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Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis

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ABSTRACT

We describe the use of gel electrophoresis in studies of equilibrium binding, site distribution, and kinetics of protein-DNA interactions. The method, which we call protein distribution analysis, is simple, sensitive and yields thermodynamically rigorous results. It is particularly well suited to studies of simultaneous binding of several proteins to a single nucleic acid. In studies of the lac repressor-operator interaction, we found that binding to the so-called third operator site (O3) is 15-18 fold weaker than operator binding, and that the binding reactions with the first and third operators are uncoupled, implying that there is no communication between the sites. Pseudo-first order dissociation kinetics of the repressor-203 bp operator complex were found to be temperature sensitive, with ΔE_a of 80 kcal mol^{-1} above 29°C and 26 kcal mol^{-1} below. The half life of the complex (5 min at 21°C) is shorter than that reported for very high molecular weight operator-containing DNAs, but longer than values reported for much shorter fragments. The binding of lac repressor core to DNA could not be detected by this technique: the maximum binding constant consistent with this finding is 10^5 M^{-1}.

INTRODUCTION

The extremely high binding affinities observed for many protein-nucleic acid interactions makes study by conventional physical methods impractical. One technique extensively used in this field is the filter binding assay, originally developed by Bourgeois and her collaborators (1), which relies on the ability of proteins to induce attachment of DNA to a nitrocellulose filter. A weakness of this method is that it does not generally allow analysis of the detailed composition of the products of the binding reaction. Interaction of a single protein with a DNA molecule is usually sufficient to cause filter retention, so it is frequently impossible to determine directly the extent to which a single DNA species binds several proteins. This is a serious limitation when the objective is to characterize ternary complexes between two proteins and a single DNA

fragment, for example.

We present here results illustrating the use of gel electrophoresis to characterize the interaction of E. coli lac repressor with DNA restriction fragments containing specific repressor binding sites. The protein is mixed with one or more DNA fragments, and protein-DNA complexes are resolved as discrete bands by polyacrylamide gel electrophoresis, with mobilities decreasing as a function of the number of proteins bound to a given DNA fragment. Low ionic strength conditions, and the gel matrix itself stabilize the complex against dissociation. Microdensitometry of stained gels and gel autoradiograms allows quantitation of the protein and DNA present in each complex. The method allows determination of the stoichiometric ratio of protein specificly bound to DNA, and permits accurate measurement of the relative binding affinities of a given protein for two different nucleic acids. Dissociation kinetics are readily measured for protein-DNA complexes with lifetimes greater than about one minute. Although DNA binding by lac repressor core (2) is too weak to be detected by this technique, results obtained for the E. coli cyclic AMP receptor protein (M. Fried and D.M. Crothers, to be published) indicate that the extremely high DNA binding affinity and slow dissociation kinetics of lac repressor are not essential for its application. In addition to use for quantitative binding studies, this technique should simplify identification of proteins that bind specifically to a given DNA sequence, or of restriction fragments containing sequences recognized by a particular protein.

Several unexpected results appeared in the course of our experiments, some of which raise significant questions for further study. First, we found repressor-DNA complexes to be very labile to mechanical mixing. Even a few seconds of vortex-mixing irreversibly dissociates a substantial fraction of the complex, while causing no detectable harm to samples of the pure protein or pure nucleic acid treated separately. Secondly, a surprisingly large fraction of a typical repressor preparation appears to be incapable of specific sequence recognition. Finally, the kinetics of exchange reactions in which protein is transferred from one DNA molecule to another appear to be slowed by one to two orders of magnitude in the gel, compared to the same ionic conditions in free solution. Even though interpretation of some of these phenomena remains open, we present a series of results at this stage in order to allow others to make use of the method.

MATERIALS AND METHODS

Isolation and 5' end-labeling of restriction fragments. E. coli strain MM294 carrying plasmid pOP 203 (UV5)-1 was the gift of F. Fuller. Plasmid DNA was prepared by a standard cleared lysate procedure (3) and purified by banding twice in CsCl gradients. Restriction enzymes Eco R_1 and Hpa II were purchased from New England Biolabs, and were used as directed. The 203 bp Eco R_1 fragment containing the entire lac promoter-operator region was isolated by preparative polyacrylamide gel electrophoresis (4) and labeled with ^{32}P at 5' termini according to Maxam and Gilbert (5). Subsequent digestion with Hpa II gave two fragments of 121 bp and 82 bp, in equimolar amounts (figure 1). The 82 bp fragment contains the entire lac operator (O1), while the 121 bp fragment contains both the CAP[1] recognition sequence and a secondary binding site for lac repressor, the pseudo-operator O3 (6,7).

Formation of repressor-DNA complexes. Wild type (Q) lac repressor, and lac repressor tryptic core were prepared according to published procedures (8,9) and were the kind gift of M. Leahy. The concentration of stock solutions of DNA were determined spectrophotometically using ϵ_{260} = 1.3 x 10^4 M^{-1} cm^{-1} (per mole bp). The concentrations of lac repressor and repressor core were determined using ϵ_{280} (tetramer) values of 8.88 x 10^4 M^{-1} cm^{-1} and 7.56 x 10^4 M^{-1} cm^{-1} respectively. Repressor-DNA complexes were formed by addition of appropriate amounts of the protein to a solution containing operator DNA at a final DNA concentration of 1.85 x 10^{-8}M. Binding reactions were carried out in 10 mM Tris (pH 7.4 at 21°C)- 1mM EDTA - 50 mM KCl. Inclusion of BSA (0.2 mg/ml), glycerol (20%) or DMSO (5%) in the reaction mixture had no detectable effect on the compositions or stability of the complexes formed. Tubes were gently swirled by hand to effect mixing. After equilibration for 20 min at 21°C solutions were diluted 10-fold with 10 mM Tris (pH 7.4 at 21°C) -1mM EDTA - 100 mg/ml BSA - 10 µg/ml xylene cyanol FF, -5% glycerol, and aliquots loaded immediately on polyacrylamide gels. Care was taken in all pipetting and

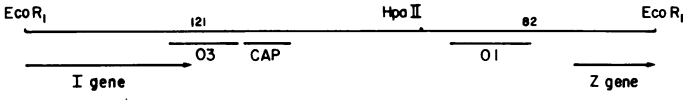

Figure 1: Partial restriction map of the lac promoter-operator region (redrawn from reference 8).

mixing operations to minimize generation of shear.

