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Characterization of the Primary Immunity Region of the *Escherichia coli* Linear Plasmid Prophage N15

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N15 is the only bacteriophage of *Escherichia coli* known to lysogenize as a linear plasmid. Clear-plaque mutations lie in at least two regions of the 46-kb genome. We have cloned, sequenced, and characterized the primary immunity region, *immB*. This region contains a gene, *c*B, whose product shows homology to lambdoid phage repressors. The *c*B3 mutation confers thermoinducibility on N15 lysogens, consistent with CB being the primary repressor of N15. Downstream of *c*B lies the locus of N15 plasmid replication. Upstream of *c*B lies an operon predicted to encode two products: one homologous to the late repressor of P22 (Cro), the other homologous to the late antiterminator of ϕ82 (Q). The Q-like protein is essential for phage development. We show that CB protein regulates the expression of genes that flank the *c*B gene by binding to DNA at symmetric 16-bp sites. Three sites are clustered upstream of *c*B and overlap a predicted promoter of the *cro* and *Q*-like genes as well as two predicted promoters of *c*B itself. Two sites downstream of *c*B overlap a predicted promoter of a plasmid replication gene, *repA*, consistent with the higher copy number of the mutant, N15*c*B3. The leader region of *repA* contains terminators in both orientations and a putative promoter. The organization of these regulatory elements suggests that N15 plasmid replication is controlled not only by CB but also by an antisense RNA and by a balance betweem termination and antitermination.

N15 is unique among known bacteriophages of *Escherichia coli* in that it lysogenizes cells as a linear plasmid (40). Despite this unusual mode of lysogenization, numerous properties of N15 resemble those of lambdoid phages, which, as prophages, are normally integrated into a bacterial chromosome. N15 is similar to λ with respect to length of the genome (46.3 kb, N15; 48.5 kb, λ), morphology of phage particles and plaques, burst size, and lysogenization frequency (27). N15 phage DNA, like λ phage DNA, is a double-stranded molecule with single-stranded, cohesive ends (40). In both cases, phage and prophage DNAs are circularly permuted with respect to each other. Kinship of N15 and λ is suggested on the basis of cross-hybridization of their DNAs (29). Cells lysogenized with N15 are unable to adsorb bacteriophages N15, T1, and ϕ80 (28). In this respect, they resemble lysogens of another lambdoid phage, ϕ80 (15–17). This lysogenic conversion depends upon *cor* (Fig. 1) (20), which is homologous to the ϕ80 gene with a similar function (44).

The N15 prophage is maintained in cells as a low-copy-number plasmid and is stable (29). Its DNA is a double-stranded linear molecule with covalently closed ends (40). The ends have a telomeric structure similar to those found in the DNAs of poxviruses, African swine fever virus, mitochondria of cells of the yeast genus *Pichia*, and linear plasmids found in spirochetes of the genus *Borrelia* (11, 12, 18). Each telomere of N15 consists of a palindromic terminal hairpin loop and a 28-bp inverted repeat (19).

Ravin and Shulga (29) isolated temperature-sensitive early mutants of N15 defective in phage replication. They were also temperature sensitive for replication of plasmid prophage. This result suggests that some of the genes of N15 are involved directly or indirectly in both plasmid and lytic replication. The presence of cohesive ends in N15 phage DNA suggests that, as in λ, the replicative form of phage DNA is a circular molecule (40).

Regions of the N15 genome important for lytic growth or lysogeny were identified by mutational analysis (Fig. 1) (32, 38, 43). The central part of the genome, including approximately 70% of the DNA, can be deleted from N15 without affecting its maintenance as a linear prophage. Genes essential for both phage development and plasmid maintenance are located in a 4.7-kb-long fragment (plasmid map coordinates, 4.1 to 9.9 kb) (Fig. 1). The minimal plasmid replicon contained within this region can drive replication of a circular plasmid (38).

In order to identify genes involved in the repression of lytic functions of N15, mutants that form clear plaques were isolated (38, 39). They were classified into three complementation groups. A temperature-sensitive mutant of one group was characterized. The mutation is located in a gene called *c*B (Fig. 1). It causes inability of N15 to lysogenize at elevated temperatures and at temperatures permissive for lysogenization, it causes an increase of the N15 plasmid copy number.

In order to elucidate the role of *c*B in the regulation of N15 functions, we cloned and characterized the region of N15 DNA that spans *c*B. We show here that CB protein is a homolog of primary repressors of lambdoid phages and that it exerts its regulatory function by binding to operators flanking the *c*B gene.

MATERIALS AND METHODS

Media. Bacteria were grown in Luria-Bertani (LB) liquid medium or on LB agar plates unless indicated otherwise (21). Antibiotics were added, as appro-

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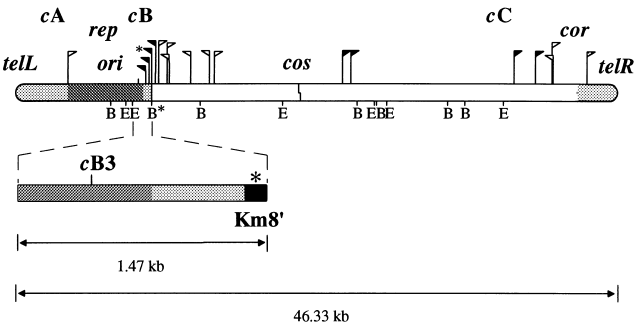

FIG. 1. The genome map of the N15 prophage. Rounded ends represent telomeric structures. The positions of known genes (*c*A, *c*B, *c*C, *rep*, and *cor*) and sites (*telL*, *telR*, *ori*, and *cos*) are indicated above the genome (18–20, 38, 40, 43). Regions that can be deleted from a prophage without affecting its maintenance as a linear plasmid are shown open; those that cannot be deleted are shown in light or dark gray (according to reference 38). A region that can drive replication of a circular DNA molecule is shown in dark grey (38). Flags represent mini-transposons in different insertional mutants of N15 (reordered on the basis of sequencing results presented here) (32). The orientations of the flags show the orientations of the antibiotic resistance genes of the minitransposons. The solid flags indicate insertions that block phage formation, and the open flags indicate those that do not. The positions of the *c*B3 mutation and the Km8 insertion in the N15*c*B3Km8 mutant prophage are indicated (32, 38). E and B below the map indicate the positions of sites recognized by restriction endonucleases *Eco*RI and *Bam*HI, respectively (40). The *Bam*HI site of the mini-kan transposon used in the cloning of the *immB* region of the N15*c*B3Km8 mutant is indicated by an asterisk. The fragment cloned is shown below the genome map.

priate, at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 25 μg/ml; and chloramphenicol, 25 μg/ml.

