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Sensitivity of virally-driven luciferase reporter plasmids to members of the steroid/thyroid/retinoid family of nuclear receptors

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Abstract

During a series of transfection experiments, the pRSV-luc plasmid used as an internal control was found to be sensitive to co-transfection with expression vectors for several members of the steroid/thyroid/retinoid superfamily of nuclear receptors. Therefore, a survey of the effect of these expression vectors on the activity of four reporter plasmids was conducted. In CV-1 cells, the activity of pRSV-luc, which contains the *P. pyralis* luciferase gene, was repressed by co-transfection of PPARα and ARP-1 and was activated by COUP-TFI. Expression of pSV40-luc, containing the same luciferase gene, was repressed by PPARα and HNF-4 and activated by both COUP-TFI and ARP-1. All four of these expression vectors reduced the expression of the pRL-TK plasmid, which contains the luciferase gene from *Renilla reniformis*. RXR expression vectors had no effect on luciferase activity in CV-1 cells but induced luciferase activity in H4IIEC3 hepatoma cells. This activation was blocked by the addition of ligand, 9-*cis* retinoic acid. pSV2-CAT, which contains the chloramphenicol acetyltransferase gene, was insensitive to all receptor expression vectors tested. Both the *P. pyralis* and *R. reniformis* luciferase genes appear to contain sequences that render them responsive to steroid/thyroid/retinoid nuclear receptors.

1. Introduction

In transfection assays, an internal control plasmid is commonly used to correct for plate-to-plate variations in transfection efficiency [1]. These plasmids typically use a viral promoter to drive transcription of a gene encoding an enzyme whose activity is easy to assay, such as luciferase, chloramphenicol acetyltransferase (CAT), or β-galactosidase (β-gal). As long as the transcription of these internal control plasmids is unaffected by the experimental conditions, they provide a convenient method of normalizing reporter activity.

This laboratory has been investigating transcriptional regulation of several genes using CAT reporter constructs in transfection experiments, and a luciferase vector was chosen as the internal control. The regulatory sequences in our promoter–reporter constructs contain potential DR-1 sites, a direct repeat of the consensus sequence (A/G)G(G/T)TCA, with one intervening base. This binding motif is favored by certain members of the steroid/thyroid/retinoid superfamily of nuclear receptors. In particular, it is bound by retinoid X receptors (RXRs), the peroxisome proliferator activator receptor (PPAR, whose heterodimeric partner is RXR), hepatocyte nuclear factor-4 (HNF-4), chicken ovalbumin upstream promoter transcription factor (COUP-TFI), and apolipoprotein A1 regulatory protein-1 (ARP-1) [2]. The latter two are so-called "orphan" receptors, transcription factors for which no ligands have yet been identified. When the functional consequences of co-transfecting expression vectors for these receptors with promoter–reporter constructs were examined, it was discovered that the internal control plasmid was sensitive to the presence of some of the nuclear receptors. We, therefore, extended this finding

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Table 1
Effects of co-transfection of receptor expression vectors on internal control plasmid expression in CV-1 cells[a]

Co-transfected expression vector	Internal control plasmid			
	pRSV-luc	pSV40-luc	pRL-TK	pSV2-CAT
None[b]	100 ± 20	100 ± 11	100 ± 23	100 ± 20
PPARα[b]	43 ± 13*	29 ± 5*	8 ± 5*	103 ± 30
HNF-4[b]	98 ± 30	26 ± 7*	7 ± 3*	113 ± 28
COUP-TFI[c]	657 ± 181*	359 ± 78*	19 ± 12*	243 ± 76
ARP-1[b]	51 ± 13*	346 ± 86*	5 ± 1*	107 ± 19

[a] Luciferase activity was measured as light units/μg protein and CAT activity as % conversion of chloramphenical to acetylated products/μg protein, and the values are expressed in the table relative to the control with no co-transfected receptor expression vector. *$P < 0.05$ compared to activity of reporter plasmid with no co-transfected nuclear receptor expression vector. [b]$n = 5$. [c]$n = 7$ for pRSV-luc, $n = 5$ for all others.

and found that three luciferase plasmids are significantly activated or repressed by the presence of various members of the steroid receptor family.

