Purification and Characterization of the Repressor of the Shiga Toxin-Encoding Bacteriophage 933W: DNA Binding, Gene Regulation, and Autocleavage

Astrid P. Koudelka, Lisa A. Hufnagel, and Gerald B. Koudelka

Department of Biological Sciences, University at Buffalo, Buffalo, New York

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The genes encoding Shiga toxin (stx), the major virulence factor of Shiga toxin-encoding Escherichia coli (STEC) strains, are carried on lambdoid prophages resident in all known STEC strains. The stx genes are expressed only during lytic growth of these temperate bacteriophages. We cloned the gene encoding the repressor of the Shiga toxin-encoding bacteriophage 933W and examined the DNA binding and transcriptional regulatory activities of the overexpressed, purified protein. Typical of nearly all lambdoid phage repressors, 933W repressor binds to three sites in 933W right operator (OR). Also typical, when bound at OR, 933W repressor functions as an activator at the P_{RM} promoter and a repressor at the P_r promoter. In contrast to other lambdoid bacteriophages, 933W left operator (OL) contains only two repressor binding sites, but the OL-bound repressor still efficiently represses P_r transcription. Lambdoid prophage induction requires inactivation of the repressor's DNA binding activity. In all phages examined thus far, this inactivation requires a RecA-stimulated repressor autoproteolysis event, with cleavage occurring precisely in an Ala-Gly dipeptide sequence that is found within a "linker" region that joins the two domains of these proteins. However, 933W repressor protein contains neither an Ala-Gly nor an alternative Cys-Gly dipeptide cleavage site anywhere in its linker sequence. We show here that the autocleavage occurs at a Leu-Gly dipeptide. Thus, the specificity of the repressor autocleavage site is more variable than thought previously.

Shiga toxins (stx) are the major virulence factors in enterohemorrhagic Escherichia coli infections, causing such diseases as hemorrhagic colitis, infantile diarrhea, and hemolytic uremic syndrome. In virtually all known Shiga toxin-encoding E. coli strains, the genes encoding Shiga toxins are carried on lambdoid prophages (8, 19, 31, 34, 45) as part of an operon whose activity is ultimately regulated by the bacteriophage repressor (32, 33, 46, 47).

Lambdoid phage genomes contain two operator regions, OR and OL, each of which includes promoters whose expression is controlled by the binding of the bacteriophage repressor to multiple binding sites found in each operator region. Efficient functioning of the genetic switch between lysis and lysogeny depends on the ability of the repressor to bind with different affinities to each of the individual sites within OR and OL (40). The repressor directs the establishment and maintenance of the lysogenic state by simultaneously repressing transcription of the genes needed for lytic phage growth and activating transcription of its own gene, the only gene needed for maintenance of the lysogenic state (40).

The E. coli O157:H7 strain EDL933 is considered to be the reference strain for disease-causing O157:H7 isolates. Incubating this strain with agents that trigger induction of resident prophages causes this strain to express the disease-causing stx2 gene product and to produce a lambdoid bacteriophage (38). Sequence analysis of the bacterial strain and the liberated bacteriophage reveals that the stx2 gene is carried by the lyogenic phage, 933W (37). The stx2 gene in bacteriophage 933W is part of an operon controlled by the bacteriophage P_r promoter (24, 46). In a lysogen, the expression of this operon, and consequently the toxin gene, is prevented by a strong transcription terminator. In lambdoid phages, this operon is expressed only in the presence of the phage-encoded antiterminator protein Q (7). Expression of Q is repressed via a regulatory cascade that is ultimately controlled by the binding of the phage repressor protein to its DNA sites. During lysogen induction, the repressor is inactivated, leading to expression of Q. Subsequently, the operon controlled by P_r is transcribed and, in the case of the stx-carrying phages, leads to expression of Shiga toxin.

Analysis of the sequences of the 933W left operator regions and the sequence of the 933W repressor gene reveal potentially significant deviations from the well-established lambda paradigm. First, whereas the other lambdoid bacteriophages contain at least three repressor binding sites in OR, sequence analysis suggested that bacteriophage 933W contains only one repressor binding site in this region (10, 38). Given the essential role that cooperative binding of repressor to two adjacent sites plays in regulating the bacteriophage's lysis-lysogeny decision (9, 17, 18), this observation suggests that the 933W phage may use a unique mechanism to regulate repressor occupancy of the sites at OR. Second, in all phages examined thus far, inactivation of the repressor protein occurs by autoproteolytic cleavage that is stimulated by the DNA damage-induced activated form of the host RecA protein (1, 23). The RecA-stimulated autocleavage of these proteins occurs precisely in an Ala-Gly or Cys-Gly dipeptide sequence that is found within a "linker" region that joins the two domains of these proteins (7). Lysogens bearing 933W are inducible by DNA-damaging...
agents (e.g., mitomycin C and certain antibiotics), suggesting that RecA also stimulates the autocleavage of this phage repressor (38). However, 933W repressor has neither an Ala-Gly nor a Cys-Gly dipeptide anywhere in its linker sequence. This observation suggests that the cleavage site sequence, the mechanism of autocleavage, or the effect of RecA on the 933W repressor may differ from that of the other self-cleaving proteins.

