PROBING THE BINDING OF ESTROGEN AND GLUCOCORTICOID RECEPTORS ON CLASSICAL AND NON-CLASSICAL RESPONSE ELEMENTS AND INFLUENCE OF HMGB-1

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ABSTRACT

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Estrogen receptor (ER) was known to be ligand-inducible enhancer protein that belonged to member of nuclear hormone receptor super family. Estrogen receptors known to be shared a highly conserved structure with other members of the steroid receptor super family and a common mechanism, regulating gene transcription. Estrogen receptors reside in the nucleus and in the absence of hormone signal were shown bind to other proteins. However, in the presence of hormone, the receptor dissociates from the other proteins and dimerizes. The dimeric form of estrogen receptor was the active form which binds to a specific DNA sequence, known as the estrogen response element (ERE) in the regulatory region of the target gene. The estrogen response element (ERE) composed of asymmetric or pseudo asymmetric, palindromic repeat of two half-site sequences (cHERE) 5’-AGGTCA-3’, separated by 3bps.

HMG domain proteins identified as architectural proteins involved in chromatin function and have been shown to stabilize the ER/ERE binding. One aims of this thesis was to determine how differences in spacer length between the ERE half-site affect on ER binding affinity in the presence and absence of the coactivator protein, HMGB-1. The binding affinity and selectivity of the two forms of the estrogen receptor (α and β) and glucocorticoid receptor (GR) for cHERE, in three different orientations (direct repeats, inverted repeats and everted repeats) were studied by using the gel mobility shift assay (EMSA). ERs, in contrast to GR, showed a strong cooperativity when interacting with direct repeats, inverted repeats as well as everted repeats.
This work is dedicated to my parents and family members
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CHAPTER I

Introduction to Nuclear Receptor Super Family

Nuclear receptors are one of the most abundant classes of transcriptional regulators found in animals. They regulate diverse biological functions, including homeostasis, development, reproduction and bone metabolism (1-3). Nuclear hormone receptors function as ligand-inducible transcription factors and are located in either cytoplasm or in nucleus of cell in the absence of their cognate hormone. They provide a direct link between signaling (hormone) molecules and transcriptional responses. A large number of nuclear receptors have been identified through amino acid sequence similarity to known receptors and they fall into three different homology classes.

Nuclear receptor super-family consists of three receptor subclasses: 1) the steroid hormone receptors which bind progesterone (PR), estrogen (ER), glucocorticoids (GR), androgens (AR), or mineralocorticoids (MR); 2) class II receptors for thyroid hormone (TR), retinoids (RAR and RXR), vitamin D3 (VDR), prostaglandins (PPAR), oxysterols, and bile acids; and 3) orphan receptors for which no endogenous ligand has been identified (4-7). Each of the receptor subclasses is characterized by a unique mechanism of action with respect to the stoichiometry of the activated complex and DNA sequence recognition. Steroid receptors form homodimers that optimally recognize hexameric DNA elements arranged as inverted repeats separated by three unspecified base pairs. PR, GR, AR, and MR bind to the core hexamer 5’-AGAACA-3’, whereas, ER recognizes the sequence 5’-AGGTCA-3’ (5). Class II receptors preferentially function as heterodimers with RXR and recognize the sequence 5’-AGGTCA-3’ hexamer arranged as direct repeats (DRs). The variable spacing between the direct repeats (DRs) determines the
RXR heterodimer binding specificity. Class II receptors, particularly TR, can also recognize an inverted repeat as homodimers, or half-sites as monomers. Orphan receptors can bind to the 5’-AGGTCA-3’ hexamer arranged either as a direct repeat, palindrome, or half-site as hetero-dimers with RXR, homodimers, or monomers (4, 8–10).

**Estrogen receptor (ER)**

Estrogen receptor (ER) is a transcription factor that is activated by estrogen. It was initially assumed that ER is a cytoplasmic protein in the absence of hormone. However, it is now clear that it occurs primarily in the nucleus as an inactive complex in combination with heat shock proteins (hsp) and immunophilin proteins (11). ER binds to the DNA palindrome 5’-AGGTCA-3’, which differs from other steroid receptors that bind to derivatives of a common response element, 5’-AGAACA-3’.

Estrogens are essential hormones for successful reproduction in mammals. Although produced locally by the ovary, estrogens circulate systemically and exert selective effects on target tissues. This action is mediated by the presence of estrogen receptors (ER). Estrogen receptors are members of a family of nuclear transcription factors including receptors for sex steroids, thyroid hormone, vitamin D, retinoids, as well as many orphan receptors, for which no ligands have been identified (12). The estrogen receptor was discovered ERα while, the second ER gene was cloned from prostate tissue in 1996 (13), and thus there are two ER receptors, the originally described (ERα) and now also ERβ.

By comparing the ER sequences, it is apparent that both share a general domain structure common to ligand-modulated nuclear receptor transcription factors (14). The current understanding of ER mechanisms of action can be summarized with reference to the
overall structure of the receptors (Figure 1). The functions of some regions of the ER molecules have been defined using deletion and mutational analysis as well as structural analysis.

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ERα/ERβ homology 97% 60%

Figure 1: Comparison of domain structures of ERα and ERβ.

The estrogen receptors share a domain structure, depicted schematically above. The ERs have six domains, A–F, and the number of amino acids in these domains, as well as the functions associated with some of these domains, are indicated for each form of ER. AF-1 and AF-2 refer to regions that mediate the transcriptional activation functions of the ERs. The numbers of amino acids contain in A/B, C, D, E and F domains of for ERα comprise 183, 83, 39, 251 and 43 amino acids, respectively and for ERβ are 142, 84, 32, 245 and 27 amino acids respectively. The degree of homology between ERα and ERβ in the DNA binding domain (DBD) and the ligand binding domains (LBD) are indicated in figure 1. The DNA binding domain includes a zinc containing motif (C domain, Figure 1), which binds with high affinity and specificity to estrogen response elements (EREs) in target genes, and a ligand-binding domain (domain E), which binds estrogen as well as
other estrogenic ligands. The consensus ERE is a 13-base pair inverted repeat sequence (5’-GGTCAnnnTGACC-3’), however, the majority of ERE sequences contain one or more variations from the consensus. In vitro DNA-binding studies have indicated that the ER binds as a dimer (15), with one ER molecule contacting each 5-base pair-inverted repeat (16). Although DNA binding is a dimerization initiator, amino acid sequences in the ligand-binding domain are also involved in dimerization (12), and crystallized truncated ER containing only the ligand-binding domain is clearly shown to be a dimer in the presence of an agonist ligand (17). The AF-1 region in the amino terminus and the AF-2 region within the ligand-binding domain are involved in ligand-independent and ligand-dependent transcriptional activation, respectively, as deletion or mutations of these (AF-1 and AF-2) regions result in a diminished ability to regulate estrogen-responsive genes (18, 19). The mechanism by which transcription is mediated by the ER is thought to involve interaction of AF-1 and AF-2 with the transcriptional machinery.

**Glucocorticoid receptor (GR)**

Glucocorticoids are steroid hormones synthesized and secreted by the adrenal cortex. They regulate a variety of physiologic functions and play an important role in maintaining basal and stress-related homeostasis. At the cellular level, the glucocorticoid receptor (GR) also belongs to the nuclear receptor super family of ligand-dependent transcription factors. The human glucocorticoid receptor (hGR) gene is located on chromosome 5 and consists of nine exons. In the absence of ligand, hGR proteins reside mostly in the cytoplasm of cells as part of a large multi protein complex, which consists of the receptor polypeptide, two molecules of hsp90 and several other proteins (20).
Upon hormone binding, the receptor undergoes an allostERIC change, which results in dissociation from hsp90 and other proteins, and phosphorylation occurs at five serine phosphorylation sites. The phosphorylated, ligand-bound GR translocates into the nucleus, where it binds as homodimer to GREs located in the promoter regions of target gene. hGRα then communicates with the basal transcription machinery and regulates the expression of target gene positively or negatively, depending on the GRE sequence and promoter context (21).

**Naturally occurring ligand for steroid hormone receptors**

ERα and ERβ are similar in those parts of the LBD involved in the actual binding of ligand and have similar binding specificities (22). Ligand stabilizes ER–ERE binding (23). Although, unliganded ER binds EREs *in vitro*, ligand binding further affects the migration of the ER–ERE complex in EMSA experiments, indicating a role for ligand in altering ER conformation, as anticipated from crystal structure studies (24-27)
Structural organization of class I and class II nuclear receptors

Nuclear receptors of the classes I & II share a common structural organization, they all contain six domains designated from A to F. The different regions of homology (A-F) of nuclear receptors were originally identified by alignment of the sequences of the human and chicken estrogen receptors (28).

The A/B domain comprising the N-terminal region is highly variable and contains the first ligand-independent transactivation region (AF-1), whereas, the second activation function (AF-2) is located in the ligand binding domain (LBD) in region E. The DBD
Hormone response element interact with nuclear receptor

Hormone response elements are generally composed of two repeats of core sequences arranged as palindromes, direct repeats, or sometimes as inverted repeats, reflecting the fact that dimerization is important for efficient DNA binding by most nuclear receptors (NRs). The number of nucleotides separating the two repeats influences the efficiency of DNA binding, with differing requirements displayed by different NRs. A dimerization interface referred to as the D-box is present within the DBD at the N-terminal half of the second Zn motif. Dimerization via the DBDs facilitates cooperative DNA binding, and the interactions of the D-boxes further stabilize NR-DNA contacts (29). A small subset of NRs interacts with DNA as monomers. These receptors recognize and bind to isolated half sites, generally with a requirement for specific nucleotides.

The consensus ERE is composed of two core sequences, organized as a palindrome and with the two half-sites separated by three nucleotides. The three-dimensional structures
of ER DBD bound to an ERE and of GRDBD bound to a GRE have been determined (30-33). ER and GR show both similarities and differences in amino acid residues within their respective DBDs that make direct contact with nucleotides of their cognate DNA response elements, and these findings provide a basis for understanding the selective recognition of response elements by NRs. Cooperative interactions between the two DBDs is facilitated by the spacing between the two core elements, which enhances the exposure of the dimerization interfaces of each subunit to one another. Dimerization of the ERα DBDs enhances binding to imperfect EREs, thus contributing to increase the number of sequences with which ERα can interact (18). The D-boxes differ considerably between NRs, perhaps reflecting the different modes of DNA-binding displayed by various NRs.

**Interaction of full length human estrogen receptor (ER) α, β with cERE, and 0n-4n cERE**

In the past, a limited number of estrogen response elements (EREs) have been functionally identified in the promoter regions of estrogen regulated genes. From those novel response elements, a consensus estrogen response element cERE has identified that is identical to the *Xenopus laevis vitellogenin A*2 gene promoter, in which 5’-AGGTCA-3’ and 5’-TGACCT-3’ are separated by three nonspecific nucleotides (5’-AGGTCAnnnTGACCA-3’). To date, all but one intracellular receptor have been found to bind to DNA as dimmers interacting with elements composed of two recognition half-sites arranged as direct repeats, palindromes, or inverted palindromes (34-37). But specificity is determined by the orientation and spacing of the two half-sites for a
complete element (38-41). Activated estrogen receptors (ER) bind as homodimers to estrogen response elements (ERE) composed of a palindromic (inverted repeat) arrangement of core motifs, 5’-AGGTCAnnnTCCAGT-3’, with a fixed spacing of three nucleotides between the half-sites. In many cases, ERE half sites, which are either proximal or distal halves of the palindrome, are also involved in estrogen response (16). Specific contacts between the ER dimer and the sugar phosphate backbone of the ERE are important in sequence recognition and high affinity binding (42). Each monomer is bound to DNA in the major groove with the ER dimer located predominantly on the same face of the DNA helix. These specific amino acids within the ‘P box’ of zinc motif C1 interact in the major groove in a sequence-specific manner. The fourth base pair of the ERE half site (AGGTCA) provides a positive contact for the P-box, whereas, the third base pair (AGGTC) provides the binding energy (44-46). The CII zinc motif is involved in half-site ERE spacing recognition and ER dimerization (47). Phosphate methylation interference assay showed that ERα forms the strongest interaction with the underlined nucleotides: 5’-GGTCAGCGTGACC-3’. Whereas, ethylation and thymine interference assays indicate ERα contacts the underlined nucleotides in the chicken vitellogenin II ERE: 5’-CTGGTCACGCTGACCG-3’ (48). Thus the technique used to analyze ER-DNA contact indicates differences in nucleotide recognition by ERα.

**High mobility group proteins**

HMGB 1 and HMGB 2 proteins are highly abundant, ubiquitous, architectural proteins (which can induce flexibility upon interact with minor groove of DNA) in DNA and . With in each mammalian nucleus containing there are on 10⁵ to 10⁶ molecules of HMGB,
equivalent to about 1 molecule for every 10 nucleosomes (49). There are three families of HMG proteins, HMGA, HMGB & HMGN which have recently been renamed with systematic reference to their structural properties (50) to avoid confusion between unrelated proteins. HMGA proteins are very small (in the range of 10 kDa) and contain AT-hooks, allows binding to A/T-rich stretches of DNA. HMGB proteins have a molecular weight of ~25 kDa and contain HMG boxes; HMGN proteins have intermediate molecular weights (between 10 and 20 kDa) and bind directly to nucleosomes, between the DNA spiral and the histone octamer. A general review on HMG proteins has been provided by Bustin (51); Thomas and Travers (52) have focused on HMGB proteins and (53) on HMGA proteins.

