Comparison of the responsiveness of the pGL3 and pGL4 luciferase reporter vectors to steroid hormones

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The ovalbumin gene (Ov) *gene is responsive to estrogen, glucocorticoid, androgen, and progesterone. In our efforts to characterize the regulation of the* Ov *gene by steroid hormones, we have repeatedly observed that many vector backbones and promoters are responsive to steroids. In order to determine which vectors are most suitable for these types of analyses, vectors from Promega's pGL3 and newly engineered pGL4 Dual-Luciferase® Reporter Assay System were tested with both estrogen and/or corticosterone. The results confirmed that both series are induced by glucocorticoids in transient transfections of primary oviduct tubular gland cells, which contain glucocorticoid receptors, but not in MCF-7 cells, which do not. Modest effects that were dependent upon backbone and promoter context were observed with both series of vectors with estrogen. Thus, use of these vectors for experiments analyzing the effects of steroid hormones, especially glucocorticoids, should be done with caution. However, the new pGL4 series does have some advantages over the older series, and a comparison of transcription factor binding sites is reported.*

INTRODUCTION

The ovalbumin (*Ov*) gene has served as a unique model system in which to study tissue-specific gene expression and steroid hormone gene regulation for over 25 years. After initial stimulation by estrogen, this gene is responsive to four classes of steroid hormones: estrogen, androgen, glucocorticoid, and progesterone. Historically, we have used pBLCAT to analyze the *cis*-acting elements in the 5´ flanking region of this gene and the effects of overexpressing *trans*-acting factors. In an effort to increase the sensitivity of our analyses, limit the use of radioactive material, and decrease the time required to obtain experimental results, the pGL3 Dual-Luciferase® Reporter Assay System was evaluated for use in our system.

While the pGL3 vectors are designed to be transcriptionally neutral, proper controls are necessary to ensure that experimental treatments and/or overexpression of transcriptional regulators do not affect the promotorless vectors or the vectors containing promoters that will be used as internal controls (1). Numerous reports document the transcription factors and experimental treatments that transactivate or repress promoterless reporter vectors and internal control vectors containing promoters such as cytomegalovirus (CMV), herpes simplex virus thymidine kinase (HSV-TK), and simian virus 40 (SV40) (2–7). Here we report that the promoterless pGL3-basic and pRL-CMV vectors are transactivated by glucocorticoid treatment in primary oviduct tubular gland cell culture, while the promoterless pRL null vector remains unaffected by treatment. Due to this observation, pGL4 vectors were obtained and evaluated for estrogen and glucocorticoid responsiveness in two different cell culture systems. The results of this analysis are presented herein.

MATERIALS AND METHODS

Oviduct Tubular Gland Cell Culture and Transfection

White leghorn chicks were treated with estrogen in the form of diethystilbesterol (DES; Hormone Pellet Press, Leawood, KS, USA). DES pellets (20 mg) were implanted subcutaneously in the neck for 2 weeks, and the pellets were subsequently withdrawn 2 days prior to isolation of tubular gland cells as previously described in detail (8). Primary oviduct tubular gland cells were transfected with a total of 1 ug DNA using the Effectene® transfection reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's directions. DNA concentrations were held constant by the addition of appropriate amounts of pBlue-TOPO® (Invitrogen, Carlsbad, CA, USA). Cells were cultured for 24 h in F12 (Gibco, Grand Island, NY, USA)/Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) with 50 ng/mL insulin (Sigma) either alone or with 100 nM 17β-estradiol (Sigma), 1 µM corticosterone (Sigma), or 17β-estradiol and corticosterone. Following transfection, the cells were harvested and then lysed with 200 µL Passive Lysis Buffer (Promega, Madison, WI, USA). Twenty microliters cell extract

