DNA-Stimulated Assembly of Oligomeric Bacteriophage 434 Repressor: Evidence for Cooperative Binding by Recruitment†

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ABSTRACT: Typical of many transcriptional regulatory proteins, the lambdoid bacteriophage repressors bind cooperatively to multiple sites on DNA. This cooperative binding is essential for establishment and maintenance of phage lysogeny. In the phage, two repressor homodimers, one bound at each of the adjacent operator sites, interact to form the tetramer that is necessary for the cooperative binding of the repressor. Bacteriophage 434 repressor does not form tetramers in the absence of DNA, and the mechanism by which the tetramer assembles on the two adjacent sites is unknown. Hence DNA binding may stimulate the repressor to form tetramers and formation of a repressor oligomer (≥ 3 monomers) on a single DNA sites may precede multisite binding. Consistent with these ideas, a complex containing three repressor molecules readily assembles on a single operator (O_R1) site. Mutations that inhibit cooperative tetramer binding to the adjacent O_R1 and O_R2 sites also block formation of this complex. Together with other evidence, these findings show that the complex that forms on a single site assembles using the same interface as does the tetramer assembled on adjacent operator sites. Adding additional O_R1 DNA dissociates the oligomeric repressor−DNA complexes into dimeric repressor−O_R1 complexes. In contrast, adding O_R2 to these complexes results in the formation of a repressor oligomer containing an O_R2 and an O_R1 site. The observation that a repressor oligomer bound to two O_R1 sites is less stable than the one formed between repressor dimers bound to O_R1 and O_R2 implies that DNA allosterically influences the structure of the 434 repressor. Together these findings suggest that an O_R1-bound repressor may cooperatively help repressor bind to O_R2 by recruiting an additional repressor molecule from solution that subsequently occupies O_R2.

The cooperative binding of regulatory proteins to nucleic acids is a feature of the genetic regulatory circuitry of virtually all organisms. Protein−DNA complexes assembled via cooperative binding interactions are nucleated by the strong interaction of one or a few proteins with the DNA. Subsequent binding of additional proteins, which alone have lower affinity for the DNA, is then stabilized by protein−protein contacts with the tightly bound protein.

Despite the demonstrated importance of cooperative binding of proteins to nucleic acids, the mechanism by which cooperative complexes assemble is unclear. On the basis of studies of several systems, two alternative models have been proposed. In one model, the presence of high affinity binding sites near those that have low protein affinity is proposed to increase the local concentration of the binding protein. This increased local concentration is thought to enhance the binding of the protein to the low affinity sites (*1*). An alternative model postulates that unique protein−protein complexes, which are nucleated by the tightly bound protein and not normally present in solution, form specifically and only on DNA. Such a protein assemblage is particularly structured to facilitate occupancy of the weak site. This model of cooperative interactions is often referred to as recruitment. Although many transcriptional activators have been postulated to stimulate transcription by recruiting elements of the basal transcription machinery to the promoter (for review see ref *2*), direct demonstration of recruitment is lacking.

The lysis−lysogeny decision of bacteriophage 434 critically depends on the differential affinities of 434 repressor for three sites in the O_R region, the so-called O_R1, O_R2, and O_R3 sites of the phage chromosome. Cooperative binding of two repressor dimers to the adjacent O_R1 and O_R2 sites is required for the repressor to establish and maintain bacteriophage lysogeny. On independent sites, 434 repressor binds with lowest affinity to O_R2, 2-fold more tightly to O_R3 and with yet a 6-fold higher affinity to O_R1. By contrast, in intact O_R, repressor binds O_R1 and O_R2 with an almost equal affinity, and subsequently binds O_R3 with 8-fold lower affinity. Thus, in intact O_R, 434 repressor dimers do not bind independently to the three sites in intact O_R; rather they cooperatively bind to the adjacent O_R1 and O_R2 sites, while binding O_R3 independently. Repressor occupancy of O_R2 is crucial to the phage's decision between lytic and lysogenic growth. The observation that a bacteriophage encoding a repressor that is unable to form DNA bound tetramers (*3*) cannot lysogenize *Escherichia coli* (*4*) emphasizes the importance of this cooperative binding (*5*, *6*).

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Similar to the repressors of other temperate lambdoid phages, the 434 repressor can be functionally and structurally divided into two domains (*7*−*9*). The smaller amino (N-) terminal domain contains a helix−turn−helix structural motif that is responsible for mediating all specific and nonspecific contacts between the protein and DNA (*10*−*14*). In the 434 repressor, the carboxyl (C-) terminal domain encompasses approximately the last 115 amino acids of the repressor. This domain stabilizes formation of repressor dimers. It also mediates formation of repressor tetramers (*15*−*17*) and is therefore required for the cooperative binding of two repressor dimers to adjacent DNA sites. The two domains are joined by a "linker" of ~40 amino acids in length. The three-dimensional structure of the C-terminal domain of the repressor of bacteriophage λ has recently been determined (*18*), but no structure of any of the intact repressors has been determined either in the absence or presence of DNA. Consequently, although we know that dimer and tetramer formation by both λ and 434 repressors are accompanied by marked changes in protein conformation (*19*, *20*), structural insights into interactions between the N-terminal and C-terminal domains and the linker between them, either in the dimeric or DNA-bound tetrameric repressor, remain unknown. Nonetheless, extensive genetic studies of Hochschild and co-workers (*16*, *21*) identified amino acids that direct the cooperative specificity of λ repressor. We showed that changing one of the amino acids in the homologous position in 434 repressor decreased the stability of the cooperative tetramer (see below). Thus, 434 and λ repressor appear to use homologous regions of the protein structure to form tetramers.

Tetramers formed by the repressors of bacteriophages 434 and λ are both capable of cooperatively binding to adjacent sites on DNA. Despite their similarity in structure and function, λ repressor, but not 434 repressor, forms a tetramer at high concentrations in the absence of DNA. These tetramers form in a concentration range that closely approaches that needed for site occupancy of O_R2 alone (*22*, *23*), suggesting that λ repressor may bind DNA as a preformed tetramer. However, these concentration-induced forms do not appear to have the conformation appropriate for specific DNA binding, because added DNA destabilizes these concentration-induced forms (*24*).

Despite its ability to bind cooperatively to O_R1 and O_R2 in the nanomolar concentration range, intact 434 repressor does not form tetramers in the absence of DNA, even at millimolar concentrations (G. Koudelka, A. Donner, unpublished results). Thus, 434 repressor likely does not bind DNA as a preformed tetramer. How then does repressor bind cooperatively to adjacent sites on DNA? Two alternative pathways have been proposed to explain how O_R1-bound repressor "helps" repressor bind at O_R2 (*22*, *23*, *25*, *26*). First, in a model that is a variant of the local concentration model, repressor bound at O_R1 may stabilize a repressor dimer that transiently interacts with the adjacent O_R2 site. This view requires that the repressor bind to O_R2 prior to associating with the O_R1-bound repressor, and thus formation of the repressor tetramer requires the presence of an adjacent repressor binding site. Alternatively, O_R1-bound repressor may recruit free repressor to the vicinity of O_R2 thus providing an additional interaction site for the incoming repressor. In this model, the formation of repressor tetramers precedes interaction of the repressor with O_R2, and hence tetramer formation does not require the presence of an adjacent repressor binding site.

To distinguish between these two alternatives and to provide insight into the role that cooperativity plays in the maintenance and establishment of lysogeny, we addressed two major questions. First, can a repressor tetramer form on a single DNA binding site? The answer to this question establishes the potential order of protein−protein and protein−DNA contacts events at O_R. Second, how is the specificity for cooperative binding of repressor O_R1 and O_R2 generated? This second question addresses how 434 repressor binds cooperatively to the three binding sites in an operator region in an alternate pairwise fashion, i.e., to O_R1 and O_R2 or to O_R2 and O_R3, but not to O_R1 and O_R3.

EXPERIMENTAL PROCEDURES

Media and Cell Growth Conditions. Liquid cell cultures were grown in Luria broth (*27*) at 37 °C. Both Luria broth and Luria agar were supplemented with ampicillin at 100 μg/mL.

Bacterial Strains. *E. coli* XA90 (*28*) was used for protein purification. Plasmids were prepared from JM101.

Plasmids. Construction of plasmids containing single naturally occurring 434 operators (O_R1, O_R2, and O_R3) (*29*) and intact O_R (*30*) have been described previously. For construction of the pGEM434D173G plasmid encoding the D173G mutant repressor, a two-step PCR approach was followed as described (*31*). In the first step, two amplification reactions were performed using pGEM434 repressor (*32*), which encodes the wild-type 434 repressor, as a template. In one reaction, the forward universal M13 primer 17-mer 5′-GTAAAACGACGGCCAGT-3′ and a primer annealing inside the 434 repressor coding sequence (sequence corresponding to amino acids 94−102) 5′-CGAACACTTGCCTACGATATCAAGGAC-3′ were used for amplification. In the second reaction, the reverse universal M13 primer 17-mer 5′-AAACAGCTATGACCATG-3′ and the mutagenic 19-mer primer D173G 5′-GACCGCCTCCTATGACCAG-3′ were used. The amplification products from both reactions were individually gel isolated. After purification of the sample by phenol extraction and ethanol precipitation, 50 ng of each product was combined and used as template for another amplification reaction in the presence of 1 μg each of the forward and reverse universal M13 primers. After gel purification, the product of this second PCR reaction was digested with *Xba* I and *Bam* HI *(*New England Biolabs) and ligated into a pGEM434 vector cut with the same enzymes. After transformation into XA90, plasmid DNA was extracted and sequenced to confirm the presence of the mutation.

