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DNA recognition by thyroid hormone and retinoic acid receptors: 3-4-5 rule modified

Theresa Q. Phan¹, Margaret M. Jow^{1,2}, and Martin L. Privalsky^{1,*}

¹Department of Microbiology College of Biological Sciences University of California at Davis

²Currently at the Department of Cell and Molecular Biology San Francisco State University

Abstract

It has been proposed that retinoic acid receptors (RARs) and thyroid hormone receptors (TRs) both bind to AGGTCA “half-site” sequences, but distinguish their different target genes by recognizing different half-site spacings. We report here that artificial DNA binding sites based on these AGGTCA half-sites confer high affinity, but poor specificity, and that spacing alone does not account for the divergent DNA recognition properties of TRs and RARs. Instead, we have determined that the non-consensus half-sites that are present in naturally occurring RAR and TR target genes play a crucial role in defining receptor DNA recognition specificity, and work together with flanking sequences and half-site spacing to produce receptor-specific DNA binding *in vitro*. We also provide evidence that auxiliary proteins in cells generate an additional layer of receptor-specific target gene recognition, in part by destabilizing the binding of nuclear receptors to the “wrong” response elements.

Keywords

TRs; RARs; DNA recognition; half-site; DNA binding; nuclear receptor

1. INTRODUCTION

Nuclear receptors are ligand-regulated transcription factors that bind to specific DNA sequences (denoted hormone response elements) and modulate the expression of adjacent target genes [Escriva et al., 2004, Flamant et al., 2006, Germain et al., 2006, McEwan, 2009]. The nuclear receptor family includes endocrine receptors, such as the thyroid hormone receptors (TRs), retinoic acid receptors (RARs), estrogen receptors (ERs), vitamin D3 receptors (VDRs), glucocorticoid receptors (GRs) and androgen receptors (ARs), as well as receptors that sense metabolic lipids, respond to xenobiotics, or have no known ligand. Many nuclear receptors can both activate and repress target gene expression by alternatively tethering accessory proteins denoted coactivators and corepressors [McKenna and O'Malley, 2002, Privalsky, 2008, Tsai and Fondell, 2004]. Coactivators are typically recruited in response to ligand agonists, and include acetyltransferases, methyltransferases, chromatin remodelers, and components of the general transcriptional machinery [McKenna

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*To whom correspondence should be addressed: Address: Department of Microbiology One Shields Avenue University of California at Davis Davis, CA 95616 **Phone:** (530) 752-3013 **Fax:** (530) 752-9014 mlprivalsky@ucdavis.edu .

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and O'Malley, 2002, Privalsky, 2008, Tsai and Fondell, 2004]. Corepressors typically associate with unliganded nuclear receptors or in response to ligand antagonists, and mediate effects opposite to those of the coactivators [McKenna and O'Malley, 2002, Privalsky, 2008, Tsai and Fondell, 2004].

Despite the diversity of their ligands, virtually all members of the nuclear receptor family possess a common zinc-finger DNA binding domain comprised of two α -helical domains that are folded and stabilized through interactions with coordinated zinc ions (reviewed in [Claessens and Gewirth, 2004, Khorasanizadeh and Rastinejad, 2001]). The "P-box" amino acids in the first α -helical domain make major groove contacts with a hexanucleotide sequence, denoted a half-site, on the target DNA. Most nuclear receptors contact their DNA binding sites as protein dimers; therefore two half-sites are required to create a functional hormone response element (e.g. [Brent et al., 1992, Forman et al., 1992, Hirst et al., 1992, Kurokawa et al., 1993, Lazar et al., 1991, Näär et al., 1991, Perlmann et al., 1993, Umesono et al., 1991, Wahlstrom et al., 1992]). The second α -helical domain in the zinc-finger region stabilizes the overall structure and can also contribute to receptor dimerization. In several nuclear receptors a third α -helical domain (the "A/T box") C-terminal of the zinc-finger domain interacts with the minor groove of the DNA to recognize nucleotides flanking the core half-site sequence [Claessens and Gewirth, 2004, Khorasanizadeh and Rastinejad, 2001].

Idealized half-sites have been elucidated experimentally for many nuclear receptors, but surprisingly tend to distribute into just two generic groupings: AGGTCA (e.g. for RARs, TRs, and ERs) and AGAACA (e.g. for ARs and GRs) [Claessens and Gewirth, 2004, Khorasanizadeh and Rastinejad, 2001]. Similarly, the P-box motifs within the nuclear receptors parallel this binary grouping of the DNA sequences they contact, and are typically comprised of EGCKG or GSCKV sequences, respectively [Claessens and Gewirth, 2004, Escriva et al., 2004, Flamant et al., 2006, Germain et al., 2006, Khorasanizadeh and Rastinejad, 2001, McEwan, 2009]. Given vertebrates encode approximately 50 different nuclear receptors, each with its own characteristic panel of target genes, this raises the question of how adequate target gene specificity is achieved by the different nuclear receptors. A paradigm has emerged proposing that it is the spacing of the half-sites in a response element that defines its specificity [Forman et al., 1992, Kurokawa et al., 1993, Näär et al., 1991, Perlmann et al., 1993, Umesono et al., 1991, Vivanco Ruiz et al., 1991]. In this model, VDRs, TRs, and RARs recognize direct repeats of the same AGGTCA half-sites, but require different spacings between the half-site (a DR3, DR4, or DR5 respectively). Orientation of the half-sites can also play a role, ERs, for example, binds to inverted AGGTCA repeats with a 3 base spacer [Claessens and Gewirth, 2004].

Although this "3, 4, 5 rule" provides an extremely important insight, it falls short of fully explaining the specificity of DNA recognition and target gene regulation by the nuclear receptors. Naturally-occurring hormone response elements can contain half-sites that diverge significantly from the AGGTCA consensus sequences that helped define the spacing rule (e.g. [Williams and Brent, 1995]). Reciprocally, many nuclear receptors are unexpectedly promiscuous in their abilities to recognize half-sites displayed in a variety of spacings or orientations (e.g. [Forman et al., 1992, Hua et al., 2009, Lazar et al., 1991]). Further, as additional nuclear receptors have been identified, the number of possible half-site topologies has proven inadequate to conceptually assign each to a specific receptor.

