Characterization of the lysogenic repressor (c) from transposable Mu-like bacteriophage D108

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ABSTRACT

The c gene products from related, transposable phages Mu and D108 encode lysogenic repressors which negatively regulate transcription and transposition. Using the gel shift assay to monitor c-operator specific DNA-binding activity, the 19.5 kDa D108 c repressor was purified to homogeneity. Sequence analysis of the N-terminus confirmed the identity of the purified protein as the repressor and ascribed its ATG initiation codon to base pair 864 from the D108 left end. Analytical gel filtration and dimethyl suberimidate cross-linking of repressor at 0.1–0.5 μM concentrations revealed that the repressor protein could form oligomers in the absence of its DNA substrate. From DNase I footprinting and gel mobility shift analyses, the D108 repressor only bound to two operators (01 and 02) which, as in Mu, flank an Integration Host Factor (IHF) binding site. In contrast to Mu, an 03 site in D108 was not found. Moreover, D108 repressor first bound operator 02, while occupancy of 01 required higher protein concentrations. The implications of these results on the D108 regulatory system are discussed.

INTRODUCTION

Temperate bacteriophage D108 functions as both a transposon and as a virus during its life cycle (1) and, although heteroimmune, is closely related to bacteriophage Mu (for review see 2, 3). During their lytic or lysogenic cycles, infecting phage DNA conservatively integrates to random locations. During lytic growth, these phages amplify their genomes by replicatively transposing to new locations within the host chromosome. In the lysogenic state, the prophage lytic functions are repressed by the immunity region at the left end of each phage's genome. Mu and D108 are approximately 90% homologous at the DNA level (4). Their heteroimmunity is a consequence of the lack of homology over their respective immunity regions, which roughly span base pairs 300 to 1500 from their respective left ends (5). This region encodes the repressor (c), ner, and the amino terminus of the transposase gene product (6,7). Transcriptional organization of the D108 left-end regulatory region (Figure 1) resembles Mu's (8–11). The D108 c repressor is transcribed toward the genome's left extremity from promoter Pc, while the early operon, subject to c and ner control, is transcribed in the opposite direction from promoter Pe (11). Furthermore, the activities of the two divergent lytic and lysogenic operons, in both Mu and D108, are influenced by the Escherichia coli protein Integration Host Factor (IHF) (10–14).

The heterologous phages each encode two negative regulatory proteins of transcription, the products of genes c and ner, which bind to specific sites in their respective intergenic regions (15–21). Ner is functionally analogous to λ Cro in that it negatively regulates transcription from Pc and autoregulates Pe activity (22–25). Mu c repressor, which has been overexpressed and its gene product purified (16), binds to three distinct operators termed O1, O2, and O3 within this region. As the immunity repressor, Mu's c protein inhibits transcription from Pe, which partially overlaps O2, thereby preventing expression of early lytic functions. A region spanning Mu's O1 and O2 operators also functions as a cis-acting enhancer of transposition, and is utilized by the Mu A protein to enhance formation of an important intermediate in transposition (26,27). The Mu A protein contains a DNA-binding domain at its amino terminus that is homologous to the amino terminus of Mu repressor, and it is believed that this region of Mu A protein mediates its binding to the Mu operators. Mu repressor can thus negatively regulate transposition in vitro by competing with Mu A for operator DNA-binding (27). The presumed DNA-binding domain at the amino terminal segment of the D108 c repressor gene product is highly homologous to the amino terminus of the D108 transposase gene product (7), and since the heterologous transposases are, to a certain extent, functionally interchangeable (28), the D108 transposase may also require interactions over a region spanning the D108 c operator.

