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GRANT NUMBER DAMD17-98-1-8195

TITLE: Chromatin HMG-I (Y) as a Co-regulatory Protein for Estrogen Receptor Action in Breast Cancer Cells

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REPORT DATE: May 1999

TYPE OF REPORT: Annual Summary

19990929 048

PREPARED FOR: Commanding General
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

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Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 1999	Annual Summary (1 Apr 98 - 31 Mar 99)

4. TITLE AND SUBTITLE
Chromatin HMG-I (Y) as a Co-regulatory Protein for Estrogen Receptor Action in Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-98-1-8195

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Colorado Health Sciences Center
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Through sequence analysis of several estrogen responsive gene promoters, we found that many of estrogen response elements (EREs) are composite elements for ER and potential adjacent or overlapping sites for the non-histone chromatin protein HMG-I(Y). Therefore, we propose that depending on the context of the target gene promoter, HMG-(Y) may either enhance or blunt the activity of ER and could contribute to cellular resistance to estrogen and antiestrogen frequently observed in breast tumors. Two estrogen responsive gene promoters, human C3 and rat prolactin which contain EREs with putative HMG-I(Y) binding sites flanking or overlapping the ERE, were used as a model. Oligonucleotides containing ERE and flanking sequences from both promoter were used in *in vitro* DNA binding assays. A synthetic ERE containing oligonucleotide which has no HMG-I(Y) binding sites was used as control. HMG-I(Y) was found to bind efficiently to both oligonucleotides containing ERE from C3 and prolactin promoter but did not bind to the synthetic EREs oligonucleotide. The binding of HMG-I(Y) to C3 and prolactin promoters appeared to prevent ER from binding whereas HMG-I(Y) had little effect on ER binding to the synthetic ERE oligonucleotides. These results suggest that the presence of HMG-I(Y) binding sites negatively modulates ER DNA binding

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
19

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Background

Estrogen plays an important role in cell proliferation, differentiation and metastatic potential of breast cancer cells (1, 2). Its effects are mediated by estrogen receptor (ER) which belongs to the steroid hormone receptor superfamily of ligand-dependent transcription factors. Steroid hormone receptors (SR) are modular proteins composed of ligand binding, DNA binding and transactivation domains (3), and they bind to specific recognition sequences of steroid regulated genes, termed hormone response elements (HREs). The mechanism by which the DNA-bound SR control gene transcription is not well understood. A number of recent studies have shown that the liganded SR recruits other proteins which can function as coactivators or corepressors (4-7) by transmitting signals from the SR to the basal transcriptional machinery (8). Coactivators and corepressors may also facilitate remodeling of chromatin that is associated with transcription, since they can recruit histone acetylases (HATs) or deacetylases to the promoter (9, 10)that in turn affect nucleosome conformation (11-13).

High mobility group (HMG) proteins are among the best characterized nonhistone chromosomal proteins. This group of proteins includes three different families: HMG-1/-2, HMG-14/-17 ,and HMG-I(Y) family (14). A general property of HMG proteins is an ability to recognize and manipulate distortions in DNA structures which have led to the idea that they are architectural factors required for essential processes that require transient manipulation of DNA structure such as repair, recombination, chromatin packaging, and transcription (15, 16). HMG-1/2 have been implicated as co-factors for transcription regulation since they have been shown to facilitate binding of several sequence-specific transcription factors (17-19) and to enhance transcription activation of these activators including OCT, p53, HOX etc. Disruption of the yeast homologs of HMG-1/2 results in reduction or ablation of selected yeast transcription activators but not all (20). HMG-I(Y) family, including HMG-I(Y) which is the product of two alternative splice variants of a single gene and HMGI-C, have also been implicated as cofactors in activator-mediated transcription (14). Although HMG-I(Y) has a strong preference for recognition of DNA structures more so than nucleotide sequences, preferred consensus recognition sequences for HMG-I(Y) binding have recently been deduced and described (21, 22). Each HMG-I(Y) molecule contains three DNA-binding domains (AT-hooks) which mediate high affinity binding to the minor groove of multivalent A/T rich DNA sequences (21, 23, 24). HMG-I(Y) does not act as a transcription factor itself, but has been shown to be an important co-regulatory factor that can enhance or inhibit activities of other sequence-specific transcription factors, depending on the context of the promoter. To date all the transcription factors which have been described to be modulated by HMG-I(Y) are associated with the immune system. Several promoters contain composite elements with overlapping transcription factor and HMG-I(Y) binding sites. For example, in the human interferon ß (IFN-ß) and interleukin-2 receptor α (IL-2Rα) promoter , HMG-I(Y) binds to several composite elements on the promoter, physically interacts with other transcription factors, bends DNA, and enhances gene transcription (25-27). In contrast, in the interleukin-4 (IL-4) promoter, HMG-I(Y) interferes with DNA binding of NF-AT factors and inhibits the induction of IL-4 gene transcription(28).

Unlike HMG-1/2 which is constitutively expressed, HMG-I(Y) is a highly regulated proteins. HMG-I(Y) is a phosphoprotein and the level of phosphorylation is regulated during the cell cycle (29, 30). In addition, HMG-I(Y) is upregulated during cell proliferation. The expression of HMG-I(Y) mRNAs are very low to nondetectable in quiescent cells and increase in proliferating cells (31). There are several reports that HMG-I(Y) is overexpressed in transformed cells (32, 33). The levels of HMG-I(Y) mRNAs and proteins in tumor cells were estimated to be 15-20 times higher than those of normal cells (34). The expression of HMG-I(Y) also correlates with increased metastatic potential in certain tumor models. For example, HMG-I(Y) mRNAs are highly elevated as the tumors become more metastatic in the Dunning rat model for prostate cancer progression (35) and in regions with high Gleason grade prostate cancer in human (36). Similar findings were also found in malignant human thyroid neoplasias (33). In mouse mammary epithelial model for mammary tumorigenesis, increased levels of HMG-I(Y) mRNAs are directly correlated with the degree of neoplastic transformation and metastatic progression of the cells (37). In addition, alteration in HMG-I(Y) gene loci in preneoplatic cells is associated with the conversion of normal mammary epithelium to the preneoplastic immortalized state (37). In addition, the levels of HMG-I(Y) are increased in human uterine leiomyomas (38) which express high levels of PR (39) and are progesterone responsive (40). These data suggest a link between overexpression of

HMG-I(Y) in tumors of endocrine origin and sensitivity to steroid hormones. Overexpression of HMG-I(Y) in tumor cells and their ability to function as transcriptional coregulators have raised the question of whether HMG-I(Y) proteins broadly effect patterns of gene expression and contribute to tumor phenotypes.

