Record, M. T., Jr., Lohman, T. M., & deHaseth, P. L. (1976) *J. Mol. Biol. 107*, 145.
Record, M. T., Jr., Anderson, C. F., & Lohman, T. M. (1978) *Q. Rev. Biophys. 72*, 103.
Revzin, A., & von Hippel, P. H. (1977) *Biochemistry 16*, 4769.
Richter, P. H., & Eigen, M. (1974) *Biophys. Chem. 2*, 255.
Riggs, A. D., Bourgeois, S., & Cohn, M. (1970) *J. Mol. Biol. 53*, 401.
Sadler, J. R., Tecklenburg, M., & Betz, J. L. (1980) *Gene 8*, 279.
Schranner, R., & Richter, P. H. (1978) *Biophys. Chem. 8*, 135.
Schurr, J. M. (1979) *Biophys. Chem. 9*, 413.
von Hippel, P. H. (1979) in *Biological Regulation and Development* (Goldberger, R. F., Ed.) pp 279–347, Plenum Press, New York.
von Hippel, P. H., Revzin, A., Gross, C. A., & Wang, A. C. (1975) in *Protein-Ligand Interactions* (Sund, H., & Blauer, G., Eds.) pp 270–288, Walter de Gruyter, Berlin.
Wilemshi, G., & Fixman, M. (1974) *J. Chem. Phys. 60*, 866.
Winter, R. B., & von Hippel, P. H. (1981) *Biochemistry* (second paper of three in this issue).
Winter, R. B., Berg, O. G., & von Hippel, P. H. (1981) *Biochemistry* (third paper of three in this issue).

Diffusion-Driven Mechanisms of Protein Translocation on Nucleic Acids. 2. The *Escherichia coli* Repressor–Operator Interaction: Equilibrium Measurements†

Robert B. Winter‡ and Peter H. von Hippel*

ABSTRACT: In this paper the equilibrium binding of *lac* repressor to operator sites has been studied as a function of monovalent salt concentration, of length of the DNA molecule containing the operator, and (by using various natural *lac* "pseudo"-operators) of operator base pair sequence. The nitrocellulose filter assay has been used to obtain values of repressor–operator association constants (K_{RO}), both directly and as ratios of association to dissociation rate constants (k_a/k_d). Measurements of K_{RO} have been made in the absence of Mg^{2+} or other divalent ions, allowing a direct estimate [Record, M. T., Jr., Lohman, T. M., & deHaseth, P. L. (1976) *J. Mol. Biol. 107*, 145] of the contribution of electrostatic (charge–charge) interactions to the stability of the RO complexes. Using *lac* operator containing DNA restriction fragments of known size, we have shown the following: (i) The magnitide of the RO interaction is salt concentration dependent. A plot of log K_{RO} vs. log [KCl] is linear over the 0.1–0.2 M KCl range, and from the slope of this plot, we can determine that RO complex formation involves six to seven charge–charge interactions. This value is independent of operator type and of DNA fragment size for fragments greater than ~170 base pairs in length. (ii) This number of charge–charge interactions is appreciably less than the 11 such interactions involved in RD complex formation [deHaseth, P. L., Lohman, T. M., & Record, M. T., Jr. (1977) *Biochemistry 16*, 4783; Revzin, A., & von Hippel, P. H. (1977) *Biochemistry 16*, 4769], suggesting that repressor binds to operator and to nonoperator DNA in different conformations. (iii) The RO interaction involves a substantial (>50%) nonelectrostatic component of the binding free energy, in contrast to the RD interaction for which all the binding free energy appears to be electrostatic in nature. (iv) The binding constant (K_{RO_2}) for the secondary (*lacZ* gene) pseudooperator is 5-fold weaker than K_{RO_1} for the primary (physiological) operator when both are measured on separate pieces of DNA. When both operators are on the *same* piece of DNA, the measured value of K_{RO_2} is ~25-fold smaller than that of K_{RO_1}. (v) K_{RO_3}, the binding constant for the tertiary (I gene) pseudooperator, has been estimated at $<10^{10}$ M^{-1} at salt concentrations where $K_{RO_1} \simeq 10^{13}$ M^{-1}. (vi) K_{RO_1} for repressor binding to short DNA fragments is smaller than that for binding to long DNA fragments under the same environmental conditions. Several of these findings, together with others in the literature, are suggestive of "long-range" effects on RO binding constants; possible molecular bases for such effects are discussed. These measurements provide the equilibrium "underpinnings" of our analysis of RO kinetic binding mechanisms [Winter, R. B., Berg, O. G., & von Hippel, P. H. (1981) *Biochemistry* (following paper in this issue)] and also allow comparisons of repressor binding affinities for operator, pseudooperator, and nonoperator DNA. In addition, these results further demonstrate the importance of the surrounding (nonspecific) DNA in controlling the equilibrium stability as well as the rates of formation and dissociation of RO complexes.

In the preceding paper (Berg et al., 1981), we described and quantitatively formulated theoretical models for mechanisms of diffusional (thermal fluctuation driven) translocation of proteins on nucleic acids. As pointed out in that paper, in order to test the applicability of these theories to a real system, one must determine the equilibrium and kinetic parameters for the binding of the protein to specific target sites and to nonspecific sites as a function of salt concentration and of the length of the DNA molecules that contain the target sites. In this paper we report some relevant equilibrium measurements for the *Escherichia coli lac* repressor–operator system; additional equilibrium measurments on this system have also recently been presented by Barkley et al. (1981). Equilibrium pa-

† From the Institute of Molecular Biology and Department of Chemistry, University of Oregon, Eugene, Oregon 97403. *Received April 20, 1981*. A portion of this work was presented by R.B.W. to the Graduate School of the University of Oregon in partial fulfillment of the requirements for the Ph.D. degree in Chemistry. This research was supported in part by U.S. Public Health Service Grant GM-15792 (to P.H.v.H.). R.B.W. was a predoctoral trainee on U.S. Public Health Service Training Grants GM-00715 and GM-07759.

‡ Present address: Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO 80302.

0006-2960/81/0420-6948$01.25/0

rameters for the *lac* repressor nonspecific DNA interaction have been measured and described elsewhere (deHaseth et al., 1977; Revzin & von Hippel, 1977). The related *kinetic* measurements and the conclusions we come to concerning translocation mechanisms are discussed in the following paper (Winter et al., 1981).

Although determination of these equilibrium repressor–operator (RO) binding constants is crucial to a quantitative testing of protein translocation theories as applied to the *lac* system, additional molecular information can also be gleaned from these measurements. The interaction of *E. coli lac* repressor with its operator site has been extensively studied in order to describe molecular mechanisms whereby a protein can preferentially recognize a particular DNA sequence. Determination of the *lac* operator DNA sequence (Gilbert & Maxam, 1973), and its subsequent chemical synthesis (Bahl et al., 1976; Yansura et al., 1977), has allowed a systematic replacement of base pairs in defined regions of the operator and has thus facilitated identification of a number of the functional groups important in repressor binding (Goeddel et al., 1978). Purine methylation (Gilbert et al., 1979a) and phosphate alkylation (Maxam & Gilbert, 1977b)[1] of operator sequences to which repressor is bound and UV cross-linking of repressor to operator (Ogata & Gilbert, 1977) have also defined possible contact points between protein and operator DNA. In addition, studies with genetically altered (Müller-Hill, 1975; Miller et al., 1975), chemically modified (Manly & Matthews, 1979), and enzymatically cleaved repressors (Jovin et al., 1977; Ogata & Gilbert, 1979; Matthews, 1979) have been carried out to determine which portions of the repressor protein are involved in the specific and nonspecific binding interactions. The measurements of binding constants for operators of various sequence described here, together with the effects of DNA fragment length on the stability of these interactions, provide additional information on the detailed nature of the RO interaction.

The physiological importance of repressor binding to nonoperator DNA has been previously emphasized (von Hippel et al., 1974, 1975; Lin & Riggs, 1975; Kao-Huang et al., 1977). Nonspecific (RD) binding appears to serve as a crucial "buffer" for determining levels of free and bound repressor in the cell and for controlling the level of repression in vivo. Binding parameters for the RD interaction have been previously investigated (Butler et al., 1977; deHaseth et al., 1977; Record et al., 1977; Revzin & von Hippel, 1977); these data, together with the observations reported here and in the following paper (Winter et al., 1981), allow us to make additional comparisons of the "operator" and "nonoperator" binding modes of repressor and to demonstrate further the role of RD interactions in both equilibrium and kinetic aspects of RO complex formation.

Materials and Methods

All chemicals used were reagent grade, and solutions were prepared in double distilled water. Buffer solutions for nitrocellulose filter assays were passed through type HA Millipore filters (0.45-μm pore diameter) prior to use. *E. coli* DNA polymerase I was purchased from Worthington, [α-^{32}P]dATP was from New England Nuclear, and *Eco*RI restriction enzyme was purified by the method of Modrich & Zabel (1976) and kindly supplied by Dr. C. Woodbury. *Hae*III and *Hpa*II restriction enzymes were from New England Biolabs; agarose (Sea Kem) for preparative gel electrophoresis was from Marine Colloids, Inc.

Repressor Preparation. The source of the wild-type (SQ) *lac* repressor was the overproducing *E. coli* strain CSH46. Cells were grown, and the repressor was purified as described by Butler et al. (1977), except that phenylmethanesulfonyl fluoride (PMSF) was added at 50 μg/mL to the initial cell lysing buffer. The chemical purity of the peak fractions eluted from a phosphocellulose column was assayed by sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis (Laemmli, 1970); in all cases the protein was greater than 90% pure by this criterion. Repressor concentrations were determined spectrophotometrically by using an extinction coefficient (per mole of repressor tetramer at 280 nm) of 9×10^4 M^{-1} cm^{-1} (Butler et al., 1977).

DNA Preparation. The source of wild-type *lac* operator DNA was the transducing phage λCI857p*lac*5 (kindly provided by Dr. Benno Müller-Hill). Phage were purified by poly(ethylene glycol) precipitation, followed by a cesium chloride block gradient and a cesium chloride equilibrium gradient. DNA was then phenol extracted from the purified phage. An alternative source of *lac* operator DNA was a Col E1-*lac* hybrid plasmid. This was constructed by inserting a 4.2×10^6 dalton *lac* operator fragment, resulting from *Eco*RI cleavage of λp*lac*5 DNA, into the unique *Eco*RI site of the Colicin E1 plasmid. Transformants were screened for constitutive β-galactosidase synthesis on X-Gal indicator plates (X-Gal is 5-bromo-4-chloro-3-indolyl β-D-galactoside). *lac* plasmid DNA was isolated by cesium chloride/ethidium bromide density gradient centrifugation of cleared lysates as described by Clewell & Helinski (1970).

lac operator DNA restriction fragments were prepared as follows: ColE1-*lac* DNAs (2 mg) or λp*lac*5 DNA (20 mg or more) were digested to completion with either *Eco*RI or *Hae*III restriction endonucleases for 15–20 h at 37 °C. The digest was diluted with binding buffer (BB) containing 10 mM KCl and 1 mM $MgCl_2$ to make stock solutions containing operator at concentrations of no less than 5×10^{-9} M. A 2-fold excess of active repressor was added, and the mixture was incubated at room temperature for 30 min, filtered through a type HA Millipore filter (45-mm diameter), and washed once with an equal volume of BB. The DNA retained on the filter was eluted for several hours at room temperature in 50 mM NH_4HCO_3 (pH 8), 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 0.1% $NaDodSO_4$ with gentle shaking. The elutate was collected and concentrated by two ethanol precipitations, phenol extracted, and dialyzed against 10 mM tris(hydroxymethyl)aminomethane (Tris) (pH 8) and 1 mM EDTA. Yields of DNA fragments by this method were about 50%, with the largest loss probably occurring at the filter binding step since *lac* operator containing DNA cannot be quantitatively retained on the filters even when saturating amounts of repressor are added.

