APPLICATION FOR FEDERAL ASSISTANCE

SF 424 (R&R)

2. DATE SUBMITTED	Applicant Identifier
3. DATE RECEIVED BY STATE	State Application Identifier

1. * TYPE OF SUBMISSION

❍ Pre-application ❍ Application
● Changed/Corrected Application

4. Federal Identifier
GM054357

5. APPLICANT INFORMATION *** Organizational DUNS:**617407325

* Legal Name: BOWLING GREEN STATE UNIV BOWLING GREEN
Department: Sponsored Programs & Research
Division:
* Street1: 106 University Hall
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* City: Bowling Green
County:
* State: OH: Ohio
Province:
* Country: USA: UNITED STATES
* ZIP / Postal Code: 43403

Person to be contacted on matters involving this application

Prefix:	* First Name:	Middle Name:	* Last Name:	Suffix:
	Cynthia	A	Price	

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Email: spar@bgsu.edu

6. * EMPLOYER IDENTIFICATION NUMBER *(EIN) or (TIN)*:
346402018

7. * TYPE OF APPLICANT
H: Public/State Controlled Institution of Higher Education

8. * TYPE OF APPLICATION: ❍ New
❍ Resubmission ● Renewal ❍ Continuation ❍ Revision

Other (Specify):

Small Business Organization Type
❍ Women Owned ❍ Socially and Economically Disadvantaged

If Revision, mark appropriate box(es).
❍ A. Increase Award ❍ B. Decrease Award ❍ C. Increase Duration
❍ D. Decrease Duration ❍ E. Other *(specify)*:

* Is this application being submitted to other agencies? ❍ Yes ● No
What other Agencies?

9. * NAME OF FEDERAL AGENCY:
National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER:
TITLE:

11. * DESCRIPTIVE TITLE OF APPLICANT'S PROJECT:
Influence of HMGB1, Nucleosomes and SWI/SNF on Estrogen Receptor Binding

12. * AREAS AFFECTED BY PROJECT *(cities, counties, states, etc.)*
NA

13. PROPOSED PROJECT:

* Start Date	* Ending Date
07/01/2008	06/30/2011

14. CONGRESSIONAL DISTRICTS OF:

a. * Applicant	b. * Project
OH-005	OH-005

15. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix:	* First Name:	Middle Name:	* Last Name:	Suffix:
	William		Scovell	

Position/Title: Professor
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Division: Arts & Sciences
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* State: OH: Ohio
Province:
* Country: USA: UNITED STATES
* ZIP / Postal Code: 43403
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16. ESTIMATED PROJECT FUNDING

a. * Total Estimated Project Funding	$204,795.00
b. * Total Federal & Non-Federal Funds	$204,795.00
c. * Estimated Program Income	$0.00

17. * IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?

a. YES ○ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:

DATE:

b. NO ● PROGRAM IS NOT COVERED BY E.O. 12372; OR

○ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

18. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

● * I agree

** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.*

19. Authorized Representative

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*** Signature of Authorized Representative**

Cynthia Price

*** Date Signed**

10/26/2007

20. Pre-application File Name: Mime Type:

21. Attach an additional list of Project Congressional Districts if needed.

File Name: Mime Type:

424 R&R and PHS-398 Specific Table Of Contents

RESEARCH & RELATED Project/Performance Site Location(s)

Project/Performance Site Primary Location

Organization Name: Bowling Green State University

* Street1: 209 Physical Science Building		Street2:
* City: Bowling Green	County:	* State: OH: Ohio
Province:	* Country: USA: UNITED STATES	* Zip / Postal Code: 43403

File Name	Mime Type

Additional Location(s)

RESEARCH & RELATED Other Project Information

1. * Are Human Subjects Involved? ❍ Yes ● No

1.a. If YES to Human Subjects

Is the IRB review Pending? ❍ Yes ❍ No

IRB Approval Date:

Exemption Number: _ 1 _ 2 _ 3 _ 4 _ 5 _ 6

Human Subject Assurance Number

2. * Are Vertebrate Animals Used? ❍ Yes ● No

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ❍ Yes ❍ No

IACUC Approval Date:

Animal Welfare Assurance Number

3. * Is proprietary/privileged information included in the application? ❍ Yes ● No

4.a. * Does this project have an actual or potential impact on the environment? ❍ Yes ● No

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ❍ Yes ❍ No

4.d. If yes, please explain:

5.a. * Does this project involve activities outside the U.S. or partnership with International Collaborators? ❍ Yes ● No

5.b. If yes, identify countries:

5.c. Optional Explanation:

6. * Project Summary/Abstract	2470-Abstract.pdf	Mime Type: application/pdf
7. * Project Narrative	4655-Narrative.pdf	Mime Type: application/pdf
8. Bibliography & References Cited	9219-References.pdf	Mime Type: application/pdf
9. Facilities & Other Resources	9959-Resources.pdf	Mime Type: application/pdf
10. Equipment		
11. Other Attachments	7978-Manuscript..pdf	Mime Type: application/pdf

Abstract

The regulation of activated transcription in eukaryotic cells involves an exquisite network of sequence-specific DNA-protein interactions working in concert with the timely recruitment of a multitude of coactivator proteins to modify chromatin structure and communicate with the preinitiation complex. HMGB1 protein exhibits context-dependent regulatory properties in that it can act as a coactivator (nuclear hormone receptors) or as a general repressor (on TBP). We have found that HMGB1 interaction with the estrogen receptor (ER)-DNA interactions broadly expands the spectrum of ER binding sites and strongly enhances their binding affinity to imperfect estrogen response elements (EREs), ERE half-sites (cHEREs), EREs with various spacers (cEREn), direct repeats, everted repeats and well-separated inverted repeats. Preliminary data also show that HMGB1 strongly enhances ER binding to cERE or cHERE to comparable levels within a phased nucleosome by a nonenzymatic mechanism. In addition, we have initiated transient transfection studies using luciferase reporter gene assays to begin to compare the activity of different EREs in activate transcription. These findings, together with recent findings from ChIP-chip experiments and genomic searches, bring into question the current paradigm for ER binding and activity. It also appears that the C-terminal extension (CTE) in ER may play a vital role in ER interactions with its response element. We shall use the very sensitive hydroxyl footprinting procedure to investigate whether the ER-CTE is involved in the DNA interaction, which would further change the current model for ER binding. In addition, we will investigate more systematically the role of HMGB1 in acting as a chromatin remodeling complex (CRC) in a phased nucleosome that contains a spectrum of single and multiple EREs. The remodeling activity of HMGB1 will be compared to the activity of the established ATP-dependent SWI/SNF CRC, in addition to determining if these two very different CRCs act independently or cooperatively. Chromatin immunoprecipitation experiments will determine if HMGB1 colocalizes with ER in EREs of estrogen-responsive genes in MCF-7 cells. Luciferase reporter genes assays will be used to compare the relative transcriptional activity of a spectrum of ER binding sites.

Project Narrative: Relevance to Public Health

Our findings on estrogen receptors will lead to a deeper understanding of the mechanism by which estrogen affects normal physiology and development, in addition to the role the estrogen receptors play in the development of majors diseases, including cancer, atherosclerosis, osteoporosis and dementia.

Facilities & Resources

Laboratory

1200 sq. ft, 2 fume hoods, adjacent dark room, including a Konica QV-60A Automatic Film Developer, Hitachi HPLC unity with dual pumps/UV detection, small fraction collector; Power supply for SDS-PAGE/Western blots, EMSA & DNase I/hydroxyl footprinting; electrophoretic transfer unit, Sorval Superspeed and IECCR-8000 refrigerated centrifuges with a variety of fixed and swing bucket rotors, in addition to tabletop Eppendorf centrifuges; -80 Freezer, small incubator; Barnstad Lab Line MonoQ Environmental Shakers, BioRad MJ Mini Personal Thermal Cycler; 150 sq. ft cell culture lab with pneumatic hood, two water-jacketed CO2 incubators and a small refrigerator.

Office

The PI's office (10x15) is adjacent to the lab on the second floor of the Physical Science Building.

Computers

iMAc 4.1 and lap top in office, with a Dell computer in the lab. All are connected to the WWW and Ethernet. Printer and scanner accompany both computers.

Major & Shared Equipment

150 sq. ft cold room, Molecular Dynamics Phosphoimager with quantitative (Image Quant) software; complete lab for photography and autoradiography; 2 Beckman ultracentrifuges, TI-80 & TI-45 rotors; 2-Beckman Liquid Scintillation Counters; -80 Freezers; UV-visible spectrophotometers, including a Varian 219A & HP Diode Array; Brucker-Dalonics MALDI-TOF Mass Spectrometer facilities.

Other

Secretaries, electronics shop, machine shop & personnel are excellent and provide continuous support for the teaching and research effort; BGSU has a strong tradition in supporting an academic environment that encourages baccalaureates to pursue careers in biochemistry, chemistry, biology, biomedical and behavior sciences. Our estimate is that more than 700 BGSU graduates have gone onto to complete professional advanced degrees in the health/physical sciences in the past 10-15 years. This figure includes 30-40% of the graduates who have pursued advanced degrees (MS, Ph. D, MD, DD or Ph.D/MD) in biochemistry or aspects of molecular biology. I have attached an (incomplete) list of alumni from just my research group, of which about 50% of the students were undergraduates. The students have gone onto to receive advanced degrees at The Johns Hopkins U., Stanford U., U. California Institute of Technology, Cornel U. School of Medicine (Memorial Sloan Kettering Cancer Center), U. North Carolina, Chapel Hill, Case Western U., Ohio State U, Emory U. School of Medicine, Purdue U., Indiana U., U. Wisconsin, UC Berkeley, UC San Diego, U. Oregon and U. Toledo. Many of these former students hold faculty positions in major research universities, schools of medicine and national labs.

In an effort to further strengthen and underscore the University's commitment to undergraduate research, BGSU has established an Office of Undergraduate Research, with Dr. J. Farver as the Director (letter attached). It has supported undergraduate research throughout the academic year and awarded stipends for outstanding undergraduates to do research during the summer months. Rich Housman, a student in my group, received a summer stipend in 2005, is now pursuing an MS degree in chemical engineering, with plans to then pursue a Ph. D. in physical biochemistry.

The Plasticity of Estrogen Receptor-DNA Complexes: Binding Affinity and Specificity of Estrogen Receptors to Estrogen Response Element Half-sites Separated by Variant Spacers

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Abstract

The consensus estrogen response element (cERE) contains a palindromic sequence of two six-base pair half-sites separated by a spacer size of 3 bps. This study investigates the extent

to which estrogen receptors, ERα and ERβ, can bind target sequences not considered as conventional EREs. We investigated the effect of spacer size (n = 0, 1, 2, 3, 4) on the binding affinity and conformation of ERα and ERβ in these complexes and the effect of HMGB1 on the complexation. We find 1) both receptors bind similarly and with progressively reduced affinity to cEREn, as n differs from 3; 2) however, both receptors bind as strongly to the cERE with no spacer (cERE0) as to cERE3; 3) HMGB1 enhances ER binding affinity in all complexes, resulting in strong binding in all complexes examined; 4) the full-length ER binding differs strikingly from similar binding studies for the ER DNA binding domain (ERDBD), with the full length ER dimer exhibiting strong binding affinity, enormous plasticity and retaining binding cooperativity as the spacer size varies; 5) both protease digestion profiles and monoclonal antibody binding assays indicate the conformation of the receptor in the ER/ERE complex is sensitive to the spacer size; 6) the ER/cERE0 complex appears to be singularly different than the other ER/cEREn complexes in binding and conformation. These findings reinforce the notion of the plasticity in ER binding and lead to the hypothesis that in most cases, the minimum requirement for estrogen receptor binding is the ERE half-site, in which one or more cofactors, such as HMGB1, can cooperate to decrease ER binding specificity, while increasing its binding affinity.

Key words: estrogen receptors; estrogen response element half-sites; HMGB1; F domain

1. Introduction

The current paradigm for binding selectivity for steroid hormone receptors to their bipartite hormone response elements (HRE) in DNA emphasizes the importance of 1) the

nucleotide sequence of the half-site, 2) its inverted repeat orientation and 3) a spacer size of 3 bps between the half-sites [1]. The receptors bind as homodimers to palindromic or imperfect palindromic sequences, with ER binding to consensus half-sites (cHERE) of 5'-AGGTCA-3', while the other steroid hormone receptors bind to the consensus half-site, 5'-AGAACA-3'(cHSRE). To date, perhaps greater than 50, predominantly imperfect palindromic EREs, have been studied (many more reported; 2), with most findings generally revealing no (linear) correlation between ERα binding affinity and the level of transcriptional activation [1, 3]. Although ER interaction with these "classical" EREs can explain its action on many estrogen-responsive genes, it is becoming evident that there is a broader diversity of binding and mechanisms by which ER can influence transcriptional activities [4-7]. For example, the presence of HMGB1, a ubiquitous, highly conserved and highly abundant (>500,000 copies per cell) nuclear coactivator protein [6, 8] increases the binding affinity of ER to a spectrum of EREs [9-11], with its strongest effect on the consensus half-site (cHERE) [6]. These cHEREs are found in promoters of an increasing number of genes that are responsive to estradiol [7, 12-15], in addition to the cHERE being the predominant sequence found by a genetic screen in yeast [16]. In contrast to the steroid hormone receptors, non-steroid receptors bind predominantly as heterodimers to these same HEREs (5'-AGGTCA-3') that are part of a direct repeat, with heterodimer binding selectivity determined by the size of the spacer between the half-sites. Orphan nuclear receptor typically bind to this same half-site as monomers or dimers, and require an AT-rich sequence 5'- to the half-site [17, 18]. Interestingly, while GR exhibits binding to direct repeats, ER is reported to exhibit only weak binding [19]. In studies that used only the ER DNA-binding domain (DBD), ERDBD bound as a monomer to cEREn in which the spacers sizes were n= 0-2 & 4-5, with n = 3 being the sole cERE in which a stable DBD dimer was observed [20]. The crystal structures of the ERDBD binding to cERE and to a nonconsensus ERE showed the interactions important for dimer stability and strong DNA binding and how ERDBD changes its interactions to adapt to a nonconsensus ERE [21, 22]. Furthermore, the crystal structure for GRDBD binding to the

consensus glucocorticoid response element (cGRE) revealed specific interactions which were altered when GRDBD bound to the GRE that contained a 4 bp spacer [23]. Unlike ERDBD binding to cERE4, the GRDBD remained a dimer and bound to GRE4 with one monomer binding specifically to one half-site, while the partner monomer was out of register with the other half-site and bound nonspecifically to the DNA [20, 23, 24]. This resultant (GRDBD)2-GRE4 structure was reportedly due to the relative strength of the dimer interface, which was more important to complex formation than were specific interactions between GRDBD and its cognate half-site, which was displaced and rotated from its consensus structure in cGRE3. Another indication of the plasticity of steroid receptor binding was revealed recently when the androgen receptor DBD was shown to bind strongly in a head-to-head manner to a direct repeat of half-sites for the androgen response element, with a 3 bp spacing [25]. Increasingly, more crystallographic data are becoming available to show the structural plasticity of the DBD interactions, which will provide the first level of understanding for full-length receptor interactions with their response elements. One may anticipate modifications of this basic model and increasing evidence for a greater plasticity in the full-length receptor/ERE interaction.

ER occurs in two isoforms, ERα & ERβ. Although the full length ERs differ significantly in their protein sequence, the sequence in the 66 bp core DBDs is highly conserved and differs by only 2 residues. As a result, there are very few distinctive differences in the binding characteristics of the two isoforms for a variety of classical EREs. Although ERα binding on ERE produces a bend in DNA, while ERβ does not, there are not distinguishing differences in their binding or transactivation profiles [26, 27], with the exception that ERα binds to steroidogenic factor 1 response element (SFRE), while ERβ does not [28]. On the other hand, ERβ exhibits preferential recruitment of mammalian mediator subunit, TRAP220, with evidence that the F domain in ERα is responsible for inhibiting its interaction [29], suggesting that perhaps much of their differences occur subsequent to ERE binding.

In this work, we compare the binding profiles for both ERα and ERβ on cERE(n)s that have spacers with n = 0-4, in the presence and absence of HMGB1. The binding affinity of both isoforms of ER for a cEREn are similar, gradually decreasing as the size of the spacer (n) changes, with the exception of cERE0, which shares many similarities with cERE3. The conformation of ER in the complexes is a sensitive function of the spacer as revealed by protease digestions and antibody binding. HMGB1 enhances ER binding affinity to all cERENs, with its greatest effect on those cEREn elements in which ER bound weakest in the absence of HMGB1. In the presence of HMGB1, the Kd values for all ER/cEREn complexes are in the range of 10 +/- 5nM, indicating that the spacer size has only a marginal effect on binding for complexes in the series. Evidence is presented that suggests that the character of the F domain of ERα in the weakest complexes clearly differs from those complexes in which ERα binds strongly.

2. Materials and Methods

2.1 Estrogen Receptors, HMGB1, and Oligonucleotides

ERα and ERβ were purchased from Panvera/Invitrogen. HMGB1 was isolated and purified from calf thymus as previously outlined [30]. Oligonucleotides were purchased from IDT Technologies and the sequences are shown in Table 1. Oligonucleotides that involved substituted base pairs in the ERE (altered cEREn for n = 0, 1) are designated by the star, in which the formal spacer and/or some of the second half-site was changed to bps that had the potential to inhibit ER binding [6, 30.]

Insert Table 1

2.2 Electrophoretic Mobility Shift Assays (EMSA)

The EMSA experiments were carried out as outlined [6]. The Kd values were determined by a titration of 100 pM DNA with increasing concentrations of ER after equilibration for 20 mins at 4^{o} C. The dried gel was exposed to a Phosphor Imager screen that was scanned using the

Molecular Dynamics Phosphorimager system. The Molecular Dynamics ImageQuant software program was used to measure the band intensities for DNA and the complex and the percent complex plotted versus ER concentration. The best fit of the data was derived using Sigma Plot with points from multiple experiments and the Kd value determined as previously described [6].

2.3 Protease Digestions of Estrogen Receptor/DNA Complexes

Trypsin and chymotrypsin digestion profiles were carried out after a constant amount of the ER (slightly above the Kd values) was incubated with 100 pM of cEREn for 20 minutes. After equilibrium was established, a constant volume of increasing amounts of protease was added and the reaction continued for another 10 minutes at room temperature. The reaction tubes were then placed in ice and the samples were immediately loaded on the gel.

2.4 Antibody Supershift Assays

Antibody binding studies were done similarly, but with the addition of 1 ug of monoclonal antibody to the equilibrated samples for an additional 10 minutes at 4^{o}C before loading on the gel. In experiments in which HMGB1 was present, the concentration was at 400 nM. ER antibodies were kindly provided by A. Nardulli and G. Greene.

3. Results

3.1 Comparative EMSA Profiles for ER/cEREn Complexes

ERα and ERβ bind strongly to the palindromic sequence in cERE in which two inverted consensus half-sites (cHERE) are separated by 3 bps, and with a decreased affinity for imperfect palindromic sequences [1, 4]. We examine the binding characteristics of ERα and ERβ to a series of palindromic consensus sequences (cEREs) in which the spacer sizes differ in the number of bps (n = 0-4; i.e., cERE0, cERE1, cERE2, cERE3, cERE4), in addition to a cHERE in which the 3'-ERE half-site in cERE3 was completely changed to greatly reduce ER binding [31].

Figure 1A & B compares the general binding profiles for ERα & ERβ in this series of cEREns. Qualitatively, as the spacer was changed by 1 bp from n = 3 to either n = 2 or 4, the extent of binding decreased. The removal of another bp to n = 1 produces an even greater decrease in binding affinity. However, this decreasing trend abruptly changes at n = 0, at which point the binding affinity for both ERα and ERβ is sharply increased, with a binding profile similar to that for cERE3. Lastly, the weak binding affinity for both ERs to cERE1 is actually comparable to that of the consensus half-site (cHERE), as previously reported [6]. These binding profiles qualitatively indicate that although binding is sensitive to spacer size, for n = 0-4, the spacer presents a significant barrier to ER binding for n =1, only a modest barrier to binding for n = 2 & 4, but no apparent barrier for n = 0. It is also evident that the binding profiles for ERα and ERβ are comparable and parallel each other in their binding affinities.

Insert Figure 1 A & B

3.2 The Effect of HMGB1 on Binding Affinity

Since HMGB1 and HMGB2 enhance the binding of ERα to EREs and is essential in facilitating strong binding to an ERE half-site (cHERE) [6, 10], the effect of HMGB1 on the binding affinity of ERα to cEREn (n = 1- 4) was determined and shown in Figure 2. In all cases, the presence of HMGB 1 increases the binding affinity of ERα. However, its effect is significantly greater on cEREns with n values other than 3 and 0, with a range of effects for n = 1, 2, 4 and cHERE, which are the cEREs that exhibited the weakest ER binding affinity in the absence of HMGB1. The binding profiles for ERβ are similar and parallel those for ERα (data not shown).

Insert Figure 2 A-F

Table 1 compares the apparent Kd values determined for ERα and ERβ binding in the cEREn series and shows the effect of HMGB1 on the Kd.

Insert Table 1

The Kd values for both ERα & ERβ range from ca. 7-100 nM in the absence of HMGB1 or about 10-fold, with the order of increasing Kd being n = 3 ~ 0 < 4 ~ 2 < 1 ~ cHERE. In the presence of HMGB1, the binding affinity for every complex increases, with the Kd values being in a much more limited range (about 3-4 fold), varying from 4-16 nM. In agreement with our previous findings for ER binding to EREs and ERE half-sites [6], the effect of HMGB1 on ER binding affinity shows a general trend, with its greatest effect on the complexes that are weakest in the absence of a HMGB1. Importantly, the presence of HMGB1 not only increases the binding affinity, but at the same time it reduces the binding specificity. In addition, the presence of HMGB1 does not change the order of the binding affinities for the complexes in the series. The general trend in the influence of HMGB1 on enhancing the binding affinity is:

$$\text{cHERE} \sim 1 > 2 > 4, 0 > 3$$

3.3 Protease Digestion Profiles for ER/cEREn Complexes

To investigate the global conformation of the estrogen receptor in the ER/ERE complexes, protease digestion profiles of the complexes were obtained with chymotrypsin and trypsin. The ability of the protease to gain access to and cleave at its specific sites in the ER/cEREn complex will directly reflect the conformation of bound ER [32, 33]. If the ER interactions are dissimilar in the different cEREn complexes, the ER conformation may be altered, leading to a change in the digestion profile and/or the extent of the digestion. Figure 3 shows the trypsin and chymotrypsin digestion profile for the ER/cERE(n) complexes. Both the trypsin (A) and the chymotrypsin (B) profiles lead to the same general conclusions for both ERα and ERβ (data not shown), with similar profiles shown side-by-side. The trypsin digestion for n = 1 & cHERE (ii & v) are virtually identical (measured band positions and general profile), suggesting that the conformations are very similar. The digestion profiles for n = 2 & 4 (iii & vi) are also very similar to each other, but clearly different that those for n = 1 & cHERE. The profiles for n = 0 & 3 (i & iv) are also distinct from the others, but also different from each other,

suggesting that, in these two complexes in which ER exhibits strong and comparable binding affinities, the conformations is clearly different than those found in the weaker complexes. Collectively, the data indicate that ER can rearrange itself and interact with EREs in more than one way to lead to stable binding interactions. Interestingly, the digestion profiles in the presence or absence of HMGB1 are not significantly different. In addition, these findings parallel the binding affinity results in that the conformation of ER in the complexes for n = 1 & HERE are similar, those in the n = 2 & 4 complexes can be generally grouped together, while ER in the complexes for n = 0 and n = 3 are not only different from the others, but also different from each other.

Insert Figure 3A & B

3.4 Antibody Supershift Profiled for ER/cEREn Complexes

The ability of monoclonal antibodies (mAb) to bind ER epitopes in the complexes can provide an alternate, and more focused, examination on the nature of the conformation of ER in the complexes. We used seven mAbs that bind in each domain of the receptor, with the location of the epitopes shown in Figure 4A. Figure 4B shows the results for the series of mAbs interacting with the ERα/cERE3 complex, which generally represents what is seen with most of the complexes. A supershift is observed for ER21, H222, D75, H226 and D547, but not for P1A3 and MA1-310. This suggests that when ERα is bound to the normal consensus ERE sequence, the epitopes in domains A, B, D, E and F are accessible, but the epitope for P1A3 in the C domain, the DBD domain that interacts directly with the ERE, is inaccessible. In addition, MA1-310, which targets the C-terminal region of the ERDBD, fails to supershift any of the complexes indicating that this epitope is inaccessible and suggests that it is presumably involved in tight binding within the complexes. On the other hand, a slightly different picture is derived from the supershift data for cERE1, which is a complex in which both ER isoforms exhibit the weakest binding. Figure 4C shows that antibody D75, which interacts in the F domain, supershifts the

complex band (lane 5 & 14), but also produces a band of higher mobility. This may indicate that because of this weak ER binding to cERE1, the D75 antibody successfully competes with one of the ER monomers and stabilizes a monomeric complex on the DNA. In addition, P1A3 (lanes 8 & 17), which targets an epitope directly in the DBD, also shows a weaker band at this same position, consistent again with a more accessible epitope because of the weaker ER/cERE1 interaction. To further explore this unusual character of the D75 interaction in this complexes, the supershift data for D75 on the cHERE and all the cEREn complexes are collectively shown in Figure 4D. Both cEREs for n = 0 and 3, for which there is strong ER binding show a normal supershift with D75. In contrast, the other four complexes show a supershift, in addition to the band of greater mobility. Interestingly, the relative intensity of this higher mobility band in the four complexes also shows a general inverse correlation with the binding affinity of ER in the complex (n = 1, HERE > 4, 2). Finally, the presence of HMGB1 made no significant effect on the interaction of the mAb.

Insert Figure 4 A-D

The nature of the ER binding varies in this series of complexes, but may, to a first approximation, be considered to fit into either of two extreme models. In Model 1, the ER dimer and its (dimer) interface can be considered flexible, acting perhaps like an “accordion”. As such, each ER monomer can twist, and extend or contract, so that each ER may bind strongly to a cHERE, irrespective of the n value in this series. The alternative, Model 2, assumes that the ER dimer interface is rigid, as in the $(GRDBD)_2$-GRE4 case, with one ER monomer binding specifically to a cHERE, while the other cHERE is out of register and the partner ER must bind nonspecifically to base pairs that are strictly 3 bps from the 5’-cHERE. The ER binding in the latter cases has a reduced affinity and exhibit different degrees of “wobble” in their DNA interactions.

3.5 ER Binding to *cERE0 and *cERE1

To test whether ER in these complexes might better fit into one or the other of these models, we carried out additional ER binding experiments on cERE0 and cERE1 and compared the binding affinities with the same response elements in which the 2 or 3 bps 3'- to the 5'- cHERE were changed. The altered cERE0 and cERE1, referred to as *cERE0 and *cERE1 in Table 2, would be expected to be the complexes in this series that are most sensitive to these changes. The changes in the sequence are those that are expected to greatly reduce the binding affinity to the consensus ERE half-sites, as outlined previously [6, 31]. In Model 1, these changes would be expected to greatly reduce ER binding, while on the other hand, there would be little or no reduction in the binding affinities if ER binds as in Model 2.

Figure 5 compares the ERα binding profiles for cERE0 & *cERE0 and those for cERE1 and *cERE1. The binding affinity to *cERE0 is dramatically reduced from that of cERE0 as a result of the change. Although the Kd is about 10 nM for cERE0, there is no detectable binding to *cERE0 up to as high as 180 nM ER. We estimate that the Kd value is increased by 30-fold or greater. On the other hand, when the same experiment was carried out with the cERE1 and *cERE1 complexes, the binding affinity is only marginally reduced. Although we were able to only extend the study to 180 nM ER, we estimate that the Kd for *cERE1 is approximately 300 nM, an increase of only about 3-fold from that in cERE1. This indicates that changing the sequence immediately 3'- to the cHERE in cERE0 has an enormous effect on ER binding, while the similar change in the cERE1 complex has very little influence. We conclude that ERα binding is distinctively different in the cERE0 and cERE1 complexes.