However, HMGB proteins bind to linear DNA non-specifically with a short residence time (54). They have been implicated in bending or increasing the flexibility of DNA so as to facilitate the assembly of nucleoprotein complexes and nucleosomal remodeling (55). The HMG B domain untwists the DNA by binding in the minor groove and also introduces a sharp 90°–100° bend within a single double-helical turn (56). DNA binding by HMG boxes widens the minor groove and causes a significant bend in the DNA strand (28). This induced bend is stabilized by the basic region, which neutralizes the adjacent negative charges on the sugar-phosphate backbones in the opposing compressed major groove (56). The role of the acidic tail, in contrast, remains an enigma. In vitro this tail usually decreases the affinity of the protein for DNA (57). A common feature in both regulatory roles is the ability of HMGB1 to mediate a stronger binding affinity of the transcription factor for its cognate site, which in many cases correlates with increased transcriptional activation. HMGB proteins exhibit selectivity in their interactions with
elements of the nuclear hormone receptor (NHR) super-family in that they enhance class I receptor binding to their cognate response element, but exert no effect on class II receptors (55). The HMGB proteins distort, bend or modify the structure of DNA complexed with transcription factors or with histones (55). HMGB proteins have also been implied in the determination of nucleosomes structure and stability (chromatin remodeling) as well as in transcription and replication and efficient V (D) J recombination, as the sequences recognized by recombinase RAG1/2 is made accessible by HMGB1/2 even when packaged in nucleosomes.

The ability of HMGB1 to bind irreversibly to chromatin during apoptosis is a specific behavior important for chromatin remodeling. The self-motivated behavior of HMGB1 changes completely during the apoptosis, and its movement within the cell is blocked (58). This phenomenon is specific for HMGB1; other proteins do not show any reduction in mobility as a result of the characteristic chromatin condensation that is one of the hallmarks of apoptosis. HMGB1 is not modified post-translationally during apoptosis; rather, the causes of HMGB1 binding to chromatin are chemical and/or structural alterations in the chromatin itself. In particular, histone H4 is deacetylated during apoptosis, and the inhibition of deacetylation by trichostatinA abolishes HMGB1 binding to chromatin (58). The high affinity of HMGB1 to hypoacetylated chromatin has not yet been explained: one possibility is that HMGB1 establishes direct contacts with under-acetylated histone tails; alternatively, histone deacetylation might determine structural changes in chromatin architecture.
Steroid receptor binding to non-classical response elements

The mouse mammary tumor virus promoter has been shown to be inducible by glucocorticoids and progesterone (59-61). Although steroid hormone receptors bind with high affinity to palindromic response elements, the hormone responsive region of the mouse mammary tumor virus promoter contains a pair of directly repeated half-sites that are important for hormone inducibility. Recent experiments have also indicated that direct repeats can function as estrogen response elements (59, 60).

However, it should be pointed out that most GREs and EREs defined to date are not perfectly palindromic and are therefore bound more weakly by their corresponding receptors.

Hormone response elements for steroid receptors have been characterized as palindromic sequences, containing two half-sites separated by 3 base pairs. The estrogen response element (ERE) in the vitellogenin A2 gene of *Xenopus laevis* contains the perfectly palindromic element AGGTCAnnnTGACCT (61).

A number of estrogen-responsive genes contain imperfect palindromic EREs either in isolation or in multiple arrays (62–67). Similarly, the imperfect palindromic glucocorticoid response element (GRE) AGAACAnnnTGTTCT has been found in the rat tyrosine aminotransferase gene (68). Altering the spacing between half-sites disrupts the binding of steroid receptors to palindromic response elements (69, 70). The binding of either the ER or the GR to direct repeats is weaker than to palindromes (71).

It is noteworthy that molecular genetic experiments in yeast have identified a number of DNA sequences that bind ER very weakly as potential EREs (72). In a similar study, a direct repeat of AGGTCA motifs separated by 3 bps mediated an estrogen response (73).
Direct repeats, like imperfect palindromic elements, can be found in multiple arrays upstream of target promoters (74-76). Multiple imperfect palindromes of glucocorticoid- or estrogen-responsive promoters have been shown to combine to give a synergistic response to hormone (77-79). In the MMTV promoter, proximal response sequences, containing the sequences studied here, combine with more distal sites for an optimal response to glucocorticoid (80). Similarly, there is strong synergism between the four AGGTCA half-sites found far upstream of the chicken ovalbumin gene (81).

**Consensus**

A single sequence defined from an alignment of multiple constituent sequences that represents a "best fit" for all those sequences. This procedure is used to determine which residue is placed at a given position in the event that not all of the constituent sequences have the identical residue at that position.

The table I shows introns of five human globin genes have been searched for the rat and mouse introns. The human globin (table III) consensus structure is similar to that of the rat and mouse (data not shown). IVS refers to intervening sequence.

Tabel I. The Potential 3' splice signals in introns of human globin genes (43)

<table>
<thead>
<tr>
<th>Intron</th>
<th>Distance</th>
<th>Signal</th>
<th>%</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-2-Globin</td>
<td>17</td>
<td>C-T-C-A-C</td>
<td>95</td>
<td>31</td>
</tr>
<tr>
<td>IVS-2</td>
<td>16</td>
<td>C-T-G-A-C</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>β-Globin</td>
<td>35</td>
<td>C-T-G-A-C</td>
<td>100</td>
<td>32</td>
</tr>
<tr>
<td>δ-Globin</td>
<td>33</td>
<td>C-T-G-A-C</td>
<td>100</td>
<td>33</td>
</tr>
<tr>
<td>γ-A-Globin</td>
<td>28</td>
<td>C-T-G-A-C</td>
<td>100</td>
<td>34</td>
</tr>
<tr>
<td>IVS-2</td>
<td>38</td>
<td>C-T-C-A-C</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>ε-Globin</td>
<td>17</td>
<td>C-T-G-A-T</td>
<td>88</td>
<td>35</td>
</tr>
<tr>
<td>IVS-2</td>
<td>35</td>
<td>C-T-C-A-A</td>
<td>80</td>
<td></td>
</tr>
</tbody>
</table>

Consensus structure of primary signals with flanking regions

A 1 4 — 2 10 1 1 4 — 1
G 3 — 3 — 5 — — 1 — 5
C 5 5 1 9 — 3 — 7 4 4 — 4
T 2 4 2 1 10 — — 2 5 1 10 —

C-T-G-A-C
Cooperativity Index ($Ci$)

$Ci = \text{Concentration of ER at (80\%-20\% \text{) complex}}$

Without HMGB-1 \hspace{2cm} Ci = (M4 – M3)

With HMGB-1 \hspace{2cm} Ci = (M2-M1)
CHAPTER II
MATERIALS AND METHODS

A. Proteins and DNA Oligonucleotides used in EMSA Studies

I. Isolation and purification of HMGB1 and HMGB-2 proteins from calf thymus

This method was based on salt extraction, selective precipitation with ammonium sulfate and ion exchange chromatography. All the steps in the isolation were done at 4 deg C.

Isolation of Nuclei (Ghattamaneni. R, 2005 MS thesis, BGSU)

1. A 200gms of calf thymus (Pel-Freez, Arkansas), stored at -80 deg C, was slowly thawed in the cold room at 4°C.

2. The calf thymus was then blended to homogeneity, in approximately 250 ml of buffer A (20 mM Tris.HCl, 50 mM NaCl, 1 mM EDTA, 5 mM BME, 10% glycerol, 0.5 mM PMSF, pH 8.8) for 2 minutes at full speed in a blender.

3. The thick homogenate was filtered through two layers of cheesecloth.

4. The relatively thick filtrate was transferred into 10 autoclaved 50 ml plastic centrifuge tubes and sedimented at 14000 x g (Sorvall superspeed RC-2, SS-34 rotor) for 40 minutes.

5. The supernatant was discarded and the pellet is repeatedly washed with 50 ml buffer A until no floating lipid was observed on the supernatant. This usually required around 4 washes.

6. The pellet was then resuspended in two pellet volumes (about 300 mL) of buffer B (20 mM Tris.HCl, 5 mM BME, 0.5 mM PMSF, pH 7.2) taken in 500 ml of Erlenmeyer flask. The suspension was stirred at moderate speed with magnetic stirrer and a stir bar.
7. A 0.1 volume of total suspension of 1.65 M ammonium sulphate (AS) (all AS solutions were prepared in buffer B) was slowly added, over a few minutes with moderate stirring to the suspended nuclei.

8. The suspension is gently stirred for an additional 30 minutes at 4°C and then sedimented at 13000 x g for 20 minutes at 4 deg C in a Sorvall centrifuge with SS-34 rotor. The supernatant containing HMGB1/2 protein was saved and the pellet was extracted twice more with buffer B containing 0.15 M AS.

9. The supernatants were pooled together and the final AS concentration was brought to 2.6 M AS by very slow addition of solid AS (0.4 g of salt per mL) after which the solution was stirred gently for one hour and sedimented at 100 KG (25,000 rpm) for 20 minutes at 4 deg C in the Beckmen L8-M ultracentrifuge using the SW-28 rotor.

10. The supernatant was then transferred to ¼ inch dialysis tubing [12 KDa molecular weight cut off (MWCO)] and dialyzed exhaustively against 4 L of buffer.

11. The dialysis was done six times, each time in a fresh buffer to reduce the AS concentration to lower than 20 µM range.

12. The protein solution was then concentrated to ca. 10 ml by ultrafiltration (10 kDa MWCO, Diaflow by Amicon) using N2 pressure unit (Amicon) at 4°C.

13. It was further concentrated to 4 ml by using Millipore centrifuge concentrator (10 kDa MWCO, 1600 rpm, 219 rotor, IECCR centrifuge) at 4°C.

14. The HMGB-1 and HMGB-2 were further purified by using HPLC. However, I did not take part in the final stages.
II) Estrogen receptor (ER) α and β

The recombinant full length human ERα was purchased from Invitrogen (Cat # P2187) (66.4kDa, 559 amino acid residues). The protein was expressed and purified from recombinant baculovirus-infected insect cells. The purity was greater than 80% as determined by a Coomassie blue-stained SDS-PAGE mini gel by Invitrogen. The protein was stored in storage buffer containing 50mM Tris-HCl (pH 8.0), 500mM KCl, 2mM DTT, 1mM EDTA, 1mM sodium orthovanadate, 10% glycerol.

The recombinant full length human ERβ was purchased from Invitrogen (Cat #P2466) (53.4 kDa, 530 amino acid residues). The protein was expressed and purified from recombinant baculovirus-infected insect cells. The purity was greater than 80% as determined by a Coomassie blue-stained SDS-PAGE mini gel by Invitrogen. The protein was stored in storage buffer containing 50mM Tris-HCl (pH 9.0), 400mM KCl, 2mM DTT, 1mM EDTA, and 10% glycerol.

Table II shows the lot number, total protein concentration, specific activity and functional receptor concentration for each of the lots used in the research

**Table II: Estrogen receptor α and β (Invitrogen)**

<table>
<thead>
<tr>
<th>Receptor (Lot number)</th>
<th>Total protein concentration (mg/ml)</th>
<th>*specific activity pmol/mg</th>
<th>*functional receptor concentration (pmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα (3421A)</td>
<td>0.24</td>
<td>8700</td>
<td>2088</td>
</tr>
<tr>
<td>ERβ (23348G)</td>
<td>0.44</td>
<td>10,200</td>
<td>4500</td>
</tr>
</tbody>
</table>

* Functional assays: the concentration of active receptor is determined by quantitation of $[^3H]$- esteraiol:receptor complexes using hydroxyapatitle assay.
III) Glucocorticoid receptor (GR)

The recombinant full length human GR was purchased from Invitrogen as partially purified nuclear extract (Cat # P2812) 85.6kDa). The protein was expressed and partially purified from recombinant baculovirus-infected insect cells. The partially purified protein was stored in storage buffer containing 10mM potassium phosphate (pH 7.4), 5mM DTT, 0.1mM EDTA, 10mM sodium molybdate, 10% glycerol. Except when added separately, all assays were performed without hormone.

Table III below shows the lot numbers, total protein concentration, specific activity and functional receptor concentration for each of the three lots used in the research.

Table III: Glucocorticoid receptor (Invitrogen)

<table>
<thead>
<tr>
<th>Lot number</th>
<th>Total protein concentration (mg/ml)</th>
<th>Specific activity (pmol/mg)</th>
<th>Functional receptor concentration (pmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>36761B</td>
<td>8.55</td>
<td>15.2</td>
<td>130</td>
</tr>
<tr>
<td>26331B</td>
<td>8.3</td>
<td>11.8</td>
<td>98</td>
</tr>
<tr>
<td>34242E</td>
<td>9.6</td>
<td>25</td>
<td>240</td>
</tr>
</tbody>
</table>

* Functional assays: the concentration of active receptor is determined by quantitation of \[^3\text{H}\]- Dexamethasone: receptor complexes using charcoal assay.

IV). Optikinase

Optikinase (USB Co.; Cat # 7833Y) catalyzes the transfer of the terminal phosphate of ATP to 5'-hydroxyl termini of single-stranded and double-stranded DNA. 1,000 units of
95% pure (by SDS-PAGE determined by USB Co.) optikinase was purchased where one unit is amount of enzyme required to incorporate 1nmole of $[^{33}]P$ from $[^{γ-33}]P$ onto 5'-OH of DNA in 30 minutes at 37°C. Optikinase was stored in 50mM Tris-HCl, pH 7.5, 1mM EDTA and 50% glycerol and supplied with the 10X reaction buffer (500mM Tris-HCl, pH 7.5, 100mM MgCl$_2$, 50mM DTT).