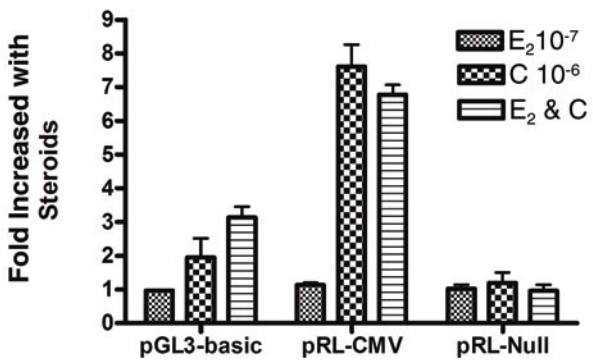

Figure 1. Transactivation of pGL3 luciferase reporter vectors in the presence of 17β-estradiol (E_2) and corticosterone (C) in oviduct cells. Oviduct cells were transfected with pGL3 luciferase reporter plasmids and cultured alone or in the presence of 100 nM E_2, 1 µM C, or E_2 and C. Cells were transfected with 1 µg promoterless vector (pGL3-basic and pRL null) or 5 ng vector (pRL-CMV) containing a promoter. Luciferase activity was measured 24 h after transfection and is reported as the average fold change relative to basal activity for each vector. The relative light units (RLUs) from three separate experiments with duplicate dishes per treatment were averaged and analyzed by analysis of variance (ANOVA). The SEM is indicated by the error bars, and the *P* value for each is <0.0001.

were assayed for luciferase activity using the Dual Luciferase Reporter Assay kit (Promega). Relative light units (RLU) were determined using 100 µL of the appropriate substrate and a Lumat LB 9507 luminometer (EG&G-Berthold, Bad Wildbad, Germany). The concentrations of the cell extracts were determined using the Bradford method, and the raw RLUs were normalized to protein concentration. The luciferase values represent the light emitted over a period of 10 s and are expressed as fold change with steroid treatment for each vector.

MCF-7 Cell Culture and Transfection

MCF-7 cells (ATCC, Manassas, VA, USA) were maintained in minimum essential medium (MEM; Gibco), 10% fetal bovine serum (FBS; Tissue Culture Biological, Tulare, CA, USA), 10 µg/mL bovine insulin (Sigma), and 50 U and 50 µg/mL PenStrep (Invitrogen). Twenty-four hours prior to transfection, the cells were cultured in MEM (minus phenol red), 10% charcoal-stripped FBS, insulin, and PenStrep. Cells were transfected with a total of 400 ng DNA using Effectene transfection reagent according to the manufacturer's directions. DNA concentrations were held constant by the addition of appropriate amounts of pBlue-TOPO. Cells were cultured for 24 h in MEM (minus phenol red), 10% charcoal stripped FBS, insulin, and PenStrep either alone or with 1 nM 17β-estradiol, 10 nM hydrocortisone (HCT; Sigma), or 17β-estradiol and HCT as indicated. Following transfection, the cells were harvested and lysed, and the protein extracts were assayed for luciferase activity as described above.

Plasmids

The pGL3 luciferase vectors were obtained from Promega, and the pBlue-TOPO vector was obtained from Invitrogen. The pGL3 vectors consist of pGL3 basic (firefly), pRL null (*Renilla*), and pRL-CMV. The pGL4 vectors, provided as a generous gift by Promega, include pGL4.10 [*luc* 2] (firefly null), pGL4.13 [*luc* 2/SV40], pGL4.70 [*hRluc*] (*Renilla* null), pGL4.75 [*hRluc*/CMV], pGL4.73 [*hRluc*/SV40], and pGL4.74 [*hRluc*/TK]. Control vectors used to determine estrogen or glucocorticoid responsiveness include 1100 bp of the vitellogenin regulatory region and the TK promoter subcloned into pGL3 (pGL3-TK101) and the mouse mammary tumor virus (MMTV) promoter subcloned into pGL3 (pGL3-MMTV).

Sequence Analysis

The sequences of pGL3-basic, pGL4.10 [*luc* 2], pRL null, and pGL4.70 [*hRluc*] were analyzed for the presence of transcription factor binding sites using the TRANSFAC program at bimas.dcrt.nih.gov/molbio/signal. The sequences were then analyzed for changes in transcription factor binding sites..

RESULTS AND DISCUSSION

The pGL3 luciferase reporter vectors are routinely used for the analysis of steroid hormone responsive genes. While the pRL-TK (*Renilla* luciferase gene controlled by the TK promoter) has been observed to be responsive to dihydrotestosterone and dexamethasone (9), the effects of estrogen and natural glucocorticoids remain unreported. For the present study, we transfected primary chicken oviduct tubular gland cells with pGL3 basic, pRL null, and pRL-CMV and evaluated the effects of 17β-estradiol, corticosterone, or the combination of 17β-estradiol and corticosterone on these vectors (Figure 1). While 100 nM17β-estradiol had little or no effect on these vectors, the addition of 1 µM corticosterone transactivated pGL3-basic firefly 2-fold, and the combination of 17β-estradiol and corticosterone activated this promoterless vector 3-fold. The promoterless pRL null *Renilla* vector was unaffected by steroid hormone treatment, however, the addition of the CMV promoter (pRL-CMV) to this vector resulted in a 7- to 8-fold induction of *Renilla* activity in the presence of corticosterone, while no additional increase was observed with the additon of 17β-estradiol. This result indicates that the CMV promoter is responsive to glucocorticoid treatment, and therefore, pRL-CMV is not an appropriate vector for use as an internal control in studies involving the use of glucocorticoids. Furthermore,