Insert Figure 5 A & B

4. Discussion

Both ERα and ERβ show similar binding affinities to the respective cEREn target sites, with parallel trends in their interaction along the series of cEREns. Table 2 shows the strongest binding for the ER isoforms occurs for the already recognized consensus ERE, cERE3. The Kd

value increases by a factor of 3 as n is changed by 1 (to 2 or 4 bps) and then an additional factor of 3-4 as the spacer size is decreased by another bp to 1 bp. However, the binding affinity abruptly increases at n = 0, becoming comparable to that for a spacer with n = 3. In all cases, the presence of HMGB1 increases the binding affinity, with the greatest effect being observed in the weakest ER/cEREn complexes (n = 1, 4) in the absence of HMGB1. The effect of HMGB1 is consistent with previous findings that the stronger the binding affinity is in the absence of HMGB1, the smaller the effect of HMGB1 has on increasing the binding affinity [6]. However, irrespective of the complex, it is important to note that the presence of HMGB1 decreases the Kd value for every complex into the range, 4-15 nM, which is comparable to the Kd value for ER binding to cERE in the absence of HMGB1. This indicates that all cEREns investigated are viable sites for strong binding in the presence of the coactivator protein, HMGB1 [8]. These findings also support the contention that the binding affinity of ER to EREs represents only the first level of selectivity, with additional levels (other cooperating cofactors, chromatin structure, etc) being essential in providing the resultant functional specificity.

The manner in which HMGB1 affects the increased ER binding affinity is unsettled, but it clearly behaves like an “allosteric cofactor”. It has been proposed that HMGB1 interacts in the minor groove and facilitates the C-terminal extension (CTE) of ER to interact more effectively in the minor groove to provide ER with a second interface with the DNA, adding to its binding interaction in the major groove [11]. A proposal also suggested that HMGB1 binds only transiently in the minor groove [34, 35] which “pries it open” to permit the CTE to more readily bind [6]. In addition, the increased thermodynamic driving force for this interaction may include a significant entropic contribution, similar to that for GRDBD binding to cGRE [36]. A “spine” of immobile waters is found along the minor groove of the B-form DNA [37, 38]. The HMGB1 interaction in the minor groove may well displace bound waters, which would lead to both an increase in entropy and a greater binding affinity for ER.

Digestion profiles by trypsin and chymotrypsin show that the conformation of ERα in the ER/EREn complexes is a sensitive function of the spacing in each complex. In this series of six complexes, the digestions profiles for the weakest complexes, those with n = 1 and a singular half-site (cHERE), are comparable, indicative of similar ER conformations in these complexes. This is likewise true for the digestion profiles for ER/cEREn complexes with n = 2 and 4, which exhibit moderate binding affinities. This suggests that the ER conformation in these complexes is similar, but that they differ from the others in the series. The profiles for the complexes with n = 0 and 3 do not fit into these previous two groups and also exhibit differences between each other. The differences in digestion profiles for the corresponding ERβ complexes (data not shown) parallel those for ERα. These findings suggest that these six complexes can be viewed generally in four groups. The ER in complexes that exhibit the strongest binding, n = 0 and 3, have unique structures that differ from the others, with those with moderate binding being similar (n = 2 & 4), but different than those exhibiting the weakest binding (n = 1 & cHERE).

Seven antibodies were used to probe the accessibility of each domain in ERα. The MA1-310 mAb, with its epitope at the border of the C/D domains (DNA binding/hinge), did not supershift any of the complexes, indicating that, irrespective of the binding affinity, its vicinity to the ERDBD/DNA interface makes it inaccessible in all complexes. On the other hand, each of the mAbs - ER21(A domain), H226 (B domain), D547 (D domain, hinge region) and H222 (E domain) - produced a supershift in all the complexes indicating that these epitopes are accessible to the antibodies. Figure 4D showed that D75, which binds in the F domain, which lies immediately to the carboxyl side of AF-2 in the H12 helix, produces a supershifted band in all the complexes. However, an additional band of greater mobility is observed for only the four complexes in which ER exhibits weak binding. This suggests that the dimeric ER complex has possibly been disrupted by the interaction. The relative intensity of this band in the n = 1, 2, 4 and cHERE complexes generally correlates inversely with the binding affinity of ER in the complexes

and is absent in the n = 0 and 3 complexes. The D75 binding to the F domain in cEREn, where n = 1, 2 & 4, suggests that it could influence the strength of dimerization in the complex, as reflected in the unusual band of increased mobility. With the F domain being so "distant" from the ER DBD, it suggests that it may interact in the vicinity of the DBD domain and represent an indicator of the conformation and binding interaction between ERα and the DNA.

The structure and function of the F domain is unclear. The crystal structure of the ER LBD does not include the F domain and so its relative position and interactions within ER is not known [39]. However, previous findings have suggested that the F domain may function to inhibit dimerization and that it also plays a key modulating role in functional differences between antagonist and agonists, thereby influencing its transcriptional activation [40-42].

Interestingly, the character of the EMSA binding profiles for both full length ERα and ERβ differ sharply from those reported for ERα DBD binding to a similar series of cEREns [20]. Although ERDBD binds to cERE3 as a dimer, ERα monomer binding is found exclusively for n = 1, 2, 4, 5 and 6, with both monomer and noncooperative dimer binding observed for n = 0, 7 and 8. These previous findings reinforce reports that the D-box interactions between DBD monomers provide stability for the DBD dimer when bound to the cERE3. However, comparison of our data with that from the ERDBD binding study indicates that the D box apparently provides only a very weak interface between ER monomers to stabilize the dimer. When just one bp is changed in the spacer, a 3.4 A translation and a 36^{o} rotation occurs and the interface at the D-boxes is insufficient to retain DBD dimer stability when bound to DNA. On the other hand, for the full length ER, the dimer stability is retained, presumably due to the more extensive protein-protein interactions that involve sequences in the E domain. [43, 44]. These comparative results also bring into question the extent to which the D-box interactions drive ER dimer formation and serve to measure the distance between half-sites in the full-length estrogen receptors.

GRDBD binding to GRE in which the spacer size was 4 bps (cGRE4) is the only crystal structure that has addressed the nature of a steroid hormone receptor DBD binding to its response element in which the spacer size is not 3 bps. The crystallographic data for the GRDBD/cGRE4 showed that one GR monomer bound specifically to a half-site, while the partner monomer bound nonspecifically. Parallel solution studies however, indicate that although GRDBD monomers do bind to cGRE4, they bind noncooperatively [23, 45].

Our data cannot unequivocally distinguish between the two proposed simple models and more structural characterization is required to define the nature of ER binding in these complexes. However, the ER binding affinity and conformational data suggest that the series of complexes fall into at least two groups; the weak complexes with cEREn with n = 2, 4, 1 and cHERE and the strong binding complexes in which n = 0 and 3. To help to distinguish between the two extreme ER binding models, the DNAs for the cEREn in which n = 0 & 1, which are expected to be the most sensitive to bp changes, were changed (see Table 1). The bp changes 3'- to the 5'-cHERE resulted in a significant reduction in ER binding affinity for cERE0 (> 30-fold). This is consistent with a flexible ER dimer, in which both ER monomers bind strongly to the cHEREs in the ER/cERE0 complex (Model 1). On the other hand, changes in 2 bps in the spacer in cERE1 lead to only about a 3-fold reduction in binding affinity, suggesting that the ER dimer is much more "rigid" and these bps are not involved in significant interactions, acting effectively as only part of a 3 bp spacer. This is consistent with what was observed with GRDBD with GRE4 [23]. If this result can be extended to the weak binding complexes (n = 1, 2 & 4), it would suggest that the 3 bps immediately 3'-to the 5'cHERE do not play an important role in ER binding in these complexes, implying that one ER monomer binds specifically to a half-site, while the partner monomer binds nonspecifically. However, ER binding to the cERE0 target sequence appears distinctly different, with the bps immediately 3'- to the 5'-cHERE contributing significantly to the stability and conformation of the complex.

These findings may have some relevance to regulatory regions in estrogen-responsive genes already reported. A response element in the promoters for the human TGF-α and the rat luteinizing hormone β genes have a 4 bp and 5 bp spacer, respectively [3, 46]. cERE0 is one of the important target sites in the estrogen responsive human Na^+/H^+ exchanger regulatory factor gene in human breast cancer cells [7]. The thyroid hormone receptor binds to both cERE0 (also called TREpal) and cERE3 [47-49] and our findings of strong ER binding to cERE0 adds further support that competitive receptor binding to these cEREs may play a role in a mechanism for the cross-talk between overlapping gene networks [48, 50, 51].

In a majority of simple EREs, the presence of one consensus HERE is very common. Those estrogen-responsive genes with regulatory elements of two or more HEREs in direct or everted repeats (estrogen response units) also contain one or more consensus HEREs [3, 4, 7, 52]. Collectively, this is consistent with the notion that, in many cases, the basic target element for ER is the cHERE. This may offer a number of potential advantages. It can provide a symmetrical ER dimer with an asymmetric target that inherently helps to facilitate directionality in some regulatory sites. The content of the adjacent bps could further help to regulate the extent of activated transcription by modulating the binding affinity to the target sequence, in addition to influencing the conformation of the ER/ERE that controls its interaction with coactivators and corepressors. In addition, it has been suggested that the greater the plasticity in regulatory factor binding, the more readily a network may adapt to new environments [53].

It should also be pointed out that this binding plasticity for estrogen receptors may not generally extend to other steroid hormone receptors. We carried out a parallel study (data not shown) on progesterone receptor (PR-B) binding to a similar series of cGREn. We confirm that PR binds strongly to cGRE3 (Kd = 3 nM). However, no binding was observed to cGREns, for n = 0-2 and 4 at PR levels as high as 80 nM.

These findings indicate that the ER binding affinity *in vitro* varies significantly with unconventional ERE sites and half-sites (HERE). However, in the presence of HMGB1, the binding affinities of ER for all cEREns examined were significantly increased into a range that are comparable to the binding affinity for ER binding to cERE in the absence of HMGB1. This is consistent with findings that nonconventional EREs and, especially ERE half-sites, are prevalent and functional in many regulatory regions of genes in the human genome [3, 4, 7, 15, 52] and suggests a potentially important role for the ubiquitous and abundant cooperating cofactor, HMGB1. This is also the first case, thus far, in which strong ER binding is being assisted by a nonspecific DNA-binding protein. This leads to the hypothesis that although ER activity occurs utilizing classical pseudopalindromic EREs, ER binding appears to be considerably less restrictive and less specific than previously considered and suggests that HMGB1 and these nonconventional EREs may play an increasingly significant role in the regulation of estrogen-responsive genes.

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Figure Legends

Figure 1. Comparison of ERα and ERβ binding profiles to cEREn, n = 0, 1, 2, 3, cHERE and 4. A 100 pM of cERE0 (lanes 1-5), cERE1 (lanes 6-10), cERE2 (lanes 11-15), cERE3 (lanes 16-20), cHERE (lanes 21-25) and cERE4 (lanes 26-30) is incubated with increasing levels of either (A) ERα or (B) ERβ at 0 nM (lanes 1, 6, 11, 16, 21 & 26), 5 nM (lanes 2, 7, 12, 17, 22 & 27), 10 nM (lanes 3, 8, 13, 18, 23 & 28), 15 nM (lanes 4, 9, 14, 19, 24, & 29) and 20 nM (5, 10, 15, 20, 25, & 30).

Figure 2. The binding profiles for ERα binding to (A) cERE0, (B) cERE1, (C) cERE2, (D) cERE3, (E) cHERE & (F) cERE4. A 100 pM of is reacted with ER at 0 nM (lanes 1 & 11), 1.4 (lanes 2 & 12) , 2.7 (lanes 3 & 13), 4.5 (lanes 4 & 14), 6.8 (lanes 5 & 15), 9.5 (lanes 6 & 16), 12.7 (lanes 7 & 17), 16.4 (lanes 8 & 18; omitted for cERE3), 20.5 (lanes 9 & 19) and 25 nM (lanes 10 & 20), in the absence (lanes 1-10) and presence (lanes 11-20) of 400 nM HMGB1.

Figure 3. Protease digestion profiles for ER/cEREn complexes. Trypsin (A) and chymotrypsin (B) digestion profiles for ERα complexed with (i) cERE0, (ii) cERE1, (iii) cERE2, (iv) cERE3, (v) cHERE, (vi) cERE4. A 100 pM of each DNA is reacted with 0 nM (lanes 1,10), 25 nM (lanes 2-9, A, C, D & F) and 50 nM (lanes 2-9, B & E) of ER for 20 minutes at 4^{o} C. A. The complexes were treated with 0 ng (lanes 1 & 2), 0.05 ng (lane 3), 0.1 ng (lane 4), 0.2 ng (lane 5), 0.4 (lane 6), 0.8 ng (lane 7), 1.6 ng (lane 8) and 3.2 ng (lane 9 & 10; lane 9 is omitted in iii & vi with trypsin) of trypsin and incubated for an additional 10 minutes at room temperature. The reactions were then set on ice to stop the reaction and immediately loaded on the gel. B. Chymotrypsin digestions were similar with 0 ng (lanes 1 & 10), 7.8 pg (lane 3), 15 pg (lane 4), 31 pg (lane 5), 62 pg (lane 6), 125 pg (lane 7), 250 pg (lane 8) and 250 pg (lane 9).

Figure 4. A. Schematic representation of epitope locations for the monoclonal antibodies (mAb) in the domains of ERα. B & C. EMSA band shift data for a series of mAb interacting with (B) ERα/cERE3 and (C) ER/cERE1 complexes, in the absence (lanes 1-9) and in the presence of 400 nM HMGB1 (lanes 10-18). The ERα complex was treated with 1 ug of the mAbs ER21

(lanes 3 &12), H222 (lanes 4 & 13), D75 (lanes 5 & 14), H226 (lanes 6 & 15), D547 (lanes 7 & 16), P1A3 (lanes 8 & 17) and MA1-310 (lanes 9 & 18). D. EMSA band shift data for D75 mAb interacting with ER complexed with cEREn and cHERE. ER is complexed with cERE3 (lane1-3), cERE0 (lanes 4-6), cERE1 (lanes 7-9), cERE2 (lanes 10-12), cERE4 (lanes 13-16) and cHERE (lanes 16-18). Lane 1 in each series is the DNA, with the ER/cEREn complex in Lane 2.

Figure 5. Comparison of ERα binding profile to cEREn (n = 0, 1) and the corresponding (altered) *cEREn DNA. The binding profiles for ERα to (A) cERE0 (●) and (altered) *cERE0 (▲) and (B) cERE1 (●) and *cERE1 (▲).