Bacteria and bacteriophage strains. *E. coli* K-12 strain DH5 (*supE44 hsdR17 recA1 gyrA96 thi-1 relA1*) was used as a host for plasmid and phage propagation and for cloning experiments (30). Cell lysates for CB activity assays were prepared from strain UT5600. It was obtained from Michael Lundrigan via Susan Gottesman as *leu proC trpE thiA rpsL* Δ(*ompT-fepA*) but is described in detail in the catalog of New England Biolabs (Beverly, Mass.). Mutants of N15 bacteriophage N15*c*3Cm1, N15*c*3Km8, and N15*c*3Km19 (referred to here as N15*c*B3Cm1, N15*c*B3Km8, and N15*c*B3Km19) were described previously (32). Positions of the *c*B3 and Km8 mutations in the genome of N15 prophage are indicated in Fig. 1.

Plasmids. Plasmid pB2D (kindly provided by A. Vostrov) was constructed by insertion of the 1.47-kb *Eco*RI-*Bam*HI fragment (Fig. 1) of the N15*c*B3Km8 mutant prophage in place of the *Eco*RI-*Bam*HI fragment of the pUC19 plasmid polylinker. In the resultant plasmid, the terminal 71 bp of the insert containing the recognition site for *Bam*HI endonuclease are derived not from N15 DNA but from the mini-kan transposon of the mutant (Fig. 1). Plasmid pRPG48 (Ap^r) is a vector derived from pBR322 (30) and carries the *trp* promoter-operator region of *Serratia marcescens* (22). Plasmid pRPG18 is derived from pACYC184 (30) and carries the *E. coli trpR* gene. Both plasmids were gifts from Robert P. Gunsalus.

Thermal induction of N15*c*B3 prophage. Lysogenic bacteria in logarithmic growth at 30°C were collected by centrifugation, washed in fresh LB medium to remove free bacteriophages, and resuspended at a low cell density in LB medium. Aliquots (1 ml) were incubated at 30°C with or without a prior 20-min incubation at 37, 40.5, or 42°C. Samples were withdrawn periodically for assay of CFU at 30°C or for assay of PFU at 37°C. The assay of PFU was performed with DH5/pBR322 being used as the indicator on LB medium with ampicillin to prevent the growth of the lysogens tested. The shift to 40.5 or 42°C caused significant killing of N15*c*B3 lysogens and a release of phage particles. The shift to 37°C prevented growth in the number of N15*c*B3 lysogens and a negligible or minor release of phage. Neither growth inhibition nor phage release occurred in N15*c*B^+ lysogens that were otherwise isogenic. Comparable results were obtained with lysogens of both *recA* strain DH5 and an unrelated rec^+ strain and independently of whether N15 carried the Km19 or Cm1 marker.

Plasmid and phage techniques. Plasmid DNA was prepared by the mini-prep alkaline lysis procedure described by Sambrook et al. (30) or, when necessary, by use of a Qiagen plasmid minikit (Qiagen, Inc., Chatsworth, Calif.). Restriction digestions and ligations were performed according to standard protocols. Plasmid DNA was introduced into bacteria by transformation of $CaCl_2$-treated cells (30).

DNA sequencing. DNA sequences were determined by the dideoxy chain termination method of Sanger et al. (31), using a PCR AmpliTaq Cycle Sequencing Kit (Bethesda Research Laboratories, Bethesda, Md.) as described in the supplier's protocol. Primers for sequencing were 5′ end labeled with [γ-^{33}P]ATP (1,000 to 3,000 mCi/mmol; DuPont, Wilmington, Del.). We initiated sequencing of the insert of N15*c*3Km8 in the pB2D plasmid from one end with a commercial pUC19 primer (cat. no. 1224; New England Biolabs). Sequencing was continued using the following clockwise (cw) or counterclockwise (ccw) primers complementary to the N15 fragments of the insert: N15P5 (5′-TTCGGCTGCTACG GTCTGGTTCA, cw), N15P7 (5′-CCAGTACATCCTCACCCGGTAAA, cw), N15P16 (5′-CCGATCTCCAGCGACGGCATCAC, cw), N15P17 (5′-TGACAC CCGGCGCAGTCTAT, cw), N15P10 (5′-TCCAGCTGGAGCAGCTGCGTCT C, cw), N15P2 (5′-CCCGTTCGTTATCACGTTGGTAA, cw), N15P11 (5′-AATCGTTCATGGTTGAGGGTAGA, ccw), N15P22 (5′-GAGTCAACGGAA CACCGTTTAC, ccw), N15P15 (5′-CCGTCGCTGGAGATCGGGCTATC, ccw), N15P8 (5′-ATTGATAGACTGCGCCGGGTGTC, ccw), and N15P6 (5′-GTATCGCCGATGCAGATCGTTCT, ccw). To find the location of the *c*B3 mutation in the pB2D plasmid, we used DNA of the N15Cm1 prophage, which is wild type with respect to *c*B, as a template for sequencing with the primers described above.

Sequence analysis. DNA and predicted protein sequences were analyzed with the programs of the Genetics Computer Group (University of Wisconsin). Searches for homologous proteins in a database were performed with the help of the FASTA and TFASTA programs of this package. Optimal pairwise alignments of protein sequences were generated with the PILEUP (Genetics Computer Group) and MACAW programs (35). The program PCSEARCH (a version of TARGSEARCH) served to find putative σ^{70} promoters (23). The rho-independent transcription termination sites were predicted with the TERMINATOR program (Genetics Computer Group) (3).