We overexpressed and purified the 933W repressor, characterized its DNA binding to both its natural and synthetic sites, and determined the site of repressor autocleavage. Our results show that contrary to earlier predictions, 933W O\textsubscript{r} contains two repressor binding sites. We also show that 933W repressor possesses a unique sequence at its autocleavage site.

**MATERIALS AND METHODS**

**Bacterial strains and DNA.** All plasmids were propagated in JM101 (30). 933W repressor was purified from the E. coli strain BL21(DE3)-pLyS\textsubscript{S} (Novagen, Madison, Wis.) bearing a plasmid that directs its overexpression (see below). Binding site and transcription template DNAs were generated by PCR from plasmids bearing the desired regions of the bacteriophage 933W genome constructed as described below.

A plasmid that directs the overproduction of 933W repressor, p933WR, was constructed by amplifying the region corresponding to the 933W repressor gene from the genomic DNA of the E. coli O157 strain EDL933, a 933W lysogen (38). Amplification of this DNA was carried out using primers (IDT Technologies, Coralville, Iowa) with the sequences GGAATTCCATATGGTTCAGAATGAA and CCAAGGATCCCTAAGGACCATCTAG and CCAATGTCGTCAAACGCGGGA. The resulting DNA was cleaved with HindIII and EcoRI and inserted into pUC18 (30) that had previously been cleaved with the same enzymes.

DNA bearing the 933W O\textsubscript{r} region was obtained by amplifying this region of the EDL933 genomic DNA using the primers CCACCAATGTCGTCGACCT and CCAATGTCGTCGACCT. The resulting DNA was cleaved with HindIII and EcoRI and inserted into pUC18 (30) that had previously been cleaved with the same enzymes.

**Purification of 933W repressor.** A saturated overnight culture of BL21(DE3)-pLyS\textsubscript{S} cells bearing p933WR was diluted 1:50 into 3 liters of prewarmed Luria broth supplemented with 100 \mu g of ampicillin/ml and 20 \mu g of chloramphenicol/ml. After 2 h of growth at 37°C, production of the 933W repressor was induced by adding 0.5 mM IPTG (isopropyl-\beta-D-thiogalactopyranoside) to the cultures. After an additional 5 h of growth at 37°C, the induced cells were harvested by centrifugation at 10,000 g for 10 min, and the cell pellet was suspended in 25 ml of lysis buffer (100 mM Tris [pH 7.5], 200 mM NaCl, and 10 mM EDTA) and protease inhibitors (5 \mu g of leupeptin/ml, 50 \mu g of benzamidine/ml, 10 \mu g of apotinin, 5 \mu g of pepstatin/ml, and 5 \mu g of TPCK [tosylphenylalanyl chloromethyl Ketone]). All subsequent procedures were performed at 4°C. Cells were lysed in a French press, and the resulting lysate was diluted to 100 ml with lysis buffer. Cellular debris was removed from the diluted lysate by centrifugation at 12,000 x g for 20 min. Polyclonal imine was added to the cleared lysate to a final concentration of 0.6%, and the precipitated nucleic acids were removed by centrifugation at 10,500 x g for 10 min. Ammonium sulfate was added to the resulting supernatant to a final concentration of 0.4 g/ml and centrifuged at 10,500 x g for 30 min to precipitate the repressor. The resulting repressor-containing pellet was dissolved in 15 ml of standard phosphate buffer (SPB\textsubscript{Ac}, \textsubscript{200}) containing 50 mM Na\textsubscript{2}PO\textsubscript{4} (pH 6.8), 200 mM NaCl, and 1 mM EDTA and dialyzed against three changes of 1 l of the same buffer. After removal of insoluble debris from the dialysate by centrifugation at 12,000 x g for 10 min, the protein was loaded onto a 1- to 10-cm carboxymethyl ion exchange column (Bio-Rad, Hercules, Calif.) equilibrated with SPB\textsubscript{Ac}, \textsubscript{200}. The elution was eluted from this column with a linear salt gradient from 0.2 to 1 M NaCl. Repressor-containing fractions were pooled, concentrated, and loaded onto a 1- by 50-cm Sepharose S200HR (Amersham Biosciences, Piscataway, N.J.) size exclusion column equilibrated with SPB\textsubscript{Ac}, \textsubscript{200}. The elution was monitored by determining the affinity of repressor for DNA binding under standard conditions.