2. Methods and materials

2.1. Materials

Most chemicals and supplies were purchased from Sigma Chemical Company (St. Louis, MO). Agarose, trypsin, all restriction endonucleases, DNA modifying enzymes, and tissue culture media were purchased from GIBCO BRL (Gaithersburg, MD). Fetal bovine serum charcoal-stripped of lipids was purchased from Hyclone Laboratories (Logan, UT). All radioisotopes were purchased from DUPONT NEN Research Products Inc (Boston, MA).

2.2. Plasmids

pRSV-luc and pSV40-luc were generously provided by S. Nordeen and pSV2-CAT by D. Spandau. pRL-TK was purchased from Promega (Madison, WI). Expression vectors were the kind gifts of F. Sladek (HNF-4), R. M. Evans (PPARα), P. Chambon (RXRs), and H. Nakshatri (COUP-TFI and ARP-1). All constructs were transformed into competent *E. coli* by the heat shock method [3]. Plasmids were isolated by alkaline lysis and their identity verified by restriction mapping.

2.3. Transfection of tissue–culture cells

CV-1 and H4IIEC3 cells were cultured in Delbecco's modified Eagle's medium (DMEM) supplemented with 5–10% fetal bovine serum (FBS), 100 μg/ml streptomycin, and 63 μg/ml penicillin G. The day before transfection, the cells were plated at 10^6 cells/100 mm dish. The cells were transfected with internal control plasmids and a receptor expression plasmid by calcium phosphate precipitation [4] in the following amounts: PPARα, 10 μg/plate; pSV2-CAT, RXR, COUP-TFI, and ARP-1, 5 μg/plate; pSV40-luc and HNF-4, 1.0 μg/plate; and pRSV-luc and pRL-TK, 0.5 μg/plate.

Four hours later the cells were exposed to PBS containing 15% glycerol for 3 min. The cells were rinsed with PBS and fresh DMEM with charcoal-stripped serum was added. Twenty-four hours after transfection, some plates were treated with 100 nM 9-cis retinoic acid. Forty-eight hours after transfection, the cells were washed with PBS and lysed in 150 μl of Reporter Lysis Buffer (Promega). Thirty-five μl of cell extract was incubated with luciferase assay reagent based on the original protocol of deWet [5]. The number of relative light units was determined with a 3 s delay and a 30 s incubation. CAT activity was measured as described previously [6]. The conversion of chloramphenicol to its acetylated products was quantified on an AMBIS β-scanner. Protein content was determined using the Bio-Rad (Hercules, CA) Protein Assay according to manufacturer's instructions.

2.4. Statistical analyses

Luciferase and CAT activities were normalized to cell extract protein content. All transfections were done in duplicate and averaged for each data point. Results are expressed as a percentage of normalized activity of the reporter plasmid without co-transfection of the receptor expression vector ± standard error. Significance of the differences was determined by 2-tailed *t*-tests (Microsoft Excel, Version 7.0).

3. Results

The original investigation was designed to determine whether potential DR-1 sites in the ALDH2 [7] and preproenkephalin A [8] promoters were involved in the regulation of these genes. Initial co-transfection experiments in CV-1 cells included a CAT promoter-repor-

ter construct and expression vectors for PPARα, the three isoforms of RXR, HNF-4, COUP-TFI, or ARP-1. pRSV-luc, which contains the Rous sarcoma virus long terminal repeat (LTR) driving transcription of the luciferase gene, was included as the internal control. Consistent changes in the level of activity of the internal control plasmid were noted and, therefore, a systematic examination of the sensitivity of pRSV-luc to co-transfection of the nuclear receptor expression vectors was performed in CV-1 cells. To avoid problems of effects of the receptors on other internal controls, the activity of the reporters was normalized to the amount of protein present in the cell extracts. Co-transfection of the receptors with a luciferase plasmid lacking all promoter elements did not activate expression of the luciferase cassette (not shown). However, co-transfection of PPARα or ARP-1 with pRSV-luc decreased luciferase activity by 57 and 49%, respectively, while COUP-TFI stimulated the expression of luciferase from this plasmid by about 6-fold (Table 1). HNF-4 did not have a statistically significant effect on pRSV-luc expression. In CV-1 cells, none of the isoforms of RXR appeared to have an effect on expression of pRSV-luc or any of the other internal control plasmids tested in the absence of 9-*cis*-retinoic acid (data not shown).