Cloning of the *c*B gene. N15 plasmid DNA and two primers, N15P18 (5′-ATATGGATCCCCAGCTATAAAGAGATCATTTATGA, complementary to N15 from base 872 to base 896 [Fig. 2]), and N15P19 (5′-ATATATGTCGAC GATCGGATTAAACCAGAATTATAGT, complementary to N15 from base 219 to base 244 [Fig. 2]), carrying a synthetic *Bam*HI site and a *Sal*I site, respectively (underlined), were used in a PCR to amplify a 627-bp DNA fragment of N15 containing the *c*B gene without its promoter (Fig. 2). To give rise to a construct in which the transcription of the *c*B gene is under the control of the *trp* promoter, the amplified DNA fragment was digested with *Bam*HI and *Sal*I and appropriately inserted into the pRPG48 (Ap^r) vector carrying the promoter region of the *trp* operon of *S. marcescens*. To avoid possible toxic effects of excess CB protein, the DH5 cells that were transformed with the ligation mixture carried pRPG18 (Cm^r), which provides several copies of the *E. coli trp* repressor gene. Transformants were selected on a medium with ampicillin and chloramphenicol. When used as indicators for the plating of N15*c*B3Cm1 at temperatures above 37°C, transformants that contained the cloned *c*B gene were distinguished by their ability to confer turbidity to the phage lysis zone. A pRPG48 plasmid that carried the *c*B gene on the basis of the test described above was named pMLO121 and was used for further studies.

Overexpression of the cloned *c*B gene. Cells of UT5600 transformed with pMLO121 plasmid were used to overproduce CB protein. In UT5600, an *ompT* mutation renders the major periplasmic protease, OmpT, defective and improves the efficiency with which many proteins can be isolated. To achieve maximal expression of *c*B from the *trpPO-c*B fusion of pMLO121, we followed the procedure described by Nichols and Yanofsky (24). Cells of UT5600 carrying pMLO121 were grown at 30°C in M63 minimal medium (21) supplemented with 0.5% glucose, 0.05% Casamino Acids, and ampicillin and leucine (20 μg/ml), proline (20 μg/ml), thiamine (20 μg/ml), and tryptophan (20 μg/ml) to satisfy the nutritional requirements of the strain. The 30°C temperature was chosen to overcome the apparently toxic effects of excess CB. Cells bearing pMLO121 grew very poorly in minimal medium at 37°C, in contrast to cells bearing control plasmid pRPG48. After the culture reached a density of approximately 3×10^8 to 5×10^8 cells per ml, bacteria were harvested by centrifugation at room temperature, washed, and resuspended in the same medium, except that 20 μg of a tryptophan analog, 3-β-indoleacrylic acid, per ml was substituted for tryptophan to cause a mild tryptophan deficiency and consequently derepression of transcription from the *trp* promoter. Under these conditions, cultures of cells carrying the pMLO121 plasmid did not grow but an increase of CB synthesis was induced (data not shown). After 3 h of incubation in 3-β-indoleacrylic acid-containing medium, cells were used to prepare cell lysates.

Preparation of cell lysates. Cells from 250-ml cultures grown as described above were harvested by centrifugation, washed twice in 50 mM Tris-HCl (pH 8.0)–10 mM EDTA, and resuspended in 50 mM Tris-HCl (pH 8.0)–10% (wt/vol) sucrose to an A_{600} of 300 to 400. Lysis was started by the addition of an equal volume of the same buffer containing 58.2 mg of $(NH_4)_2SO_4$ per ml, 400 μg of egg white lysozyme per ml, 40 mM spermidine HCl, 20 mM EDTA, 4 mM dithiothreitol, and 100 μM phenylmethylsulfonyl fluoride, a protease inhibitor. After a 30-min incubation of the samples at 4°C with repeated inversion, Brij 58 was added to 0.1% (vol/vol). Samples were incubated in ice for an additional 30 min and then frozen in liquid N_2, thawed, refrozen, and thawed again to complete lysis. Cell debris was removed from lysates by centrifugation in a Beckman TLA 45 rotor at 50,000 × *g* for 1 h.

Determination of the N-terminal sequence of CB. The amino acid sequence of the N-terminal fragment of CB was determined as described by Hubbard and Klee (14) with a Model 477 Applied Biosystems amino acid sequenator. Lysates of cells (UT5600/pMLO121) overproducing CB, prepared as described above, served as a source of CB protein for further purification and sequencing. Lysate proteins (53 μg) were separated by electrophoresis in a 12.5% acrylamide-