Three changes of 20% glycerol, prior to freezing and storage at ~70°C. As judged by silver staining of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel of the purified repressor, the repressor is >98% pure. The overall yield of 933W repressor was ~10 mg/liter of bacterial culture.

**Gel mobility shift assays.** Gel mobility shift assays were performed essentially as described previously (6). DNA containing 933W repressor binding sites were obtained by PCR using pB933Wor or pB933Wol as templates and the standard forward and reverse M13 sequencing primers. Following isolation from agarose gels, the DNA fragments were radioactively labeled at their 5’ ends by incubating the DNA with [\gamma\textsuperscript{32P}]ATP (3,000 Ci/mmol) (Perkin-Elmer, Boston, Mass.) in the presence of T4 polynucleotide kinase (Epipcentre, Inc., Madison, Wis.). The labeled DNA was incubated with the specified concentrations of 933W repressor protein in binding buffer (10 mM Tris [pH 8.0], 50 mM KCl, 5% glycerol) for 10 min on ice. The protein-DNA complexes were resolved on 5% polyacrylamide gels at 4°C. The electrophoresis buffer was 1 x TBE (89 mM Tris [pH 8.9], 89 mM borate, 1 mM EDTA). The dried gels were analyzed with a Molecular Dynamics PhosphorImager (Amersham Biosciences).

**DNase I footprinting.** DNase I protection assays were performed as described previously (20). DNA templates were obtained as described above. These DNAs were digested with either EcoRI or HindIII restriction endonucleases, and the cleaved binding-site-containing DNA fragments were isolated from agarose gels. The DNA fragments were radioactively labeled at their 3’ ends by incubating the DNA with [\gamma\textsuperscript{32P}]ATP (3,000 Ci/mmol) (Perkin-Elmer) in the presence of the Klengow fragment of DNA polymerase I (Epipcentre, Inc.). The labeled DNA fragments were incubated with increasing amounts of 933W repressor in buffer (10 mM Tris [pH 8.0], 50 mM KCl, 1 mM Mg\textsubscript{2}C\textsubscript{2}O\textsubscript{4}) for 5 min at 25°C prior to addition of sufficient DNase I to generate, on average, one cleavage per DNA template. The DNA fragments were then separated from the cleavage products by gel electrophoresis (SDS-PAGE) gel of the purified repressor, the repressor is >98% pure. The overall yield of 933W repressor was ~10 mg/liter of bacterial culture.

**In vitro selection of 933W repressor binding sites.** Fifty nanomolar 933W repressor was incubated in binding buffer (see above) with a twofold excess of a 65-bp double-stranded DNA molecule radioactively labeled at its 5’ ends that contains a 25-bp random sequence segment embedded between fixed sequences. The fixed sequences serve as annealing sites for two 20-base PCR primers. The repressor-bound DNA was separated from the unbound DNA by gel electrophoresis as described above. The repressor-bound DNA was eluted from the gel and amplified by PCR with the appropriate primers. This process of selection and amplification was reiterated six times, lowering the concentration of 933W repressor present in the reaction by twofold at each iteration. The progress of the selection was monitored by determining the affinity of repressor for bulk DNA binding by gel mobility shift assay (see above) and was stopped when the affinity of 933W repressor for the selected population of DNA did not increase in two successive rounds of selection. At this point, the selected pool of binding-site containing DNAs was inserted into the SmaI site of pUC18 (30), and 15 individual clones were sequenced. Multiple repeats of three sequences were obtained, indicating that we had adequately sampled the sequences of the selected population of DNAs.

**Filter binding assays.** The affinity of 933W repressor for the selected binding site DNAs was determined by filter binding essentially as described previously (26). Briefly, \textsuperscript{32P}-labeled DNA at a concentration of ≤0.1 nM was incubated at 25°C with increasing concentrations of 933W repressor in a buffer containing 10 mM Tris (pH 7.5), 50 mM KCl, 100 \mu g of bovine serum albumin ml\textsuperscript{-1}, and 1 mM dithiothreitol (DTT). Following vacuum filtration through nitrocellulose and DEAE paper (49), values of the dissociation constant (K\textsubscript{D}) were determined by nonlinear squares fitting of the filter binding data to a hyperbolic equation with Prism 3.0 software (GraphPad Software Inc., San Diego, Calif.). Each dissociation constant was determined from at least four replicate measurements. The standard errors of the K\textsubscript{D} values are below 10% for all reported values.