All *lac* operator DNAs were ^{32}P labeled by nick translation with [α-^{32}P]dATP as described by Maniatis et al. (1975). Generally reactions were carried out by using 2–5 μg of DNA. Unreacted dATP was removed by Sephadex G-50 chromatography in 0.1 M triethylamine carbonate at pH 9.5, and the recovered DNA was subjected to electrophoresis on a 1% agarose gel (for the *Eco*RI 6700-base-pair *lac* fragment) or a 7% acrylamide gel (for the *Hae*III 170- and 203-base-pair *lac* fragments) in TBE (89 mM Tris–borate and 2.5 mM EDTA, pH 8.5) buffer. DNA was visualized by autoradiography. The desired bands were cut out of the gel and electroeluted into dialysis bags, and the DNA was concentrated, phenol extracted, and dialyzed as before. DNA electroeluted

[1] Maxam, A., & Gilbert, W. (1977b), as cited in von Hippel (1979).

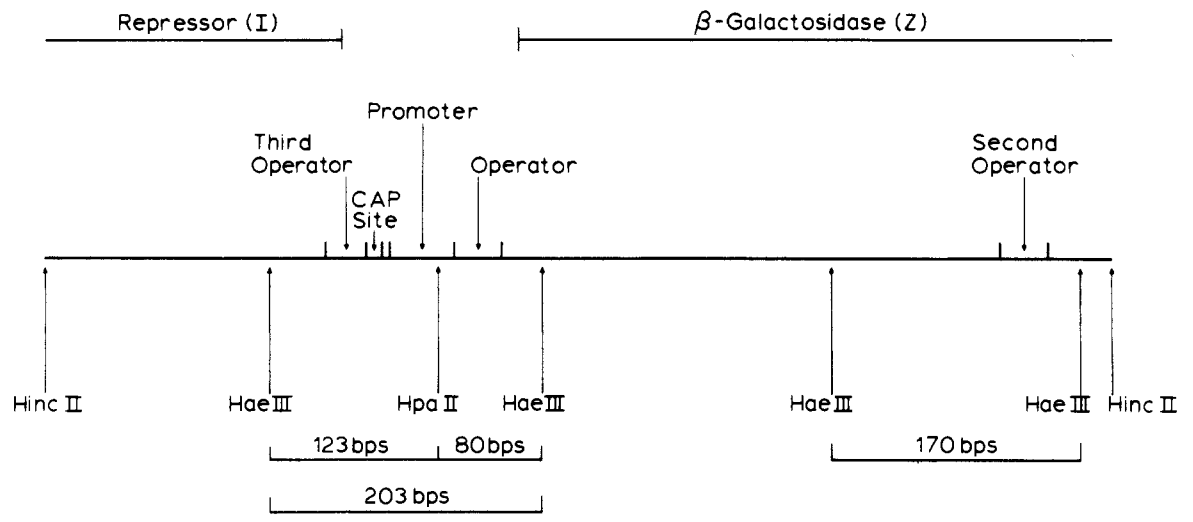

FIGURE 1: Locations of some of the restriction endonuclease cleavage sites within the *E. coli lac* control region that allow separation of operator and "pseudo"-operator containing DNA sequences. Functional regions and DNA fragments are drawn roughly to scale (after Gilbert et al., 1975).

from agarose gels was absorbed onto a small hydroxylapatite column equilibrated with 0.1 M KH_2PO_4–K_2HPO_4 buffer (pH 7.0) and eluted with 1 M KH_2PO_4–K_2HPO_4 buffer (pH 7.0). Quantitative elution of DNA from the hydroxylapatite column occasionally required 0.2 M EDTA to remove tightly bound material.

The single-stranded integrity of the small *lac* operator fragments was checked by strand separation and denaturing acrylamide gel electrophoresis (Maxam & Gilbert, 1977a). DNA labeled by this procedure did not undergo extensive single-strand "nicking". Specific activities of 5×10^6–10^7 cpm/μg were routinely obtained.

Concentrations of ^{32}P-labeled fragments were determined spectrophotometrically at 260 nm or by monitoring ethidium bromide fluorescence enhancement (LePecq & Paoletti, 1966) in a Hitachi MPF-2A fluorometer using λp*lac*5 DNA as a standard. The latter method, which was particularly useful for the shorter *lac* operator fragments, is sensitive to DNA concentrations as low as 0.1 μg/mL (15 ng of DNA).

Binding Measurements. RO binding assays were performed in binding buffer (BB; pH 7.5, 10^{-4} M EDTA, 10^{-4} M dithiothreitol, 0.01 M Tris, 5% Me_2SO, and 50 μg/mL bovine serum albumin) as described by Riggs et al. (1970), except that Mg^{2+} was omitted and KCl concentrations were adjusted as required. Either BA-85 nitrocellulose filters (Schleicher & Schüll; pretreatment with dilute KOH), or HA Millipore filters were used. Prior to use, filters were soaked in filter buffer (BB containing 50 mM KCl, but without dithiothreitol or BSA). Binding curves were generated by adding increasing concentrations of repressor to a fixed concentration of *lac* operator DNA. For the smaller *lac* operator fragments (170 and 203 base pairs), DNA concentrations varied from 0.5×10^{-12} to 2×10^{-12} M in operator; for the larger (*Eco*RI) fragment, operator concentrations ranged from 1×10^{-14} to 3×10^{-14} M. Reaction volumes varied from 0.6 to 3.1 mL; triplicate samples were filtered, washed with an equal volume of BB of the same KCl concentration as the sample, dried, and counted by liquid scintillation.

Binding curves were analyzed in two ways. Generally a standard Scatchard analysis for single-site binding, utilizing data points obtained at 30–80% saturation, was performed. This analysis is complicated somewhat by the presence of the *lacZ* gene "pseudooperator" site on the *Eco*RI *lac* operator fragment (see Results). It is assumed that all DNA is bound to repressor under saturating conditions [the efficiency of the filter binding process is taken into account; see Riggs et al. (1970) for a discussion of this point]. In some cases binding data were fitted by using a binding polynomial procedure, developed for use with data generated by the nitrocellulose filter assay method, which accounts directly for the efficiency of protein-DNA complex retention, as well as for multiple protein binding (C. P. Woodbury, Jr. and P. H. von Hippel, unpublished results). Binding isotherms were calculated by using a Varian 620i computer. Good agreement was obtained between the two methods (within a factor of 2 in K_{RO} for single-site binding to *Hae*III 170 and 203 base-pair (bp) *lac* operator fragments), indicating that the assumptions involved in applying the Scatchard analysis are generally appropriate.

Determination of Repressor Activity. Stoichiometric titrations were carried out as described by Riggs et al. (1970). Each *lac* DNA fragment at $\sim 1 \times 10^{-11}$ M in BB containing 0.1 M KCl was titrated with increasing amounts of *lac* repressor until saturation was reached. Our repressor preparations were 20–30% active in operator binding by this criterion, and activity estimates did not vary significantly when measured with different *lac* operator containing DNA fragments.

Results

Measurements of repressor binding to operator sequences have been performed on a variety of operator-containing DNA fragments. As Figure 1 shows, the entire operon contains not only the "primary" (wild-type) repressor sequence, but also two "pseudooperators", defined as DNA sites which contain most of the specific operator sequence but have no known physiological role. These sites occur at the 5′ end of the β-galactosidase (Z) gene (secondary operator) and at the 3′ end of the structural (I) gene for repressor (tertiary operator). Both these pseudo operators and the primary operator can be obtained separately on discrete restriction fragments (Figure 1), and their affinities for repressor have been determined separately.

Binding constants of repressor to various operator and pseudooperator-containing fragments have been measured, both by direct equilibrium techniques and as ratios of apparent association and dissociation constants [for methodological details of rate constant measurements, see Winter et al. (1981)]. The results are presented in the following sections, and important conclusions from these and other studies are summarized in Table I.

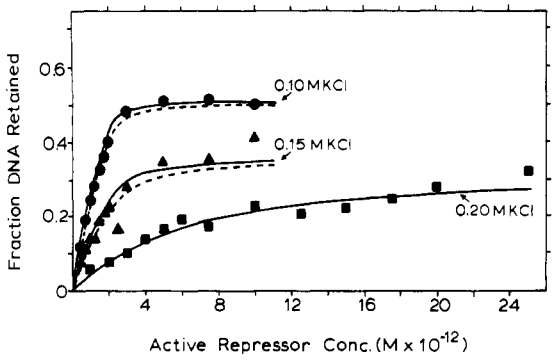

FIGURE 2: Binding of *lac* repressor to *Hae*-203 *lac* fragment. Experimental procedures are described under Materials and Methods. Solid and dashed curves are computer-generated binding isotherms used to fit the data points (see Materials and Methods). The conditions for each set of data are (●) BB + 0.1 M KCl and [DNA] = 2×10^{-12} M in operator. For the dashed curve, we used $K_{RO} = 1 \times 10^{13}$ M^{-1}; for the solid curve, $K_{RO} = 2 \times 10^{13}$ M^{-1}. (▲) BB + 0.15 M KCl and [DNA] = 2×10^{-12} M in operator. For the dashed curve, $K_{RO} = 2 \times 10^{12}$ M^{-1}; for the solid curve, $K_{RO} = 3.5 \times 10^{12}$ M^{-1}. (■) BB + 0.2 M KCl and [DNA] = 3×10^{-12} M in operator. $K_{RO} = 2.5 \times 10^{11}$ M^{-1} (solid curve).

