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pRL-TK Induction Can Cause Misinterpretation of Gene Promoter Activity

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The Dual-Luciferase® Reporter assay system (Promega, Madison, WI, USA) is a widely used gene reporter system that facilitates the simultaneous expression and measurement of the firefly and *Renilla* luciferase enzymes in many eukaryotic systems (7). In this system, the firefly luciferase gene is linked to the gene promoter being investigated, while the *Renilla* luciferase gene (*Rluc*) is present in pRL vectors, which provide an internal control for transfection efficiency. The study of gene regulation often requires that the effect of adding certain transcription factors on promoter activity be evaluated in this reporter system. Hence, expression of *Rluc* should not be influenced by the transcription factor being studied. Here we report a 12-fold induction of *Rluc* expression from one of the common control vectors called pRL-TK (Promega) by the transcription factor Sp1. We also report a modi-

fication of the pRL-TK vector that reduces the level of induction to 3-fold.

The pRL-TK vector contains the herpes simplex virus-thymidine kinase (HSV-TK) promoter region upstream of *Rluc*. Numerous transient transfections of breast and skin cancer cell lines with pRL-TK and pCMV-Sp1, displayed apparent Sp1-induced expression of *Rluc*. These exaggerated levels of *Renilla* expression prevented accurate normalization of transfection efficiencies in these studies. Analysis of the HSV-TK promoter region, described at <http://transfac.gbf.de/TRANSFAC/>, revealed eight potential Sp1 transcription factor binding sites spanning the entire promoter region.

Removal of five potential Sp1 binding sites was the first strategy in desensitizing the pRL-TK vector to Sp1 induction, since previous studies have shown that multiple Sp1 consensus sites are required to give efficient Sp1 induction of promoter activity (4). This modification was carried out by digesting pRL-TK with *Bgl*II and *Sma*I, followed by a "fill-in" reaction using DNA Polymerase I, Large Klenow fragment (New England Biolabs, Beverly, MA, USA) to generate blunt ends of the vector for re-ligation. The resulting vector, pRL-TK Δ 5Sp1 (Figure 1), was sequenced to confirm the removal of the five Sp1 consensus sequences. The second strategy in the adjustment of this vector was to remove the HSV-TK promoter completely. Previous studies (1) demonstrated that a promoterless vector was less affected by the genetic system being studied, while allowing a measurable level of *Renilla* luciferase expression to maintain a control for transfection efficiency. Removal of the promoter region from the pRL-TK vector involved a *Bgl*II-*Hind*III double digest, followed by a Klenow fill-in reaction, to enable re-ligation of the vector. This promoterless vector, pRL-TK Δ prom (Figure 1), was sequenced to confirm deletion of the HSV-TK promoter.

To investigate the activity of the modified pRL-TK vectors, transient transfections of the pRL-TK, pRL-TK Δ 5Sp1, and pRL-TK Δ prom vectors were carried out in the malignant melanoma skin cancer cell line, MM96L. These cells were maintained

Figure 1. Structure of the promoter regions in pRL-TK, pRL-TKdel5Sp1, and pRL-TKdelprom vectors. The HSV-TK promoter region is contained within a 750-bp *Bgl*II-*Hind*III fragment that controls expression of the *Rluc* gene in the pRL-TK vector. Dashed lines represent regions of DNA excised from the pRL-TK vector to construct the pRL-TKdel5Sp1 and pRL-TKdelprom vectors by deleting the 275-bp *Bgl*II-*Sma*I fragment and the entire promoter region, respectively. The Sp1 binding sites are depicted as small black squares, revealing three consensus Sp1 sites remaining in pRL-TKdel5Sp1 and none in the pRL-TKdelprom vector.

in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS (JRH Biosciences, Melbourne, Australia) and antibiotics. The transfections involved 40 ng control vector, 400 ng Sp1 expression vector, pCMV-Sp1, and 400 ng pcDNAIII (Invitrogen) to maintain a constant transfected DNA level. DNA was transfected into 110 000 MM96L cells/well in antibiotic-free RPMI 1640 media using LipofectAMINE™ 2000 (Invitrogen) in 24-well tissue culture plates (Nalge Nunc International, Mt. Waverley, Victoria, Australia). The three control vectors were transfected in triplicate with pCMV-Sp1 and separately with pcDNAIII to provide base line *Rluc* expression. The cells were lysed 24 h after transfection using 100 µL passive lysis buffer (Promega) via gentle rocking at room temperature for 30 min. The lysed cells were centrifuged briefly at 18 000× *g* to remove cell debris.

Benchmarks

Figure 2. Sp1-induced expression of the *Renilla* luciferase gene from the pRL-TK, pRL-TKdel5Sp1, and pRL-TKdelprom vectors. Results show a 12-fold induction of *Renilla* luciferase expression in the pRL-TK vector by Sp1 and a 3-fold induction of *Renilla* luciferase expression in the pRL-TKdel5Sp1 vector by Sp1. *Renilla* luciferase expression from the pRL-TKdelprom vector was not above background levels. RLU, *Renilla* luciferase units.

Twenty microliters of lysate were assayed using the Dual-Luciferase reporter assay system according to the manufacturers instructions in a Wallac Victor²™ multi-label counter (Perkin Elmer Life Sciences, Turku, Finland).

The levels of *Renilla* luciferase expression are shown in Figure 2. The pRL-TK vector produced baseline levels of 3776 *Renilla* luciferase units and Sp1-induced levels of 47 039 *Renilla* luciferase units. The pRL-TKdel5Sp1 vector produced baseline levels of 2945 *Renilla* luciferase units and Sp1-induced levels of 8280 *Renilla* luciferase units. The pRL-TKdelprom vector only produced background *Renilla* luciferase expression levels in the MM96L cell line, indicating that promoterless *Rluc* vectors are not viable as controls in all eukaryotic cell lines. Results from the pRL-TK and pRL-TKdel5Sp1 vector show a 12-fold and 3-fold induction by Sp1, respectively, indicating that removal of the five Sp1 consensus sequences did not totally prevent Sp1-induced *Renilla* luciferase expression. Therefore, an alternative means of normalization needs to be considered.

These results highlight the importance of testing transfection normalization control plasmids to determine if they are responsive to any co-transfected transcription factors. Other reports have shown that the pRL-TK control

plasmid is also induced by other factors such as dexamethasone (3), the 12S E1A oncogene (9), and the Nurr family of transcriptional activators (6). Sp1 is a transcription factor that binds to many gene promoters (8) and is therefore the focus of numerous transcription regulation studies. The Sp1 dependence of the HSV-TK promoter as described here has been indicated previously (5) and is also strongly influenced by the extent of cytosine methylation of the Sp1 binding sequences (2). This study represents the first time that the widely used reporter vector pRL-TK has been subjected to a specific analysis that shows it is significantly regulated by Sp1. Our results

demonstrate that the use of the pRL-TK plasmid as a normalization control for Sp1 induction studies should be undertaken with caution to prevent misinterpretation of gene promoter activity data. In addition, all researchers using pRL-TK should realize that it may not be an inert reporter vector, but expression levels may also reflect cellular fluctuations in Sp1 activity that may be cell or growth dependent. Thus, the Sp1 regulation of pRL-TK may have consequences for any experiment using pRL-TK as a reporter, in addition to those applications that are directly focused on examining Sp1 function.

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