Purification of Wild-Type and Mutant 434 Repressor Proteins. Intact 434 repressor proteins were purified from *E. coli* XA90 cells bearing the desired expression vector as described elsewhere (*33*). The C-terminal domains were isolated from the same strain as described in refs *15* and *32*.

Fluorescence Polarization Spectroscopy. The fluorescence data were obtained using a SLM8100 L-format spectrofluorometer equipped with a 750 W Xenon arc lamp and UV transmitting Glan-Thompson polarizers. G- factor corrections

were considered in all calculated anisotropy values. For these experiments, 10 individual readings were taken at each of the four orientations of the UV polarizers. Three types of fluorescence polarization experiments were performed. In the first type of experiment, the anisotropy of the intrinsic tryptophan fluorescence of repressor was measured (r) as the concentration of repressor was increased in solution. The excitation wavelength was set 295 nm and the polarized emission intensities were recorded at 350 nm. To derive from the isothermal polarization Perrin plot the limiting anisotropy r_o, we measured the tryptophan anisotropy of 750 nM repressor at 5, 15, 25, and 37 °C, in the absence or presence of 5, 10, and 20% glycerol. The inverse of the measured anisotropy ($1/r$) was plotted versus T/η (the ratio between temperature and viscosity) in an isothermal polarization Perrin plot, and the data were fitted by linear regression. According to modified the Perrin equation (*34*, *35*), the value of the intercept with the ordinate gives the value of the inverse of apparent limiting anisotropy ($1/r_0^{app}$). These measurements yielded an apparent limiting anisotropy $r_0^{app} = 0.154117$. We substituted this value into the Perrin equation written as a function of rotational correlation time θ:

$$r = \frac{r_0}{1 + (\tau/\theta)} \quad (1)$$

Using this r_0^{app}, together with the lifetime (τ) of repressor's tryptophan fluorescence (3.3 ns) (*34*), we can estimate the size of the concentration-induced species.

In the other two experiments, we recorded the anisotropy of a double-stranded 20-base pair oligonucleotide encoding the O_R1 sequence (15 nM), labeled at its 5′ end with fluorescein (*19*) as a function of repressor concentration and/or added unlabeled DNA. For these measurements, the excitation wavelength was set at 492 nm and the emission intensities recorded at 520 nm. To examine the effect of repressor concentration on protein−DNA complex size, increasing concentrations of wild-type or mutant 434 repressor proteins were incubated in binding buffer (10 mM Tris HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA) in a 750 μL, 1 cm path length, quartz cuvette with 15 nM fluorescein-labeled DNA. Readings were taken after a 5 min incubation at 25 °C at each protein concentration step. To examine the effect of DNA concentration and sequence on the stability of repressor−DNA complexes, different fixed amounts of fluorescein-labeled O_R1−repressor complexes were formed and subsequently increasing amounts of 20-base pair double-stranded unlabeled oligonucleotides encoding either the O_R1 or the O_R2 sequence were added. The sequences of the DNA oligonucleotides used for fluorescence polarization were the following:

O_R1 top 20-mer 5′ TATACAAGAAAGTTTGTACT 3′

O_R1 bottom 20-mer 5′ AAGTACAAACTTTCTTGTAT 3′

O_R2 top 20-mer 5′ TATACAAGATACATTGTATG 3′

O_R2 bottom 20-mer 5′ CATACAATGTATCTTGTATA 3′

Analysis of Fluorescence Anisotropy Data. All data analysis was performed using Origin 7.0 (OriginLab Corporation) or Maple 5.0 software (Waterloo Maple, Inc). Equations and strategies used for simulations and fitting of the anisotropy data are given in Results.

Gel Mobility Shift Assays. These experiments were performed essentially as described in ref *19*. Plasmids bearing either O_R1 or intact O_R operator were digested with *Eco* RI and *Hind* III restriction endonucleases, and the operator-containing DNA fragments were isolated from agarose gels. The DNA fragments were radioactively labeled at their 3′ ends by incubating the DNA with α-[^{32}P]-dATP (3000 Ci/mmol) (Perkin-Elmer, Boston, MA) in the presence of Klenow fragment of DNA polymerase I (Epicentre, Inc. Madison, WI). The labeled DNA was mixed with specified concentrations of repressor protein without or with unlabeled competing oligonucleotide in binding buffer plus 5% glycerol for 10 min at 37 °C. The protein−DNA complexes were resolved on 5% polyacrylamide gels at room temperature. The electrophoresis buffer was 0.5× TBE (45 mM Tris, pH 8.9; 45 mM borate; 0.5 mM EDTA). The dried gels were analyzed using a Phosphor Imager (Molecular Dynamics).

DNase I Footprinting. DNase I protection assays were performed as described (*36*). The DNA probe used in these assays was obtained by digestion of pJX (*30*), a plasmid that contains the intact 434 O_R region, with *Hind* III and *Pvu* II. After gel isolation, the 160 bp fragment was labeled at its 3′ end as described above. The labeled fragment was incubated with increasing amounts of either wild-type 434 repressor or D173G mutant protein for 15 min at 25 °C prior to addition of sufficient DNase I to generate, on average, one cleavage per DNA molecule in 5 min of additional incubation. The cleavage reactions were terminated by precipitation with ethanol, and the DNA was dissolved in 90% formamide solution containing tracking dyes. The products of the reaction were resolved on 7.5% acrylamide gels containing: 8 M urea, 89 mM Tris-HCl pH 8.9, 89 mM borate, 1 mM EDTA. The cleavage fragments were visualized using a Phosphor Imager (Molecular Dynamics). Affinities of the various repressors for the binding sites in O_R were determined from quantitative analysis of the phosphorimage using ImageQuant (Molecular Dynamics).

N-Ethyl Maleimide (NEM) Modification of Repressor. A stock solution containing 450 μM 434 repressor was mixed with a solution of 5 mg/mL NEM in 0.1 M $NaPO_4$ pH 7.0 in 1:4 volume ratio and incubated overnight at 4 °C. Following the reaction, the sample was exhaustively dialyzed at the same temperature against 10 mM Tris-HCl, pH 7.5, 50 mM NaCl for 24 h to remove unreacted NEM. The extent of NEM modification of accessible sulfhydryl groups was nearly complete as determined by comparing the reaction of modified and unmodified repressor with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB).

RESULTS

Added DNA Stimulates Repressor Oligomer Formation. As a first step in examining repressor oligomer formation, we monitored the concentration dependence of 434 repressor tryptophan anisotropy in the absence of DNA. As the repressor concentration is increased from 0.1 to 15 μM, the measured anisotropy increases and plateaus at ~10 μM repressor (Figure 1). No additional increases in anisotropy are observed at higher concentrations.

The dependence of the repressor anisotropy as a function of repressor concentration was fitted to eq 2:

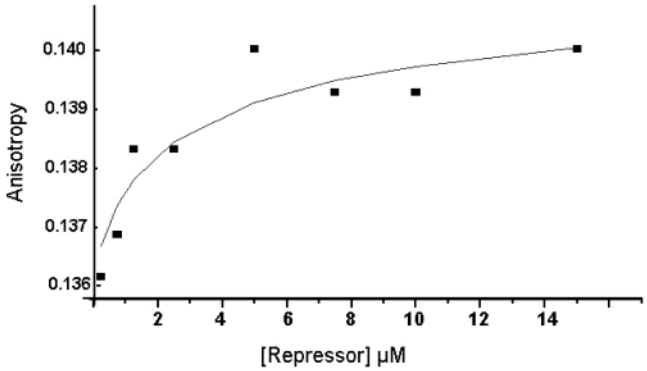

FIGURE 1: Concentration-dependent oligomerization of 434 repressor. The increase in repressor anisotropy is plotted as a function of 434 repressor concentration. The anisotropy levels were determined by monitoring the intrinsic tryptophan fluorescence of repressor with excitation at 295 nm and emission intensity detected at 350 nm. The line represents the results of fitting these data to an equation describing the relationship $2R \rightleftarrows R_2$ (eq 2) with a $K_{Dim} = 2.15 \pm 0.6$ μM (see text).

$$r_{obs} = r_i + (r_{sat} - r_i)\left\{\frac{K_{Dim} + 4[R]_{tot} - \sqrt{K_{Dim}{}^2 + 8K_{Dim}[R]_{tot}}}{8[R]_{tot}}\right\} \quad (2)$$

where r_{obs} is the anisotropy obtained from measurements, r_i is the initial anisotropy obtained at the lowest concentration of repressor, r_{sat} is the anisotropy at the plateau, $[R]_{tot}$ is the total concentration of repressor present in solution at each data point expressed as molar concentration of monomeric species, K_{Dim} is the dimerization dissociation constant.

