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

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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 (O_R). Also typical, when bound at O_R, 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 (O_L) contains only two repressor binding sites, but the O_L-bound repressor still efficiently represses P_L 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, 35, 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, O_L and O_R, 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 O_R and O_L (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 lysogenic prophage, 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 O_L, 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 O_L. 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

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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_L 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)::pLysS (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 AAAGTGCGG and GGTTACAAGCTTTGTGACGATGAAG. Subsequent to purification, the resulting 708-bp DNA fragment was cleaved with NdeI and HindIII (New England Biolabs) and inserted into pET17b (Novagen) that had been previously cleaved with the same enzymes.

DNA bearing the 933W O_R region was obtained by amplifying this region of the EDL933 genomic DNA using the primers CCACAAGCTTTCGCAACT TCAG and CCATGTTCATCAAGACCAGCTT. The resulting DNA was cleaved with HindIII and EcoRI and inserted into pUC18 (30) that had been previously cleaved with these enzymes, creating the plasmid p933WO_R. The O_L region was subcloned into the SmaI site of pUC18 (30) following its amplification from EDL933 with primers with the sequences CTTTGCCTAACGTTCGCCC and CCCAAAGTTAACTTTGGTTATTGCG to create p933WO_L.

Purification of 933W repressor. A saturated overnight culture of BL21(DE3):: pLysS cells bearing p933WR was diluted 1:50 into 3 liters of prewarmed Luria broth supplemented with 100 μg of ampicillin/ml and 20 μg 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-β-D-thiogalactopyranoside) to the cultures. After an additional 4 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 μg of leupeptin/ml, 50 μg of benzamidine/ml, 10 U of aprotinin, 5 μg of pepstatin/ml, and 5 μg of TPCK [tosylsulfonyl phenylalanyl chloromethyl ketone]/ml). 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 10,500 × *g* for 20 min. Polyethylene 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 × *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 × *g* for 30 min to precipitate the repressor. The resulting repressor-containing pellet was dissolved in 15 ml of standard phosphate buffer (SPB_{50+200}) containing 50 mM $NaPO_4$ (pH 6.8), 200 mM NaCl, and 1 mM EDTA and dialyzed against three changes of 1 liter of the same buffer. After removal of insoluble debris from the dialysate by centrifugation at 12,000 × *g* for 10 min, the protein was loaded onto a 1- by 10-cm carboxymethyl ion exchange column (Bio-Rad, Hercules, Calif.) equilibrated with SPB_{50+200}. Repressor 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_{50+200}. Repressor-containing fractions from this column were pooled, concentrated, and dialyzed against three changes SPB_{50+200} supplemented with 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 p933WO_R or p933WO_L 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 [γ-^{32}P]ATP (3,000 Ci/mmol) (Perkin-Elmer, Boston, Mass.) in the presence of T4 polynucleotide kinase (Epicentre, 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× 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 [α-^{32}P]dATP (3,000 Ci/mmol) (Perkin-Elmer) in the presence of the Klenow fragment of DNA polymerase I (Epicentre, 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 $MgCl_2$) for 5 min at 25°C prior to addition of sufficient DNase I to generate, on average, one cleavage per DNA molecule in 5 min of additional incubation. The cleavage reactions were terminated by precipitation with ethanol, and the DNA was dissolved in 90% formamide solution containing tracking dyes. The products of the reaction, together with the products of chemical sequencing reactions (27) derived from the same templates, were resolved on 7.5% acrylamide gels containing 8 M urea, 89 mM Tris-HCl (pH 8.9), 89 mM borate, and 1 mM EDTA. The cleavage fragments were visualized with a Molecular Dynamics Phosphorimager (Amersham Biosciences). Affinities of the various repressors for the binding sites in O_R were determined by quantitative analysis of the phosphorimage using ImageQuant (Amersham Biosciences).

