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Pitfall of an Internal Control Plasmid: Response of *Renilla* Luciferase (pRL-TK) Plasmid to Dihydrotestosterone and Dexamethasone

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ABSTRACT

The thymidine kinase promoter-*Renilla* luciferase reporter plasmid (pRL-TK) is commonly used as a control for transfection efficiency in the Dual-Luciferase® Reporter Assay System. While investigating hormone response elements in the promoters of the androgen-dependent, epididymis-specific EP2 gene, we found that hormone treatment affected the luciferase activity of pRL-TK-transfected cells. In African Green Monkey Kidney (CV-1) cells, cotransfected transiently with a hormone-responsive promoter-firefly luciferase reporter plasmid and with pRL-TK, *Renilla* luciferase activity increased in response to dihydrotestosterone (DHT) and decreased in response to dexamethasone (DEX). When a thromboxane synthase promoter *Renilla* luciferase plasmid (pRL-TS) was used in place of pRL-TK, *Renilla* luciferase activity remained constant in CV-1 cells treated with DHT but decreased in CV-1 cells treated with DEX. In transfection studies, internal control plasmid expression in response to treatment must be carefully monitored to ensure proper interpretation of normalized results.

INTRODUCTION

EP2 is an androgen-dependent, epididymis-specific gene (9). To study the androgen response elements (AREs) in this gene, candidate promoter sequences were cloned upstream of the simian virus 40 (SV40) promoter in the pGL3-promoter vector (Promega, Madison, WI, USA), which expresses firefly luciferase as a reporter enzyme. In such studies, potential variability in transfection efficiency is controlled for

by the use of the Dual-Luciferase® Reporter (DLR™) Assay System (Promega). In the DLR assay, cells are cotransfected with the experimental pGL3-promoter plasmid that encodes firefly luciferase as a reporter and with the control promoter plasmid that encodes *Renilla* luciferase as a reporter. As each luciferase enzyme has a distinct substrate, reporter activity from each plasmid can be measured sequentially in the same cell lysate sample (8).

Firefly luciferase expression, driven

by the experimental promoter plasmid, should depend on the experimental treatment. *Renilla* luciferase expression, driven by the control plasmid, should be independent of the experimental treatment (8) and the enzyme activity measured should depend only on the number of cells that took up plasmid DNA. To correct experimental promoter activity for potential variations in transfection efficiency, firefly luciferase activity is divided by *Renilla* luciferase activity. Therefore, any treat-

