AN ABSTRACT OF THE THESIS OF

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The complex repression of lytic gene expression in P1 phage is due in part to the phage encoded repressor, C1, acting at many widely separated promoter-operators. Unlike some other prokaryotic regulatory DNA binding proteins, C1 repressor appears to act as a monomer, and its contact with DNA apparently occurs by a means other than the helix-turn-helix motif.

Another P1-encoded protein, Bof, has been implicated in performing a variety of regulatory phenomena, including phage immunity and positive and negative regulation of specific genes. The *bof* gene has been isolated and its sequence determined. By measuring efficiency of initiation of transcription at promoter-operators from several P1 genes, fused to *lacZ*, Bof was shown to act exclusively as a corepressor with C1, i.e. it increased the ability of C1 repressor to directly regulate genes under C1 control (e.g. *ref*, *c*1). Since the *c*1 gene has been shown to autoregulate its expression, it too is subject to Bof action. By involvement in *c*1 autoregulation, Bof is able to positively influence the expression of certain genes under C1-control indirectly (e.g. *bac-1 ban*), by modulating C1 repressor concentrations.

There are two classes of P1 operators: those that contain a single asymmetric operator site and bind one repressor molecule, and those that contain two partially overlapping (pseudo-dyad) sites and bind two repressors. Studies using purified proteins and proteins synthesized *in vitro* have suggested that Bof increases the affinity of C1 for both single and pseudo-dyad operators, without itself possessing significant affinity for operator sequences. The sensitivity of an operator to Bof-enhanced C1 binding appears to depend on the precise sequence of the "consensus sequence" for the C1-operator site, rather than on some region outside of the operator. Bof-mediated increases in C1 affinity for operator sites may reflect an allosteric modification of C1 by Bof, or simply tandem binding of C1 and Bof, increasing the contact points with DNA.

The Bacteriophage P1 Bof Protein.

A Corepressor Whose Action Can Both

Negatively and Positively Affect Phage Gene Expression.

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Chapter I. Introduction-Review of Literature

The operon theory of Jacob and Monod (21) was a monumental advance in the understanding of gene regulation. The relevance of the operon to bacteria and bacteriophages was immediately appreciated, and only later was it shown to be a widely used means of gene expression in many diverse organisms. The operon, in a classical sense, is a gene or set of genes, whose expression is regulated in a concerted manner. Upstream of the structural gene(s) is a region of DNA called the promoter, the point at which RNA polymerase (RNAP) initially contacts the DNA. Genes regulated in a negative fashion usually possess another sequence called an operator, at which a regulatory protein, a repressor, can bind. In many instances, the promoter and operator overlap or are in close proximity to one another. In the simplest model of negative regulation, occupancy of the operator by a repressor protein precludes the binding of RNAP to the promoter and prevents transcription.

The initial step in expression of a gene is transcription of the sequence information into RNA. The process of transcription may be divided into three phases: initiation, elongation, and termination. Initiation, the most complex of the three, involves the interaction of RNA polymerase (RNAP) with the DNA (28). Localized melting of the DNA generates an open complex. At this point, the first several ribonucleotides are incorporated into a polyribonucleotide. Elongation of the

nascent mRNA strand continues with the incorporation of successive ribonucleotides. Termination of transcription in *E. coli* occurs in one of two ways. Either the polymerase complex disassociates from the DNA by interaction with a termination protein, Rho, or in a Rho-independent reaction, upon encountering a region of secondary structure.

Two features of the operon afford a simple and efficient means of regulating gene expression in prokaryotes. Prokaryotic genes are often transcribed polycistronically, generating long mRNA molecules which contain the information for more than one polypeptide. Since these mRNA molecules originate from a common promoter, the regulation of several genes can be achieved simultaneously by the regulation of one transcript. Also, genes encoding proteins of common function or members of a pathway (eg. biosynthesis of an amino acid) are often clustered together. This organization of genetic information is also seen in bacteriophages; genes encoding structural components of the virus particle are often closely associated. Since the proteins encoded by these genes are needed concurrently, organization into operons provides an efficient means of jointly regulating their synthesis. This organization assures that gene products will be present at the proper time and quantity.

Since conversion of the information encoded by a gene into a protein product is energetically quite costly, it is not surprising that the first step in this process is highly regulated. In the case of biosynthetic or catabolic operons, the regulatory mechanism assures that the genes of the operon are only expressed when necessary.

In prokaryotes, negative genetic regulation at the level of transcription takes many forms. These range from the competition between a repressor protein and RNAP for a single site on the DNA, to multiple operator sites which bind repressor molecules in a cooperative fashion. Representative examples of well characterized negative regulation are reviewed below.

A familiar paradigm of simple negative regulation is the *lac* operon of *E.coli* [for review (6)]. The *lac* operon encodes three contiguous genes (*lac*ZYA) involved in the transport and catabolism of the disaccharide lactose. These genes are transcribed polycistronically from a promoter preceding *lac*Z. In the absence of lactose, expression of the *lac* operon is minimal, accomplished by a repressor protein encoded by the *lac*I gene. The lac repressor binds to an operator sequence which overlaps the *lac* promoter. In the absence of lactose, the low expression of the *lac*ZYA genes results from a *lac* repressor bound to the operator, which prevents the binding of RNAP. Expression of the *lac* genes can be induced by the addition of lactose, allolactose, or several other lactose analogs. These molecules bind to the *lac* repressor, preventing it from occupying the operator site and thus permitting transcription of the *lac* genes (6). Therefore, by this negative mechanism, the genes of the *lac* operon are only expressed at a significant level when lactose (or a lactose analog) is present.

Regulation of the tryptophan operon has also been extensively studied. Although it is a paradigm of attenuation-antitermination, the *trp* promoter is also subject to a classic example of feedback inhibition. The *trp* promoter region

contains an operator to which the product of the (unlinked) *trp*R gene can bind. By occupying this operator, the TrpR repressor prevents transcription of the genes whose products are involved in the biosynthesis of the amino acid tryptophan. Unlike the repressor of the *lac* operon, the TrpR repressor requires the presence of a second molecule for repressor activity; tryptophan itself (29). Thus, when cellular levels of tryptophan are adequate, the TrpR-tryptophan complex represses the transcription of the *trp* operon, preventing unnecessary synthesis of the *trp* genes.

Certain gene products regulate their own expression (18, 24, 26). The cI repressor of bacteriophage is one example of this type of regulation. In a lambda lysogen, continued expression of the *cI* gene is essential for maintenance of lysogeny. If cI repressor levels fall below a critical level, productive growth of the phage by lytic growth ensues. Transcription of most of the bacteriophage genome depends on initiation from two divergent master promoters which flank the *cI* gene. Associated with each of these two promoters, p_R and p_L (promoter right and left, respectively) are three adjacent cI operator sequences, O_{R1}, O_{R2}, and O_{R3}, each with a different affinity for repressor. Initially, the *cI* gene is transcribed from a promoter, designated p_{RE}, which allows the cI repressor to accumulate. The accumulation of cI repressor results in the binding of repressor to the operator sites. O_{R1} has the highest affinity for repressor; it is occupied first, followed rapidly by a second molecule at O_{R2}, due to the cooperative nature of repressor binding. Occupancy of O_{R1} and O_{R2} by cI has two effects: one positive and one negative. A dormant promoter (p_{RM}) to the left of O_{R3} becomes active through an

interaction between RNAP and bound cI repressor. The p_{RM} promoter maintains synthesis of adequate cI repressor for maintenance of lysogeny. cI protein bound to O_{R1} and O_{R2} also negatively effects transcription from p_R, by interfering with the binding of RNAP. Thus, occupied O_{R1} and O_{R2} enhance transcription in one direction, and disallow it in the other. It should be noted that initiation at p_{RM} (and hence C1 production) is depressed when the repressor level increases to high levels. Repressor bound to O_{R3} not only precludes the positive enhancement of RNAP at p_{RM}, but also prevents its binding to the promoter. This arrangement therefore maintains relatively constant repressor concentrations.

In all of the examples cited thus far, the repressor has acted by binding in close proximity to the promoter. These simple examples are by no means the only modes of repressor action. Studies involving the *gal* operon suggest that repressor proteins can act by binding to sites not closely associated with the promoter.

The galactose operon encodes three genes, in the order *gal*ETK, whose products are involved in the utilization of galactose. The operon contains two tandem promoters, both of which are negatively regulated by the GalR repressor protein, the product of the (unlinked) *gal*R gene. Certain mutations resulting in constitutive expression of *gal* operon genes were mapped to a location outside of the known *gal* repressor-operator site (20). This genetic evidence suggested that two *gal* operators were involved in repression of the operon. This promoter-operator configuration is novel in that neither operator overlaps the promoter region. In fact, the two operators are separated by a distance greater than 100 bp, and one of the

operators is located in the coding sequence of the *galE* gene, well downstream of the promoter (20). This prompted a model to explain how two operators, separated by a substantial distance and flanking the promoter region, can repress transcription. The model postulates the formation of a "DNA loop", using repressor proteins bound to each of the operator sequences. This looped-out region of DNA, containing the *gal* promoters, presumably results in a structure which precluded interaction between RNAP and the promoter sequences.

This repression-at-a-distance is not unique to the *gal* operon. Other bacterial operons believed to utilize this mechanism include *ara*BAD (13, 23) and *deo* (9, 10, 40). The lactose operon, described above as an example of simple RNAP/repressor competition, has been shown to contain two additional operator sites, one located on either side of the promoter. Studies *in vivo* have suggested that there is a cooperative interaction between repressor molecules bound to these secondary operator sequences (15, 27). Results of other experiments also suggested that *lac* repressor can bind in a cooperative manner to well-separated operator sites. For example, a wild-type copy of the *lac* operator sequence upstream of an operator-constitutive mutant sequence, was shown to result in repression of the *lac* genes (25).

Repressor molecules have also been shown to act during message elongation. A *lac* operator placed within the coding sequence of a reporter gene causes in lac-repressor-dependent termination of transcription (11). This effect is readily reversible by the addition of the gratuitous inducer IPTG, which prevents lac

repressor binding. Thus, a repressor-operator complex appears capable of directly blocking the translocation of RNAP.

To summarize, a repressor can exert its negative regulatory action in a number of ways. Repressor occupancy of an operator site can prevent initiation of transcription, by blocking the access of RNAP to an overlapping or closely associated promoter. Accessory molecules, such as tryptophan or IPTG, can interact with the repressor, increasing or decreasing its affinity to bind its cognate operator site. In certain instances, bound repressor appears to facilitate the binding of additional repressor molecules at neighboring and even widely separated operator sites. This apparent communication between bound repressor molecules presumably is manifested as formation of a higher-order structure. This looping-out of DNA can apparently disallow the formation of a stable RNAP-promoter complex. Repressor bound to its cognate operator also appears to be capable of directly blocking the progress of a transcribing RNAP molecule.

Bacteriophage P1 encodes a repressor protein, C1, which binds to numerous operator sites, negatively regulating the expression of many associated phage genes. P1 encodes a second protein, Bof, which itself apparently has little specificity for operator sequences, but acts as a corepressor in the presence of C1. Regulatory control of phage P1 gene expression is the subject of the current study.

Bacteriophage P1 is a temperate phage with a chromosome of about 90 kilobasepairs (47) (Fig. I.1). Upon infection of a susceptible host, P1 chooses between lytic or lysogenic growth. P1 can produce numerous phage particles by the lytic

pathway, or become a quiescent prophage. In contrast to lambdoid phages, which integrate into the host genome, P1 prophages are unit-copy extrachromosomal plasmids (26). After injection into a cell, its linear phage DNA is efficiently circularized. A phage-encoded recombinase, Cre catalyzes the cyclization between two *lox* sites (38). P1 plasmid dimers which result from DNA replication are resolved to monomers by Cre-*lox* recombination.

Prophage P1 is stably maintained at approximately one copy per bacterial chromosome (19); prophages are lost at a frequency of less than 10^{-5} (30). This low frequency is a result of an active P1-encoded plasmid partitioning system, which involves both *cis* and *trans*-acting components (1, 2).

P1 has both plasmid and phage characteristics. P1 is incompatible with other plasmids of incompatibility group Y (16), including other P1 plasmids. P1 prophages also possess immunity functions analogous to those of other bacteriophages, such as the lambdoid phages and phage P22. Thus, P1 lysogens are immune to P1 superinfection. Functions encoded by three regions of the P1 genome have been shown necessary for superinfection immunity (36) (Fig. I.2). The C1 repressor is encoded by the ImmC region, the *c4-ant(reb)* functions are encoded by the ImmI region, and Bof (*c6*, *lxc-1*, *f2*) is encoded by the ImmT region. Studies of P1 immunity have been facilitated by use of phage P7. Electron microscopy has shown P1 and P7 to be physically very similar [over 90% homology] (47). Despite this, P1 lysogens are not immune to infection by P7, and a P1 phage can grow on a P7 lysogen, i.e. P1 and P7 are heteroimmune. However, this normal immunity is

"extended" by certain P1 mutations. For example, lysogens of mutants P1*dpro*11a (36), and P1*bof* ⁻ (39) are immune to P7 as well as to P1 phages. The feature common to all of these latter mutants is a defect in the ImmT region.

The expression of at least two P1 immunity genes, *c*1 and *c*4, is necessary for prophage maintenance. Mutations in either gene result in phage with virulent phenotypes. Both genes encode repressor molecules, but the modes of repressor action are quite different. The *c*1 gene, part of the ImmC region, also encodes a repressor protein which binds to many widely separated operators. Analysis of 17 operators a consensus binding sequence ATTGCTCTAATAAATTT has been derived [4, 7, 14, 41, (M. Velleman, personal communication)]. The operators consist of either a single asymmetric site, or a two partially overlapping (pseudo-dyad) sites (Fig I.3). C1 operators are located at or near the promoters of several lytic genes believed to be under C1 control (eg. *ban*, *ref*, *dam*).

The function of the *c*4 gene product is regulation of the expression of the closely linked *ant* gene (3). The *ant* (*ant*irepressor) gene is believed to encode a repressor *ant*agonist, a protein whose mode of action has not been clearly defined. One study suggested that the presence of a *cis*-loading site (*sas*) was necessary for Ant action (37). Recently, Schuster and colleagues have shown that purified Ant protein interferes with the action of C1 repressor (personal communication). The *c*4 "repressor" has been shown to be an antisense RNA (8). This RNA binds to a homologous region on the *ant* mRNA, thus preventing its translation. P1-P7 heteroimmunity has been thought to be due to differences between *c4* genes of P1

and P7, because all but one P1 mutations resulting in a clear-plaque phenotype can be complemented by P7 phages (35); the exception is a P1*c4* mutation which is not complemented by P7. The difference between P1 *c4* and P7 *c4* immunity functions appears to be the result of several nucleotide differences in the coding region for *c*4 (8). Apparently this minor difference is sufficient to make P1 and P7 phages unable to repress the synthesis of one another's *ant* genes.

The mutation *vir*C, which allows phages to plate on P1 lysogens, appears to cause uncontrolled expression of an ImmC component that is not the C1 repressor. *vir*C mutations are suppressed by second-site mutations which map to orf-4, one of several small open reading frames upstream of the *c*1 gene, designated *coi* (C one inactivator). The product of the *coi* gene is responsible for the virulent phenotype of *vir*C phages (5, 17). Expression of the *coi* gene from a multicopy plasmid results in the induction of a resident prophage (5, 17). Partially purified Coi protein has been shown to interfere with the ability of C1 repressor to interact with operator sequences (17). The *coi* gene is normally transcribed from a C1-controlled promoter; the *vir*C mutation generates a promoter, farther upstream, that is no longer under C1 control [N. Sternberg cited in (46)].

Results from several laboratories have independently implicated the ImmT region in a wide number of functions. Each study will be discussed below, in rough chronological order. In each instance, the ImmT gene or gene product discussed (*c6, f2, lxc-1, dpro11a*) is believed to be the *bof* gene. The activities of the *bof* gene product are the major emphasis of this work.

Scott and Kropf described (32) a leaky temperature-sensitive P7 clear-plaque mutant (*c*6) which appeared to be part of a previously undescribed cistron. The fact that the mutant yielded clear plaques indicated that the gene affected was important in the maintenance of lysogeny. Since a *c*6 mutant released only 4 to 10 phage per lysogenic cell upon thermal induction (31), the *c*6 gene product was believed not to be a primary repressor. In contrast, a P1 mutant encoding a temperature-sensitive C1 repressor (*c*1.100) has been shown to release 100-200 upon thermal induction (31). P7*c*6 mutations were complemented by P1 phages, suggesting that P1 encoded a similar gene (31).

Two years later, Touati-Schwartz described a pleiotropic amber mutant, *bof*, which displayed, among its many phenotypes, an increase in superinfection immunity (39). P1*bof*$^-$ prophages are "superimmune" to infection by heteroimmune P7, as well as being immune to P1 phages, but they paradoxically form clear plaques at high temperatures. Although Bof appears not to be essential for the establishment of lysogeny by wild-type phages, it is necessary for lysogeny by P1 phages with temperature-sensitive *c*1 repressor (*c*1.100). P1*c*1.100 phages normally establish stable lysogeny at 30°C; prophage can be induced by a increase temperature to 42°C. In contrast, P1*bof-1* *c*1.100 double mutants lysogenize efficiently only at temperatures below 25°C and are lytically induced at 30°C. Thus, the threshold temperature for induction of a P1*c*1.100 lysogen is dramatically reduced in the absence of Bof, suggesting that interaction of Bof with C1 protein might stabilize the *c*1.100 repressor.

The designation *bof* arises from the inability of P1*bof-1 bac-1* double mutants to produce sufficient P1 Ban protein (constitutively expressed in P1*bac-1* prophages), to complement *E. coli dna*B (Ts) mutations at nonpermissive temperatures (39). This observation suggested that Bof was necessary for the expression of the *ban* gene. Thus, the designation *ban* on function (39).

The simultaneous stable lysogeny of the same cell by two P1 prophage is prevented by P1 plasmid incompatibility functions. P1*bof*$^-$ phages form double lysogens at a frequency two orders of magnitude higher than their *bof*$^+$ counterparts (39). Thus, the *bof* gene product was also implicated in plasmid incompatibility.

Systematic dissection and reconstruction of the P1 immunity system was performed by Sternberg *et al.* (36). Recombinant lambda phages containing components of the P1 immunity system were constructed. The P1 immunity components were tested separately and in combinations, using appropriate single or multiple lambda lysogens. The lambda-P1 hybrids were also used to complement immunity-defective P1 lysogens. P1*dpro*11a (Fig. I.4) lysogens exhibit extended ("super") immunity to P7 as a result of a deletion in the ImmT region. Normal P7 sensitivity was restored to a P1*dpro*11a lysogen by presence of a λ-P1 hybrid encoding P1 *Eco*RI-2 (R1-2 is the second largest fragment generated by restriction of P1 with *Eco*R1). The influence of some component encoded by EcoR1-2 on P1 immunity was also shown using isolated P1 fragments. A double λP1:*c*1/λP1:*c*4 lysogen displayed extended immunity, but the presence of a third lambda prophage

containing P1 *Eco*RI-2 restored P7 sensitivity (normal immunity). It was suggested that a factor encoded by P1 *Eco*RI-2, *f2*, was necessary for normal (P7-sensitivity) P1 immunity, and that its absence resulted in extended immunity. The *f2* factor, presumably Bof, was also thought to interfere with the expression or activity (41) of the C1 repressor because of the observation that λP1:*c1* lysogens were immune to wild-type P1, but λP1:*c1*/λP1:*f2* double lysogens was not immune.

In addition to evidence implicating Bof in P1 immunity, results of two studies have suggested involvement of Bof in direct regulation of phage gene expression. P1 is believed to encode an analog of the *E. coli* single-stranded DNA binding protein (*ssb*A). Certain mutant P1 prophages, designated *lxc-1*, are able to complement *E. coli ssb*A(Ts) mutants for growth at nonpermissive temperatures, unlike wild-type P1 prophages. Thus P1*lxc-1* (22) appear to constitutively express a yet unmapped gene that encodes an Ssb analog. A connection between *lxc* and *bof* resulted from studies of the P1 *ref* gene.

The *ref* gene product stimulates certain homologous recombination events in *E. coli* (44). The *ref* gene seems to be a lytic gene; its activity in a wild-type P1 lysogen is negligible, consistent with the presence of a C1 repressor binding site located in the *ref* promoter region (45) (Fig. I.5). Both P1*bof*$^-$ and P1*lxc-1* prophages were shown to be derepressed for *ref* expression. This and other phenotypic similarities between P1*lxc-1* and P1*bof*$^-$ prophages suggested that the mutations were allelic (44).

Despite nearly ten years of research, the role of Bof in P1 physiology remains

poorly understood. Seemingly contradictory results include the properties of P1*bof*$^-$ mutants that suggest Bof increases C1-mediated repression, but also somehow interferes with the P1 immunity system. P1*bof*$^-$ mutants have a clear plaque phenotype, suggesting that Bof is important in maintenance of lysogeny. However, the extended immunity seen with P1*bof*$^-$ lysogens suggests that the presence of Bof lowers the immunity of P1 to superinfecting phages, most probably by altering C1 repressor expression or function. The notion that Bof interacts with C1 is consistent with the fact the absence of Bof results in a lower threshold for thermal induction in P1*c*1.100 *bof-1* mutants. Another paradox is posed by the role of Bof in lytic gene expression. The *bof-1* mutation was isolated on the basis of its ability to lower the expression of *ban* in Ban-constitutive *bac-1* mutants. In contrast, expression of *ref* (and presumably the P1 *ssb* analog) is increased in P1*bof*$^-$ prophages. Thus, Bof appears to *positively* regulate *ban* while *negatively* regulating *ref* and *ssb.*

The apparently paradoxical nature of P1*bof*$^-$ mutants has provided the impetus for this study. The *bof* gene has been isolated from P1 and its DNA sequence determined. Using operon fusions to promoter signals, the role of Bof in the regulation of several P1 genes has been determined, and shown to be direct in some cases, and indirect in others. In the case of one gene, *ref*, results obtained *in vivo* were confirmed using purified proteins *in vitro.* The results presented here resolve many of the paradoxes resulting from the previous analysis of various *bof*$^-$ P1 prophages.

Figure I.1. Circular Map of bacteriophage P1.(reproduced from reference 46)

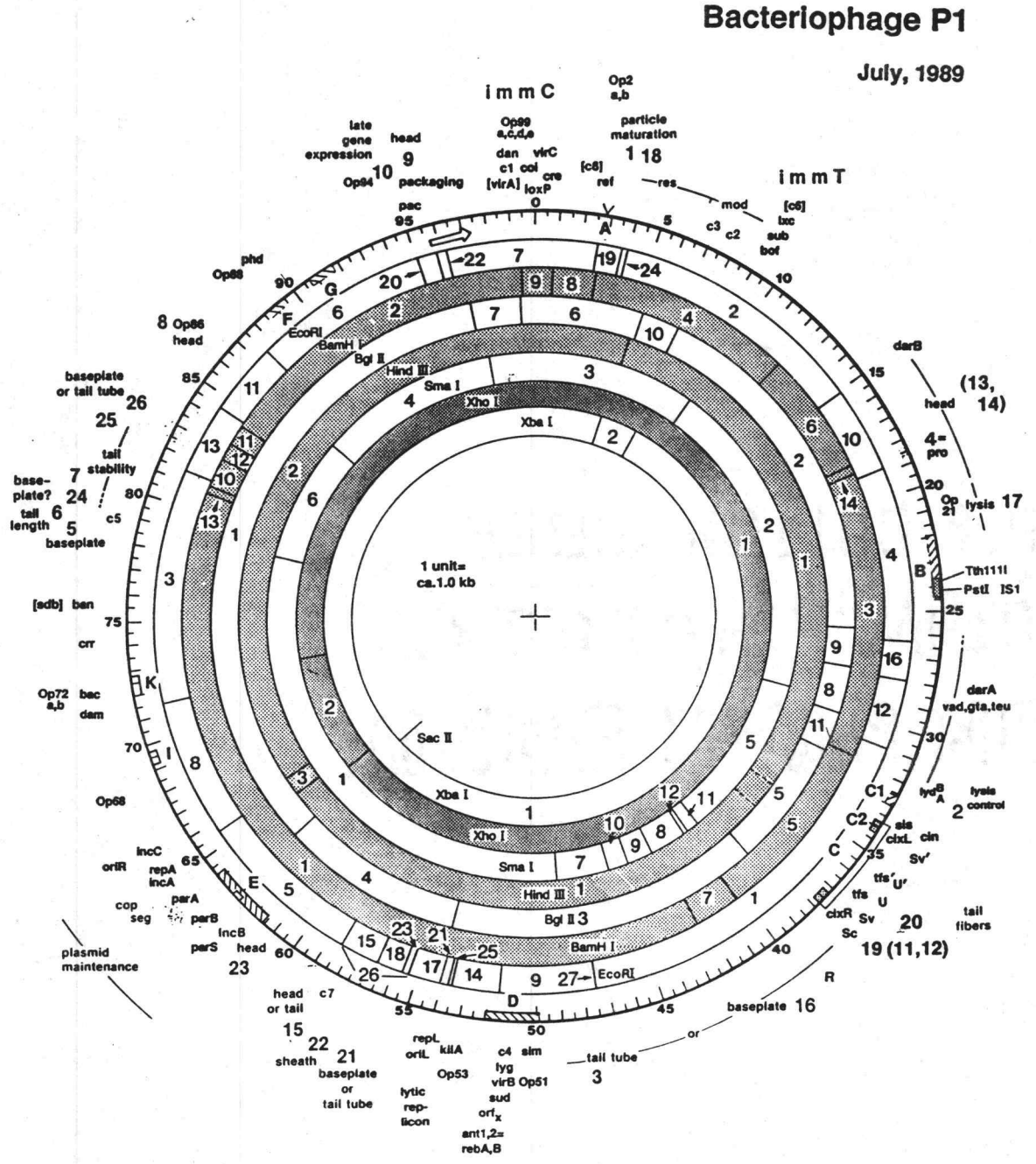
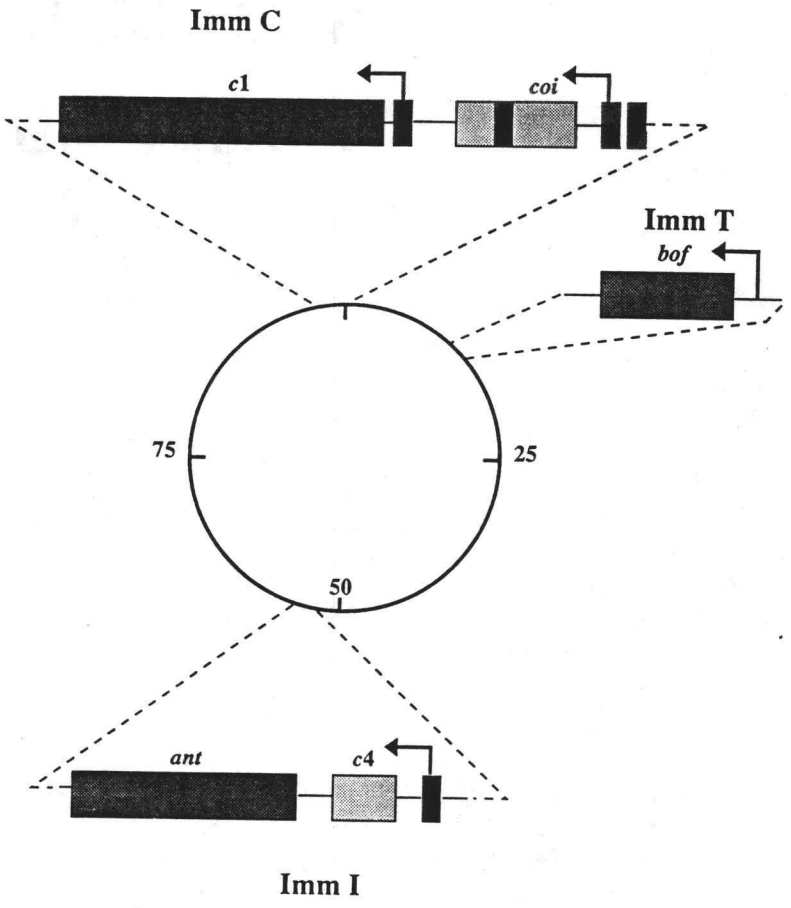
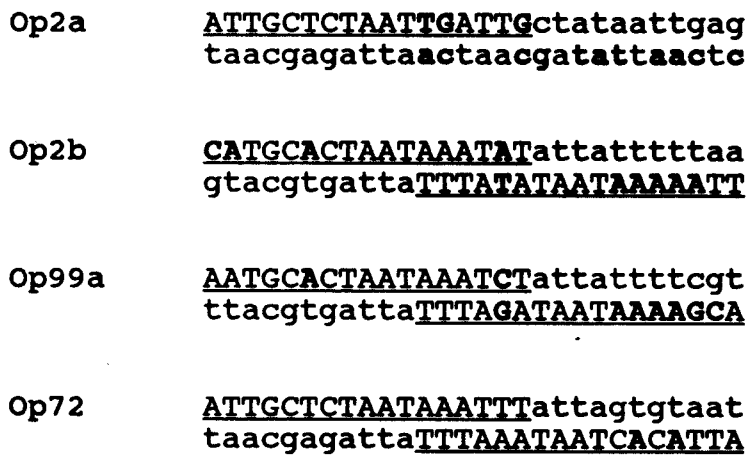


Figure I.2. Map of P1 Immunity Regions



P1 Immunity Regions. Genes involved in P1 immunity are depicted by elongated boxes (stippled or shaded). C1 repressor binding sites are represented by thin black boxes. Points and direction of transcription initiation are indicated by arrows.

