DNA Binding Specificity and Function of Retinoid X Receptor α*

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Retinoid X receptors are members of the erbA superfamily of ligand-inducible transcription factors. Similar to several other members of this gene family, retinoid X receptors are known to bind to the hexameric DNA sequence AGGTCA. After binding to a direct repeat of this hexamer with a one-base pair spacer, retinoid X receptor homodimers are able to activate transcription in the presence of the ligand 9-cis-retinoic acid. However, it is not known if AGGTCA represents the highest affinity binding site for retinoid X receptors. A combination of the electrophoretic mobility shift assay and polymerase chain reaction was used to isolate from a pool of random DNA those sequences that bind retinoid X receptors with highest affinity. This approach, combined with mutational analysis and DNA footprinting, led to the identification of the seven-base pair sequence GGGGTCA as the highest affinity retinoid X receptor binding site. A direct repeat of this sequence is substantially more active than a direct repeat of AGGTCA as a retinoid X response element.

Retinoid X receptors (RXRs) are ligand-inducible transcription factors that, along with the receptors for vitamin D (VDR), thyroid hormone (TR), and retinoic acid (RAR), belong to the erbA superfamily. RXR was first described by Hamada et al. (1) when RXRβ, referred to as H2RIIBP, was found to bind and activate transcription of the murine major histocompatibility complex class I genes. Presently three isoforms termed α, β, and γ have been identified (2-4). Compared with mouse RXRs, the β and γ isoforms share 92 and 95% amino acid homology in the DNA binding domain, and 87 and 86% amino acid homology in the ligand binding domain. The N-terminal regions of these isoforms show no conservation.

RXRs can heterodimerize with TRs, RARs, and VDR and thereby enhance the DNA binding of these receptors to their respective response elements. This enhanced DNA binding leads to increased thyroid hormone (T3), retinoic acid (RA), and vitamin D-mediated transcriptional activation (5-9). In addition to the heterodimer properties described above, RXRs also trans-activate specific RXR responsive target genes in the presence of the ligand 9-cis-RA (10, 11).

It has been demonstrated that RXRs, TRs, RARs, and VDR can all bind to direct repeats of the sequence AGGTCA (12-14). For these receptors, the specificity of DNA binding is determined by the spacing between the AGGTCA half-sites. Thus, a direct repeat of AGGTCA with a one-base pair spacer (DR+1) is a response element for RXR (RXRE) (14), whereas DR+3, DR+4, DR+5 are response elements for VDR, TR, RAR, respectively (12, 13).

However, although RXRs are known to bind to the hexamer AGGTCA, it is not known if this sequence represents the optimal binding site. Currently, few RXR responsive genes have been characterized, and the determination of the optimal binding site from such a limited number of genes would be difficult. Therefore the goal of the present work was to identify the optimal DNA sequence for RXR binding. A nonbiased approach was taken similar to that employed by Blackwell et al. (15) to study the DNA binding of c-Myc. By using random DNA pool selection, competition assays, DNA sequencing and footprinting, mutational analysis, and transient transfections, we were able to identify the optimal RXRs binding site as the heptamer 5'-GGGGTCA.

MATERIALS AND METHODS

Production and Purification of RXRs—The mouse RXRa cDNA was ligated into frame into the Escherichia coli expression vector pMAL (New England Biolabs Inc.) to produce a fusion protein consisting of maltose binding protein (MBP) followed by the recognition sequence for the protease factor Xa, fused to RXRs. Expression and purification of recombinant RXRa were performed according to the vendor’s protocol. Briefly, E. coli strain XL1 was transformed with the expression plasmid, MBP-RXRs expression was induced with isopropyl-1-thio-β-D-galactopyranoside, and the recombinant fusion protein was purified by amyllose affinity chromatography. Purity was confirmed by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie Blue staining, which revealed only a single band of ~88 kDa, consistent with the expected combined mass of MBP (42 kDa) plus RXRa (46 kDa) (data not shown). Incubation with factor Xa was then performed to release RXRs from MBP. The cleaved MBP was not removed, since control studies indicated it does not bind DNA. The final products were shown by SDS-PAGE to be of the appropriate size.

