Appendix D - **UNIVERSITY HONORS PROGRAM**
SENIOR PROJECT - APPROVAL

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College: Arts & Sciences Department: BCMB

Faculty Mentor: John Koontz, Ph.D.

PROJECT TITLE: Assessing steroid action with the Green Fluorescent Protein Reporter Gene: Construction of Eukaryotic Expression Vectors

I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

Signed: John W. Koontz, Faculty Mentor

Date: 12-18-98

Comments (Optional):

I have been impressed by Erin's persistence and desire to successfully complete this project. Her work has been characterized by attention to detail, a clearly developed strategy and a willingness to put considerable time towards its completion. I feel fortunate that she chose to do this project in my lab.

Assessing steroid action with the Green Fluorescent Protein Reporter Gene: Construction of Eukaryotic Expression Vectors

Erin N. Kahler

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Assessing Steroid Action with the Green Fluorescent Protein Reporter Gene: Construction of Eukaryotic Expression Vectors

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ABSTRACT: Green fluorescent protein (GFP) is fast becoming a widely used reporter of gene expression and regulation. By fusing the genes encoding GFP and various steroid hormone-responsive elements, we have created a set of reporter constructs that can be visualized directly without addition of exogenous substrates or cofactors. Two constructs were created by inserting the gene encoding green fluorescent protein 3' of the gene encoding either glucocorticoid-responsive elements (GRE) or GAL 4-binding elements responsive to progesterone (GAL). A third estrogen-responsive construct was created by inserting the gene for the estrogen-responsive element (ERE) into the commercially available vector EGFP-N1 (Clontech). The effectiveness of each vector in assessing steroid action was demonstrated by transfection of Chinese hamster ovary (CHO) cells and treatment with steroid hormones. We have found that induction of gene expression with Dexamethasone for the GRE and GAL constructs and Estradiol for the ERE construct stimulates transcription of GFP, allowing fast analysis of the genes of interest by looking at expression *in vivo* under a fluorescence microscope. Because agonist must be present for the receptor to bind ligand and stimulate transcription, there is low constitutive background fluorescence. These constructs have potential application for studies on the interactions of various compounds with steroid hormones, and if incorporated into a stably-transfected cell line, as environmental screening agents of estrogen mimics.

Assessing Steroid Action with the Green Fluorescent Protein Reporter Gene: Construction of Eukaryotic Expression Vectors

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Green fluorescent protein (GFP) is fast becoming a widely used reporter of gene expression and regulation. By fusing the genes encoding GFP and various steroid hormone- responsive elements, we have created a set of reporter constructs that can be visualized directly without addition of exogenous substrates or cofactors. Two constructs were created by inserting the gene encoding green fluorescent protein 3' of the gene encoding either glucocorticoid-responsive elements (GRE) or GAL 4-binding elements responsive to progesterone (GAL). A third estrogen-responsive construct was created by inserting the gene for the estrogen-responsive element (ERE) into the commercially available vector EGFP-N1 (Clontech). The effectiveness of each vector in assessing steroid action was demonstrated by transfection of Chinese hamster ovary (CHO) cells and treatment with steroid hormones. We have found that induction of gene expression with Dexamethasone for the GRE and GAL constructs and Estradiol for the ERE construct stimulates transcription of GFP, allowing fast analysis of the genes of interest by looking at expression *in vivo* under a fluorescence microscope. Because agonist must be present for the receptor to bind ligand and stimulate transcription, there is low constitutive background fluorescence. These constructs have potential application for studies on the interactions of various compounds with steroid hormones, and if incorporated into a stably-transfected cell line, as environmental screening agents of estrogen mimics.

Key Words: Green Fluorescent Protein (GFP);reporter gene; Glucocorticoid-responsive element (GRE); steroid hormone; estrogen

Green fluorescent protein (GFP)[2] has recently become an important reporter molecule of gene expression and regulation (Kain *et al,* 1995). Currently used methods to monitor gene expression often utilize the formation of fusion proteins encoding either β-galactosidase, firefly luciferase, or bacterial luciferase, which require the addition of exogenous substrates of cofactors

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[2] Abbreviations used: GFP, Green fluorescent protein; GRE, glucocorticoid-responsive element; CAT, chloramphenicol acetyltransferase; CHO, Chinese Hamster Ovary; ER, estrogen receptor; DMEM, Dulbecco's modified Eagle's medium

(Chalfie *et al*, 1994). This limits their use with living tissue so that a simpler, quicker detection method for viewing gene expression *in vivo* is desirable. First cloned and sequenced in the early 1990s from the jellyfish *Aequorea victoria*, GFP has been used for rapid expression screening of cells containing various genes of interest (Cassata *et al*, 1998). Because GFP fluorescence does not require a substrate or cofactor, it is species-independent and can be used in both prokaryotic and eukaryotic cells and even whole organisms that are near-transparent, such as *C. elegans* (Plautz *et al*, 1996). Fluorescence is stable and can be visualized non-invasively in living cells, eliminating the need to "fix" the cells or use antibodies (Kain *et al*, 1995; Rizzuto *et al*, 1995). In addition, GFP maintains its fluorescent properties in the presence of strong denaturing agents, exhibits a broad range of pH stability, and is extremely thermostable, able to withstand temperatures up to 65°C (Leffel *et al*, 1997). Because green fluorescent protein is non-toxic and nonradioactive, is is a promising candidate for monitoring gene expression (Garamszegi *et al*, 1997).

Chalfie *et al* first demonstrated GFP's use as a reporter of gene expression *in vivo* in their studies with the nematode *Caenorhabditis elegans*, an organism often used for developmental studies (Chalfie *et al*, 1994). Wild type GFP is a 27-kDa monomer composed of 238 amino acid residues (Leffel *et al*, 1997). When excited with UV or blue light, it emits a bright green light with λ maxima at 509 nm (Kain *et al*, 1995). The entire protein is required for fluorescence, but the minimal chromophore responsible for light emission has been identified as a hexapeptide within the protein containing the amino acid

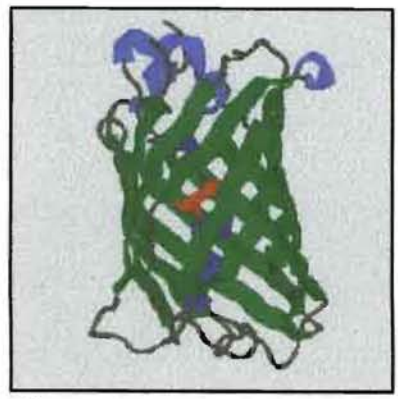


Figure 1

trimer Ser-dehydroTyr-Gly.(Chalfie *et al*, 1994). **Figure 1** shows the tertiary structure of the protein and depicts the amino acid trimer in red. This trimer is cyclized and oxidized during post-translational modification to yield a chromophore that emits light, but how this occurs is unknown (Kain *et al,* 1995; Leffel *et al*, 1997).