D108 c repressor has previously been cloned and overexpressed under the control of the lacUV5 transcriptional promoter (11). Footprinting analysis, using E. coli crude extracts containing overexpressed D108 c protein, delineated the location of D108's operator to between base pairs 863 and 940 from the D108 left end, and wholly encompassing the IHF binding site (11). In order
to precisely define the nature of D108 repressor-operator interactions, we describe here the purification of the D108 repressor protein. We demonstrate that it protects two distinct blocks from DNase I cleavage, termed O1 and O2, which, as in Mu, flank either side of an IHF site. However, unlike Mu, D108 lacks an O3 site, and repressor occupation of O1 occurs only after site O2 is occupied. In contrast, Mu repressor binds simultaneously to O1 and O2, while at higher concentrations, presumably through cooperative interactions, occupies site O3 (12). The components of the left-end regulatory regions in phages D108 and Mu are strikingly analogous. The organization of repressor operators, which in Mu flank an IHF site to comprise the transpositional enhancer, indicate that the heterologous repressors may control transposition in similar manners. However, the differing affinities of the repressors for their respective sites, and the lack of an O3 site in D108, suggest that though the mechanism and regulation of transposition appears to be conserved between the two phages, there are significant differences in the regulation of Pe and Pc transcription by their heterologous c repressors.

MATERIALS AND METHODS

Chromatography Material, Enzymes and Reagents

Phosphocellulose (P-11, Whatman), DEAE-Sephadex A-50, CM-Sephadex C-50, Sephadex G-200 SF, Sephacryl S-200 HR, and heparin-Sepharose CL-6B (Pharmacia) were prepared according to the manufacturers' instructions. Restriction endonucleases and T4 polynucleotide kinase were from New England BioLabs. E. coli polymerase I Klenow fragment and bacterial alkaline phosphatase were obtained from Gibco-BRL. DNase I was purchased from Worthington. [α-32P]dNTPs (3000 Ci/mmol) and [γ-32P]ATP (5000 Ci/mmol) were obtained from Amersham. Dimethyl suberimidate (DMSI) was obtained from Pierce. IHF was purified according to the published procedure (29) from strain K5746, which was kindly provided by Dr. H. I. Miller.

DNA Fragments and End labeling

DNA fragments containing the D108 c-operator were isolated from plasmid pUD78, which carries the leftmost 1414 bp of the D108 genome (18). The D108 repressor-specific 348 base pair (bp) pUD78 Rsal-BglII restriction fragment was labeled by ‘backfilling’ the recessed 3'-BglII end with [α-32P]dATP and the Klenow fragment of DNA polymerase I (30). The 217 bp HpaII restriction fragment from pBR322 (labeled at the 3' ends with [α-32P]dCTP) was used as a non-specific substrate lacking the repressor-operator. Fragments containing truncated repressor-binding sites were isolated by secondary digestion of the 348 bp Rsal-BglII fragment; the 249 bp Rnal fragment was labeled by backfilling with [α-32P]dTTP, whereas the 93 bp Alul-NalIII fragment was treated with bacterial alkaline phosphatase and then labeled with [γ-32P]ATP using T4 polynucleotide kinase. Radioactive DNA fragments were subjected to electrophoresis on 5% polyacrylamide gels, located by autoradiography, and extracted by the ‘crush and soak’ procedure as previously described (31).

Operator Binding Assay

Specific binding of D108 repressor was detected using the gel electrophoresis shift method (32,33). The buffer used in the binding reactions contained 20 mM Tris-HCl pH7.5, 1 mM EDTA, 10 mM 2-mercaptoethanol, 150 mM NaCl, 100 μg/ml bovine serum albumin, 2×10^−5 M bp (14 μg/ml) sonicated calf thymus DNA and 0.02% (v/v) NP-40. Protein was added to the binding buffer, containing radioactive DNA, to bring the final reaction volume to 20 μl and incubated at room temperature for the appropriate amount of time. One microliter of binding buffer, containing 0.05% (w/v) bromophenol blue, was added to the samples, which were then subjected to electrophoresis on 5% polyacrylamide gels (29:1 w/w ratio of acrylamide to bis-acrylamide in 89 mM Tris-borate pH 8.3, 2 mM EDTA buffer) for 1 to 1.5 h at 10 V/cm.