Figure I.3. DNA Sequences of P1 Operators Op2a, Op2b, Op99a, and Op72



DNA sequences of P1 operators Op2a, Op2b, Op99a, and Op72. Underlined regions represent consensus or near-consensus C1 operator sequences. Letters in bold represent mismatches from the consensus ATTGCTCTAATAAATTT (4)(7)(12)(14).

Figure I.4. Linear Map of Bacteriophage P1. (reproduced from reference 35)

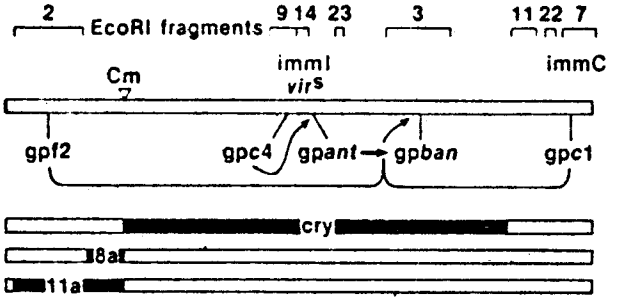
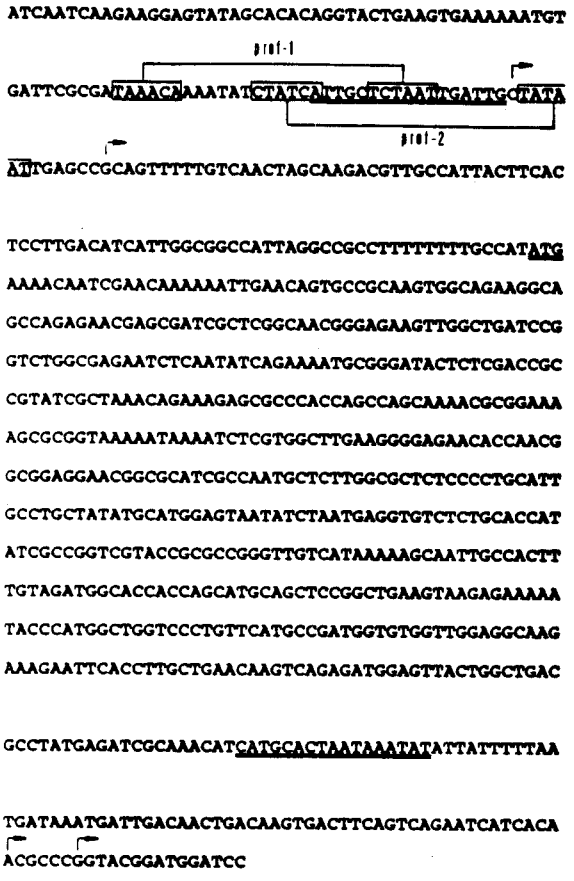


FIG. 1. P1 genome with the positions of the two immunity regions and the Cm (Tn9) transposon (3, 4) above the bar and pertinent genes, whose products are designated in the figure, below the bar. The extent of the deletions in the P1*cry* (5), P1*dpro8a* (6), and P1*dpro11a* (6) prophages are indicated by the black areas. The location of *Eco*RI-generated P1 fragments is based in part on the map of Bächi and Arber (7) and on our own marker rescue experiments using *am* mutations (8). The *vir*^{s} mutation, located in the *imm*I region (2, 9), permits the phage to express gp*ant* constitutively even in the presence of gpc4. Thus, when a P1 lysogen is infected with P1*vir*^{s}, gp*ant* is expressed, repression of lytic functions is lifted, and the infecting phage grows. For the same reason, P7, whose *ant* gene expression is insensitive to repression by gpc4 of P1, will grow in a P1 lysogen.

Figure I.5. The sequence of the ref gene region of P1.



Consensus promoter sequences of p_{ref-1} and p_{ref-2} are boxed and connected by a solid line as indicated. Transcription initiation points are depicted by rightward arrows. Operators Op2a, Op2b, and *ref* initiation codon (ATG) are double underlined. (From Lu et.al. and Windle and Hays)

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CHAPTER II. The *bof* gene of bacteriophage P1. DNA sequence and evidence for roles in regulation of phage *c*1 and *ref* genes.

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ABSTRACT

The C1 repressor of bacteriophage P1 acts via fourteen or more distinct operators. It represses its own synthesis as well as that of other gene products. Mutation of an auxiliary regulatory gene, *bof*, has previously been shown to increase expression of some C1-regulated P1 genes (e.g. *ref*) but decrease expression of others (e.g. *ban*). Here the *bof* gene was isolated on the basis of its ability to depress stimulation of *E. coli* chromosomal recombination by the P1 *ref* gene, if and only if a source of C1 was present. C1 alone, but not Bof alone, was partially effective. The *bof* DNA sequence encodes an 82-codon reading frame that begins with a TTG codon and includes the sites of the *bof-1*(Am) mutation and a *bof*::Tn5 null mutation. Expression of *ref*::*lacZ* and *c1*::*lacZ* fusion genes was partially repressed in *trans* by a P1*bof-1* prophage or by plasmid-encoded C1 alone, in agreement with effects on Ref-stimulated recombination and with previous indirect evidence for *c1* autoregulation. Repression by plasmid-encoded C1 + Bof or by a P1*bof*$^+$ prophage, of both fusion genes, was more complete. When the C1 source included as well an 0.7-kb region upstream of C1 which encodes the *coi* gene, repression of both *c1*::*lacZ* and *ref*::*lacZ* by C1 alone or C1 + Bof was much less effective, as if Coi interfered with C1 repressor function.

INTRODUCTION

Upon infection, temperate phages respond to physiological signals from their hosts and choose between establishment of stable repressor synthesis (lysogeny), and irreversible commitment to lytic-gene expression. Bacteriophage P1 maintains stable lysogeny in host bacteria as other temperate phages do, by binding its primary repressor, C1, to promoter-operators for lytic genes (43). Prophage P1 is a (unit-copy) plasmid rather than a part of the host chromosome, and its regulatory apparatus is unusually complicated - the C1 repressor acts at numerous sites scattered throughout the genome (including the *c*1 gene itself). P1 differs from most temperate phages in two major respects.

First, in contrast to phage λ and its relatives, whose two promoter-operators each contain three symmetric sequences that bind repressor dimers, there are at least fourteen C1 binding sites, in ten different regions of the P1 genome (5). The C1 operators, all of which include an asymmetric sequence identical or close to the consensus ATTGCTCTAATAAATTT, are numbered according to their location on the P1 map (42) (Fig. 1), e.g. Op2, Op21. Operators near the same map coordinate are lettered, e.g. Op99a...Op99e. The P1 operators are almost all oriented in the same direction relative to associated promoters, but their locations relative to putative "-35" and "-10" sequences vary considerably (2,5,7,11,39). [The C1 repressor of the closely related but heteroimmune phage P7 appears to be identical to the P1

repressor (4,36).]

The λ repressor controls its own "maintenance" synthesis during lysogeny by means of the O_R operators, which overlap the maintenance promoter region (25). However, a critical factor in the lysis-lysogeny decision is initiation of transcription of the λ repressor gene at an "establishment" promoter, not associated with any repressor binding site and positively regulated by the λ cII protein (40). In contrast, both of the promoters which might logically be expected to initiate P1 *c*1 transcription are associated with C1 binding sites (7). Thus P1 must employ different regulatory mechanisms than λ during establishment of lysogeny, and most likely during maintenance as well.

The actions of C1 repressor at its own and other genes are modulated by auxiliary elements, organized into three regions (43). The *immC* region, at coordinates 99-0, includes the *c*1 gene and upstream elements: two promoter-operators, another operator, and three small open reading frames. The order (counter-clockwise on the P1 map) is P-Op99d *orf-2 orf-3 orf-4* Op99c P-Op99ba *c*1. The *virC* mutation, which maps upstream of these and appears to make expression of at least one of these *orf*s constitutive (10) might be considered part of *immC*. Overexpressed *orf* protein is thought to cause the phenotype of P1*vir*C, by acting at the level of C1 function (43) rather than *c*1 gene expression (10,27).

The *immI* region, coordinates 50-52, is responsible for the synthesis and control of a function or functions [*ant* (36); *rebA,B* (27)] that antagonize C1, and for P1-P7 heteroimmunity (29). Expression of *ant/reb* is regulated by the *c4* gene. The

mechanism of antagonism, though not well understood, appears not to involve direct inactivation by Ant/Reb of C1, unlike the anti-repressor mechanism of phage P22 (33).

The *immT* region is defined by the *bof* gene (35) at coordinate 9.5; its product is yet another modulator of C1 action. The Bof $^-$ phenotype is complicated and somewhat paradoxical, suggesting a correspondingly complex mechanism of action. A *positive* role for Bof in expression of the C1-controlled P1 *ban* gene, which encodes an analog of the *E. coli* DnaB protein, was suggested by the inability to express Ban activity of second-site mutants of certain prophages (P1 *bac-1*) that were normally Ban-constitutive. The Ban$^-$ phenotype of the doubly-mutant P1 *bof-1 bac-1* prophages motivated the designation Ban-on-function (35). [*bac-1* proves to be a mutation in C1 binding site Op72a associated with the *ban* promoter (11).] In contrast, a *negative* role for Bof in the regulation of some P1 genes is suggested by the markedly increased levels of two activities in P1 *bof-1* prophages: Ref, which stimulates certain RecA-dependent homologous recombination processes in *E. coli* chromosomes (18,38) and plasmids (17), and P1 Ssb, which suppresses the defects of *E. coli ssbA*(Ts) mutants at non-permissive temperatures (15,38). [P1 *lxc* mutations confer the same phenotypes, and appear to be allelic with *bof-1* (15).] Bof effects on immunity establishment and/or maintenance appear similarly contradictory. The immunity of P1 prophages against superinfection (by P1 *vir*^{S} and P7) seems to be *weakened* by Bof (34,35), but the stability of P1 *c1*.100(Ts) prophages appears *strengthened* by Bof (35). *c6* may be allelic with *bof* (28).

Although C1 is necessary for regulation of *ref* gene expression, the Ref-constitutive and Ssb-constitutive phenotypes of P1 *bof-1* and *lxc* prophages demonstrate that it is not sufficient (38). Here we have used depression of Ref recombination-stimulation activity, in the presence of C1, as an assay for isolation of the *bof* gene, and have determined the *bof* DNA sequence. Using the isolated *bof* gene and *ref*::*lacZ* and *c1*::*lacZ* fusion genes, we show that Bof is a negative effector of both *c1* and *ref* transcription, if and only if C1 is present as well.

MATERIALS AND METHODS

Bacteria, bacteriophages, and plasmids. These are described in Table 1. Stocks of λ bacteriophages were prepared by growth to confluent lysis on S-plates, as described (32). Stocks of P1 bacteriophages were prepared by confluent lysis on R-plates (22). Lysogens of P1CmO phages were prepared by spotting phage lysates onto appropriate bacterial lawns and streaking turbid centers onto LB plates with chloramphenicol (10 μg per ml). λ bacteriophages encoding *ref*::*lacZ* and *c1*::*lacZ* gene fusions were prepared by plate-stock growth of λRS88 on MPh30($Plc^+r^-m^-$) bacteria harboring appropriate plasmids, and identified as prophages in MPh30 bacteria spread onto X-Gal plates (blue colonies). Plasmids were extracted from bacteria by rapid-boiling (14) or alkaline-lysis techniques (21) and purified by equilibrium sedimentation (4.5 h at 90,000 rpm: 340,000 x g) in CsCl plus ethidium bromide, using 3.5-ml tubes in a Beckman TL-100 table-top ultracentrifuge.

Media and Buffers. TBE-buffer: 89 mM TrisBase, 89 mM Boric Acid, 2 mM Na_2EDTA; pH 8.0. TE-buffer: 10 mM Tris-HCl, 1 mM Na_2EDTA; ph 8.0. Z-buffer: 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol; pH 7.0. TBY-broth: 1% Bacto-tryptone (Difco), 0.5% Yeast extract (Difco), 1% NaCl. M9-medium: 42 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.5 mM NaCl, 18.7 mM NH_4Cl, 10 mM$CaCl_2$, 0.2% glucose, 0.001% thiamine. LB plates: TBY-broth solidified with 1.5% agar. MacConkey-lactose plates: 4% MacConkey agar base

(Difco), 1% lactose. R-plates: 1% Bacto-tryptone, 0.1% Yeast extract, 0.8% NaCl, 2 mM $CaCl_2$, 0.1% glucose, 1.2% agar. S-plates: 1% Bacto-tryptone, 0.5% NaCl, 0.075 mM $CaCl_2$, 0.004 mM $FeCl_3$, 10 mM $MgSO_4$, 0.3% glucose, 0.001% thiamine, 1% agar. TCMB plates: 1% trypticase (Baltimore Biol. Labs), 0.5% NaCl, 10 mM $MgSO_4$, 0.0001% thiamine, 1.1% agar. X-Gal plates: LB plates with 40 μg/ml 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Antibiotics were used in plates at the following concentrations: ampicillin [Ap], 75 μg per ml; neomycin [Nm], 50 μg per ml; chloramphenicol [Cm], 10 μg per ml.

General recombinant DNA techniques. Restriction endonucleases were purchased from New England Biolabs [NEB], United States Biochemical Corporation or Bethesda Research Laboratories [BRL], T4 DNA ligase from NEB, and exonuclease III, S1 nuclease, DNA polymerase I large (Klenow) fragment from BRL. All enzymes were used as specified by the manufacturers. Nested deletions were generated by exonuclease III digestion at 5'-overhang restriction-enzyme termini (in the presence of exoIII-resistant 3'-overhang termini), as described by Henikoff (13). Electrophoresis of DNA fragments was performed in 0.7 to 1.0% agarose or 12% polyacrylamide gels in TBE-buffer; fragments were isolated from gels by electroelution techniques. Bacteria were transformed with plasmids by the $CaCl_2$ technique (20) or by an adaption of other procedures (21): resuspension of cells in an equal volume of cold 0.1M MOPS, pH 6.5, 50 mM $CaCl_2$, 10 mM $RbCl_2$, 15% glycerol; incubation on ice for 1hr; centrifugation of cells and resuspension in 1/10 volume of the same buffer; freezing of cells at -80°C or immediate use.

DNA Sequence Determinations. The dideoxy-chain termination method of Sanger (26) was applied to duplex plasmid DNA primed with synthetic oligonucleotide primers, as described by Zhang *et al.* (44), using modified phage T7 DNA polymerase (Sequenase; United States Biochemical Corp.) and [^{35}S]dATP (New England Nuclear). Sequencing reactions were electrophoresed in 6-8% polyacrylamide gels. Gels were rinsed in 5% methanol/5% acetic acid, transferred to 3MM filter paper, and dried. X-ray film was exposed to the filter paper for 20 to 70 h, as needed.

The sequence of the entire 0.93-kb *Bam*HI-*Dra*I fragment of plasmid pTS882 was obtained using a variety of primers, with plasmid templates derived by subcloning various fragments [approximate regions sequenced, and direction of dideoxy synthesis, are indicated in parentheses, e.g. (0.44-0.72) implies synthesis left-to-right (Fig. 2), yielding the indicated 280 bp of sequence]. "Left and right" refer to Fig. 2. The pUC "universal" primer CAGCACTGACCCTTTTG, corresponding to 17-nt of *lacZ* sequence downstream of the "multi-linker" region of pUC plasmids, was used with a nested series of deletion derivatives spanning the entire fragment [generated from pTS881 by the Henikoff (13) technique (*Bam*HI + *Sal*I restriction, exoIII digestion of 3'-ended strand at *Bam*HI end, treatment with S1 nuclease and DNA polymerase I (Klenow fragment), ligation], to generate the (0.0-0.9) sequence. The pUC "universal" primer and "reverse" primer AACAGCTATGACCATG (corresponds to 16-nt of sequence that is just upstream of "multi-linker" region in pUC plasmids and spans *lacZ* translation start signal) were used with the *Bam*HI(*a*)-*Fsp*I fragment of pTS882 replacement-inserted into the *Bam*HI and *Hind*II sites of

pUC19 to generate (0.70-0.45) and (0.0 to 0.26), respectively, and with the *Pst*I-*Fsp*I fragment (from plasmid pTS882) inserted into the *Pst*I and *Hinc*II sites of pUC19, to generate (0.66-0.93) and (0.93-0.67), respectively. The pUC universal primer was used with plasmid pTS882 to generate (0.0-0.25). The 17-nt primer (GGGAAAGGTTCCGTTTC) corresponding to a conserved sequence in the right and left IS*50* arms of Tn*5*, was used with fragments containing respectively *bof* DNA left of the insertion [associated with the left *Sal*I half of Tn*5*], and *bof* DNA right of insertion [associated with the right (neomycin-resistance(Nm^r)-encoding) *Sal*I half of Tn*5*], after the fragments were generated by deleting the *Sal*I-*Sal*I fragment encoding the right (Nm^r) half and adjacent P1 and pACYC184 DNA from pTS8745, then inserting that fragment into pACYC184, to obtain respectively (0.56-0.90) and (0.56-0.33). Primers TS4 (GCCAGTCCCTGAAGA) and TS3 (TGATGATCTGCTCCC) corresponding to locations in the *bof* gene region, were used with plasmid pTS882 to generate respectively (0.43-0.72) and (0.73-0.41).

Measurement of β-galactosidase expression by fusion genes. *Uninduced plasmids and prophages.* M9-medium (supplemented with 5% TBY, 0.02% Casamino Acids, 0.2% glucose, and appropriate antibiotics for selecting for plasmid maintenance) was inoculated directly with colonies of bacteria (MPh30 derivatives), and cultures were grown for about 8 h with shaking at 32°C. At A_{600} about 0.5, cultures were diluted in Z-buffer, usually 1:2 or 1:10, and 1-ml aliquots mixed with 0.05 ml of 0.1% sodium dodecyl sulfate plus 0.1 ml $CHCl_3$, in 1.9 ml microcentrifuge tubes. At time zero, samples were mixed with 0.2 ml of 0.4% ortho-nitrophenyl-β-D-

galactopyranoside [ONPG] and warmed to 37°C. After appropriate incubation times (usually 5 to 20 min.), samples were mixed with 0.5 ml 1M Na_2CO_3 and centrifuged for 1 min. in a microfuge. Supernatants (about 1 ml) were transferred to disposable microcuvettes for determination of A_{420} (ONPG absorbance) and A_{550} (cell-turbidity correction). β-galactosidase units were calculated as described by Miller (22). *IPTG-induced pMV1w.* M9-medium [with supplements described above, plus isopropylthio-β-D-galactopyranoside (IPTG) was inoculated with an aliquot of a colony resuspended in broth or sterile saline, and cultures grown up and assayed as described above. Multiple aliquots of the same suspension were used for replicate determinations.

Measurement of *lac*$^-$ x *lac*$^-$ recombination. *Lac*$^+$ *papillation assay*: growth of bacteria (KS391 derivatives) streaked onto MacConkey-lactose plates) for 48 h at 32°C; counting of Lac$^+$ papillae in about 5-7 colonies. *Assay for preexisting Lac*$^+$ *colonies*: growth of bacteria (KS391 derivatives) in LB broth for about 18 h; serial dilution of cultures in 0.85% NaCl; spreading onto LB plates (to determine cell concentrations) and onto MacConkey-lactose plates (about 10^5-10^6 colonies per plate) (to determine Lac$^+$ cell concentration); about 20 h incubation at 32°C.

RESULTS

Requirement for both *c1*-encoding and *bof*-encoding DNA fragments to suppress Ref recombination-stimulation activity. Previous observations that P1*c1*$^+$*bof-1* mutants were derepressed for Ref activity (38) and that *c*1-encoding DNA segments did not repress *ref* expression in *trans* [Lu, S.D., D. Lu and M.E. Gottesman, unpublished observations; Windle, B.E., Ph.D. thesis, 1986] together suggested that in addition to C1, the product of the *bof* gene, or of one or more genes controlled by it, was required to fully repress *ref* gene expression. The *bof-1* mutation maps to P1 coordinate 9.5 (35,43) (Fig. 1). We tested wild-type P1 *Bam*H1 fragment 4 [P1:B4], which spans coordinates 3 through 12, for its ability, when inserted into the plasmid vector pPR110 (37), to complement a P1*bof-1* prophage for repression of *ref* expression. Ref activity was estimated indirectly, as stimulation of *lac*$^-$ x *lac*$^-$ chromosomal recombination [manifested as increased frequency of Lac$^+$ papillae on colonies of appropriate derivatives of strain KS391 (38)]. Under conditions (see "Materials and Methods") such that KS391 averaged about one Lac$^+$ papilla per colony, KS391(P1*bof-1*) and KS391(P1*lxc-1*) averaged over 30 Lac$^+$ papillae. In the presence of fragment P1:B4 (on a multi-copy plasmid), the latter two lysogens showed only 1-2 and 2-4 papillae, respectively i.e., P1:B4 sufficed for complementation.

To further define requirements for regulation of *ref* expression, we inserted

the 9.5-kb P1:B4 fragment into pACYC184 (plasmid pTS871) and tested the effect of this plasmid and the compatible plasmid p*c*1 Δr (Sternberg, N., unpublished), which bears only the P1 *c*1 gene and the immediately associated promoter-operator [P-Op99ba (7)]. Ref activity was assayed as the frequency of Lac^+ cells in cultures of appropriate KS391 derivatives. Although C1 function alone partially depressed Ref activity, C1 plus a source of Bof were necessary (and sufficient) for full repression (Table 2). The assay is not precise enough for the small decrease in Ref activity in the presence of plasmid-borne P1:B4 without C1 to be considered significant.

Localization of the *bof* gene. The localization of the *bof* gene by Tn*5* mutagenesis and deletion experiments is summarized in Figure 2. Bof activity, defined as suppression, in the presence of p*c*1 Δr, of λ-P1:B8-prophage-stimulated *lac*$^-$ x *lac*$^-$ chromosomal recombination, was expressed by a series of subfragments of P1:B4, inserted into plasmids. Details are provided in the Fig. 2 legend. All constructs shown in Fig. 2, except pTS8745, expressed full Bof activity. A series of unidirectional deletions of pTS5881 DNA, beginning at the point where *Bam*HI linkers had been inserted at the *Sma*I site (designated SMB; see structure of pTS5872 in Fig. 2), and extending increasingly farther into P1 sequence. Bof activity was eliminated when these deletions extended about 0.7 kb or more from the SMB site (estimated by restriction analyses). In pTS8745, the Tn*5* insertion, which eliminated Bof activity, was mapped by restriction analyses to be 0.6 to 0.9 kb from the SMB site.

The DNA sequence of the *bof* gene. Direct dideoxy plasmid-DNA sequencing,

using a variety of oligonucleotide primers, was used to identify the *bof* gene, as described under "Materials and Methods". There were five independent sources of *bof* $^{+}$ sequence information for the critical region that proves to encode the presumptive translation start codon (below). The sequence of the *bof-1*(Am) gene in plasmid pMV15RD (kindly provided by H. Schuster), was determined by extension of primer TS3.

The DNA sequence of the *bof* gene region of phage P1 appears in Fig. 3, numbered counter-clockwise from the center of the *Sma*I site near P1 map position 10. (The *Bam*H1-linker nucleotides in plasmid pTS873 and derivatives are not numbered.) The DNA sequence between bp 388 and 931 has been determined by Schuster and colleagues (12), and is in complete agreement with the sequence presented here. We identify the open reading frame commencing with the TTG at bp 541-543 as *bof*, for the following reasons: (i) The TAC at bp 553-555 in *bof* $^{+}$ is a TAG in *bof-1*(Am). (ii) There is a TGA at bp 511-513 in-frame with the bp 553-555 TAC. There is no ATG between bp 513 and 553, but the bp 541-543 TTG is preceded by a reasonable ribosome binding site (30). (iii) The reading frame includes bp 601-609, which flank (in duplicate) the *bof*::Tn*5* insertion. The *bof* reading frame terminates with a TAA at bp 787-789, thus encoding an 82-amino-acid polypeptide, in agreement with the M_r 9600 protein identified as Bof by Schuster and coworkers (12). About 1% of *E. coli* proteins initiate translation at a TTG codon (9). Schuster and co-workers (Velleman, M., M. Heirich, A. Günther and H. Schuster, manuscript submitted) have identified the TATGAG at bp 482-487 as a transcription signal ("-

10") required for *bof* expression.

Bof-plus-C1-mediated repression of *ref*::*lacZ* expression. Inhibition of Ref-stimulation of *E. coli lac*$^-$ x *lac*$^-$ recombination by Bof activity in the presence of C1 activity could in principle reflect interference with the recombination process rather than repression of *ref* gene transcription. Furthermore, the relationship between *ref* gene transcription and the frequency of Ref-stimulated recombination is not known. In order to measure directly the effect of Bof and C1 on *ref* transcription, we constructed a *ref*::*lac*Z fusion gene (Fig. 4). The 0.25-kb *Bam*H1-*Sau*3a fragment from pUC19r81 (17), encodes a few vector bp plus the following: the *ref* promoter-operator region (17) - dual tandem promoters, C1 recognition sequence; the *ref* attenuator (39); the first 65 bp of the *ref* reading frame. This fragment was fused (out of frame) to the *Bam*H1 site of pRS415 (31), thus placing the *ref* transcription-initiation signals upstream of a promoterless *lac*ZYA segment [a few bp of *trp* sequence (lacking expression signals) are 5' to *lac*ZYA]. The fusion gene was transferred to phage λRS88 (31) by homologous recombination, and the λ *ref*::*lac*Z phage used to lysogenize the Δ*lac* strain MPh30.

MPh30 (λ*ref*::*lac*Z) lysogens synthesized only about 1/6 as much β-galactosidase as *lac*$^+$*Z*$^+$ bacteria (data not shown). [There is a rho-independent attenuator in the *ref* leader sequence (17).] Expression of *ref*::*lac*Z was repressed twice as well by P1*c*$^+$ prophages as by P1*bof-1* and P1*lxc-1* prophages (Table 3A lines 5,3,2) in qualitative agreement with their effects on Ref-stimulated recombination (38). P1*c1*.100 prophages suppressed *ref*::*lac*Z expression much less

effectively than P1*c*$^+$ prophages, also in agreement with recombination studies; the *c*1.100 data were highly variable (sometimes cultures showed partial lysis), but supported the notion that *ref* is partially derepressed in *c*1.100 prophages (38).

We tested a plasmid encoding an isolated *bof* gene, and two plasmid sources of the *c*1 gene, for regulation of *ref*::*lacZ* expression (Table 3B). C1 alone (in the presence of *bof*::Tn*5* plasmids) resulted in partial repression, (Table 3B, lines 2,5); the addition of Bof increased repression (lines 3,6). The Bof + C1 combination was about as effective as an intact P1*c*$^+$ prophage (compare line 5, Table 3A, with lines 3 and 6, Table 3B).

Modulation of *c1* expression and C1 activity. C1 appears to regulate its own synthesis (43,23). The increased superinfection immunity shown by *bof-1* mutants (35) suggested that Bof might be a co-regulator of *c*1 expression. We constructed a *c*1::*lacZ* fusion gene (Fig. 4) and assayed for Bof and C1 effects. The *c*1 portion is a 74-bp *Ball* fragment that encodes associated near-canonical (Op99a) and degenerate (Op99b) C1 operators (Fig. 4) and no upstream P1 DNA. A P1*c*$^+$ prophage suppressed *c*1::*lacZ* expression about four times as well as a P1*bof-1* prophage (Table 4A, lines 4,2) indicating that C1 synthesis is indeed elevated in *bof* mutants. There appears to be little autoregulation in *c*1.100 prophages.

