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Activation of pRL-TK by 12S E1A Oncoprotein: Drawbacks of Using an Internal Reference Reporter in Transcription Assays

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ABSTRACT

In transient expression assays for transcription, a vector carrying the experimental reporter is usually co-transfected with a second vector containing a distinct reporter gene as a control. The second reporter is linked to a constitutive promoter driving a low-level transcription that is unresponsive to the experimental trans-acting transcription factors used. The use of dual reporters

enables the normalization of the experimental gene transcription with respect to the control reporter transcription. This method is expected to minimize the inherent variability in transfection data caused by changes in cell density and viability, cell lysis, and the recovery of samples at various stages of the experiment. Here, we report that one of the most widely used internal control reporters, the Renilla luciferase plasmid (pRL-TK), is unresponsive to human tumor suppressor protein p53, a potent transcriptional regulator; however, the reporter transcription is enhanced by another well-known transcriptional regulator, the adenoviral 12S E1A oncoprotein, thereby disqualifying pRL-TK as a universal internal control reporter for transcription assays. Our results reveal the necessity of stringent experiments to test the responsiveness of internal control plasmids to transcriptional regulators present in the assay to avoid the misinterpretation of transcriptional analysis data.

INTRODUCTION

Dual reporters are commonly used in transient expression assays in cultured cells in which one vector carrying an experimental reporter is co-transfected with a second vector containing a distinct reporter gene serving as a control. The second reporter is linked to a constitutive promoter that is considered neutral in response to various transcriptional regulators. This method helps in the estimation of transcriptional properties of the test gene promoter without being affected by experimental variations, such as cell density and viability, cell lysis, and the recovery of samples at various stages of the experiment. The Dual-Luciferase® reporter technology (Promega, Madison, WI, USA) (7), which integrates the firefly luciferase assay with the *Renilla* luciferase assay, is a widely used dual reporter system to assay transcription in

eukaryotic cells. The *Renilla* luciferase, cloned from *Renilla reniformis*, has been used to construct internal control vectors (called pRL vectors) in several promoter configurations. pRL-TK (Promega) is one such vector frequently used as an internal reference promoter in combination with the firefly luciferase reporter in the Dual-Luciferase assay (7). Because these two reporters produce luciferase enzymes that are different from each other in substrate specificity, transcriptional activity from each reporter can be measured sequentially in the same cell lysate sample.

We have been analyzing mechanisms by which human tumor suppressor protein p53 and adenoviral oncoprotein 12S E1A regulate transcription of the human proliferating cell nuclear antigen (PCNA) gene. Although internal control plasmids used in the Dual-Luciferase assay are supposed to correct for experimental variations by

virtue of their constitutive expression unperturbed by transcriptional regulatory factors, we have encountered a major problem in the use of pRL-TK as the internal reference reporter plasmid in our transient transfection experiments. pRL-TK, while nonresponsive to transcriptional regulator p53, was found to be sensitive to another potent transcriptional regulator, the adenoviral 12S E1A oncoprotein. Based on those results, we concluded that pRL-TK is not an appropriate internal control plasmid reporter for certain transcription assays.

MATERIALS AND METHODS

Plasmids

The -1265PCNA-luc reporter plasmid has been previously described (4). The p53 expression construct pRc/CMVhp53 and the 12S E1A (E1A

243R) expression constructs (4) were kindly provided by Drs. A.J. Levine (Rockerfeller University) and E. Moran (Temple University School of Medicine), respectively.

Cell Line

Saos-2 (human osteosarcoma) cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM (cellgro®; Mediatech, Herndon, VA, USA) supplemented with 10% FBS (Life Technologies, Rockville, MD, USA).

