Published in final edited form as:

J Mol Biol. 2013 November 15; 425(22): . doi:10.1016/j.jmb.2013.07.033.

Dissection of androgen receptor-promoter interactions: Steroid receptors partition their interaction energetics in parallel with their phylogenetic divergence

Rolando W De Angelis, Qin Yang, Michael T Miura, and David L Bain*

Department of Pharmaceutical Sciences, University of Colorado Anschutz Medical Campus, Aurora, Colorado 80045, United States

Abstract

Steroid receptors comprise a homologous family of ligand-activated transcription factors. The members include androgen receptor (AR), estrogen receptor (ER), glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and progesterone receptor (PR). Phylogenetic studies demonstrate that AR, GR, MR and PR are most closely related, falling into subgroup 3C. ER is more distantly related, falling into subgroup 3A. To determine the quantitative basis by which receptors generate their unique transcriptional responses, we are systematically dissecting the promoter-binding energetics of all receptors under a single “standard state” condition. Here we examine the self-assembly and promoter-binding energetics of full-length AR and a mutant associated with prostate cancer, T877A. We first demonstrate that both proteins exist only as monomers, showing no evidence of dimerization. Although this result contradicts the traditional understanding that steroid receptors dimerize in the absence of DNA, it is fully consistent with our previous work demonstrating that GR and two PR isoforms either do not dimerize or dimerize only weakly. Moreover, both AR proteins exhibit substantial cooperativity between binding sites, again as seen for GR and PR. In sharp contrast, the more distantly related ER- dimerizes so strongly that energetics can only be measured indirectly, yet cooperativity is negligible. Thus homologous receptors partition their promoter-binding energetics quite differently. Moreover, since receptors most closely related by phylogeny partition their energetics similarly, such partitioning appears to be evolutionarily conserved. We speculate that such differences in energetics, coupled with different promoter architectures, serve as the basis for generating receptor-specific promoter occupancy and thus function.

Keywords

steroid receptors; thermodynamics; protein-DNA interactions; quantitative footprinting; transcriptional regulation

© 2013 Elsevier Ltd. All rights reserved.

*To whom correspondence should be addressed: David L Bain, PhD, Department of Pharmaceutical Sciences, University of Colorado Anschutz Medical Campus, 12850 E Montview Blvd, Aurora, CO 80045, Phone: 303-724-6118, Fax: 303-724-7266, david.bain@ucdenver.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

The androgen receptor (AR) is a member of the steroid receptor family of ligand-activated transcription factors.¹ The remaining members include the estrogen receptor (ER), glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and the progesterone receptor (PR). ER exists naturally as two isoforms (ER- α and ER- β) as does PR (PR-A and PR-B). Phylogenetic studies indicate that although all receptors descend from a common ER-like ancestor, the family eventually branched into two distinct subgroups.² As shown in Figure 1A, these correspond to subgroup 3A (ER- α and ER- β) and 3C (AR, GR, MR and PR).³ One of the key differences between the subgroups is that the ER isoforms recognize a consensus AGGTCA_{nnn}TGACCT hormone response element (HRE), whereas the more closely related AR, GR, MR and PR recognize a consensus AGAAC_{nnn}TGTTCT.

The generally accepted model of receptor function is that upon binding hormone, the receptors dimerize via their C-terminal hormone-binding domains, bind HREs within upstream promoter sites, and recruit coactivator proteins to activate transcription.¹ Although this model provides a strong qualitative understanding of receptor function, it nonetheless remains incomplete. Specifically lacking is a quantitative framework for considering receptor-specific gene regulation: How does a homologous family of transcription factors, capable of binding identical or nearly identical response elements, regulate different gene networks? Although we have qualitative insight into aspects of this question, a quantitative and mechanistic understanding has yet to be determined.

As a step toward addressing receptor-specific transcriptional behavior, we are systemically dissecting the promoter-binding energetics of all the human steroid receptors. We are attempting to do so at a single “standard state” condition under which the receptors are amenable to rigorous analysis and thus meaningful comparison (pH 8.0, 100 mM NaCl, 4 °C). Our rationale is based on previous work demonstrating that receptor-DNA interaction energetics are the primary determinant of sequence-specific transcriptional activity within the cell.⁴ This finding suggests that energetics should play an important role in generating receptor-specific function as well. However, only by dissecting out each individual energetic contribution to receptor-promoter assembly (receptor dimerization, DNA binding, cooperative stabilization and coactivator recruitment, for example) is it possible to identify which parameter(s) might be most critical to specificity. By comparison, more traditionally measured terms such as an apparent affinity (K_{app}) or Hill Coefficient (n) are composite values typically lacking in molecular insight.

Shown in Figure 1B are representative assembly states and interaction energetics for receptor assembly at a simple promoter containing two hormone response elements (HRE₂). Energetics are expressed as affinity constants to facilitate comparison to other reports. By the traditional model, receptors dimerize in the absence of DNA (k_{dim}) and bind to response elements as pre-formed dimers (k_{int}). These terms – all microscopic interaction constants with clear molecular interpretations – can also be expressed as part of a macroscopic constant, K_{tot} . This parameter describes the total affinity of loading two monomers onto a response element, regardless of whether assembly occurs via a pre-formed dimer as described here or via successive assembly of monomers.⁵ Thus using the values in Figure 1B, $K_{tot} = k_{dim} \cdot k_{int}$. Finally, as shown in Figure 1C, assembly at a multi-site promoter such as HRE₂ may also be coupled to inter-site cooperativity (k_c).

In our earlier studies of steroid receptor assembly at the HRE₂ promoter, we first noticed that dimerization energetics (k_{dim}) vary enormously. For example, if expressed as dissociation constants, ER- α dimerizes with an affinity of 0.35 nM under conditions in which the PR isoforms dimerize with values of 1–2 μ M, or ~ 1,000-fold weaker.⁶⁻⁸

Surprisingly, GR showed no evidence for dimerization, allowing us to place only a lower limit of 100 μM , or at least 100,000-fold weaker than ER- α .⁹ Inter-site cooperative binding (k_c) also varied significantly and trended inversely with dimerization. For example, ER- α exhibits essentially no cooperativity ($k_c = 1.4$) whereas GR and PR-B maintain substantial cooperative stabilization ($k_c = 70$).^{6; 9} Thus, ER- α , a receptor affiliated with subgroup 3A, partitions its interaction energetics in such a way to generate exceptionally strong dimerization but little or no cooperativity. By contrast, receptors in subgroup 3C such as GR and PR-B exhibit distinct behavior – dimerization is weak or non-existent but inter-site cooperativity is strong.