The major obstacle in treatment of breast cancer with endocrine therapies such as estrogen antagonists is that tumors continue to express ER but acquire resistance to anti-hormone upon prolonged treatment. The molecular basis for this resistance is not well understood. The resistance most likely involves changes in expression or activity of steroid receptor coregulatory proteins (41-43). Although a great deal of attention on the molecular mechanism of SR has been focused on recently discovered nuclear receptor coactivators and corepressors, we propose that EREs of certain hormone responsive genes are composite elements that contain binding sites for ER and HMG-I(Y), and HMG-I(Y) could modulate the activity of receptors on these promoters. As a result, alteration of cellular HMG-I(Y) may either inhibit or enhance the sensitivity of receptors to hormone agonists and antagonists. To test these hypotheses, two estrogen responsive gene promoters, human complement 3 (C3)(44) and rat prolactin (45),were used as models to examine whether and how HMG-I(Y) effects ER binding to ERE in these promoters *in vitro*. These ER responsive gene promoter contain putative HMG-I(Y) binding sites either flanking (C3 promoter) or overlapping the ERE (rat prolactin promoter). In addition, we also plan to investigate the functional role of HMG-I(Y) on the transcriptional activity of ER in response to estrogens and estrogen in mammalian cell transfection assays.

Methods

Expression of recombinant human estrogen receptor in the baculovirus system

A recombinant baculovirus transfer vector for human ER was provided by Bert O'Malley and Nancy Weigel, Baylor College of Medicine. The vector was constructed by inserting the human ER cDNA into pBlueBacHis2 (Invitrogen, San Diego CA) in-frame with amino-terminal plasmid sequences that contain an ATG translation start site, six sequential histidine residues and enterokinase cleavage site.

To construct recombinant viruses encoding human ER protein, *Spodoptera frugiperda* insect cells (Sf9) were cotransfected with the baculovirus transfer plasmid with wild type AcNPV baculovirus DNA as previously described (46). Recombinant viruses were identified by visual inspection under a reverse-phase light microscope and individual viruses were plaque purified. Viruses were screened for their ability to express protein by Western blot analysis of infected Sf9 cells. ER proteins were produced in Sf9 cells in 500 ml spin-bottles. Cells were grown in Grace'insect medium (Gibco-BRL) supplemented with lactalbumin hydrolysate, yeastolate, 0.1% Pluronic F68, 10% heat inactivated fetal bovine serum (Hyclone Labs) and 50 µg/ml gentamycin. Cells were grown at 27 C to a density of 1.5 to 1.8 x 10^6 cells/ml, at which time they were inoculated with virus at an MOI of 1.0 and were allowed to grow for an additional 32-36 hr at 27 C. Estradiol was added to the culture for the last 6-8 hr of infection. Cells were harvested by centrifugation at 1500 rpm for 15 min in 50 ml aliquots, washed once in TG buffer (10 mM Tris-HCl, pH 8.0 and 10% glycerol) and frozen as pellets at 80 C.

Purification of polyhistidine tagged estrogen receptor by metal ion affinity columns

Sf9 insect cells were lysed in the following buffer: 20 mM Tris-HCl, pH 8.0, 350 mM NaCl, 10 mM imidazole, 5% glycerol and a cocktail of protease inhibitors (47). All procedure were done at 0-4 C. Cell lysates were centrifuged at 100,000 x g for 30 min and the supernatant taken as a soluble whole cell extract. Whole cell extract was passed over Ni-NTA resin (Qiagen) column at a flow rate of 1-2 ml/min. The resins were washed with lysis buffer until the OD at 280 nM returned to buffer baseline. Bound receptors were eluted under non-denaturing conditions by competition with 100 mM imidazole. Receptor was eluted into siliconized tubes to prevent binding to receptors surface and 1 mM DTT, 1 µM zinc chloride, and $MgCl_2$ were added immediately to stabilize DNA binding activity. Samples were stored at -80 C in aliquots.

SDS polyacrylamide gel electrophoresis and Western blots

Purified ER was analyzed by electrophoresed on 7.5% polyacrylamide SDS gel as described previously (48) and by silver staining as previously described. Proteins were also transferred to supported nitrocellulose membrane, Immobilon-P (Millipore Corp, Bedford MA) and incubated with human ER monoclonal antibody (h151) overnight at 4 C. Immunoblot detection was carried by using ECL kit according to manufacturer's procedures (Amersham).

Electrophoretic gel mobility shift assay (EMSA)

ER-DNA binding by EMSA was performed under the same condition as previously described (49). Purified HMG-I protein and anti-HMG-I antibody were provided by R. Reeves, Washington State University, Pullman WA. Briefly, purified hER (amounts per assay are indicated in Figure legends) was incubated for 1 hr. at 0-4 C with 0.3 ng of an end-[^{32}P] labeled oligonucleotide (specific activity of 100,000 to 300,000 cpm/ng) in a DNA binding buffer containing 10 mM HEPES, pH 7.8, 50 mM KCl, 4 mM $MgCl_2$ and 12% glycerol. The binding reaction also contained 1 µg of ovalbumin as a carrier protein and 100 ng of poly dI-dC/ poly dI-dC to reduce non-specific-DNA binding. After 1 hr, DNA binding reactions were electrophoresed on 5% polyacrylamide (40:1 acrylamide:bis) gel and 2.5% glycerol in 0.25 X TBE buffer (0.02 M Tris-borate, pH 8.3, 0.02 M boric acid, 0.5 M EDTA) with cooling to maintain gel temperature at 4C. Gels were dried and autoratiographed. Sequences of oligonuleotides used in this study are shown below. Bold letters indicate ERE. Underline indicates putative HMG-I(Y) binding sites.

