, and $[DNA_t]$ is the total substrate concentration.

tion (data not shown). The binding of CI to the DNA segment was completed in 20 min (at least 80% of the radioactivity applied was retained by the filters).

The specificity of the binding between the *cI-cro* intergenic segment and CI was tested by determining the ability of unlabeled DNA molecules to act as competitors. Nonspecific DNA [poly(dI)-poly(dC)] ranging from 10 pg to 200 ng only partially abolished the binding, even at concentrations 400-fold in excess (Fig. 2C). On the contrary, when the competitor DNA was specific (i.e., a nonlabeled 183-bp segment), competition was readily observed (Fig. 2D). A 10-fold excess of specific competitor DNA reduced CI-DNA complex formation to background levels.

Cooperative binding of CI to the *cI-cro* intergenic region.

The rate of CI-DNA complex formation was determined as a function of CI concentration (Fig. 3A). The dependence of the DNA retention on protein concentration appeared to follow a sigmoidal curve. The apparent equilibrium constant (K_{app}) of the CI-*cI-cro*-DNA complex is estimated to be 80 nM at pH 7.5 and 30°C, whereas the K_{app} for nonspecific DNA is about 100 μM . The sigmoidal form of the curve suggested that the CI protein binds cooperatively to the *cI-cro* intergenic DNA segment.

To measure the affinity of CI for its cognate site by a different method and to learn about the type of complexes formed, an EMSA was used. Pure CI became specifically bound to the DNA segment, originating two bands (Fig. 3B) with retarded electrophoretic mobilities. Complex I was preferentially

formed at low protein concentrations, whereas at high protein concentrations only complex II was detected. The rate of CI-DNA complex formation was determined as a function of CI concentration (Fig. 3C). The dependence of the DNA retardation on the concentration of the protein followed a sigmoidal curve, from which a K_{app} of 7 nM at pH 7.5 and 30°C was deduced. The slope of the curve was similar to the one observed from the filter binding experiments (Fig. 3A), indicating again a cooperative binding of CI, although its K_{app} is about 10 times higher than when the EMSA was used. As a means to determine which of the K_{app} values is closer to reality, we measured CI-DNA complex formation under conditions in which the amount of substrate DNA was varied in the presence of a constant concentration of CI. As shown in Fig. 3D, the data were plotted as the total protein concentration divided by the concentration of bound substrate versus the inverse of the total DNA concentration. The slope of the line provided the dissociation constant ($K_D = 10$ nM) for CI binding, assuming that each CI monomer had one functional DNA binding site. This value is very similar to the one obtained by EMSA at different concentrations of CI.

A quantitative measurement of the cooperative binding of CI to the DNA was obtained by calculating the cooperativity factor, τ , according to the following equation: $\tau = 4(F)(2R)/(1R)^2$ (34). The advantage of this method is that it allows for internal controls, because all the necessary data are obtained from within a single lane. Therefore, τ can be calculated for each of the lanes, simply by quantifying the amount of DNA:

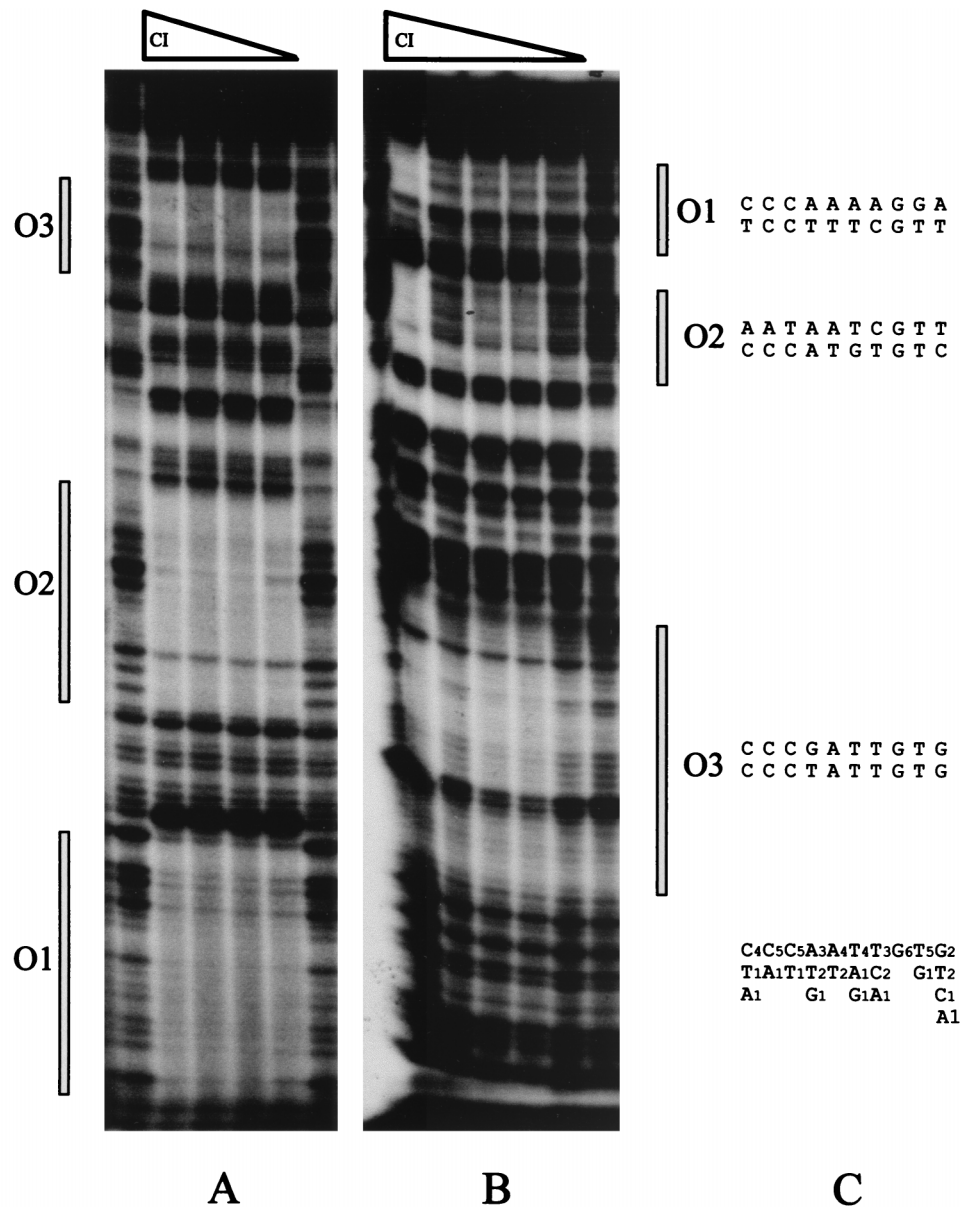


FIG. 4. DNase I footprinting analysis of CI-DNA complexes. (A) The 172-bp *EcoRI-SphI* DNA fragment (top strand) from plasmid pUO183 containing the 153-bp *cro-cl* intergenic region from A2 was incubated with 3.5, 7, 14, and 28 nM CI. (B) The 166-bp *HindIII-KpnI* segment (bottom strand) was incubated with 3.5, 7, 14, 28, and 56 nM CI. The DNA fragments were end labeled at the *HindIII* or *EcoRI* restriction sites and partially digested with DNase I. The operator sites are indicated. A DNA sequencing ladder was used to determine the DNA fragment lengths. (C) The DNA half-sites of the inverted repeated sequences of the CI proposed operators are aligned. Conserved bases and their frequencies are indicated below the sequences.

unbound (F), bound to one molecule of CI ($1R$), and bound to two or more molecules of CI ($2R$). The cooperativity factor is defined as the extent to which the formation of the doubly bound complex exceeds the formation of the singly bound complex. Theoretically, a value of τ greater or less than 1 indicates, respectively, positive or negative cooperativity, whereas a value equal to 1 indicates a lack of cooperativity. The value calculated from the data presented in Fig. 3B ($\tau = 9.97 \pm 0.59$) indicates that CI probably is a protein that binds cooperatively to its cognate sites.

CI binds to three discrete sites. For determination of the precise location of the sequences recognized by CI, the CI-DNA complexes formed with the *cl-cro* intergenic region were analyzed by DNase I footprints. The top strand contained three domains of CI protection of 22, 34, and 38 bp.

These were separated from each other by 12- and 38-bp intervals (Fig. 4A; also see Fig. 8). Within this interspaced region, phosphodiester bonds hypersensitive to DNase I cleavage were identified. One of them was located between O_1 and O_2 . The several hypersensitive sites between O_2 and O_3 are separated from each other by 10 ± 1 nucleotides, which is about one helical turn (assuming 10.5 bp per turn) in double-stranded DNA.

The bottom strand also exhibited three protected stretches (Fig. 4B). Here again, the protected domains are interspaced by sites hypersensitive to DNase I cleavage. These periodic anomalies in the DNase I cleavage pattern imply that the DNA is deformed by CI binding (reviewed in reference 30).

