DNA Intersegment Transfer, How Steroid Receptors Search for A Target Site*

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The mammalian nucleus contains 6 billion base pairs of DNA, encoding about 100,000 genes, yet in a given cell steroid hormones induce only a handful of genes. The logistical difficulties faced by steroid receptors or other transcription factors of sorting through this much genetic information is further increased by the density of nuclear DNA (approximately 10–50 mg/ml). Standard models propose that steroid receptors find target elements by repeated cycles of dissociation and reassociation until a high affinity site is found (cycling model) and/or by conducting a one-dimensional search along the DNA (sliding model). A third model proposes that steroid receptors search for target sites in the genome by DNA intersegment transfer. In this model, receptor dimers bind nonspecific DNA sequences and search for a target site by binding a second strand of DNA before dissociating from the first, in effect moving through the genome like Tarzan swinging from vine to vine. This model has the advantage that a high concentration of DNA favors, rather than hinders, the search. The intersegment transfer model predicts, in contrast to the cycling and sliding models, that the dissociation rate of receptor from DNA is highly dependent on DNA concentration. We have employed the purified DNA binding domain fragment from the rat glucocorticoid receptor to perform equilibrium and kinetic studies of the DNA dependence of receptor-DNA dissociation. We find receptor dissociation from DNA to be highly dependent on the concentration of DNA in solution, in agreement with the intersegment transfer model. We also find that this interaction is primarily electrostatic, because DNA-like polyanion chains (e.g. heparin and polyglutamate) can mediate the transfer. These studies provide evidence that direct DNA transfer aids the target site search conducted by steroid receptors in their role as inductive transcription factors.

Many eukaryotic transcription factors function by binding to specific DNA sequences upstream of transcription start sites. In order to bind these DNA elements, the protein must first locate these sites in the genome. In humans this search entails locating a few functional binding sites from over 6 billion base pairs of DNA, requiring the protein to sample a vast number of possible binding sites in a very short period of time. Neverthe-

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chosen to study the well-characterized rat glucocorticoid receptor DNA binding domain protein fragment (GRdbd). This fragment, which contains amino acids 440–525 of the rat GR, contains two zinc finger motifs and has strong DNA sequence binding specificity (13). The solution structure of the GRdbd has been determined by NMR, and the crystal structure of the GRdbd bound to DNA has been deduced (14–20). The GRdbd is composed of two zinc finger-stabilized α-helices. The first helix is responsible for specific DNA contacts (14), whereas the second is positioned away from the DNA at right angles to the first helix and making no DNA contacts at a target site. The GRdbd is a monomer in solution and has been shown to bind to DNA both as a monomer and cooperatively as a dimer (15, 21).

This paper addresses not only the question of whether GRdbd, as a model of the steroid receptor family of transcription factors, may employ an intersegment transfer mechanism as part of its target site search strategy but the mechanistic details of this transfer as well. These questions touch directly upon the ability of regulatory proteins to mediate their effects by rapidly locating specific binding sites in the genome.

EXPERIMENTAL PROCEDURES

Materials—Bacterially expressed, purified GRdbd (an 88-residue peptide encompassing the rat GR DNA binding domain from amino acid 440 to amino acid 525) was generously provided by Dr. Len Freedman, Cell Biology and Genetics Program, Sloan-Kettering Cancer Institute, New York, NY. Purified oligonucleotide DNA was purchased from the University of Colorado Cancer Center, Denver, CO, and DNA Express, Colorado State University, Fort Collins, CO. All other reagents were obtained from Sigma, Fisher, and Boehringer-Mannheim unless otherwise noted.

DNA mimics (from Sigma) were heparin (H3125), heparin disaccharide (H9267), poly-l-proline (P2129), poly-l-glutamate (P4886), and 1:1 poly-l-glutamate/glutamate-O-ethyl (P4785).

DNA oligonucleotides:

Full site: 5’ AGGGCGTTTTTTGAGGACAAACTGTCTCCTAAACCCG 3’

GCGAAAACCTTTGTTTGACAAGGATTTTGCGGGGA

SEQUENCE I

Half site: 5’ AGGGCGTTCGGTGCAACTGTCTCCTAAACCCG 3’

GCGAAAACAGTCATCGGACAACGATTTTGGCGGGGA

SEQUENCE II

Nonspecific: 5’ AGGGCGTTTTGTGCACTGAAGACGGTGCTATCTATCTGGGGGA

SEQUENCE III

Gel Mobility Shift Assay—The conditions described by Alroy and Freedman (10) were adapted for these studies. Briefly, complementary strands of oligonucleotide DNA were allowed to slowly anneal for 2 h in TEK buffer from 90°C to room temperature. Annealed oligonucleotides were labeled with 32P-ethyl (P4785).

