Isolation of λ Repressor Mutants with Defects in Cooperative Operator Binding†

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ABSTRACT: A hybrid operator–promoter region was designed to aid in a screen for cooperativity mutants of the λ repressor. In this system, λ repressor mutants with defects in pairwise cooperative binding are unable to act as efficient transcriptional repressors. Four single amino acid substitutions in the C-terminal domain of the repressor were isolated. Studies of the DNA binding properties of the purified mutant proteins show that a repressor bearing the Gly147 → Asp mutation binds with normal affinity to single operator sites but is defective in pairwise cooperative site binding. Quantitative footprinting studies show that the free energy of interaction between repressor dimers bound at operator sites O_R1 and O_R2 is reduced from −2.4 kcal/mol for the wild-type repressor to 0 kcal/mol for the GD147 mutant.

The λ repressor exhibits cooperative binding to adjacent DNA sites in the O_R and O_L operators of bacteriophage λ (Johnson *et al.*, 1979, 1981; Ptashne, 1986). Each of these regulatory regions contains three 17-base-pair operator sites, spaced at 3–7-base-pair (bp) intervals. Individual operator sites serve as binding sites for λ repressor dimers, and cooperative interactions between adjacently bound dimers can stabilize the protein–DNA complex by 2–3 kcal/mol. Cooperative interactions between DNA-bound dimers have also been shown to stabilize DNA loop formation in artificially constructed operators with sites separated by five or six turns of the DNA helix (Hochschild & Ptashne, 1986; Griffith *et al.*, 1986). The importance of cooperativity in the biological function of the λ repressor has not been experimentally tested because mutants that are defective in cooperative binding but competent in other repressor functions such as dimerization and binding to single DNA sites have not been available. For example, the SN228 mutant described by Hochschild and Ptashne (1988) is defective both in cooperativity and in single-site DNA binding. Quantitative modeling, however, suggests that the cooperativity of λ repressor binding is required for maintenance of the bacteriophage in the lysogenic state and for the efficient switch from lysogenic to lytic development (Ackers *et al.*, 1982; Shea & Ackers, 1983).

In this paper, we describe a genetic screen for λ repressor mutants that may be defective in pairwise cooperative binding. Using this screen, we have isolated four mutations in the C-terminal domain of the λ repressor. Studies of the corresponding repressor mutants *in vitro* show that at least one of these mutations (Gly147 → Asp) confers a cooperativity-defective phenotype.

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EXPERIMENTAL PROCEDURES

Strains. Escherichia coli strain X90 is *ara* Δ(*lac–pro*) *nalA argE*am *rif thi-1*/F′ *lacI*^{Q} *lac*$^+$ *pro*$^+$ (Amman *et al.*, 1983). Strain X90K is a *lacZ*$^-$, kanamycin-resistant derivative of X90 in which the *lac–pro* region of the F′ is disrupted by transposon Tn5 (M. Mossing, unpublished results). Standard methods of microbiology and molecular biology were performed as described in Miller (1972) and Maniatis *et al.* (1982).

Enzymes and Chemicals. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs, Inc. The large fragment of DNA polymerase (Klenow fragment) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) were obtained from Boehringer Mannheim Biochemicals. Isopropyl β-D-thiogalactoside (IPTG) was obtained from Sigma. Hydroxylamine was from Fisher Scientific Co. [α-^{32}P]dATP (400 Ci/mmol) used for DNA sequencing and [α-^{32}P]dNTPs (3000 Ci/mmol) used for end labeling of DNA were from Amersham Corp. Unlabeled dideoxy- and deoxynucleotide triphosphates were from P-L Biochemicals. Acrylamide, bisacrylamide (BioRad), and urea (Pierce) were electrophoresis grade. All chemicals used in preparation of buffers were reagent or analytical grade.

Synthesis and Purification of Oligonucleotides. DNA oligonucleotides were synthesized on a Systec Microsyn 1450A DNA synthesizer using phosphoramidites from American BioNuclear. The oligonucleotides were purified on a 20% acrylamide (acrylamide:bisacrylamide = 30:1) slab gel containing 7 M urea. Fragments were visualized by UV shadowing, and gel slices were excised, crushed, and soaked overnight in 100 mM Tris (pH 7.5)/1 mM EDTA at 37 °C to elute the DNA. Gel fragments were removed by centrifugation, and the resulting supernatant was passed over a Schleicher and Schuell elutip column. DNA was eluted from the column in 1 M NaCl and precipitated by addition of three volumes of 100% ethanol.

