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The operator-early promoter regions of Shiga-toxin bearing phage H-19B

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Summary

Genes (*stx*) encoding Shiga toxins (Stx), major virulence factors in some pathogenic strains of *Escherichia coli* (STEC), are located in prophages of the λ family. Agents that induce prophages lead to high levels of Stx, suggesting a role for the prophage in *stx* expression. Activation of the phage regulatory cascade has been shown to contribute to Stx production and release. Therefore, repressor–operator interactions that maintain prophage repression appear important in regulating expression of a major bacterial virulence factor. To determine if the operators of an *stx*-bearing phage have distinctive features, we characterized the operator regions of H-19B, a lambdoid phage carrying *stx1* genes. H-19B mutants that grow in the presence of repressor (classically called virulent mutants) were selected and the mutations definitively identified the operators. The H-19B operators, as those in other lambdoid phages, comprise variations of an inverted repeat. Four repeats were identified in O_R rather than the three found in each of the operators of other lambdoid phages. Primer extensions identified the transcription start sites of P_R and P_{RM}, the two promoters in O_R regulated by repressor.

Introduction

The *stx* genes, encoding Shiga toxins (Stx), are carried on resident prophages in some, if not all, of the strains of *Escherichia coli* that produce Stx (STEC) (O'Brien *et al*., 1984; Huang *et al*., 1987; Mizutani *et al*., 1999). One subset of STEC, enterohaemorrhagic *E. coli* (EHEC), has been identified as one of the newly emerging pathogens (Kaper and O'Brien, 1998). Because of the release of Stx, EHEC infections can result in serious sequelae such as haemorrhagic colitis and haemolytic uraemic syndrome, which are caused by Stx. All of the phages identified as carriers of *stx* genes are members of the Lambda family of temperate phages (Karch *et al*., 1999; Mizutani *et al*., 1999).

For the most part, lambdoid phages share common regulatory schemes and genome arrangements. Genes having similar activities are located at analogous positions on the phage genomes, but genes having the same functional activity and genome position may have significant sequence differences. Highly conserved sequences scattered throughout these genomes allow recombination between the lambdoid phages, explaining why genome alignments reveal that the various lambdoid phage genomes appear to be mosaics generated by assortment from a common pool of genes (Susskind and Botstein, 1978; Campbell, 1988; Juhala *et al*., 2000).

After infection of an *E. coli* host, lambdoid phages can proceed via either the lysogenic or lytic pathway (Ptashne, 1992). Phages following the lysogenic pathway synthesize a limited number of functions that include repressor and integrase that turn off expression of most phage functions and integrate the phage DNA into the bacterial genome respectively. The integrated phage genome (the prophage) is thus replicated as a set of largely unexpressed genes on the bacterial chromosome. Phage following the lytic pathway express, in a regulated manner, functions required for replication, packaging and release of phage. The repressed prophage remains silent until the bacterial host existence is threatened by damage to its DNA; the resulting SOS response results in prophage induction (Little, 1995) and the induced prophage excises from the bacterial chromosome and enters the lytic pathway (Weisberg and Landy, 1983). Although λ and its family of phages have served for decades as tools for studying major questions in molecular biology, only relatively recently has it been found that some members of this family of temperate phages contribute significantly to the virulence of pathogenic *E. coli* (reviewed in Waldor, 1998).

Shiga toxins (Stx) are a family of closely related toxins that were first identified in *Shigella dysenteriae* (reviewed in O'Brien and Holmes, 1987; 1996; Acheson *et al*., 1991). Stx are composed of two subunits, a single A subunit that disrupts protein synthesis in eukaryotic cells by cleaving the 28S ribosomal RNA and a B subunit that forms a pentameric structure that facilitates entrance of the A subunit into the eukaryotic cell. Two major groups of Stx are found in STEC, Stx 1 and Stx 2, which are related by

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varying similarities to the toxin found in *S. dysenteriae*. Analysis of the DNA sequences (Neely and Friedman, 1998a; Plunkett *et al*., 1999) of *stx*-carrying phages H-19B (*stx1*) *and* 933 W (*stx2*) located the *stx* genes downstream of the phages' late P_R' promoters (Neely and Friedman, 1998b; Wagner *et al*., 2001) and upstream of the lysis genes (see Fig. 1). Subsequently, a search of *stx* and surrounding sequences indicated that, in all of the STEC strains examined, *stx* genes are carried either by complete or defective prophages (Mizutani *et al*., 1999; Unkmeir and Schmidt, 2000).

There are two primary ways induction of a prophage with *stx* genes located downstream of P_R' could lead to increased expression of *stx* genes. First, induction leads to replication of the prophage genome and, thus, to a significant increase in the copy number of the *stx* genes. Second, induction leads to transcription from phage promoters, including P_R'. When RNA polymerase initiating transcription at P_R' is modified by the Q gene product, there is high-level expression of downstream genes (see Fig. 1) (Roberts, 1992; Roberts *et al*., 1998), which would, when present, include the *stx* genes. Because of their location, the *stx* genes as well as the downstream phage lysis genes are part of the Q-dependent P_R' transcription unit (Fig. 1) (Neely and Friedman, 1998b; Wagner *et al*. 2001). A number of studies have shown that treatment of STEC with agents that induce prophages result in production and release of high levels of Stx (Kimmitt *et al*., 1999; Matsushiro *et al*., 1999; Zhang *et al*., 2000).

Based on these considerations and experiments with phages that carry *stx* genes, we have proposed that significant levels of *stx* are expressed and/or released from a subpopulation of the bacteria in which the prophage has been induced (Neely and Friedman, 1998b; Wagner *et al*. 2001). In view of the fact that an apparent mechanism for Stx release could not be identified (O'Brien and Holmes, 1987), we additionally proposed that expression of phage encoded lysis functions after prophage induction could explain how Stx production and release might be coordinated. Therefore, the nature of the operator–repressor interaction is likely to be an important consideration in evaluating regulation of Stx production and release in STEC infections.

Based largely on studies with λ (Ptashne, 1992), details of the design of lambdoid phage operator regions and the interactions of the operators with repressor have been elucidated. The λ operator regions, located on either side of the *c*I (repressor) gene, regulate transcription initiating from the P_L and P_R promoters. Each operator region has three copies of variations of a 17 nucleotide sequence with partial dyad symmetry, and each of the 17 bp sequences binds a dimer of repressor protein. Binding of repressor to the operator regions exhibits what is described as 'alternate pairwise cooperativity'; e.g. dimers at O_R1 cooperatively bind with dimers at O_R2. Dimers bound at O_R2 cooperatively bind with dimers bound at O_R3 when O_R1 is mutationally inactivated. In addition to repressing transcription from P_R, repressor also activates transcription from P_{RM}, the promoter used for transcription of the *c*I gene from the repressed prophage. Repressor bound at O_R2 stimulates transcription of the *c*I gene P_{RM}. Thus, in the prophage state, repressor bound at O_R1 and O_R2 represses transcription of lytic genes expressed from P_R and activates transcription of the *c*I gene expressed from P_{RM} (Gussin *et al*., 1983; Ptashne, 1992).

In this study, we report results of genetic and biochemical experiments demonstrating that sites identified using sequence comparison with other lambdoid phages are the operators of H-19B; similarly the product of the *c*I gene is proven to encode the repressor protein. We find that the organization of the right operator region of H-19B differs from that of λ, having four functional repressor binding sites in O_R rather than the three found in the λ O_R. Our studies also identify the P_R and P_{RM} promoters in the H-19B immunity region.

Results

Identification of operator sites and putative promoters

Based on our sequence data (Neely and Friedman, 1998a) and the extensive information on the nature of operator-promoter sites in other lambdoid phages (Ptashne, 1992), we identified sequences likely to be the O_L and O_R regions of H-19B and the associated P_L and P_R promoters (Fig. 2A). These sites resemble operator regions found in other lambdoid phages in their locations relative to the *c*I gene, and in having variations of a consensus sequence (Fig. 2A and C) consisting of an inverted repeat of 17 bp (as in the λ operators) with partial dyad symmetries that are typically separated by seven or eight bp (Ptashne, 1992).