1. Synthetic palindromic ERE oligonucleotide

5'-gatcTCTTGATC**AGGTCA**CT**GTGACC**TGACTT-3'
3'-AGAACTAG**TGGAGT**GA**CACTGG**ACTGAActag-5'

2. Human complement 3 (C3) promoter

5'-gatcAAC**GGCATGTTGGCC**CCAGGGACTGAAAAGCTTAGGAAATGGTATTGAAAT-3'
3'-TTG**CCGTACAACCGG**GGTCCCTGACTTTTCGAATCCTTTACCATAACTTTActag-5'

3. Rat prolactin promoter

5'-gatcTGCATTAAAAAATGCATTT**TGTCACTATGTCCT**AGAGTGC-3'
3'-ACGTAATTTTTTACGTAAA**ACAGTGATACAGGA**TCTCACGctag-5'

Cell culture and transient transfection

COS-1 cells were maintained in DMEM (Gibco-BRL) supplemented with 10% fetal bovine serum (Hyclone Labs) and were plated in multiwells dishes(6 well dishes, Falcon plastic) at density of 165,000 cells/well. Hela cells were plated at the same density in MEM supplemented with 5% fetal bovine serum. Twenty four hours after plating cell were transfected with plasmid DNAs using Lipofectamine for COS-1 cells and Lipofectin for Hela cells (Life Technologies) according to manufacturer's guidelines. A CMV-ß-gal reporter gene was included in all transfection as an internal control for transfection efficiency for each well. Five hours after, the DNA/Lipid mixture was removed and cells were fed with phenol red-free media containing 5% charcoal stripped serum. Twenty four hours after transfection, cells were treated with 17ß-estradiol or 4-OH tamoxifen. Forty eight hours after transfection, cells were harvested and extracts were assayed for luciferase and ß-gal activity as described (49).

Results

Purification of recombinant human ER (hER) for *in vitro* DNA binding studies

Preliminary results in my post-doctoral fellowship application were all done with highly purified progesterone receptor (PR) and target progesterone responsive gene with putative composite HMG-I(Y) binding sites. Because the main focus of the Edward's lab is PR. The reagents and methodologies were readily available to generate preliminary data on HMG-I(Y) and steroid hormone receptor interaction using PR. In order to develop a more independent direction from other lab projects, I chose to study ER-HMG-I(Y) interaction. In this first year of my fellowship, this switch from PR to ER necessitated a considerable amount of development time optimizing conditions for expression and purification of ER and for mammalian cell transfections to assay ER transcriptional activity. Figure 1 shows purification of full length human ER which expressed in Sf9 cells from a baculovirus vector with a N-terminal polyhisditine tag. Sf9 insect cells expressing hER were lysed in buffer containing 350 mM NaCl and low concentration of imidazole (15 mM) to reduce binding of non-specific proteins to the nickel resins. The lysate was passed over a column containing a metal ion affinity resin (Ni-NTA, Qiagen), and bound receptor were eluted by competition with 100 mM imidazole. As judged by silver stained SDS polyacrylamide gel electrophoresis and Western blot (Figure 1), to confirm the identity of protein bands, this method of purification yielded intact his-tagged hER $\approx$ 70 kDa at approximately 50% purity and another major band of slightly slower mobility. In addition to expressing full length hER in the baculovirus system, the hER DNA binding domain (DBD) (amino acid 189-288) has been cloned into pGEX2T (Pharmacia) , expressed as GST tagged protein in bacteria. and purified. This purified ER-DBD will be used in future *in vitro* DNA binding experiments.

HMG-2 but not HMG-I facilitates the DNA binding activity of recombinant purified hER to a synthetic ERE oligonucleotide.

To test whether purified hER was functionally active and capable of binding to target EREs (consensus ERE oligonucleotide), EMSAs were performed in the presence or absence of purified recombinant his-tagged HMG-2. As previously reported (49), addition of purified HMG-2 substantially enhanced the formation of ER-DNA complexes (Figure 2). The DNA complex stimulated by HMG-2 contained ER as demonstrated by supershifting with a monoclonal antibody to hER (h151). In contrast to the stimulatory effect of HMG-2, addition of HMG-I had little effect on binding of purified hER to synthetic consensus ERE probe (Fig 2B). This is not surprising since this synthetic ERE is not predicted to have HMG-I(Y) binding sites.

To examine further the effect of HMG-I on ER-DNA binding to synthetic ERE, two concentrations of hER were used. At low (0.3 µg per reaction) or high concentration of hER (1.0 µg per reaction) of hER , addition of purified HMG-I from 10 ng to 1 µg per reaction had minimal to no effect on ER binding to ERE. In addition, ER-DNA complexes were supershifted only by ER monoclonal antibody but not by HMG-I antibody indicating that HMG-I was not part of ER-DNA complexes (data not shown).

HMG-I inhibits hER binding to ERE from human complement 3 (C3) and rat prolactin promoter.

As purposed in the grant application, there are several putative HMG-I(Y) binding sites overlapping or flanking EREs in certain estrogen responsive promoters. To test whether HMG-I(Y) can bind to these putative binding sites and have any effects on ER binding to ERE in these promoters, ERE from human C3 and rat prolactin promoter were synthesized. The sequences of these oligonucleotide and predicted HMG-I(Y) binding sites are shown in the method section. Purified hER (500 ng per reaction) was able to bind to C3 and prolactin probes (Figure 3). However, addition of increasing amount of HMG-I(Y) abolished hER binding to both C3 and prolactin promoter at the expense of the formation of HMG-I-DNA complexes (Figure 3)

To examine whether ER can compete with HMG-I(Y) for binding to these oligonucleotides, a constant amount of purified HMG-I (50 ng/reaction) was incubated with increasing amount of purified hER (100 ng to 1.2 µg). As shown in figure 4, high dose of hER failed to compete with HMG-I for DNA binding. To determine whether hER and/or HMG-I were part of the upshifted DNA complexes, DNA complexes were incubated with hER antibody or antibody for HMG-I. The complexes were supershifted with HMG-I(Y) antibody but not with hER antibody indicating that only HMG-I bound to DNA and the binding of HMG-I appeared to exclude the binding of hER (Figure 4 A&B).