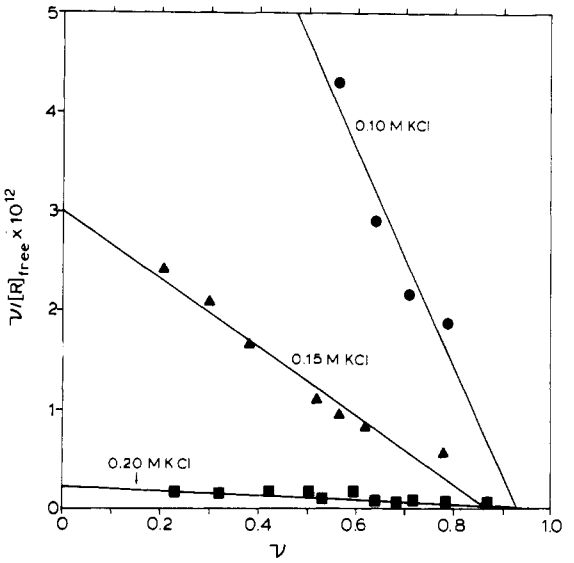

FIGURE 3: Scatchard plots of *lac* repressor binding to the *Hae*-203 *lac* fragment. Solid lines represent linear least-squares fits of the data points. (Same data as in Figure 2.) (●) Binding in 0.1 M KCl; $K_{RO} = 1.1 \times 10^{13}$ M^{-1}. (▲) Binding in 0.15 M KCl; $K_{RO} = 3.4 \times 10^{12}$ M^{-1}. (■) Binding in 0.2 M KCl; $K_{RO} = 2.2 \times 10^{11}$ M^{-1}.

Repressor Binding to the HaeIII (203-Base-Pair) Operator Fragment. Extensive restriction mapping of the *lac* control region (Gilbert et al., 1975), summarized in Figure 1, has facilitated the generation of DNA fragments of defined length containing the *lac* operator. A 203-bp fragment resulting from the cleavage of λp*lac*5 DNA with *Hae*III (the *Hae*-203 fragment) contains the entire *lac* operator/promoter region along with some flanking sequences and provides a well-defined DNA segment for initial measurements of the *lac* repressor–operator interaction (Figure 1).

The binding of repressor to the *lac* operator site of the *Hae*-203 fragment as a function of active repressor concentration at three different KCl concentrations (in the absence of Mg^{2+}) is shown in Figure 2, and Scatchard plots derived from these data are presented in Figure 3. Values of K_{RO}, calculated from these data and others over the 0.05–0.2 M KCl concentration range, are presented as a plot of log K_{RO} vs. log [KCl] in Figure 4. This log–log plot is linear, though only over a restricted range of ionic conditions (0.125–0.2 *M*

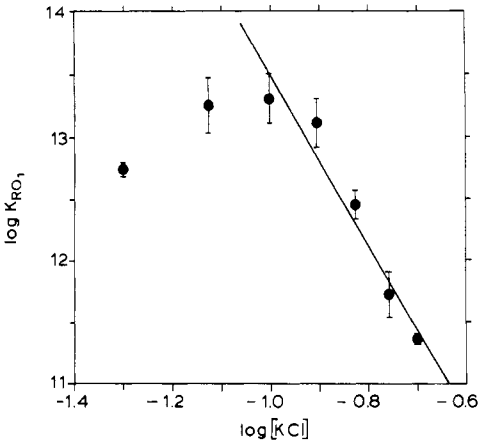

FIGURE 4: Log K_{RO} vs. log [KCl] for *lac* repressor binding to the *Hae*-203 *lac* fragment. K_{RO} was determined by a Scatchard and curve-fitting analysis (see Materials and Methods) for experiments at >0.125 M KCl (Figure 3). Curve fitting (Figure 2) was used to determine K_{RO} at salt concentrations ≤0.125 M KCl. The solid line represents the linear least-squares fit to data points obtained in the range 0.1–0.2 M KCl.

KCl); the slope of such plots ($-\partial \log K_{RO}/\partial \log [KCl]$) is defined as $m'\psi$, where m' designates the number of ion pairs formed between repressor and operator and ψ is the number of counterions thermodynamically bound to the DNA backbone (in the absence of repressor) per DNA phosphate (Record et al., 1976). Over the linear range (Figure 4), we calculate $-\partial \log K_{RO}/\partial \log [KCl] = 6.9 \pm 0.5$, corresponding (for $\psi = 0.88$) to the formation of 7.8 (±1.0) charge–charge interactions between repressor and operator in the binding process.[2]

Extrapolation of the linear portion of the data of Figure 4 to 1 M KCl gives a value of $K_{RO,1M} \simeq 4 \times 10^6$ M^{-1}. This corresponds to a standard free energy of formation of the RO complex at 1 M KCl ($\Delta G°_{RO,1M}$) of −8.4 (±0.4) kcal/mol at 25 °C. Lohman et al. (1980), utilizing data for the binding of pentalysine to DNA, have estimated the standard free energy of formation of a *single* lysine–phosphate charge–charge interaction in 1 M KCl as +0.2 (±0.1) kcal/mol. Correcting our extrapolated value of $\Delta G°_{RO,1M}$ by this factor [−8.4 − (8)(+0.2)], we obtain a value of −10.0 (±1.0) kcal/mol for the *nonelectrostatic* binding free energy of RO complex formation. This component represents 50% or more of the total binding free energy, even at low ionic strengths where electrostatic interactions are strongest. The fraction of the total binding free energy represented by this nonelectrostatic component is, of course, larger still at higher salt concentrations.

In contrast, the RD interaction involves the formation of 11–12 charge–charge interactions between basic protein residues and DNA phosphates, and extrapolation of these data to 1 M KCl indicates that $\Delta G°_{RD,1M} \simeq 0$ for nonspecific binding (after correction for the standard free energy of formation of the charge–charge interactions at 1 M KCl—see above—protonation of the repressor, etc.; for details, see de Haseth et al. (1977); Revzin & von Hippel, 1977). Thus the free energy of the RD interaction appears to be totally electrostatic whereas the RO interaction involves both electrostatic and nonelectrostatic components; the latter is presumed to be primarily responsible for the *specificity* of the RO interaction. The decrease in the number of charge–charge interactions in forming the RO (as opposed to the RD) complex, as well as

[2] This value of m' is comparable to that calculated by Record et al. (1977), after correcting (for the presence of Mg^{2+}) the much less extensive RO binding data measured by Riggs et al. (1970) with λp*lac*5 DNA. It is somewhat smaller than the value of m' recently estimated for the RO interaction with λp*lac*5 DNA by Barkley et al. (1981).

the need to make base-specific contacts with the operator, indicates that the binding geometries of the two types of complexes may be quite different.

In interpreting the salt dependence of RO complex formation, we have assumed that any anion-specific effects do not significantly alter the number of charge–charge interactions (m') involved in the repressor–operator binding interaction. The same assumption was made by Record et al. (1977) in recalculating the RO complex salt dependence data of Riggs et al. (1970). Experimentally this assumption means that the *slopes* of plots of log K_{RO} vs. log [KX] (where X represents various anions) should be independent of the specific anion used. This has been shown to be the case for *lac* repressor binding, both to operator and to nonoperator DNA (deHaseth et al., 1977; Barkley et al., 1981). The substitution of various anions for chloride has been shown by these workers to change appreciably the *magnitudes* of K_{RD} and K_{RO} but to have little or no effect on the *slopes* of the log–log plots.[3] This indicates that (unlike cations) anions are *not* displaced in either RO or RD complex formation (see Discussion).

Possible pH effects on the magnitudes of K_{RO} and on the slopes of log–log plots of K_{RO} vs. salt concentration must also be considered. deHaseth et al. (1977) have shown that K_{RD} increases with decreasing pH, suggesting that the protonated form of His-29 is involved in charge–charge interactions with backbone phosphates in nonspecific complex formation. However, the pH dependence of the RO interaction is very small (Riggs et al., 1970; Barkley et al., 1981), suggesting that changes in the state of protonation of repressor do not significantly affect K_{RO}. Extrapolations of K_{RO} values to 1 M salt to determine nonelectrostatic free energies of RO complex were carried out on data obtained at various concentrations of KCl at pH 7.5; thus these values may require further correction if anion binding or protonation effects do need to be taken into account.

As Figure 4 shows, at KCl concentrations below ~0.125 M, K_{RO} for repressor binding to the *Hae*-203 fragment does not appear to continue to increase with decreasing [KCl] but rather remains relatively constant down to 0.05 M KCl. This low salt behavior is also evident in the ratios of association and dissociation rate constants seen at these salt concentrations [see Table I and Winter et al. (1981)]. Theoretical considerations (Manning, 1978) and experimental measurements (Anderson et al., 1978; Bleam et al., 1980) suggest that an approximately constant number of counterions should be associated with the DNA (per phosphate) over an extensive range of salt concentrations (~3 mM to >1 M NaCl; see Discussion). As a result, the number of cations released upon RO complex formation should be independent of salt concentration, and a linear increase in K_{RO} with decreasing [KCl] should be observed. This appears to be the case, down to quite low salt concentrations, in the presence of Mg^{2+} (Record et al., 1977). However, no quantitative data for the dependence of either K_{RO} or K_{RD} on monovalent salt concentrations below 0.1 M have previously been reported. Due to the strong binding of repressor to the *Hae*-203 fragment at <0.1 M KCl and the limits of detection of the filter binding assay (about 5×10^{-13} M in operator at the ^{32}P specific activities used here; for the *Hae*-203 fragment this corresponds to about 9.5×10^{-15} mol of operator or about 0.19 ng of DNA), the data in this

[3] We emphasize that this is not always the case; e.g., the slopes of log–log plots of binding constants for phage T4 coded gene 32 protein to nucleic acid lattices are strongly dependent on the anion used in measuring the salt dependence of the binding (Kowalczykowski et al., 1981).