Construction of a λ Operator–Promoter–cat Fusion. A λ operator–promoter region (designated O_SP_S; Figure 1A) was constructed from six DNA oligonucleotides. The 5′ termini of four of the oligonucleotides were phosphorylated using T4 polynucleotide kinase, and the six oligonucleotides were then

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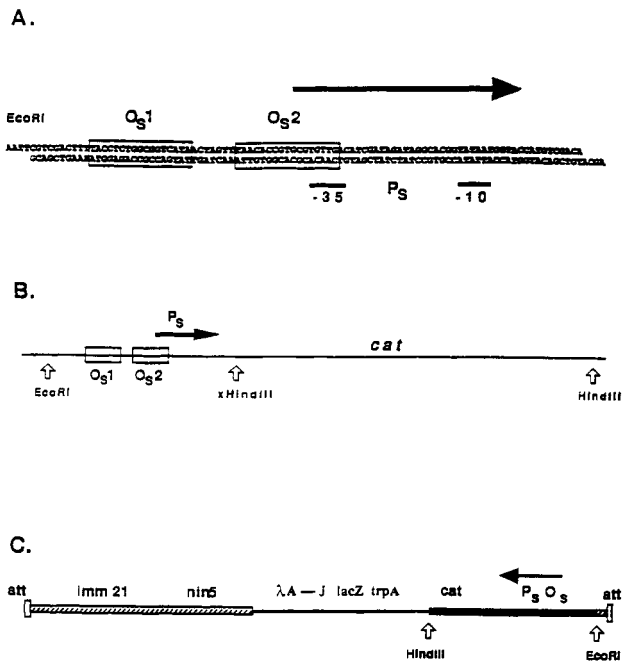

FIGURE 1: (A) Sequence of the O_SP_S operator–promoter region. The O_S1 and O_S2 operator sites are boxed. The O_S2 site overlaps the −35 region of the P_S promoter. (B) Fusion of the O_SP_S operator–promoter region to the chloramphenicol acetyltransferase (*cat*) gene. Ligation of the right end of the fragment shown in (A) to the left end of the *Hin*dIII fragment from pCM7 destroys this *Hin*dIII site. (C) Structure and prophage orientation of the λ112–O_SP_S–*cat* fusion phage.

joined at equimolar concentrations using T4 DNA ligase. The oligonucleotides at the 5′ termini of the O_SP_S insert were not phosphorylated to avoid production of multimers of the O_SP_S fragment. Fusion of the O_SP_S fragment to the chloramphenicol acetyltransferase (*cat*) gene was achieved by ligation of the 100-bp synthetic insert to the 260-bp and 3-kbp fragments obtained from *Hin*dIII and *Eco*RI restriction of the plasmid pCM7 (Close & Rodriguez, 1982; obtained from US Biochemicals). In the resulting construct, chloramphenicol acetyltransferase is expressed from the synthetic promoter P_S. Chloramphenicol-resistant transformants of strain X90 were selected, and plasmid DNA was isolated and analyzed by restriction digestion and sequencing. A plasmid with the expected structure was designated pO_SP_S–*cat*.

λ112 is an *imm21* derivative that contains a λ P_{RM}–*cI*–*trpA*–*lacZ* fusion in the *b*2 region of the phage (Maurer *et al.*, 1980). λ112 DNA was cleaved with *Hin*dIII to generate a 25-kbp left arm fragment containing the *lacZ* and *trpA* genes. The right arm of the phage was generated by cleavage of the phage with *Eco*RI. The purified arms were ligated to a *Hin*dIII–*Eco*RI partial digestion product of pO_SP_S–*cat*. The ligation mix was packaged using packaging extracts (Promega Biotech) according to the instructions of the manufacturer. Lysogens of phage λ112–O_SP_S–*cat* were obtained by plating the phage on strain X90K using plates containing chloramphenicol (100 μg/mL), kanamycin (50 μg/mL), and Xgal. The lysogens (X90K/λ112–O_SP_S–*cat*) present in the turbid centers of blue plaques were further purified by restreaking on media containing Xgal and chloramphenicol.

Mutagenesis. The source of DNA for the λ repressor gene *cI* was pFG600 (Gimble & Sauer, 1989). This plasmid is a pBR322 derivative that carries the λ *cI* gene expressed from the *lac* UV5 promoter, an M13 origin of replication, and an ampicillin resistance gene. Plasmid DNA was treated with 0.5 M hydroxylamine in 0.25 M potassium phosphate (pH 6.0)/2.5 mM EDTA, at 67 °C, for 80–100 min. The mutagenized plasmid was then cleaved with *Sph*I to obtain a fragment that encodes residues 93–236 of the λ repressor protein. The mutagenized fragment was ligated to an unmutagenized *Sph*I backbone fragment of pFG600, and the ligation mixture was transformed into strain X90. Pools of the plasmid DNA were prepared from these transformants and used to transform the tester strain X90K/λ112–O_SP_S–*cat*.

Immunity Tests. Transformants of the X90K/λ112–O_SP_S–*cat* tester strain expressing wild-type or mutant repressors from pFG600 were cross-streaked against $>10^6$ plaque-forming units of clear or virulent derivatives of phage λ on LB plates containing ampicillin (100 μg/mL). After incubation for 5–6 h at 37 °C, the plates were examined. Survival of a strain to cross-streaking indicates immunity of that strain to superinfection by a phage of given virulence. The phages used here—λ KH54, λ *c*Ic17, λ vir, λ 3v, λ 4v, and λ 5v—form a series of increasing virulence (Eliason, 1985). For example, a cell containing lysogen levels of λ repressor (ca. 200 molecules) is immune to λ KH54 but sensitive to the remaining phages, while a cell containing 100-fold higher levels of repressor is immune to λ KH54, λ *c*Ic17, λ vir, λ 3v, and λ 4v but sensitive to λ 5v. Hence, for a strain expressing the wild-type repressor, the level of immunity provides a rough measure of the intracellular concentration of repressor (Hecht & Sauer, 1985). For strains expressing mutant repressors, the immunity test provides information about the operator binding capacity of the mutant repressor relative to that of the wild-type repressor. A reduced immunity may reflect a reduced capacity to bind the operator and/or a reduced intracellular concentration of the mutant repressor.