The amount of CI required to protect the three cognate sites is at least threefold smaller than when EMSA experiments

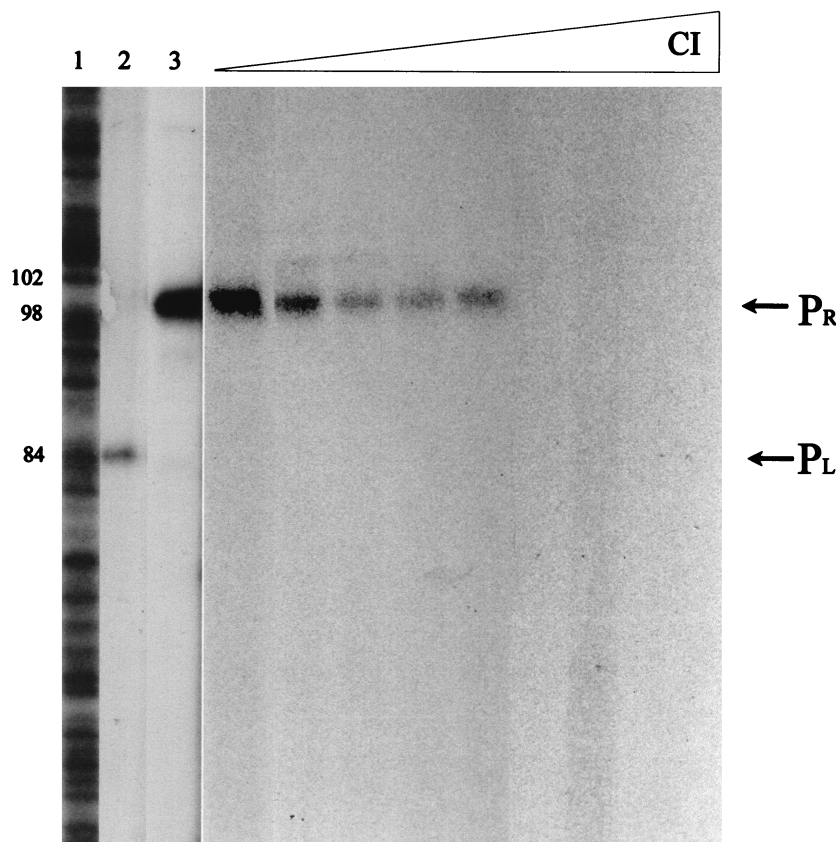


FIG. 5. Location of the transcription start sites in the *cro-cl* intergenic segment of phage A2 and role of CI in regulation of the P_R promoter. *B. subtilis* σ^A -RNAP (30 nM) and linearized pUO183 DNA (3 nM) were used for the in vitro generation of *cl* (lane 2) and *cro* (lane 3) runoff transcripts, and the resulting mRNAs were subjected to primer extension with reverse transcriptase in the presence of [α - 32 P]dATP. Maxam and Gilbert (G+A) sequencing reactions were used as size standards (lane 1). The sizes of the cDNAs obtained are indicated. Successive lanes show the effects of increasing concentrations of CI (3.7, 7.4, 29.6, 59.2, 118.5, 177, 237, and 474 nM, respectively) on *cro*-specific transcript generation.

were performed (3.5 nM, versus 10 nM in EMSA). Under these conditions about 40 CI monomers per DNA molecule (81 pM) were present in the footprint reaction.

To identify the peculiar characteristics in the DNA sequences that determine CI binding, these three protected regions were examined for common sequence elements. Three sets of inverted repeated regions that are not perfectly symmetric were identified. Regardless of whether we split each arm of the repeat into two half-sites (as done in the case of the lambdaoid operator sites; see reference 27) and align them as shown in Fig. 4C, a recognition pattern emerges: $C_4C_5C_5A_3A_4T_4T_3G_6T_5G_2$. We call these unique inverted repeated sequences O_1 , O_2 , and O_3 , and the intergenic region between *cl* and *cro* is termed the genetic switch region, in accordance with the λ nomenclature (reviewed in reference 27).