Random DNA Pool Construction—Oligonucleotide primers A (5'-TCGATTCTCCTAG) and B (5'-AGACGGATCCATTGCA) were synthesized. A pool of oligonucleotides of 49 nucleotides in length was synthesized containing the primer A sequence at the 5'-end, an internal random sequence of 18 nucleotides, and the reverse complement of primer B at the 3'-end. This internally random sequence oligonucleotide pool was converted to double-stranded DNA by annealing with primer B and filling-in with a Klenow reaction. The double-stranded DNA pool was purified by PAGE.

Electrophoretic Mobility Shift Assays (EMSA)—Protein-DNA binding reactions were performed in 35 μl of 20 μM HEPES, pH 7.8, 20% glycerol, 1.4 μg of poly(dI-dC), 1 mM MgCl₂, 50 mM KCl (in the latter steps of the selection process up to 400 mM KCl was employed), 0.1% Nonidet P-40, 32P-labeled DNA, and E. coli-expressed RXRs. The amounts of RXRs and 32P-labeled DNA are specified in subsequent sections. Reactions were incubated at room temperature for 45 min prior to electrophoresis. Electrophoresis was carried out on 0.25 x TBE...
Probe: Random Pool
RXRa: + mM KCl: 50
Lane: 1

6th Round Pool
- +
- +
3 4

FIG. 1. Selection of RXRa binding sequences by EMSA. Recombinant RXRs was incubated with radiolabeled random pool DNA or the DNA pool obtained following six rounds of selection, and protein-DNA complex was analyzed by EMSA.

**TABLE I**

| Sequence conservation amongst 19 clones selected for RXR α-monomer binding |
|---------------------------------------------------------------|
| The sequences shown contain the conserved portions of the selected random 18-mers. Conserved nucleotides are numbered 1 through 7, with surrounding nonconserved nucleotides numbered -3 through -1 and 8 through 10. The number of clones containing either A, C, G, or T at each position is indicated, and the consensus sequence is shown. |

| A | 5 4 5 2 3 0 0 0 0 | 19 2 3 3 |
| C | 3 6 6 2 1 0 0 0 0 | 19 0 9 5 7 |
| G | 6 7 4 15 15 19 17 | 0 0 0 9 4 |
| T | 5 2 4 0 0 0 0 2 0 | 19 0 8 2 5 |

Consensus: GGGGTTCA

**FIG. 2.** Affinity of RXRa for selected DNA sequences analyzed by competition EMSA. 32P-labeled clone 29 was incubated with 5 ng of RXRa plus the indicated amounts of nonradiolabeled competitor DNAs, and protein-DNA complexes were analyzed by EMSA. The dose of competitor DNA that competes 50% of the RXRa-32P clone 29 complex (C0.5) was determined by densitometry, and used as a measure of relative affinity. A competition assay using both a high affinity clone (clone 48; C0.5, 2.4 ng) and a low affinity clone (clone 38; C0.5, 3 ng) is shown. The samples were analyzed within the same experiment but were electrophoresed on separate gels.

**TABLE II**

| Table of Sequences of the conserved portions of RXRa-binding clones and associated C0.5 values |
|---------------------------------------------|
| Sequence | No. of Clones isolated | C0.5 (ng) |
|------------------------------------------------|
| GGGTCTCA | 11 | 3.4 |
| GAGTCTCA | 2 | 7.8 |
| CCGTCTCA | 1 | 9.5 |
| AGTCTCA | 1 | 10.9 |
| CCGTCTCA | 2 | 12.6 |
| GGCTCTCA | 2 | 12.7 |

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RESULTS

Selection of High Affinity RXRα Monomer-binding DNA Pool—The selection process utilized a pool of random double-stranded 18-bp oligonucleotides flanked by known sequences (total length, 49 bp). After 8 rounds of selection using EMSA and PCR amplification of gel eluted DNA, a DNA pool that bound RXRα with high affinity was obtained (Fig. 1).

The RXRα monomer-binding DNA pool was subcloned, and 19 different clones were studied. As shown in Tables I and II, 11 of these 19 clones contained the sequence GGGGTCA. However, neither the position nor orientation of this heptamer within the 18-bp random sequence was conserved nor was there conservation of sequence outside the heptamer. In EMSA competition assays, the clones containing the heptamer GGGGTCA showed the highest affinity for RXRα with a C50 of 3.4 ± 0.3 ng (mean ± S.E.); the other 8 clones including those containing the idealized hexamer AGGTCA had lower affinities with C50 values ranging from 7.8 to 12.7 ng (Table II and Fig. 2).