DNA Sequencing. DNA was sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977). Sequencing of the O_SP_S–*cat* fusion was performed on double-stranded plasmid DNA prepared by the rapid alkaline lysis method (Maniatis *et al.*, 1982). Sequencing of λ repressor mutants was performed on single-stranded plasmid DNA prepared after infection with the M13 helper phage RV1 as described (Vershon *et al.*, 1986).

Protein Purification. The mutant λ repressor genes were fused to the *tac* promoter by ligating the 700-bp *Nsi*I–*Cla*I restriction fragment from the appropriate pFG600 derivative to the 5.5-kbp *Nsi*I–*Cla*I backbone fragment of pMH236 (Hecht *et al.*, 1986), which carries the N-terminal portion of the wild-type repressor gene under control of the *tac* promoter. This construction transfers any λ repressor mutation present between residues 56 and 236 from *lac* promoter to *tac* promoter control. The wild-type and mutant repressors were purified from strain X90 as described (Johnson *et al.*, 1980). The purified repressors were estimated to be >95% pure as judged by Coomassie Brilliant Blue staining of samples electrophoresed on SDS/polyacrylamide gels (Laemmli, 1970). The concentrations of the repressors were determined by UV absorbance (A_{280} = 1.18 cm^{-1} for a 1 mg/mL solution; Sauer & Anderegg, 1978).

Preparation of DNA Fragments. Plasmids pOR1, pBJ301, pBJ303, pBJ304, pBJ306, and pKB252 (Johnson, 1980; Meyer *et al.*, 1980; Backman *et al.*, 1976) were used for preparation of operator DNA fragments for binding experiments. The 570-bp fragment of pOR1 which contains the single operator site O_R1 was obtained by digestion of the plasmid with *Eco*RI. For pBJ301, pBJ303, pBJ304, pBJ306, and pKB252, an 1107-base-pair fragment containing the λ O_R operator region was obtained by double digestion of the plasmid with *Pst*I and

*Bgl*II. The λ O_R region from pBJ301 contains the *vs387* mutation which greatly reduces the affinity of the λ repressor for the O_R1 operator site (Ordal & Kaiser, 1973). The λ O_R region from pBJ303 is O_R1^- and O_R3^-. The λ O_R regions from pBJ304 and pBJ306 are O_R1^- and O_R2^-, respectively. The λ O_R region from pKB252 is the wild type. Restriction digestion products of the plasmids were separated by electrophoresis on a 1% agarose gel (BioRad). The fragments were visualized by ethidium bromide staining, electroeluted, and then passed over NACS prepac columns (BRL). DNA fragments were stored at −70 °C in 10 mM Tris (pH 7.2)/0.1 mM EDTA. Restriction fragments were labeled for binding assays by end-filling with [α-^{32}P]dNTPs and the Klenow fragment of DNA polymerase I.

Nitrocellulose Filter Binding. Nitrocellulose filter binding was performed as described in Senear *et al.* (1986). The binding mixtures were prepared in buffer containing 10 mM Bistris (pH 7.0 at 20 °C), 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 100 μg/mL bovine serum albumin, and 2 μg/mL sonicated calf thymus DNA. The mixtures were incubated at 20 °C for at least 1 h prior to filtration. Nitrocellulose filters (BA25) were obtained from Schleicher and Schuell and were soaked once in distilled water for 45 min and twice in wash buffer for 45 min prior to filtration. Wash buffer is binding buffer without the bovine serum albumin and calf thymus DNA. The filters were dried at room temperature, dissolved in 4 mL of Aquasol (National Diagnostics), and counted in an LKB-Pharmacia rack-β scintillation counter.

Nitrocellulose filter binding data obtained from titration of the O_R1 template were analyzed using a nonlinear least-squares parameter estimation method (Straume *et al.*, 1991) that employs a modified version of the Gauss–Newton procedure (Hildebrand, 1956). The data were fit to the binding equation

$$\bar{Y} = \frac{[R]^2}{K_1K_D + [R]^2}$$

where K_1 is the equilibrium dissociation constant for the repressor dimer–operator complex, K_D is the equilibrium dissociation constant for the repressor dimer, and [R] is the concentration of free repressor monomer. The K_D values for wild-type and mutant proteins were assumed to be identical for analysis of the nitrocellulose binding data, and the O_R1 binding data were fit directly for the Gibbs free energy of binding of the dimer to the site. In the experiments described here, the concentration of the operator fragment is significantly lower than the total repressor concentration so that

$$[R]_T = [R] + 2[R_2] + 2[R_2O] \approx [R] + 2[R_2]$$

DNAse I Footprinting. DNase I footprinting was performed as described by Brenowitz *et al.* (1986) in buffer containing 10 mM Bistris (pH 7.0 at 20 °C), 200 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 100 μg/mL bovine serum albumin, and 2 μg/mL sonicated calf thymus DNA. Reaction mixtures containing repressor and 15 000–25 000 cpm of end-labeled operator DNA were incubated in a volume of 200 μL at 20 °C for 1 h, before adding DNAse I at a concentration of 0.17 μg/mL. After 1 min the DNAse reactions were stopped by addition of 0.7 mL of a solution containing 50 μg/mL tRNA (Sigma), 0.4 M ammonium acetate, and 95% ethanol. Cleavage products were displayed by autoradiography following electrophoresis on 8% acrylamide gels containing 8 M urea. The operator concentration was sufficiently low in all footprinting experiments to assume that $[R]_T \approx [R] + 2[R_2]$.