The O_R region of H-19B (Fig. 2A), like those of λ and other lambdoid phages (Fig. 2B) has three inverted repeats, corresponding to O_R1, O_R2, and O_R3, that are spaced, respectively, eight and seven nucleotides apart. However, a fourth site, O_R0, having significant homology with the consensus H-19B operator sequence, is located downstream of the putative O_R1. The one bp spacing between O_R1 and O_R0 is unusual. The H-19B O_L region has three repressor binding sites as does the O_L of λ and other lambdoid phages. It differs from λ in the spacing between the O_L2 and O_L3 inverted repeats, 15 bp in H-19B rather than the 7 bp in λ.

Strategy for isolation of virulent mutants of H-19B

The genetic identification of the operator regions of H-19B

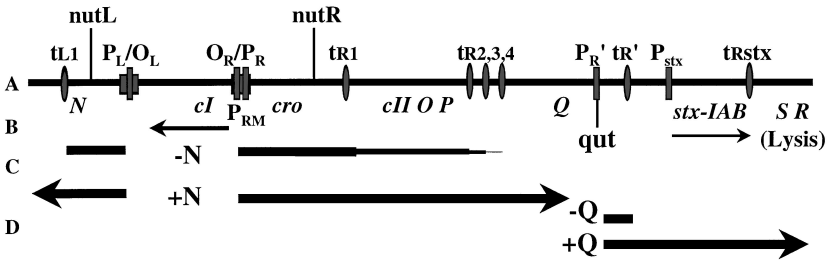

Fig. 1. Arrangement of regulatory signals, genes, and transcription patterns in the early region of H-19B.
A. Above and within the line are shown locations of promoter, operators, *nut*, *qut*, and transcription terminators. Below the line are shown the locations of relevant genes.
B. Transcription from the P_{RM} promoter, which is responsible for expression of maintenance levels of repressor protein and P_{STX}.
C. Transcription from early P_L and P_R promoters in the presence and absence of N. The change in the thickness of the line indicates different levels of readthrough of transcription terminators.
D. Transcription from P_R' in the presence and absence of Q.

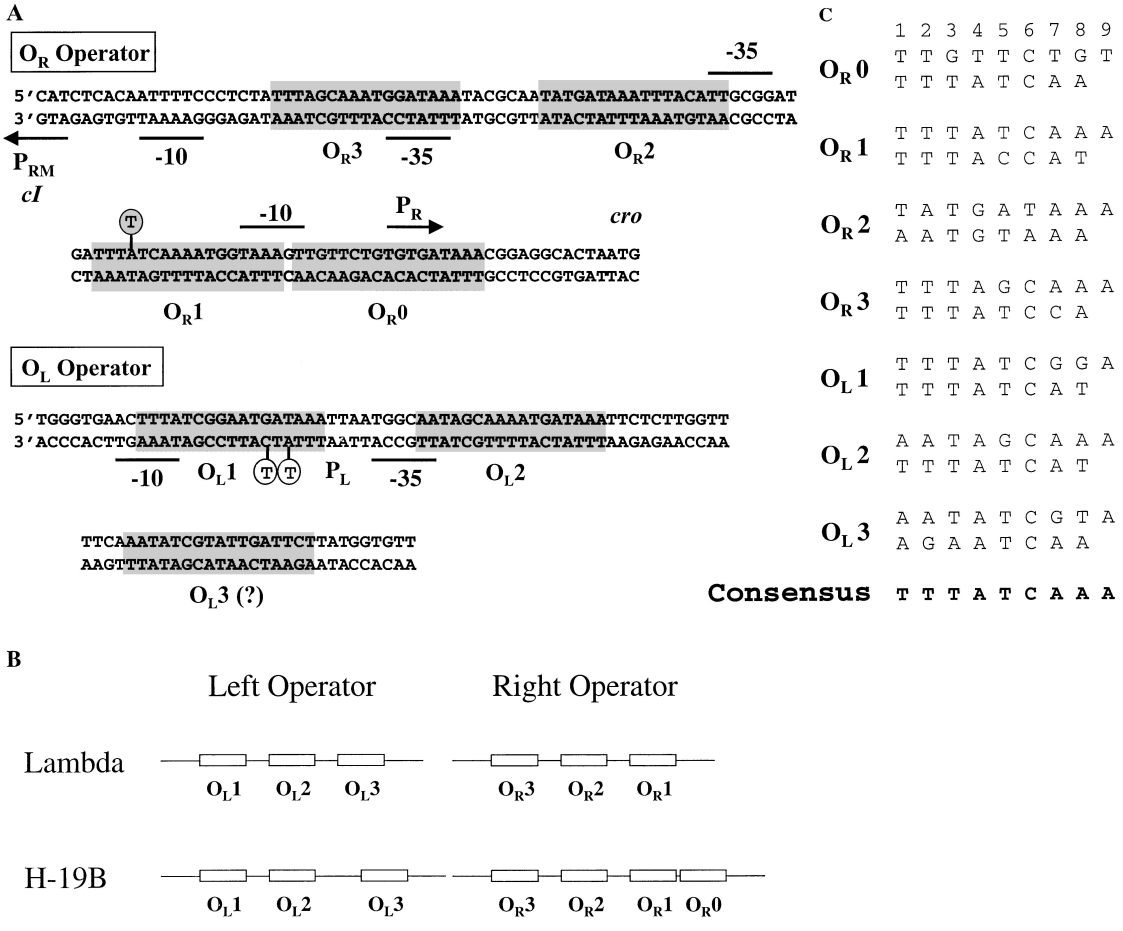

Fig. 2. A. Left and right operator sequences of H-19B. Highlighted are the imperfect inverted repeats corresponding to the consensus operator sequence that were definitively identified by DNase I protection studies. The arrows indicate the transcription start sites of the P_{RM} and P_R transcripts and point in the direction of transcription. The putative −10 and −35 regions of the P_{RM} and P_R promoters are indicated above and below the DNA sequence respectively. Nucleotide changes in the operators found in the H-19Bvir mutant are placed above and below the wild-type sequence.
B. Diagram showing the relative positions of the operator binding sites in the λ and H-19B operators.
C. Alignment of H-19B operator half-sites. The upper sequences are from the left half of the upper strands and the lower sequences are from the right half of the lower strands. Shown at the bottom is the derived consensus operator site sequence.

was based on the studies that identified the operators of λ (reviewed in Ptashne *et al*., 1980; Gussin *et al*., 1983; Ptashne, 1971; 1992). In those classic studies, a λ mutant that grew in the presence of repressor was isolated as a variant that formed plaques on a lawn of a λ lysogen (Jacob and Wollman, 1954). Subsequently, it was shown that this mutant, called virulent (*vir*), had mutations in sites (operators) on either side of the *c*I gene. It should be noted that this use of the term virulent (as well as the term virulence) should be distinguished from its use to describe the pathogenic nature of infectious bacteria. Hopkins and Ptashne (1971) determined that three of the four mutations initially identified in this virulent mutant were necessary for virulence. The mutations, two in O_R and one in O_L, reduce repressor binding at those sites and thus permit sufficient transcription from P_R and P_L in the presence of repressor to support phage growth. Transcription from P_R is required for expression of nearly all genes whose products are involved in lytic growth, whereas transcription from P_L is required for expression of N, which is required for transcription initiating at P_R to proceed through transcription terminators into downstream genes (Fig. 1). Because H-19B has a gene arrangement in this region similar to that of λ (Neely and Friedman, 1998a), we adopted a working model that H-19B virulent mutants would require at least two mutations reducing repressor binding, one in O_R and one in O_L. Based on these assumptions, we used a two-step strategy for isolating virulent mutants of H-19B; selecting first for a mutation(s) in O_R and then for a mutation(s) in O_L.