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 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, ^{32}P-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 μg of bovine serum albumin ml^{-1}, and 1 mM dithiothreitol (DTT). Following vacuum filtration through nitrocellulose and DEAE paper (49), values of the dissociation constant (K_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_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 PvuII sites found in both p933WO_R and p933WO_L. 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 $MgCl_2$, and 10 mM DTT. RNA polymerase was added to a final concentration of

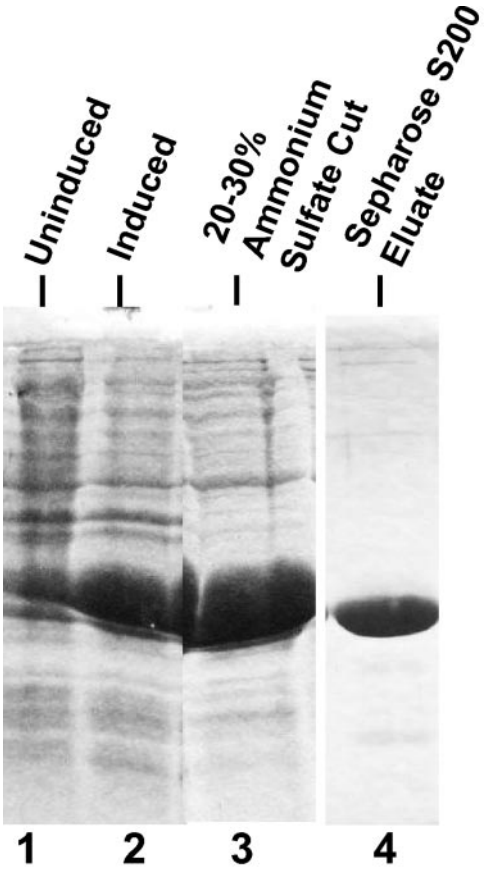

FIG. 1. Purification of 933W repressor. Pellet fractions of uninduced (lane 1) and IPTG-induced (lane 2) cultures of BL21(DE3):: pLysS cells bearing p933WR (see Materials and Methods) and purified cellular extracts after ammonium sulfate precipitation (lane 3) and column chromatography (lane 4) were dissolved in sample buffer and fractionated on SDS–13.5% PAGE discontinuous gels (21). Shown is a photograph of the Coomassie brilliant blue-stained gel.

50 nM, and incubation was continued for 15 min at 37°C to allow the formation of open complexes. The transcription reaction was started by the addition of 0.25 mM ATP, GTP, and CTP, 0.04 mM UTP, 10 μCi of [α-^{32}P]UTP, and 0.1 mg of heparin/ml. After 10 min of further incubation, the reactions were stopped by addition of formamide dye mix (90% formamide and 1× TBE) and fractionated on 6% denaturing gels. The positions and amounts RNA transcripts were visualized by Phosphorimager analysis (Amersham Biosciences).

RecA- and pH-stimulated autocleavage of 933W repressor. To examine RecA-stimulated autocleavage of 933W repressor, activated RecA filaments were formed by mixing 1.25 μM RecA (Epicentre), 5 mM γ-S-ATP (Sigma), and 1.5 μM oligo (dT_{20}) (IDT Technologies) in standard reaction buffer (50 mM KCl, 15 mM Tris [pH 7.5], 2 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT) and incubating this mixture at room temperature for 10 min. Subsequently, 20 μM 933W repressor was added, and this mixture was incubated for various times at 37°C. pH-induced repressor autocleavage was effected by incubating 20 μM 933W repressor for various times in a buffer containing 50 mM 3-(cyclohexylamino)-1-propane sulfonic acid (CAPS)–NaOH (pH 10.3) and 50 mM KCl at 37°C. The cleavage reaction was quenched by adding an SDS-containing sample loading buffer, and the reaction products were separated on 15% Tris–Tricine polyacrylamide gels and visualized by staining with Coomassie brilliant blue.

Protein sequencing. To determine the sequence of the internal 933W cleavage site, the products of the RecA- and pH-stimulated autocleavage reactions were first fractionated on an SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane by electroblotting (15). The positions of the cleavage products on the membrane were visualized by using Ponceau S, and these products were excised from the membrane. N-terminal sequencing of these reaction products was performed at the Harvard Microchemistry Facility (Cambridge, Mass.).

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_R. To begin characterizing the DNA binding properties of the purified 933W repressor, we isolated the region corresponding to 933W O_R from strain EDL933. We used this DNA to assess 933W repressor binding in a gel mobility of shift experiment.