Localization and characterization of the promoters of the genetic switch region. Northern blot hybridization analysis of the mRNAs produced from the genetic switch region, together with a visual inspection of its nucleotide sequence, suggested that the promoters of *cl* and *cro* were located in this intergenic region and were divergently orientated (20) (Fig. 1). To map the transcription start points of the two promoters, in vitro transcription runoff experiments were performed with σ^A -RNAP and linearized pUO183, which contains the 153-bp segment of the genetic switch region, as a template DNA. After primer extension of the in vitro-transcribed products with the oligonucleotides described in Materials and Methods, we obtained two cDNA bands corresponding to segments of 84

and 100 nucleotides from the left- and right-oriented promoters, respectively (Fig. 5). These promoters were consequently named P_L and P_R . The cDNA bands seemed to indicate that the transcript from P_R would be about 10 times more abundant than the one from P_L . The 5' ends of transcription of these mRNA species map at guanosine nucleotides located at positions 12 and 130 (see Fig. 8). In both cases, 7 bp upstream from the transcription start sites, extended -10 consensus sequences (with TG dinucleotides at positions -14 and -15) were observed. These were separated by canonical 14-bp stretches from -35 consensus hexamers. Stretches of about 20 bp rich in A+T nucleotides could be predicted immediately upstream of the -35 hexamers (12). Consequently, all the crucial elements for RNAP promoter recognition in gram-positive bacteria, located near positions -59 to -38 , -35 , -14 , -15 , and -10 relative to the transcription start sites, were identified at both P_L and P_R (12, 15, 23, 33) (see Fig. 8). It is likely, therefore, that the data obtained from this work with the *B. subtilis* σ^A -RNAP would correspond to those that might be obtained if RNAP from *Lactobacillus* was used.

As shown in Fig. 1, P_R would transcribe the early lytic operon, including *cro* and *ant*, while P_L would be responsible for *cl* expression.

CI represses transcription from the P_R promoter. The CI repressor of lambdaoid phages is known to repress the transcription of early lytic genes and stimulate its own transcription by binding to its operator sites (reviewed in reference 27). Consequently, a likely role for the A2 CI protein would be to

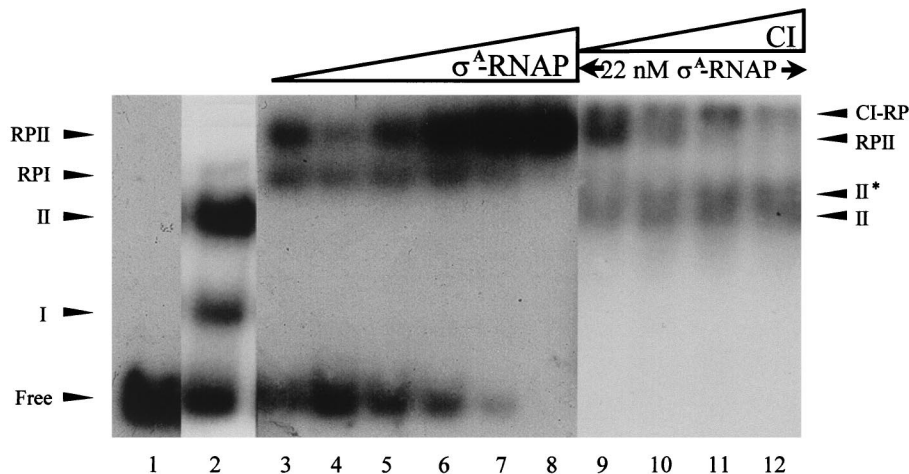


FIG. 6. Binding of CI and σ^A -RNAP to their cognate binding sites. The α - 32 P-labeled 183-bp DNA segment (81 pM) was incubated with CI (6 nM) (lane 2) and increasing concentrations of σ^A -RNAP (13, 17.5, 22, 26.5, 34, and 44 nM) (lanes 3 to 8) or with 22 nM of σ^A -RNAP and increasing CI concentrations (2.7, 5.6, 11, and 22 nM). The unbound probe (Free), the CI-DNA complexes (I and II), σ^A -RNAP-DNA (RPI and RPII), and the ternary complex CI- σ^A -RNAP-DNA (II* and CI-RP) are indicated. Lane 1, unbound DNA probe.

repress the transcription of the P_R promoter. To address this question, we used *B. subtilis* σ^A -RNAP and linearized pUO183 DNA (3 nM) to follow, by in vitro transcription assays, the expression of P_R in the presence of increasing amounts of CI. As shown in Fig. 5, CI reduced P_R utilization, in a concentration-dependent process, until no expression was detected. We estimated that 2.5 CI monomers (7.4 nM) per DNA molecule were enough to detect some repression from P_R , while about 60 polypeptides (180 nM) were needed to block it completely.

CI concentrations in excess of those that repress P_R (up to 480 nM) did not affect the expression of an unrelated promoter (data not shown), therefore ruling out the possibility that the repression effect on P_R was due to a nonspecific binding of CI to DNA or to the presence of contaminating RNases in the CI protein preparation.