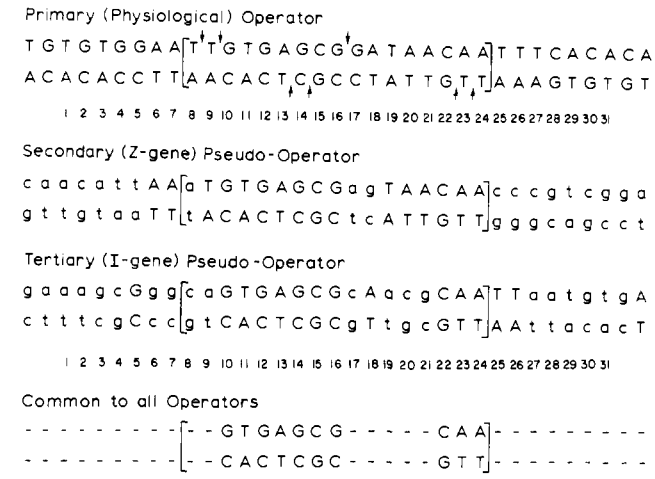

FIGURE 5: Comparison of wild-type and "pseudo"-operator sequences. Upper case letters indicate the positions of identical base pairs in the wild-type and the respective "pseudo"-operator sequences; lower case letters identify positions of base pairs which differ from the wild-type sequence. Arrows (in the wild-type sequence) indicate positions of phosphates shielded from nitrosourea reaction by bound repressor (Maxam & Gilbert, 1977b).[1]

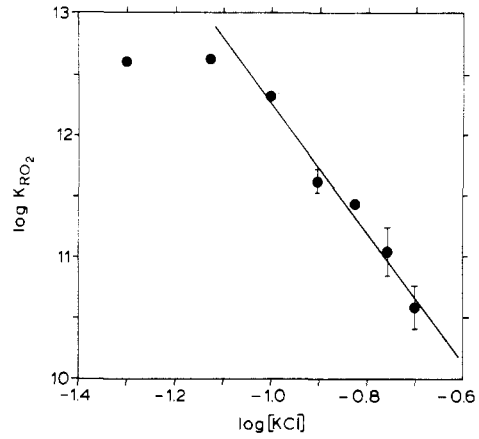

FIGURE 6: Log K_{RO_2} vs. log [KCl] for *lac* repressor binding to the *Hae*-170 (*lacZ*) pseudooperator fragment. Scatchard analyses similar to those described with respect to Figure 3 were used to determine K_{RO_2}. The solid line represents a linear least-squares fit to data points obtained in the salt concentration range 0.1–0.2 M KCl.

salt range are not of high precision [±30–50%; see also Barkley et al. (1981)]. Nevertheless there is no doubt (see Figure 4) that the dependence of K_{RO} on [KCl] is very small at salt concentrations below 0.1 M. This point is considered further in Winter et al. (1981).

Repressor Binding to Pseudooperator Sequences. An "operator-like" sequence located ~300 nucleotides to the 3′ side of the *lac* operator (within the coding region for β-galactosidase) was first detected by Reznikoff et al. (1974). Equilibrium competition experiments suggested the affinity of repressor for this site to be 25–30-fold less than for the "primary" operator. Subsequent sequence analysis by Gilbert et al. (1976b) of this pseudooperator identified the base pair changes relative to the wild-type sequence (see Figure 5).

We have examined the salt dependence of the affinity of *lac* repressor for this operator site by using a 170-base-pair *Hae*III restriction fragment (the *Hae*-170 fragment) which carries this secondary operator sequence (Figure 1). The data are shown in Figure 6; clearly the log of the binding constant for the secondary operator (K_{RO_2}) also varies approximately linearly with log [KCl] at the higher salt concentrations. A slope ($-\partial \log K_{RO_2}/\partial \log [KCl]$) of 5.4 (±0.9) is calculated for the data between 0.1 and 0.2 M KCl; this corresponds to

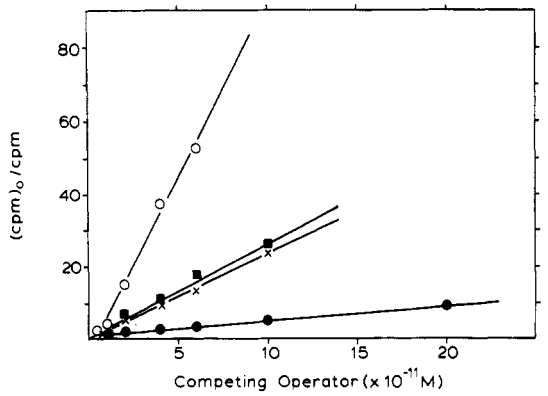

FIGURE 7: Equilibrium competition experiments with various *lac* operator containing fragments. ^{32}P-Labeled λp*lac*5 DNA or ^{32}P-labeled *lac* operator fragments were added to a final concentration of 2×10^{-11} M, along with the indicated amounts of competing (unlabeled) λp*lac*5 DNA. Active repressor was added to 1.2×10^{-11} M, and the mixture was incubated at room temperature for 30 min prior to filtering. $(cpm)_0$ represents the amount of ^{32}P-labeled λp*lac*5 DNA or ^{32}P-labeled *lac* operator fragments retained in the *absence* of unlabeled λp*lac*5 DNA; (cpm) is the amount of ^{32}P-labeled *lac* operator DNA retained in the *presence* of varying amounts of competitor. Labeled DNA: λp*lac*5 DNA (●); *Hae*-203 *lac* fragment (■); *Hae*-203 + *Hae*-170 *lac* fragments (equimolar concentrations of each) (×); *Hae*-170 *lac* fragment (○).

6.2 (±1.0) charge–charge interactions formed on repressor binding to this (secondary operator) site. Comparison of the linear portions of the *Hae*-203 (primary operator) data shows that the salt dependences of the two are about the same but that binding of repressor to the secondary operator fragment is 5–10-fold weaker (7-fold average) than binding of repressor to the primary operator fragment at the various salt concentrations examined. This corresponds to a destabilization of ~1 kcal/mol for the secondary operator–repressor complex, relative to the complex with primary operator for operator-containing DNA fragments of comparable size. This destabilization is primarily in the nonelectrostatic component of the binding free energy, as described above, and must involve base-pair changes within the recognition sequence. It is striking, however, that repressor binding to this site is *stronger* than that observed for the weakest (single base-pair change) *lac* operator constitutive (O^c) mutation (Jobe et al., 1974), despite the presence of *three* base pair changes (at positions 8, 17, and 18) within a 17-base-pair sequence in the secondary operator (Figure 5). Apparently these positions are not importantly involved in the energetics of the specific binding process, even though UV cross-linking (Ogata & Gilbert, 1977) and methylation (Gilbert et al., 1976b) probes indicate the proximity of bound repressor to these loci. In keeping with this conclusion, Goeddel et al. (1978) have shown that base substitutions at these positions do not dramatically alter repressor binding.

We also note, as with the *Hae*-203 fragment, that repressor binding to this pseudooperator site does *not* increase further at salt concentrations less than 0.1 M KCl, with a maximum of 50% of the binding free energy (based on extrapolation to 1 M KCl concentration) being electrostatic in origin.

We have confirmed the relative affinities for repressor of the primary and secondary operators by equilibrium competition with unlabeled λp*lac*5 as competitor. In Figure 7 we plot $(cpm)_0/cpm$ (the ratio of DNA counts retained in the absence and presence of unlabeled competitor) against the concentration of competing operator. Linear plots are obtained [see Bourgeois & Riggs (1970)] and show that the *Hae*-203 fragment competes for repressor about 4-fold better than does the *Hae*-170 fragment. This suggests a 4-fold greater affinity for the primary relative to the secondary operator, in reasonable accord with the direct equilibrium measurements described above. In Figure 7 we also note that the *Hae*-203 fragment competes about 7-fold less effectively than does intact λp*lac*5 DNA for repressor, suggesting a weaker intrinsic affinity of repressor for an operator located on the small fragment than for the same operator site within a large DNA. In addition, an equimolar mixture of *Hae*-203 and *Hae*-170 fragments appears to be as effective in a competition experiment as is the same concentration of *Hae*-203 fragments alone (Figure 7), in keeping with the weaker binding affinity of the secondary operator.

An additional "operator-like" sequence, the *lacI* gene pseudooperator (or tertiary operator), which overlaps the CAP protein binding site (Gilbert et al., 1976b; see Figure 5), can be isolated by digestion of the *Hae*-203 fragment with *Hpa*II to give an 80-base-pair primary operator fragment and a 123-base-pair fragment containing the tertiary *lac* (*I* gene) pseudooperator. Attempts to measure binding to the latter site were unsuccessful. Under conditions where binding to the primary and secondary operator sites is stoichiometric (i.e., at 0.1 M KCl and operator concentrations of 10^{-11} M), no binding is detected to the tertiary operator site, even in the presence of a 30-fold excess of active repressor. Concentrations of the 123-base-pair fragment of 10^{-10} M and above are required to detect any repressor binding at all, and the interaction appears to be IPTG sensitive. However, at these operator concentrations, the binding data are not sufficiently reliable to allow us to estimate K_{RO_3} for repressor binding to the *lacI* pseudooperator located on a separate DNA fragment. On the basis of these observations, we conclude that the value of K_{RO_3} (for repressor binding to the *lacI* pseudooperator site located on a separate DNA fragment) at 0.1 *M* KCl is probably near 10^9 M^{-1} and is unlikely to be as high as 10^{10} M^{-1}; this makes the RO_3 interaction about 10^3–10^4 times weaker than the primary operator (RO_1) interaction. (In making this estimate, we assume that the RO_3 complex is retained on the filter as efficiently as are the primary or the secondary operator complexes under appropriate concentration conditions). However, we note that K_{RO_3} is still 100–1000-fold larger than K_{RD} under similar ionic conditions (Revzin & von Hippel, 1977).

Repressor Binding to Large lac Operator DNA. The *Eco*RI *lac* operator fragment (~6700 base pairs, spanning most of the repressor and the β-galactosidase genes) was selected to test the effect of a large amount of nonoperator DNA on the electrostatic and nonelectrostatic components of the RO interaction. The slope of the linear portion of a plot of log K_{RO} vs. log [KCl], for the binding of the *Eco*RI fragment to repressor over the 0.1–0.2 M KCl range, yields a slope of 5.1 (±1.2); (data not shown). This slope corresponds to a value of m' of 5.8 (±1.4) charge–charge interactions and agrees, within experimental error, with the slope measured for the primary operator with the *Hae*-203 fragment and for the secondary operator with the *Hae*-170 fragment. In addition, the value for K_{RO} deduced for the *Eco*RI fragment data (see below) is about 3–6-fold greater than that for the *Hae*-203 fragment at all salt concentrations tested. Since electrostatic contributions to binding are about the same for the two DNA substrates, repressor binding to the *Eco*RI fragment appears to involve an increase in the free energy of the nonelectrostatic component of the RO interaction of about −1.0 kcal/mol at 25 °C. This increase in apparent binding affinity for the larger DNA fragments must be due to the presence of the extra (vicinal) nonoperator DNA. The *Eco*RI fragment binds repressor with about the same affinity (and salt dependence)

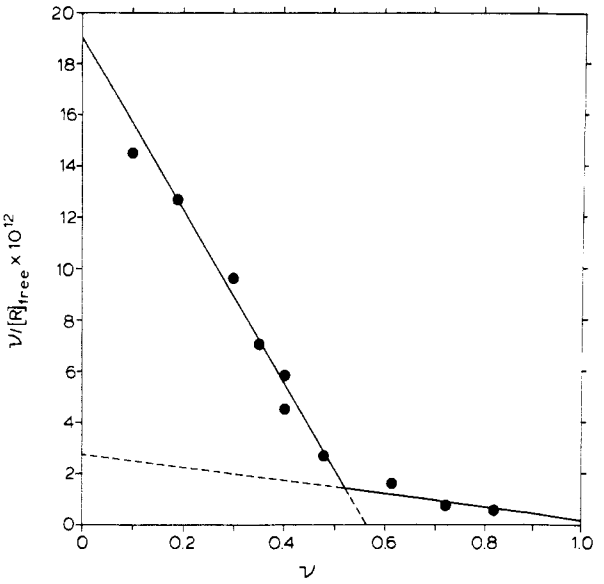

FIGURE 8: Scatchard plot for the binding of *lac* repressor to the *Eco*RI *lac* fragment in BB + 0.1 M KCl. Experimental procedures are described under Materials and Methods and Results. Analysis by the Klotz & Hunston (1971) technique. The best linear fit to the low saturation ($\nu < 0.5$) data gives a slope of -3.4×10^{13} and a y axis intercept of 1.9×10^{13}. The best linear fit to the high saturation ($\nu > 0.5$) data gives a slope of $\sim -5 \times 10^{12}$ and a y axis intercept of $\sim 5 \times 10^{12}$.