Two-dimensional optical scans of footprint autoradiographs were obtained using an Eikonix 1412 charge coupled device (CCD) camera. Transmittance units obtained from the CCD camera were converted to optical density values relative to the output of the Aristogrid Model T light box used as a transmissive source for the optical scans. Optical density (OD) values were normalized to a 255 gray scale range prior to use in the gel blocking program and calculation of fractional operator occupancy as described by Brenowitz *et al.* (1986). Data files were checked to ensure that the maximum optical density values did not exceed the linear range of the CCD camera (2.2–2.4 OD units, determined by scanning a Kodak precalibrated step wedge) or the X-ray film.

Energetic parameters for the interaction of the repressor with the right operator were estimated by simultaneous nonlinear least-squares analysis of data obtained from wild-type and mutant templates. The data were fit to the eight-species model described by Ackers *et al.* (1982) and an alternative model described in the Results. Calculations were performed on a Hewlett-Packard 9000 computer.

RESULTS

We constructed the O_SP_S transcriptional control region (see Figure 1A) to aid in a search for λ repressor mutants defective in cooperative operator binding. The sequence of O_S1 is identical to the sequence of the natural operator O_R1, and that of O_S2 is identical to that of O_R2. Moreover, the two sites are separated by seven base pairs in both cases. Thus, on the basis of the known properties of repressor binding to O_R1 and O_R2 (Johnson *et al.*, 1979), the intrinsic affinity of the wild-type repressor should be greater for O_S1 than for O_S2 and binding to the two sites should be cooperative. The major difference between the O_SP_S and O_RP_R regions is in the relationship of the promoter to the operator sequences. In O_RP_R, both O_R1 and O_R2 overlap the P_R promoter and occupancy of either site leads to repression. In O_SP_S, the P_S promoter overlaps the right end of the O_S2 site but does not overlap O_S1 (Figure 1A). As a consequence, repression of the P_S promoter requires only that the repressor occupy the lower affinity O_S2 site. Under normal conditions of cooperative binding, the O_S1 site will influence repression by allowing the O_S2 site to be occupied at lower repressor concentrations. However, any loss of cooperative binding free energy will increase the concentration of repressor necessary to repress P_S, and the system should therefore be sensitive to defects in pairwise cooperative binding.

The O_SP_S promoter–operator region was placed upstream of the chloramphenicol acetyltransferase (*cat*) gene and cloned into a λ *imm21* phage (Figure 1B,C). In the resulting construct, transcription of both the *cat* and *lacZ* genes is initiated from the P_S promoter so that levels of β-galactosidase and chloramphenicol resistance provide measures of repression at O_SP_S. Single-copy derivatives of the fusion phage were isolated as lysogens of the strain X90K and were designated X90K/λ112–O_SP_S–*cat*. In addition to the O_SP_S–*cat* fusion, tester strains contained wild-type or mutant variants of plasmid pFG600 and an F′ episome encoding the Lac repressor. Expression of the λ repressor from pFG600 is controlled by a *lac* UV5 promoter. Thus, the level of the λ repressor can be regulated by the Lac repressor expressed from the F′ episome, which, in turn, can be controlled by the inducer IPTG.

Mutagenesis and Screening for Mutants. Mutagenesis was directed to the C-terminal region of the λ repressor gene, since this region is thought to mediate cooperativity (Pabo *et al.*, 1979; Johnson *et al.*, 1979). The λ repressor producing

Table I: Properties in Vivo of Wild-Type and Mutant Proteins

repressor	phage sensitivity level[a] –IPTG	phage sensitivity level[a] +IPTG	chloramphenicol resistance (μg/mL)
wild type	2/3	6	10
EK102	2/3	6	20
GD147	1	3/4	200
SN198	2/3	5	20
SN228	1	2/3	200
no repressor	1	1	200

[a] Phage sensitivity levels: (1) λ KH54; (2) λ *c*Ic17; (3) λ vir; (4) λ 3v; (5) λ 4v; (6) λ 5v.