We therefore performed a detailed study on the DNA recognition properties of TRs and RARs, two receptors with different physiological functions and distinct target gene repertoires in cells, yet with closely related response elements as defined by the 3,4,5 rule [Forman et al., 1992, Kurokawa et al., 1993, Näär et al., 1991, Perlmann et al., 1993,

Umesono et al., 1991, Vivanco Ruiz et al., 1991]. We report that the experimentally derived AGGTCA half-site creates high affinity, but low specificity DNA binding sites that fail to adequately discriminate between TRs and RARs whether presented as a DR4 or a DR5. Instead, it is the non-consensus half-sites found in physiologically-relevant hormone response elements that are essential for receptor specificity, with TRs preferentially binding response elements comprised of AGGACA, and RARs preferentially binding response elements comprised of AGTTCA. DNA sequences flanking these half-sites, and half-site spacing, further contribute to the specificity of DNA binding by these receptors in a combinatorial fashion. Interestingly, only a subset of the DNA elements that bind a given receptor *in vitro* mediate transcriptional regulation *in vivo*, and we provide evidence for the ability of auxiliary cellular proteins to destabilize the binding of nuclear receptors to the “wrong” DNA response elements. Our results help clarify how different nuclear receptors mediate their different biological roles.

2. MATERIALS AND METHODS

2.1. Oligonucleotides and molecular clones

Annealed oligonucleotides representing either single (for EMSA) or double (for luciferase assays) copies of each response elements were designed to incorporate either a 5' *XhoI/SalI* (5'-tcga-3') or *HinDIII* (5'-agct-3') overhang to facilitate both radiolabeling with Klenow polymerase and cloning into reporter vectors. The original M-pTK-Luciferase reporter was digested with *HinDIII* and the direct-repeat-4 element was discarded prior to introduction of the response elements described here [Vivanco Ruiz et al., 1991]. The expression vectors for wild-type human TR α and RAR α were described previously [Hauksdottir and Privalsky, 2001, Rosen and Privalsky, 2009]. All constructs were verified by DNA sequencing.

2.2. Protein Expression

Native, full-length human TR α 1, RAR α , RXR α were expressed using a recombinant baculovirus system [Chen and Privalsky, 1993]. Briefly, 1.0×10^7 Sf9 cells were infected at high multiplicity with the recombinant baculovirus of interest. After 48 h incubation, infected Sf9 cells were washed in Dulbecco's Phosphate-Buffered Saline (Mg⁺⁺ and Ca⁺⁺ free, Gibco Inc, Grand Island NY) and harvested. The infected Sf9 cells were incubated on ice for 15 min. in 40 μ l Buffer A [10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA (pH 8.0), 0.1 mM EGTA (pH 8.0), 1mM DTT and 1x Complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN)]. NP-40 was added to 0.6% to lyse the cells, the nuclei were pelleted and were resuspended in 20 mM HEPES (pH 7.9), 400 mM KCl, 0.1 mM EDTA (pH 8.0), 0.1 mM EGTA (pH 8.0), 10% glycerol, 1mM DTT and 1x Complete protease inhibitor cocktail. After 15 min of incubation on ice with shaking, the nuclear extracts were cleared by centrifugation for 10 min at 20,800 x g at 4° C. Receptor yield was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot as described previously [Hauksdottir et al., 2003], using bacterially expressed GST-TR α or GST-RAR α as concentration standards. CV-1 cell nuclear extracts were prepared in a similar manner, except they were resuspended in Buffer A and were lysed by Dounce homogenization instead of NP-40. The lysates were incubated on ice for 25 min before centrifugation at 5000 rpm for 5 min. to isolate the nuclei. The pellet was resuspended in 20 mM HEPES (pH 7.9), 420 mM KCl, 25% glycerol, 1.0 mM EDTA (pH 8.0), 1mM DTT and 1x Complete protease inhibitor cocktail and incubated on ice for 45 min. The nuclear extracts were then cleared by centrifugation at 20,800 x g for 20 min at 4° C prior to use.

GST proteins employed as protein standards (GST-TR α , GST-RAR α) or for EMSA supershift (a GST-SRC1 representing codons 568-891, or a GST-CBP representing codons

1-451) were isolated from transformed *Escherichia coli* strain BL-21 by a modification of [Guan and Dixon, 1991]. Protein yield was quantified by SDS-PAGE and Coomassie Brilliant Blue staining, using a Fluorochem 8900 densitometer and AlphaEaseFC software (Alpha Innotech Corp., San Leandro, CA). Defined domains of these coactivators were used in these experiments because the high molecular weight of the native SRC1 and CBP proteins makes obtaining full-length products problematic in most recombinant expression systems.

2.3. Electrophoretic Mobility Shift Assays (EMSA)

Oligonucleotides representing the different response elements were annealed to form double-stranded DNA and were radiolabeled using Klenow polymerase with ^{32}P - α -deoxy-GTP (3000 Ci/mMol). EMSAs were performed by combining 2 μl nuclear extract and 4 μl radiolabeled probe (200,000 cpm, 4 pmol total DNA) in binding buffer and incubating at 25° C for 15 min. For supershift experiments, recombinant SRC1 or CBP proteins were added after the DNA probe and receptor preparations were mixed. Binding buffer for receptor homodimers contained 7.5 mM Tris-HCl (pH 7.5), 2.3% glycerol, 10 mg/ml bovine serum albumin, 200 mM KCl, 1.5 mM MgCl_2 , 0.66 μg poly(deoxyinosine-deoxycytosine), and 2 μM dithiothreitol. Binding buffer for receptor heterodimers contained 20mM HEPES, 80 mM KCl, 2.5 μg poly(deoxyinosine-deoxycytosine), 1 μM dithiothreitol, 0.1% NP-40, and 10% glycerol as previously described [Umesono et al., 1991]. This buffer was employed to allow a better comparison of heterodimer formation in our study with heterodimer formation analyzed in the Umezono et al. publication; homodimer formation was essentially the same in either buffer system. The resulting DNA-protein complexes were resolved by electrophoresis in a 5% polyacrylamide (29:1 acrylamide/bisacrylamide) gel buffered with 0.5x TBE (45mM Tris-borate, 1mM EDTA) at 180V for 90 min (5% glycerol was included in the polyacrylamide gel for the receptor heterodimer assays). Free and bound DNA probe were visualized by STORM PhosphorImager analysis and quantified using ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA). Probe input for each experiment was arbitrarily defined as 1 unit. For EMSA supershifts, the coactivator of interest was added to the binding buffer and nuclear extracts and was incubated for 15 min at 25° C prior to gel electrophoresis; 1 μM triiodothyronine (T3), *all-trans* retinoic acid (ATRA), or LG69, or an equivalent volume of ethanol carrier, were added to the binding reaction as indicated.