Transient Transfections

Saos-2 cells were seeded onto six-well, flat-bottom culture plates (Falcon®; Becton Dickinson, Franklin Lakes, NJ, USA) at a density of 6×10^4 cells/well. After 24 h, the cells were transfected by the calcium phosphate

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co-precipitation method (1) with 1 μ g -1265PCNA-luc plasmid reporter with or without 30 ng internal control plasmid reporter pRL-TK in combination with 1.24 ng p53 and 625 ng 12S E1A expression constructs. For experiments in which activation of the PCNA promoter by p53 and repression of activation by 12S E1A were analyzed, 1.24 ng p53, 873 ng 12S E1A, 1 μ g -1265PCNA-luc, and 30 ng pRL-TK were transfected into Saos-2 cells. The final concentration of the plasmids was made up to 2.5 μ g with pUC119. Three independent precipitations for three transfections were made for each combination of plasmids. Sixteen hours after transfection, the cells were washed with PBS containing 2 mM EGTA and fed with fresh medium. After 24 h, the cells were harvested using the respective lysis buffers for firefly luciferase and Dual-Luciferase assays. The cell lysates were first frozen in liquid nitrogen and stored at -80°C until processed for luciferase assays. The firefly luciferase and Dual-Luciferase assays were performed using 10 μ L of the lysates, according to the recommendations of the manufacturer. The luciferase activities were measured with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA).

Sequence Analysis of pRL-TK Vector

To find out if there is any DNA sequence on the pRL-TK plasmid that is homologous to the known E1A response region (5), an analysis on this vector sequence was done at <http://www.ncbi.nlm.nih.gov/BLAST/index.compat.html>.

RESULTS AND DISCUSSION

When Saos-2 cells were transfected with p53 expression construct and -1265PCNA-luc reporter plasmid, PCNA transcription was activated by p53 about 5-fold, based on the direct measurement of firefly luciferase activity (Table 1). Similar enhancement of transcriptional activation (5-fold) was observed when the experiment was performed in the presence of the internal control reporter plasmid pRL-TK (i.e., Dual-Luciferase assay), when transcrip-

Table 1. Activation of PCNA-luc Reporter Plasmid by p53 and 12S E1A: Comparison of Results Obtained with Luciferase and Dual-Luciferase Assays

	Luciferase assay	Dual-Luciferase assay		
	PCNA Firefly luc reporter activity	PCNA Firefly luc reporter activity	pRL-TK <i>Renilla</i> luc reporter activity	Ratio PCNA luc/pRL-TK activity
-p53	283.3 $\pm$ 14.24	229.3 $\pm$ 10.26	19.69 $\pm$ 5.35	12.19 $\pm$ 3.07
+p53	1509 $\pm$ 41.4	1751 $\pm$ 397.5	27.34 $\pm$ 7.01	64.38 $\pm$ 2.04
Fold activation	5.3			5.3
-12S E1A	114.7 $\pm$ 11.4	328.7 $\pm$ 93.89	16.74 $\pm$ 5.234	19.79 $\pm$ 0.824
+12S E1A	310.7 $\pm$ 31.86	1161 $\pm$ 162.6	85.03 $\pm$ 8.421 ^a	13.62 $\pm$ 0.777
Fold activation	2.7			0.7

^aElevated activity of pRL-TK internal reference promoter.

Saos-2 cells were transfected with 1265PCNA-luc plasmid reporter with and without the p53 expression construct. In another experiment, Saos-2 cells were transfected with -1265PCNA-luc plasmid reporter with and without 12S E1A expression construct. Transcriptional activity was measured by luciferase and Dual-Luciferase assays. The assay values shown are averages of activity from three independent transfections.

tional activity of -1265PCNA-luc reporter plasmid was normalized to pRL-TK activity. Transcriptional activation of pRL-TK *Renilla* luciferase activity remained almost the same, both in the absence and presence of the transfected p53 expression construct. Therefore, pRL-TK is suitable as an internal control reporter when p53 is the only transcriptional regulator tested in the assay.

When the above experimental situation was repeated with adenoviral 12S E1A expression construct in place of p53, it activated the PCNA promoter about 2.7-fold in the absence of the internal control plasmid pRL-TK (Table 1), which is consistent with earlier published reports (4,5). However, with the Dual-Luciferase assay, where transcriptional activity of the -1265PCNA-luc reporter plasmid was normalized to pRL-TK activity, 12S E1A caused an apparent repressive effect on the basal transcription of PCNA promoter. This discrepancy is the consequence of activation of the pRL-TK internal control plasmid promoter by 12S E1A, leading to the increase in *Renilla* luciferase activity, which, in turn, causes a decrease in the ratio between the activities of the firefly luciferase and *Renilla* luciferase. The sequence analysis of the pRL-TK vector did not reveal any DNA sequence

of significant similarity with a known E1A response region on PCNA gene, indicating that 12S E1A may mediate the transcriptional activation of the pRL-TK internal control plasmid by some other unidentified sequence on this plasmid.