Protein distribution analysis by gel electrophoresis. Polyacrylamide slabs (4.94% acrylamide, 0.06% bisacrylamide), were cast as described by Maxam and Gilbert (5) except that the gel buffer was 10 mM Tris (pH 7.4 at 21°C) - 1 mM EDTA (TE buffer). Following polymerization, gels were equilibrated overnight in contact with TE buffer. Electrophoresis was carried out in a vertical device modified for buffer circulation. Before loading samples, gels were run until the conductivity was invariant with time. Due to the low conductivity of the gels, voltage gradients of up to 16 V cm^{-1} could be obtained without significantly raising the gel temperature. Protein bands were stained with Coomasie blue R-250 according to Weber and Osborn (10). Autoradiograms were obtained by exposing Dupont Cronex X-ray film to the gel at -20°C. Stained gels and autoradiograms were scanned with a Joyce-Loebl scanning microdensitometer. Peak areas were determined by planimetry. Typically, scans through different sections of an autoradiogram band gave areas differing by less than 5%. Replicate planimetric measurements varied by less than 2%.

Measurement of dissociation Kinetics. Repressor-DNA complexes were formed at an input molar ratio (protein: DNA) of $r \approx 3$ as described above, and equilibrated at the measurement temperature for 20 minutes. To initiate the reaction, excess unlabeled 203 bp operator DNA, also at the measurement temperature, was added. The mixture was agitated gently, and returned to the incubator. Samples were taken at intervals, diluted 1:10 with TE-10% glycerol at 4°C, and applied immediately to a running PDA gel. With practice, we found that the sampling operation could be completed in less than 15 sec. In experiments measuring the temperature dependence of dissociation rates, all buffer solutions were adjusted to pH 7.4 at the desired temperature prior to use.

RESULTS

The protein-DNA ladder. Titration of the 203 bp operator fragment with lac repressor produces a ladder of bands, revealing a maximum of eight discrete protein-DNA complexes (figure 2a). Further addition of protein produced very high molecular weight aggregates that were not resolved by the gel. We used quantitative gel scanning to determine the relative ratio of protein to DNA in each of the bands. We observed a linear relationship between coomassie blue stain intensity and amount of lac repressor applied to a gel. A similar, linear relationship between stain intensity

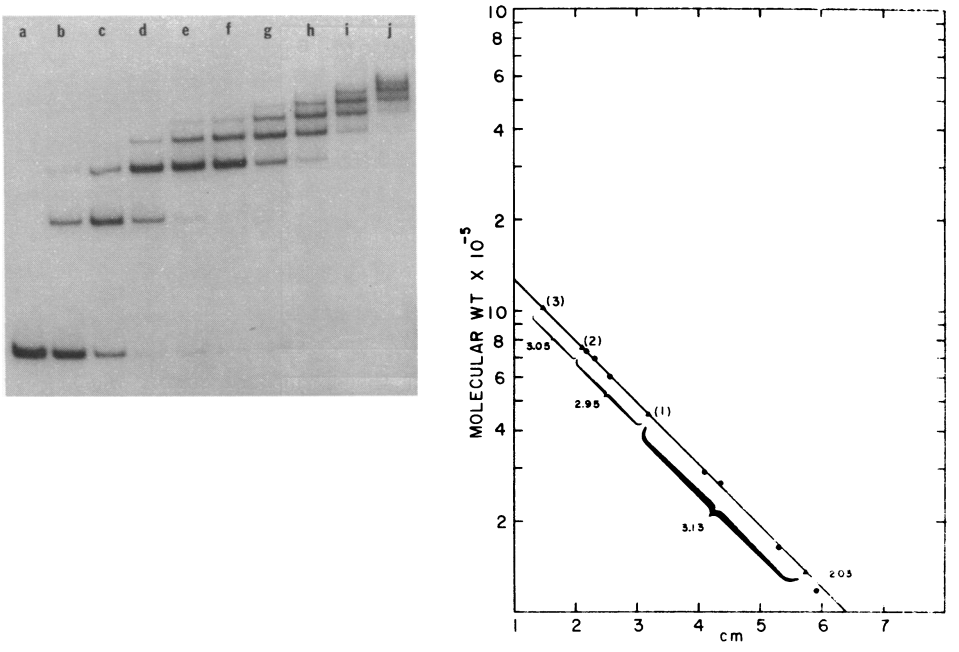

Figure 2: A: Titration of the 203 bp operator fragment with lac repressor. Values of r_{added} for lanes a-j respectively: 0, 0.67, 1.34, 2.0, 2.67, 3.33, 4.0, 5.34, 6.67. Polyacrylamide gel run in TE buffer at room temperature, band detection by autoradiography.
B: Increments in apparent molecular weight for the protein-DNA ladder. Gel electrophoretic mobilities for the 203 bp fragment and complexes of n = 1, 2, and 3 (▲). Standard curve: Hae III digest of Col E_1 (●) (32). Brackets indicate apparent molecular weight increments.

and amounts of protein loaded has been reported for another gel system (11). As summarized in table 1, band 2 contains twice the molar ratio of lac repressor (tetramer) to DNA as band 1. Within experimental error, the ratio in band 3 is tripled. The simplest interpretation of this result is that band 1,2,3, ...n contains 1,2,3,...n moles of lac repressor tetramer per mole of DNA.