Since the -35 hexamer of P_R is embedded in O_2 and O_1 is located immediately after its transcription start (see Fig. 8), it might be possible that CI repressed transcription from P_R , either by excluding the RNA polymerase (steric hindrance) or by holding it at the promoter in such a way that it could not start transcription. To analyze these two hypotheses EMSA experiments were performed (Fig. 6). When σ^A -RNAP (13 to 34 nM) was incubated with the 153-bp A2 DNA segment (81 pM) two complexes (RPI and RPII) were readily detected, but at a high protein concentration (44 nM) only the RPII complex was observed. The K_{app} of the σ^A -RNAP-DNA complex, which was estimated to be at a concentration of 20 nM at pH 7.5 and 30°C, is about threefold higher than the K_{app} values obtained for the CI-DNA complexes (Fig. 3C). When the σ^A -RNAP concentration was limiting (22 nM), the rate of protein-DNA complex formation was enhanced by the addition of CI at concentrations as low as 2.7 nM (Fig. 6, lanes 2, 5, and 9), with the subsequent formation of complex II (CI-DNA), RPII, and a novel low-mobility complex termed CI-RP. At a CI concentration approaching the K_D (11 nM) (Fig. 6, lane 11), diffuse complexes II (II + II*) and CI-RP complexes were observed. This result was obtained independently of the order of addition of the proteins; i.e., CI and σ^A -RNAP could remain bound to the DNA substrate. It is likely, therefore, that (i) σ^A -RNAP binds to two discrete, nonoverlapping sites in the DNA fragment, (ii) both CI and σ^A -RNAP coexist on the

intergenic 153-bp *ci-cro* DNA fragment, and (iii) CI and σ^A -RNAP interact.

CI relocates the RNA polymerase to the *ci-cro* intergenic region. The P_R and P_L divergent promoters are 81 bp apart and thus are located on the same face of the DNA helix. To investigate the type of complexes formed by CI, σ^A -RNAP, or both proteins on the intergenic 153-bp *ci-cro* DNA fragment, DNase I footprinting experiments were performed. The binding of CI to DNA gives a characteristic DNase I protection pattern already shown in Fig. 4 (Fig. 7A), while σ^A -RNAP shows an extended protection (about 40 bp), in the region where O_2 and the P_R promoter overlap, at concentrations of 15 nM and up (Fig. 7B).

In the presence of a constant amount of σ^A -RNAP (31 nM) and increasing concentrations of CI, a protection pattern is observed that differs from the picture obtained with each of the proteins (Fig. 7C). σ^A -RNAP does not alter the CI footprint at the O_1 and O_2 sites; this means that the polymerase becomes displaced from its binding site at P_R (which overlaps with O_2) even at low concentrations of the repressor (3.5 nM). However, the polymerase competes with CI at the O_3 site (which covers most of P_L), as is indicated by the change in the pattern of CI protection in the presence of σ^A -RNAP (a typical CI footprint at the O_3 site requires that its concentration be about eightfold higher [28 nM] than when σ^A -RNAP is not present in the reaction mixture [Fig. 7C and 8]). It is likely, therefore, that σ^A -RNAP interacts with the P_R promoter in the absence of CI but that in its presence, at a low concentration, the polymerase would become relocated to interact with the P_L promoter.

The combined data of gel retardation and DNase I footprint experiments suggest that, in the absence of CI, σ^A -RNAP interacts first with P_R , forming the complex RPI (Fig. 6). As its concentration increases, the polymerase would bind both promoters (RPII). However, in the presence of CI at a low concentration (from 2.7 nM), σ^A -RNAP would become displaced from P_R , due to the higher affinity of the repressor for O_1 and O_2 . As a consequence, transcription from the early P_R promoter would be repressed. CI, in turn, would interact with σ^A -RNAP to relocate it, by an as yet unidentified mechanism, on the P_L promoter originating the complex CI-RP (Fig. 6) and presumably activating transcription from P_L . Finally, when the