plasmid, pFG600, was treated with hydroxylamine, and the mutagenized plasmid was restricted with *Sph*I, generating a fragment encoding the C-terminal domain (residues 93–236). This mutagenized fragment was then ligated to an unmutagenized backbone fragment of pFG600 and transformed into *E. coli* strain X90. Plasmid pools were isolated from the resulting transformants and used to transform the tester strain X90K/λ112–O_SP_S–*cat*. Transformants were initially selected on media containing 50 μg/mL ampicillin and 50 μg/mL Xgal. Dark blue candidates were then picked and screened by replica plating on media containing chloramphenicol at concentrations ranging from 10 to 200 μg/mL. Candidates that were resistant to higher concentrations of chloramphenicol than the wild-type control were further screened for resistance to superinfection by clear and virulent derivatives of phage λ to ensure that mutant repressors had some ability to bind operator DNA. The intracellular concentrations of the mutant candidates were determined after electrophoresis of lysates on SDS/polyacrylamide gels, and candidates displaying wild-type levels of the repressor protein were sequenced. In one screen 58 independent blue colonies were subjected to the chloramphenicol resistance screen and the immunity tests. Nineteen of the isolates exhibited partial immunity and resistance to higher levels of chloramphenicol than the strain expressing the wild-type repressor. Wild-type levels of the repressor protein were observed in 13 of the 19 immune isolates, and sequencing of a subset of these candidates resulted in identification of 3 of the mutants described below.

Four different mutants, each containing a single amino acid change in the C-terminal domain of the repressor, were obtained. One independent isolate of each mutant was obtained. The mutations were Glu102 → Lys (EK102), Gly147 → Asp (GD147), Ser198 → Asn (SN198), and Ser228 → Asn (SN228). The ability of the mutants to repress *cat* expression from O_SP_S and to confer immunity to phage λ derivatives is tabulated in Table I. The EK102 and SN198 mutants repress *cat* synthesis nearly as well as the wild-type repressor (cells containing these mutants are resistant to chloramphenicol levels only twice as high as cells containing the wild-type repressor) and have immunities similar to or slightly lower than the wild type. By these criteria, the EK102 and SN198 mutants appear to have only minor defects in operator binding. The remaining two mutants (GD147 and SN228) are poor repressors of O_SP_S. Cells containing these mutants are resistant to 20-fold higher levels of chloramphenicol than the wild-type control and, in fact, are as resistant as cells containing no repressor (Table I). Cells containing the GD147 and SN228 mutants are also considerably less immune than the wild type (Table I), suggesting that these mutants are defective in some aspect of DNA binding.

Purification and DNA Binding Measurements. The EK102, GD147, and SN228 mutant proteins were purified to allow assays of DNA binding *in vitro*. During purification the wild type, EK102, and GD147 proteins behaved almost identically, whereas the SN228 protein behaved differently. When the wild-type repressor and the EK102 and GD147 mutants were chromatographed on CM-Sephadex columns, the elution profiles were asymmetric, with a sharp leading edge and a long trailing edge. Since monomers, dimers, and higher oligomers of the repressor are in dynamic equilibrium, this behavior is expected if the different oligomeric species have different affinities for the column. The SN228 mutant, by contrast, eluted from CM-Sephadex as a symmetric peak. This may indicate that the Ser228 → Asn mutation alters one or more of the equilibria for repressor oligomerization.

The binding of the purified proteins to restriction fragments containing one or more operator sites was assayed by the nitrocellulose filter binding method. A restriction fragment containing the single-site O_R1 was used for one set of experiments, since repressor binding to this DNA is determined only by the free energy of the repressor dimer–operator interaction and does not depend upon cooperative interactions. Binding was also measured to a restriction fragment from pBJ301 that contains the wild-type O_R2 and O_R3 sites and a mutant O_R1^- site. Repressor binding to this fragment depends both on the intrinsic free energies of repressor binding to the O_R2 and O_R3 sites, which are similar, and on the cooperative free energy of binding. Although the nitrocellulose filter binding technique does not permit resolution of the cooperative and intrinsic free energies, results obtained from such measurements can provide initial biochemical evidence that a mutant may be defective in cooperativity. A pure cooperativity mutant will bind to the O_R1 template with the same affinity as does the wild-type repressor and to the O_R1^- O_R2^+ O_R3^+ template with lower overall affinity than the wild-type repressor.

The results of binding experiments with the O_R1 template are shown in Figure 2A and Table II. The GD147 and wild-type repressors bind this DNA fragment with similar affinities, the EK102 mutant binds somewhat better, and the SN228 mutant binds with significantly reduced affinity. The results of binding measurements made on the O_R1^- O_R2^+ O_R3^+ template are shown in Figure 2B. The binding of the EK102 mutant and wild-type repressor to this template are similar, the GD147 mutant binds less well, and the SN228 mutant binds with dramatically reduced affinity. Since the GD147 repressor binds with wild-type affinity to the single-site template but with reduced affinity to the O_R1^- O_R2^+ O_R3^+ template, this mutant is clearly a promising candidate for a cooperativity mutant. The SN228 mutant binds with reduced affinity to both templates, although the defect seems larger with the O_R1^- O_R2^+ O_R3^+ template than with the O_R1 template. While this could indicate a cooperativity defect, the SN228 mutation must also affect other repressor functions and thus is unlikely to be a pure cooperativity mutant. The EK102 mutant binds the single-site template slightly better than the wild type and binds the multiple-site template with an affinity similar to that of the wild type. This small difference could indicate a weak cooperativity defect.