Based on analysis of the bacteriophage 933W sequence, this fragment should contain three binding sites for the 933W repressor (10, 38). Consistent with this idea, when increasing concentrations of purified 933W repressor are incubated with radioactively labeled 933W O_R, 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

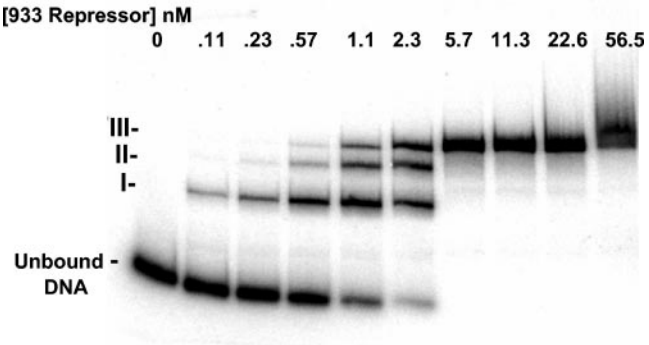

FIG. 2. 933W repressor binds to three binding sites in bacteriophage 933W O_R. A radioactively labeled DNA fragment containing wild-type 933W O_R was incubated with increasing concentrations of the 933W repressor. The concentration of the repressor was increased in twofold steps starting at 0.11 nM. 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 O_R as do most other lambdoid phages (5, 20, 39, 48), we suggest that complex I represents 933W repressor bound at O_R1, complex II is formed by repressor bound at O_R1 and O_R2, and complex III is formed when repressor simultaneously binds all three sites in O_R, O_R1, O_R2, and O_R3.

Although the results shown in Fig. 1 suggest that the repressor binds to three sites in O_R, 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 O_R by DNase I footprinting. Adding increasing concentrations of 933W repressor to 933W O_R 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 (K_D^{App}) of repressor. 933W repressor binds a region predicted to correspond to O_R1 with a K_D^{App} of 1 nM. The K_D^{App} of 933W repressor for the region corresponding to O_R2 is 2.5 nM, while its affinity for O_R3 is 7 nM. Due to the difference in temperature of the two assays, the K_D^{App} values determined by DNase I footprinting do not precisely correspond to estimates of K_D^{App} for formation of complexes I, II, and III (Fig. 2). However the relative values of K_D^{App} for O_R1, O_R2, and O_R3 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 O_R 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 O_R 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 O_R1 and O_R2. 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 binding site has a partially rotationally symmetric consensus sequence: T/C_1 G_2 A_3 A_4 C_5 C/T_6 T/G_7 A/T_8 $A/C_{7'}$ $A/G_{6'}$ $G_{5'}$ $T_{4'}$ $T_{3'}$ $C_{2'}$ $A/G_{1'}$. 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 O_R. 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 O_R, 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 nonspecific 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 nonconsensus 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 O_R2. 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 O_R1 and O_R2 or altering repressor's ability to regulate transcription from P_R and/or P_{RM}.

Control of transcription at O_R by 933W repressor. Having established the sequence and location of the naturally occurring binding sites in O_R, 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 P_R and P_{RM} when bound at wild-type 933W O_R. At 933W O_R in the absence of repressor,