2.4. Reporter gene assays

CV-1 cells were plated in 24-well culture plates (3×10^5 /well) in Dulbecco's modified Eagle medium formulated with high glucose, L-glutamine (DMEM; Invitrogen, Carlsbad, CA), and 5% heat-inactivated fetal bovine serum (HI-FBS; Hyclone, Logan, UT). Cells were maintained at 37° C in a humidified 5% CO_2 atmosphere. Twenty-four hrs after plating, the medium was replaced with DMEM containing 5% hormone stripped HI-FBS; 0.5 ng of pSG5-TR α , pSG5-RAR α , or an empty pSG5 control were introduced per well together with 200 ng of luciferase reporter and 50 ng of a pCH110- β -galactosidase vector using Effectene and the manufacturer's protocol (QIAGEN, Valencia, CA). The medium was replaced 24 hrs later with fresh medium containing 1 μM T3-thyronine, *all-trans* retinoic acid (ATRA), or ethanol-vehicle alone and the incubation continued for an addition 24 h before harvesting. Absolute luciferase activity was measured using a Promega luciferase assay system (Promega, Madison, WI) and a TD 20/20 luminometer (Turner Design, Sunnyvale, CA). Relative luciferase was calculated by normalization to β -galactosidase activity, measured using chlorophenol-red- β -D-galactopyranoside (CPRG) substrate (Roche, Indianapolis, IN) and a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale CA).

3. RESULTS

3.1. AGGTCA half-sites confer high affinity but low specificity receptor binding, whereas naturally-occurring half-sites confer much higher specificity

We first explored the ability of TR α or RAR α to bind to artificial DR4 and DR5 response elements comprised of AGGTCA consensus half-sites (Fig. 1A). We used an electrophoretic mobility shift assay (EMSA) and a range of TR α and RAR α concentrations; the DR4 and DR5 DNA elements were identical except for their spacing (Fig. 1B). Both TR α and RAR α bound strongly, but near equally, as homodimers to both the DR4 and DR5 elements (Fig. 1B and quantified in Fig. 1C; the identity of these complexes was previously confirmed by antibody supershift). A similar lack of specificity was observed whether TR α and RAR α were isolated from recombinant baculovirus-infected Sf9 cells or from transfected mammalian cells, and over a series of buffer conditions, running temperatures, and poly dI-dC concentrations (data not shown). Neither TR α nor RAR α bound to a GR response element (an AGAATA inverted repeat 0) and no mobility shifts were observed using Sf9 lysates infected by a non-recombinant baculovirus vector (Figure 1B and data not shown).

We next turned to two naturally-occurring response elements, a TR binding site in the α MHC promoter (a DR4) and an RAR binding site in the β RARE promoter (a DR5) [Brent et al., 1992, de The et al., 1990] (Fig. 2A). Although these elements were bound by their cognate receptors slightly less strongly than were the artificial AGGTCA elements (e.g. compare TR α 1 in Fig. 2B and Fig. 1C), they were bound with much greater specificity (compare Fig. 2B to Fig. 1C). To explore the basis of this specificity, we also created a derivative of the MHC site with a 5 base spacer, and a derivative of the β RARE with a 4 base spacer (Fig. 2A). Interestingly, both the DR4 and DR5 α MHC derivatives retained preferential binding by TR α , and both the DR4 and DR5 β RARE derivatives retained preferentially binding by RAR α (Fig. 2B). However, when comparing the DR4 and DR5 versions of the same element, TR α exhibited a moderate preference for the DR4 α MHC element, and RAR α displayed a slight preference for the DR5 (Fig. 2B). We conclude that half-site spacing contributes to DNA recognition by these receptors, but is not the dominant discriminatory factor under these conditions.

Both the MHC and the β RARE natural elements contain non-consensus hexanucleotide half-sites, as well as additional DNA sequence divergences in the regions flanking the half-sites themselves (Fig. 2A). To test the contributions of these different sequences to specificity, we next replaced the non-consensus half-sites in these elements with AGGTCA sequences, but retained the α MHC and the β RARE flanking sequences (Fig. 3A). Virtually all of the specificity observed for the native elements was lost by these AGGTCA substitutions: both the TR α and RAR α bound near equally to both the α MHC (AGGTCA) and the β RARE (AGGTCA) elements, with spacing having only a modest (or no) effect (Fig. 3B).

We next exchanged the native non-consensus half-sites between the α MHC and β RARE elements, testing both the DR4 and DR5 spacings (Fig. 4A). The α MHC half-sites conferred strong TR recognition when transferred to the β RARE background whether presented as a DR4 or a DR5, whereas the β RARE half-sites severely destabilized binding of TR α when inserted into the α MHC background, again whether presented as a DR4 or DR5 (Fig. 4B). The reversal of DNA recognition by TR α through swapping of the half-sites alone indicates that these non-consensus half-sites are the primary arbiter of TR α specific DNA recognition in these native response elements, with the spacing and flanking sequences playing secondary roles. Similarly, inserting the α MHC half-sites into the β RARE background greatly decreased RAR α binding (compare Fig. 4B to Fig. 3B and Fig. 2B). Unlike the case with TR α , however, this exchange of non-consensus half-sites did not fully reverse RAR α recognition, but instead produced response elements near equally recognized by RAR α (Fig.