The chromophore in GFP is intrinsic to the primary structure of the protein, due to modified amino acid residues within the polypeptide (Prasher *et al*, 1992). Cnidarians use green fluorescent proteins as energy-transfer acceptors in bioluminescence (Prasher *et al*, 1992). One GFP from the jellyfish *Aequorea victoria* fluoresces *in vivo* after receiving energy from the Ca^{2+}-activated photo-protein aequorin (Kain *et al,* 1995), transducing the blue chemiluminescence of aequorin into green light (Leffel *et al*, 1997). Purified GFP has similar spectral properties, absorbing blue light and emitting a green light that is detectable by using a fluorescence microscope, UV light box, or fluorescence-activated cell sorting (FACS) (Kain *et al,* 1995).

GFP has much potential, not only in monitoring gene expression, but also for studying intracellular protein distribution and protein trafficking (Fejes-Tóth *et al*, 1998). Current methods in use for detecting protein-protein interactions commonly involve radiolabeled or biotinylated probes, which are costly (Garamszegi *et al*, 1997). Fusion constructs with a protein of interest can be used to analyze molecular interactions, if both the protein of interest and GFP behave as independent functional domains (Garamszegi *et al*, 1997; Glantschnig *et al*, 1998; Richards *et al*, 1996; Meng *et al*, 1997). Much research is currently focusing on new ways to approach this, such as using a bifunctional reporter or two-color reporter system (Quaedvlieg *et al*, 1998).

Such a two-color reporting system is achieved by a blue-shifted variant of GFP that was created by substituting a histidine or tryptophan for a tyrosine at position 66 in the chromophore.

These mutant proteins, called BFPs (blue fluorescent proteins), can provide a second color distinct from GFP for *in vivo* visualization of subcellular activities. (Leffel *et al*, 1997) A similar mutation has been induced to enhance the signal given by GFP by exchanging the amino acid serine 65 to cysteine (S65C) and threonine (S65T) (Helm *et al*, 1995). The fluorescent signal of this enhanced protein is of greater magnitude than wild-type GFP, and further mutations have improved folding of the protein as much as 19-fold (Crameri *et al*, 1996; Reichel *et al*, 1996). Many such improved versions of GFP are now offered commercially, such as the one used in this laboratory, Enhanced Green Fluorescent Protein, or EGFP (Clontech).

We have constructed a series of GFP eukaryotic expression vectors responsive to various steroid hormones to provide a simple means of assessing steroid action in transfected cells. This method allows fast analysis of genes of interest by looking at expression in vivo using their putative promoter region to control the expression of GFP (Htun *et al*, 1996). Glucocorticoids, estrogens, and progesterones bind receptors in the cytoplasm and cause them to translocate to the nucleus (Meng *et al*, 1997). Because a ligand is required for these receptors to act as transcription factors, translocation to the nucleus occurs only upon binding the agonist (Carey *et al*, 1996; Fejes-Tóth *et al*, 1998). Our system takes advantage of this by using artificial agonist such as dexamethasone or estradiol to induce expression of EGFP-steroid responsive chimeras in mammalian cells. We have created three such constructs: one that is responsive to estrogens and two that are responsive to glucocorticoids, including one that is fused with the GAL 4 binding domain. These vectors allow fast analysis of steroid action through GFP expression. The effectiveness of hormones in inducing expression is measured by the amount of protein being

expressed. The assay is simply to view the cells with such a construct under a fluorescence microscope and see what percentage of cells are fluorescing.

MATERIALS AND METHODS

All enzymes were obtained from either Promega (Madison, WI) or New England Biolabs (Beverly, MA). Cell culture reagents were obtained from Sigma Chemicals Corp. (St. Louis, MO). CHO cells were obtained from American Tissue Culture and Cells (Atlanta, GA).

Plasmid Constructions

$pGRE_7105tkCAT$ and $pGAL_7tkCAT$ were constructed previously in this laboratory (Pan and Koontz, 1995) and contained seven copies of the double-stranded oligonucleotides 5'-GCTGTACAGGATGTTCTAG-3' and 5'-CTAGACCGGAGGACTGTCCTCCGGT-3', respectively. To construct $pGRE_7GFP$ (See **Figure 2**), the XhoI/HpaI fragment of $pGRE_7105tkCAT$ was replaced with the XhoI/HpaI fragment of EGFP-N1 (Clontech, Palo Alto, CA). This fragment contained the sequence encoding enhanced green fluorescent protein. EGFP-N1 was chosen because it contained a neomycin/kanamycin resistance cassette in