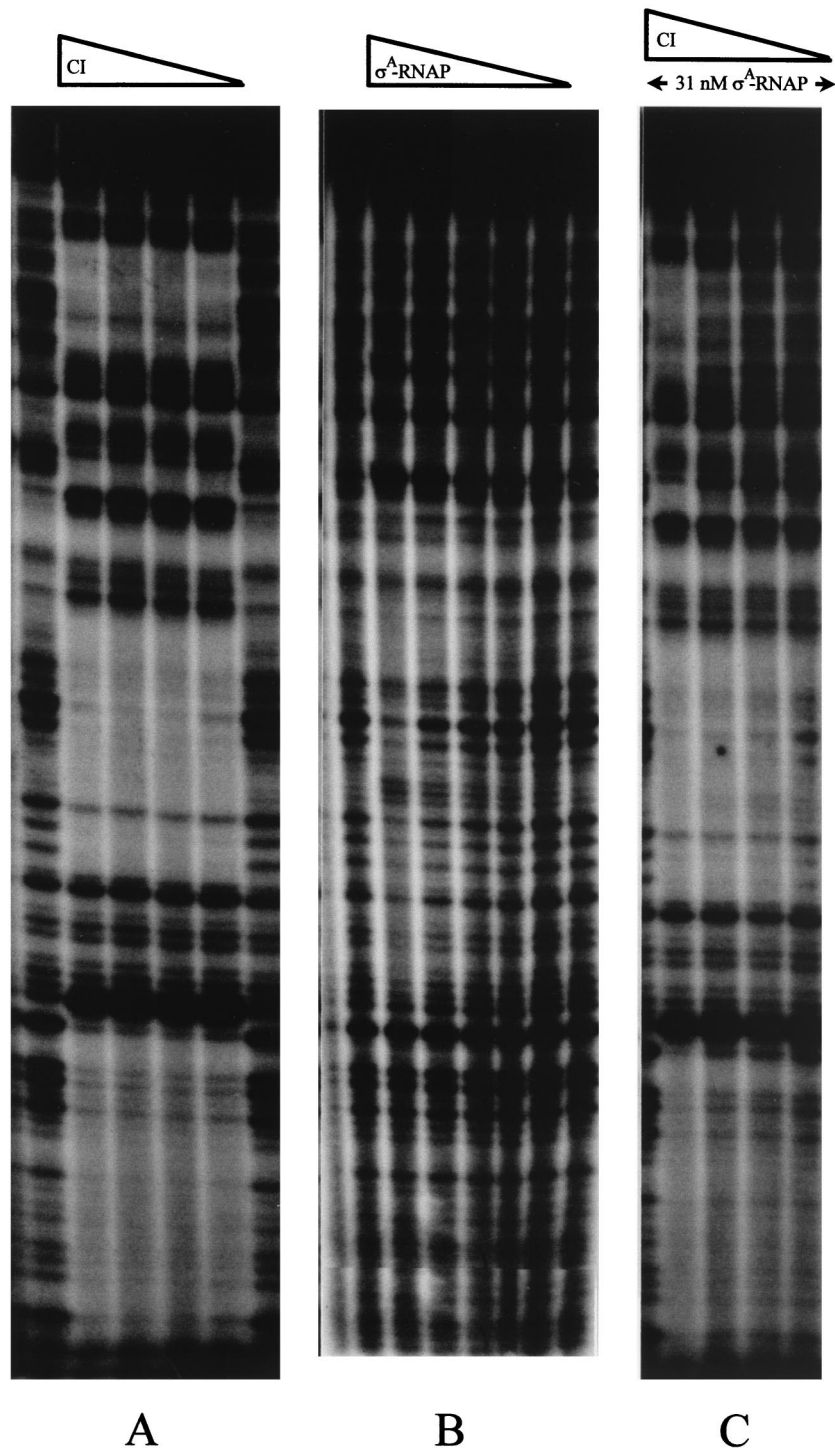


FIG. 7. Effect of CI on σ^A -RNAP binding to the intergenic *ci-cro* segment of A2, as seen through DNase I footprinting analysis. The 172-bp *EcoRI-SphI* DNA fragment (top strand) from plasmid pUO183 was end labeled at the *EcoRI* restriction site and incubated with 3.5, 7, 14, or 28 nM CI (A), with 3.9, 7.8, 15.5, 31, or 62 nM σ^A -RNAP (B), or with 31 nM σ^A -RNAP plus 3.5, 7, 14, or 28 nM CI (C). In this last case the RNAP was incubated with the DNA for 15 min prior to the addition of CI.

CI concentration increases to cause occupancy of O_3 , transcription from P_L would become prevented.

DISCUSSION

The deleterious effect of bacteriophages on industrial processes that rely on the activity of lactic acid bacteria is fuelling

the interest in their biology as a first step towards devising rational interference systems that abolish phage development. Furthermore, phages may be a source of genetic tools for these bacteria. The utility of the CI product in strain defense (reference 20 and unpublished data) has moved us to undertake an in-depth study of the regulation of the early processes that lead to either the lytic or lysogenic cycle of phage A2.