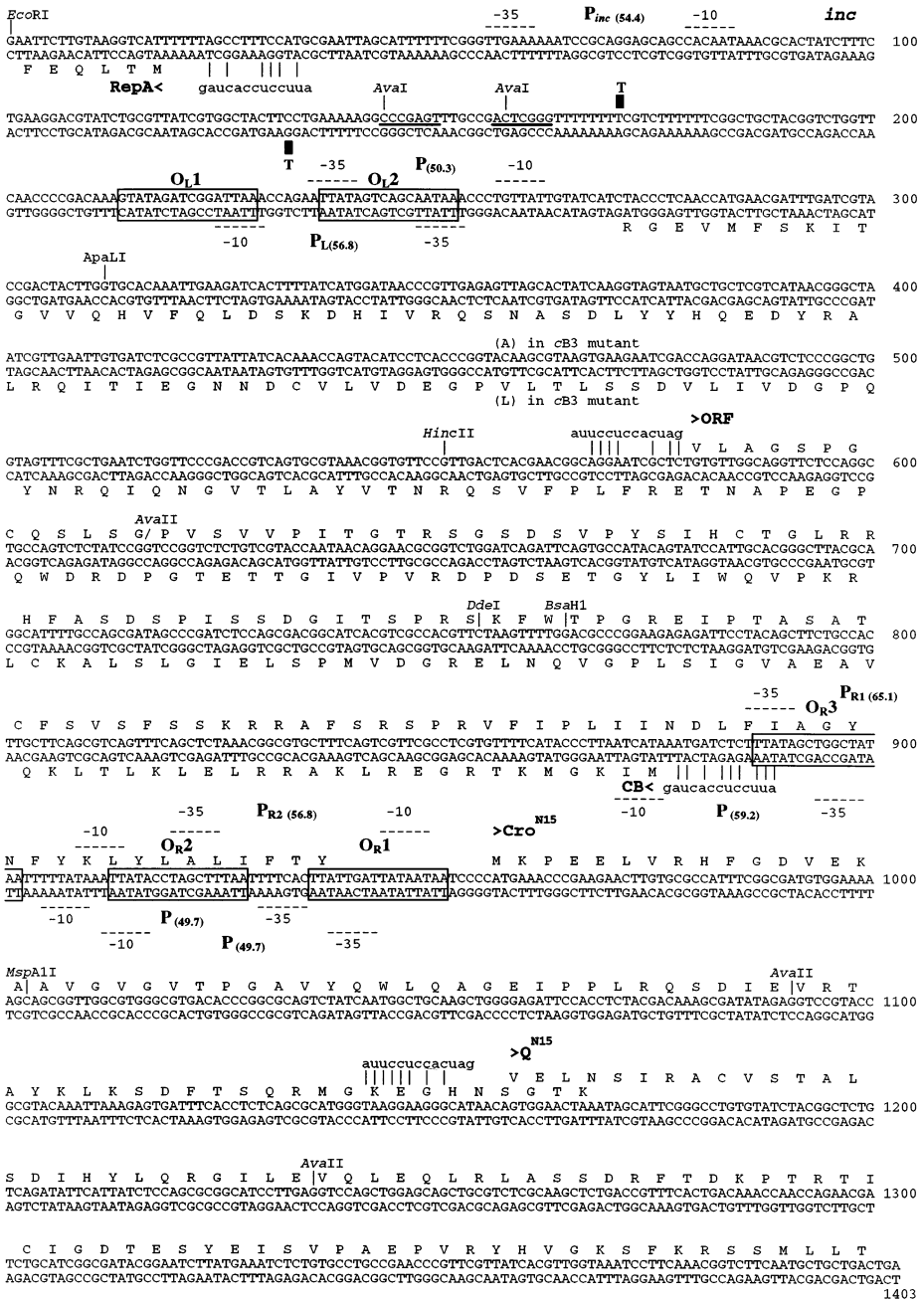

FIG. 2. Nucleotide sequence of the N15 *c*B gene region. The sequence starts at the *Eco*RI site of the pBD2 plasmid insert and ends at a junction between the DNA of N15 and the mini-kan transposon in the N15*c*B3Km8 mutant. Amino acid sequences of predicted proteins are indicated above the DNA sequence for genes read from left to right and below the DNA sequence for genes read in the opposite direction. Regions of homology to the 3′ end of 16S rRNA (lowercase letters) corresponding to ribosome binding sites are shown in front of each protein sequence. Dashed lines indicate the −10 and −35 regions of putative promoters. Numbers in parentheses next to promoter names show a calculated homology (homology score) for each of the predicted promoters to the consensus sequence for the σ^{70} promoters (the homology score of the $lacp_1$ promoter to the consensus, calculated by the same method, is 49.7%). The solid rectangles marked "T" indicate predicted rho-independent transcription termination sites (see Materials and Methods). Regions of inverted repeats within the predicted terminators are underlined. The CB-binding operators are boxed.

bisacrylamide (30:1) gel in 25 mM Tris–250 mM glycine–0.1% sodium dodecyl sulfate (SDS) buffer (pH 8.3) as recommended by Sambrook et al. (30). After separation, proteins were electrotransferred to an Immobilon transfer membrane (polyvinylidene difluoride; pore size, 0.45 μm) (Millipore, Bedford, Mass.) with a Protean Cell electroblotting apparatus (Bio-Rad, Richmond, Calif.). The transfer was performed for 25 min at 300 mA and 4°C in 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) equilibrated to pH 11 with NaOH and containing 10% methanol (5). Membranes were stained with Coomassie brilliant blue and destained as recommended by Cook (5). The region of the membrane containing CB protein (verified on the basis of the absence of this protein in a control lysate prepared from cells with the vector plasmid instead of pMLO121; see Fig. 5) was cut out and used as a source of CB for sequencing reactions.

DNA binding assay. The binding of CB to different restriction fragments of N15 DNA was tested by a band shift assay. DNA of pB2D was digested with appropriate restriction endonucleases. After separation in 4% NuSieve agarose gel (FMC BioProducts) in 40 mM Tris-acetate–1 mM EDTA buffer (pH 8.0), each fragment to be tested was purified from the gel with a Geneclean kit (Bio

101, La Jolla, Calif.) according to the supplier's protocol. Fragments were 3′ end labeled with [α-^{32}P]dATP or [α-^{32}P]dCTP (6,000 mCi/mM; Amersham, Arlington Heights, Ill.), using the Klenow fragment of DNA polymerase I as described by Sambrook et al. (30). After being labeled, the DNA was extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and passed twice through 1-ml columns of Sephadex G-25 equilibrated with 10 mM Tris-HCl (pH 8.0)–0.1 mM EDTA (5-Prime, 3-Prime, Inc., Boulder, Colo.). Reaction mixtures (20 μl) containing 5 fmol of [α-^{32}P]DNA, 25 μg of sonicated calf thymus DNA, 40 μg of bovine serum albumin, and cell lysate (1 to 4 μg of protein) in a binding buffer (50 mM Tris-HCl [pH 8.0]–5% glycerol–50 mM KCl–2 mM dithiothreitol) were assembled in ice, incubated for 15 min at 30°C, and loaded onto a 5% acrylamide-bisacrylamide gel (37.5:1) (30) in 45 mM Tris-borate–1 mM EDTA buffer (pH 8.3). The gel was run at 7 V/cm at 4°C, dried, and exposed to X-ray film (X-Omat AR; Kodak).

Footprinting of CB on DNA. DNA for footprinting experiments was prepared by digestion of pB2D with an appropriate restriction enzyme and then by ^{32}P labeling of the 3′ ends with the Klenow fragment of DNA polymerase I as described above. To obtain fragments labeled only at one end, labeling was followed by a second enzyme digestion. Fragments were separated by electrophoresis in a 5% acrylamide-bisacrylamide gel (37.5:1) in 45 mM Tris-borate–1 mM EDTA buffer (pH 8.3). Fragments to be tested were cut out of the gel and purified by the procedure described by Sambrook et al. (30). CB-DNA complexes were assembled in ice in 50 μl of binding buffer (50 mM Tris-HCl [pH 8.0]–5% glycerol–50 mM KCl–2 mM dithiothreitol) containing an α-^{32}P-, 3′-end-labeled fragment of DNA, 10 μg of sonicated calf thymus DNA, and 5 μg of cell lysate protein. Samples were incubated for 10 min at 30°C and then for 5 min at 37°C. DNase digestion was initiated by the addition to each sample of 5 μl of a mix containing 0.05 U of DNase (Promega, Madison, Wis.) in 5 mM $CaCl_2$–0.5 mM magnesium acetate–250 ng of bovine serum albumin in binding buffer. After 2.5 min, the reactions were stopped by the addition of 4.5 ml of 250 mM EDTA–5% SDS. Proteinase K (1.25 ml of a 10-mg/ml solution) was added to each sample. Samples were incubated overnight at 37°C, extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) mixture, precipitated with 2.5 volumes of 100% ethanol, washed with 70% ethanol, dried, dissolved in a formamide loading dye (30), denatured at 95°C for 3 min, and loaded on a sequencing gel (6% acrylamide-bisacrylamide–7 M urea–0.45 M Tris-HCl [pH 8.3]). After separation of the fragments, the gel was dried (30) and exposed to X-ray film (BioMax AR; Kodak).