These findings demonstrated that: 1) a homologous family of receptors partitions its promoter-binding energetics quite differently, 2) differences in partitioning span many orders of magnitude, and 3) receptors most closely related in phylogeny partition their energetics similarly. Taken together, these results suggested to us that differences in promoter binding energetics are an evolutionarily conserved feature of the steroid receptor family, and thus critical to receptor-specific function. Such an interpretation further predicts that human AR, a subgroup 3C receptor, should display promoter-binding energetics most similar to those of other 3C receptors and distinct from ER- α . To test this, we rigorously dissected the self-assembly and promoter-binding energetics of full-length human AR under conditions identical to those of our earlier studies. To independently confirm our results, we also analyzed T877A, a well-known functional mutant associated with advanced prostate cancer.^{10; 11} T877A is one of the few mutations found in prostate cancer for which the functional change in mechanism is well understood, and the protein itself is amenable to biochemical and structural analysis.¹²

Using analytical ultracentrifugation, we find that both AR proteins exist only as monomers, showing no evidence of dimerization up to and above micromolar concentrations. Using quantitative footprint titrations, we find that both proteins also exhibit strong inter-site cooperativity. Thus as predicted, AR partitions its promoter-binding energetics in line with other closely related family members. This finding suggests a basis for driving receptor-specific transcriptional control. Specifically, large differences in receptor-specific energetics define a series of receptor-specific energetic profiles; coupling these profiles to different promoter architectures may create a molecular framework for generating receptor-specific promoter occupancy and thus receptor-specific gene regulation. Importantly, such a framework predicts preferential promoter binding even if multiple receptors are competing for identical response elements, as is the case for the 3C subgroup.

Results

Wild-type (wt) AR and T877A were expressed as hexahistidine-tagged proteins in baculovirus-infected Sf9 cells. Each receptor was isolated to greater than 90 % purity as judged by quantification of Coomassie-stained SDS-PAGE (Figure 2A). Average yields were 1 mg/L of cell culture. Since we found that even a single freeze-thaw cycle initiated irreversible AR aggregation and polydispersity, only freshly prepared receptor was used for all analyses. Finally, although we have not directly tested whether the hexa-histidine tag influences AR function, our work on ER- α , GR, and both PR isoforms has shown that neither a FLAG-tag nor His-tag has any effect.^{6; 8; 9; 13-16}

Analytical ultracentrifugation demonstrates that AR exists as a monomer

Sedimentation velocity was first used to characterize the hydrodynamic and self-association properties of wt AR and T877A. Like all other receptors we have examined, AR is most soluble at high salt concentrations. Therefore, we first sedimented both proteins at 500 mM NaCl and over a ten-fold protein concentration range. Shown in Figure 2B are representative

absorbance scans of wt AR at 1.4 μM ; the solid lines represent the best fit by $c(s)$ analysis. Figure 2C shows the corresponding sedimentation coefficient distributions determined for wt AR and T877A at concentrations ranging from 0.14 to 1.4 μM . It is evident that both receptors sediment as a single species regardless of protein concentration, with a buffer and temperature-corrected sedimentation coefficient ($s_{20,w}$) of 4.8 S . The absence of a concentration-dependent change in the sedimentation coefficient indicates neither receptor undergoes reversible self-association over this concentration range. With regard to assembly-state, $c(M)$ analysis of the 4.8 S peak yields an average molecular mass of 94 ± 5 kDa for wt AR and 84 ± 14 kDa for T877A, indicative of AR monomer (calculated molecular mass of 104 kDa).

For both proteins, we also observe two minor peaks at ~ 2.8 and 6.5 S . Each peak represents $\sim 5\%$ of the total $c(s)$ distribution regardless of receptor concentration, indicating that they represent irreversibly formed conformers or aggregate. The 2.8 S species undoubtedly represents a misfolded or partially unfolded monomer, since simple hydrodynamic calculations show that a fully unfolded, random coil AR dimer could sediment no slower than 3.4 S . Using a similar approach, we find that the 6.5 S species must represent a higher order aggregate since it sediments too quickly to represent an AR monomer, regardless of conformation. To test this latter interpretation biochemically, we electrophoresed wt AR under non-denaturing conditions (Figure 2D). As seen by immunoblot analysis, we do indeed detect a high molecular mass species (small arrow) in addition to the primary AR species (large arrow). Quantification of the former species indicates that it represents 15 % of the population, in reasonable agreement with the percentage seen by sedimentation velocity. We address the exact stoichiometry of this larger species in the sedimentation equilibrium studies described below.

To rigorously determine the molecular mass and assembly-state of wt AR and T877A, we carried out sedimentation equilibrium studies under conditions identical to those of sedimentation velocity. Shown in Figure 3A are sedimentation equilibrium scans for wt AR carried out at three concentrations and three rotor speeds. Analogous scans for T877A are shown in Figure 3B. For both receptors, fitting the data either individually or globally to a single species model always resolved a molecular mass slightly above AR monomer. However, the quality of the fit was poor and generated non-random residuals (data not shown). Global fitting using equilibrium self-association models (monomer-dimer, monomer-trimer, etc) resolved similarly poor fits and non-random residuals.

Based on the sedimentation velocity results indicating the presence of an irreversible 6.5 S aggregate, we therefore fit the data to a non-interacting, two species model. (It is unnecessary to account for the 2.8 S partially unfolded monomer, since being the same mass as the 4.8 S monomer it would be invisible by sedimentation equilibrium). As seen by the lines through the data and random residuals, this model readily described all datasets for both receptors. Moreover, for wt AR, the fit resolved a species 1 molecular mass of 104 ± 1 kDa indicative of AR monomer, and a stoichiometry for species 2 of 3.0 ± 0.1 , indicative of AR trimer. An essentially identical result was seen for T877A, resolving a species 1 molecular mass of 104 ± 1 kDa and a stoichiometry of 2.6 ± 0.2 . These results were invariant of analysis regardless of how the data were edited or which subsets of data were analyzed. Thus sedimentation velocity and sedimentation equilibrium studies independently confirm that wt AR and T877A exist only as monomers with a small amount of irreversibly formed trimer – contrary to dogma we find no evidence of AR dimerization.