Plasmid-encoded *c*1 genes directly repressed *c*1::*lacZ* transcription (Table 4B), confirming previous evidence that *c*1 is autoregulated (23). Autorepression by p*c*1Δr, which encodes only P-Op99a *c*1, was about three times as effective as autorepression by pMV1w, which includes most of the *immC* region (P-Op99c *orf-2*

orf-3 orf-4 Op99c P-Op99a *c*1) (Table 4B, lines 2,5). In both cases, addition of plasmid-encoded Bof further repressed *c*1::*lacZ* expression (Table 4B, lines 3,6).

The P1 *virC* mutation, which makes the synthesis of one or more small proteins constitutive, maps just upstream of the *orf*-2,3,4 region (10). The *coi* mutation, which suppresses *vir*C, maps in the *orf*-2,3,4 region (27). On this basis, Coi has been proposed to be a product of one of these reading frames, and an antagonist of C1-mediated repression. Plasmid pMV1w, but not p*c*1Δr (or the *c*1::*lacZ*-encoding pTS321) would be expected to encode the *coi* gene. The lesser ability to repress *ref*::*lacZ* and *c*1::*lacZ* expression shown by pMV1w is consistent with the presence of a pMV1w-encoded C1 antagonist, but might also be ascribed to other plasmid-specific effects on *c*1 expression. (p*c*1Δr and pMV1w copy numbers appeared approximately equal.) The presence of a p_{tac} promoter in pMV1w, positioned to drive the upstream open reading frames as well as *c*1 itself, provided an opportunity to rule out plasmid-specific effects and perform a preliminary *cis-trans* test for Coi function. We measured *c*1::*lacZ* and *ref*::*lacZ* expression, first in the presence of the moderate levels of C1 and putative Coi protein provided by uninduced pMV1w, and then in the presence of pMV1w expression induced by various concentrations of IPTG (Fig. 5). The expression of both *ref*::*lacZ* and *c*1::*lacZ* was markedly increased by induction of putative Coi function. Since induction would be expected to concomitantly *increase* synthesis of the C1 repressor, the data suggest that Coi acts in *trans* to inhibit C1 activity.

DISCUSSION

The inference drawn from previous studies (38) with P1 *bof*(*lxc*) and *c*1(Ts) mutants - that C1 and Bof are co-regulators of *ref* gene expression - has been supported here by experiments with isolated *c*1 and *bof* genes. We demonstrated Bof (plus C1)-mediated repression of both Ref-stimulated recombination and *ref*::*lacZ* transcription. How might Bof act with C1 to regulate *ref*? Since multiple copies of the *bof* gene appear to have little or no regulatory effect alone, Bof seems not to act in this case as an independent repressor. Bof could in principle decrease *ref* transcription indirectly, by causing an increase in C1 levels. However, we have shown here that Bof activity actually represses *c*1::*lacZ* transcription (in the presence of C1), and Schuster and coworkers have found that Bof depresses the levels of immunoassayable C1 (12). Bof might modify the C1 protein to a $C1^*$ form with increased affinity for operators, or form a C1•Bof complex with similarly altered affinity. These experiments do not distinguish between these alternatives, but it should be possible to do so using appropriate *in vitro* assays. Bof-mediated alteration of C1 susceptibility to antagonism by the *ant*(*rebA*,*rebB*) product(s) has been proposed previously (35), but our experiments with isolated *bof* genes show that this cannot be the universal mechanism for Bof action.

The observation that C1 itself and (indirectly) Bof are co-regulators of *c*1::*lacZ* transcription may point the way to a resolution of the paradox posed by the

bof-1 mutation, which negatively affects Ban activity, but positively affects *ref* (and *ssb*) gene expression. Thus (Bof + C1)-regulated levels of C1 might be high enough to suppress *ban* gene expression in wild-type but not *bac-1* prophages, but in the absence of Bof, C1 levels might increase to the point that the *ban* gene is repressed in *bac-1* prophages as well. It thus appears likely that C1 tightly represses the *ban* gene without direct participation by Bof. However, the data of Tables 3B and 4B do not rule out small positive effects by Bof in the absence of C1. Final determination of the mechanisms by which Bof affects *ban* gene expression requires studies with *ban*::*lacZ* and (*bac-1*)*ban*::*lacZ* fusions.

These data do not provide an obvious explanation for the ability of P1*c*1.100 *bof-1* prophages to be induced at lower temperatures than P1*c*1.100 prophages (35), since one expects *bof-1* prophages to make more, not less, repressor. Nor is there an apparent biological rationale for enhancement of C1-mediated repression of some genes by Bof, at the same time that it causes C1 synthesis to decrease. The latter paradox may be related to a need to fine-tune C1 levels, so that lytic genes are tightly repressed (with the help of Bof), but genes that contribute to plasmid maintenance (in at least some P1 hosts) are only partially repressed.

The evidence that *bof* translation initiates with a TTG codon is compelling but circumstantial, and remains to be confirmed by N-terminal analysis of purified Bof protein. The putative 82-amino-acid polypeptide (Fig. 3) would have an unusual secondary structure. The high content of basic amino acids corresponds to a pI of 10.76. The hydrophilic residues are roughly grouped into four domains of eleven or

so units each, separated by a few neutral residues. Many of the hydrophobic amino acids are contained in a ten-residue C-terminal tail. In contrast to Bof, seen here to act with C1 as a co-repressor, some activity expressed by plasmid pMV1w, but not by p*c*1Δr, appears to interfere with C1 repressor activity. It seems logical to associate this apparent C1-antagonizing activity with the Coi protein(s), whose constitutive synthesis is thought to be responsible for the phenotype of *vir*C mutants (27). The *vir*C-suppressing *coi* mutation maps in the *orf*-2,3,4 region (27) and Baumstark (Baumstark, B.R., S.R. Stovall and P. Bralley, manuscript submitted) has obtained evidence that Coi is the product of *orf-4*, which is encoded by pMV1w. At moderate levels of C1 and Coi (intermediate IPTG concentrations in Fig. 5), Coi seems to have the upper hand over C1. At high IPTG concentrations it appears that enough C1 is made to establish repression of Coi synthesis, restoring C1-mediated repression of other genes, but we cannot rule out some competitive inhibition of β-galactosidase by residual IPTG. Orf-4, believed to encode the putative Coi protein, is expected to produce a highly acidic protein with a pI of 3.68 (7). We propose that the two auxiliary proteins, Bof and Coi, act by binding to C1. A C1•Bof complex would have a significantly more positive charge than C1 alone, and might be expected to bind more tightly to DNA. Conversely, a C1•Coi complex would be more negatively charged, and thus bind to DNA less well. This would explain the apparent opposite effects of Coi and Bof on expression of C1-controlled genes. Since Bof can enhance C1-mediated repression in the presence of Coi, the two proteins may compete for the same site on C1 or bind to one another. Some genes, *ban* for

example, appear to be repressed tightly by C1 alone. This model can readily be tested by biochemical experiments with purified C1, Bof, and Coi proteins.

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Table II.1a. Bacterial Strains

Designation	Genotype	Source/Reference
C600	*lac*Y1 *leuB*6 *supE*44 *thr-1 thi-1 tonA*21	(1)
JM101	Δ(*lac-proAB*) *thi supE* [F'*tra*36 *proAB lacI*^{q} *lacZ* ΔM15]	(39)
KS391	HfrH *lacZ*M5286 (Φ80dII*lacZ*BK1)	(16)
MPh30	Δ(*argF-lacZ*) U169 *pho*A::Tn5	from R. Wolf
TSS301	MPh30(λTS301)	This work
TSS321	MPh30(λTS321)	This work

Table II.1b. Bacteriophage Strains

Designation	Genotype/Remarks	Source/Reference
λRS88	`*bla*`*lacZ lacY lacA imm*^{434} *ind*$^-$	(31)(Fig. 5)
λ301	`*bla* Tl_4 P1:*ref*::*lacZlacY lacA imm*^{434}*ind*$^-$ [a] (λRS88 x pTS301 recombinant)	This work
λ321	`*bla* Tl_4 P1:*c1*::*lacZlacY lacA imm*^{434}*ind*$^-$ (λRS88 x pTS321 recombinant)	This work
P1CmO*r*$^-$*m*$^-$	Lacks P1 restriction-modification	M. Gottesman
P1CmO*c1*.100*ref*::Tn*5*-101	Ref$^-$	(38)
P1CmO*bof-1*	Derepressed for *ref*, putative *ssb*	N.Sternberg(35)
P1CmO*lxc-11*	Derepressed for *ref*, putative *ssb*	L. Rosner[b]

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[a] Tl_4 is tandem tetramer of *E. coli rrnB* transcription terminator, oriented so as to block transcription of *lac*ZYA from upstream promoters.

[b] This phage appears to have undergone a rearrangement that inactivated the *bof* gene. *Bam*H1 restriction fragments 3 and 4 (P1 coordinates 3-12 and 18.5-31.5, respectively), and *Sma*I fragments 2 and 3 (P1 coordinates 10-29 and 97-100 respectively) are altered, and there is a novel joint that fuses *bof* DNA 3' to bp 656 to P1 DNA of unknown origin.

Table II.1c. Plasmids

Designation	Description/Remarks	Source/Reference
pACYC184	Tc^r Cm^r	(3)
pAC*lac*19	*Ava*II-*Bgl*II fragment from M13m19 (encoding *lacPO*, "polylinker" and additional 5' bp of *lacZ* replacement-inserted into *Hin*dII-*Bam*H1 sites (*tet* gene) of pACYC184.	T. Schaefer (unpublished)
pBR325	Ap^r Cm^r	(24)
p*c1*Δ*r*	Encodes Op99ba *c1* of phage P1.	N. Sternberg (unpublished)
pMV1w	*Pvu*II-*Bcl*I fragment of P1c^+ in pJF118EH,[a] such that p_{tac} drives (in order) *orf-2, orf-3, orf-4, orf-5,* and *c1*.	H. Schuster
pPR110	Encodes (λcI857 p_r)-"multi-linker"-*lacZ*.	(37)
pRS415	Encodes *bla*Tl_4 (no promoter) *lac*`*ZYA*.	(30)
pTS-4-6	P1:B4 in *Bam*H1 site of pPR110, Bof^+.	This work
pTS301	*Eco*RI-*Sau*3a fragment of plasmid	This work
pUC19r81	(38), encoding *ref* promoter-operator,	

Table II.1c (continued)

	attenuator and 21 N-terminal amino acids, in *Sma*I site of pRS415.	
pTS321	*Ball-Ball* fragment of pMV1w, encoding Op99ab p_{c1}, in *Sma*I site of pRS415.	This work
pTS871	P1:B4 in *Bam*H1 site of pACYC184. Bof^+Tc^S	This work
pTS872	*Bgl*II-*Bam*H1 fragment of pTS871 in *Bam*H1 site of pACYC. Bof^+Tc^S.	This work
pTS873	8-bp *Bam*H1 linker [d(CGGATCCG), New England Biolabs] in unique *Sma*I site of pTS872. Bof^+Tc^S.	This work
pTS874	Bof^+ derivative of pTS873.	This work
pTS8745	*bof*::Tn*5* derivative of pTS874. *Bof*$^-$ Tc^S.	This work
pTS881	Bof^+ derivative of pTS874, pAC*lac*19.	This work
pTS882	Bof^+ derivative of pTS881, pAC*lac*19.	This work
pTS891	Bof^+ derivative of pTS882, pUC19.	This work
pTS894	Bof^+ derivative of pTS882, pUC19.	This work

Tc^S, tetracycline-sensitive. Ap^r, ampicillin-resistant.

Cm^r, chloramphenicol-resistant.

[a] Reference (8).

[b] See note *a*, Table 1B.

Table II.2. Suppression of Ref activity by *trans*-acting P1 genes.

C1 source	Bof source	Relative frequency of Lac^+ cells in Ref^+ bacteria[a]
none[b]	none[b]	25
p*c1Δr*	none[b]	15
none[b]	pTS871	21
p*c1Δr*	pTS871	0.9
P1*c*$^+$	P1*c*$^+$	0.6

[a] The frequency of *lac*$^-$ x *lac*$^-$ chromosomal recombination in plasmid-bearing derivatives of strain BW2060, which is KS391(λ-P1:B8), was assayed by measuring the frequency of preexisting Lac^+ cells in overnight cultures, as described under "Materials and Methods". A relative frequency of 1.0 corresponds to the value for strain KS391, 7×10^{-4}.

[b] Vector plasmid(s) only.

Table II.3a. Regulation of *ref*::*lacZ* expression by P1 prophages.

P1 prophage	Relative β-galactosidase expression by λ*ref*::*lacZ* prophage[a]	Average Bof^+/Bof^- ratio[b]
none	(100)	
P1*lxc*11CmO	58 ± 11	0.32 ± 0.06
P1*bof-1*CmO	56 ± 9	0.33 ± 0.13
P1*c1*.100CmO	93 ± 24	
P1c^+CmO	17 ± 7	

[a] Strain TSS301 was lysogenized with the indicated P1 phages. β-galactosidase was measured in exponential-phase 32°C cultures as described under "Materials and Methods". Measurements were made on five different sets of lysogens of TSS301, usually in duplicate. Enzyme incubations were usually performed for two different durations; there was no systematic variation with duration. Relative expression equals β-galactosidase activity [Miller units (22)] normalized by activity for no P1 prophage (about 300), times 100. Relative activity for MPh30 without λ or P1 prophage was 1. Data correspond to averages and standard deviations for fourteen determinations.

[b] The ratios of *ref*::*lacZ* expression in the presence of Bof (supplied by P1c^+) to that in the absence of Bof (*lxc*$^-$1 or *bof-1*) were determined for each set of lysogens, and averages and standard deviations calculated.

Table II.3b. Regulation of λ*ref*::*lacZ* prophage expression by plasmid-encoded P1 genes.

C1 Source	Bof Source	"Coi" Source	Relative β-galactosidase[a] expression	Average ratios Bof$^+$/Bof^b	Coi$^+$/Coi^{0c}
none[d]	none[d]	none[d]	(100)		
p*c1*Δr	none[f]	none[d]	13 ± 3.5		
p*c1*Δr	pTS874	none[d]	7.5 ± 1.6	0.61 ± 0.24	
none	pTS874	none[e]	119 ± 17		
pMV1w	none[f]	pMV1w	68 ± 20	5.4 ± 1.4	
pMV1w	pTS874	pMV1w	29 ± 15	0.45 ± 0.24	4.0 ± 2.1

[a] Strain TSS301 was transformed with the indicated plasmids and β-galactosidase determined in exponential-phase cultures, as described under "Materials and Methods". Relative expression equals β-galactosidase activity [Miller units (22)] normalized by activity in the absence of other factors (about 300) times 100. Average relative activity for strain MPh30 without any prophage was 1. Data correspond to averages and standard deviations for twenty-eight determinations (six different sets of transformants, duplicate samples, two different enzyme-assay durations).

Table II.3b (continued)

[b] Ratios of *ref*::*lacZ* expression in the presence of the same C1 source, but *bof*$^+$ *vs.* *bof*::Tn5, were calculated for each of the twenty-eight determinations and averaged (standard deviations indicated).

[c] Ratios of *c*1::*lacZ* expression in the presence of pMV1w (Coi$^+$) *vs.* p*c*1Δr (Coi^0), in the presence or absence of Bof were calculated for each of the twenty-eight determinations and averaged (standard deviations indicated).

[d] No plasmid. [e] pBR325 or pK04 [f] pTS8745 (*bof*::Tn*5*)

Table II.4a. Regulation of *c*1::*lacZ* expression by P1 prophages.

P1 Prophage	Relative β-galactosidase expression from λ*c*1::*lacZ* prophage[a]	Average Bof$^+$/Bof$^-$ ratio[b]
none	100	
P1*bof-1*CmO	91 ± 7	
P1*c*1.100CmO	112 ± 12	
P1*c*$^+$CmO	61 ± 11	0.56 ± 0.12

[a] Strain TSS321 was lysogenized with indicated P1 prophages, and β-galactosidase determined in exponential-phase cultures, as described under "Materials and Methods". Relative expression equals β-galactosidase activity [Miller units (22)] normalized by activity for no P1 prophage (about 4000) times 100. Average relative expression for strain MPh30 was 3. Values correspond to averages and standard deviations for eighteen determinations using three different sets of lysogens of TSS321 (duplicate samples, three assay durations).

[b] Ratios of *c*1::*lacZ* expression in the presence of P1*c*$^+$ to that in the presence of P1*bof-1* were determined for three different sets of lysogens, and averages and standard deviations calculated.

Table II.4b. Regulation of λ*c*1::*lacZ* prophage expression by plasmid-encoded P1 genes.

Source of C1	Source of Bof	Source of "Coi"	Relative β-galactosidase expression[a]	Average ratios Bof$^+$/Bof$^-$[b]	Coi$^+$/Coi^0[c]
none[d]	none	none	(100)		
p*c1*Δr	none[f]	none	14 ± 7		
p*c1*Δr	pTS874	none	6 ± 4	0.43±0.13	
none[e]	pTS874	none	101 ± 9		
pMV1w	none[f]	pMV1w	72 ± 10		6.4±2.0
pMV1w	pTS874	pMV1w	39 ± 9	0.54±0.11	9.7±5.4

[a] Strain TSS321 was transformed with the indicated plasmids and β-galactosidase determined in exponential-phase cultures, as described under "Materials and Methods". Relative expression equals β-galactosidase activity [Miller units(22)] normalized by activity for no P1 prophage (about 4000) times 100. Average relative activity for strain MPh30 without any prophage was 0.4. Data correspond to averages and standard deviations for fourteen determinations (five different sets of transformants; multiple samples and time points).

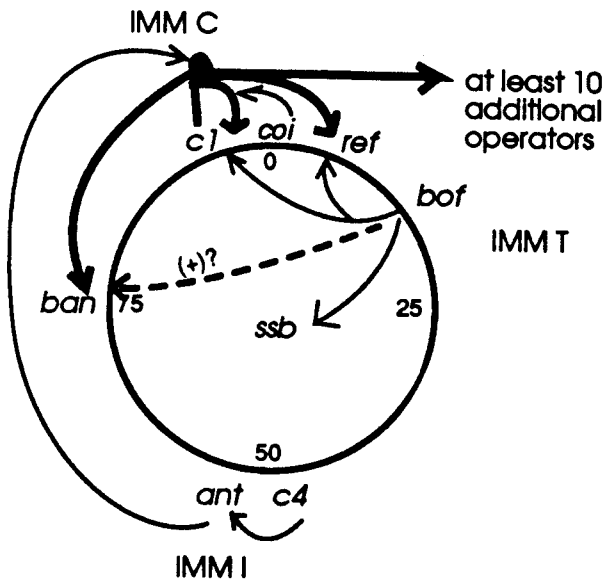
Table II.4b (continued)

[b] Ratios of *c*1::*lacZ* expression in the presence of the same C1 source, but *bof*$^+$ *vs.* *bof*::Tn5, were calculated for each of the fourteen determinations and averaged (standard deviations indicated).

[c] Ratios of *c*1::*lacZ* expression in the presence of pMV1w (Coi$^+$) *vs.* p*c*1Δr (Coi^0), *bof*$^+$ or *bof*::Tn5, were calculated for each of the fourteen determinations and averaged (standard deviations indicated).

[d] No plasmid. [e] pBR325 or pK04 [f] pTS8745 (*bof*::Tn5)

Figure II.1. Regulatory circuits of bacteriophage P1.



Regulatory circuits of bacteriophage P1. Important regulatory elements in the *immC* [*c1 coi*], *immI* [*ant*(*reb*A,B), *c4*] and immT[*bof*(*lxc*, *c6*)] regions, and some regulatory targets, are indicated. Positions relative to P1 map coordinates 0-100 (inside circle) are from Yarmolinsky and Sternberg (43); the location of the putative P1 *ssb* gene (15) is not known. (▬), C1-mediated negative-control interactions inferred from presence of C1 operators, and in some cases [*ban* (19), *ref* (38), *c*1 (this work)] directly demonstrated. (—), negative-control or antagonistic interactions demonstrated by or inferred from existing data. (- -), possible positive-control interaction, based on the Ban-constitutive phenotype of *bof-1* prophages (35).

Figure II.2. Localization of *bof* gene. In each case, constructs containing P1 DNA fragments were tested for Bof activity: the ability, in concert with the *c*1-encoding plasmid p*c*1Δr, to suppress Ref-stimulated *lac*$^-$ x *lac*$^-$ chromosomal recombination in strain BW2060 [assayed as frequency of Lac$^+$ papillae per colony (see Table 2)], and analyzed by restriction and electrophoresis. All plasmids shown, except pTS8745, expressed full Bof activity. Left-to-right corresponds to counter-clock-wise direction on P1 map, from *Bam*HI site at coordinate 12 to*Bam*HI site at coordinate 2.5 (Fig. 2). Construction of plasmids was as follows. pTS871: isolation of 9.5-kb P1:B4 fragment and insertion into unique *Bam*H1 site in *tet* gene of pACYC184; pTS872: isolation of P1:B4 and *Bgl*II restriction; insertion of 4.5-kb *Bam*HI-*Bgl*II fragment into *Bam*H1 site of pACYC. pTS873: ligation of 8-nt *Bam*H1 linker(s) into the unique *Sma*I site of pTS872 (SMB). pTS874: *Bam*H1 restriction of pTS873; purification of larger fragment and re-ligation. pTS8745: infection of C600(pTS874) with λ::Tn*5 b221 Oam Pam* andselection for stable Cm^{r}Nm^r colonies; extraction of plasmids, transformation of strain C600 and selection for chloramphenicol and neomycin resistance; approximate localization of Tn*5* insertion point in 7.9-kb fragment by *Bam*H1 and *Hin*dIII restriction (one, two cuts in Tn5 respectively) mapping. pTS881: *Bam*H1 plus *Eco*RV (site in pACYC184 188-bp from *Bgl*II/*Bam*H1 joint) restriction of pTS874 and isolation of smaller (2.5-kb) DNA fragment; replacement cloning into *Bam*HI and *Hin*cII sites of pAC*lac*19./ pTS882: isolation of

Figure II.2 (continued)

smaller *Hin*dIII-*Bam*H1 fragment from pTS874; *Dra*I restriction and isolation of (926-bp) *Dra*I-*Bam*H1 subfragment; insertion into *Hin*cII and *Bam*HI sites of pAC*lac*19. Abbreviations of restriction-site designations (only those relevant to construction steps indicated): B, *Bam*HI; BG, *Bgl*II; SM, *Sma*I; RV, *Eco*RV; DR, *Dra*I. (=) P1 DNA; (–) pACYC184 DNA; (••••) pAC*lac*19 DNA. Sizes (kb) refers to Bof-encoding fragments indicated by heavy line, and corresponds to DNA sequence information in the case pTS882, and to estimates based on electrophoretic mobility in other cases.

Figure II.2. Localization of the *bof* gene.

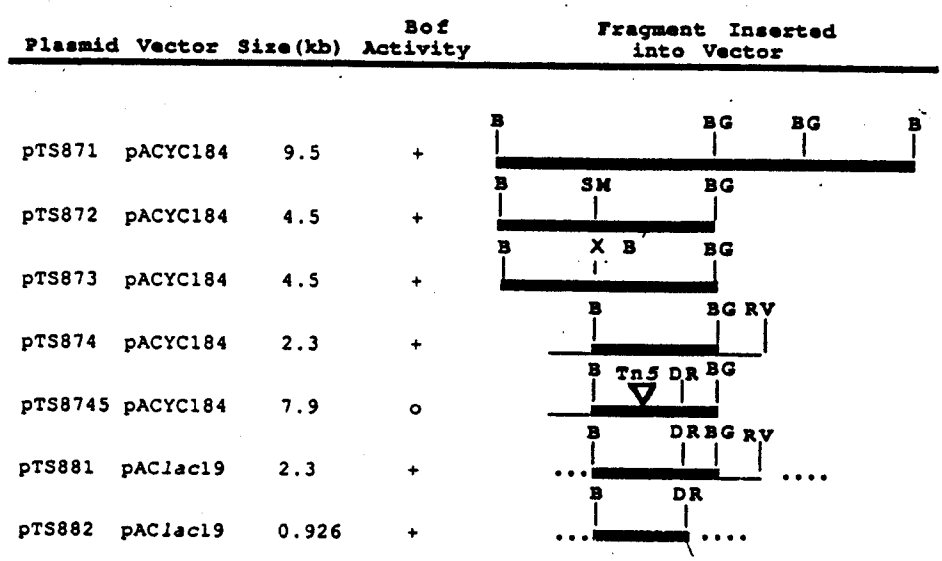
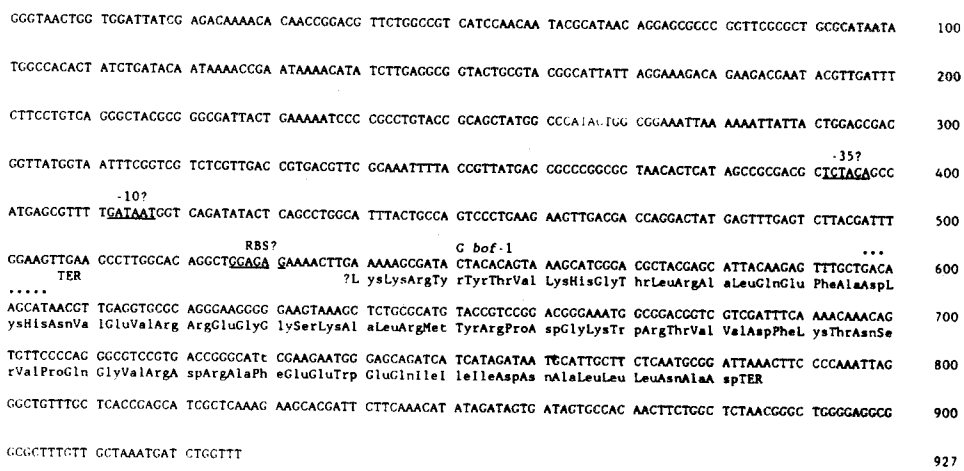


Figure II.3. Sequence of the *bof* region of bacteriophage P1.



The DNA sequence of 931 bp of P1 DNA, beginning at the *Sma*I site near P1 map coordinate 10, was determined as described under "Materials and Methods". We have not directly demonstrated translation from the presumptive TTG initiation codon, nor the activity of the putative translation (29) signal [RBS, ribosome binding site]. The C→G mutation in *bof-1*(Am) and the repeated sequence (• • • •) that flanks the insertion in *bof*::Tn*5* are indicated. This sequence has been submitted to the GenBank/EMBL data base under the accession number M32689.

Figure II.4. Construction of *ref*::*lacZ* and *c*1::*lacZ* fusion phages. *ref*::*lacZ* constructs: *Eco*RI digestion of plasmid pUC19r81 [BamH1-BamH1 fragment from *ref*-encoding plasmid pPR110r81 (17), inserted into pUC19 *Bam*HI-site] and electrophoretic isolation of smaller (*ref*$^+$-encoding) (0.7-kb) fragment; digestion of 0.7-kb fragment with *Sau*3a and electrophoretic isolation of two subfragments, one of which was expected to encode the *ref* promoter-operator region; replacement-insertion of each sub-fragment into pRS415 (31) at the latter's *Bam*HI site, and screening of transformants of MPh30(P1*c*$^+$*r*$^-$*m*$^-$) for Lac$^+$ phenotype so as to identify *ref*::*lacZ* constructs (e.g. pTS301) in which *lacZ* was expressed via *ref* transcription signals; verification of novel joints in plasmid pTS301 by dideoxy-sequencing, using a *lacZ*-sequence primer with sequence *complementary* to pUC19 "universal" primer; preparation of λRS88 plate-growth stock on MPh30(P1*c*$^+$*r*$^-$*m*$^-$)(pTS301) bacteria, to accomplish *bla* x *`bla* and *lacZYA* x *lac`ZYA* recombination between λRS88 and pTS301; lysogenization of MPh30 bacteria with the phage plate stock and spreading on X-Gal plates for identification of MPh30(λ301) lysogens (light blue colonies). *c*1::*lacZ* constructs: *Bal*I restriction of plasmid pMV1w [*Pvu*II-*Bcl*I subfragment from *immC* region of P1*c*$^+$, inserted into expression vector pJF118EH (6) (H. Schuster, personal communication)] and isolation of 74-bp fragment encoding P-Op99ba; insertion into *Sma*I site of pRS415 and identification of Lac$^+$ construct pTS321 by transformation of MPh30(P1*c*$^+$*r*$^-$*m*$^-$) bacteria; verification of structure of P-Op99ba

Figure II.4 (continued)

-*lacZ* novel joint by direct dideoxy sequencing on plasmid pTS321 using "universal" synthetic primer; preparation of λRS88 plate-growth stock on MPh30(P1$c^+r^-m^-$)(pTS321) bacteria to accomplish `*bla*x *bla* and *lac*ZYA x *lac*`ZYA recombination; infection of MPh30 bacteria with phage plate stock and identification of Lac^+ MPh30(λ321) lysogens as blue colonies on X-Gal plates.