**Transcription in vitro.** Transcription reactions were performed essentially as described previously (50) using templates prepared by PCR with primers that anneal near the two P\textsubscript{r} sites found in both pB933Wor and pB933Wol. Following purification of these DNA templates, a 5 nM concentration of each was incubated without or with various amounts of 933W repressor for 10 min at 37°C in transcription buffer containing 100 mM KCl, 40 mM Tris (pH 7.9), 3 mM Mg\textsubscript{2}C\textsubscript{2}O\textsubscript{4}, and 10 mM DTT. RNA polymerase was added to a final concentration of
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Characterization of Bacteriophage 933W Repressor

Sequence comparisons and analysis. Homologies between the sequences of the various bacteriophage repressors and selected 933W repressor binding sites were examined by using the PILEUP subroutine included within the GCG package, version 10.3 (12) (Accelrys, San Diego, Calif.). Promoter regions were identified by using neural network promoter prediction software (41).

RESULTS

Using PCR, we amplified the region corresponding to the 933W repressor gene from the genomic DNA of the E. coli O157 strain EDL933 (see Materials and Methods), a 933W lysogen (38). Subsequent to restriction cleavage, this fragment was inserted into the plasmid pET17b, thereby placing expression of the 933W repressor gene under the control of the T7 RNA polymerase promoter. When transformed into the E. coli strain BL21(DE3)::pLysS (43), this plasmid directed the overexpression of the 933W repressor (Fig. 1, lanes 1 and 2) upon addition of the inducer IPTG. The resulting protein was purified to near homogeneity by conventional methods (Fig. 1, lanes 3 and 4) (see Materials and Methods). The yield is 10 mg of purified 933W repressor per liter of induced cell culture.

Repressor binding to O\textsubscript{R}. To begin characterizing the DNA binding properties of the purified 933W repressor, we isolated the region corresponding to 933W O\textsubscript{R} from strain EDL933. We used this DNA to assess 933W repressor binding in a gel mobility shift experiment.

Based on analysis of the bacteriophage 933W sequence, this fragment should contain three binding sites for the 933W repressor. A radioactively labeled DNA fragment containing three and only three complexes of decreasing mobility are sequentially formed in gel mobility shift assays (Fig. 2). These findings are in agreement with those reported by Tyler et al. (44). Complex I is formed in the presence of 0.11 nM 933W repressor and grows to half-maximal intensity with ~0.3 nM repressor prior to disappearing completely at 5.7 nM repressor. Complex II appears in the gel in the presence of ~0.23 nM protein and reaches half-maximal intensity in the presence of ~0.8 nM repressor. Complex III does not appear until both complexes I are II are formed in the presence of 0.23 nM repressor and reaches its

![Unbound DNA](http://jb.asm.org/)

FIG. 2. 933W repressor binds to three binding sites in bacteriophage 933W O\textsubscript{R}. A radioactively labeled DNA fragment containing wild-type 933W O\textsubscript{R} was incubated with increasing concentrations of the 933W repressor. The concentration of the repressor was increased in twofold steps starting at 0.11 nM. Shown is a native gel of the resulting complexes visualized by phosphorimaging. The positions of the three complexes and the unbound DNA are indicated.
half-maximal intensity with 2.0 nM repressor. All of the DNA is shifted into complex III by 5.7 nM repressor. Assuming that 933W repressor displays the same relative affinities for its naturally occurring sites in OR as do most other lambdoid phages (5, 20, 39, 48), we suggest that complex I represents 933W repressor bound at OR1, complex II is formed by repressor bound at OR1 and OR2, and complex III is formed when repressor simultaneously binds all three sites in OR1, OR2, and OR3.

Although the results shown in Fig. 1 suggest that the repressor binds to three sites in OR, we wished to confirm this interpretation. Moreover, we also wished to determine the exact location of the repressor binding sites on DNA and the precise order in which repressor binds to these sites. To this end, we studied the binding of 933W repressor to its sites in OR by DNase I footprinting. Adding increasing concentrations of 933W repressor to 933W DNA results in a progressive protection of three individual regions of the DNA from DNase I cleavage (Fig. 3). Under the conditions of these experiments, the concentration of repressor that half-maximally protects the DNA from DNase I cleavage corresponds to the apparent dissociation constant (KD) of repressor. 933W repressor binds a region predicted to correspond to OR1 with a KD of 1 nM. The KD of 933W repressor for the region corresponding to OR2 is 2.5 nM, while its affinity for OR3 is 7 nM. Due to the difference in temperature of the two assays, the KD values determined by DNase I footprinting do not precisely correspond to estimates of KD for formation of complexes I, II, and III (Fig. 2). However, the relative values of KD for OR1, OR2, and OR3 obtained by DNase I footprinting do correspond to the relative amounts of 933W repressor needed to half-maximally form complexes I, II, and III, respectively (Fig. 2). Hence, the quantitative and qualitative analysis of the findings shown in Fig. 3 support the idea that 933W repressor binds to three and only three binding sites in the OR region of bacteriophage 933W.