Isolation of virulent mutants of H-19B

In selecting mutations affecting repressor binding at O_R, we assumed that, as in λ, the only function expressed from P_L required for H-19B lytic growth would be N (Court and Sato, 1969; Sly *et al*., 1971). The selecting bacterium, K9368, has an H-19B prophage that blocks growth of an infecting H-19B and, because it carries the *plac*-N(H-19B) plasmid, constitutively expresses H-19B N. If our assumption that transcription from P_L is only needed to supply N is correct, then an H-19B variant with a mutation reducing binding of repressor at O_R, thus freeing P_R from repressor control, should grow in K9368. Although repressor will bind at O_L and block transcription from P_L, the N supplied from the plasmid will obviate the need for P_L transcription. A directed mutagenesis strategy was used to create a pool of H-19B enriched for mutations in the O_R region (see *Experimental procedures*). This pool was used to isolate an H-19B derivative that forms plaques on a lawn of K9368. As expected, this mutant phage is not fully virulent because it is unable to form plaques on K7523, an H-19B lysogen that does not provide H-19B N. DNA sequencing of the immunity region revealed that this partially virulent phage has a single mutation in O_R1 (see Fig. 2A). The mutation, an AT to TA transversion in the fourth position, was named O_R1A4T. The phage carrying the O_R1A4T mutation was named H-19BvirR-1.

To begin the selection for full virulence, H-19BvirR-1 was grown in the mutD5 (Fowler *et al*., 1974) strain, K7045. The resulting mutagenized lysate was used to obtain fully virulent H-19B by selecting for derivatives of H-19BvirR-1 that form plaques on an H-19B lysogen, K7523. DNA sequencing of the operator region revealed that in addition to the O_R1A4T mutation identified in H-19BvirR-1, this fully virulent phage has two mutations in O_L1 (see Fig. 2A). The changes are an AT to TA transversion at position four (the same as the O_R1A4T mutation) and a CG to TA transition at position six. These mutations were named, respectively, O_L1A4T and O_L1C6T.

We next considered whether the acquisition of the two mutations in O_L was fortuitous or was required for full virulence. Using site-directed mutagenesis, we constructed derivatives of H-19BvirR-1 that contained either O_L1A4T or O_L1C6T (see *Experimental procedures*). As shown in Table 1, a fully virulent derivative could be constructed from H-19BvirR-1 that also carried either of the two changes in O_L found in the original fully virulent mutant. H-19B, unlike the classical λ virulent mutant

Table 1. Plating of H-19B virulent mutants and effectiveness of H-19B CI proteins.

Strain	Bacteria Source of H-19B CI	Efficiency of plating of listed phages H-19B	H-19Bvir[a]	H-19Bvir1[b]	H-19Bvir2[c]
K3443	No CI	1	1	1	1
K9866[d]	H-19B Prophage	$< 10^{-7}$	1	1	1
K9153[d]	Cloned H-19B*cltrc*[e]	$< 10^{-7}$	1	1	1
K9710[d]	Cloned H-19B*c*[e]	$< 10^{-7}$	1	1	1

a. Virulent mutant with oR1A4T, oL1A4T, and oL1C6T mutations.
b. Virulent mutant with oR1A4T and oL1A4T mutations.
c. Virulent mutant with oR1A4T and oL1C6T mutations.
d. Isogenic with K3443 except for indicated added genome.
e. Expressed CI proteins were His tagged.

(Hopkins and Ptashne, 1971), only requires two mutations for virulence, one in O_L1 and one in O_R1.

Cloning and expression of the H-19B full size and a truncated repressor

The H-19B *c*I-repressor gene was cloned in an expression vector so that the expressed CI protein has a polyhistidine tail that facilitates isolation of repressor. Previous work from this laboratory, using fragments from the H-19B genome cloned into a plasmid vector, identified a 4.3 kb fragment that conferred the same immunity characteristics as an H-19B prophage (Neely and Friedman, 1998a). *Escherichia coli* carrying the plasmid with this fragment are immune to infection with H-19B, but do support growth of other lambdoid phages. Based on this result, we identified the putative gene encoding the H-19B CI repressor and cloned the putative *c*I gene downstream of the T5 promoter and in-frame with a sequence encoding a poly histidine tail in pQE-60 generating plasmid pQE-H-19BcI. An H-19B *c*I gene truncated at the 3′ end was similarly cloned in pQE-60 generating pQE-H-19BcItrc. The expressed truncated repressor protein, CI-trc, is missing the 55 carboxy-terminal amino acids of the full sized repressor protein. This protein was produced to assess the functional role of the carboxy portion of the CI protein.

Escherichia coli derivatives carrying plasmids expressing either the full-sized or truncated CI protein fail to support growth of infecting H-19B, showing that the His-tag does not substantially interfere with the biological activity of either of these H-19B repressor proteins, and that the truncated CI protein is functional (Table 1). The fact that the truncated protein has repressor activity indicates that, like repressors of other lambdoid phages (Ptashne, 1992), the DNA binding domain of the H-19B repressor is probably located in the amino portion of the repressor protein.

Gel mobility shifts with O_R DNA

Gel mobility shift assays with full-size repressor protein demonstrate that H-19B repressor binds to the region of the H-19B genome identified by sequence analysis as the right operator and the site is specific for that protein (Fig. 3A). Lanes 1–6 show gel mobility shifts with a labelled 240 bp fragment containing the four putative O_R operator sites from a wild-type operator. With increasing concentrations of repressor, bands are observed at three positions. Although the identification of bands at three positions in the gel is consistent with three operator sites in O_R, we show below in the footprint analysis that there is a fourth potential repressor binding site in O_R. Presumably, the strength of binding at this site is insufficient to be maintained during electrophoresis.

Gel mobility shift assays were also used to examine binding of repressor to a variant of the O_R DNA fragment with the O_R1A4T mutation identified in the H-19B virulent mutant described above. A higher concentration of repressor is required to shift the mutant probe, suggesting that the O_R1A4T mutation reduces the affinity of O_R for repressor (compare lanes 2, 3 and 4 with 10, 11 and 12). The gel mobility shifts also indicate another way that binding to the mutant probe differs from binding to the wild-type probe. At low repressor concentration, a single band is observed initially at position 1 (lanes 11 and 12) and at higher concentrations bands are observed at positions 1 and 3, but not at position 2 (lanes 13 and 14). This suggests that even though binding at O_R1 is weakened by the O_R1A4T mutation, at higher repressor concentrations either the mutant O_R1 or the putative fourth O_R site, O_R0 participates in binding of repressor.

To determine if binding at O_R is specific for the H-19B CI protein, we determined if two other proteins, λ repressor (lane 7) and BSA (lane 8), would shift the probe. Both of these proteins failed to shift any of the probe. Therefore, we concluded that the H-19B O_R region specifically interacts with H-19B repressor.

Gel mobility shifts with O_L DNA

Gel mobility shift assays demonstrate that the region of the H-19B genome identified using sequence analysis as the left operator specifically binds H-19B repressor (Fig. 3B). Lanes 1–6 show band shifts with a labelled 200 bp fragment containing wild-type O_L operator sites in the presence of twofold increases in repressor concentration. Two bands are observable suggesting that O_L has two binding sites. However, as discussed below, the DNase I protection studies are consistent with a third operator site in O_L. As was discussed above for the fourth site in O_R, we assume the strength at the third site in O_L of binding is insufficient to be maintained during electrophoresis.

Lanes 9–14 show gel mobility shifts with an O_L variant probe that is identical to the wild-type O_L probe used above except that it contains the O_L1A4T and O_L1C6T mutations identified in the H-19Bvir mutant described above. Only one band is observed, even at repressor concentrations that shifts all of the probe. This suggests that the O_L mutations eliminate binding at O_L1. Moreover, the repressor concentrations required to shift the mutant probe are slightly higher than those required to shift the wild-type probe. This data also suggests that O_L has two repressor binding sites rather than the three predicted by the sequence analysis. However, as reported below, the DNase I protection studies provide evidence that there are three repressor binding sites in the H-19B O_L region.