V) DNA Oligonucleotides used in EMSA Studies

Oligonucleotides used in EMSA assays were obtained from Integrated DNA Technologies, Inc. (IDT) Table IV indicates the name, sequence, supplier, reference number  (IDT ) and size of oligonucleotides used in my research. (Ghattamaneni. R. 2005, MS thesis, BGSU)

B. End-labeling of DNA (82)

i) End-labeling of single-stranded 33 base pair DNA (cERE, GRE) and 55 base pair DNA

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5' --&gt; 3'</th>
<th>Source</th>
<th>Reference #</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=0</td>
<td>5' TGATGCCTCCAGGTGATGACTGAATCCAACCCAA 3'</td>
<td>IDT</td>
<td>6605069/6605070</td>
<td>30</td>
</tr>
<tr>
<td>n=1</td>
<td>5'-TGATGCCTCCAGGTGCACATGACTGAATCCAACCCAA 3'</td>
<td>GL</td>
<td>7658489/7658490</td>
<td>31</td>
</tr>
<tr>
<td>n=2</td>
<td>5'-TGATGCCTCCAGGTGCAGATGACCGATGACTGAATCCAACCCAA 3'</td>
<td>GL</td>
<td>7658495/7658496</td>
<td>32</td>
</tr>
<tr>
<td>cERE</td>
<td>5'-TGATGCCTCCAGGTGCACTGACCTGAATCCAACCCAA 3'</td>
<td>IDT</td>
<td>7658493/7658494</td>
<td>33</td>
</tr>
<tr>
<td>n=4</td>
<td>5'-TGATGCCTCCAGGTGCACTGACCTGAATCCAACCCAA 3'</td>
<td>IDT</td>
<td>13456079/13456080</td>
<td>30</td>
</tr>
<tr>
<td>cERE</td>
<td>5'-TGATGCCTCCCAACACCTGGTGGGCAACCTAGGTGCACATGATCCCCGAACCCAA 3'</td>
<td>IDT</td>
<td>15699672/15699673</td>
<td>55</td>
</tr>
<tr>
<td>cERE</td>
<td>5'-TGATGCCTCCCAACACCTGGTGGGCAACCTAGGTGCACATGATCCCCGAACCCAA 3'</td>
<td>IDT</td>
<td>15699670/15699671</td>
<td>55</td>
</tr>
<tr>
<td>2cERE</td>
<td>5'-TGATGCCTCCCAACACCTGGTGGGCAACCTAGGTGCACATGATCCCCGAACCCAA 3'</td>
<td>IDT</td>
<td>1733633 order No</td>
<td>55</td>
</tr>
<tr>
<td>DR1-3</td>
<td>5'-TGATGCCTCCCAACACCTGGTGGGCAACCTAGGTGCACATGATCCCCGAACCCAA 3'</td>
<td>IDT</td>
<td>16584565/16584566</td>
<td>55</td>
</tr>
<tr>
<td>DR26</td>
<td>5'-TGATGCCTCCCAACACCTGGTGGGCAACCTAGGTGCACATGATCCCCGAACCCAA 3'</td>
<td>IDT</td>
<td>16594563/16594564</td>
<td>60</td>
</tr>
<tr>
<td>DR1</td>
<td>5'-TGATGCCTCCCAACACCTGGTGGGCAACCTAGGTGCACATGATCCCCGAACCCAA 3'</td>
<td>IDT</td>
<td>14855547/14855548</td>
<td>55</td>
</tr>
<tr>
<td>DR2</td>
<td>5'-TGATGCCTCCCAACACCTGGTGGGCAACCTAGGTGCACATGATCCCCGAACCCAA 3'</td>
<td>IDT</td>
<td>14855553/14855554</td>
<td>55</td>
</tr>
<tr>
<td>EvR1</td>
<td>5'-TGATGCCTCCCAACACCTGGTGGGCAACCTAGGTGCACATGATCCCCGAACCCAA 3'</td>
<td>IDT</td>
<td>14855549/14855550</td>
<td>55</td>
</tr>
<tr>
<td>IR1</td>
<td>5'-TGATGCCTCCCAACACCTGGTGGGCAACCTAGGTGCACATGATCCCCGAACCCAA 3'</td>
<td>IDT</td>
<td>14855551/14855552</td>
<td>55</td>
</tr>
</tbody>
</table>
(Direct repeats, everted repeats and inverted repeats) for EMSA studies.

About 1 L of water was taken in a 2L beaker and was set to boil on a hot plate. An aliquot of 2.0 µL of the non-coding strand of the oligo (50 ng/µL), 17.0µL of ddH₂O, 2. µL of 10X opti-kinase reaction buffer (from USB: 500 mM Tris-HCl, 100 mM MgCl₂, 50 mM DTT, pH 7.5), 2.5 µL γ-(³²P)-ATP and 1.0 µL Optikinase (USB: 10U/µL) was taken into a 500µL Eppendorf tube. The mixture was incubated at 37°C for 30 minutes in a water bath. This reaction was stopped by the addition of 2.0 µL of 0.5 M EDTA (pH 8.0) and the mixture was heated in the boiling water bath for 3-4 minutes to denature the kinase.

ii) Formation of double stranded DNA

A 2.3 µL of the coding strand (50 ng/µL) was added to the labeling mixture and was incubated at 37°C for an additional 5 minutes and heated in the boiling water bath for additional 2-3 minutes. The beaker containing the Eppendorf was then removed from the hot plate and slowly permitted to cool to room temperature to re-anneal the two strands. After the beaker had cooled a few hours (ca. 4-5 hours), the labeled DNA was placed in the refrigerator (4°C) overnight.


The labeled DNA was separated from the γ-(³²P)-ATP through the use of G-50 Sephadex beads. An aliquot of 0.2 grams of G-50 Sephadex beads were swollen in 10 mL of distilled water at room temperature overnight to obtain 4 mL of bead slurry. A spin column was prepared in a 1-mL syringe by plugging the bottom of the spin column with a piece of glass
wool and filling the column with swollen beads. The column was spun at 1600xg (IEC centrifuge: 219 rotor at 2600 rpm) for 2.5 minutes. The syringe was filled with swollen beads until the column volume reached 1 mL. The column was subsequently washed three times with 100 μL portions of TEN buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 8.0). The volume of the DNA reaction mixture was made up to 100 μL with TEN buffer, and the reaction mixture was loaded to the column. The column was spun at 1600xg (IEC centrifuge: 219 rotor at 2600 rpm) for 2.5 minutes, and the 100 μL of the first flow through was collected in a 2 mL microfuge tube. The first fraction usually contained entire end-labeled DNA and this is used for studies and stored at -20°C.

D. Electrophoretic mobility shift assay (EMSA)

Polyacrylamide gels separate DNA and protein according to charges, size and shape. EMSA or band shift analysis is based on the observation that stable protein-DNA complexes migrate through polyacrylamide gels more slowly than free DNA fragments. The DNA molecules can be labeled with radioactive γ-(32 P)-ATP which enables us to observe on a autoradiograph or on a screen using phosphoimager. The assay allows us to determine the amount of DNA bound by the protein as a fraction of protein concentration. This in turn allows the equilibrium dissociation constant (K_D) to be calculated. The DNA sequences were end-labeled with radioactive γ-(32 P)-ATP and were reacted with proteins to form a complex.
ii). Gel preparation and electrophoresis

Native gels 5% acrylamide (29:1 w/w, acrylamide: bis-acrylamide) or 4% acrylamide (49:1, acrylamide: bis-acrylamide) were used for the EMSA studies. To prepare a 5% polyacrylamide gel that was 0.25X TBE, a 7 mL aliquot of 5X TBE buffer (450 mM TrisHCl, 450 mM boric acid, and 10 mM EDTA), 13.5 mL 30% (29:1 w/w) acrylamide, 78.5 mL distilled water and 1 mL of 5% Igepal (Sigma I-3021) were dispensed into a 500 mL flask and degassed for 15 minutes.

To prepare 4% polyacrylamide gel that was 0.25X TBE, a 7 mL aliquot of 5X TBE buffer (450 mM TrisHCl, 450 mM boric acid, and 10 mM EDTA), 8 mL 50% (49:1 w/w) acrylamide, 84 mL distilled water and 1 mL of 5% Igepal were dispensed into a 500 mL flask and degassed for 15 minutes. The final concentration of salts in the gel would be 31.5 mM TrisHCl, 31.5 mM boric acid, and 0.007 mM EDTA.

In the meantime the cassette was prepared with glass plates (19 x 18 and 19 x 16 cm) and plastic spacers (1.5 mm in thickness), clipped and put into a 45 degree inclined position in a bucket.

After degassing, 500 μL of 10% (w/v) ammonium persulfate and 100 μL of TEMED were added and mixed. The gel solution was poured into the cassette and within 15-20 minutes polymerization occurred. If the gel was to be stored, it would be sprinkled with water and stored at 4°C with a Saran wrap around it. The pre-made gel was attached to the power supply and the upper and lower buffer chambers were filled with 0.35X TBE. The gel was pre-electrophoresed at 100 V for 1.5-2 hours or until the current dropped to half its starting value, which was typically 11 mA.
iii). Preparation of ER samples

The preparation of samples for the binding reaction of estrogen receptor (ER) with the responsive elements is given below.

The entire sample preparation was done at 4°C. The ER dilution buffer was first prepared by adding 990 µL of ER binding buffer (80mM KCl, 10% glycerol, 15 mM Tris HCl pH 8.0, 0.2 mM EDTA, 0.4 mM DTT) and 10 µL of 10 mg/mL bovine serum albumin (BSA).

The protein stock solutions ER and HMGB-1 were diluted with ER dilution buffer (80mM KCl, 10% glycerol, 15 mM Tris HCl pH 8.0, 0.2 mM EDTA, 0.4 mM DTT and 100ng/µL BSA) to nanomolar (nM) concentrations (typically 0-90 nM) could be used in each reaction. A typical scheme for making ER dilutions is given below in table V.

ERα dilution: Stock 2088 nM of ERα

**Tabel V: ERα dilution series**

<table>
<thead>
<tr>
<th>Quantity of ER</th>
<th>Dilution buffer</th>
<th>Concentration of ER</th>
<th>ER concentration in the final reaction mixture (2 µL into10 µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.9µL of 2088nM</td>
<td>27.1µL</td>
<td>200nM</td>
<td>40nM</td>
</tr>
<tr>
<td>5µL of 200nM</td>
<td>5µL</td>
<td>100nM</td>
<td>20M</td>
</tr>
<tr>
<td>5µL of 100nM</td>
<td>5µL</td>
<td>50nM</td>
<td>10nM</td>
</tr>
<tr>
<td>5µL of 50nM</td>
<td>5µL</td>
<td>25nM</td>
<td>5nM</td>
</tr>
<tr>
<td>5µL of 25nM</td>
<td>5µL</td>
<td>12.5nM</td>
<td>2.5nM</td>
</tr>
<tr>
<td>5µL of 12.5nM</td>
<td>5µL</td>
<td>6.25nM</td>
<td>1.25nM</td>
</tr>
</tbody>
</table>
Table VI: A typical sample preparation for ER (α and β) interaction with cERE with different spacers (n=0-4) and with direct repeats in the presence and absence of HMGB-1 for EMSA studies (Volumes in µl)

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>ER Dil. Buf.</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
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</tr>
<tr>
<td>2.5 nM ER</td>
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<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5 nM ER</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
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<tr>
<td>10 nM ER</td>
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<tr>
<td>400 nM HMGB1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>2</td>
<td>2</td>
<td>2</td>
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<td>2</td>
</tr>
</tbody>
</table>

iv). Preparation of GR samples

Dexamethasone solution was prepared to be used as the hormone in the reaction mixture. 39.25µg of pure dexamethazone (Sigma Cat # D1756, M.W 392.5Da) was dissolved in 100µL of 100% ethanol to obtain a 200µM solution and then diluted with ddH2O (10µL of stock + 90 µL) to make it 20µM.

 Entire sample preparation was done at 4°C. The BSA in dilution buffer was not needed because GR stock is a partially purified extract consisting of only 30% GR and 70% of other proteins. The protein stock solutions GR was diluted with binding buffer (80mM KCl, 10% glycerol, 15 mM Tris HCl pH 8.0, 0.2 mM EDTA, 0.4 mM DTT). The protein was diluted to nanomolar (nM) concentrations (typically 0-20nM) to be used in each reaction.
The GR dilution buffer was prepared by adding 800µL of ER binding buffer (80mM KCl, 10% glycerol, 15 mM Tris HCl pH 8.0, 0.2 mM EDTA, 0.4 mM DTT), 20µL Dex (20µM), 8.0 µL of 200 ng/µL of poly (dI/dC) and 172.0 µL of ddH2O to get 0.4µM Dex. Finally the concentration of dexamethzone in the reaction mixture was 160nM. A typical scheme for making GR dilutions is given below in table VII.

GR dilution: Stocks were, 240nM, 130 nM and 98nM,

A new stock solution was prepared, (concentration was 50nM) by using each of GR stock solution (240nM, 130 nM or 98nM). This solution (50nM) was used to prepare series of GR solutions with different concentrations as shown in following table.

**Tabel VII: GR dilution series**

<table>
<thead>
<tr>
<th>Quantity of GR</th>
<th>Dilution buffer</th>
<th>Concentration of GR</th>
<th>GR concentration in the final reaction mixture (2 µL into10 µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4µL of 50nM</td>
<td>3.6µL</td>
<td>20nM</td>
<td>4nM</td>
</tr>
<tr>
<td>2.1µL of 50nM</td>
<td>3.9µL</td>
<td>17.5nM</td>
<td>3.5M</td>
</tr>
<tr>
<td>1.8µL of 50nM</td>
<td>4.2µL</td>
<td>15nM</td>
<td>3nM</td>
</tr>
<tr>
<td>1.5µL of 50nM</td>
<td>4.5µL</td>
<td>12.5nM</td>
<td>2.5nM</td>
</tr>
<tr>
<td>1.2µL of 50nM</td>
<td>4.8µL</td>
<td>10nM</td>
<td>2nM</td>
</tr>
<tr>
<td>1µL of 50nM</td>
<td>8.5µL</td>
<td>65nM</td>
<td>1nM</td>
</tr>
</tbody>
</table>
Table VIII: A typical sample preparation for GR interaction with DNA in presence
and absence of HMGB-1 for EMSA studies. (Volumes in µL)

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
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</thead>
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<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Dil. Buf.</td>
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<td>4</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>2</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1.5 nM GR</td>
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<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>2 nM GR</td>
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<tr>
<td>400 nM HMGB1</td>
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<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

v). Preparation DNA working solution

The DNA dilution, with $\gamma^{(32)}$-ATP end-labeled DNA was prepared by taking an aliquot of 2.4 µL of 33-mer HRE (2ng/µL) and 4.8µL of 200 ng/µL of poly (dl/dC) diluted up to 960µL with binding buffer so that 100pM concentration of DNA could be obtained in the final reaction mixture. Each reaction was performed in a 500 µL Eppendorf tube, which was maintained at 4°C. A typical reaction consisted of 2µL of diluted protein, 4µL of dilution buffer and 4µL of DNA dilution in a total of 10 µL reaction mixtures. Preparation of a typical reaction mixture is shown below in table.

vi). Preparation of reaction mixture

The DNA was added to the dilution buffer in the tube and then the proteins (ER dilutions or HMGB-1 400nM) were added simultaneously. The entire mixture was then incubated for 20 minutes at 4°C. After incubation a 10 µL aliquot of reaction mixture was loaded on each
well at 250 V. after loading, the voltage was lowered to 200V and the gel was electrophoresed at 200V for 90 minutes at 4°C.

vi). **ERs and GR binding to DNA double stranded oligonucleotides.**

The entire sample preparation was done at 4°C. The protein stock solutions GR and ER were diluted with GR dilution buffer and ER dilution buffer, respectively as described above.

