components of an RT kit to buffer the heparinase reaction. This is a quick and easy way to treat RNA samples potentially contaminated with heparin, such as RNA samples collected from lung or liver tissues or from unclotted samples of blood.

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REFERENCES

1. Holodniy, M., S. Kim, D. Katzenstein, M. Konrad, E. Groves, and T.C. Merigan. 1991. Inhibition of human immunodeficiency virus gene amplification by heparin. *J. Clin. Microbiol.* 29:676-679.
2. Izraeli, S., C. Pfeleiderer, and T. Lion. 1991. Detection of gene expression by PCR amplification of RNA derived from frozen heparinized whole blood. *Nucleic Acids Res.* 19:6051.
3. Imai, H., O. Yamada, S. Morita, S. Suehiro, and T. Kurimura. 1992. Detection of HIV-1 RNA in heparinized plasma of HIV-1 seropositive individuals. *J. Virol. Methods* 36: 181-184.
4. Jung, R., C. Lubcke, C. Wagener, and M. Neumaier. 1997. Reversal of RT-PCR inhibition observed in heparinized clinical specimens. *BioTechniques* 23:24-28.
5. Beutler, E., T. Gelbart, and W. Kuhl. 1990. Interference of heparin with the polymerase chain reaction. *BioTechniques* 9:166.
6. Bai, X., S. Fischer, S. Keshavjee, and M. Liu. 2000. Heparin interference with reverse transcriptase polymerase chain reaction of RNA extracted from lungs after ischemia-reperfusion. *Transpl. Int.* 10:146-150.

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Unexpected sensitivity of synthetic *Renilla* luciferase control vectors to treatment with a cyclopentenone prostaglandin

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Internal control reporter vectors are widely used in transient transfection assays to correct for inherent variations in transfection efficiency between experiments. The ideal control reporter vector should provide stable and high

expression of the reporter gene, and the expression should not be affected by experimental conditions, such as cotransfected vectors or the agonists used. However, internal control vectors do not necessarily provide an unbiased

Figure 1. Luciferase activity of pHRG-TK and pHRG-B transfected into HEK 293 cells in response to treatment with 15d-PGJ₂, interleukin 1 β (IL-1 β), or tumor necrosis factor- α (TNF- α). HEK 293 cells (ATCC, Manassas, VA, USA) were grown to 90% confluence in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) and transfected with 0.5 μ g plasmid DNA (pHRG-TK or pHRG-B) using 2 μ L LIPOFECTAMINETM 2000 (Invitrogen) according to the manufacturer's instructions. After 4 h, the cells were washed and treated with the indicated concentrations of 15d-PGJ₂ or a corresponding amount of methyl acetate (MA) carrier, media alone (control), IL-1 β (1 ng/mL), or TNF- α (10 ng/mL) in serum-free medium for 20 h. The cells were lysed, and the luciferase activity was measured using the Dual-Luciferase[®] Reporter Assay System (Promega). The luciferase activity was normalized to the protein concentration (μ g/mL) of the lysate. Data are presented as the mean ($\bar{x} \pm$ SEM) of experiments done in triplicate and are representative of at least three independent experiments. Data were examined for significant differences using analysis of variance (ANOVA), followed by individual comparisons with the Bonferroni post-test. $P < 0.05$ was considered significant. (A) Dose-dependent effect of 15d-PGJ₂ on pHRG-TK activity. $*P < 0.05$; $**P < 0.01$ versus MA. (B) Effect of IL-1 β and TNF- α on pHRG-TK activity. (C) Dose-dependent effect of 15d-PGJ₂ on pHRG-B activity. $*P < 0.001$ versus MA. (D) Effect of IL-1 β and TNF- α on pHRG-B activity.

standard to normalize plate-to-plate variations. For example, the firefly luciferase reporter plasmid pRSV-luc and the native *Renilla* luciferase reporter plasmid pRL-TK were found to be sensitive to cotransfection with certain nuclear receptors (1,2). pRL-TK was also affected by dihydrotestosterone and dexamethasone treatment (3). β -galactosidase reporter vectors were shown to repress platelet-derived growth factor β polypeptide (PDGFB) regulatory element reporter gene expression (4), were sensitive to cotransfection of the Fli-1 transcription factor (5), and were enhanced by angiotensin II (6). All of these studies suggest that using internal

control reporter systems in certain experiments may lead to a misinterpretation of the results.