Establishment of Conditions for Determination of Dissociation Constants—The GRdbd has been shown to exist as a monomer in solution, but it can bind to DNA cooperatively as a dimer (13, 23–28). This is presented schematically in Fig. 1. An intersegment transfer mechanism can be distinguished from cycling via repeated dissociation and reassociation with DNA by assessing whether the dissociation of the bound GRdbd from DNA is influenced by the concentration of DNA in solution.

An intersegment transfer mechanism predicts that increasing DNA will increase the rate of dissociation, whereas increasing DNA will have no effect if a cycling mechanism is used. Experimentally, we measure the loss of the receptor-oligonucleotide complex (R-D) with time at different concentrations of unlabeled DNA. First, GRdbd is bound to labeled DNA to equilibrium, and then disequilibrium conditions are imposed so that reestablishment of R-D occurs. Generally, this is accomplished by blocking reassociation with a large molar excess of unlabeled DNA. However, this strategy is invalid if DNA itself influences the apparent dissociation constant. We have therefore determined the association and dissociation constants for monomer (k1, k–1) and dimer (k2, k–2) and established conditions wherein we can measure the apparent dissociation constant in the presence of different concentrations of DNA.

In Fig. 2 are presented equilibrium binding experiments at GRdbd concentrations ranging from 5 to 250 nM (1–50 ng) and oligonucleotides containing either a full glucocorticoid response or a 10-fold molar excess of the full binding site oligonucleotide to prevent reassociation of the GRdbd. Protein-DNA complexes were resolved on a precast 10% nondenaturing polyacrylamide gel (75.1 acrylamide) and run at 400 V for 2.5 h in 0.5× TBE buffer (40 mM Tris base, 1 mM EDTA, pH 8.0, 40 mM borate) at 0–4°C. There was a delay of ~15 s between dilution of complexes and the time the complexes entered the gel for the nominal zero time point. Gels were fixed for 10 min in 7% acetic acid followed by 10 min in 100% methanol (to prevent cracking). Dried gels were exposed to a phosphorimager screen for 24–48 h, and bound and free DNA was quantified on a Molecular Dynamics PhosphorImager, model 300E (Sunnyvale, CA).

Sheared E. coli DNA—A 50 ml overnight bacterial culture (DH5) in nutrient broth (22) was centrifuged at 3500 rpm (2000 × g) for 10 min to form a pellet. Cells were resuspended in 13.3 ml of TE. To this was added 10% SDS to a final concentration of 0.5%, proteinase K to 200 µg/ml, and RNase to 80 µg/ml. The cells were incubated at 37°C for 1 h, and then NaCl was added to 0.7 M and polyethylene glycol 8000 to 0.5% final concentration; the solution was then shaken at 65°C for 10 min. The mixture was then left at room temperature for 30 min. The resulting DNA was extracted once with an equal volume of chloroform and three times with phenol/chloroform (1:1) and precipitated with 2 volumes of EtOH at −20°C overnight. The DNA pellet was centrifuged for 15 min at 14,000 × g, washed with 70% ethanol, dried, and resuspended in TE. The A260 was determined, and the DNA was diluted to 5 mg/ml. The DNA was sheared by 20 passages through a 25 gauge needle; it ranged in size from 500 to 20,000 bp as determined by gel electrophoresis on a 0.8% agarose gel.

RESULTS

Overall GRdbd Binding Scheme

FIG. 1. Kinetic description of the binding of the GRdbd to DNA and the intersegment transfer mechanism. R, GRdbd protein; D*, labeled specific oligonucleotide; D, unlabeled DNA. Association constants and dissociation constants are indicated lower case. The equilibrium constants are K1 for monomer binding and K2 for dimer binding. The apparent off-rate of GRdbd dimer from D* is a function of k–2 and k–3. In the intersegment transfer model, unlike the cycling model, k–3 is greater than zero.

The abbreviations used are: GRdbd, glucocorticoid receptor DNA binding domain; GR, glucocorticoid receptor; OEt, O-ethyl; TEK buffer, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 100 mM KCl; TE, 1 mM Tris-HCl, pH 8.0, 1 mM EDTA; bp, base pairs.