Effects of HMG-I on ER-mediated transcription in mammalian cells

To investigate whether HMG-I(Y) can modulate ER transactivation of C3 promoter within mammalian cells, ER negative COS-1 and Hela cells were used in transfection experiments. Cells were transfected with mammalian expression vector for full length ER ,pSVMT-wER (a gift from C. Smith, Balyor College of Medicine) and increasing amount of expression vector for HMG-I, pHMG-I (a gift from R. Reeves, Washington State University, Pullman, WA) along with the reporter gene containing C3 promoter linked to luciferase gene (pC3-Luc)(50). Cells were then treated with vehicle (ethanol), 20 nM 17ß-estradiol, 100 nM 4-OH tamoxifen (4HT) or the combination of both ligands. Cotransfection of pHMG-I at all doses tested had minimal to no effect on ER-mediated gene transcription in Hela cells. Similar results was observed in COS-1 cells (data not shown).

Conclusions and Discussion

We have successfully expressed full length hER in the baculovirus expression system. In addition, we also purified hER using non-denaturing condition; however, the receptor preparation still had significance amount of other proteins. We are now working on improving the purity by eluting the bounded receptor from the column by gradient of immidazole or by increasing the concentration of immidazole step-wise during the eluting processes and passing the purified hER over DNA-cellulose column to enrich functionally active receptors.

Previously we showed that HMG-1 and highly related HMG-2 enhanced DNA binding activity of hER and other steroid hormone class of receptors but did not effect DNA binding of nonsteroid nuclear receptors (49). In this studies, we showed that HMG-I(Y) can affect hER binding to ERE; however, the effect seemed to depend on the presence of HMG-I(Y) binding sites flanking or overlapping EREs. In case of the synthetic ERE oligonucleotide which have no predicted HMG-I(Y) binding site, HMG-I(Y) did not have any effects on hER binding to ERE even at high doses (1-3 µg per reaction). In contrast, oligonucleotides derived from human C3 or rat prolactin promoter which contained predicted HMG-I(Y) binding sites flanking or overlapping the ERE, binding of HMG-I(Y) to DNA prevent hER from binding to the ERE even at a very low concentration (10 ng per reaction). However, HMG-I(Y) have minimal to no effect on ER transactivation of C3-reporter gene in Hela and COS-1 cells. This could due to several factors . Although the expression of HMG-I(Y) is highly regulated, HMG-I(Y) is expressed in most cells and is expressed at high level during cell proliferation. Transient transfection experiments used in this study were carried out in active proliferating cells. The expression of HMG-I(Y) from transfected DNA may not be high enough to overcome the background expression of endogenous HMG-I(Y) with in the cells. Therefore, it will be important to examine whether the expression of antisense-HMG-I(Y) mRNA to inhibit endogenous HMG-I(Y) affect ER transactivation of C3 promoter. In addition, HMGI(Y) can physically interact with cell type specific transcription factors (25, 26), therefore, it is possible that HMG-I(Y) may required other cell type specific transcription factors to perform its function. We are now working on testing the effect of HMG-I(Y) on ER dependent gene transcription using endogenous hER in human breast cancer cells. Finally, since there is evidence that HMG-I(Y) could facilitate the assembly of higher nucleosome complexes (51), the effect of HMG-I(Y) might be best seen on stable transfected reporter gene which can assume chromatin structure and nucleosome complexes which are absence in transfected episomal plasmid DNA.

Although HMG-1/2 and HMG-I(Y) are not related at the primary sequence level, they do exhibit similar DNA binding properties (14). HMG-I(Y) recognizes DNA structure, binds in the

minor groove and has been shown to function as an architectural cofactor in the transcriptional regulation of several cytokine genes. HMG-I(Y) through the combined effect of altering DNA conformation and direct protein interaction, facilitates the assembly of higher order of nucleoprotein complexes or enhanceosomes (51). Therefore, it is possible that binding of HMG-I(Y) to sequences flanking or overlapping the ERE in C3 and prolactin promoter might alter the DNA structure of the ERE enough such that the DNA structure can no longer be recognized by hER or become a low affinity site for hER.

HMG-I(Y) differs from HMG-1/2 in that it independently binds an A-T rich recognition sequence and HMG-I(Y) have three DNA binding domains termed A-T hooked that recognized a variety of multivalent A-T tracts of 5 to 8 in length (52). The recent solution structure of the DNA binding domain of HMG-I(Y) complexed to DNA revealed that the AT-hook adopted a well defined extended conformation that lies along the minor groove over consecutive A-T tract base pair. This extended loop conformation is strikingly similar to the carboxyl terminal extension (CTE) of the DNA binding domain (DBD) of an orphan receptor, Rev-Erb-α, referred to as Grip box (53). Orphan receptors are members of nuclear receptor superfamily. The crystal structure of Rev-Erb-α DBD complexed to DNA revealed that Grip box, like the A-T hook of HMG-I(Y), forms an extended loop structure that lies along the minor groove (53). The consensus Grip box sequence (RXGRZP, X=F/G/R, Z= any hydrophobic residue) is conserved in orphan receptors and forms an interaction with the 5'-A-T flanking sequences at the minor groove and is required for high affinity binding of the receptor monomers to the extended half-site DNA response elements. It is interesting to note that certain orphan receptors such as NGFI-B, SF-1, RORα, and Rev-Erb, bind as monomer to an ERE half-site with a 5'-flanking A-T rich sequence specificity (54-57).