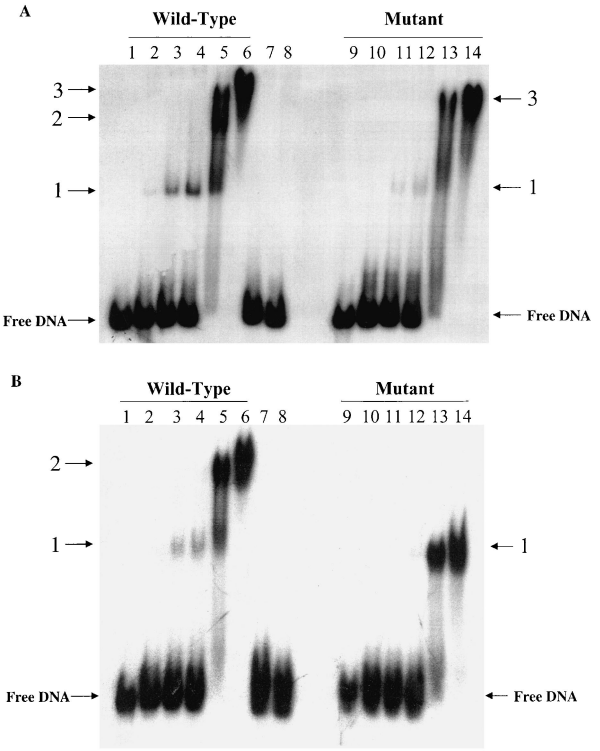

Fig. 3. Binding of H-19B repressor to operator sites as assessed using gel mobility shifts. DNA fragments containing either the O_R or O_L sites were end labelled with ^{32}P and incubated in the absence of repressor with twofold increases of concentrations (60, 220, 450, 900 and 1800 nM) of H-19B repressor or with control proteins (1500 nM). Reaction mixtures were analysed using electrophoresis in a non-denaturing polyacrylamide gel (see *Experimental procedures* for details). Arrows indicate positions of bands.
A. Probes were DNA fragments containing the O_R or a variation of the O_R sequence. The probe used in gel shifts shown in lanes 1–6 had the wild-type O_R sequence and the probe used in gel shifts shown in lanes 9–14 had a variant O_R sequence with the O_R1A4T mutation. Proteins used in gel shifts: lanes 2–6 and 10–14, H-19B repressor; lane 7, λ repressor; lane 8, BSA. Lanes 1 and 9, DNA probe incubated in the absence of protein.
B. Probes were DNA fragments containing the O_L sequence or a variation of the O_L sequence. The probe used in gel shifts shown in lanes 1–6 had the wild-type O_L sequence and the probe used in gel shifts shown in lanes 9–14 had a variant O_L sequence with the O_L1A4T and O_L1C6T mutations. Proteins used in gel shifts: lanes 2–6 and 10–14, H-19B repressor; lane 7, λ repressor; lane 8, BSA. Lanes 1 and 9, DNA probe incubated in the absence of protein.

DNase I protection of O_R

The nature of the repressor binding sites in the H-19B O_R region was examined by DNase I protection using purified His-tagged H-19B repressor and 240 bp ^{32}P-labelled DNA fragments containing either the wild-type or the O_R1A-T mutant O_R regions.

Looking at protection of the top strand (Fig. 4A, lanes 1–7), the protected regions correspond to at least three operator sites as suggested by the gel mobility shift experiments, with a suggestion of protection above the O_R1 site, which we call O_R0 (see section below). However, because these sequences are located in the compressed part of the gel, it is not possible to assess the significance of this protection. Although there appears to be better protection of O_R1 and O_R2 at lower repressor concentrations than O_R3, effective protection of O_R3 requires, at most, a twofold higher concentration of repressor for protection (compare lanes 3 and 4).

A different DNase I protection pattern was observed with a labelled mutant DNA fragment with the O_R1A-T *vir* mutation (Fig. 4A, lanes 8–14). Looking at protection of the top strand, there is, as expected, reduced protection of O_R1. The protection at O_R2 does not appear to be different from that seen with the wild-type fragment, whereas protection at O_R3 was somewhat enhanced (compare lanes 4 and 11).

DNase I protection of the bottom strand is consistent with that seen with the top strand, except that the protection of the O_R0 site is now apparent (Fig. 4B). Protection at O_R0 is observed at repressor concentrations higher than those required for protection at O_R1 and O_R2. However, O_R0 is protected at repressor concentrations that are about the same as those required for protection of O_R3. As was seen with the top strand, the O_R1A-T mutation reduces protection at O_R1, does not appear to significantly affect protection at O_R2, and increases protection at O_R3. There also appears to be a reduction in protection of the O_R1A-T mutant fragment at O_R0.

DNase I protection of O_L

Results of the DNase I protection assays of ^{32}P-labelled 200 bp DNA fragments, containing either the wild-type O_L region or a variant with the O_L1A4T and O_L1C6T mutations, were consistent with the H-19B O_L region having the three operator sites shown in Fig. 2A. As with our analysis of O_R, the DNase I protection results differed from the gel mobility shift assays, which were consistent with two binding sites in O_L.

Looking at DNAse I protection of the top strand (Fig. 5A), the protected regions correspond to two operator sites as suggested by the gel mobility shift experiments. Although an extended protected region immediately below the O_L1 site can be observed, protection above O_L2 expected for an O_L3 site cannot be observed. However, this is in the compressed part of the gel and evidence of protection may be obscured. As shown in Fig. 5A (lanes 7–12), the O_L1 mutations eliminate repressor binding at O_L1. However, in the absence of an effective O_L1 site, the protection indicates that O_L2 binds repressor with the nearly the same affinity as when O_L1 is intact, consistent with the results of the gel mobility shift assays.

Protection of the bottom strand (Fig. 5B), in addition to duplicating the identification of the O_L1 and O_L2, also identified the third repressor binding site in the H-19B O_L region at the position of the O_L3 sequence shown in