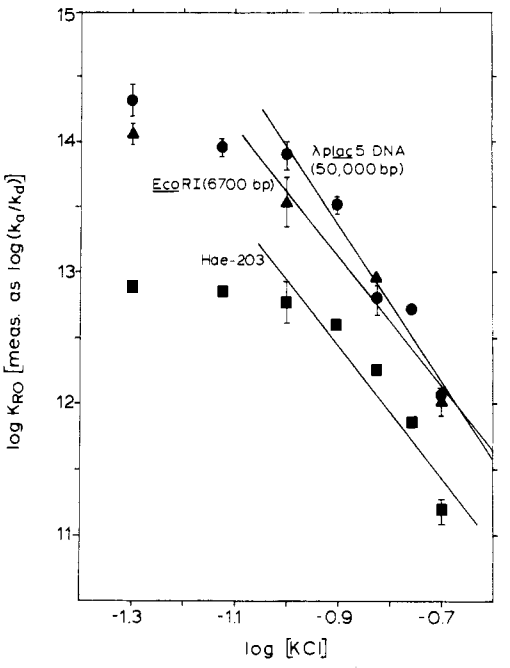

FIGURE 9: Plot of log K_{RO} (calculated as k_a/k_d) vs. log [KCl]. Straight lines represent best fits through the high salt (>0.1 M KCl) points. (●) λp*lac*5 DNA (50 000 base pairs); (▲) *Eco*RI fragment (6700 base pairs); (■) *Hae*-203 fragment. m' from these data is 6.8 for λp*lac*5 DNA, 5.6 for the *Eco*RI fragment, and 5.7 for the *Hae*-203 fragment.

as intact λp*lac*5 DNA (~50 000 base pairs); thus *additional* nonoperator DNA (beyond the 6700-base-pair level) does not further stabilize the interaction (K_{RO} for λp*lac*5 DNA–repressor interaction is estimated from the ratio of association and dissociation rate constants; see Table I).

Binding measurements to the *Eco*RI fragment are complicated by the presence of the *lacZ* pseudooperator sequence on the same fragment as the primary site. If we assume that the two sites bind repressor separately and independently, we can derive two apparent binding constants from curved Scatchard plots such as that presented in Figure 8. Following the treatment of Klotz & Hunston (1971), we calculate that $K_{RO_1} = (5 \pm 1.5) \times 10^{13}$ M^{-1} and $K_{RO_2} = (2 \pm 1) \times 10^{12}$ M^{-1} for binding in 0.1 M KCl from three sets of such experiments using either the *Eco*RI fragment of λp*lac*5 DNA. This value for K_{RO_1} is close to the value estimated straightforwardly from these data by using only the slopes at low levels of saturation [$(5 \pm 1) \times 10^{13}$ M^{-1}]. The binding constant for the second operator calculated in this way is the same as that measured directly on the separate (*Hae*-170) fragment (see above). This result suggests either that, unlike the primary operator–repressor complex, K_{RO_2} is *not* sensitive to DNA fragment length or, more likely, that the intrinsic value of K_{RO_2} (after correction for the fragment length effect seen with K_{RO_1}) is decreased at least 5-fold relative to the value of K_{RO_2} measured directly on the separate (*Hae*-170) fragment. This latter interpretation suggests that the binding affinity of repressor for a secondary operator located on a piece of DNA which contains an already repressor-saturated primary operator site may be appreciably weakened by the presence of the neighboring of the RO_1 interaction, implying an "allosteric" (negatively cooperative) interaction between the two operator sites. This may explain why the *lacZ* pseudooperator site was not detected in binding assays until the primary site had been eliminated from the target DNA. Other related evidence leading to similar conclusions will be considered under Discussion.

Binding Constants to Operator-Containing DNA Fragments Determined from the Ratio of Association and Dissociation Rate Constants. Apparent binding constants for repressor to operator sites may also be obtained by using the ratio of rate constants for the overall reaction:

$$R + O \underset{k_d}{\overset{k_a}{\rightleftharpoons}} RO \qquad (1)$$

Procedures for making such measurements are described in detail in the following paper (Winter et al., 1981), and typical values of K_{RO} derived from kinetic measurements over the 0.1–0.2 M KCl concentration range for the *Hae*-203, the *Eco*RI *lac* (~6700 base pairs), and the λp*lac*5 (~50 000 base pairs) fragments are presented in Figure 9. The slopes of the log–log plots and the values of the binding constants obtained from such kinetic data are quite comparable to those measured directly by the equilibrium technique; this strengthens our confidence in the filter binding technique as used here and also reinforces our underlying assumption that parameters reflecting the same overall process are measured by both the equilibrium and the kinetic techniques, at least at salt concentrations > 0.1 M KCl.[4]

In Figure 10 we plot the measured values of K_{RO} (obtained by both equilibrium and kinetic methods), for the various operator-containing DNA fragments, against fragment size at three different salt concentrations. We see that K_{RO} begins to decrease as the DNA fragment size drops below a few hundred base pairs; when 80-base-pair fragments are reached, K_{RO} has dropped 10–30-fold. [This effect becomes somewhat more pronounced at the lower ionic strengths, but is quite evident at all the salt concentrations examined (0.1–0.2 M KCl).] K_{RO} drops an additional factor of 10 for fragments

[4] We note that the standard errors for the *individual* kinetic measurements are generally smaller than those for the direct equilibrium determinations. As a consequence we consider the values of K_{RO} determined as ratios of rate constants (Figure 9 and Table I) to be as reliable as those measured directly.

Table I: Summary of Measured Values of K_{RO} (M^{-1}) vs. Salt Concentration for Various *lac* Operator Containing DNA Molecules

[KCl] or [NaCl] (M)	DNA used							
	λ*plac*5 [a]	λ*plac*5 [b]	λ*plac* [c]	λ*plac*5 [d]	*Eco*RI fragment [e]	*Eco*RI fragment [f]	*Hae*-203 fragment [g]	*Hae*-203 fragment [h]
0.05		1×10^{13}		2.1×10^{14}		1.2×10^{14}	5.5×10^{12}	7.8×10^{12}
0.06			3.1×10^{15}					
0.10		4.3×10^{11}		8.0×10^{13}	5.0×10^{13}	3.4×10^{13}	2.0×10^{13}	5.9×10^{12}
0.11			4.6×10^{13}					
0.125				3.2×10^{13}			1.3×10^{13}	4.0×10^{12}
0.13	3.0×10^{13}							
0.15	8.2×10^{12}			6.4×10^{12}	1.8×10^{13}	9.1×10^{12}	2.8×10^{12}	1.9×10^{12}
0.16		9.0×10^{10}	3.4×10^{12}					
0.17	2.3×10^{12}							
0.175				5.3×10^{12}			5.2×10^{11}	7.1×10^{11}
0.19	1.1×10^{12}							
0.20	4.7×10^{11}			1.1×10^{12}	1.3×10^{12}	1.1×10^{12}	2.3×10^{11}	1.6×10^{11}
0.21			4.9×10^{11}					
$m'\psi$ [o]	9.3	4.1	7.0	6.0	5.1	4.9	6.9	5.0
m' [p]	10.6	4.8	8.0	6.8	5.8	5.6	7.8	5.7

[KCl]	DNA used					
	(*Hae-Hpa*)-80 fragment [i]	29-base-pair fragment [j]	26-base-pair fragment [k]	21-base-pair fragment [l]	*Hae*-170 fragment [m]	(*Hae-Hpa*)-123 fragment [n]
0.05	6.3×10^{12}	1.5×10^{11}			4.0×10^{12}	
0.06						
0.10	2.3×10^{12}	5.9×10^{10}			2.1×10^{12}	$<10^{10}$
0.11						
0.125					4.2×10^{11}	
0.13						
0.15		2.6×10^{10}			2.7×10^{11}	
0.16			1.0×10^{11}	7.1×10^{10}		
0.17						
0.175					1.1×10^{11}	
0.19						
0.20					3.8×10^{10}	
0.21						
$m'\psi$ [o]		1.6	1.5	1.6	5.4	
m' [p]		1.8	1.7	1.8	6.2	

[a] Data of Barkley et al. (1981). K_{RO} was determined from the ratio of on to off rates. Experiments were carried out in phosphate buffer, pH 7.4; the monovalent cation varied was Na^+. [b] Data of O'Gorman et al. (1980). K_{RO} was determined from direct equilibrium measurements. Experiments were carried out in Tris buffer, pH 7.5. [c] Data of Riggs et al. (1970). K_{RO} was determined from direct equilibrium measurements. Experiments in Tris buffer, pH 7.4, and in the presence of 10 mM $MgCl_2$. Values presented are corrected for the presence of Mg^{2+} by eq 5 of Record et al. (1977). The Riggs et al. data obtained at an ionic strength of 0.05 (corresponding to [KCl] = 0.01 M) have not been included. [d] Data of Winter et al. (1981). Experiments were performed as described under Materials and Methods. K_{RO} from ratio of on to off rates. $m'\psi$ calculated from 0.1 to 0.2 M KCl. [e] Data from this paper. K_{RO} determined from direct equilibrium measurements. $m'\psi$ calculated from 0.1 to 0.2 M KCl. [f] Data of Winter et al. (1981). K_{RO} determined from ratio of on to off rates. [g] Data from this paper. K_{RO} determined from direct equilibrium measurements. [h] Data of Winter et al. (1981). K_{RO} from ratio of on to off rates. [i] Data of Winter et al. (1981). K_{RO} from ratio of on to off rates. [j] Data of O'Gorman et al. (1980). K_{RO} was determined by direct equilibrium measurements. [k,l] Data of Goeddel et al. (1977). K_{RO} was determined from the ratio of on to off rates. Experiments were performed in Tris buffer, pH 7.4, and in the presence of 10 mM $MgCl_2$. m'_{RO} was calculated from eq 10 of Record et al. (1977) by using $m'_{RD} = 12$, $\partial \log K_{RD}/\partial I^{1/2} = -11$, $\partial \log K_{RO}/\partial I^{1/2} = -1.6$ for the 26-base-pair fragment, and $\partial \log K_{RO}/\partial I^{1/2} = -1.7$ for the 21-base-pair fragment. Values of K_{RO} were corrected for the presence of Mg^{2+} by using eq 5 of Record et al. (1977) and values of $\log K_{RO,T}$ of 9.8 for the 26-base-pair fragment and 9.6 for the 21-base-pair fragment ($K_{RO,T}$ is the thermodynamic binding constant). Data of Goeddel et al. (1977) at 0.05 M ionic strength (corresponding to [KCl] = 0.01 M) are not included. [m] *lacZ* pseudooperator fragment. Data from this paper. K_{RO} determined from direct equilibrium measurements. $m'\psi$ calculated from 0.1 to 0.2 M KCl. [n] *lacI* pseudooperator fragment. K_{RO} estimated as described in text (this paper). [o,p] $m'\psi$ is the number of counterions released from DNA upon formation of RO complex. m' is the number of ion pairs formed between repressor and operator DNA [see Record et al. (1976)].

that are 21–26 base pairs in length (Figure 10). This loss of RO complex stability with decreasing DNA fragment size is also reflected directly in the kinetic measurements; the association rate decreases and the dissociation rate increases with decreased DNA length [see Winter et al. (1981)].