While investigating the regulation of the interleukin-8 (*IL-8*) gene promoter by the cyclopentenone prostaglandin 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2 (15d-PGJ₂) (Cayman Chemical, Ann Arbor, MI, USA), we consistently observed changes in the activity of the second-generation *Renilla* reporter vectors phRG-TK and phRG-B (Promega, Madison, WI, USA), which were used as internal control plasmids for *IL-8* promoter constructs. Because these second-generation *Renilla* vectors were engineered to reduce the risk of anomalous transcriptional behavior (<http://www.promega.com/tbs/tm237/tm237.pdf>), we undertook the present study to formally assess the sensitivity of phRG-TK and phRG-B to 15d-PGJ₂ and the proinflammatory cytokines IL-1 β and tumor necrosis factor- α (TNF- α) (both from R&D Systems, Minneapolis, MN, USA).

Here we show that 15d-PGJ₂ dose-dependently inhibited the expression of phRG-TK (Figure 1A) and phRG-B (Figure 1C), while its solvent, methyl acetate, had little effect (Figure 1, A and C). The maximum inhibition of *Renilla* expression for both vectors (5- to 6-fold) was achieved with concentrations of 15d-PGJ₂ between 7.5 and 10 μ M. These 15d-PGJ₂ concentrations were non-toxic, but cellular toxicity characterized by cell shrinkage, development of cytoplasmic vesicles, and detachment from the culture flasks was apparent at concentrations of 15 μ M or greater. The inhibition of *Renilla* expression was stimulus-specific because IL-1 β

and TNF- α had no significant effect on phRG-TK or phRG-B activity (Figure 1, B and D, respectively).

To determine whether 15d-PGJ₂ also affected firefly luciferase, first-generation *Renilla* luciferase, and nonluciferase plasmids, we tested the response of pGL3-Basic, pRL-TK, and pSV- β -galactosidase (all from Promega) to the prostaglandin. At concentrations between 7.5 and 10 μ M, 15d-PGJ₂ attenuated the expression of pGL3-Basic and pRL-TK by approximately 2-fold (data not shown). Similarly, 15d-PGJ₂ had a small inhibitory effect on pSV- β -galactosidase, with a maximum inhibition of 36% at 10 μ M (Figure 2A). Unexpectedly, IL-1 β and TNF- α increased β -galactosidase activity 2.2- to 3.3-fold, respectively (Figure 2B).

Presently, there are no known reports that the synthetic second-generation *Renilla* co-reporter vectors phRG-TK and phRG-B are sensitive to cotransfected plasmids or chemical or cytokine agonists. However, the cyclopentenone prostaglandin 15d-PGJ₂ significantly inhibited the expression of phRG-TK and phRG-B at concentrations that were not cytotoxic to the recipient cells. Although 15d-PGJ₂ also attenuated the expression of the native *Renilla* vector pRL-TK, the firefly luciferase vector pGL3, and the non-luciferase vector pSV- β -galactosidase, the degree of inhibition was less pronounced than for the synthetic *Renilla* genes, which suggests vector-specific sensitivity. While these data demonstrate that phRG-TK and phRG-B can respond inappropriately to a specific experimental stimulus in the context of HEK 293 transfection, other stimuli and cell lines will need to be tested to determine the extent of this response.

The precise site of action and mechanism of inhibition of phRG-TK and phRG-B by 15d-PGJ₂ remains unclear. This effect does not appear to be specific for the vectors' promoter sequences because although phRG-TK and phRG-B are both derived from the pGL3-Basic vector backbone, phRG-TK contains the herpes simplex virus thymidine kinase promoter, whereas phRG-B is promoterless. Given the disparity in the degree of prostaglandin-mediated inhibition of pGL3-Basic compared to phRG-TK and phRG-B, it

Figure 2. β -Galactosidase activity of pSV- β -galactosidase transfected into HEK 293 cells in response to treatment with 15d-PGJ₂, interleukin 1 β (IL-1 β), or tumor necrosis factor- α (TNF- α). HEK 293 cells were transfected with 0.5 μ g of the β -galactosidase vector pSV- β -galactosidase and treated with the indicated concentrations of 15d-PGJ₂ or a corresponding amount of methyl acetate (MA) carrier, media alone (control), IL-1 β (1 ng/mL), or TNF- α (10 ng/mL). β -galactosidase activity was measured according to the Promega Protocols and Application Guide (3rd edition) after the cells had been lysed in a buffer containing 40 mM tricine, pH 7.8, 50 mM NaCl, 2 mM EDTA, 1 mM MgSO₄, 5 mM dithiothreitol (DTT), and 1% Triton® X-100. β -galactosidase activity was expressed as the value of A₄₂₀, normalized to the protein concentration (mg/mL) of the lysate. Data are presented as the mean ($\bar{x} \pm$ SEM) of experiments done in triplicate and are representative of at least three independent experiments. Data were examined for significant differences using analysis of variance (ANOVA), followed by individual comparisons with the Bonferroni post-test. $P < 0.05$ was considered significant. (A) Dose-dependent effect of 15d-PGJ₂ on pSV- β -gal. * $P < 0.01$ versus MA. (B) Effect of IL-1 β or TNF- α on pSV- β -gal. * $P < 0.001$ versus control.