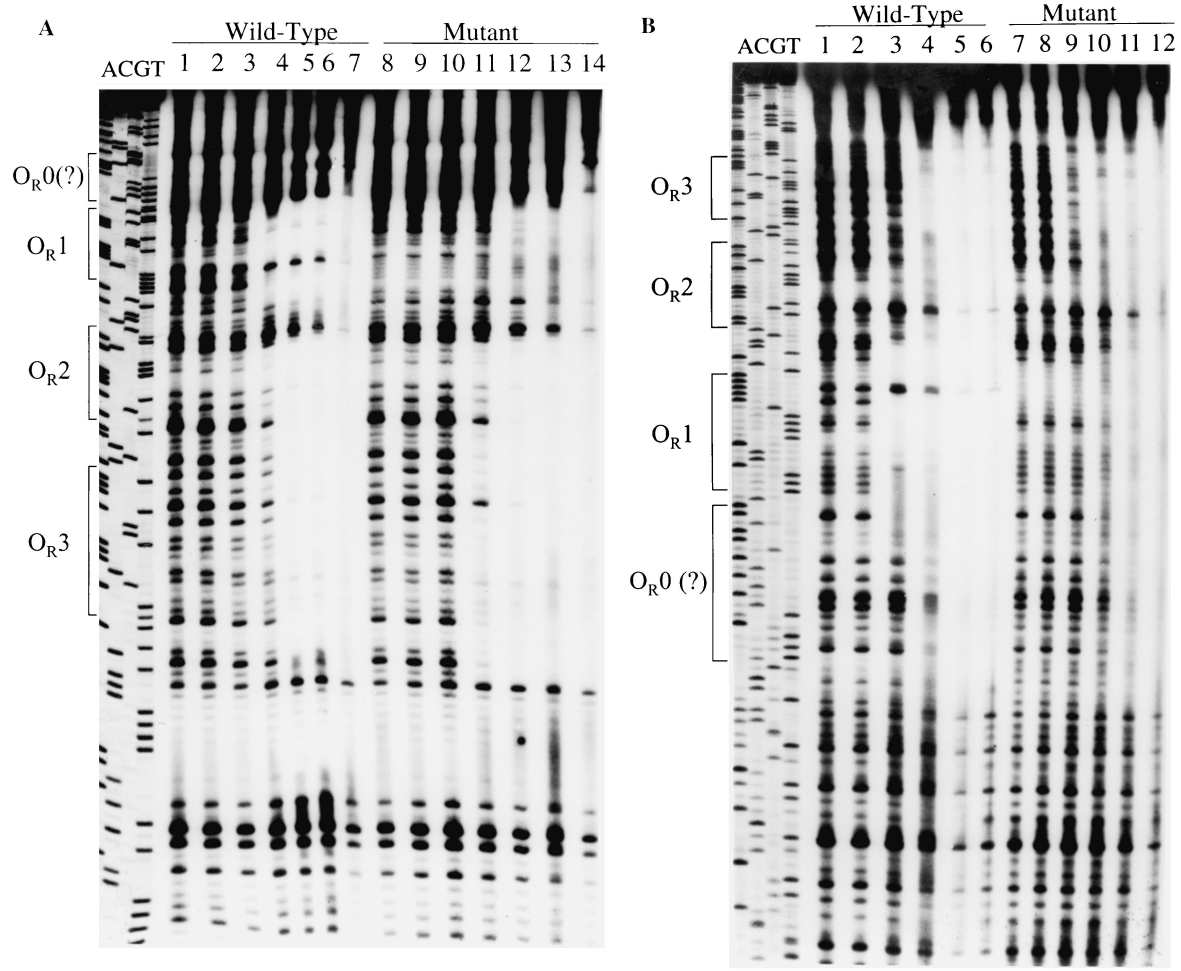

Fig. 4. DNase I protection experiment identifying repressor binding sites in the H-19B O_R region. Details of procedure are found in *Experimental procedures*. DNA sequencing ladders are shown on the left. The 240 bp ^{32}P-labelled DNA fragments contained either the wild type or the O_R1A-T mutant O_R regions. Protected sites are identified on the left.
A. Protection of top strand (corresponds to top strand of O_R in Fig. 2A). Lanes 1 and 8, cleavage without repressor, lanes 2–7 and 9–14, cleavage in the presence of twofold increases in repressor concentrations (80, 160, 320, 640, 1280 and 2560 nM). Lanes 1–7, the DNA fragment contained the wild-type O_R sequence; lanes 8–14, the DNA fragment contained the O_R1A4T mutation.
B. Protection of bottom strand (corresponds to bottom strand of O_R in Fig. 2A). Lanes 1 and 7, cleavage without repressor, lanes 2–6 and 8–12, cleavage in the presence of twofold increases in repressor concentrations (160, 320, 640, 1280 and 2560 nM). Lanes 1–6, the DNA fragment contained the wild-type O_R sequence and lanes 7–12 the DNA fragment contained the O_R1A4T mutation.

Fig. 2A. The binding at O_L3 appears to be different from that at O_L1 and O_L2; the binding is weaker and O_L3 has two hypersensitive sites not seen at O_L1 and O_L2. Moreover, the 15 basepair spacing between these sites differs from the 7–8 basepair spacing between the other operator repeats excluding the 1 basepair spacing between O_R0 and O_R1.

Binding of a truncated repressor protein

The DNA binding domains of repressors of other lambdoid phages are located in the amino domain of the protein. To determine if the DNA binding domain of the H-19B repressor is located in the amino portion of the protein, we assessed the effectiveness, *in vivo* and *in vitro*, of a truncated H-19B repressor protein missing its 55 carboxy-terminal amino acids. As discussed, when expressed from a plasmid, the truncated repressor is active *in vivo* (Table 1). To assess the action of the truncated protein, we used DNase I protection to examine repressor binding at the H-19B O_R region. The truncated repressor binding appears very similar to binding of the full-sized repressor (data not shown).

Identification of P_R *and* P_{RM} *promoters*

The identification of a possible additional operator site, O_R0 located between O_R1 and *cro* (Fig. 2A), a site without a homologue in λ, raised the question of whether the positions of other regulatory elements located in the H-19B

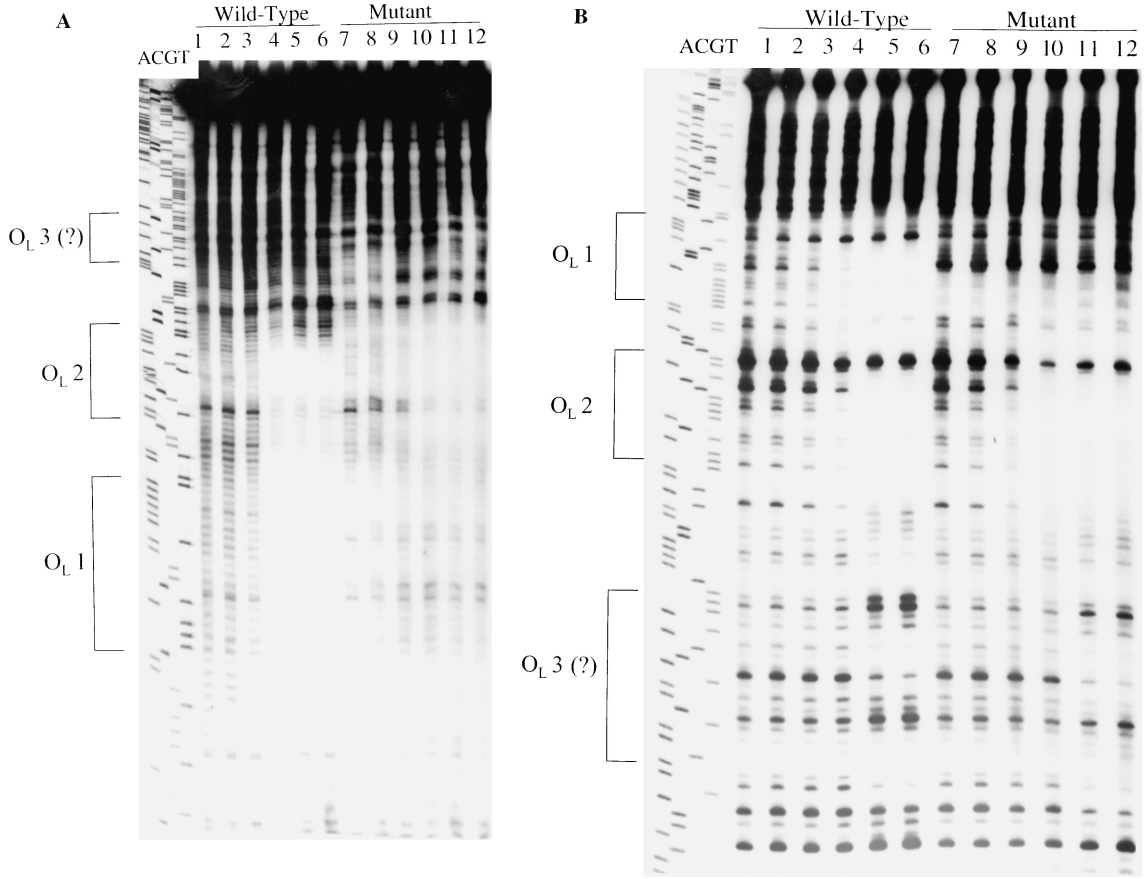

Fig. 5. DNase I protection with H-19B repressor identifying binding sites in the H-19B O_L region. Details of procedure are in *Experimental procedures*. DNA sequencing ladders are shown on the left. Lanes 1 and 7, cleavage without repressor, lanes 2–6 and 8–12, cleavage in the presence of twofold increases in repressor concentrations (160, 320, 640, 1280 and 2560 nM). Lanes 1 – 6, the DNA fragment contained the wild-type O_L sequence and lanes 7–12 the DNA fragment contained the O_L1A4T and O_L1C6T mutations. Protected sites are identified on the left.
A. Protection of top strand (corresponds to top strand of O_L in Fig. 2A.
B. Protection of bottom strand (corresponds to bottom strand of O_L in Fig. 2A).

O_R region differ from the positions of their λ homologues. Primer extension was used to locate the P_R and P_{RM} promoters (Fig. 6).

To identify the start site of the P_R transcript, RNA isolated after an infection of K37 with H-19B was used as template for the primer extension. The primer extensions (as shown in Fig. 6A) consistently produced a wide band covering four nucleotides, suggesting the possibility of multiple transcription starts at P_R and, obviously, making it difficult to identify a specific start site for P_R. However, based on the primer extensions, a sequence conforming to the consensus −10 sequence was identified at the O_R1 sequence (Fig. 2A). This −10 sequence is located at precisely the same position relative to O_R1 as is the λ P_R −10 sequence (Ptashne, 1992).