The DNA dilution, with $\gamma$-(32 P)-ATP end labeled DNA was prepared by taking an aliquot of 2.4 µL of 33-mer HRE (2ng/µL) and 4.8 µL of 200 ng/µL of poly(dI/dC) diluted up to 960µL with binding buffer so that 100pM concentration of DNA could be obtained in the final reaction mixture. Each reaction was performed in a 500 µL Eppendorf tube, which was maintained at 4°C. A typical reaction consisted of 2 µL of each of the diluted proteins, 2µL of GR-dilution buffer and 4µL of DNA dilution in a total of 10µL reaction mixture. Preparation of a typical reaction mixture is shown below in table

The GR dilutions were added to the GR-dilution buffer in the tube and incubated for 20 minutes at 4°C, ER dilutions were added subsequently. DNA dilution was then added immediately and the entire mixture was incubated for further 20 minutes at 4°C. After incubation a 10 µL aliquot of reaction mixture was loaded on each well at 250 V. after loading, the voltage was lowered to 200V and the gel was electrophoresed at 200V for 90 minutes at 4°C.
E. Gel drying

After electrophoresis, the gel was carefully transferred to a 3M Whatman filter paper, covered with Saran Wrap and placed in a gel dryer. Vacuum was applied to the gel. A dry ice/isopropanol mixture was used in the vacuum trap chamber, with heating for 30 minutes.

F. Autoradiogram

After the gel was completely dried, it was transferred to an X-Ray cassette. X-ray film (Kodak or Fuji) was placed on the gel in the dark and the cassette cover containing Dupont intensifying screen was shut tightly. The gel was exposed at −80°C for various lengths of time depending on the activity of the radio-labeled oligonucleotide. The cassette was removed from the −80°C freezer and allowed to reach room temperature. The film was developed in the dark by immersing and gently agitating the film for 5 minutes in developer solution (Sigma Co. Cat # 190 0984). The film was then rinsed for 10-30 seconds in distilled water and finally it was placed in fixer solution (Sigma Co. Cat # 190 2485) for 1-2 minutes. The film was then rinsed finally with distilled water and then hung for drying. Both developer and fixer solutions were prepared by taking 100 mL of the concentrated solution and diluting it with ddH₂O to 500 mL.

For quantitative measurements using a phosphoimager, the gels were exposed to a special phosphoimager screen (Amersham Biosciences. Cat # 63003487), which absorbs the radiation temporarily. It was kept at room temperature for 2-3 days depending on the activity of the DNA. The screens were then scanned on phosphoimager for quantitative purposes at department biological science of BGSU.
G. Quantification of bands to find Kd values.

The phosphoimager screen which was exposed to the polyacrylamide gel was scanned on a phosphoimager through Storm Scanner software and the image was saved on the hard disc. The image was then opened with Image Quant software to quantify the bands using the analysis tool. A box was drawn around each selected band on the autoradiogram. The band intensity value corresponding directly to the amount of radioactive material was read within each box as counts per band surface and was recorded in Excel sheet. Similarly a grid was prepared around the other free and complex DNA bands to report the intensity in the same Excel sheet. The K_d values of the particular protein-DNA interaction are calculated as per the following calculations.

i) K_d values (83)

The DNA concentration was kept constant (100 pM) at a level well below the concentration of the protein (1nM or higher for ERβ). The point at which 50% of the labeled DNA is bound with protein corresponds to the apparent K_d as shown by the following.

Protein-DNA interactions can be represented as an equilibrium reaction:

\[ \text{[protein-DNA complex]} \overset{k_{\text{On}}}{\rightleftharpoons} \text{[protein}_{\text{free}}\text{]} + \text{[DNA}_{\text{free}}\text{]} \]

\[ K_{D} = \frac{[\text{protein}_{\text{free}}\text{]} [\text{DNA}_{\text{free}}\text{]} }{[\text{protein-DNA complex}]} = \frac{k_{\text{Off}}}{k_{\text{On}}} \]
Where $K_D$ is the apparent equilibrium dissociation constant ($K_D$), $k_{off}$ is the dissociation rate constant, and $k_{on}$ is the association rate constant.

When 50% of the protein is bound to DNA,

\[ [\text{DNA}_{\text{free}}] = [\text{protein-DNA complex}], \]

and the above equation simplifies to $K_{eq} = [\text{protein}_{\text{free}}]$. Percentage complex formed was determined from the band intensities of the complex and free DNA measured by phosphoimager as. % complexation = [complex] / [complex + DNA]. A graph of protein concentration versus percentage complex was prepared on Sigma Plot and the $K_{50}$ was read at the 50% complexation. $K_{50}$ is defined as follows.

\[ K_{50} = \frac{(C_1+C_2) \times 100}{(C_1+C_2+\text{DNA})} \]

Where, C1 and C2 are Complex 1 and 2 respectively.
CHAPTER III

RESULTS

The research aimed to achieve three primary aims.

A). The first aim was to study the binding affinity and selectivity of ERα and ERβ for consensus ERE (cERE) variable length spacers between the ERE half-sites. This study was carried out in the presence and absence of HMGB-1 protein. This spacer valued in length between 0 and 4bps. (This study was previously done by Marzouk. M, 2004 MS thesis, BGSU)

B). The second part of the research focused on determining the binding affinity and selectivity of ERα and ERβ to cHERE half-sites in which formed in either direct repeats, inverted repeats, or everted repeats in the presence and absence of HMGB-1 protein. (This study was previously done by Ghattamanenin. R, 2005 MS thesis, BGSU)

C). The final objective of the research dealt with the determination of the binding affinity and selectivity of GR to cGRE, cHERE, cERE, and a series of direct repeats, inverted repeats and everted repeats of cHERE in the presence and absence of the transcriptional coactivator, HMGB-1.
**ERα and ERβ binding to cERE with various spacer sizes (n=3)**

Figure 4 and 5 shows ERs (α and β) binding to cERE (n=3) in the presence and absence of HMGB-1. cERE DNA duplex fragment contains two perfect half sites containing AGGTCA and TGACCT motifs separated by 3ps.

In the absence of HMGB-1, 75% of DNA was bound to ERα/DNA complex at 14nM ERα, whereas, for ERβ it was at 30nM. The extent of ERs binding was increased with increasing the ERs level in the presence of HMGB-1. In the presence of HMGB-1, 50% binding occurs for ERα at 5.8nM whereas, for ERβ, 12.5nM. This shows that ERα bound to cERE stronger than ERβ in the presence of HMGB-1. In the presence of HMGB-1, the equilibrium binding profile for ERα shows a significant shift towards the lower concentration of ERα, since K_D value is reduced from 10.5nM to 5.7nM, whereas, for ERβ change in K_D value was from 16nM to 12nM.
Fig 4: **ERα binding to cERE (n=3) in the absence and presence of HMGB-1.**

A 100 pM probe of cERE (n=3) was incubated with 0.25, 0.5, 1, 2, 4, 6, 8, and 14 nM, of ERα, respectively in the absence (lanes 2-9), and in the presence of HMGB-1 (lanes 10-17). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.

Fig 5: **ERβ binding to cERE (n=3) in the absence and presence of HMGB-1.**

A 100 pM probe of cERE (n=3) was incubated with 1, 2, 4, 5, 6, 8, 10, 15, 25, and 30 nM, of ERβ, respectively in the absence (lanes 2-11), and in the presence of HMGB-1 (lanes 12-21). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.
Figure 6: Equilibrium binding profile of ER\(\alpha\) and ER\(\beta\) binding to cERE (\(n=3\)) in the absence and presence of HMGB-1. The percent of ER\(\alpha\) complex bound in the presence (●) or absence (▲) of HMGB-1 obtained from EMSA profiles are plotted as a function of ER\(\alpha\) concentration.
Figures 7 and 8 show ERα and ERβ binding to cERE with n=0 (cERE0) in the presence and in the absence of HMGB-1, respectively. For ERα, the first ERα/ERE complex was observed at 4.5nM ERα, whereas, in the presence of HMGB-1, the first complex was detected at 1.5nM ERα. For ERβ, in the absence of HMGB-1, the first ERβ/ERE complex was detected at 1.5nM ERβ. As was found previously for ERα, in both figures, lane (11-19), HMGB-1 has enhanced binding affinity of both ERα and ERβ. Figure 9 shows the equilibrium binding profiles of ERα and ERβ binding to cERE with n=0 (cERE0) in the absence and presence of HMGB1. The percent of receptor complex bound to cERE in the presence or absence of HMGB1 was obtained when % complex profile is plotted as a function of receptor concentration using the Sigma Plot. Approximately a 3- fold increase in binding affinity was observed with in the presence of HMGB-1 as the $K_D$ decreased from 7nM to 2.4nM for ERα. This $K_D$ value (7nM) for ERα binding to cERE (cERE, n=0) is lower than value observed for cERE (cERE3). Under the same conditions, HMGB-1 was capable of enhancing the binding affinity of ERβ to cERE with n=0 (cERE0) and the insignificant effect in binding affinity was detected since, $K_D$ value decreased from 2.6nM to 2.4nM. Difference in conformation of ERα and ERβ bound to cERE (n=0) may account for the observed even small difference in $K_D$ values. Finally, the results reveal that the presence of 0bp spacer enhances the binding affinity of ERα and ERβ to cERE (n=0) and the enhancement in binding by HMGB-1 was more in case of ERα than for ERβ.
Fig 7: ERα binding to cERE (n=0) in the absence and presence of HMGB-1. A 100 pM probe of cERE (n=0) was incubated with 1.5, 3, 4.5, 7, 9, 13, 16, 21 and 25 nM, of ERα, respectively in the absence (lanes 2-10), and in the presence of HMGB-1 (lanes 11-19). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.

Fig 8: ERβ binding to cERE (n=0) in the absence and presence of HMGB-1. A 100 pM probe of cERE (n=0) was incubated with 1.5, 3, 4.5, 7, 9, 13, 16, 21 and 25 nM, of ERβ, respectively in the absence (lanes 2-10), and in the presence of HMGB-1 (lanes 11-19). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.
Fig 9: Equilibrium binding profile of ERα and ERβ binding to 0 n ERE in the absence and presence of HMGB-1. The percent of ER complex bound in the presence (■) or absence (▲) of HMGB-1 obtained from EMSA profiles are plotted as a function of ER concentration.
Figures 10 and 11, show ERα and ERβ binding to cERE with n=1 (cERE1) in the presence and absence of transcriptional coactivator HMGB-1, respectively. For ERα, the first ERα/ERE complex was detected at 4.5nM of ERα, whereas, in the presence of HMGB-1, the same complex was detected at 1.5nM ERα. For ERβ and in the absence of HMGB-1, the first ERβ/ERE band observed at 1.5nM ERβ while, in the presence of HMGB-1, the initial complex was detected at 1.5 nM ERβ. In both the figures, lane (11-19), the binding affinity was enhanced by HMGB-1 for both ERα and ERβ. The figure 12 shows the equilibrium binding profiles of ERα and ERβ binding to cERE with n=1 (cERE1) in the absence and presence of HMGB1. The percent of receptor complex bound to cERE in the presence or absence of HMGB1 was obtained when % complex profile is plotted as a function of receptor concentration using the Sigma Plot. Approximately two- fold increase in binding affinity was observed in the presence of HMGB-1 as the $K_D$ decreased from 14.8nM to 7.4nM for ERα. This $K_D$ value (7.4nM) for ERα binding to cERE (cERE, n=1) is lower than value observed for cERE (cERE3) in the absence of HMGB-1. Under the same conditions, HMGB-1 could also enhance the binding affinity of ERβ to cERE with n=1 (cERE1) and the 4- fold increase binding affinity was observed in the presence of HMGB-1, as the $K_D$ decreased from 17.5nM to 4.6 nM for ERβ. When the percent complex formation for both receptors were compared, HMGB-1 showed more stimulation on ERβ than on ERα. Further results reveal that the presence of 1bp spacer enhances the binding affinity of ERα and ERβ to ERE and the enhancement in extent of binding by HMGB-1 was more in case of ERβα than that of ERα., as the concentration of for 50% complexation for ERα was reduced from 14.8 nM
to 7.4 nM whereas, the stimulation was 2-fold, dropping the $K_D$ value from 15nM to 4nM for ERβα.
**Fig 10:** ERα binding to cERE (n=1) in the absence and presence of HMGB-1. A 100 pM probe of cERE (n=1) was incubated with 1.5, 3, 4.5, 7, 9, 13, 16, 21 and 25 nM, of ERα, respectively in the absence (lanes 2-10), and in the presence of HMGB-1 (lanes 11-19). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.