Although compositional analysis cannot rule out other multiples of the molar ratio, such as 2:2 for band 1, the relatively high electrophoretic mobilities and lack of evidence for simultaneous repressor binding to more than one DNA molecule, make such interpretations unlikely. The regularity of the apparent molecular weight increments between ladder

TABLE I:

Ratio of Bound Protein to DNA

r_{added}	Complex n = 1	n = 2	n = 3
0.985	1.01	-	-
1.932	0.93	2.16	-
3.70	1.04	1.91	3.2

Complexes were formed in 10mM Tris - 50 mM KCl - 1mM EDTA (pH 7.4 at 21°C) Gels were run in TE buffer.

Quantitation of lac repressor was by densitometry of coomassie blue stained gels. Reference gels containing known amounts of lac repressor were stained and destained simultaneously with the gel containing protein-DNA complexes, to allow estimation of the amounts of protein present in each complex. Quantitation of DNA present in each complex was by densitometry of autoradiographic films; care was taken to ensure that film exposure was within the region of linear dose-response.

steps (figure 2b) also supports a simple one protein per step model. Finally, from the observed saturation at n = 8, the assumption of one repressor tetramer per step yields a site size of 25 bp per tetramer, in good agreement with other work (12,13). Taken together, these factors strongly support the simple interpretation of an incremental protein-DNA complex ladder for the gel in figure 2a.

Sensitivity of the complex to vigorous mixing. An unexpected problem we encountered in these experiments was the irreversible destruction of repressor-DNA complexes by vigorous mixing, presumably due to shear forces either in solution or at a surface. Figure 3 shows an experiment in which shear was intentionally generated by mixing in a vortex mixer for different periods of time. Complexes of n=1 and n=2 are approximately equally sensitive to disruption, judging by the time course of complex loss. No reassociation was observed after shearing, even after prolonged incubation. Simple surface denaturation of lac repressor is unlikely to be important in this process, since inclusion of carrier protein (BSA, 0.2 mg/ml) in the reaction mixture does not suppress degradation (data not shown.) However, denaturation by shear at the surface cannot be excluded.

We found that it was difficult to avoid shear degradation entirely,

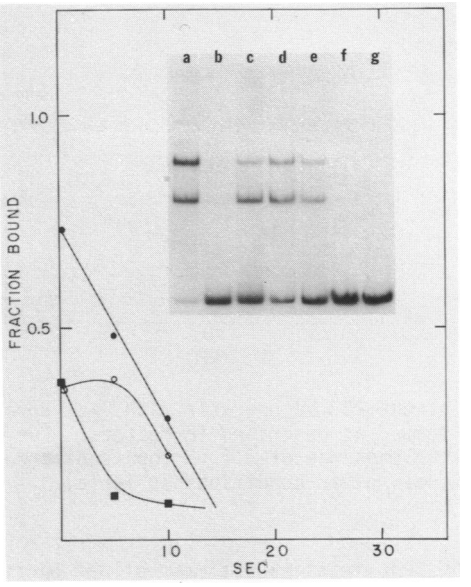

Figure 3: Sensitivity of lac repressor-DNA complexes to vigorous mixing. Fraction of DNA bound in all species (●), as n = 1 complex (■), as n = 2 complex (○). Inset lane a, no mixing; b, vortexed 30 sec followed by 30 min incubation at 21°C; c-h, vortexed for 1, 5, 10, 30, 120 sec respectively, gel loaded immediately.

even with careful hand mixing and slow pipetting onto the gels. This is probably one reason why it often requires a significant excess of protein to remove completely the free DNA band from the complex ladder. Therefore, in quantitative use of this technique, one should not rely on the observed intensity of any band present in small amount when the bulk of the complex has a larger molar ratio of protein to DNA. The intensity of such bands can be increased significantly as a result of small amounts of shear degradation of higher complexes.

Measurement of DNA binding and sequence recognition activities. The DNA binding and sequence recognition properties of lac repressor are known to be labile, and preparations may be partially inactive in specific DNA recognition, or even in DNA binding. Once the identification of bands n= 1,2,3... is accepted, the gel method permits quantitative determination of the protein: DNA ratio, as demonstrated in figure 4. Plotting the fraction of DNA radioactivity observed as complex against the nominal input ratio

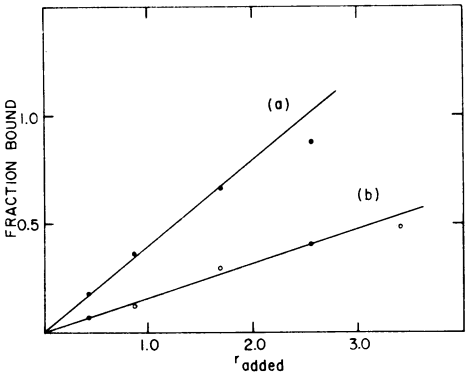

Figure 4: Titration of the 203 bp operator with lac repressor. Curve a: complexes formed as described in Methods. Curve b: complexes formed in the presence of 3.7 μM (bp) sonicated chicken erythrocyte DNA; all other conditions as in (a).

of protein to DNA (r_{added}) yields an estimate of 39% purity in DNA binding activity (curve a). Conventional filter binding assay of the same sample yielded 50% activity (M. Leahy, personal communication).

Sequence recognition by a DNA binding protein in vivo must occur in the presence of a vast excess of competing non specific DNA. We therefore repeated the titration in the presence of a 10-fold excess (in terms of phosphate) of chicken erythrocyte DNA (curve b). This assay reveals that only 16% of the added protein is able to bind specifically in the presence of competing DNA. This fraction was only reduced to 14% in the presence of a 50-fold excess of competing DNA, consistent with the existence of highly stable repressor-operator complexes. No change in the fraction of tightly bound repressor was found when the competing DNA used was from calf thymus or E. coli. Thus only 16/35 or 41% of the DNA binding activity of this preparation appears to be highly specific for the lac operator sequence.