Nucleotide sequence accession number. The DNA sequence reported here is available in GenBank under accession number U45242.

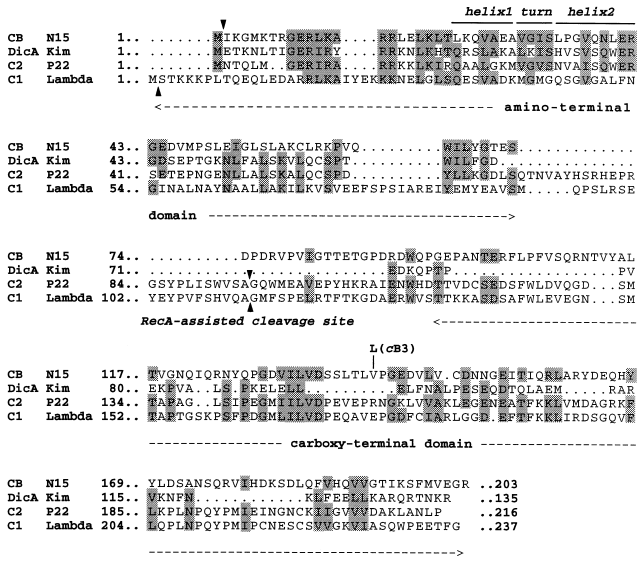

FIG. 3. Comparison of the amino acid sequence of the CB protein of N15 with those of homologous proteins DicA of *E. coli* cryptic prophage Kim (2), C2 repressor of phage P22 (34), and C1 repressor of phage λ (33). Identical or functionally similar residues found in at least three sequences are shown on a grey background. Allowed conservative replacements are I/L/V, T/S, A/S, R/K, and D/E. The domains of λ repressor C1 are indicated below the sequences. The helix-turn-helix DNA binding motif is indicated above the sequences. Arrowheads mark cleavage sites in particular proteins. The site of RecA-stimulated autocatalytic cleavage in C1 and C2 repressors (26) is distinguished from sites cleaved during posttranslational processing in CI (33) and CB. The lengths of CI and CB correspond to the lengths of the proteins prior to posttranslational processing. The position of the *c*B3 mutation in CB is indicated.

RESULTS

Pleiotropy of the *c*B3 mutation. Clear-plaque mutations in N15 were mapped to three different genes: *c*A, *c*B, and *c*C (Fig. 1) (38). The c3 mutation has proven to be useful in the isolation of N15 plasmid DNA because it increases plasmid copy number (39). Since the c3 mutation has been mapped to *c*B (38), we will refer to it hereafter as *c*B3. The *c*B gene is located between an N15 region encoding the replication functions and a region essential for phage development. The *c*B3 mutation is pleiotropic (38, 39). At 30°C, a temperature that is permissive for lysogenization by N15*c*B3, the copy number of the mutant prophage is about 10 times higher than that of the wild type, suggesting that the *c*B gene product is a repressor of N15 plasmid replication. At an elevated temperature, N15*c*B3 lysogens die. We find that the N15*c*B3 lysogens can be induced to yield a burst of phage by a transient exposure to an elevated temperature, in contrast to lysogens of an N15*c*B$^+$ control (see Materials and Methods). These results indicate that the *c*B gene product is also a repressor of lytic functions. The pleiotropy of the *c*B3 mutation suggests that *c*B plays a central role in prophage maintenance and phage development and prompted us to examine the region within which *c*B lies.

Immunity of the cloned *c*B3 region. Plasmid pB2D contains the *Eco*RI-*Bam*HI fragment of the N15*c*B3Km8 mutant prophage (Fig. 1) inserted into a pUC19 cloning vector. The insert was expected to contain the *c*B3 allele of the *c*B gene and a part of the gene which lies to the right of *c*B and which is inactivated by the Km8 insertion. At 30°C, cells with the pB2D plasmid were immune to superinfection by N15, consistent with the suggested lytic repressor function of the *c*B gene product. Immunity was not expressed at temperatures above 37°C, as was expected from the thermosensitive phenotype of the *c*B3 mutant. We designate the *c*B gene region *immB*.

Structure of the *immB* region. Sequence analysis of the cloned fragment (Fig. 2) revealed the centrally located presence of a 203-codon open reading frame (ORF) whose predicted product is homologous to lambdoid phage repressors (C2 of P22 [34], C1 of λ [33], and DicA of the *E. coli* cryptic prophage Kim [1, 2, 7]) (Fig. 3). We identified this ORF as the *c*B gene on the basis of a comparison of the sequence of the cloned fragment of the *c*B3 mutant with that of the corresponding fragment of the N15Cm1 prophage, which is wild type with respect to *c*B. The *c*B3 mutation causes a replacement of valine with leucine at residue 141 of the protein (prior to posttranslational processing).

The *c*B gene is partially overlapped by a 120-codon ORF in the opposite orientation to that of *c*B. This ORF is preceded by a putative translation initiation signal and a sequence typical of σ^{70} promoters. The predicted product of this ORF has no obvious homology to any known protein, and its expression is uncertain.

To the left of *c*B, 251 nucleotides downstream, is a methionine codon, which represents the beginning of an ORF transcribed in the same direction as *c*B. Only six codons of this ORF are present in the cloned fragment. Data to be published elsewhere indicate that these six codons represent the beginning of a replication gene, *repA*, capable of encoding a protein with 642 amino acid residues. The methionine codon is preceded by a sequence similar to those of known translation initiation signals, a sequence resembling that of a strong rho-independent transcription terminator, and a sequence resembling that of a σ^{70} promoter. Within the predicted leader sequence of this gene, there is a sequence that could encode an

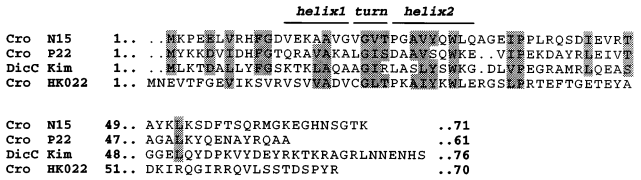

FIG. 4. Comparison of the amino acid sequence of the putative Cro protein of N15 with those of homologous proteins Cro repressor of phage P22 (25), Cro repressor of phage HK022 (GenBank accession number, V02466), and DicC protein of *E. coli* cryptic prophage Kim (2). The position of the helix-turn-helix motif, known for Cro of P22 and predicted for Cro of N15, is indicated above the sequence. Identical or functionally similar residues found in at least three sequences are shaded.

antisense RNA approximately 80 nucleotides in length (marked as *inc* in Fig. 2).