In contrast to orphan receptors, the DBD of steroid hormone receptors appear to interact with DNA only in the major groove. There are no sequences in the DBDs of most steroid hormone receptor including glucocorticoid receptor (GR), progesterone receptor (PR), androgen receptor (AR), and mineralocorticoid receptor (MR) that have sequence homology to the Grip box. However, a short sequence in the C-terminal of the core DBD of ER (RRGGRM) has a striking similarity to the Grip box and the A-T hook of HMG-I(Y) (Figure 5A). If the Grip box in ER DBD is functional and binds to the minor groove, HMG-I(Y) could potentially compete with ER for minor groove binding and prevent ER from binding to ERE with high affinity. To date, the crystal structure and NMR solution structure of the ER DBD were done with DBDs that do not contain enough CTE to cover the entire Grip box sequence (58, 59). Therefore, there is no conclusive evidence that the Grip box in the ER DBD is functional and behave similar to Grip box in orphan receptors.

Future plans and statement of work

In the first year of this grant, I have accomplished part of the experiments purposed for aim #1and #2. For aim #1, I have successfully expressed and purified of hER and perform EMSA analysis of oligonucleotides containing ERE and putative HMG-I(Y) binding sites with purified hER and/or HMG-I protein. For aim #2, I performed transient transfection experiments with sense HMG-I mammalian expression vector and determine estrogen and tamoxifen responsiveness from C3-reporter construct. As stated in the result section, due to the some technical difficulties in switching from PR to ER system, I was not able to finish all of the experiments proposed in the submitted statement of work for the first year. However, I am still pursuing the original aims and time table proposed in my grant application.

With this new information regarding the structure of the Grip box of orphan receptors and the A-T hook of HMG-I(Y), I would like to propose a new extension aim to test whether the ER DBD contains a functional Grip box using two approaches. 1) The sequence of the Grip box in ER DBD will be mutated. The putative Grip box in ER, RRGGRM, will be mutated to RRLLLM since the second G in the Grip box is required for minor groove interaction as implicated by the crystal structure (53). 2) Domain swapping experiments. The core DBD of ER will be fused to the CTE of PR or Rev-Erb. Conversely, the PR core DBD will be fused to the CTE of ER (Figure

5B). These chimeric DBDs will be expressed, purified, and compared with wild type ER and PR DBD for DNA binding affinity in the presence and absence of HMG-I. If the CTE of ER contains a functional Grip box, it should exhibit high affinity recognition of ERE half-sites with an A-T rich 5' sequence similar to that of orphan receptor. Mutation of ERE DBD Grip box or swapping ER CTE with the CTE of PR which lacks the Grip box should decrease ER binding affinity. If HMG-I(Y) prevents ER binding to ERE by competing with the minor groove binding of ER DBD Grip box, HMG-I(Y) should have very little or no effect on the affinity of the Grip box mutant or the chimera DBD. These experiments could bring about a significant finding in term of ER functions. The findings will help explain the molecular mechanism by which HMG-I(Y) prevent ER binding to ERE. In addition, if ER contains a functional Grip box in its DBD and can bind with high affinity to ERE half-sites with A-T rich 5' sequence, this suggests that ER can functionally bind and activate gene transcription through these previously unrecognized EREs.

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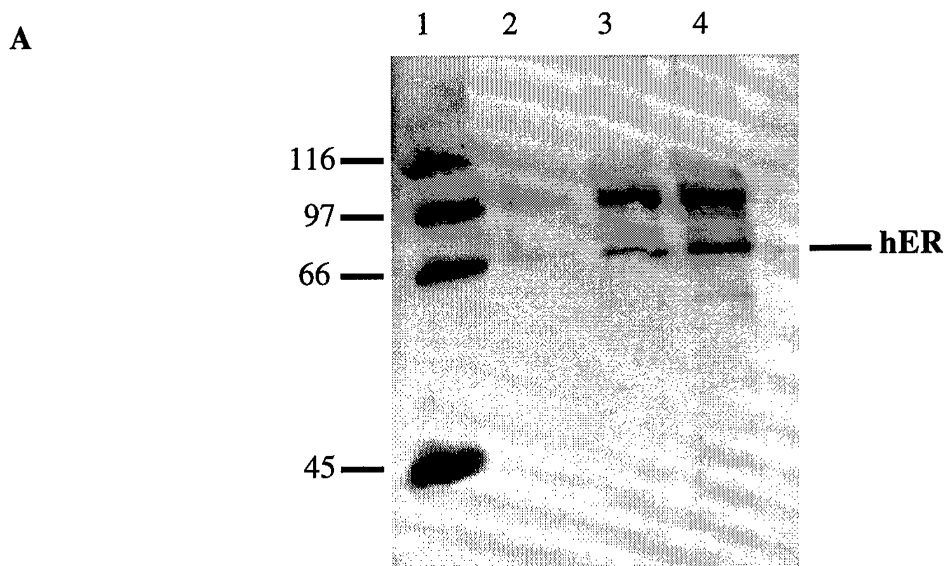