Estimation of relative binding constants. An equilibrium competition assay allows comparison of the stability of a lac repressor-DNA complex with a similar interaction between repressor and a standard ligand. Analysis of the partition equilibrium is simple as long as binding to the competing standard ligand is weak compared to binding to the DNA site of interest, thus avoiding the problems of neighbor exclusion and saturation of the competing ligand. We chose poly d(A-T) as the competing ligand, because its interaction with repressor has been extensively characterized

(15-17), and because it binds lac repressor strongly enough to compete effectively with operator DNA. Poly d(A-T) is not, however, a good choice as a competing ligand for weaker DNA sites, since its high affinity leads to saturation under competition conditions, as shown below.

Figure 5 illustrates a poly d(A-T) competition titration. Complexes formed at r_{added} = 3.5 (repressors per operator fragment) were equilibrated with increasing concentrations of poly d(A-T), establishing the equilibrium

$$OR_n + D \rightleftharpoons OR_{n-1} + RD$$

in which OR_n and OR_{n-1} are the n^{th} and $n-1^{th}$ repressor-operator complexes, and D is poly d(A-T). At the midpoint of a given titration step, $[OR_n] = [OR_{n-1}]$, and it may be shown that

$$\frac{K'_n}{K_D} = \frac{[D]}{[DR]}$$

where K'_n and K_D are the apparent equilibrium binding constants for the species in question. (See Appendix for a more precise definition of K'_n.) The concentration [DR] can be readily determined from the input repressor concentration, less the repressor bound to operator DNA at the transition

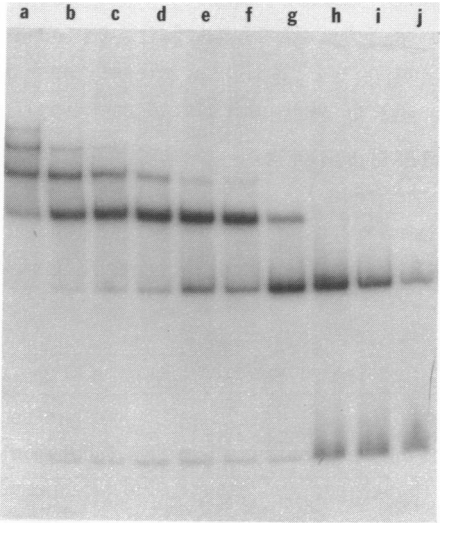

Figure 5: Poly d(A-T) titration of a repressor-203 bp operator complexes. Repressor and operator were combined at r = 3.5 as described. The solution was subdivided, and aliquots of poly d(A-T) stock added to give the indicated ratios (bp) poly d(A-T)/operator (lanes a-j respectively): 0, 2.63, 5.28, 10.5, 21.1, 105.5, 422, 843, 1688.

midpoint. Specifically,

$$[DR] = [O_o]\left[\frac{\sum_{n=o}^{n} I_n(o)}{\sum_{n=o} I_n(o)} - \frac{\sum_{o}^{n} nI_n}{\sum_{n=o} I_n}\right]$$

where $[O_o]$ is the input molar concentration of operator DNA and I_n is the autoradiographic film intensity of the n^{th} complex band. $I_n(o)$ is the corresponding band intensity in the absence of poly d(A-T). Use of the normalizing factors $\sum_{n=o} I_n$ and $\sum_{n=o} I_n(o)$ compensates for variable gel loading volumes.

The free site concentration [D] can be determined without complications only when the competing ligand D is in large excess so that [D] can be replaced by its input basepair concentration $[D_o]$. A first order correction for saturation of D can be made by estimating the number of base pairs m_D occupied on D by R, and setting $[D] = [D_o] - m_D [DR]$. However, because of neighbor exclusion effects, the effective binding constant K_D decreases as saturation is approached, and in the absence of an accurate full binding isotherm for D-R, we can only set an upper limit on K'_n.

Table 2 shows the results calculated from the titration shown in Figure 5. The ratio of poly d(A-T) basepairs to bound repressor is close to 7 for the n = 5 and n = 4 complexes, clearly near to saturation. The columns labeled $[D]^*$ and K'_n were calculated assuming 7.0 bp/repressor for saturation of poly d(A-T), with $K_D = 5 \times 10^7\ M^{-1}$ as determined by Lin and Riggs (14). Since the true K_D near saturation must be below this value, the calculated K'_n values represent upper limits. In the case of the n = 1 complexes, however, D is in large excess, and it makes no significant

TABLE II: Titration data for points at which $[OR_n] = [OR_{n-1}]$

n	$[D]_{input}$ M bp	[DR] M	$\frac{[D]_{input}}{[DR]}$	$[D]^*$ M	$K_{n_1}^*$ M^{-1}	$[D]^{**}$ M	$K_{n_1}^{**}$ M^{-1}
5	2.72×10^{-9}	3.59×10^{-10}	7.6	2.14×10^{-10}	$<5 \times 10^7$	0	$<5 \times 10^7$
4	8.4×10^{-9}	1.16×10^{-9}	7.2	2.80×10^{-10}	$<5 \times 10^7$	0	$<5 \times 10^7$
3	2.57×10^{-8}	1.87×10^{-9}	13.7	1.26×10^{-8}	3.3×10^8	0	$<5 \times 10^7$
2	1.02×10^{-7}	2.99×10^{-9}	34.1	8.10×10^{-8}	1.35×10^9	2.72×10^{-8}	4.55×10^8
1	4.04×10^{-6}	3.52×10^{-9}	1142.2	4.02×10^{-6}	5.70×10^{10}	3.95×10^{-6}	5.61×10^{10}

*$n_D = 7$; $[D]^* = [D]_{input} - m_D [DR]$; **$n_D = 25$; *$K_{O1}/K_{O3} = 42$; **$K_{O1}/K_{O3} = 123$;
Reaction buffer was 10 mM Tris (pH 7.4 at 21°C) - 50 mM KCl - 1 mM EDTA

difference whether one assumes m_D = 7 or 25, the latter value found in the case of the 203 bp fragment. (Compare $K_1'^*$ with $K_1'^{**}$.)