Figure 1A

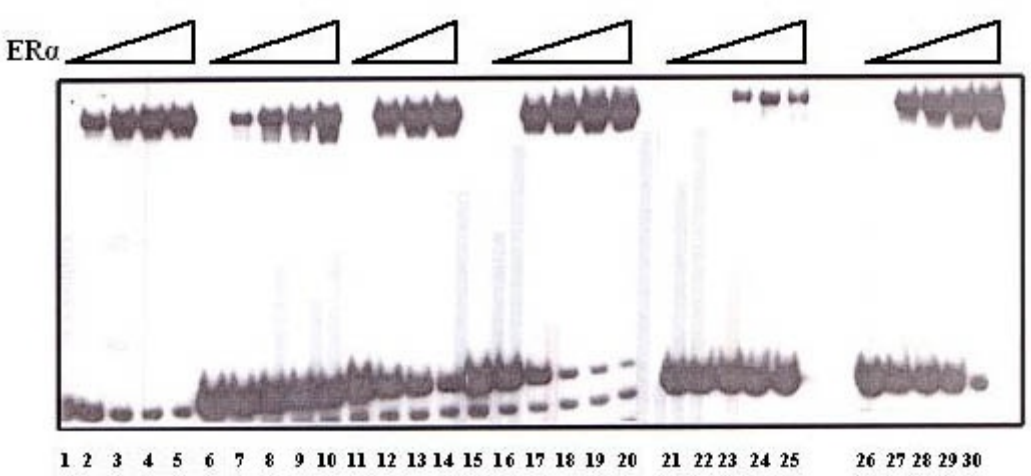


Figure 1B

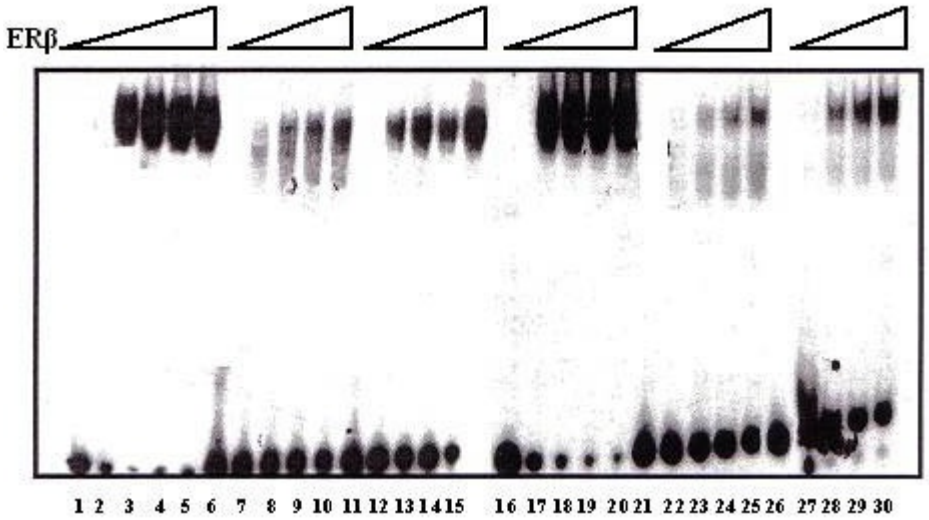


Figure 2A

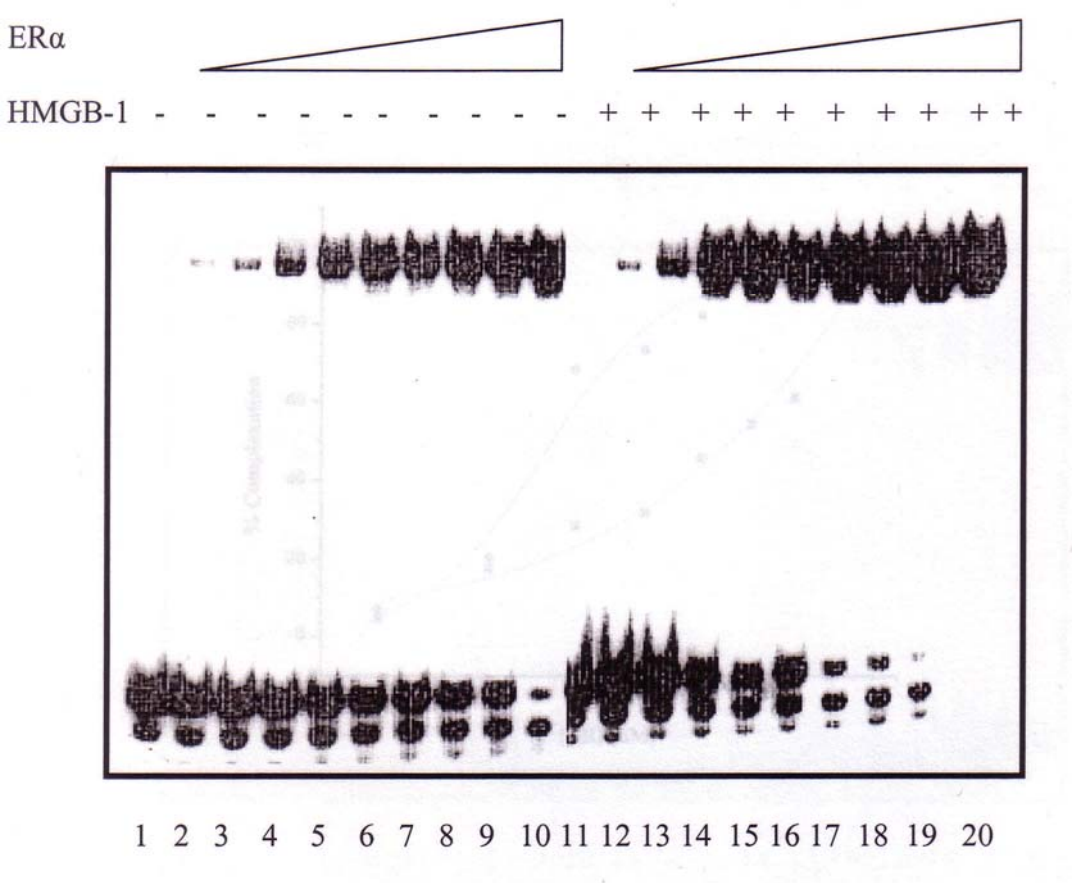


Figure 2B

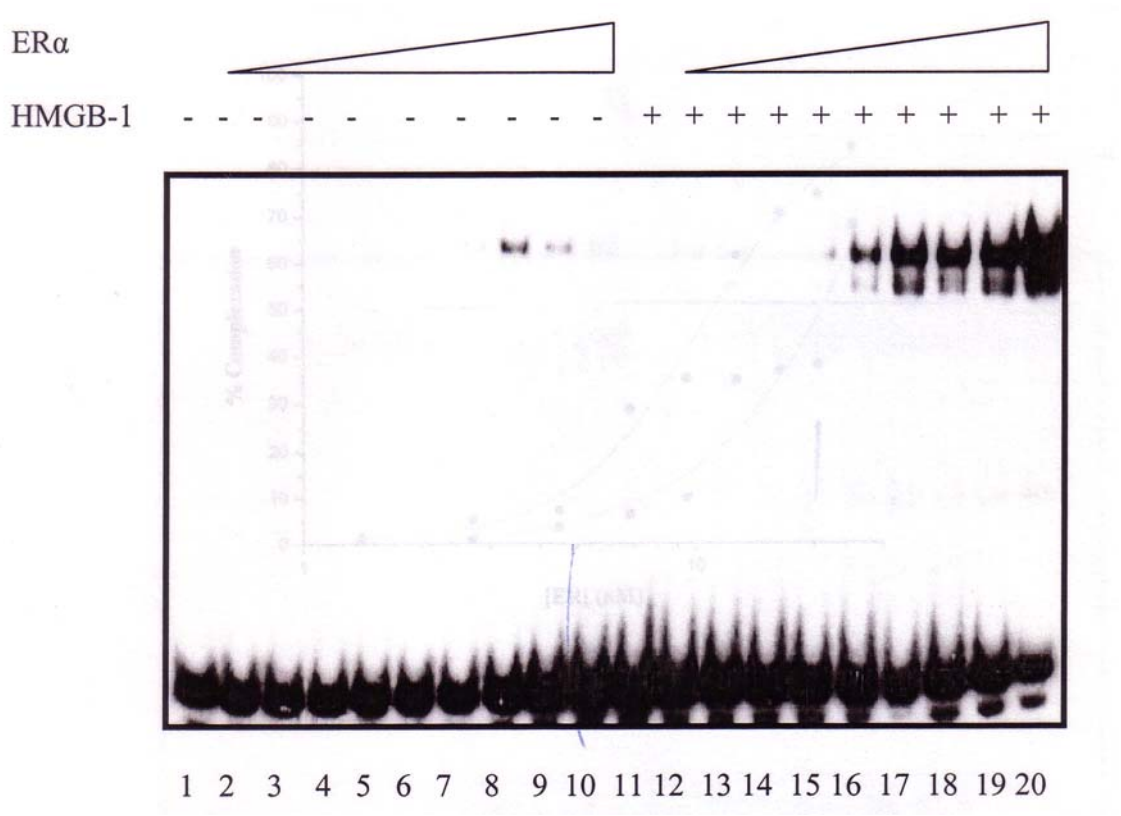


Figure 2C

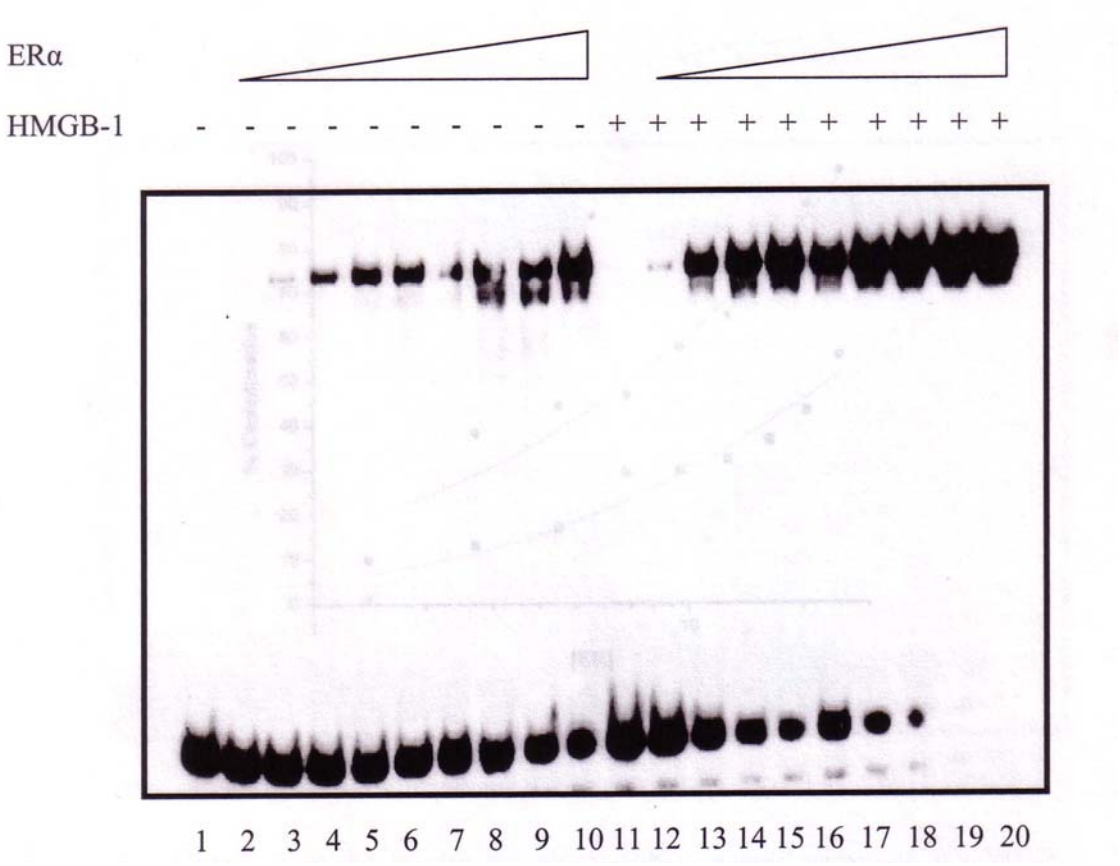


Figure 2D

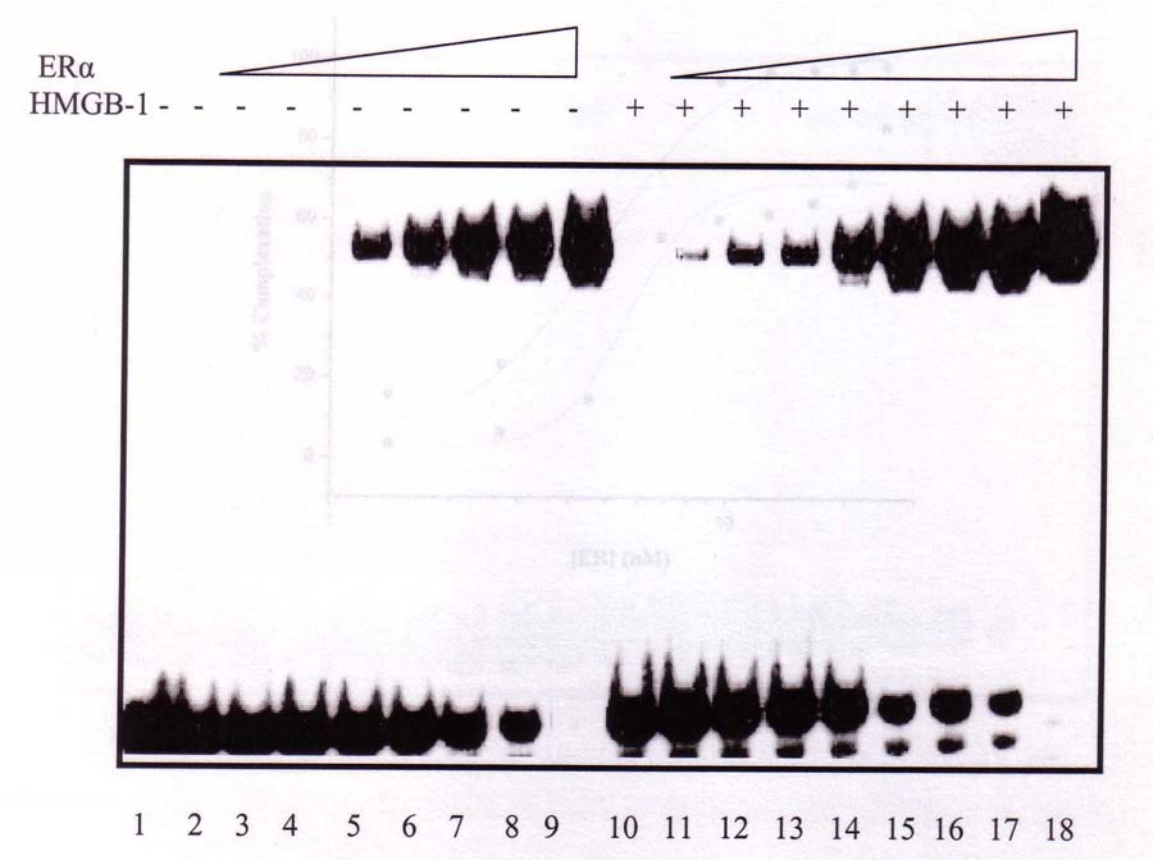


Figure 2E

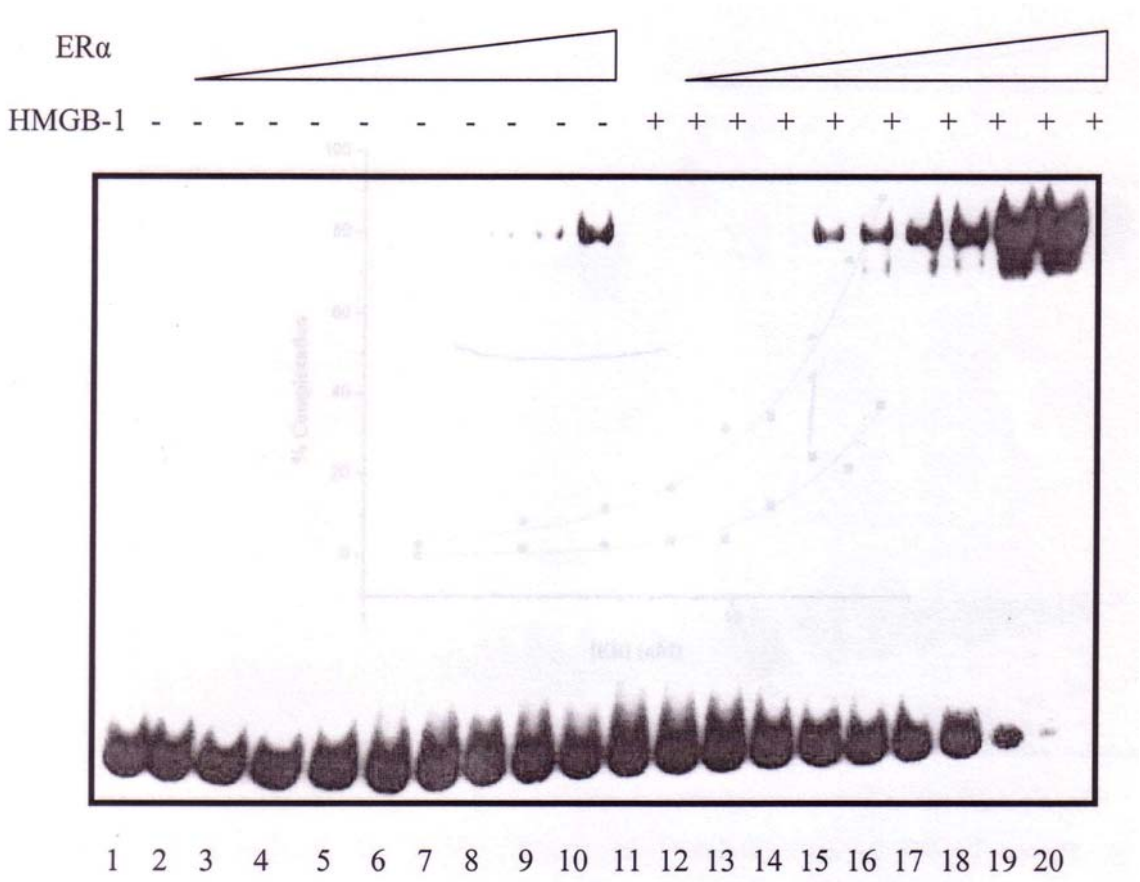


Figure 2F

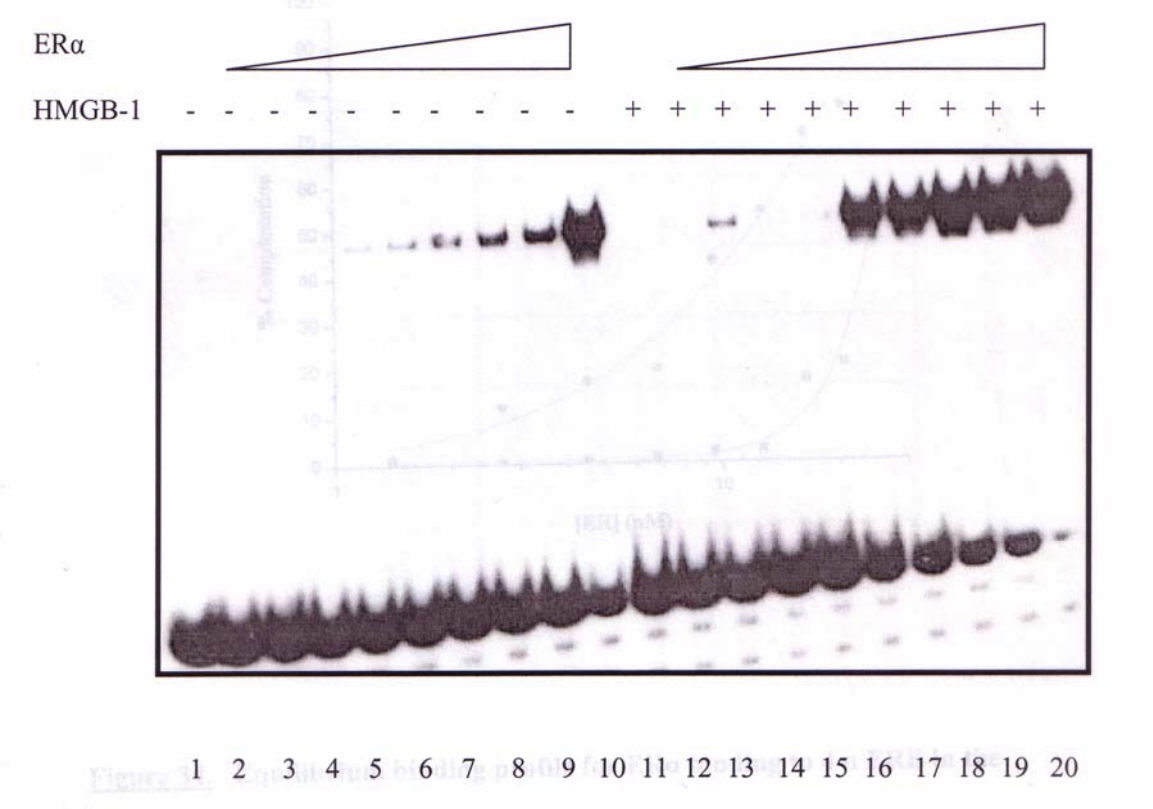


Figure 3A

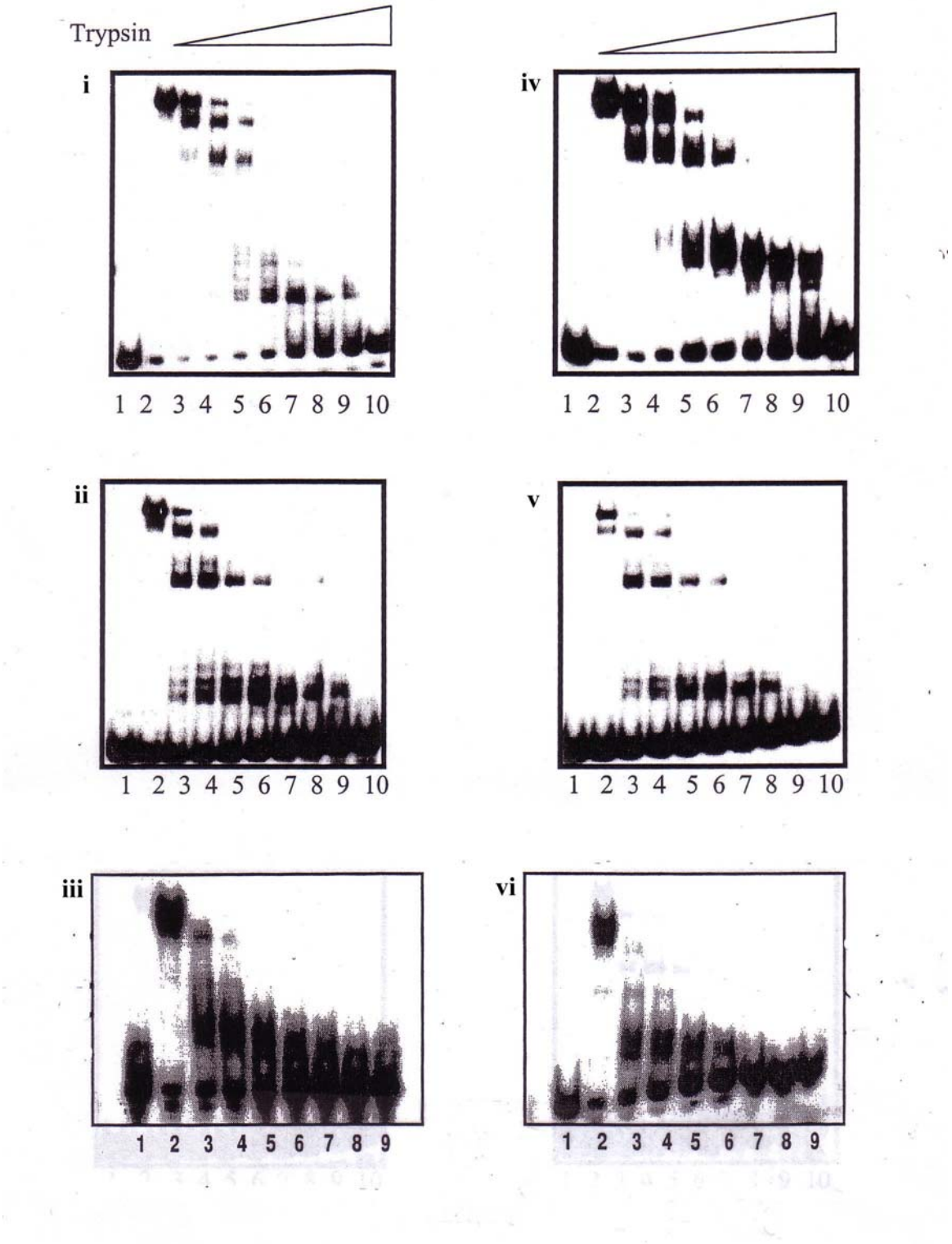
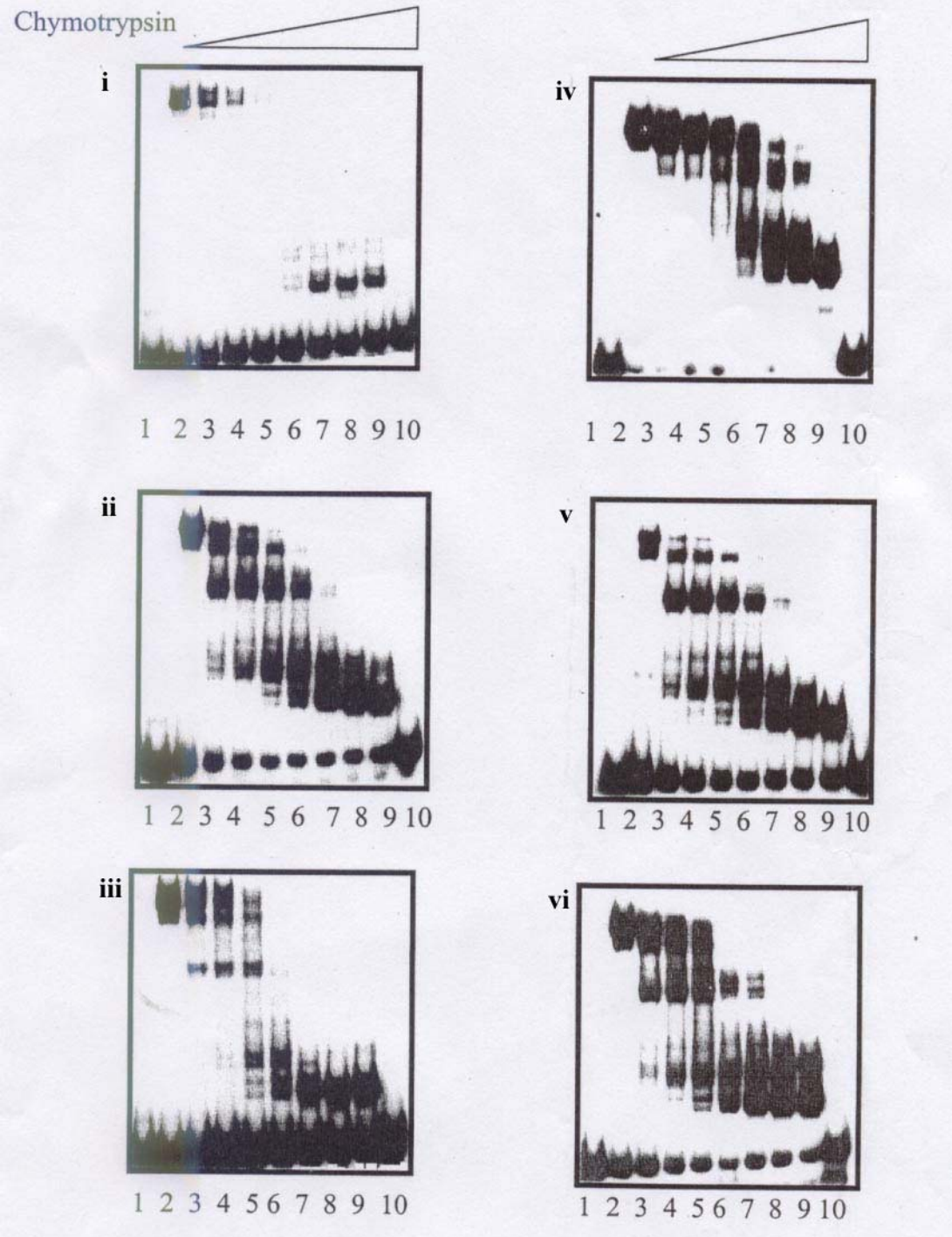

Trypsin
i
1 2 3 4 5 6 7 8 9 10
iv
1 2 3 4 5 6 7 8 9 10
ii
1 2 3 4 5 6 7 8 9 10
v
1 2 3 4 5 6 7 8 9 10
iii
1 2 3 4 5 6 7 8 9
vi
1 2 3 4 5 6 7 8 9

Figure 3B


Chymotrypsin
i
1 2 3 4 5 6 7 8 9 10
ii
1 2 3 4 5 6 7 8 9 10
iii
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v
1 2 3 4 5 6 7 8 9 10
vi
1 2 3 4 5 6 7 8 9 10

Figure 4A

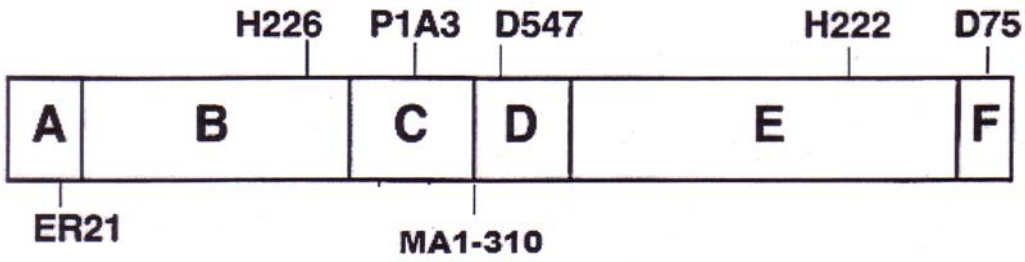

Figure 4B cERE3

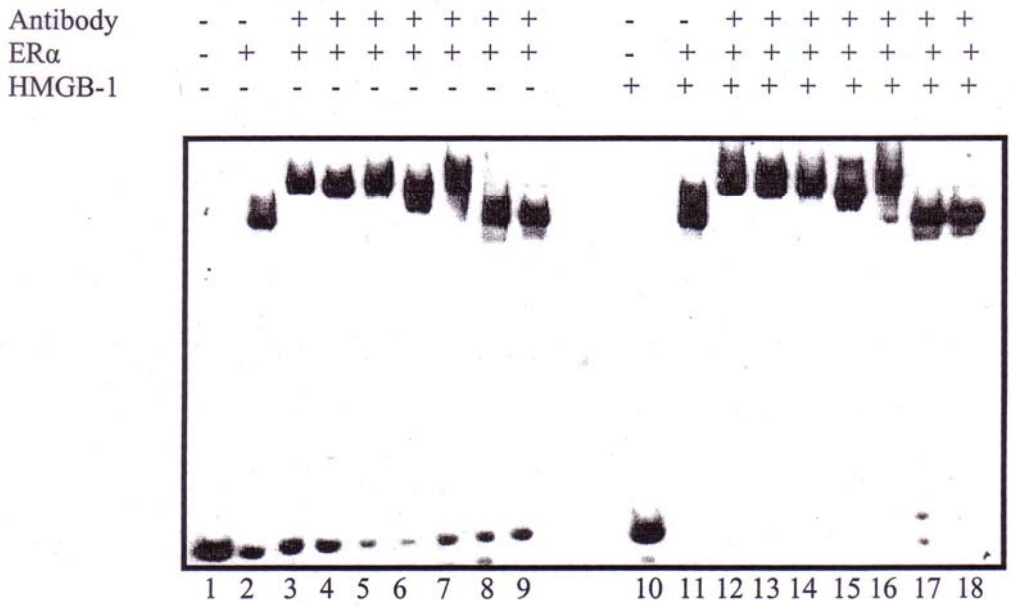

Figure 4C cERE1

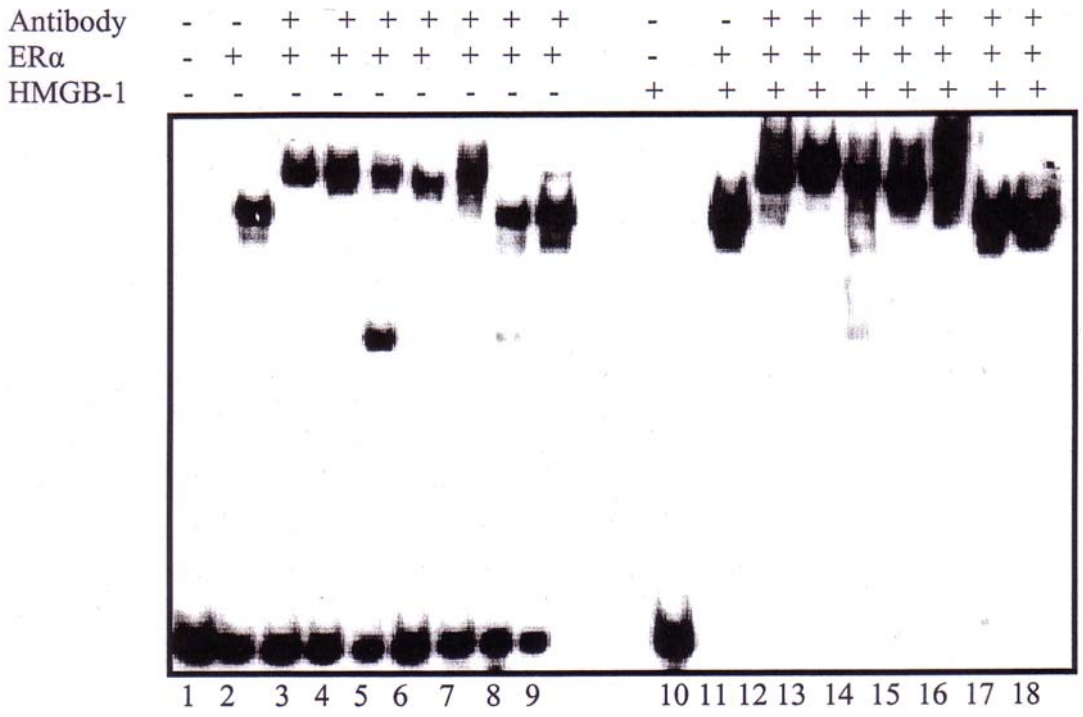

Figure 4D D75

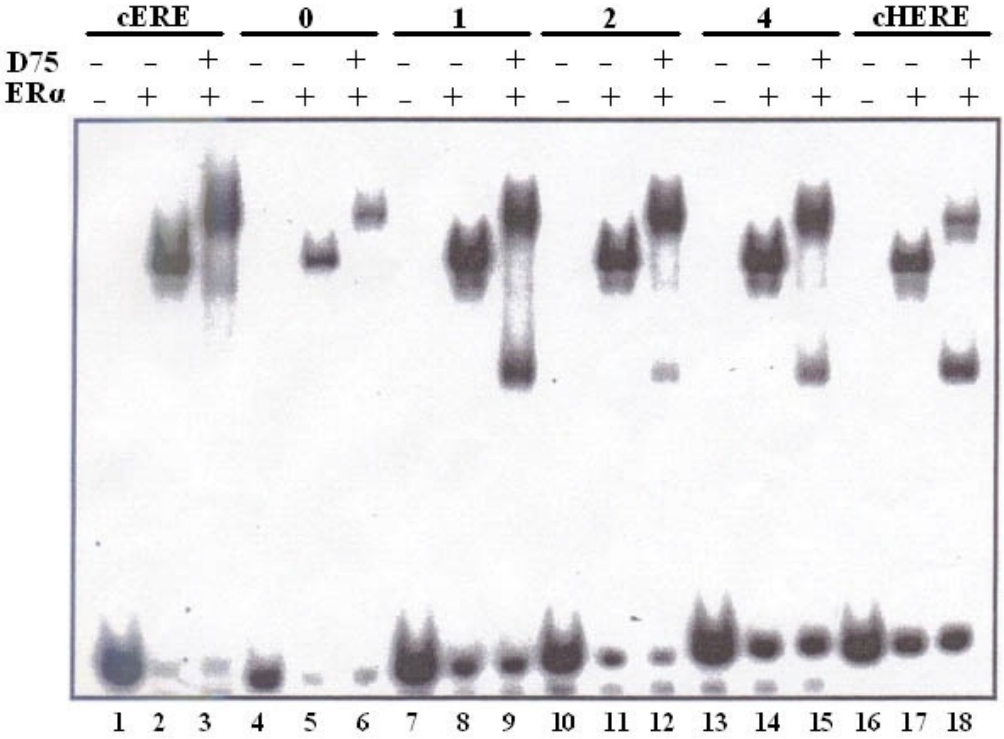

Figure 5A

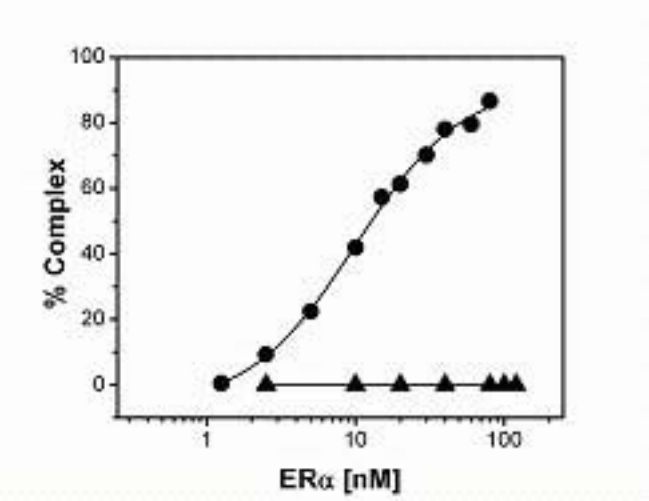

Figure 5B

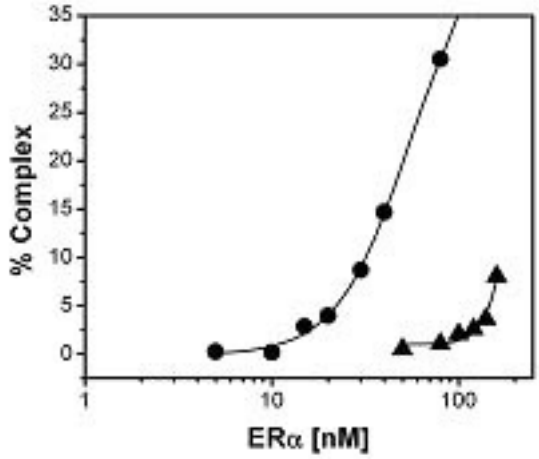

Table 1. Oligonucleotides

cEREn	Sequence		# bps
cERE0	5’-TGATGCCTCC**AGGTCATGACCT**CAACCCAA-3’		30
cERE1	5’-TGATGCCTCC**AGGTCA**c**TGACCT**CAACCCAA-3’		31
cERE2	5’-TGATGCCTCC**AGGTCA**cg**TGACCT**CAACCCAA-3’		32
cERE3	5’-TGATGCCTCC**AGGTCA**ctg**TGACCT**CAACCCAA-3’		33
cERE4	5’-TGATGCCTCC**AGGTCA**ctag**TGACCT**CAACCCAA-3’		34
cHERE	5’-TGATGCCTCC**AGGTCA**ctgGTTGGGCAACCCAA-3’		33
*cERE0	5’-TGATGCCTCC**AGGTCA**xxx**CCT**CAACCCAA-3’	xxx = GTT	30
*cERE1	5’-TGATGCCTCC**AGGTCA**cxx**ACCT**CAACCCAA-3’	xx = GT	31

The complete or partial cHEREs are denoted in underlined bold type

Table 2 Kd Values (nM) for ERα and ERβ binding to cEREn and cHERE

	ERα			ERβ		
cEREn	HMGB1		Effect	HMGB1		Effect
	-	+		-	+	
n value						
0	10	4.5	2	10	4.4	2
1	80	15	5-6	80	16	5-6
2	25	7.3	3-4	32	8.4	4
3	7.4	5.1	1-2	8.4	5.2	1-2
4	25	12	2	36	14	2-3
cHERE	80	15	5-6	100	13	7-8

RESEARCH & RELATED Senior/Key Person Profile

PROFILE - Project Director/Principal Investigator

Prefix	* First Name	Middle Name	* Last Name	Suffix
	William		Scovell	

Position/Title: Professor
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Division: Arts & Sciences
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Street2:
* City: Bowling Green
County:
* State: OH: Ohio
Province:
* Country: USA: UNITED STATES
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419 372-8293		wscovel@bgnet.bgsu.edu

Credential, e.g., agency login: WSCOVELL

*** Project Role:** PD/PI
Other Project Role Category:

	File Name	Mime Type
***Attach Biographical Sketch**	1944-ScovellBioSketch.pdf	application/pdf
Attach Current & Pending Support		

PROFILE - Senior/Key Person 1

Prefix	* First Name	Middle Name	* Last Name	Suffix
	Ronald	Clifford	Peterson	

Position/Title: Professor
Department: Chemistry & Biochemistry
Organization Name: Ohio Northern University
Division:
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Street2: Meyer Hall of Science 269
* City: Ada
County:
* State: OH: Ohio
Province:
* Country: USA: UNITED STATES
* Zip / Postal Code: 45810

*Phone Number	Fax Number	* E-Mail
419 772-2338		r-peterson@onu.edu

Credential, e.g., agency login:

*** Project Role:** Consultant
Other Project Role Category:

	File Name	Mime Type
***Attach Biographical Sketch**	2993-PetersonBioSketch.pdf	application/pdf
Attach Current & Pending Support		

	File Name	Mime Type

ADDITIONAL SENIOR/KEY PERSON PROFILE(S)

Additional Biographical Sketch(es) (Senior/Key Person)

Additional Current and Pending Support(s)

William M. Scovell, Professor of Chemistry

Education: B. S. in Chemistry, 1965, Lebanon Valley College, Annville, PA
Ph. D. in Chemistry, 1969, University of Minnesota, Minneapolis, MN; advisor, Stuart Tobias

Positions:

Teaching Appointments

1a) Professor, Bowling Green State University, 1979-
1b) Adjunct Professor, Biological Sciences Department, Bowling Green State University, 1980-
1c) Adjunct Professor, Biochemistry Department, Medical College of Ohio, Toledo, Ohio, 1981-
1d) Associate Professor, Bowling Green State University, Bowling Green, Ohio, 1974-79
2) Assistant Professor of Chemistry, State University of New York at Buffalo, Buffalo, New York, 1972-74
3) Instructor in physical (1970-71) and general chemistry (1971-72), Princeton University, 1970-72

Research Appointments

1) NIH Fellow, Pathology Department, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA, 1984-85
2) Post Doctoral Research Associate, Princeton University, 1969-70
3) Research Chemist, E. I. DuPont de Nemours & Co., Inc., summer,1965

Recent Sabbatical Leave

1) Lab of Eddie Sanchez, Department of Pharmacology, University of Toledo Health Science Center, Toledo, OH, Jun-July, 2006.
2) Lab of Jason Lieb, Biology Department and the Center for Genomics Research, University of North Carolina, Chapel Hill, NC, Jan-Feb, 2007

Publications (pertinent publications of the 44; 4 books)

1. W. M. Scovell, F. Collart, Unwinding of Supercoiled DNA by Cis-and Trans Diamminedichhloro-platinum (II): Influence of the Torsional Strain of DNA Unwinding, Nucleic Acids. Res. 13, 2881-2895 (1985).
2. W. M. Scovell, N. Muirhead & L. R. Kroos, Cis-Diamminedichloroplatinum(II) Crosslinks High Mobility Group 1, 2 and E Proteins to DNA in Micrococcal Nuclease Accessible Regions of Chromatin, Biochem. Biophys. Res. Commun. 142, 826-835 (1987).
3. W. M. Scovell, The Structural and Possible Functional Alterations on DNA and Cellular Chromatin Resulting from Cis-Pt$(NH_3)_2Cl_2$ Modification, Tokyo Seminar on Macromolecular-Metal Complexes. ,J. Macromol. Sci. (Chemistry) A26 (2 &3) 455-480 (1989).
4. J. J. Hayes & W. M. Scovell, Cis-Diamminedichloroplatinum(II) Modified Chromatin and Nucleosomal Core Particle Probed With DNase I, Biochim. Biophys. Acta (Gene Expression)1088, 413-418 (1991).
5. J. Hayes & W. M. Scovell, Cis-Diamminedichloroplatinum(II) Modified Chromatin and Nucleosomal Core Particle, Biochim. Biophys. Acta, 1089, 377-386 (1991).
6. W. M. Scovell, Cis-Diamminedichloroplatinum(II) Preferentially Cross-Links High Mobility Group Proteins 1 and 2 to DNA in Chromatin in Micrococcal Nuclease Sensitive Regions, Regulation of Eukaryotic mRNA Expression, Cold Spring Harbor Symposium, 185 (1991).
7. Y. B. Chao, W. M. Scovell & S. B. Yan, High Mobility Group Protein, HMG-1, Contains Insignificant Glycosyl Modification, Protein Science 3, 2452-2554 (1994).
8. Marmillot & W. M. Scovell, Enhancement of Transcription Factor, USF, Binding to the Adenovirus Major Late Promoter: Effect of Dithiothreitol and High Mobility Protein-1, Biochim. Biophys. Acta 1395, 228-236 (1998).
9. Ranatunga, J. Lebowitz, B. Axe, P. Pavlik, S. R. Kar & W. M. Scovell, Reexamination of the High Mobility Group-1 Protein For Self-Association and Characterization of Hydrodynamic Properties, Biochim. Biophys. Acta 1432, 1-12 (1999).
10. Lu, R. Peterson, A. Dasgupta & W. M. Scovell, Influence of HMG-1 and Adenovirus Oncoprotein E1A on Early Stages of Transcriptional Preinitiation Complex Assembly, J. Biol. Chem. 275, 35006-35012 (2000).