A complete summary of the values of K_{RO} measured in our studies and by others is presented in Table I. The molecular implications of these parameters and of their dependence on salt concentration, DNA fragment length and operator composition are considered under Discussion.

Discussion

Several generalizations emerge from a perusal of the results of this work as summarized in Table I. These generalizations not only provide the equilibrium "underpinnings" of the kinetic measurements described and interpreted in the following paper (Winter et al., 1981), but also yield considerable insight into some molecular details of the RO interaction.

Formation of the RO Complex Involves Six to Eight Charge–Charge Interactions. Our data for the binding of *lac* repressor to the primary (physiological) operator site in whole λ*plac*5 DNA (50000 base pairs), in the *Eco*RI fragment (6700 base pairs), and in the *Hae*-203 fragment show a constant slope of the log K_{RO} vs. log [KCl] plots at salt concentrations between ~0.1 and 0.2 M, indicating that m' is the same for all these interactions and is equal to approximately six to eight charge–charge interactions [see also Record et al. (1977); Barkley et al. (1981)]. This number, considering the assumptions made in obtaining it, is surprisingly consistent with the determination of Maxam & Gilbert (1977b)[1] that seven

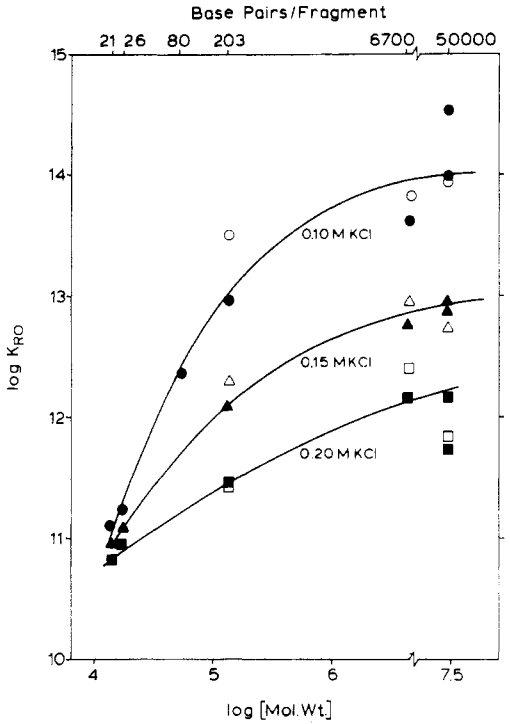

FIGURE 10: Plots of the logarithm of the measured values of K_{RO} vs. the logarithm of the molecular weight of the operator-containing DNA fragment. (Fragment size in base pairs is indicated at the top of the graph; the lines drawn through the data serve only to connect the points and have no theoretical significance.) Open symbols indicate direct equilibrium measurments; closed symbols represent K_{RO} values calculated from k_a/k_d ratios. (○, ●) BB + 0.1 M KCl; (△, ▲) BB + 0.15 KCl; (□, ■) BB + 0.2 M KCl. Some of the λ*plac*5 DNA data are from Riggs et al. (1970), as corrected by Record et al. (1977), and from Barkley et al. (1981). The 21- and 26-base-pair data are from Goeddel et al. (1978) and have been corrected for the presence of Mg^{2+} and extrapolated to different salt concentrations as described in footnotes *k* and *l* to Table I. All the rest are from this study.

phosphate groups within the specific *lac* operator sequence are partially protected by bound repressor from alkylation by ethylnitrosourea. The simplest interpretation of these facts is to attribute the electrostatic component of the binding of repressor to operator primarily to displacement of bound counterions (here K^+) from these seven phosphates. We note that this value of m' is considerably smaller than the value of ~11 charge–charge interactions determined for the repressor-nonspecific DNA interaction by Revzin & von Hippel (1977) and deHaseth et al. (1977) on the basis of the salt dependence of RD complex formation.

Two classes of binding models can be invoked to explain these differences. In the first model [and the one we favor; see also Winter et al. (1981)], it is assumed that the *same* number of subunits per repressor tetramer are involved in both RO and RD complex formation, with the subunits (and perhaps the DNA?) changing conformation to alter the number of charge–charge interactions per subunit. In the second class of models, the *number* of charge–charge interactions with the DNA per repressor subunit stays constant, but *different* numbers of subunits are involved in the two complexes.

In the former class of models, we assume that the "nonoperator-DNA binding form" of the repressor allows maximum interaction with the sugar–phosphate backbone, whereas the "operator binding form" maximizes specific nucleotide interactions at the expense of some electrostatic contacts. These changes in conformation presumably represent the "extremes" of the spectrum of binding interactions available to the repressor. A third conformation, the "inducer binding form" may then represent an intermediate state in which *some* specific contacts have been disrupted. Recent data of O'Gorman et al. (1980) indicate that the salt concentration dependence of binding to operator of repressor complexed with inducer is very similar to that of the RO complex itself, supporting the notion that the "induced repressor" maintains essentially the same electrostatic interactions with operator as does the noninduced form. [See von Hippel (1979) for one specific formulation of this "conformation-change" model, though note that the difference in the number of charge–charge interactions between the specific and nonspecific binding forms of repressor is not considered there.]

At present it is not known with certainty how many repressor subunits are directly involved in *either* operator or nonoperator DNA binding. The suggested 2-fold symmetry of the repressor tetramer (Steitz et al., 1974) is consistent with the involvement of two repressor subunits in the RO interaction, and studies with hybrid repressors generated by brief tryptic digestion (Kania & Brown, 1976; Geisler & Weber, 1976) also suggest that as few as two subunits are sufficient for operator binding. Ogata & Gilbert (1979) have also utilized chemical modification data to argue that two subunits are in contact with operator DNA.

If two subunits *are* involved in RO complex formation, then each must be responsible for three to four charge–charge interactions with operator phosphates. Then if the *same* number of charge–charge interactions *per subunit* is to be maintained in the RD complex, *three* to *four* repressor subunits must bind to the DNA in this latter reaction. Proposals along these lines have been put forward by Dunaway et al. (1980). [We note that the amino-terminal 59 residues of each repressor subunit contain only 8 basic residues—see Revzin & von Hippel (1977)—indicating that more than one subunit *must* be involved in RD complex formation if only basic groups on the N-terminal peptide are involved in charge–charge interactions. In keeping with this latter point, O'Gorman et al. (1980) have recently demonstrated that operator DNA–repressor "core" interactions show no salt dependence.]

We emphasize that our estimate of the number of charge–charge interactions involved in repressor–operator complex formation is based on data obtained over the 0.1–0.2 M KCl concentration range; repressor binding at KCl concentrations lower than ~0.1 M (at least in the absence of Mg^{2+}) appears to be less sensitive to salt concentration (Figures 4, 6, and 9). As a consequence, electrostatic contributions to RO binding do not exceed 50% of the total binding free energy, even at fairly low ionic strengths.

Nevertheless, ionic interactions are important in specific binding. Under approximately in vivo conditions (~0.2 M KCl; Kao-Huang et al., 1977), electrostatic (salt-dependent) interactions contribute 32% (±6%) to the overall binding free energy of repressor to the *Hae*-203 fragment, 26% (±9%) for binding to the (*lacZ* gene) secondary operator interaction, and 21% (±10%) for binding to the *Eco*RI fragment. [The *differences* between these values for different DNA fragments are not statistically significant, due to variations in the slopes of the log/log plots and hence to variations in $\Delta G°_{RO,1M}$ (obtained by extrapolation to 1 M KCl).]

Definition of Electrostatic and Nonelectrostatic Binding Components in RO Complex Formation. As pointed out under Results, we have followed the approach of Record et al. (1976) in differentiating electrostatic and nonelectrostatic components of the binding free energy of a ligand with a polyelectrolyte lattice. These workers plot the logarithm of the observed binding constant against the logarithm of the salt concentra-

tion; the slope of this log–log plot establishes the *salt concentration dependence* of the interaction and is defined on this basis as the electrostatic component of the binding process. The (corrected and extrapolated; see Results) value of the binding constant at 1 M salt, also by definition, is used to establish the *nonelectrostatic* component of the binding interaction, i.e., that part not due to the charge–charge interactions. Effects of anions, pH, etc., on the binding constant, which in other approaches are often considered to represent perturbations of electrostatic interactions, are here treated as effectors of the electrostatic component only if they alter the *slope* of the log–log plot. We use this definition of electrostatic interactions throughout this and the accompanying papers (Berg et al., 1981; Winter et al., 1981).

In practice [see Record et al. (1976)], this means that electrostatic effects are considered only to be those in which the *number* of counterions (thermodynamically) bound either to DNA (cations) or to protein (anions) (or to both) is *altered* as a consequence of complex formation. Thus, for example, if anions are displaced from (or bound to) the protein in the protein–nucleic acid interaction process (demonstrated by a dependence of the slope of the log–log plot on anion *type*; see footnote 2), this is considered an electrostatic effect. On the other hand, if replacement of one anion by another changes the *absolute value* of the binding constant [perhaps by bringing about a conformational change in the protein; for data on this point, see deHaseth et al. (1977); Barkley et al., 1981) but not the *slope* of the log–log plot, such an effect is considered here to be *nonelectrostatic*.

The latter situation seems to apply in both the RD (deHaseth et al., 1977) and the RO interaction; in particular, Barkley et al. (1981) have shown that the measured value of K_{RO} can vary by factors of up to 10^5 with different anions but without a change in the slope of the log–log plot. Those anions which bind most tightly to functional groups of macromolecules [eg., SCN^-, I^-, etc.; see von Hippel & Schleich (1969)] appear to have the greatest effect in decreasing K_{RO}.