4B). Changing the spacing of the half-sites did not further influence RAR α recognition of these chimeric response elements (Fig. 4B), indicating that the flanking sequences (the only other difference between the less-specific α MHC element possessing β RARE half-sites and the more specific native β RARE) must also contribute to the specificity of DNA recognition by RAR α .

3.2. The naturally-occurring, non-consensus half-sites work together with flanking DNA sequences and spacing to confer receptor-specific DNA recognition

To further explore the contributions of the different components of the DNA response element to receptor recognition, we returned to the study of artificial response elements based on AGGTCA repeats and manipulated the half-site, flanking sequences, and spacing individually (Fig. 5A). Substitution of the two AGGTCA consensus half-sites in these elements with two AGGACA half sites (a reiterated form of the non-consensus half-site found in the MHC element) maintained TR α binding and destabilized RAR α recognition (Fig. 5B). Reciprocally, substitution of two AGTTCA half sites into the artificial element (a reiterated form of one of the non-consensus half-sites found in the β RARE element) slightly reduced RAR α binding but more severely destabilized TR α binding (Fig. 5B). The effect of these half-site substitutions were observed in both the DR4 and DR5 contexts (Fig. 5B). These results confirm that the third and fourth positions in the hexanucleotide half-site play a dominant role in defining the divergent DNA recognition properties of TR α and RAR α , can do so in a different flanking sequence context than that observed in the native α MHC and β RARE elements, and can operate largely independent of half-site spacing.

We next tested the effect of altering the flanking sequences on receptor specificity. We focused on the (−2) position: (a) the sequence at this position differs between the native α MHC and β RARE elements, and (b) prior studies have shown differences in DNA recognition by RAR α and TR α at this position [Hauksdottir and Privalsky, 2001, Judelson and Privalsky, 1996]. TR α prefers an T at (−2) [Judelson and Privalsky, 1996], and substitution of this position with an A resulted in slightly reduced binding of TR α to both the AGGTCA half-site elements and the AGGACA half-site elements (Fig. 5C). Notably, however, this unfavorable A at (−2) severely destabilized the binding of TR α to the AGTTCA half-site elements (Fig. 5C); thus two base substitutions, each moderately unfavorable to TR α binding, work together to strongly interfere with TR α recognition. RAR α , conversely, favors an A at (−2) [Hauksdottir and Privalsky, 2001]. Response elements bearing an T at this position were bound less efficiently by RAR α than were the equivalent response elements bearing a T at (−2), and response elements bearing both the T at (−2) and an AGGACA half-site were particularly unfavorable for RAR α binding (compare Fig. 5B and 5C). We conclude that for RAR α , as for TR α , the identity of the flanking sequences can further enhance the effects of non-consensus sequences in the half-sites themselves to generate receptor-specific response elements.

The role of half-site spacing was also evaluated in the context of these different half-sites and flanking sequences. For many of the artificial half-site and flanking sequences tested, little or no difference was seen in the ability of a given receptor to bind to the DR4 versus the DR5 version of the corresponding response element (Fig. 5B and C). Intriguingly, however, a subset of combinations of half-site and flanking sequence demonstrated a spacing preference consistent with the 3,4,5 rule. For example, a (−2A) AGTTCA DR5 was preferentially bound by RAR α compared to an (−2A) AGTTCA DR4 (Fig. 5B and C). Some of these effects of spacing were enhanced when analyzing TR α or RAR α as heterodimers with RXR α , below.

3.3. Heterodimers of TR α or RAR α with RXRs display higher affinity, but lower specificity than do the corresponding homodimers

Both TR α and RAR α can heterodimerize with other members of the nuclear receptor family, and heterodimer formation with RXRs, in particular, has been proposed to be an important, but not exclusive mode of DNA recognition by these receptors [Glass, 1996, Mangelsdorf and Evans, 1995]. We therefore next extended our studies on DNA binding specificity to RXR α /TR α and RXR α /RAR α heterodimers.

As previously reported (e.g. [Harbers et al., 1996, Marks et al., 1992, Nelson et al., 1996, Olson and Koenig, 1997]), the heterodimeric forms of both TR α and RAR α displayed an elevated overall affinity for many of the response elements tested compared to the corresponding TR α or RAR α homodimers (note lower concentrations of receptor were employed in Figure 6 than in the prior figures). RXR α itself was able to homodimerize on several of these elements, but at very low efficiencies (data not shown). Somewhat unexpectedly, however, the specificity of DNA binding was reduced in the heterodimers. The RXR α /TR α heterodimers retained a preferential interaction with the naturally occurring DR4 α MHC versus DR5 β RARE elements, but the difference between the two was less than that observed for TR α homodimers (compare Fig. 6A to Fig. 2B). Similarly, RXR α /RAR α heterodimers bound the DR5 β RARE better than the DR4 α MHC, but with a lower specificity than did the RAR α homodimers (Fig. 6A).

Notably, all three components of the response element, half-site sequence, flanking sequence, and half-site spacing, worked together to confer response element recognition by these heterodimers. For example, artificial DNA response elements containing a T at the (−2) flanking sequence and consensus AGGTCA half-sites were bound by RXR α /TR α and RXR α /RAR α near equally, and this was largely independent of half-site spacing (Fig. 6B). However, the same (−2T) flanking sequence linked to AGGACA half-sites retained binding to RXR α /TR α heterodimers, but significantly destabilized binding to RXR α /RAR α heterodimers, an effect that was further enhanced by a DR4 versus a DR5 spacing (Fig. 6B). Conversely, RAR α prefers an A at the (−2) position, and RXR α /RAR α heterodimers efficiently recognized (−2A) elements containing AGTTCA half-sites with a DR5 spacing (Fig. 6C). Therefore, although half-site sequence alone confers little or no DNA binding discrimination for RAR α versus TR α heterodimers, the combining of receptor-selective half-sites with appropriate flanking sequences and half-site spacings generates detectable DNA binding specificity.