Purification of D108 Repressor

Strain LF2100 harbours plasmid pDL100, which contains the left most 866 bp DraI fragment of the D108 genome and expresses D108 c+ under the control of the lacUV5 promoter (17). This strain overproduces repressor and was used as the source for its purification. Fifteen liters of LB broth grown cells (supplemented with 40 μg/ml of ampicillin and 1 mM isopropyl-β-D-thiogalactoside) were used to prepare crude extracts as previously described (18). From 52 grams of wet cells, a 100 ml crude extract containing 1.8 g of protein was obtained (fraction I) and dialyzed against buffer A (25 mM Tris-HCl pH 7.5, 1 mM EDTA, 200 mM NaCl and 10 mM 2-mercaptoethanol) at 4°C. A 20% (w/v) solution of streptomycin sulfate was added dropwise to the dialysate to a final concentration of 4% (w/v). Precipitated nucleic acids were pelleted by centrifugation at 15,000 g for 40 minutes at 4°C and the D108 repressor-containing supernatant fluid was dialyzed overnight at 4°C against buffer B [25 mM Tris-HCl pH 7.5, 1 mM EDTA, 5% (v/v) glycerol, 10 mM 2-mercaptoethanol] containing 25 mM NaCl. Ammonium sulfate was added to 30% saturation and the precipitated material was pelleted by centrifugation at 10,000 g for 20 minutes at 4°C. Additional ammonium sulfate was added to the supernatant fluid to achieve 50% saturation, the precipitated repressor was recovered by centrifugation, and then resuspended and extensively dialyzed at 4°C against buffer B containing 50 mM NaCl (fraction II: 40 ml, 250 mg protein).

Fraction II was loaded onto a 1.6 cm × 10 cm phosphocellulose column equilibrated in buffer B plus 50 mM NaCl, washed with 180 ml of the same buffer, and then eluted with a 200 ml linear gradient of 50–750 mM NaCl in buffer B. Active fractions, which eluted over the range of 160–420 mM NaCl, were pooled and dialyzed against buffer B containing 50 mM NaCl (fraction III: 40 ml, 30 mg protein).

Figure 1. Map of the Left-end Regulatory Region of D108. The transcription initiation sites for Pe and Pc are indicated with arrows (11). Ner (21) and IHF binding sites are designated by solid and hatched boxes, respectively. Open reading frames for the c and ner genes are represented with open boxes. Numbers represent base pairs from the left end of the D108 genome (7). Also shown are restriction enzyme sites which were used to isolate fragments for binding studies.
Fraction III was loaded onto a DEAE-Sephadex column (1 cm x 7 cm) previously equilibrated with 50 mM NaCl in buffer B and the D108 repressor DNA-binding activity was located in the flow through (fraction IV: 80 ml, 9 mg protein).

Fraction IV was loaded onto a CM-Sephadex column (1 cm x 4 cm) equilibrated with 50 mM NaCl in buffer B. The column was developed with a linear gradient of 50-600 mM NaCl in buffer B. D108 repressor activity eluted between 200 and 350 mM NaCl (fraction V: 5 ml, 8 mg protein).

Fraction V was applied to a Sephadex G-200 Superfine column (2 cm x 40 cm), eluted with 200 ml of 150 mM NaCl in buffer B, and fractions containing D108 repressor activity were pooled (fraction VI: 12 ml, 6 mg protein). At this point, D108 repressor was greater than 95% pure as judged by overloading and silver staining of an SDS-PAGE gel.

In order to separate D108 repressor from a faintly visible polypeptide of 70 kDa, fraction VI was loaded onto a heparin-Sepharose column (1 cm x 4 cm), equilibrated with buffer B plus 150 mM NaCl, and eluted with a 30 ml linear gradient of 150-450 mM NaCl in buffer B. Active fractions, which eluted at ~300 mM NaCl, were examined by SDS-PAGE, and the trailing edge of this peak contained a single 19.5 kDa polypeptide. These fractions were pooled and dialyzed against buffer C (25 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 25% (v/v) glycerol; fraction VII: 2 ml, 2 mg protein) and stored at -20°C. D108 repressor binding activity was stable for many months.