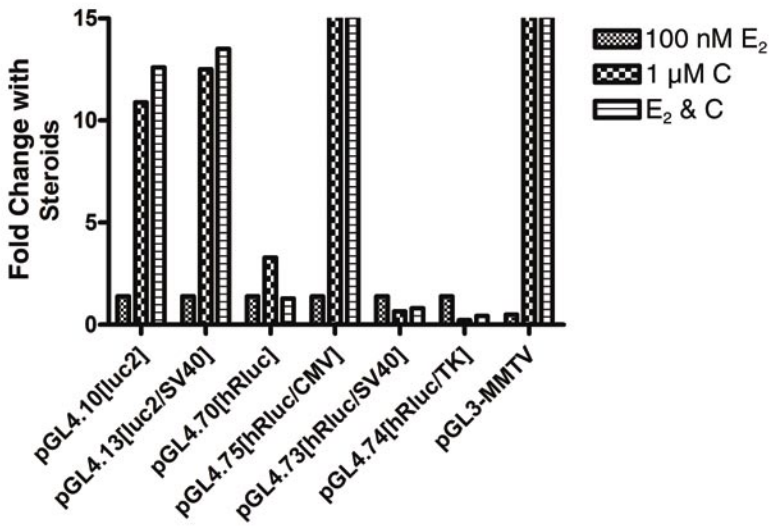

Figure 2. Transactivation of pGL4 luciferase reporter vectors in the presence of 17β-estradiol (E_2) and corticosterone (C) in oviduct cells. Oviduct cells were transfected with pGL4 reporter plasmids and cultured alone or in the presence of 100 nM E_2, 1 µM C, or E_2 and C. Cells were transfected with 1 µg promoterless vector (pGL4.10 [*luc* 2] and pGL4.70 [*hRluc*]) or 5 ng vectors (pGL4.13 [*luc* 2/SV40], pGL4.75 [*hRluc*/CMV], pGL4.73 [*hRluc*/SV40], and pGL4.74 [*hRluc*/TK]) containing promoters. Luciferase activity was measured 24 h after transfection and is reported as the fold change relative to basal activity for each vector. This is one experiment done in duplicate that has been repeated with similar results at least twice.

Table 1. Putative Transcription Factor Binding Sites Deleted or Added to pGL4 Vectors Compared with pGL3 Vectors

TF	Luc 2/pGL3-basic	hRLuc/pRL null
α-CBF	2/5	6/15
α-IRP	2/5	6/15
AP1	0/0	4/7
c-Myb	1/3	5/7
c-Myc	4/8	0/2
CBF-B	3/5	6/15
CCAAT-BF	4/6	7/16
CDP	3/5	6/15
CP1	2/19	15/55
CP2	3/6	6/16
CREB	3/9	1/24
CTF	2/6	7/18
GATA-1	21/35	7/21
GATA-2	2/2	3/3
GATA-3	0/0	4/4
GR half site	8/46	8/47
GRE	0/1	2/5
H1TF-2	4/5	6/15
H4TF-1	+3	1/2
HNF-1	3/3	1/1
NF-1	15/96	+7
NF-Y	3/5	6/15
Pit 1	11/19	6/23
SP1	+60	+21
SRF	4/6	9/18
TATA	0/0	2/3
TBP	7/11	3/8
TFIID	4/5	2/3

Columns 2 and 3 reflect the number of sites deleted in pGL4 compared with the total number of sites in pGL3 vectors or the number of sites added to pGL4 vectors. None of these sites have been tested for activity. TF, transcription factor.

the use of pGL3 basic to analyze glucocorticoid-responsive genes under these conditions would result in a 3-fold inflation of the true transactivation achieved with hormone treatment.