We also examined if hormone ligand altered the specificity of DNA recognition by these RXR α /TR α and RXR α /RAR α heterodimers. No significant difference was observed in the recognition of the α MHC or β RARE elements in the presence or absence of T3 or all-*trans* retinoic acid (ATRA), nor was an effect observed if LG68, an RXR α agonist, was added to the binding reactions alone, or together with T3 or ATRA (data not shown). We conclude that *in vitro*, binding of cognate ligand to RXR α /TR α or to RXR α /RAR α does not alter DNA recognition compared to that observed for the corresponding unliganded receptors.

3.4. Half-site spacing contributes an additional level of specificity for transcriptional activation in cells beyond that observed for DNA binding by TR α and RAR α *in vitro*

To evaluate the ability of our different DNA response elements to confer receptor-specific transcriptional regulation in cells, we inserted these elements into a thymidine-kinase promoter-luciferase reporter gene (tk-Luc) and introduced them, together with expression vectors for TR α or RAR α , into CV1 cells by transient transfections. We chose CV1 cells because they contain almost no endogenous TRs and relatively low levels of endogenous RARs [Lee and Privalsky, 2005] CV1 cells express endogenous RXRs, and we assume that

most of the transcriptional activation we observe in these reporter assays is mediated by RXR/TR α and RXR/RAR α heterodimers (based on the higher affinity of heterodimers for DNA, their stability in the presence of hormone, the absence of a further effect of ectopic RXR when co-transfected, and the effects of RXR knockouts in other cell systems) [Andersson et al., 1992, Chiba et al., 1997, Forman et al., 1992, Galli et al., 1998, Lazar et al., 1991, Yen et al., 1992]. Nonetheless we cannot exclude the possibility that TR α homodimers or RAR α homodimers may also contribute to reporter gene regulation in this assay (e.g. [Velasco et al., 2007, Diallo et al., 2007]).

In the absence of a response element, the tk-luciferase reporter displayed a low level of basal expression that was relatively unaltered by introduction of either nuclear receptor or hormone status (Fig. 7 and data not shown). Introduction of the α MHC-DR4 into the tk-Luc reporter resulted in a strong, TR α -mediated response to T3, whereas introduction of the β RARE-DR5 into the tk-Luc reporter resulted in a strong, RAR α -mediated response to ATRA (a more modest ATRA response, mediated by endogenous RARs in these cells, was also observed in the absence of the ectopic RAR α expression vector) (Fig. 7). As expected, response elements poorly bound by a given receptor *in vitro* were poor at conferring reporter gene activation in cells (e.g. the α MHC-DR4-TK-Luc reporter unresponsive to RAR α , and the β RARE-DR5-TK-Luc reporter was relatively unresponsive to TR α) (Fig. 7). The half-site sequences themselves clearly contributed to this specificity; substitution of the α MHC half-sites into the β RARE-DR4 element permitted significant TR α -mediated reporter expression, and substitution of the β RARE half-sites into the α MHC-DR5 element permitted significant RAR α -mediated reporter expression (Fig. 7). *In vivo*, as *in vitro*, the flanking sequences also contributed to receptor specificity: (a) the elements with swapped half-sites activated less strongly than did the native elements, and (b) consensus AGGTCA half-sites conferred stronger TR α activation when inserted into the α MHC-DR4 element background (i.e. linked to α MHC flanking sequences) than when inserted into the β RARE-DR4 element background (i.e. linked to β RARE flanking sequences).

Unexpectedly, not all response elements that bound a given receptor strongly *in vitro* conferred transcriptional regulation in cells. Most striking was the much greater role of half-site spacing in reporter gene activation *in vivo* versus receptor binding *in vitro*. TR α was essentially inactive in the luciferase assay on all of the DR5 elements tested, despite the ability of TR α homo- and heterodimers to bind to both the DR4 and DR5 versions of these element *in vitro* (Fig. 7). Similarly, RAR α displayed much weaker activity in the luciferase assay on the DR4 version of the β RARE element compared to the DR5 version the same was true of the DR4 versus DR5 versions of the response elements bearing β RARE half-sites in the α MHC background (Fig. 7). Parallel results were observed using integrated DR4 and DR5 versions of the β RARE-TK-Luc reporter instead of the transiently transfected reporters (data not shown). Interestingly, neither the DR4 nor DR5 versions of a consensus AGGTCA half-site functioned with RAR α in this context; although unexpected, this further highlights the importance of the non-consensus half-sites present in the native β RARE for RAR α recognition and function (Fig. 7). We conclude that an additional level of specificity operates in cells to further restrict transcriptional activation to a subset of the response elements that can be recognized by TR α or RAR α *in vitro*, and that the dependence on half-site spacing invoked by the 3,4,5 rule is manifested most strongly at this cellular level.

3.5. Auxiliary proteins in cells can destabilize nuclear receptor binding to the wrong response elements

We next tested if the dependence on half-site spacing for reporter gene activation in cells was observable as a difference in coactivator binding *in vitro*. We employed an EMSA supershift protocol to compare the ability of an SRC1 coactivator construct (representing the receptor-interaction domain of this coactivator; [McKenna and O'Malley, 2002]) to

recognize RXR α /TR α or RXR α /RAR α heterodimers bound to DR4 or DR5 versions of the α MHC and β RARE elements (all in the presence of cognate agonist) (Fig. 8A). Notably the SRC1 coactivator construct was able to interact with, and supershift, the receptor complexes assembled on these DNA elements independent of half-site spacing (Fig. 8A). We conclude that the DR4/DR5 specificity observed in cell transfections did not reflect the ability of these half-site spacings to recruit our p160 SRC1 coactivator construct *in vitro*.