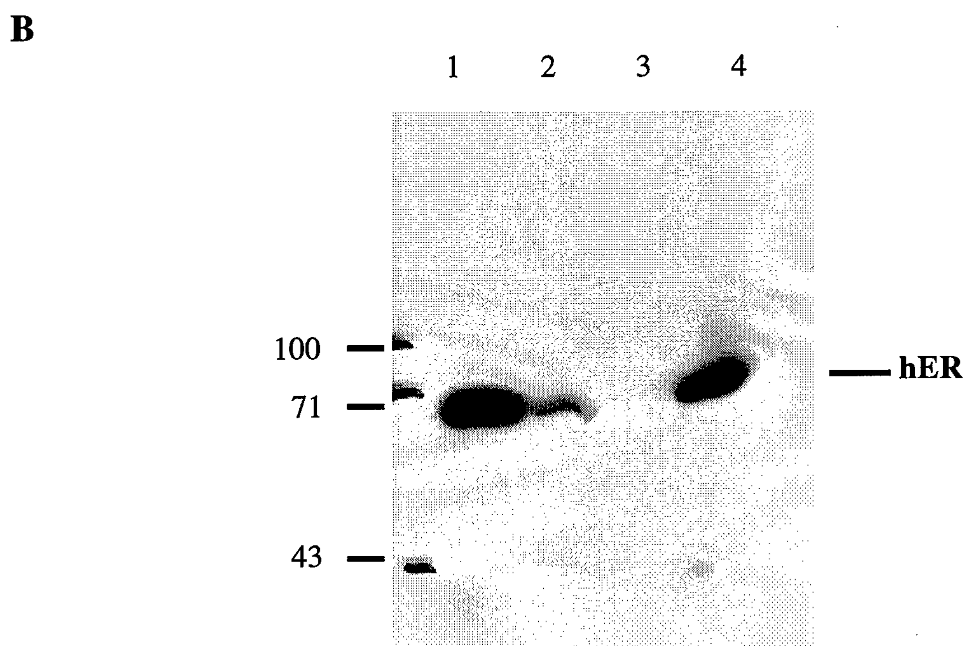

Figure 1 Purification of baculovirus expressed human estrogen receptor (hER). Human ER were expressed as polyhisditine tagged proteins in baculovirus and were purified by metal ion affinity column (Ni-NTA). **A)** Silver stained polyacrylamide SDS gel electrophoresis of hER purification fraction. Lane 1, Molecular weight markers; Lane 2-4, purified fraction of hER (10, 20, and 30 μl , respectively). **B)** Western blot of purified hER. Lane 1, Sf9 whole cell extract (5 μl); Lane 2, Nickel resin flow-through (5 μl); Lane 3, Column wash (50 μl); Lane 4, Purified hER 100 mM imidazole eluate (10 μl).

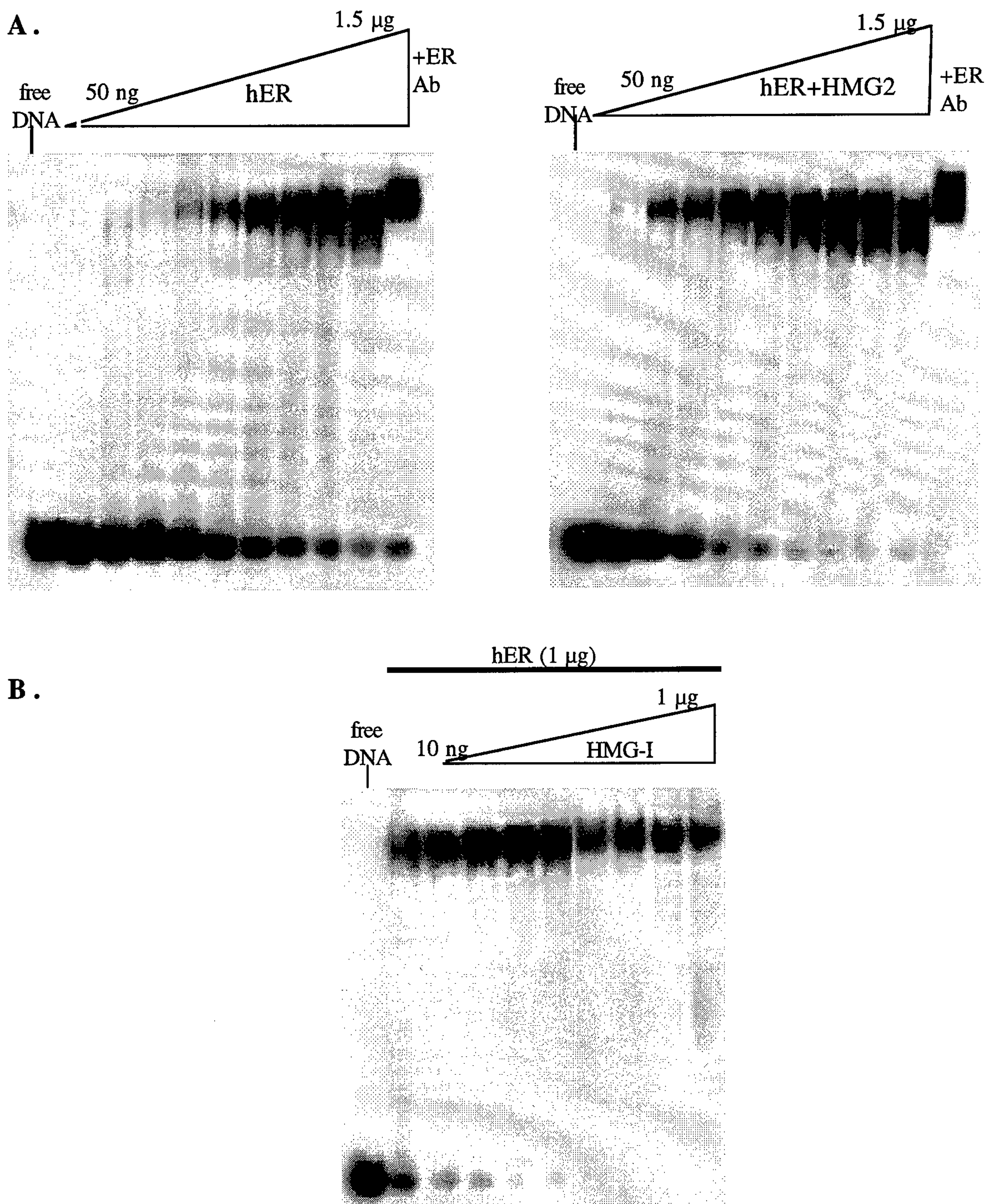

Figure 2 A) HMG-2 increased the binding activity of hER to a consensus ERE probe. 50 to 1.5 μg of purified hER were varied in DNA binding reactions against a constant amount (0.3 ng) of [^{32}P] labeled synthetic ERE oligonuleotide and DNA binding was analyzed by EMSA. The receptor was assayed alone (left panel) or with addition of HMG-2 (500 ng) (right panel). Purified hER was quantitated by Bradford assay and silver stained SDS gels comparing receptor band intensities with those of known amounts of purified bovine serum albumin. **B)** HMG-I has little to no effect on ER binding to synthetic consensus ERE probe. A constant amount of hER (1.0 μg) was incubated alone (lane 2) or with varied amounts of HMG-I (10 ng to 1 μg) (lane 3-10) in DNA binding reaction against a [^{32}P] labeled synthetic consensus ERE oligonuleotide and DNA binding was analyzed by EMSA.