The estimated value of 5.7 x 10^{10} M^{-1} for the first binding step is about an order of magnitude smaller than that measured at comparable salt concentration by other methods for an operator site contained in high molecular weight DNA (1), and about 3 times smaller than that estimated from kinetic data for a small fragment by Goeddel et. al. (18). It is difficult to be certain that these differences are significant until direct comparisons have been made using the same technique for all fragments; however, it is likely that weaker binding is a general characteristic of short fragments containing the operator (18).

The operator-like sequence O3, located at the distal end of the i gene, is a strong candidate for the site of the second binding interaction to the 203 bp fragment (6,7). Apparently the requirement for a large excess of D is not met for poly d(A-T) titration of this complex, hence the 3-fold difference between $K_2'^*$ and $K_2'^{**}$. In spite of this uncertainty, it is clear that the second interaction is significantly stronger than those with higher values of n. Use of a competing ligand with lower repressor binding affinity than poly d(A-T) could allow the criterion of excess D to be met for titrations of repressor-operator complexes of n = 2 and above. This would permit more rigorous estimation of the relative stabilities of the higher complexes.

Interaction between two repressor binding sites. Although lac repressor has been shown to bind specifically to more than one site near the lac promoter (6,7), the regulatory role of the secondary binding sites remains unknown. To determine whether operator binding influences occupancy of the secondary site O3, and vice-versa, we compared repressor distribution between sites O1 and O3 contained on a single fragment with its distribution when the sites are on separate fragments.

As outlined in the appendix, the ratio of binding constants K_2 (site O3) and K_1 (site O1) for the repressor-203 bp fragment interaction may be approximated by the ratio of band intensities

$$\frac{I_2 I_0}{(I_1)^2} \simeq \frac{K_2}{K_1}$$

Values calculated for several r are given in table 3, in which we show $K_1/K_2 \simeq 15$. To the best of our knowledge, this represents the first direct measurement of the relative binding constant of site O3 with respect to

TABLE III: Titration of 203 L8 Fragment with lac Repressor

r	normalized band intensities band 0	1	2	$\frac{I(2)\ I(0)}{[I(1)]^2} = \frac{K_2}{K_1}$	$\frac{K_1}{K_2}$
1.69	0.70	0.30	8.3×10^{-3}	6.42×10^{-2}	15.57
2.56	0.575	0.40	1.84×10^{-2}	6.42×10^{-2}	15.55
3.41	0.487	0.476	3.24×10^{-2}	7.82×10^{-2}	14.36

Repressor-203 bp Operator complexes were formed in the presence of a 10-fold molar excess (bp) of chicken erythrocyte DNA over operator. Reaction buffer was 10 mM Tris (pH 7.4 at 21°C)-50 mM KCl-1 mM EDTA. Gels were run in TE buffer.

01, and yields a value significantly higher than previously estimated (6).

A titration of an equimolar mixture of the 121 bp (03) fragment and the 82 bp (01) fragment is shown in Figure 6a. The ratio [82] bound/ [121] bound is plotted as a function of repressor added in Figure 6b. As described in the appendix,

$$\lim_{r \to o} \frac{I_1(82)}{I_1(121)} = \frac{K_1}{K_2}$$

Extrapolation to r = 0 yields $K_1/K_2 = 18.2$.

Pfahl et. al. (19) have shown that deletion of the secondary sites does not alter the filter binding of λ phage DNA containing the lac operator. Their findings imply no interaction between the sites, as is confirmed directly by our results. Our observation that 03 binding is unperturbed by occupancy of 01 indicates that the reactions are not coupled, and limits the potential role of 03 in regulation of the promoter.

Technique Sensitivity. Detection of ^{32}P by autoradiography is the process determining the lower limit of sensitivity of this technique. In order to establish the lower limits of detection, several rapid 10-fold dilutions into TE buffer were performed on a repressor-operator complex containing 3.7×10^{-13} moles of 203 bp operator DNA (40,000 cpm), $r_{added} = 5.7$. Film was exposed for 21 days, approximately 1.5 half lives of the ^{32}P label. The highest dilution yielding detectable film darkening corresponded to a factor of 10^{-5}, placing the lower limit of detection at 10^{-18} moles. Thus, in a typical sample volume of 20 µl, the lowest detectable DNA concentration is of the order of 10^{-12} M. Complex band n=1 could still be observed at this dilution, in about the same ratio to free DNA as at

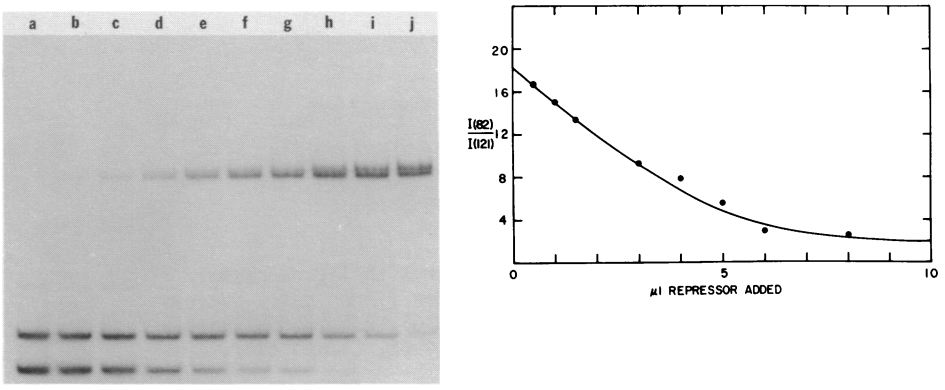

Figure 6: A: Titration of an equimolar mixture of 82 bp (01) and 121 bp (03) fragments with lac repressor. Binding buffer was 10 mM Tris (pH 7.4 at 21°C) - 1 mM EDTA - 1.3 μM (bp) sonicated chicken erythrocyte DNA. Each sample contained 1.96 x 10^{-13} moles of each fragment, to which was added (lanes a-j respectively) 0, 0.5, 1, 2, 3, 4, 5, 6, 8, and 10 μl of lac repressor stock (0.233 mg/ml, 4.95 μM).
B: Dependence of the ratio of normalized band intensities on added repressor. Data of A, above.

higher total concentration. Complex band n=2 was barely visible. Highly labelled DNA, produced for example by nick translation, could, of course, be detected at even lower concentrations.