11. Das & W. M. Scovell, The Binding Interaction of HMG-1 with the TATA-Binding Protein/TATA Complex, J. Biol. Chem. 276, 32597-35605 (2001). Selected as "Hot Paper" in Chromatin Structure and Function.
12. A. Dasgupta & W. M. Scovell, Competition Between HMGB1 and EIA with TFIIA in the Early Stages of Assembly of the Transcriptional Preinitiation Complex, Biochim. Biophys. Acta, 1627, 101-110 (2003).
13. D. Das, R. Peterson & W. M. Scovell, HMGB1 Proteins Facilitate Strong Estrogen Receptor Binding to Classical and Half-sites Estrogen Response Elements and Relax Binding Selectivity, Mol. Endocrinol, 18, 2616-2632 (2004).

Manuscripts in preparation

1. The Binding of Estrogen Receptor Alpha and Beta to Direct Repeats of the Estrogen Receptor Element Half-Sites, with R. Ghattamanini and Y. Sarpong.
2. Sequence-specific Protein-DNA Binding; The Reality or the Myth?
3. The Binding of Estrogen, Glucocorticoid, and Progesterone Receptors to Recognition Elements Rotationally Phased andTranslationally Positioned Within a Nucleosome, with Y. Sarpong

Seminars (>200 total;* denotes written abstract)

1. A Model for the Role of HMG-1 in the Regulation of Eukaryotic Transcription, Department of Biochemistry and Immunology, Michigan State University, East Lansing, MI., April 3, 2000.
*2. Interaction of HMG-1 Protein with the TATA-Binding Protein, with D. Das, NIH, Workshop on The Role of HMG Proteins in Chromatin Structure, Gene Expression and Neoplasia, Bethesda, MD, May 1-2, 2000.
*3. The Role of HMG-1 and E1A in Preinitiation Complex Formation with TATA-Binding Protein, TFIIA and TFIIB, with A. Dasgupta, NIH, Workshop on The Role of HMG Proteins in Chromatin Structure, Gene Expression and Neoplasia, Bethesda, MD, May 1-2, 2000.
*4. Influence of HMG-1 and Adenovirus Oncoprotein, E1A, on the Early Stages of Assembly of the Preinitiation Complex, with W. Lu & R. Peterson, NIH, Workshop on The Role of HMG Proteins in Chromatin Structure, Gene Expression and Neoplasia, Bethesda, MD, May 1-2, 2000.
*5. The Role of HMG-1 in Repression of Eukaryotic Transcription, FASEB Summer Research Conference on Transcriptional Regulation During Cell Growth, Differentiation and Development, Snowmass/Aspen, CO, June 177-22, 2000.
*6. A Model for the Repressor/Coactivator Role for HMG-1 in Transcriptional Regulation, Cold Spring Harbor Symposium, Cancer Genetics & Tumor Suppressor Genes, Cold Spring Harbor, NY, Aug. 16-20, 2000.
7. A Model for the Role of HMG-1 in the Regulation of Eukaryotic Transcription, Department of Biological Sciences, University of Toledo, Toledo, OH., Sept., 29, 2000.
8. The Interaction of HMGB-1 with the TBP/TATA Complex, with D. Das, Symposium on Mechanisms of Transcriptional Regulation, Department of Biochemistry, Michigan State University, East Lansing, MI, June 16, 2001.
9. The Competition Between HMGB-1 and TFIIA for the TBP/TATA Complex, with A. Dasgupta, Symposium on Mechanisms of Transcriptional Regulation, Department of Biochemistry, Michigan State University, East Lansing, MI, June 16, 2001.
10. Understanding the Dual Repressor/Coactivator Role of HMGB-1 in the Regulation of Eukaryotic Transcription, Department of Biochemistry & Molecular Biology, Colorado State University, Fort Collins, CO, July 6, 2001.
11. The HMGB-1/TBP/TATA Complex and Its Implications in Transcriptional Control, FASEB Summer Conference on Chromatin and Transcription, Snow Mass, CO July 7-12, 2001.
12. HMGB-1/TBP/TATA Complex and Its Implications in Transcriptional Control, Cold Spring Harbor Symposium on Mechanism of Eukaryotic transcription, Aug. 29-Sept. 2, 2001..
13. Coactivator HMGB1 Enhances Estrogen Receptor Binding Affinity and Facilitates Reduced Binding Specificity at Estrogen response Element Half-Sites, Cold Spring Harbor Laboratory Symposium on Mechanisms of Eukaryotic Transcription, Aug. 27-31, 2003.

14. Coactivator HMGB1 Enhances Estrogen Receptor Binding Affinity and Facilitates Reduced Binding Specificity at Estrogen Response Element Half-Sites, First Annual Great Lakes Nuclear Receptor Conference, Medical College of Ohio at Toledo, Nov. 15, 2003.
15. HMGB1 Proteins Facilitate Strong Estrogen Receptor Binding to Classical and Half-sites Estrogen Response Elements and Relax Binding Selectivity, Keystone Symposium on Nuclear Hormone Receptors, Keystone, CO, Feb 28-Mar. 3, 2004.
16/17. Case Western University Symposium on Cancer Frontiers, Cleveland, OH, May, 2005
    1. The Influence of Spacer Size on the Binding Specificity of Estrogen Receptor on Consensus Estrogen Response Elements, with R. Ghattinimini
    2. Exposing the Promiscuous Binding Specificity of the Glucocorticoid Receptor, with N. Agarwal.
18. Cold Spring Harbor Symposium, Mechanisms for the Regulation of Eukaryotic Transcription, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, Aug. 29-Sept 2, 2005. The Plasticity in the Binding Specificity of Estrogen Receptor and the Influence of HMGB1, with D. Das.
19. Keystone Symposium on Nuclear Hormone Receptors, Taos, NM, Mar. 20-24, 2007. The Influence of Spacer Size on the Binding Specificity of Estrogen Receptor on Consensus Estrogen Response Elements, with R. Ghattinimini
20. Cold Spring Harbor Symposium, Mechanisms for the Regulation of Eukaryotic Transcription, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, Nov. 2-5, 2006. The Plasticity in the Estrogen Receptor Binding Specificity and the Influence of HMGB1.
21-23. Pennsylvania Summer 26th Symposium on "Chromatin and Epigenetic Regulation of Eukaryotic Transcription", June 19-22, 2007.
    1. Invited Presentation. HMGB1 Facilitates Estrogen Receptor Binding to Consensus and Half-site Estrogen Response Elements in Rotational Phased Nucleosomes, W. M. Scovell
    2. The Interaction of HMGB1 with Nucleosomes and Its Influence on Estrogen Receptor Binding to the Estrogen Response Element in Nucleosomal DNA, with Y. Sarpong.
    3. A Preliminary Look at the Influence of HMGB1 on Transcriptional Activation Driven by a Series of Estrogen Response Elements, with S. Joshi.

Honors & Awards

NIH Study Group for AREA Grant Proposals, 2005, 2007.
Distinguished Alumni Citation, Lebanon Valley College, 1985; Invited Speaker in international conferences: Tokyo Seminar on Macromolecular-Metal Complexes, U. Tokyo, July 14, 1987; Local Changes in DNA Structure and Their Biological Implications, Brno, Czech, July 6-9,1988, sponsored by Czech Acad. Sciences; 14th Intl. Congress of Biochemistry, Prague, Czech.
Professional Fellow at the Research Science Institute at Center for Excellence in education, George Washington U, Wash. DC, June 2-27, 1980.

American Chemical Society (ACS) Positions

Editor, Concepts in Biochemistry Features, J. Chem. Educ., (1986-present); Chair, Task Force for Biochemistry, Div. Chem. Ed of ACS, 1998-2000.
Chair of ACS committee to write national ACS exam in Biochemistry for ACS Div. Chem. Educ, 1999-2002.

Former Research Students (1974-)

1. **Timothy O'Connor** – MS, BGSU; **Professor of Biology**, City of Hope Medical Center, Duarte, CA.
2. **Christopher Johnson** – BS, BGSU**;** Chemical Engineer
3. **Ronald Reoach –** BS, BGSU**;** M., BGSU; Quality Control Manager, Dow Chemical Co.
4. **Mohammed El-Etri –** MS, BGSU; Research Associate, Cell Biology, U. Cincinnati Medical School
5. **Bahne Cornelius – Professor**, Chemistry Department, Michigan Technical College; Post-doctoral Fellow in my lab, 1975
6. **Vincent Capponi** – BS, BGSU; MS, BGSU; Director of Operation, Pharmacia Deltec
7. **Sue Miller –** BS, BGSU**;** Ph. D. Biochemistry, Molecular & Cellular Biology, University of Oregon
8. **Frank Collart –** BS, BGSU**;** MS, BGSU;, Ph.D. Medical College of Ohio at Toledo; **Senior Scientist**, Gene Expression in Carcinogenesis Group, Argonne Natl. Labs, Argonne, IL
9. **Becky Vonicus –** BS, BGSU; Ph. D. Biochemistry, University of Maryland

10. **Joe Knezetic** – BS, BGSU; Ph. D., U. Cincinnati School of Medicine; Post-Doctoral Fellow, NIH, Gary Felsenfeld; **Associate Professor**, Biochemistry, Creighton University School of Medicine, Omaha, NE
11. **Lee Kroos –** BS, BGSU; Ph. D., Stanford U. School of Medicine; Helen B. Hayes Whitney Fellow, Harvard U**; Professor**, Department of Biochemistry, Michigan State University, East Lansing, MI
12. **Venitha Veraserian –** MS, BGSU; Research Associate, Oil Co. in Texas
13. **Tedd Hupp –** BS, BGSU; Ph. D. Michigan State University; **Received the British Association Cancer Research/Zeneca Young Investigator of the Year Award for 1998**; **Lecturer**, Cellular & Molecular Pathology, U. of Dundee, Ninewells Hospital and Medical School, Dundee DD 19SY, Scotland, UK
14. **Jeff Hayes –** BS, MS, BGSU; Ph.D. Johns Hopkins University; Post Doctoral Fellow, NIH, **Professor**, Department of Biochemistry & Biophysics, U. Rochester School of Medicine, Rochester, NY
15. **Scott Schlemmer –**BS, BGSU**;** Ph. D., Pharmacology, Cornell Medical School/Memorial Sloan Kettering Cancer Center; Research Associate, Lineberger Cancer Center, UNC, Chapel Hill, NC
16. **Steve Leidich –** BS, BGSU; Ph. D., Biochemistry, U. Illinois; **Assistant Professor**, Cayahoga Community College, Cleveland, OH
17. **Derrick Jacobs –** BS, BGSU**;** M.D., U. Pennsylvania School of Medicine
18. **Adam Schwarz –** BS, BGSU; M. S. BGSU; M.D., Case Western University School of Medicine
19. **Douglas Long –** M. D., Medical College of Ohio at Toledo.
20. **Jenny Schroeder –** BS, BGSU; M. D., U. Wisconsin School of Medicine
21. **Scott Valentine –** BS, BGSU; Ph. D., Chemistry, UCLA
22. **Maria Pena –** BS, BGSU; MS & Ph.D., BGSU; Post-doctoral Research Fellow, University of Michigan School of Medicine; **Associate Professor**, University of Charleston, Charleston, SC.
23. **Shawn Lucas –** BS, BGSU**;** M. D., U. Cincinnati Medical School; Anesthesiologist, California
24. **Petr Pavlik –** MS, BGSU**;** returned to Prague, Czech Republik, Foreign Services
25. **Li Fan –** MS, BGSU; Ph.D., Biochemistry, Michigan State U.
26. **Marci Glavic –** BS, BGSU**,** Ph. D., Molecular Biology, U. California, San Diego
27. **Vince Pallotta –** BS, BGSU**;** Ph.D., Biochemistry, The Ohio State University
28. **Tom Kowski –** BS, BGSU**;** MS, BGSU; Associate Scientist, Immunex Pharmaceuticals, Seattle, WA.
29. **Devin Kothari –** BS, BGSU; M. D., The Ohio State University School of Medicine, 1999.
30. **Adam Kennah –** BS. BGSU**;** M. D., U. Pittsburgh School of Medicine, 1999.
31. **Wei Lu –** MS, BGSU**;** Ph. D., Biochemistry & Molecular Biology, The Johns Hopkins School of Medicine; post doc, Harvard U. School of Medicine; Senior Scientist, Merck.
32. **Shiranthi Keppetipola –** MS, BGSU**;** Ph. D. candidate, Biological Sciences, BGSU
33. **Nadege Mix –** BS, BGSU**;** Ph. D., Purdue University; Research Nutritionist, Kraft Foods.
34. **Wasantha Ranatunga** – MS, BGSU**;** Ph. D., Chemistry, University of Toledo; Post doc, Salk Institute
35. **Dave Roberts –** BS, BGSU, Goldwater Scholar**;** Ph. D. Biochemistry, UNC; Post Doc, UNC
36. **Jennifer Huntley-Aurandt –** BS, BGSU; Ph. D., Biological Sciences, U. Michigan School of Medicine, Ann Arbor, MI; **Assistant Professor** ,Chemistry & Biochemistry, Kettering University, Flint, MI
37. **Ryan Ceiply** – BS, BGSU; M. D. program, U. Toledo School of Medicine, 2003-.
38. **Kapila Navaratne**, MS, BGSU, Associate Research Scientist, Cleveland, Clinic, Cleveland, OH
39. **Dweepanita Das** - MS. & Ph. D., BGSU; Post-doc, U. Michigan School of Medicine, 2004-.
40. **Saad El Marzouk**, MS, BGSU, 2005; Ph. D. candidate, U. Illinois, Champagne-Urbana. IL, 2006-
41. **Erika Scheufler** -BS, BGSU; MS, BGSU, 2003, Advisor, College of Health & Human Services, BGSU.
42. **Atreyi Dasgupta**-Ph. D., Biological Sciences, BGSU, 2004; Post doc, Cleveland Clinic, Cleveland, OH.
43. **Nitin Agarwal** - MS, 2006, BGSU, Ph. D., U. Wisconsin School of Medicine, Milwaukee, WI, 2006-
44. **Depack Dash**, MS, BGSU, 2004, Research Assistant, Biotech Co, California.
45. **Sudath Dhanayaka**, MS, 2006; Resch Tech, U Texas Southwestern Medical Center, Dallas, TX
46. **Richard Housman**, BS, BGSU, 2006; MS, Chemical Engineering, U. Toledo, Toledo, OH.
47. **Jacintha Odafe,** BS, BGSU; M. D., Wright State U. School of Medicine, (4 year scholarship), 2006-
48. **Ramesh Ghattamanini**, M. S., BGSU, 2005; Research Assistant, Duke U. School of Medicine, 2006-
49. **Yaw Sarpong**, MS, BGSU, 2006, currently Ph.D. candidate, Biological Sciences, BGSU, 2007-
50. **Sachandra Joshi**, currently Ph. D candidate, Biological Sciences, BGSU, 2007-

About 50% of these research students received their BS degrees at BGSU

Ronald Clifford Peterson **Professor of Chemistry, Ohio Northern University**

Education: B.S., Chemistry, 1973, Northern Illinois University, Dekalb, Illinois
Ph.D., Biochemistry, 1977, University of Chicago, Chicago, Illinois
Ph.D. Advisor: John H. Law

Positions: Ohio Northern University, Ada, OH
Professor of Biochemistry 9/1997 - present
Associate Professor of Biochemistry 9/1990 - 8/1997
Assistant Professor of Biochemistry 9/1987 - 8/1990
Uniformed Services University of the Health Sciences, Bethesda, MD
Assistant Professor of Biochemistry 9/1980 - 6/1987
University of Texas Southwestern Medical Center, Dallas, TX
Visiting Professor of Biochemistry (sabbatical leave) 9/2006-720/07
Bowling Green State University, Bowling Green, OH
Summers 1996-1999, 2001-2005
Carnegie Institution of Washington, Post-doctoral Research Fellow
With Donald D. Brown, 9/1977 - 9/1980

Publications:

Das, D., Peterson, R.C., and Scovell, W.M., (2004) "HMGB Proteins Facilitate Strong Estrogen Receptor Binding to Classical and Half-site Estrogen Response Elements and Relax Binding Selectivity," Mol. Endocrinol., 18, 2616-2632.

Lu, W., Peterson, R., Dasgupta, A., and Scovell, W.M., (2000) "Influence of HMG-1 and Adenovirus Oncoprotein E1A on Early Stages of Transcriptional Preinitiation Complex Assembly," J. Biol. Chem., 275, 35006-35012.

Wood, L., Hatzenbuhler, N., Peterson, R., and Vogeli, G. (1991) "Isolation of a mouse genomic clone containing four $tRNA^{cys}$-encoding genes," Gene, 98, 249-252.

Jacobo-Molina, A., Peterson, R., Yang, D.C.H. (1989) "cDNA Sequence, Predicted Primary Structure, and Evolving Amphipathic Helix of Human Aspartyl tRNA Synthetase," J. Biol. Chem., 264, 16608-16612.

Chang, L.M.S., Rafter, E., Rusquet-Valerius, R., Peterson, R.C., White, S.T. and Bollum, F.J., (1988) "Expression and Processing of Recombinant Human Terminal Transferase in the Baculovirus System," J. Biol. Chem., 263, 12509-12513.

Peterson, R.C., (1988) "Prediction of the Frequencies of Restriction Endonuclease Recognition Sequences Using Di- and Mononucleotide Frequencies," BioTechniques, 6, 34-40.

Peterson, R. C., (1987) "Sequence and Transcription of the $tRNA^{val}$ Gene from Xenopus laevis," Biochem. Biophys. Acta, 908, 81-91.

Isobe, J., Heubner, K., Erikson, J., Peterson R.C., Bollum, F.J., Chang, L.M.S. and Croce, C.U., (1985) "Chromosome Localization of the Gene for Human Terminal Deoxynucleotidyltransferase to Region 10q23-q25," Proc. Natl. Acad. Sci. USA, 82, 5836-5840.

Peterson, R.C., (1985) "Purification and Properties of the Juvenile Hormone Carrier Protein from the Hemolymph of Manduca sexta," Meth. Enzymol., 111, 482-487.

Peterson, R.C., Cheung, L.C., Mattaliano, R.J., White, S.T., Chang, L.M.S. and Bollum, F.J., (1985) "Expression of Human Terminal Deoxynucleotidyl Transferase in Escherichia coli," J. Biol. Chem., 260, 10495-10502.

Peterson, R.C., Cheung, L.C., Mattaliano, R.J., Chang, L.M.S. and Bollum, F.J., (1984) "Molecular Cloning of Human Terminal Deoxynucleotidyltransferase," Proc. Natl. Acad. Sci. USA, 81, 4363-4367.

Peterson, R.C., Dunn, P.E., Seballos, H.L., Barbeau, B.K., Keim, P.S., Riley, C.T., Heinrikson, R.L. and Law, J.H., (1982) "Juvenile Hormone Carrier Protein of Manduca sexta Haemolymph. Improved Purification Procedure; Protein Modification Studies and Sequence of the Amino Terminus of the Protein," Insect Biochem., 12, 643-650.

Peterson, R.C., Doering, J.L. and Brown, D.D., (1980) "The Characterization of Two Xenopus Somatic 5S DNAs and One Minor Oocyte-Specific 5S DNA," Cell, 20, 131-141.

Brown, D.D., Korn, L.J., Birkenmeier, E., Peterson, R.C. and Sakonju, S., (1979) "The in vitro Transcription of

Xenopus 5S DNA" in Eukaryotic Gene Regulation, Axel, R., Maniatis, T. and Fox, C.F., eds., Academic Press, New York, pp. 511-519.
Peterson, R. C., Reich. M.F., Dunn, P.E., Law, J.H. and Katzenellenbogen, J.A., (1977) "Binding Specificity of the Juvenile Hormone Carrier Protein from the Hemolymph of the Tobacco Hornworm Manduca sexta Johannson," Biochemistry 16, 2305-2311.
Kramer, K.J., Dunn, P.E., Peterson, R.C., Seballos, H.L., Sanburg, L.L. and Law, J.H., (1976) "Purification and Characterization of the Carrier Protein for Juvenile Hormone from the Hemolymph of Tobacco Hornworm, Manduca Sexta Johannson," J. Biol. Chem. 251, 4979-4985.
Kramer, K.J., Dunn, P.E., Peterson, R.C. and Law, J.H., (1976) "Interaction of Juvenile Hormone with Binding Protein in Insect Hemolymph" in The Juvenile Hormones, Gilbert, L.I., ed., Plenum Press, New York, p.327.

Patents:

Bollum, Frederick J., Chang, Lucy M.S., and Peterson, Ronald C., "Recombinant DNA Molecules for Producing Terminal Transferase-like Polypeptides" U. S. Patent #5,037,756, issued August 6, 1991, licensed to Fermentas UAB and Invitrogen Corp.

Professional Societies:

American Chemical Society, Officer of the Northwest Central Ohio Section, 1989 - 2005
American Association for the Advancement of Science
Council on Undergraduate Research

Honors:

Biggs Chair in Chemistry, Ohio Northern University, 1992-93, 2001-02
Helen Hay Whitney Post-doctoral Fellowship, 1977-80

PHS 398 Cover Page Supplement

OMB Number: 0925-0001
Expiration Date: 9/30/2007

1. Project Director / Principal Investigator (PD/PI)

Prefix:
* First Name: William
Middle Name:
* Last Name: Scovell
Suffix:

* New Investigator? ● No ○ Yes

Degrees: Ph.D.

2. Human Subjects

Clinical Trial? ○ No ○ Yes

* Agency-Defined Phase III Clinical Trial? ○ No ○ Yes

3. Applicant Organization Contact

Person to be contacted on matters involving this application

Prefix:
* First Name: Cynthia
Middle Name: A
* Last Name: Price
Suffix:

* Phone Number: 419 372-2481
Fax Number:
Email: spar@bgsu.edu

* Title: Assoc.VP for Research Admin.

* Street1: 106 University Hall
Street2: Sponsored Programs & Research
* City: Bowling Green
County:
* State: OH: Ohio
Province:
* Country: USA:
* Zip / Postal Code: 43403

PHS 398 Cover Page Supplement

OMB Number: 0925-0001
Expiration Date: 9/30/2007

4. Human Embryonic Stem Cells

* Does the proposed project involve human embryonic stem cells? ●No ❍Yes

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://stemcells.nih.gov/registry/index.asp . Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the registry will be used:

Cell Line(s): ❑ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

PHS 398 Modular Budget, Periods 1 and 2

OMB Number: 0925-0001
Expiration Date: 9/30/2007

Budget Period: 1

Start Date: 07/01/2008 End Date: 06/30/2011

A. Direct Costs

	Funds Requested ($)
* Direct Cost less Consortium F&A	150,000.00
Consortium F&A	
* Total Direct Costs	150,000.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	MTDC	39.00	140,500.00	54,795.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number) DHHS: Henry Williams: 214 767-3261

Indirect Cost Rate Agreement Date 02/23/2004

Total Indirect Costs 54,795.00

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($) 204,795.00

Budget Period: 2

Start Date: End Date:

A. Direct Costs

	Funds Requested ($)
* Direct Cost less Consortium F&A	
Consortium F&A	
* Total Direct Costs	

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.				
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number)

Indirect Cost Rate Agreement Date

Total Indirect Costs

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($)

PHS 398 Modular Budget, Periods 3 and 4

OMB Number: 0925-0001
Expiration Date: 9/30/2007

Budget Period: 3

Start Date: End Date:

A. Direct Costs

Funds Requested ($)

* Direct Cost less Consortium F&A
Consortium F&A
* Total Direct Costs

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.				
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number)

Indirect Cost Rate Agreement Date

Total Indirect Costs

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($)

Budget Period: 4

Start Date: End Date:

A. Direct Costs

Funds Requested ($)

* Direct Cost less Consortium F&A
Consortium F&A
* Total Direct Costs

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.				
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number)

Indirect Cost Rate Agreement Date

Total Indirect Costs

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($)

PHS 398 Modular Budget, Period 5 and Cumulative

OMB Number: 0925-0001
Expiration Date: 9/30/2007

Budget Period: 5

Start Date: | End Date:

A. Direct Costs

	Funds Requested ($)
* Direct Cost less Consortium F&A	
Consortium F&A	
* Total Direct Costs	

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.				
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number)

Indirect Cost Rate Agreement Date | Total Indirect Costs

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($)

Cumulative Budget Information

1. Total Costs, Entire Project Period

* Section A, Total Direct Cost less Consortium F&A for Entire Project Period	$ 150,000.00
Section A, Total Consortium F&A for Entire Project Period	$
* Section A, Total Direct Costs for Entire Project Period	$ 150,000.00
* Section B, Total Indirect Costs for Entire Project Period	$ 54,795.00
* Section C, Total Direct and Indirect Costs (A+B) for Entire Project Period	$ 204,795.00

2. Budget Justifications

Personnel Justification	3370-PersonnelJustification.pdf
Consortium Justification	
Additional Narrative Justification	

Attachments

PersonnelJustification_attDataGroup0

File Name	Mime Type
3370-PersonnelJustification.pdf	application/pdf

ConsortiumJustification_attDataGroup0

File Name	Mime Type

AdditionalNarrativeJustification_attDataGroup0

File Name	Mime Type

Personnel Justification

William Scovell, PI (1.35 months AY all years; 0.90 months Summer Y1, 1.80 months Summer Y2 & Y3). My main role will be coordinate activities in the lab, carefully and critically design and evaluate experimental approaches, interpret the findings and help students develop a deeper understanding and critical judgment in the evaluation of data and how the data in hand leads to the logical next set of experiments.