Identification of the 933W binding site sequence. The DNase I results clearly show that the 933W repressor binds three sites in the 933W OR region. However, as can be seen by a comparison of the relative positions of the protected regions (Fig. 3A and B) with respect to the predicted binding sites (10), the precise locations of the observed binding sites do not completely correspond to the predicted locations of these binding sites. This problem is particularly acute in the region of OR2. These discrepancies could be due to inaccuracies in the predicted site locations or to problems inherent in using DNase I footprinting to define the positions and/or extent of a protein binding site. As a result of these discrepancies, we were unsure of the sequence recognized by the 933W repressor. Consequently, we used in vitro selection to more precisely define the sequence of the 933W repressor binding site. We used 933W repressor to select binding sites from a 25-bp-long randomized sequence (see Materials and Methods). After six rounds of selection, we subcloned the resulting DNA and obtained eight unique, 15-bp-long putative 933W repressor binding sites (Fig. 4). Analysis of the sequences of these sites reveals that the 15-bp site has a partially rotationally symmetric consensus sequence: T/C1 G2 A3 A4 C5 C/T6 T7 G8 A/T9 A/C10 A/C11 G12 T13 C14 C15. 933W repressor exhibits particularly strong base sequence preferences at the symmetrically related positions 2 to 5 and 2′ to 5′. In subsequent analyses (below), we define this region as the core sequence. The overall consensus sequence selected by 933W repressor is similar to that predicted from a bioinformatics analysis of the naturally occurring 933W repressor binding sites (10). Using the information gathered from the selection analysis, we located the binding site sequences in OR. The positions of these sites are indicated in Fig. 3B. The main differences between the two consensus sequences derived from selection and bacteriophage sequence analysis are in the extent of conservation at two symmetrically related positions, 3 and 3′ (Fig. 4). The selection results indicate that repressor strongly prefers an A·T pair at positions 3 and 3′. However, in the naturally occurring sites in OR, A·T and T·A pairs occur at these positions with equal frequency.

We wanted to verify that the selection procedure actually identified the sequence of the 933W repressor binding site. To do this, we measured the affinity of 933W repressor for the selected sites in a filter binding assay. All the selected binding sites bind 933W repressor with dissociation constants between 0.17 and 125 nM (Fig. 4), whereas the protein binds a non-specific DNA fragment containing no 933W binding site only at concentrations exceeding 10 μM (data not shown). This finding indicates that all the selected sequences contain 933W repressor DNA binding sites.

In large measure, the selected binding sites containing non-consensus bases in the core sequence bind repressor with a substantially lower affinity than do sites bearing the consensus base sequence in this region. Moreover, the relative repressor affinities of the sites containing nonconsensus bases in the core sequence region are correlated with the number of bases that do not match consensus. The only exception to this pattern is found in site 1, which binds repressor with a relatively low affinity despite having a completely consensus core sequence. For example, sites 7 and 8, which contain one and two changes from the core consensus sequence, respectively, bind repressor with 4- and 17-fold-lower affinities than does site 6, a binding site that contains a perfect match to the consensus sequence. Hence, the findings shown in Fig. 4 firmly establish the identity of the 933W repressor binding site sequence.

Interestingly, all virulent mutant 933W bacteriophages isolated by Tyler et al. (44) contain a mutation at position 3 in OR2. Given the relatively minor effect of changing position 3 away from consensus on the formation of 933W repressor-DNA complexes (Fig. 4, compare sites 6 and 7), the apparent importance of this position to virulence is surprising. This finding may indicate that, in addition to contributing to phage virulence by decreasing the DNA binding affinity of 933W repressor, changes at this position may also affect other functions of the 933W repressor-DNA complex, e.g., by affecting cooperative interactions between 933W repressors bound at OR1 and OR2 or altering repressor’s ability to regulate transcription from PR and/or PRM.

Control of transcription at OR by 933W repressor. Having established the sequence and location of the naturally occurring binding sites in OR, we wished to examine the ability of 933W repressor to function as a regulator of bacteriophage transcription initiation. We examined the ability of repressor to control in vitro transcription from PR and PRM when bound at wild-type 933W OR. At 933W OR in the absence of repressor,
only transcripts resulting from RNA polymerase initiating at the P_R promoter are detectable (Fig. 5, lane 1). Adding increasing amounts of 933W repressor to the reaction inhibits transcription from P_R and stimulates transcription from P_RM (Fig. 5, lanes 2 to 4). Given the marked temperature and salt dependence of repressor’s DNA affinity (data not shown), the higher salt concentration and temperatures used in the transcription reactions require that the concentrations of repressor needed to activate P_RM and repress P_R are slightly higher than those needed to occupy O_R1 and O_R2 (Fig. 3). Despite this difference, the results shown in Fig. 5 illustrate the positive transcriptional regulatory function of repressor at P_RM and the negative effect of repressor on P_R transcription that is common to all known lambdoid bacteriophage repressors (2, 40). Also as anticipated, adding higher concentrations of repressor results in repression of P_RM transcription. We suggest that at these excess repressor concentrations, O_R3 is occupied by repressor and prevents the binding of RNA polymerase to the promoter. Thus, 933W repressor behaves as a typical lambdoid bacterio-
phage repressor with respect to transcriptional control of the promoters in 933W OR, functioning as a repressor and an activator at PRM and a repressor at PR.