**Fig 11:** ERβ binding to cERE (n=1) in the absence and presence of HMGB-1. A 100 pM probe of cERE (n=1) was incubated with 1.5, 3, 4.5, 7, 9, 13, 16, 21 and 25 nM, of ERβ, respectively in the absence (lanes 2-10), and in the presence of HMGB-1 (lanes 11-19). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.
Fig 12: Equilibrium binding profile of ERα and ERβ binding to cERE (n=1) in the absence and presence of HMGB-1. The percent of ER complex bound in the presence (■) or absence (▲) of HMGB-1 obtained from EMSA profiles are plotted as a function of ER concentration.
Figures 13 and 14, show ERα and ERβ binding to cERE with n=2 (cERE2) in the presence and absence of HMGB-1, respectively. For ERα, a less intense ERα/ERE complex band was observed at 4.5nM ERα, whereas, in the presence of HMGB-1, the intensity of this band dramatically enhanced for ERα. For ERβ, in the absence of HMGB-1, the very first ERβ/ERE complex observed at 3 nM ERβ, and in the presence of HMGB-1 a little intense band was observed at 1.5 nM. While, both the gel profiles show that, lane (11-19), binding affinity was enhanced by HMGB-1 for both ERα and ERβ. The figure 15 shows the equilibrium binding profiles of ERα and ERβ binding to cERE with n=2 (cERE2) in the absence and presence of HMGB1. The percent of receptor complex bound to cERE in the presence or absence of HMGB1 was obtained and plotted as a function of receptor concentration using the Sigma Plot. No significant change in binding affinity of ERα was observed in the presence of HMGB-1, since the $K_D$ decreased from 5.1nM to 4.7 nM ERα. This $K_D$ value (5.1nM) for ERα binding to cERE (cERE, n=2) is lower than value observed for cERE (cERE3) in the absence of HMGB-1. When the percent of total complex formation for both receptors were compared, HMGB-1 showed more stimulation on ERβ than on ERα for cERE2, since the concentration of for 50% complexation for ERβ was reduced to 9 nM to 4.1 nM, whereas, in case of ERα, the stimulation was not significant but it caused to bring down the $K_D$ value from 5.1 nM to 4.7 nM. Results indicate that HMGB-1 has a greater effect on ERβ than on ERα. It can be concluded that both specificity and binding constant decreased for both receptors in presence of HMGB-1.
**Fig 13: ERα binding to cERE (n=2) in the absence and presence of HMGB-1.** A 100 pM probe of cERE (n=2) was incubated with 1.5, 3, 4.5, 7, 9, 13, 16, 21 and 25 nM, of ERα, respectively in the absence (lanes 2-10), and in the presence of HMGB-1 (lanes 11-19). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.

**Fig 14: ERβ binding to cERE (n=2) in the absence and presence of HMGB-1.** A 100 pM probe of cERE (n=2) was incubated with 1.5, 3, 4.5, 7, 9, 13, 16, 21 and 25 nM, of ERβ, respectively in the absence (lanes 2-10), and in the presence of HMGB-1 (lanes 11-19). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.
Fig 15: Equilibrium binding profile of ERα binding to c ERE (n=2) in the absence and presence of HMGB-1. The percent of ER complex bound in the presence (▲) or absence (●) of HMGB-1 obtained from EMSA profiles are plotted as a function of ER concentration.
Figures 16 and 17, show ERα and ERβ binding to cERE with n=4 (cERE4) in the presence and absence of HMGB-1, respectively. As expected, the mobility of two complexes for ERα and ERβ was the same, because of their comparable molecular weights. For ERα, a ERα/ERE complex was observed at 3 nM ERα, whereas, in the presence of HMGB-1 the very first band was detected at 1.5 nM ERα. For ERβ, in the absence of HMGB-1, the foremost ERβ/ERE complex observed at 4.5 nM ERβ, and in the presence of HMGB-1 it was detected at 3 nM ERβ. HMGB-1 enhanced the extent of binding of both ERα and ERβ, and this is shown in both figures, lane (11-19).

Equilibrium binding profiles of ERα and ERβ binding to cERE with n=4 (cERE 4) in the absence and presence of HMGB1 is shown in figure 18. The percent of receptor complex bound to cERE in the presence or absence of HMGB1 was determined and equilibrium binding profile is shown in figure 18. Significant changes in the extent of binding was observed in the presence of HMGB-1 for ERα as the K_D decreased from 11 nM to 5.1 nM. This K_D value (11 nM) for ERα binding to cERE (cERE, n=4) is lower than value observed for cERE (cERE3) in the absence of HMGB-1. When the percent of total complex formation for both receptors were compared, HMGB-1 showed a greater stimulation on ERβ than on ERα to cERE4, as the concentration of for 50% complexation for ERα was reduced to 11 nM to 5.1 nM whereas, in case of ERβ, the stimulation was 3.7-fold, decreasing the K_D value from 9.8 nM to 2.6 nM. This indicates that when compare two receptors, enhancement in binding by HMGB-1 was greater for ERβ than ERα. It can be concluded that both specificity and binding constant decreased for both the receptors in the presence of HMGB-1. These findings are consistent with previous reports in our lab (Marzouk S. 2003 MS. thesis BGSU)
Fig 16: ERα binding to cERE (n=4) in the absence and presence of HMGB-1. A 100 pM probe of cERE (n=4) was incubated with 1.5, 3, 4.5, 7, 9, 13, 16, 21 and 25 nM, of ERα, respectively in the absence (lanes 2-10), and in the presence of HMGB-1 lanes(11-19). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.

Fig 17: ERβ binding to cERE (n=4) in the absence and presence of HMGB-1. A 100 pM probe of cERE (n=4) was incubated with 1.5, 3, 4.5, 7, 9, 13, 16, 21 and 25 nM, of ERβ, respectively in the absence (lanes 2-10), and in the presence of HMGB-1 lanes(11-19). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.
Fig18: Equilibrium binding profile of ERα and ERβ binding to cERE (n=4) in the absence and presence of HMGB-1. The percent of ER complex bound in the presence (▲) or absence (■) of HMGB-1 obtained from EMSA profiles are plotted as a function of ER concentration.
Table IX: $K_D$ for ER$\alpha$/ERE (n) interaction in the presence and absence of HMGB-1.

<table>
<thead>
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<th>Oligonucleotides</th>
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<th>HMGB effect</th>
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<td>2.9</td>
</tr>
<tr>
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</tbody>
</table>

Table IX shows the $K_D$ values for the interactions of ER$\alpha$ with various cERE (n), in which n= 0, 1, 2, 3 and 4bps. $K_D$ values were obtained from equilibrium binding profiles and correspond to 50% complexation. $K_D$ values were reported as a function of ER$\alpha$ concentration in nM, in the presence and absence of 400nM HMGB-1

Table X: $K_D$ for ER$\beta$/ERE (n) interaction in the presence and absence of HMGB-1

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Without HMGB1</th>
<th>With HMGB1</th>
<th>HMGB effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 n cERE</td>
<td>2.6</td>
<td>2.4</td>
<td>1.1</td>
</tr>
<tr>
<td>1 n cERE</td>
<td>17.5</td>
<td>3.8</td>
<td>4.6</td>
</tr>
<tr>
<td>2 n cERE</td>
<td>9</td>
<td>4.1</td>
<td>2.2</td>
</tr>
<tr>
<td>3 n cERE</td>
<td>15.3</td>
<td>12</td>
<td>1.3</td>
</tr>
<tr>
<td>4 n cERE</td>
<td>9.8</td>
<td>2.6</td>
<td>3.7</td>
</tr>
</tbody>
</table>
Table X shows the $K_D$ values for the interactions of ER$\beta$ with various cERE (n), in which $n= 0, 1, 2, 3$ and $4$bps. $K_D$ values were obtained from binding plots and correspond to 50% complexation. $K_D$ values were reported as a function of ER$\beta$ concentration in nM, in the presence and absence of $400nM$ HMGB-1.

B) **Comparison of ER$\alpha$ and ER$\beta$ interaction with cERE, 2cERE (refer to table III), cHERE and DR (1)**

The purpose of this study was to compare the binding affinity of baculovirus expressed full length hER$\alpha$ and hER$\beta$ to cERE, 2cERE, cHERE and DR (1) each on a 55bps DNA. This section further explains briefly what experiment is going to be done during the research. Figures 19 and 20 show a general comparative titration of cERE, 2cERE, cHERE and DR(1) with ER$\alpha$ and ER$\beta$, respectively.

Consensus ERE (cERE) is composed of two classical cHERE half-sites separated by three base pairs. The 2cERE contains two tandem EREs are separated by 22bps, center-to-center. The cHERE DNA contains only one perfect half-site while, the other one is completely mutated. DR(1) DNA contains two perfect half sites (AGGTCA), separated by 15 bps with a head to head orientation. From lane (2-5), the extent of binding of ER$\alpha$ and ER$\beta$ to cERE was increased as increasing the receptor concentration. The mobility is the same for complexes in both figures. This further confirms that, both receptors, bind to respective DNA as dimers, since ERs binds to cERE as dimer. The gel for 2cERE shows two ER/DNA complexes are denoted as C1 and C2. Both C1 and C2 bands appeared simultaneously at 5nM ERs ($\alpha$ and $\beta$). These data suggested that the binding of both receptors ER ($\alpha$ and $\beta$) to 2cERE were very similar.
Previous studies in our lab have reported that (Das, A, 2002 Ph.D thesis BGSU) ERα and ERβ bind with ERE2/ERE1 cooperatively, explaining that the binding of receptors at one site stimulate the binding at the second site as receptor concentration increased. These results are comparable and consistent with previous findings of the cooperativity of two receptors (α and β) on tandem EREs.
Fig 19: Titration of ERα with cERE, 2cERE, cHERE and DR (1). A 100pM probes of cERE (lanes 2-5), 2cERE (lanes 6-9), cHERE (lanes 10-13) and DR (1) (lanes 14-17) was incubated with 5, 10, 20, and 25nM of ERα from lane 1 to 17. The lane 1 contains only free DNA.

Fig 20: Titration of ERβ with cERE, 2cERE, cHERE and DR (1).

A 100pM probes of cERE (lanes 2-5), 2cERE (lanes 6-9), cHERE (lanes 10-13) and DR (1) (lanes 14-17) was incubated with 5, 10, 20, and 25nM of ERα from lane 1 to 17. The lane 1 contains only free DNA.
Figures 21 and 22, lane (2-9) and lane (10-17) show the ERα and ERβ binding to DR (1) in the presence and absence of HMGB-1, respectively. The vitellogenin B1 ERE contains the two imperfect EREs (ERE2/ERE1) separated by 20 bps, center-to-center (80, 81). Because the sequences in the individual half-sites for both ERE2 and cERE1 differ, ERs binding at these sites will not be equivalent.

DR (1) DNA duplex fragment contains two perfect 5’ half sites (AGGTCA), followed by three nonspecific nucleotides and the both the 3’ half sites, have been completely mutated (GTTGGG). In both figures, lane 2-9, the extent of binding increased as the concentration of ER increased. In the absence of HMGB-1, ERα and ERβ produced a major complex (C1) followed by second protein-DNA complex C2. DR (1) contains two perfect 5’ half sites, suggest that ER (α and β) preferentially bind on them as a dimer, the faster mobility complex (C1) contains an ER dimer, whereas, the slower mobility complex contains two dimer or a higher order ER oligomer. The both complexes were obtained in the absence of HMGB-1, indicating that they do not represent ER complexes containing HMGB-1.

Both figures show that at low concentrations of ERs (α and β), HMGB-1 stimulate the formation of the both complexes, but at high receptor concentrations, HMGB-1 predominantly stimulate the slower mobility complex (C2) concomitant with a decrease of the faster mobility complex (C1). ERα and ERβ bind to single half site (C1) with the $K_D$ of 20nM and 22nM, respectively. This difference in affinity is consistent with previous findings in our lab, in which ERα and ERβ bind to single half site (C1) with the $K_D$ of 7nM and 8nM respectively, with ERα, having a higher affinity for DR(1) than ERβ. The strong cooperativity of binding could observe in the presence of HMGB-1.
**Fig 21: ERα binding to DR (1) in the absence and presence of HMGB-1.** A 100 pM probe of DR(1) was incubated with 2, 8, 16, 20, 25, 30, 45, and 52 nM, of ERα, respectively in the absence (lanes 2-9), and in the presence of HMGB-1 (lanes 10-17). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.

**Fig 22: ERβ binding to DR (1) in the absence and presence of HMGB-1.**

A 100 pM probe of DR(1) was incubated with 2, 8, 16, 20, 25, 30, 45, and 52 nM, of ERβ, respectively in the absence (lanes 2-9), and in the presence of HMGB-1 (lanes 10-17). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.
Fig 23: Equilibrium binding profile for ERα and ERβ binding to DR1, respectively, in the absence and presence of HMGB1. Percent ER binding at one half-site (C 1) and two half-sites (C 2), in the absence of HMGB-1, 400nM.
Figures 24 and 25 show the binding profile of ERα and ERβ bound to DR2 in the presence and absence of HMGB-1 respectively. DR (2) DNA duplex fragment contains two perfect half sites containing TGACCT motifs were separated by 15bps. The both the 3’ half sites, have been completely mutated (AGGTCA). In both figures, lane 2-9, the extent of binding increased as the concentration of ER increased. However, in the absence of HMGB-1, ERα and ERβ produced two protein-DNA complexes, denoted as C1 and C2. DR (2) contains two perfect 5’ half sites, suggests that ER (α and β) preferentially bind on them as a dimer, the faster mobility complex (C1) contains an ER dimer, whereas, the slower mobility complex contains two dimer or a higher order ER oligomer. The both complexes were obtained in the absence of HMGB-1, indicating that they do not represent ER complexes containing HMGB-1. At low concentrations of ER (α and β), addition of HMGB-1 stimulated the formation of both mobility complexes, but at high receptor concentrations, HMGB-1 predominantly stimulate the slower mobility complex (C2) concomitant with a decrease of the faster mobility complex (C1). ERα and ERβ bind to single half site (C1) with the $K_D$ of 11.2nM and 17.5nM respectively. This affinity difference is consistent with previous findings in our lab (Ghattamaneni. R. 2005, MS. thesis BGSU), in which ERα and ERβ bind to single half site (C1) with the $K_D$ of 4nM and 9.6nM respectively, clearly explained that ERα show higher affinity for DR(2) than ERβ.
**DR2**

5' TGATGCCTCCCCAACctgTGACCTCAACCTCCCCAACctgTGACCTCTTTAGTTGG 3'

![Image of gel electrophoresis with lanes labeled 1 to 17, showing bands labeled C1, C2, C3, and C4.]