Gel dead time. The time required for electrophoresis of free DNA into the gel imposes a lower limit on the time resolution of studies utilizing this method. We evaluated this limit by determining the time required for a sample of 203 bp operator DNA to migrate from the loading well completely into the gel under the influence of a typical, 10 v/cm voltage gradient. The process appeared complete in 80 sec, with a characteristic $t_{\frac{1}{2}}$ of 17 ± 2 sec.

Does lac repressor tryptic core bind DNA? The location and role of DNA binding sites in lac repressor is a subject under investigation in several laboratories. In particular, the question of whether the DNA binding site is contained within the amino-terminal 59 residues, or whether other regions of the protein are involved, has stimulated debate (20-22). Our attempt to demonstrate DNA binding activity in lac repressor core is shown in figure 7. Parallel titrations of the 203 bp operator were carried out with lac repressor and tryptic core. No DNA binding by tryptic core was

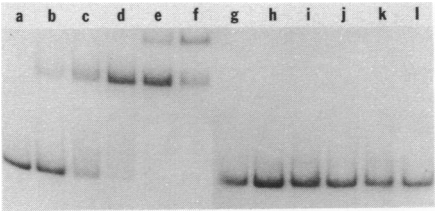

Figure 7: Titration of the 203 bp operator with lac repressor (lanes a-f) and with tryptic core (lanes g-l). Values of r_{added} are: 0, 0.59, 1.06, 1.67, 2.5, 3.85 (lanes a-f); and 0, 0.47, 1.05, 1.81, 2.87, 4.65, (lanes g-l), respectively.

detected.

Under the conditons of this experiment, we can detect bands containing as little as 0.05% of the total DNA. At r = 1 both core and operator are present at 10^{-7} M concentrations. Thus if

$$\frac{[CD]}{[C]\,[D]} = K_{core} \text{ and } \frac{[CD]}{[D]} < 5 \times 10^{-4}$$

where [C] is the tryptic core concentration, then $K_{core} < 5 \times 10^{3}\ M^{-1}$. Dissociation of such weakly bound complexes during electrophoresis may reduce the sensitivity of the gel method. Thus we believe that the probable binding constant is no greater than $10^{4} - 10^{5}\ M^{-1}$.

Kinetics and activation energy of dissociation. The dissociation rate of the n=1 complex was measured at six temperatures from 0° to 37°C, in the presence of a 135-fold excess of unlabeled 203 bp operator. Identical rates were obtained with 60, 135, and 270-fold excesses of competing operator DNA (data not shown), indicating that pseudo first-order kinetics obtained. The solution half life of the n=2 and higher complexes proved too short to measure, although in the gel they are sufficiently stable to be resolved as sharp bands. A typical experiment is shown in figure 8 frames a and b. Dissociation rate constants (k_d) were determined by linear least squares analysis of ln (fraction bound) vs time. At 21°C K_d was 0.144 ± .008 min^{-1}, corresponding to a half life of 5 minutes. This half life is only one-fourth that reported by Riggs et. al. (23) for dissociation of the wild type repressor-λφ 80 d lac complex, under similar buffer conditions. Significantly, even shorter half lives of 37 seconds and 46 seconds have been reported for 21 bp and 26 bp operator fragments (24). Thus, it is probable that the length of the operator-containing DNA