D108 repressor concentrations were determined using a calculated molar extinction coefficient at 280 nm of 1.65 x 10$^4$ M$^{-1}$cm$^{-1}$ (34). Automated sequence analysis of purified repressor was performed on an Applied Biosystems Model 470A gas phase microsequencer using a Model 120A PTH-amino acid analyzer.

Measurement of equilibrium constants

To various concentrations of D108 repressor, which had been equilibrated at room temperature for 30 min in 15 µl of binding buffer, was added 5 µl of binding buffer containing 2 x 10$^{-11}$ M labeled operator fragments. After a further 1 hr incubation at 24°C, bound and free DNA were resolved on a 5% polyacrylamide gel and quantitated by Cerenkov counting of gel slices. The apparent equilibrium constant, $K_{app}$, is the concentration of D108 repressor required to give 50% free DNA and 50% complexed DNA and was determined from the averages of three separate experiments with each fragment.

Gel filtration

The apparent molecular weight of native D108 repressor was determined by analytical Sephacryl S-200 HR chromatography. D108 repressor, at the indicated concentrations in 600 µl of buffer B containing 200 mM NaCl, was loaded onto a 1.6 cm x 45 cm column previously calibrated with Blue Dextran 2000 and the following marker proteins (with Stokes radius in Å in parentheses): aldolase (48.1), bovine serum albumin (35.5), ovalbumin (30.5), chymotrypsinogen A (20.9), and RNaseA (16.4). Protein was eluted in the same buffer and 2 ml fractions collected at a flow rate of 22 ml/hr until the A$_{280}$ (as measured with an ISCO-UA5 U.V. monitor) returned to baseline. Aliquots of each fraction were assayed for the presence of D108 repressor by (i) the operator binding assay, and (ii) by visualization on silver stained SDS-PAGE gels. Gel filtration data are presented in terms of $K_{av} = V_e - V_o/V_t - V_o$, where $V_e$ = elution volume of a given protein, $V_o$ = void volume of the column, and $V_t$ = total volume of the gel bed (35).

Dimethyl suberimidate (DMSI) crosslinking

Approximately 500 pmol of D108 repressor in various volumes of crosslinking buffer (80 mM triethanolamine-HCl pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.1 mM dithioerythritol) was incubated at 24°C for 30 min to allow the protein to equilibrate at these concentrations. A freshly prepared 30 mg/ml solution of dimethyl suberimidate (pH 8.0) in the same buffer was added, such that the final DMSI concentration was 5 mg/ml, and incubated at room temperature for 45 min. The reaction was terminated by the addition of 1/5th volume of sample buffer [50 mM NaPO$_4$ pH 7.0, 5% (w/v) SDS, 50% (v/v) glycerol, 20% (v/v) 2-mercaptoethanol, 0.1 mg/ml bromophenol blue] followed by heating at 90°C for 5 min. Protein which was crosslinked in volumes exceeding 100 µl was first concentrated by ultrafiltration in Amicon Centricon-10 centrifugal microconcentrators, and then subjected to electrophoresis on 15% polyacrylamide/0.1% SDS gels (36) and stained with silver (37).

Protection experiments

DNase I protection experiments were performed as previously described (38). Briefly, the radioactively labeled 348 bp Rsal-BglII DNA fragment, containing the D108 repressor-operators, was incubated with various concentrations of D108 repressor in operator binding buffer containing 10 mM MgCl$_2$ and 5 mM CaCl$_2$ for 45 min at room temperature. DNase I (2482 units/mg) was added for 30 seconds to a final concentration of 120 ng/ml. The reaction was terminated by adding 25 µl of stop solution (3 M ammonium acetate; 250 mM EDTA; 150 µg/ml sonicated calf thymus DNA). The DNA was extracted with phenol:chloroform:isoamyl alcohol (45:45:10 v/v/v) followed by ethanol precipitation, and then subjected to electrophoresis on 15% polyacrylamide/0.1% SDS gels.