Figure II.4. Construction of *ref*:*lacZ* and *c*1::*lacZ* fusion phages.

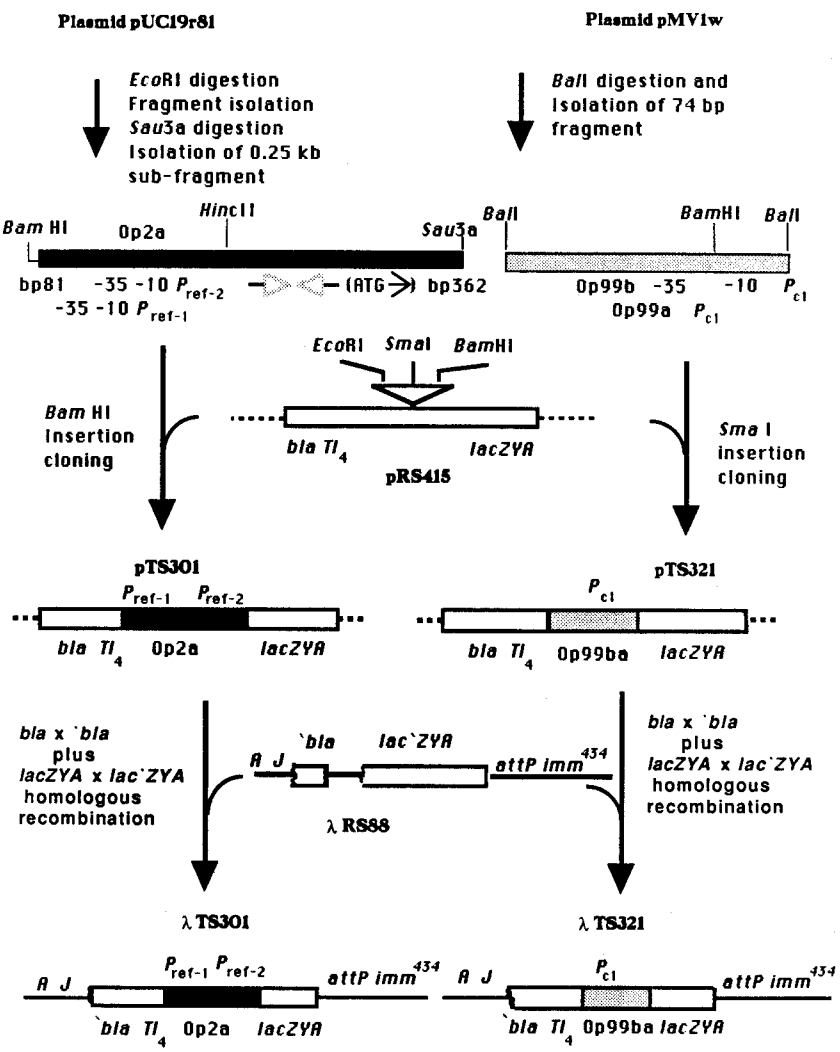
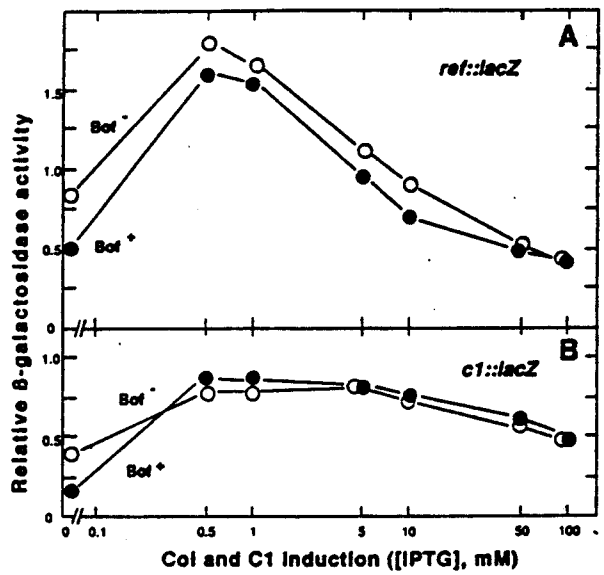


Figure II.5. Effect of induction of "Coi" and additional C1 activity on C1-regulated *ref* and *c*1 expression.



Cultures of TS301 (upper panel) or TS321 (lower panel) bacteria, previously transformed with [pTS874 plus pMV1w (●)] or [pTS8745 plus pMV1w (○)] were grown up in supplemented M9 medium plus indicated concentrations of IPTG and assayed for β-galactosidase activity, as described under "Materials and Methods". Relative activities have been normalized for activity of parallel cultures of TS301 or TS321 bacteria (not transformed with any plasmids), grown without IPTG. The curves shown are representative. In other experiments the zero-IPTG levels were higher or lower, and/or the Bof effects greater or lesser, but the IPTG effects were quite similar.

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CHAPTER III. The *bof* paradox resolved: Bacteriophage P1 Bof protein indirectly derepresses, but directly represses, transcription of the phage *bac-1 ban* gene$^+$

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ABSTRACT

Previous genetic studies have suggested that the Bof protein of bacteriophage P1 can act as both a negative and a positive regulator of phage gene expression: in *bof-1* prophages the *ref* gene and a putative phage *ssb* gene are derepressed, but expression of an operator-semi-constitutive variant (*bac-1*) of the phage *ban* gene is markedly reduced. An explanation of this seemingly paradoxical duality is suggested by recent reports that Bof co-represses genes that are regulated by the phage C1 repressor, including the autoregulated *c*1 gene itself. Here we show by means of operon fusions to *lacZ* that the balance points between Bof-mediated decreases in *c*1 expression and Bof-mediated increases in C1 efficacy are different among various C1-regulated genes. Thus, Bof affects some genes (e.g. *bac-1 ban*) positively, and others (e.g. *ref*) negatively. Even at *bac-1 ban*, where the positive indirect effect of Bof is physiologically dominant, Bof acts as a co-repressor when C1 is supplied from a non-autoregulated source.

INTRODUCTION

Prophage P1 is a unit-copy extrachromosomal plasmid (12). Stable lysogeny requires repression of many widely dispersed P1 lytic-gene operators by the phage C1 repressor. There are at least fourteen C1 operators, numbered according to their approximate map locations, e.g. Op2a, Op21, Op51. The asymmetric operator sequences (consensus ATTGCTCTAATAAATTT) (2,3,5,6) are almost always oriented in the same direction relative to associated promoters, but operator positions relative to "-10" and "-35" sequences vary from promoter to promoter. Prophage P1 encodes an analog of the *E.coli* replication protein DnaB (4,17). The product of the phage *ban* (DnaB analog) gene complements *E.coli dna*B(Ts) mutations at nonpermissive temperatures. P1*bac* (*ban* control) mutations result in constitutive expression of the *ban* gene by P1 prophages (4). Genetic evidence (1) and DNA sequence analysis (14,22) have demonstrated that the P1 *ban* gene is transcribed from a C1-controlled promoter p_{ban}, associated with Op72. This operator is unique for two reasons (10). First, it consists of two partially overlapping operators, Op72a and Op72b (Fig. 1), arranged so as to create a site that is more nearly palindromic than most other C1 operators. Second, Op72a is the only operator thus far found to match every base of the C1-consensus sequence. The *bac-1* mutation is the result of a single base change in Op72a (22).

Second-site mutations which abolished the ability of P1*bac-1* lysogens to

complement *dna*B (Ts) mutants were designated *bof* (Ban on function) (21); *bof* mutations mapped to P1 coordinate 9.5 (21). Because Ban levels appeared to be reduced in the absence of Bof, it was suggested that Bof was a positive effector of *ban* expression (21).

Subsequently however, Bof was found to be a negative regulator of the P1 *ref* gene (19). Ref activity, which enhances homologous recombination in *E. coli*, was found to be very low in wild-type prophages (24), but high in *bof-1* prophages. Furthermore, at least one additional P1 gene appeared to be negatively regulated by Bof: P1 *bof-1* prophages, unlike wild-type prophages, complemented *E.coli ssb*(Ts) mutations at nonpermissive temperatures (24). Thus the paradox: Bof appeared to be a positive effector of *ban* expression but a negative regulator of *ref* (and P1 *ssb*) expression.

A quantitative understanding of the role of Bof in regulation of *ref* and autoregulation of the *c*1 repressor gene has emerged from recent studies with corresponding operon fusions (19). P1 *bof*$^+$ prophages repressed *ref*::*lacZ* expression significantly better than P1 *bof-1* prophages (83% and 44% repression, respectively), and *bof*$^+$ prophages repressed *c*1::*lacZ* expression better than *bof-1* prophages (39% repression versus 9%). Similarly, multicopy *c*1-encoding plasmids *in trans* partially repressed both *ref*::*lacZ* and *c*1::*lacZ*, but addition of a compatible multi-copy *bof* plasmid resulted in more complete repression of both fusion genes. Multicopy *bof* plasmids had very little effect in the absence of C1. These results thus clearly showed Bof to be a negative effector of transcription of the *ref* and (autoregulated)

*c*1 genes. Velleman *et al.*(23) further implicated Bof in *c*1 regulation, by demonstrating that a *bof*-encoding plasmid caused a decrease in the amount of immunoassayable C1 protein expressed from a *c*1-encoding plasmid.

This role of Bof protein in autoregulation of the C1 repressor (19) suggests a resolution of the *bof* paradox. Although C1 repressor levels normally appear insufficient to tightly repress *bac-1 ban* gene expression, the elevated amount of C1 repressor expected in *bof* prophages might cause Ban activity to fall below the level required to complement *dna*B(Ts) mutations. Thus Bof would be an indirect positive effector of *bac-1 ban* expression. This hypothesis assumes that p_{ban} Op72 differs from the *ref* and *c*1 operator-promoters, to the extent that any direct Bof co-repressor effect on *ban* and *bac-1 ban* expression is not physiologically significant. Here we have tested this indirect-positive-effector hypothesis, using *ban*$^+$::*lac*Z and *bac-1 ban*::*lac*Z fusion genes. Bof indeed modulates C1 levels so as to derepress *bac-1 ban*, but not *ban*$^+$ transcription; in the absence of Bof, C1 levels rise to the point that *bac-1 ban* expression is also significantly repressed.

MATERIALS AND METHODS

Bacteria, bacteriophages,and plasmids. These are described in Table 1. Stocks of P1 or λ bacteriophages were prepared by confluent lysis on R-plates or S-plates respectively, and P1 lysogens were prepared by streaking turbid centers of plaques onto LB chloramphenicol plates (30 μg/ml), as described previously (19). Plasmid DNA was extracted by boiling (11) or alkaline-lysis (15) techniques. Plasmids used for DNA sequencing were subsequently purified by isopycnic sedimentation in CsCl/ethidium bromide.

Media and Buffers. TBY-broth, LB-plates, X-gal plates, R-plates, S-plates, TCMB plates, and Z-buffer, were as previously described (19). Antibiotics were used at the following concentrations: ampicillin [Ap], 75μg/ml; neomycin [Nm], 50μg/ml; chloramphenicol [Cm], 30μg/ml.

Recombinant DNA techniques. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs or United States Biochemical Corporation and used as recommended by the suppliers. Gel electrophoresis, electroelution of DNA restriction fragments, ligations and DNA transformations were performed as described (19).

Construction of multicopy and single-copy operon fusions. The promoterless *lacZ* fusion-vector plasmid pRS415 was restricted with *Sma*I endonuclease and ligated to 315-bp *Hinc*II fragments containing p_{ban}Op72 or $p_{bac\text{-}1ban}$ Op72, previously isolated from plasmid pSS2 (*ban*$^+$) or pSS2-1 (*bac-1 ban*). The ligation

products were transformed into Δ*lac* bacteria (MM2838) containing either a P1*c*$^+$ prophage or plasmid pAM2b as a source of C1 repressor. Transformants forming blue colonies on (ampicillin-containing) X-gal plates were used to isolate plasmids. Plasmids were screened, by restriction analysis, for inserted fragments of the appropriate size. The orientation of the inserts in *ban*::*lacZ* (pTS341) and *bac-1 ban*::*lacZ* (pTS351) plasmids was verified by direct double-stranded DNA sequencing (25), using the M13 "universal" sequencing primer and Sequenase (United States Biochemical Corp). Fusion genes were transferred from plasmids to λ phages by homologous recombination, during plate-stock growth as previously described (19), or single-cycle lytic growth of the vector phage λRS88 on MM2838 bacteria harboring pTS341 or pTS351. In the single-cycle procedure, plasmid-containing bacteria were adsorbed to λRS88 at a multiplicity of 0.1 phage per cell at 37°C for 15 minutes and TBY broth added to each mixture. The cultures were incubated for an additional 90 minutes at 37°C and then treated with $CHCl_3$. Lysates were plated with MM2838 bacteria on X-gal plates. Blue plaques were purified three times and used to prepare phage stocks by confluent lysis. Stocks were used to lysogenize MM2838. Light-blue lysogens were repeatedly streaked until stable on X-gal plates. Single and multiple lysogens were distinguished by a Ter-test, as described (20). Single lysogens were used for all subsequent experiments.

Measurement of β-galactosidase. Bacteria lysogenic for fusion-gene-encoding λ prophages were assayed in log-phase liquid cultures as described previously (19). Cells were diluted with 9 volumes of 0.85% NaCl prior to dilution in Z-buffer. Assay

mixtures were warmed to 37°C before addition of orthonitrophenyl-β-D-galactopyranoside (ONPG) substrate, then incubated at 37°C for 5 to 20 minutes, depending on activity levels.

RESULTS

In order to mimic the expression of *ban* genes in unit-copy P1 prophages, we isolated bacteria lysogenic for single λ prophages encoding *ban*$^+$::*lacZ* or *bac-1 ban*::*lacZ* fusion genes. The respective λ phages were obtained by homologous recombination between the vector phage λRS88 (Table 1B) and the corresponding plasmids. The latter had been constructed by inserting 315-bp DNA fragments encoding the promoter-operator regions of P1 wild-type and *bac-1* phages (Fig. 1). Expression of λ-prophage-encoded fusion genes was measured in the presence of wild-type or mutant P1 prophages, or in the presence of multi-copy plasmids supplying C1 or Bof activity, or both of the latter.

In the presence of a P1*c*$^+$ prophage, optimal transcription of the P1 *bac-1 ban* gene requires Bof activity. Either wild-type or P1*bof-1* prophages *in trans* completely blocked expression of β-galactosidase from *ban*$^+$::*lacZ* fusion genes (Table 2, column 2). This suggests that unlike the *c*1 and *ref* genes, the *ban* gene is repressed very tightly by C1 alone, so that Bof function has no physiologically significant direct role at p_{ban} Op72. As expected, the *bac-1* mutation rendered *ban* gene expression relatively insensitive to C1 repression (Table 2, column 3). Evidently the level of *bac-1 ban* expression in the presence of a wild-type P1 prophage, seen here to be 34% of derepressed levels, suffices for complementation of *dna*B(Ts) mutations (4); under the same conditions *ban*$^+$::*lacZ* expression was negligible (Table

mutations (4); under the same conditions *ban*$^+$::*lacZ* expression was negligible (Table 2, column 2). In bacteria lysogenic for P1*bof-1*, the absence of any Bof contribution to *c*1 autorepression would be expected to elevate C1 levels. In agreement with this prediction, *bac-1 ban*::*lacZ* expression was reduced to 10% of maximal in such lysogens (Table 2, line 3). Evidently this level of expression corresponds to too little Ban protein for complementation of *dna*B(Ts) mutations.

When *ban*$^+$::*lacZ* and *bac-1 ban*::*lacZ* expression were compared with one another in parallel experiments, the fully derepressed activity of the mutant gene was only about two-thirds that of the wild-type *ban* gene (Table 3, line 1). Thus the proposal that the *bac-1* mutation [a C→A transversion at position 5 of Op72 (Fig. 1)] should increase absolute promoter strength, by making the "-35" region of the promoter more nearly resemble the *E.coli* consensus sequence (10,14), was not supported.

The levels of C1 repressor expressed by multi-copy plasmids suffice to reduce both *ban*$^+$ and *bac-1 ban* expression from λ prophages by 96 to 98% (Table 3, lines 2 and 4). The difference between negligible expression of *ban*$^+$::*lacZ* in the presence of P1 prophages (Table 2) and low but measurable expression in the presence of plasmid-encoded C1 (and Bof) (Table 3) may reflect a minor regulatory role by some additional P1 element. In the absence of C1, Bof alone appeared to repress both *ban*$^+$ and *bac-1 ban* expression (Table 3, line 3), but the data are not precise enough for this small effect to be considered significant. It is clear however, that Bof is not a direct positive effector of *ban* expression, either alone, or in the

in the presence of a multi-copy Bof source, are higher than prophage-maintained C1 levels [compare *bac-1 ban* expression shown in Table 2, line 2, with that shown in Table 3, lines 2 and 4].

Bof is a direct negative regulator of expression of *bac-1 ban::lacZ* expression. In order to determine whether Bof possesses any intrinsic ability to act as a corepressor at the wild-type *ban*$^+$ or mutant *bac-1 ban* promoter-operators, as it does at the *ref* and *c*1 promoter-operators, it was necessary to eliminate the effect of Bof on *c*1 autorepression. This was accomplished by placing the *c*1 structural gene under the control of the p_{tac} promoter. Transcription of the latter is blocked by *lac* repressor, but is inducible by IPTG. Even in the absence of IPTG, the multi-copy p_{tac}-*c*1 plasmid completely (>99%) blocked *ban*$^+$::*lacZ* expression, in the presence or absence of Bof (data not shown). However, *bac-1 ban::lacZ* expression was significant in the absence of IPTG; it was further repressed by IPTG induction of additional C1 synthesis (Table 4). At every level of C1 synthesis, the presence of Bof activity caused a further decrease (3 to 7-fold) in *bac-1 ban::lacZ* expression (Table 4). Thus even though the strong negative effect of Bof on *c*1 expression causes it to be an indirect positive effector of *bac-1 ban* expression *in vivo*, Bof is intrinsically a direct negative effector of *bac-1 ban* transcription.

DISCUSSION

The genomic organization of bacteriophage P1 is quite different from that of λ, the temperate phage paradigm. In contrast to two divergent master promoters with associated operators, seen in lambdoid phages, the P1 operators are widely separated; the phage functions that they control are numerous and varied. P1 operator-promoters appear to differ widely among themselves with respect to their affinity for the C1 repressor, and in their dependence on the Bof co-repressor for maximum down-regulation of transcription. Maximum repression by C1 of its own synthesis requires Bof; this provides a mechanism for further fine tuning of regulatory circuits. Since prophage P1 is a plasmid, rather than an integral part of the bacterial chromosome, as the lambdoid prophages are, it seems likely that expression of some P1 genes must be delicately adjusted to levels intermediate between those corresponding to wide-open lytic-growth levels and to total shutdown. Recent studies have provided some clues as to how this fine tuning might be accomplished.

The *ban* wild-type and mutant (*bac-1*) promoter-operators investigated here increase to four the number of P1 transcription-regulation elements studied quantitatively by operon-fusion techniques. The *ban* and *bac-1 ban* genes provide a good example of the possibilities for subtle adjustment of P1 gene expression.

The *ban*$^+$ promoter-operator, p_{ban} Op72, differs from those of other genes

studied by operon fusion techniques (*bac-1 ban, ref, c*1) in that *ban*$^+$ expression is completely repressed by low levels of C1 alone; thus Bof, a co-repressor of several other C1-regulated genes, is irrelevant to regulation of *ban*. Even the presumably low levels of C1 expressed by *lacI*^{q} p_{tac}-*c*1 plasmids, in the absence of IPTG, repress *ban*$^+$::*lacZ* expression by more than 99%, despite the high intrinsic strength of p_{ban}. We do not know whether or not Bof would act as a co-repressor at p_{ban} Op72 at even lower *in vivo* C1 concentrations, but recent *in vitro* work suggests that this might be the case. Velleman *et al.* (23) showed that purified Bof protein slightly enhanced the ability of low levels of purified C1 protein to retard a *ban*$^+$ promoter-operator DNA fragment during electrophoresis; even lower C1 levels might well have revealed a greater Bof effect.

What might account for the high C1 affinity of Op72? Two aspects of the *ban* promoter-operator architecture may be significant. Its two tandem overlapping C1 binding sites distinguish it from single-C1-site promoter-operators, such as p_{ref} Op2a; the two p_{ban} Op72 sites are designated Op72a and Op72b. Although some other P1 promoter-operators [e.g. Op99a(b)] (23) display this overlapping-tandem C1-site motif, Op72a is the only P1 operator to provide a perfect match to the consensus C1 site (the match is 15 of 17 for Op72b). In contrast, the matches are 15 of 17 for Op99a and only 11 of 17 for the quasi-site that overlaps Op99a. These differences may be sufficient to account for the observations that even very low concentrations of C1 suffice for full repression at Op72 without co-repression by Bof, whereas full repression at Op99a requires Bof (19), even when C1

concentrations are very high (data not shown).

The *bac-1* mutation, a single base change in Op72a, reduces the intrinsic promoter strength of $p_{\text{bac-1 ban}}$ to 70% that of p_{ban}. However, the mutation simultaneously reduces the affinity of Op72 for C1 to the point that, even in the presence of a P1 prophage, *bac-1 ban::lacZ* expression is 0.34 of the derepressed level (as compared to <0.01 for *ban*$^+$::*lacZ*). Thus in *bac-1* lysogens the concentration of *ban* transcripts would be expected to be at 24% of the concentration corresponding to a fully derepressed *ban*$^+$ gene (0.34 x 70%). This would account for the ability of the mutant prophages to complement *E. coli dna*B(Ts) mutations. It seems clear that C1 still binds to the mutant operator, however; *bac-1 ban::lacZ* transcription is repressed 67% and 90% by (single-copy) wild-type and *bof-1* prophages respectively, is 95% repressed by a multi-copy *c*1-encoding plasmid, and is over 98% repressed by a C1-overproducing plasmid. The sensitivity of *bac-1 ban* expression to C1 levels fortuitously set the stage for discovery of the *bof* gene (21). The *bof-1* mutation causes steady-state C1 levels to increase to the point that *bac-1 ban::lacZ* expression is reduced to 0.10 of the derepressed value. This corresponds to *ban* transcripts in *bac-1 bof-1* lysogens at only 7% of derepressed *ban*$^+$ levels, evidently not enough to complement *E. coli dnaB*(Ts) mutations.

Thus the window between *dna*B(Ts) complementation at 24% *ban* expression, and lack of complementation at 7% *ban* expression, made it possible to isolate the *bof-1* mutation on the basis of its apparent Ban$^-$ phenotype. Although Bof was originally proposed to be a positive effector of *ban* expression, the Ban$^-$ phenotype

of *bof* mutants seems entirely a result of the role of Bof in C1 autoregulation. Bof is not a positive effector of either *ban::lacZ* or *bac-1 ban::lacZ* expression in the presence or absence of C1. In the presence of non-autoregulated C1, Bof is clearly seen to be a direct negative regulator of *bac-1 ban::lac* expression, as it is of *ref* and *c*1 expression. Neither this direct Bof effect at *bac-1 ban*, nor a possible direct Bof effect at *ban*$^{+}$, seem physiologically important, being masked by the role of Bof in C1 autoregulation.

The results presented here show the original *bof* acronym Ban-on-function to be a misnomer. Since P1 *lxc* mutations, which confer constitutive expression of *ref* and a putative *ssb* gene, appear to be allelic with *bof* (24), it has been suggested that *lxc* be reinterpreted as lowers expression of *c1* (23). [The *lxc* designation was originally by way of analogy with *E. coli lexC* mutations, on the basis of the ability of P1 *lxc* prophages to suppress *E. coli ssb*(Ts) mutations (13).] However, Bof down-regulates expression of genes other than *c*1 (19), so a more general acronym seems appropriate. We suggest that the designation *bof* be retained, but reinterpreted as bolsters C-one function. This would be consistent with the co-repressor activity of Bof seen at most P1-promoter-operators tested thus far, and with the apparent absence of significant Bof function in the absence of the C1 repressor.

The dozen or two P1 genes controlled by the phage primary (C1) repressor seem capable of being expressed simultaneously at quite different levels. The Bof protein modulates C1-mediated repression by acting as a co-repressor, thus exerting a direct negative effect on expression of some genes; Bof simultaneously

downregulates C1 levels, thus exerting an indirect positive effect on other genes. It will be of interest to elucidate the role of this versatile fine-tuning element in regulation of other C1-controlled genes.

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Table III.1a. Bacterial strains.

Strain	Genotype	Source or Reference
MM2838	Δ(*lac-proAB*)*thi supE*	(7)
TSS341	MM2838 (λ341)	This work
TSS351	MM2838 (λ351)	This work

Table III.1b. Bacteriophages.

Strain	Genotype/remarks	Source/Reference
λRS88	`*bla* `*lacZ lacY lacA imm*^{434} *ind-*	R.W. Simons(20)
λ341	`*bla Tl*$_4$ *ban::lacZ lacY lacA imm*^{434} *ind*$^-$	This work[a]
λ351	`*bla Tl*$_4$ *bac-1ban::lacZ lacY lacA imm*^{434} *ind*$^-$	This work[a]
P1Cm*Or*$^-$*m*$^-$	lacks P1 restriction, modification	M. Gottesman
P1 Cm*Obof*-1	Derepressed for *ref*, and for putative *ssb*	N. Sternberg

[a] Constructed by homologous recombination between λRS88 and plasmid TSS341 or TSS351, as described under "Materials and Methods"

Table III.1c. Plasmids.

Plasmid	Description/remarks	Source/reference
pRS415	Encodes *bla* Tl_4 (no promoter) `*lacZYA*.	(20)
pSS2	Phage P1 *Eco*RI-*Sph*I fragment, encoding p_{ban}/Op72, inserted into pBR325.	(9)
pSS2-1	as pSS2, but $p_{bac-1ban}$/Op72.	(9)
pTS341	315-bp *Hinc*II fragment encoding p_{ban}/Op72 from pSS2 inserted into unique *Sma*I site of pRS415[a].	This work
pTS351	315-bp *Hinc*II fragment encoding $p_{bac-1ban}$/Op72 from pSS2-1 inserted into unique *Sma*I site of pRS415[a].	This work
pAM2b	*Pvu*II-*Bcl*I subfragment of P1 *Eco*RI fragment 7 inserted into *Dra*I restricted pKT101 (see reference 3 for details). Encodes Op99e Op99d p_{coi} Op99c Op99a p_{c1} *c*1 (Nm^r).	(3)
pTS874	Phage *Bam*H1-*Bgl*II fragment inserted into *Bam*HIrestricted pACYC184. Encodes *bof*$^+$. Compatible with ColE1-derived plasmids (Cm^r).	(19)
pTS8745	as pTS874, but *bof*::Tn5 (Nm^r).	(19)
p*c*1Δr	Encodes Op99a p_{c1} *c*1 of phage P1 in plasmid pKO-4(Ap^r).	N.Sternberg

Table III.1c (continued)

pKO-4	*gal*K promoter-cloning vector (Ap^r).	(16)
pTS500	*Bam*HI-*Sal*I fragment containing the *c*1 gene from fragment from pMV1w (4) inserted into corresponding sites of pJF119EH (7). Encodes $lacI^q$ p_{tac} *c*1 (Ap^r).	This work

[a]Construction described under "Materials and Methods"

Table III.2. Regulation of *ban*$^+$::*lacZ* and *bac-1 ban*::*lacZ* expression by P1 prophages.

	Relative β-galactosidase activity	
P1 prophage	λ*ban*$^+$::*lacZ* fusion	λ*bac-1 ban*::*lacZ* fusion
none	(100)	(100)
P1*c*$^+$	<1	34 ± 9
P1c$^+$*bof-1*	<1	10 ± 3

Bacteria lysogenic for single λ*ban*$^+$::*lacZ* (TSS341) and λ*bac-1 ban*::*lacZ* (TSS351) prophages were lysogenized with P1*c*$^+$ or P1*c*$^+$*bof-1* phages. Lysogens were grown at 37°C to exponential phase in TBY broth supplemented with chloramphenicol. β-galactosidase activity was measured as described (18). Experiments with *ban*$^+$ or *bac-1 ban* fusions were performed separately and are presented together for clarity. The *ban*$^+$ data represent the averages of four independent experiments (a total of 26 determinations). Relative expression equals β-galactosidase activity divided by activity for TSS341 with no prophage (11.1 ± 1.8 x 10^3 Miller Units) multiplied by 100. The *bac-1 ban* data represent the average and standard deviations for five independent experiments (a total of 28 determinations). Relative expression equals β-galactosidase activity divided by activity of TSS351 in the absence of P1 prophages (8.6 ± 1.7 x10^3 Miller Units) multiplied by 100.