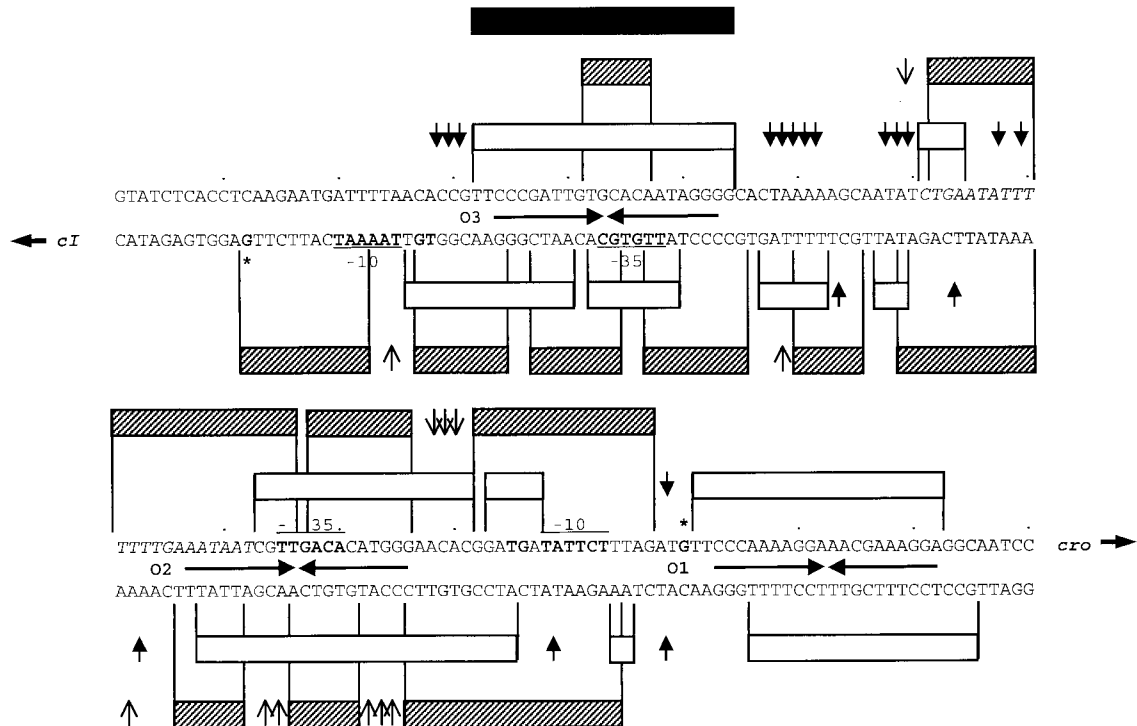


FIG. 8. Scheme of the stretches protected by CI (white bars) and σ^A -RNAP (cross-hatched bars) in the *cI-cro* intergenic region of phage A2, based on the data shown in Fig. 4 and 7. In addition, the start points of transcription (*), the -10 and -35 regions of the P_R and P_L promoters (from the primer extension data shown in Fig. 5), and the UP-like sequence of P_R (nucleotides in italics) are indicated. The inverted repeated sequences of the operators are shown by converging arrows, and hypersensitive sites are indicated by pointing arrows (filled for CI and open for σ^A -RNAP). The black bar indicates the σ^A -RNAP protected region in the presence of low CI concentrations.

Northern blots from the A2 immunity region made *in vivo* (20) and *in vitro* transcription experiments showed that it is centered around a 153-bp segment comprised of the structural portions of the divergently transcribed *cro* and *cI* genes (Fig. 1). In front of these genes there appeared the corresponding promoters, P_R and P_L, respectively. Interspersed with these two promoters are three discrete, nonoverlapping, operator sites (O₁, O₂, and O₃) to which the CI protein was shown to bind specifically. Each of these operator sequences contains a 20-bp imperfect inverted repeated sequence with a dyad axis of symmetry. This organization suggests that CI, which is a monomer in solution, could dimerize on the DNA through recognition of each of the sides of the inverted repeats. The relative locations of promoters and operators are as follows: O₁ is centered 13 bp downstream from the transcription start point of P_R, while O₂ and O₃ have their axes of symmetry 33.5 and 86.5 bp upstream from that point and have embedded the -35 hexamers of P_R and P_L, respectively (Fig. 8). At low concentrations, CI formed two complexes with the intergenic *cI-cro* DNA segment, while only the most retarded one was observed at high CI concentrations (Fig. 3). The absence of a third intermediate complex suggests the strong cooperative binding of CI to two operator sequences, probably O₁ and O₂, based on its higher affinity to them than to O₃ (Fig. 4 and 7). This cooperative binding of CI probably provokes bending of the DNA, as judged by the pattern of increased sensitivity to DNase I cleavage with a periodicity of about 10 bp that occurs between the O₂ and O₃ sites (Fig. 4). However, the O₁ and O₂ sites are separated by a nonintegral number of turns in the DNA helix (the center-to-center distance is 46.5 bp, which means 4.4 turns), so we have to assume that the helix undergoes an unfavorable twisting process upon CI binding. At present the en-

ergetic cost of looping between these two high-affinity operator sites is unknown. On the other hand, O₂ and O₃ lay on the same face of the DNA, thus favoring DNA looping (the center-to-center distance is about 52.5 bp, i.e., 5 helix turns).

