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FOREWORD

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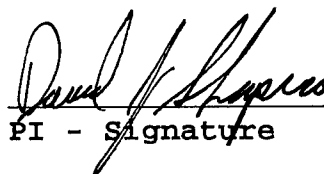
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INTRODUCTION

Estrogens, acting through the estrogen receptor (ER), play a critical role in regulating the growth and metastases of breast cancers. Growth promotion by estrogen is thought to involve direct estrogen receptor-mediated regulation of the expression of several genes important in cell growth, including those encoding some growth factors (such as TGF- α , IGF1, and their receptors), some early response genes (such as c-myc, and cyclin D1), and other genes, including the progesterone receptor gene. Interference with estrogen activity, usually based on antiestrogens, such as tamoxifen, or on aromatase inhibitors, therefore represents a mainstay in breast cancer treatment. While antiestrogen therapy is often effective initially, the tumors almost always eventually progress to estrogen-independent growth. This limits the long-term utility of endocrine therapies.

It is usually accepted that the ability of the 17 β -estradiol-ER complex to influence the growth and metastasis of breast cancer cells is due to its ability to regulate the expression of specific genes. The estradiol-ER complex acts directly to induce the expression of a set of "early genes" including c-myc, cyclin D1 and TGF- α , and other genes important in cell growth (1-16) by directly interacting with these genes. These early genes, and the products of other directly regulated genes, such as the progesterone receptor, may also initiate a regulatory cascade leading to the regulation of downstream genes important in growth control in breast cancer cells.

It has been widely proposed that the 17 β -estradiol-ER complex induces breast cancer cell growth by directly or indirectly regulating the expression of genes important in cell growth control. **If genes critical to growth control in breast cancer cells are directly induced by the estradiol-ER complex, then repression of estrogen-dependent and estrogen-independent transcription of these genes should block estrogen stimulated growth of the breast cancer cells.** Although it is known that estrogen growth autonomous cells synthesize high levels of growth factors that are normally under estrogen regulation (2-4,6,12,13,17-21), the hypothesis that the high level expression of growth factor genes is responsible for growth of these cells has never been tested directly. We will test this hypothesis by repressing the transcription of these growth factor genes and determining the effect on breast cancer cell growth. If this hypothesis is correct, suppression of the expression of these genes should result in the loss of estrogen-independent growth of these cells, and possibly in their death. In addition, identification of genes as critical for estrogen-dependent or estrogen-independent tumor cell growth provides a basis for the identification of additional agents to suppress their activity.

Background

The KRAB repressor system.

When tethered to a DNA binding domain the KRAB repressor, a 75 amino acid segment found in a number of DNA binding proteins, can efficiently suppress transcription of synthetic genes containing strong binding sites for the protein (22-28). Efficient repression of the natural HIV TAT gene has also been reported (27).

The P22 challenge phage system.

Any sequence-specific DNA binding protein can act as a repressor of transcription if its binding site is close to the transcription initiation site. In our application of the P22 challenge phage system (29, 30), we generated a recombinant phage with an ERE close to the transcription initiation site of the phage P22 Ant gene, whose expression results in lysis of the bacterial host cell. If a steroid receptor mutant with sufficient affinity binds to this ERE it will block the transcription of the gene, allowing the growth of bacterial colonies. Although conceptually quite simple, work with the challenge phage system is quite complex and it required a major development effort to modify it so that it could be used for the first time with a vertebrate protein

Project Overview

We developed a phage selection system which allows us to select estrogen mutants with an enhanced ability to bind to the estrogen response element. The enhanced affinity mutants will therefore bind efficiently to the relatively weak, imperfect, EREs present in most estrogen-regulated genes. In parallel we introduced into various forms of the ER the KRAB box which has been shown to be a potent repressor of transcription of genes to which they are bound. The resulting novel, ligand regulated, ER chimeras are termed estrogen regulated gene-repressors, (ERG-repressors). The ERG-repressors have the capacity to efficiently repress both estrogen-dependent and estrogen-independent transcription from ERE-containing genes in both ER-dependent and ER-independent breast cancer cells. The development of the ERG-repressors, which is detailed below, formed the core objective of our first years work.

In subsequent studies we will express the most effective ERG-repressor in breast cancer cells and determine whether repression of the expression of estrogen-regulated genes blocks the growth of estrogen-dependent breast cancer cell lines, and of our tamoxifen resistant and estrogen (E_2) growth-autonomous lines of MCF-7 cells, and of ER negative MDA-MB-231 cells, both in cell cultures and in tumors formed in immune suppressed mice. These studies will serve as a base for a potential gene therapy approach to breast cancer treatment. In addition these studies will test the hypothesis that expression of ER-regulated genes in breast cancers, is critical to estrogen-independent and estrogen-dependent tumor growth.

BODY

In the first section of the Progress Report which corresponds to Experimental Methods we describe the construction of the numerous estrogen receptor-KRAB chimeras developed and tested in the first year of this project. This section also contains descriptions of other experimental methods used in this work. In the second section of the Progress Report we describe the results obtained when we tested the ability of the candidate ERG-repressors to suppress expression of estrogen-regulated genes.

Materials and Experimental Methods

Construction of ERG-repressors.

p18 KRAB Constructions.

Dr. Luigi Lania (University of Naples 'Frederico II', Italy) provided us with the cDNA of p18KRAB. This protein only contains the KRAB A domain. The KRAB domain consisting of aa 180-235 of the p18 gene was cloned either on the N-terminus or the C-terminus of the human estrogen receptor and a number of transcriptionally inactive dominant negative mutants in the following ways.

P18 KRAB on the N-terminus of hER, hERS554FS and hERL540Q.

The p18 KRAB domain was PCR amplified with the following primers

Forward: TAGAATTCATGCTCCTAACAGCCCAG

Reverse: GAGGGTCATGGTCATGGTCTCCCATTC

The product was then digested with MspI and ligated to the N-terminal MspI/FseI. The resulting ligation product was then digested with EcoRI/NotI. Upon gel purification the resulting fragment was ligated into the pCMV5hER backbone that was ligated with EcoRI/NotI. To obtain the KRAB ER chimeras of the dominant negative mutants hERS554FS and hERL540Q (31, 33), the wild type ligand binding domain was exchanged as an XbaI/BamHI fragment.

KRAB on the C terminus of hER, hERL540Q and Δ A/B-hER

Using the Quikchange mutagenesis strategy (Stratagene) a unique NheI site was introduced at the C-terminal end of the hER and hERL540Q.

Forward: GAGGCAGAGGGTTTCCTGCTAGCTGCCACAGTCTGAG

Reverse: CTCAGACTGTGGCAGCTAGCAGGAAACCCTCTGCCTC

The p18 KRAB domain was PCR amplified using the following oligonucleotides:

Forward: TGAGAGCTAGCAGCCCAGCCCCAGGAG

Reverse: GATCAGCTAGCCCTCGGTCATGGTCTCCCAT

The resulting PCR product was digested with BamHI/XbaI and ligated into the NheI/BamHI digested backbone of hER and hERL540Q. The Ligand binding domain of the resulting ER-KRAB chimeras was obtained as an XbaI/BamHI fragment and ligated

into the backbone of similarly digested $\Delta A/B$ -hER. This same procedure was also followed with wild type ligand binding domain KRAB fusion and the N-terminal KRAB hER backbone to construct an hER-KRAB chimera containing KRAB domains at both the N- and C-terminus.