Table III.3. Regulation of expression of *ban*$^+$::*lacZ* and *bac-1 ban*::*lacZ* fusion genes by plasmid-encoded Bof and C1 activities

		Relative β-galactosidase activity	
C1 source	Bof source	λ*ban*$^+$::*lacZ*	λ*bac-1 ban*::*lacZ*
none	none	(100)	(100)[a]
pc1Δr	none[b]	2.4 ± 2.4	4.8 ± 2.1
none[c]	pTS874	77 ± 22	82 ± 16
pc1Δr	pTS874	3.5 ± 2.4	3.8 ± 3.5

Bacteria lysogenic for single λ*ban*$^+$::*lacZ* (TSS341) or λ*bac-1 ban*::*lacZ* (TSS351) prophages were transformed with the indicated plasmids. Transformants were grown to exponential phase in TBY broth supplemented with Ap, Cm and Km (for selection of pTS8745) at 37°C. β-galactosidase activity was measured as described (18). The data represent the averages and standard deviations for six independent experiments (a total of 32 determinations). Experiments with both fusions were performed simultaneously: relative expression equals β-galactosidase activity for indicated constructs divided by activity for TSS341 or TSS351 containing no plasmids multiplied by 100.

[a]Absolute expression of β-galactosidase from λ351 (*bac-1 ban*::*lacZ*) prophages was 0.70 ± 0.13 of expression from λ341 (*ban*$^+$::*lacZ*) prophages, in the absence of C1 and Bof sources.

[b]plasmid pTS874

Table III.3 (continued)

[c]plasmid pKO-4

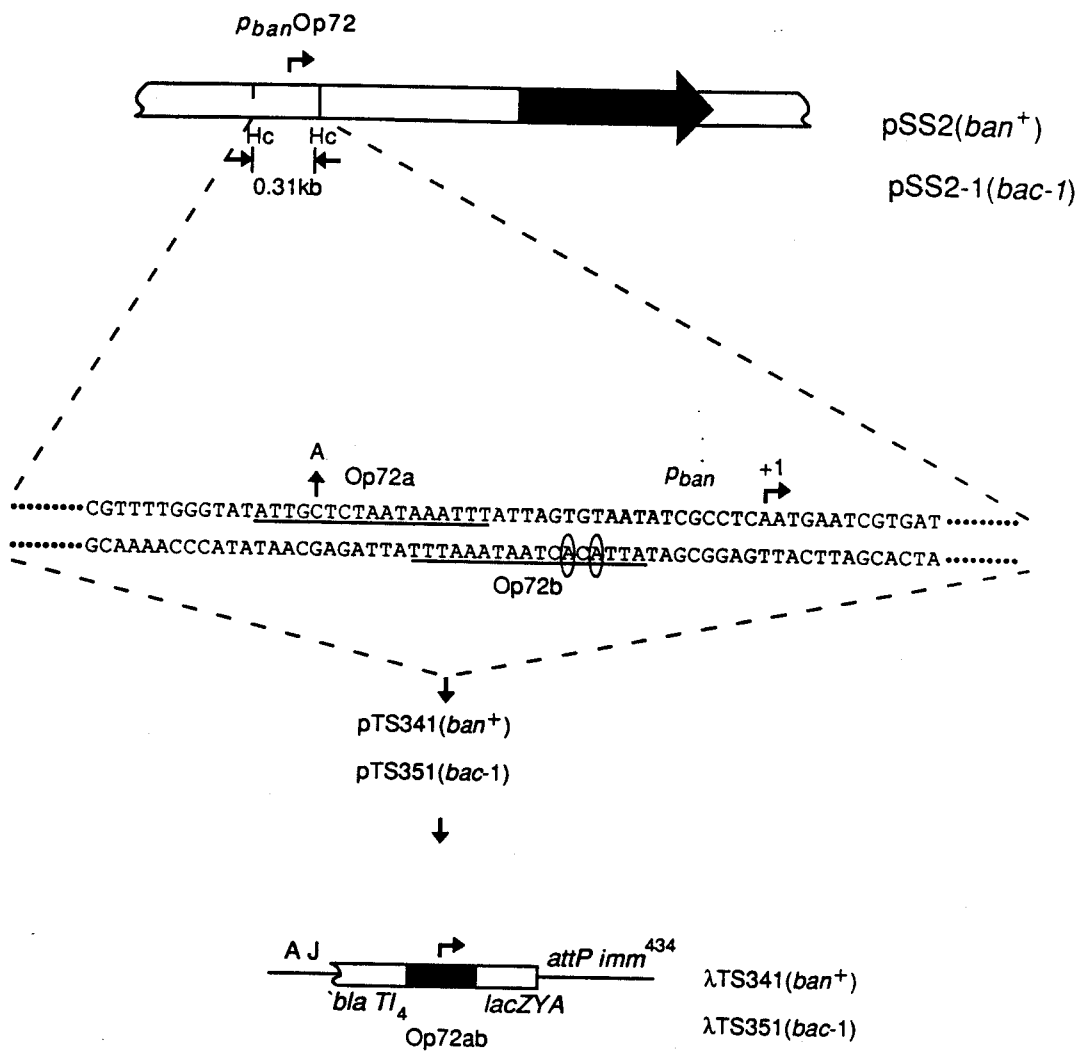
Table III.4 Effect of Bof on *bac-1 ban::lacZ* expression in the presence of non-autoregulated C1 synthesis

	Relative B-galactosidase activity		
[IPTG]	pTS8745(*bof*::Tn5)	pTS874(*bof*$^+$)	$\frac{bof^+}{bof^-}$ Ratio
	present	present	
0	11 ± 1.2	1.7 ± 0.43	0.15
10μM	3.9 ± 0.5	1.0 ± 0.26	0.26
30μM	2.5 ± 0.43	0.84 ± 0.14	0.34
100μM	1.7 ± 0.24	0.59 ± 0.22	0.35

Bacteria lysogenic for single λ*bac-1ban::lacZ* prophages (TSS351) and harboring a non-autoregulated (p_{tac}-controlled) source of *c*1(pTS500) were transformed with one of the two plasmids indicated. Single transformants were resuspended in 0.85% NaCl. Aliquots were transferred to TBY broth with Ap, Cm, and Nm (for selection of pTS8745) containing 0, 10μM, 30μM, or 100μM IPTG. Cultures were grown to mid-log phase at 37°C and assayed for β-galactosidase activity as described (19). Data represent averages and standard deviations for quadruplicate determinations. Relative expression equals β-galactosidase activity divided by the activity for bacteria lysogenic for λ351, but containing no plasmids, multiplied by 100. The data presented are representative of similar experiments performed on different days. The effect of Bof observed in other experiments was slightly greater or less than that shown here.

Figure III.1. Figure III.1 depicts the region of P1 DNA analyzed by operon-fusion techniques. The 0.31 kb *Hinc*II (Hc) fragment containing the wild-type *ban*$^+$ (from pSS2) or mutant *bac-1 ban* (from pSS2-1) promoter-operator region (indicated by the small rightward arrow above the fragment) was ligated to *Sma*I-linearized pRS415, yielding plasmids pTS341 and pTS351, as described under "Materials and Methods". The heavy rightward arrow represents the *ban* structural gene. The sequence of the p_{ban}Op72 promoter-operator region is shown (10,22). The location and nature (22) of the single base transversion which results in the *bac-1* phenotype is indicated by an upward arrow. The underlined sequences illustrate the C1 repressor-binding sites Op72a and Op72b; circled nucleotides represent deviations from the derived C1 consensus sequence (2,3,5,6). The rightward small arrow designated +1 represents the nucleotide at which transcription initiates *in vitro* (10). A representative example of a recombinant lambda phage containing the P1 p_{ban}Op72 operon fusion is shown at the bottom.

Figure III.1. P1 DNA encoding p_{ban} and $p_{bac-1\ ban}$ analyzed by operon fusion.



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CHAPTER IV. The enhancement of C1 repressor binding by Bof protein

INTRODUCTION

The bacteriophage P1 repressor acts at many widely separated operators to prevent significant transcription of genes involved in lytic growth (2)(4)(7)(22). The presence of another P1 protein, Bof, is necessary for complete repression of certain P1 genes under C1 control (13)(26). P1*bof*$^-$ prophages are pleiotrophic (21). The Bof protein apparently acts exclusively as a negative regulator, despite certain seemingly paradoxical phenotypes of P1*bof*$^-$ mutants. Previous studies of the regulation of the *ref* and *c*1 genes suggested that Bof itself has little, if any, capacity to regulate transcription. However, Bof enhances the repression of the corresponding promoters in the presence of C1, suggesting an interaction between C1 and Bof proteins (15).

In contrast to Bof, which strengthens C1-mediated repression, the Coi protein appears to antagonize repressor function (3)(12). Expression of Coi is constitutive in P1 *vir*C prophages, resulting in a virulent phenotype (17). The *coi* gene is located directly upstream of *c*1 (3)(12). Studies of the regulation of *ref*::*lacZ* and *c*1::*lacZ* expression revealed multicopy plasmids that encoded the *coi* gene as well as *c*1 were five-fold less effective in repression than Coi$^-$-*c*1 plasmids (15). This suggested that Coi interfered with the action of C1 repressor (15), in agreement with previous speculations (18). Despite antagonism of C1-mediated repression by Coi, Bof remained capable of enhancing residual C1 repression *in vivo*.

Thus, the C1 repressor appears to interact with at least two auxiliary proteins, Bof and Coi. The former appears to strengthen the repressor action of C1, and the latter to weaken it. I have analyzed the ability of Bof and Coi proteins to alter binding of C1 repressor to the operators associated with the P1 *ref* gene *in vitro*, using purified C1 and Bof, and partially purified Coi proteins. The results suggest that Bof enhances C1 repressor activity stoichiometrically rather than catalytically, via a C1-Bof-operator DNA complex.

MATERIALS AND METHODS

1. Bacteria and plasmids Bacteria strains TSS301 and TSS321 are strain MM2838 (Δ*lac)* lysogenic for lambda prophages containing, respectively, *ref*::*lacZ* and *c*1::*lacZ* operon fusions (15). Plasmid DNA was routinely propagated in MM2838 (8) or JM101 (28). Plasmids pBS+ (Stratagene), pJF199EH (9), pTS500 (Chapter II), pUC19r81 (27), and pTS8745 (15) have been described previously. The construction of plasmid pTS600 is described below and is depicted in Figure IV.1.

2. Media and Buffers. Denaturing Buffer: 0.25 M NaOH, 0.25 mM EDTA; TE: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA; RI/B buffer: 20 mM Tris-HCl (pH 7.6), 0.1 mM EDTA, 1 mM DTT, 50 mM NaCl, BSA 100 μg/ml, glycerol 10% v/v; Buffer A: RI/B buffer with 250 μg/ml sonicated salmon sperm DNA. Buffer W: 25 mM Tris-HCl (pH 8.5), 1 mM EDTA, 10% glycerol, 1 mM DTT; Buffer X: 25 mM Tris-HCl (pH 8.5), 50 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT; Buffer Y: 25 mM Tris-HCl (pH 8.5), 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT; Buffer Z: 25 mM Tris-HCl (pH 8.5), 250 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT; RNA polymerase buffer: 70 mM Tris-HCl (pH 8.3), 105 mM KCl, 15 mM dithiothreitol, 4 mM $MgCl_2$.

3. Measurement of B-galactosidase. Bacteria lysogenic for λphages containing gene fusions were grown to log phase in liquid cultures, and lysed as described previously (15). Cells were diluted in Z-buffer and warmed to 37°C before addition of

orthnitrophenyl-B-D-galactopyranoside (ONPG) substrate, then incubated at 37°C for 5 to 20 minutes.

4. Purified proteins. Bof and C1 were purified by M. Velleman as described (6) (23). Coi protein was partially purified as described (12) by T. Heinzel.

5. Plasmid denaturation Plasmid DNA to be used for sequencing (2.5 μg) or DNase I protection experiments (15 μg) was denatured in 4 volumes of Denaturing Buffer for 10 minutes at 30°C. After addition of 0.1 volume 2 M Ammonium acetate (pH 4.8), denatured DNA was precipitated by the addition of 2.5 volumes of 95% ethanol and incubation at -20°C for 30 minutes. DNA was recovered by centrifugation in a microfuge for 30 minutes at 4°C. After disposal of the supernatant, the pellet was rinsed with 1.0 ml 70% ethanol (-20°C) and cetrifuged an additional 10 minutes at 4° C. The plasmid pellet was then dried for 5 minutes in a Speed-Vac centrifuge.

6. Preparation of duplex DNA for DNAse I digestion. To generate duplex DNA for use as substrates for Dnase I protection, an end-labeled oligonucleotide, annealed to denatured plasmid DNA, was extended using Klenow fragment. Plasmid DNA pUC19r81 or pOp2b was denatured (as described in 5), and incubated for 45 minutes at 37°C in Klenow polymerase buffer with one of the following ^{32}P end-labeled primers: ["universal", 5'd(GTAAAACGACGGCCAGT); "reverse", 5'd(AACAGCTATGACCATG); or TS5, 5'd(CCACTTGCGGCACTG)] as indicated in figure legends. All four dNTPs were added, to final concentrations of 30 μM, and extension begun by addition of 15 units of DNA polymerase I Klenow fragment (New England Biolabs). After incubation at 30°C for 30 minutes, additional dNTPs were

added to a final concentration of 150 μM, and the reaction allowed to continue at 30°C for 30 minutes. Reaction mixtures were extracted with equal volumes of TE-equilibrated phenol, and the phenol back-extracted with 1.5 volumes R1 buffer. Aqueous phases from both extractions were combined and extracted twice with 3 volumes of diethylether. Residual ether was allowed to evaporate and RI buffer added to a final volume of 0.63 ml. Aliquots of 20 μl (500 ng) were frozen at -20°C until further use.

7. DNase I Protection DNA substrates were prepared as described above. BSA was added to each 20 μl aliquot to a final concentration of 100 μg/ml. Purified Bof and C1 proteins were added, alone or together, at concentrations indicated in figure legends (typical reaction mixtures contained 0-240 ng of C1 repressor and 0, 50, or 750 ng of Bof). The components were gently mixed and incubated for 15 minutes at 30° C. The reactions were cooled on wet ice and DNaseI added to a final concentration of 50 ng/ml. Reaction mixtures were transferred to a 30°C waterbath for 5 minutes, then rapidly returned to wet ice. In succession, ammonium acetate and tRNA were added to final concentrations of 2 M and 100 μg/ml, respectively. DNA was precipitated by addition of three volumes of cold (-20°C) 95% ethanol, followed by incubation for 30 minutes at -70°C. The samples were recovered by centrifugation, washed with 1.0 ml 95% ethanol (-20°C) and dried. Portions of the samples were subjected to electrophoresis on 6% sequencing gels, dried, and subjected to autoradiography.

8. Determination of *ref* gene transcriptional initiation sites. Extension of primers

annealed to transcripts generated *in vitro* was performed essentially as described (12). Briefly, plasmid pUC19r81 (2 μg), containing the *ref* gene and promoter region, was transcribed *in vitro* with *E. coli* RNA polymerase (4 units), in RNA polymerase buffer containing 0.2 mM rNTPs and RNase inhibitor, at 37°C for 15 minutes. Transcription experiments employing C1 repressor only (342 ng), Bof only (160 ng), or both proteins was performed in parallel. 5'-^{32}P-labeled oligonucleotide TSS5 was annealed to the RNA products, and dNTPs added to 0.1 mM. Primers were extended with reverse transcriptase for 20 minutes at 37°C. Products from these reactions were electrophoresed in parallel with products of dideoxy sequencing reactions performed on the same plasmid substrate using the TSS5 primer.

9. DNA sequencing. DNA sequencing was performed using the dideoxy chain termination method of Sanger (14). Denatured plasmid substrates were prepared as described in **5.**

10. Gel Electrophoresis of Proteins. Analysis of proteins was performed on discontinuous-SDS-polyacrylamide gels as described (1) at 200 volts.

11. Recombinant DNA Techniques. Gel electrophoresis, DNA ligation reactions, and DNA transformations were performed as described (15). Electroelution of DNA fragments was performed as described (15) or by using a Biotrap (Schleicher and Schuell) apparatus. Restriction enzymes, T4 DNA ligase, T4 DNA kinase, MMTV-reverse transcriptase, RNA polymerase, DNaseI, were used as recommended by the suppliers.

12. Construction of recombinant plasmids. Construction of plasmid pOp2ba. Plasmid

pUC19r81 was restricted with *BamH*I and *Eco*RI and the 158-bp fragment containing Op2b isolated. This fragment was ligated to pUC19 restricted with *BamH*I and *Eco*RI. Construction of plasmid pTS600. The 472-bp *Hinc*II-*Pst*I fragment from pTS882 (15) encoding the *bof* gene was ligated to plasmid pBS+ (Stratgene) which had been treated with *Sma*I and *Pst*I. In the product plasmid pBS*bof*, a portion of the *bof* gene promoter was removed and an *Eco*RI site placed proximal to the *Hinc*II/*Sma*I junction. The *bof*-encoding *Eco*RI-*Pst*I (490bp) fragment from pBS*bof* was isolated and ligated to pJF119EH DNA which had been treated with *Eco*RI and *Pst*I. The resulting plasmid, pTS600, which has the genetic organization *bla* $lacI^q$ p_{tac} *c*1, is shown in Fig. IV.1.

13. Interaction of purified proteins with operator-encoding DNA fragments. The retardation of DNA-operator fragments by proteins following electrophoresis was performed with isolated DNA fragments and purified Bof and C1 proteins, plus in some experiments, partially purified Coi containing extract. The 136-bp *Nru*I-*Nde*I restriction fragment encoding Op2a was isolated from pUC19r81. The 158-bp *Eco*RI-*Bam*HI restriction fragment containing Op2b was isolated from pOp2b. In a typical reaction volume of 20 µl, 50-75 ng of operator fragment were incubated with combinations of C1, Bof and Coi extract (as indicated in the appropriate figure or figure legend). The operator fragment and protein(s) were mixed and incubated for 15 minutes at 37°C in RI-B buffer. Three microliters of 30% ficoll were gently added to each sample. The reaction products were separated by electrophoresis on 5% polyacrylamide gels (previously warmed by electrophoresis at 125 volts for 15

minutes) at 125 volts. The DNA bands were visualized by ethidium bromide and photographed.

14. Interaction of proteins made *in vitro* with operator-encoding DNA fragments. The retardation of DNA-operator fragments by proteins following electrophoresis was performed using C1 and Bof proteins synthesized *in vitro* (described in **15**). The DNA operator fragments were labeled by "filling in" ends generated by restriction enzyme digestion using ^{32}P-dTTP and the Klenow fragment of DNA polymerase I followed by isolation by electroelution. The 136-bp *Nru*I-*Nde*I restriction fragment encoding Op2a was isolated from plasmid pUC19r81. The 329-bp *Eco*RI and *BamH*I fragment containing Op72a(b) was isolated from pTS341. Incubation of C1 and Bof translation products (described below) with DNA fragments and separation by electrophoresis were performed as described in **13.** The gels were transferred to 3 MM Whatman paper, dried under vacuum, and autoradiography performed.

15. Synthesis of C1 and Bof proteins *in vitro*. DNA from plasmid pTS500 () and pTS600 were transcribed and translated using a DNA-directed prokaryotic translation kit from Amersham. Bof protein used for DNA binding experiments was synthesized in the presence of [35-S] methionine. Unlabeled methionine (5mM) was added instead during synthesis of C1 protein to be used in DNA-binding experiments. The transcription-translation reactions were performed as suggested by the supplier. Briefly, plasmid DNA (3μg) and components of the kit were mixed and incubated for 1 hour at 37°C, followed by the addition of a cold methionine chase and further incubation for 5 minutes. Five microliters of 10X Buffer A was added and sterile

glycerol to a final concentration of 27%. Approximately ten aliquots of 5 µl each were frozen at -70° C.

Bof protein used for DNA binding experiments was separated from other radiolabeled proteins that might have been synthesized during transcription-translation, by ion-exchange chromotography, and concentrated by centrifugation in a Centricon column, as described below. Four pTS600-directed trancription-translation mixtures (approximately 200 µl) were pooled and diluted 1:2 in buffer W. An 0.5 ml DEAE column was prepared using a 1.0 ml tuberculin syringe. The column was washed with 3 ml buffer W (no added NaCl). The Bof-containing sample was added and allowed to enter the column. This was followed by 2 successive 0.6 ml washes with buffer W. A 3.0 ml salt gradient (50-250 mM NaCl) was applied to the column and a total of 56 fractions (2 drops, 75µl per fraction) collected. Aliquots from every third or fourth fraction were analyzed by polyacrylamide gel electrophoresis and autoradiography. Fractions 23-36 (Bof-A) and 37-45 (Bof-B) were pooled. The Bof-A pool (0.96 ml) was concentrated using a Centricon 10 microconcentrator (Amicon). The column was first washed with 0.5 ml buffer Y (100 mM NaCl) and centrifuged at 5000 rpm (3400 x g) for 20 minutes. The Bof-A sample was then applied and centrifuged at 5000 rpm for 1 hour. To elute the column rententate, the column was inverted, 100 µl buffer Y (100 mM NaCl) applied, and the column was centrifuged an additional 30 minutes at 2500 rpm (1000 x g). The resulting fraction was divided into 20 (5 µl) aliquots and frozen at -70°C.

RESULTS

Bof-enhanced, C1-mediated repression of transcription initiating at p_{ref} and p_{c1} was roughly two-fold, using a plasmid in which C1 was capable of autoregulation [Chapter II (15)]. Since Bof was shown to play a direct role in *c*1 autoregulation, a plasmid was constructed in which the *c*1 promoter had been removed, eliminating the effect of Bof on *c*1 expression. In this plasmid (pTS500), the *c*1 gene was transcribed via the heterologous p_{tac} promoter. Plasmid pTS500 also encodes the *lacI*^{q} gene and thus overproduces the lac repressor which in turn blocks transcription initiation at p_{tac}. The addition of IPTG, to cultures of cells harboring this plasmid, results in the derepression of p_{tac} and consequent induction of *c*1 synthesis. Using pTS500 as a source of C1, transcription initiated at the *ref* and *c*1 promoters was analyzed in the absence or presence of Bof.

Significant enhancement by Bof of C1-mediated repression of transcription initiated at p_{ref} and p_{c1} was seen when various concentrations of IPTG were used to induce C1 synthesis (Fig IV.2). At every level of IPTG-induced C1 repressor, the presence of Bof reduced expression at the *ref*::*lacZ* gene fusion by 5 to 8-fold. Expression of the *c*1::*lacZ* fusion gene was analyzed in a similar manner (Fig. IV.2). At every level of C1 repressor the presence of Bof further reduced expression of *c*1::*lacZ*, by 1.1 to 8 fold.

Elevated C1 repressor concentrations cannot compensate for the absence of Bof. In the absence of Bof, each successive increase in IPTG concentration resulted in a decrease in *ref*::*lacZ* expression, from 87% of the unregulated level at no IPTG to 35% at 3 mM IPTG. In the presence of Bof, the amount of C1 synthesized without induction (no IPTG) resulted in a more complete repression of *ref*::*lacZ* than the amount of C1 produced under fully induced conditions (3 mM), in the absence of Bof (compare triangle, 3 mM to circle, 0mM in Fig. IV.2). This supports the results described in Chapter II that complete repression of *ref* requires the presence of both C1 and Bof, even at very high C1 repressor concentrations.

Purified Bof protein enhances C1 repressor-mediated retention of operator-containing fragments. The ability of purified C1 and Bof proteins to bind to the putative operators associated with the *ref* gene was tested using an electrophoretic mobility-shift assay (Fig. IV.3). A 136-bp DNA fragment containing Op2a was incubated with increasing concentrations of C1 repressor in the presence or absence of a constant amount Bof protein (representing a three-fold molar excess of Bof with respect to operator DNA). At each repressor concentration, there was an increase in the amount of DNA-operator fragment with lower mobility (position A) when Bof protein was included in the reaction mixture. Although the addition of Bof protein resulted in more DNA fragment retained than in the presence of C1 repressor alone, no prominent additional band appeared, i.e. there was no evidence for distinct C1-DNA and C1-Bof-DNA complexes. However, the single prominent

retarded band displayed a slightly lower mobility when Bof protein was present. At the two highest C1 repressor concentrations employed (C1:operator mole ratios of 2.5:1 and 3.8:1, respectively), a second band (position B) with even lower mobility appears. In contrast to the band at position A, the intensity of the second band does not appear to be Bof-dependent. In the absence of C1, Bof protein did not result in any band with reduced mobility, although the addition of 20 ng of Bof alone resulted in a slight smearing of the (unretarded) Op2a-operator-DNA band.

Enhancement by Bof of C1-mediated retardation of Op2b DNA was also observed (Fig. IV.4). Unlike Op2a, Op2b has a psuedo-dyad C1-binding site, as do at least two other P1 operators [e.g. Op99a(b)] (23) and Op72 (11); see Fig. I.2). Incubation of C1 repressor with the 158-bp fragment containing Op2b resulted in retarded bands displaying two distinct mobilities (positions A and B). At the highest level of C1, a third very faint band was observed. At each amount of C1, the addition of Bof protein resulted in broadened bands at shift positions A and B, and a further decrease in the amount of the free (unretarded) operator DNA. As seen with the Op2a fragment, Bof protein was unable, in the absence of C1 repressor, to cause any appreciable shift in the mobility of the Op2b operator DNA fragment; only a slight smearing of the unretarded fragment was observed.

Bof-enhancement of C1-mediated mobility-shift is maximal at C1-Bof molar parity. Having demonstrated the ability of Bof protein to increase the affinity of C1 (Fig. IV.3 and IV.4), I next attempted to determine the stoichiometric relationship between C1 and Bof, by the titration of a known amount of C1 with Bof protein.

Equimolar amounts of the 136-bp Op2a DNA fragment and C1 repressor were incubated together with increasing amounts of Bof protein (Fig. IV.5). The Bof:C1 molar ratio ranged from 0.2:1 to 2:1. C1 repressor, when equimolar with Op2a, caused no significant shift when Bof protein was absent. However, addition of Bof at even the lowest level (DNA:C1:Bof mole ratio of 1:1:0.2) resulted in a visible band of reduced mobility. The intensity of this band was maximum at a C1:Bof mole ratio of 1:1.5; further increases in Bof protein resulted in no increase in the intensity of the retarded band. The retarded-band maximum intensity occured at a Bof level at which all three components of the reaction were roughly at molar parity (assuming C1 to be a monomer). Thus, an amount of C1 repressor insufficient for retardation of the Op2a fragment was sufficient to do so when Bof protein was present. However, at Bof:C1 levels greater than unity, further increases in Bof levels caused no increase in band-shift, suggesting that the C1 repressor was limiting at this point. The ability of Bof to increase the efficacy of C1 repressor was also measured by comparing the effects of C1 concentration in the presence of Bof to those in its absence (Fig. IV.5). Incubation of C1 (25 ng) with Bof (6 ng) [operator:C1:Bof mole ratio 1:1:0.8] results in a nearly equivalent amount of retarded operator fragment to that seen using four times as much C1 repressor [operator:C1 mole ratio of 1:4] in the absence of Bof (compare lanes 6 and 12).

An analogous experiment was performed with the Op2b-containing fragment (Fig. IV.6). In this case an amount of C1 repressor equimolar with Op2b fragment was sufficient to cause a significant band retardation, in the absence of Bof. The

retarded bands at the A and B positions appeared to be of roughly equal intensity; their intensity remained unchanged upon the addition of low amounts of Bof protein (lanes 2-4). However, at the point of operator:C1:Bof molar parity (lane 6), the band at position A became more prominent. Further increases in Bof protein above this point also resulted in the predominance of the position A band. In the absence of Bof, C1 repressor at higher concentrations (C1:operator mole ratios of 2:1 and 5:1) resulted in a prominent shift of operator fragment to position B (lanes 10 and 11). The highest levels of C1, representing 10:1 and 20:1 C1:operator mole ratios, respectively, resulted in a "ladder" of bands, corresponding to operator fragments with a sequential increase in the number of C1 molecules bound. Since, the point of maximal Bof-enhancement of C1-mediated retardation of both Op2a and Op2b occurs at molar parity, it appears that Bof acts stoichiometrically rather than catalytically, at a 1:1 mole ratio with C1 repressor.