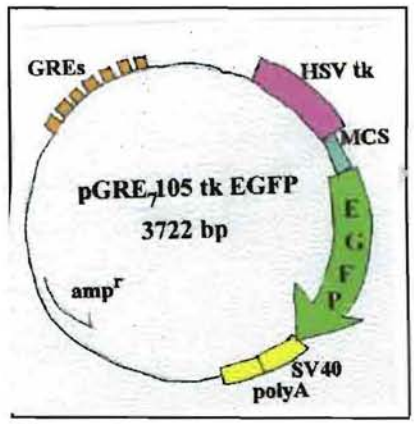


Figure 2

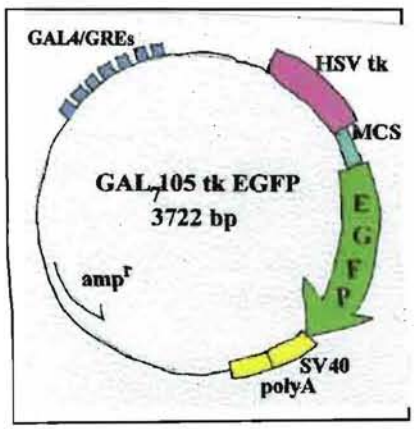


Figure 3

addition to the immediate-early promoter of the cytomegalovirus (CMV). This allowed for constitutive expression of GFP fusions in mammalian cells (Pan and Koontz, 1995). To construct $pGAL_7GFP$ (See **Figure 3**), the XhoI/HpaI fragment of $pGAL_7105tkCAT$ was replaced with the above mentioned XhoI/HpaI fragment of EGFP-N1. pERE-GFP (See **Figure 4**) was constructed by inserting the SalI/BamHI fragment of ERE-TATA-CAT (Tora *et al*, 1989) into the SalI/BamHI-cut multiple cloning site of EGFP-N1. The ERE fragment contained the synthetic oligonucleotide responsive to estrogen: 5'-CTAGAGGTCACAGTGACCGGCG-3'. All recombinants were constructed using standard procedures (See **Appendix**) and were verified by gel electrophoresis (Maniatis *et al*, 1982).

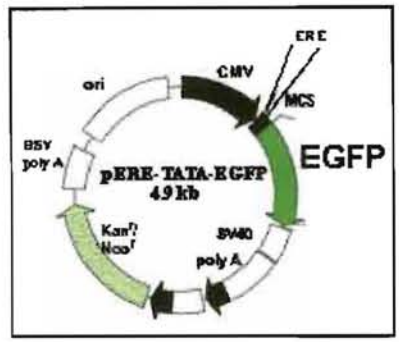


Figure 4

Cell culture

CHO cells were maintained in Ham's F-12 medium (Sigma) containing 5% Fetal Bovine Serum (FBS) at 37°C in a humidified atmosphere with 10% CO_2. Cells were plated onto two-well LabTek chamber dishes and returned to the incubator for 24 hours prior to transfection.

Transient Transfection

Cells were transfected with 2 μg DNA per well for 5 hours as detailed by Life Technologies (See **Appendix**). For each transfection, either 2 μg of $pGRE_7GFP$, 1 μg of $pGAL_7GFP$, or 1 μg of

pERE-GFP was used. In addition, pERE-GFP was co-transfected with 1 μg of HEO (an estrogen receptor) and $pGAL_7GFP$ was co-transfected with 1 μg of pGgalG (a GAL 4 receptor). 10 μg of Lipofectamine (Life Technologies, Grand Island, NY) was added to the DNA and the mixture was diluted into 100 μl of serum-free medium. The final volume added to each well of the cell culture was 2 ml. Cells were incubated 5 hours at 37°C in a CO_2 incubator and media was replaced with fresh, complete medium. 24 hours after transfection media was changed and cells were treated with either dexamethasone (1 μM), Estradiol (20 nM), or neither hormone.

Microscopy

Ham's F-12 medium was used for cell culture because DMEM exhibited a high background fluorescence and contained endogenous glucocorticoids. Immediately before viewing, media was aspirated and the cells were mounted and examined by epifluorescence and bright field microscopy. Cells were examined using an Olympus epifluorescence microscope with a 20x objective lens. For each construct, cells were randomly selected from those that were fluorescent. Images were captured with a Hamamatsu color 3CCD camera and collected in Adobe Photoshop. Exposure time was 0.3 seconds. The pictures in **Figures 5-8 a, b** were generated by superimposing a bright field image on a fluorescent image. **Figures 5-8 c** show the fluorescent image by itself. Cells were observed 24 hours after transfection (before induction) and 48 hours after transfection (post-induction).

RESULTS

Tagging of Steroid Hormone-Responsive Elements with GFP. We constructed a series of plasmids with different steroid hormone-responsive elements. $pGRE_7GFP$ contains seven copies of a synthetic glucocorticoid-responsive element upstream of the promoter. This number of GREs was chosen because it yields the maximum induction when expression is induced (Pan and Koontz, 1995). $pGAL_7GFP$ contains seven copies of the gene encoding the yeast GAL 4 protein for the same reasoning. Induction of $pGAL_7GFP$ by dexamethasone requires co-transfection with G-GAL-G, a human glucocorticoid receptor-GAL 4 chimeric protein in which the DNA binding domain of the glucocorticoid receptor is replaced by that of GAL 4. This protein functions the same as wild-type glucocorticoid receptor, but instead of binding DNA recognition sequences of the GREs it binds the recognition sequences for the GAL 4 protein. pERE-TATA-GFP contains a synthetic oligonucleotide responsive to estrogen. This construct requires an estrogen receptor to be present for hormone to induce expression. The reporter gene for all three constructs is EGFP. Antibiotic resistance enabled selection of only the desired constructs, and once selected, the linearized constructs were run on an agarose gel next to restriction-digested vector. This further verified that the plasmids were correct by size comparison. The vectors for $pGAL_7GFP$ and $pGRE_7GFP$ (XhoI/HpaI cut $pGAL_7105tkCAT$ and $pGRE_7105tkCAT$, respectively) were 2815 base pairs. The new GFP constructs are 3722 base pairs. EGFP-N1, the vector for pERE-TATA-GFP, is 4733 base pairs. The ERE fragment added approximately 150 base pairs. This was difficult to distinguish electrophoretically, so direct transfection was used to confirm this plasmid.