To the right of *c*B, 93 nucleotides upstream of it, are two tandem ORFs apparently forming an operon. They are transcribed in the direction opposite to that of *c*B and are preceded by two sequences typical of those of σ^{70} promoters. A predicted product of the first ORF, a 69-residue protein, shows homology to the Cro proteins of phages P22 (25) and HK022 (GenBank accession number, V02466) and also to the regulatory protein DicC, a Cro homolog encoded by the cryptic prophage Kim of *E. coli* (1, 2, 7) (Fig. 4). The second ORF is incomplete in the cloned fragment. It represents the gene interrupted by the Km8 insertion (Fig. 1) in the parental N15*c*B3Km8 plasmid. The N-terminal part of the predicted product of this gene encoded by the fragment present in the insert shows a modest homology to the N-terminal part of the Q antiterminator of phage ϕ82, a protein which activates transcription of late phage genes (9). The DNA encoding the remainder of this ORF has been sequenced and shown to have sufficient homology to the Q of ϕ82 to warrant referring to this ORF, at least tentatively, as Q^{N15} (17a).

Expression of the *c*B gene. To obtain a source of wild-type CB protein, we amplified a fragment containing the predicted *c*B gene. We used as a template the N15Cm1 plasmid, which is wild type with respect to *c*B. The fragment, which lacks the native promoter of *c*B, was placed in a multicopy plasmid (pRPG48) containing the *trp* promoter so that the expression of *c*B was under the control of this promoter in the resulting construct (pMLO121; see Materials and Methods). To prevent any toxic effects of CB overproduction, the cells of DH5 that were transformed with pMLO121 carried plasmid pRPG18, which overexpresses the repressor, TrpR, of the *trp* promoter. Cells with the pMLO121 plasmid (and with pRPG18 present or not) appeared to be immune to superinfection by N15, which is consistent with *c*B being an immunity determinant.

At a high multiplicity of infection by N15*c*B3Cm1, sufficient to overcome the immunity conferred by pMLO121, the cloned wild-type *c*B complemented the *c*B3 mutation carried by the phage. Whereas N15*c*B3Cm1 forms a clear zone of lysis at temperatures above 37°C on a layer of DH5 cells carrying the vector pRPG48, under similar conditions, it forms a turbid zone of lysis on a layer of DH5 cells bearing the clone of wild-type *c*B in pRPG48 (pMLO121).

Under conditions of *trp* promoter derepression, cells carrying pMLO121 synthesized a protein that migrated in an SDS–15% polyacrylamide gel to the position of a 26-kDa marker, in approximate agreement with the predicted molecular mass of the CB protein, 23 kDa (Fig. 5). N-terminal sequencing revealed that the N terminus was either of two amino acids, methionine or isoleucine, and that the successive peaks of

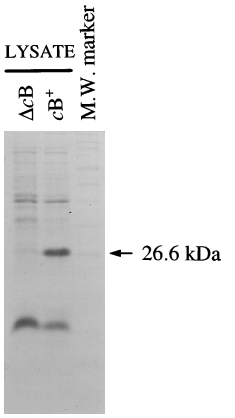

FIG. 5. Detection of CB protein in cells overexpressing the *c*B gene. Cell lysates were prepared as described in Materials and Methods. Lysate proteins were separated in a 12.5% acrylamide-bisacrylamide gel (30:1) in 25 mM Tris–250 mM glycine–0.1% SDS buffer (pH 8.3) and stained with Coomassie brilliant blue, as described by Sambrook et al. (30). Bio-Rad protein markers were used as molecular weight (M.W.) markers. The lane labeled *c*B$^+$ was loaded with lysate proteins of UT5600 carrying the *trpPO-c*B fusion plasmid (pMLO121). The Δ*c*B lane was loaded with lysate proteins of UT5600 carrying the control plasmid (pRPG48).

amino acids appeared in pairs. These pairs were related to one another, consistent with their provenance from a mixture of two forms of protein differing by the presence of the first amino acid. The sequence representing the longer protein form, MIKGM-TRGERL--RRL-L-, in which hyphens represent unidentified residues, corresponds to the predicted N-terminal sequence of the CB protein. The sequence representing the shorter protein form, IKGM-T-GERL-ARRL-L-K, corresponds to the predicted sequence of CB minus the first methionine. We conclude that CB protein within a cell undergoes posttranslational processing. The first methionine is cleaved off the protein. In this respect, CB resembles the homologous CI repressor of λ phage, which, as isolated from cells, lacks the first methionine (33).

Identification of operator sites that bind CB. Known lytic repressors of lambdoid phages exert their regulatory effect by binding to operators flanking the repressor genes. DNA band shift assay experiments with different restriction fragments of the cloned *immB* region and lysates of cells overexpressing CB

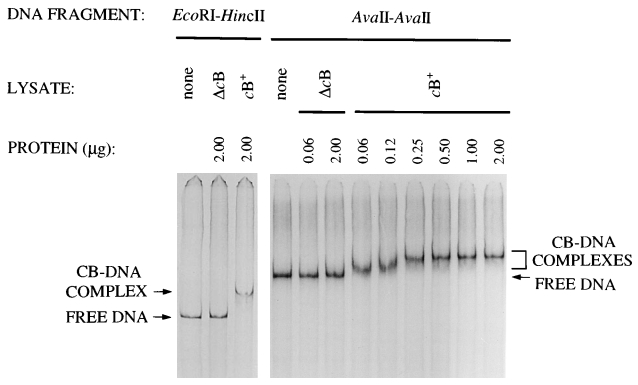

FIG. 6. Binding of CB to α-^{32}P-, end-labeled fragments of *c*B gene region DNA, as measured by a band shift assay. Lysate of cells of UT5600 carrying pMLO121 and overexpressing *c*B served as a source of CB protein. The assay conditions are described in Materials and Methods. The locations in the *c*B region of the DNA fragments that bind CB can be seen in Fig. 2.