Intriguingly, repeating this experiment with a different transcriptional coactivator, CBP [McKenna and O'Malley, 2002], produced an unexpected result (Fig. 8B). A CBP construct (representing the N-terminal receptor interaction domain) did not generate a dose-dependent supershift with any response elements tested (Fig. 8B). Instead, addition of the CBP construct selectively destabilized the binding of the receptor heterodimers to the elements representing the “wrong” spacings. For example, the CBP construct strongly interfered with the ability of the RXR α /TR α heterodimer to bind to the α MHC-DR5 element, but only modestly interfered with RXR α /TR α binding to the “correctly spaced” α MHC-DR4 element (Fig. 8B). Conversely, the CBP construct interfered with the ability of the RXR α /RAR α heterodimer to bind to the β RARE-DR4 element, but displayed little or no ability to interfere with its binding to the “correctly spaced” β RARE-DR5 element (Fig. 8B). A similar spacer-specific destabilization was observed using the CBP construct on RAR α homodimers (data not shown). T3 disrupts the ability of TR α homodimers to bind to DNA, preventing us from testing the effect of CBP in the TR α homodimer context [Andersson et al., 1992, Yen et al., 1992]. We suggest that at least one component of the additional selectivity for half-site spacing observed in cell transfections may arise from the presence of auxiliary cell proteins that can destabilize binding to the wrong spacings. CBP may be such a protein, or there may be additional proteins yet to be elucidated that are more relevant to the *in vivo* context.

4. DISCUSSION

4.1. Half-site sequence, not spacing, dominates in defining the specificity of DNA recognition by RAR α and TR α *in vitro*

Nearly 50 mammalian nuclear receptors have been identified, and yet to function properly, each must be able to specifically recognize its own distinct panel of DNA binding sites and target genes [Escriva et al., 2004, Flamant et al., 2006, Germain et al., 2006, McEwan, 2009]. Complicating any simple explanation for this selectivity, many nuclear receptors share identical P-box amino acid sequences in their zinc-finger domains and recognize identical consensus DNA half-sites [Claessens and Gewirth, 2004, Khorasanizadeh and Rastinejad, 2001]. A major advance in understanding this phenomenon came with the realization that the spacing of the two half-sites in a DNA response element plays an important role in its recognition, an observation subsequently codified as the 3, 4, 5 rule [Forman et al., 1992, Kurokawa et al., 1993, Näär et al., 1991, Perlmann et al., 1993, Umesono et al., 1991, Vivanco Ruiz et al., 1991]. Interestingly, a contribution of half-site sequence was noted in these or in subsequent publications (e.g. [Katz et al., 1995, Mader et al., 1993, Nelson et al., 1996, Umesono et al., 1991, Williams et al., 1992]), but was often not explored in detail and has frequently been overlooked in subsequent interpretations of this pioneering work. We report here a re-examination of these issues by a detailed analysis of the roles of half-site spacing, half-site sequence, and flanking DNA sequence in response element recognition by RAR α and TR α , two closely related receptors with different target genes and different biological roles.

Significantly, neither RAR α nor TR α displayed the ability to discriminate between a DR4 and a DR5 spacing of consensus AGGTCA half-sites, despite this sequence being among the highest affinity DNA binding sites known for these receptors. In contrast, two naturally-occurring response elements, obtained from the T3-regulated α MHC promoter and the

ATRA-regulated β RARE promoter, display a strong preference for their cognate receptors. Exchanging the different components of these natural response elements with one another, and with artificial AGGTCA-based DR4 and DR5 elements, demonstrated that it is the non-consensus nature of the half-sites in the α MHC and β RARE elements, not their spacing, that plays the dominant role in their specific recognition *in vitro*. The α MHC element is comprised of AGGTCA and AGGACA half-sites, whereas the β RARE element is comprised of GGTTCa and AGTTCA half-sites. Significantly, reiteration of these non-consensus half-sites (AGGACA for TR α or AGTTCA for RAR α) within an artificial response element conferred or further enhanced the receptor specificity observed with the natural elements. In contrast, changing the spacing in these natural elements had only modest effects on the ability of their cognate receptors to bind to these sequences.

We suggest that AGGTCA sequences generate high affinity, but low specificity receptor recognition and that the “suboptimal” half-site sequences such as are found in the α MHC and β RARE elements (and in many other naturally-occurring response elements) confer slightly lower affinity for their cognate receptors, but are far more disfavorable for binding by non-cognate receptors, thereby helping to confer specificity. Although we analyzed in depth AGTTCA and AGGACA half-sites, additional base changes at other positions in naturally-occurring half-sites may further modify the specificity conferred by these non-consensus position 3 and 4 positions [Williams and Brent, 1995].

The P-box amino acids that mediate the major groove contacts responsible for half-site recognition are identical between TR α and RAR α (EGCKG). How then do these receptors preferentially recognize different half-sites, as described here? Differences do exist in the other amino acid sequences that comprise the zinc-finger domains of these two receptors, and we suggest that these differences alter the precise position or conformation of the P box recognition helices themselves. In this fashion receptor amino acids that do not contact the DNA half-site directly can nonetheless influence its recognition. Amino acid sequence differences in the N-terminal domains of TR α and RAR α , mapping outside of the zinc-finger domains, may also contribute to this phenomenon, most likely by a similar, indirect effect on P-box conformation [Chen et al., 1993, Wong and Privalsky, 1995]. In support of these proposals, our preliminary studies on chimeric constructs of RAR α and TR α demonstrate that the differing recognition properties of these receptors are encoded by multiple determinants mapping within both N-terminal and zinc-finger domains (TQP and MLP, unpublished observations).

4.2. Flanking sequences provide additional response element specificity

Sequences flanking the hexanucleotide half-sites also contributed to receptor-specific DNA recognition in our study. Although observable to some extent when tested with consensus AGGTCA half-sites, the effects of these flanking sequences were particularly evident when used together with the corresponding, receptor-selective half-site sequences. For example, the β RARE half-sites did not fully confer RAR α -specific recognition unless the flanking sequences were also derived from the β RARE element. We conclude that flanking and half-site sequences work together to generate receptor-specific DNA recognition. Flanking sequences contribute to DNA binding by other nuclear receptors, and it has been proposed that the “true” nuclear receptor half-site is therefore eight bases long (e.g. [Harbers et al., 1996, Hsu et al., 1998, Olson and Koenig, 1997, Wilson et al., 1992]). However, given the -2 and -1 position are recognized through minor groove contacts mediated by a receptor “AT-box” α -helix lying outside of the zinc finger domain itself [Claessens and Gewirth, 2004, Khorasanizadeh and Rastinejad, 2001], we have treated this aspect of DNA recognition as distinct from the major groove, P-box contacts involved in recognition of the +1 to +6 half-site sequences.