933W repressor binding to OL. Virtually all lambdoid bacteriophages contain at least three repressor binding sites in OL (11). Binding of repressor in this region represses transcription initiation from PL. Strong repression of PL transcription is crucial for the establishment and maintenance of lysogeny. In other phages, complete repression of PR transcription requires the cooperative repressor binding to two adjacent DNA sites in OL. Moreover, cooperative binding interactions between repressors bound at all three sites in OR and OL of bacteriophage λ regulate the activity of phage promoters (9). However, sequence analysis suggested that bacteriophage 933W contains only one repressor binding site in this region (10, 37, 38). Given the importance of multisite binding in OL to transcriptional regulation in related phages, we wished to experimentally determine the number of repressor binding sites in the 933W OL region.

When increasing concentrations of purified 933W repressor are incubated with radioactively labeled DNA containing 933W OL, two and only two complexes of decreasing mobility are formed sequentially in gel mobility shift assays (Fig. 6). Complex I is formed in the presence of 0.024 nM 933W repressor and grows to half-maximal intensity with 0.56 nM repressor prior to disappearing completely with 2.3 nM repressor. Complex II appears in the presence of 0.23 nM protein and reaches half-maximal intensity in the presence of ~0.8 nM repressor. Assuming that 933W repressor displays the same relative affinities for its naturally occurring sites in OL as do most other lambdoid phages, we suggest that complex I represents 933W repressor bound at OL1 and that complex II is formed by repressor bound at OL1 and OL2.

To confirm the suggestion that 933W OL contains two repressor binding sites and to determine the exact locations of these sites, we studied the binding of 933W repressor to its sites in OL by DNase I footprinting. Adding increasing concentrations of 933W repressor to 933W OL DNA results in protection of two individual regions of the DNA from DNase I cleavage (Fig. 7). Consistent with the results shown in Fig. 6, this finding indicates that 933W OL contains two repressor binding sites. Repressor occupies both of these sites at identical concentrations. The KD App of 933W repressor for the two sites in OL derived from DNase I cleavage is ~2 nM. This observation suggests either that the 933W repressor has identical intrinsic affinities for these two sites or that repressor binding to these sites is strongly cooperative. Given that the gel mobility shift results show that 933W repressor sequentially forms two complexes, we favor the latter interpretation.

**Control of transcription at OL by 933W repressor.** We examined the ability of 933W repressor to control transcription on September 28, 2017 by guest
The overall organization of the 933W repressor is similar to that of the other phage repressors (Fig. 9). However, while lysogens of bacteriophage 933W are inducible by DNA-damaging agents (38), 933W repressor does not contain an Ala-Gly dipeptide anywhere in its putative linker region (Fig. 9). It also does not contain a Cys-Gly sequence, the cleavage site utilized by other, related autolysing proteins (Fig. 9). Moreover, the position of the putative active-site lysine is not conserved among the other bacteriophage repressors. We wished to know whether these differences indicate that bacteriophage 933W uses an alternative induction strategy.

To answer this question, we examined the ability of activated RecA protein (see Materials and Methods) to stimulate autoproteolysis of purified 933W repressor in vitro. Incubating 20 μM 933W repressor with RecA results in the appearance of two protein fragments of lower molecular weight (∼13 and 9 kDa) than 933W repressor (Fig. 10, lanes 3 and 4). However, no lower-molecular-weight protein fragments are observed when 933W repressor was incubated in buffer in the absence of RecA (Fig. 10, lanes 1 and 2). These findings suggest that despite lacking the consensus Ala-Gly dipeptide cleavage site, 933W does undergo RecA-stimulated autoproteolysis.