DNase Footprint Titrations. The interaction of the λ repressor with the three sites of the O_R operator can be described by the intrinsic free energy of binding to each individual site and by the two cooperative free energies associated with the binding of repressors to adjacent operator sites. These energetic terms can be resolved by analysis of DNAse footprinting data using O_R^+, O_R1^-, O_R2^-, and $O_R1^-3^-$ templates (Brenowitz *et al.*, 1986). Figure 3 shows results of analysis of DNAse footprint titrations of the O_R^+ template

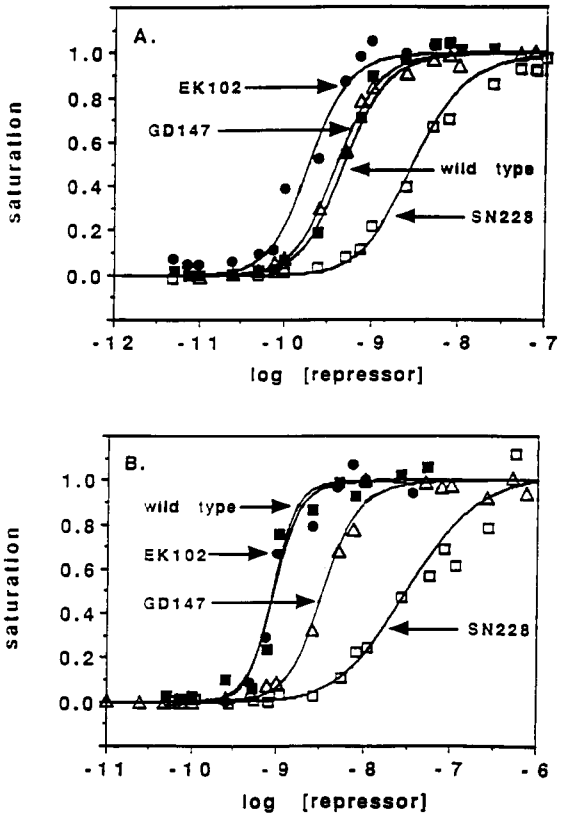

FIGURE 2: (A) Binding isotherms obtained from titration of the pO_R1 570-base-pair fragment with the wild-type and mutant repressors using the nitrocellulose filter binding method: wild type (■), EK102 (●), GD147 (△), SN228 (□). The solid lines represent theoretical curves simulated using the best fit parameters for binding of the repressor dimers to the operator site. (B) Binding curves obtained from titration of the 1107-base-pair pBJ301 (O_R1^-) fragment with the wild-type and mutant repressors. The symbols are the same as those in (A). The solid lines are simulations obtained using approximate values for two-site cooperative binding. In both (A) and (B), the total repressor concentration is expressed in terms of monomer equivalents.

Table II: O_R1 Binding Data

repressor	$\Delta G_1 + \Delta G_D$[a,b] (kcal/mol)	K_1K_D[a] (M^2)
wild type	−25.0	1.7×10^{-19}
EK102	−26.4(0.4)	1.4×10^{-20}
GD147	−25.4(0.1)	9.5×10^{-20}
SN228	−23.0(0.2)	5.8×10^{-18}

[a] ΔG_1 and ΔG_D are the Gibbs free energies for binding of the repressor dimer to O_R1 and repressor dimerization, respectively. K_1 and K_D are the equilibrium dissociation constants for binding of the repressor dimer to site O_R1 and dimerization, respectively (see Data Analysis section of the Materials and Methods). A dimerization constant of 5.6 nM was assumed for all four proteins. [b] Standard Gibbs free energies at 20 °C with 65% confidence intervals in parentheses. Values were calculated from the DNA binding data shown in Figure 2A.

with the wild-type and GD147 repressors. The protein concentrations required to fill the individual operator sites differ significantly for the two repressors. Specifically, titrations of O_R1 and O_R2 with the wild-type repressor occur at similar protein concentrations while a large difference in the protein concentrations required to fill these two sites is observed in the GD147 titrations. Simultaneous analysis of the protection data for all four templates using the eight-species model (Ackers *et al.*, 1982) yields the free energy values shown in Table III. Parameters obtained from this analysis clearly indicate that the GD147 mutant is defective in cooperativity. An alternative more general model for the