Primer extension was also used to identify the P_{RM} promoter. RNA isolated from K9949, the H-19B lysogen containing pImmH-19B, a plasmid with a 4.3 kb DNA fragment encoding the immunity region of H-19B (see *Experimental procedures* for details). As shown in Fig. 2A, the start site of transcription, like that of λ P_{RM} (Ptashne, 1992) is located at the ATG translation initiation codon of the *c*I gene. A sequence conforming to the consensus −10 sequence is located six nucleotides from this transcription start site (Fig. 2A); the same arrangement as observed for the λ P_{RM} promoter. However, the position of the putative H-19B P_{RM} differs from the position of the λ P_{RM}, in that the H-19B P_{RM} −10 element is located six nucleotides outside O_R3 (Fig. 2A), whereas the λ P_{RM} −10 is located partially within the terminus of O_R3.

Induction of prophage

To assess how the properties of the H-19B operator–repressor interactions influence induction of the H-19B prophage, we compared spontaneous and mitomycin C-induced phage production from H-19B and λ lysogens constructed from strain K37. As shown in Table 2, there is not a significant difference in the spontaneous or induced yields of phage between these lysogens.

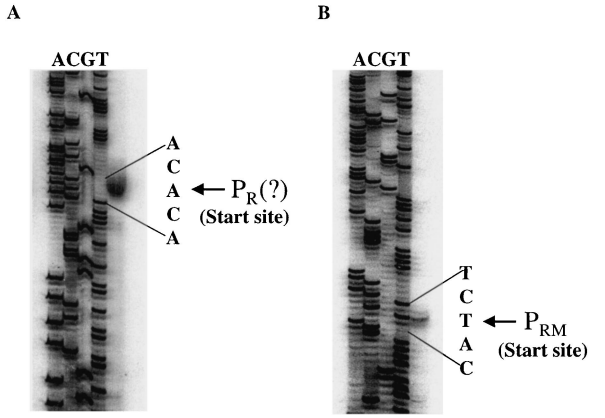

Fig. 6. Primer extensions identifying the start sites of transcription from the P_R and P_{RM} promoters. Details are outlined in *Experimental procedures*. DNA sequences were obtained using the same primers used to determine the transcription start sites. Arrows point to the start sites of transcription derived from the primer extension products.
A. Extension with a primer directed toward the P_R promoter. A precise band indicating the start site of transcription was not attained.
B. Extension with a primer directed toward the P_{RM} promoter.

Discussion

Largely through the studies from the Ptashne laboratory, the λ operator-repressor duo has served as the basis for understanding the regulatory scheme of lambdoid phages, referred to as a 'genetic switch' (Ptashne, 1992). This regulatory mechanism determines the pathway of phage development, lytic or lysogenic. Attention has focused on the right operator, which controls transcription from both P_R, the promoter directly or indirectly responsible for expression of nearly all genes encoding functions required for lytic growth, and P_{RM}, the promoter that maintains repressor expression in the lysogen (Johnson *et al*., 1981). Repressor binding at the operator sites also regulates its own expression by activating transcription from P_{RM} (Gussin *et al*., 1983). We have constructed an H-19B virulent mutant that differs from the classical λ virulent mutant in having one mutation each in O_L and O_R, rather than the two mutations in O_R and one mutation in O_L (Hopkins and Ptashne, 1971). Therefore, there appears to be a difference in the required mutations for virulence in λ and H-19B. A later study revealed that λ virulent mutants could be obtained that had either two mutations in O_R and one in O_L1 or one mutation in O_R and two in O_L2 (Flashman, 1978), indicating that virulence in λ requires three mutations, two in one operator and one in the other, but the two can be in either O_R or O_L. Studies with other lambdoid phages have defined the mutational requirements for virulence in O_R. P22 and HK022, like the classical λ virulent mutant, require two mutations in the O_R region (Poteete *et al*., 1980; Carlson and Little, 1993a), 434 and 21 require only one mutation in O_R (Poteete *et al*., 1980; Wharton *et al*., 1984).

Isolation of an H-19B virulent mutant coupled with repressor binding studies allowed us to definitively identify the operator regions and repressor (*c*I) gene of this phage. We have named the imperfect inverted repeat in the O_R region second from the *cro* gene O_R1 (Fig. 2A). Although the repeat closest to the *cro* gene is typically given that name, the inverted repeat we have named O_R1 has the functional characteristics of the O_R1 inverted repeats of other lambdoid phages (Bushman, 1993). In addition to being the site of a point mutation sufficient to eliminate effective repression at O_R, the O_R1 inverted repeat binds repressor more effectively than does the O_R0 inverted repeat (Figs 4 and 5). Moreover, our primer extension studies locate the H-19B P_R promoter in what we identify as O_R1 precisely as the λ P_R −10 is positioned relative to the λ O_R1 sequence. If this analysis is correct, the functional role, if any, for O_R0 is unclear.

The gel mobility shift experiments are consistent with the presence of two repressor binding sites in the O_L region and three in the O_R region. The DNase I protection studies identify the specific repressor binding sites within the operator regions: three in O_L and four in O_R. The fourth site, O_R0, located between O_R1 and *cro*, is observed in the DNase I protection of the bottom strand. The fact that the protection observed in the bottom strand covers the entire O_R0 inverted repeat suggests that O_R0 is a bona fide repressor binding site. However, we cannot rule out the possibility that the repressor binding at O_R0 does not result from binding to DNA, but results from a protein–protein interaction with repressor bound at O_R1; this type of binding has been reported for the HK022 repressor (Carlson and Little, 1993a). A fourth operator site has been identified to the right of the *c*I gene of phage HK022 (Carlson and Little, 1993b). This operator site, O_{FR}, is also located to the *cro* side of O_R1 but, instead of being directly adjacent to O_R1, O_{FR} is separated from O_R1 by the *cro* gene. O_{FR} is biologically important because it is a necessary component along with a mutation in either O_R1 or O_R2 of some HK022 virulent mutants. However, in any comparison with HK022, it must be kept in mind that because of the peculiarity of antitermination in the early operons of HK022 (Weisberg *et al*., 1999), virulence in that

Table 2. Comparison of phage production by H-19B and λ lysogens.

	Phage ml^{-1}[a] produced by lysogens[b]	
Method of Induction	K9951 (λ)	K9774 (H-19B)
Spontaneous	7.6 (± 0.5) X 10^5	1.2 (± 0.1) X 10^5
Mitomycin C	5.2 (± 1.3) X 10^9	6.2 (± 1.8) X 10^9

a. See *Experimental procedures* for details. Numbers are average of three individual experiments. Standard deviations are shown in parenthesis.
b. Strains are lysogens of K37 containing the prophage identified in the parenthesis.

phage only requires elimination of effective repressor binding at O_R (Carlson and Little, 1993a). The added requirement for a mutation in O_{FR} for virulence is postulated to serve as a mechanism to reduce the chances of HK022 mutating to virulence.

The DNase I protection studies suggest that repressor binding at H-19B operators may be somewhat unusual. We briefly consider these results, bearing in mind that the results could be influenced by the tag located on the carboxy terminus of the repressor used in our studies. As discussed above, studies on λ, as well as other lambdoid phages, have focused on repressor binding at O_R regions because of the important regulatory activities occurring within those regions. In the O_R regions of these phages (e.g. λ, 434, and HK022), cooperative binding at O_R1 and O_R2 results in tight binding of repressor at those sites (Ptashne, 1992; Carlson and Little, 1993a; Koudelka and Lam, 1993), with substantially weaker binding at O_R3. Our DNase I protection studies suggest the possibility of a different mode of repressor binding in the H-19B O_R region. Although repressor binding appears to occur first at the O_R1 site, binding at the O_R2 and O_R3 sites occurs at only a slightly higher repressor concentration. Moreover, the strength of binding at O_R2 appears to be independent of repressor binding at O_R1. For λ, 434 and HK022, the binding at O_R1 and O_R2 stimulates *c*I transcription from P_{RM}, whereas binding at all three operators turns off transcription from P_{RM} (Hochschild *et al*., 1983;Cam *et al*., 1991; Bushman, 1993; Carlson and Little, 1993a). A different picture was observed with φ80 (Ogawa *et al*., 1988), one more closely resembling that which we observe with H-19B. Tightest repressor binding in each of the φ80 operator regions is to the second repeat: i.e. O_R2 and O_L2. Although this binding pattern differs from that of the λ paradigm, the following argument was raised suggesting that regulation of transcription from P_{RM} in φ80 might not differ from that in λ. Because repressor binding to O_R2 occurs before binding to O_R3, repressor binding at O_R2 could activate transcription from P_{RM} even if binding at O_R3 blocks transcription from P_{RM}. A similar role for binding at O_R2 could explain how transcription from P_{RM} could be regulated by repressor binding in H-19B.