1 Graduate Research Assistant, TBN. (1.50 months Summer, all years) The GA will work on one or more of the AIMS outlined in the proposal.

1 Undergraduate Research Assistant, TBN. (1.50 months Summer, all years) This person will take part in the research, working closely with a graduate (Ph. D.) student on one or more AIMS outlined in the proposal. The initial project will be to carryout EMSA analyses, which will subsequently lead them into more involved experiments.

Dr. Ronald Peterson, Consultant (as needed: 0.0 person-months). Ron is a highly trained, well-organized biochemist/molecular biologist who has contributed greatly to the progress in our studies through his ability to design definitive experimental approaches, his laboratory skills and his contributions in interpreting data. He and I are like-minded in that we emphasize and demand that the students have an essential contribution into all aspects of preparation for the lab experiment, carrying out the lab manipulations, and evaluation of the data. His ever-present summer activities in the lab add enormously to the development of the students during the laboratory experience. I know that his day-in and day-out presence has a lasting impression on all the students as they develop into critically thinking scientists. Ron has been a coauthor on two major papers in the last 6 years (J. Biol. Chem., 2001; Mol. Endocrinol, 2004). In addition, he just returned from a one-year sabbatical leave at the University of Texas Southwestern Medical Center where he worked in the lab of Dr. Steven McKnight, an internationally recognized leader in the area of regulation of gene expression.

OMB Number: 0925-0001
Expiration Date: 9/30/2007

PHS 398 Research Plan

1. Application Type:

From SF 424 (R&R) Cover Page and PHS398 Checklist. The responses provided on these pages, regarding the type of application being submitted, are repeated for your reference, as you attach the appropriate sections of the research plan.

*Type of Application:

❍New ❍ Resubmission ● Renewal ❍ Continuation ❍ Revision

2. Research Plan Attachments:

Please attach applicable sections of the research plan, below.

1. Introduction to Application (for RESUBMISSION or REVISION only)	
2. Specific Aims	4920-SpecificAims.pdf
3. Background and Significance	3140-Background.pdf
4. Preliminary Studies / Progress Report	6233-PreliminaryFindings.pdf
5. Research Design and Methods	7724-ResearchDesign_Methods.pdf
6. Inclusion Enrollment Report	
7. Progress Report Publication List	

Human Subjects Sections

Attachments 8-11 apply only when you have answered "yes" to the question "are human subjects involved" on the R&R Other Project Information Form. In this case, attachments 8-11 may be required, and you are encouraged to consult the Application guide instructions and/or the specific Funding Opportunity Announcement to determine which sections must be submitted with this application.

8. Protection of Human Subjects	
9. Inclusion of Women and Minorities	
10. Targeted/Planned Enrollment Table	
11. Inclusion of Children	

Other Research Plan Sections

12. Vertebrate Animals	
13. Select Agent Research	
14. Multiple PI Leadership	
15. Consortium/Contractual Arrangements	
16. Letters of Support	2561-SupportLetters.pdf
17. Resource Sharing Plan(s)	

18. Appendix

Attachments

IntroductionToApplication_attDataGroup0

File Name	Mime Type

SpecificAims_attDataGroup0

File Name	Mime Type
4920-SpecificAims.pdf	application/pdf

BackgroundSignificance_attDataGroup0

File Name	Mime Type
3140-Background.pdf	application/pdf

ProgressReport_attDataGroup0

File Name	Mime Type
6233-PreliminaryFindings.pdf	application/pdf

ResearchDesignMethods_attDataGroup0

File Name	Mime Type
7724-ResearchDesign_Methods.pdf	application/pdf

InclusionEnrollmentReport_attDataGroup0

File Name	Mime Type

ProgressReportPublicationList_attDataGroup0

File Name	Mime Type

ProtectionOfHumanSubjects_attDataGroup0

File Name	Mime Type

InclusionOfWomenAndMinorities_attDataGroup0

File Name	Mime Type

TargetedPlannedEnrollmentTable_attDataGroup0

File Name	Mime Type

InclusionOfChildren_attDataGroup0

File Name	Mime Type

VertebrateAnimals_attDataGroup0

File Name	Mime Type

SelectAgentResearch_attDataGroup0

File Name	Mime Type

MultiplePILeadershipPlan_attDataGroup0

File Name	Mime Type

ConsortiumContractualArrangements_attDataGroup0

File Name	Mime Type

LettersOfSupport_attDataGroup0

File Name	Mime Type
2561-SupportLetters.pdf	application/pdf

ResourceSharingPlans_attDataGroup0

File Name	Mime Type

Appendix

File Name **Mime Type**

AIMS

The human genome contains more than 3600 binding sites for estrogen receptor α (ER) that control the expression of estrogen (E2)-responsive genes. They act in concert in intricate transcriptional networks to regulate a great deal of human physiology, including sexual development, proliferative activities and cellular differentiation. Our knowledge of the mechanistic details of how ER regulates these genes and drives these networks is critical to understanding not only normal cellular functioning, but also how changes in these control mechanisms lead to diseases, including the development of cancer, osteoporosis, heart disease and dementia. The four proposed AIMS will test specific hypotheses that will help us gain a deeper understanding of the role of ER and HMGB1 in the regulation of E2-responsive gene expression.

AIM1. Determine the extent to which the C-terminal extension (CTE) of ER contributes interactions, and therefore important additional binding affinity in ER complexation with estrogen response elements (EREs) and the level to which HMGB1 enhances these interactions.

AIM2. Characterize 1) the effect of HMGB1 on the binding affinity of ER on rotationally phased and translationally positioned EREs within a nucleosome; 2) the effect of HMGB1, and HMGB1 and ER in combination, on the stability of the nucleosome and 3) the effect of a human chromatin remodeling complex (CRC), SWI/SNF, on the dynamics of the nucleosome, in the presence and absence of ER, and the extent to which HMGB1 enhances or inhibits nucleosome remodeling.

AIM3. Determine if HMGB1 is physically associated (colocalization) with 1) "uncomplexed" ER prior to and after productive transcription and 2) ER at four different types of regulatory elements in E2-responsive genes in human MCF-7 human breast cancer cells. These findings will begin to reveal how inclusive or restrictive the role of HMGB1 is in transcriptional regulation. The regulatory elements include an imperfect ERE, a cERE half-site (cHERE), direct repeats (DRs) and an element that contains a zero spacer in the cERE (cERE0).

AIM4. Compare the relative transcriptional activity of a series of ER binding sites found in *in vitro* binding assays - cERE, cHEREs, DRs, & tandem EREs - in *in vivo* assays. These elements will be used to drive a transiently transfected luciferase reporter gene. The effect of HMGB1 overexpression on transcriptional activity will be determined.

Background & Significance The regulation of eukaryotic transcription involves an exquisite network of sequence-specific and conformationally sensitive interactions (1-3). At the same time, the pathways within the network must provide enormous flexibility to insure that changes in environmental conditions adjust the temporal and gene-specific expression patterns. (4, 5). The action of nuclear hormone receptors (NHR) on hormone responsive genes is considered the best current model for ligand-activated transcription. Hormones, like many global ques, stimulate programs of genes directed to numerous physiological, developmental and metabolic processes. The combinational model for gene regulation proposes that transcription factors (TF) act in partnership and use cooperative binding at regulatory sites to mediate concerted and selective activation of gene expression (6, 7). The human genome has been sequenced (8) and chromatin immunoprecipitation coupled with DNA microarrays (ChIP-chip assays) have begun to define the regulatory targets for many TFs within living cells (1, 9-14). Rapid progress has been made in defining a spectrum of the putative cis-acting elements for estrogen (E2)-responsive genes that now include an increasing number of nonconventional binding sites for ER, in addition to the conventional (im)perfect palindromic sequences (12, 13). Many of these estrogen response elements (EREs) have been mapped, together with the changes in chromatin structure that occur and correlate with the activation or repression of E2-responsive genes (12, 13, 15-18). E2-activated ER directly regulates a wide spectrum of genes, including those that encode TFs (FOXA1, GATA-3, NF-κB), hormones (oxytocin, prothymosin), proteases (cathepsin D), cell proliferation factors (cyclin D), cell survival proteins (Bcl-2, PI9) and angiogenesis proteins (VEGF) (15, 19, 39, 46).

Significance

High mobility group protein B1 (HMGB1) is an coactivator protein actively involved in the regulation of selective transcription by nuclear hormone receptors (20-29). It is overexpressed in many cancer cell types, strongly implicating it in the development of human cancers, including breast, colon, melanoma and others (30-32, reviewed in 34). Not only has HMGB1 been shown to activate or repress transcription (20-30, 35), but it has been shown to play a role in inhibiting apoptosis and in DNA repair (36, 135, 136). Estrogen receptor is a central target in the treatment of breast cancer. An understanding of the roles and activity of these gene products and how their activities are regulated is essential for future improvements in clinical treatments.

Classification of Nuclear Hormone Receptors (NHRs) The binding sites for all NHRs are bipartite elements composed of hexameric core half-site motifs. The consensus sequences form either an inverted or direct repeat, which consist of the two half-sites separated by a specific number of bps (spacer DNA) (37). The identity of the response element is determined by 1) the nucleotide sequence of the half-sites, 2) the number of bps separating them & 3) the relative orientation of the half-sites. The NHRs can be divided into three subclasses based on their binding properties (38-40). **Class I** NHRs include steroid hormone receptors (SHR) for 17-β-estradiol/estrogen (ER; NR3A1), progesterone (PR; NR3C3), glucocorticoids (GR;NR3C1), androgens (AR;NR3C4) and mineralocorticoids (MR;NR3C2) (41). These receptors bind as homodimers to a palindromic or (im)perfect palindromic sequence, with the consensus estrogen response element (cERE) being (5'-**AGGTCAnnnTGACCT**-3'), with any 3 nucleotide spacer between the half-sites (cHERE). Interestingly, all other SHRs bind to a consensus palindrome with a different half-site sequence (5'-**AGAACA**-3'). **Class II** NHRs contain the nonsteroid receptors, retinoic acid (RAR), thyroid (TR) and others. They bind as either homodimers or heterodimers [with the heteropartner being retinoid receptor X (RXR)] to direct repeats (DRs) of the same half-site as ER, but with binding specificity determined by the number of bps between the half-sites. The last class contains the **orphan receptors** (originally discovered by genomic searches, and without any known activating ligand), with the DNA binding site being an extended cHERE. In many cases, the sequence is 5'-TCA**AGGTCA**-3'. Our findings show that ER binding does not require an (im)perfect palindromic sequence, since we show that ER binds also to half-sites (cHERE), direct repeats (DR), everted repeats (EVR), widely separated inverted repeats (wsIR) and consensus palindromic EREs with spacer bps varying from 0-4, cEREn (in which n = 0-4, the number of bps in the spacer). The presence of HMGB1 enhances the binding affinity for all these ER binding sites and facilitates cooperative binding in DRs, EVR and multiple cEREs (two or more cEREs in tandem) (42; **Prelim Findings & attached manuscript).** These findings suggest that the current classification for ER is far too limiting, must be reconsidered and modified.

Characteristics of HMGB1 Protein and Its Role in Transcription HMGB1 is a highly conserved, ubiquitous protein with three structural domain. The A & B domains are highly positively charged and facilitate its nonspecific binding to DNA, while the C-domain contains a stretch of 34 acidic residues that can interact

with positively charged proteins, such as core histones (20, 43). HMGB1 binds in the minor groove of DNA, with intercalation to widen or expand this groove, leading to enormous bends toward the major groove (44-47, 106-107). The interaction of HMGB1 with DNA is notably transient and produces wide-spread flexure in the DNA (49, 108). As a result, HMGB1 is considered an "architectural" factor, which interacts with, and functions to facilitate the assembly of higher-order nucleoprotein complexes (49, 50, 55). We believe that these known fundamental properties are largely responsible for the affect that HMGB1 has on enhancing ER binding to EREs in DNA and nucleosomes (**Prelim Findings**). With respect to transcription, HMGB1 interacts with "**HMGB1-sensitive regulatory factors**" that includes steroid hormone receptors, Oct-family proteins, the tumour suppressor gene products, p53 and p73, HOX D proteins, rel proteins, viral proteins, TAFII130, TATA-binding protein (hTBP)/TATA complex, topoisomerase II and at least one chromatin remodeling complex (CHRAC) (21-30, 35, 42, 51-57). It also exhibits characteristics of a "pioneer" protein that can dominantly enter inactive chromatin to "open up" chromatin and initiate regulatory events (12, 13, 58). There is evidence that the negatively charged C-domain of HMGB1 binds to the Q-tract in the N-terminus of human TBP in the TBP/TATA complex (35), while the A &/or B domains bind DNA (23, 44, 106).

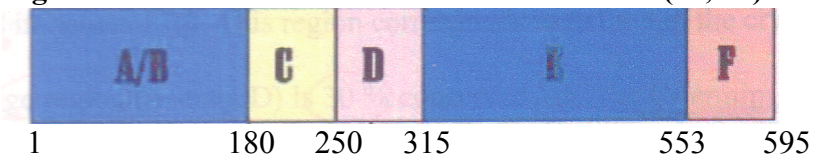
In addition to our findings with HMGB1 (**Prelim Findings),** it is interesting to note that HMGB2 (60), a protein distinct, but similar to HMGB1, required 1.2 micrograms of rER (100 uM in a 20 ul volume) to produce 80% binding on free cERE/DNA (my estimate from gel figure). This is 10^{+4} greater than found for HMGB1 (23, 42, 93-95). They also find that HMGB2 produces an additional low mobility EMSA band, besides that for the ER/ERE complex, suggesting that HMGB2 is stably bound in the ER/cERE complex. In addition, HMGB2 effect on ER binding to nucleosomes is apparently extremely weak, requiring ca. 3 ugs to produce what is indicated as a weak ER/nucleosome complex. All these findings for HMGB2 are in stark contrast to what we observe for HMGB1 in our results.

Estrogen Receptors Bind to Estrogen Response Elements (ERE) in DNA

Treatment of cells with E2 converts inactive ER to an active homodimer, $(ER)_2$, that binds an ERE in the promoters of E2-responsive genes. The ER/ERE complex is thought to trigger recruitment of (co)activators, chromatin remodeling complexes (CRCs) and enzymes that are involved in posttranslational modifications to overcome the chromatin barrier to transcription (61,6,72-82). These, and other factors, remodel chromatin and then recruit components of the preinitiation complex (PIC) to activate transcription (70, 74, 77)**.**

ER, like all steroid hormone receptors, has a modular structure, with six structural/functional domains (A-F) (38-41). The C domain is the most conserved in evolution and contains the core DNA binding domain (DBD; 70 residues, 180-250), which is the minimum required for ER binding to ERE. The ligand-binding domain (LBD), which is designated as domain E, is also highly conserved, binds E2, contains a strong dimerization domain and interacts with coactivators. The D domain (250-315) is a less conserved, flexible hinge region that binds to CRCs and also contains the C-terminal extension (CTE; 251-288). The CTE is found to be essential for stable **class II** non-steroid receptor binding to their response element, with the CTE targeting the bases and phosphates in the minor groove. Only recently has evidence suggested that the CTE may play an important role in binding with **Class I** steroid receptors (26, 63). See **AIM1.** The variable A/B domains contain a ligand-independent activation functions.

Figure 1 Schematic of the domains of ERα (26, 40) [DBD-CTE] = 180-288



Two crystal structures of the ER DBD (dimer) binding to ERE have been determined (64-66). One structure has the ERDBD dimer complexed with a cERE DNA, while the second one has the DBD binding to an imperfect ERE. In each case, each DBD monomer unit binds to nucleotides within the major groove of each ERE half-site (cHERE), with an inherent plasticity in the complexes evident by the observed changes in ERDBD/ERE interactions in the two different structures (64-66). The crystal structure for GRDBD dimer binding to the GRE3 (i. e., GRE with a 3 bp spacer) shows a similar structure, but with different interactions

that provides the basis for the different binding selectivity for GR and ER (67). However, the GRDBD (dimer) also binds to a GRE with a four bp spacer [GRE4] instead of three, demonstrating that one GRDBD monomer binds specifically to a single GRE half-site, while the other DBD binds nonspecifically to DNA as a result of the increase of one bp in the (longer) spacer (68). These studies collectively emphasize the plasticity in interactions between SHRs and various response elements and for GR, a stable interaction with only one half-site. We have shown that in the presence of HMGB1, ER binds strongly to a half-site, cHERE, (42). Edwards has presented evidence that the CTE in the SHRs may contribute significant interactions with the SHR elements (23-28)**.** In addition, the first structural data, a crystal structure for the truncated progesterone receptor that contains the CTE, complexed with its response element (PRE), the [(PRDBD-CTE)/PRE] complex, reinforced the notion that, in addition to DBD interactions in the major groove, the CTE interacts in the minor groove immediately adjacent to the PRE to add increased binding affinity in the complex (63).

ER can cooperate with a number of sequence-specific TFs, such as AP1, Sp1, c-myc and NF-kB to expand its specificity for regulatory sequences that either do not contain an ERE or contain only a half-site (7, 40). Our finding that coactivator, HMGB1, enhances the binding of ER to a spectrum of EREs serves as the only current example of a non-specific DNA binding protein that cooperates with ER to enhance its binding affinity. This finding may open up yet another avenue for regulating the ER binding, and perhaps extend to other "HMGB1-sensitve TFs" (42, **Prelim Findings**).

ER Binding to Chromatin and the Effect of Chromatin Remodeling Complexes (CRCs)

In the nucleus of the cell, DNA is packaged with an octamer of core histone proteins to form nucleosome repeating units, which are further organized into a higher-order chromatin structure. This assembly of the DNA within nucleosome subunits impedes TFs from gaining access to and binding to regulatory sequences &/or promoters, thus serving generally to repress transcription (69, 70). The ERE in the pS2 promoter in MCF-7 breast cancer cells is one of the few regulatory elements that has been mapped, with the ERE located on the edge of the nucleosome (71). It is increasing evident that eukaryotic cells possess the enzymatic machinery to selectively modify nucleosomes & play a vital role in the dynamics of chromatin in the regulation and activation of transcription (72-77). Extensive efforts are directed at characterizing ATP-dependent chromatin/nucleosome remodeling complexes (CRCs) that facilitate an avenue for TFs and other cofactors to gain access to their regulatory sequences. The three classes of enzymatic CRCs include 1) SWI/SNF, 2) ISWI and the 3) Mi-2 types, which are distinguished by the identity of their ATPase subunit (78). The SWI/SNF complex, which we will investigate, is a highly conserved CRC, originally discovered in yeast and is involved in the initiation of transcription (79). Although the ATPase subunit can remodel chromatin on its own, the subunits modulate this activity (78). This CRC binds strongly to both DNA and nucleosomes in an ATP-independent manner and appears to interact with the minor groove and, in this regard, resemble that of an HMG-box containing protein (75, 80, 81).

Although extensive studies have focused on 1) how CRCs target a specific gene promoter in a nucleosome, 2) its relative activity within different contexts and 3) the nature of the resulting nucleosomal DNA that the TFs gain access to, the mechanism(s) of action remains unknown. For SWI/SNF, current evidence suggests that its action can produce sliding of histone octamer along DNA in *cis* or dissociation of the histone octamer from the nucleosome (77). Hypotheses for SWI/SNF targeting of regulatory sequences within a nucleosome and its subsequent remodeling activity include that it 1) binds through a random, non-specific manner (prior to or after activator binding to its recognition site); 2) is recruited to the recognition site by interactions with a gene-specific activator; 3) utilizes an intrinsic DNA binding activity, as observed for HMG box protein, BAF57, which is a subunit in the human SWI/SNF complex. BAF57 contains an acidic tail (-28 charge) and a single HMG box that may bind nonspecifically to DNA. Both characteristics are remarkably similar to HMGB1 (87, 118). In addition, it was shown that BAF57 is recruited to the pS2 promoter (pS2 is a gene directly regulated by E2 in the human breast cancer cell line, MCF-7) and interacts directly with ER, both interactions being E2-dependent. BAF57 also interacts with the family of p160 coactivator proteins and potentiates transcriptional activity (82, 83). A key point is that since the HMG box protein, BAF57, mediates interactions with DNA and nucleosomes and also between ER and p160, it is conceivable that HMGB1 may either enhance remodeling and transcriptional activity, or alternatively compete with this interaction and act as an inhibitor, especially when overexpressed (118). Our recent findings on the nonenzymatic nucleosome remodeling activity of HMGB1 to facilitate ER binding to cERE within a nucleosomes suggest that comparing

the individual and combined effect of SWI/SNF and HMGB 1 would be an especially fruitful avenue to pursue. A second key point is that it is not clear if CRCs actually bind first to facilitate TF binding or alternatively, whether CRCs target promoters because TF already bind to the promoter (77). These interactions may well be context dependent (134). Our finding that HMGB1 nonenzymatically facilitates ER binding without any enzymatic CRC activity may provide a general mechanism to facilitate either route and help resolve this present conundrum since HMGB1 may act in a concerted manner to facilitate binding of both ER and possibly a CRC **(Prelim Find & AIM2)**.

ChIP assays have shown that BRG-1, the ATPase subunit of human SWI/SNF, is localized on E2-responsive promoters for cathepsin D and pS2. On the other hand, BRG-1 is not found at promoters for the RARβ, β-actin gane and a region near cathepsin D, gene which is distant from the promoter and not associated with ER (59). It is also reported that HMGB1 enhances the ability of another class of CRCs, CHRAC/ACF [at mole ratio (HMGB1/CHRAC) ~ 200], to bind to nucleosomal DNA and increase "sliding" activity (57). These findings stimulate our interest to determine if HMGB1 is colocalized with ER at E2-responsive genes **(AIM3)**.

Rotationally phased DNA within nucleosomes are prepared by incorporating DNA that contains nucleosome positional sequences (NPSs) into a nucleosome. These NPSs influence the rotational orientation of the DNA as it curves and bends around the octamer surface. This is due to the anisotropic bending properties of the DNA. We have used the NPSs, in conjunction with an ERE, to produce a nearly homogeneous population of nucleosomes in which the ERE is rotationally phased so that the major grooves of the ERE face out from the nucleosome and are in the optimum orientation for strong binding interaction with ER.

Rotationally phased nucleosomes that are used in remodeling studies exhibit a characteristic DNase I 10 bp cutting pattern, which is disrupted in nucleosomes that are remodeled by a CRC. Although universally used as a test, perhaps the classic example of this was shown in the effect of SWI/SNF on the TATA-binding protein (TBP) binding to the TATA box within nucleosomal DNA (84). Although TBP binds to TATA box in DNA with a Kd ~ 1 nM (35), it does not bind TATA within a rotationally phased nucleosomes as evidenced by a lack of a DNase I footprint (FP), with as high as 50 uM TBP, which is four-orders greater than needed to bind DNA. Additional DNase I cuts were observed and the 10 bp pattern was partially lost, however, indicating perhaps nonproductive, nonspecific interactions of TBP with the nucleosomes (84).

Incubation of SWI/SNF with the TATA-containing nucleosomal DNA produced a clear ATP-dependent disruption of the DNase I 10 bps pattern, with the new pattern looking more like that of free DNA. EMSA showed that the remodeled nucleosomes exhibited the same mobility as the untreated nucleosomes, indicating that the histones remained associated with the DNA. When TBP was added to the remodeled nucleosomes, an ATP-dependent footprint of the TATA box is produced. Thus, SWI/SNF altered nucleosomal DNA to facilitate specific binding of TBP to TATA. The TBP binding was also found to be dependent on the rotational phasing. In addition, the many DNase I cleavages outside of the footprint remained with TBP binding. Similar studies investigated the binding affinity of other TFs (including Sp1, Myc/Max, GAL4, GAL4-VP16, NF1, GR, PR, NF-κB, Amt1DBD) to nucleosomes and/or whether a CRC facilitated binding (85-92). In contrast to many TFs, GR binds comparably to GRE in DNA and in a nucleosome. It is found that the presence of GR, however, actually mediating SWI/SNF activity (88, 89, 102-104). A general summary of the relative binding affinity of TFs for free DNA and nucleosomal DNA is shown in Table 1.

Table 1 Classification of TFs According to Relative Binding Affinities to DNA and Nucleosomal DNA

	TFs	Binding Affinity Ratio - [Nucleosomal DNA/DNA]
Class A	**GR & NF-κB, Amt1DBD**	**Similar, ca. 1**
Class B	**Sp1**	**Reduced, ca. 0.1-0.05**
Class C	**TBP, NF1, Gal4-VP16 (ER & PR, our work)**	**Markedly Reduced, ca. 1/100 or smaller**

Transient transfection assays (TTA) have provided a valuable means to evaluate the relative strength of a series of promoter sequences, discern essential cofactors and reveal the effect that overexpressed cofactors have on transcriptional activity. A number of natural and artificial EREs have been examined and, in many

cases, showed a general correlation between *in vitro* binding affinities and transcriptional activities. (93-95). TTA has also been used to show that HMGB1 enhances transcriptional activities of steroid hormone receptors on a limited number of EREs (23-25, 30). Our proposed studies will expand this to many nonconventional EREs. In previous studies that used a BRG1/BRM deficient cell line, TTA have also been used to show that these catalytic SWI/SNF subunits potentiate transcriptional activation of ER and other nuclear hormone receptors (96, 97).

Genomic Approaches Although a number of genomic interrogations have cataloged consensus and imperfect ERE (15, 98), the most informative and inclusive approach has been a genomic-wide (1,500 Mb of nonrepetitive DNA) analysis to generate an unbiased examination of all actual ER and RNA pol II binding sites in an authentic breast cancer cell line, MCF-7, at 35 bp resolution using a ChIP-chip assay. In addition to defining functional EREs and their locations within the genome, it is becoming increasingly apparent that, in many cases, transcriptional activation involves a combination of cooperating TFs, with FoxA1, a pioneer protein, being the first one discovered (12, 13).

Preliminary Findings (AREA grant, Sept. 2005-July 2008)

AIM1 (D. Das, R. Ghattamaneni, S. Joshi; undergraduates - J. Odafe, N. Berger) The current model for steroid hormone receptors indicates that they bind as a homodimers to a bipartite element composed of two hexameric core half-site motifs, separated by a 3 bp spacer that is critical for dimer formation and binding affinity to the response element. For ER, the consensus ERE, cERE, is 5'AGGTCAnnnTGACCT-3', in which n is any base pair (nucleotide here) in the 3 bp spacer (39, 40).

HMGB1 is isolated and purified from calf thymus as previously outlined (42). The oligonucleotides are purchased from IDT Technologies, ^{32}P-end-labeled using [γ]-^{32}P-ATP/polynucleotide kinase, annealed to produce double stranded DNAs and separated from ATP by spin column (42). Human recombinant hormone receptors, ERα (P2187), ERβ (P2466) and PRB (P2835), expressed in baculovirus/insect cells were purchased from Invitrogen.

We believe that the findings presented here indicate that in the presence of HMGB1, an abundant, ubiquitous, nuclear architectural protein, the classification of ER and perhaps other NHRs requires a closer reevaluation.

ER binds *in vitro* to its cERE with a Kd ~ 7 nM, but binds with a reduced affinity as the sequence in one of the ERE half-sites becomes more imperfect (as one or more bps is changed). Studies have determined the extent to which ER binding was reduced as the bp at each position in an cERE half-site is changed. This led to the prediction that the greatest reduction in binding would occur if a half-site had the sequence, 5'-GTTGGC-'3 (99). The consensus half-site, cHERE, is 5'-AGGTCAnnnGTTGGC-3'. We find that HMGB1 strongly enhances ER binding to every ERE that we have tested. Although it enhances binding to cERE by 2-3x, the effect of HMGB1 on ER binding affinity becomes greater as a half-site in the ERE sequence becomes less perfect, with the greatest effect on an ERE that contains only one consensus half-site, the cHERE. The Kd for cHERE is reduced from ~90-100 nM (no HMGB1) to 15 nM in the presence of HMGB1, a 6-fold change in the presence of 400 nM HMGB1 (cf. Kd ~ 7nM for cERE) (42). **Conclude:** ER binds strongly to a cERE half-site (cHERE) in the presence of a cofactor, HMGB1. This is inconsistent with the current model.