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element site (right) or a half-site (left). The gel mobility shift assay was used to separate bound complex from free DNA, and each was quantified by phosphorimager analysis. A half-site oligonucleotide was used to measure the equilibrium binding constant $K_1$ for GRdbd monomer binding to DNA. We were able to measure nearly pure monomer complex at concentrations of GRdbd up to 50 nM (10 ng/20 μl). Using the experimentally determined values of free DNA and GRdbd/DNA complex, the equilibrium binding constant $K_1 = [R(D)]/[D][R] = 1.8 \times 10^4$ M$^{-1}$ was derived.

The dimer equilibrium constant $K_2$ was then determined by measuring the overall equilibrium constant from experiments in which GRdbd was bound to a full, palindromic binding site and solving $K_{overall} = K_1K_2 = [R_2D]/[D][R]^2$ for $K_2$. A value of $3 \times 10^8$ M$^{-1}$ was determined for the equilibrium dimer binding constant $K_2$. The observation that $K_2$ is greater than $K_1$ is indicative that dimer binding is cooperative. Data presented in Fig. 2 were used to determine a Hill coefficient (Fig. 3). A coefficient of 1.5 was determined for the binding of the GRdbd to the full-site oligonucleotide. A Hill coefficient for dimer binding of between 1 and 2 is indicative of positive cooperativity (29).

To study dissociation of a binding protein from DNA, reassociation must be prevented. This is often accomplished by adding excess, unlabeled competitor DNA to inhibit reassociation with labeled DNA. However, this will give incorrect estimates of dissociation rates if the added DNA itself influences the apparent rate of dissociation, as would be predicted for an intersegment transfer mechanism. Gel shift experiments are often done in conditions of protein excess to maximize signal. However, this also means that high excesses of unlabeled DNA must be used to soak up the excess binding protein, thereby exacerbating the possibility that the apparent dissociation rates are being influenced by the added DNA. An alternative strategy to prevent reassociation is to greatly reduce the concentration of the bound complex by dilution, shifting the equilibrium to favor dissociation. Experimentally, this is limited by the ability to detect binding in extremely dilute solutions.

We have established that we can readily assess GRdbd binding following a 100-fold dilution of our standard binding condition. To test the extent to which reassociation occurs at this dilution, we mixed DNA and GRdbd at concentrations equivalent to the 100-fold diluted standard condition and showed that some binding was still detectable (about 15% of that seen with undiluted extract). Therefore, some reassociation can occur even after dilution. To prevent this small amount of reassociation, we examined adding a small amount of specific oligonucleotide to the dilution buffer. In a binding reaction in which GRdbd is bound to labeled DNA at a 1:1 molar ratio, addition of an equimolar amount of unlabeled specific oligonucleotide reduces binding by precisely one-half and a 10-fold excess reduces binding by 90%, a concentration at which nonspecific oligonucleotide does not compete (Fig. 4A). In Fig. 4B we show that addition of a 10-fold molar excess of specific oligonucleotide to the dilution buffer completely prevents any reassociation. As subsequent experiments will confirm, addition of this concentration of specific oligonucleotide is still 200-fold below levels at which effects on dissociation rates are observed. Therefore, in all subsequent experiments, dissociation is initiated by 100-fold dilution into a 10-fold excess of specific oligonucleotide. Other nucleic acids or test compounds were added as indicated.

**Dissociation Kinetics of GRdbd/DNA Complex**—Using the dilution condition established above, we examined the dissociation of monomer and dimer GRdbd. As can be seen in Fig. 5, dissociation of monomer from DNA, unlike that of dimer, is quite rapid. Dissociation constants were calculated according to the integrated first-order kinetic equation $-k_{d,t} = \ln(R_t/R_0)$, where $k_{d,t}$ is the dissociation constant, $t$ is time, $R_t$ is the complex remaining at time $t$, and $R_0$ is the initial bound GRdbd (taken as bound counts at time 0). In all of the succeeding figures, dissociation data is presented as $\ln(R_t)$ versus time, and the absolute value of the slope of the best fit line represents the apparent $k_{d,t}$. As shown in Fig. 5, the dissociation rate of monomer GRdbd from the half-site ($k_1$) is $1.2 \times 10^{-3}$ s$^{-1}$ (−88 s), and dimer GRdbd from the full site ($k_2$) is $2.2 \times 10^{-4}$ s$^{-1}$ (−77 min). Thus, the half-life of the monomer complex (ln(2)/$k$) is about 60 s, and for the dimer complex the half-life approaches 60 min.