DNaseI protection of Op2a and Op2b. Having demonstrated a specific Bof-enhanced interaction between C1 repressor and Op2a or Op2b, I wished to determine the regions of DNA bound by these proteins using the technique of protection against DNase I digestion. Duplex DNA substrates were prepared by extension of a 5'-^{32}P-labeled primer annealed to alkali-denatured operator-encoding plasmid by Klenow fragment. The *ref* gene promoter-operator was first analyzed by this method by synthesizing radiolabeled DNA colinear with the template (anti-sense) strand (Fig. IV.7B). The duplex DNA polymerase product was incubated with combinations of purified C1 repressor and Bof protein, prior to treatment with DNase I. At the

lowest concentration of C1 repressor employed (30 ng) (lane 10), virtually no change in the DNase I cleavage pattern was observed. However, the same amount of C1 repressor in the presence of added Bof protein (50 ng) (lane 11) resulted in a decrease in the intensity of many prominent DNase I cleavage bands. At every C1 concentration, the presence of Bof reduced the intensity of the DNase I-generated bands; i.e. Bof plus C1 conferred greater protection from DNase I cleavage than C1 alone. The presence of Bof also appeared to increase the size of the protected region. In the absence of C1 repressor, Bof protein, either 50 ng (lane 18) or 750 ng (lane 19), caused no significant change in the DNase I cleavage pattern.

The DNase I cleavage pattern of the radiolabeled opposite strand (colinear with the *ref* sense strand) was also analyzed in this manner (Fig. IV.8). Addition of C1 repressor in increasing amounts resulted in a gradual increase in protection (lanes 6, 8, 10, and 12). The addition of Bof at each repressor level resulted in a more complete protection (lanes 7, 9, 11, 13). The lowest concentration of C1 repressor, in the presence of Bof, resulted in a protection about equal to that produced by the highest level of C1 alone (compare lanes 7 and 12). As seen for the opposite DNA strand, the region of DNA protected in the presence of Bof protein appeared to be several nucleotides larger than the region protected by C1 alone Again, even 750 ng (lane 19) of Bof protein did not afford significant protection in the absence of C1.

Analysis of Op2b yielded two results different from those observed with Op2a. On either strand of Op2b, the lowest level of C1 (30 ng), the addition of Bof

made no difference in the amount of protection or intensity of the DNase-generated bands (Fig. IV.9 and IV.10, lanes 6 and 7). Doubling the concentration of C1 (60 ng) also had no effect (lane 8). However, at 60 ng C1 (lane 9), the addition of Bof dramatically increased protection. At high levels of C1, Bof did not enhance protection. Also, unlike the situation with Op2a, the presence of Bof did not alter the size of the protected region at Op2b. In fact, at C1 levels higher than 60 ng (with or without Bof), the protected region of the Op2b sense strand (Fig. IV.10, lanes 10-13) was larger than that seen at 30 ng C1 in the presence of Bof (50 ng) (lane 9). The ability of high levels of C1 repressor, in the absence of Bof, to expand the protected region probably reflects the binding of two C1 molecules. Low levels of Bof alone (50 ng) caused no DNase I protection, but, at 750 ng (lane 19), a slight decrease in the intensity of several bands was observed.

C1 alone, and C1 plus Bof, reduce transcription initiated at p_{ref} *in vitro*. Analysis of the DNA sequence upstream of the *ref* gene revealed two tandem sequences resembling promoter consensus sites (27). Using a primer extension technique, the points of initiation of *ref* gene, and the extent of *ref*-specific mRNA *in vitro* in the presence of C1 and Bof proteins were determined. Plasmid pUC19r81, containing the *ref* gene and putative promoter sequences was transcribed *in vitro* with *E. coli* RNA polymerase in the presence of C1 or Bof, or both proteins together. An end-labeled oligonucleotide primer (TS5), expected to be complementary to the *ref* mRNA, was annealed to RNA product. Following extension of the primer with reverse transcriptase, the products were separated by electrophoresis (Fig. IV.7A). The

position of the band relative to bands on a DNA sequencing "ladder" revealed the point of transcription initiation, and the relative intensity provided a rough estimate of the amount of *ref*-specific mRNA. Two transcripts of very different intensity were produced in the absence of any regulatory proteins. The points of initiation of the transcripts, at positions 185 and 172, corresponded well to the previously predicted promoter sequences p_{ref-2} and p_{ref-1}, respectively (Fig. I.4) (27). When C1 repressor was added prior to transcription, the band corresponding to the predominant RNA product initiated from p_{ref-2} was significantly reduced (lane 3). Addition of both Bof and C1 proteins resulted in nearly complete loss of this band (lane 2). Bof protein alone had little effect (lane 1). The intensity of the less prominent band, corresponding to transcripts initiated from p_{ref-1}, was unchanged upon the addition of C1, Bof, or both proteins together. Results obtained *in vitro* with the prominent band, believed to correspond to transcripts initiated from p_{ref-2}, are in good agreement with the ability of C1 and Bof together to reduce *ref* operon fusion expression more completely than either C1 or Bof alone.

The effect of Coi protein on Bof-enhanced C1-mediated retardation of operator encoding fragments.

Using *ref*:*lacZ* and *c1*::*lacZ* operon fusions, we have previously shown that when C1 is supplied from a construct that also encodes the the closely linked *coi* gene, C1 and C1 plus Bof repress transcription less effectively [Chapter II (15)]. It has since been demonstrated that the product of the *coi* gene interferes directly

with C1 activity, most likely through a protein-protein interaction (12). Since Bof enhancement of C1-mediated repression nevertheless occurs in the presence of Coi, we have suggested [Chapter II] that the Bof and Coi proteins were mutual antagonists, perhaps competing for the same site on C1 protein. The availability of purified Bof and C1 protein, and partially purified Coi protein made it possible to test this hypothesis directly.

Bof-dependent, Coi-resistent, C1-operator complex. Bof protein, in the presence of Coi-containing extract, was tested for its ability to increase the amount of C1-mediated DNA fragment containing Op2a retarded, as demonstrated above (Fig. IV.11). Bof and C1 were used at concentrations previously shown to result in a complete retardation of all Op2a fragments present. As expected, nearly all the operator fragment was retarded (position A), in the absence of Coi, regardless of the order of addition of C1 and Bof proteins (lanes 3 and 9). However, if Coi protein was added, along with operator DNA, to a mixture of C1 and Bof proteins, there was no retardation of the DNA fragment (compare lane 3 to 4-6). Similarly, there was no operator DNA retardation when C1 and Coi were incubated together before the addition of Bof and operator DNA (compare lane 9 to 10-12). Thus, the ability of Coi protein to interfere with C1 repressor binding seems to be dominant over the enhancement of C1 binding by Bof.

Dramatically different results were obtained when the order of addition of reaction components was altered. Incubation of the operator fragment with both C1 and Bof proteins prior to challenge with Coi resulted in retardation of all operator

DNA, regardless of the amount of Coi protein (Fig. IV.12, lanes 4-6). In contrast, C1 protein alone, at the same concentration, incubated with the operator before the addition of Coi, failed to significantly retard operator DNA (lane 7). This result suggests the formation of an operator-C1-Bof complex that is resistent to the action of Coi protein. When the operator DNA fragment was incubated with C1 and Coi before the addition of Bof, different proportions of retarded versus unretarded fragment were observed with different concentrations of Coi protein. With each successive increase in Coi, the amount of retarded operator DNA was reduced (lanes 10-13). At lower Coi concentrations, Bof appeared to enhance the binding of C1 (lanes 10 and 11). However, at the highest concentration of Coi, the majority of the operator fragment remained unretarded, i.e. Coi seemed dominant over Bof.

Operator DNA-binding of C1 and Bof proteins synthesized *in vitro*. The results presented thus far have clearly demonstrated the ability of Bof protein to increase the ability of C1 repressor to bind to the operators associated with the *ref* gene. Bof appears unable to bind DNA in the absence of C1 repressor, and its presence in a complex with C1 at an operator, although suggested by the ability to form a Bof-dependent Coi-resistent C1-complex, has not been unequivocally demonstrated. To address the question of participation of Bof in a C1-Bof-DNA complex, C1 and Bof proteins were produced using an *in vitro* system that facilitated the radiolabeling of Bof protein with ^{35}S-methionine. If radiolabeled-Bof is present in a complex, following electrophoresis of mixtures containing operator DNA, C1 and Bof, its presence should be detectable in bands retarded by the proteins.

DNA-directed synthesis of C1 repressor and Bof protein. Plasmid pTS500($c1^+$) (Fig. IV.1) was used to direct *in vitro* transcription-translation as described in "Materials and Methods". Three prominent ^{35}S-labeled protein bands were visible (Fig. IV.13A) upon analysis by electrophoresis under denaturing conditions. The M_r of the middle band was in good agreement with that predicted of the P1 C1 repressor (33 kD) (6). The assumption that this band corresponds to C1 protein was tested by programmming a parallel transcription-translation reaction with DNA in which the coding sequence of the *c*1 gene had been disrupted by *Bgl*II restriction of pTS500 DNA. This control reaction did not produce a 33 kD protein, but did yield the two other bands, presumably corresponding to the proteins encoded by the *bla* and *lacI*^{q} genes present on pTS500.

Bof protein was similarly synthesized *in vitro*. DNA-directed transcription-translation of pTS600 (Fig. IV.1) resulted in at least four protein products that could be detected by electrophoresis under denaturing conditions (Fig. IV.13B). The M_r of the most prominent band corresponded well to a molecular weight of 7 kD, in good agreement with the value for Bof previously reported (23). Prior cleavage of pTS600 with *Eco*RI endonuclease, which separates the p_{tac} promoter signals from the *bof* structural gene, eliminated the 7 kD protein from the products. *Eco*RI treatment also caused loss of the 3 kD band which may correspond to a degradation product of Bof, or to a peptide produced from an internal ATG in the *bof* sequence. As with synthesis reactions programmed with pTS500, two additional higher molecular weight protein products were present, presumably the products of the *bla* and *lacI*^{q}

genes.

In order to eliminate other radiolabeled proteins from the Bof-synthesis reactions, the transcription-translation products were subjected to a DEAE-fractionation step. Figure IV.13C depicts the electrophoretic analysis of the DEAE-purified product. Virtually all remaining radiolabeled protein migrates at a position corresponding to Bof protein.

C1 protein produced *in vitro* is functional. The activity of C1 repressor protein synthesized by *in vitro* transcription-translation was measured using an operator-DNA mobility-shift assay. Products from transcription-translation reactions programmed with pTS500 DNA, and with pTS500 DNA that had been restricted with *Bgl*II [pTS500(B2)], were tested. These products were incubated with a DNA fragment encoding the promoter-operator from the P1 *ban* gene (Op72a(b)) (Fig. IV.14). Op72 was chosen because of its sensitivity to C1 repressor [Chapter II and (11)]. Each putative C1 repressor concentration tested (lanes 1-4) resulted in significantly more retardation of the Op72a(b) operator fragment than resulted from a control mixture with no added protein (lane 5) At the lowest concentration employed, a single band of reduced mobility was observed; increasing the C1 concentration resulted in the appearance of a second band with even lower mobility. In contrast, no concentration of a control-synthesis product, programmed with pTS500(B2), caused a retardation of the Op72 operator fragment (lanes 6-9). Thus, C1 repressor synthesized *in vitro* appears to be functional; its incubation with an

Op72-encoding DNA fragment resulted in two bands of reduced mobility following electrophoresis, in accordance with results using purified proteins (11).

The 136-bp Op2a operator fragment was also tested for decreased mobility in the presence of C1 synthesized *in vitro* (Fig. IV.15). Two-fold serial dilutions of C1 were incubated with a constant amount of operator DNA. Incubation of Op2a DNA with undiluted C1 protein resulted in a single prominent retarded band. A very faint second band with even lower mobility was also seen (lane 2), consistent with the second band seen at higher concentrations using purified C1 (Fig. IV.3) Decreasing the concentration of C1 resulted in the absence of the faint second band, and the gradual disappearance of the position A band.

Bof-enhanced C1-mediated retention of Op2a fragment. The Op2a-containing DNA fragment was incubated with various concentrations of *in vitro*-synthesized C1, in the absence or presence of ^{35}S-labeled *in vitro*-synthesized Bof protein (Fig. IV.16). Putative Bof protein alone retarded a small amount of Op2a fragment (lane 2). At each concentration of C1 added (without Bof) there was a single retarded band (position A) (lanes 3, 5, and 7), greater in intensity but of similar mobility as the band seen in the presence of Bof protein alone. Quite unexpectedly, at each concentration of C1, addition of Bof resulted in the appearence of a band of slightly lower mobility than the band caused by binding of C1 (or Bof) alone (position A'). At the highest C1 levels, the addition of Bof produced, in addition, a very faint third band of even lesser mobility. The appearence of this distinct, Bof-dependent retardation product (position A') is the most substantial evidence, to date, for the

existence of an operator-C1-Bof complex.

DISCUSSION

The effect of Bof protein on C1-mediated repression of gene expression was studied both *in vivo* and *in vitro*. Results obtained *in vivo* with *ref*::*lacZ* and *c1*::*lacZ* operon fusions, demonstrating enhancement of C1-mediated repression by Bof, reinforced those previously reported, in which complete repression of transcription of p_{ref} and p_{c1} was found to require both Bof and C1 repressor [Chapter II (15)]. Experiments using purified proteins, and experiments using proteins synthesized *in vitro*, demonstrated that Bof increases the affinity of C1 repressor for operator sites. Two results suggested that Bof participates in a C1-Bof-operator complex were presented: a distinct Bof-dependent C1-mediated retardation product was formed using Bof and C1 proteins synthesized *in vitro*, and using purified C1 and Bof, Bof enhanced the ability of C1 repressor to interact with operator-containing DNA fragments, an interaction which was resistent to the action of Coi protein, unlike the interaction between operator DNA and C1 repressor alone.

We previously reported that Bof reduced C1-regulated expression of *ref*::*lacZ* and *c1*::*lacZ* genes *in vivo* by about two-fold [Chapter II (15)]. The *in vitro* results presented here suggested that Bof has a more dramatic effect on C1 binding, and that the small enhancement of C1-mediated repression observed previously *in vivo* was due to the negative effect of Bof on autoregulated C1 synthesis, which would be expected to partially counteract the negative effect of Bof on expression of C1-

regulated promoters. Elimination of the *c*1 auto-regulatory loop by replacing the *c*1 promoter-operator with p_{tac}, resulted in a considerable increase in the *in vivo* Bof effect (Fig. IV.2): Bof caused as much as an 8-fold decrease in expression of both *ref*::*lacZ* and *c*1::*lacZ*. Some C1 is produced from the p_{tac}-*c*1 construct (pTS500) under uninduced conditions (no IPTG) because of the "leakiness" of the p_{tac} promoter. This small amount of C1 decreased *ref*::*lacZ* expression by 13% in the absence of Bof and 87% in the presence of Bof, demonstrating the dramatic effect of Bof on the repression of expression from the *ref* promoter at limiting C1 concentrations. These experiments reinforce the notion that full repression of certain P1 promoter-operators (e.g. p_{ref}) requires Bof.

The Bof-enhancement of C1 mediated repression of p_{c1}-initiated transcription is comparable to that at p_{ref}, but there are two major differences in the repression charateristics of these two promoter-operators. First, at very low C1 levels (uniduced p_{tac}-*c*1), the presence of Bof makes little difference in the expression of *c*1::*lacZ*, in contrast to the dramatic negative Bof effect on expression of *ref*::*lacZ*. Second, in the absence of Bof, the c1 promoter appears to be slightly more sensitive to C1 repressor than p_{ref}. Thus induction of p_{tac}-*c*1 expression resulted in repression of *ref*::*lacZ* expression 13% to 65% (0 and 3 mM IPTG, respectively), compared to an increase from 32% to 83% repression of *c*1::*lacZ*.

Consistent with the enhancement of C1-mediated repression of *ref*::*lacZ* expression seen by Bof *in vivo*, the presence of C1 and Bof proteins inhibited transcription of the *ref* gene by RNA polymerase *in vitro*: as the amount of mRNA

that could be subsequently copied using a *ref*-specific primer and reverse transcriptase was decreased by the presence of C1, and C1 plus Bof. Upstream of the *ref* gene are two promoter structures in tandem, p_{ref-1} and p_{ref-2} (27). The five-prime ends of the two *in vitro ref* transcripts correspond quite well to the initiation points predicted for these structures at positions 172 (p_{ref-1}) and 185 (p_{ref-2}) (Fig. IV.7a)(27). The transcript initiating at p_{ref-2} was considerably stronger than that of p_{ref-1}. The addition of purified C1 protein significantly reduced the p_{ref-2} signal, while the addition of both C1 and Bof together abolished it. The level of the p_{ref-1} band remained unchanged, regardless of added C1 and Bof. This latter result is surprising, since the p_{ref-1} transcript initiates within the DNA sequence protected from DNase I by C1 plus Bof. The apparent repression-resistant basal level of transcription initiated at p_{ref-1} may explain the inability of even multicopy plasmids encoding *c*1 and *bof* to totally repress *ref*::*lacZ* expression *in vivo* (Chapter II, and Fig. IV.2) Even though the p_{ref-2} transcript is initiated at a point outside of the major C1-plus-Bof protected region, it is inhibited by C1 plus Bof. This may be due to steric hindrance of RNAP binding by regulatory protein(s) bound outside of the promoter.

Both Op2a, associated with the *ref* promoters, and Op2b, located at the three-prime end of the *ref* gene coding sequence, appear to be sensitive to the action of Bof protein *in vitro*. At a given concentration of C1 repressor, the presence of Bof appeared to increase C1 binding to Op2a or Op2b operators (Fig. IV.3-6). Thus Bof increased the fraction of operator-containing fragments that was

retarded by C1 during electrophoresis, and enhanced the protection of both operators against DNase I digestion. Upon incubation of Op2a and Op2b fragments with repressor, one and two prominent retarded species were observed respectively, consistent with their corresponding single-site and double-site (psuedo-dyad) operator configurations.

In the case of the single-site operator Op2a addition of Bof resulted in a significantly greater intensity of the shifted bands at each concentration of C1 (Fig. IV.3). Results with the single operator Op2a (Fig. IV.5) using constant equimolar amounts of C1 and operator DNA while increasing the Bof level suggested that Bof was needed in amounts equimolar with C1 repressor since Bof-enhancement of C1-mediated operator-fragment retardation was maximal when C1 and Bof were equimolar.

At Op2b, two molecules of C1 repressor appear to bind in a cooperative manner, regardless of the presence of Bof. Presumably one C1 molecule first binds to the higher affinity site (i. e. more consensus-like), facilitating binding of a second molecule to the weaker site. In principal Bof could enhance the binding of one or both of these C1 molecules at Op2b. Two results suggest that Bof preferentially enhances C1 binding to one of these sites. In the experiment shown in Figure IV.4 at the three lowest concentrations of C1 employed (10, 25, and 50 ng), the addition of Bof resulted in an increase in the intensity of the (lower) position A band (these represent C1:Bof mole ratios of 0.2:1, 0.4:1, and 0.8:1, respectively). However, at C1 levels higher than 50 ng, the position A band became progressively faint and the

position B band correspondingly increased in intensity. These results, especially at the lower concentrations of C1 (where Bof was in molar excess over C1), were similar to those seen when increasing amounts of Bof were used with constant equimolar amounts of C1 and operator DNA (Fig.IV.6). The position A band became the most prominent when Bof was present at a level equimolar or higher with respect to C1, presumably because the binding of C1 repressor is mostly at one of the operator sites. Speculation that Bof influences the binding of repressor to one half of the double (pseudo-dyad) operator sites suggests that the Op2b operator can bind C1 by two mechanisms. In the absence of Bof, binding of a single C1 molecule is followed cooperatively by a second molecule. In the presence of Bof, the binding of the first C1 molecule occurs at a lower C1 concentration, and the first bound C1 cooperatively enhances binding of the second C1. Consequently, full occupancy of the operator occurs at a lower repressor concentration than would otherwise occur with C1 alone.

In the operator DNA-retention experiments describe above, there was little evidence for the presence of Bof in a C1-operator complexes. In several instances (Figs. IV.3 and IV.4), Bof caused a slight further retention of the C1-retarded band but no prominent Bof-dependent band was observed. DNase I footprinting did reveal an enlargement of the region protected at Op2a when Bof protein was also present. Whether this increase in protected area was due to the binding of Bof to DNA adjacent to bound C1, or to overlapping of bound Bof with bound C1, or to a Bof-mediated allosteric change in C1, resulting in additional contacts with the DNA, is

unknown.

Additional circumstantial evidence for a stable C1-Bof complex comes from the band-retardation experiments using Coi protein. Coi protein has been shown capable of preventing C1 from binding to operator sequences (12), presumably by means of Coi-C1 interactions. The results of *in vivo* studies using *ref*::*lacZ* and *c*1::*lacZ* operon fusions [Chapter II (15)] suggested that Bof enhanced the ability of C1 to bind to operator sequences, even in the presence of Coi. When Bof and C1 were incubated *in vivo* with Op2a operator fragments, prior to addition of Coi, the resulting complex was resistent to the action of Coi protein (Fig. IV.12). However, C1 and Bof were incubated together, before the addition of Coi and operator DNA, there was no complex formation (Fig. IV.13). Thus the interaction between C1 and Bof resulting to form a Coi-resistant complex seems to require operator DNA, i.e. this suggests the formation of a C1-Bof-DNA complex.

The ability of the Bof synthesized *in vitro* to generate a second retention product when added to a mixture of C1 and Op2a DNA, is in contrast to the slight Bof-induced broadening of the C1-Bof band when purified Bof and C1 were used (Fig. IV.16). There are several differences between the two systems. Besides the different origins of the protein products, the gels employed for electrophoretic analysis were quite different. The gels used to analyze the reactions employing proteins synthesized *in vitro* were much smaller in length, width, and thickness than those used for the reactions with purified proteins. Since the same voltage was used for both experiments, the field strength (volts per centimeter) was substantially

higher in the former experiments. The consequence of this might be an alteration in the effect of the gel on the migration of the DNA-protein complexes. This "caging" effect may result in a higher resolution of the retention products. An alternate explanation for the additional Bof-dependent band observed using the proteins synthesized *in vitro* would be a requirement for some *E. coli* gene product present in the protein-synthesizing extract.

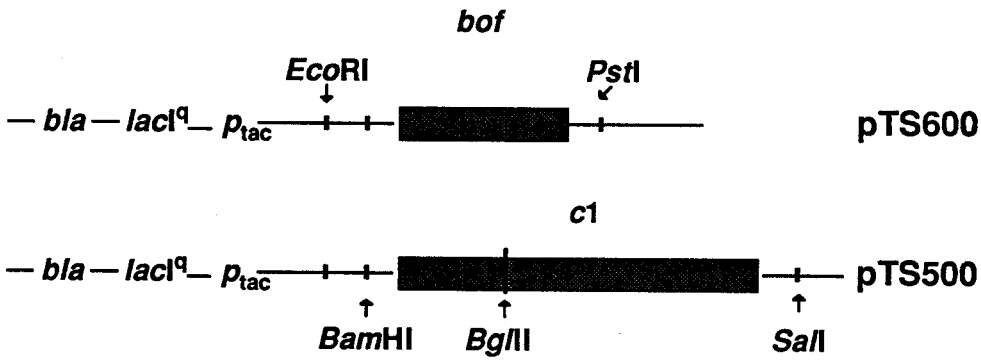
The second more retarded band (position A), observed in the presence of Bof protein synthesized *in vitro*, is not seen at all in the absence of Bof, and has a slightly lower mobility than the C1-operator band. This is the strongest evidence yet for the existence of Bof in the C1-operator complex. Since the Bof protein in these experiments was labeled with [35S-methionine], its presence in a shifted operator complex was expected to be readily detectable by the scintillation counting of excised bands. However, the presence of Bof in the second retarded band has, thus far, not been unequivically detected. The non-denaturing conditions used for the electrophoresis of operator-protein reactions resulted in a smearing of an ^{35}S-labeled product throughout the lane. Since denaturing-electrophoresis of the Bof employed in these experiments resulted in a single band, this ^{35}S background is presumably Bof. This ^{35}S background results in a very low signal-to-noise ratio making a Bof-specific signal, relative to the non-specific background, difficult to detect.

The location of Op2b near the 3'-terminus of the *ref* gene raises the question of the utility of this C1 operator. Its location is probably not appropriate for the

blocking the advancement of RNAP as it transcribes *ref*. Op2b might serve, however, as a "sink" for C1, assuring that the local concentration of repressor in the vicinity of the *ref* gene is high as postulated for the *deo* operon (5). Alternatively, repressor molecules bound at Op2b might positively influence the binding of C1 to Op2a by cooperative binding at a distance, or by means of a higher order structure involving repressor proteins bound at both operators. The latter event would result in a looping out of the entire coding sequence of the *ref* gene. However, most known C1 operator sites are associated with promoters, and this might also be the case for Op2b. Two transcripts which initiated slightly downstream of Op2b (data not shown)(see Fig. I.5 to the right of Op2a) were observed using primer extension analysis of transcipts generated *in vitro*, on plasmid pOp2ba-encoding Op2b and about 140-bp of surrounding DNA. The abundance of both transcripts was reduced partially in the presence of C1 and almost completely eliminated in the presence of C1 plus Bof. Thus there may be a gene downstream of *ref*, negatively regulated by C1 and Bof. Is there any other evidence for such a gene? Genetic characterization of P1 mutants affected in phage morphogenesis led to the assignment of linkage clusters, the genes of which were believed important for phage morphology (24). Mutants defective in gene(s) of linkage cluster I, were noninfectious, and tails of corresponding virions were heterogenous in length (25). The six mutations comprising this linkage cluster were unable to complement one another for the ability to produce infective phage but a multicopy plasmid containing P1 fragment *Eco*R1-19 (0.9-kb) complemented all linkage cluster I mutants. The gene postulated to be

affected in linkage cluster I mutants has been designated gene 1; its gene product is believed to be involved in the maturation of phage particles. Since the Op2b operator is also located on the small *Eco*R1-19 fragment, gene 1 seems a logical candidate for a lytic gene under control of Op2b.

Figure IV.1. Linear maps of pTS500 and pTS600.



Linear maps of pTS500 and pTS600. A) The construction of pTS600 is desrcibed in "Materials and Methods". The construction places the *bof* gene (with minimal additional P1 sequences) under the control of the p_{tac} promoter. B0 The construction of plasmid pTS500 was described previously (Chapter III). In this plasmid, the *c*1 gene is under control of p_{tac}. Relevant restriction sites are shown.

Figure IV.2. Effect of Bof on the *ref*::*lacZ* and *c*1::*lacZ* expression in the presence of non-autoregulated C1. Top Panel. Bacteria lysogenic for single *ref*::*lacZ* prophages (TSS301) and harboring a non-autoregulated source of *c*1 (pTS500) were transformed with one of the two plasmids indicated. Single transformants were resuspended in 0.85% NaCl. Aliquots were transferred to TBY broth with AP, Cm and Nm (for selection of pTS8745) containing 0, 10uM, 30uM 100uM 1mM, or 3mM IPTG. Cultures were grown to mid-log phase at 37 C and assayed for B-galactosidase activity as described. Relative expression equals B-galactosidase activity divided by the activity for bacteria lysogenic for 301, containing no plasmids, multiplied by 100. Data points represent averages of quadruplicate determinations. The curves presented here are representative of similar experiments performed independently. B. Bottom panel Bacteria lysogenic for single *c*1::*lacZ* prophages (TSS321) and were determined as described above. Data points represent the averages of duplicate determinations. The curves presented here are representative of similar experiments performed independently.