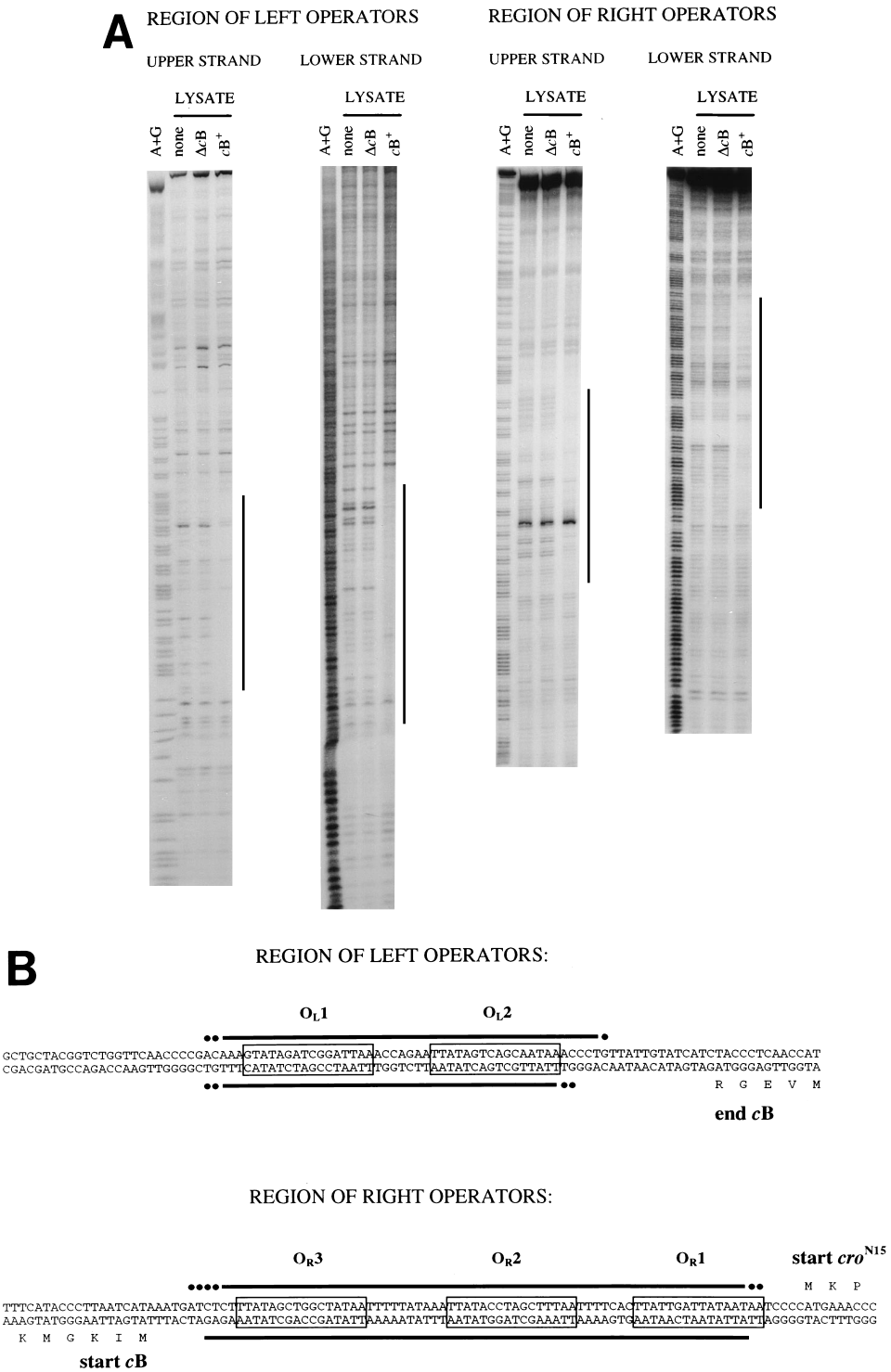

FIG. 7. (A) DNase I footprints of CB at operators flanking the *c*B gene. (B) Summary of the results. In the description of the footprints shown in panel A, the lower and upper strands represent the DNA strands shown in panel B. In the footprint of CB to DNA of the left operators, the upper strand is the *Ava*I-*Bsa*HI fragment α-^{32}P labeled at the *Ava*I end and the lower strand is the *Apa*LI-*Pvu*I fragment labeled at the *Apa*LI end. In the footprint of CB to DNA of the right operators, the upper strand is the *Bsa*HI-*Msp*A1I fragment labeled at the *Bsa*HI end and the lower strand is the *Ava*II-*Dde*I fragment labeled at the *Ava*II end (Fig. 2). In the summary of the results, bases protected from DNase I digestion by CB are indicated by lines above (upper strand) or below (lower strand) the sequence. Ambiguities in the precise boundaries of the protected regions (●) are due to the failure of DNase I to cut between certain bases in the control digests.

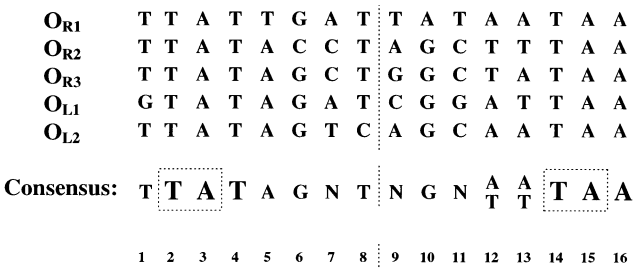

FIG. 8. Comparison of the CB-binding operator sequences. The dashed line separates symmetrical half sites of operators. The bases that are conserved in all half sites (in strands read from 5′ to 3′) are boxed.

showed that CB could bind to fragments containing either the region to the left or to the right of the *c*B gene (Fig. 2 and 6). A search for operatorlike sites in the tested fragments revealed three 16-bp palindromic sequences upstream of the *c*B gene and two similar sequences downstream of the *c*B gene (Fig. 2 and 7). Both regions of the predicted operators (Fig. 8) were protected from DNase digestion in the presence of CB, confirming that CB binds to them.

The two operators downstream of *c*B overlap the predicted promoter of the N15 *repA* gene, implying that binding of CB at these operators represses transcription of *repA*. This supposition is in agreement with an increased plasmid copy number conferred by the *c*B3 mutation. The three operators upstream of *c*B overlap the predicted promoter of the *c*B gene itself and the predicted promoters of the operon containing *cro*^{N15} and Q^{N15}. We conclude that CB, by binding to these operators, represses both its own transcription and transcription of *cro*^{N15} and Q^{N15}. The function of CB as a negative regulator of the expression of genes presumed to be involved in lytic development is consistent with the clear-plaque phenotype of the N15*c*B3 mutant phage.