The association rate for monomer ($k_1$) was too rapid to be measured by gel shift analysis, and because the association rate for dimer ($k_2$) is dependent on the formation of monomer (the GRdbd is a monomer in solution), a direct measurement of association was not possible. However, using the relation between the equilibrium constant and the kinetic constants ($K_1 = k_1/k_{d,t}$, and similarly, $K_2 = k_2/k_{d,t}$) we calculate the values for $k_1$ and $k_2$ as $2.1 \times 10^9$ and $5.6 \times 10^9$ s$^{-1}$ for monomer and dimer, respectively. The equilibrium constant for dimer binding is −17 times greater than that for monomer binding, indicative of strongly cooperative binding. This is despite the fact that association of monomer is 3−4-fold faster than that of dimer. Thus, the cooperative effect can be attributed to stabilization of the DNA-bound complex upon dimer formation that...
results in an almost 60-fold reduction in off-rate compared to monomer.

**Effect of DNA on Dissociation of GRdbd Dimer from DNA**—Having established the kinetics of this system, we then measured the effect of additional DNA on the dissociation rate of dimer. Because the intersegment transfer mechanism predicts that increasing concentrations of DNA should increase the apparent dissociation rate of dimer (in contrast to the cycling model), we tested the ability of sheared *E. coli* DNA and specific or nonspecific oligonucleotides to increase the apparent dissociation rate of GRdbd bound to a specific oligonucleotide. As seen in Fig. 6, increasing amounts of sheared *E. coli* DNA in the dissociation dilution mix increased the measured off-rate from $2.7 \times 10^{-4}$ to $3.6 \times 10^{-3}$ s$^{-1}$ (from $t_{1/2} = 42.8$ min to $t_{1/2} = 3.2$ min) over the concentration range of $0-3.0 \times 10^{-4}$ Mbp (0–200 µg/ml). This is a 13-fold increase in the apparent dissociation rate of GRdbd dimer. The same effect can be demonstrated for specific and nonspecific oligonucleotides (data not shown). Increasing concentrations of oligonucleotide DNA from $1.0 \times 10^{-7}$ to $1.0 \times 10^{-5}$ x increases the off-rate up to $5.8 \times 10^{-3}$ s$^{-1}$ ($t_{1/2} = 2.0$ min) for specific oligonucleotide, and $6.0 \times 10^{-5}$ s$^{-1}$ ($t_{1/2} = 1.9$ min) for nonspecific oligonucleotide. Note that the size of the oligonucleotide used here is 34 base pairs, so the final concentration of added oligonucleotide DNA falls between $3.4 \times 10^{-6}$ and $3.4 \times 10^{-4}$ Mbp (between 2.2 and 220 µg/ml), an effective range similar to that obtained with sheared *E. coli* DNA. These data suggest that the bound GRdbd dimer can transfer between two separate DNA molecules. In addition, the similarity between specific and nonspecific oligonucleotide results suggests that intersegment transfer occurs via non-site-specific binding of DNA. This observation has implications for the binding mechanism employed, an issue addressed further below and under “Discussion.”

**GRdbd Monomers Are Capable of Intersegment Transfer**—Although our initial expectation was that intersegment transfer would occur by a strand invasion mechanism whereby one monomer of a bound dimer would transiently bind two separate molecules of DNA, the failure of specific oligonucleotide to increase the apparent off-rate any differently than nonspecific oligonucleotide or bulk DNA led us to question whether a domain other than the site-specific DNA binding domain was mediating intersegment transfer and therefore whether a monomer was itself capable of intersegment transfer.

To test this hypothesis, the half-site oligonucleotide was used at concentrations determined to permit exclusively monomer formation (see Fig. 2). We observe indications that monomer complex can undergo intersegment transfer even at added DNA concentrations at the low end of those at which dimer dissociation was affected. As shown in Fig. 7, nonspecific DNA increased the monomer dissociation rate to the limit of our systems measurement ($t_{1/2} \approx 20$ s). At DNA concentrations greater than $1 \times 10^{-6}$ Mbp (22 µg/ml), dissociation was faster than could be measured. Similar results were obtained with specific oligonucleotide. These data suggest that monomers as well as dimers of GRdbd can transfer from one DNA strand to another.