This suggested that the spacer size (n = 3) may not be as critical for ER binding as previously thought. In all these studies, we also tested both forms of the estrogen receptor, ERα and ERβ, side-by-side, for their binding to cEREns. (in all these discussions, ER = ERα & ERβ). The resulting Kd values obtained for ER binding to this series of cEREns and cHERE is displayed in Table 2. The spacer size, n, in this series varied from n = 0-4. Both forms of ER exhibit strong binding to the normal consensus, cERE3, with binding affinity progressively decreasing as n decreases from n = 3 to n = 1. Weaker binding is also observed for n=4. Surprisingly, ER binds to cERE0 as strongly as to cERE3. cERE0 is an actual ER binding site in an E2-responsive gene (see **AIM3**) and is also a response element for thyroid hormone receptor (TR), which suggests a mechanism for cross-talk between these two nuclear hormone receptors (100). In the presence of HMGB1, the Kd values for binding to all cEREn are comparable (5-15 nM) and consistent with our other data above, in that HMGB1 enhances ER binding inversely to the Kd value observed in the absence of HMGB1. **Conclude:** These findings indicate that all cERE (n = 0-4) sites are very flexible in the presence of HMGB1 as reflected by the comparable ER binding affinities at all sites (Kd = 5-15 nM). Therefore the spacer size is not restrictive to strong ER binding. In addition, Table 2 shows there is very little difference in the binding affinities for ERα and ERβ. The Kd values have an uncertainty of 10% from 3 independent runs.

Table 2 Kd Values (nM) for ERα & ERβ Binding to cEREn (n = 0-4) and the cHERE

	ERα			ERβ		
cEREn	**HMGB1**		**Effect (x)**	**HMGB1**		**Effect (x)**
	-	+		-	+	
n = 0	10	4.5	2	10	4.4	2
1	80	15	5-6	80	16	5-6
2	25	7.3	3-4	32	8.4	4
3 (cERE)	7.4	5.1	1-2	8.4	5.2	1-2
4	25	12	2	36	14	2-3
cHERE	80	15	5-6	100	13	7-8

Using individual protease digestion profiles (trypsin, chymotrypsin, proteinase K), in addition to a series of six monoclonal antibodies binding to the ERα/cEREn complexes, we also find that the spacer size strongly influenced the global conformation of ER bound to each of the cEREn sites. Interestingly, although full length ER bound to all cEREns as a dimer, ERDBD binds as a dimer to only cERE3, while binding as a monomer to all other sites (64). This indicates that the strong dimerization function in the E domain of ER is essential for dimer binding to this series of nonconventional binding sites and that full length ER displays enormous binding flexibility. Findings at the ERDBD level therefore provide an incomplete picture of the ER interaction with EREs. **Conclude:** Although the 3 bp spacer restricts binding at the ERDBD level, it has little or no effect with the full length ER (our findings). All these findings are inconsistent with the current model. A manuscript that contains these data, conclusions and discussion is appended to the proposal (submitted to J. Steroid Biochem. Mol. Biol.).

Since ER binds strongly to cHERE in the presence of HMGB1, we investigated whether HMGB1 would also facilitate ER binding to a direct repeat (DR; -> ->) – which is essentially two cHEREs (half-sites) oriented in the same direction. A preliminary report had suggested that ER binding to a few DRs was weak (no quantitation or binding profiles were shown)(101). To our surprise, we find that ER binds strongly to one half-site in the DRs, even in the absence of HMGB1. The position of the EMSA band is consistent with ER binding as a dimer. We further show that strong binding appears to have little or no dependence on the spacer size. ER also binds strongly to everted repeats (EVR; <- ->) and inverted repeats that have large spacers (LSIR; ->…...<-). In the presence of HMGB1, ER dimers bind to both half-sites and binding to the two cHEREs is strongly cooperative. A manuscript of these findings is in preparation. Table 3 shows the complied data for ERα and ERβ binding to two half-sites positioned in six different orientations. These represent four direct repeats (DR1, DR2, DR1-26 & DR1-3) an everted repeat (EvR1), and a long inverted repeat (IR1). In all cases, one finds that the ER binding is surprisingly strong for both ER forms and the presence of 400 nM HMGB1 enhances the binding affinity. What is not apparent from the tabulation is the strongly cooperative binding that is observed in the presence of HMGB1 and the fact that two complexes are formed in the presence of HMGB 1, presumably one ER dimer binding at each half-site.

The ERE2/ERE1 at the top of the Figure 2a was used as the template from which we derived the spacing between half-sites since it is a naturally found response element that contains two tandem repeats, the vitelligenin B2 gene. (42). The (------) at which another half-site should be position contains the sequence 5'-GTTGGC-3'. In all cases, except for DR26, the spacing is the same as in ERE2/ERE1. In the case of DR26, the spacing between the DRs was increased by 5 bps to determine the effect of the distance (& rotation) change. There is no significant affect as a result of this chan e.

Table 3 Kd Values (-HMGB1) and K50 Values (+HMGB1) For Two ERE Half-sites in Different Orientations

	ERα	ERβ	ERα	ERβ	
	(- HMGB1)		(+HMGB1)		
DNAs	Kd values		K50 Values		In the absence of HMGB1, one ER
DR1	7.0	8.0	4.2	7.0	dimer binds to one cHERE and Kd
EvR1	7.2	8.5	7.0	7.5	values are calculated.
IR1	12.5	16.5	4.0	9.0	In the presence of HMGB1, an ER
DR2	4.0	9.6	3.0	8.0	dimer binds to each of the two cHEREs
DR1-26	13	23	4.5	10.	The K50 = [ER] at which 50% of the
DR1-3	11	20	4.8	4.0	DNA is complexed with ER.

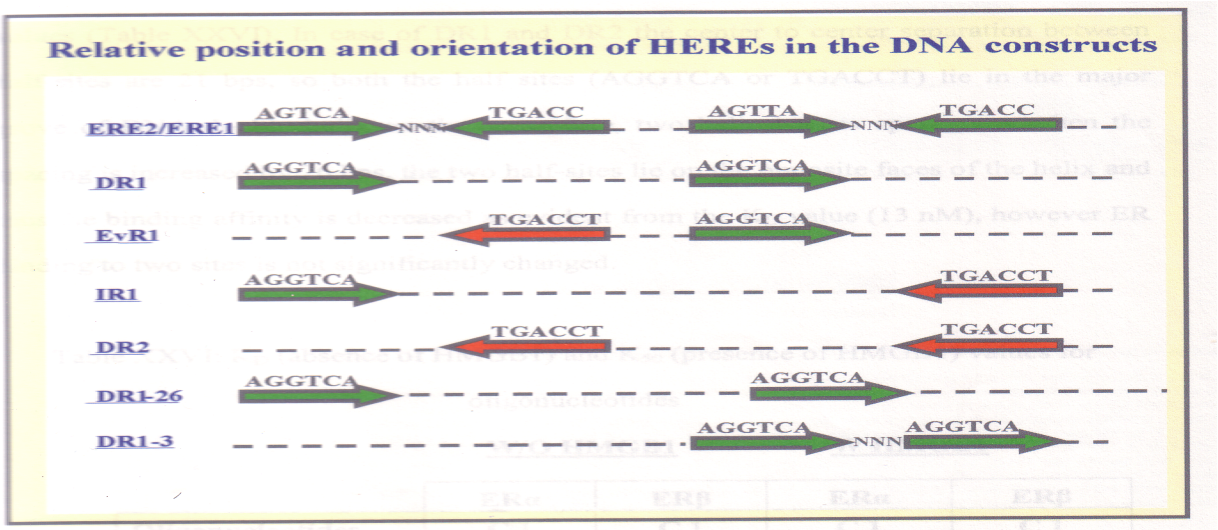


Figure 2a. Schematic drawing of the relative orientations of cHERE in DNAs studied

Figure 2b shows a representative EMSA profile for ERα binding to DR1, with the accompanying figure to its right side showing the percent species of complex 1 (C1) and complex 2 (C2) as a function of ERα concentration, in the presence and absence of HMGB1. In the absence of HMGB 1, only one complex, C1, is progressively formed as levels of ER increase. Virtually all complex exists at and above 11 nM ER. In the presence of 400 nM HMGB1, two complexes are formed, with C2 being the predominant complex at ER levels at and above 11 nM. Essentially all DNA is in the form of a complex at 6 nM ER.

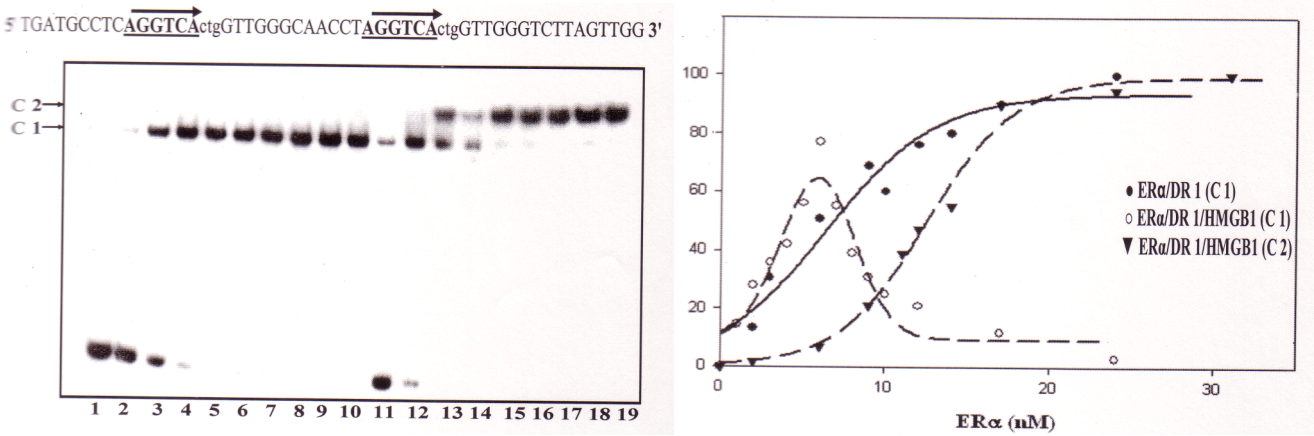


Figure 2b. EMSA binding profile for ERα binding to DR1 and percent species (complexes) formed. Lanes 1 contains DR1 DNA only. Lanes 2-10 and 11-19 have increasing levels of ER, from 2, 6, 11, 17 24, 31, 38, 45 and 52 nM ER. Lanes 11-19 also contain 400 nM HMGB1. Note the second complex (C2) forming in lanes 12-19 and the sharp binding cooperativity.

Conclude: This collection of findings reveals two points. 1) ER binds strongly to a very broad range of nonconventional sites, clearly indicating that the current paradigm for ER binding is far too restrictive, especially in the presence of a ubiquitous transcriptional coactivator, HMGB1. 2) These findings suggest that HMGB1 may play an important role in E2-responsive gene regulation.

AIM2 (Y. Sarpong, R. Peterson; undergraduates - D. Taylor, L. Bisachi) DNA is complexed in nucleosomes within the nucleus of the cell and this provides a layer of transcriptional repression such that the binding sites for many transcription factors become much less accessible than in free DNA. We have started to examine the effect of HMGB1 on the binding affinity ER at EREs that are rotationally phased and translationally positioned such that the major groove of the ERE is facing out from the nucleosomes to provide the optimum orientation

for strong binding. Our results led us to the important question of how HMGB1 facilitates ER binding to nucleosomes via a nonenzymatic manner. We also wanted to compare our ER studies with similar studies for GR binding to GRE (89, 102-104) and to PR binding to PRE (PR and GR have the same response element, GRE=PRE) (127). These studies, and ours, incorporate four-20 bp nucleosome positioning sequences [NPS = (5'-TCGGTGTTAGAGCCTGTAAC-3')] in the DNA with a single hormone receptor response element (XRE; X= E, P). We prepared our 161 bp DNAs using a modified procedure developed by Wrange (89). The pGEM-Q2 plasmid, obtained from Wrange was cut at its unique AvaI site (CPyCGPuG). Because of the recognition site redundancy, the 20 bp inserts (NPS or EREs) can be prepared with asymmetric ends and can be directionally inserted. As a result, a series of plasmids can be directly prepared (one cloning step, instead of multiple steps as done in the Wrange procedure) so that any ERE (cHERE, multiple sites, etc) can be inserted readily at any 20 bp interval. Using this new procedure, we initially constructed five DNA fragments, two with the cERE (one with cERE at the dyad axis & the other at 40 bps from the dyad) and two similar DNAs containing cPRE. In addition, a nonbinding control DNA (called A5) contains five NPSs, but without an ERE, was constructed. These were used to determine 1) how strongly ER (PR) binds to an "outward phased" ERE (PRE) 2) if binding was influenced by the position of the cERE within the nucleosome and 3) if HMGB1 influenced the binding affinity within the nucleosome. The 161 bp DNA is cut out of the plasmid by double digestion with restriction enzymes, EcoRI & Hind III, purified and then radiolabeled on either both strands (Kd detn.) or on individual strands for obtaining a DNase I 10 bps pattern (indicator of rotational phasing) and/or DNaseI FTs (indicator of DNA protection by ER). For the latter labeling, one end of the plasmid is cut with Eco RI, dephosphorylated, the DNA purified and then ^{32}P-end labeled. Excess ATP is eliminated by two ethanol precipitation steps and the use of a spin column. The DNA is cut with Hind III, run on a gel and the 161 bp band excised and the DNA purified. Labeling the alternate DNA strand is prepared in the same manner, but the order of the restriction enzyme digestions is reversed. Radiolabeled nucleosomal DNA is then prepared by histone octamer transfer. Oligonucleosomes (10-30 mers) are first produced by controlled micrococcal nuclease digestion of calf thymus nuclei and then depletion of H1 histone by Sephadex chromatography in 0.65 M salt buffer. These oligonucleosomes are the source of the core histone proteins for the mononucleosome. Oligonucleosomes are mixed with the 161 bp DNA in 1 M salt buffer and sequentially diluted to yield mononucleosomes in physiological salt buffer. The mononucleosomes are fractionated from free DNA by centrifugation in a 5-30% sucrose gradient. The fractions were monitored by running aliquots on gels to insure that free DNA and nucleosomal DNA were clearly separated. The nucleosomes were stored at -20^{o}C. ER (& PR with PRE in nucleosome) was reacted with the nucleosomes at 25^{o} C to obtain Kd values and a DNase I 10 bp repeat pattern, with the latter pattern being a confirmatory test for a homogeneous population of rotationally phased nucleosomes (84). Figure 3 shows the DNase I 10 bp pattern for ERE within a nucleosome, in the absence and presence of HMGB1. A clear 10 bp pattern is apparent under both conditions and therefore indicates that at these HMGB1 levels, in the absence of ER, does not disrupt the rotational phasing within the nucleosome. This may indicate that the combined interactions of ER and HMGB1 are required to disrupt the nucleosome.

We find that ER (& PR) exhibits no detectable binding to nucleosomal cERE (PRE) at the dyad or 40 bps from the dyad, with [ER] (or [PR]) as high as 200 nM. We estimate both Kd values ~300 nM. This indicates that both receptors have extreme difficulty gaining access to their DNA binding partners, even when the major groove is phased in an optimum (outward) manner. Binding is not simply a "docking" interaction. This suggests that the complete ER binding site may extend beyond the major groove in the cERE and part of it may be thermodynamically inaccessible, consistent with interactions outside the major groove of cERE (& possibly involving the CTE binding in the minor groove outside the cERE) being important for stable binding (**Proposed AIM1**). In the presence of HMGB1, ER binds with a Kd ~40-50 nM, while PR remains unaffected and does not bind (positive control for PR on the same gel shows that PR binds free DNA with Kd ~ 2 nM). In addition, ER does not bind to PRE and PR does not bind cERE, as expected. Both results are obtained for the XRE (X= E, P) at the dyad and 40 bp from dyad and therefore the binding result is position-independent. Therefore, HMGB1 has a distinctly different effect on ER and PR, suggesting that the nature of their binding to nucleosomal DNA is significantly different. In contrast to both ER and PR, GR binds comparably to GRE in DNA and nucleosomal DNA (89). This suggests that these three steroid hormone receptors, ER, PR and GR, certainly bind with distinctive differences at the nucleosome level and probably at the DNA level as well. In addition, we

find ER does not bind cHERE in the nucleosome up to 200 nM, but in the presence of 400 nM HMGB1, ER binds with a Kd ~60-70 nM. This is very interesting and surprising since ER binding to cHERE is not much weaker than found for the full cERE, which I quite different that found on free DNA! This may suggest that within a nucleosome, stable ER binding requires little more than a ERE half-site. One may also speculate from the data in hand that GR binding involves only 'docking" strictly interacting within the major groove of DNA, while ER and PR require additional interactions, with HMGB1 able to facilitate ER binding, while HMGB 1 is not able to accomplish this with the PR/PRE interaction.

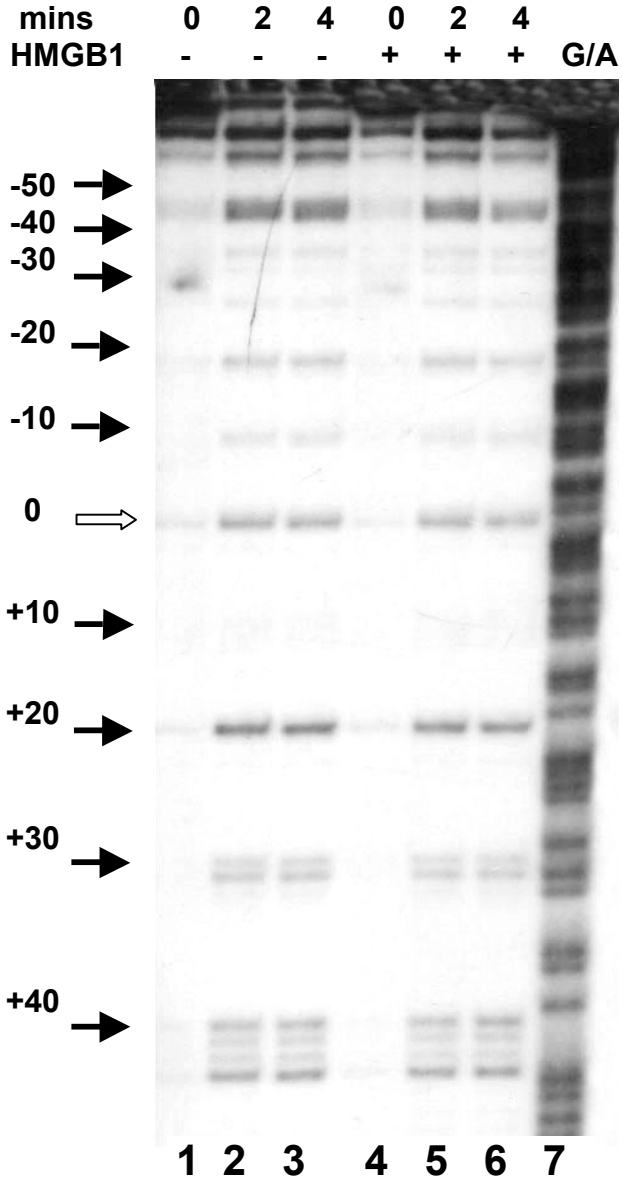


Figure 3. DNase I 10 bps pattern for cERE/nucleosome +/- HMGB1
DNase I digestion profiles on a nucleosome for 0, 2 & 4 minutes. The DNase I 10 bp pattern is observed for a nucleosome containing the 161 bp DNA, with cERE at 40 bps from the dyad axis and four 20-bp nucleosome positioning sequences. Lanes 1-3 show the cutting pattern on the nucleosomal DNA in the absence of HMGB1, while lanes 4-6 are in the presence of 400 nM HMGB1. Lane 7 is the G/A ladder for the DNA.

These data are considered preliminary, require closer scrutiny, but provide a strong basis for subsequent studies.

A relatively insensitive test (EMSA bands) to determine if HMGB1 facilitates octamer dissociation from the nucleosome showed no evidence for free, dissociated DNA as HMGB1 levels were increased to 500 nM

(**Proposed AIM2 contains a far more definitive test**). This work remains in progress and a manuscript is presently premature. However, the findings and proposed studies suggest that a deeper understanding about ER & PR binding on nucleosomes is forthcoming, together with bringing out major difference between ER, PR and GR binding characteristics.

Findings from both AIMS1/2 and previous work lead us to propose a working model for a mechanism for HMGB1 action at the nucleosome level. Nucleosomes undergo spontaneous conformational fluctuations in which stretches of DNA are transiently exposed (105). HMGB1 binds nonspecifically in the minor groove of DNA to widen it (45, 106,107) and produces enormous bending and wide-reaching flexure in the DNA (49) by its transient interactions (108). We envision that without HMGB1, ER "samples" the nucleosomal DNA, with little success in establishing a stable binding interaction. HMGB1 binds randomly to DNA to produce dynamic fluctuations in the DNA. This greatly reduces some of the structural stiffness and energetic constraints that are inherent in DNA and in DNA-histone octamer interactions within the nucleosome. HMGB1 shifts the equilibrium (DNA**wrapped** = DNAstretches **exposed**) toward DNA**exposed**. As ER "samples" nucleosomal DNA binding sites stochastically, HMGB1 interactions weaken (destabilizes) histone-DNA interactions "globally" and a more dynamic nucleosome results (somewhat like acetylation of histones that reduces DNA-histone interaction locally and enhances TF binding in nucleosomes (109,110). The flexure also helps in the transient realignment of the bps for stronger ER binding interactions in both DNA and nucleosomal DNA. In addition, any HMGB1 interaction in the minor groove in the vicinity of ERE will "pry open" the minor groove and could facilitate a much more favorable thermodynamically avenue for the CTE to gain access to and bind in the minor groove, therefore facilitating increased further ER binding affinity. **Conclude**: HMGB1 appears to have an enormous effect on ER binding to EREs in DNA and nucleosomes in expanding its specificity and facilitating strong binding to nonconventional EREs. If the working model and mechanism of action is not significantly modified on further study, HMGB 1 would be expected to facilitate binding site access to a very wide spectrum of TFs and be of much wider utility and significance in remodeling nucleosomes than these current findings indicate. From the current data, the three steroid hormone receptors appear to have very different requirements in their selective binding interactions. PR would appear to require much more extensive interactions with DNA than even ER does, that HMGB1 at these levels cannot provide.

AIM3 (S. Joshi) It is important to determine if the spectrum of *in vitro* EMSA binding sites translates into functional sites for E2-responsive transcription in cells. Transient transfection studies with reporter gene assays were initiated. U2-OS cells (ER^-) were obtained from A. Nardulli and maintained in culture according to her protocol (111). After some initial experiments, we changed from our original proposal of using a CAT reporter gene to using a more sensitive luciferase reporter. We have preliminary results using a plasmid (pGL2-TATA-Inr-Luc; from D. McDonald, Duke U.Med.) that contains 3cEREs to drive the reporter (see Figure 4). We are in process of reconstructing this reporter plasmid by excising the 3cERE (Xho I/Bgl II) and ligating in other ERE binding sites (ERE oligonucleostides that have Xho I/Bgl II cut ends; synthesized by IDT) to compare the relative capabilities of these EREs to drive luciferase activity, in the presence and absence of overexpressed HMGB1. We have subcloned cERE, cHERE and 2 separated cEREs, transfected them into JM109 and are currently doing PCR on the transfected colonies to identify the colonies that have incorporated ERE in the plasmid. The DNA from the positive colonies will then be purified, sequenced by Retrogen and then used in luciferase reporter gene assays.

We have carried out luciferase assays for a number of transfections, using transfectamine, with 0-200 ng ERα expression vector (pCMVflag:ERα; from Nardulli) for 1 x 10^5 cells, and found that optimum reporter activity occurs with 10 ng ERα (Figure 5). In initial studies, 3 ng pHMGB1 (from Edwards) was transfected. However, we are currently determining the optimum level of HMGB1 to be used. At the current 3 ng level for HMGB1, together with the 1 ng of each Firefly luciferase (FL) reporter and Renilla luciferase (RL), we find high levels of E2-dependent luciferase activity with 3cERE (15-fold over no E2 and mock transfection) in our initial experiments. The calculated fold-induction of FL was normalized for RL and expressed as a ratio between treatment groups. We consider these findings only preliminary until the levels of HMGB1 are optimized. **Conclude;** The maintenance and handling of U2-OS cells, initial transient transfections and 3cERE luciferase reporter gene assays have been successfully done and we are expanding the series of EREs that drive

the luciferase assay (**proposed AIM4**). Our progress toward accomplishing all the three AIMS set out in the 2005-2008 proposal are progressing as outlined in our original timeline.

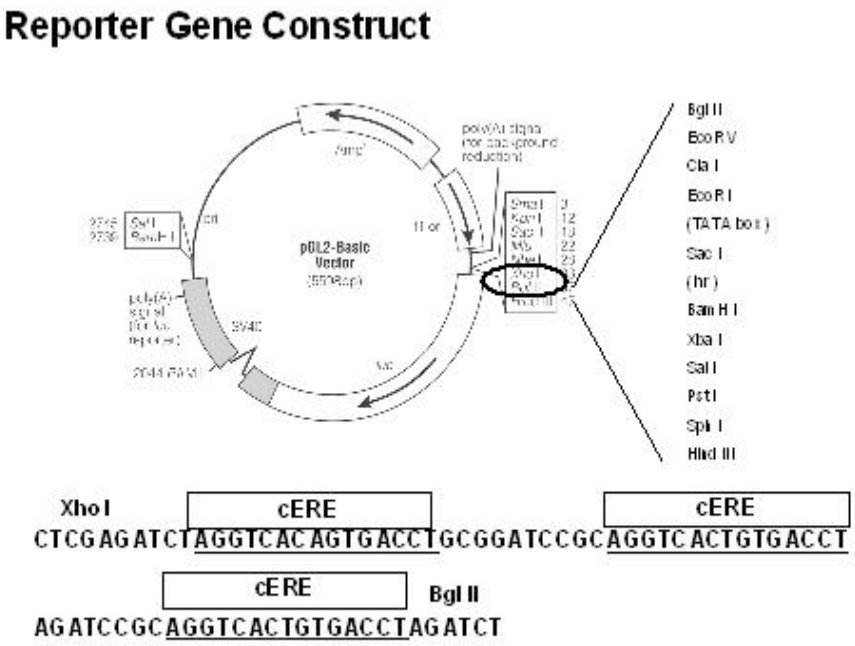


Figure 4. The pGL2-Basic vector, showing the 3cERE insert

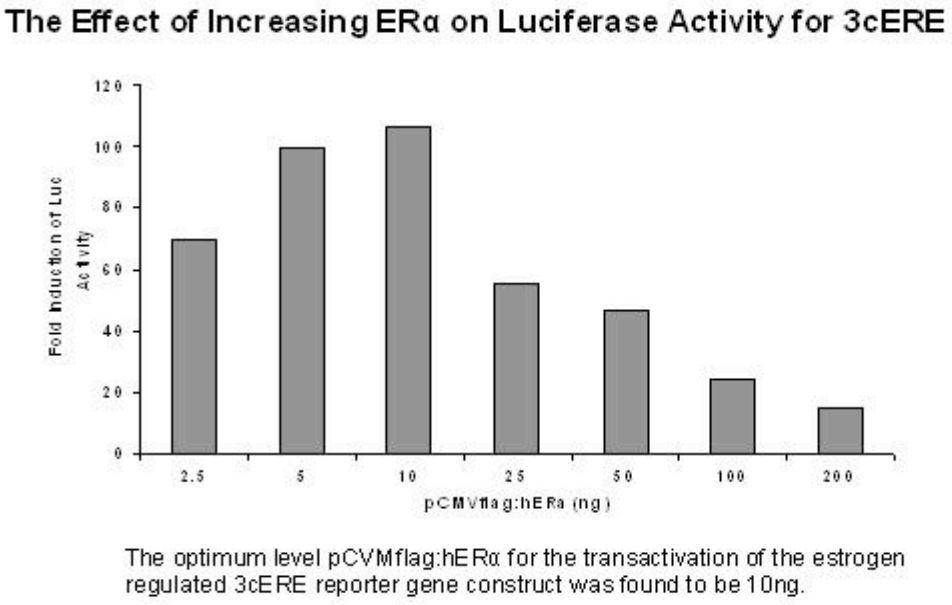


Figure 5. Plot of luciferase activity as a function of the amount of pCMVflag:ERα expression vector transfected into 10^{+5} U2-OS (ER^{-}/$HMGB1^{+}$) cells.