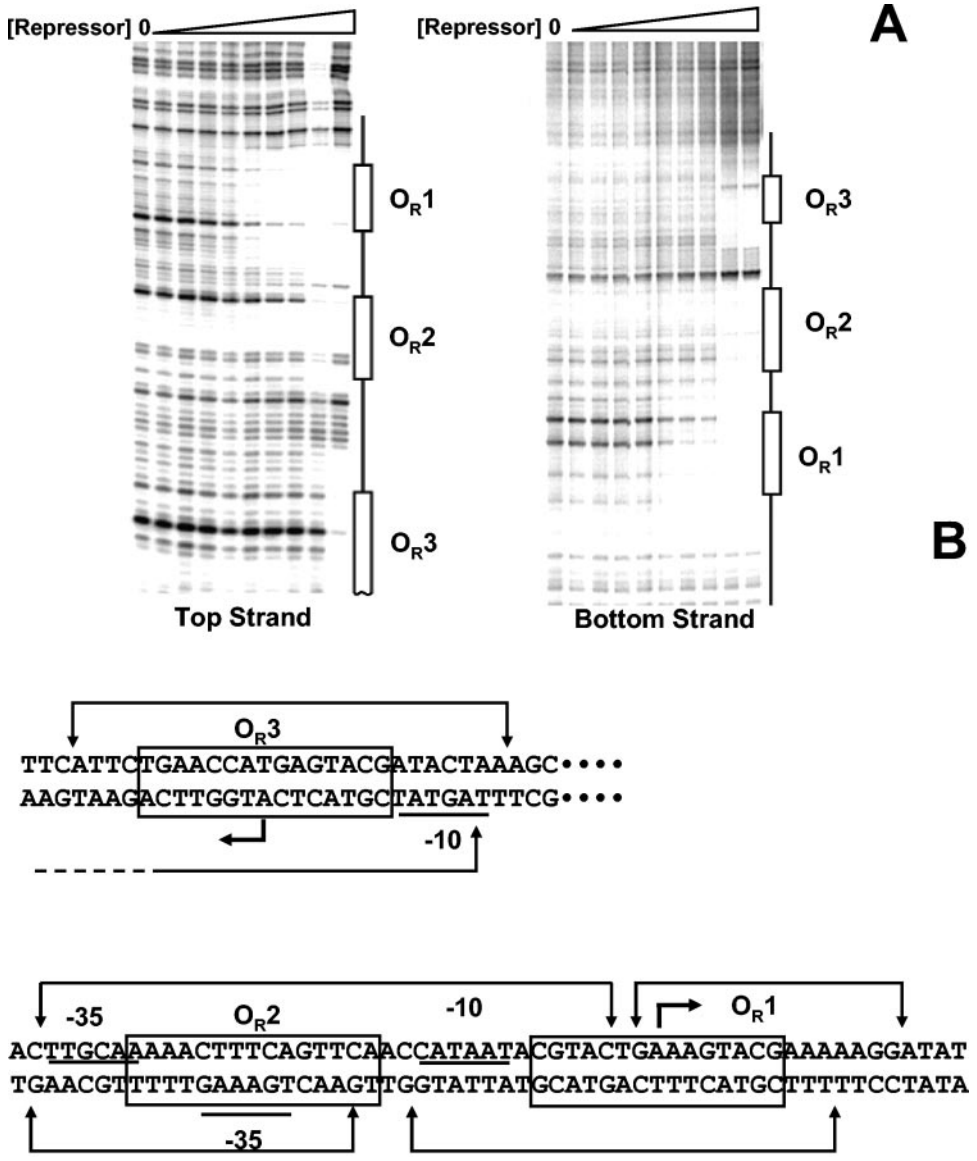

FIG. 3. (A) DNase I footprinting of complexes between 933W repressor and 933W O_R. DNA templates containing 933W O_R radioactively labeled on the top (left panel) or bottom (right panel) strand were partially digested by DNase I in the presence of increasing amounts of the 933W repressor. The leftmost lane (lane 1) of each panel shows the DNase I cleavage pattern of the DNA in the absence of added repressor. In lanes 2 to 10, repressor concentrations were increased in 2.5-fold steps starting at 0.05 nM protein. (B) Disposition of 933W binding sites and P_R and P_{RM} promoters within 933W O_R. The positions of the binding sites (boxed) are predicted from the consensus sequence derived as discussed in the legend to Fig. 4. The arrows above and below the sequences depict the regions of DNA protected from DNase I digestion in the presence of 933W repressor. The dashed line indicates that the region of protection ran to the end of the DNA fragment (see panel A, lane 10). The positions of the transcription start points were calculated from the length of runoff transcripts (see Fig. 5). The positions of the −10 and −35 promoter elements were determined by sequence analysis (41).

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-

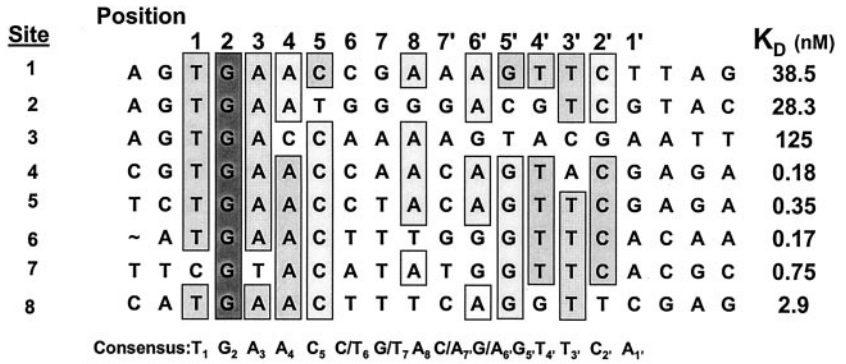

FIG. 4. Sequences and repressor affinities of in vitro selected 933W repressor binding sites. In vitro selection was performed as described in Materials and Methods. Sequences were aligned using Pileup (12). Differential coloration reflects degrees of sequence conservation among the individual sites (dark shading, completely conserved; medium gray, ≥80% conserved; light shading, ≥50% conserved).

phage repressor with respect to transcriptional control of the promoters in 933W O_R, functioning as a repressor and an activator at P_{RM} and a repressor at P_R.