Both pRSV-luc and pSV40-luc contain the luciferase gene from *P. pyralis* (firefly). To determine if the sequences conferring sensitivity to the nuclear receptors were present in the luciferase gene or viral promoter, the effect of the receptors on pSV40-luc was tested. pSV40-luc, in which luciferase expression is driven by the simian virus 40 early promoter, was found to be repressed by PPARα and HNF-4 and stimulated by both COUP-TFI and ARP-1. The effect of the nuclear receptors was then tested on a vector containing a different luciferase gene. pRL-TK, containing *Renilla* luciferase driven by the herpes simplex thymidine kinase promoter [9] was found to be the most sensitive to nuclear receptors of the three plasmids, with PPARα, HNF-4, COUP-TFI, and ARP-1 all profoundly suppressing luciferase activity (Table 1).

pSV2-CAT, which contains the simian virus 40 early promoter driving transcription of the bacterial (CAT) gene, was then tested to see if the SV40 promoter contained nuclear receptor response elements. CAT activity was not significantly affected by co-transfection of any of the receptor expression vectors, although there was a trend toward activation by COUP-TFI (Table 1). This suggested that the effect of the receptors on the luciferase–containing plasmids was dependent upon luciferase, rather than viral promoter, sequences.

pSV40-luc expression was also sensitive to the presence of the nuclear receptors in H4IIEC3 cells. Co-transfection of HNF-4 expression vector repressed luciferase activity by 64%, while the presence of COUP-TFI, ARP-1, RXR-α, RXR-β, or RXR-γ all resulted in robust activation. Furthermore, the increases seen with all three isoforms of RXR were suppressed by the addition of their ligand, 9-*cis* retinoic acid (Table 2). PPARα was not tested in H4IIEC3 cells.

Table 2
Effects of co-transfection of receptor expression vectors on SV40-luc in 'H4IIEC3 cells[a]

Co-transfected expression vector	Relative luciferase activity	
	No Addition	9-cis RA (100 nM)
None	100 ± 6	$138 \pm 13^{*}$
HNF-4	$36 \pm 10^{*}$	–
COUP-TFI	$10{,}827 \pm 1007^{*}$	–
ARP-1	$11{,}153 \pm 3352^{*}$	–
RXR-α	$1072 \pm 119^{*}$	$194 \pm 34^{**}$
RXR-β	$2785 \pm 801^{*}$	$932 \pm 320^{**}$
RXR-γ	$5642 \pm 1446^{*}$	$1472 \pm 364^{**}$

[a] Luciferase activity was measured as light units/μg protein and the values are expressed in the table relative to the control with no co-transfected receptor expression vector Each experiment was repeated 4–7 times. 9-*cis* RA denotes 9-*cis* retinoic acid. $^{*}P < 0.05$ compared to activity of reporter plasmid with no co-transfected nuclear receptor expression vector. $^{**}P < 0.05$ compared to activity of reporter plasmid with co-transfected RXR in absence of 9-*cis* RA.

These data suggested that the nuclear receptors exert their effects by binding to sequences in the two luciferase genes, rather than in the promoters. Although analysis of the sequences using the software program *TFSearch* did not disclose any pertinent binding motifs, a visual inspection proved more informative. In the firefly luciferase gene a sequence was found (bp 1206–1218) which differs from the DR-1 element in the chicken ovalbumin gene by only two bases in the 3′ half-site and the intervening base. The DR-1 site present in the ovalbumin gene was the original binding site identified for COUP-TFI [10]. It has since been shown to bind PPAR/RXR heterodimers as well [11]. Furthermore, all six purine residues in the ovalbumin gene DR-1 sequence identified by methylation interference assays as important for DNA-protein contacts [12] are preserved in the luciferase sequence. Neither the RSV-LTR nor the SV40 promoter appeared to contain direct repeat sequences. No obvious DR-1 sites were found in the *Renilla* gene, but it contains