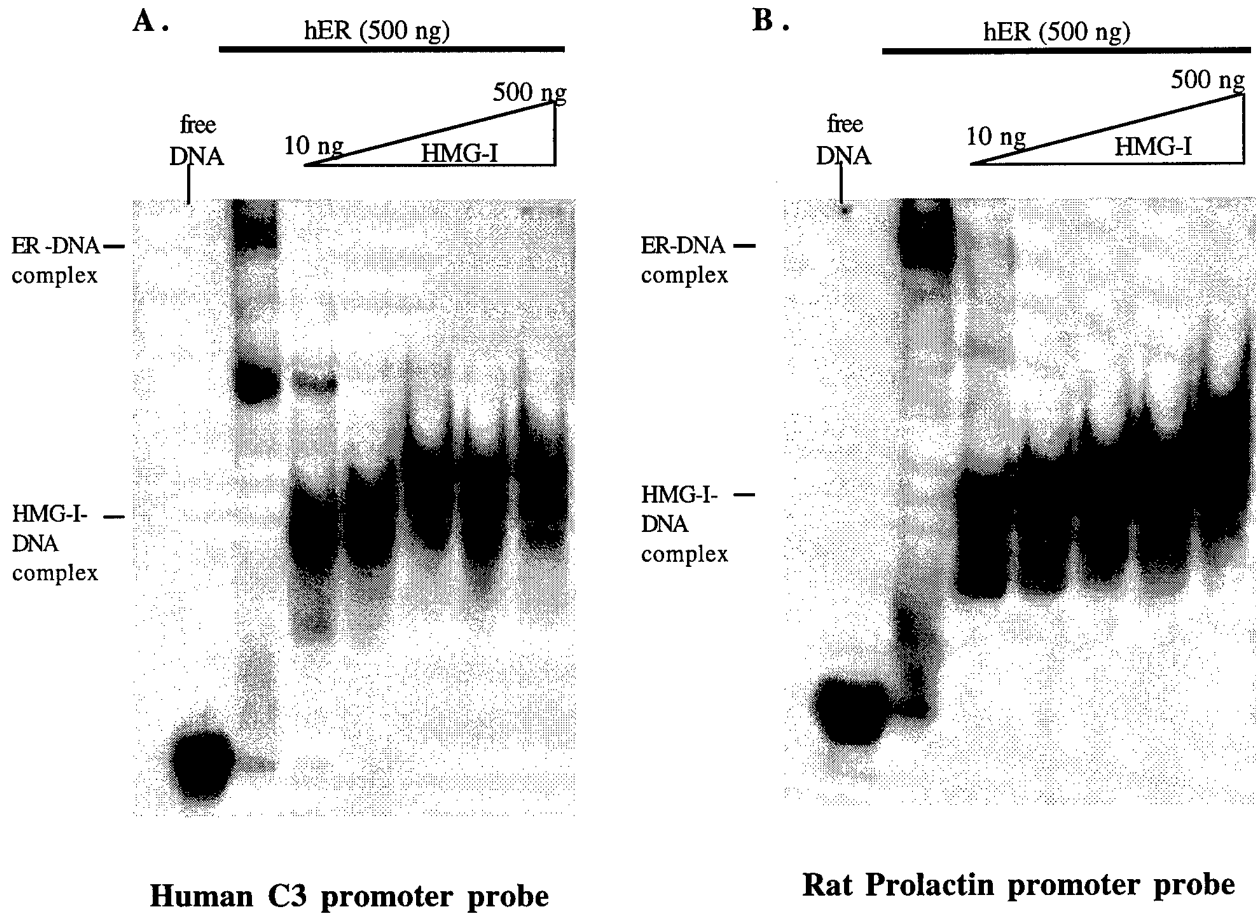

Figure 3 HMG-I inhibits hER binding to ERE in oligonucleotides from human C3 promoter and rat prolactin promoter. A constant amount of purified hER (500 ng) were incubated with a constant amount of [^{32}P] labeled oligonucleotides containing ERE from **A)** human C3 promoter or **B)** rat prolactin promoter and DNA binding was analyzed by EMSA. The receptor was assayed alone (lane 2) or with addition of varied amounts of HMG-I (10 ng to 500 ng) (lane 3-7).