4.3. Heterodimerization with RXR α uncovers a role for half-site spacing in receptor-specific DNA recognition

RAR α and TR α can bind to DNA as heterodimers with other nuclear receptors, including RXRs [Glass, 1996], although the relative proportion of homo- and hetero-dimers is likely to vary at different target genes, in different cells, or under different hormone ligand conditions (e.g. [Hsu et al., 1995, Leid et al., 1992, Olson and Koenig, 1997, Schröder et al., 1993]). Notably RXR heterodimers displayed a somewhat greater dependence on half-site spacing than did homodimers, although only when combined with appropriate receptor-selective half-site and flanking DNA sequences. For example, RXR α /TR α heterodimers more strongly recognized agAGGACA half sites when presented as a DR4 than as a DR5. Conversely, RXR α /RAR α heterodimers preferred binding to agAGTTCA half-sites presented as a DR5 versus a DR4. In contrast, spacing alone was insufficient to confer selective recognition of AGGTCA half-site elements by either RXR α /TR α or RXR α /RAR α heterodimers. We conclude that the spacing of the half-sites does play a role in DNA recognition by TR α and RAR α , but that this is most evident for RXR heterodimers and operates together with the half-site and flanking sequence contributions we elucidated for the corresponding receptor homodimers.

Although RXR/TR heterodimers have been reported to bind to certain DNA elements more strongly than do TR homodimers [Harbers et al., 1996, Marks et al., 1992, Nelson et al., 1996, Olson and Koenig, 1997], other studies have indicated that heterodimerization with RXR narrows the DNA binding specificity of these receptors [Yu et al., 1991, Zhang et al., 1992]. One explanation for this apparent contradiction may be the differing sources of receptor used; earlier studies often employed *in vitro* translated protein, in contrast with our use of baculovirus or mammalian cells expression systems (notably this also permitted us to use a greater range of receptor protein concentrations than those available in these prior studies). The precise nature of the response elements tested may also offer an explanation: the broadening effect of RXR on specificity was most clearly observed on response elements that bound receptor homodimers poorly, whereas less of a difference was observed on elements that bound homodimers strongly.

4.4. The 3, 4, 5 rule resurgent: additional determinants of specificity exist in cells

We examined the ability of our response elements to activate reporter gene expression in cells. As expected, response elements that were bound poorly by a given receptor *in vitro* also typically mediated poor transcriptional activation *in vivo*. Significantly, the corollary was not true; several elements that were strongly bound by TR α or RAR α *in vitro* were very poor at mediating reporter gene activation *in vivo*. Half-site spacing had a particular impact in this context: efficient TR α -mediated reporter regulation required a DR4 and efficient RAR α -mediated reporter regulation required a DR5. This dependence on half-site spacing was observed both on transiently introduced and stably integrated reporter. We conclude that an additional layer of specificity is imposed on nuclear receptor-mediated transcriptional regulation in cells beyond that observed for DNA binding *in vitro*, and may be the predominant basis for the 3,4,5 rule.

Disparities between DNA binding and transcriptional activation has been observed before for various nuclear receptors, although the actual mechanisms underlying these disparities have often remained poorly understood (e.g. [Ikeda et al., 1996, La Vista-Picard et al., 1996, Meijnsing et al., 2009, Nagpal et al., 1992, Olson and Koenig, 1997]). In our hands, the ability of RXR α /TR α and RXR α /RAR α heterodimers to bind to a p160 coactivator *in vitro* was the same for DR4 and DR5 elements. We cannot rule out, however, that yet-other coactivators in cells may display a stricter requirement for a given half-site spacing before they can be recruited by their nuclear receptor partners.

Intriguingly, we found that CBP, a coactivator/transcriptional integrator, selectively destabilizes binding of receptors to “wrongly” spaced DNA elements, such as RXR α /TR α to DR5 elements and RXR α /RAR α to DR4 elements. We suggest that when CBP associates with these receptors, it imposes a specific topology on the receptor dimer that is incompatible with the incorrect spacing. In this model, DNA binding is mediated by a nuclear receptor/coregulator complex, not by the receptor alone, and DNA specificity is a superposition of the recognition properties of the receptor and the associated coregulator complex. There may also be additional proteins in cells that are not coactivators or corepressors, *per se*, but that serve primarily to modulate the DNA recognition properties of the nuclear receptors. These specificity-conferring proteins, by defining the recognition of the DNA sequence itself, may operate in addition to factors, such as FoxA1, that help open the chromatin structure and confer access to the DNA [Carroll et al., 2005, Dong et al., 2009, Nielsen et al., 2008]. Interactions between the multiple response elements that often are present within each target gene may further contribute to receptor-specific binding and transcriptional regulation. Conversely, the same mechanisms described here that help explain receptor-specific DNA recognition also help account for how composite response elements, known to permit regulatory crosstalk on genes targeted by multiple members of the nuclear receptor family, can function (e.g. [Adan et al., 1993, Hua et al., 2009, Kato et al., 1995, Nakshatri and Chambon, 1994]).

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Figure 1. TR α and RAR α bind with high affinity, but low specificity to DR4 and DR5 spacings of AGGTCA consensus half-sites

A. The nucleotide sequences of the consensus half-site DR4 and DR5 response elements are shown. Arrows indicate the hexanucleotide half-sites. **B.** TR α and RAR α bind near equally to both DR4 and DR5 spacings of the AGGTCA consensus half-sites. A representative EMSA gel is presented. Identical two-fold dilution series (25–400 ng protein per pane) of each nuclear receptor, obtained from a recombinant baculovirus/Sf9 expression system, were mixed with a fixed amount of DR4 or DR5 radiolabeled DNA probe. The resulting receptor/DNA complexes (arrows) and free DNA probe (fp) were resolved by native polyacrylamide gel electrophoresis and were visualized by phosphorimager scanning.