**Fig 24: ERα binding to DR (2) in the absence and presence of HMGB-1.**

A 100 pM probe of DR (2) was incubated with 2, 8, 16, 20, 25, 30, 45, and 52 nM, of ERα, respectively in the absence (lanes 2-9), and in the presence of HMGB-1 (lanes 10-17). The concentration of HMGB-1 was 400 nM.

![Image of gel electrophoresis with lanes labeled 1 to 17, showing bands labeled C1, C2, C3, and C4.]

**Fig 25: ERβ binding to DR (2) in the absence and presence of HMGB-1.**

A 100 pM probe of DR (2) was incubated with 2, 8, 16, 20, 25, 30, 45, and 52 nM, of ERβ, respectively in the absence (lanes 2-9), and in the presence of HMGB-1 (lanes 10-17). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.
Fig 26: Equilibrium binding profile for ERα and ERβ binding to DR2, respectively, in the absence and presence of HMGB-1. Percent ER binding at one half-site (C1) and two half-sites (C2), in the presence of HMGB-1 400nM.
Figures 27 and 28 show binding profile of ERα and ERβ bound to DR (3) in the presence and absence of HMGB-1 respectively. DR (3) oligonucleotide is composed of two perfect half sites (AGGTCA), directed in head to tail orientation. The direct repeats of two AGGTCA motifs are separated by 3-bps.

In figure 29, in the absence of HMGB-1, two complexes, C1 and C2 were observed, whereas, at low ERα concentrations, the faster mobility complex (C1) is predominant. One complex C1 is for an ER dimer binding to one AGGTCA motif, while, the second complex C2 containing ERα binding to both AGGTCA motifs as dimer. This behavior hardly observed for ERβ in the absence of HMGB-1. In both figures 26 and 27, in the presence of HMGB-1, both receptors showed two complexes at very low receptor concentration. The absence of HMGB-1, C2 shows less co-cooperativity for ERα and start forming C2 around 25nM and increased between 16nM and 52nM. In the presence of HMGB-1 formation of C2 was more co-operative for both ERs, showing a steep rise in binding between 10nM and 15nM of the receptor concentration and saturated around 18nM. It was surprising that, faster mobility, less intense two bands are denoted as C3 and C4 were observed at very low concentration of receptors only in the presence of HMGB-1. Whereas, the intensity of C3 and C4 are being increased over initial proteins concentration and then start decreasing suddenly as the C1 and C2 start forming.
**Fig 27: ERα binding to DR1-3 in the absence and presence of HMGB-1.**
A 100 pM probe of DR1-3 was incubated with 2, 8, 16, 20, 25, 30, 45, and 52 nM, of ERα, respectively in the absence (lanes 2-9), and in the presence of HMGB-1 (lanes 10-17). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.

**Fig 28: ERβ binding to DR1-3 in the absence and presence of HMGB-1.** A 100 pM probe of DR1-3 was incubated with 2, 8, 16, 20, 25, 30, 45, and 52 nM, of ERα, respectively in the absence (lanes 2-9), and in the presence of HMGB-1 (lanes 10-17). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.
Fig 29: Equilibrium binding profile for ERα and ERβ binding to DR1-3, respectively, in the absence and presence of HMGB-1. Percent ER binding at one half-site (C 1) and two half-sites (C 2), in the absence of HMGB-1, 400nM.
Figures 30 and 31 show binding profile of ER (α and β) binding to EvR1 in the presence and absence of HMGB-1 respectively. EvR1 oligonucleotide is composed of two perfect half sites separated by 5bps directed in head to tail orientation. The percent complexation of ERs/EvR1 as a function of ER concentration, in the presence and absence of HMGB-1 was determined by phosphoimage quantitation of band intensities and plotted using Sigma Plot. The equilibrium binding profiles are shown in figure 32. In figure 30, in the absence of HMGB-1, although two complexes, C1 and C2 were observed, C1 was the predominant complex over broad range of ERα, whereas, in figure 31, the both bands were detected starting from the third lane. It was surprising that cooperativity was observed for the both receptors, even in the absence of HMGB-1, but strong cooperativity was detected for ERβ in the absence of HMGB-1.

At low concentrations of ER (α and β), HMGB-1 stimulate the formation of both mobility complexes, but at high receptor concentrations, HMGB-1 predominantly stimulate the formation of slower mobility complex (C2) concomitant with a decreasing the intensity of the faster mobility complex (C1). In the absence of HMGB-1, ERα and ERβ bind to single half site (C1) with the Kᵝ values of 14.2nM and 12.5nM respectively, whereas, in the presence of coactivator protein Kᵝ values were 13.4nM and 12.4nM for ERα and ERβ respectively.
**EvR1**

5' TGATGCCTCCCCAACctgTGACCTCAACCTAGGTCAtgGTTGGGTCTTAGTTGG 3'

![Image of gel with bands labeled C1 and C2]

**Fig 30: ERα binding to EvR1 in the absence and presence of HMGB-1.** A 100 pM probe of EvR1 was incubated with 2, 8, 16, 20, 25, 30, 45, and 52 nM, of ERα, respectively, in the absence (lanes 2-9), and in the presence of HMGB-1 (lanes 10-17). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.

![Image of gel with bands labeled C1 and C2]

**Fig 31: ERβ binding to EvR1 in the absence and presence of HMGB-1.** A 100 pM probe of EvR1 was incubated with 2, 8, 16, 20, 25, 30, 45, and 52 nM, of ERβ, respectively, in the absence (lanes 2-9), and in the presence of HMGB-1 (lanes 10-17). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.
Fig 32: Equilibrium binding profile for ERα and ERβ binding to EvR1, respectively, in the absence and presence of HMGB-1. Percent ER binding at one half-site (C 1) and two half-sites (C 2), in the absence of HMGB-1, 400nM.
Figure 33 and 34 show the ERα and ERβ binding to direct repeat, DR1 (26), both the half-sites, with a center to center separation of 26 bps. DR1-26 DNA duplex has two perfect 5’ half-sites (AGGTCA) separated by 19 bps. Figures 33 and 34, lanes (2-9) show as the concentration of ERα and ERβ increases, binding affinity is increased showing two complexes C1 and C2. DR (26) is composed of two perfect half sites, suggest that ER (α and β) preferentially bind on them as a dimer. The faster mobility complex (C1) contains an ER dimer, whereas, the slower mobility complex contains two dimer or a higher order ER oligomer. Both complexes were observed in the absence of HMGB-1, indicating that they do not represent ER complexes containing HMGB-1. In the absence of HMGB-1, ERα showed some co-cooperativity in binding to DR (26), as C2 formation was increased between 20nM and 52nM of the receptor concentration, which partially saturated at 52nM. This cooperativity was further enhanced in the presence of HMGB-1 as shown in the equilibrium binding profile between 10nM and 20nM. Equilibrium binding profile for the both receptors revealed that binding saturation for C2 formation was achieved ca. 25nM. In figure 32, C2 was hardly observed at low receptor concentration in the absence of HMGB-1, whereas, C2 complex can be seen even at very low concentration of ERα. In addition to that, in the presence and absence of HMGB-1 for ERβ, the extent of C2 formed, relative to C1 was much lower than that of ERα.
**DR (26)**

5’TGATGCCCTCAGGTCActgGTTGGGTGGGCAACCTAGGTCActgGTTGGTTTAGTTGG3’

**Fig 33: ERα binding to DR1-26 in the absence and presence of HMGB-1.**

A 100 pM probe of DR1-26 was incubated with 2, 6, 16, 20, 25, 30, 45 and 52 nM, of ERα, respectively, in the absence (lanes 2-10), and in the presence of HMGB-1 (lanes 10-17). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.

**Fig 34: ERβ binding to DR1-26 in the absence and presence of HMGB-1.**

A 100 pM probe of DR1-26 was incubated with 2, 6, 16, 20, 25, 30, 45 and 52 nM, of ERβ, respectively in the absence (lanes 2-10), and in the presence of HMGB-1 (lanes 10-17). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.
Fig 35: Equilibrium binding profile for ERα and ERβ binding to DR26, respectively, in the absence and presence of HMGB-1. Percent ER binding at one half-site (C1) and two half-sites (C2), in the absence of HMGB-1, 400nM.
Figure 36 and 37 show ERs binding to the inverted repeat 1 (IR1), with two perfect half-sites. These two perfect half-sites are symmetrically oriented 10bps from both ends and are separated by 24 bps. IR1 has the one perfect 5’ half-site (AGGTCA) intact and one perfect 3’ half-site (TGACCT), organized in head to tail orientation and the rest of the half-sites have been completely mutated. Right panel of the both figures, illustrates the effect of HMGB-1 on binding affinity, whereas, the left panel shows the binding affinity of estrogen receptors on IR in the absence of HMGB-1. For the both receptors, in the absence of HMGB-1 protein, the fast moving band C1 is indicative of binding of ERs at one half-site. In the presence of HMGB-1, at very low levels of ERs, the second band (C2) was observed indication of binding ERs at the other half-site. The band intensities (C1) for both receptors, in the presence of HMGB-1, were increased with increasing levels of ERs and then decrease with increasing receptor’s concentration, with the concomitant increase in the intensity of the second band (C2) representing ERs binding at the both half-sites. The C2 band is observed for the both receptors, in the presence of coactivator protein HMGB-1 approximately at 6nM ERs and then becomes the prominent band at progressively higher ER levels. Figure 38 shows the binding profile for complexation at increasing receptor concentrations, in the absence and presence of HMGB-1, was determined and quantitated by phosphoimager and plotted using Sigma Plot.
Fig 36: ERα binding to IR in the absence and presence of HMGB-1. A 100 pM probe of IR1 was incubated with 2, 6, 16, 20, 25, 30, 45 and 52 nM, of ERα, respectively in the absence (lanes 2-10), and in the presence of HMGB-1 (lanes 10-17). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.

Fig 37: ERβ binding to IR in the absence and presence of HMGB-1. A 100 pM probe of IR1 was incubated with 2, 6, 16, 20, 25, 30, 45 and 52 nM, of ERβ, respectively in the absence (lanes 2-10), and in the presence of HMGB-1 (lanes 10-17). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.
Fig 38: Equilibrium binding profile for ER\(\alpha\) and ER\(\beta\) binding to IR1, respectively, in the absence and presence of HMGB-1. Percent ER binding at one half-site (C 1) and two half-sites (C 2), in the absence of HMGB-1, 400nM.
Table XI: $K_{50}$ (nM) values obtained for oligonucleotides in the presence and absence of HMGB-1

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Without HMGB-1</th>
<th>With HMGB-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ER$\alpha$</td>
<td>ER$\beta$</td>
</tr>
<tr>
<td>DR(1)</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>DR(2)</td>
<td>11.2</td>
<td>17.5</td>
</tr>
<tr>
<td>DR(3)</td>
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<tr>
<td>DR(26)</td>
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<td>EvR(1)</td>
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<td>10.3</td>
</tr>
<tr>
<td>IR(1)</td>
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<td>31</td>
</tr>
</tbody>
</table>
C) GR binding to cGRE, cHERE, cERE, direct repeats, inverted repeats and everted repeats of cHERE

The objectives are to determine the binding affinity and selectivity of GR to cGRE, cHERE, cERE, direct repeats, inverted repeats and everted repeats of cHERE in presence and absence of HMGB-1. The glucocorticoid and estrogen response elements consist of palindromic sequence composed of two half-sites separated by three non-specific base pairs and only two nucleotides per half-site differ between functionally define cERE and cGRE. In part B, DNA binding experiment have revealed that ERs (α and β) bind to cERE, direct repeats, inverted repeats and everted repeats (EvR) of cHERE in presence and absence of HMGB-1 protein. This flexibility was further emphasized in this study. These observations revealed that GR can bind to cERE, direct repeats, inverted repeats and everted repeats of cHERE. This study further highlighted the considerable binding affinity of GR in the presence of HMGB-1 protein. Previous studies have demonstrated that cooperative binding of steroid receptor DBDs to palindromic response elements is controlled by a dimerization domain in the second zinc motif of the DBD, known as the D-box, but no significant evidence observed that cooperative binding of GR to cERE, direct repeats, inverted repeats and everted repeats of cHERE either in presence or absence of HMGB-1 protein (34, 35, 36).

Virtually all the naturally occurring bipartite EREs contain imperfect palindromes (84). Although ER binds with only slightly lower affinity to these sites than to cERE (3), the in vitro ER binding affinity to ERE half-sites drops precipitously (85-88). Since, HMGB-1 protein increased ER binding affinity to consensus and imperfect EREs, hence, it was
interest to determine whether HMGB1 is able to increase the weak binding affinity of GR to ERE half-sites.

The figure 39 shows that GR binding to cGRE in the presence and absence of HMGB-1. The percent of receptor complex bound to cGRE in the presence or absence of HMGB1 was obtained when % complex profile is plotted as a function of receptor concentration using the Sigma Plot. The figure 40 shows the equilibrium binding profiles of GR binding to cGRE in the absence and presence of HMGB1. In the absence of HMGB-1, the first GR/GRE complex was observed at 1.5nM GR, whereas, in the presence of HMGB-1, a faint complex was detected at 1.0 nM GR. As expected, lane (10-17), HMGB-1 has enhanced binding affinity of GR binding to cGRE. The $K_D$ value for the GE/cGRE complex was 3.4nM, is comparable to values reported previously in our lab (Nitin A, 2004 thesis, BGSU). The binding affinity of GR/cGRE was increased in the presence of HMGB-1 since, the $K_D$ values decreased from 3.4nM to 2.8nM. The dissociation profiles for the complex differ only slightly, indicating that the presence of HMGB-1 has little effect on complex formation.
**Fig 39: GR binding to cGRE in the absence and presence of HMGB-1.** A 100 pM probe of cGRE was incubated with 0.25, 0.5, 1.0, 1.5, 2, 2.5, 3, and 4 nM, of GR, respectively in the absence (lanes 2-10), and in the presence of HMGB-1 (lanes 11-19). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.