933W repressor binding to O_L. Virtually all lambdoid bacteriophages contain at least three repressor binding sites in O_L (11). Binding of repressor in this region represses transcription initiation from P_L. Strong repression of P_L transcription is crucial for the establishment and maintenance of lysogeny. In other phages, complete repression of P_R transcription requires the cooperative repressor binding to two adjacent DNA sites in O_L. Moreover, cooperative binding interactions between repressors bound at all three sites in O_R and O_L 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 O_L to transcriptional regulation in related phages, we wished to experimentally determine the number of repressor binding sites in the 933W O_L region.

When increasing concentrations of purified 933W repressor are incubated with radioactively labeled DNA containing 933W O_L, 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 O_L as do most other lambdoid phages, we suggest that complex I represents 933W repressor bound at O_L1 and that complex II is formed by repressor bound at O_L1 and O_L2.

To confirm the suggestion that 933W O_L 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 O_L by DNase I footprinting. Adding increasing concentrations of 933W repressor to 933W O_L 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 O_L contains two repressor binding sites. Repressor occupies both of these sites at identical concentrations. The K_D^{App} of 933W repressor for the two sites in O_L derived from the DNase footprinting 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 O_L by 933W repressor. We examined the ability of 933W repressor to control transcription

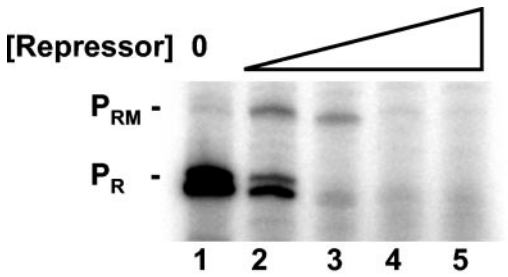

FIG. 5. Transcriptional control of P_{RM} and P_R promoters in 933W O_R. Transcription reactions were performed as described in Materials and Methods. Repressor concentrations were increased in fourfold steps starting at 9 nM. RNA polymerase was present at 50 nM. Positions of transcripts corresponding to the transcription from P_{RM} and P_R are indicated.

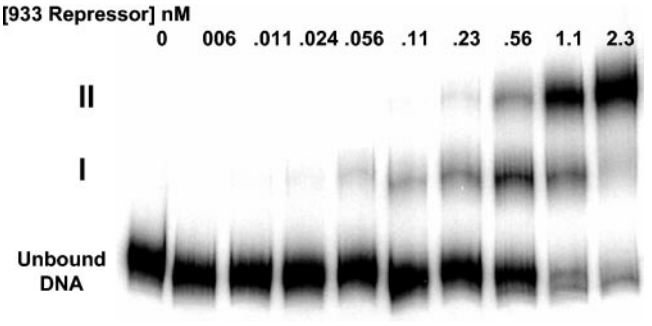

FIG. 6. 933W repressor binds to two binding sites in bacteriophage 933W O_L. A radioactively labeled DNA fragment containing wild-type 933W O_L was incubated with increasing concentrations of the 933W repressor. The concentration of the repressor was increased in twofold steps starting at 0.006 nM. Shown is a native gel of the resulting complexes visualized by phosphorimaging. The positions of the two complexes and the unbound DNA are indicated.

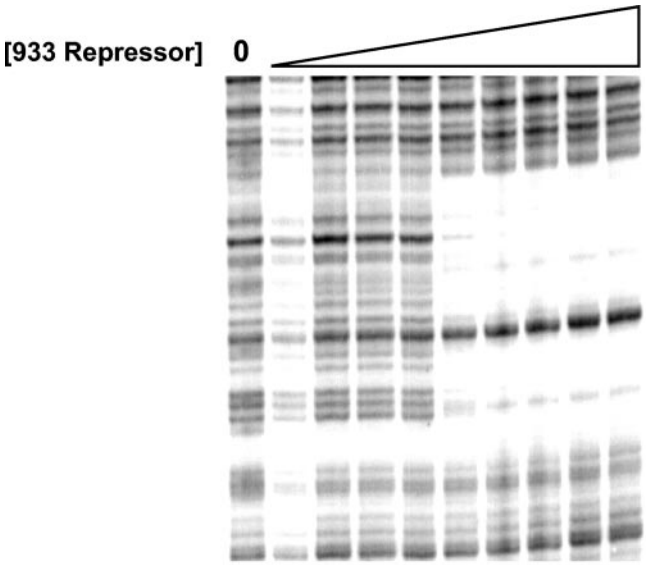

FIG. 7. DNase I footprinting of complexes between 933W repressor and 933W O_L. DNA templates containing 933W O_R radioactively labeled on the top (coding) strand were partially digested by DNase I in the presence of increasing amounts of the 933W repressor. The leftmost lane (lane 1) shows the DNase I cleavage pattern of the DNA in the absence of added repressor. In lanes 2 to 10, repressor concentrations were increased in twofold steps starting at 0.05 nM protein.