Cloning of ER KRAB chimeras with ZNF10 (Kox1), ZNF133 and ZNF140 KRAB

The following KRAB domains containing both the KRAB A and KRAB B domain were also used to generate more potent ER KRAB chimeras. The ZNF10 KRAB cDNA (25) was a kind gift of Dr. Hans-Jürgen Thiesen (University of Rostock, Germany), while the ZNF133 and ZNF140 cDNAs (24, 26) were sent to us by Dr. Henrik Vissing (Novo Nordisk, Denmark).

The following fragments from plasmid pCMV5hER were subcloned into vector pGEM11Zf(+) (Promega): 1) the N-terminal fragment EcoRI/NotI; 2) the NotI/HindIII fragment containing the ligand binding domain; 3) The HindIII/BamHI C-terminal fragment of pCMV5hER and pCMV5hERL540Q (31, 33). Quikchange mutagenesis was employed to generate unique NheI sites in these fragments to generate the vectors pG11EnsNhe, pG11EnhNhe, pG11EbhNhe and pG11QbhNhe respectively.

Quikchange primers for pG11EnsNhe

Forward: GCCCGCGGCCACGGACCGCTAGCAATGACCATGACCCTCCA
Reverse: TGGAGGGTCATGGTCATTGCTAGCGGTCCGTGGCCGCGGGC

Quikchange primers for pG11EnhNhe

Forward: AAGTATGGCTATGGAGCTAGCCAAGGAGACTCGCTA
Reverse: TAGCGAGTCTCCTTGGCTAGCTCCATAGCCATACTT

To introduce the NheI site into pG11EbhNhe and pG11QbhNhe the same oligos were used as described above for the cloning of the C-terminal p18KRAB fusion.

Thermocycler sequencing with the BigDye kit (ABI Prism) was employed to verify that the sequences were correct.

For the N-terminal KRAB-ER chimeras the following oligonucleotides were used for PCR amplification using Platinum Taq DNA polymerase(GibcoBRL):

ZNF10 (aa 1-91)

Forward: CAGAATTCATGGATGCTAAGTCACTAAC
Reverse: TATCTAGAAATGCAGTCTCTGAATCAG

ZNF133 (aa 1-119)

Forward: CAGAATTCATGGCATTTCAGGGATGTG
Reverse: TATCTAGAGGCTGGATGTTACCTTCTG

ZNF140 (aa 1-106)

Forward: CAGAATTCATGTAGGGGTCAGTGAC
Reverse: TATCTAGAATTCTTCCATGATCAAATAC

The amplified products were subcloned into the pGEM T-vector (Promega) and the sequence was verified using Thermocycler sequencing with the BigDye kit (ABI Prism). The inserts were obtained as EcoRI/XbaI fragments and together with either the NheI/NotI fragment of plasmid pG11EnsNhe cloned into pCMV5hER digested with EcoRI/NotI or with the NheI/HindIII fragment of pG11EnhNhe into pCMV5hER digested with EcoRI/HindIII. These manipulations generated the following clones:

K10-ER	K10-ΔA/B-ER
K133-ER	K133-ΔA/B-ER
K140-ER	K140-ΔA/B-ER

For the C-terminal KRAB-ER chimeras the following oligonucleotides were used for PCR amplification using PlatinumTaq (GibcoBRL):

ZNF10 (aa 1-91)

Forward:	CTTCTAGATATGGATGCTAAGTCACTAAC
Reverse:	ATGGATCCTAAATGCAGTCTCTGAATCAG

ZNF133 (aa 1-119)

Forward:	CTTCTAGATATGGCATTTCAGGGATGTG
Reverse:	ATGGATCCTAAGGCTGGATGTTACCTTCTG

ZNF140 (aa 1-106)

Forward:	CTTCTAGATATGTAGGGGTCAGTG
Reverse:	ATGGATCCTATCTTTCCATGATCAAATACTG

The amplified products were subcloned into the pGEM T-vector (Promega) and the sequence was verified using Thermocycler sequencing with the BigDye kit (ABI Prism). The inserts were obtained as XbaI/BamHI fragments and ligated into NheI/BamHI digested plasmids pG11EbhNhe and pG11QbhNhe, respectively. The ER LBD-KRAB fusions were obtained as XbaI/BamHI fragments and cloned into pCMV5hER and pCMV5ΔA/B-hER digested with XbaI/BamHI as well as their similarly digested N-terminal chimeras generating the following chimeras:

ERK10	ERQK10
ERK133	ERQK133
ERK140	ERQ140
KERK10	KERQK10
KERK133	KERQK133
KERK140	KERQK140
ΔA/B-ERK10	ΔA/B-ERQK10
ΔA/B-ERK133	ΔA/B-ERQK133
ΔA/B-ERK140	ΔA/B-ERQK140

K- Δ A/B-ERK10
K- Δ A/B-ERK133
K- Δ A/B-ERK140

K- Δ A/B-ERQK10
K- Δ A/B-ERQK133
K- Δ A/B-ERQK140

Flag-GAL4-KRAB constructions

To serve as controls a number of Flag-GAL4-KRAB chimeras were constructed. Dr. Cheng Ming Chiang (University of Illinois) provided us the Flag-GAL4-VP16 fusion protein cloned into the bacterial expression plasmid pET11d (Novagen). For our purpose we sub cloned the coding sequence into a mammalian expression vector for mammalian expression in the following way: the parent plasmid was digested with NcoI and filled in with Pfu polymerase. Subsequently the insert was liberated with a BamHI digest. The gel purified fragment was then ligated into the vector pCDNA 3 (Stratagene) that was digested with HindIII, filled in with Pfu polymerase and subsequently digested with BamHI to generate plasmid pFGVP16. Subsequently the C-terminal end in conjunction with a polylinker was obtained as a PCR fragment from the plasmid pM (Clontech) in which the Dam methylation sensitive BclI site was changed into an ApaI site. The PCR fragment was digested with XhoI/ApaI and ligated into the similarly digested plasmid pFGVP16 to generate plasmid pFGmcs. The N-terminal KRAB domains of ZNF10, ZNF133, ZNF140 were obtained as EcoRI/BamHI fragments and ligated into plasmid pFGmcs, which provided the stop codon generating the vectors pFGK10, pFGK133 and pFGK140, respectively.

Reporter plasmids

To serve as an indicator of repression the following plasmid containing an estrogen responsive promoter displaying high endogenous activity in the absence of estrogen receptor was constructed. The estrogen response elements were obtained from plasmid (ERE)₄-TATA-CAT (33), which was digested with HindIII, blunt ended with Pfu polymerase and religated to generate an NheI site. Subsequently an NheI/BglII digest was performed to liberate the EREs. This fragment was then ligated into the similarly digested vector pGL3-Control (Promega). A similar plasmid was generated for the Flag-GAL4-KRAB constructs in the following way. Five GAL4 binding sites were lifted from the plasmid pG5E1b (34) as an XhoI/BamHI fragment and inserted into either XhoI/BglII or BglII/Sall digested plasmid pGL3-Control to generate plasmids 5'-G5-pGL3-Control and 3'-G5-pGL3-Control, respectively.

Cell maintenance and transfection

The human hepatoma cell line HepG2 and Simian COS-7 cell line were maintained at 37 °C, 5% CO₂ in Dulbecco's Minimal Essential medium (Sigma) supplemented with 10% charcoal dextran stripped fetal bovine serum (Atlanta Biologicals) and 50,000 U/I Penicilline and 50 mg/I Streptomycin (Gibco/BRL).