"Long-Range" Effects in RO Complex Formation. Four types of observations suggest that the value of the binding affinity of repressor for operator involves "long-range" effects in addition to direct (contact) interactions between functional groups of the repressor and those of the operator DNA which lies directly beneath it. (i) The observed values of K_{RO} decrease with decreasing length of the operator-containing DNA fragment (see Figure 10). (ii) The value of K_{RO} for repressor binding to the secondary (Z gene) operator seems to depend on whether repressor is bound to a primary operator site on the same piece of DNA (assuming that K_{RO_2} decreases with DNA fragment length in parallel with K_{RO_1}; see Results). (iii) Sadler et al. (1980) have shown that in plasmids ("relaxed" by γ irradiation) which carry tandem operators, the binding of repressor to one operator site excludes binding to vicinal operator sites over distances as large as 120–160 base pairs.[5] (iv) Chan & Wells (1974) and Chan et al. (1977) have presented evidence suggesting that a specific nick, introduced by a single-strand-specific nuclease into a double-stranded *lac* operator containing DNA fragment at a position which is at least 100 base pairs away from the operator, results in a reduced affinity of repressor for the operator site on the fragment.

All these effects have in common the feature that events located 100 or more base pairs (300–400 Å) away from the operator alter the strength of the RO interaction, whether the event comprises binding of another repressor to a second operator site, removal of vicinal DNA, or enzymatic nicking of the sugar–phosphate backbone.

Three types of mechanisms can, in principle, account for these observations:

(i) The effect could be attributed to a conformational change that occurs in the vicinal DNA as a consequence of the above events and is "projected" into the operator via twisting, base tilting, or other distortions of the intervening DNA (telostability?). Such distortion of the operator could put it into a less favorable conformation for repressor binding, resulting in an apparent decrease in K_{RO}. Long-range effects of this sort are clearly possible as a consequence of superhelix formation in a closed-circular DNA[6] and have been suggested in linear DNA [e.g., see Wells et al. (1977)]. However, certain types of "projected" conformational distortions clearly are *not* possible in linear (or nicked-circular) DNAs since the distortions, in effect, can "run out of the ends" of the linear molecules. Thus the intrinsic binding constant of DNA for ethidium bromide (which binds by intercalation between base pairs and brings about massive local distortion of the double helix) is a strong function of the dye binding density in closed-circular DNA; on the other hand, the magnitude of this binding constant is approximately *independent* of the degree of binding saturation (after correction for nearest neighbor exclusion effects) in linear DNA. [For a recent summary of such data, see Cantor & Schimmel (1980).]

(ii) Long-range "electrostatic" interactions between distant charges on the DNA backbone and bound repressor might also be considered, although (e.g.) the calculations of Stigter (1977) suggest that such effects should be screened out over distances of no more than ~25 Å in moderate (0.1 M) salt concentrations. However, a part of the DNA-length-dependent decrease in K_{RO} (Figure 10) *could* be due to such effects.

(iii) An overall *bending* of the DNA double helix as a consequence of repressor binding to the operator is also possible. The wrapping of DNA around histone octamers in nucleosome formation, bringing pieces of DNA which are separated by ~100 base pairs along the contour length into reasonable proximity, provides a possibly relevant model. Such monotonic distortion of the backbones of the double helix *could* bring sections of the DNA molecule which are separated by 100 base pairs or more close enough together to make long-range "electrostatic" effects of type ii nonnegligible. No direct evidence for or against such "higher order" structural consequences of *lac* repressor binding to operator exist at this time.[7]

Thus at present we have a number of lines of experimental evidence that strongly implicate "long-range" effects in repressor–operator interactions. No unequivocal molecular explanation of these effects is currently available.

Salt Dependence of Repressor Binding to Small Operator-Containing DNA Fragments. We have not rigorously shown that repressor binding to operator-containing DNA fragments *less* than ~170 base pairs in length involves 6–8

[5] The operators cloned in tandem by Sadler et al. (1980) are each located on DNA fragments that are 40 base pairs in length. These workers showed that only one repressor is bound to plasmids containing one to four such operators.

[6] In fact, Wang et al. (1974) have shown that the *lac* repressor–operator complex formation does involve some "unwinding" of the operator DNA and that as a consequence, K_{RO} can be increased 10-fold or more by using an operator located in an "underwound" (superhelical) circular DNA molecule.

[7] Recently Chao et al. (1980) have shown that under some conditions the incorporation of operator-containing DNA into nucleosomes can alter the strength of the specific repressor–operator interaction.

charge–charge interactions since our measurements with the 80- and 123-base-pair fragments (Table I) as a function of salt concentration are not sufficiently precise to yield an unambiguous measure of m' for these moieties. Data of Goeddel et al. (1977) and O'Gorman et al. (1980) on the salt dependence of the binding of repressor to synthetic operator fragments 21–29 base pairs in length suggest that m' for these interactions is ~2 (see Table I), a value much smaller than that obtained with operator-containing pieces of DNA longer than 170 base pairs. This may reflect a real difference in repressor–nucleic acid charge–charge interactions for these very small DNA fragments [though see the interpretation of Goeddel et al. (1977) data put forward by Lohman et al. (1978)].

If indeed the seven phosphates (see Figure 5) identified by the protection experiments of Maxam & Gilbert (1977b)[1] are those involved in charge–charge interactions in RO complex formation, then all fall within the synthetic operator sequences examined by Goeddel et al. (1977) and by O'Gorman et al. (1980). On the other hand, the fragment studied by the latter workers consists only of a central double-stranded sequence (the "sticky ends" of the *Eco*RI "linker") protruding from the 5′ termini on each end. It is known (Record & Lohman, 1978) that DNA phosphates located near the ends of DNA fragments bind counterions less effectively than do those in the middle of a long DNA strand and thus show less counterion displacement on repressor binding. Four of the seven phosphates identified by Maxam & Gilbert (1977b)[1] as being shielded by repressor do indeed occur at the ends of the specific operator sequence. Such effects could account for a part of the decreased salt dependence of binding of these small fragments; it is also possible that some of the charge–charge interactions involved in the RO interaction lie somewhat beyond the outer edges of the operator-specific base-pair sequence.

The Operator Sequence Can Experience Substitutions at 4–5 Base Pairs and Still Retain Most Recognition Features. Maximum homology between the "primary" and the "pseudo"-operator sites occurs within a 17-base-pair sequence (see Figure 5) previously identified as absolutely required for repressor interaction (Bahl et al., 1977). Outside this interval there is little homology between the primary and the pseudooperator sites. Thus the energetically important specific base pair contacts involved in RO formation are doubtless located between positions 8 and 24. Therefore the unfavorable (relative to primary site binding) change in free energy observed when repressor complexes with the *lacZ* pseudooperator can be viewed as being distributed over three base-pair alterations (positions 8, 17, and 18), with each "incorrect" pair destabilizing the interaction by about +0.4 kcal/mol. [We assume that the destabiliztion can be equipartitioned over these three base changes; in support of this assumption, Goeddel et al. (1978) found little difference in repressor binding to synthetic operators with different bases substituted into position 8, 17, or 18]. This is a relatively small perturbation compared to those characteristic of O^c mutations (Jobe et al., 1974), which disrupt wild-type repressor binding by +1.8 to +2.6 kcal/mol. Thus, repressor binding to the *lacZ* pseudooperator provides additional evidence that not all base pairs in the *lac* operator sequence contribute an equal amount of free energy to the RO interaction [see also Goeddel et al. (1978)].

We can examine repressor interaction with the *lacI* pseudooperator site in a similar fashion. This site carries six base-pair changes between positions 8 and 24 of the operator sequence (Figure 5); five of these occur at loci where no important contacts with repressor have yet been documented. However, the transversion T·A → A·T at position 19 occurs at a known O^c site where the transition T·A → C·G has been observed to weaken repressor binding by about +2 kcal/mol (Jobe et al., 1974). The overall weakening of repressor binding to the *lacI* "pseudo"-operator site is about +4 to +5 kcal/mol relative to wild-type interactions, suggesting that five of these changes could contribute ~ +0.4 kcal/mol each to the binding free energy and that the transition at position 19 disrupts RO complex formation by +2 to +3 kcal/mol in addition.

The behavior of the E40 mutation in the *lacI* pseudooperator described by Pfahl (cited in Bourgeois & Pfahl, 1976), which *increases* repressor affinity to this site to about 20-fold less than wild type, is consistent with these calculations. *If*, for example, the E40 mutation were to represent the reversion of A·T → T·A at position 19, the remaining estimated loss in binding free energy (about +2 kcal/mol according to the above model) would indeed result in about a 20-fold weaker binding of repressor to this site relative to repressor binding to the primary (wild-type) opeator. It therefore appears that at least some operators, carrying as many as five base pair changes within the operator sequence, still retain sufficient essential (hydrogen-bonding) contacts to permit specific repressor binding; we emphasize that the binding of repressor to the *lacI* pseudooperator is still about 4 orders of magnitude tighter than that to random nonoperator DNA.

The *lac* operator represents an "overspecified" sequence (von Hippel, 1979), i.e., that portion of the DNA represented by the *lac* operator (Figure 5) is longer than the minimum length of DNA required to specify a "unique" site along the *E. coli* genome (about 12 base pairs). Such overspecification is required to avoid the occurrence of a large number of nearly identical sequences within the *E. coli* chromosome (i.e., sequences with one or two base-pair changes relative to the wild type) which would occur at random for a minimum length sequence and then would compete effectively with the "real" operator site for repressor.

Using a conditional probability approach (von Hippel, 1979), we calculate that the *lacZ* pseudooperator, with three incorrect base pairs in the *lac* operator sequence, has only a 3% change of arising at random within the *E. coli* genome, whereas the *lac* I pseudooperator, with six incorrect base pairs, has an 84% probability of arising at random. Thus coincident with the increased probability of operator-like sequences occurring at random is the decreased affinity of repressor for these sites. Clearly it is crucial for the correct intracellular distribution and in vivo functioning of repressor that this protein not show an appreciable affinity (compared to that for the operator sequence) for a large number of other related nonoperator DNA sequences [see also von Hippel et al. (1974)].

Can Repressor Bind to More Than One DNA Fragment at a Time? Certain protein translocation models described in the companion papers (Berg et al., 1981; Winter et al., 1981) involve the transient formation of doubly bound repressor–DNA complex intermediates, i.e., the binding of repressor to more than one DNA molecule or segment simultaneously. The formation of such intermediates is obviously possible, in principle, for repressor models in which two (or less) subunits are involved in binding to one operator or DNA sequence.