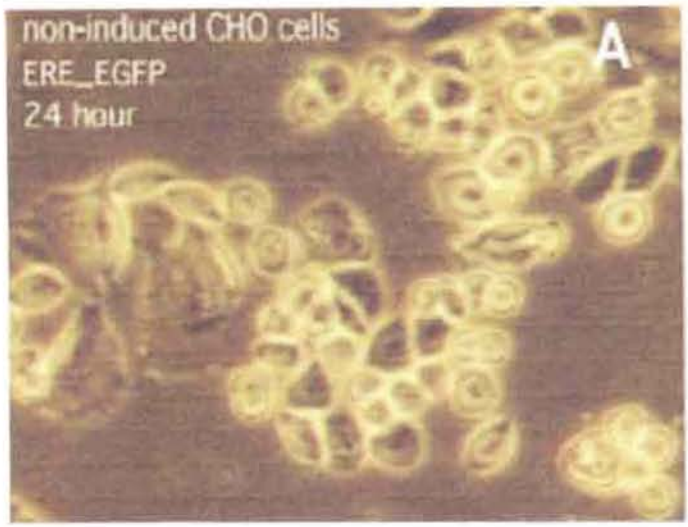
GFP Expression in CHO Cells. GFP expression in $pGRE_7GFP$- and $pGAL_7GFP$-transfected cells was induced with dexamethasone. Non-induced controls showed no expression of green fluorescent protein (**Figures 5a, 6a, and 7a**), meaning that there is low background in using this assay. $pGAL_7GFP$-transfected cells lacking the glucocorticoid receptor-GAL 4 chimera also showed a lack of fluorescence, which confirms that no endogenous receptor was present and that receptor was needed to activate transcription (See **Figure 7c insert**). Addition of dexamethasone caused an induction in gene expression that led to visible levels of green fluorescent protein in transfections where both the reporter construct and glucocorticoid receptors were present (**Figures 6c and 7c**). Cells transfected with pERE-TATA-GFP also did not show expression of GFP unless the receptor, HEO, was present, and cells were treated with hormone (**Figure 5a-c**). Transfection efficiency was low for all cells, and the cells were not very confluent even 48 hours after transfection. No quantitative analysis on level of GFP expression was performed at this time.

DISCUSSION

The presence of positively-transfected cells, by virtue of their fluorescence, indicates that the constructs were made successfully and contain both the region encoding EGFP and the region encoding a steroid-responsive element, be it GRE, ERE, or GAL. Were more time available, a time course study of GFP expression may yield interesting data, and an SDS-PAGE and Western Blot of the protein would allow quantification of the amount of protein present. Conditions to optimize transfection efficiency would also be of value, as well as further purification of the DNA used for transfection. The lower number of cells producing a fluorescent signal may be partially

attributable to the amount of time required for posttranslational maturation of the GFP chromophore, which can be lengthy and varies according to cell type (Kain *et al,* 1995). This suggests that an inadequate time interval for chromophore formation may result in a fluorescent signal that is below the level of detection. Despite a relatively low level of transfection, our results demonstrate the efficacy of the GFP reporter and the ease in using it.

We have found green fluorescent protein to be a highly effective reporter in assessing steroid action and an attractive alternative to traditional methods of monitoring gene expression *in vivo*. GFP can be used to study interactions between steroid hormones and any compound of interest, or for detecting estrogen mimics in the environment, though this would require a stably-tranfected cell line. GFP has utility as a reporter not only in gene expression and protein localization, but also in cell- population dynamics, evolution, ecology and pathogenesis (Carey *et al*, 1996; Rizzuto *et al*, 1995; Kain *et al,* 1995). It may be useful in recognizing genetically modified cells in physiological studies as well as monitoring complex phenomena such as changes in shape and distribution of organelles in living cells (Rizzuto *et al*, 1995). The type of construct we have made can be applied to a wide range of gene expression studies since any gene can be used to drive GFP expression. One example of this is a method for monitoring HIV infection that has been developed using a stable T-cell line containing a plasmid encoding GFP driven by the HIV-1 long terminal repeat (Gervaix *et al*, 1997). Because it is non-toxic, simple to assay, and does not require any substrates or cofactors, GFP is a powerful tool for monitoring gene expression. The potential dual application of multiple variants of GFP brings complex gene transcription studies closer to the present. These unique features make green fluorescent protein a valuable tool for gene expression studies.



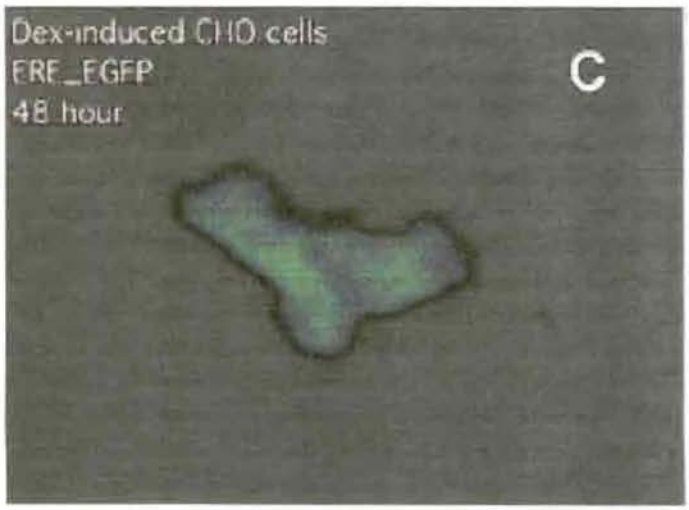


Figure 5. CHO cells transfected with pERE_EGFP and pHEO. A, Fluorescence image of non-induced CHO cells superimposed on bright-field image 24 hours after transfection. B, Fluorescence image of Estradiol-induced CHO cells superimposed on bright field image 48 hours after transfection. C, Fluorescence image of single Estradiol-induced CHO cell 48 hours after transfection.