The hamster cell line CHO was maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12, 1:1 (Sigma), 29.2 mg/I L-Glutamine (Sigma), 5% charcoal dextran stripped newborn bovine serum (Atlanta Biologicals), 50,000 IU/I Penicilline and 50 mg/I Streptomycin

Cells were transfected with the calcium phosphate coprecipitation method as published previously. Briefly, Cells were plated in 60mm dishes at a density of 4.5×10^5 for HepG2 cells and 2×10^5 for CHO cells, respectively. Forty hours after plating the media was replaced and 2-6 h later the Calcium phosphate crystals were added. The next morning cells were shocked for three minutes with 10% glycerol in a Tris buffered saline solution (TBS). The cells were harvested 48 h later by scraping with a rubber policeman.

Reporter gene assays

For the experiments involving the p18 KRAB-ER chimeras CAT reporter constructs were used, while pCMV-Luciferase served as an internal control. CAT activity was determined by the quantitative mixed-phase assay as described previously (35). The more recent experiments involving the Kox1, ZNF133 and ZNF140 KRAB ER chimeras the dual luciferase assay (Promega) was used according to manufacturers instructions with the PRL-SV40 serving as an internal control.

Results

To identify the most potent ERG-repressor, we separately co-transfected into CHO cells the constitutively active EREVITCAT promoter (36) and each of 7 different p18 KRAB repressors (Fig. 1). The basal activity of the EREVITCAT promoter in the absence of transfected reporter was established as a reference. Wild-type ER in the presence of ligand elicited a 2.2 fold increase compared to the no ligand control. The ERK repressor was the most effective repressing the basal activity of the promoter by 54% (Fig. 1). To examine repression in more detail we performed a dose response curve EREVITCAT promoter and the ERK and $\Delta A/B$ -ERK repressors. Clearly, ERK is the most potent of the two yielding 85% repression at the 100 ng dose (Fig. 2). While these data suggested that ERK would be a potent ERG-repressor, subsequent studies demonstrated that ERK would not provide a generally useful ERG-repressor. ERK failed to repress activity of the constitutively active 2ERETKCAT promoter (37) when wild-type ER was present, producing only a 38.5% inhibition of promoter activity at a 20:1 ratio of ERK:ER (Fig. 3).

Although the p18 repressors were effective under some conditions, repression was neither as universal nor as potent as desired. The KRAB domain can be subdivided into two sub domains the KRAB A and the KRAB B domain. The KRAB A domain is absolutely required for repression activity, while the KRAB B domain can further potentiate this action. The p18 KRAB domain only consists of the KRAB A domain. Earlier studies using GAL4 binding domain fusions compared p18 KRAB with other KRAB domains containing both the A and B sub domains (22), appeared to indicate that at higher levels of transfected KRAB the levels of repression converged. However, we could not exclude the possibility that the KRAB A domain by itself would be substantially weaker than the A + B domains together in a situation where the KRAB domain had to compete with the endogenous activation domains present in the ER to which it was fused. We therefore requested the cDNA clones of the Kox1 (or ZNF10, see ref. 25), ZNF133 and ZNF140 genes (24,26), all of which contain KRAB A+B

repression domains. Kox1 has been described most widely in literature. However, work by Vissing et al. (26) demonstrates that all three of these candidates should exhibit approximately equal potency as gene repressors. To exclude the possibility that lack of repression was due to an unusual interaction with the estrogen receptor, we also cloned GAL4-KRAB fusions and tested these on the G5-pGL3-Control vector in HepG2 cells (Fig. 4). The GAL4-Kox1 fusion potently repressed transcription. In this experiment the ZNF133 domain had approximately equal to slightly lower potency. We therefore are focusing our studies on the Kox1 variants of the various ERG repressors that we have recently cloned recently.

Our next experiments were then to establish whether the ER-Kox1 variant would also prove to be a potent transcription repressor (Fig. 5). In the first experiment we set out to establish a dose response curve for the KER10 variant. At 5 ng of transfected receptor we achieved 57% repression, with repression reaching a maximum of 71% at 40 ng of transfected receptor. At higher levels KER10 begins to affect the internal standard as well, which is reflected in the graph by the fact that the amount of repression appears to diminish, while in absolute terms repressor was more potent. The Kox1 receptors clearly are more potent at lower doses than the p18 KRAB constructs in CHO cells. This is particularly true since higher levels of expression are usually achieved in CHO cells from the CMV promoter compared to HepG2 cells.

To determine whether ablation of AF2 (as in KERFS10) or AF1 (as in $\Delta A/B$ -KER10) function, or the addition of a second KRAB domain (as in KERK10) could further potentiate repression, we compared the potency of a sub set of constructs at a single dose. While differences are small, the results show that both the deletion of AF1 and the addition of a second KRAB further potentiate repression (Fig. 6). The lack of potentiation by the ablation of AF2 function may result from the fact that this particular mutation causes the receptor to have a lowered affinity for its response element (32). The next obvious question then became whether the repressor would function in a ligand dependent fashion. HepG2 cells do not contain endogenous estrogen receptor. In the absence of transfected receptor the absence or presence of ligand (10^{-8} M Moxestrol, a slowly metabolized estrogen) has no influence on the level of activity of the promoter. Estrogen receptor robustly activated transcription in the presence of ligand alone. The KERK10 repressor potently repressed transcription in the presence of ligand, but only marginally in the absence of ligand (Fig. 7).

Finally, the KERK10 repressor can also potently repress both the ability of the wild-type ER to activate transcription from the EREs in the promoter and the constitutive activity of the promoter itself in the presence of wild type estrogen receptor. We compared the levels of repression in the presence and absence of 10 ng of cotransfected hER. The results demonstrate that at higher doses of KERK repression is hardly affected by the presence of estrogen receptor (Fig. 8). We would therefore expect the KERK10 to be an effective ERG-repressor in both the presence and absence of estrogen receptor.

Recommendations in Relation to the Statement of Work

The statement of work was prepared for a three year time frame with the first task to be completed in year one and task 2 within the first 18 months.

Task 1: Months 1-12: The preparation of potent repressors of estrogen regulated genes.

1. The use of the bacteriophage P22 challenge phage system to generate ER mutants with an enhanced affinity for the ERE. This task is essentially completed. We have identified ER-DBDs with >10 fold higher affinity for the ERE compared to the wild type ER DBD and full length receptors with up to 5-fold increase in affinity (TABLE 1). These mutants are also very effective in binding to a naturally occurring non consensus binding site, the ERE in the PS2 gene.
2. The insertion of the KRAB repressor domain at either the N- or C-terminus or both of an inactive estrogen receptor mutant. This task has been completed not only for the p18 KRAB domain but also for the Kox1, ZNF133 and ZNF140 KRAB domain. We will now focus our attention on the Kox1-ER chimeric repressors. We have not yet established the effectiveness of all the many Kox1-ER chimeras we constructed, but have shown that the sub set we tested are extremely efficient.
3. The replacement of the wild-type DNA binding domain of the KRAB repressor-ER construct with the DNA binding domain with the most strongly enhanced affinity for consensus and imperfect EREs. Our work in the P22 challenge phage system has demonstrated that there is no direct correlation between the potency of binding of the DBD mutants vs. The full length receptors. We have identified full-length receptors with an increased affinity and will construct KRAB chimeras with these. Interestingly we find that when fused to the KRAB domain these mutated DBDs can form effective repressors. While under certain circumstances it may be advantageous to have potent repressors that do not require ligand to induce the repressive phenotype, the use of full-length ER-KRAB chimeras may be warranted since recent reports have indicated that the ER not only acts directly by binding to the ERE, but can also act for instance through the AP1 (38) and the raloxifene response element (39), presumably via (an) intermediary protein(s). The A/B domain has been implicated in the interaction of ER with the c-Jun protein. It is not clear whether these are unique cases or whether this forms an emerging trend. It may well be that more promoters will be identified where ER acts through a similar mechanism, especially taking in mind that the presence of EREs has not been established in all estrogen regulated genes. In fact, our DBD-KRAB constructs may prove to be essential tools in identifying genes that are activated through those kind of mechanisms.