Low salt sedimentation studies confirm the presence of AR monomer and absence of dimer

To determine AR assembly stoichiometry under our “standard state” low salt conditions, we repeated the sedimentation velocity studies at 100 mM NaCl. Shown in Figure 4 are the $c(s)$ results for wt AR and T877A. For both proteins, the major species now sediments with an $s_{20,w}$ of 5.1 S (wt AR) to 5.0 S (T877A) regardless of receptor concentration. Thus the decrease in NaCl concentration causes AR to undergo slight compaction. It is also evident that the change in buffer conditions also causes the two minor peaks to increase from 5 to 10% of the total $c(s)$ distribution. Their increased presence increases the overall heterogeneity of the AR sample, making $c(M)$ analysis less reliable.^{17; 18} However, assuming the 5.0-5.1 S species is indeed the AR monomer, the frictional coefficient for wt AR is calculated to be 9.5×10^{-8} g/s and 9.6×10^{-8} g/s for T877A. The Stokes radii are 50 Å and 51 Å for wt and T877A, respectively. Modeling the 5.0-5.1 species as a monomeric prolate ellipsoid predicts an axial ratio of 8:1. The high level of asymmetry seen for both proteins has been observed previously for ER-, GR and both PR isoforms;⁶⁻⁹ it is most consistent with biochemical and structural studies demonstrating that the N-terminal regions of these proteins are natively unfolded.¹⁹ Finally, we speculate that the decreased NaCl concentration reduces ionic screening within and between AR monomers, thus promoting aggregation and formation of the secondary species.

To confirm our interpretation that AR is again only monomeric at 100 mM NaCl, we attempted sedimentation equilibrium studies. Under these low salt conditions, wild-type AR slowly and irreversibly aggregates over the multi-day time frame required for sedimentation equilibrium, making it impossible to collect reliable data. We were however able to collect complete data sets for T877A. We again globally fit the data using a non-interacting, two-species model. All data were well described using such an approach, resolving a species 1 molecular mass of 96 ± 2 kDa and a stoichiometry of 5.0 ± 0.3 (see Supplementary Figure 1). However, the data collected at 100 mM NaCl were not as robust as the 500 mM data, thus the stoichiometry of species 2 ranged from three to seven (trimer to heptamer), depending upon how data were edited or which subsets were analyzed. However, there was never a change in the species 1 molecular mass, indicating that under our standard state conditions, T877A exists as a monomer with a slightly increased amount (~10%) of an irreversible, non-dimeric aggregate. Noting that wt AR displayed an essentially identical $c(s)$ distribution, we are confident in stating that it too is a monomer with a similar amount and type of aggregate. A summary of all hydrodynamic parameters for wt AR and T877A is shown in Table 1.

AR assembles onto a two-site promoter with substantial cooperativity

We next investigated the energetics of wt AR and T877A binding to the HRE₂ promoter using quantitative footprint titrations. Shown in Figure 5A is a representative titration of the HRE₂ promoter with wt AR. Receptor binding is highly specific, and sequencing studies confirm that the protected regions correspond to the two HREs. Receptor-induced hypersensitivity adjacent to each site is also apparent. Hypersensitive regions have been observed in our work on the other steroid receptors and are likely due to receptor-induced DNA bending.^{20; 21}

The individual-site binding isotherms for wt AR and T877A assembly at the HRE₂ and HRE₁ promoters are shown in Figure 5B. Our standard approach to quantifying receptor interactions at these promoters has been to resolve the energetics of preformed dimer assembly at each palindromic binding site: As shown schematically in Figure 1B and described in more detail elsewhere¹³, a dimerization constant (k_{dim}) is independently determined by analytical ultracentrifugation and then used as a fixed parameter for the

footprint titration data, allowing resolution of an intrinsic binding affinity (k_{int}). Unfortunately, this approach was not possible here since we observed no evidence of AR dimerization. Nor was a model in which AR monomers sequentially bind to palindromes, since monomer binding to an individual palindrome is highly cooperative. (Fitting the AR:HRE₁ data to the Hill Equation resolved a Hill coefficient of 2.0 ± 0.2 (data not shown)). Instead, we globally fit the HRE₂ and HRE₁ binding isotherms to Eq 5 and 6, allowing us to resolve the total affinity for assembling two AR monomers on a single palindromic HRE regardless of pathway (K_{tot}), and the cooperativity between AR dimers bound to adjacent HREs (k_c).

For wt AR, we found that the receptor assembles onto a palindromic HRE with a K_{tot} of $2.1 \pm 0.2 \times 10^{13} \text{ M}^{-2}$. This corresponds to a binding free energy of $-16.9 \pm 0.1 \text{ kcal/mol}$. By contrast, the apparent affinity of binding (K_{app}) is 220 nM or -8.4 kcal/mol . The reason for this large discrepancy is that K_{app} does not take into account the dimeric stoichiometry of AR assembly at the palindromic HRE. T877A generated comparable results, with a K_{tot} of $9.0 \pm 0.2 \times 10^{12} \text{ M}^{-2}$ or $-16.4 \pm 0.1 \text{ kcal/mol}$. Finally, noting the leftward shift and increased steepness of the HRE₂ isotherms relative to HRE₁, there must be significant cooperativity between binding sites. Global fitting of the wt AR data resolves a cooperativity term (k_c) of 69 ± 15 , which translates to a free energy of $-2.3 \pm 0.1 \text{ kcal/mol}$. For T877A, the value was 96 ± 35 or $-2.5 \pm 0.2 \text{ kcal/mol}$. The energetic parameters for wt AR and T877A promoter binding are summarized in Table 2.

Discussion

Hydrodynamic and thermodynamic properties of human androgen receptor

Here we have examined the physical and functional attributes of human AR using a rigorous and quantitative approach and under a standardized set of conditions. In doing so, we hope to gain insight into the quantitative mechanisms by which steroid receptors generate receptor-specific gene regulation. We first discovered that both wt AR and T877A exist only as monomers, showing no evidence of dimerization up to micromolar concentrations. We note that this result is at odds with the traditional model of receptor function¹, and with early semi-quantitative studies indicating that AR formed disulfide-linked dimers.²² We still detect a small amount (5-10 %) of an irreversible aggregate that corresponds to AR trimer. Notably, we also observe this species using a non-equilibrium assay (native gel electrophoresis) similar to one first used to report the existence of disulfide-linked dimers.²² Indeed, we find that if DTT levels are decreased to less than 1 mM, the percentage of this aggregate increases significantly, indicating that it is disulfide-linked (data not shown). Unfortunately, decreased DTT levels also increase AR polydispersity, making it intractable for rigorous analysis and interpretation. (Freeze-thawing of purified AR generated a similar result, presumably by decreasing DTT activity within the buffer). Taken together, we therefore postulate that the irreversible AR aggregate observed here is functionally irrelevant, and that the monomer should be considered as the active species.