**Comparison of Various DNA-like Molecules on Dimer Dissociation Kinetics**—To test the specificity of the transfer mecha-
nism, we employed several compounds that would mimic the long chain polyanionic character of DNA and several control compounds as well (Fig. 8). Three compounds with polyanionic character, heparin, polyglutamate, and ribo-poly(A)-poly(U) could promote dissociation of GRdbd from DNA. However, dissociation was not affected by a neutral macromolecule, polyproline, nor the polyanion subunits, glutamate and heparin disaccharide. Two cations, arginine and spermidine, likewise had no effect on dissociation. These data indicate that the interaction of GRdbd that permits intersegment transfer is primarily electrostatic, requiring a long chain polyanion to promote the dissociation of GRdbd from DNA.

A counterion condensation layer is present around DNA and other long chain polynucleotides of sufficient charge density. Displacement of this charge condensation layer provides an entropic gain for protein binding to DNA. Because the intersegment transfer reaction appears to be via nonspecific interactions with negatively charged polyanions, our results suggest that the counter-ion condensation layer around the DNA may play a role in direct intersegment transfer (see “Discussion”). We wished to test whether there was a density of negative charge necessary to promote the intersegment transfer mechanism. Using poly-Glu and a 1:1 random copolymer of Glu/Glu-OEt, we examined the ability of each compound to mediate intersegment transfer. The results shown in Fig. 9 indicate that the transfer reaction is severely inhibited by the reduction of the average charge of the glutamate backbone by 1/2. Poly-Glu increased the off-rate over 80-fold at the highest concentration tested, whereas the Glu-Glu-OEt copolymer increased it only 3-fold. These data indicate the importance of the density of negatively charged residues along the chain and implicates the need for the formation of a counter-ion condensation layer. The residual effect of the poly-Glu/Glu-OEt may be attributable to the fact that because it is a random copolymer, there will be an occasional region of high charge density due to the clustering of Glu residues in the polymer. Nevertheless, it is clear that charge density is important for the transfer of GRdbd bound to DNA.

**DISCUSSION**

**Cooperative Binding by GRdbd**—The present study confirms earlier work indicating that GRdbd exhibits positive cooperativity in binding to DNA as a dimer. Härä et al. (23) estimated by fluorescent spectroscopy that the cooperative effect was about 10-fold for a somewhat larger GRdbd fragment (115 versus 85 amino acids) binding at high salt conditions (270 mM). More recently, this group updated its findings at lower salt conditions (187 mM), estimating a 25–50-fold cooperativity (17, 18). In our work, the equilibrium constant for dimer binding $K_2$ exceeded that of monomer binding $K_1$ by 15-fold. Moreover, the
kinetic work presented here indicates that the cooperative effect is driven by stabilization of the bound dimer \( t_{1/2} = 60 \text{ min} \) for dimer dissociation versus \( t_{1/2} = 60 \text{ s} \) for monomer dissociation.

**Search Mechanisms in Chromatin**—Since the initial observations by Riggs et al. (2) in the early 1970s that *E. coli* lac repressor associated with a DNA 100-fold faster than simple diffusion would allow (2), a great deal of theoretical and experimental work has been conducted on search mechanisms for binding sites in chromatin. The theoretical framework for diffusion-controlled association of protein with DNA has been detailed extensively (31–34). Based on von Smoluchowski diffusion theory (35), these authors describe the macromolecular and micromolecular interactions that occur when DNA-binding proteins interact with the DNA chain. Briefly, as a DNA-binding protein diffuses through a solution, it undergoes collisions with the DNA chain where nonspecific electrostatic contacts are made (macromolecular collisions). Electrostatic stabilization (with counter-ion release) can then allow the protein to be localized near the DNA chain; this results in multiple short-range contacts (micromolecular collisions). Thus, the protein can sample several binding orientations before diffusing back along the DNA strand before the protein dissociates.