In some lambdoid phages competition between P_R and P_{RM} for binding of RNAP could influence expression of transcription from P_{RM}. The distance between the two promoters would appear to be a significant factor in this competition. Promoter competition is observed between P_R and P_{RM} in lambdoid phages 434, in which the transcription start sites are separated by 66 bp, and P22, in which the transcription start sites are separated by 52 bp (Bushman, 1993; Strainic *et al*., 2000; Xu and Koudelka, 2000). Competition between P_R and P_{RM} for RNAP binding is not observed in λ, in which the transcription start sites are separated by 83 bp. However, binding of RNAP at P_R does modulate activity of P_{RM} (Hershberger and deHaseth, 1991; Fong *et al*., 1993). It is unlikely that competition for RNAP binding plays a role in regulating H-19B repressor expression because the start sites of the H-19B P_{RM} and P_R promoters are separated by ~90 bp (Fig. 2A), substantially greater than the separations between the P_{RM} and P_R promoters of other characterized lambdoid phages. However, we can not rule out the possibility that binding of RNAP to P_R modulates RNAP binding at P_{RM}.

The repressor used in these *in vitro* studies, although carrying a His-tag on its carboxy terminus, is active *in vivo*, conferring immunity from infection by H-19B. The *in vivo* studies also show that this immunity results from physiologically correct repressor action; although the bacterium expressing the His-tagged repressor fails to support infection with H-19B, it does support infection with H-19Bvir and lambdoid phages with immunities differing from that of H-19B. Therefore, although suggesting that the tag does not have a significant effect on repressor interactions, this conclusion must be tempered by additional findings with H-19B and λ repressors. First is our finding that a His-tagged repressor truncated by 55 amino acid residues at the carboxy terminus is also active *in vivo*. Thus, either the terminal 55 amino acids, missing in the truncated repressor protein, are not functionally important or our immunity assay does not provide a complete assessment of repressor interactions. Second is the finding that, when expressed from a plasmid, a mutant λ repressor with a defect in cooperative binding confers immunity to λ infection (Astromoff and Ptashne, 1995). Thus, some of the novel aspects of our findings could reflect the fact that the purified His-tagged repressor we used in these studies may not behave like wild-type repressor.

Any differences in the nature of the mechanisms controlling maintenance of repressor expression observed between H-19B and the λ paradigm is not reflected in the maintenance of repression as measured by spontaneous or mitomycin C-stimulated prophage induction.

Experimental procedures

Bacteria, bacteriophages and plasmids

Plasmids. pREP4 (Qiagen) is a derivative of pACYC that overexpresses *lac* repressor. pQE-60 (Qiagen) was used as the expression vector for the His-tagged H-19B CI proteins. This plasmid has a T5 promoter regulated by two *lac* operators followed by a multicloning region with a sequence encoding a six histidine tag at its 3′ end. plac-N(H-19B), a derivative of pGB2, was used to supply H-19B N (Neely and Friedman, 1998b). pQE-H-19B *c*I and pQE-H-19B*c*Itrc are derivatives of pQE-60 with the cloned fragments encoding the full-sized H-19B CI protein and a truncated H-19B CI protein missing the carboxy terminal 55 amino acids respectively (see

Table 3. Bacterial strains.

Strain	Relevant genotype/plasmid/prophage	Parent/original name	Source/reference
K37	Wild type	N99	This laboratory
K3443	Wild type	N100	NIH collection
K7045	mutD5	LE30	H. Echols, Fowler *et al.* (1974)
K7523	H-19B	C600	O'Brien *et al.* (1984)
K7878	pREP4	K3443	This study
K9153	pQE-H-19Bcltrc	K7878	This study
K9193	Δ(*recC ptr recB recD*)::P_{lac}-*bet exo kansbcB15 sbcC201*	KM20	Murphy (1998)
K9710	pQE-H-19Bcl	K7878	This study
K9368	H-19B/plac-N(H-19B)	K7523	This study
K9496	λattL int-redγ-N pL cI857 [Δcro-bio]	DY329	D. Court, Yu *et al.* (2000)
K9759	Δ(*recC ptr recB recD*)::P_{lac}-*bet exo kansbcB15 sbcC201* H-19B	K7523/P1transduction fromK9193	This study
K9774	H-19B	K37	This study
K9866	H-19B	K3443	This study
K9949	H-19B, pImmH-19B	K7523	This study
K9951	λ lysogen	K37	This study

below for details on their construction). pImmH-19B is a derivative of pBR325 with a 4.3 kb fragment that includes the immunity region of H-19B (Neely and Friedman, 1998a).

Phage. H-19B phage was obtained by mitomycin C induction of the H-19B lysogen K7523.

Bacteria. The relevant genotypes and sources of bacterial strains used in this study are listed in Table 3.

Media

LB (Luria–Bertani), TB (Tryptone) broth and plates and top agar have previously been described (Miller and Friedman, 1980).

Construction of H-19B c I *expression plasmids*

Polymerase chain reaction (PCR) was used to synthesize DNA fragments encoding either the full-sized or a truncated form of the H-19B repressor. The primers in the PCR reactions contained *Bam*H1 or *Nco*1 sites. The template was DNA isolated from the H-19B lysogen K7523. Standard cloning procedures were used to place the PCR products in the pQE-60 expression vector positioned so that the *c*I gene is transcribed from the T5 promoter. Primers used in constructing the fragment encoding the full-sized H-19B repressor were (5′-CCCGCGGATCCCGAACTTTTCAGCCA CTCCCTTGCCT-3′) and (5′-CATGCCATGGAAAACAAAGA TATTCG-3′). Primers used in constructing the fragment encoding the truncated form of the H-19B repressor were (5′-CGGGATCCTATGCCAAGAACTTTGATCATG-3′) and (5′-CATGCCATGGAAAACAAAGATATTCG-3′).

Phage techniques

Efficiency of plating and determination of phage titres have been described elsewhere (Friedman *et al.*, 1984; Mozola *et al.*, 1979).

Mutagenesis

Construction of H-19B derivatives with an O_R mutation. A recombination system based on the λ Red functions (Murphy, 1998) was used to enhance homologous recombination between a mutagenized collection of DNA fragments, created using PCR-generated random mutagenesis (Zhou *et al.*, 1991) and an H-19B prophage. K9759, the H-19B lysogen that can be induced to express λ recombination functions, was grown in LB made 2 μM in IPTG (isopropylthiogalactoside) prior to being made competent for electroporation. The template used in the PCR reactions was a DNA fragment containing the wild-type H-19B O_R region. The linear 1 kb collection of mutagenized PCR products were electroporated into the competent bacteria. Transformed bacteria were grown in LB for 3 h at 37°C and then for another 2 h in the presence of 2 μg ml^{-1} mitomycin C to induce the H-19B prophage. The resulting lysate was used to select a partially virulent phage with a mutation(s) in O_R. The mutant phage, H-19BvirR-1, was identified as a variant that

formed a plaque on K9368, an H-19B lysogen containing the N expressing plac-N(H-19B) plasmid.

Construction of a fully virulent H-19B. A lysate of the H-19BvirR-1 mutant propagated in the mutator strain K7045 was used to generate fully virulent H-19B mutants, isolated by their ability to form plaques on a lawn of the H-19B lysogen K7523.