Task 2: Months 6-18: The Characterization of the ability of the ERG repressors to suppress transcription of synthetic reporter genes and of endogenous ER-regulated cellular genes.

1. Transient transfections to introduce the ERG repressors and reporter genes onto mammalian cell and into ER⁺ MCF7 and ER⁻ MDA-MB-231 breast cancer cells to determine their ability to repress transcription of synthetic reporter genes and of some endogenous genes including growth factor and growth factor receptor genes known to be under estrogen control. Our initial transient transfection studies using synthetic promoters have established the effectiveness of a sub set of the ERG repressors in HepG2 cells in both the absence and presence of ER. We are currently starting experiments in ER⁺ MCF7 and ER⁻ MDA-MB-231 breast cancer cells and expect to finish these studies within the projected time frame.
2. The construction and evaluation of the suppression by additional ERG-repressors. We have obtained the Kox1, ZNF133 and ZNF140 KRAB repressor domains and demonstrated that these are more potent than the p18 KRAB domain. We do not expect it to be necessary to construct further repressors containing the polycomb repressor (40).

CONCLUSIONS

The work carried out so far shows that KRAB-ER chimeras form extremely potent repressors that can effectively repress transcription from estrogen responsive genes, even in the presence of liganded wild-type estrogen receptor. While our initial constructs with the p18 KRAB were neither as potent nor as universal as hoped, the construction of the Kox1 KRAB chimeras has yielded powerful controllable ligand-dependent ERG-repressors.

Related work with the P22 challenge phage system has yielded estrogen DBD mutants that display an enhanced affinity for the estrogen response element. So far we have constructed DBD-KRAB chimeras with two of the most promising DBD mutants. In our initial test (Fig 9) these mutants proved to be very effective repressors of transcription. While the DBDs display up to a many fold higher affinity for the ERE, the full-length constructs have up to a 5-fold higher affinity. We will construct chimeric ER-KRAB constructs to test whether this raised affinity for the ERE will result in a more potent repressor.

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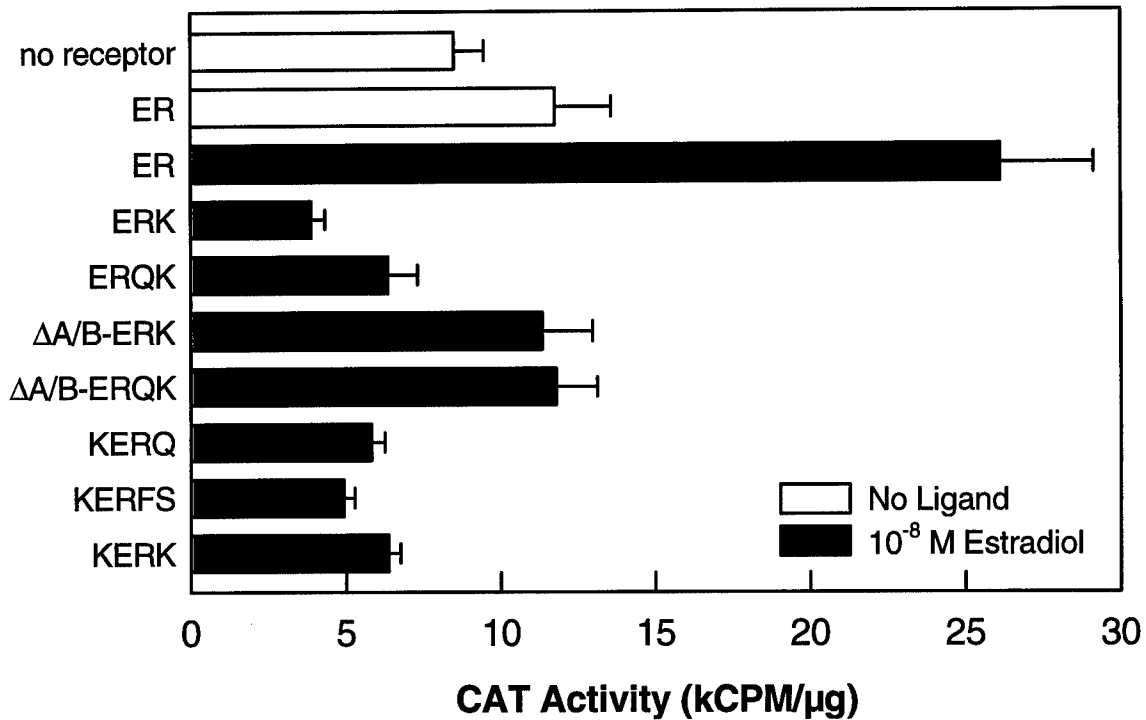


Figure 1. Effect of various p18KRAB-ER chimeras on EREVITCAT in CHO cells
In this Experiment the various chimeras were transfected at a 10ng dose. Plasmid CMV Luciferase (50 ng) served as an internal control and EREVITCAT (2 g) served as the reporter.