Figure 2. Purification of D108 Repressor Monitored by the Operator Binding Assay. Panel A, silver stained SDS/15% PAGE gel containing 1-2 µg of total protein from successive steps during the purification. Lane 1, fraction I (crude protein extract from strain LF 2100); 2, fraction II (after NH$_4$SO$_4$ precipitation); 3, fraction III (after phosphocellulose chromatography); 4, fraction IV (DEAE-Sephadex flow through); 5, fraction V (following CM-Sephadex chromatography); 6, fraction VI (after Sephadex G-200 gel filtration); and 7, fraction VII (following heparin-Sepharose chromatography). The markers (lane M), shown in decreasing molecular weight, are phosphorylase B, bovine serum albumin, ovalbumin, α-chymotrypsigen, β-lactoglobulin and lysozyme. Panel B, Operator-binding assay performed with two DNA fragments: the specific DNA substrate (S) is the 348 bp Rsal-BglII fragment from the D108 left end; the non-specific DNA substrate (NS) is the 217 bp HpaII restriction fragment from pBR322. Lanes 1-6, respectively, contain 0, 0.1, 0.5, 3, 8, and 15 nM purified repressor protein.
precipitation with ethanol. Reaction products were subjected to electrophoresis on DNA sequencing gels (31) which were then exposed to Kodak XAR-5 film under Dupont Cronex intensifying screens at -70°C.

RESULTS

Purification of the D108 c repressor

The purification procedure exploited the DNA-binding properties of this protein in a gel shift assay to detect specific binding of the D108 repressor to its binding site in the D108 early region. Following precipitation of nucleic acids from extracts by streptomycin sulfate and ammonium sulfate precipitation of proteins, D108 repressor adsorbed to a cellulose-phosphate column and eluted at a NaCl peak of about 290 mM. The repressor protein flowed through a DEAE column, and by this point was at least 80% pure (Figure 2A, lane 4). The remaining steps removed all of the other proteins present in minor quantities. Purified repressor retained binding activity for a DNA fragment from the D108 early region, yet at these concentrations did not bind to a non-specific fragment (Figure 2B). The results of NH2-terminal sequencing of purified D108 repressor showed that the first 21 residues perfectly coincided with the predicted amino acid sequence (7). The first codon (ATG) of the D108 repressor gene begins at bp 864 from the left end of the genome and encodes a 174 amino acid polypeptide with a predicted and observed molecular mass of 19.5 kDa.

DNA-Repressor Complex Formation

Purified D108 repressor was assayed for DNA binding using three different radiolabeled restriction fragments containing various segments of the D108 immunity region. Complex formation with each of these substrates was monitored using the operator-binding assay as detailed in Materials and Methods. The 348 bp Rsal-BglII restriction fragment (bp 803–1150 of the D108 genome) spans the entire left-end intergenic region. Figure 3A shows that, with increasing amounts of repressor, two protein-DNA complexes formed with this fragment. At low concentrations one complex was formed, and then a second complex of slower migration gradually appeared as the concentration of repressor was increased. Since the formation of two different protein-DNA complexes correlated with the amount of repressor used, we propose that this represents the binding of the D108 repressor to two different sites on this fragment. The first complex reflects binding to a high affinity site, and the second complex reflects subsequent binding to a second site of lower affinity.

Figure 3. Equilibrium Binding of D108 Repressor to Different Operator Containing Fragments. The assay is detailed in Materials and Methods. Lanes 1 to 20 contain 0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.35, 0.38, 0.5, 0.62, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 5.0, 7.5 and 12.5 nM D108 repressor. Panels A, B and C were assays performed using the 348 bp Rsal-BglII, 249 bp Rmal and 93 bp Alul-NalIII restriction fragments, respectively. Panel D. Data points obtained by Cerenkov counting of bound and free DNA from three separate experiments using the different fragments. Curves A–C correspond to panels A–C above.