Although the finding that AR does not readily dimerize contradicts previous interpretations, it is fully consistent with crystallographic work demonstrating that the isolated hormone-binding domain – the domain thought to mediate dimerization for other receptors – crystallizes as a monomer.¹² Our findings are also consistent with genome-wide studies indicating that AR, much more so than other receptors, preferentially binds to and transcriptionally activates at half-sites rather than palindromes.²³ Thus under the conditions examined here, AR shows no evidence of reversible self-association. However, processes such as DNA binding, presence of accessory proteins, or molecular crowding events could result in self-association via thermodynamic coupling. With regard to the strong AR cooperativity observed here, a number of biochemical studies have indicated that the

receptor is capable of cooperative interactions²⁴⁻²⁶, thus confirming the results seen here. However, differences in experimental approach, receptor preparation and data analysis make it difficult to directly compare those findings to the values reported here. Finally, as seen in Table 2, wt AR and T877A share essentially identical energetic parameters, suggesting that interaction energetics do not play a role in explaining the unique functional properties of the mutant.¹² However, as evidenced by our 100 mM NaCl sedimentation equilibrium studies, T877A is more stable than wt. This could suggest that another mechanism by which T877A contributes to prostate cancer is via enhanced stability, thus leading to inappropriate persistence of function. This possibility is currently being investigated.

Energetic comparison to other receptors

As outlined in the introduction, we are attempting to systematically dissect the energetics of steroid receptor-promoter interactions under a rigorously controlled set of conditions. For receptors such as ER- and the two PR isoforms, this corresponds to the traditional understanding that monomers dimerize in the absence of DNA (k_{dim}) and assemble as pre-formed dimers capable of engaging in inter-site cooperativity (k_c). These values are plotted in Figure 6. Also shown are the cooperativity terms resolved for wt AR, T877A and GR. However, since these latter receptors do not show any evidence for dimerization, we can only plot lower limits as estimated from our analytical ultracentrifugation studies. For example, noting that our studies should be able to easily detect 10% dimer, and the highest wt AR concentration we examined was 1.4 μM , this places a lower limit for dimerization (as emphasized by the downward arrow) at 14 μM . A similar approach was used for T877A and GR. Note that all parameters are plotted in log units on the y-axis.

Despite being a homologous family of transcription factors, the results in Figure 6 make clear that steroid receptor dimerization and cooperative energetics vary enormously. In particular, dimerization energetics (k_{dim}) are generally inversely proportional to cooperative energetics (k_c). Thus, ER- displays a sub-nanomolar dimerization affinity but generates statistically non-existent cooperativity. At the opposite extreme are the AR proteins and GR, which have dimerization limits that are four to five orders of magnitude weaker than ER- , and cooperativity values two orders of magnitude greater. The second observation is that the inverse relationship between dimerization and cooperativity trends along evolutionary lines. Thus PR-B, wt AR, T887A and GR – all closely related, subgroup 3C receptors – partition their dimerization and cooperative energetics similarly. In sharp contrast, ER- , the more distantly related subgroup 3A receptor, maintains a distinct distribution. (It is also clear that PR-A, a subgroup 3C receptor, is not fully consistent with this argument since it exhibits weak cooperativity. We note that PR-A is still capable of generating 1000-fold cooperative stabilization on a different promoter architecture.²⁷ We find similar promoter-specific differences in cooperativity for other subgroup 3C receptors via manipulation of HRE phasing and periodicity. Moreover, the extent of cooperativity now varies between these receptors, suggesting a critical role for cooperativity in generating receptor-specific transcriptional responses. By contrast, ER- cooperativity is not detectable on seven promoter architectures we have tested to date (Connaghan and Bain, manuscript in preparation)).

The consequences of such large differences in energetics can be better appreciated in Figure 7. Shown are the probabilities of observing the fully ligated HRE₂ promoter (i.e. the presumed transcriptionally active microstate) as a function of total receptor concentration under our standardized conditions. Overlaid are the probabilities of observing receptor dimer, calculated from either an experimentally determined dimerization constant (ER- , PR-A and PR-B) or an estimated lower limit (wt AR, T877A and GR). All probabilities were calculated using the energetics shown in Figure 6 and equations S1 and S2 (see Supplementary Data, Eq 1 and 2). It is immediately obvious that for the subgroup 3A

receptor, ER- α , a significant dimer population exists over the entire concentration range in which receptor binds to the promoter. (Interestingly, the strong dimerization energetics of ER- α predict an abundance of functionally active full-sites rather than half-sites throughout the genome, which indeed is the case.²³) By contrast, for all subgroup 3C receptors, little dimer exists from low to full DNA saturation. Although these results do not allow us to address whether DNA binding occurs via a pre-formed dimer or successive monomer assembly reaction, they nonetheless predict a deficiency of subgroup 3C dimers under *in vitro* DNA binding conditions and raise questions about the relevance (or existence) of such dimers in cellular function.

Has evolution tinkered with the functional energetics of steroid receptors?

Our results demonstrate that steroid receptor family members differentially partition their microscopic interaction energetics, and do so by multiple orders of magnitude. Furthermore, the manner in which energetics are partitioned mirrors the evolutionary divergence of the receptor family. Noting that all the receptors descend from an ER-like ancestral protein, it may be the case that the 3C subgroup receptors (AR, GR, MR and PR) lost the ability to strongly dimerize but gained cooperative binding. This may seem unlikely since sequence-specific transcription factors typically maintain strong dimerization as a tool for increasing DNA binding affinity and specificity. However, a redistribution of interaction energetics may serve a more important role by generating receptor-specific promoter occupancy. For example, simulations⁹ indicate that if a loss in receptor dimerization is compensated by inter-site cooperativity, it is not only possible to maintain promoter occupancy, but also preferential promoter occupancy – that is, occupancy of a particular receptor even in the presence of competitive binding by other receptors with similar DNA binding affinities. Second, experimental studies demonstrate that it is receptor monomers rather than (the previously assumed) dimers that assemble at clusters of half-sites, and that monomer binding is highly cooperative.²⁷ Moreover, multiple types of cooperativity exist – between pairs of monomers, monomers and dimers, and the pairs of dimers seen here. Taken together, these results suggest that receptor assembly-state, promoter binding stoichiometry, and the number and layout of promoter binding sites are critical factors for specifying receptor occupancy at a promoter.

In summary, our findings suggest that nature has selected for receptor-specific differences in energetics as a basis for generating receptor-specific transcriptional activation. Specifically, the combination of different interaction energetics and different promoter architectures may serve as a combinatorial framework for generating receptor-specific promoter occupancy, and thus receptor-specific transcriptional activity. This is an appealing hypothesis for explaining how receptors such as AR, GR, MR and PR can bind identical response elements yet regulate different subsets of genes.²⁸⁻³⁰ Moreover, it is consistent with the recent finding that despite the apparent complexity of gene regulation, DNA binding energetics are the dominant factor in controlling transcriptional activity at different response elements.⁴ Finally, such a hypothesis implies that steroid receptors are energetically poised to carry out function, and predicts a multidimensional “binding affinity landscape” for each receptor.³¹

Materials and Methods

Expression and purification of full-length human AR proteins

An expression plasmid encoding the wild-type version of full-length human AR (residues 1-919) was donated by Dr. Elizabeth Wilson (University of North Carolina, Chapel Hill). The AR-encoding region was PCR amplified and cloned into a pBAC baculovirus vector (EMD, formerly Novagen) encoding an N-terminal hexahistidine tag. Site-directed mutagenesis was then used to create the T877A mutation. Both receptors were expressed in

baculovirus-infected Sf9 insect cells using a multiplicity of infection of 1. 24 hour post-infection, cells were treated with 1 μ M of the synthetic androgen R1881. Cells were harvested 24 h later.