Extracts of Sf9 cells infected with non-recombinant virus was used as a negative control (lanes 1, 7). n.s. refers to a non-specific band. **C.** A quantification of the results from panel B is presented. Three EMSA experiments were performed and quantified; the mean and standard error are presented.

Figure 2. TR α and RAR α bind with lower affinity, but higher specificity, to native α MHC and β RARE elements

A. The nucleotide sequences of the native MHC (a DR4) and β RARE (a DR5) elements are presented, as are an artificial DR5 version of the MHC element and an artificial DR4 version of the β RARE element. **B.** TR α and RAR α bind specifically to their cognate response elements. The EMSA protocol in Figure 1B was repeated using the native α MHC or β RARE sequences in panel 2A as DNA probes. Three EMSA experiments were performed and quantified; the mean and standard error are presented.

Figure 3. Substitution of AGGTCA consensus half-sites into the α MHC and β RARE sequences abolished specific recognition by TR α and RAR α

A. Consensus AGGTCA sequences were introduced into the α MHC and β RARE elements in place of the native half-sites, using both a DR4 and a DR5 spacing. Arrows indicate the hexanucleotide half-sites. **B.** TR α and RAR α bind with little or no specificity to the AGGTCA consensus versions of the α MHC and β RARE sequences. The EMSA protocol in Figure 1B was repeated using the AGGTCA versions of the α MHC or β RARE DNA probes indicated in panel 3A. Three EMSA experiments were performed and quantified; the mean and standard error are presented.

Figure 4. Exchanging the native half-sites between the α MHC and β RARE elements partially exchanges their recognition by TR α and by RAR α

A. The native α MHC half-sites were introduced into the β RARE sequence background, and the native β RARE half-sites were introduced into the α MHC sequence background as shown, using both a DR4 and a DR5 spacing. Arrows indicate the hexanucleotide half-sites.

B. Exchanging the native half-sites between the α MHC and β RARE elements results in a partial exchange in their recognition by TR α and RAR α . The EMSA protocol in Figure 1B was repeated using the interchanged half-site versions of the α MHC or β RARE DNA probes indicated in panel 4A. Three EMSA experiments were performed and quantified; the mean and standard error are presented.

Figure 5. Half-site sequence, flanking sequence, and half-site spacing all contribute to specific DNA recognition by TR α and by RAR α

A. The nucleotide sequences of the DNA response elements used in the EMSA experiments are presented. Different half-sites, flanking sequences (at the -2 position), and different half-site spacings, as indicated, were tested. Arrows indicate the hexanucleotide half-sites.

B, C. Specific DNA recognition by TR α and by RAR α depends on half-site sequence, half-site spacing, and the identity of the nucleotide at the (-2) position in the flanking region of the response element. The EMSA protocol in Figure 1B was repeated using the different DNA probes indicated in panel 5A with either a T (panel B) or an A (panel C) at the -2 position. Three EMSA experiments were performed and quantified; the mean and standard error are presented.

Figure 6. Specific DNA recognition by RXR α heterodimers of TR α and RAR α is dependent on all three parameters: half-site sequence, flanking sequence, and half-site spacing
A. RXR α /TR α and RXR α /RAR α heterodimers display higher affinity, but less specificity on the native MHC α and β RARE response elements than do TR α or RAR α homodimers. An increasing amount of RXR α and TR α , or RXR α and RAR α (approximately 12-200 ng of each nuclear receptor) was mixed with the radioactive DNA probes indicated, and the resulting receptor/DNA complexes were resolved and quantified using the same overall EMSA protocol as in Figure 1B and 1C. Three EMSA experiments were performed; the mean and standard error are presented. **B, C.** An AGGACA half-site with a T in the (-2) position displays specificity for RXR α /TR α compared to RXR α /RAR α . The protocol in panel 6A was repeated using the radioactive DNA probes indicated, containing either a T (panel B) or an A (panel C) at the -2 position.

Figure 7. The ability of TR α and RAR α to activate reporter gene expression in cells reveals an additional layer of DNA recognition specificity

The response elements indicated (center panel) were engineered into a tk promoter-luciferase reporter and introduced into CV-1 cells by transient transfection, together with an expression vector for TR α (left panel “+”) or for RAR α (right panel “+”). A reporter without a response element (“-”) was used as a negative control. Cells were treated with 1 μ M T3 (left panel) or 1 μ M ATRA (right panel) for 24 hrs, the cells were harvested, and luciferase activity was determined relative to a β -galactosidase internal control. Fold activation was calculated as the ratio of luciferase in the presence versus the absence of hormone. The means and standard error of three independent experiments are shown.

Figure 8. SRC1 coactivator is recruited to TR α and RAR α independent of half-site spacing, whereas CBP destabilizes nuclear receptor binding to non-cognate half-site spacings

A. SRC1 coactivator is recruited by nuclear receptors to both DR4 and DR5 response elements. A single concentration of RXR α /TR α or RXR α /RAR α heterodimers was mixed with the DNA probes indicated above the panel, together with cognate hormone and increasing amounts of an SRC1 (codons 568-891) construct was then added. The complexes were resolved by native acrylamide gel electrophoresis as in Figure 1B. A representative electrophoretogram is shown (left panel) and the amount of receptor/DNA complex supershifted by SRC1 (arrow), or not (arrowhead), was quantified (right panel). The mean and standard deviation of three independent experiments are presented. **B.** CBP destabilizes nuclear receptor binding to non-cognate half-site spacings. A single concentration of RXR α /TR α or RXR α /RAR α heterodimers was mixed with the radiolabeled DNA probes indicated above the panel, together with cognate hormone and increasing amounts of a CBP (codons 1-451) construct. The complexes were resolved by native acrylamide gel electrophoresis as in Figure 1B. A representative electrophoretogram is shown (left panel) and the amount of receptor/DNA complex formed was quantified (right panel). The means and standard deviation of five independent experiments are presented.