Figure 4. DNase I Protection of the Intergenic Region by D108 Repressor. Lanes GA and TC are the Maxam and Gilbert (31) sequencing reactions. Lane 1, DNase I cleavage in the absence of repressor. Lanes 2–10 show the DNase I cleavage in the presence of 0.05, 0.15, 0.3, 0.4, 0.5, 1, 2, 3 and 4 μM D108 repressor, respectively.
This is supported by the independent isolation of each of these two sites on smaller restriction fragments and their subsequent use in the operator-binding assay. The higher affinity binding site can be localized to a 249 bp Rmal restriction fragment spanning bp 874–1133 from the left end of the D108 genome. Only one bound complex formed with this substrate in the operator-binding assay using an identical range of protein concentrations (Figure 3B). The apparent equilibrium dissociation constant, as determined from the binding curve B in Figure 3D, revealed a \( K_{\text{app}} \) of \( 9 \times 10^{-10} \) M, which is slightly larger than the \( 6 \times 10^{-10} \) M obtained for binding of D108 repressor to the full length Rsal-BglR fragment (Curve A, Figure 3D).

The lower affinity binding site can be isolated on a 93 bp Alul-NlaW restriction fragment (base pairs 823–915 in D108). In an operator-binding assay, this particular substrate (Figure 3C) also formed a single bound complex with D108 repressor, but at protein concentrations of repressor that corresponded to the quantity needed for formation of the second complex with the full length intergenic fragment (Figures 3A and 3C, lanes 13–20). The apparent equilibrium dissociation constant for this site, as determined from curve C in Figure 3D, revealed a \( K_{\text{app}} \) of \( 4 \times 10^{-9} \) M.

Footprinting of the D108 c Repressor

In order to determine the precise boundaries of the repressor binding sites, DNase I footprinting was performed on the 348 bp Rsal-BglII fragment containing the two putative operators Figure 4). As the concentration of repressor is increased (lanes 2–6) a 56 bp region, termed O2 and extending from bp 925–980, was protected from DNase I cleavage. With greater concentrations (lanes 7–11) of protein, a second region termed O1, and spanning bp 844–890, was also protected from DNase I attack. The location of the high affinity O2 and lower affinity O1 sites correspond to their mapped assignments as determined from the restriction fragments used in the binding studies in Figure 3. Situated between the O1 and O2 operators is an IHF binding site (7,11). This organization is identical to the O1 and O2 repressor sites which flank the IHF box in bacteriophage Mu and, although the sequence and extent of binding differs between the heterologous proteins, we have chosen to maintain the same terminology.

Mu repressor binds its O1 and O2 sites simultaneously and IHF has no effect on this interaction (12), while D108 repressor had different affinities for its two sites. We thus explored IHF’s effect on D108 repressor binding to its O1 and O2 operators. Figure 5 displays D108 repressor’s DNase I protection pattern in the presence of 40 nM IHF. Lane 2 depicts the protection, spanning base pairs 888–932, conferred by IHF alone. The concentration of D108 repressor required to protect O2 from DNase I cleavage did not change (remaining in the 0.4–0.5 \( \mu \)M range) in the presence of IHF. However, the amount of D108 repressor required to protect O1 site decreased from 2 \( \mu \)M to 1 \( \mu \)M in the presence of IHF. Although the effect of IHF on D108 repressor binding to O1 was reproducible, it was small and its significance is not obvious at this time.

![Figure 5. DNase I Protection of the Intergenic Region by D108 repressor in the Presence of IHF. Lanes GA and TC are the sequencing reactions. Lane 1, DNase I cleavage pattern without either protein; lane 2, with \( 4 \times 10^{-8} \) M IHF; lanes 3–11 all contained \( 4 \times 10^{-8} \) M IHF and, in addition, contain 0.05, 0.15, 0.3, 0.4, 0.5, 1, 2, 3 and 4 \( \mu \)M D108 repressor, respectively.](image)

![Figure 6. Sephacryl S-200 HR Analytical Gel Filtration. Panel A, Measurement of specific DNA binding in fractions 19–38 eluted from the column using the operator-binding assay. Panel B, the \( A_{260} \) elution profile of each fraction with vertical arrows denoting the peak elution of the markers M1, blue dextran 2000; M2, aldolase (ALD); M3, bovine serum albumin (BSA); M4, ovalbumin (OVA); M5, \( \alpha \)-chymotrypsinogen (CHYA); and M6, RNase A. Panel C, silver stained SDS/15% PAGE gel of samples from fractions 19–38.](image)
Physical Properties of D108 Repressor