The fact that transcription from pRL-TK is responsive to 12S E1A compromises one of the necessary characteristics of an internal control plasmid reporter—to be nonresponsive to co-transfected transcriptional regulators. Therefore, pRL-TK cannot be considered a universal internal control plasmid for transcription assays.

The inappropriate use of reference reporters and their responses to various transcriptional regulators has not been rigorously investigated, which can cause serious misinterpretations of transcriptional data. Our results on the effect of 12S E1A on transcriptional activation of the PCNA gene by p53 in Saos-2 cells exemplify such a situation. Mutational analyses on PCNA promoter have revealed a p53-binding site (6,8). Although E1A cannot bind DNA directly, an E1A-responsive region required for both basal and 12S E1A-induced PCNA expression has been identified (5). Our studies using firefly luciferase assays showed that the PCNA gene is transactivated several fold by p53 and moderate-

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Table 2. Effect of Normalization to pRL-TK Internal Control Plasmid Reporter on Data on Repression of p53-Mediated Activation of PCNA Promoter by 12S E1A

Transcriptional regulators	PCNA-luc reporter activity	Fold activation of PCNA basal transcription	Fold repression of p53-mediated activation	pRL-TK ref. reporter activity	Ratio PCNA-luc/pRL-TK activity	Fold activation of PCNA basal transcription (normalized)	Fold repression of p53-mediated activation (normalized)
-p53	435.1 ± 126.8			25.52 ± 5.44	16.83 ± 1.60		
+p53	2225 ± 586.4	5.1		28.36 ± 8.18	78.97 ± 3.78	4.67	
+p53							
+12S E1A	795.7 ± 84.23	1.8 ^a	2.8 ^a	55.08 ± 4.87 ^c	14.43 ± 0.32	0.86 ^b	5.43 ^b

^aNormal values from -1265PCNA-luc reporter.
^bAberrant values obtained after normalization to pRL-TK internal reference reporter.
^cElevated activity of pRL-TK internal reference reporter.

Saos-2 cells were transfected with -1265PCNA-luc plasmid reporter with and without p53 expression construct or with p53 and 12S E1A expression constructs. Transcriptional activity was measured by luciferase and Dual-Luciferase assays. The assay values shown are averages of activity from three independent transfections.

ly by the 12 S E1A adenoviral oncoprotein, whereas, when they are both present, 12S E1A can repress transactivation of the PCNA gene by p53 (4). When those experiments were repeated using the Dual-Luciferase assay, PCNA transcription was found to be repressed (i.e., compared to the level of basal transcription in the absence of any activators) (Table 1), contrary to the modest activation observed in earlier experiments without the use of the Dual-Luciferase assay (4,5). Therefore, the use of pRL-TK internal control plasmid would result in the misinterpretation of results as follows: (i) 12S E1A is a modest repressor rather than an activator of PCNA transcription, and (ii) 12S E1A-mediated repression of PCNA transcriptional activation by p53 will be exaggerated because of higher transcriptional activation of the internal control plasmid reporter by 12S E1A (Table 2).

Since Saos-2 cells have been widely used to analyze transcriptional roles of factors such as p53 and 12S E1A in various combinations, our studies with these cells are relevant to the design and interpretation of various experiments using not only Dual-Luciferase assays but also other reporter systems involving internal control reporter plasmids untested for responsiveness to particular factors and experimental conditions. The issue becomes more important in light of a recent report that pRL-TK is responsive to dihydrotestos-

terone and dexamethasone in CV-1 cells (3). Further, it has been reported that co-transfection into HeLa cells of an experimental reporter can affect transcription from pRSVβgal, another widely used reference reporter in transfection assays (2). Our data also suggest that relying on statistical analysis of results from multiple experiments may be more accurate in cases when the appropriate internal control plasmid reporters are not available.

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