The overall organization of the genetic switch region appears to be significantly similar to the functionally homologous stretches of other bacteriophages, although in most of these the operators are uniformly spaced (14, 18, 26). However, the lambdoid phages HK022 and ϕ 80 also show asymmetric spacing (8, 25). Furthermore, A2 operators partially differ in their sequences, a feature previously thought to be important in phage λ for the lysis-lysogeny decision, since they seem to be involved in the different affinities of CI and Cro for these sites.

The phage A2 CI protein represses P_R through binding to O₁ and O₂, which results in the displacement of the RNAP from its normal position, from nucleotide -2 to -50 upstream of the transcription start point of P_R, to place it on the P_L promoter, which presumably would result in the enhancement of *cI* transcription. However, at high concentrations of CI, even O₃ becomes occupied, which in turn provokes displacement of the RNAP from P_L, resulting in repression of *cI*.

The general arrangement of the operator sites relative to their promoters indicates that repression of the lytic cycle in phage A2 occurs by a principle similar to that proposed for the lambdoid phages (7, 27) but with clear mechanistic differences. First, the promoters of the lytic-lysogenic commitment of the A2 phage have four elements identified in bacterial promoters: the crucial -35 and -10 hexamers, the -14 to -15 TG dinucleotide which appears to be a basic third element found in a large portion of gram-positive bacterial promoters (15, 33), and a sequence rich in A+T called the UP element, located upstream from the -35 hexamer (6, 23, 28). The UP element,

to which the C-terminal domain of the RNAP α subunit (α -CTD) binds, has been located at positions -59 to -38 relative to the transcription start and has the consensus sequence 5'-nnAAA(A/T)(A/T)T(A/T)TTTTnnAAAAnnn-3' (10). A poor match with an UP element could be predicted in the -59 to -38 interval of P_L (8 of 17 nucleotides), whereas an almost perfect match (15 of 17 nucleotides) was observed in the case of the P_R promoter (Fig. 8). These last two elements are not present in the promoters of the genetic switch region of bacteriophage λ .

Second, *in vitro* transcription of the *cI* gene from the P_L promoter of phage A2 takes place in the absence of CI, albeit about 10-fold less abundantly than *cro* expression which occurs from the P_R promoter (Fig. 5). This contrasts with the fate of the repressor synthesis from the P_{RM} promoter in phage λ (13), which requires the presence of the repressor itself.

Third, in the case of phage A2 the spacer regions have 26 bp between O_1 and O_2 and 32 bp between O_2 and O_3 (Fig. 8), while in λ these are reduced to 7 and 6 bp, respectively. The λ CI protein activates transcription of *cI* when bound at O_{R2} (which is centered at position -42 of promoter P_{RM}) (19, 21, 27). It has been recently shown, by using artificial constructs, that the λ CI repressor bound to O_{R2} at position -62 upstream of the promoter transcription start point does not activate RNA synthesis, because λ CI cannot contact the σ subunit of RNAP (9). In the case of the A2 phage, we have shown that the CI repressor bound to the O_1 and O_2 sites (centered at positions -131 and -84.5 of promoter P_L) helps in the binding of RNAP to the P_L promoter. This might be effected through contact between the α subunit of the *B. subtilis* RNAP and CI (our unpublished data) which, in the end, would enhance the expression from P_L . It is likely, therefore, that this situation is reproduced *in vivo* when an A2 genome enters a new host cell. As a result of early transcription, CI would bind the O_1 and O_2 sites, provoking repression of the transcription from the P_R promoter by covering part of the surface that must be occupied by σ^A -RNAP, leading the phage into a lysogenic cycle. The CI dimers bound to O_1 - O_2 help to position σ^A -RNAP at the P_L promoter, further enhancing the synthesis of CI until its concentration allows the occupancy of O_3 , which would result in a halt of transcription from P_L . In this way, as it occurs in λ , A2 CI would regulate its own synthesis and the maintenance of the lysogenic state of the cells.

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