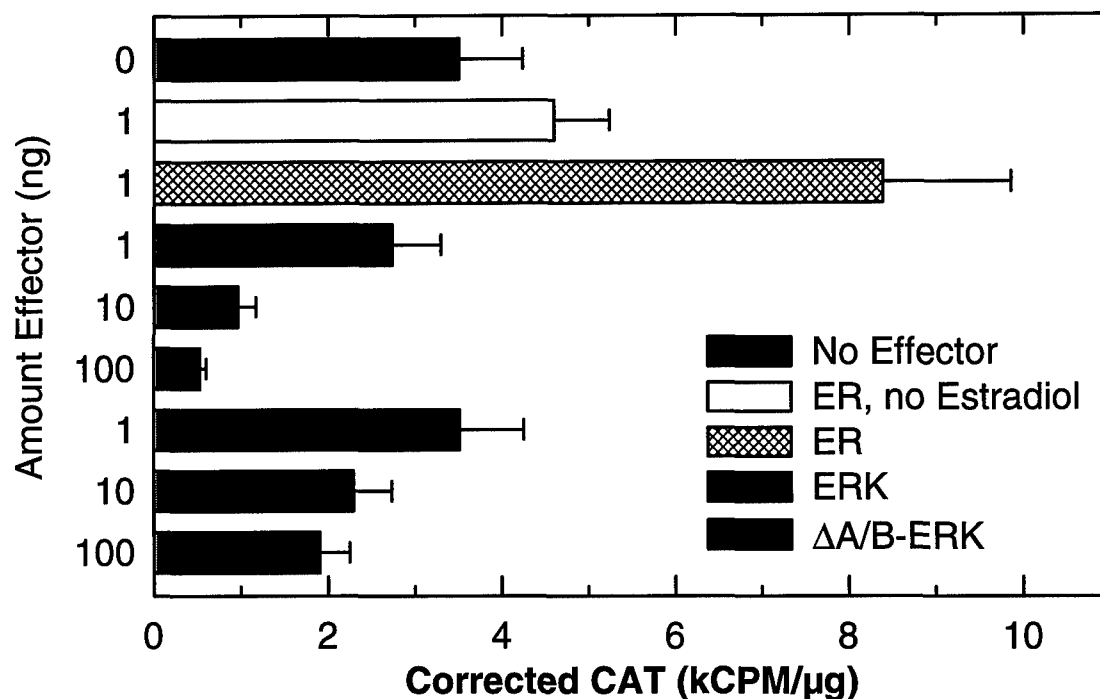


Figure 2. Transcription Repression by p18KRAB-ER chimeras ERK and Δ A/B-ERK on EREVITCAT in CHO cells. In this experiment the chimeras were transfected at doses varying from 1-100ng to establish a dose response curve. The basal activity of the promoter is established by transfection of the reporter in the absence of Effector (bar marked Alone). ERK was a more potent repressor that Δ A/B-ERK and achieved 85% repression. Estrogen receptor (1 ng) in the absence and presence of 10^{-8} M Estradiol served as a positive control. Plasmid CMV Luciferase (50 ng) served as an internal control and EREVITCAT (2 μ g) as the reporter.

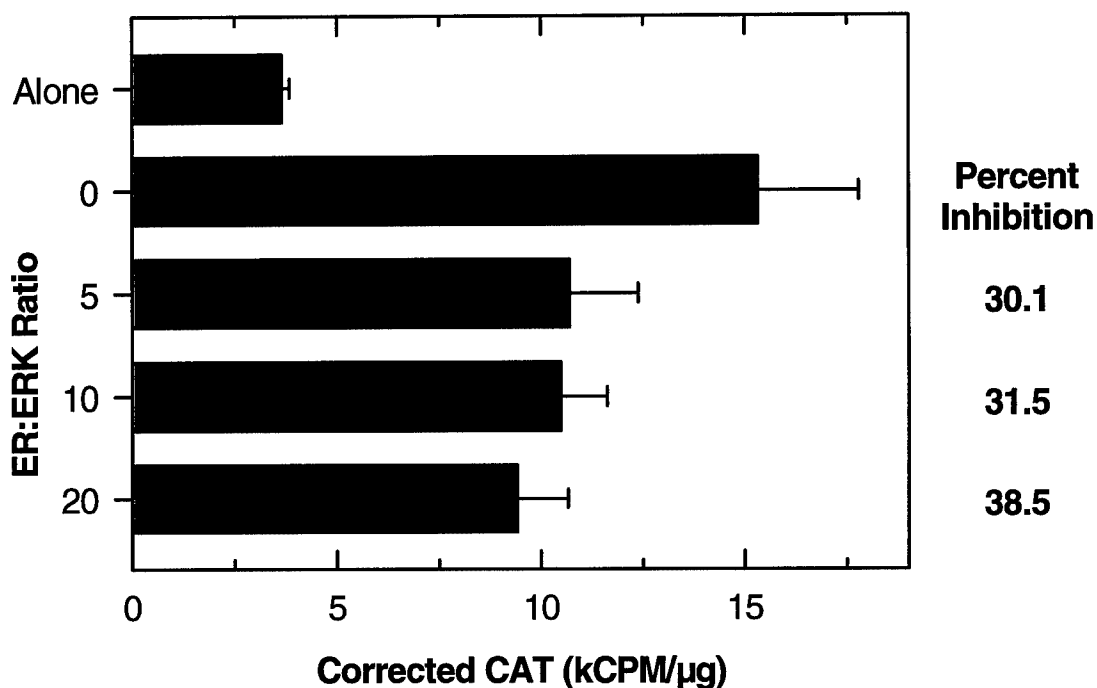


Figure 3. Repression of p18KRAB-ER chimera ERK on (ERE)₂-TK-CAT in the presence of ER in CHO cells. Increasing amounts of ERK were cotransfected with 1ng ER at ratios indicated. The basal activity of the promoter is established by transfection of the reporter in the absence of Effector (bar marked Alone). While the promoter activity is decreased in the presence of ERK, a maximum of only 38.5% inhibition is achieved. Plasmid CMV Luciferase (50 ng) served as an internal control and EREVITCAT (2 g) as the reporter.