The sizes of the 933W repressor fragments formed in the presence of RecA are consistent with those that would be expected from autoproteolytic cleavage occurring in the putative linker region of the 933W repressor protein. To verify that the autocleavage reaction takes place in this region, we sequenced the N-terminal 10 residues of each of the two cleavage products. The sequence of the smaller fragment corresponded to that of the N-terminal end of the repressor protein. The N-terminal sequence of the larger fragment is GVGDGA IEMTE, which corresponds to residues 108 to 117 of the 933W repressor sequence, showing that the formation of the two protein fragments is caused by an internal cleavage. This sequence lies in the predicted linker region of the 933W repressor protein’s structure, and the sequencing results show that repressor cleaves itself between amino acids Leu 107 and Gly 108. Hence, although it contains a unique Leu-Gly dipeptide sequence, the 933W repressor has the ability to catalyze RecA-stimulated autocleavage in its linker region.

These findings indicate that despite the lack of a consensus autocleavage site, inactivation of bacteriophage 933W repressor likely occurs through RecA-mediated autocleavage. All proteins that undergo RecA-stimulated autocleavage can also undergo autoproteolysis at high (≥10.3) pH in the absence of RecA (23). We determined whether 933W repressor also cleaves itself under these conditions. Incubating 20 μM 933W repressor at pH 10.5 results in the appearance of two protein fragments of lower molecular weight (∼13 and 9 kDa) than 933W repressor (Fig. 10, lanes 5 and 6). These fragments are
of identical sizes and sequences as the ones formed in the presence of RecA. These lower-molecular-weight protein fragments were not formed when 933W repressor was incubated for an extended period at neutral pH. These findings indicate that the mechanism of 933W repressor autocleavage is similar to that used by the other bacteriophage repressors and their homologues. Consistent with this assertion, a 933W bacteriophage bearing a mutation in one of the residues predicted to catalyze 933W repressor autocleavage is not inducible by DNA-damaging agents (44).

DISCUSSION

Despite the differences between bacteriophage 933W and other lambdoid bacteriophages, the mechanisms that the 933W repressor uses to control the lysis-lysogeny decision of its cognate phage are generally similar to those used by the repressors of the well-studied 434, λ, and P22 bacteriophages. Specifically, 933W repressor sequentially binds to three rotationally symmetric sites in O₉. Binding to O₉1 and O₉2 turns off transcription from P₉ and activates transcription from P₉M. Repressor occupancy of O₉3 inhibits transcription from P₉M. 933W repressor also binds to sites in O₉ and negatively regulates transcription from the P₉ promoter. The bacteriophage repressor also undergoes autocleavage at a site within its linker region.

The differences between the right operator regions of 933W and those of the other bacteriophages allow us to gain insight into the mechanism of gene control by 933W repressor. Using sequence analysis (41) and the positions of the transcription start sites derived from our runoff transcription assays, we tentatively identified the positions of the −10 and −35 promoter elements of the OR2 site and the P₉M promoter is distinctly different from

FIG. 8. Transcriptional control of the P₉ promoters in 933W O₉ (A) and disposition of 933W binding sites, P₉, and other control elements promoters within 933W O₉ (B). (A) Transcription reactions were performed as described in Materials and Methods. Repressor concentrations were increased in twofold steps starting at 2 nM. RNA polymerase was present at 50 nM. (B) The positions of the binding sites (bars) were determined as described in the text. The arrows above and below the sequences depict the regions of DNA protected from DNase I digestion in the presence of 933W repressor. The positions of the transcription start points were calculated from the lengths of runoff transcripts. The positions of the −10 and −35 promoter elements were determined by sequence analysis (38, 41).
that found in the \( \lambda \) and 434 bacteriophages and remarkably similar to that observed in the bacteriophage P22. This observation suggests that 933W repressor may activate \( \text{PR} \) transcription using residues in positions homologous to those of P22 repressor. Consistent with this suggestion, 933W repressor contains a series of negatively charged residues at the C-terminal end of the putative \( \alpha_3 \) DNA recognition helix. Mutations that decrease the negative charge in the homologous region of P22 repressor decrease the ability of protein to stimulate transcription (16). In contrast, the N-terminal end of 933W repressor’s putative \( \alpha_2 \) contains several positively charged residues, whereas the ability of the \( \lambda \) and 434 repressors to activate \( \text{PR} \) transcription requires the presence of negatively charged residues in this region of these proteins (3, 4, 16).

The relative positioning of repressor binding sites with respect to the \( \text{PR} \) promoter elements appears to determine the mechanism the phage repressor uses to repress \( \text{PR} \) transcription (51). In bacteriophage 434, the −35 and −10 regions of the \( \text{PR} \) promoter surround, but do not overlap, the \( \text{OR}2 \) site (51). Nonetheless, 434 repressor binding to \( \text{OR}2 \) is necessary and sufficient to prevent transcription from \( \text{PR} \). Thus, in phage 434, the \( \text{OR}2 \)-bound repressor blocks transcription initiation by blocking the transition of a \( \text{PR} \)-bound RNA polymerase from a stable closed complex to an open complex (52). The position-
ing of the \( P_R \) promoter elements with respect to the 933W repressor binding sites in \( O_R \) resembles that found in bacteriophage 434, suggesting that 933W repressor represses initiation of transcription at \( P_R \) using a mechanism similar to that used by 434 repressor.