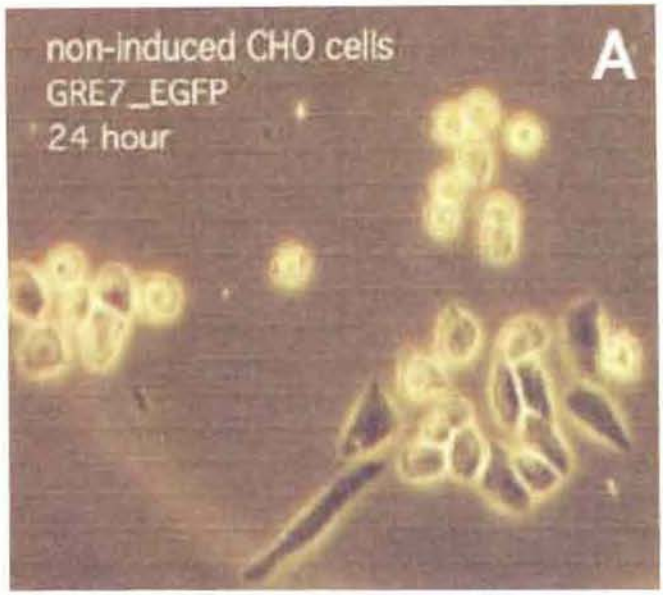
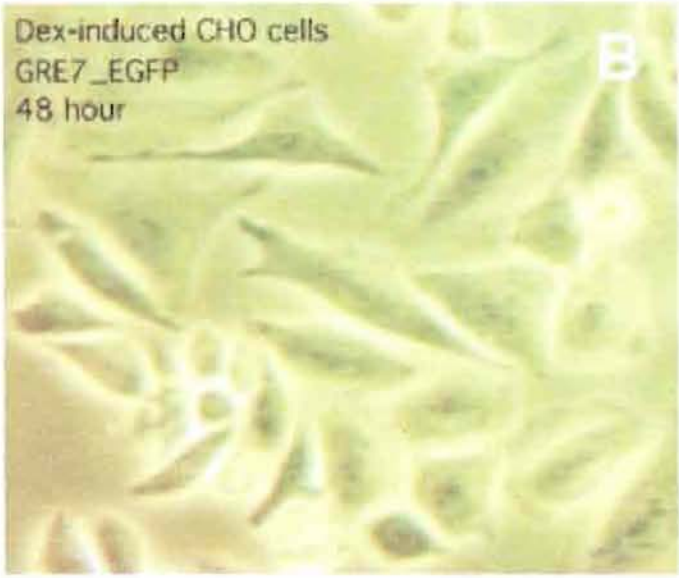
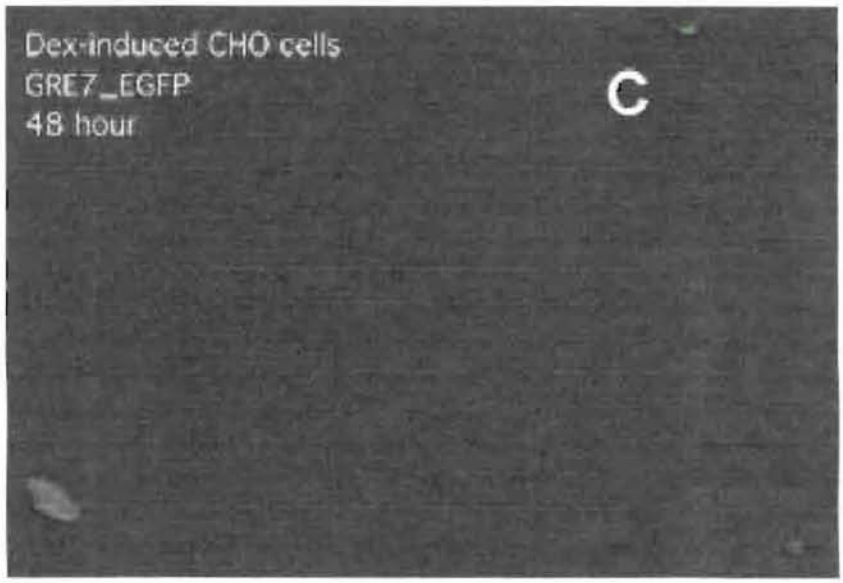
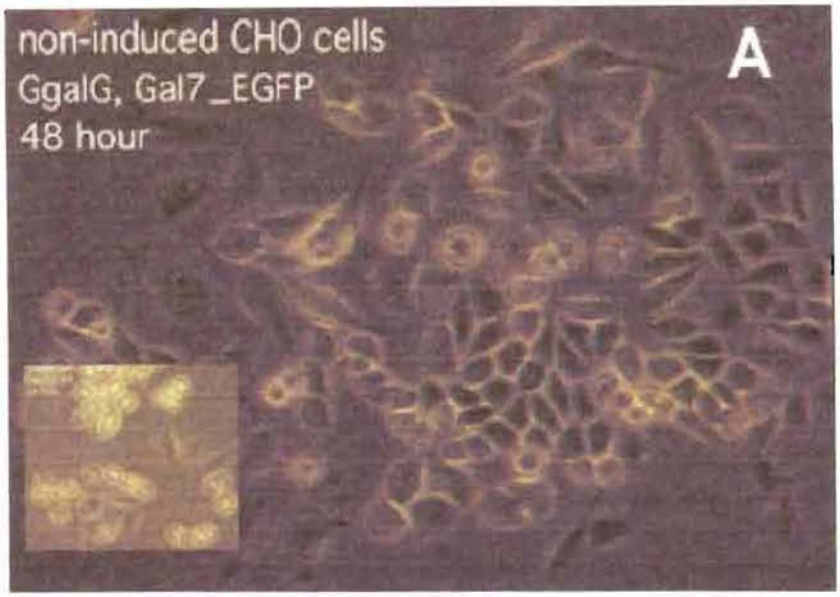
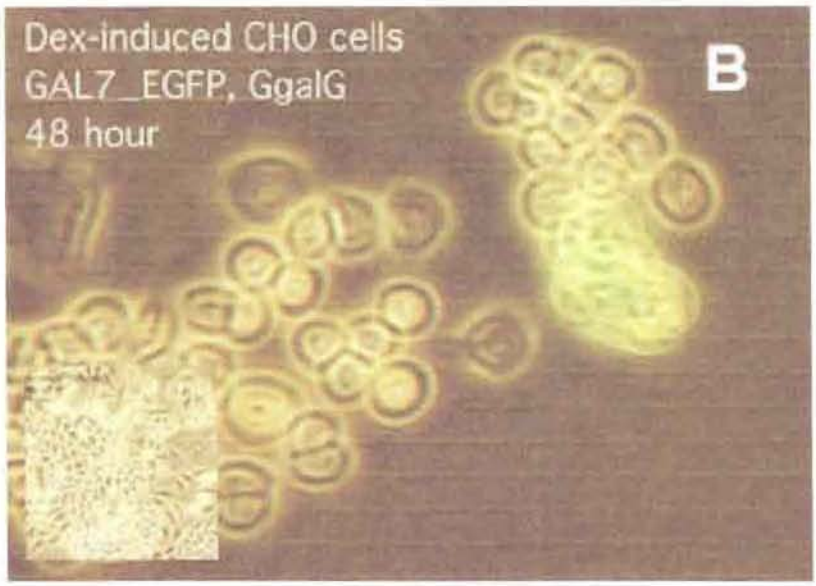


Figure 6. CHO cells transfected with pGRE7_EGFP. A, Fluorescence image of non-induced CHO cells superimposed on bright-field image 24 hours after transfection. B, Fluorescence image of Dexamethasone-induced CHO cells superimposed on bright field image 48 hours after transfection. C, Fluorescence image of Dexamethasone-induced CHO cells 48 hours after transfection.