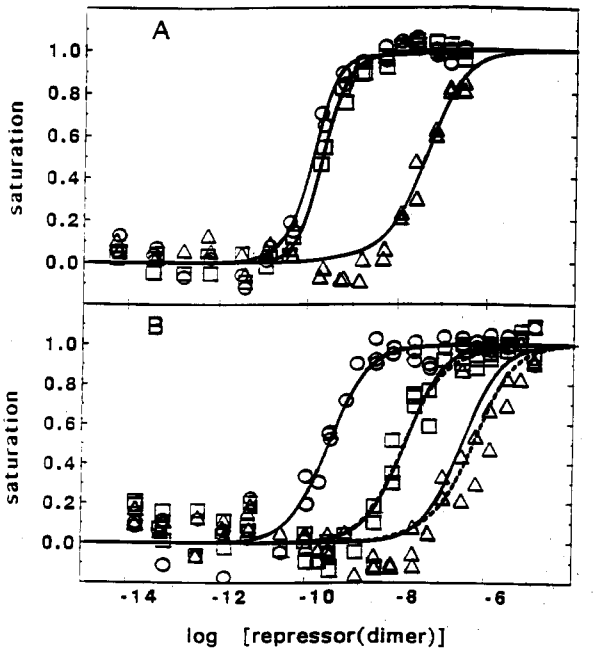

FIGURE 3: DNAse I footprint titrations of the O_R^+ template with (A) wild-type repressor and (B) the GD147 repressor: (○) site O_R1, (□) site O_R2, (△) site O_R3. The solid lines correspond to the best fits from simultaneous analysis of O_R^+, O_R1^-, O_R2^-, and $O_R1^-3^-$ footprint titration data to the eight-species model (Ackers *et al.*, 1982); the dashed line corresponds to the best fit from simultaneous analysis to an alternate model (Senear & Ackers, 1990, Appendix) described in the text (Results).

λ repressor–O_R interaction includes an additional cooperative free energy term, ΔG_{123}, which denotes a nonspecified distribution of the interaction free energy between dimers bound simultaneously at sites O_R1, O_R2, and O_R3 (Senear & Ackers, 1990, Appendix). This general model was found to more accurately describe the GD147 titration data. The cooperative free energy terms resolved for the mutant using either model verify that GD147 is defective in pairwise cooperative binding to the λ right operator region.

The data in Table III were calculated assuming that the dimerization constants for the wild-type and GD147 repressors are the same (Beckett *et al.*, 1991). This assumption seems reasonable since a defect in GD147 dimerization would be expected to result in reduced single-site binding which is not observed. Moreover, the calculation of the cooperative free energy is independent of the dimerization energy. If the dimerization properties of the GD147 mutant differed from those of the wild-type repressor, this would only affect the ΔG_1, ΔG_2, and ΔG_3 values. The cooperativity values, ΔG_{12} and ΔG_{23}, would not change.

DISCUSSION

We designed a hybrid operator–promoter region, O_SP_S, to help identify λ repressor mutants defective in pairwise cooperative operator binding. High-level repression of the P_S promoter requires that a repressor dimer bound at the weak operator site, O_S2, be stabilized by cooperative interactions with a repressor dimer bound at the strong operator site, O_S1. Hence, by screening for λ repressor mutants that could not fully repress O_SP_S, we hoped to enrich for cooperativity mutants. In all, four mutants with single amino acid substitutions in the C-terminal domain of the λ repressor were found to result in increased *cat* expression from O_SP_S. The λ repressor prevents transcription by a simple steric blockade mechanism (Hawley *et al.*, 1985), and thus the phenotypes

Table III: Resolved Gibbs Free Energies[a] of Interaction of the Wild-Type and GD147 Repressors with Sites in λ O_R[b]

	ΔG_1[c]	ΔG_2	ΔG_3	ΔG_{12}	ΔG_{23}	ΔG_{123}	σ[d]
wild type	−12.8(0.2)	−11.1(0.2)	−9.9(0.2)	−2.4(0.4)	−3.2(0.5)		0.072
GD147	−12.4(0.2)	−10.4(0.2)	−8.8(0.2)	0.0(0.3)	−1.0(0.3)		0.068
GD147	−12.4(0.2)	−10.4(0.1)	−9.1(0.2)	0.0(0.3)	−0.7(0.3)	0.8(0.5)	0.064

[a] The binding free energies were estimated from simultaneous analysis of O_R^+, O_R1^-, O_R2^-, and $O_R1^{-}3^-$ footprint titration data. A dimerization constant of 5.6 nM was used for the wild-type and GD147 repressors. The GD147 data were analyzed using both the eight-species alternate pairwise cooperativity model (Ackers et al., 1982) and a more general model (Senear & Ackers, 1990) (see text). [b] ΔG_1, ΔG_2, and ΔG_3 are the free energy changes for binding of a repressor dimer to operator sites O_R1, O_R2, and O_R3, respectively. ΔG_{12} is the cooperative free energy for repressor dimers interacting between sites O_R1 and O_R2. ΔG_{23} is the cooperative free energy for dimers interacting between sites O_R2 and O_R3 when site O_R1 is not occupied. ΔG_{123} denotes a nonspecified distribution of the cooperative free energy between dimers bound simultaneously at sites O_R1, O_R2, and O_R3. [c] Standard Gibbs free energies (kcal/mol) (with 65% confidence limits). [d] Square root of the variance of the fitted curves.

of these mutants are most easily explained if they are defective in some aspect of operator binding. Contacts between the λ repressor and operator DNA are mediated through the N-terminal domain (Sauer *et al.*, 1979; Jordan & Pabo, 1988), making it unlikely that these C-terminal mutations affect DNA binding directly. The C-terminal domain of the repressor is required for strong dimerization and for pairwise cooperativity (Pabo *et al.*, 1979; Johnson *et al.*, 1979), and thus we expected that the mutants would be defective in cooperativity and/or dimerization.