All purification steps were carried out at 4 °C and in the presence of 10 μ M R1881. Cells were Dounce-homogenized in a buffer containing 20 mM Tris (pH 8.0 at 4 °C), 10 % glycerol, 500 mM NaCl, 10 mM β -ME, 10 μ M R1881, 25 mM imidazole and protease inhibitors (Complete, EDTA-free, Roche). Following centrifugation, the AR-containing supernatant (either wild-type or T877A) was incubated for 1 h with Ni-NTA agarose (Qiagen). The AR-bound resin was washed extensively with homogenation buffer and receptor was eluted using the same buffer now containing 250 mM imidazole. Eluted receptor was then chromatographed on a Sephacryl S-400 size exclusion column (GE Healthcare) equilibrated in 20 mM Tris (pH 8.0 at 4 °C), 500 mM or 100 mM NaCl, 1 mM CaCl_2 , 2.5 mM MgCl_2 , 1 mM DTT and 10 μ M R1881. The identity of wt AR and T877A were confirmed by MALDI-TOF mass spectrometry, and concentrations were determined using a calculated extinction coefficient of 93,170 $\text{M}^{-1} \text{cm}^{-1}$.³² Finally, because we found that even a single freeze-thaw cycle generated AR aggregation and polydispersity, only freshly purified protein was used in the experiments shown here.

Sedimentation velocity

Sedimentation velocity experiments were carried out using a Beckman XL-A analytical ultracentrifuge equipped with absorbance optics, using two-sector epon centerpieces and an An-50 Ti rotor. Each AR protein was sedimented in a buffer containing 20 mM Tris (pH 8.0 at 4 °C), 1 mM CaCl_2 , 2.5 mM MgCl_2 , 1 mM DTT, 10 μ M R1881 and NaCl concentrations of either 100 or 500 mM. Other than steroid type, these conditions were identical to our previous work on GR, the two PR isoforms and ER- α .⁶⁻⁹ Receptors were sedimented at concentrations ranging from 0.11 to 1.5 μ M at 50,000 rpm and 4 °C. Samples were monitored at 230 nm and scans were collected as quickly as the instrument would allow. Sedimentation coefficient (*s*) distributions were calculated using the program Sedfit¹⁷ and corrected to standard conditions (water at 20 °C).³³ The frictional coefficient (*f*) was calculated using the Svedberg equation:

$$f = \frac{M(1 - \bar{v}\rho)}{N_A s} \quad (1)$$

Where *M* is the calculated AR monomer molecular mass, $\bar{v}$ is the AR partial specific volume³⁴, ρ is the solvent density³⁵ and N_A is Avogadro's number. The density was calculated from the buffer composition and temperature³⁵, and the partial specific volume was calculated by summing the partial specific volumes of the individual amino acids (0.7171 mL/g for both receptors).³⁴

Sedimentation equilibrium

For T877A, sedimentation equilibrium studies were carried out under the identical 100 and 500 mM NaCl buffer conditions as used in the velocity studies. For wt AR, studies were carried out only at 500 mM NaCl since the receptor slowly and irreversibly aggregates after ~24 hours under low salt conditions. All samples were allowed to reach sedimentation equilibrium using six-channel Epon centerpieces. Wild-type AR was loaded at three concentrations, 0.3, 0.4 and 0.6 μ M. T877A was loaded at 0.3, 0.5 and 1.3 μ M. Samples were equilibrated at 14,000, 17,000 and 21,000 rpm and judged to be at equilibrium by successive subtraction of scans. Data sets were analyzed individually and globally using nonlinear least squares analysis as implemented in the program Scientist 3.0 (Micromath).

Models were constructed using the following equation to resolve the reduced molecular mass (σ_i):

$$Y_r = \delta + \sum_{i=1}^n \alpha_i \exp\left(\sigma_i \frac{r^2 - r_0^2}{2}\right) \quad (2)$$

Where Y_r is the absorbance at radius r , δ is the baseline offset, n is the number of non-interacting species and α_i is the absorbance of the i th species at the reference radius, r_0 . The reduced molecular mass of the i th species is defined as:

$$\sigma_i = \frac{M(1 - \bar{v}\rho)\omega^2}{RT} \quad (3)$$

Where M is the weight-average molecular mass, $\bar{v}$ is the partial specific volume of AR, ρ is the solvent density, ω is the angular velocity, R is the gas constant and T is the absolute temperature.³⁶

DNA preparation for DNase I footprint titrations

A promoter vector encoding two tandemly linked hormone response elements (HRE₂; see Figure 1B) was donated by Dr Kathryn Horwitz (University of Colorado Anschutz Medical Campus). Each HRE corresponds to an imperfect palindrome derived from the tyrosine aminotransferase promoter, AGAACAaggTGTACA³⁷, spaced 25 bp apart. A reduced valency template (HRE₁) containing a C-to-A point mutation in each half site of the distal HRE (designated as site 1) was created in-house. Each template was excised from its respective vector using *HindIII* and *AatII* to generate a 1 kb promoter fragment and end-labeled with ³²P using a Klenow fill-in reaction. The proximal HRE of each fragment (site 2) was positioned 100 bp from the 3' end of the labeled strand. The HRE₂ promoter is identical to that of the previously named GRE₂ and PRE₂ promoters used in our earlier work on GR and the two PR isoforms.^{9; 13; 14} The promoter differs only in response element sequence when compared to the ERE₂ template used in our work on ER- α .⁶

