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Use of a Promoterless *Renilla* Luciferase Vector as an Internal Control Plasmid for Transient Co-Transfection Assays of Ras-Mediated Transcription Activation

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One problem in studies of gene regulation is selecting an appropriate internal control for transfection efficiency

and extract preparation whose expression remains constant during treatments that cause changes in the expression of the gene being studied (2,6). This is an especially difficult problem

with studies involving activated *ras*, because most internal control reporters are driven by viral promoters and almost all of these are transactivated by oncogenic *ras* or other nonnuclear

Figure 1. Ras-mediated transactivation of the human MCSF receptor promoter using either pRL-0 or pRL-CMV as an internal control plasmid. (A) The MCSF receptor promoter in pXP2 and the CMV promoter in pRL-0 are both activated by oncogenic *ras*, whereas the promoterless vector pRL-0 is not. For transactivation assays, 2×10^5 CV1 cells (CCL-70; ATCC, Rockville, MD, USA) were plated in 4 mL of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in 60-mm tissue culture plates 18 h before transfection. Cells were transfected by the calcium phosphate method as previously described (4,14) with: (i) the reporter plasmid: 10 µg of the promoterless firefly luciferase vector pXP2; 10 µg of the 159-bp human MCSF receptor promoter (pMCSFR) (-88 to +71 with respect to the major monocytic transcription start site) in pXP2; 0.002 µg of the CMV promoter in the firefly luciferase vector pXP2; 0.002 µg of an internal control plasmid using a CMV promoter subcloned in pRL-0 (*Renilla* luciferase gene driven by the CMV promoter) (11) or 0.2 or 0.8 µg of the pRL-0 vector for *Renilla* luciferase, which lacks eukaryotic promoter and enhancer elements upstream of *Renilla* luciferase; (ii) 1 µg of the expression vector: oncogenic pMT3-H-Ras(L61) or the empty expression vector; and (iii) sheared salmon sperm DNA to a total of 15 µg DNA per plate. The medium was changed after 16 h, and firefly luciferase activities for pXP2 alone and pM-CSFR or pCMV in pXP2 and *Renilla* luciferase activities for pCMV in pRL-0 and pRL-0 alone were determined 40 h after the initiation of the transfection protocol with the Dual-Luciferase™ Reporter Assay System (Promega). Results were normalized for extract protein content and are given as mean plus standard error of the mean (SEM) of at least 6 independent experiments. (B) Using pRL-0 as an internal control plasmid results in an accurate calibration of the 4-fold transactivation of the MCSF receptor promoter by activated *ras*, whereas pRL-CMV can not be used as an internal control. Firefly luciferase activities of pMCSFR in pXP2 were normalized to the *Renilla* luciferase values of either pCMV in pRL-0, 0.2 µg pRL-0 or 0.8 µg pRL-0 (data from Panel A).

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oncogenes such as receptor and nonreceptor tyrosine kinases, serine/threonine kinases or guanine triphosphate (GTP)-binding proteins (6). Thus, some investigators have used "minimal" promoter constructs, such as pTKhGH containing the herpes simplex virus (HSV) thymidine kinase promoter, as internal control plasmids for studies of *ras*-mediated transcription activation (5). However, results of other groups (6) and our own findings (data not shown) suggest that even minimal promoter constructs, such as pBLCAT2 (9) or pTK81-luc (10), which contain the HSV thymidine kinase promoter, are *ras*-responsive in the absence of inserted binding sites and, therefore, not useful as internal control plasmids for analysis of *ras*-mediated transactivation. For these reasons, in transient co-transfection assays investigating transcription activation by *ras* or

other oncogenes, an internal control plasmid is often omitted. Instead, transfections have to be repeated many times, and the values have to be normalized for extract protein content (6). Overall, the co-transfection assays of *ras*-mediated transcription activation without an internal control plasmid become very time-consuming, and the results are difficult to interpret. This is especially important when comparing results among different cell lines.