This equation describes the equilibrium:

$$2R \rightleftarrows R_2$$

The results of this fitting are shown as the line in Figure 1 with fit values $Chi^2 = 2.1132 \times 10^{-7}$, and $R^2 = 0.8684$. Attempts to fit the data either to formation of trimeric or tetrameric repressor species yielded fits that were statistically insignificant, arguing against these models (data not shown). Several lines support the conclusion that the overall change in anisotropy value observed in Figure 1 results from concentration-dependent repressor dimerization. First, as discussed above, using the limiting anisotropy of repressor determined in an isothermal polarization experiment (see Experimental Procedures) (*37*), we calculated the rotational correlation time (θ) of the species formed at $\geq$ 10 μM repressor concentration as being 30.35 ns using eq 1. This value of θ is similar to those of other globular-shaped proteins that have molecular weights between 42 and 47 kDa (*38*). Since the molecular weight of the repressor dimer is ~45 kDa, this finding suggests that the oligomeric state of repressor at $\geq$ 10 μM is a dimer. Second, the K_{Dim} obtained from the fitting the data to eq 2 was 2.15 ± 0.6 μM, a value that is in excellent agreement with that determined using size exclusion chromatography (*39*). Third, cross-linking showed that repressor forms a dimer in this concentration range, without the formation of higher order repressor species (*15*, *32*, *39*). It is important to note that no further increase in anisotropy is seen if the protein concentration is increased to 15 μM (Figure 1). Thus, in the concentration range of up to 15 μM, 434 repressor does not appear to form oligomers with higher order than a dimer in the absence of DNA. These observations are both qualitatively and quantitatively consistent with our previous studies of 434 repressor oligomerization behavior (*39*).

Although repressor binds adjacent DNA sites as a tetramer, Figure 1 shows that the repressor does not form this oligomeric species in the absence of DNA, at least up to 15 μM. We wished to examine whether repressor binding to DNA would stimulate the formation of repressor oligomers containing more than two subunits. We cannot monitor this process by simply observing repressor anisotropy. Therefore, we followed repressor oligomer assembly on DNA by measuring the anisotropy of a fluorescein-labeled 20 bp oligonucleotide encoding the O_R1 site, to which repressor binds specifically. Repressor DNA binding and oligomer formation are then detected as an increase in anisotropy of this labeled DNA molecule.

Adding increasing concentrations of repressor to a constant amount of fluorescein-labeled O_R1 increases the anisotropy of the DNA, reflecting repressor binding to O_R1. Inspection of these data indicated that the increase in DNA anisotropy is biphasic (Figure 2). To confirm this observation, we first attempted to fit these data to models that describe the single-step binding of various oligomeric forms of repressor to DNA (Figure 2A). Such models predict a highly sigmoidal dependence of the O_R1 anisotropy on repressor concentration. Comparison of the fitted and experimental data in Figure 2A shows that the dependence of the O_R1 anisotropy on repressor concentration is not sigmoidal, and as judged by the quality of the fit of the data to equations derived from these models, such models do not accurately describe the dependence of O_R1 anisotropy on repressor concentration.

To determine the stoichiometry of the repressor−DNA complexes that are formed in each of the two phases of the biphasic dependence of O_R1 anisotropy on repressor concentration, we sequentially fitted these data to equations that describe each of the two phases of the reaction. The first phase is best described by the binding of a repressor dimer to O_R1. This phase saturates at ~50 nM 434 repressor. The behavior of this phase is described by the expression:

$$2R \overset{K_{D1}}{\rightleftharpoons} R_2 + O_R1 \overset{K_{D2}}{\rightleftharpoons} R_2(O_R1)$$

To fit the anisotropy data of this phase, we first analytically evaluated eq 3 to give f_c the fraction of DNA engaged in the complex $R_2(O_R1)$ species present in reaction as a function of repressor concentration and the known, fixed total amount of O_R1 DNA present in solution:

$$[D]_{tot}\left\{\frac{[R]_{tot}}{2[D]_{tot}} + \frac{K_{D1}}{8[D]_{tot}} - f_c - \frac{1}{8}\sqrt{\frac{8[R]_{tot}K_{D1}}{[D]_{tot}{}^2} + \left(\frac{K_{D1}}{[D]_{tot}}\right)^2 - 16\frac{K_{D1}}{[D]_{tot}}f_c}\right\}(1 - f_c) - K_{D2}\, f_c = 0 \quad (3)$$

where $[R]_{tot}$ is the total concentration of repressor present in solution at each data point expressed as molar concentration of monomeric species, K_{D1} is the repressor dimerization dissociation constant in the presence of O_R1 DNA, K_{D2} is

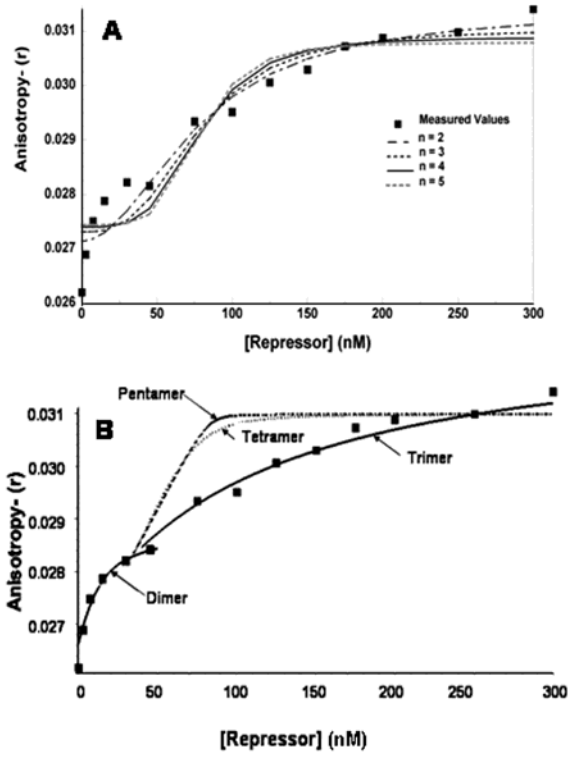

FIGURE 2: Assembly of multiple oligomeric species of 434 repressor on O_R1. The increase in anisotropy of a 20-base pair fluorescein-labeled oligonucleotide 434 O_R1 measured as a function of added 434 repressor. The concentration of O_R1 is 15 nM. The values of anisotropy correspond to emission intensity detected at 520 nm. (A) Observed anisotropy data (■) are represented together with our attempts to fit these data single-step binding of various oligomeric forms of repressor to DNA. The fittings were obtained for various cooperativity coefficients *n* expressing the association between repressor monomers $n = 2, 3, 4, 5$. (B) The same anisotropy data points as in A. The lines represent fittings of the data to two sequential binding events. The solid line between data points between 0 and 50 nM repressor represents fits to equations describing the binding of a repressor dimer to O_R1. The lines between 60 and 300 nM represent simulated data obtained using analytical solutions of equilibrium expressions where O_R1 is bound to a trimer (−), a tetramer (- - -), or a pentamer (− - − -). As can be seen, only fits to the trimer gave statistically significant ($R^2 = 0.97$, S. D. of residuals $= 2.46 \times 10^{-4}$) fits to the observed data.

the dissociation equilibrium constant of repressor dimers to O_R1 DNA, $[D]_{tot}$ is the total concentration of fluorescein O_R1 DNA present in solution, $f_c = [R_2(O_R1)]/[D]_{tot}$ the fraction of DNA present in the complex $R_2(O_R1)$ from the total amount of O_R1 DNA in reaction. Subsequently, the analytical solutions of eq 3 were introduced into the anisotropy eq 4.

$$r_{obs} = r_i + f_c(r_{sat} - r_i) \quad (4)$$

where r_{obs} is the anisotropy obtained from measurements, r_i is anisotropy of fluorescein-labeled O_R1 DNA in absence of repressor, r_{sat} is the anisotropy of the dimeric repressor−O_R1 complex. The best fit of data to eq 4 using the analytical solutions of eq 3 yielded a $K_{D1} = 52 \pm 12$ nM, $K_{D2} = 5.6 \pm 0.67$ nM, $r_{sat} = 0.028173$, with fit values $Chi^2 = 1.817 \times 10^{-9}$, and $R^2 = 0.96$ and is shown together with our data in Figure 2B. Analytical expressions assuming binding of monomeric, trimeric, or tetrameric repressor species to O_R1 gave poor fits to the first phase of the data. In addition to the finding that this model is the only one that gave statistically significant fits to the data, several lines of evidence support the idea that the first phase of the anisotropy increase results from binding of a repressor dimer to DNA. First, the fitted value of equilibrium dissociation constant (K_{D2}) for the binding of a repressor dimer (R_2) to O_R1 is 5.6 ± 0.67 nM, a value that is nearly identical with that measured in independent experiments (*29*, *40*). Second, the fold change in DNA anisotropy is what would be expected for this interaction (eqs 3 and 4). Third, the repressor concentration dependence of this phase is consistent with the sequence-specific binding of a repressor dimer to DNA. Moreover, no increase in anisotropy is seen in this concentration range if repressor is added to a fluorescein-labeled DNA bearing a sequence to which repressor does not bind specifically (*19*).