Individual-site binding experiments

DNA-binding experiments were conducted using quantitative DNase I footprint titrations as described by Ackers and co-workers with minor modifications.³⁸⁻⁴⁰ All reactions were carried out in a buffer containing 20 mM Tris (pH 8.0 at 4 °C), 100 mM NaCl, 1 mM CaCl₂, 2.5 mM MgCl₂, 1 mM DTT, 10 μ M R1881, 100 μ g/mL BSA and 2 μ g/mL salmon sperm DNA. Other than BSA and salmon sperm DNA, this buffer is identical to that used in our 100 mM NaCl sedimentation studies. Each reaction mixture contained 15,000 cpm of freshly labeled HRE₁ or HRE₂ promoter. Freshly prepared AR (either wild-type or T877A) was added to each reaction sample, covering a concentration range from nanomolar to micromolar. Samples were allowed to equilibrate at 4 °C for at least 1 h. Promoter DNA concentrations (~10 pM) were estimated to be well below AR DNA binding affinity, justifying the assumption that $AR_{free} \approx AR_{total}$. Denatured DNA fragments were electrophoresed on 6% acrylamide-urea gels and visualized using phosphorimaging. Band densities were determined using the program ImageQuant TL 7.0 (GE Healthcare). Individual-site binding isotherms were calculated as described previously.³⁹

Resolution of microscopic interaction free energies

Footprint titrations resolve the fractional occupancy of binding ($\bar{Y}$) at each HRE. The statistical thermodynamic expressions that describe the resultant individual-site binding isotherms are constructed by summing the probabilities of each microstate that contributes

to binding at each site. A detailed description for constructing these expressions has been presented elsewhere.⁴¹ In short, the probability (f_s) of each microstate is defined as:

$$f_s = \frac{e^{(-\Delta G_s/RT)} x^j}{\sum_{s=1}^j e^{(-\Delta G_s/RT)} x^j} \quad (4)$$

where G_s is the free energy of configuration of energy state s relative to that of the unliganded reference state, x is the free AR monomer concentration, j is the stoichiometry of AR monomers bound to a response element, R is the gas constant and T is the absolute temperature. The relationship between each free energy change and its association constant is described by the equation $G_i = -RT \ln k_i$.

Isotherms were analyzed to resolve the total binding affinity (K_{tot}) for assembling two AR monomers at a palindromic HRE, and the inter-site cooperativity (k_c) between AR dimers bound at separate HREs (see Figures 1B and 1C). Equation 5 describes AR binding by such a model to an HRE₁ isotherm:

$$\bar{Y}_{HRE_1} = \frac{K_{tot} x^2}{1 + K_{tot} x^2} \quad (5)$$

where x is the free AR monomer concentration. Using a similar approach, equation 6 describes AR binding to each isotherm of the HRE₂ promoter:

$$\bar{Y}_{HRE_2} = \frac{K_{tot} x^2 + K_{tot}^2 k_c x^4}{1 + 2K_{tot} x^2 + K_{tot}^2 k_c x^4} \quad (6)$$

To resolve K_{tot} and k_c , the isotherms from all footprint titrations were analyzed globally using Scientist 3.0 (Micromath). The apparent binding affinity (K_{app}) was determined by fitting the HRE₁ isotherms to the Langmuir binding equation:

$$\bar{Y} = \frac{K_{app} x}{1 + K_{app} x} \quad (7)$$

Finally, since receptor binding at specific DNA sites does not result in complete protection from DNase, all data were treated as transition curves ($\bar{Y}_{app}$) fit to upper (m) and lower (b) end points:

$$\bar{Y}_{app} = (m - b) \bar{Y} + b \quad (8)$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Keith Connaghan, James Lambert and N. Karl Maluf for insightful discussions. This work was supported by grants from NIH (DK88843) and the Avon Foundation for Women.

Abbreviations

AR androgen receptor

ER-	estrogen receptor alpha isoform
ER-	estrogen receptor beta isoform
PR-A	progesterone receptor A isoform
PR-B	progesterone receptor B isoform
GR	glucocorticoid receptor
MR	mineralocorticoid receptor
HRE	hormone response element

References

1. Tsai MJ, O'Malley BW. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu. Rev. Biochem.* 1994; 63:451–486. [PubMed: 7979245]
2. Thornton JW. Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions. *Proc. Natl. Acad. Sci. U. S. A.* 2001; 98:5671–5676. [PubMed: 11331759]
3. Nuclear Receptors Nomenclature Committee. A unified nomenclature system for the nuclear receptor superfamily. *Cell.* 1999; 97:161–163. [PubMed: 10219237]
4. Bain DL, Yang Q, Connaghan KD, Robblee JP, Miura MT, Degala GD, Lambert JR, Maluf NK. Glucocorticoid receptor-DNA interactions: Binding energetics are the primary determinant of sequence-specific transcriptional activity. *J. Mol. Biol.* 2012; 422:18–32. [PubMed: 22698871]
5. Wyman, J.; Gill, SJ. Binding and linkage: Functional chemistry of biological macromolecules. University Science Books; Mill Valley, CA: 1990.
6. Moody AD, Miura MT, Connaghan KD, Bain DL. Thermodynamic dissection of estrogen receptor-promoter interactions reveals that steroid receptors differentially partition their self-association and promoter binding energetics. *Biochemistry.* 2012; 51:739–749. [PubMed: 22201220]
7. Connaghan-Jones KD, Heneghan AF, Miura MT, Bain DL. Hydrodynamic analysis of the human progesterone receptor A-isoform reveals that self-association occurs in the micromolar range. *Biochemistry.* 2006; 45:12090–12099. [PubMed: 17002309]
8. Heneghan AF, Berton N, Miura MT, Bain DL. Self-association energetics of an intact, full-length nuclear receptor: The B-isoform of human progesterone receptor dimerizes in the micromolar range. *Biochemistry.* 2005; 44:9528–9537. [PubMed: 15996107]
9. Robblee JP, Miura MT, Bain DL. Glucocorticoid receptor-promoter interactions: Energetic dissection suggests a framework for the specificity of steroid receptor-mediated gene regulation. *Biochemistry.* 2012; 51:4463–4472. [PubMed: 22587663]
10. Gaddipati JP, McLeod DG, Heidenberg HB, Sesterhenn IA, Finger MJ, Moul JW, Srivastava S. Frequent detection of codon 877 mutation in the androgen receptor gene in advanced prostate cancers. *Cancer Res.* 1994; 54:2861–2864. [PubMed: 8187068]
11. Taplin M-E, Bubley GJ, Shuster TD, Frantz ME, Spooner AE, Ogata GK, Keer HN, Balk SP. Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. *N. Engl. J. Med.* 1995; 332:1393–1398. [PubMed: 7723794]
12. Sack JS, Kish KF, Wang C, Attar RM, Kiefer SE, An Y, Wu GY, Scheffler JE, Salvati ME, Krystek SR, Weinmann R, Einspahr HM. Crystallographic structures of the ligand-binding domains of the androgen receptor and its T877A mutant complexed with the natural agonist dihydrotestosterone. *Proc. Natl. Acad. Sci. U. S. A.* 2001; 98:4904–4909. [PubMed: 11320241]
13. Heneghan AF, Connaghan-Jones KD, Miura MT, Bain DL. Cooperative DNA binding by the B-isoform of human progesterone receptor: Thermodynamic analysis reveals strongly favorable and unfavorable contributions to assembly. *Biochemistry.* 2006; 45:3285–3296. [PubMed: 16519523]
14. Connaghan-Jones KD, Heneghan AF, Miura MT, Bain DL. Thermodynamic analysis of progesterone receptor-promoter interactions reveals a molecular model for isoform-specific function. *Proc. Natl. Acad. Sci. U. S. A.* 2007; 104:2187–2192. [PubMed: 17277083]