The GD147 mutant is unable to repress *cat* expression from O_SP_S *in vivo* and confers reduced immunity to phage λ superinfection when compared with the wild type (Table I). The purified GD147 mutant was found to bind with wild-type affinity to a DNA fragment bearing the O_R1 operator site, suggesting that the mutation does not affect the protein's ability to dimerize or contact DNA. The mutant does, however, bind less well to an operator bearing adjacent sites of comparable affinity, as would be expected for a cooperativity mutant. DNase footprint analysis of the binding of GD147 to sites in λ O_R shows that the intrinsic affinities of the protein for the O_R1, O_R2, and O_R3 sites are similar to those of the wild type, but the cooperative free energy of interaction between repressor dimers bound at O_R1 and O_R2 is reduced from −2.4 kcal/mol for the wild-type λ repressor to 0 kcal/mol for the GD147 mutant, while the O_R2–O_R3 interaction energy is reduced from −3.2 to −1 kcal/mol (Table III). These data directly show that the GD147 protein is defective in pairwise cooperative binding.

The GD147 mutation has previously been isolated as a mutation that renders the λ repressor resistant to RecA-mediated cleavage (Gimble & Sauer, 1985, 1986). Phage λ bearing the GD147 mutation makes lightly turbid plaques, suggesting that the mutant repressor does not allow efficient lysogen formation. Moreover, lysogens of phage bearing the GD147 mutation are less immune than the wild type and show extremely high levels of spontaneous induction. These results, taken with those presented here, suggest that the reduced operator binding capacity indicated by the results of the *in vivo* tests is due to a defect in cooperativity, and that the ability of the repressor to bind cooperatively is important for its ability to establish and maintain stable lysogens. Two other position 147 sequence changes (GN147 and GV147) have been isolated as mutations that make the λ repressor resistant to inactivation by P22 antirepressor (DeAnda, 1985). However, phage bearing either of these mutations forms normal turbid plaques and prophage affords immunities similar to those of the wild type. By these criteria, the GN147 and GV147 mutants have no obvious defect in DNA binding or in cooperativity. Thus, the defect in the GD147 protein is likely to result from introducing aspartic acid at position 147 rather than from replacing the glycine. Moreover, since asparagine and aspartic acid are isosteric, it appears to be the introduction of the negative charge of Asp147 rather than its bulkier side chain that results in the cooperativity defect.

The SN228 mutation, like GD147, results in complete derepression of *cat* expression from O_SP_S. However, the purified SN228 protein binds significantly less well than either the wild type or GD147 to a DNA template bearing the single operator site, O_R1. This reduced binding to O_R1 is likely to result from a dimerization defect (Table II). The behavior of the mutant SN228 protein during purification is also consistent with a reduction in its ability to dimerize or form higher-order oligomers. The SN228 mutation has also been isolated by Hochschild and Ptashne (1988), who have shown that the mutant protein displays noncooperative binding to the O_R1 and O_R2 operator sites in footprinting experiments. They also noted that SN228 was less active than the wild-type repressor in binding to a single operator site, and ascribed this to a mutant protein preparation of relatively low activity. Such reduced activity could result from a defect in dimerization. Benson and Youderian (personal communication) have isolated a Ser → Arg mutation at position 228. Biochemical studies of this mutant show that this protein is also defective both in dimerization and in pairwise cooperative binding (in preparation).

The properties of the GD147 and SN228 mutants indicate that the O_SP_S screen alone does not distinguish between the GD147 repressor that may be a pure cooperativity mutant and the SN228 repressor that is defective in individual site binding (although perhaps defective in cooperativity as well). The phenotypes in the chloramphenicol screen of the GD147 and SN228 mutants are identical while the immunity of the first mutant is slightly greater than that of the second. Although it remains to be seen whether such subtle differences in immunity will serve as a reliable indicator of pure cooperativity mutants, complete thermodynamic analysis of two additional mutants that demonstrate the same phenotype as the GD147 mutant indicate that they are, indeed, defective primarily in cooperativity (D. S. Burz *et al.*, manuscript in preparation).

The EK102 and SN198 mutations were also isolated using the O_SP_S screen. However, these mutations cause only mild derepression of *cat* expression from the O_SP_S control region *in vivo* and confer superinfection immunities similar to those of the wild-type repressor. These data suggest that the DNA binding properties of the EK102 and SN198 mutants must be reasonably similar to those of the wild-type λ repressor. This expectation is confirmed by studies of the EK102 protein *in vitro*. Compared with the wild-type repressor, the purified EK102 protein shows slightly improved binding to the O_R1 operator site and similar binding to an operator containing functional O_R2 and O_R3 sites. These data suggest that use of the O_SP_S–*cat* screen to identify potential cooperativity mutants will be most useful when mutations with strongly derepressing phenotypes are selected.

The current view is that pairwise cooperativity is mediated by protein–protein interactions between the C-terminal domains of repressor dimers bound at adjacent operator sites (Ptashne, 1986). Studies of the pH and salt dependence of λ repressor binding to O_R indicate that the cooperative free energy terms are largely independent of pH and ionic strength (Senear & Ackers, 1990; Koblan & Ackers, 1991). This suggests that ionic interactions are not involved in stabilizing cooperatively bound dimers. The identification of residues like Gly147, which are likely to be near the cooperativity interface, in combination with structural information about the C-terminal domain should permit a deeper understanding of the structural and energetic basis of the cooperative binding of the λ repressor to adjacent operator sites.

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