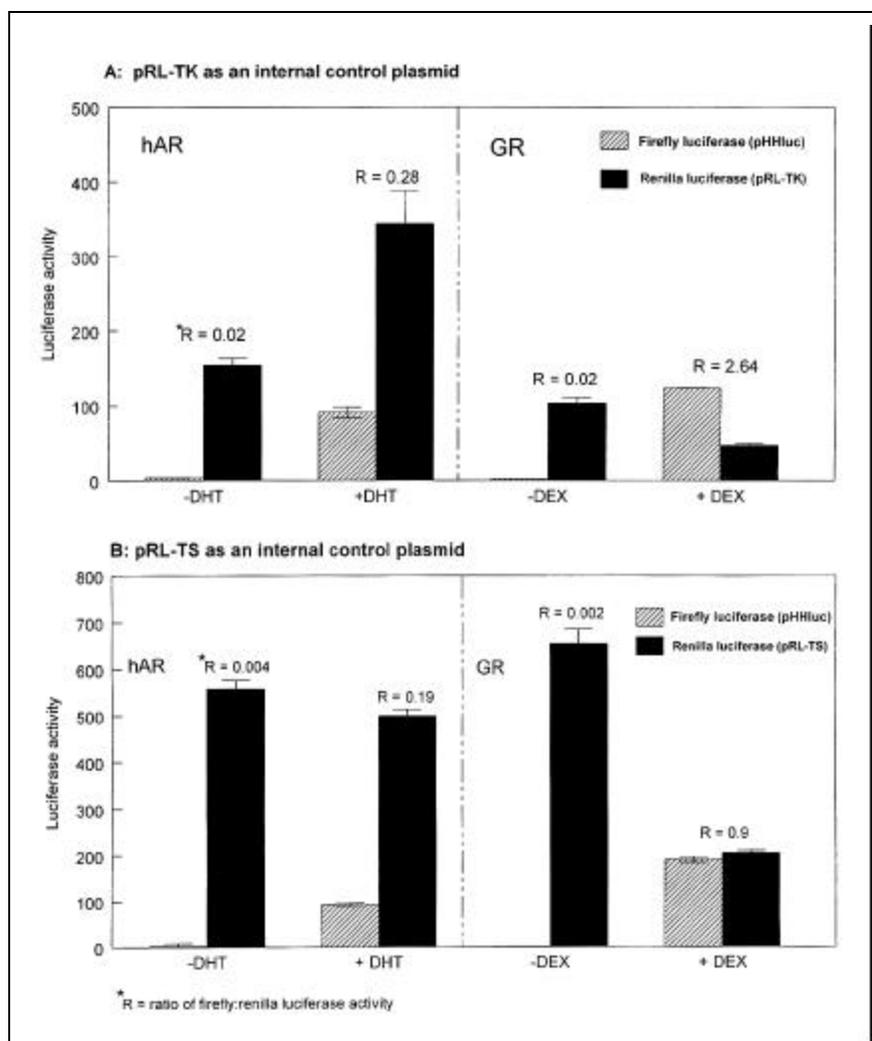


Figure 1. Effects of DHT and DEX on luciferase activity in CV-1 cells. CV-1 cells, cotransfected with experimental plasmid pHHluc, control plasmids pRL-TK or pRL-TS, pCMVhAR (hAR; androgen receptor) or pCMVhGR (GR; glucocorticoid receptor) were lysed and luciferase activity measured as described in Materials and Methods. Briefly, the CV-1 cells were seeded at a density of 60 000 cells/well in 12-well cell culture clusters (Corning Costar, Corning, NY, USA). After 20–24 h in culture, cells were transfected and luciferase activity was measured as luminescence units using the Turner Design TD-20/20 luminometer. Data represent three independent experiments and are expressed as mean \pm SEM luciferase activity ($n = 5$). The calculated ratios of firefly luciferase activity to *Renilla* luciferase activity (R) are shown above the bars representing luciferase activity values.

ment-induced change in *Renilla* luciferase activity would result in misinterpretation of the normalized results. We report here that in African Green Monkey Kidney (CV-1) cells the expression of the *Renilla* luciferase from the control plasmid pRL-TK, which uses the constitutively active herpes simplex virus thymidine kinase (HSV-TK) promoter, changes in response to both dihydrotestosterone (DHT) and dexamethasone (DEX).

MATERIALS AND METHODS

The DLR Assay System was used to measure luciferase activity expressed by the experimental plasmid, pHHluc (ATCC no. 37581; ATCC, Manassas, VA, USA), and by the internal control *Renilla* luciferase-expressing plasmids pRL-TK or pRL-TS. pHHluc is a firefly luciferase-expressing plasmid driven by the mouse mammary tumor virus (MMTV) promoter that contains glucocorticoid, progesterone and androgen response elements (2,5,7). pRL-TS was generated by cloning the 120-bp thromboxane synthase promoter (-90 bp to +30 bp) sequence (R. Sheng, University of Maryland, College Park, MD, USA) into the *Hind*III site of the pRL-null vector (Promega). CV-1 cells (ATCC no. CCL-70) were grown in 12-well plates in 1.5 mL minimum essential medium (MEM; Life Technologies, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) and phenol red. They were transfected in replicates of five wells with 375 ng experimental plasmid pHHluc, 75 ng control plasmid pRL-TK or control plasmid pRL-TS, and 25 ng either human androgen receptor expression plasmid pCMVhAR (E. Wilson, University of North Carolina, Chapel Hill, NC, USA) or glucocorticoid receptor expression plasmid pCMV4/neo/rGR (D. Pearce, University of California, San Francisco, CA, USA).