The single band of purified repressor observed on denaturing 15% SDS-PAGE has an approximate molecular weight of 19,500 (Figure 2). The apparent molecular weight of the native repressor, at two different concentrations, was determined by analytical Sephacyl S-200 HR chromatography. A comparison of the elution volume of the repressor, loaded onto the column at an initial concentration of 50 μM (Figure 7), with those of reference proteins indicated an apparent molecular weight for the repressor of about 100,000 and a Stokes radius of 41 Å (Data point 1, Figures 8A and 8B). However, the peak elution volume of repressor increased when it was loaded onto the column at a 100-fold lower initial concentration (0.5 μM). At this lower concentration, the apparent native molecular weight was approximately 80,000 with a corresponding Stokes radius of 36 Å (Data point 2, Figures 8A and 8B). This data suggests that D108 repressor conformation and/or subunit composition can vary at differing concentrations.

To further examine this effect, cross-linking studies with repressor were undertaken. Treatment of repressor protein (at 0.1 μM and 1 μM) with dimethyl suberimidate (40), followed by denaturing SDS-PAGE, revealed four major covalently linked species (Figure 8). The four observed bands may be the result of partial cross-linking of adjacent subunits and thus represent monomer, dimer, trimer and tetramer forms of the protein. However, the two largest cross-linked species migrate faster than the uncross-linked monomeric markers, and could represent either different subunit composition or conformational isomers which may be constrained from fully unfolding during denaturation (41). Cross-linking repressor at concentrations exceeding 5 μM resulted in the additional appearance of a smeared band(s) in the 100—120 kDa range (data not shown).

DISCUSSION

One of the physiological functions of the D108 repressor is to repress transcription of the early lytic functions from the Pe promoter (11). The results presented here showed that purified and functional repressor protein is encoded by an open reading frame starting with the methionine at base pair 864 of the D108 genome, as originally predicted by Mizuuchi and co-workers (7). At a concentration of 10^-7 M, which is the approximate physiological concentration of other phage repressors in a lysogen (42,43), the 174 amino acid D108 repressor chromatographed and cross-linked as an oligomer. A tetrameric form of repressor is consistent with both the gel filtration and cross-linking studies, though other hypotheses, such as altered repressor structure, cannot be ruled out at this time. At high protein concentrations, the presence of repressor binding sites of the D108 genome is indicated by the putative Pe -35 and -10, plus the Pc-10 regions (11) are highlighted by asterisks; the transcription start sites from these two promoters are designated by open arrows, and broken arrows represent this motif with a single nucleotide difference.
reppressor appeared to exist as a larger species, a characteristic common to many proteins which bind DNA cooperatively.

Purified D108 repressor bound to two operator sites, termed O1 and O2, in the D108 left-end intergenic region (Figure 9). Half-maximal binding of repressor to the O2 site, as determined from the footprinting studies, can be estimated to occur in the 150–300 nM concentration range whereas the $K_{\text{app}}$ value obtained with the gel shift assay was 0.9 nM. This difference could be due to the different chemical kinetic conditions employed in the two assays (44). One possibility is that D108 repressor has a quick on-off rate, and the gel shift assay provided a 'caging effect' (32,33) through the gel matrix so that dissociated components could not diffuse away. In this regard, a fast dissociation rate was observed (greater than 90% of repressor-O2 complexes dissociated in 1 min) following the addition of a 1000-fold molar excess of unlabeled operator fragment (data not shown). Alternatively, the $K_{\text{app}}$ value obtained by DNase I footprinting is equally valid and may reflect repressor structural differences at higher protein concentrations (see above paragraph) which yield tight binding to its operators. A $K_D$ value representative of in vivo conditions may thus require a more extensive kinetic analysis.