**Figure 40: Equilibrium binding profile of GR binding to cGRE in the absence and presence of HMGB-1.** The percent of GR complex bound in the presence (■) or absence (●) of HMGB-1 obtained from EMSA profiles are plotted as a function of GR concentration.
Figures 41 and 42 show GR binding to HERE in the presence and absence of HMGB-1 and the equilibrium binding profiles of GR binding to cHERE in the presence and of HMGB1, respectively. It was observed that GR binding to ERE half-sites is weak and exhibits nearly a 10-fold higher $K_D$ than that observed for ER binding to cERE. We observed that GR does bind strongly to HERE, exhibiting a $K_D$ value of about 2.1 nM to 1.5 nM in the presence and absence of HMGB-1 respectively. This is comparable to the $K_D$ values previously reported in our lab (Nitin A. 2004, MS thesis BGSU). However, in the presence of HMGB-1, slightly change the GR binding profile, with complex formation evident at GR level as low as 1.5 nM, is comparable to the $K_D$ value for GR binding to GRE in the presence of HMGB-1. This indicates that HMGB-1 protein has no significant effect on GR binding to ERE half-sites.
**Fig 41: GR binding to HERE in the absence and presence of HMGB-1.** A 100 pM probe of HERE was incubated with 0.5, 1.0, 1.5, 2, 2.5, 3, 3.5 and 4 nM, of GR, respectively in the absence (lanes 2-10), and in the presence of HMGB-1 (lanes 10-17). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.

**Figure 42:** Equilibrium binding profile of GR binding to HERE in the absence and presence of HMGB-1. The percent of GR complex bound in the presence (●) or absence (▲) of HMGB-1 obtained from EMSA profiles are plotted as a function of GR concentration.
Figures 43, shows GR binding to cERE in the presence and absence of HMGB-1 and the figure 44 shows equilibrium binding profiles of GR binding to cERE in the absence and presence of HMGB-1. A very first faint GE/cERE band was observed at 2 nM of GR in the absence of HMGB-1, whereas, in the presence of HMGB-1, the first complex was detected at 1.0 nM of GR. These results revealed that HMGB-1 enhanced the binding affinity of GR binding to cERE but much less than expected. However, when the GR concentration increases, the extent of GR binding to cERE is increased in the presence and absence of HMGB-1. Furthermore, HMGB-1 had no significant effect on the binding affinity of GR to cERE and this is in contrast to our findings with ERs binding to cERE. As reported in the previous studies, the wild type GRDBD was found to bind with highly affinity to palindromic GRE but had no measurable affinity for the cERE.
Fig 43: GR binding to cERE in the absence and presence of HMGB-1.
A 100 pM probe of cERE was incubated with 0.25, 0.5, 1.0, 1.5, 2, 2.5, 3, 3.5 and 4 nM, of GR, respectively in the absence (lanes 2-10), and in the presence of HMGB1 (lanes 11-19). The concentration of HMGB1 was 400 nM.

Figure 44: Equilibrium binding profile of GR binding to cERE in the absence and presence of HMGB1. The percent of GR complex bound in the presence (■) or absence (▲) of HMGB1 obtained from EMSA profiles are plotted as a function of GR concentration.
Both ER and GR belong to same super-family of the steroid receptors, but bind to different DNA response elements that differ by 4bps. My previous experiments clearly observed that ERs binds to a spectrum of ERE binding sites as half-sites arranged in direct repeats, inverted repeats and everted repeats. This is an attempt to investigate that whether GR has a broad affinity, to direct repeats, inverted repeats and everted repeats. Figure 45 shows GR binding to DR (1) in the presence and absence of HMGB-1. DR1 (1), has two half-sites, composed of two AGGTCA motifs with a center to center separation of 20 bps. The GR/DR (1) complex was more spread-out or diffused than what was observed for ERs, perhaps suggesting that faster on-off or less stable complex. In figures 45, lane (2-9) and lane (10-17), observed that the extent of binding increased as the concentration of GR increased. However, in the absence of HMGB-1, GR produced two protein-DNA complexes over a small range of GR, denoted as C1 and C2. The both complexes were obtained in the absence of HMGB-1. In addition to that, the figure 45 shows that at low concentrations of GR, in the presence of HMGB-1 stimulate the formation of the both mobility complexes, at high receptor concentrations, HMGB-1 predominantly stimulate the slower mobility complex (C2) associated with a decrease of the faster mobility complex (C1). Furthermore, the cooperative binding was not as significant as ERs binding to DR (1) but a little cooperativity was detected even at lower concentration of GR in the absence of HMGB-1. The percent of receptor complex bound to DR (1) in the presence or absence of HMGB-1 was determined by phosphoimager quantitaion of band intensities and % complex profile is plotted as a function of GR concentration by using the Sigma Plot. Significant changed in extent of GR binding was
observed in the presence of HMGB-1 since, the $K_D$ values decreased from 1.5 nM to 0.5 nM GR.

![Figure 45: GR binding to DR (1) in the absence and presence of HMGB-1.](image)

A 100 pM probe of DR (1) was incubated with 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 nM, of GR, respectively in the absence (lanes 2-9), and in the presence of HMGB1 (lanes 10-17). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM

![Figure 46: Equilibrium binding profile of GR binding to DR (1) in the absence and presence of HMGB1.](image)

Figure 46: Equilibrium binding profile of GR binding to DR (1) in the absence and presence of HMGB1. The percent of GR complex bound in the presence (■) or absence (▲) of HMGB1 obtained from EMSA profiles are plotted as a function of GR
Figure 47 shows GR binding to DR (2) in the presence and absence of HMGB-1. DR (2) DNA duplex fragment is composed of two perfect half sites containing two TGACCT motifs were separated by 15bps.

Figure 47 shows the enhancement in binding affinity of GR in a HMGB-1 concentration dependent manner and optimal enhancement was observed with 400 nM of HMGB-1. Neither secondary complex nor diffused was detected for the GR/DNA binding. The percent complexation of GR/DR (2) as a function of GR concentration, in the presence and absence of HMGB-1 was determined by phosphoimager quantitation of band intensities and plotted using a Sigma Plot. The quantitative examination of band at lower GR levels reveals an increased level of complex formation in the presence of HMGB-1 proteins. The KD for GR/DR (2) was found to be 1.2nM and HMGB-1 reduced it to 0.8nM, and it appears that HMGB-1 enhances the binding affinity for GR. It was surprising that the GR/DR (2) complex was neither spread-out nor diffused as observed for GR/DR (2). As expected, band intensity of the GR/DR (1) complex was increased with increasing the concentration of GR, in the presence and absence of HMGB-1. An interesting point to be noted that in the presence of HMGB-1, 90% of DNA saturation was observed at 1.5nM of GR, whereas, in the absence of HMGB-1, it was detected at 2.5nM of GR.
Fig 47: **GR binding to DR (2) in the absence and presence of HMGB-1.**

A 100 pM probe of DR (2) was incubated with 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 nM of GR, respectively in the absence (lanes 2-9), and in the presence of HMGB-1 (lanes 10-17). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.

Figure 48: Equilibrium binding profile of GR binding to DR (2) in the absence and presence of HMGB-1. The percent of GR complex bound in the presence (■) or absence (▲) of HMGB-1 obtained from EMSA profiles are plotted as a function of GR concentration.
Figure 49 shows GR binding to DR (3) in the presence and absence of HMGB-1. DR (3) DNA duplex fragment contains two perfect half sites containing two AGGTCA motifs were separated by 3ps. As expected, the extent of GR binding was increased with increasing the GR level in the presence and absence of HMGB-1. The absence of coactivator protein HMGB-1, 90% of DNA was bound to GR/DNA complex at 1.5nM GR, whereas, in the presence of HMGB-1 it was observed at 1nM GR. This shows that GR bound to DR (3) slightly better in the presence of HMGB-1. Equilibrium binding profiles of GR, in the absence and presence of HMGB-1, binding to DR (3) is shown in figure 50. The binding profile for complexation with increasing GR concentrations, in the absence and presence of HMGB-1, was determined and quantitated by phosphoimager and plotted using Sigma Plot. It was surprising that the GR concentration at which 50% complexation occurs at 1.2nM in the presence and absence of HMGB-1.
Fig 49: GR binding to DR (3) in the absence and presence of HMGB-1.

A 100 pM probe of DR (3) was incubated with 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 nM, of GR, respectively in the absence (lanes 2-9), and in the presence of HMGB1 (lanes 10-17). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM

Figure 50: Equilibrium binding profile of GR binding to DR (3) in the absence and presence of HMGB-1. The percent of GR complex bound in the presence (■) or absence (▲) of HMGB-1 obtained from EMSA profiles are plotted as a function of GR concentration.
Figure 51 shows the titration of DR (26) with GR in the presence and absence of HMGB-1. DR1 (26) is composed of two AGGTCA motifs and they are apart from each other with a center to center separation of 26 bps. GR binding to DR (26) was observed by incubating the different concentrations of GR with DR (26) at 40°C. The complexes were resolved on a 4% native gel. A single GR/DR (26) complex was detected. The first GR/DR (26) complex was readily detected at 2.5 nM GR in the absence and presence of HMGB-1. The percent complexation of GR/DR (26) as a function of GR concentration, in the presence and absence of HMGB-1 was determined by phosphoimager quantitation of band intensities and plotted using Sigma Plot. Equilibrium binding profile for the GR/DR (26) interaction is shown in figure 52. The $K_D$ value for GR/DR (26) binding was 3nM and 2.7nM in the absence and the presence of HMGB-1 respectively.
Fig 51: GR binding to DR (26) in the absence and presence of HMGB-1.
A 100 pM probe of DR (26) was incubated with 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 nM, of GR, respectively in the absence (lanes 2-9), and in the presence of HMGB-1 (lanes 10-17). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.

Figure 52: Equilibrium binding profile of GR binding to DR (26) in the absence and presence of HMGB-1. The percent of GR complex bound in the presence ■) or absence (▲) of HMGB-1 obtained from EMSA profiles are plotted as a function of GR concentration.
Figure 53 shows GR binding to the inverted repeat 1 (IR1) in the absence and presence of HMGB-1. IR1 is composed of perfect half–sites (AGGTCA) being separated by 24 bps. IR1 has the one perfect 5’ half-site (AGGTCA) intact and one perfect 3’ half-site (TGACCT), organized in head to tail orientation and the rest of the half-sites have been completely mutated. Right panel of figures 53 indicates the effect of HMGB-1 on binding profile, whereas, left panel shows binding affinity of GR receptors on IR in the absence of HMGB-1. A very small complex (ca. 5% less) was detected when GR concentration was 2.5nM, whereas, in the presence of HMGB-1 a little intense band was observed at 2nM GR. However, in the absence of HMGB-1 about 90% binding of GR to IRI occurs at 4 nM, is comparable to the GR binding IRI at 3 nM in the presence of HMGB-1. This experiment shows that HMGB-1 stimulates GR binding to IRI.
**Fig 53: GR binding to IR in the absence and presence of HMGB-1.**

A 100 pM probe of IR was incubated with 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 nM of GR, respectively in the absence (lanes 2-9), and in the presence of HMGB1 (lanes 10-17). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.

Figure 54: Equilibrium binding profile of GR binding to IR in the absence and presence of HMGB-1. The percent of GR complex bound in the presence (▲) or absence (●) of HMGB-1 obtained from EMSA profiles are plotted as a function of GR concentration.
Figure 53 shows GR binding to the everted repeat1 (EvR1) in the presence and absence of HMGB-1. EvR1 contains one perfect 3’ half-site (TGACCT) intact with one perfect 5’ half-site (AGGTCA). As expected, the extent of GR binding to EvR1 was increased with increasing level of GR from 0nM to 4nM in the absence of HMGB-1. The binding affinity was further enhanced in the presence of HMGB-1. The Sigma Plot for percent complexation as a function of GR concentration in the presence and absence of HMGB-1 protein is shown in figure 54. In the absence of HMGB-1, 50% complexation occurs at 2.2nM GR, whereas, in the presence HMGB-1 GR concentration was decreased to 1.4nM. I emphasized that HMGB-1 has effect on binding interaction between GR and EvR1. The fold of increment was ca. 1.6-fold.
Fig 55: GR binding to EvR in the absence and presence of HMGB-1.
A 100 pM probe of EvR was incubated with 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 nM, of GR, respectively in the absence (lanes 2-9), and in the presence of HMGB-1 (lanes 10-17). The lane 1 contains only free DNA and the concentration of HMGB-1 was 400 nM.

Fig 56: Equilibrium binding profile of GR binding to IR in the absence and presence of HMGB-1. The percent of GR complex bound in the presence (■) or absence (▲) of HMGB-1 obtained from EMSA profiles are plotted as a function of GR concentration.
Table XII: $K_D$ values for GR/cGRE, cERE, cHERE, DR, EvR and IR interaction in the presence and absence of HMGB1

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Without HMGB1</th>
<th>With HMGB1</th>
<th>HMGB effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>cGRE</td>
<td>3.4</td>
<td>2.8</td>
<td>1.20</td>
</tr>
<tr>
<td>cERE</td>
<td>2.5</td>
<td>2.3</td>
<td>1.0</td>
</tr>
<tr>
<td>cERE (mutant)</td>
<td>2.1</td>
<td>1.5</td>
<td>1.08</td>
</tr>
<tr>
<td>DR(1)</td>
<td>2.5</td>
<td>0.9</td>
<td>1.40</td>
</tr>
<tr>
<td>DR(2)</td>
<td>1.4</td>
<td>1</td>
<td>1.4</td>
</tr>
<tr>
<td>DR(3)</td>
<td>1.1</td>
<td>1.1</td>
<td>1</td>
</tr>
<tr>
<td>DR(26)</td>
<td>3</td>
<td>2.7</td>
<td>1.10</td>
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<tr>
<td>EvR(1)</td>
<td>2.2</td>
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<td>1.6</td>
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<tr>
<td>IR</td>
<td>2.8</td>
<td>2.6</td>
<td>1.07</td>
</tr>
</tbody>
</table>

Table XII shows the approximate $K_D$ values for the interactions of GR with various Direct repeats, inverted repeats and everted repeats. $K_D$ values were obtained from binding plots and correspond to 50% complexation
CHAPTER IV

DISCUSSION AND CONCLUSION

Estrogen receptors binding to non classical estrogen receptor element

Estrogen receptor resides in the cell nucleus bound to other proteins such as heat shock proteins (hsp) and immunophilins. When estrogen enters the nucleus, and binds ER, and the other proteins dissociate from ER dimerizes. The dimeric form of the estrogen receptor ($\alpha$ & $\beta$) binds to estrogen response element (ERE) in the regulatory region of the target gene. This binding selectivity is generally considered to be governed by three major factors: 1) the sequence of the ERE half sites; 2) the orientation of the half-sites being inverted repeats or direct repeats; and 3) the number of base pairs (i.e. spacer) between the half sites (89). The first part of my research demonstrates how binding selectivity of estrogen receptor is affected by modifying the consensus estrogen response element, cERE, by inserting with spacer of variable size, $n = 0, 1, 2, 3$ and $4$. The second part of the research demonstrates binding of steroid receptors ($\alpha$ & $\beta$) to direct repeats with different half site spacing, in which two AGGTCA sequences are separated by more than five nucleotides.