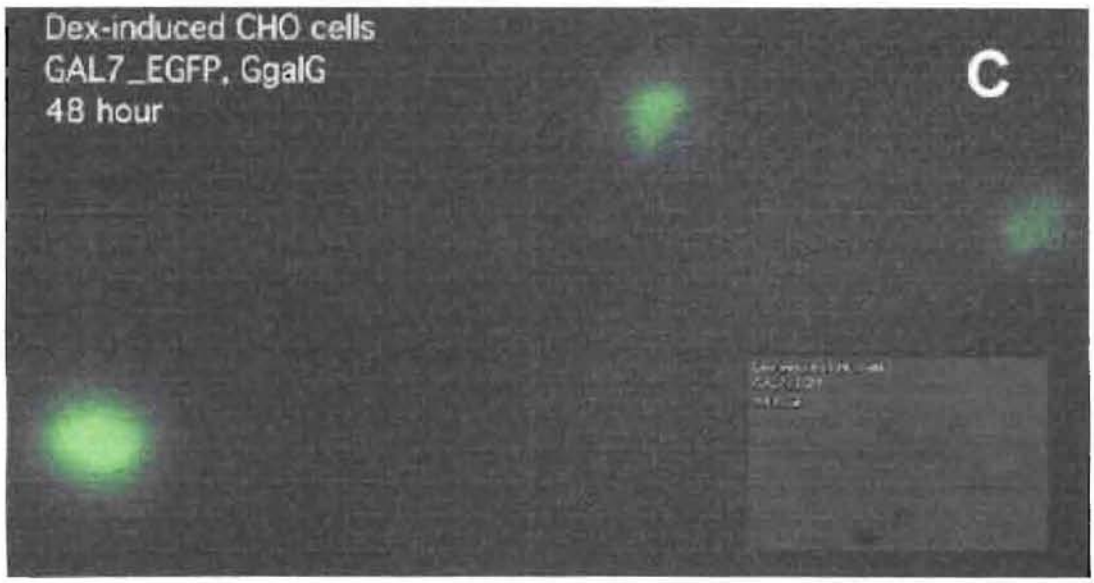


Figure 7. CHO cells transfected with pGAL7_EGFP. A, Fluorescence image of non-induced CHO cells, co-transfected with pGGALG, superimposed on bright field image 24 hours after transfection. The insert represents a similar image taken from the control plate without co-transfection. B, Fluorescence image of Dexamethasone-induced CHO cells, co-transfected with pGGALG, superimposed on bright field image 48 hours after transfection. The insert represents a similar image taken from the control plate without co-transfection. C, Fluorescent image of Dexamethasone-induced CHO cells, co-transfected with pGGALG, 48 hours after transfection. The insert represents a similar image taken from the control plate without co-transfection.

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APPENDIX:
Experimental Protocols

A. Sample Restriction Digest

In microcentrifuge tube, add:

for:	ERE-GFP (.658 μg/ml)	GAL_7-GFP (.203 μg/ml)	GRE_7-GFP (.479 μg/ml)
ddH_20	4 μl	4 μl	2 μl
BSA (10x)	1 μl	2 μl	1 μl
DNA	3 μl	10 μl	4 μl
Buffer D	—	2 μl	1 μl
SalI	1 μl	----	----

mix and spin down, add restriction enzyme (keep on ice):

XhoI	----	2 μl	2 μl
Sal I	1 μl	----	----

Incubate 1-5 hours at 37°C. Add 1 μl .5 M EDTA to stop reaction. Store tubes in -20.

B. Gel Electrophoresis

Weigh out amount of agarose needed- for example, if 40 ml volume total, weigh .48g for a 1.2% gel. Add 40 ml TAE and microwave until almost boiling. Solution should be clear.

Let cool until not hot to touch. Pour into taped gel cast with gel comb inserted. Allow gel to set. When ready, load gel with samples. Ratio of dye:sample is approximately 1 μl:5 μl. Load ladder on gel. Run until loading dye front about 1 cm from edge of gel.

Place gel in plastic box with TAE from gel. Add 10 μl of Ethidium Bromide (careful!) and let stain for 15-30 minutes. Destain in ddH_20 for 5 minutes and view under UV light.

C. Sample Ligation Reaction

In 1.5 ml microcentrifuge tube, add:

ddH_20	4 µl
10X ligase buffer	3 µl
ERE DNA (.06 µg/µl)	15 µl
GFP DNA (.117 µg/µl)	5 µl
Ligase (3 U/µl)	3 µl

Allow reaction to go for 4-16 hours 4-16°C. Stop reaction with 1 µl .5M EDTA. Store tubes in -20°C freezer.

D. Ethanol Precipitation of DNA

Add 6 µl 1M NaCl to 20 µl DNA (1/10 volume 3M). Mix, add 2 volumes absolute ethanol. Precipitate on dry ice 30 minutes.

Centrifuge in table-top cfg highest speed 5 min. in cold room. Wash pellet with 70% ethanol two times. Centrifuge again and pour off supernatant.

Allow pellet to air dry and redissolve in sterile water.

E. Miniprep of Plasmid DNA

1-5 ml of overnight LB culture (10 ml) centrifuged in table-top centrifuge at top speed 5 minutes and supernatant poured off. Resuspend pellet in 100 µl cold lysis buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8.0) and incubated at room temp for 5 minutes.

200 µl cold solution B (0.2 N NaOH, 1% sodium dodecyl sulfate (SDS)), made fresh, were added, mixed by inversion, and the samples left of ice for 5 minutes.