Figure IV.2

Effect of Bof on λ *ref* :: *lac* Z and λ *c1*::*lac* Z
expression in the presence of non-autoregulated C1

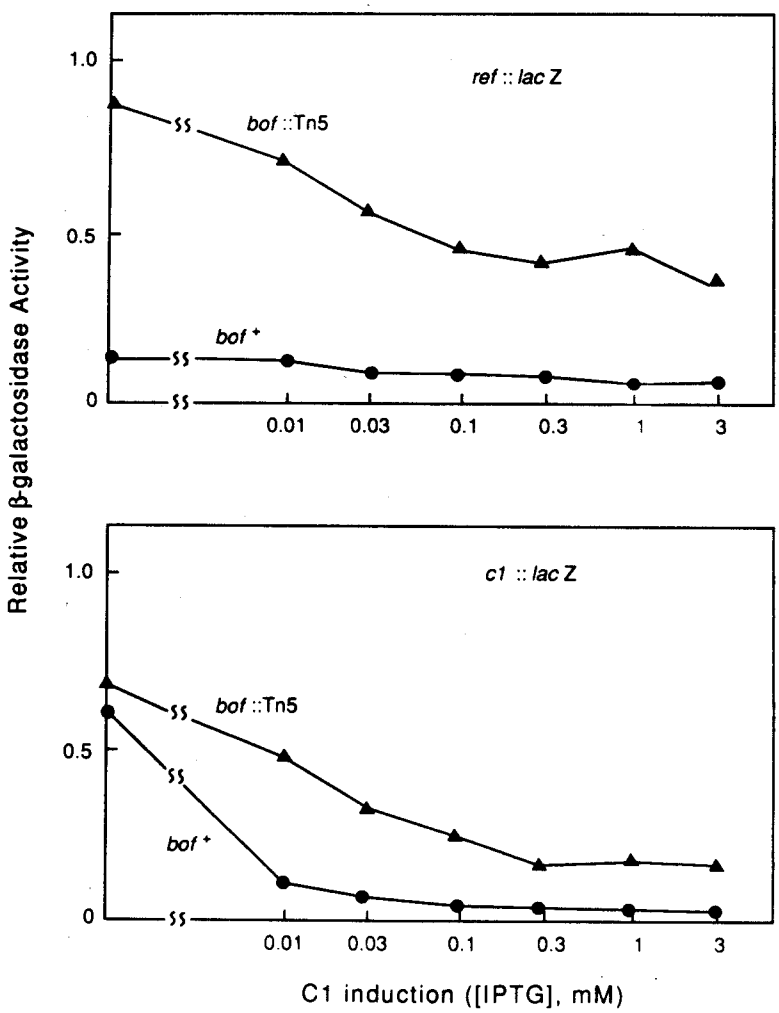
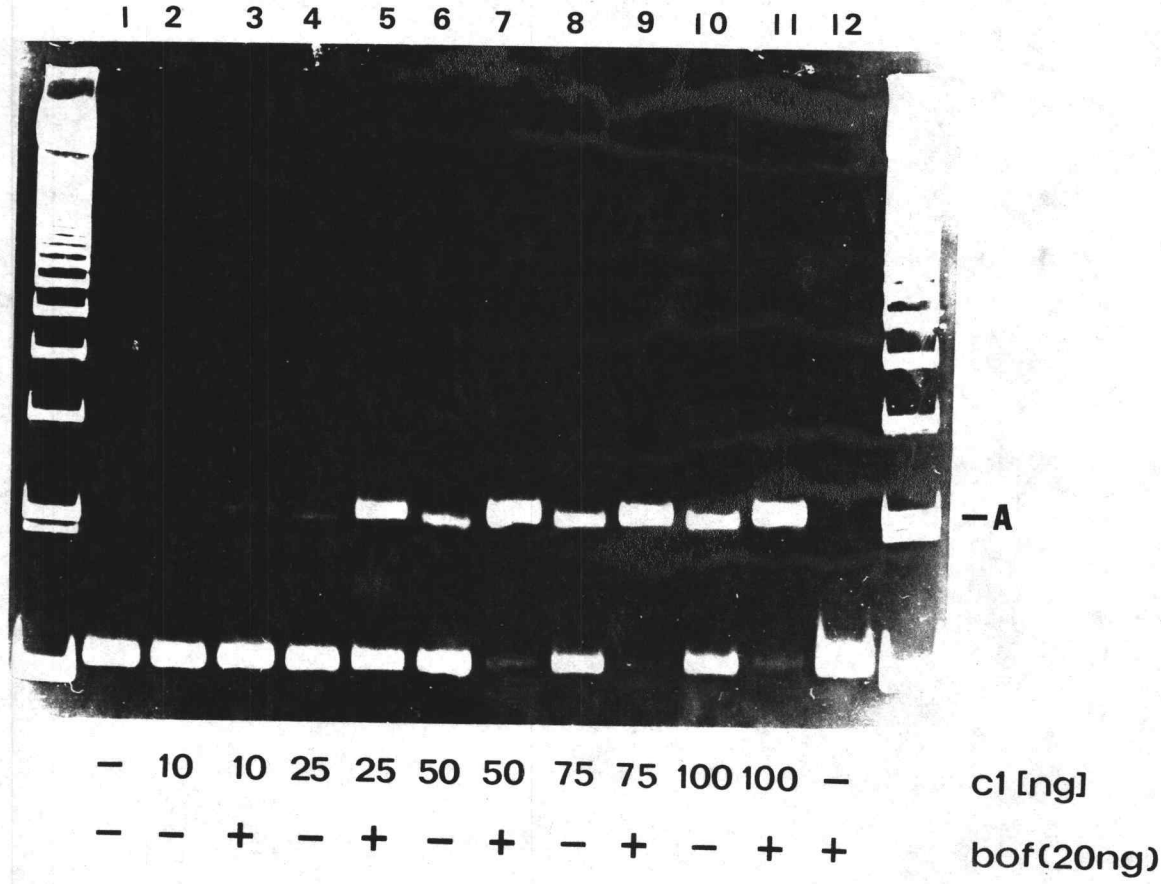
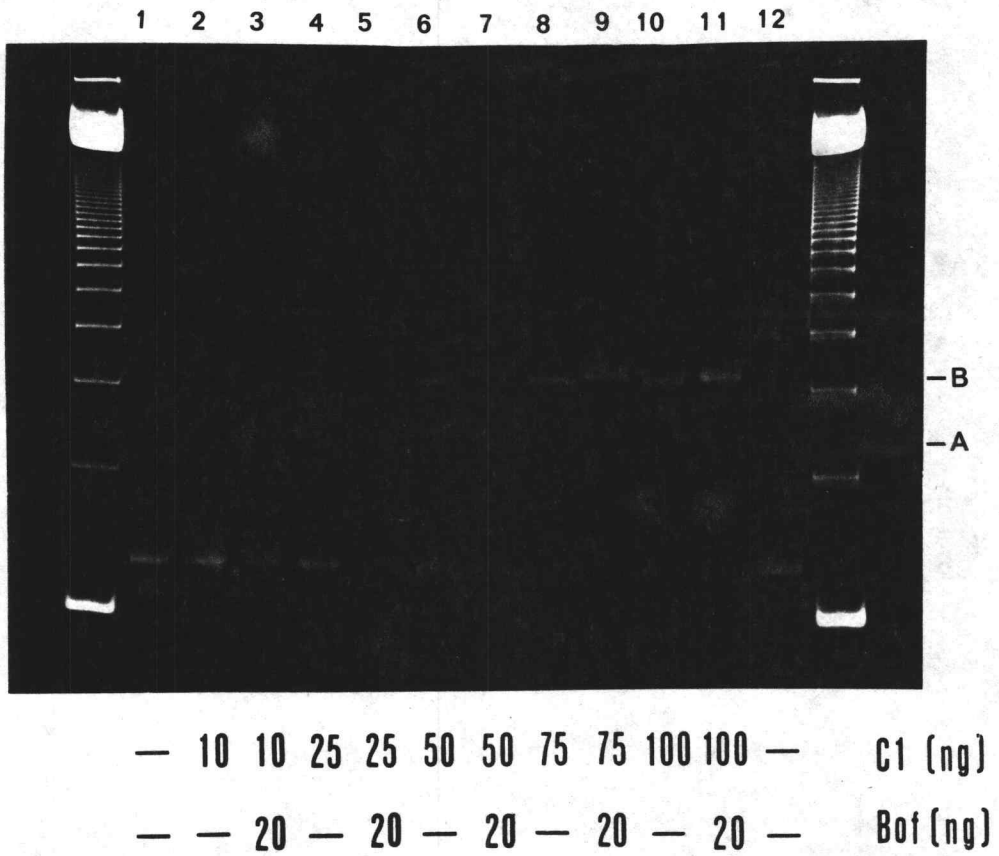


Figure IV.3. Bof-enhanced C1 retardation of Op2a operator fragment.



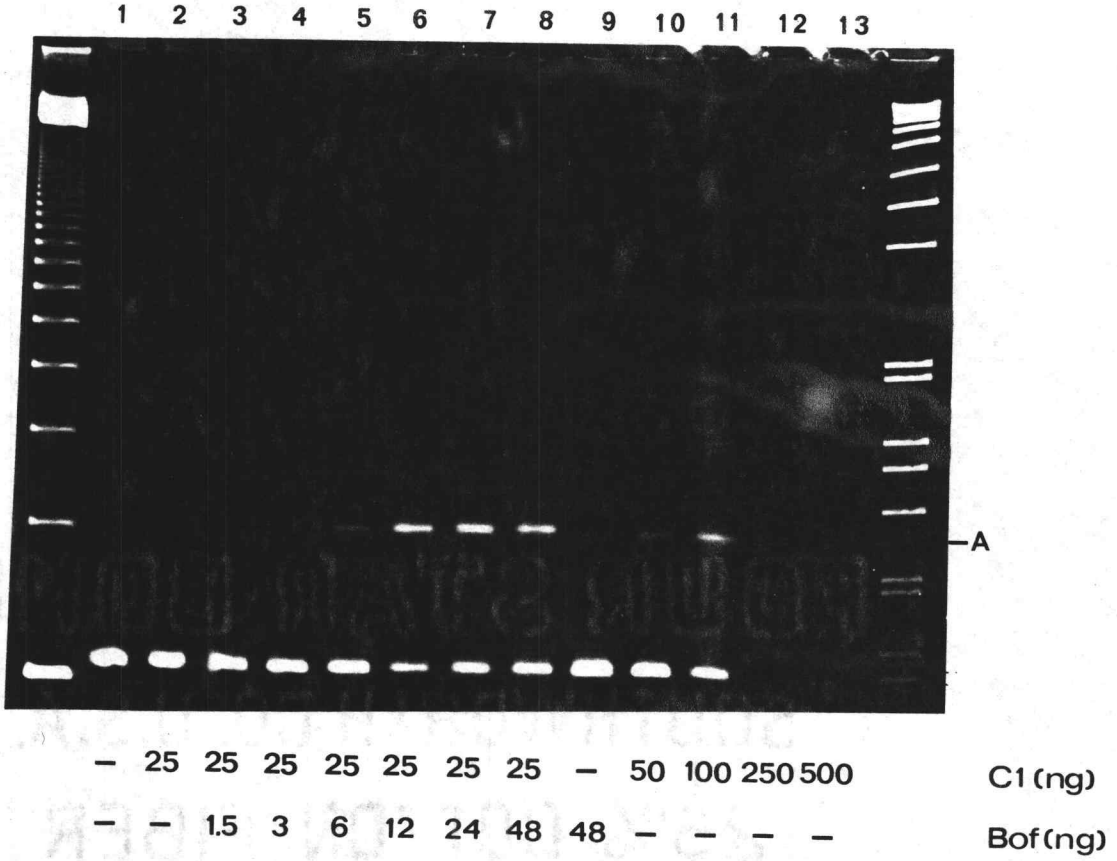
Bof-enhanced C1 retardation of Op2a operator fragment. The 136-bp Op2a-containing fragment (75ng) was incubated with the proteins at the concentrations indicated in the figure. The reactions were otherwise performed as described in "Materials and Methods".

Figure IV.4. Bof-enhanced C1 retardation of Op2b operator fragment.



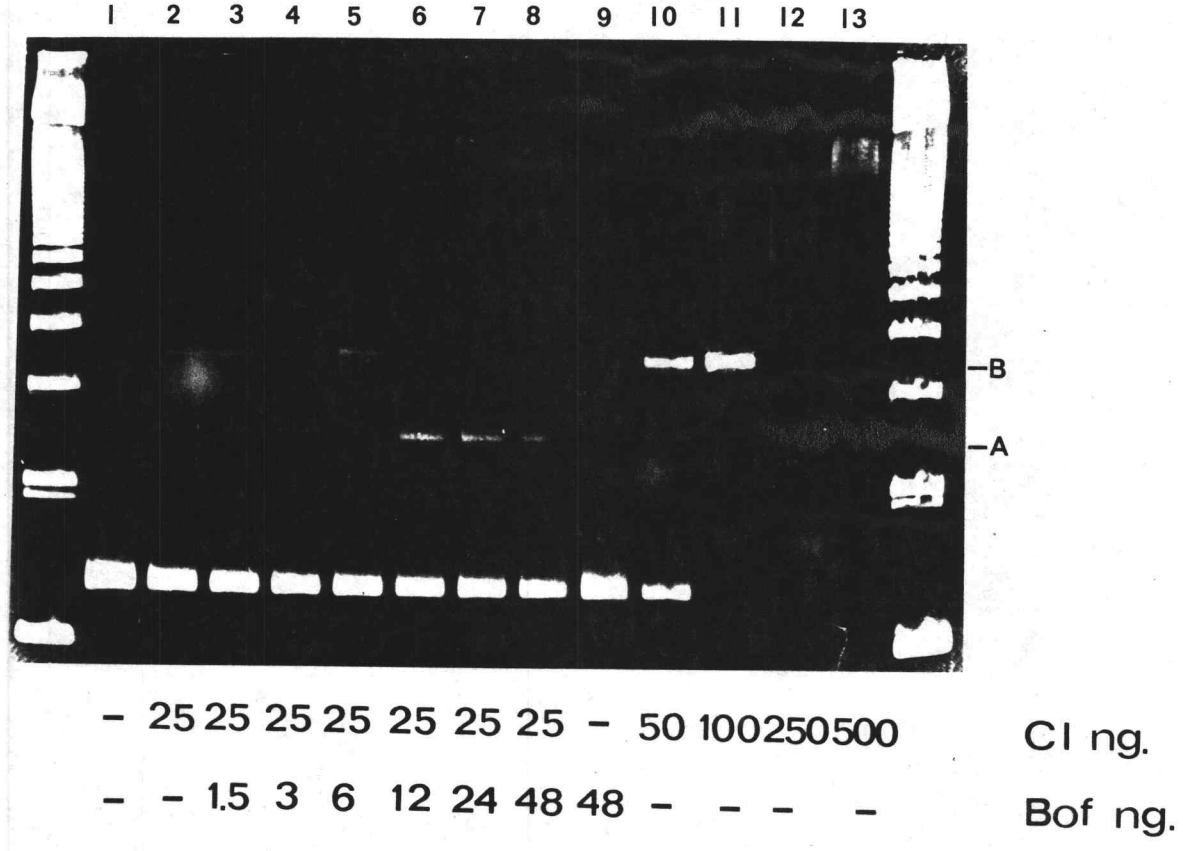
Bof-enhanced C1 retardation of Op2b operator fragment. The 158-bp Op2b-containing fragment (75ng) was incubated with the proteins at the concentrations indicated in the figure. The reactions were otherwise performed as described in "Materials and Methods".

Figure IV.5. Titration of C1-mediated repressor activity with Bof protein at Op2a.



Titration of C1-mediated repressor activity with Bof protein at Op2a. Lanes 2-8; equimolar amounts of the 136-bp Op2a fragment (75ng) was incubated with C1 repressor (25ng). Bof protein was added at the concentration indicated in the figure. Lanes 10-13; the operator fragment with increasing amounts of C1 repressor. The reactions were performed under conditions described in "Materials and Methods"

Figure IV.6. Titration of C1-mediated repressor activity with Bof protein at Op2b.



Titration of C1-mediated repressor activity with Bof protein at Op2b. Lanes 2-8; equimolar amounts of the 158-bp Op2a fragment (75ng) was incubated with C1 repressor (25ng). Bof protein was added at the concentration indicated in the figure. Lanes 10-13; the operator fragment with increasing amounts of C1 repressor. The reactions were performed under conditions described in "Materials and Methods"

Figure IV.7

A) The effect of C1 and Bof proteins on the initiation of p_{ref} transcription in vitro. Primer extension assays were performed on transcripts generated from plasmid pUC19r81 as described in "Materials and Methods". Proteins were included in the reactions in the following amounts: lane 1, Bof (160ng);lane 2 (160ng), C1 (342ng); lane 3, C1 (342ng); lane 4, no proteins. B) Protection of Op2a non-coding strand from DNAse I by C1 and Bof proteins. Protection assays were performed as described in "Materials and Methods". Lanes 1-4 are dideoxy sequencing reactions (CATG, respectively) performed on the pUC19r81 with TSS5 primer. The DNAse protection reactions included the following amounts of C1 and Bof proteins; lanes 9 and 20 contained no protein. C1 repressor was added to lanes 10 and 11 (30ng); 12 and 13 (60ng); 14 and 15 (120ng); and to lanes 16 and 17 (240ng). Bof protein (50ng) was included in lanes 11, 13, 15, and 17, and (750ng) in lane 19.

Figure IV.7 a) The effect of C1 and Bof proteins on the initiation of p_{ref} transcription *in vitro*. b) Protection of Op2a non-coding strand from DNAse I by C1 and Bof proteins.

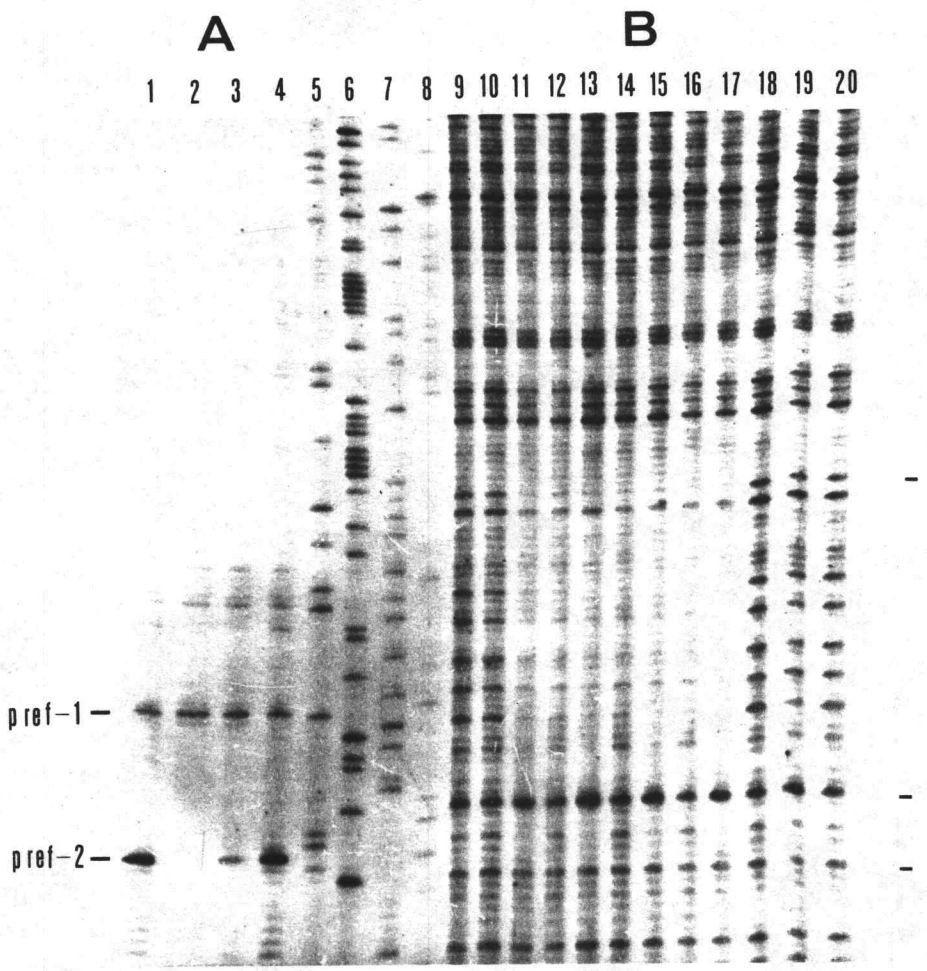


Figure IV.8

Protection of Op2a coding strand from DNAse I by C1 and Bof proteins. Protection assays were performed as described in "Materials and Methods". Lanes 1-4 are dideoxy sequencing reactions (CATG, respectively) performed on the pUC19r81 with the "universal" primer. The DNAse protection reactions included the following amounts of C1 and Bof proteins; lanes 5 and 16 contained no protein. C1 repressor was added to lanes 6 and 7 (30ng); 8 and 9 (60ng); 10 and 11 (120ng); and to lanes 12 and 13 (240ng). Bof protein (50ng) was included in lanes 7, 9, 11, and 13, and (750ng) in lane 15.

Figure IV.8. Protection of Op2a coding strand from DNAse I by C1 and Bof proteins.

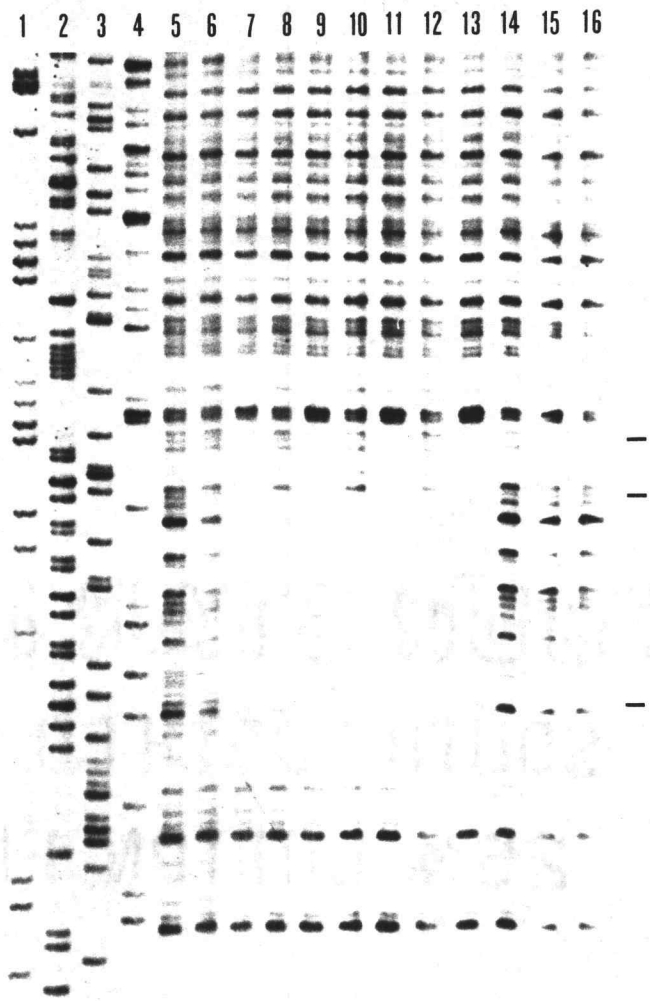


Figure IV.9

Protection of Op2b non-coding strand from DNAse I by C1 and Bof proteins. Protection assays were performed as described in "Materials and Methods". Lanes 1-4 are dideoxy sequencing reactions (CATG, respectively) performed on the pOp2ba with the "universal" primer. The DNAse protection reactions included the following amounts of C1 and Bof proteins; lanes 5 and 16 contained no protein. C1 repressor was added to lanes 6 and 7 (30ng); 8 and 9 (60ng); 10 and 11 (120ng); and to lanes 12 and 13 (240ng). Bof protein (50ng) was included in lanes 7, 9, 11, and 13, and (750ng) in lane 15.

Figure IV.9. Protection of Op2b non-coding strand from DNAse I by C1 and Bof proteins.

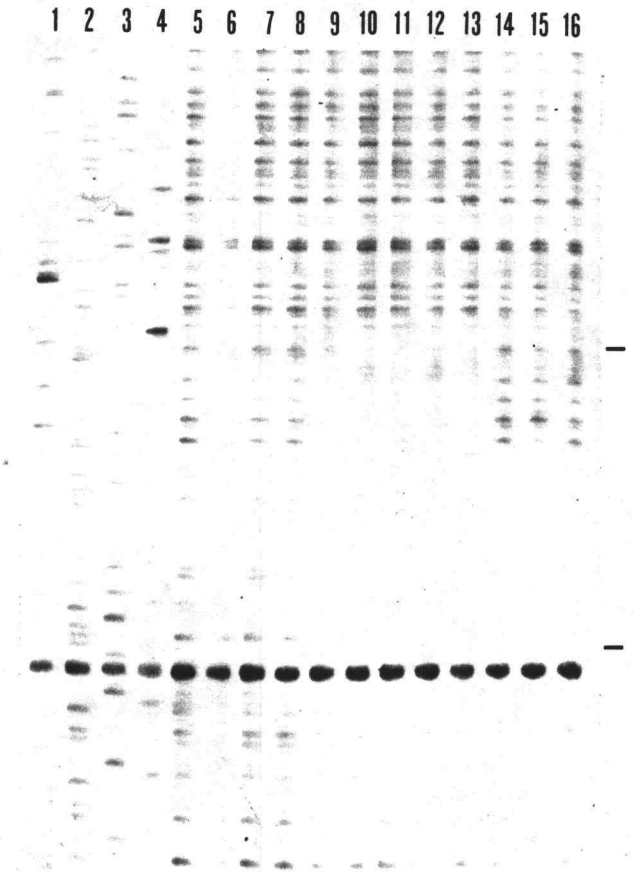


Figure IV.10

Protection of Op2b coding strand from DNAse I by C1 and Bof proteins. Protection assays were performed as described in "Materials and Methods". Lanes 1-4 are dideoxy sequencing reactions (CATG, respectively) performed on the pOp2ba with the "reverse" primer. The DNAse protection reactions included the following amounts of C1 and Bof proteins; lanes 5 and 16 contained no protein. C1 repressor was added to lanes 6 and 7 (30ng); 8 and 9 (60ng); 10 and 11 (120ng); and to lanes 12 and 13 (240ng). Bof protein (50ng) was included in lanes 7, 9, 11, and 13, and (750ng) in lane 15.

Figure IV.10. Protection of Op2b coding strand from DNAse I by C1 and Bof proteins.