15. Bain DL, Franden MA, McManaman JL, Takimoto GS, Horwitz KB. The N-terminal region of the human progesterone A-receptor. Structural analysis and the influence of the DNA binding domain. *J. Biol. Chem.* 2000; 275:7313–7320. [PubMed: 10702302]
16. Bain DL, Franden MA, McManaman JL, Takimoto GS, Horwitz KB. The N-terminal region of human progesterone B-receptors: Biophysical and biochemical comparison to A-receptors. *J. Biol. Chem.* 2001; 276:23825–23831. [PubMed: 11328821]
17. Schuck P. Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and lamm equation modeling. *Biophys. J.* 2000; 78:1606–1619. [PubMed: 10692345]
18. Philo JS. A method for directly fitting the time derivative of sedimentation velocity data and an alternative algorithm for calculating sedimentation coefficient distribution functions. *Anal. Biochem.* 2000; 279:151–163. [PubMed: 10706784]
19. Kumar R, McEwan IJ. Allosteric modulators of steroid hormone receptors: Structural dynamics and gene regulation. *Endocr. Rev.* 2012; 33:271–299. [PubMed: 22433123]
20. Petz LN, Nardulli AM, Kim J, Horwitz KB, Freedman LP, Shapiro DJ. DNA bending is induced by binding of the glucocorticoid receptor DNA binding domain and progesterone receptors to their response elements. *J. Steroid Biochem.* 1997; 60:31–41.
21. Nardulli AM, Greene GL, Shapiro DJ. Human estrogen receptor bound to an estrogen response element bends DNA. *Mol. Endocrinol.* 1993; 7:331–40. [PubMed: 8483477]
22. Liao M, Zhou Z.-x. Wilson EM. Redox-dependent DNA binding of the purified androgen receptor: Evidence for disulfide-linked androgen receptor dimers. *Biochemistry.* 1999; 38:9718–9727. [PubMed: 10423251]
23. Tang Q, Chen Y, Meyer C, Geistlinger T, Lupien M, Wang Q, Liu T, Zhang Y, Brown M, Liu XS. A comprehensive view of nuclear receptor cancer cisomes. *Cancer Res.* 2011; 71:6940–6947. [PubMed: 21940749]
24. Cleutjens KBJM, van Eekelen CCEM, van der Korput HAGM, Brinkmann AO, Trapman J. Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter. *J. Biol. Chem.* 1996; 271:6379–6388. [PubMed: 8626436]
25. Huang W, Shostak Y, Tarr P, Sawyers C, Carey M. Cooperative assembly of androgen receptor into a nucleoprotein complex that regulates the prostate-specific antigen enhancer. *J. Biol. Chem.* 1999; 274:25756–25768. [PubMed: 10464314]
26. Kasper S, Rennie PS, Bruchovsky N, Sheppard PC, Cheng H, Lin L, Shiu RP, Snoek R, Matusik RJ. Cooperative binding of androgen receptors to two DNA sequences is required for androgen induction of the probasin gene. *J. Biol. Chem.* 1994; 269:31763–31769. [PubMed: 7989349]
27. Connaghan-Jones KD, Heneghan AF, Miura MT, Bain DL. Thermodynamic dissection of progesterone receptor interactions at the mouse mammary tumor virus promoter: Monomer binding and strong cooperativity dominate the assembly reaction. *J. Mol. Biol.* 2008; 377:1144–1160. [PubMed: 18313072]
28. Monroe DG, Getz BJ, Johnsen SA, Riggs BL, Khosla S, Spelsberg TC. Estrogen receptor isoform-specific regulation of endogenous gene expression in human osteoblastic cell lines expressing either ER α or ER β . *J. Cell. Biochem.* 2003; 90:315–326. [PubMed: 14505348]
29. Richer JK, Jacobsen BM, Manning NG, Abel MG, Wolf DM, Horwitz KB. Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. *J Biol. Chem.* 2002; 277:5209–5218. [PubMed: 11717311]
30. Wan Y, Nordeen SK. Overlapping but distinct gene regulation profiles by glucocorticoids and progestins in human breast cancer cells. *Mol. Endocrinol.* 2002; 16:1204–1214. [PubMed: 12040008]
31. Segal E, Widom J. From DNA sequence to transcriptional behaviour: A quantitative approach. *Nat Rev Genet.* 2009; 10:443–456. [PubMed: 19506578]
32. Gill S, von Hippel P. Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* 1989; 182:319–326. [PubMed: 2610349]
33. Van Holde, KE. Physical biochemistry. Prentice-Hall; Englewood Cliffs, CA: 1971.
34. Cohn, EJ.; Edsall, JT. Proteins, amino acids and peptides. Reinhold; New York: 1943.

35. Laue, TM.; Shah, BD.; Ridgeway, TM.; Pelletier, SL. Analytical ultracentrifugation in biochemistry and polymer science. Harding, SE.; Rowe, AJ.; Horton, JC., editors. Royal Society of Chemistry; Cambridge: 1992.
36. Laue TM. Sedimentation equilibrium as thermodynamic tool. *Methods Enzymol.* 1995; 259:427–452. [PubMed: 8538465]
37. Jantzen HM, Strahle U, Gloss B, Stewart F, Schmid W, Boshart M, Miksicek R, Schutz G. Cooperativity of glucocorticoid response elements located far upstream of the tyrosine aminotransferase gene. *Cell.* 1987; 49:29–38. [PubMed: 2881624]
38. Brenowitz M, Senear DF, Shea MA, Ackers GK. Footprint titrations yield valid thermodynamic isotherms. *Proc. Natl. Acad. Sci. U. S. A.* 1986; 83:8462–8466. [PubMed: 3464963]
39. Brenowitz M, Senear DF, Shea MA, Ackers GK. Quantitative DNase footprint titration: A method for studying protein-DNA interactions. *Methods Enzymol.* 1986; 130:132–181. [PubMed: 3773731]
40. Connaghan-Jones KD, Moody AD, Bain DL. Quantitative DNase footprint titration: A tool for analyzing the energetics of protein-DNA interactions. *Nat. Protocols.* 2008; 3:900–914.
41. Hill, TL. An introduction to statistical thermodynamics. Dover Publications; New York: 1960.