150 µl cold 3 M $KC_2H_3O_2$ were added, mixed and incubated in the same manner.

Centrifuge 5 minutes at 4°C (12000 x G) and transfer supernatant to new tube.

The solution was extracted once with .5 ml phenol:chloroform:isoamyl alcohol and once with .5 ml chloroform: isoamyl alcohol.

Two volumes of EtOH were added and samples precipitated in dry ice (or -80) for 15 minutes. Centrifuge at 12,000 x G for 15 minutes at room temperature.

The pellet was washed with 0.5 ml 70% ice-cold ethanol, recentrifuged, decanted, vacuum dried 5 minutes and dissolved in .5-1ml TE containing 100 µg/ml DNAse-free RNAse.

DNA was quantitated by measuring the optical absorbance at 260 nm (A_{260} = 1.0 = 50 µg DNA/ml).

F. Maxiprep of Plasmid DNA

One liter of LB conaining 50 μg/ml ampicillin in a 4 L flask was inoculated with transformed bacteria from the remainder of the appropriate miniprep culture and incubated 24 hours at 37°C with vigorous shaking. The culture was centrifuged at 4°C for 10 minutes (3,000 x G) (JA-10 rotor, 4200 rpm) and the pellet resuspended in 10 ml cold solution A (50 mM sucrose, 10 mM EDTA, 25 mM Tris, pH 8.0, 4 mg/ml lysozyme) and incubated at room temp for ten minutes.

10 ml cold solution B (0.2 N NaOH, 1% sodium dodecyl sulfate (SDS)), made fresh, were added, mixed by inversion, and the samples left of ice for 10 minutes.

15 ml cold solution C (3 M $KC_2H_3O_2$) were added, mixed and incubated in the same manner. The 47,000 x G x 20 minute supernatant (Ti-60 rotor, 21K rpm, 4°C) was precipitated with 0.6 volumes of 100% isopropanol at room temperature for 30 minutes, then centrifuged 30 minutes at room temperature (12,000 x G, 9400 rpm with JA-17).

The pellet was washed once with cold 70% ethanol, vacuum dried, dissolved in 1.5 ml TE containing 10 μl of 10 mg/ml DNAse-free RNAse and incubated 1-2 hours at 37°C. The solution was extracted twice with 2 ml phenol:chloroform:isoamyl alcohol and once with 2 ml chloroform: isoamyl alcohol and brought to 1.8 ml with 360 μl 5 M NaCl and TE.

The solution was then spun through a pZ523 column (5'→3', Inc.) at 1,100 x G for 12 minutes (See Protocol below). The eluate was precipitated with 0.6 volumes of 100% isopropanol at room temperature for 20 minutes and centrifuged at 12,000 x G for 30 minutes at room temperature.

The pellet was washed with 0.5 ml 70% ice-cold ethanol, recentrifuged, decanted, dried and dissolved in .5-1ml TE. DNA was quantitated by measuring the optical absorbance at 260 nm (A_{260} = 1.0 = 50 μg DNA/ml).

G. pZ523 Spin Column Procedure

1. Remove top (large) and bottom closures from column and carefully pour off column storage buffer. Do not strike or bang column as this will cause column failure.

2. Place column in one of the collection tubes provided and centrifuge at 1100 x G for 1 min at 2/3 speed in a swinging bucket rotor.

3. Discard collected effluent and collection tube. Place column in second collection tube. (Note: it is normal for the resin to appear dry and pack down to approximately 1 cm below the upper column disk (frit)).

4. Carefully load all of the 1.8 ml sample prepared as described above into the reservoir portion of its respective column. This sample volume is fixed for each column, not each preparation, and should not be altered.

5. Centrifuge the loaded column/collection tube assembly at 1100 x G for 12 minutes at 2/3 speed in a swinging bucket rotor.

6. Determine the volume of the collected column effluent. This volume should be approximately 1.8-2 ml.

H. Preparation of Competent Cells

Wash Buffer	*for 200 ml:*
5 mM Tris-HCl, pH 7.6	1 ml 1 M Tris-HCl, pH 7.6
10 mM $MgCl_2$	667 µl 3 M $MgCl_2$
50 mM NaCl	4 ml 2.5 M NaCl
Calcium Buffer	*for 100 ml:*
10 mM Tris-HCl, pH 7.6	1 ml 1 M Tris-HCl, pH 7.6
10 mM $MgCl_2$	333 µl 3 M $MgCl_2$
100 mM $CaCl_2$	3.33 ml 3 M $CaCl_2$
Glycerol Stock Storage Buffer	*for 25 ml:*
	21.5 ml Calcium Buffer
	3.5 ml glycerol

1. 100 ml LB preheated to 37°C and inoculated with 1% volume of an overnight culture.

2. Grow to a cell density of $O.D._{600}$ of ~0.4. Put on ice for 15 minutes. Before proceeding, make sure that the culture is ice-cold.

3. Transfer to an autoclaved 250 ml centrifuge bottle and pellet the cells for 5 minutes at 10,000 x G. (8750 rpm, JA-17)

4. Resuspend cells very gently in 50 ml Wash Buffer and spin as before.

5. Repeat step 4.

6. Resuspend the pellet in 25 ml Calcium Buffer. Store on ice for 30 minutes.

7. Spin as before and resuspend the cells in 4 ml Storage Buffer.

8. Store in 250 µl aliquots at -80°C for up to 3 months.

I. Transformation of Competent Bacteria with Plasmid DNA

Luria Broth (LB)	*for 1 L:*
Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g

H_2O to 1 L
NaOH to pH = 7.5

0.5 μg of plasmid DNA (containing a constitutively expressed ampicillin resistance gene) was mixed with a thawed aliquot of $CaCl_2$-prepared JM109 and allowed to stand on ice for 30 minutes. The suspensions were warmed to 37°C for 5 minutes, then 1 ml of LB was added to each and they were allowed to incubate 1 hour at 37°C. 2 ml of LB top agar (LB + 0.7% agar) warmed to 45°C were added, mixed quickly and spread on LB plate agar (LB + 1.5% agar) containing 50 μg/ml ampicillin. Plates were incubated upside down at 37°C overnight.