Construction of fully virulent mutants with a single mutation in O_L. DNA fragments with only one of the two mutations in the O_L1 were generated by directed mutagenesis using splicing by overlap extension (Horton *et al*., 1990) to synthesize O_L DNA fragments with either the O_L1A4T or O_L1C6T mutations. DNA isolated from K7523, an H-19B lysogen, served as the template. Primers used for generating the O_L1A4T mutation were: (5′-ATAAAAACAACCGTTAAC GT-3′), (5′-TTTATCGGAATAATAAATTAATG-3′), (5′-CGTAA AGAGCTACATTTGGA-3′) and (5′-CATTAATTTATTATTCC GATA AA-3′).

Primers used for generating the O_L1C6T mutation were: (5′-ATAAAAACAACCGTTAACGT-3′), (5′-TTTATCGGAAT GAAAAATTAATGGC-3′), (5′-CGTAAAGAGCTACATTTG GA-3′) and (5′-GCCATTAATTTTTCATTCCGATAAA-3′). The PCR-generated DNA fragments with the O_L mutations were crossed onto the genomes of the partially virulent phage, H-19Bvir-1, using a variation of the method developed by Yu *et al*. (2000). Strain K9496 infected with the H-19Bvir-1 mutant was incubated on ice for 20 min to allow adsorption. The infected bacteria were then incubated at 37°C for 5 min, shifted to 42°C for 10 min (to induce expression of the λ recombination functions), put on ice and then made competent for electroporation. The constructed fragments with O_L mutations were electroporated into the competent bacteria. The bacteria were diluted into LB and grown at 37°C for approximately 3 h. Fully virulent H-19B mutants were isolated from the supernatants as phages that form plaques on strain K7523, an H-19B lysogen.

Isolation and purification of the H-19B CI repressor proteins

One litre cultures of bacteria, K9153 (truncated CI) or K9710 (full-sized CI), were grown in LB with vigorous shaking to an OD_{600} of ~0.7. IPTG was added to a final concentration of 1–2 mM to turn on expression of the T5 RNA polymerase, resulting in transcription of the cloned *c*I gene and production of CI protein. The incubation was continued for another 2–3 h. Harvested bacteria were resuspended in wash buffer (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl, 10 mM imidazole) and stored at −70°C until use. Bacterial suspensions were lysed using a French Pressure cell and the His-tagged derivatives of the H-19B CI protein were isolated using a Ni^{++} column as per the manufacturer's instructions (Qiagen).

Preparation of ^{32}P-labelled DNA fragments

Fragments (~200 bp in length) were generated by PCR using H-19B DNA as the template. The 5′ end of one of the primers was labelled using [γ-^{32}P]-ATP and T4 polynucleotide kinase (Life Technologies). The labelling reaction mixture, containing 10 pmol of primer, forward reaction buffer [70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 100 mM KCl and 1 mM 2-mercaptoethanol], 20 units of T4 polynucleotide kinase, 50 μCi of [γ-^{32}P]-ATP in a total volume of 50 μl, was incubated at 37°C for 30 min and then at 70°C for 10 min. The total labelling-reaction mix was added to a PCR reaction mix containing the second primer, and the combined mixture used directly in a PCR reaction to generate the end-labelled probe. The labelled probes, used in the gel mobility shift and DNase I protection assays, were purified using a PCR purification kit according to the manufacturer's directions (Qiagen). The primers used for generating the right operator (O_R) probes were: (5′-CCCA AGCTTACGCCAGATTCGA-3′) and (5′-CGGGATCCTCAC--CGGGATCCTCACTAGAAGCCTC). The primers used for generating the left operator (O_L) probes were: (5′-CCCAA GCTTGGGTGAGTAGTGCGT-3′) and (5′-CGGGATCCAGA TCTCCCTCAAATCTCTTTAT-3).

Gel mobility shifts

The procedure was performed essentially as previously described (Crawford *et al*., 1998) with only slight modifications. The binding reaction was carried out in a total volume of 20 μl of binding buffer containing 10 μg ml^{-1} salmon sperm DNA. The concentrations of H-19B repressor protein in the binding reaction mixtures varied by twofold increments that ranged from 60 nM to 1800 nM. The concentration of λ repressor and BSA in the control reactions was ~1500 nM 20 000 c.p.m. of ^{32}P-labelled DNA fragments was used in each reaction and the binding reaction was incubated at 37°C for 30 min. Samples were electrophoresed in a 6% non-denaturing polyacrylamide gel. The resulting gel was exposed to film with intensifying screen overnight.

DNase I protection

DNase I protection assay used the same binding buffer as DNA gel mobility shift assay. The binding reactions were performed as previously described (Crawford *et al*., 1998). The reaction mixture containing 14 μl of 5× binding buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% glycerol, 50 μg ml^{-1} BSA, 500 mM KCl), 7 μl of salmon sperm DNA (0.1 mg ml^{-1}), 1 μl of probe (70 000 c.p.m., ~15 ng of ^{32}P-labelled fragment) and the desired amount of H-19B repressor was made to a final volume of 70 μl with H_2O. After a 30 minute incubation at 37°C, 17 μl of H_2O, 11 μl of 50 mM $MgCl_2$, 2 μl of 50 mM $CaCl_2$, and 1 μl of DNase I (0.01 unit μ^{-1}l) were added to the mixture, which was gently mixed and incubated for 2 min at room temperature. DNase I digestion was stopped by adding 100 μl of stop solution (200 mM NaCl, 2 mM EDTA, 1% SDS) to the mixture, which was then extracted twice with equal volumes of phenol and chloroform: isoamyl alcohol (24:1) to remove the protein. The DNA fragment was precipitated with 1 ml 100% cold ethanol at −70°C for at least 1 h, collected by centrifugation, washed with 70% ethanol once and resuspended in 5–10 μl of formamide stop dye. The precipitated DNA fragment was electrophoresed on a denaturing polyacrylamide-urea (6.88 M) gel for ~3 h. The gel was dried for 1 h at 80°C and exposed to film at −70°C with intensifying screens overnight.

^{33}P-labelled sequencing ladders covering H-19B operator regions were run in parallel.

Primer extension

The procedure was essentially that described by Yu and DiRita (1999). The source of RNA for the primer extension to determine the start site of transcription from P_{RM} was K9949, an H-19B lysogen with pImmH-19B. Some modification was required in isolating the RNA for the primer extension to determine the start site of transcription from $P_{R.}$ In this case, bacteria infected with H-19B were used as the source of the RNA. An overnight culture of *E. coli* strain K37 grown in LB was diluted 1:100 into fresh LB medium and grown to log phase. The cells were collected by centrifugation, resuspended at one tenth volume in 0.01 M Mg^{++}, infected with H-19B at a m.o.i. of 10, and incubated on ice for 20 min to allow adsorption. The volume of the infected bacteria mixture was brought to 50 ml in LB, and the diluted bacteria were incubated with shaking at 37°C for 30 min. The infected cells were collected and treated according to the method outlined by Yu and DiRita (1999).

The sequence used as the primer (5′-TCTGGTCAAGATA GCCAACTGAAGTTTTCGCTAATGCGGC-3′) to determine the start of transcription of P_R was that of the sense strand beginning 60 bp downstream of the 5′ end of the *cro* gene. The sequence used as the primer (5′-GCTTATCGGCAAAGCTTG CATTAGTGTTACCCGCGATTTT-3′) to determine the start site of transcription from P_{RM} was that of the sense strand beginning 57 bp downstream of the 5′ end of the *c*I gene.

DNA sequencing

DNA sequences were obtained using the Thermosequenase kit (USB).

Phage production by lysogens

Overnight cultures of lysogens grown in LB broth were diluted 1:100 into LB broth and grown to an OD_{600} of ~0.45. One aliquot was immediately treated with chloroform, bacteria were sedimented by centrifugation and the supernatant assayed for phage titre. The second aliquot was made 4 μl ml^{-1} in mitomycin C per ml and incubated with shaking for an additional 2 h. The culture was treated with chloroform, sedimented by centrifugation and the supernatant assayed for phage titre.

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