Mutation Analysis—To confirm the above results, a series of mutant oligonucleotides was synthesized, and their affinities were tested by EMSA competition. Changing any of the 4 guanines to thymines within the heptamer significantly decreased the affinity for RXRα (Fig. 3). Thus, the GGGGTCA-containing competitor oligonucleotide had a C50 of 0.6 ng (lanes 2–4), whereas the C50 values for oligonucleotides with mutations at the first, second, or third plus fourth guanines were 4, >8, and >8 ng, respectively (lanes 6–10, 11–14, and 16–19). However, mutation of the two nucleotides immediately 3’ to the heptamer did not affect the affinity of RXRα binding (data not shown).
DNA Footprinting—DNA footprinting analysis was performed using a high affinity clone containing the conserved sequence GGGGTCA. Methylation interference was used to identify critical guanine residues, and uracil interference was used to identify critical thymine residues. As depicted in Fig. 4, when any of the guanine residues from either strand in the heptamer sequence was methylated, RXRα binding was impaired. In a similar way, loss of a thymine 5-methyl group by changing the bottom strand thymine to uracil within the heptamer interfered with RXRα binding. No footprinting was noted outside the conserved heptamer. These data, combined with the random selection results and mutational analysis, indicate that the heptamer GGGGTCA represents the highest affinity RXRα binding sequence.

Transient Transfections—To test the function of the optimal RXR binding site, transient transfections were performed in JEG-3 cells. Potential response elements were ligated upstream of a basal thymidine kinase promoter driving expression of chloramphenicol acetyltransferase. In particular, we wished to determine whether the heptamer GGGGTCA is a stronger RXRE than the idealized hexamer AGGTCA. Initially we studied both GGGGTCA and AGGTCA as potential single-site response elements with co-transfected RXRα or RXRβ. However, neither sequence supported 9-cis-RA induction of chloramphenicol acetyltransferase (data not shown), indicating that a single RXR binding site is unable to function as a response element under these conditions. Therefore we studied multiple copies of RXR binding sites. Since it had previously been shown that the optimal spacing for an RXRE is a DR+1 (14), we constructed oligonucleotides that contained a DR+1 of AGGTCA (R8) or of GGGGTCA (R7) (in the latter case, the first G of the 3'-half-site functions as the spacer, since the sequence is a heptamer rather than a hexamer). EMSAs show RXRα monomers and homodimers bind with higher affinity to R7 than to R8 (Fig. 5). Two copies of either the AGGTCA DR+1 (R8AA) or the GGGGTCA DR+1 (R7AA) were ligated into pUTKAT3 and tested for 9-cis-RA induction. Transient transfection of JEG-3 cells with RXRα demonstrated that R7AA is ~6-fold more potent an RXRE than is R8AA (Fig. 6A). Similar results were obtained when RXRβ was used instead of RXRα; the 9-cis-RA induction of chloramphenicol acetyltransferase activity was ~4-fold greater for R7AA than for R8AA (Fig. 6B). To confirm these data, a separate series of transfections was performed comparing R7AA to the RXRE of the rat cellular retinol binding protein II (CRBPII) gene (14). This natural RXRE contains four AGGTCA-like half-sites arranged as a series of DR+1s (Table III). Consistent with the previous data, transfection with RXRα demonstrated that 9-cis-RA induction of chloramphenicol acetyltransferase was 3.7-fold greater for R7AA than for CRBP (Fig. 6C). Thus these data demonstrate that not only does RXRα bind to GGGGTCA better than to AGGTCA, but also the heptamer is more active as an RXRE half-site.