J. QIAEX II Agarose Gel Extraction Protocol

1. Excise the DNA band from the agarose gel with a clean, sharp scalpel.
   Minimize the size of the gel slice by removing excess agarose. Use a 1.5 ml microfuge tube for processing up to 250 mg agarose.

2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QX1 to 1 volume of gel for DNA fragments 100 bp-4kb; otherwise follow below:
   For example, add 300 µl of Buffer QX1 to each 100 mg of gel.

DNA fragments <100 bp	Add 6 volumes Buffer QX1
DNA fragments >4 kb	Add 3 volumes Buffer QX1 plus 2 volumes of H_2O
≥2% or Metaphor agarose gels	Add 6 volumes of Buffer QX1

3. Resuspend QIAEX II by vortexing for 30 sec. Add QIAEX II to the sample according to the table below and mix.

≤2 µg DNA	Add 10 µl of QIAEX II
2-10 µg DNA	Add 30 µl of QIAEX II
Each additional 10 µg DNA	Add additional 30 µl of QIAEX II

4. Incubate at 50°C for 10 minutes to solubilize the agarose and bind the DNA. Mix by vortexing every 2 min to keep QIAEX II in suspension. Check that the color of the mixture is yellow.
   If the color of the mixture is orange or purple, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color should turn to yellow. The incubation should then be continued for an additional 5 min at least. The adsorption of DNA to QIAEX II particles is only efficient at pH ≤7.5. Buffer QX1 now contains a pH indicator which is yellow at pH ≤7.5, and orange or violet at a higher pH, allowing easy determination of the optimal pH for DNA binding.

5. Centrifuge the sample for 30 sec and carefully remove supernatant with a pipet.

6. Wash the pellet with 500 µl of Buffer QX1.
   Resuspend the pellet by vortexing. Centrifuge the sample for 30 sec and remove all traces of supernatant with a pipet. This wash step removes residual agarose contaminants.

7. Wash the pellet twice with 500 µl of Buffer PE.
   Resuspend the pellet by vortexing. Centrifuge the sample for 30 sec and carefully remove all traces of supernatant with a pipet. These washing steps remove residual salt contaminants.

8. Air-dry the pellet for 10-15 minutes or until the pellet becomes white.
   If 30 µl of QIAEX II suspension are used, air-dry the pellet for approximately 30 min. Do

not vacuum dry, as this may cause overdrying. Overdrying the QIAEX II pellet may result in decreased elution efficiency.

9. To elute DNA, add 20 µl of 10 mM Tris-HCl, pH 8.5 or H_2O and resuspend the pellet by vortexing. Incubate according to the table below.

DNA fragments $\leq$ 4 kb	Incubate at room temp for 5 min
DNA fragments 4-10 kb	Incubate at 50°C for 5 min
DNA fragments > 10 kb	Incubate at 50°C for 10 min

Elution efficiency is dependent of pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water for elution, make sure that the pH is within this range, and store DNA at -20°C as DNA may degrade in the absence of a buffering agent.

10. Centrifuge for 30 sec. Carefully pipet the supernatant into a clean tube.
    The supernatant now contains the purified DNA.

11. Optional: repeat steps 9 and 10 and combine the eluates.
    A second elution step will increase the yield by approximately 10-15%.

K. GCG Commands

stringsearch This searches the various databases, genebank, embl, etc. for files that have the text ftring for which you ask the program to search. This file will be stored with the name you give it. You can view the information by typing in **more** and then the filename. (filename.strings)

fetch (genbank code) filename.gb_sy Allows you to retrieve a specific sequence file from among the many that stringsearch identified.

translate Converts DNA sequence in the "fetched" DNA sequence into protein sequence. This is stored in a new file. (filename.gb_pr)

peptidestructure This takes the file with the "translated" protein sequence and predicts secondary structure, hydrophobicity, antigenicity, etc. and stores this information in a new file.

plotstructure (filename.p2s) This takes the information in **peptidestructure** and graphs it depending upon the plot you specify.

view Allows you to view any file.

mapsort This shows the restriction enzymes that cut a certain sequence; can sort them by number of times they cut, and it will draw a map of all the sites.
filename.gb_sy -onc maxc=2 filename.mapsort

ls List files.

Printing:
postscript lzr 1200 LaserWriter
LN03 Script Printer
LPS20
Colorscript 100
EPSF

choose (laserwriter)-d
port - 1pr-P bicclw
language- POSTSCRIPT
device - EPSF
port - PLOT.PS

hpgl for any non-postscript file
lpr - Pbicclw filename (text files)
lpr - P printer filename.dat

L. Cell Culture

Remove aliquot of cells from liquid nitrogen, add to plate containing 10 ml medium containing serum but no antibiotic. Let subculture grow 5-6 hours, change medium. Aspirate old medium off of subculture plate and add 4 ml ESPG (EDTA, Saline, Phosphate, Glucose) to wash, aspirate off immediately. Add 2 ml T-ESPG (ESPG + trypsin) and aspirate off immediately. Shake cells loose by tapping plate against palm of hand until all cells visibly loose. Resuspend cells in 10 ml of medium, spin at 1/3 speed in swinging-bucket centrifuge for 5 minutes. Resuspend cells in medium and aliquot 50 µl to each chamber of LabTek chamber/slides. Change media after 24 hours.

M. Transfection Protocol (Life Technologies)

1. Prepare the following solution in 12 x 75 mm sterile tubes:

   Solution A: For each transfection, dilute 1-2 μg DNA into 100 μl serum-free DMEM.

   Solution B: For each transfection, dilute 5 μl of Lipofectamine Reagent (10 μg) into 95 μl of serum-free medium.

2. Combine the two solutions, mix gently, and incubate at room temperature for 15-45 min. to allow DNA-liposome complexes to form. The solution may appear cloudy, although this will not impede transfection. While complexes form, rinse the cells once with 2 ml of serum-free medium.

3. For each transfection, add 0.8 ml of serum-free medium to the tube containing the complexes. Mix gently and overlay the diluted complex solution onto the rinsed cells. Do not add antibacterial agents to media during transfection.

4. Incubate the cells with the complexes for 2-24 hours at 37°C in a CO_2 incubator.

5. After 72 hours, add α-estradiol (20 nM) or dexamethasone.