In addition to simple diffusion and sliding, a third mechanism, intersegment transfer, was proposed to facilitate the location of specific sites in the genome. This mechanism envisions that a protein bound to one DNA site transfers directly to a second site via an intermediate stage in which it is transiently bound to two molecules of DNA (5, 8, 34, 37). This strategy reduces the dimensions of the search space and allows the protein to conduct a rapid search for a binding site by maintaining a close association with the DNA matrix. This mechanism also has the advantage that increasing DNA concentration increases the rate of search by enhancing the apparent off-rate of the protein, thus allowing a more rapid sampling of potential binding sites. Solutions to the kinetics equations for a model protein suggest that the rate of site sampling can be increased by 3 orders of magnitude.2

**GRdbd Employs Intersegment Transfer as a Search Mechanism**—Glucocorticoid hormone must initiate a series of steps to convert its receptor to an active transcription factor, including dissociation of the receptor from heat shock proteins, translocation of the receptor to the nucleus, location of binding sites on a target promoter, and assembly of a transcription complex. Glucocorticoids can modify rates of transcription within minutes (38), necessitating that the receptor rapidly locate and bind target sites. Our data are not consistent with the concept that the receptor finds these sites by sampling through repeated rounds of association and dissociation. The intrinsic dissociation rate of GRdbd dimer from DNA is slow; however, the apparent dissociation rate is greatly enhanced by DNA, a result consistent with our interpretation that the GRdbd is employing intersegment transfer as a search mechanism. This enhancement was detected at DNA concentrations ranging from 2 to 200 \( \mu \text{g/mL} \). The concentration of DNA in the nucleus of a eukaryotic cell is estimated at \( \sim 10–50 \text{ mg/mL} \). This presents a physical obstacle to diffusion-controlled search strategies and an aid to an intersegment transfer mechanism. Even acknowledging that a fraction of DNA may be packaged so that it is effectively inaccessible to receptor, intersegment transfer is a particularly effective mechanism for exploiting the circumstances posed by the eukaryotic nucleus in undertaking a search for a target site.

Analysis of the crystal structure of the GRdbd-DNA complex indicates that specific DNA contacts are made via an \( \alpha \)-helical domain present at the C-terminal end of the first zinc coordination finger (14). The intersegment transfer mechanism requires that GRdbd transiently bind two separate molecules of DNA. It is a straightforward assumption that one monomer of a DNA-bound GRdbd dimer could release from its site and bind a second DNA followed by transfer of the other half of the dimer to the second DNA. However, this supposition is inconsistent with the finding that the dissociation from a specific target is enhanced equally by a nonspecific oligonucleotide and by one containing a specific target site. If transfer involves the DNA binding domain that mediates specific binding, one would anticipate that transfer would be less favored if specific contacts

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2 J. R. Cann, unpublished data.
could not be made. Indeed, transfer can be mediated by a variety of polyanions. Moreover, our data indicate that the dissociation of monomer from DNA is enhanced by DNA, suggesting that the monomer itself can transiently bind separate molecules of DNA. Therefore, the GRdbd monomer must posses a second domain capable of nonspecific interaction with DNA. There are two possibilities for candidates for this domain. The crystallography data (14) indicate that the α-helix at the C-terminal end of the second zinc finger makes no DNA contacts and that when the GRdbd is bound to a specific site, the second helix is positioned away from and at right angles to the first helix. A peptide comprising the second zinc finger can bind DNA (39), and helix-disrupting mutations in this helix abrogate function in the context of the intact receptor (40). Thus, this helix is potentially responsible for mediating transfer through nonspecific DNA binding. A second domain that could assist with or mediate nonspecific DNA binding is a highly basic region near the C terminus of the GRdbd that was not localized in the crystal structure. In preliminary studies, a GRdbd fragment lacking this region (courtesy of T. Kerppola) failed to bind DNA.

**Specificity of GRdbd Intersegment Transfer**—During a search for target sites in vivo, a glucocorticoid receptor would first sample and transfer from many nonspecific sites before locating a specific target site. Binding of GRdbd to nonspecific DNA was too low affinity to permit its quantification and therefore assess whether DNA could influence the apparent off-rate. Therefore, the experiments above were conducted by initially binding GRdbd to a palindromic target site. Thus, it is clear that DNA at moderate to high concentrations enhances the apparent off-rate of GRdbd from specific target sites, as well as from nonspecific sites. It is possible that assembly of the receptor into a transcription complex at its target site abrogates intersegment transfer, perhaps because the second DNA binding domain necessary for the transient, simultaneous binding to two DNA molecules is involved in protein-protein interactions in such a complex. Alternatively, cycling on and off a target site may be critical to receptor activation. One can envision mechanisms whereby the receptor acts as a single-shot effector that must be removed and reloaded onto a target site before it is capable of again fulfilling its activation function. In this case, intersegment transfer may be critical not just to the search for target sites but to the transcription activation function of the receptor at the target site as well.