is unlikely that vector backbone confers the major response to 15d-PGJ₂. This, along with the observation that the native *Renilla* gene is inhibited less than the synthetic genes, suggest that the re-engineered *Renilla* luciferase gene may be the source of enhanced sensitivity to 15d-PGJ₂. Because 15d-PGJ₂ is a natural ligand of peroxisome proliferator activated receptor (PPAR)- γ (7), we scanned the sequences of native and synthetic *Renilla* genes for potential PPAR- γ binding motifs using either TF-Search (<http://www.cbrc.jp/research/db/TFSEARCH.html>) or visual inspection; however, no typical PPAR- γ response elements were found. This does not exclude the participation of PPAR- γ in the inhibitory effect. For example, activated PPAR- γ can compete for limiting amounts of coactivator proteins necessary for certain transcription factors (8). Altering the availability of a cofactor required for *Renilla* expression could potentially explain the observed inhibition. Furthermore, 15d-PGJ₂ readily

binds protein sulfhydryl groups (9), and in this way, could directly modify or inactivate cofactors needed for *Renilla* transcription or translation.

β -galactosidase reporter vectors have been the subject of concern as internal controls in transfection assays because of cross-talk with cotransfected plasmids (4–6). Adding to this concern, we found pSV- β -galactosidase expression to be significantly increased by IL-1 β or TNF- α treatment. It has been suggested that transfecting β -galactosidase protein may be a better way to monitor transfection efficiency. Assuming a similar transfection efficiency for protein and DNA constructs and acknowledging the potential influence of cell type, by bypassing transcription and translation, this technique should not be affected by transcription factors and inducing agents (10).

The inclusion of control reporter vectors in transient transfection assays is routine for the determination of transfection efficiencies. The choice of a control vector appears to be a critical factor to avoid potentially misleading results. Strong inhibition of normalization controls may falsely suggest an increase in the activity of test constructs, while up-regulation of internal control activity may artificially blunt true increases. In complicated and varied experimental conditions, we recommend pretesting several control vectors to find one best suited to serve as the internal control for a particular study. As an alternative choice to using control vectors, one could consider normalizing transfection assay results to protein concentration in the cell lysate and performing a larger number of replicates.

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REFERENCES

1. Everett, L.M. and D.W. Crabb. 1999. Sensitivity of virally-driven luciferase reporter plasmids to members of the steroid/thyroid/retinoid family of nuclear receptors. *J. Steroid Biochem. Mol. Biol.* 70:197-201.
2. Matuszyk, J., E. Ziolo, M. Cebraf, I. Kochel, and L. Strzadala. 2002. Nurrl affects pRL-TK but not phRG-B internal control plasmid in genetic reporter system. *Biochem. Biophys. Res. Commun.* 294:1036-1039.
3. Ibrahim, N.M., A.C. Marinovic, S.R. Price, L.G. Young, and O. Frohlich. 2000. Pitfall of an internal control plasmid: response of *Renilla* luciferase (pRL-TK) plasmid to dihydrotestosterone and dexamethasone. *BioTechniques* 29:782-784.
4. Adam, G.I., S.J. Miller, E. Ulleras, and G.C. Franklin. 1996. Cell-type-specific modulation of PDGF-B regulatory elements via viral enhancer competition: a caveat for the use of reference plasmids in transient transfection assays. *Gene* 178:25-29.
5. Bergeron, D., B. Barbeau, C. Leger, and E. Rassart. 1995. Experimental bias in the evaluation of the cellular transient expression in DNA co-transfection experiments. *Cell. Mol. Biol. Res.* 41:155-159.
6. Huszar, T., I. Mucsi, T. Terebessy, A. Masszi, S. Adamko, C. Jeney, and L. Rosivall. 2001. The use of a second reporter plasmid as an internal standard to normalize luciferase activity in transient transfection experiments may lead to a systematic error. *J. Biotechnol.* 88:251-258.
7. Kliewer, S.A., J.M. Lenhard, T.M. Willson, I. Patel, D.C. Morris, and J.M. Lehmann. 1995. A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. *Cell* 83:813-819.
8. Daynes, R.A. and D.C. Jones. 2002. Emerging roles of PPARs in inflammation and immunity. *Nat. Rev. Immunol.* 2:748-759.
9. Perez-Sala, D., E. Cernuda-Morollon, E. Pineda-Molina, and F.J. Canada. 2002. Contribution of covalent protein modification to the anti-inflammatory effects of cyclopentenone prostaglandins. *Ann. NY Acad. Sci.* 973:533-536.
10. Howcroft, T.K., S.L. Kirshner, and D.S. Singer. 1997. Measure of transient transfection efficiency using beta-galactosidase protein. *Anal. Biochem.* 244:22-27.

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