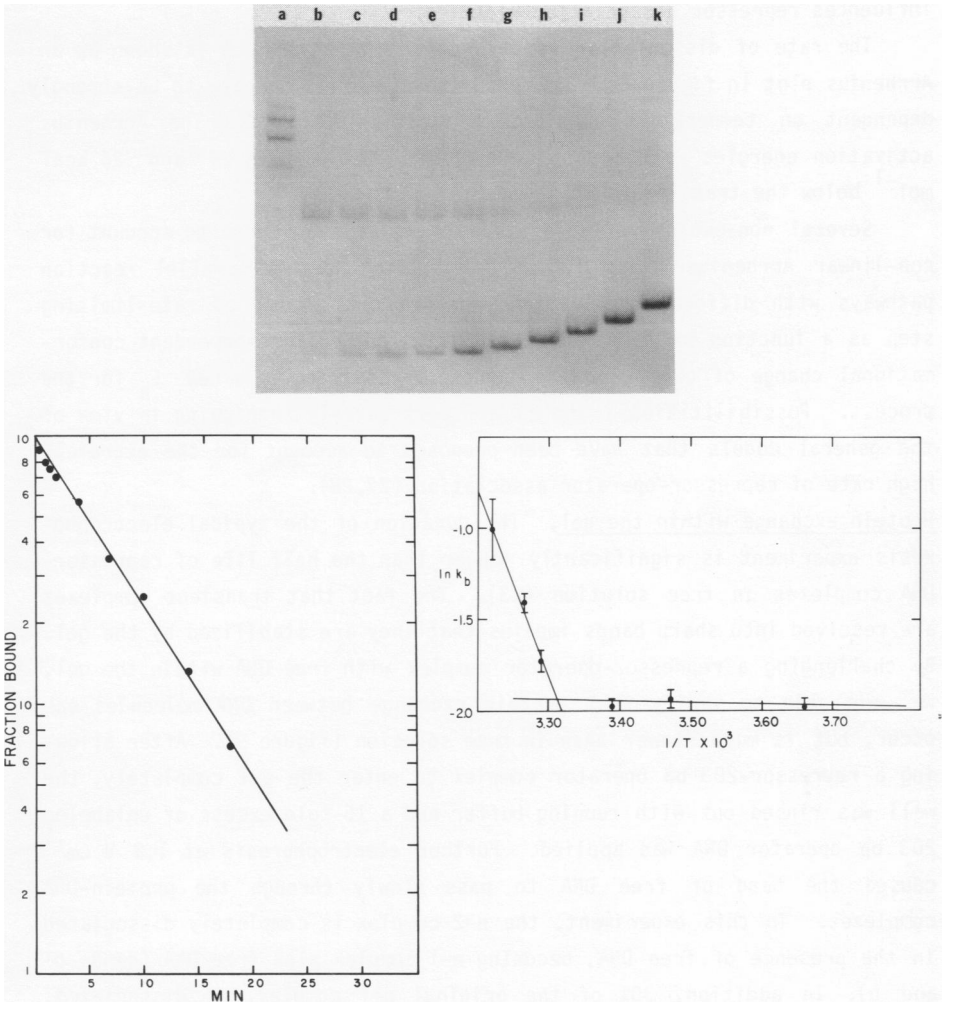

Figure 8: A: Measurement of the dissociation kinetics of the lac repressor 203 bp operator complex at 21°C. Length of reaction with competing DNA is: 0, 0.5, 1, 1.5, 2, 4, 7, 10, 14, 18, 22 min for lanes a-k repectively.
B: Analysis of the kinetics data from figure 8A. Linear least squares analysis gives k_d = 0.144 min^{-1} at 21°C.
C: Arrhenius plot for dissociation of the lac repressor - 203 bp operator n = 1 complex.

influences repressor dissociation kinetics.

The rate of dissociation as a function of temperature is shown as an Arrhenius plot in figure 8c. We found the dissociation rate to be strongly dependent on temperature, with an inflection at 29°C. The Arrhenius activation energies were found to be 80 kcal mol^{-1} above 29° and 26 kcal mol^{-1} below the transition.

Several non-exclusive explanations have been advanced to account for non-linear Arrhenius plots (25,26), including i) two parallel reaction pathways with different activation energies, ii) change of rate-limiting step as a function of temperature, and iii) temperature-dependent conformational change of one or more components, resulting in a new E_a for the process. Possibilitities i and ii are particularly intriguing in view of the general models that have been proposed to account for the extremely high rate of repressor-operator association (27,28).

Protein exchange within the gel. The duration of the typical electrophoresis experiment is significantly longer than the half life of repressor-DNA complexes in free solution (23). The fact that transient complexes are resolved into sharp bands implies that they are stabilized by the gel. By challenging a repressor-operator complex with free DNA within the gel, we were able to verify that protein exchange between DNA molecules can occur, but is much slower than in free solution (figure 9). After allowing a repressor-203 bp operator complex to enter the gel completely, the well was rinsed out with running buffer and a 15-fold excess of unlabeled 203 bp operator DNA was applied. Further electrophoresis at 1.3 V cm^{-1} caused the band of free DNA to pass slowly through the protein-DNA complexes. In this experiment, the n=2 complex is completely dissociated in the presence of free DNA, becoming n=1 complex plus free DNA (bands c' and b). In addition, 30% of the original n=1 complex has dissociated. Based on relative mobilities, and band width at half height, we estimate an interaction time of 90 ± 5 minutes, during which protein exchange with free DNA could take place. Assuming psuedo first-order dissociation kinetics, k_d (gel) for O1 is approximately 4×10^{-3} min^{-1}, roughly 40-fold slower than the rate in free solution.

DISCUSSION

Detection of kinetically labile complexes. The dissociation kinetics experiment shown in figure 8a reveals that the complexes with $n \geq 2$ have lifetimes shorter than 1 min. Since the time required to run the gel is

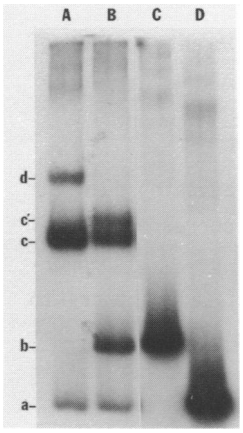

Figure 9: Protein exchange within the gel.
A: repressor-203 bp operator complex formed at r = 3.3.
B: Same complex following exchange in the gel with a 15-fold excess of unlabeled 203 bp operator, added after the complex had entered the gel.
C: free ^{32}P-labeled operator DNA loaded simultaneously with the competing DNA in lane B.
D: free ^{32}P-labeled operator DNA loaded simultaneously with complexes of lane A
Band assignments: a, free operator DNA present in initial mixture; b, free operator formed by protein exchange; c, the n = 1 complex; c' (lane B), n = 1 complex formed by loss of repressor from the n = 2 complex; d, the n = 2 complex.

several hours, it is clear that kinetic stability is not a requirement for detection of a complex. The gel method is probably more sensitive to the existence of such complexes than the filter binding assay, because of the tendency for kinetically labile complexes to dissociate during washing of the filter. Repressor-operator complexes formed in the presence of IPTG, for example, can be detected by the gel method (data not shown), but not by filter binding.

Stabilization of the complex in the gel. At least two factors act to stabilize protein-DNA complexes in the gel. A simple influence is the low salt buffer used in the gel, which serves to increase the binding affinity. As long as the free protein concentration in a gel band is small compared to the complex concentration (eg 1/100), there will be little loss of protein as the band moves. In addition, the volume excluded by the gel is inaccessible to reaction components, thus increasing local concentrations of reactants, favoring association.

There appears to be an additional effect reflected in the slow exchange rate revealed in figure 9, which may result from a decrease of the complex dissociation rate due to properties of the gel. It is possible that the equilibrium binding constant is increased by interactions with the gel. The detection of weak DNA-protein complexes in the serial dilution experiment at component input concentrations significantly below the equilibrium binding constant (figure 9) supports this idea. Another possible explanation is that the gel provides a "cage" so that dissociated components generally cannot diffuse away, and hence must recombine with the same

partners.