The DNA mimic experiments (Figs. 8 and 9) indicate that there is surprisingly little constraint on the structure of the molecule that will promote dissociation of GRdbd from DNA. RNA, heparin, and polyglutamate all increase the apparent off-rate. This result supports the hypothesis that the second interaction is primarily electrostatic, requiring a long chain polyanion. Decreasing charge density inhibits the ability of the polyanion to mediate the transfer (Fig. 9). Based on theoretical analyses (41, 42), the amount of thermodynamically bound counterions around a long chain molecule (e.g., DNA) is dependent on the average charge separation along the chain. Release of counter-ions from the DNA by protein binding has been shown to create an entropic stabilizing force for nonspecific protein-DNA interactions (41, 42). For double-helical B-DNA this charge separation is 1.7 Å (43), and results in a fractional charge neutralization (\(\psi\)) of 0.88. The structure of poly-Glu is not known, but if we assume an extended chain, then the average charge separation can be represented by the α-carbon of the side chain projected back to a central line along the polypeptide backbone, and the charge separation should be about 3.62 Å (30). This gives a linear charge density of \(\xi \sim 1.96\) (43), and the fractional charge neutralization (\(\psi\)) is \(1 - (1/1.96) = 0.49\), indicating that poly-Glu has the capacity to form a thermodynamically bound ion condensation layer. The substituted polypeptide (1:1 poly-Glu/Glu-OEt, in which half of the charged carboxyl groups have been replaced with an ester linked ethyl group, has an average charge separation of \(\sim 7.2\) Å and thus should have no counter-ion condensation layer associated (\(\xi \sim 1.0; \psi = 0\)) (43). This random copolymer retains some small ability to mediate transfer, probably through scattered regions of clustered Glu residues, where the local charge density is high enough to create regions of organized counterions.

**Dissociation Measurements and Competitor DNA**—Dissociation measurements with the GRdbd also point out a potential problem encountered by experiments designed to measure dissociation rates of DNA-binding proteins. Many researchers use a large excess of specific competitor DNA in their experiments to prevent reassociation of the protein with a labeled specific DNA fragment. Because we have shown that the concentration of competitor DNA added to a protein-DNA binding reaction can potentially affect the rate of apparent dissociation, experiments using excess competitor DNA to measure dissociation must experimentally determine the minimal amount of necessary competitor in order to avoid a systematic error. In our experiments, with the GRdbd at 1:1 molar ratio to DNA, we determined that 2-fold excess specific competitor was sufficient to disrupt the binding equilibrium, and 10-fold excess specific competitor was sufficient to prevent reassociation to labeled DNA (Fig. 4). This latter concentration of specific competitor DNA (50 nm for standard conditions and 1/100 of that for the diluted dissociation conditions) is below that seen to influence the off-rate by intersegment transfer (>10^{-7} m or 2–3 × 10^{-6} Mbp). For example, if a typical experiment using 1 ng of bound 30-mer oligonucleotide was competed with 400-fold competitor, then a 20-μl sample volume would contain 10^{-13} m oligonucleotide (3 × 10^{-6} Mbp), a concentration sufficient to substantially influence the dissociation rate of GRdbd. If the binding reaction contained nonspecific competitor, such as poly(dI-dC), which is often used to reduce the level of nonspecific protein binding from crude protein preparations, then this also could influence the measured equilibrium and dissociation kinetics.

We have presented data that are compatible with the theory that the GRdbd is able to transfer directly from one DNA molecule to another using an intersegment transfer mechanism. We propose that the active full-length GR is able to sample potential target sites rapidly by a similar mechanism. At a target promoter, the association of GR with transcriptional coactivators may stabilize GR dissociation from the site by abrogating intersegment transfer, perhaps because the protein-protein interactions sterically hinder the nonspecific DNA binding site needed to conduct intersegment transfer. Alternatively, receptor could activate transcription by a single-shot mechanism so that rapid transfer from DNA would be required to remove receptor from a target site in order for it to be reloaded and again fired. Although the intersegment transfer mechanism must still be shown with the full-length receptor, we are attracted to the idea of the receptor swinging through the nuclear jungle from DNA to DNA in search of a specific binding site.

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