As repressor concentration is increased beyond ∼50 nM, a second phase of anisotropy increase is observed, and the plateau of this second phase is attained at ∼300 nM of added 434 repressor. Due to the limiting amount of DNA in the reaction, and knowing that the first plateau in the anisotropy data represents the binding of a repressor dimer to O_R1, the second phase of the anisotropy increase (i.e., [434 repressor] > 50 nM) must be due to the binding of additional repressor molecules to the initial dimeric repressor−O_R1 complex. Since the oligonucleotide used in our anisotropy experiments is 20 base pairs in length and since repressor contacts the DNA phosphate backbone over 18 base pairs, the region of the DNA where repressor specifically contacts DNA would be largely covered by the initially bound repressor dimer (*12*). Thus, an additional repressor molecule could associate with the initial complex by binding DNA in a nonspecific fashion, or by interacting with the DNA-bound repressor dimer. Two observations indicate that the additional repressor molecule that joins the repressor dimer−O_R1 complex is not interacting with the DNA. First, although the second anisotropy change plateau at ∼300 nM repressor, nonspecific binding of repressor to DNA is not observed unless substantially higher repressor concentrations are added (*29*). Second, the C-terminal domain of 434 repressor, a protein that lacks any DNA binding activity, is able to form a complex with the specifically bound repressor dimer (see Figure 4, below). This finding shows that additional repressor molecules can associate with the initial O_R1 repressor dimer complex by interacting with the DNA-bound repressor dimer via the C-terminal domain. Since higher order repressor oligomers (>2 repressor monomers) do not form in the absence of DNA (Figure 1), the findings in Figure 2 suggest that DNA induces an allosteric change in the repressor that allows the formation of such complexes.

Knowing that the first plateau in the anisotropy data represents the binding of a repressor dimer to O_R1, the equilibrium expression accounting for all DNA-containing repressor species is:

$$R_2{\cdot}O_R1 + (n-2)R \rightleftarrows R_n{\cdot}O_R1$$

where R is repressor and *n* is the number of repressor monomers bound to O_R1 in the oligomeric complex. Having obtained the anisotropy of the dimeric repressor−O_R1 complex from analysis of the data from the first phase data using eqs 3 and 4 we calculated the concentration of this species and used this information to analyze the second phase

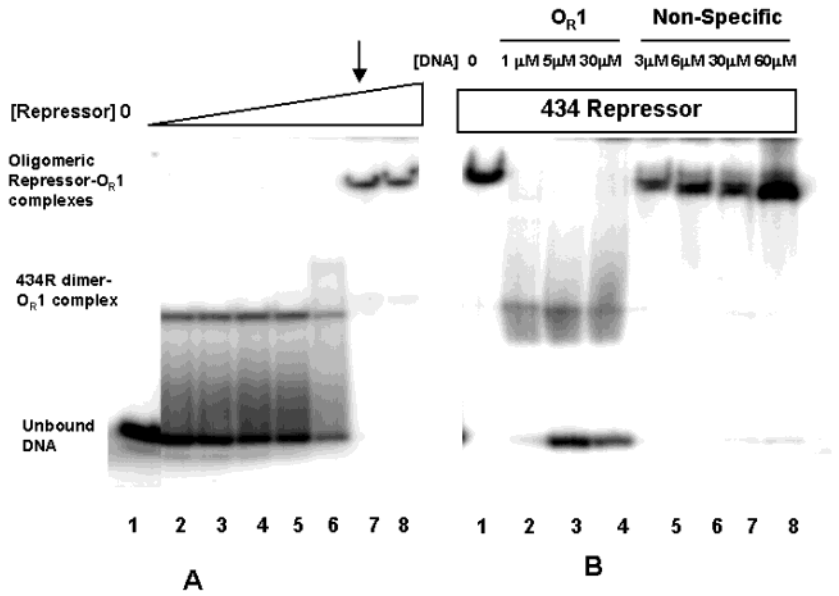

FIGURE 3: Specific binding of higher order repressor oligomers to a single O_R1 site. (A) A labeled 76-base pair DNA fragment bearing one 434 O_R1 site was incubated with increasing concentrations of 434 repressor. Shown is a native gel of the complexes formed under these conditions. The initial concentrations of DNA and repressor were 5 nM and concentration of repressor was increased in 2-fold steps. (B) The complex formed in lane 7 of panel A (indicated by the arrow) was incubated with increasing concentrations of a 76-base pair unlabeled DNA fragment bearing the O_R1 sequence (lanes 2−4) or 76-base pair fragment that does not contain any sequences to which 434 repressor binds specifically (lanes 5−8).

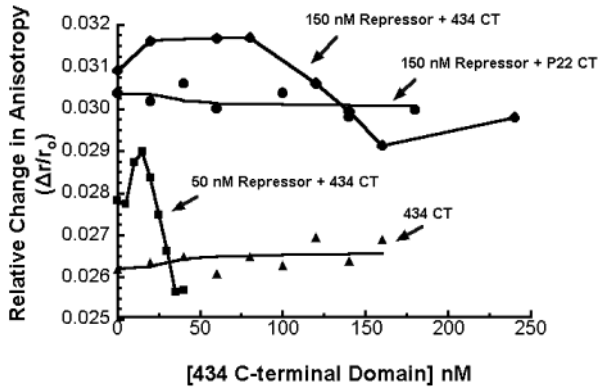

FIGURE 4: Binding of additional 434 repressor molecules to a repressor O_R1 complex is mediated by protein−protein interactions, not DNA binding. The anisotropy ($\Delta r/r_o$) of a 20-base pair fluorescein-labeled 434 O_R1 oligonucleotide alone or in complex with 434 repressor was measured as a function of added C-terminal domains of 434 or P22 repressor. 15 nM fluorescein-labeled 434 O_R1 DNA was equilibrated without (▲) or with 50 nM (■) or 150 nM (♦) intact 434 repressor and increasing concentrations of the C-terminal domain of 434 repressor or P22 repressor (●).

anisotropy increase to identify the number of repressor monomers that add to the initial complex at higher repressor concentrations. Thus, we started with the mass balance equation (eq 5) describing the second phase of the binding isotherm (see Figure 2)

$$[R_2(O_R1)]_{tot} = [R_2(O_R1)] + [R_n(O_R1)] \quad (5)$$

where $[R_2(O_R1)]$ represents the concentration of the repressor dimer−DNA complex present at the beginning of the second phase reaction, and $[R_n(O_R1)]$ is the concentration of oligomeric−DNA complex in the second phase reaction. To fit the anisotropy data of the second phase, we first analytically evaluated eq 6, which describes the dependence of the repressor dimer−O_R1 complex as a function of repressor concentration:

$$[R]_{tot} = \left\{\frac{K_{D3}\{[R_2(O_R1)]_{tot} - [R_2(O_R1)]\}}{[R_2(O_R1)]}\right\}^{(1/n-2)} + 2[R_2(O_R1)] + n\{[R_2(O_R1)]_{tot} - [R_2(O_R1)]\} \quad (6)$$

where K_{D3} is an apparent equilibrium dissociation constant that describes the concentration of repressor $[R]_{tot}$ (expressed as the total concentration of repressor monomers) that gives half-maximal binding in the second phase binding isotherm. We obtained analytical solutions for the binding of three, four, and five repressor monomers and substituted these into the anisotropy expression for formation of the oligomeric ($n \geq 2$) repressor−O_R1 complex eq 7:

$$r_{obs} = r_i\frac{[D]}{[D]_{tot}} + r_D\frac{[R_2(O_R1)]}{[D]_{tot}} + r_{Oc}\frac{[R_n(O_R1)]}{[D]_{tot}} \quad (7)$$

where r_i is the initial anisotropy of fluorescein-labeled O_R1 measured in the absence of repressor, r_D (= 0.028173) is the anisotropy of dimeric repressor−O_R1 as determined from the fits to eq 4, r_{Oc} is the anisotropy of oligomeric repressor ($n \geq 2$)−O_R1 complex, and D and D_{tot} represent the concentration of DNA in complex with repressor and the total concentration of DNA, respectively. The results of the solutions for the binding of three, four, and five repressor monomers to DNA are depicted graphically in Figure 2B. As shown in the figure, the second phase of the observed anisotropy data is best described by the analytical solution where the repressor−O_R1 complex contains three repressor monomers (see Figure 2B and Figure 2, legend). Hereafter we will refer to this complex as the higher order or oligomeric repressor−DNA complex, reflecting the observation that this repressor−DNA complex contains three repressor monomers.

To begin to investigate the mechanism by which the higher order repressor−DNA complexes form, we repeated the experiments shown in Figure 2, and detected repressor complex formation by gel electrophoresis on native polyacrylamide gels. Similar to the anisotropy results, adding increasing concentrations of repressor to a 76-base pair ^{32}P-labeled DNA molecule bearing a single O_R1 site results in the sequential formation of two complexes (Figure 3A). At repressor concentrations between 5 and 50 nM, a protein−DNA complex of intermediate mobility is formed. We showed previously that this complex corresponds to the binding of a repressor dimer to O_R1 (Figure 3A, lanes 2−6) (*40*). At repressor concentrations above 100 nM an additional supershifted complex is observed (Figure 3, lanes 7−8). Since this supershifted complex forms at a repressor concentration similar to that at which the higher molecular order repressor−DNA complex identified in the anisotropy measurements (Figure 2) forms, we suggest that this supershifted complex represents the higher order repressor−DNA complex.

We examined the stability of the higher molecular order repressor−DNA complex in the presence of increasing concentrations of DNA that either does or does not contain a sequence to which repressor binds specifically. This experiment allowed us to test whether the higher order complex forms by association of additional repressor monomers with a DNA-bound repressor dimer or by binding nonspecifically to the DNA in the complex. The higher order repressor−complex readily dissociates in the presence of a 10-fold excess of DNA containing the O_R1 sequence (Figure 3B, lanes 1−4). By contrast, the high molecular weight complex is stable even in the presence of a 600-fold molar excess of nonspecific DNA (Figure 3B, lanes 5−8). The slight increase in mobility of the protein−DNA complex in these lanes is due to the high DNA concentrations, and not to dissociation of proteins from the complex. These data strongly suggest that additional 434 repressor molecules bind to the repressor−dimer−O_R1 complex via protein−protein interactions and not by binding nonspecifically to DNA.