Table 3
Potential COUP-TFI/ARP-1 binding sites in the pRL-TK vector

Base Pair♯	Motif	Nucleotide sequence	Vector region
414–419	Half-site	GGGTCA	HSV-tk promoter
974–979	Half-site	AGTTCA	Chimeric intron
1429–1445	DR-5	GCATCAagataAGATCA	Luciferase gene
1440–1458	DR-7	AGATCAaagcaatAGTTCA	Luciferase gene

both potential DR-5 and DR-7 sequences, other motifs to which COUP-TFI can bind [13]. In addition, the vector sequences contain two perfect half-sites (Table 3). It should also be noted that COUP-TFI and ARP-1 are considered "promiscuous" transcription factors. Although they have a high affinity for DR-1 motifs, they bind a diversity of elements including direct repeats with varying spacer lengths, as well as inverted and everted repeats and palindromic sequences [14]. The pSV2-CAT plasmid did not appear to contain any direct repeat binding motifs except for a potential DR-0 site located in the sequence containing the SV40 polyadenylation signal. However, this sequence is present in all of the internal control plasmids tested. Since pSV2-CAT activity was unaffected by these nuclear receptors, this sequence is not likely to mediate their effects.

4. Discussion

These data indicate that members of the steroid/thyroid/retinoid superfamily of nuclear receptors affect the expression of several different luciferase plasmids, making luciferase vectors unsuitable for internal control purposes. Their use could result in misinterpretation of data due to under- or over-estimation of promoter–reporter activity. This study did not identify the sequences in the luciferase genes that mediate the effect of the receptors. We propose that the receptors may bind to sites within the coding sequence for this enzyme and, through subsequent interactions with co-activators or co-repressors, modulate transcriptional activity of the viral promoter present in the plasmids. However, the possibility should not be ruled out that the regulatory effects described herein are mediated not by direct DNA binding, but by protein–protein interactions, as appears to be the case for COUP-TFI activation of the vHNF-1 promoter [15] and the HIV-1 LTR [16].

It was somewhat surprising that the luciferase plasmids were activated by some of the receptors and repressed by others. This effect appeared to depend upon the particular viral promoter involved, since pRSV-luc, whose activity was repressed by ARP-1, and pSV40-luc, whose activity was activated, are identical vectors except for their promoter sequences. This is consistent with the reports that the effect of either COUP-TFI [17,18] or ARP-1 [19] on promoter–luciferase reporter plasmid was reversed when portions of the promoter were progressively deleted.

It was also observed that the effect of the receptors on the luciferase differed in the two cell types tested. In particular, the RXRs had no effect on expression of pSV40-luc in CV-1 cells, but they were highly stimulatory in a hepatoma cell line. Other investigators have reported similar findings. A tk-CAT reporter containing the arrestin promoter was activated by ARP-1 in CV-1 cells but unaffected in NG108 neuronal cells [20]. In HepG2 cells, COUP-TFI induced a CAT reporter containing the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase promoter while suppressing activity of the same reporter in R2C (rat Leydig tumor) cells [21]. These differences are likely due to the variable expression of co-activators, co-repressors, and accessory proteins that interact with the various transcription factors and components of the transcriptional machinery.

The discovery that certain nuclear receptors affected luciferase expression from viral promoters prompted a review of the relevant literature. Luciferase vectors do not appear to have been used as internal controls; however, the widespread use of β-gal vectors may be a cause for concern, since the pCOUP-TF expression vector was reported to repress β-gal activity [13]. This caveat concerning experimental artifacts must be extended to reporter plasmids as well, and reports of activation by COUP-TFI [22] and ARP-1 [23] of promoters linked to luciferase may have to be re-examined. In addition, Kadowaki et al. have discovered a potential COUP-TFI binding site (DR-5) in the MMTV promoter downstream from the transcriptional start site and have reported activation by COUP-TFI of an MMTV-CAT reporter in the absence of upstream enhancer elements [15]. Thus, the present study re-emphasizes the importance of testing vectors for responses to exogenous stimuli and to co-transfected transcription factors.

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