Sabbatical Leave Experience

a) S. Joshi & I spent June-July, 2006 in the lab of Eddie Sanchez (Pharmacology Department, U. Toledo Health Science Center, Toledo, OH, just 25 miles north of Bowling Green) working on cellular aspects of glucocorticoid receptors. We gained valuable hands-on experience in cell culture, mammalian cell transfections and luciferase reporter gene assays.

b) I spent Jan-Mar,2007 in the lab of internationally recognized genomics researcher, Jason Lieb, Biology Department & Center for Genomics Research at University of North Carolina, Chapel Hill. We initiated a project using chromatin immunoprecipitation & DNA microarrays (ChIP-chip) using yeast to determine the residence times of TF, RAP1, at each of its recognition sites in over 300 genes. I completed 2 replicates of these experiments, which involved growing up genetically engineered yeast cells, Western blots analysis using ECL detection, ChIP experiments with two antibodies (anti-myc & anti-HA), use of QIAquick DNA purification kits, ligand-mediated PCR, conjugation of dyes to the DNA, hybridization of the "chipped" DNA to DNA microarrays and the analysis of the chip data. Although the two months leave impeded immediate productivity on the proposed work, the experience were extremely valuable and puts my lab in a position to greatly extend our studies into new areas, including ChIP, ChIP-chip, & genomic areas and the possibilities of new and valuable collaborations.

AIM1. Determine the extent to which the C-terminal extension (CTE) of ER contributes interactions, and therefore important additional binding affinity in ER complexation with estrogen response elements (EREs) and the level to which HMGB1 enhances these interactions.

Our findings suggest that the binding characteristics of steroid hormone receptors (SHR), ER, GR & PR, are quite different, opening up the current paradigm to significant changes. In addition, Edwards (21, 23-28, 63) has presented evidence that further questions the current model that indicates that only **class II** receptors require the CTE for stable binding, while **class I receptors** (SHRs) do not. Although the crystal structures for ERDBD/ERE show ERDBD interactions in the major groove (64-66), the findings suggest that SHRs also require additional CTE interactions in the minor groove, similar to that observed in Class II non-steroid receptor interactions (23-28). A crystal structure shows the CTE in PRDBD-CTE interacts with the minor groove (63). Experiments in **AIM1** will provide evidence to address this question and further support or help to remold the current paradigm for ER. In addition and to the point of this work, previous data from my lab indicates that ERDBD has a relatively high Kd value compared to ER for cERE binding (150 vs 5 nM). This clearly indicates that regions outside the ERDBD contribute significantly in stabilizing the ER/cERE complex.

Hydroxyl Radical Footprints. Hydroxyl radical footprinting (HRFP) and the missing nucleoside strategy (112,113) will be used to identify potential changes in six protein/DNA binding interactions. These involve the binding of ERDBD, ERDBD-CTE and ER to both the consensus ERE (cERE) and the consensus half-site (cHERE), in addition to the effect of HMGB1 on ER binding. HRFP may also help define 1) the extent of major protein-nucleotide contacts within the cERE; 2) the contributions generally from CTE and how these may modulate interactions in the major groove and 3) how other domains in (holo)ER that further contribute and/or modulate ER/ERE interactions may affect complex stability. It is estimated that differences of ca. 20% in the HRFP can be readily detected in the HRFP profile (112, J. Hayes, personal communication). Figure 1 shows the domains for ERα.

These studies will parallel previous studies that used HRFP in defining whether sequence-specific TFs, such as homeodomain proteins and the pioneer protein, FOXA1, utilize regions other than their DBD in binding DNA (58, 114). Although my lab has experience with HRFP, Jeff Hayes, a former student of mine (BS & MS at BGSU) has agreed to serve as an advisor in these experiments (see attached letter). The advantages of HRFP are that ·OH is a very small, highly reactive molecule that exhibits no sequence preference, cleaves all exposed nucleosides and produces a smooth, continuous and high resolution band pattern in the FP that depends on the level of accessibility by the hydroxyl radical. DNase I, in contrast, is a large nuclease, cuts with some sequence preference to produce relatively few cuts in the DNA, leaving many "blank" uncut regions and yielding an artificially extended FP (112,114-116). It should also be emphasized that although ·OH footprinting is sensitive to binding in the major groove, its is especially sensitive to any changes in accessibility in the minor groove which can influence band intensities in the major groove (112).

I have requested the bacterial expression vectors for the ERDBD (180-250), ERDBD-CTE (180-288) from Schwabe (MRC, Cambridge) and Edwards (Baylor School of Medicine), respectively. These will be purified by standard procedures (63-65). The 33 bp oligonucleotides for cERE and cHERE (42) will be purchased from IDT Technologies, the strands annealed and then subcloned into our pGL2-TATA-Inr-Luc vector (as above) and transfected into JM109 cells. The JM109 cells will be grown up, the plasmid isolated and purified and the 115 bp fragment excised by Xho I/Bgl II digestion. To label one strand, the plasmid can be cut with Xho I, ^{32}P-end labeled, the DNA is gel purified, cut with the second restriction enzyme and then purified. The alternate strand is labeled similarly, with the order of cutting reversed.

Initial experiments (EMSA) will determine the Kd values for DBD, DBD-CTE and ER, binding to the labeled cERE and cHERE DNA fragment, as noted previously (42). This will define the Kd values for the three forms of ER under the same conditions and give us a general indication of which transition (DBD --> DBD-CTE or DBD-CTE --> ER) may produce the greater changes in HRFP.

The detailed experimental procedure for the HRFP, including potential problems, has been outlined in detail (115). The Fenton reaction used to generate the HRs is: $Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + {\cdot}OH$, with ascorbate to regenerate Fe(II). Optimum conditions are empirically determined, depending on the buffer components and require some preliminary experiments. However, typically ca. 10 uLs of 10X stock solution of the three major components-$(NH_4)_2Fe(SO_4)_2$/EDTA, H_2O_2 and ascorbate are "dotted" as individual spots on the side of the tube in which the DNA (ca. 50-200 kdpms) and ER (+/- 400 nM HMGB1) are incubated in

binding buffer (with ca. 0.5 ug nonspecific DNA) until equilibrium is established at 25°C. A quick spin is used to then react all components (ca. 100 uL) and start the cleavage reaction. The study will be done in conditions to insure >95% complex formation. Reactions with ·OH are run for 1-3 mins and stopped by addition of radical scavenger (thiourea)/EDTA. The DNA is purified by standard phenol extractions, dried, dissolved in loading buffer, heated and loaded on a denaturing gel. Quantitation of band position and intensities will be carried out as outlined using the Image Quant software, which is part of the phosphoimager package (42). The intensity of the bands for nucleotides distant (ca. 30 bps) from the ERE will not be affected by changes (or be least affected) and will be used as internal intensity controls to give at least a semiquantitative measure of the changes. To insure that the best quality footprint is being obtained, a number of samples from the first experiments will also be run on an EMSA gel, the ER/ERE complex band excised from the gel, the DNA eluted and purified and then run on a denaturing gel. This gel patterns for the two approaches will be compared to determine if the initial EMSA separation step is needed in subsequent experiments.

A great deal can be learned from a comparison of the differences in the phosphoimager band positions and intensities in the ERDBD, ERDBD-CTE and ER with cERE or cHERE in the presence and absence of HMGB1. A comparison of the phosphoimager scans for DBD-CTE with the DBD will reveal any interactions that change as a result of the presence of the CTE. These will include changes outside the ERE that may be directly attributed to CTE/DNA interactions, while changes in the DBD/ERE interactions may be expected, due to the CTE modulating the binding interactions in the major groove. The (holo)ER binding interactions will, for the first time, extend the map for additional interactions from domains outside the DBD. By comparison of the "step-wise" changes in the HRFP profiles as the complexity of "ER" increases, one may expect a new level of detail revealed (contributions from A, B, E &/or F domains) that was previously unrecognized, due to using the less-sensitive DNase I FP technique. If the crystal structure for the PRDBD-CTE may be used as a general guide to possible interactions (63), the band intensity changes are expected to be greatest in the ERE and within ca, +/-5-10 bp. The comparison of the HRFP profiles for each form of ER, in the absence and presence of HMGB1, will be an indicator of the influence of HMGB1 on these interactions.

The next set of experiments will focus on the effect of HMGB1 on the binding of ER and ERDBD-CTE (26, 28, 42, 63). Initial control experiments with the DNA fragments (cERE & cHERE), in the absence and presence of HMGB1, will be done to determine if transient, non-specific HMGB1 interactions induce any changes on the HRFP on free DNA. Using this as a base line, the effect of HMGB1 on the HRFP for binding of ER and its truncated forms will be examined in the same manner. Since the binding affinity is increased, additional interactions may result in all three forms of ER. A comparison of the Kd values and the HRFPs for the three forms of ER will permit us to determine the relative contribution of CTE and other domains to any increased binding affinity. Comparison of the effect of HMGB1 on the incremental changes (i. e., DBD to DBD-CTE) will also show which region or regions are most responsible for the "HMGB1 binding enhancement effect".

Since all three forms of ER do not bind to the ERE <u>half-site</u> (cHERE) in the absence of HMGB1, only the complexes that occur in the presence of HMGB1 can be examined. Again, EMSA will be run to determine the Kd values to quantitate the relative binding affinities for each complex and then use levels of ER that produce >95 % complex. Progressive changes that occur on the hydroxyl radical phosphoimager band profile will be measured and compared as we progress from ERDBD to ERDBD-CTE to ER binding.

An objective in these studies will also be to determine the extent of protection found at these EREs to reveal the real hydroxyl radical footprint for each form of ER. Previous work reported that ERDBD and ER have essentially the same DNase I FP (42). This was, perhaps not unexpected, and most likely resulted from the lack of sensitivity and the relatively few bands that can be used to define the DNase I FP.

<u>Missing Nucleoside Experiment</u> The "missing nucleoside" experiment (113, 116) is a second and alternate approach to gain further evidence for the specific bps in DNA that contribute energetically important contacts with a DNA-binding protein. This missing nucleoside (hydroxyl radical assay) strategy has been successfully used previously to determine if ERα and ERβ make different nucleotide contacts when binding to cERE and also to an ERE with one bp change in one of the half-sites (138). With the same DNA fragments used above, hydroxyl radicals are generated that act on DNA to remove a single nucleoside from an individual DNA strand. This generates DNA fragments with random one nucleoside gaps (missing nucleoside), prior to reaction with

ER. This then allows the analysis of protein-DNA interactions at single nucleotide resolution. Similar to the strategy used by Maxam Gilbert sequencing reactions, cleavage conditions will be determined that, on average, permit one nucleoside cleavage per DNA molecule. The technique is based on the premise that if a base in DNA that makes an important contribution to protein binding is missing (cleaved off), the protein binding will be significantly reduced or it will not bind. Individual strands of DNA are end-labeled, cleaved with hydroxyl radicals and then precipitated twice with ethanol/salt, dried and then dissolved in reaction buffer (112). ER will be reacted with the labeled, gapped DNA at relatively low levels (~30% complexation) so that only the strongest interaction will dominate in the complex. The ER-bound ERE complex is separated from the unbound free DNA by nondenaturing (EMSA) PAGE. Radioactive bands that contain the ER/ERE complex and free DNA are excised from the gel, eluted and then subjected to denaturing (sequencing) PAGE for band analysis. The individual band intensities will then be quantified by phosphoimager analysis. DNAs that have missing nucleosides that had contributed important (energetically favorable) interactions to the binding affinity in the complex will migrate with the free DNA since they will not bind. The DNAs that bind to ER have missing nucleosides that had not contributed strong interactions and therefore do not significantly reduce the stability of the complex.

A typical gel will contain a G/A ladder, the ER/ERE and free DNAs from the three (ERDBD, ERDBD-CTE & ER) complexes that are isolated from the gel. A comparison of the phosphoimager band intensity results will then be made for any one set of the free and ER/ERE complexes (e. g. free ERE vs.DBD/ERE bands). The ratio of the intensities, [(free DNA)/(ER/ERE)], for each band (base) will be complied for nucleotides in and about the ERE region. The higher the ratio is at a particular base, the greater that base will contribute to the approximate binding affinity. Each complex (DBD, DBD-CTE and ER) will be examined individually and then a comparison will be made between complexes to determine how the additional region of ER influences binding, within the ERE and beyond the major groove.

The results from both strategies will provide a two-pronged approach to clarify important interactions that are derived directly from the DBD, CTE and generally from other domains outside the DBD.

Potential Problems:

1) In only a few cases has it been shown that the reagents for HRFP affect protein binding. In those cases, H_2O_2 was the problem. This is tested by doing a DNase I FP in the presence and absence of the three individual reagents in the hydroxyl generating system.

2) The most avid scavenger of hydroxyl radicals is glycerol. Less than 0.5% (v/v) is recommended in the binding buffer. We will reduce our 2% glycerol in our reaction to 0.5% and do not expect significant changes in binding affinity.

3) The analysis for both strategies will require high quality gels and careful phosphoimager analysis to sort out the interactions. We believe that coupling the two strategies and analyzing them simultaneously will provide the best means to defining the observed interactions.

AIM2. **Characterize 1) the effect of HMGB1 on the binding affinity of ER on rotationally phased and translationally positioned EREs within a nucleosome; 2) the effect of HMGB1, and HMGB1 and ER in combination, on the stability of the nucleosome and 3) the effect of a human chromatin remodeling complex (CRC), SWI/SNF, on the dynamics of the nucleosome, in the presence and absence of ER, and the extent to which HMGB1 enhances or inhibits nucleosome remodeling. The ER target sequences will be cERE, cHERE, two cEREs & two cHEREs.**

a. Determine the effect of HMGB1 and ER on nucleosome integrity and stability (No SWI/SNF)

ER binds very weakly to a rotationally phased cERE (K_d ~ 300 nM) at the dyad axis or 40 bps from the dyad in nucleosomal DNA, while 400 nM HMGB1 enhances ER binding affinity 5-6 fold at both sites, with a Kd ~50-60 nM. The DNase I 10 bp pattern is observed for the nucleosome and is not significantly disrupted in the presence of 400 nM HMGB1. A preliminary experiment (not especially sensitive) was done to detect if 500 nM HMGB1 facilitates octamer dissociation. No evidence for nucleosome disruption (**Prelim. Findings**). Assuming 500,000 HMGB1 copies/nucleus and a nuclear diameter of 6 um (137), the approximate HMGB1 concentration in the nucleus may be estimated as high as 7000 nM. That being the case, we will also examine

the effect of higher HMGB1 levels produces on the integrity of the nucleosome and on the extent of ER binding to EREs within a nucleosome.

i. Effect of increasing HMGB1 levels on the integrity of the nucleosome

The integrity of the nucleosome appears intact with 400 nM HMGB1, as monitored by its DNase I 10bp pattern. We will test its stability and integrity initially at HMGB1 levels up to 1000 nM. A distinct DNase I 10 bp pattern is indicative of a rotationally phased nucleosome, while Exo III digestion is diagnostic of the size of the DNA that is wrapped tightly around the nucleosome and is inaccessible to exonucleolytic cleavage. Changes in these two characteristics, or alternatively, evidence for dissociated histones from the nucleosomes, are common indicators of disruption of DNA-histone interactions in nucleosomes.

We have DNAs with cERE (& PRE) at the dyad axis and 40 bps from the dyad, with Kd values for ER binding in the presence of 400 nM HMGB1 (PR does not bind). We shall use our standard protocols to construct DNAs that contain 4 NPSs, with cERE & cHERE at 20 bps from the dyad in the 161 bp DNAs. In addition, we will incorporate 1) two cEREs and 2) two cHEREs, symmetrically at +/- 40 bps from the dyad, with three NPSs centered at the dyad axis of the DNA. The DNAs will then be sequenced. The series of DNAs will be ^{32}P-end-labeled at either end and reacted with ER at 200, 400, 600, 800 and 1000 nM HMGB1 to compare the effect of increasing HMGB1 concentration on nucleosome stability (buffer contains 10 uM BSA to keep a total protein level). The nucleosomal DNA will be digested with DNase I, as outlined (**Prelim Find**). The primary DNAs will contain cERE and cHERE at the dyad and at 40 bps from the dyad, in addition to the two cEREs (cHEREs)**.** DNAs with EREs at 20 bps from the dyad will be examined if we find a difference between the dyad and 40 bp. This strategy will be used throughout **AIM2**. The 10 bp band pattern produced in the absence of HMGB1 will be the reference against which those incubated with HMGB1 will be compared. The band positions and intensities will be quantitatively measured by phosphoimager analysis to detect any disruption in rotational phasing and the level of HMGB1 at which it may occur. A disrupted pattern usually includes many more bands and looks more like that observed for DNase I cutting free DNA, which will also be run simultaneously on the gel. Parallel studies will also be done with Exo III digestions to reveal the size of the DNA in the nucleosome that is protected from nucleolytic digestion. Any decrease in the size of the DNA (from the standard) will be a signal that HMGB1 interactions have altered the translational position and facilitated movement of the ERE DNA within the nucleosome. One may predict that the fewer NPSs included in the nucleosome that contains two cEREs (cHEREs) may enhance the dynamic aspects of the nucleosome and facilitate more facile disruption of DNA-histone interactions.

The effect of HMGB1 on nucleosome integrity will be similarly examined utilizing EMSAs to detect disruption and/or dissociation of histone octamer from radiolabeled nucleosomal DNA by the appearance of a band that results if free radiolabeled DNA (released from nucleosome) is observed. Our preliminary data indicate little or no disruption by HMGB1 up to levels of 600 nM. However, a clearer assessment of nucleosome stability can be obtained if there is a competitor DNA (a "histone sink") that any dissociated histone octamer can transfer to. **(cf. Section iv)** In the proposed experiment, a 10, 100 and 1000-fold excess of cold DNA, the A5 DNA (**PRELIM. FIND; AIM2**) that does not contain an ERE will serve as a competitor for any dissociated histones to associate with (77, 118). The amounts of cold DNA needed can be quantified by taking 1 uL aliquot of the radiolabeled DNA, apply to a Skatron filter, wash with cold ethanol, then ethanol dry and determine dpm/ug DNA (scintillation counter) prior to nucleosome assembly. After nucleosome assembly, an aliquot can be taken and the dpms measured again to determine the amount of nucleosomal DNA. This will be used to determine the amount of cold DNA to be used. If HMGB1 causes nucleosome disruption to occur, radiolabeled DNA will be released from the nucleosome and become free, as has been previously observed (77, 118). The relative level (band intensities) of radiolabeled DNA that is free and in nucleosomal DNA can be measured accurately by phosphoimager analysis to determine the extent to which HMGB1 disrupts nucleosomes. The effect of ERE (cHERE) position and the effect of HMGB1 levels can be readily determined. In addition, the effect that two cERE (cHEREs) and one less NPS has on nucleosome stability will be revealed. The levels of HMGB1 in subsequent experiments may be altered or extended if higher levels of HMGB1 produce dramatically different results.

ii. Effect of increasing HMGB1 levels on ER binding affinity Since 400 nM HMGB1 facilitates ER/cERE binding in a nucleosome, we will determine if increasing levels of HMGB1 further weakens the DNA-histone interactions in the nucleosome and facilitates stronger ER binding. The DNAs in section (**ai)** will

be used here also. In parallel with the experiments in **(i)**, the series of DNAs will be ^{32}P-end-labeled and reacted with ER at 200, 400, 600, 800 and 1000 nM HMGB1 to compare the Kd values (EMSA) as a function of HMGB1 levels. If increasing HMGB1 levels do destabilize the DNA-histone interactions, one may predict that increasing HMGB1 will produce increased accessibility and lead to an increase in ER binding affinity to each site. This set of experiments will test Widom's proposal (105) that suggests that it should be easier to unwrap the DNA ends (& therefore bind) than disrupt those segments at or near the dyad axis. Our preliminary data are not consistent with this and suggest that HMGB1 exerts virtually the same effect on ER binding to cERE at the dyad and 40 bp from the dyad. In addition, using a number of different DNAs - those with a cERE, a half-site and then two EREs - may reveal that ER access to their sites may be context dependent and not consistent with an all-inclusive generalization. This expanded series of sites will very carefully test Widom's proposal. In addition, this will be the first study to investigate the extent to which binding at one ERE can influence the binding to the second site (any cooperative binding between the two ERE sites) within a nucleosome and the effect that HMGB1 exerts. The bands will be quantitatively analyzed by phosphoimager, looking for cooperativity and calculating K_{50} (concentration at which 50% of DNA is bound by ER) for each binding interaction. It will be particularly interesting to also compare ER binding affinity at multiple cERE or half-sites (cHERE) as compared to that at a single whole (half)-site and determine if cooperativity between two separated sites is in line with the finding that two or more sites act synergistically in transcriptional activation (117).

iii. Compare the DNase I footprint for ER bound to DNA and nucleosomal DNA

The DNase I FP for ER binding to the series of four ER binding sites will be obtained for free DNA (+/- 400 nM HMGB1) and for nucleosomal DNA in the presence of 400 nM HMGB1. We want to compare the FPs of cERE, cHERE in free DNA, both of which we have published (42) to FPs for two cEREs (& two cHEREs) separated (center-to-center) by 8 bps in DNA and nucleosomal DNA. This spacing is similar to that found in the natural *Xenopus* vitellogenin B1 promoter (vit B1) that we previously studied (42). In these DNAs with multiple EREs, this technique will also reveal any cooperativity in ER binding in DNA or nucleosomes, as we found for the imperfect palindromes in free (vit B1) DNA (42). In the case with GAL4, one finds that GAL4 binding affinity was the same (unaffected) when binding to a single or five sites on DNA, but GAL4 binding affinity was increased when there were multiple sites in a nucleosome (119).

The 161 bp DNAs will be radiolabeled on one strand and the annealed DNA, or the nucleosome, will be reacted with 1) ER (>95 % complex) and then digested with DNase I from 1-3 min or alternatively 2) increasing ER levels for a constant level of DNase I for ca. 3 mins. Both of these procedures are expected to yield a clear FP and possibly show DNase I hypersensitive sites that are indicative of an unusual local structure in DNA as a result of, perhaps unique bending, resulting from multiple ERs binding. The strategy will be similar to those already reported (42), with comparison of the difference in the FP for cERE and cHERE, both in DNA and in the nucleosomes. The size of the DNase I FP for the cHERE is expected to be smaller, with only partial protection over the "non-specific " half-site (42). The individual DNA FPs for the 2 cEREs and two cHERE will be examined for binding cooperativity as ER levels increase and whether a similar pattern extends to nucleosomes. The footprints for cERE and cHERE will be particularly revealing in light of our finding that indicates that they have comparable Kd values in the presence of HMGB1 (50 vs 80 nM, respectively; **Prelim. Findings).**

iv. Determine the combined effect of HMGB1 and ER on nucleosome integrity

TF binding to nucleosomal DNA is a competition between TF and octamer binding for the DNA. TF binding can destabilize the nucleosome, but in most cases, requires the assistance of cofactors or CRCs (reviewed in (77). In the case of GR binding, the GR/GRE-nucleosome complex remains intact. GAL4 proteins bind to nucleosomes containing GAL4 binding sites and do not dissociate histones. However, the histones in the GAL4-bound nucleosome are different than those in the unbound nucleosomes as revealed by the histones in the former being susceptible to transfer onto a competitor DNA (120). We know that ER binds to nucleosomes in the presence of 400 nM HMGB1, indicating some form of nucleosome remodeling that facilitates ER much greater access to the cERE. These experiments and analysis will parallel those in **(section i)**, but will reveal the effect of ER binding in combination with HMGB1. Three experiments will be carried out on ER binding to the series of nucleosomes in the presence of HMGB1. A DNase 10 bp pattern, an Exo III digestion pattern and an EMSA study to determine if increasing HMGB1 levels (400-1000 nM) produces dissociation of histones from the nucleosomes, in the presence and absence of increasing levels of competitor A5 DNA.

b. Determine the effect of human SWI/SNF complex, HMGB1 and ER interactions on nucleosome stability

Human SWI/SNF has been implicated in remodeling nucleosomes that contain binding sites for many TFs and particular ER and other steroid hormone receptors (72-84, 96, 104, 121). Since SWI/SNF is directly involved in ER f\unction and since ER and SWI/SNF can both remodel ERE-containing nucleosomes, we want to determine their individual (relative) remodeling activities and whether they function independently or cooperatively.

i. Determine the effect of hSWI/SNF on nucleosome dynamics

Human SWI/SNF complex (with BRG and hBrmATPase) will be prepared by the step-by-step procedure sent to us by S. Sif (121). Briefly, a cell line, FL-Inil-11, was created that expresses the Flag-tagged BGG1/hBrm hSWI/SNF subunits. This cell line has been extensively used in a wide variety of mechanistic studies. The cells (10 liters) will be purchased from NCCC (National Center for Cell Culture) and 5 Ls used in an individual isolation. Nuclei are prepared according to the Dignam procedure (129). Protein concentration is determined and about 60 mg of protein is incubated with 1 mL anti-Flag M2 affinity gel (Kodak) for 8-12 hrs at 4^oC. The beads are loaded into a column, washed extensively with buffer and then eluted with 20-fold excess of Flag peptide (Kodak). The concentration is determined by Bradford assay and proteins analyzed by SDS-PAGE & silver staining. This single affinity step yields high purity. Generally 12-250 mL fractions are collected, with fractions 2-6 having very high activity (ca. 2 uL needed for ATP-dependent disruption of the 10 bp pattern of 3 ng phased nucleosome). Fractions 6-12 also exhibit activity, but require about 5-6 uL/ reaction. Anthony Imbalzano, a contributing author in this work, has consented also to serve as a consultant in this work (letter attached) and Said Sif (Ohio State U.) has indicated that he will provided any needed help also. The same nucleosomal DNAs prepared in **section ai** will be used as outlined. This first test will determine if the different nucleosomal DNAs (different ERE and translational positions) prepared under identical conditions require significantly different levels of hSWI/SNF complex to disrupt the 10 bp pattern. Increasing levels of hSWI/SNF (+/- 4 mM Mg-ATP) will be added to the nucleosomes to determine the level at which the nucleosomes are disrupted (84). Disruption will be evident by the increased number of bands that more resemble the DNase I digestion profile for free DNA. The levels of hSWI/SNF found here will serve as a "standard or unit level" in section **bii**. This will answer the question of whether hSWI/SNF activity is affected by nucleosome position, type of ERE and numbers of EREs within the nucleosome. I am unaware of any series of nucleosomal DNAs that have been examined to address this question in this way.

ii. Determine the effect of HMGB1 on hSWI/SNF activity

HMGB1 remodels nucleosomes nonenzymatically to permit ER to bind to ERE in nucleosomal DNA. We will determine what effect HMGB1 has on hSWI/SNF activity. The experiments outlined above will be repeated with 400 nM HMGB1 and possibly higher HMGB1 levels with the DNase I 10 bp band pattern and intensities monitored as a function of hSWI/SNF levels. These will then be compared to those in **section bi**. We have shown that HMGB1 promotes ER binding by some manner of nucleosome remodeling, but alternatively, it is possible that it may inhibit hSWI/SNF binding by competing in some way with BAF57, the HMG box subunit (binding to the DNA). If HMGB1 is found to stimulate ATP-dependent hSWI/SNF activity, the hSWI/SNF levels used will be reduced accordingly to produce a nucleosomes disruption pattern that is comparable to that observed in nucleosomes in the absence of HMGB1 (the "unit level"). The level to which SWI/SNF needs to be reduced to reach the "unit level" will be used then to assess the synergic effect that HMGB1 exerts on SWI/SNF activity. Alternatively, if HMGB1 inhibits SWI/SNF activity, the level of SWI/SNF used will be increased to get to the level of activity observed in the absence of HMGB1. Therefore, the magnitude of its inhibitory effect can be determined. This is the first time that the combined action of CRCs (one enzymatic and the other nonenzymatic) on nucleosome stability will be examined. The four ERE that have been constructed will be examined in this manner to determine how the different EREs influence combined [HMGB1-SWI/SNF] activity. The outcome of these studies will provide an important starting point for section **(iii**).

iii. Determine the effect that hSWI/SNF activity, and HMGB1 & hSWI/SNF activities in combination, exerts on ER binding affinity

HMGB1 has a positive effect on ER binding to nucleosomal DNA and its effect on SWI/SNF will be determined in section **(ii)**. We will determine the effect that 1) enzymatic hSWI/SNF activity has on facilitating ER binding and 2) how HMGB1 modulates this activity. This will provide the first comparison of the nonenzymatic HMGB1 CRC activity relative to the ATP-dependent SWI/SNF activity and then the combined activity of both on a TF (ER) binding to its response element.