In order for a lambdoid bacteriophage to establish and maintain lysogeny, the repressor must occupy \( O_R 1 \) and \( O_R 2 \) at a lower concentration than it binds to \( O_R 3 \). In all known lambdoid phages, the cooperative binding of repressor to \( O_R 1 \) and \( O_R 2 \) allows repressor to bind \( O_R 1 \) and \( O_R 2 \) at nearly identical concentrations. However, inspection of Fig. 3 reveals that three- to fourfold-higher 933W repressor concentrations are required to bind \( O_R 2 \) than are needed to bind \( O_R 1 \). We speculate that the inability of 933W repressor to bind these two sites with identical affinities may be due to either one or both of two situations: (i) weak inherent cooperative interactions formed by the repressor; and (ii) a large difference in the affinities of two situations: (i) weak inherent cooperative interactions and (ii) a large difference in the affinities of repressor for \( O_R 1 \) and \( O_R 2 \). Although we do not yet know the affinities of 933W repressor for the individual naturally occurring binding sites, both \( O_R 1 \) and \( O_R 2 \) are poor matches to consensus (compare Fig. 1 and 4), suggesting that these sites would have a relatively low affinity for repressor.

933W repressor binds the two sites in \( O_2 \) with identical affinities, suggesting that this protein is capable of strong cooperative binding. Hence we favor the second explanation.

Regardless of the reason, the relatively weak binding of repressor to \( O_R 2 \) in intact \( O_R \) allows repressor to bind this site at a concentration that is only twofold lower than that needed to occupy \( O_R 3 \). Since repressor synthesis in a lysogen is stimulated by an \( O_R 2 \)-bound repressor but inhibited by an \( O_R 3 \)-bound molecule, we anticipate that 933W prophages would direct the synthesis of a tightly regulated amount of repressor. Also, because of the low affinity of repressor for \( O_R 2 \), a small drop in repressor concentration would lead to a sharp drop in occupancy of \( O_R 2 \). Thus, consistent with recent observations (24), we would predict that 933W lysogens would be relatively unstable.

The discovery that 933W uses a Leu-Gly sequence at its autocleavage site expands the repertoire of sequences found at the cleavage sites that can support RecA-mediated autocatalysis in vivo. At this point, three sequences occur naturally at the cleavage sites that can support RecA-mediated autocatalysis: Ala-Gly, Cys-Gly, and Leu-Gly. This survey led us to examine the roles that these amino acid pairs play in the autocleavage reaction. The universal selection of a glycine at the second position of the cleavage site is likely due to the unusual conformation it must assume in order for the scissile bond to be appropriately positioned within the active site (25). Consistent with this idea, Gly-Glu and Gly-Asp mutants of \( \lambda \) and lexA repressors, respectively, do not undergo autocleavage, and a \( \lambda \) repressor bearing a Gly-Asp change cleaves itself, but at a substantially slower rate than does the wild-type protein (14, 22).

Structural analysis predicts that the side chain of the first residue in the cleavage site in these proteins penetrates into the hydrophobic core of the protein and thereby stabilizes the loop of polypeptide that contains a cleavage site within the active site (25). Consistent with this idea, a \( \lambda \) repressor bearing glycine at this position, which can make no contacts within the hydrophobic core, while capable of undergoing pH- and RecA-mediated autocleavage, undergoes autoproteolysis at a rate too slow to support normal lysogen induction (13, 14). Among the proteins containing alanine in the first position of the cleavage site sequence, the residues that are contacted, or predicted to be contacted, by this residue are nearly completely conserved (42). These observations indicate that efficient autocleavage requires structural complementarity between the residue in the first position of the cleavage site and residues in the core of the proteins. This suggestion is supported by two observations. First, changing the alanine in the cleavage site to threonine totally blocks pH- and RecA-catalyzed autodigestion of \( \lambda \) repressor. Second, several of the residues that contact alanine differ in UmuD and 933W repressor, two proteins that have a residue with a side chain different from and larger than alanine in the first position of the cleavage site. We suggest that these sequence differences accommodate the larger side chains at this position in UmuD (Cys-Gly cleavage site sequence) and 933W repressor (Leu-Gly cleavage site sequence). We also suggest that the larger side chains present at the first position of the cleavage site in these proteins stabilize the scissile bond within the active site and lead to the enhanced rates and efficiencies of autocleavage displayed by these proteins (28, 29; G. Koudelka and L. Hufnagel, unpublished data).

We speculate that the latter property also enhances the sensitivity of the lysis-lysogeny switch of 933W lysogens (24).

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