Carboxyl Terminal Domain of Repressor Binds to an O_R1-Bound Repressor Dimer. To further demonstrate that higher order complexes of 434 repressor form on a single DNA site via protein−protein contacts, we assessed the ability of O_R1-bound intact repressor to form complexes with the purified C-terminal domain of 434 repressor (434 CT) (*15*). The C-terminal domain fragment lacks the DNA binding domain of the repressor and does not interact with DNA, either specifically or nonspecifically. However, the 434 C-terminal domain retains the ability to form dimers with itself and to bind intact repressor forming a hemidimer (*39*). Interestingly, this protein fragment also has the ability to form homotetramers (A. Donner, J. Urbauer, and G. Koudelka, unpublished results).

Adding 434 CT to fluorescein-labeled O_R1 does not alter the anisotropy of the DNA (Figure 4). This finding confirms our earlier observation that 434 CT does not bind DNA. Reflecting formation of a dimeric repressor−DNA complex, adding 50 nM of intact 434 repressor to the fluorescein-labeled O_R1 increases its anisotropy (Figure 4, compare anisotropy value of DNA in absence of any protein vs that in the presence of repressor alone, see also Figure 2). At low concentrations (5−15 nM), 434 CT causes the anisotropy of the dimeric repressor−O_R1 complex to increase. Since 434 CT alone does not bind DNA, the increase in anisotropy reflects the association of 434 CT with the DNA-bound repressor. Adding higher concentrations of 434 CT to the dimeric repressor−O_R1 complex decreases the anisotropy of the complex (Figure 4). The decrease in anisotropy was consistent with our earlier findings showing that at higher concentrations of added 434 CT, intact 434 repressor and the C-terminal domain fragment form hemidimers and that these hemidimers are incapable of binding DNA (*15*). Hence, the anisotropy decrease seen at higher 434 CT concentrations results from formation of such hemidimers, causing the consequent dissociation of the intact repressor dimer from DNA.

Since we do not know the mechanism or affinity of hemidimer formation, we are unable to accurately simulate the data depicted in Figure 4. However, two lines of evidence support the conclusion that the 434 CT-dependent increase in anisotropy of the protein−DNA complex seen in Figure 4 reflects formation of a higher order oligomeric repressor species assembled via protein−protein contacts on a single DNA site. First, adding increasing concentrations of the C-terminal domain of bacteriophage P22 repressor (P22 CT) to the 434 repressor−O_R1 complex does not affect the anisotropy of the associated DNA (Figure 4). Since we showed previously that P22 CT does not form complexes with 434 repressor (*32*), this finding indicates that the anisotropy increase observed in the presence of 434 CT is due specific protein−protein interactions. Second, the concentration of 434 CT needed to decrease the anisotropy of O_R1 in the dimeric repressor−DNA complex increases with increasing repressor concentrations. The data in Figure 4 show that increasing the concentration of intact repressor from 50 to 150 nM intact repressor increases the amount of 434 CT needed to cause the increase in anisotropy from 15 to 75 nM. At 150 nM repressor, 75 nM of 434 CT is approximately equimolar to the concentration of the intact repressor dimer (Figure 4) present under these conditions. The only way that the repressor concentration could affect the dependence of anisotropy on 434 CT concentration is if 434 CT is associating with repressor bound to the fluorescein-labeled O_R1.

Binding of Additional Repressor Monomers to the Repressor Dimer-O_R1 Complex Is Mediated by the Repressor's Tetramerization Interface. Since additional repressor molecule(s) appear to associate with the repressor dimer−O_R1 complex by protein−protein interactions in the C-terminal domain, we wished to identify the surface of the C-terminal domain that is involved in forming these complexes. The anisotropy results indicate that the higher order oligomer contains three repressor monomers. Thus, formation of a repressor oligomer bound to O_R1 results from the binding of one monomer to the DNA-bound repressor dimer. It seemed likely that the tetramerization interface used in cooperative binding of the repressor to two adjacent sites on DNA may also mediate this interaction. To test this idea, mutations and/or chemical modifications were introduced into this surface of repressor. Two types of modifications in the tetramerization interface were examined. In one protein, we covalently modified Cys189 of 434 repressor with *N*-ethyl malemide (NEM), a modification that we previously showed prevents the cooperative binding of repressor to O_R1 and

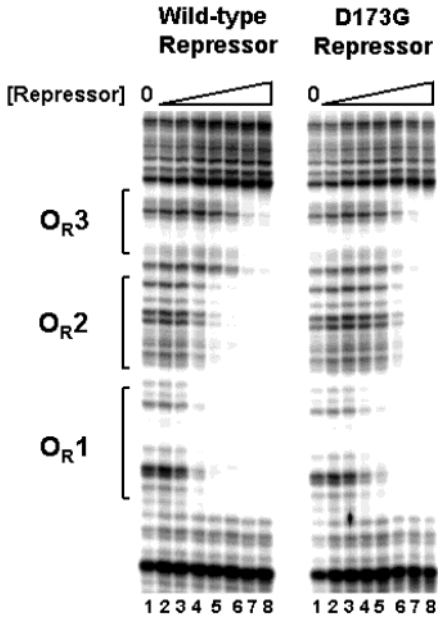

FIGURE 5: The D173G 434 repressor is partially defective in cooperative binding to O_R1 and O_R2. The binding of wild-type (A) and D173G mutant (B) 434 repressor to a 160 bp ^{32}P-labeled DNA fragment containing the 434 O_R region 434 examined by DNase I footprinting as described in Experimental Procedures. In panels A and B, the 0.1 nM DNA fragment was incubated in the absence (lane 1) or presence (lanes 2−8) of wild-type or mutant 434 repressor Concentrations were increased in 2-fold steps starting from 5 nM.

O_R2 (*41*). In a second protein, we changed aspartic acid 173 to a glycine. Whipple et al. (*21*) showed that changing this residue at the homologous position in λ repressor, aspartate 197, to glycine decreases the cooperative binding of a λ repressor tetramer to two adjacent sites in λO_R.

To ensure that the D173G mutation confers a tetramerization defect on 434 repressor, we examined the ability of the D173G mutant repressor protein to bind cooperatively to the adjacent O_R1 and O_R2 in intact 434 O_R. In the presence of 50 nM wild-type 434 repressor O_R1 is completely protected from DNase I digestion and both O_R1 and O_R2 sites are entirely protected at 100 nM repressor (Figure 5). Since the intrinsic affinities of repressor for O_R1 and O_R2 differ by 18-fold (*29*), these findings show that wild-type repressor bound at O_R1 cooperatively assists binding of another repressor dimer to O_R2. Similar to the wild-type 434 repressor, the D173G mutant protein occupies O_R1 at 100 nM repressor (Figure 5), indicating that the mutation does not dramatically affect DNA binding of a repressor dimer. However, in contrast to the wild-type protein, O_R2 occupancy by the D173G repressor requires ≥5-fold more protein than is required to completely fill the O_R1 site. This finding shows that, as in λ repressor, the D → G mutation at this position partially abrogates cooperative tetramer DNA binding by 434 repressor.

We examined the ability of both the 434 D173G repressor mutant and the NEM modified 434 repressor to form DNA-induced higher order oligomers using fluorescence anisotropy. Incubating increasing amounts of either NEM-modified or D173G repressor protein with fluorescein-labeled 434 O_R1 increases the anisotropy of the DNA (Figure 6). In contrast to the case of the wild-type, unmodified repressor, the anisotropy increases seen with both modified proteins

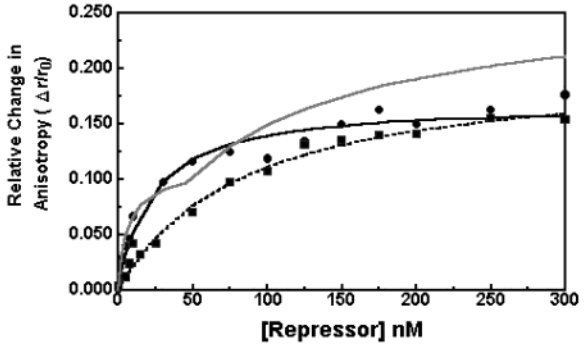

FIGURE 6: Assembly of multiple oligomeric species of 434 repressor on O_R1 is inhibited by mutations that compromise cooperative binding of 434 repressor. The relative increase in anisotropy ($\Delta r/r_o$) of a 20-base pair fluorescein-labeled oligonucleotide 434 O_R1 was measured as a function of added D173G (●) or NEM-modified (■) 434 repressor. For reference, the gray line represents the results of this experiment for wild-type repressor as reported in Figure 2. The concentration of O_R1 is 15 nM. The values of anisotropy correspond to emission intensity detected at 520 nm. The lines represent a hyperbolic fit to the data. In the case of D173G mutant 434 repressor (dotted line), the fit yielded a global apparent binding constant $K_D = 78.56 \pm 4.05$ nM ($R^2 = 1.6 \times 10^{-3}$, correlation = 0.98), while for NEM modified repressor (solid line) the fit generated a global apparent binding constant $K_D = 83 \pm 13.6$ nM ($R^2 = 9.6 \times 10^{-4}$, correlation = 0.99).

appeared to be monophasic, indicating that preponderantly one type of repressor−O_R1 complex is formed by these proteins as their concentration increases. The overall increase in anisotropy plateaus at a level ∼35% lower than that of the wild-type protein, showing that the complexes formed at high concentration of the modified proteins are smaller than those formed with the wild-type repressor. These data sets where fitted to simple rectangular hyperbolas describing monophasic repressor binding to O_R1. Examination of the fitting statistics (see Figure 6 legend) and the quality of the fitted line with respect to the observed data in this concentration range indicates that unlike wild-type repressor, the two modified repressors do not form considerable amounts of oligomers with higher order than a dimer in this concentration range. No increase in anisotropy is seen in this concentration range if these repressors are added to a fluorescein-labeled DNA bearing a sequence to which they do not bind specifically (data not shown), indicating that the anisotropy increases are due to specific binding of the repressor to DNA. Similar results were obtained if the complexes were visualized on native gels (data not shown). Thus, both the mutant 434 repressor D173G and NEM modified 434 repressor have a reduced tendency to form higher order oligomers than does the wild-type protein (compare Figures 2 and 6). Since these proteins both bear modifications in the tetramerization interface, we conclude that formation of DNA-induced higher order repressor oligomers is mediated, at least in part, by the same surface of repressor that is responsible for mediating the side by side cooperative binding of repressor to adjacent sites.