Glucocorticoids regulate transcription by activating the glucocorticoid receptor, which binds glucocorticoid response elements in promoters of target genes. Glucocorticoid receptors also associate with transcription factors such as COUP-TFII, AP1, NF-1, NF-κB, and SP1, among others, to antagonize or stimulate the activation potential of these transcription factors (10). The glucocorticoid receptor can interact with NF-1 and SP1 to enhance activation potential, and mutation of an NF-1 binding site in the MMTV long terminal repeat (LTR) abolishes the ability of glucocorticoids to activate this promoter (11). Our results show that pGL3-basic and pRL-CMV are glucocorticoid-responsive in primary oviduct tubular gland cell culture. These results confirm that the pGL3 basic vector and CMV promoter both contain sequences that are transactivated by glucocorticoid treatment. Therefore, newly engineered pGL4 vectors were evaluated for estrogen- and glucocorticoid-responsiveness.

In an effort to identify luciferase reporter vectors that are suitable for our system, pGL4 vectors were obtained from Promega and evaluated for steroid hormone responsiveness in primary oviduct tubular gland cells and MCF-7 cells. Control vectors used to determine estrogen or glucocorticoid responsiveness were pGL3-TK101 and pGL3-MMTV, respectively. Glucocorticoid-responsive primary oviduct tubular gland cells were transfected with the pGL4 vectors and treated with 100 nM 17β-estradiol, 1 μM corticosterone, or 17β-estradiol and corticosterone (Figure 2). The promoterless pGL4.10 [*luc* 2] firefly vector was transactivated 11- to 12-fold with corticosterone or with estradiol and corticosterone. This is a 4-fold greater induction than that observed with pGL3-basic. Analysis of the vector sequence revealed numerous NF-1 sites and additional SP1 sites in pGL4.10 [*luc* 2], which may explain the increase in activation of this promoterless vector in the presence of glucocorticoid (Table 1). Addition of the SV40 promoter to pGL4.10 [*luc* 2] does not affect the glucocorticoid responsiveness of this vector (pGL4.13 [*luc* 2/SV40]), indicating that the SV40 promoter is not glucocorticoid-responsive. The pGL4.70 [*hRluc*] promoterless *Renilla* vector is slightly responsive to glucocorticoid treatment but is unaffected by the combination of estradiol and corticosterone. The addition of the CMV promoter, which contains numerous NF-1 and SP1 binding sites, to pGL4.70 [*hRluc*], significantly increases the glucocorticoid responsiveness of this vector, more so than observed with pRL-CMV (Figure 1). The addition of TK and SV40 promoter sequences to pGL4.70 [*hRluc*] results in repression of luciferase activity in oviduct cells. The pGL4.74 [*hRluc*/TK] vector is significantly repressed, compared with the promotorless vector, by 17β-estradiol, corticosterone, and the combination of 17β-estradiol and corticosterone. The pRL-TK *Renilla* vector was previously reported to be repressed by dexamethasone treatment (9). We observe not only significant repression of pGL4.74 [*hRluc*/TK] by glucocorticoid but also repression of this vector in the presence of 17ß-estradiol, which is a new observation. The addition of the SV40 promoter to the pGL4.70 [*hRluc*] vector (pGL4.73 [*hRluc*/SV40]) results in a slight repression of *Renilla* activity in the presence of either 17β-estradiol, corticosterone, or both. The pGL4 vectors pGL4.10 [*luc* 2] and pGL4.75 [*hRluc*/CMV] exhibit increased glucocorticoid responsiveness compared with their pGL3 counterparts, pGL3-basic, and pRL-CMV in glucocorticoid-responsive oviduct tubular gland cells. Unfortunately, these results show that pGL4 vectors, like pGL3 vectors, are glucocorticoid-responsive. However, other changes in the vector sequences (Table 1) may permit these vectors to be used in overexpression studies and under experimental conditions for which the pGL3 vectors are not suited.

As expected, MCF-7 cells are responsive to estrogen but not to glucocorticoid as shown by the 6-fold increase in luciferase activity of the pGL3-TK101 vector in the presence of 17β-estradiol and no induction of pGL3-MMTV luciferase activity in the presence of HCT (Figure 3). These cells were transfected with the pGL4 vectors and treated with 1 nM 17β-estradiol,10 nM HCT, or both. The promoterless pGL4.10 [*luc* 2] and pGL4.70 [*hRluc*] vectors are slightly repressed by 17β-estradiol, HCT, and the combination of 17β-estradiol and HCT as compared with pGL3-basic, which is unaffected by steroid hormone treatment in these estrogen-responsive cells. The addition of the SV40 promoter to the pGL4.10 [*luc* 2] firefly vector (pGL4.13 [*luc* 2/SV40]) results in a modest relief of the repression observed with the promoterless vector in the presence of 17β-estradiol and HCT. The presence of the CMV or SV40 promoters in the pGL4.70 [*hRluc*] *Renilla* vector abolishes the repression observed with 17β-estradiol treatment. However, treatment of pGL4.73 [*hRluc*/SV40] with the combination of 17β-estradiol and HCT results in 50% reduction in luciferase activity. The pGL4.74 [*hRluc*/TK] *Renilla* vector is only slightly repressed by 17β-estradiol and the combination of 17β-estradiol and HCT and is minimally affected by HCT alone. In contrast to the behavior of pGL4 vectors in primary oviduct tubular gland cells, the promotorless vectors, pGL4.10 [*luc* 2] and pGL4.70 [*hRluc*], are negatively regulated by estrogen treatment in the estrogen-responsive MCF-7 cell line.