Highlights

- Human androgen receptor (AR) is a ligand-activated transcription factor
- Promoter binding energetics of AR and the T877A mutant were dissected
- Energetic distributions were similar to other closely-related steroid receptors
- Distributions were opposite to that of more distantly related receptors
- Steroid receptors partition their energetics in parallel with their phylogeny

Figure 1. Steroid receptor phylogenetic tree and promoter binding energetics

(A) Phylogenetic tree representing divergence of the steroid receptor family. Filled circle represents the ER-like common ancestor for subfamily 3A (ER- α and ER- β) and subfamily 3C (PR, AR, GR and MR). The two PR isoforms are not shown since they are generated from the same gene via alternative transcriptional or translational start sites. (B) Representative dimer-binding pathway for receptor-HRE₂ assembly. Dimers are formed through microscopic equilibrium constant k_{dim} and pre-formed dimers bind response elements with intrinsic affinity k_{int} . Schematic representing the overall reaction for receptor monomer assembly at a palindromic response element regardless of pathway. Total affinity is represented by K_{tot} . (C) Potential inter-site cooperativity is represented by k_c .

Figure 2. Purification of full-length human AR and characterization using sedimentation velocity and native-PAGE

(A) Baculovirus-expressed wt AR and T877A were purified as described in Materials and Methods. Purified receptors were resolved using 10 % SDS-PAGE and Coomassie-Blue staining. Molecular mass markers are indicated on left. (B) Sedimentation velocity data for 1.4 μ M wt AR in 500 mM NaCl. Open circles represent absorbance data collected at 230 nm as a function of time and radial distance. Solid lines represent best fit from $c(s)$ analysis as implemented in the program Sedfit.¹⁷ For clarity, only every seventh data point is displayed. (C) $c(s)$ distributions for three concentrations of wt AR and T877A in 500 mM NaCl. Thick solid line (1.4 μ M), dashed line (0.7 μ M) and thin solid line (0.14 μ M). (D) Immunoblot of wt AR using native-PAGE. Lower molecular mass species is indicated by large arrow; larger mass species indicated by small arrow.

Figure 3. Sedimentation equilibrium analysis of wt AR and T877A in 500 mM NaCl

(A) wt AR sedimentation equilibrium data plotted as absorbance versus $r^2/2$ for three different concentrations of AR. From left to right: 0.6 μM , 0.4 μM , and 0.3 μM . Symbols represent three different rotor speeds: 14,000 rpm (open circles), 17,000 rpm (inverted triangles), and 21,000 rpm (open squares). Solid lines represent best global fit to a two species non-interacting model. Standard deviation of global fit was 0.0039 absorbance units. Residuals are plotted below the data and best-fit lines. For clarity, only every third data point is displayed. (B) T877A sedimentation equilibrium data plotted as absorbance versus $r^2/2$ for three different concentrations. From left to right: 1.3 μM , 0.5 μM , and 0.3 μM . Symbols, corresponding rotor speeds and solid lines are identical to those described above. The standard deviation of global fit was 0.0043 absorbance units. Residuals are plotted below. For clarity, only every third data point is displayed.

Figure 4. Sedimentation velocity analysis of wt AR and T877A in 100 mM NaCl
(A) $c(s)$ distributions for three concentrations of wt AR (top). Thick solid line (1.4 μM), dashed line (0.7 μM) and thin solid line (0.14 μM). (B) $c(s)$ distributions for three concentrations of T877A. Thick solid line (1.1 μM), dashed line (0.7 μM) and thin solid line (0.11 μM).

Figure 5. Quantitative DNase footprint titrations and individual site-binding isotherms of wt AR and T877A assembly at the HRE₂ promoter

(A) Representative autoradiogram of wt AR binding at the HRE₂ promoter in 100 mM NaCl. AR concentration increases from left to right. Positions of site 1 (solid rectangle) and site 2 (open rectangle) are depicted to right. (B) Individual site-binding isotherms constructed from analysis of wt AR and T877A footprint titration images. Filled red squares represent binding to site 1 and open red squares represent binding to site 2 of the HRE₂ promoter. Open blue circles represent binding to site 2 of the HRE₁ promoter. Red and blue lines represent best global fits to all isotherms using Eqs 5 and 6. The fit lines for each HRE₂ site overlay since the sequences of sites 1 and 2 are identical.

Figure 6. Microstate energetics of steroid receptor dimer assembly at the HRE₂ promoter
 Circles represent receptor dimerization affinity (k_{dim}) and triangles represent inter-site cooperativity (k_c). Since dimerization was not observed for wt AR, T877A and GR, downward arrows have been added to indicate that plotted values represent lower limits. Error bars represent 67% confidence intervals.

Figure 7. Probabilities of receptor dimer formation and fully-ligated HRE₂ promoter microspecies under standard state conditions

Probabilities determined from experimentally measured energetics or lower limit estimates. Blue lines represent the probabilities of receptor dimers and red lines represent the probabilities of the fully-ligated HRE₂ promoter. Because dimers have not been observed for wt AR, T877A and GR, blue lines for these proteins represent limits.

Table 1

Wild-type AR and T877A hydrodynamic parameters

	<i>500 mM NaCl</i>		<i>100 mM NaCl</i>	
	<i>WT</i>	<i>T877A</i>	<i>WT</i>	<i>T877A</i>
$S_{20,w}$ (S)	4.8	4.8	5.1	5.0
f (g/s)	1.0×10^{-7}	1.0×10^{-7}	9.5×10^{-8}	9.6×10^{-8}
f/f_0	1.53	1.51	1.44	1.45
Stokes radius (Å)	53	53	50	51
axial ratio	10:1	10:1	8:1	8:1
molecular mass (kDa) ^a	94 ± 5	84 ± 14	N/A	N/A
molecular mass (Da) ^b	$103,629 \pm 1,846$	$104,041 \pm 1,657$	N/A	$96,191 \pm 2,194$

^a Average and standard deviation estimated by $c(M)$ analysis of the major peak as implemented in Sedfit.

^b Resolved molecular mass from sedimentation equilibrium.

Table 2Microstate energetics of wt AR and T877A assembly at the HRE₂ promoter^a

	<i>wt AR</i> (kcal/mol)	<i>T877A</i> (kcal/mol)
G _{total}	-16.9 ± 0.1	-16.4 ± 0.1
G _c	-2.3 ± 0.1	-2.5 ± 0.2
SD of fit ^b	0.043	0.060

^aValues calculated using the relationship $G_i = -RT \ln(k_i)$. Errors represent one SD reported from the program Scientist.^bSD of global fit in units of fractional saturation.