DISCUSSION

We have shown here that the *c*B gene is an immunity determinant of N15 homologous to genes encoding primary phage repressors of lambdoid phages: *c*2 of P22 and *c*I of λ (33, 34). The *c*B gene is flanked by sequences typical of σ^{70} promoters and by operators to which its product binds (Fig. 9). The N15 operators upstream of the primary repressor gene form a triplet of tandem, imperfectly palindromic sites, as in λ or P22. The operators, at least O_R1 and O_R2, are sufficiently close together to permit pairwise cooperative interactions between bound repressor dimers, as described for λ (26). In contrast to λ and P22, which have three tandem operators downstream of the primary repressor gene, N15 has only two. A feature of the operators both upstream and downstream of *c*B is their association with putative divergent promoters. Beyond the operators upstream of *c*B (to its right) is a gene homologous to *cro* of lambdoid phages, in particular to *cro* of P22. Its location relative to the immunity repressor gene is typical (4). As in other lambdoid phages, the *cro*^{N15} gene and the repressor gene itself are under the control of the right operators.

The organization of N15 genes beyond the left operators and beyond *cro*^{N15} (to the right) differs from that of other lambdoid phages. In λ and P22, the gene adjacent to the left operators and controlled from them encodes an early antiterminator protein (26), whereas in N15, it encodes a replication protein, RepA. In λ and P22, terminators and several genes (including replication genes) are interposed between *cro* and a late antiterminator gene (*Q* in λ and *23* in P22), whereas in N15, there is no such separation. The need to express the early antiterminator and transcribe the interposed genes mentioned above, prior to the expression of *Q* of λ or *23* of P22, accounts for the delay before late gene expression in these phages. If the function of Q^{N15} is similar to that of the Q or 23 proteins of lambdoid phages, then transcription of late N15 genes may be more promptly responsive to control by the immunity repressor. Possibly, the sequential expression of early and late phage genes during lytic development of N15 could be achieved by the differential affinity of the CB protein for the two sets of operators flanking the *c*B gene. This mechanism would require gradual changes in the intracellular concentration of CB. Alternatively, a second regulatory mechanism might delay the expression of the Q^{N15} gene.

Sequence analysis of the predicted product of the N15 *repA* gene reveals that the RepA protein is a homolog of primases of conjugative plasmids and the primase of phage P4 (17a). The latter enzyme (the product of P4 gene α), a multifunctional replication protein with primase, helicase, and origin recognition activities, is the only phage-encoded protein essential for replication during the lytic cycle and during lysogeny as a plasmid prophage (reviewed in reference 47). The choice between lytic development and the plasmid state depends on the regulation of the expression of α (reviewed in reference 8). It is likely that in N15, a switch from lysogeny to lytic growth may depend on CB-mediated changes in the expression of *repA*. A modest decrease in CB activity, such as that which appears to result from the *c*B3 mutation at 30°C, may be inadequate to trigger lytic development but sufficient to increase plasmid copy number.

In addition to CB, at least two other factors appear to regulate the expression of *repA*. The *repA* leader sequence contains a motif typical of strong rho-independent terminators, suggesting the involvement of termination and antitermination in the regulation of the gene. In this respect, the *repA* gene resembles genes for bacterial primases and the primase of bacteriophage P4 which also contain strong terminators within their leader sequences (8, 42, 45). Also within the leader region of *repA* is a strong counteroriented promoter that appears not to be under direct CB control (Fig. 2 and 9). This strong promoter, because it is followed within about 80 bp by a strong factor-independent terminator, could initiate the transcription of a short untranslated RNA antisense to the leader sequence of *repA*. If this is the case, then the region encoding this

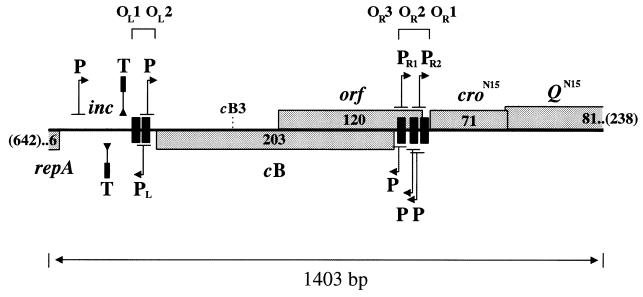

FIG. 9. Organization of the *immB* region of the N15 genome. Genes (grey rectangles), promoters (P), terminators (T), and operators (O) are indicated. Genes and sites labeled above the line representing the DNA apply to transcription from left to right, and those labeled below the line representing the DNA apply to transcription in the opposite direction. Numbers within the rectangles indicate the amino acid lengths of products or predicted products of the corresponding genes. The lengths of the products of truncated genes (on the basis of unpublished data) are given in parentheses.

transcript should be an incompatibility element. In an independent search for N15 incompatibility elements, one of us (A.N.S.) has cloned a short fragment that spans the region and found it to possess the expected property. It exerts a strong N15-specific incompatibility. Presumably, the replication of N15 is modulated by the interaction of the RNA of the leader region of the replication gene with the RNA that is antisense to it. Modulation of replication gene expression by antisense RNA is a strategy of several other plasmids (6, 13, 36, 41) and of bacteriophage P1 (10). In the case of P1, the antisense RNA modulates expression of a C1-controlled replication gene that is normally specific for lytic development but which is also capable of sustaining plasmid replication when partially derepressed (46).

Of the lambdoid phages, P22, whose *c*2 gene is the closest relative of *c*B, possesses a bipartite immunity system (reviewed in reference 37). The secondary immunity locus of P22 determines an antirepressor, Ant, that can bind to and inactivate C2. In P22 lysogens, *ant* transcription is blocked by the secondary immunity repressor, Mnt. Mutations that render Mnt thermosensitive allow P22*mnt*ts, like P22*c*2ts, to be thermally induced. Recently, we isolated a heat-inducible mutant of N15. The mutation is located in *c*A (unpublished data). It is likely that *c*A, like *mnt*, is a component of a second-order regulatory system and represses a gene whose product controls the activity of CB. The proximity of *c*B to homologs of other pivotal regulatory genes implies that *c*B, rather than *c*A, is the primary immunity repressor gene of N15.

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