at O_L by determining the effect of added repressor on in vitro transcription from promoter(s) in the O_L region. In the absence of any repressor, only a single transcript is observed in the presence of RNA polymerase (Fig. 8). Based on sequence analysis (41) of the O_L region, we suggest that this transcript results from RNA polymerase initiation at the P_L promoter (Fig. 8A, lane 1). Adding increasing amounts of 933W repressor to the reaction inhibits transcription from this promoter, and no other transcripts are observed (Fig. 8A, lanes 2 to 9). Thus, the behavior of 933W repressor at O_L is similar to that of other bacteriophage repressors at O_L, where all function only as transcriptional repressors. The concentration of repressor needed to repress transcription is similar to that needed to occupy the repressor binding sites in the O_L region (compare Fig. 6, 7, and 8). Hence, repressor occupancy of these sites is sufficient to inhibit transcription from 933W P_L.

Knowing the size of the DNA fragment used in the transcription reaction and measuring the size of the runoff transcript, we are able to approximately place the position of the P_L transcription start. The proposed start site is in good agreement with that determined by Tyler et al. (44). Together with this information and sequence analysis (41), we identified the likely position of the −10 and −35 P_L promoter elements (Fig. 8B). Comparing the positions of these elements and the transcription start point with the positions of O_L1 and O_L2 determined by DNase I footprinting shows that both of these 933W repressor binding sites overlap the P_L promoter. This juxtaposition is appropriate to explain the observed negative regulatory effect of 933W repressor binding on transcription from P_L.

933W repressor sequence and bacteriophage 933W induction. The repressors of bacteriophages λ, 434, and P22 fold into two structurally and functionally distinct domains that are joined by a linker that is ~30 amino acids in length (42). In these phages, the switch from lysogenic to lytic growth (phage induction) occurs by autoproteolytic cleavage of their repressors that is stimulated by the DNA damage-induced, activated form of the host RecA protein (1, 23). In all previously studied bacteriophage repressors, autocleavage occurs precisely within a conserved Ala-Gly dipeptide sequence that is found in a homologous position in the linker region. Chemical catalysis is carried out by a serine-lysine dyad, the position of which is also conserved among these phage repressors (22, 23, 36).

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 autocleaving 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 GVDGA 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 sequence at its cleavage site, like the other bacteriophage repressors, 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