The sequences of pGL3-basic, pGL4.10 [*luc* 2], pRL null, and pGL4.70 [*hRluc*] were analyzed for transcription factor binding sites using the TRANSFAC database. The transcription factor binding site profiles for the pGL4 vectors were compared with their respective pGL3 counterparts, and significant changes in transcription factor binding sites are described in Table 1. Of particular note, is the complete elimination or significant reduction of GATA sites in both pGL4 vectors. Additionally, background transcription may be further minimized by the reduction of TATA, TBP, and TFIID binding sites. Unfortunately, as a result of these changes, there is an increase in the number of binding sites for SP1. While these putative SP1 sites have not been tested, our results suggest that some of these sites are functional. A possible solution may be the use of vectors containing both the firefly and *Renilla* genes. Two such vectors have been described (12,13), each of which carries both luciferase genes, but the pFRL2 vector (11) orients the genes in opposite directions to minimize leaky transcription of the *Renilla* gene. Experiments are in progress to evaluate both of these vectors.

In conclusion, our results underscore the necessity of appropriate controls when using expression vectors as reporters or internal controls in transient transfection assays. A reporter vector that is influenced by treatment or overexpression of transcription factors could cause experimental results to be inflated or underreported, depending upon the transcriptional effect. Likewise, it is not safe to assume that a vector reported to be transcriptionally neutral in one cell type will behave similarly in every other cell type when subjected to like treatment. In addition to proper controls, sequence analysis of reporter vectors for transcription factor binding sites may be advisable to discern which vector and/or promoter is best suited to experimental conditions.

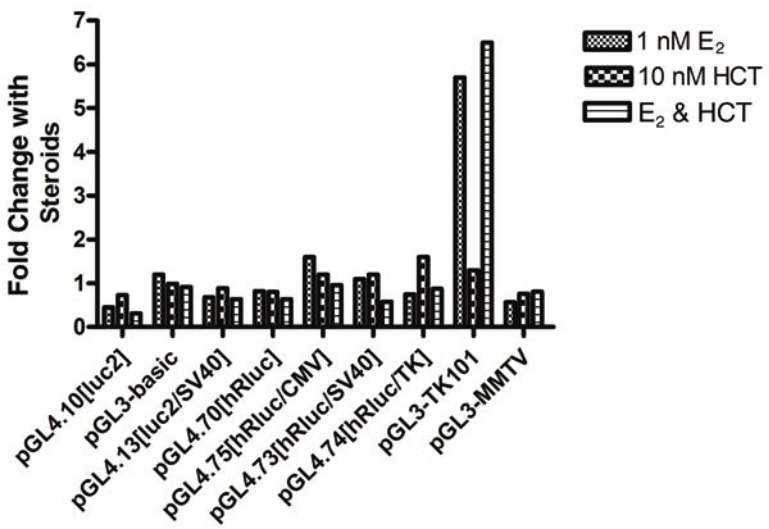

Figure 3. Transactivation of pGL4 luciferase reporter vectors in the presence of 17β-estradiol (E_2) and hydrocortisone (HCT) in MCF-7 cells. MCF-7 cells were transfected with pGL4 reporter plasmids and cultured alone or in the presence of 1 nM E_2, 10 nM HCT, or E_2 and HCT. Cells were transfected with 400 ng of promoterless vector (pGL4.10 [*luc* 2] and pGL4.70 [*hRluc*]) or 5 ng of vectors (pGL4.13 [*luc* 2/SV40], pGL4.75 [*hRluc*/CMV], pGL4.73 [*hRluc*/SV40] and pGL4.74 [*hRluc*/TK]) containing promoters. Luciferase activity was measured 24 h after transfection and is reported as the average fold change relative to basal activity for each vector. This is one experiment done in duplicate per treatment that has been repeated with similar results at least twice.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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