Flexibility of binding of steroid receptors to consensus ERE with different spacer size.

Hormone response elements for steroid receptors have been characterized as palindromic sequences, containing two half sites separated by three base pairs. However a number of estrogen responsive genes contain imperfect palindroms. The estrogen response element, ERE, in the vitellogenin A2 gene of Xenopus laevis is composed of the perfect
palindromic element AGGTCAnnnTCCAGT. It has been recorded that altering the spacing between half-sites disrupts the binding of steroid receptor to palindromic response elements (90).

**Table XIII: K$_D$ values for ERs/ERE (n) interaction in the presence and absence of HMGB-1**

<table>
<thead>
<tr>
<th>Oligos</th>
<th>ER$\alpha$</th>
<th>ER$\beta$</th>
<th>ER$\alpha$</th>
<th>ER$\beta$</th>
<th>ER$\alpha$</th>
<th>ER$\beta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 n ERE</td>
<td>7</td>
<td>2.6</td>
<td>2.4</td>
<td>2.4</td>
<td>2.9</td>
<td>1.1</td>
</tr>
<tr>
<td>1 n ERE</td>
<td>16.2</td>
<td>16.2</td>
<td>14.2</td>
<td>3.8</td>
<td>1.1</td>
<td>4.2</td>
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<tr>
<td>2 n ERE</td>
<td>5.1</td>
<td>9</td>
<td>4.7</td>
<td>4.1</td>
<td>1.1</td>
<td>2.2</td>
</tr>
<tr>
<td>3 n ERE</td>
<td>10.1</td>
<td>8</td>
<td>5.9</td>
<td>7</td>
<td>1.7</td>
<td>1.1</td>
</tr>
<tr>
<td>4 n ERE</td>
<td>11</td>
<td>10</td>
<td>5.1</td>
<td>6.3</td>
<td>2.3</td>
<td>2.5</td>
</tr>
</tbody>
</table>

The table X summarizes K$_D$ values obtained for ERs/ERE with different spacer sizes in the absence and presence of HMGB-1. A number of studies have reported that purified steroid hormone receptors bind poorly to their recognition sequences when compared with receptors associated with other cellular proteins. The fact that addition of crude cellular or purified proteins restores the ability of the purified receptors to bind to DNA (91-94) suggests that nuclear receptors do not function in isolation, but that they require the participation of other cellular proteins to efficiently bind to DNA. One protein that has demonstrated the ability to enhance binding of ER to their respective response elements is HMGB-1. This fact agrees with my results regardless of spacer size. I used electrophoretic mobility shift assay (EMSA) to observe the interaction of ERs with consensus estrogen response element, cERE with different spacer size, n =0, 1, 2, 3 and 4 in the presence and absence of HMGB-1. For both receptors, this work show that HMGB-1 facilitates ERs binding to cERE with different spacer sizes and also they reveal that the presence of HMGB-1 protein creates additional interaction that account for the
increased ER binding affinity to various spacer size. Previous studies in our lab has reported that in the absence of HMGB-1, a dissociation constant ($K_D$) value of 11nM was obtained for ER$\alpha$ interaction with consensus ERE, whereas, in the presence of HMGB-1, it $K_D$ was 4nM. Previous findings for ER$\alpha$/cERE in the presence and absence of HMGB-1 are comparable to 10.1nM and 5.9nM respectively in my observations. Calculated $K_D$ values for ER$\beta$/cERE interaction in the absence and presence of HMGB-1 were 8nM and 7nM respectively. This reveals that HMGB-1 has significant effect on ER$\beta$/cERE with respect to ER$\alpha$/cERE binding.

In fact, ER$\alpha$ binds to n=0 ERE, which is comparable to ER$\alpha$ binding to cERE with a $K_D$ value of 7 nM whereas, in the presence of HMGB-1 $K_D$ value has brought down to 2.4 nM depicting strong interaction between them. However, HMGB-1 increased the affinity of the ER$\beta$/cERE (n=0) interaction but without any significant effect on the dissociation kinetics of the complex. Previous studies have recorded that deletion or insertion of additional base pair in the spacer will alter center to center distance between half-sites by 3.4Å and an associated rotation of 36° about the DNA axis. These results are consistent with previous studies in which HMGB-1 enhanced binding of the intact ERs to cERE (n= 0, 1, 2, 3, 4), in our lab. (Ghattamanenii. R, 2005 MS thesis BGSU & Morzouk. S. 2004, MS thesis, BGSU) (data not shown).

**High mobility group protein-1 facilitates estrogen receptor binding to Direct repeats (DR), Inverted repeats (IR) and Everted repeats (EvR)**

Most estrogen responsive genes identified to date contain one or more imperfect EREs or multiple copies of the ERE half-sites rather than cERE. Recent studies demonstrated
that ER also binds to various spaced DR and IR of the ERE half-site motif, albeit with significantly lower affinity when compared with ER binding to cERE.

In addition to those facts, it has been demonstrated that Retinoid X receptors, RxRs, thyroid hormone receptor TRs, RARs, and vitamin D receptor, VDR all can bind to direct repeats of the sequence AGGTCA. 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), whereas, DR+3, DR+4, DR+5 are response elements for vitamin D receptor (VDR), thyroid hormone receptor (TR), retinoic acid receptor (RAR) respectively. A thyroid receptor element (TRE) is composed of two AGGTCA "half sites" separated by either zero or four nucleotides and these half sites of a TRE can be arranged as direct repeats (DR), pallindromes or inverted repeats (IR) (95-99)

There are two tandem copies of half-sites (AGGTCA) in DR (1), the distance between each AGGTCA motif is 15 nucleotides. Assuming 10.5 bps/turn in B-form DNA (100), this indicates that the half-sites are 1.4 helical turns apart, but both of them on the same faces of the DR(1) DNA helix. This orientation of the two half sites allows the ERs bind to the DR (1) from one side, whereas, in DR(3) both half-sites are oriented in head to head orientation with 3bps apart, and both half-sites are oriented on the same faces of the DR(3) DNA duplex. Current orientation of half-sites allows ERs to associate with DNA duplex from the same side as two dimers on each half site. In DR (26) the distance between the half-sites has increased to 20nucleotides, in which both half-sites ~2 helical turns apart and they are faced in opposite face of the DR (26) duplex.
Table XIV: $K_D$ and $K_{50}$ (nM) values obtained for oligonucleotides in the presence and absence of HMGB-1

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Without HMGB-1</th>
<th>With HMGB-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ER$\alpha$</td>
<td>ER$\beta$</td>
</tr>
<tr>
<td>DR(1)</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>DR(2)</td>
<td>11.2</td>
<td>17.5</td>
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<tr>
<td>DR(3)</td>
<td>16</td>
<td>27</td>
</tr>
<tr>
<td>DR(26)</td>
<td>18.5</td>
<td>21</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Distance between half-sites</th>
<th>Orientation of half-sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR(1)</td>
<td>15 nt</td>
<td>Same face of the duplex</td>
</tr>
<tr>
<td>DR(2)</td>
<td>15 nt</td>
<td>Same face of the duplex</td>
</tr>
<tr>
<td>DR(3)</td>
<td>3 nt</td>
<td>Same face of the duplex</td>
</tr>
<tr>
<td>DR(26)</td>
<td>20 nt</td>
<td>Opposite face of the duplex</td>
</tr>
</tbody>
</table>

DNA binding experiments have demonstrated that ER$\alpha$ binds DR of the ERE half-site AGGTCA as well as ERE palindromes (101-103). A study of 5'-AGGTCA-3' DR spacing, i.e. DR1 (where 1 refers to the number of nucleotides separating half sites), DR2, DR3, DR4, DR5, DR10, DR15, DR20, DR25, DR35, DR50, DR100, DR150 and DR 200 showed that E$_2$ stimulated transcription from all constructs in which the DR were separated by >10 bps in transiently transfected COS-1 cells. At best, E$_2$-induced
transcription ~ 6-fold from DR15 and DR20 compared to 19-fold from a perfectly palindromic ERE (103). DR separated by 35, 50, 100, 150 or 200 bps showed decreased E2-induced transcription (101). Another study reported that ERα bound specifically to DR6, but 8–15-fold less retarded ER–DNA complex was formed on DR6 than on the ERE palindrome (105). In competition binding experiments, DR6 and a single ERE half-site competed for ERα 6–10-fold less efficiently than the 13 bps palindromic ERE. Thus, specific rules defining ER–DR binding, the affinity of such interaction, and the functional consequences of ER–DR binding, i.e. transcriptional responsiveness, remain to be clarified.

The research focused on to study the effect of HMGB-1 on the binding of ERs (α and β) to various ERE sequences. As a general fact, my results show that the presence of HMGB-1 protein exert a dramatic effect on ERs (α and β) binding to half sites, and that the consensus ERE with different spacing and direct repeats. For all the direct repeats, inverted repeats and everted repeats used in this study, ERα showed a little cooperativity in the absence of HMGB-1, whereas, strong cooperativity was observed in the presence HMGB-1. This further confirms the idea that HMGB-1 by some interactions stabilizes the homodimers to interact on DNA and also with each other.
Table XV: Nucleotide sequences of direct repeats

| DR(1) | 5′–TGATGCCTCAGGTCACtgGTTGGGCAACCTAGGTCACtgGTTGGGTCTTAGTTGG-3′ |
| DR(2) | 5′-TGATGCCTCCCCAACctgTGACCTCAACCTCCCCAACctgTGACCTTCTTAGTTGG-3′ |
| DR(3) | 5′- TGATGCCTCCCCAACCTGGTTGGGCAACCTAGGTCACtgAGGTCATCTTAGTTGG-3′ |
| DR(26) | 5′-TGATGCCTCAGGTCACtgGTTGGGCAACCTAGGTCACtgGTTGGGTCTTAGTTGG-3′ |

The table XV shows 3 nucleotide sequences among the directly used for study. Each sequence is composed of two perfect half-sites separated by certain number of base pairs. However, for direct repeats, in the absence of HMGB-1, ERα produced two protein-DNA complexes, called C1 and C2. Our results suggest that ERα preferentially bind on them as a dimer, the faster mobility complex (C1) contains an ER dimer, whereas, the slower mobility complex contains two dimers or a higher order ER oligomer. This results was confirmed by the comparison studies shown in both figures 19 and 20.

It has been known for some time that estrogen regulation can occur through half-sites as well as through inverted repeats, but the way in which the ER interacts with half-sites has not been determined. From X-ray crystallography results (105), we know that two ER DNA binding domains (DBDs) interact with one consensus ERE oligonucleotide, with each DBD binding to one half-site. Also ER is known to dimerize, with the major interactions occurring through the ligand binding domain (106,24). There are several theoretical possibilities ways in which ERR could interact with half-sites:

(1) One ER monomer could interact with one half-site; (2) one ER dimer could interact with one half-site; or (3) one ER dimer could interact with two half-sites. In the second possibility, one of the ERR DBDs would interact with the half-site while, the other would
interact with the adjacent DNA. This type of interaction was observed in the crystal structure of the glucocorticoid receptor DBD binding to an oligonucleotide with two half-sites separated by four base pairs instead of the usual three (31). The results presented in ERα/DR binding interactions argue strongly for the third model.

In addition to that, our findings indicate that at low concentrations of ERα, in the presence of HMGB-1 stimulate the formation of both mobility complexes, but at high receptor concentrations, HMGB-1 predominantly stimulate the slower mobility complex (C2) concomitant with a decrease of the faster mobility complex (C1). These findings can be argued in terms of cooperativity. It has been reported that cooperative binding of steroid receptor DBDs to palindromic response elements is controlled by a dimerization domain in the second zinc motif of the DBDs, known as the D-box (107-109). However, recent studies have suggested that direct repeats of RGGTCA half-sites can function as EREs (109-111).

**Binding of the Human Glucocorticoid Receptor to Response Elements Containing Direct Repeats (DR), Inverted Repeats (IR) and Everted Repeats (EvR)**

Numerous studies have shown that steroid receptors bind to palindromic response elements as homodimers, with the subunits coupled by dimerization interfaces present in both the hormone binding domains and DNA binding domains of the receptors (112-119). Interestingly, recent studies on the estrogen-responsive chicken ovalbumin gene (54), molecular genetic experiments in yeast (120), and studies with extracts of the ER expressed in mammalian cells (91) have suggested that directly repeated half-sites, as well as palindromes, are capable of acting as EREs. I was therefore interested to know if
direct repeats could also function as GREs. One of the best characterized glucocorticoid-responsive promoters is the long terminal repeat of MMTV, which contains a number of binding sites for the GR (55, 56). The binding site most proximal to the TATA box contains the sequence TGTTCTN9TGTTCT, which is a direct repeat of consensus GRE half-sites.

Specific GR-DNA or ER-DNA complexes were observed on direct repeats with different spacers between half-sites, indicating that binding of steroid receptors to direct repeats is more flexible than binding to palindromic elements. This flexibility was further emphasized by the observation that the GR could also bind to everted repeats of \textbf{TGACCT} (TGACCT\textbf{CAACCTAGGTCA}) motifs separated by 6 base pairs. Cooperative binding was observed when ERs binding to direct repeats, however no evidence was observed for GR cooperative binding to direct repeats.
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