Such intermediates, involving two simultaneous nonspecific DNA interactions, would be very hard to detect because of the relatively low values of K_{RD}. On the other hand, it is possible to carry out two types of equilibrium experiments to look for repressors that are bound to two *operators* at the same

time, which should reflect nonspecific binding if essentially the same protein binding sites are involved in both interactions. First, very careful titrations of repressor were carried out at low salt concentrations (to ensure binding saturation) with all the operator-containing DNA fragments used in these studies. For each size of fragment (80 base pairs and larger), an unambiguous stoichiometry of 1:1 (RO) was obtained, indicating no stable 1:2 (RO) complexes were formed. (This information is, of course, also required to interpret the stoichiometry which applies to the binding isotherms measured at higher salt concentrations.) In addition, chemical cross-linking studies (Winter, 1980) were attempted to see whether 1:2 cross-linked species were demonstrated. Again none were seen, though, as expected, 1:1 cross-linked species were demonstrated. In contrast, O'Gorman et al. (1980) have obtained evidence for 1:2 (RO) complexes with 21-base-pair (synthetic) operator fragments, though not with larger operator-containing DNA molecules. While negative experiments have only limited validity, as pointed out in the preceding paper (Berg et al., 1981) these results suggest that doubly bound intermediates involving anything larger than the smallest operator fragments are probably unstable. Furthermore, for the purposes of *this* paper, these results mean that all the RO interaction systems studied here can be interpreted in terms of 1:1 (RO) complexes.

Implications for the Cellular Control of lac Operon Enzymes. The studies presented here on molecular aspects of the RO interaction allow us to compare further the interactions of repressor with operator and nonoperator DNA and to define more precisely some of the "operator-binding" form of repressor, previously described as a conformation that binds inducer more weakly than free repressor (Laiken et al., 1972; Barkley et al., 1975; Wu et al., 1976). Repressor bound to operator forms 6–8 ion pairs with operator phosphates and in addition interacts with functional groups of at least 12–13 base pairs within the operator sequence (these are the "crucial" interactions, though others may contribute secondarily to the binding free energy). The binding of inducer to the repressor appears to disrupt many of these latter (specific) contacts, without significantly perturbing charge–charge interactions. This "inducer-binding" form of repressor still binds appreciably more tightly to the operator than to nonoperator DNA. Finally, the "nonoperator" conformation derives virtually its entire binding free energy from charge–charge interactions stabilized by counterion displacement. The "coupling" of repressor–operator–inducer–DNA interactions within the cell thus involves conformational transitions of the protein between (at least) these three states. For a further consideration of this point in the context of kinetic mechanisms, see Winter et al. (1981).

Nonoperator DNA has been shown to be important in the intracellular distribution of repressor and the extent of repression in vivo in both induced and noninduced states (von Hippel et al., 1974; Lin & Riggs, 1975; Kao-Huang et al., 1977). In addition, we show here that the appearance of full repressor binding affinity depends upon the presence of contiguous nonoperator DNA sequences extending as far as 100–200 base pairs from the operator site itself. Such sequences also appear to be important in the pathways by which repressor locates the operator [see Winter et al. (1981)]. At present, there are no detailed data available to determine whether the binding of other DNA site-specific binding proteins (e.g., λ repressor, *E. coli* RNA polymerase) exhibit similar equilibrium behavior with respect to DNA vicinal to the specific target sequence. Additional discussion of this point with respect to kinetic mechanisms is presented in Winter et al. (1981).

References

Anderson, C. F., Record, M. T., Jr., & Hart, P. A. (1978) *Biophys. Chem. 7*, 301.

Bahl, C. P., Wu, R., Itakura, K., Katigiri, N., & Narang, S. A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 91.

Bahl, C. P., Wu, R., Stawinsky, J., & Narang, S. A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 966.

Barkley, M. D., Riggs, A. D., Jobe, A., & Bourgeois, S. (1975) *Biochemistry 14*, 1700.

Barkley, M. D., Lewis, P. A. & Sullivan, G. E. (1981) *Biochemistry 20*, 3842.

Berg, O. G., Winter, R. B., & von Hippel, P. H. (1981) *Biochemistry* (preceding paper in this issue).

Bleam, M. L., Anderson, C. F., & Record, M. T., Jr. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3085.

Bourgeois, S., & Riggs, A. D. (1970) *Biochem. Biophys. Res. Commun. 38*, 348.

Bourgeois, S., & Pfahl, M. (1976) *Adv. Protein Chem. 30*, 1.

Butler, A. P., Revzin, A., & von Hippel., P. H. (1977) *Biochemistry 16*, 4757.

Cantor, C. R., & Schimmel, R. R. (1980) *Biophysical Chemistry*, Vol. III, W. H. Freeman, San Francisco, CA.

Chan, H. W., & Wells, R. D. (1974) *Nature (London) 252*, 205.

Chan, H. W., Dodgson, J. B. & Wells, R. D. (1977) *Biochemistry 16*, 2356.

Chao, M. V., Martinson, H. G., & Gralla, J. D. (1980) *Biochemistry 19*, 3260.

Clewell, D. B., & Helinski, D. R. (1970) *Biochemistry 9*, 4228.

deHaseth, P. L., Lohman, T. M. & Record, M. T., Jr. (1977) *Biochemistry 16*, 4783.

Dunaway, M., Manly, S. P., & Matthews, K. S. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7181.

Geisler, N., & Weber, K. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3102.

Gilbert, W., & Maxam, A. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3581.

Gilbert, W., Gralla, J., Majors, J., & Maxam, A. (1975) in *Protein-Ligand Interactions* (Sund, H., & Blauer, G., Eds.) pp 193–210, de Gruyter, Berlin.

Gilbert, W., Maxam, A., & Mirzabekov, A. (1976a) *Control of Ribosome Synthesis*, Alfred Benzon Symp. 9, pp 139–148, Munksgaard, Copenhagen.

Gilbert, W., Majors, J., & Maxam, A. (1976b) *Organization and Expression of Chromosomes*, Life Sci. Res. Rep. 4, pp 176–178, Heyden & Son, Ltd., London.

Goeddel, D. V., Yansura, D. G., & Caruthers, M. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3292.

Goeddel, D. V., Yansura, D. G., & Caruthers, M. H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3578.

Jobe, A., Sadler, J. R., & Borgeois, S. (1974) *J. Mol. Biol. 85*, 231.

Jovin, T. M., Geisler, N., & Weber, K. (1977) *Nature (London) 269*, 668.

Kania, J., & Brown, D. T. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3529.

Kao-Huang, Y., Revzin, A., Butler, A. P., O'Conner, P., Noble, D. W., & von Hippel, P. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4228.

Klotz, I. M., & Hunston, D. L. (1971) *Biochemistry 10*, 3065.

Kowalczykowski, S. C., Lonberg, N., Newport, J. W., & von Hippel, P. H. (1981) *J. Mol. Biol. 145*, 75.

Laemmli, U. K. (1970) *Nature (London) 727*, 680.
Laiken, S. L., Gross, C. A., & von Hippel, P. H. (1972) *J. Mol. Biol. 66*, 143.
LePecq, J.-B., & Paoletti, C. (1966) *Anal. Biochem. 17*, 100.
Lin, S.-Y., & Riggs, A. D. (1975) *Cell (Cambridge, Mass.) 4*, 107.
Lohman, T. L., deHaseth, P. L., & Record, M. T., Jr. (1978) *Biophys. Chem. 8*, 281.
Lohman, T. L., deHaseth, P. L., & Record, M. T., Jr. (1980) *Biochemistry 19*, 3522.
Maniatis, T., Jeffrey, A., & Kleid, D. G. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1184.
Manly, S. P., & Matthews, K. S. (1979) *J. Biol. Chem. 254*, 3341.
Manning, G. S. (1978) *Q. Rev. Biophys. 11*, 179.
Matthews, K. S. (1979) *J. Biol. Chem. 254*, 3348.
Maxam, A., & Gilbert, W. (1977a) *Proc. Natl. Acad. Sci. U.S.A. 74*, 560.
Miller, J. H., Coulondre, C., Schmeissner, U., Schmitz, A., & Lu, P. (1975) in *Protein–Ligand Interactions* (Sund, H., and Blauer, G., Eds.) pp 238–252, de Gruyter, Berlin.
Modrich, P., & Zabel, D. (1976) *J. Biol. Chem. 251*, 5866.
Müller-Hill, B. (1975) *Prog. Biophys. Mol. Biol. 30*, 227.
Ogata, R. T., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4973.
Ogata, R. T., & Gilbert, W. (1979) *J. Mol. Biol. 132*, 709.
O'Gorman, R. B., Dunaway, M., & Matthews, K. S. (1980) *J. Biol. Chem. 255*, 10100.
Record, M. T., Jr., & Lohman, T. M. (1978) *Biopolymers 17*, 159.
Record, M. T., Jr., Lohman, T. M., & deHaseth, P. L. (1976) *J. Mol. Biol. 107*, 145.
Record, M. T., Jr., deHaseth, P. L., & Lohman, T. M. (1977) *Biochemistry 16*, 4791.
Revzin, A., & von Hippel, P. H. (1977) *Biochemistry 16*, 4769.
Reznikoff, W. S., Winter, R. B., & Hurley, C. K. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2314.
Riggs, A. D., Suzuki, H., & Bourgeois, S. (1970) *J. Mol. Biol. 48*, 67.
Sadler, J. R., Tecklenburg, M., & Betz, J. L. (1980) *Gene 8*, 279.
Steitz, T. A., Richmond, T. J., Wise, D., & Engelman, D. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 593.
Stigter, D. (1977) *Biopolymers 16*, 1435.
von Hippel, P. H. (1979) in *Biological Regulation and Development* (Goldberger, R. F., Ed.) pp 279–347, Plenum Press, New York.
von Hippel, P. H., & Schleich, T. (1969) in *Structure and Stability of Biological Macromolecules* (Timasheff S. N., & Fasman, G. D., Eds.) p 417, Marcel Dekker, New York.
von Hippel, P. H., Revzin, A., Gross, C. A., & Wang, A. C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4808.
von Hippel, P. H., Revzin, A., Gross, C. A., & Wang, A. C. (1975) in *Protein–Ligand Interactions* (Sund, H., & Blauer, G., Eds.) pp 270–288, de Gruyter, Berlin.
Wang, J. C., Barkley, M. D., & Bourgeois, S. (1974) *Nature (London) 251*, 247.
Wells, R. D., Blakesley, R. W., Hardies, S. C., Horn, G. T., Larson, T. E., Selsing, E., Burd, J. F., Chan, H. W., Dodgson, J. B., Jensen, K. F., Nes, I. F., & Wartell, R. M. (1977) *CRC Crit. Rev. Biochem. 4*, 305.
Winter, R. B. (1980) Ph.D. Dissertation, University of Oregon, Eugene, OR.
Winter, R. B., Berg, O. G., & von Hippel, P. H. (1981) *Biochemistry* (following paper in this issue).
Wu, F. Y.-H., Bandyopadhyay, P., & Wu, C.-W. (1976) *J. Mol. Biol. 100*, 459.
Yansura, D. G., Goeddel, D. V., & Caruthers, M. H. (1977) *Biochemistry 16*, 1772.