The cage effect hypothesis is supported by the apparent failure of the disproportionation reaction

$$2\ OR_n \rightleftharpoons OR_{n+1} + OR_{n-1}$$

to take place in the gel. If this reaction occured with anything near the rate of dissociation of n = 2 complexes in solution, the corresponding gel bands should rapidly broaden and produce a smear on the gel. Disproportionation would be prevented if the cage forced dissociated partners to recombine monogamously. The result shown in figure 9 demonstrates that transfer of repressor from one DNA molecule to another can occur within the gel, but the rate is slow.

The electrophoretic technique described here is simple and highly reproducible. The results obtained complement and extend those of the conventional filter binding (1) and sedimentation velocity (13) methods. Thus, the detailed equilibrium distribution for one or more proteins interacting with one or more DNA sites may be studied directly by gel electrophoresis.

ABBREVIATIONS

CAP: the cyclic AMP receptor protein of *E. coli*.
r_{added}: input molar ratio *lac* repressor tetramer: DNA fragment.
n: number of repressor tetramers bound to a DNA fragment.
BSA: bovine serum albumin

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APPENDIX

Thermodynamic analysis of band probabilities

Polyacrylamide gel analysis reveals the occupancy of protein binding sites on a DNA fragment as a number distribution: within the resolution of our present gels, all DNA molecules having the same number of bound proteins appear to have the same mobility. In this appendix we present a thermodynamic analysis which relates the fractional occupancy or probability P_n of each band to the binding constants K_i of individual sites. For simplicity, we ignore the problems of neighbor exclusion (29) and site overlap (30), a valid approach when only a few sites are occupied. In a later paper we will present the formalism for calculating the binding distribution (P_n) when both variable binding constants and neighbor exclusion effects are taken into account (to be published).

Following standard procedures (31) we set the probability of a given state j of the DNA fragment proportional to exp ($-\Delta G_j/RT$), where ΔG_j is the free energy of forming state j from the reference state, here taken to

be the DNA molecule with no proteins bound. The term $\exp(-\Delta G_j/RT)$ for binding a protein at free concentration [R] to a site with binding constant K_i is $K_i[R]$ (29). All states j of the system are represented in the partition function Q,

$$Q = \sum_j \exp(-\Delta G_j/RT) \quad (1)$$

This formalism is easier to follow with a specific example. Let the 203 bp DNA fragment have two binding sites (O1 and O3), with binding constants K_1 and K_2. Then

$$Q = 1 + K_1[R] + K_2[R] + K_1 K_2 [R]^2 \quad (2)$$

In this equation, the term 1 corresponds to the naked DNA molecule (ΔG_j=0, exp (-0) = 1), K_1 [R] represents the state with only site 1 occupied, K_2 [R] the site with only site 2 occupied, and $K_1 K_2 [R]^2$ the state with both sites occupied.

The probability of band n is equal to $\exp(-\Delta G_j/RT)/Q$, by the Boltzmann formula, where the sum is over all terms with the same number of proteins bound. For our specific example,

$$P_0 = 1/Q \quad (3)$$
$$P_1 = (K_1[R] + K_2[R])/Q$$
$$P_2 = K_1 K_2 [R]^2/Q$$

Equations (3) are of little use for PDA gels as they stand since the K_i are unknown, as is the free protein concentration [R] in the equilibrium mixture. (Since the conditions used are those of strong binding, [R] is much smaller than the input protein concentration R_0.) However, the ratios of band probabilities can be used to determine relative site affinities.

Equations (3) can be combined to yield

$$\frac{P_2 P_0}{P_1^2} = \frac{K_1 K_2}{K_1^2 (1 + K_2/K_1)^2} \quad (4)$$

when $K_2 << K_1$, equation (4) simplifies to

$$\frac{P_2 P_0}{P_1^2} = \frac{K_2}{K_1} \quad (5)$$

from which one can readily estimate the relative binding constants of the two sites. When $K_2 < K_1$, as is the case for the 203 bp fragment, $P(2)P(0)/[P(1)]^2$ approximates K_2/K_1 with small error (from 17% for K_2/K_1 = 0.1 to 2% for K_2/K_1 = 0.01).

Another useful application of this technique is to assay competition between two sites which are located on separate fragments. Consider as an example the mixture of 121 and 82 bp fragments, the former containing 03 (K_2) and the latter 01 (K_1). The partition function for the system of 2 fragments (present in equal amounts) is

$$Q = (1+K_1[R])\ (1+K_2[R]) = 1+K_1[R] + K_2[R] + K_1K_2[R]^2 \qquad (6)$$

The probability of band 0 for fragment 82 reflects the weighting factors for all states in which no protein is bound to fragment 82 (either with both sites free, or with the site on fragment 121 occupied):

$$P_0\ (82) = (\ 1 + K_2\ [R]\ )/Q$$

Similarly

$$P_1\ (82) = (K_1\ [R] + K_1\ K_2\ [R]^2)/Q \qquad (7)$$

$$P_0\ (121) = (\ 1 + K_1\ [R])/Q$$

$$P_1\ (121) = (K_2\ [R] + K_1\ K_2\ [R]^2)/Q$$

The ratio of K_1 to K_2 can be obtained by combining Eqs. (7):

$$\frac{P_1\ (82)}{P_1\ (121)} = \frac{K_1(1 + K_2[R])}{K_2(1 + K_1\ [R])} \qquad (8)$$

When [R] is small, at the beginning of the titration, Eq. (8) approaches the limit

$$\lim_{[R] \to 0} \frac{P_1\ (82)}{P_1\ (121)} = \frac{K_1\ (82)}{K_2\ (121)} \qquad (9)$$

A similar approach can be used to solve more complicated distribution problems.