O_R1- and O_R2-Bound Repressor Dimers Mediate Different Types of Protein−Protein Interactions. Although repressor can dimerize both in the absence and presence of DNA, the results discussed above show that only when repressor is bound to DNA, can repressor assemble as an oligomer containing >2 subunits. Assembly of such DNA-bound repressor higher order oligomers, specifically a tetramer, is

an essential feature of the genetic circuitry needed to maintain and establish a lysogenic 434 bacteriophage. Since formation of the DNA-bound repressor trimer observed in Figures 2, 3, and 6 utilizes the tetramer interface (Figure 5), we wanted to examine whether the trimeric repressor species functions as an intermediate in the pathway to forming a repressor tetramer bound to two DNAs. If so, we hypothesized that the O_R1-bound repressor oligomer should be capable of recruiting a DNA site from solution. To test this possibility, we measured the effect of added DNA on the anisotropy of the oligomeric repressor−DNA complex. For this experiment, we incubated a low concentration of fluorescein-labeled O_R1 (15 nM) with fixed excesses (50 or 150 nM) of wild-type or tetramerization-deficient 434 repressor and increasing concentrations of unlabeled 20-base pair oligonucleotides bearing the sequence of 434 O_R1 or 434 O_R2. We reasoned that as the concentration of added DNA increased, this DNA, perhaps together with an additional repressor molecule recruited from solution, would associate with the DNA-bound repressor trimer. This association would be detected as an increase in anisotropy of the initial trimeric repressor DNA complex.

We showed earlier that wild-type or mutant repressor increases the anisotropy of fluorescein-labeled O_R1 due to the binding of repressor dimers (50 nM repressor) or higher order oligomers (150 nM repressor) to this DNA (see Figures 2, 3, and 6). Consistent with the idea that a DNA-bound repressor oligomer can recruit additional DNA sites from solution, adding 1−5 nM of unlabeled O_R2 to the complex between O_R1 and 50 nM 434 repressor results in an initial increase in anisotropy of the complex (Figure 7A). This increase has the appearance of a "spike" that disappears at higher O_R2 concentrations. A qualitatively similar, but quantitatively different pattern of changes in anisotropy is observed when unlabeled O_R2 is added to a complex between 150 nM repressor and labeled O_R1 (Figure 7B). In this case, the appearance of the "spike" increase in anisotropy appears between 15 and 25 nM O_R2 before disappearing at higher O_R2 concentrations.

We suggest that the spike in anisotropy seen upon addition of DNA is due to the binding of the added O_R2 DNA fragment to a preformed repressor−O_R1 complex. We know from the experiments in Figures 2−4 that under the conditions of these experiments, the labeled O_R1 site is bound by a trimer of repressor, with one dimer occupying both half-sites of this site. We suggest that this configuration leaves an "unoccupied" repressor monomer that is free to interact with an additional DNA site in solution. Alternatively, it is possible that added DNA dissociates this trimeric repressor−DNA complex, and the spike in anisotropy results from the binding of two DNAs to a repressor dimer, one that is labeled and the other that is not. Since we know that formation of a DNA-bound repressor trimer utilizes the tetramerization interface normally used for cooperative DNA binding (Figures 5 and 6), our interpretation predicts that the anisotropy of complexes between O_R1 and repressors that are defective in tetramer formation would not show the "spike" in anisotropy as unlabeled DNA is added. Conversely, since the tetramerization and dimerization surfaces of repressor are independent (*18*, *39*) and Figure 5), the alternative hypothesis predicts that the anisotropy of the tetramerization interface mutants should still "spike" at low

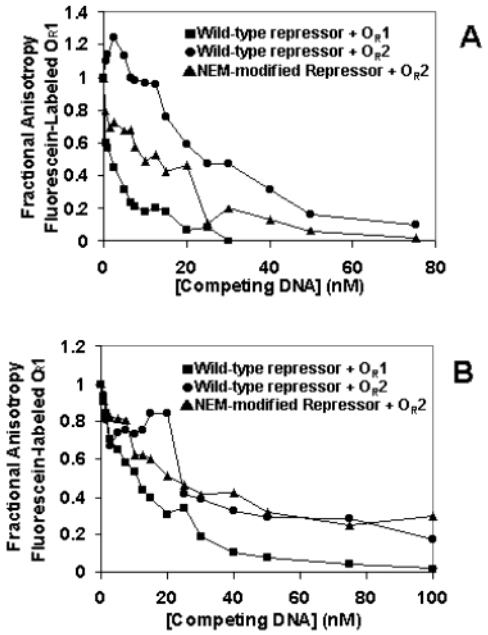

FIGURE 7: Additional DNA binds to the oligomeric repressor−O_R1 complex. The anisotropy of a 20-base pair fluorescein-labeled O_R1 oligonucleotide bound to the oligomeric 434 repressor was used to monitor the binding of additional molecules of DNA to the complex. In panel A, complexes were formed between 50 nM wild-type or NEM-modified 434 repressors (●, ■) (▲) and O_R1 were formed and the anisotropy of these complexes is assigned the relative value of unity. Subsequently the wild-type repressor−O_R1 complex was incubated with increasing concentrations of nonfluorescent 20-base pair oligonucleotides bearing the O_R1 (■) or O_R2 (●) sequence. In a similar fashion, the NEM-modified repressor−O_R1 complex was incubated with increasing concentrations of nonfluorescent 20-base pair oligonucleotides bearing the O_R2 sequence (▲). The experiments shown in panel B are identical to those in panel A, except the concentration of the repressors was increased to 150 nM.

added DNA concentrations. Indeed, we find that no increase in anisotropy occurs when O_R2 is added to the complex of O_R1 with the tetramerization defective NEM-modified repressor, instead only a decrease in O_R1 anisotropy is observed (Figure 7A,B). Identical results were obtained using the D173G mutant repressor (data not shown). Hence, the spike in anisotropy results from the binding of the added O_R2 to the repressor−O_R1 complex. Due to the complexity of the heterogeneous mixture of species present at the anisotropy "spikes", we have been unable to definitively determine whether the binding of the additional DNA to the trimeric repressor−O_R1 complex is accompanied by the binding of an additional repressor molecule thereby forming a repressor tetramer with two DNAs bound. Nonetheless, since half-site occupancy has never been reported for 434 repressor, and the affects on anisotropy are sequence-specific we suggest that binding of additional DNA to trimeric repressor−O_R1 complex is accompanied by formation of a repressor tetramer. Therefore, these findings suggest that the pathway for the assembly of repressor tetramer cooperatively bound to O_R1 and O_R2 involves the formation of a DNA-bound repressor species that can recruit a DNA molecule from solution.

When we repeated these experiments, instead adding unlabeled O_R1 to the oligomeric repressor−O_R1 complex, we obtained a surprising result: the added O_R1 does not induce any increase in anisotropy, and instead, only decreases

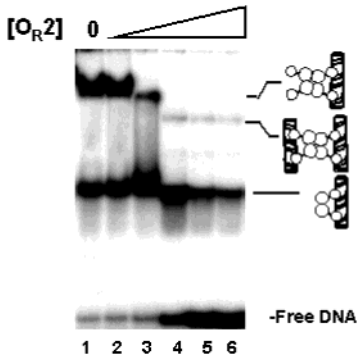

FIGURE 8: Binding of additional DNA to higher order repressor oligomers on a single O_R1 site is sequence specific. A 5 nM labeled 76-base pair DNA fragment bearing one 434 O_R1 site was incubated with 100 nM 434 repressor. These conditions are identical to that shown in Figure 3B lanes 1 and 5. Following equilibration, increasing concentrations of unlabeled O_R2 were added. A schematic of the complexes formed under these conditions is shown to the right of the gel.

in anisotropy are observed. This O_R1-mediated decrease in anisotropy results from competition between labeled and unlabeled O_R1 for binding repressor (see below). We were puzzled as to why adding O_R1 does not cause a similar anisotropy increase as does adding O_R2. The data in Figure 7 show that addition of unlabeled O_R1 completely disrupts the oligomeric repressor−O_R1 complex at substoichiometric concentrations. The efficiency of competition implies that a repressor oligomer bound to two O_R1 sites is unstable, as opposed to the relatively stable O_R1−repressor−O_R2 complex.