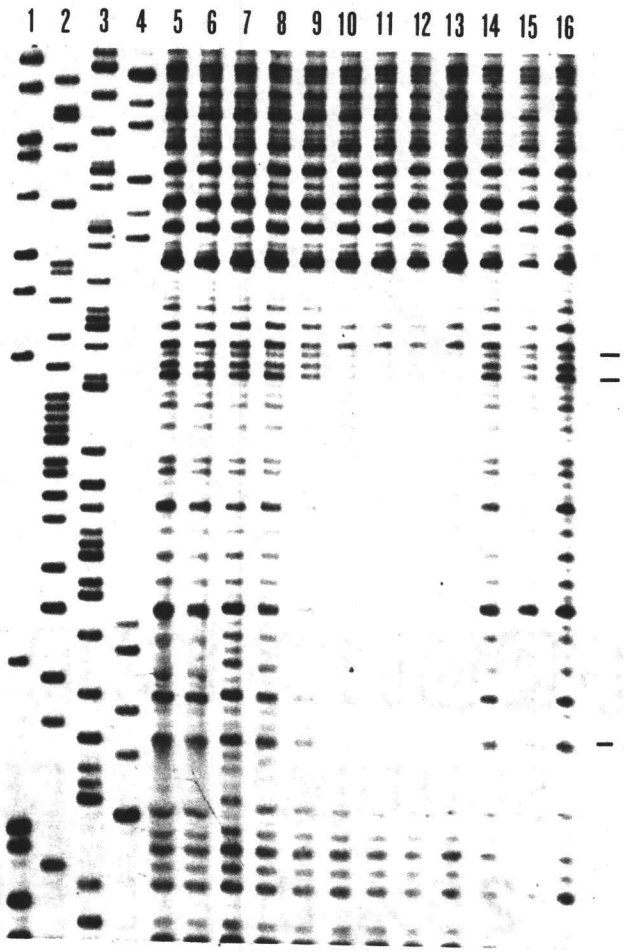
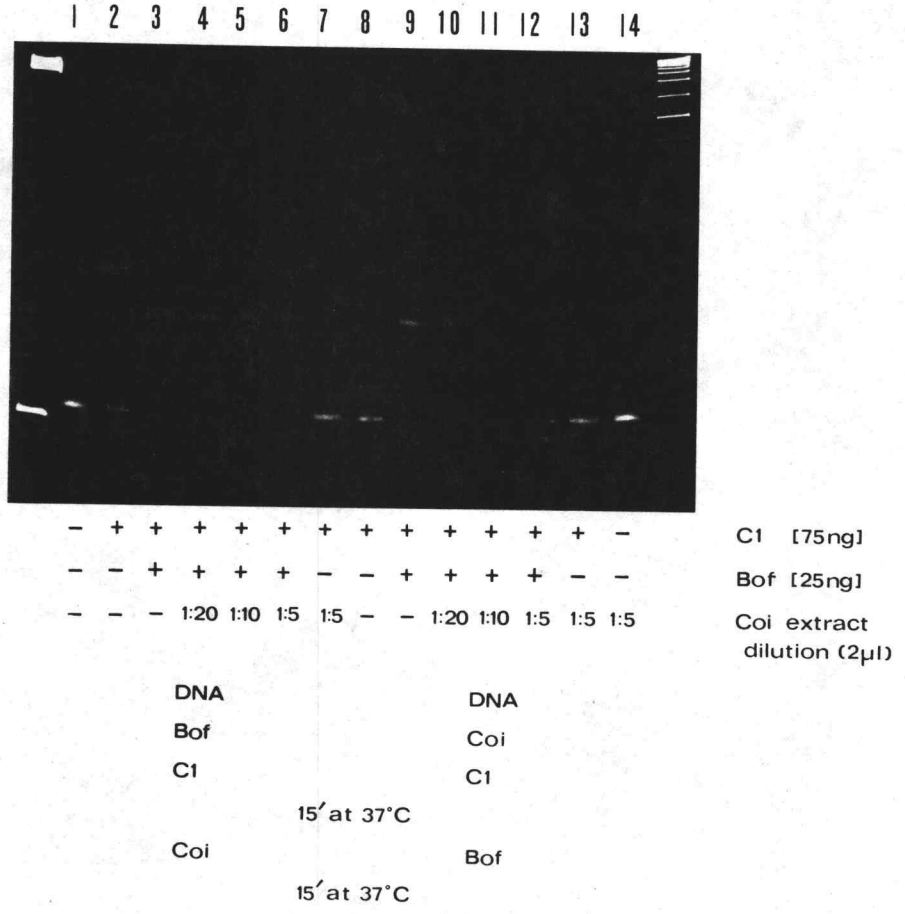
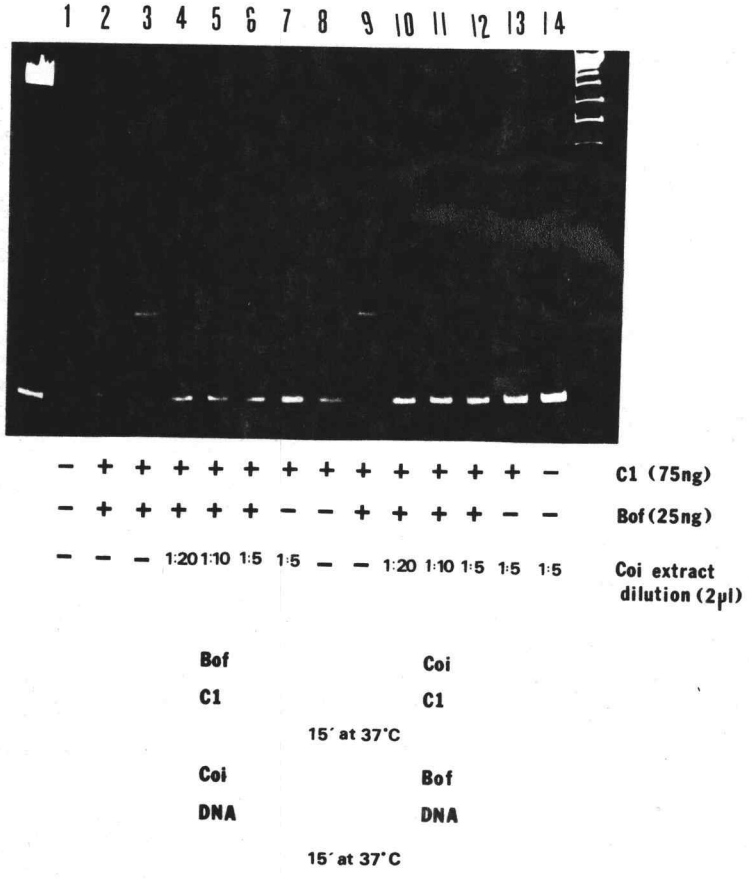


Figure IV.11. The effect of Coi protein on Bof-enhanced C1-mediated retardation of the Op2a fragment.



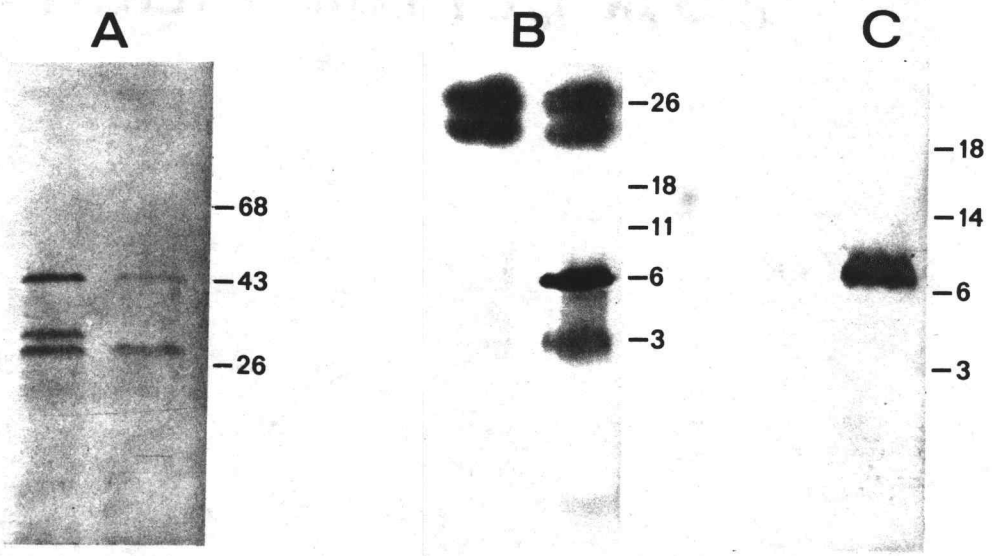
The effect of Coi protein on Bof-enhanced C1-mediated retardation of the Op2a fragment. The 136-bp Op2a fragment was incubated with combinations of C1, Bof and Coi extract at the concentration and order indicated in the figure. The reactions were performed under the conditions described in "Materials and Methods".

Figure IV.12. The effect of Coi protein on Bof-enhanced C1-mediated retardation of the Op2a fragment.



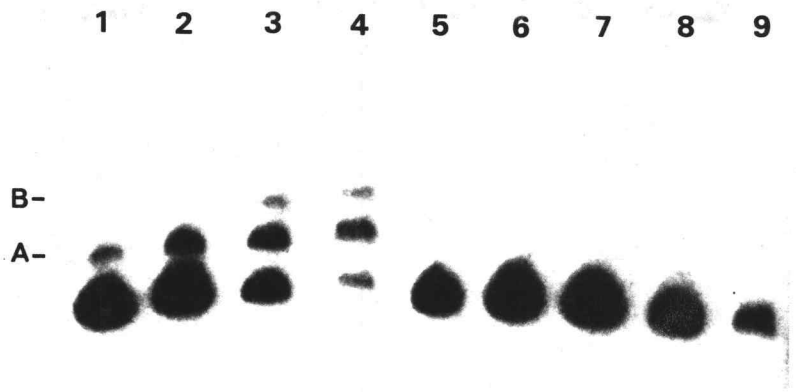
The effect of Coi protein on Bof-enhanced C1-mediated retardation of the Op2a fragment. The 136-bp Op2a fragment was incubated with combinations of C1, Bof and Coi extract at the concentration and order indicated in the figure. The reactions were performed under the conditions described in "Materials and Methods".

Figure IV.13. C1 and Bof proteins synthesized *in vitro*.



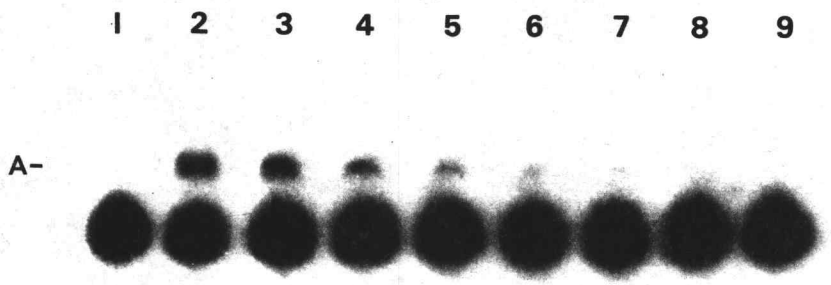
C1 and Bof proteins synthesized *in vitro*. Translation products were analyzed by 12.5% (C1) or 20% (Bof) SDS-polyacrylimide gel electrophoresis followed by autoradiography. A) *In vitro* synthesis of C1 repressor. Lanes 1 and 2 are the proteins produced by transcription-translation of pTS500 or pTS500(B2), respectively, as described in "Materials and Methods". B) *In vitro* synthesis of Bof protein. Lanes 1 and 2 are the proteins produced by transcription-translation of pTS600 or pTS600(RI), respectively, as described in "Materials and Methods". C) Bof protein following ion-exchange chromotography (DEAE) and concentration by a Centricon 10 microconcentrator as described in "Materials and Methods".

Figure IV.14. Retardation of Op72a(b) operator fragment with C1 protein produced *in vitro*.



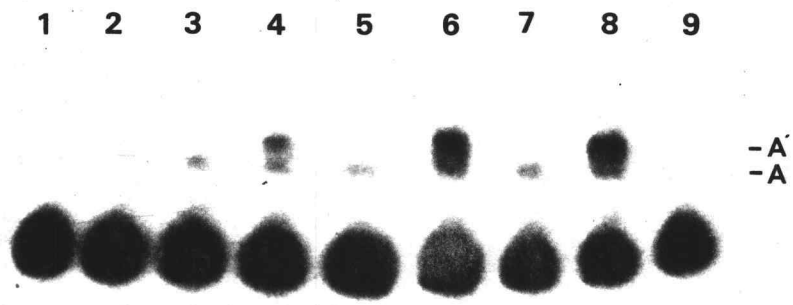
Retardation of Op72a(b) operator fragment with C1 protein produced *in vitro*. The 329-bp operator fragment containing Op72a(b) was incubated with translation products produced as described in "Materials ans Methods". Proteins were used directly or diluted with buffer A as indicated in []. Lanes 1-4 were produced from pTS500 (Lane 1 [1:8]; lane 2 [1:4]; lane 3 [1:2] lane 4, undiluted). Lane 5 contained no proteins. Lanes 6-9 were produced from pTS500 which had been restricted with BglII[pTS500(B2)] prior to translation (lane 6 [1:8]; lane 7 [1:4]; lane 8 [1:2] lane 4, undiluted). After incubation, the reactions were separated by polyacrylimide gel electrophoresis.

Figure IV.15. Retardation of Op2a operator fragment by C1 repressor synthesized *in vitro.*



Retardation of Op2a operator fragment by C1 repressor synthesized *in vitro.* The 136-bp operator fragment containing Op2a was incubated with pTS500-directed translation products produced as described in "Materials and Methods". Translation products were used directly or diluted in buffer A. Lanes 1 and 9 contain no proteins. Lane 2 is undiluted (5ul). Lanes 3-8 are a series of 2-fold serial dilutions in buffer A (each 5ul); lane 3 (1:2), lane 4 (1:4), lane 5 (1:8), lane 6 (1:16), lane 7 (1:32), and lane 8 (1:64). The reactions were incubated and separated by polyacrylimide gel electrophoresis.

Figure IV.16. Bof enhancement of C1-mediated retardation of Op2a-encoding DNA fragment.



Bof enhancement of C1-mediated retardation of Op2a-encoding DNA fragment. C1 and Bof were prepared *in vitro* as described in "Materials and Methods". Dilution factors of C1 are indicated in brackets. Bof, when present, is 1ul (undiluted). Lanes 1 and 9 contain no proteins. Lane 2 contains Bof only; lane 3 contains C1 only [1:4}; lane 4 contains C1 [1:4] and Bof; lane 5 contains C1 [1:2]; lane 6 contains C1 [1:2] and Bof; lane 7 contains C1 [0]; lane 8 contains C1 [0] and Bof. Following electrophoresis, the gel was dried and autoradiographed using two pieces of film to shield the signal generated by ^{35}S.

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CHAPTER V. Conclusions

* For the sake of simplicity, in this discussion, the respective halves of the psuedo-dyad operators will be referred to as primary and secondary half-sites. The primary half-site is defined as the cannonical C1 binding site located on the coding-strand and the one whose sequence most closely matches the derived C1-binding site. The secondary site is located on the opposite strand and overlaps the primary site by 6 nucleotides (Fig. I.3).

CONCLUSIONS

The P1 Bof protein appears capable of both positively and negatively regulating phage gene expression. Since Bof itself seems to interact with DNA rather feebly, its effect on gene expression seems to be mediated solely through the C1 repressor. Bof appears to play the role of a corepressor with C1 by increasing the affinity of C1 for operator sites; thus Bof acts as a negative effector of the expression of genes under C1 control. Since the *c*1 gene is autoregulated, Bof is also capable of indirectly increasing expression of *c*1-regulated genes, by lowering C1 repressor levels. The discovery of indirect enhancement of genes expression by Bof was pivotal in clarifying some paradoxical pleiotropic phenotypes associated with P1 *bof*$^-$ prophages. If the negative effect of Bof at a given gene (e.g. *ref*) is *greater* than the negative effect of Bof at *c*1, Bof will appear to be a negative regulator at the former gene. However, if the Bof-effect on the lytic gene (e.g. *bac-1 ban*) is *less*

than the Bof effect on *c*1, Bof will appear to be a positive regulator. Bof may, in fact, have no detectable effect at some C1-regulated genes (e.g. *ban*).

What is the role of Bof in P1 immunity? Unlike P1 c^+ lysogens, P1 *bof* $^-$ lysogens are "superimmune" to infection by the normally heteroimmune phage P7. P1*bof* $^-$ prophages are expected to produce more repressor than wild-type prophages. Since the C1 repressors of P1 and P7 are virtually identical (12)(7), the increase in C1 expected in P1 *bof* $^-$ mutants may simply influence the lytic-lysogenic decision by adding to repressor synthesed by the incoming P7 phage [a strain containing a single-(λ-*c*1) or multi-copy source of *c*1, in the absence of Bof, is immune to superinfection by P1(9) and P7(8)]. In elucidating the role of Bof in P1 immunity, one must also consider the genes of the ImmI region. Transcription of the *c4* antisense-RNA repressor, which downregulates expression of the *ant* gene, is itself regulated by a C1-controlled promoter-operator (p_{c4}-Op51)(1)(3). Depending on the relative sensitivites of the *c4* and *c*1 promoter-operators to Bof, Bof could effect the expression of *c4* (and thus indirectly of *ant*) either positively (indirectly) or negatively (directly). If the p_{c4}-Op51 operator-promoter of an incoming P7 phage, were strongly C1-repressed only in the presence of Bof, then even the increased preexisting cellular C1 expected in the absence of Bof protein would be insufficient to fully repress *c4* expression. Thus there would be better antagonism of P7 *ant* synthesis, and less chance of a decision in favor of lytic development. This scenario, in which an increase in *c4* synthesis by P1 *bof* $^-$ lysogens leads to "superimmunity", is consistent with the observation that a multi-copy plasmid source of *c4*, in the

absence of C1 or Bof, confers "superimmunity" to a strain harboring it [cited in (8)]. Conversely, if p_{c4}-Op51 were well repressed by C1 even in the absence of Bof, then the increased C1 in *bof*$^-$ lysogens would be expected to result in low *c4* expression and concommitantly increased antirepressor synthesis. Thus, Bof could contribute to P1 immunity by involvement in the regulation of components in both ImmI and ImmC. It will be necessary therefore, to determine the sensitivity of p_{c4}-Op51 to Bof in order to elucidate the role of Bof in P1 immunity.

The hypothesis that concerted action by C1 and Bof proteins is necessry for maximal regulation of certain P1 genes may explain another P1*bof*$^-$ phenotype. The lower temperature threshold for induction of a P1*c*1.100 *bof*$^-$ prophage (compared to a P1*c*1.100 *bof*$^+$ prophage) was interpreted by Velleman *et al.*(11) to mean that Bof protein interacts with the temperature-sensitive C1.100 repressor resulting in a more stable repressor. In the presence of Bof, the C1.100 repressor may be capable, at intermediate temperatures, of sufficient repression of lytic genes to maintain lysogeny. However, the increase in levels of C1.100 repressor protein expected in a P1*c*1.100 *bof*$^-$ double mutant cannot compensate for the absence of Bof protein, because of its thermolability. Thus derepression of lytic genes and induction of the prophage occurs.

What makes an operator Bof-sensitive (requiring Bof for maximal C1-mediated repression) or Bof-insensitive (regulation independent of Bof)? It has been suggested that the sensitivity of an operator to Bof is due to the presence of an additional sequence outside of the canonical C1 site (11). In many operators there are two to

six T-residues, beginning two nucleotides downstream of the C1 consensus sequence (11). The average T-stretch exceeds four for 16 primary operators analyzed (data not shown). The T-stretch hypothesis has been strengthened by DNase I "footprint" data showing that the addition of Bof protein, at some operators, extends the C1-protected region in this downstream direction (Chapter IV). However, there is little other evidence to associate this T-stretch with Bof action. In fact, several observations are inconsistent with this model. The study of the regulation of initiation of transcription of the mutant *ban* promoter (*bac-1 ban*) *in vivo* (2) suggests that the sequence of the C1 operator itself, not downstream elements, is most important in determining the Bof-dependence of C1 binding. The *bac-1 ban* mutation results in an operator which is intrinsically less sensitive to C1 repressor than is the wild-type *ban* operator, but at $p_{\text{bac-1 ban}}$ Bof enhances C1-mediated repression about as well as it enhances C1-mediated repression at p_{ref} and p_{c1}. The Bof-sensitive *bac-1 ban* and Bof-insensitive *ban*$^{+}$ operator differ only by a single nucleotide change in the primary C1-binding half-site*, and there are no differences between the putative T-stretch regions. The Bof-insensitive *ban* operator may actually have been transformed into one that is Bof-sensitive by the single change in the primary half-site (which decreases the ability of C1 repressor to bind). Alternately, Bof may actually be able to act at the wild-type *ban* promoter as well as at *bac-1 ban*, but the high intrinsic affinity of p_{ban}-Op72 for C1 repressor may make Bof effects undetectable *in vivo*. Results obtained with the *ref* promoter-operator also weaken the hypothesis of the importance of the T-stretch in operator

sensitivity to Bof-mediated C1 repression. Repression at p_{ref}-Op2a by C1 is dramatically enhanced by the presence of Bof, yet the putative T-stretch region of this operator contains only three T residues (13). The sequence beginning two nucleotides downstream of Op2a is ATAATT. Thus the primary half-site has been implicated in making the effects of Bof at an operator detectable, but there is no compelling experimental evidence to support the relevance of the T-stretch in Bof operator-sensitivity. If, in fact, a sequence outside the C1 operator site proves important for determining operator Bof-sensitivity, T and A may be equally important for this putative site, as observed at Op2a. In many instances, C1 operator sites are embeded in regions rich in A and T residues; thus A-T sequences are likely to be downstream of the operator site.

Generalization of the notion that Bof acts at Op72 leads to a hypothesis that Bof protein is intrinsically capable of interacting at all C1 operator sites. The detectability of a Bof effect *in vivo* would then depend on the innate affinity of C1 repressor for a given operator rather than some sequence outside of it. At operators with high affinity for C1, the presence of Bof would be redundant.

There are a number of pseudo-dyad C1 operators which bind two molecules of C1 repressor. In contrast to the case with wild-type Op72, C1 binding to both Op2b and Op99a is significantly enhanced by Bof. For Op2b binding was demonstrated using purified C1 and Bof proteins (Fig.IV.4 and IV.6). For Op99a Bof enhancement of C1 binding was demonstrated *in vitro* using purified proteins (11) and inferred from Bof effects on C1-mediated repression of a *c1::lacZ* fusion gene *in vivo*

[Chapter II]. The major difference among Op72, Op2b and Op99a is the extent of deviation of their respective half-sites from the consensus sequence. The number of mismatched residues in the primary half-sites ranges from none in Op72 to four in Op2b. Significant differences are observed at all three respective secondary half-sites, whose number of mismatches ranges from two in Op72 to seven in both Op99a and Op2b. There is a good correlation between the number of deviations from consensus and the sensitivity of the operator to Bof. The data for binding of C1 to Op2b and Op99a suggest that the ability of an operator to bind a second molecule of C1 at its secondary site depends not so much on the actual sequence of the secondary site, but on its close association with another C1-binding site. The Op72 operator contains the fewest total mismatches, none in the primary half-site, and is phenotypically Bof-insensitive. In contrast, the Bof-sensitive Op2b and Op99a operators contain significantly more mismatches than Op72 in both primary and secondary operators. The latter observation again supports the view that the operator site sequence is the important factor in determining the need for Bof protein for maximal C1 binding.

The three operators known to bind two molecules of C1 have now been studied *in vitro*. All three appear to bind a second molecule in a cooperative fashion, suggesting a protein-protein interaction between repressor molecules. As suggested above, a striking feature of Op2b and Op99a is their ability to bind two molecules of C1, despite a considerable deviation of the secondary half-site from consensus. In the case of Op2a and Op99a, it seems unlikely that the secondary sites, if

isolated, would be capable of repressor binding, to date, no single C1 operator sequence that contains more than five mismatches has been identified. Dissimilarity between the halves of the Op2b and Op99a operators suggests that the two repressor molecules bind to the operator half-sites nonequivalently, and that a cooperative interaction between repressor molecules is essential for the full occupancy. A recent study suggested that the two identical monomer molecules comprising a lambda phage repressor dimer bind to a "dyad" operator nonequivalently (asymmetrically) (6). In all six lambda C1 operators one half-site was always considerably closer to the consensus sequences than the other, just as with the Op2b and Op99a operators. A collection of artificial lambda O_{R1} operators was made each containing a single base substitution and tested for the ability to promote λ cI repressor binding. Alterations in the half-site closer to consensus greatly reduced lambda repressor binding, while changes in the nonconsensus half-site, for the most part, had little effect on repressor binding. Base-changes in the nonconsensus half-site, resulting in an operator closer to consensus, reduced repressor binding. Non-equivalent binding of repressor to the operator half-sites may also be the case at each of the P1 psuedo-dyad operators. The ability of purified C1 to interact with Op2b operator fragments resulting in two distinct species of retarded fragments suggested that occupancy of the primary half-site of Op2b by one C1 repressor molecule is followed by the cooperative binding of a second. This has previously been reported to be the case at Op72 and Op99a (2) (11). In the case of Op2b and Op99a, strong cooperativity might explain the ability of C1 repressor protein to bind even to their

highly aberrant secondary half-sites.

Genetic evidence suggests that even at the highly conserved Op72, C1 does not bind in a similar fashion to each of the half-sites. In all but one of twenty-seven spontaneous mutants that were constitutive for expression of the P1 *ban* gene (in the presence of the C1 repressor), the changes were in the primary half-site (2). If occupancy of both halves of Op72 by C1 is required for repression of *ban*, and C1 bound independently to each half-site with the same affinity, a more even distribution of mutations between the primary and secondary half-sites might have been expected. Thus, even in the case of Op72 operator, where the secondary half-site contains only two mismatches, the primary half-site appears critical for the binding of both C1 repressor molecules.

At Op2a, which contains a single C1 binding-site, the enhancement of C1-binding *in vitro* (gel retardation assay) is maximal when C1 repressor and Bof are equimolar. However, a different molar relationship between C1 and Bof may exist at pseudo-dyad operators such as Op99a. Using a gel retardation assay similar to the one employed above (Figs. IV.3-6), the binding of C1 and Bof to Op99a was analyzed (11). The complete shift of all operator fragment present to position 2 (corresponding to an operator with two repressor molecules bound) required C1 and Bof at a 2:1 molar ratio.

Incubation of Op99a (11) with various levels of C1 resulted in a single retarded band, thought to correspond to DNA with two molecules of repressor bound (position 2), as well as an unretarded band (no C1 bound). In the absence of Bof, no band

thought to correspond to operator DNA containing a single bound molecule of C1 repressor (position 1) was detected. However, at each level of C1 protein, the addition of Bof resulted in the appearance of a second band at position A, suggesting that Bof acts by causing a C1-Bof-operator complex to form. This result with Op99a is consistent with experiments with Op2b described above (Figs. IV.4 and IV.6) showing that the position A band becomes most prominent when Bof is present in equimolar or excess with respect to C1. Thus, Bof protein appears to influence the binding of C1 to the first half-site of pseudo-dyad operators to be occupied. Since the primary half-site of each psuedo-dyad operator is more consensus-like than the secondary half-site, it seems logical that the Bof-enhanced C1 binding occurs at the primary half-site.

Although the detailed mechanism of the Bof-C1 interaction remains to be determined, it seems useful to formulate a model of Bof-mediated C1 repression. The designation C1(Bof) will be used to describe a C1 molecule with increased operator affinity as a result of Bof action, and is not intended to specify any particular mechanism of C1-Bof interaction. Bof apparently has little inherent capacity to act as a specific repressor, and appears to act exclusively as a corepressor with C1. The presence of Bof protein dramatically increases the affinity of C1 repressor for both single and pseudo-dyad operators. At the majority of P1 operators, which contain only one C1 site, Bof simply enhances the binding of C1 to the single operator and thus Bof must be at least equimolar with C1, as observed. Pseudo-dyad operators could in principal bind two C1-Bof complexes, or bind C1(Bof)

at only one of the operator half-sites and C1 only at the other. I have argued above, on the basis of results presented here and previously reported data (2)(11), that Bof exclusively promotes C1 binding to the primary site. In the case of pseudo-dyad operators, the C1(Bof) bound to the primary (more-consensus-like) half-site seems to enhance the binding of a second C1 molecule to the secondary (less consensus-like), half-site. This is in agreement with the observation that full occupancy of Op99a by two repressor molecules requires a 2:1 C1:Bof mole ratio. In both classes of operators the result of Bof is equivalent; occupancy of the operator by C1 repressor is achieved at lower concentrations of repressor protein.

The formation of any model of Bof action would appear to be constrained by the following observations: (i.) Bof itself appears not to bind specifically to operator sequences, although a weak interaction cannot be ruled out (Figs. IV.9, IV.10, and IV.16). (ii.) There is no direct evidence for a C1-Bof heterodimer in solution; incubation of C1 and Bof together followed by sedimentation through a glycerol gradient, yielded no detectable C1-Bof complex (M. Velleman, personal communication)]. (iii.) The sequence of the C1 operator site itself, rather than sequences outside of it, appears paramount in determining whether Bof efects can be detected *in vivo*. (iv.) Bof seems intrinsically able to act at all C1 operators, including those such as p_{ban}, whose high affinity for C1 repressor makes a Bof-effect undetectable *in vivo* at physiological C1 concentrations.

The following model considers all of the points enumerated above. Bof is considerd to have *some* specificity for C1 operator sites. Bof protein alone, due to

its positive charge (pI 10.8), may have non-specific affinity for DNA [a slight band with reduced mobility was observed when Bof synthesized *in vitro* was incubated with Op2a (Fig. IV.16, lane 2)]. At high concentrations, purified Bof did slightly protect Op2b from DNase I digestion at regions abundant in A and T (Fig. IV.9 and IV.10). The AT-residues, which predominate in C1 operator sites, may provide a region to which Bof interacts weakly; weak Bof-operator complexes, although undetectable by gel-retardation or footprinting assays, could facilitate subsequent binding of C1 repressor. Thus there would be a Bof-C1-operator complex, as appeared to be the case when C1 and Bof proteins synthesized *in vitro* were incubated with DNA. This three-component complex might account for the enlarged operator region protected against DNase I digestion in the presence of Bof.

Alternatively, the first step might be formation in solution of a weak C1-Bof heterodimer, unable to withstand sedimentation in glycerol [see (ii.) above], but stabilized when bound to operator DNA. The C1 and Bof components of such a heterodimer could contact the operator DNA, or Bof might allosterically alter C1. In each case the affinity of a putative heterodimer for operator sites would be greater than that of C1 alone. If a heterodimer between C1 and Bof does exist, it would be of great interest to determine the means of the protein-protein interaction since C1 (5) and Bof proteins apparently contain no "dimerization" motifs [eg. leucine zipper (4)].

Two hypothetical models for Bof action have been presented. Given the limited biochemical information about Bof and C1 action other models cannot be excluded.

Future effort is needed to completely elucidate the mechanism of Bof interaction with C1 repressor. The concerted action of C1 and Bof proteins that enhances C1 repressor activity seems unique. Thus, studies of C1-Bof corepression and its involvement in the complex P1 regulatory circuitry, should be of interest not only to phage geneticists, but also to others involved in the study of multi-protein regulatory systems.

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