RXRs are able to heterodimerize with TRs, RARs, and VDR, increasing both DNA binding and transcriptional activation (5–9) and suggesting a diversity of RXR-dependent signaling pathways. These heterodimers are present in solution and are further stabilized by binding to their DNA response elements. In addition to their role in heterodimeric complexes, RXRs can bind as monomers and homodimers to DNA. The natural ligand for RXR is 9-cis-RA (10, 11). Thus, 9-cis-RA induction using RXR homodimers defines a second retinoid signaling pathway distinct from that using all-trans-RA and -RARs. It was assumed that the above receptors recognize the hexamer AGGTCA as an optimal binding sequence since they have identical P boxes. The P box is a region located at the base of the first zinc finger that is critical for DNA half-site recognition specificity (22). However, by using a unbiased approach, we found that the optimal DNA binding site for RXRα is the heptamer GGGGTCA. This sequence binds RXRα with higher affinity and also gives significantly higher transcriptional activation in transient transfection studies than does the hexamer AGGTCA. With a similar approach, it was shown that the optimal TRα monomer binding site is the octamer TAAGGTCA (23). These results would indicate that regions in the receptors in addition to the P box are important for half-site specific recognition. Indeed it has been shown that two other regions, denoted the T and A boxes and located 3' to the second zinc finger, play an important role in the protein-DNA interaction. The A box is important for recognition of the sequence AA at the 5'-end of the core motif for the NGFI-B/nur77 orphan receptor (24). Recently by using a (AGGGTCA motif as a half-site, it was noted that the T box for RXR and RAR and the A box for TR are important for binding to monomer sites (25).

A single copy of the optimal RXRα monomer binding sequence was unable to function as an RXRE when placed 5' to a basal thymidine kinase promoter. This is in contrast to the optimal TRα monomer binding sequence, which is a functional T3 response element, when placed as a single copy 5' to the same promoter (29). Furthermore, certain other members of the erbA superfamily, such as NGFI-B (26) and Rev-erbα (27) appear to activate transcription from monomer response elements. The reason RXRs are unable to accomplish this is not known, but it may relate to structural differences in transactivation domains or other conformational differences. Only a few RXREs have been characterized, including those found in region II of the mouse major histocompatibility class I gene locus (1), the rat CRBPII gene (14), section A of the apolipoprotein AI gene (28), and the hepatitis B virus enhancer I (HBVGelement) (29). Analysis of these 4 RXREs has revealed 10 half-sites (Table III). The perfect GGGGTCA heptamer sequence is found in only one of these 10 half-sites, located in the apolipoprotein AI RXRE. This is not contradictory to our data since half-site sequence should not be considered in isolation.
In summary, our results indicate that the optimal monomer binding site for RXRα is GGGGTCA. It is not known if this heptamer sequence also is the optimal binding site for the RXR isoforms β and γ. Functional differences have been described among these isoforms; for example, RXRα and γ are more potent than β in activating transcription of the CRBPII gene (4). Therefore it is possible that the isoforms may have slightly different optimal binding sequences.

Our data indicate that the optimal DNA half-site for RXRα binding differs from that for TRα binding (TAAGTCA) (23). In addition, the optimal binding element for VDR appears to be GGTGTCA (30) or (AG)GGGTGCA (31), and evidence suggests that the optimal element for RARα is AGAGGGTCA (32). Thus, each of these receptors may have its own unique high affinity binding sequence. These observations suggest that heterodimers of RXR with TR, VDR, or RAR may prefer to bind to imperfect direct repeats with the appropriate spacing. For example, RXR-TR heterodimers may bind best to the sequence GGGGTCAANNTAAGGTC, and this sequence may be activated preferentially by T3-occupied RXR-TR heterodimers as opposed to TR homodimers. In contrast, we speculate that a perfect direct repeat of a receptor’s optimal monomer binding site (with appropriate spacing) might be activated preferentially by the receptor homodimer (e.g. TAAGGTCANNTAAGGTC for TR homodimers). In addition, other >eRA superfamily members may form functional heterodimers with TR, VDR, or RAR on response elements that contain half-sites of appropriate sequence and spacing. The details of the importance of half-site sequence in receptor function are just beginning to be explored.

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Table III: Sequences of naturally occurring RXREs

| CRBP II   | GAGGTCGACGTTCAAGGGTCAAGGTCAAGTCCA |
| RII-MHC   | GAGGTCGAGGGTGGGGG |
| Site A-Apl  | GGGGTCAGGTTCAAGGTCAAGTCCA |
| HBV GB element | GGGGTCAGGTTCAAGGTCAAGTCCA |

The total number of half-sites, their arrangement within the response element, the similarity of each half-site to the optimal sequence, and the overall DNA context of the response element all will contribute to the inducibility and function of the response element. For example the CRBP II RXRE lacks a perfect half-site, but it contains four imperfect sites arranged as a series of DR+1 elements. Nevertheless, in a transfection analysis, the CRBP II RXRE was less potent than were two copies of the GGGGTCA DR+1.
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