To examine this idea, and to verify our interpretation of the data in Figure 7, we repeated these experiments, but detected repressor complex formation by gel electrophoresis on native polyacrylamide gels. We have already shown that this complex is stable in the presence of excess nonspecific DNA (Figure 3B, lanes 5−8). As opposed to completely dissociating the high molecular weight oligomeric repressor−O_R1 species (see Figure 3B, lanes 1−4), adding O_R2 to the complex slightly increases its mobility on gels (Figure 8). This increase in mobility is consistent with the binding of O_R2 to the oligomeric complex. Such binding would increase the net negative charge of the complex, resulting in faster electrophoretic mobility. The observation that only O_R2 can stably bind to the oligomeric repressor−O_R1 complexes suggests that a repressor tetramer can only bind to O_R1 and O_R2, not to two O_R1 binding sites. These findings suggest that the binding of a repressor tetramer to O_R1 induces conformational changes in repressor that only allow the specific binding of O_R2.

DISCUSSION

As is true of many transcriptional regulatory proteins, cooperative binding to DNA is required for gene regulatory functions of bacteriophage 434 repressor. Despite having the lowest affinity for repressor of all the sites in O_R, repressor occupancy of O_R2 is necessary and sufficient for regulating the essential promoters that direct the phage toward lysogenic growth (*42*). In the absence of cooperative interactions with a repressor dimer bound at O_R1 repressor, repressor could only bind O_R2 at substantially higher concentrations. Hence, cooperative binding of repressor is essential for the proper functioning of this genetic switch. The question at issue is, how does the O_R1-bound repressor assist repressor binding to the weaker O_R2 site? The answer to this question is not only of interest to understanding bacteriophage gene regulation but in delineating the mechanism of cooperative binding of other transcriptional regulatory proteins as well.

Two models have been proposed to account for cooperativity in DNA binding proteins (*1*), and we will consider below a third model. On the basis of their work in *E. coli lac* operon, Müller-Hill and colleagues suggested that in cases in which multiple binding sites are present within a relatively short stretch of DNA, apparently cooperative binding may occur by enhancing the local concentration of the binding protein in the vicinity of these sites (*1*). Many transcriptional control regions, including 434 O_R, contain multiple binding sites for an individual protein. However, two observations indicate that the local concentration model does not apply universally to all cooperative binding interactions. First, the local concentration of multiple binding sites drops precipitously with distance (*43*) and many cooperative binding interactions are relatively independent of the distance between the two binding sites. Second, lac repressor may not adequately model the typical cooperatively binding protein. Lac repressor is a preformed tetramer capable of binding two DNA binding sites, whereas most proteins that bind cooperatively only gain the capacity to bind multiple DNA binding sites upon forming the cooperatively bound oligomeric protein−DNA complex. If the cooperating protein does not preform a complex prior to occupancy of the strong site, the role of local concentration on the ability of a tight protein−DNA complex to stabilize interactions of the protein with weak DNA binding site is minimal. Hence, the local concentration model does not adequately account for the role of protein−protein contacts between the strongly bound and weakly bound proteins in facilitating occupancy of a weaker binding site.

As opposed to increasing the local concentration, a protein bound to the strong site also can promote binding to the weak site by providing an additional point of contact in the assembly of the cooperatively bound protein−DNA complex. In this case, assembly of the complex occurs via a sequential pathway, with protein−protein interactions driving DNA binding to the weak site. Two variants of the sequential pathway can be envisioned. In one alternative, the increased affinity of the second protein for the weaker site could result solely from the having a second "landing site" on the strongly bound protein in the vicinity of the weaker DNA binding site. This alternative implies that the protein−protein interactions that stimulate cooperative DNA binding can occur in the absence of DNA, a situation found frequently (*44*). However, this alternative does not accurately describe many other systems (see below), including the situation with cooperative binding by 434 repressor. In a second alternative, we will term "recruitment", a unique protein−protein complex, nucleated by the initial strong binding of one protein, forms specifically and *only* on DNA. The thus formed oligomeric protein−DNA complex must assume a structure that is particularly suited to occupy the weak site.

We show here that the pathway for forming a repressor bound to O_R1 and O_R2 displays the features of complex assembly by the recruitment, not the "landing site" mechanism. First, in the absence of DNA, repressor only forms a

dimer; however, upon binding of this dimer to O_R1 an additional repressor molecule is able to associate with the DNA-bound dimer, thereby assembling an oligomeric repressor species on DNA. The additional repressor monomer recruited from solution binds the dimeric repressor−O_R1 complex via residues normally used for forming tetramers bound at adjacent O_R1 and O_R2 sites. Second, the oligomeric repressor−O_R1 complex bears a second DNA binding element, to which an additional DNA molecule can and does bind.

Although we have been unable to determine the stoichiometry of repressor−DNA in the complex formed upon adding DNA to the trimeric repressor−O_R1 complex, we suggest that binding of the additional DNA molecule to this complex is accompanied by the binding of another repressor monomer to form a tetramer. Consistent with this suggestion, Figure 7 shows that the stability of the trimeric repressor−O_R1 complex depends on the sequence of the added DNA. Since repressor does not bind DNA specifically as a monomer and the trimeric repressor−DNA complex only has one DNA binding domain available for interacting with added DNA and repressor, we argue that an additional repressor monomer must bind to the complex along with the second DNA molecule, thereby providing the sequence specificity.

The observation that DNA can bind to the trimeric repressor−O_R1 complex suggests that formation of DNA-bound repressor tetramer may occur via a sequential mechanism where building of a tetramer required for cooperative O_R1 and O_R2 occupancy utilizes a trimeric intermediate. Such a mechanism would predict that neither the amount of oligomer formed in reaction nor its stoichiometry would be influenced by the amount of free repressor dimers present in solution. This idea is supported by our finding that the repressor mutant K121A, which has K_{Dimer} 60-fold lower than wild-type repressor (*39*), generates higher level of detectable oligomeric species than wild-type repressor (data not shown).

Our data show that O_R2, but not O_R1, is able to bind to an oligomeric repressor assembled on single O_R1 site (Figures 7 and 8). That is, only repressor oligomers bound to O_R1 and O_R2 are stable, and those bound to two O_R1 sites are not. We are intrigued that the favorable arrangement of DNA sites bound to an oligomeric repressor that is formed by adding DNA in trans is identical to that in cis, as is the case in wild-type O_R. These observations imply that the conformations of individual repressor dimers in the tetramers are asymmetric and *predisposed* for binding two different sequences. These findings also suggest that the identity of the DNA sequence to which repressor is bound allosterically affects the structure and function of repressor. This suggestion is consistent with our earlier findings showing that adding DNA induces repressor dimerization by altering repressor structure (*19*). Similarly, we also showed that the precise sequence of the site to which repressor binds influences its efficiency transcriptional activation (*30*). Thus, the findings reported here demonstrate that DNA sequence allosterically affects virtually all aspects of repressor function: dimerization, tetramer formation, and transcriptional activation.

Our findings show that repressor joins a growing class of transcriptional regulatory proteins whose functions are influenced by DNA binding and/or the precise sequence of the bound DNA. Among the best-studied members of this class are the nuclear receptors. In this class of proteins, DNA affects dimer formation and transcription activation by stabilizing its dimer interface (*45*−*47*) and catalyzing the exposure of an otherwise buried transcriptional activation surface (*48*, *49*). Similarly, the precise DNA sequence to which the RAR−RXR heterodimer is bound modulates the stability of its cooperative interaction with particular co-repressors in the presence of the hormone (*50*, *51*). In the Pit1/Pou class of proteins, the DNA sequence allosterically modifies the precise three-dimensional structure of the cooperative binding surface onto which co-activators and co-repressors dock, thereby leading to activation or repression, respectively. Allosteric modification of protein function is not limited to proteins derived from eukaryotic sources. Salas and co-workers (*52*, *53*) showed that the p4 protein of bacteriophage ϕ29 either activates or represses transcription depending only on the identity of the promoter sequence to which RNA polymerase is bound. That activation and repression are mediated by identical residues in RNA polymerase and the p4 protein. Thus, DNA sequence can also alter how the core transcriptional machinery responds to identical contacts mediated by the same regulator (*54*).

The sequence of events that we report here for cooperative occupancy of multiple adjacent DNA binding sites by 434 bacteriophage repressor may also be utilized by repressors of other lambdoid bacteriophages. Little and colleagues showed that the HK022 bacteriophage repressor bound to a single O_R1 site can nucleate the nonspecific binding of multiple units of HK022 repressor dimers in a phenomenon termed extended cooperativity (*55*, *56*). However, when HK022 repressor binds to O_R1 and an adjacent O_R2 site, the cooperative interactions are restricted to repressor bound at O_R1 and O_R2. Under this regimen, no further repressor dimers are "helped" to bind DNA (*57*). This latter finding indicates that the sequence specific interaction of HK022 repressor with O_R1 and O_R2 alters its conformation so that extended cooperativity is blocked.