Starting with the level of SWI/SNF that disrupted the 10 bp pattern in section **(bi)**, increasing amounts of ER will be added to determine the Kd value for ER binding. The effect of SWI/SNF on the Kd value for the other three nucleosomal DNAs will also be determined. The ER binding profile will then be repeated, but in the presence of only 400 nM HMGB1. Our current finding is that HMGB1 increases accessibility and binding to cERE ca. 5-6-fold and have a Kd = 40-50 nM. These comparisons will provide new insight in comparing the individual activities of HMGB1 and SWI/SNF and how the character of the ERE affects hSWI/SNF or HMGB1 activity, especially for binding to one or more cEREs or cHERE (half-sites). Again, it will also reveal how hSWI/SNF activity influences possible cooperative ER binding behavior to two different EREs. In this latter case, K_{50} (50% of the DNA in complex) will be determined.

The possible cooperativity that the combination of HMGB1 and SWI/SNF has on facilitating ER binding to different EREs will be most interesting. If HMGB1 has a stimulatory effect on SWI/SNF activity and greatly facilitates ER binding, a range of SWI/SNF levels, up to the (maximum) level found in the previous experiments, will be used, with 400 nM HMGB1 and increasing levels of ER. This will show, for the first time, the combined effect of two CRCs, one enzymatic and one nonenzymatic, on a TF (ER) binding to nucleosomal DNA. It is possible that the combined effect may result in a Kd value in the range found for free DNA.

If HMGB1 is found to have an inhibitory effect on SWI/SNF activity, a similar experiment will be run, a range of SWI/SNF levels will be used, but with the level found in the experiment above (SWI/SNF only) being the lowest level used.

The effect that SWI/SNF has on TF binding varies, but if the GAL4-AH (AH = alpha helix) can be a general guide, GAL4-AH binds to its site in a nucleosomes ca. 10^{+4} less strongly than in DNA, while SWI/SNF increased GAL4-AH binding affinity ca. 10^{+2} (122). Thus, SWI/SNF generally increases binding affinity, but not to the level found on free DNA. It will be of great interest to determine the relative "remodeling activity" of HMGB1 to that for SWI/SNF and then the level of enhancement or inhibition as they act in combination. The gain or loss of SWI/SNF activity as a result of the presence of HMGB1 will be a major point of interest.

If experiments go well and time permits, these studies will be extended to include PR binding to PRE and the effect that hSWI/SNF and HMGB1exerts on them. This would further reveal how similar or different these two steroid hormone receptors are.

Potential Problems We have used these techniques in the 2005-2008 grant and have a good start on this work. The isolation/fractionation of hSWI/SNF is a one-step affinity purification and we do not anticipate any major problems We plan to visit Dr. Sifs lab at OSU in summer, 2008 to do a few experiments with hSWI/SNF and become familiar with his exact isolation procedures and procedures for handling the hSWI/SNF complex.

AIM3. Determine if HMGB1 is physically associated (colocalization) with 1) "uncomplexed", ER prior to and after productive transcription and 2) ER at four different types of regulatory elements in E2-responsive genes in MCF-7 human breast cancer cells. These findings will begin to reveal how inclusive or restrictive the role of HMGB1 is in transcriptional regulation. The regulatory elements include an imperfect ERE, a cERE half-site (cHERE), direct repeats (DRs) and an element that contains a zero spacer in the cERE (cERE0).

In the presence of E2, ER binds to the pS2 promoter element in a cyclic manner in MCF-7 cells (16-18). After a nonproductive first cycle, the cyclic nature of ER binding to promoter continues with recruitment of additional regulatory factors, leading to productive transcription. The ERE in the pS2 promoter has been mapped within a phased nucleosome in the MCF-7 cell line (71).

a. Determine if HMGB1 physically associates with ER in MCF-7 breast cancer cells, in the presence and absence of E2-stimulation.

Since HMGB1 enhances *in vitro* ER binding to its response elements, it is of interest to determine if there is an association of HMGB1 with ER prior to and after E2 treatment. Using the [ER^{+}/$HMGB1^{+}$] MCF-7

breast cancer cell line, ER will be immunoprecipitated (IP), before and after E2-treatment, to determine the extent to which HMGB1 coimmunoprecipitates (coIP) with ER, when ER is not productively bound to DNA.

MCF-7 cell line (obtained from NCCC) will be maintained in E2-free media (123). Cells (ca. 10^{+7}) will be washed and 1 mL NP-40 lysis buffer added for 30 min at 4°C (all following steps are at 4°C). Cells/debris are scraped into a cold 1.5 mL tube and centrifuged for a minimum of 20 min at 12 kG. The lysate is transferred to a new tube and precleared by addition of 1 ug of control normal rabbit IgG & 20 uL of 25% (v/v) Protein A-Sepharose for 30 mins at 4° C and then spun 1 min at 1 kG. Repeat preclearing once again. Divide the SN into two and transfer ca. 100-1000 ug protein to each of two new tubes. One half (called αER) is incubated with 2 ug/mL polyclonal anti ER (Millipore), while the other (αH) is incubated with an equal amount of anti-HMGB1 (Millipore) overnight, with shaking. Add 20 uL of Protein A-Sephrose and rock for 2-6 hrs. Sediment at 1xkG for 1 min and remove SN. The beads from each are washed 3 times with wash buffer and then 40-50 uL of electrophoresis loading buffer is added to the beads and boiled for 3 mins. Each sample (αER & αH) is again divided into two (αER & αER, and αH & αH) and the four samples are loaded on individual lanes on the gel such that the individual pairs (αER & αH) are adjacent and run on SDS-PAGE. Stained MW markers are loaded on either side of each pair. The proteins will be electrophoretically transferred to PVDF membrane, the membranes are blocked for 2 hrs with 1% non-fat dry milk (1X PBS/NP40), washed three times with (TBS) and then the membrane is cut to separate the two pairs. One sample pair (αER & αH) is then incubated for 1 hr with anti-HMGB1 (2 ug/mL in PBS) to produce what we call, (αER/αH & αH/αH; 1st Ab indicates IP Ab, 2nd Ab is Western probe), while the other pair is incubated with anti-ER, called (αER/αER & αH/αER). The membrane is washed three times and then incubated with the secondary (HRP-conjugated)-antibody for 1 hr. The membrane is washed and solutions from the ECL chemiluminescence detection kit are used, as described by the supplier, to detect the bands, after short exposure on photographic film. Two lanes (αER/αER & αH/αH) will show the levels of ER and HMGB1, respectively, in the cell, while the other two samples, in which two different antibodies were sequentially used (αH/αER & αER/αH) will determine the extent of stable HMGB1 association with ER in cell lysate. These findings can be used to estimate to what extent the transcriptional coactivator, HMGB1, is associated with "unbound" ER. It is expected that the αER/αH results will be more revealing and indicative since the level of ER is expected to be limiting since it has been estimated that the level of ER is about 30,000/cell, while HMGB1 is probably greater than 100,000/cell (20, 124). A negative control, using preimmune serum, in lieu of the primary antibody, will also be run. Together with the cells that were not treated with E2, cells will also be treated with 100 nM E2 for 45 min (time when ER & other factors are bound strongly to the promoters) and run in parallel and compared to determine if the association is E2-dependent.

This experiment is designed to determine the extent to which an ER/HMGB1 complex is formed prior to targeting the ERE in DNA and whether it is E2-dependent. This will hopefully lead to insight as to whether ER/HMGB1 acts predominantly in a concerted manner to target ERE or whether ER and HMGB1 independently target ERE.

b. Determine the extent to which HMGB1 physically associates with regulatory elements in a series of established E2-responsive genes in human cells

In these initial experiments, chromatin immunoprecipitation (ChIP) assays will be used to determine if HMGB1 is associated with promoters in four well-characterized E2-responsive genes - (pS2/TFFI), Na^+/K^+ exchange regulatory factor/Ezrin Radixin-Moesin binding protein 50 (NHE-RF/EBP50), prothymosin α (PTα), proteinase inhibitor 9 (PI9) - that bind ER in the MCF-7 cell line. These findings will be compared to promoters for the retinoic acid receptor β (RARβ) & β-actin genes that are not responsive to E2. Table 4 succinctly describes the promoter regions in each gene.

Table 4 Promoter Signatures for Human E2-responsive Genes

ERE-type* - > Gene	cERE	cHERE	cERE0	DR	References
pS2	imp(2)	no	no	no	71
NHE-RF	no	13	1	yes	125
PTα	no	yes	no	no	126
PI9	v. imp(3)	yes	no	yes	127

*cERE-consensus ERE; Imp(x)-imperfect ERE with x bp changes; cHERE-ERE half-site; cERE0-cERE with zero bp spacer; DR-direct repeat. The 13 and 1 indicate the number of ERE-type elements in the promoter.

The known ER binding sites for these genes provide a diverse set of promoter/regulatory sequences that permit us to begin to define the extent to which HMGB1 associates with different regulatory sequences in E2-responsive genes. The **pS2 gene** contains an imperfect ERE at -405 to -393, approximately 375 bps from the TATA box (71). Although the **NHE-RF** regulatory region contains 13 cHEREs, the most powerful one is the proximal HERE (accounting for >63% of ER activity in reporter gene assays). This cHERE will be examined, along with cERE0, which ER binds to as strongly as it binds to its consensus sequence, cERE3 (**Prelim. Find**). This site was also shown to be the strongest binding site of all 13 regulatory sites in this control region (125). **PTα**, another primary response gene for E2, contains two cHEREs (-705 & -1051) and is considered a breast cancer tumor prognostic marker. **PI 9**, a modulator of apoptotic processes, is regulated by a very imperfect ERE, 5'-GGGGGAcccTGACCT'-3) and another cHERE upstream by 13 bps to make a DR(13) from +197 to +237 (127).

To permit our results to be integrated with previous findings with these cells, the ChIP protocol, procedures and conditions outlined by Brown and Katzenellenbogen will be closely adhered to (12. 13, 125). In the semiquantitative PCR, we will initially use PCR primers that were published by these groups or those designed by the Primer Express software (Applied Biosystems). The primer sequences will be searched against Gene Bank using BLAST will insure specificity. Thermocycling will be done for 25-30 amplification cycles, with the annealing temperature being experimentally tested, but initially done at 55^{o}C. PCR primers that have been widely used, for example, for the pS2 promoter are (12, 13, 125):

pS2 promoter forward (-448; 5'-CTAGACGGAATGGGCTTCATGAGC-3')
pS2 promoter reverse (-146; 5'-AGGATTTGCTGATAGACAGAGACGAC-3')

The step-by-step ChIP procedure has been detailed (130, 131), with an outline given here. MCF-7 cells are grown to 95% confluence (150 mm dish; ca. 1 x 10^{+7} cells is enough for 3 IPs & input DNA) in the absence of E2 and in phenol red-free Dulbecco's modified Eagle medium (DMEM), supplemented with 10% charcoal-dextran stripped fetal bovine calf serum for 3 days. Cells are treated with either 100 nM E2 or carrier for 45 mins. with shaking (all reactions). The cells are washed with PBS and cross-linked by addition of formaldehyde (1% final concentation) at 37^{o}C for 10 mins. The cross-linking reaction is stopped by addition of 0.125 M glycine and shaking for 5 min at RT. Cells are washed twice with ice-cold PBS and scraped into 1 mL ice-cold PBS. Remaining procedures are done at 4^{o}C & solutions contain protease inhibitor cocktail. Spin cells at 3 krpm for 2 min. and then resuspend pellet in 300 uL lysis buffer (1% SDS/EDTA/Tris) for 10 mins. Sonicate 3 times (15 s pulses; Virtis/microtip, setting 4) to produce DNA fragments of ca. 700 bps, followed by centrifugation at 14 kG for 10 mins. Set aside 30-60 uL of both the E2- treated and untreated sheared chromatin preparation (to undergo the same DNA purification). This is the **Input fraction**. Dilute the remaining soluble chromatin (1:10; ~250 uL in 2.25 mL) in IP buffer (Triton/EDTA/NaCl/Tris). Immunoclear 1mL of soluble chromatin with sheared salmon sperm DNA, 2 ug/mL pre-immune serum and protein-A-Sepharose (45 uL of 50% slurry) for 2 hrs at 4^{o}C. Quick spin and carefully collect supernatant. A sample is taken, and after heat reversal (see below) of crosslinks, the DNA is purified (QIAquick Spin Kit) and run on gels to confirm DNA fragment size. The remaining lysate is cleared by centrifugation and 2 ug/mL anti-HMGB1 (Millipore) is added and incubate/shake overnight at 4^{o}C. Add 45 uL of protein A-Sepharose (& 2 ug salmon sperm DNA) & incubate for 1 hrs. The Sepharose beads are washed sequentially with three different (stringency) buffers to remove unbound DNA, washed with TE and then the bound DNA fragments are eluted in 1% SDS, 0.1 M $NaHCO_3$ at room temperature for 10 mins. The pooled eluants (& Inputs) are heated overnight at 65^{o}C to reverse the crosslinks. The DNA is purified with Qiagen PCR purification kit. As a control, preimmune (normal) serum is used in parallel immunoprecipitations and control samples. Typically, 1 uL of the 50 uL DNA extraction is used for PCR. The DNA is amplified by PCR using 22-26 b primers (ca. 50-55% GC) that have been published (12, 13, 125-127) and that straddle each of the promoter regions. As a negative control, a set of primers is used that are targeted to RARβ and β-actin gene promoters. All PCR products are designed to be ca. 300-400 bps in length, which is the lower limit of the sonicated chromatin size range. The amplified DNA fragments DNA are run on a polyacrylamide gel and viewed by ethidium bromide staining. Although the measurements are semiquantitative

and less quantitative than RT-PCR, they will give a clear indication of whether HMGB1 is bound at these site that have been confirmed to have ER bound.

These experiments will be repeated using anti-ER to confirm that ER is bound to these promoters, as reported (12,13,125). These experiments will be repeated numerous times to establish the statistical significance of the findings.

Potential Problems: As with all chIP experiments, the outcome depends on the strength of the protein-protein interactions and solution conditions used. Lysis and washing will be done with weak non-ionic detergents to hopefully retain any association that exists. Utilizing the same buffers and the same antibodies to ER (purchased form the same supplier) as used by Carroll and Brown (12, 13) should be effective.

AIM4. Compare the relative transcriptional activity of a series of ER binding sites found in *in vitro* binding assays - cERE, cHEREs, DRs, & tandem EREs- in *in vivo* assays. These elements will be used to drive a transiently transfected luciferase reporter gene. The effect of overexpression of HMGB1 on transcriptional activity will be determined.

We found a spectrum of nonconventional ER binding sites by EMSA analysis, in the presence and absence of HMGB1. In the case of cHERE, HMGB1 is required for binding, while in all other cases HMGB1 enhances the binding (cERE, imperfect EREs, cEREn, n=0-4, direct repeats, everted repeats and well separated inverted repeats). In the case of tandem repeats, it produces marked cooperativity of ER binding to both sites (see Figure 2). In addition, HMGB1 facilitates binding of ER to cERE within nucleosomes (& perhaps many more nonconventional EREs). However, no studies have tested the level to which these elements actually drive E2-dependent transcription. We will determine the extent to which these binding sites are actually functional ER response elements *in vivo*. Transient transfection assays with the luciferase reporter gene will be used in a comparison of their activity relative to that for cERE.

a. Determine the relative transcriptional activity of nonconventional ER binding sites *in vivo* and the extent to which multiple sites provide a synergic effect.

We have a solid start on this work (**Prelim Find**). We will use the same procedures to construct a series of plasmids that have ER binding sites to drive the luciferase reporter. We will also use the same experimental protocols in the analyses as outlined in the **Prelim Find** section. Four series, containing twelve ERE sequences, will provide the basic framework for our comparisons and for possible further studies:

i.	cERE;	2cEREs;	3 cEREs
ii.	cHERE;	2 cHEREs;	3 cHEREs
iii.	cERE + 2cHERE;	2cHERE + cERE;	
iv.	cEREn, n = 0, 1, 2 & 4 (cERE3 = cERE)		

Series **i** will show the effect of multiple cEREs and determine if ER binding to tandem elements lead to transcriptional synergy. Series **ii** will do the same, but for half-sites, which have been found in a increasing number of genes (12, 13, 38-40, 132, 133) (effectively these are direct repeats, with half-sites aligned in the same direction). Comparison of the corresponding ERE in series **i & ii** will indicate the strength of half-sites relative to cEREs. Series **iii** will show the effect of multiple, "mixed" EREs (cHEREs & cHEREs, in different orders of alignment relative to TATA box) and how their activity compares with both series **i** and series **ii**. Series **iv** will reveal the effect of spacer size on activity and will be compared to cERE. Findings from this limited series of ERE binding sites will provide the first systematic comparison of transcriptional activity for single and multiple cERE(s) with nonconventional EREs and increase our understanding of the influence that these EREs and their relative positions exert on each other.

These oligonucleotides in series **i-iv**, with XhoI and Bgl II adapters (cut ends), will be purchased from IDT Technologies, annealed, and subcloned into XhoI/Bgl II sites in the pGL2-TATA-Inr-Luc plasmid and transfected in JM109 cells. Individual colonies will be picked, grown up, the DNA constructs purified and the insert size will be determined by PCR. The PCR primers used will produce DNA about 150 bps, making it easy to distinguish an insert. The colony(s) that have the correct insert size will be grown up, the DNA purified and then sent to Retrogen for sequencing. For example, for the cERE, the DNA fragment,
C TCGAG**AGGTCActgTGACCT**A GATCT, will be ligated into the plasmid. These sequences (or the most proximal sequence for multiple EREs) will be 30 bps upstream of the center of the TATA box. Sequences with

multiple cEREs will have an 8 bp spacer (AGATCCGC) between EREs. The cHERE will be prepared similarly, using the cERE-half-site sequence, **AGGTCA**ctgGTTGGG, with the non-binding half-site sequence chosen to minimize ER binding (99). The 3cERE DNA has been sequenced. EMSA binding studies will be done on all constructs to determine the Kd values and determine the extent of cooperativity in tandem EREs.

The U2-OS (ER^-/$HMGB1^+$) cells have been maintained and transfected, as described (**Prelim Find**). Cells (1 x 10^{+6}) will be transfected with the reporter gene constructs (pGL2-"ERE"-TATA-Inr-Luc, ca. 1 ug), pRL-TK (Renilla Luciferase, ca. 1 **n**g) and the ER expression vector (10 ng pCMVflag:hERα from A. Nardulli), with and without the expression vector for HMGB1, (pHMGB-1 from D. Edwards). The cells are treated with 100 nm E2 or ethanol vehicle 6.5 hrs after transfections. Cells are lysed after 24 hrs, treated with Reporter Lysis Buffer (Promega) and assayed for Firefly (FFL) and Renilla Luciferase (RL). The fold induction for FFL is normalized with RL and expressed as the ratio of RLU between treatment groups and control.

Before luciferase assays for the series of EREs can be compared, the level of HMGB1 expression vector (HEV) that optimizes luciferase activity for 3 cERE will be determined with the ER expression vector transfected at 10 ng. We will transfect the cells with a range (5 ng to ca. 2 ug HEV), since Edward's lab has used as high as a few ugs in some studies (23-28). Once the system is optimized, the levels of HMGB1 in a fixed number of U2-OS cells will be compared to the HMGB1 level in HMGB1 transfected U2-OS cells by Western blot to define the level of HMGB1 overexpression that is responsible for any change in activity. The anti-HMGB1 from Millipore will be used again in these studies.

The luciferase assays will then be done on U2-OS cells with transfected ER, with and without transfected HMGB1. In addition to comparisons (i-iv) indicated above, the effect of HMGB1 on the transcriptional activities will be of interest since overexpression of HMGB1 is so prevalent in cancer cells.

Potential Problems: Transient transfection assays are done on a "chromatizied" template in which the chromatin environment is not the same as that which has developed naturally about a DNA (gene) with the cell. Nonetheless, these assays have found extensive use and have provided an excellent means to obtain relative (ERE) activities for a series of promoters/regulatory elements under comparable conditions.

Anticipated Time Line

Year ->	**1**	**2**	**3**
AIM			
1	Do comparative binding (EMSA); get well into comparative HRFPs	Complete **AIM1**	----
2	Nucleosome studies	Continue SWI/SNF studies	Complete **AIM2**
3	Do very preliminary items PCR primers, maintain cells IP studies	Start studies ChIP studies	Complete **AIM3**
4	Carry out transient transfections & luciferase assays	Complete **AIM4**	**----**

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Bowling Green State University

Office of Undergraduate Research
104G University Hall
Bowling Green, Ohio 43403-0288
Phone: (419) 372-2623
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17, October 2007

Dear Madam/Sir:

As Director of the Office of Undergraduate Research I am pleased to provide my strong endorsement of this NIH proposal by Dr. Scovell. Bowling Green State University is deeply committed to undergraduate participation in meaningful research activities and we have been working to firmly establish a culture of undergraduate inquiry and research. The Office of Undergraduate Research (OUR) was created in 2004 with the charge to increase the visibility, prestige, and material support for participation in undergraduate research in order to enhance undergraduate education. In the 3 years since it's inception, programs have been developed to provide financial assistance to undergraduates who wish to do research during the academic year and to assist in travel related expenses for undergraduates presenting their research at conferences. In addition, a summer undergraduate research scholars program was initiated in 2006 that provides stipends, funds for research supplies, and tuition free credits for students to work full time with a faculty mentor for 10 weeks in the summer. Twenty-five scholars were funded in the summers of 2006 and 2007, and it is anticipate the number will grow through time. Finally, a spring symposium on undergraduate research has become an annual campus-wide event.

The PI on this proposal, Dr. Scovell, has actively involved undergraduate students in his research, including mentoring a student through the 10 weeks long Undergraduate Summer Research Scholars Program. Clearly, the PI and BGSU have a strong commitment to undergraduate research and the Office of Undergraduate Research is willing to provide assistance in support of undergraduate student involvement in this project.

With best regards,

John R. Farver
Director
Office of Undergraduate Research

UNIVERSITY OF
ROCHESTER

SCHOOL OF MEDICINE AND DENTISTRY
DEPARTMENT OF BIOCHEMISTRY
AND BIOPHYSICS

Jeffrey J. Hayes, Ph.D.
Professor
Department of Biochemistry and Biophysics
School of Medicine and Dentistry
University of Rochester
Rochester, NY 14642

October 18, 2007

William M. Scovell, Ph.D.
Professor of Chemistry
Department of Chemistry
Overman Hall
Bowling Green State University
Bowling Green, OH 43403

Dear Bill:

It is a pleasure to write in support of your proposed experiments to study estrogen receptor binding to cognate DNA sites and sites assembled into nucleosomes. I have read your proposed Aims and agree that these experiments would be worthwhile and are feasible as planned. It will be very interesting to investigate the binding of ER to cognate half sites in different defined translational positions in nucleosomes and in the presence and absence of HMGB1 and SWI/SNF activities We would be glad to offer technical expertise and support for the reconstitution of nucleosomes from purified components on defined DNA sequences, hydroxyl radical footprinting and missing nucleotide analysis. I wish you the best of luck with your proposal!

Sincerely,

Jeffrey J. Hayes, Ph.D.
Professor

THE UNIVERSITY OF NORTH CAROLINA
AT
CHAPEL HILL

Jason D. Lieb
Assistant Professor
Phone: (9l9) 843-3228
Fax: (9l9) 962-1625
Email: jlieb@bio.unc.edu

Department of Biology
203 Fordham Hall
Campus Box 3280
Chapel Hill, NC 27599

October 17, 2007

Dear Bill,

I'm writing to express my enthusiasm and support for your grant proposal that addresses the role of HMGB1 on ER binding. In living cells, protein-DNA interactions occur in the context of chromatin. Therefore, knowing the affinity of a DNA-binding protein for naked DNA sequences and the genome sequence is not sufficient to explain the pattern of loci that are bound by that factor *in vivo*. That's why the studies you propose are so important. They are rigorous because they arise from a solid foundation of traditional protein-DNA biochemistry, and innovative because they address directly the important "post-genomic" problem of how chromatin cofactors affect DNA binding affinity in a nucleosomal context. In terms of medical relevance and human biology, there is probably not a more important class of DNA binding factors than the ones you have chosen to study, the nuclear hormone receptors.

During your sabbatical in my lab I was impressed by your willingness to learn and apply new techniques to your research program. Clearly, this spirit of continuing to push forward in new directions is reflected in your proposal, and in the students that have come from your lab (like chromatin leader Jeff Hayes). If during the course of your studies there is anything I can do, from advice, protocols, or even another visit down to my lab, you know that my door is open for you and members of your group.

Best of luck with your proposal and research,

Jason Lieb

University of Massachusetts Medical School

Department of Cell Biology
University of Massachusetts Medical School
55 Lake Avenue North
Worcester, MA 01655

October 2, 2007

Dr. William Scovell
Department of Chemistry
Bowling Green State University
Bowling Green, OH 43403

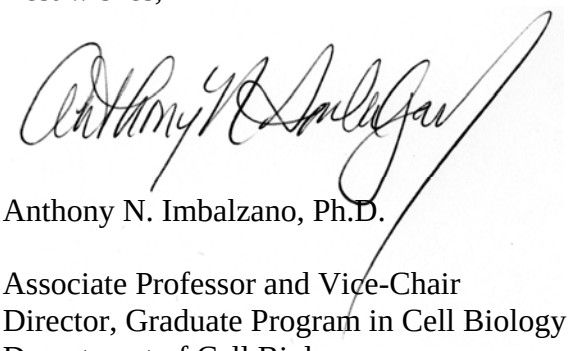
Dear Bill,

I am delighted to provide any support required for your NIH AREA grant application, "Influence of HMGB1, Nucleosomes and SWI/SNF on Estrogen Receptor Binding". The work you are doing on HMGB1 and how it effects estrogen receptor (ER) binding to DNA and to chromatin is novel and timely, and is raising a number of interesting issues in our understanding of ER function.

As you know, we have utilized a large number of protocols and assays to assemble and structurally characterize nucleosomes, nucleosomal arrays, and nucleosomal arrays containing linker histones or non-histone chromatin proteins. We have also extensively examined transcription factor binding to chromatin and the effects of specific histone modifications as well as the consequences of ATP-dependent chromatin remodeling enzymes on chromatin structure, going back to our original characterization of the mammalian SWI/SNF enzymes in 1994. Over the past 6-8 years, we have also developed a repertoire of assays to examine *in vivo* chromatin structure and factor binding to chromatin in both tissue culture cells and in developing embryonic tissue.

I would be pleased to contribute reagents, protocols, and/or technical expertise to your studies at any time.

Best wishes,



Anthony N. Imbalzano, Ph.D.

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PHS 398 Checklist

OMB Number: 0925-0001
Expiration Date: 9/30/2007

1. Application Type:

From SF 424 (R&R) Cover Page. The responses provided on the R&R cover page are repeated here for your reference, as you answer the questions that are specific to the PHS398.

* Type of Application:

❍ New ❍ Resubmission ● Renewal ❍ Continuation ❍ Revision

Federal Identifier: GM054357

2. Change of Investigator / Change of Institution Questions

❑ Change of principal investigator / program director

Name of former principal investigator / program director:

Prefix:
* First Name:
Middle Name:
* Last Name:
Suffix:

❑ Change of Grantee Institution

* Name of former institution:

3. Inventions and Patents (For renewal applications only)

* Inventions and Patents: Yes ❍ No ●

If the answer is "Yes" then please answer the following:

* Previously Reported: Yes ❍ No ❍

OMB Number. 0925-0001
Expiration Date: 9/30/2007

4. * Program Income

Is program income anticipated during the periods for which the grant support is requested?

❍Yes ●No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

*Budget Period	*Anticipated Amount ($)	*Source(s)

5. Assurances/Certifications (see instructions)

In agreeing to the assurances/certification section 18 on the SF424 (R&R) form, the authorized organizational representative agrees to comply with the policies, assurances and/or certifications listed in the agency's application guide, when applicable. Descriptions of individual assurances/certifications are provided at: http://grants.nih.gov/grants/funding/424

If unable to certify compliance, where applicable, provide an explanation and attach below.

Explanation:

Attachments

CertificationExplanation_attDataGroup0

File Name	Mime Type