The O1 and O2 operators flank a central IHF site. This organization is similar to the two left-most operators present in bacteriophage Mu's intergenic region, whose repressor binds to three sites (O1, O2, and O3) (16). Since O2 and Pe overlap in Mu, this operator is believed to be involved in repression of the early promoter. Transcription from the D108 early promoter starts at bp 1000 (11). The Pe −35 region, encompassed by the O2 operator (Figure 9), may be sequestered by bound D108 repressor to preclude RNA polymerase from binding and initiating Pe transcription.

The distance between the O1 and O2 operators in D108 spans 34 base pairs, contains an IHF site, and is identical to the spacing separating Mu’s corresponding operators. The operators in D108 are slightly smaller than their counterparts in Mu and, although the heterologous sites share little sequence homology, both sets are very AT rich. In Mu, the non-symmetric consensus sequence, 5'-CTTTPy(A/T)AANN(A/T)-3', is the proposed site required for the binding of the Mu repressor (12). This site is present once in O3, while the higher affinity O1 and O2 sites contain 4 and 5 such copies, respectively. No segment of the repressor protein binding sites in D108 conform to this consensus. However, they do possess other sequences which are repeated numerous times in their own sites. The sequence 5'-AAATC-3' is of particular interest because of its positioning in each of the operators (Figure 9). The O2 operator contains 5 such motifs, 4 of which are perfect consensus sites, and one with a single nucleotide degeneracy (5'-GAATC-3' represented by a dashed arrow in Figure 9). These five sites are positioned in O2 such that their central A's are spaced at 10–11 base pair intervals and would therefore be exposed on the same face of a conventional DNA helix. The DNase I protection pattern conferred by bound repressor revealed an enhanced cleavage site between bp 952 and 953, at the 3' end of one of these sites; this may represent the sequence specific binding of a repressor protomer to this site and the creation of a favourable environment for DNase I attack. The O1 operator only possesses 4 such consensus motifs, one of which also contains a single nucleotide degeneracy (Figure 9). As observed in O2, their positioning also follows the 10–11 bp periodicity of a B-DNA double helix. The number of consensus sites in O1 and O2, and their degree of conformity, also correlates with the binding strength of each of these operators.

The affinity of D108's repressor for O1 and O2 differs significantly. As determined from either the footprinting assay or the gel shift assay, the concentration of repressor required to observe half-maximal binding of O1 is approximately an order of magnitude greater than the amount required for O2 binding. Mu repressor binds its O1 and O2 simultaneously and perhaps cooperatively, and DNase I protection of O3 is seen only after their occupancy. The Mu O3 site overlaps the Pc promoter, which initiates leftward (c gene) transcription (12), and the binding of Mu repressor at this location is thought to autoregulate the Pc promoter. D108 repressor did not bind to any other sites in the intergenic region. D108's Pc promoter is situated to the right of the Ner-operator, as is Mu's (11,20,21) and a corresponding O3 in D108, overlapping this RNA polymerase binding site, was not detected. How D108 regulates transcription from its Pc promoter and the manner in which lysogeny and stable c expression is maintained remains to be determined.

The Mu and D108 immunity regions are non-homologous yet share some important features. Both phages require IHF for lytic development. The Mu DNA strand-transfer reaction requires IHF at 'physiological' levels of supercoiling (45) which bends the DNA between O1 and O2, facilitating transposase binding to this region and thereby promoting the formation of type I complexes (26,27). Mu and D108 transposase proteins are partially complementary in an in vivo transposition assay (28). Moreover, the putative DNA-binding domain(s) in D108 repressor share extensive homology with the amino terminal portion of D108 (but not Mu) transposase (7). Therefore, we presume that D108 transposase associates with the D108 O1 and O2 operators, in a fashion resembling Mu transposase-operator association (26), to enhance transposition.

Although the relevant nucleotide sequences have not been conserved in Mu and D108, both phages appear to possess the identical cis-acting elements required to regulate transposition; their heterologous c repressors may be involved in this process by binding to their respective O1 and O2 operators. However, the lack of an O3 site in D108, and the apparent differences in the manner with which these proteins bind their cognate sites, suggests that they may regulate Pc and Pe transcription in different ways.

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