A key feature of the recruitment model is that the cooperatively bound protein−DNA complex does not form (or is unstable) in the absence of DNA. This aspect of the recruitment assembly mechanism implies that DNA binding induces a conformational change in the protein(s) that enables subsequent protein−protein interactions. Unfortunately, lack of information concerning the three-dimensional structure of the 434 repressor, nor any other intact bacteriophage repressor, prohibits us from precisely delineating the structural basis for the DNA-induced allosteric transitions. However, the results of spectroscopic and mutagenesis studies (*15*, *32*) along with extensive sequence similarities indicates that the C-terminal domain 434 repressor shares all structural features displayed by the structurally characterized λ repressor C-terminal domain (λCTD) (*18*), UmuD′ (*58*, *59*) and lexA (*60*). On the basis of these structures, the results of our molecular modeling studies indicate that the linker region that joins the N- and C-terminal domains is not unstructured, as previously suggested, but is instead packed against the core of the C-terminal domain. In our model, the C-terminal end of the linker comes in close contact with DNA, and, hence, DNA binding by the 434 repressor would affect the structure of the linker region. As shown in the structures of the UmuD′ proteins, alteration in

the position of the linker is accompanied by changes in the relative disposition of the two halves of the C-terminal domain of this protein. Such changes in the C-terminal domain of 434 repressor would alter the relative exposure of the part of this domain that forms the dimer−dimer interface. Hence, DNA triggers a change in the structure of the linker region that allosterically affects tetramerization of repressor. The observation that 434 CT, a protein that lacks the linker polypeptide, is able to form tetramers, whereas the non-DNA bound repressor cannot, supports the suggestion that allosteric transitions mediated by the linker region regulate cooperative tetramer formation by 434 repressor.

REFERENCES

1. Muller-Hill, B. (1998) *Mol. Microbiol. 29*, 13−18.
2. Ptashne, M., and Gann, A. (1997) *Nature 386*, 569−577.
3. Hochschild, A., and Ptashne, M. (1988) *Nature 336*, 353−357.
4. Daniels, D. l., Schroeder, J. L., Szybalski, W., Sanger, F., Coulson, A. R., Hong, G. F., Hill, D. F., Petersen, G. F., and Blattner, F. R. (1983) *Lambda II*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
5. Schreiber, E., and Schaffner, W. (1989) *Somatic Cell Mol. Genet. 15*, 591−603.
6. Goodman, S. D., and Nash, H. A. (1989) *Nature 341*, 251−254.
7. Pabo, C. O., Sauer, R. T., Sturtevant, J. M., and Ptashne, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1608−1612.
8. Sauer, R. T., Ross, M. J., and Ptashne, M. (1982) *J. Biol. Chem. 257*, 4458−4462.
9. Sauer, R. T., Pabo, C. O., Meyer, B. J., Ptashne, M., and Backman, K. (1979) *Nature 279*, 396−400.
10. Mondragon, A., Subbiah, S., Almo, S. C., Drottar, M., and Harrison, S. C. (1989) *J. Mol. Biol. 205*, 189−200.
11. Anderson, J. E. (1984) Ph.D. Thesis, Harvard University, Cambridge.
12. Aggarwal, A., Rodgers, D. W., Drottar, M., Ptashne, M., and Harrison, S. C. (1988) *Science 242*, 899−907.
13. Wharton, R. P., Brown, E. L., and Ptashne, M. (1985) *Cell 38*, 361−369.
14. Wharton, R. P., and Ptashne, M. (1985) *Nature 316*, 601−605.
15. Carlson, P. A., and Koudelka, G. B. (1994) *J. Bacteriol. 176*, 6907−6914.
16. Whipple, F., Kuldell, N. H., Cheatham, L. A., and Hochschild, A. (1994) *Genes Dev. 8*, 1212−1223.
17. DeAnda, J., Poteete, A. R., and Sauer, R. T. (1983) *J. Biol. Chem 258*, 10536−10542.
18. Bell, C. E., Frescura, P., Hochschild, A., and Lewis, M. (2000) *Cell 101*, 801−811.
19. Ciubotaru, M., Bright, F. V., Ingersoll, C. M., and Koudelka, G. B. (1999) *J. Mol. Biol. 294*, 859−873.
20. Deb, S., Bandyopadhyay, S., and Roy, S. (2000) *Biochemistry 39*, 3377−3383.
21. Whipple, F. W., Hou, E. F., and Hochschild, A. (1998) *Genes Dev. 12*, 2791−2802.
22. Rusinova, E., Ross, J. B. A., Laue, T. M., Sowers, L. C., and Senear, D. F. (1997) *Biochemistry 36*, 12994−13003.
23. Senear, D. F., Laue, T. M., Ross, J. B. A., Waxman, E., Eaton, S., and Rusinova, E. (1993) *Biochemistry 32*, 6179−6189.
24. Pray, T. R., Burz, D. S., and Ackers, G. K. (1998) *J. Mol. Biol. 282*, 947−958.
25. Ptashne, M. (1986) *A Genetic Switch*, Blackwell Press, Palo Alto, CA.
26. Burz, D. S., and Ackers, G. K. (1994) *Biochemistry 33*, 8406−8416.
27. Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
28. Coulandre, C., and Miller, J. H. (1977) *J. Mol. Biol. 117*, 525−567.
29. Bell, A. C., and Koudelka, G. B. (1993) *J. Mol. Biol. 234*, 542−553.
30. Xu, J., and Koudelka, G. B. (1998) *J. Biol. Chem. 273*, 24165−24172.
31. Mikaelian, I., and Sergeant, A. (1992) *Nucleic Acids Res. 20*, 376.
32. Donner, A. L., Carlson, P. A., and Koudelka, G. B. (1997) *J. Bacteriol. 179*, 1253−1261.
33. Wharton, R. P. (1986) *Determinants of 434 Repressor Binding Specificity*, Harvard University, Cambridge.
34. Lakowicz, J. R. (1999) *Principles of Fluorescence Spectroscopy*, Kluwer Academic/Plenum, Norwell, MA.
35. Lakowicz, J. R. (1986) *Methods Enzymol. 131*, 518−567.
36. Johnson, A. D., Meyer, B. J., and Ptashne, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5061−5065.
37. Perrin, F. (1926) *Ann. Phys. 12*, 169.
38. Yguerabide, J., Epstein, H. F., and Stryer, L. (1970) *J. Mol. Biol. 51*, 573−590.
39. Donner, A. L., and Koudelka, G. B. (1998) *J. Mol. Biol. 283*, 931−946.
40. Xu, J., and Koudelka, G. B. (2000) *J. Bacteriol. 182*, 3165−3174.
41. Carlson, P. A. (1994) Ph.D. Thesis, University at Buffalo, Buffalo.
42. Xu, J., and Koudelka, G. B. (2001) *J. Mol. Biol. 309*, 573−587.
43. Rippe, K., von Hippel, P. H., and Langowski, J. (1995) *Trends Biochem. Sci. 20*, 500−506.
44. Courey, A. J. (2001) *Curr. Biol. 11*, R250−R252.
45. van Tilborg, M. A., Bonvin, A. M., Hard, K., Davis, A. L., Maler, B., Boelens, R., Yamamoto, K. R., and Kaptein, R. (1995) *J. Mol. Biol. 247*, 689−700.
46. Dahlman-Wright, K., Wright, A., Gustafsson, J.-Å., and Carlstedt-Duke, J. (1991) *J. Biol. Chem. 266*, 3107−3112.
47. Holmbeck, S. M., Dyson, H. J., and Wright, P. E. (1998) *J. Mol. Biol. 284*, 533−539.
48. Lefstin, J. A., Thomas, J. R., and Yamamoto, K. R. (1994) *Genes Dev. 8*, 2842−2856.
49. van Tilborg, M. A., Lefstin, J. A., Kruiskamp, M., Teuben, J., Boelens, R., Yamamoto, K. R., and Kaptein, R. (2000) *J. Mol. Biol. 301*, 947−958.
50. Kurokawa, R., Soderstrom, M., Horlein, A., Halachmi, S., Brown, M., Rosenfeld, M. G., and Glass, C. K. (1995) *Nature 377*, 451−454.
51. Westin, S., Kurokawa, R., Nolte, R. T., Wisely, G. B., McInerney, E. M., Rose, D. W., Milburn, M. V., Rosenfeld, M. G., and Glass, C. K. (1998) *Nature 395*, 199−202.
52. Monsalve, M., Mencia, M., Rojo, F., and Salas, M. (1996) *EMBO J. 15*, 383−391.
53. Rojo, F., Mencia, M., Monsalve, M., and Salas, M. (1998) *Prog. Nucleic. Acid. Res. Mol. Biol. 60*, 29−46.
54. Scully, K. M., Jacobson, E. M., Jepsen, K., Lunyak, V., Viadiu, H., Carriere, C., Rose, D. W., Hooshmand, F., Aggarwal, A. K., and Rosenfeld, M. G. (2000) *Science 290*, 1127−1131.
55. Carlson, N. G., and Little, J. W. (1993) *J. Mol. Biol. 230*, 1108−1130.
56. Mao, C., Carlson, N. G., and Little, J. W. (1994) *J. Mol. Biol. 235*, 532−544.
57. Liu, Z., and Little, J. W. (1998) *J. Mol. Biol. 278*, 331−338.
58. Ferentz, A. E., Opperman, T., Walker, G. C., and Wagner, G. (1997) *Nat. Struct. Biol. 4*, 979−983.
59. Peat, T. S., Frank, E. G., McDonald, J. P., Levine, A. S., Woodgate, R., and Hendrickson, W. A. (1996) *Nature 380*, 727−730.
60. Luo, Y., Pfuetzner, R. A., Mosimann, S., Paetzel, M., Frey, E. A., Cherney, M., Kim, B., Little, J. W., and Strynadka, N. C. (2001) *Cell 106*, 585−594.

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