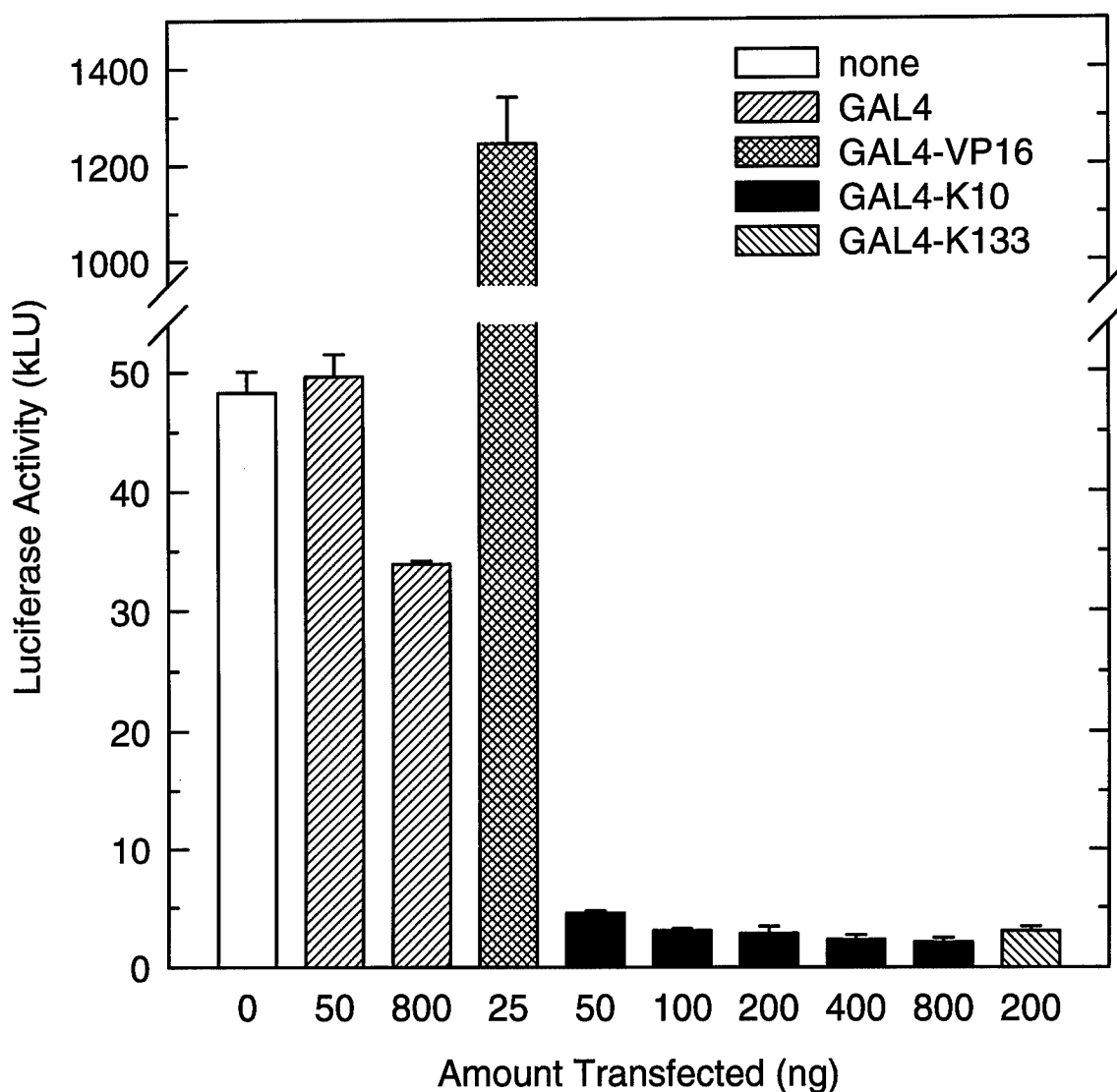


Figure. 4. Effects of various Flag-GAL4 based Effectors on the 5'-G5-pGL3-Control reporter plasmid in HepG2 Cells. The basal activity of the SV40 promoter/enhancer was determined in the absence of effector (indicated by the white bar). Two amounts (50 and 800 ng) of the Flag-GAL4 binding domain served as a negative control, while 25 ng transfected Flag-GAL4-VP16 served as a positive control. 50-800ng of Flag-Gal4-Kox1 KRAB, (black bars) was transfected and repressed transcription up to 96% repression. The Flag-GAL4-ZNF133 KRAB fusion displayed similar repression as the of Flag-Gal4-Kox1 KRAB construct.

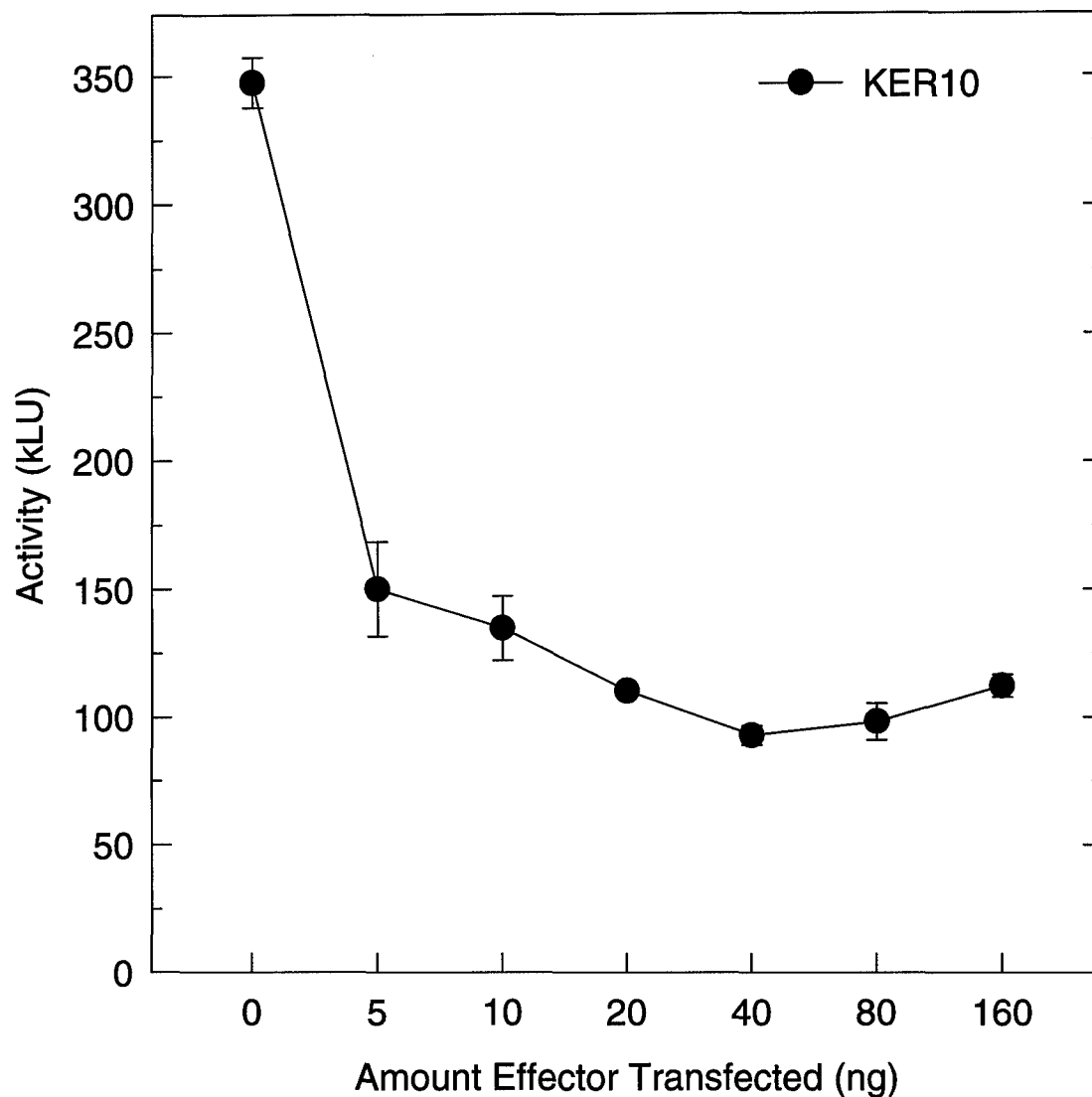


Figure 5. Repression by increasing amounts (0-160 ng) of the N-terminal Kox1 Krab ER chimera KER10 on the (ERE)₄-pGL3-Control plasmid in HepG2 Cells. A maximum of 73% inhibition was achieved at 40 ng transfected effector. Plasmid (ERE)₄-pGL3-Control (200 ng) served as reporter and PRL-SV40 (200 ng) as internal control.

