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This study was supported by grants MH48866, DAMD-17-01-J-0763, and NARSAD Independent Award. Address correspondence to Dr. Kwang-Soon Kim, Molecular Neurobiology Laboratory, MRC215, McLean Hospital, Harvard Medical School, 115 Mill Street, Belmont, MA 02478, USA. e-mail: kskim@mclean.harvard.edu

Received 29 May 2002; accepted 27 August 2002.

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pRL-TK Induction Can Cause Misinterpretation of Gene Promoter Activity

BioTechniques 33:1240-1242 (December 2002)

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 modification 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 BglII and Smal, 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-TKdel5Sp1 (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 BglII-HindIII double digest, followed by a Klenow fill-in reaction, to enable re-ligation of the vector. This promoterless vector, pRL-TKdelprom (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-TKdel5Sp1, and pRL-TKdelprom vectors were carried out in the malignant melanoma skin cancer cell line, MM96L. These cells were maintained...
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® (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 18000× g to remove cell debris.

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 BgIII-HindIII 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 BgII-SmaI 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.
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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The authors wish to acknowledge Griffith University for financial support. S.O. is a John Earnshaw Scholar of the Queensland Cancer Fund. The plasmid pCMV-Sp1 was a kind gift from Professor Guntram Suske (Philips-Universität, Marburg, Germany), and the MM96L cell line was a kind gift from Professor Peter Parsons (QIMR, Brisbane, Australia). Address correspondence to Dr. Kathryn Tonissen, School of Biomolecular and Biomedical Science, Griffith University, Nathan, Qld, Australia 4111. e-mail: k.tonissen@mailbox.gu.edu.au

Received 16 August 2002; accepted 16 September 2002.

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