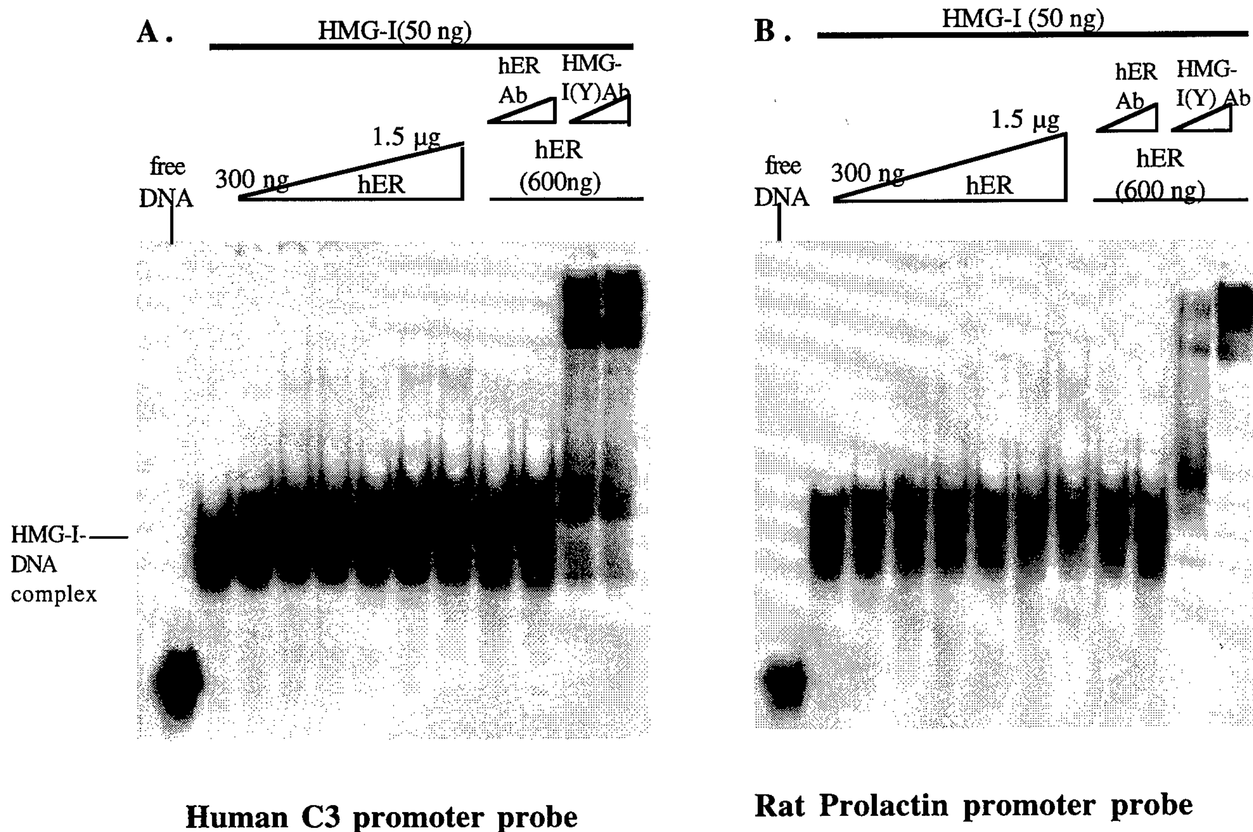

Figure 4 hER failed to compete with HMG-I for binding to DNA containing both ERE and HMG-I(Y) binding sites. A constant amount of HMG-I (50 ng) were incubated with a constant amount of [^{32}P] labeled oligonucleotide containing ERE from **A)** human C3 promoter **B)** rat prolactin promoter and the DNA binding was analyzed by EMSA. HMG-I was assayed alone (lane 2) or with the addition of varied amounts of purified hER (300 ng to 1.5 μg) (lane 3-8). hER specific antibody was added to DNA binding reactions in lane 9-10, (1 and 2 μl, respectively). HMG-I(Y) specific antibody was added to DNA binding reactions in lane 11-12 (1 and 2 μl respectively).

A.

		Core DBD (Zn++ Modules)				CTE	
Steroid Receptors	PR	C C	C C	C C	C C	GM VLGGRKFKKFNKVRVVRALDAVALPQPVGVPNESQALS	
	GR	C C	C C	C C	C C	GM NLEARKTKKKIKGIQQATAGVSQDTSENPNKTIVPAAL	
	AR	C C	C C	C C	C C	GM TLGARKLKKLGNLKLQEEGEASSTTSPTEETTQKLTVS	
	ER	C C	C C	C C	C C	GM MKGGIRKDR**RGGRM**LKHKRQRDDLEGRNEMGASGDMRA	GRIP box?
Class II Receptors	VDR	C C	C C	C C	C C	GM MKEFILTDEEVQRKREMIMKRKEEEALKDSLRPKLSEE	
	RAR	C C	C C	C C	C C	GM **SKEAVRNDR**NKKKKEVKEEGSPDSYELSPQLEELITKV	T box } Helix 3
	RXR	C C	C C	C C	C C	GM **KREAVQEER**QRGKDRNENEVEST	T box } Helix 3
	TR	C C	C C	C C	C C	GM ATDLVLD**DSKRLAKRKLIEENR EKRRREEL**QKSIGHKP	A box } Helix 3
Orphan Receptors	RevErb	C C	C C	C C	C C	GM SRDAV **RFGRIP**KREKQRM	GRIP box (RXGRZP)
	SF-1	C C	C C	C C	C C	GM RLEAVRADR M **RGGRNK**RGPMYKRD	
	NGFI-B	C C	C C	C C	C C	GM VKEVVRTDSLKG**RRGRLP**KPKQPPD	
	HMG-I/Y					RKP**RGR**PKK	A/T hook

B.

ER DBD Chimeras

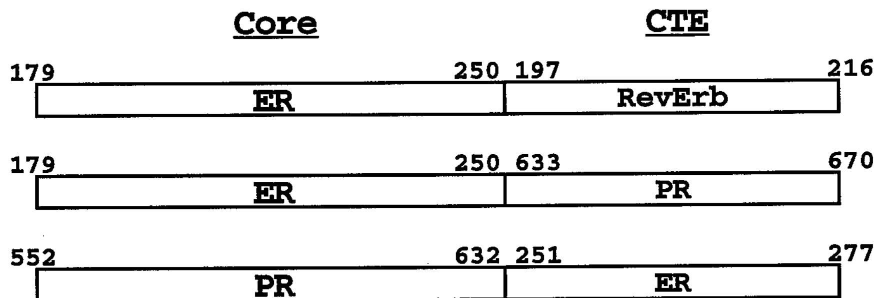

Figure 5 A) Alignment of DBDs of three different classes of nuclear receptors. GM is the conserved Gly-Met boundary between the core zinc binding module with conserve Cys residues and the C-terminal extension (CTE). The bolded sequences in the CTE show α helix 3 in class II receptors, the Grip box in orphan receptors, and the Grip box-like sequences in ER. The sequence of the A-T hook in HMG-I(Y) is shown at the bottom. **B)** Proposed core DBD-CTE chimeras