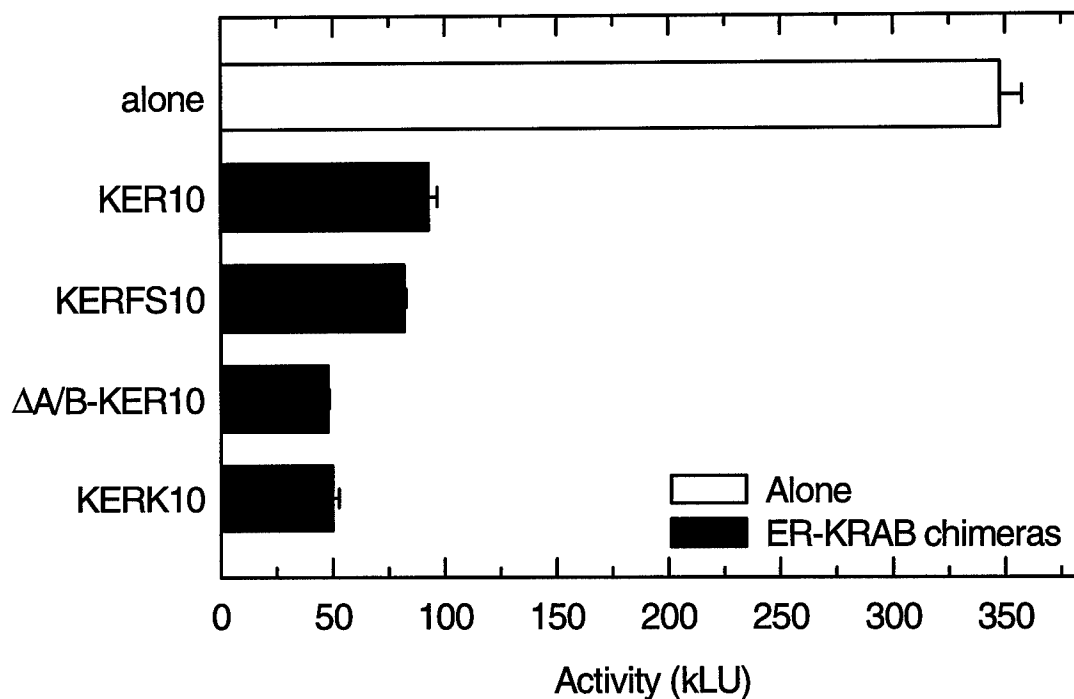


Figure 6. Repression of transcription by various ER-Kox1 KRAB chimeras on (ERE)₄-pGL3-Control plasmid in HepG2 Cells. Chimeras KER10, KERFS10, ΔA/B-ERK10 and KERK10 were transfected at 40 ng dose. Ablation of AF2 did not result in more potent repression. However, both the addition of an extra KRAB domain or the deletion of AF1 increased repression. Plasmid (ERE)₄-pGL3-Control (200 ng) served as reporter and PRL-SV40 (200 ng) as internal control.

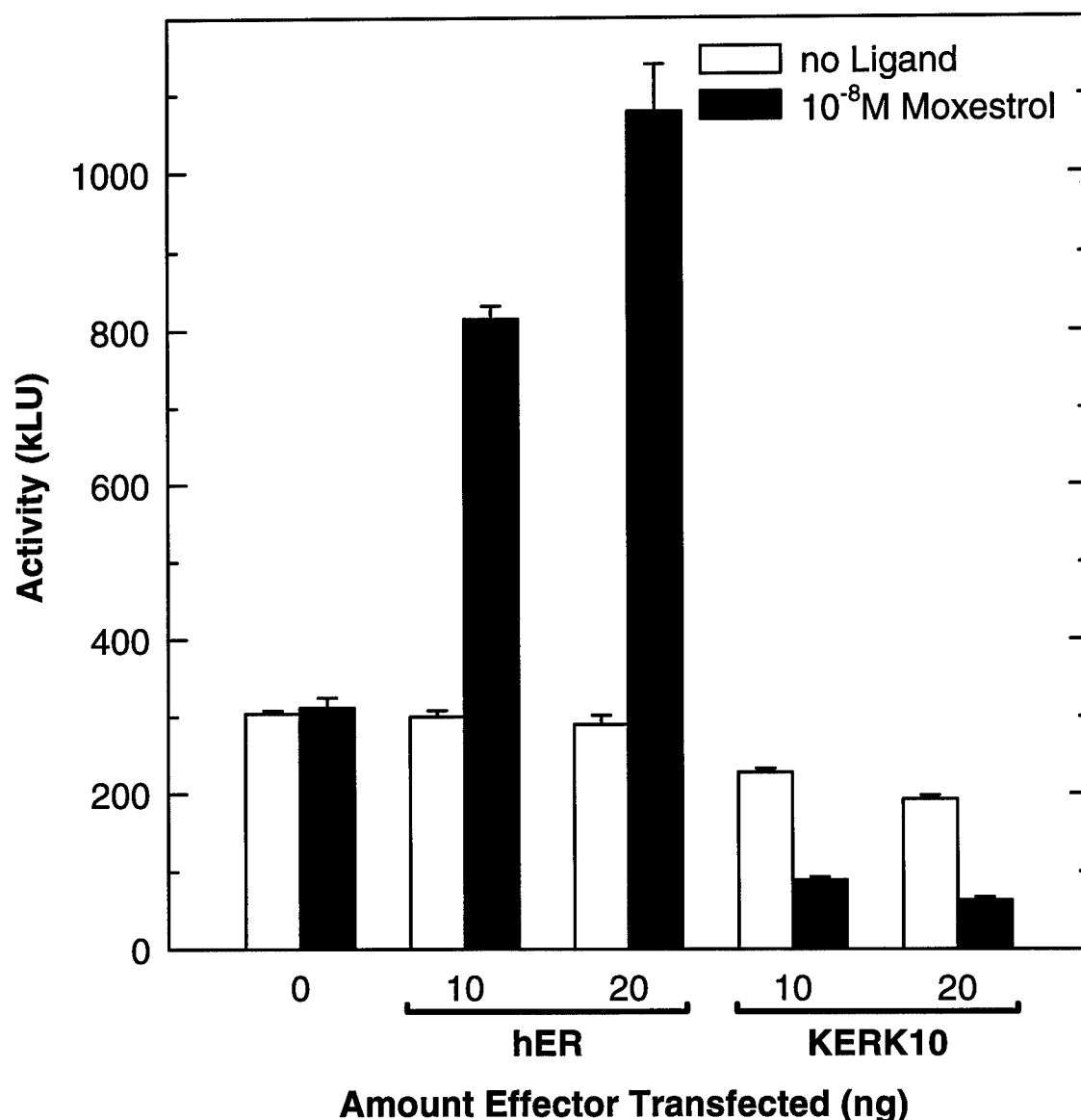


Figure 7. Ligand Inducible repression of Transcription by the ER-Kox1 KRAB chimeras KERK10 on the (ERE)₄-pGL3-Control plasmid in HepG2 Cells. In this experiment indicated amounts of hER and KERK10 were transfected in the absence or presence of ligand (10⁻⁸M Moxestrol). Activity of the promoter in the absence of ligand was not affected by hER, but was slightly repressed by KERK10. Ligand clearly induced repression. Plasmid (ERE)₄-pGL3-Control (200 ng) served as reporter and PRL-SV40 (200 ng) as internal control.