For transfection, Effectene™ transfection reagent (Qiagen, Valencia, CA, USA) at a DNA:Enhancer:Effectene ratio of 1:8:8 was added to the cells in MEM. After 24 h incubation, the cells were washed once with PBS and replenished with steroid-free (charcoal-stripped) 10% FBS, phenol red-free

MEM with or without 10 nM DHT or DEX. Cells cotransfected with the androgen receptor expression plasmid were treated with or without 10 nM DHT. Cells cotransfected with the glucocorticoid receptor expression plasmid were treated with or without 10 nM DEX. After 24 h incubation, cells were washed twice with 1 mL PBS and lysed with 250 μ L passive lysis buffer (Promega). Firefly luciferase and *Renilla* luciferase activity were measured sequentially from each well using the DLR assay reagents (Promega) and a TD-20/20 Luminometer (Turner Design, Sunnyvale, CA, USA). First, 25 μ L Luciferase Assay Reagent II were pipetted into 12 \times 55 mm polypropylene luminometer tubes (Continental Laboratory Products, San Diego, CA, USA). Second, 5 μ L CV-1 cell lysate were added, and firefly luciferase activity was measured. Third, 25 μ L Stop and Glo® Reagent (Promega) were added, and *Renilla* luciferase activity was measured. The ratio of the firefly luciferase activity to *Renilla* luciferase activity was calculated.

RESULTS AND DISCUSSION

Luciferase activity was measured in lysates of CV-1 cells cotransfected with experimental plasmid pHHluc and either control plasmid pRL-TK or control plasmid pRL-TS (Figure 1). As expected from the presence of hormone response elements (HREs) in the MMTV promoter (2,5,7), firefly luciferase expression from pHHluc increased in response to DHT and DEX treatment. When pRL-TK was used as an internal control plasmid, DHT increased the ratio of firefly luciferase activity to *Renilla* luciferase activity from 0.0225 to 0.2785, indicating an apparent 12-fold induction of pHHluc promoter activity (Figure 1A, left panel). However, in lysates of CV-1 cells cotransfected with pHHluc and pRL-TS, DHT increased the ratio of firefly luciferase activity to *Renilla* luciferase activity from 0.0040 to 0.1872, indicating a 47-fold induction of pHHluc promoter activity (Figure 1B, left panel). To resolve this discrepancy, we directly compared the absolute promoter activities of pRL-TK and pRL-TS

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in the presence and absence of DHT. In pRL-TK-transfected cells, *Renilla* luciferase activity was stimulated twofold by DHT (Figure 1A, left panel), while in pRL-TS-transfected cells it remained virtually unchanged with DHT exposure. Thus, approximately 75% of the expected MMTV promoter response to DHT was masked by the response of pRL-TK to DHT treatment.

In CV-1 cells cotransfected with pHHluc and pRL-TK or pRL-TS, DEX inhibited *Renilla* luciferase activity (Figure 1, A and B, right panels). DEX also inhibited *Renilla* luciferase activity in L6 skeletal muscle cells transfected with pRL-TK (data not shown). However, DEX did not affect *Renilla* luciferase activity in L6 skeletal muscle, LLCPK1-GR101 (LLCPK-1 cells stably transfected with glucocorticoid receptor expression vector), HepG2 or Jurkat cells cotransfected with pRL-TS (data not shown).

These data show that the regulation of the TK and TS promoters depends on the cells in which they are studied. This

cell-dependent promoter activity is best explained by assuming that the CV-1 cells and the other cell lines tested possess different combinations of transcription factors, cofactors or other auxiliary factors that interact with the androgen receptor or the glucocorticoid receptor. It is also possible that the presence of the transfected DNA or the expression level of the nuclear receptors influences the two promoters. For example, cotransfection of a second plasmid, pSV2CAT, together with pRSV β gal has been reported to decrease the amount of (β -galactosidase produced in HeLa cells, but not in keratinocytes (3).

These data also demonstrate the potential danger involved in using a control promoter plasmid to correct for possible variations in transfection efficiency during promoter activation studies. CV-1 cells have been used extensively for studying the enhancer properties of androgen response elements (6) and the properties of androgen and glucocorticoid receptors (1,4). It is therefore important to know the hormone dependence of any control promoter that might be used as an internal control in studies of the steroid hormone dependence of a gene's promoter.

We conclude that in CV-1 cells cotransfected with androgen receptor, pRL-TK *Renilla* luciferase activity increases in the presence of DHT. In CV-1 cells cotransfected with glucocorticoid receptor, pRL-TK *Renilla* luciferase activity and pRL-TS luciferase activity decrease in the presence of DEX. We suggest that these effects are cell type-specific and probably reflect different sets of transcription factors and cofactors in the different cell lines used.

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