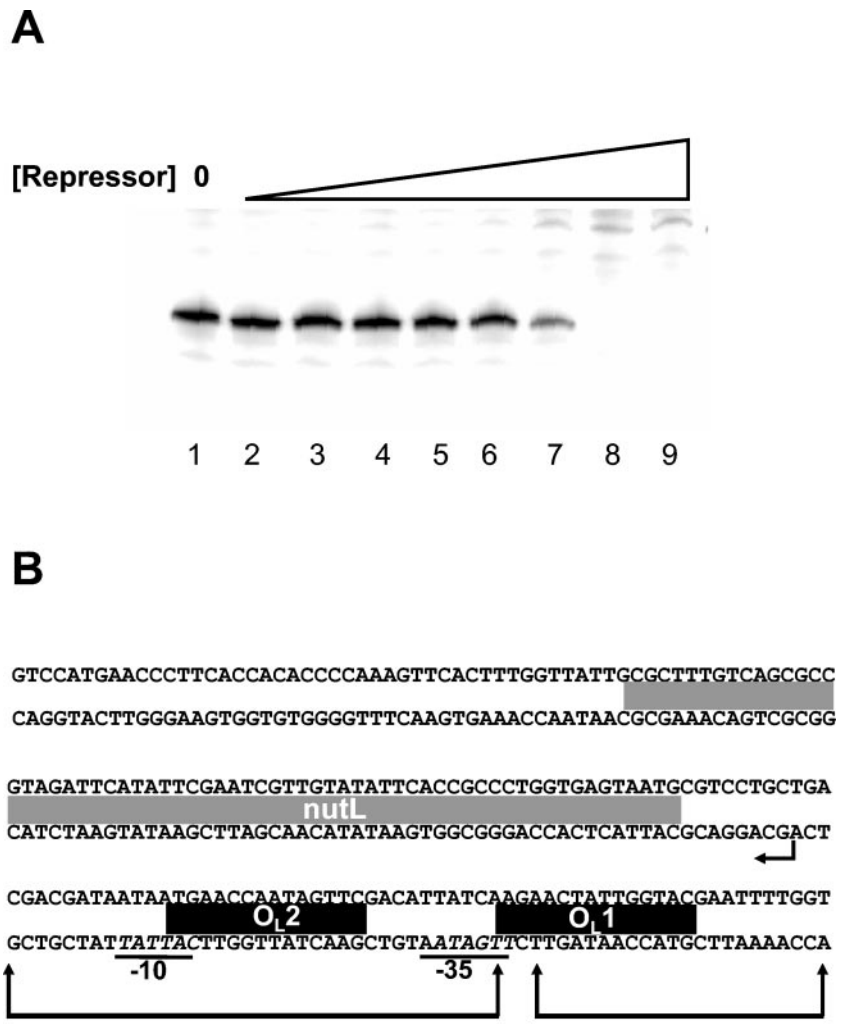

FIG. 8. Transcriptional control of the P_L promoters in 933W O_L (A) and disposition of 933W binding sites, P_L, and other control elements promoters within 933W O_R (B). (A) Transcription reactions were performed as described in Materials and Methods. Repressor concentrations were increased in twofold 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).

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 O_R. Binding to O_R1 and O_R2 turns off transcription from P_R and activates transcription from P_{RM}. Repressor occupancy of O_R3 inhibits transcription from P_{RM}. 933W repressor also binds to sites in O_L and negatively regulates transcription from the P_L 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 sequences of P_R and P_{RM} (Fig. 3B). The relative juxtaposition of the O_R2 site and the P_{RM} promoter is distinctly different from