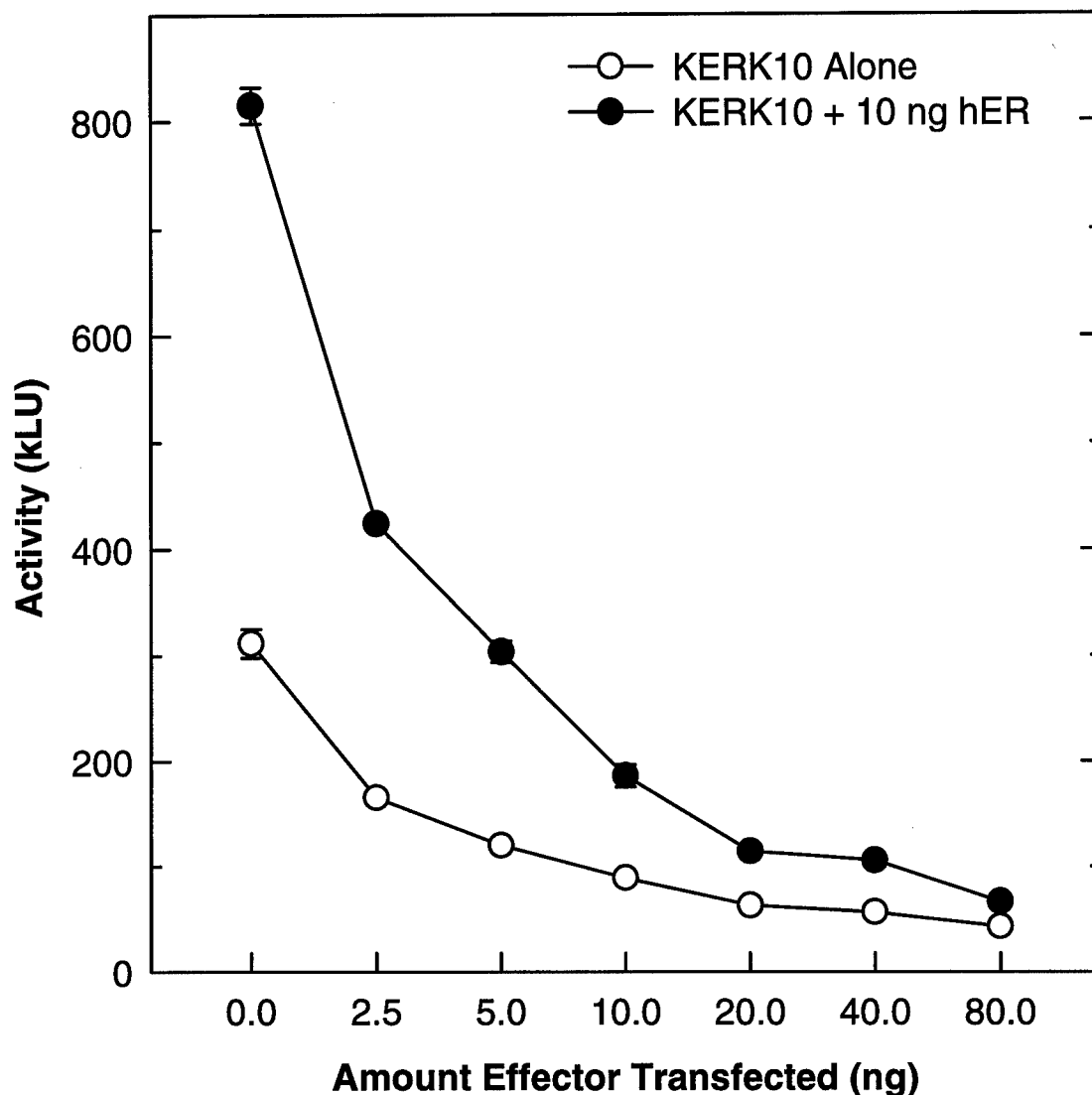


Figure 8. Repression by ER-Kox1 KRAB chimera KERK10 at varying amounts in the absence or presence of cotransfected hER on plasmid $(ERE)_4$ -pGL3-Control in HepG2 Cells. In this experiment indicated amounts of KERK10 were transfected in the absence or presence of hER (10 ng). At higher amounts of transfected KERK10 the curves converge indicating that repression potency of KERK10 is not affected by presence of hER. Plasmid $(ERE)_4$ -pGL3-Control (200 ng) served as reporter and PRL-SV40 (200 ng) as internal control.

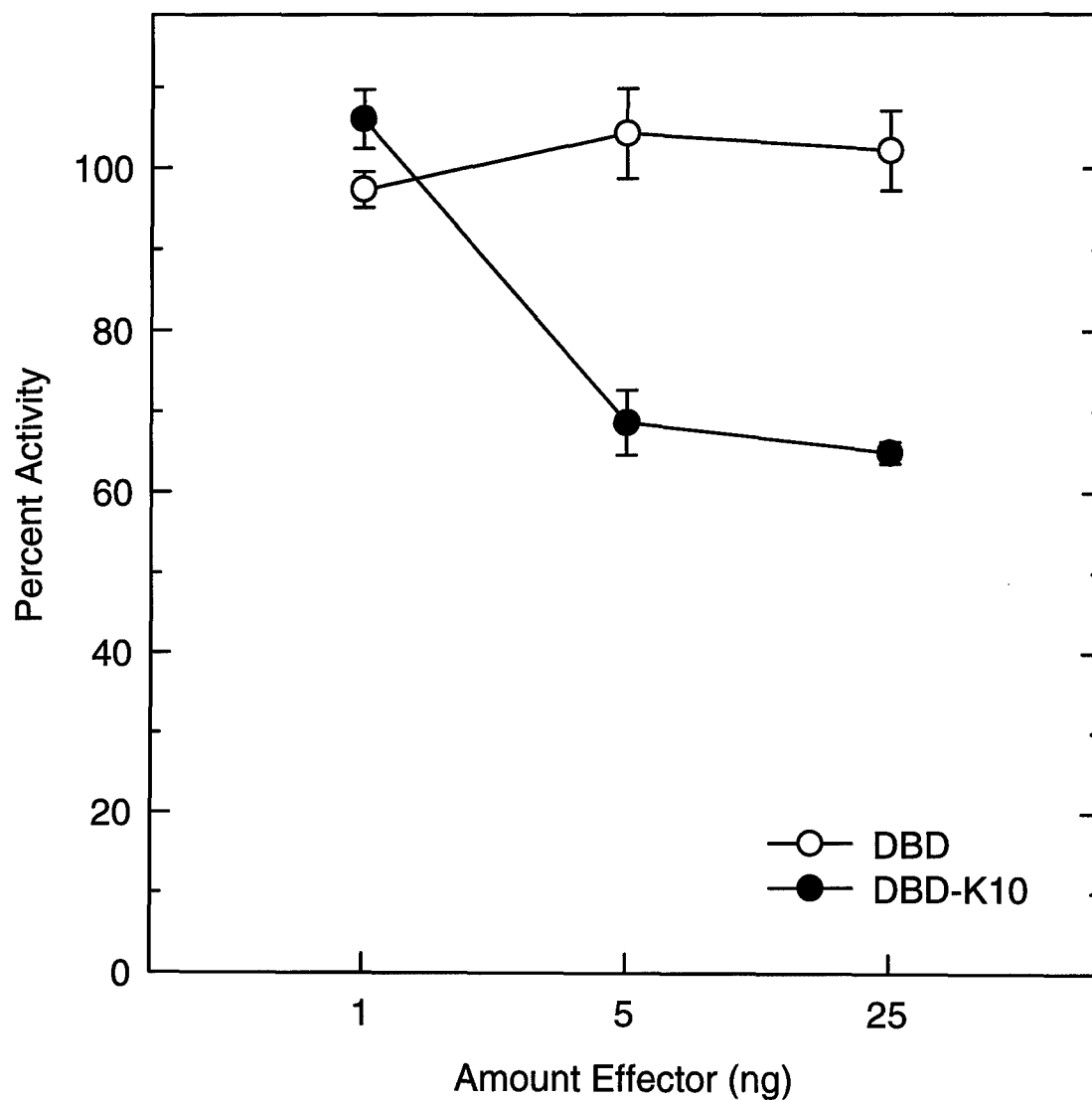


Figure 9. Repression on plasmid $(ERE)_4$ -pGL3-Control in HepG2 cells by mutant DBD #15 when fused to the Kox1 KRAB domain. Even at low dose of transfected DNA mutant DBD #15 which has a 15-fold increased affinity for the ERE (see TABLE 1) is an effective repressor of transcription. Plasmid $(ERE)_4$ -pGL3-Control (200 ng) served as reporter and PRL-SV40 (200 ng) as internal control.

TABLE 1. Affinity for the estrogen response element displayed by mutant DBDs selected in the p22 Challenge phage system directly and when inserted into the full length estrogen receptor

Clone Number	Affinity Mutant DBDs (fold over WT)	Affinity Full Length Receptor Mutants (fold over WT)
17	15.0	1.0
42	15.0	0.1
15	13.0	0.5
22	7.0	2.0
40	0.5	5.0



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US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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