Here, we demonstrate that the pRL-0 vector for *Renilla* luciferase (Promega, Madison, WI, USA) (7,8,11), which lacks eukaryotic promoter and enhancer elements upstream of the *Renilla* luciferase gene, works as an internal control plasmid and, most importantly, is not activated by oncogenic *ras* (Figure 1A). While oncogenic *ras* (L61) (3) activates 4-fold the 159-bp human macrophage colony stimulating factor (MCSF) receptor promoter (-88 to +71 with respect to the major monocytic transcription start site) (15) in the firefly luciferase vector pXP2 (10), the promoterless vector pXP2 is not activated (Figure 1A). However, although the promoterless vector pXP2 is not transactivated by *ras*, it could not easily

be used as an internal control plasmid, because the firefly luciferase activity of even 10 μ g pXP2 is only just above background, which would make comparisons in transfection efficacy between plates very difficult. As expected, a potential internal control plasmid using a cytomegalovirus (CMV) promoter subcloned in pRL-0, in which the *Renilla* luciferase gene is driven by the CMV promoter (7,11), was transactivated 4-fold by oncogenic *ras* (Figure 1A) and therefore cannot be used as an internal control plasmid for studies investigating the effect of activated *ras* on the MCSF receptor promoter (Figure 1B). The promoterless *Renilla* luciferase vector pRL-0 (11) however is not activated by oncogenic *ras*, and its *Renilla* luciferase activity can be measured high above the assay background of 500 relative light units (RLU) because of the approximately 100 $\times$ higher sensitivity of the *Renilla* luciferase assay in comparison to the firefly luciferase assay (Figure 1A). Furthermore, the luciferase activity of pRL-0 could be demonstrated to increase linearly with increasing plasmid concentrations. Thus, 0.2 μ g pRL-0 resulted in 40 000 RLU, whereas 160 000 RLU

Figure 2. Using pRL-0 as an internal control plasmid does not alter the 20-fold transactivation of the collagenase promoter by activated *ras*. For transactivation assays, 4×10^4 293E1A cells (kindly provided by Chris Carpenter, Beth Israel Deaconess Medical Center, Boston) were plated in 0.5 mL of DMEM supplemented with 10% FBS in 24-well tissue culture plates 18 h before transfection. Cells were transfected by the LIPOFECTAMINE PLUS method with 0.2 μ g of the collagenase promoter in a firefly luciferase vector (-73Col-Luc reporter) (12) either with or without activated *ras*(L61). Either no internal control or 0.2 or 0.8 μ g of pRL-0 was co-transfected. Firefly and *Renilla* luciferase activities were determined 24 h after the initiation of the transfection protocol with the Dual-Luciferase Reporter Assay System. Results were normalized for extract protein content and are given as mean plus SEM of at least 6 independent experiments.

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were measured after transfection of 0.8 µg pRL-0 (Figure 1A).

Because of the high sensitivity of *Renilla* luciferase in comparison to firefly luciferase, the ratio between control and reporter plasmid can be kept at less than 1:50. Figure 1, A and B, for example, depicts the experiment in which 10 µg of the MCSF receptor promoter driving firefly luciferase in pXP2 and 0.2 µg of the internal control plasmid pRL-0 driving *Renilla* luciferase were used. Moreover, using 0.2 or 0.8 µg of pRL-0 as an internal control plasmid did not alter the 20-fold transactivation of 0.2 µg of the collagenase promoter by activated *ras* (Figure 2). Thus, even a reporter/control DNA ratio of 1:4 does not lead to promoter competition. Both luciferase activities, the firefly luciferase for the *ras* responsive promoter being tested and the *Renilla* luciferase for the internal control plasmid, can be used in the same transfection and as-

sayed sequentially from the same extract in the same tube, minimizing variability. The firefly luciferase reporter is measured first by adding Luciferase Assay Reagent II (Promega) to generate a luminescent signal. After quantifying the firefly luminescence, this reaction is quenched, and the *Renilla* luciferase reaction is initiated simultaneously by adding Stop and Glo Reagent (Promega) to the same tube.

We have successfully used this assay as an internal control in experiments investigating activation of the transcription factors C/EBPα and PU.1 by oncogenic *ras* (1,13). Furthermore, we have used pRL-0 as an internal control plasmid for transcription activation assays by other activated signaling agonists such as activated MEKK1, in various cell types such as U937, FDCP1/FMS, F9, 293T and CV-1 cells and with different transfection methods including calcium phosphate precipitation, LIPOFECTAMINE™ and LIPOFECTAMINE Plus Reagents (Life Technologies, Gaithersburg, MD, USA) and electroporation (data not shown). In conclusion, these data show that the promoterless vector for *Renilla* luciferase (pRL-0) is a useful, convenient, relatively inexpensive, very sensitive and rapid internal control plasmid that is not affected by activated *ras* and is an extremely valuable adjunct to studies investigating transcriptional activation mediated by *ras* or other non-nuclear oncogenes.

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