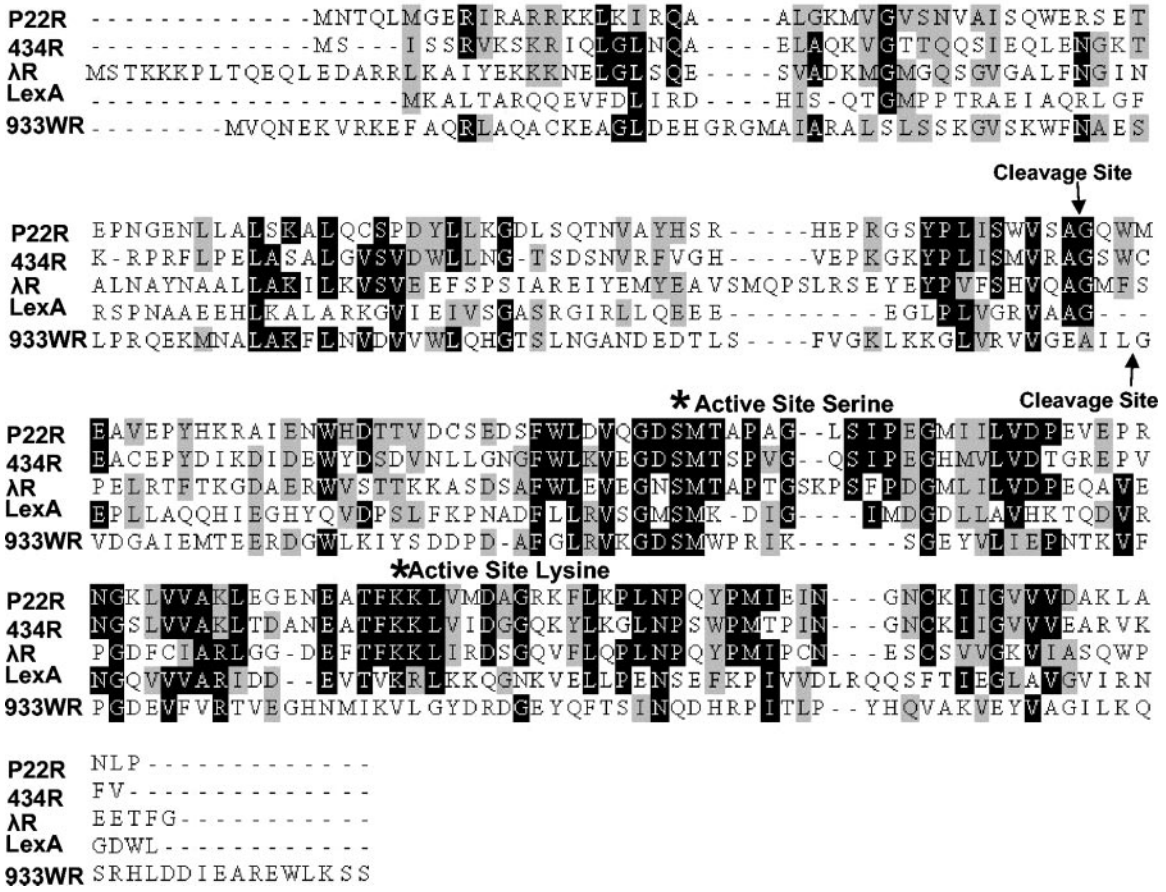

FIG. 9. Alignment of sequences of the self-cleaving DNA binding proteins from lambdoid bacteriophages and *E. coli*. The sequences are aligned by using Pileup (12). The indicated positions of the active site and cleavage site residues have been verified in all proteins except 933W repressor (see also Fig. 10). Dark shading denotes positions where identical amino acids are found in at least three of the aligned proteins. Light shading shows where homologous residues are found in at least three of the aligned proteins.

that found in the λ and 434 bacteriophages and remarkably similar to that observed in the bacteriophage P22. This observation suggests that 933W repressor may activate P_{RM} 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 α_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 α_2 contains several positively charged residues, whereas the ability of the λ and 434 repressors to activate P_{RM} 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 P_R promoter elements appears to determine the mechanism the phage repressor uses to repress P_R transcription (51). In bacteriophage 434, the −35 and −10 regions of the P_R promoter surround, but do not overlap, the O_R2 site (51). Nonetheless, 434 repressor binding to O_R2 is necessary and sufficient to prevent transcription from P_R. Thus, in phage 434, the O_R2-bound repressor blocks transcription initiation by blocking the transition of a P_R-bound RNA polymerase from a stable closed complex to an open complex (52). The position-

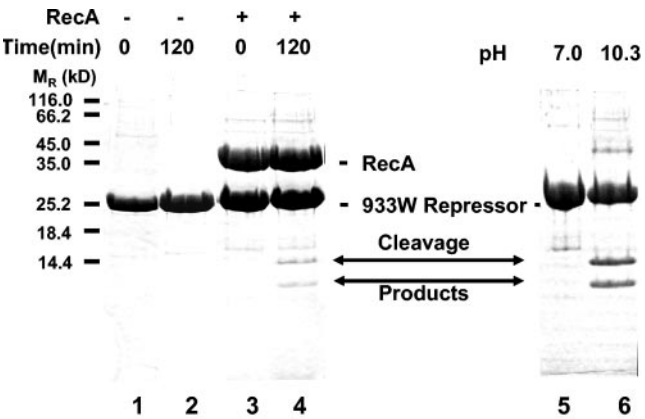

FIG. 10. RecA- and pH-stimulated autocleavage of bacteriophage 933W repressor. 933W repressor was incubated at neutral pH in the absence (lanes 1, 2, and 5) or presence (lanes 3 and 4) of activated RecA (see Materials and Methods) or in the absence of RecA at pH 10.3 (lane 6) for the indicated time. Autocleavage reactions were terminated by adding sample buffer containing SDS, and reaction contents were fractionated on a 15% Tris–Tricine discontinuous polyacrylamide gel. Shown is a photograph of the Coomassie brilliant blue-stained gel.

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_R1 and O_R2 at a lower concentration than it binds to O_R3. In all known lambdoid phages, the cooperative binding of repressor to O_R1 and O_R2 allows repressor to bind O_R1 and O_R2 at nearly identical concentrations. However, inspection of Fig. 3 reveals that three- to fourfold-higher 933W repressor concentrations are required to bind O_R2 than are needed to bind O_R1. 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 repressor for O_R1 and O_R2. Although we do not yet know the affinities of 933W repressor for the individual naturally occurring binding sites, both O_R1 and O_R2 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_L 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_R2 in intact O_R allows repressor to bind this site at a concentration that is only twofold lower than that needed to occupy O_R3. Since repressor synthesis in a lysogen is stimulated by an O_R2-bound repressor but inhibited by an O_R3-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_R2, a small drop in repressor concentration would lead to a sharp drop in occupancy of O_R2. 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 within the phage repressors and their relatives: 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 λ and lexA repressors, respectively, do not undergo autocleavage, and a λ repressor bearing a Gly→Ala 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 λ 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 λ 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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