Influence of promoter potency on the transcriptional effects of YY1, SRF and Msx-1 in transient transfection analysis

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ABSTRACT

Potent viral promoters/enhancers are often used to achieve high level expression of ectopic genes in transient transfection analysis. By using a GAL4-responsive transfection assay system, we show that the use of potent eukaryotic expression vectors can lead to biased transcriptional effects. Three functionally diverse transcription factors, YY1, SRF and Msx-1, were examined and each was found to exhibit a strong transrepression function in the context of the DNA binding domain of GAL4 when expressed from the cytomegalovirus (pCMV) or simian virus 40 (pSV) promoters/enhancers. An internal 15 amino acid domain of YY1 mediating transrepression in the viral promoter setting was identified. This GAL4-mediated transcriptional repression could, however, be completely relieved by using the yeast alcohol dehydrogenase promoter (pADH) to drive gene expression, which is ∼100-fold weaker than canonical pCMV and pSV in cultured mammalian cells. In addition, low level expression achieved with the pADH vector unveiled the intrinsic transactivation functions of YY1 and SRF previously not observed with the GAL4 assay system. Our results highlight a potential pitfall in conventional pCMV- and pSV-based transfection assays and suggest that the use of a low level expression system may be preferable in most transcriptional analysis.

INTRODUCTION

It is now clear that transcription factors can interact with one another and with co-activators in highly specific manners to modulate the activity of individual genes (1). Transient co-transfection assays have often been used to study protein–protein interactions involved in promoter targeting and transcriptional synergy or antagonism. In typical co-transfection assays, potent eukaryotic expression vectors for overexpression of transcription factors are introduced into the cell along with a promoter construct driving expression of a reporter gene. This DNA co-transfection approach is easy to perform and powerful in the sense that cis-acting promoter/enhancer elements and trans-acting activation and repression domains of a transcriptional regulator can be rapidly dissected and analyzed in a systematic fashion. A transcription inhibitory phenomenon referred to as ‘squelching’ has previously been documented in both in vivo and in vitro transcriptional studies (2–4). This inhibition is thought to be caused by the presence of large amounts of a transcription activator titrating away essential cofactors and resulting in ‘self-squelching’. Although the physiological relevance of this phenomenon remains unclear, it certainly provides evidence for common co-activators or adaptors used by multiple transcription factors. In practice, ‘squelching’ can complicate the interpretation of transient expression assays, since current gene overexpression systems are frequently based on the use of potent constitutive promoters/enhancers, such as those derived from cytomegalovirus (pCMV), simian virus 40 (pSV), Rous sarcoma virus (pRSV) or herpes simplex virus (pHSV-TK) (5,6). A transcription activator overproduced from such a potent viral promoter/enhancer may then act seemingly like a repressor or behave aberrantly. A recent report has indeed documented this pitfall in analysis of the transcriptional effects of the WT1 Wilms’ tumor suppressor protein using transient expression assays (7).

A popular transcriptional assay for examining macromolecular interaction and co-activator requirement is the gene fusion approach based on the isolated DNA binding domain of the yeast GAL4 transcription factor (4,8). We note, however, that many transcription factors examined in the context of a GAL4 fusion construct (invariably driven by a potent viral promoter/enhancer) exhibit an apparent repressor function, including but not limited to YY1 (9,10), SRF (11), the homeodomain protein Msx-1 (12), c-Fos (13), adenovirus E1A (4), growth suppressor proteins Rb and p107 (14,15), the heat shock factor HSF1 (16) and the bHLH factor Mad1 (17). These findings are somewhat surprising, since many of these transcription factors can function as a true transcription activator individually (9,13,18,19). To resolve the apparent paradox, we designed and tested an expression vector driven by the yeast alcohol dehydrogenase promoter (pADH). As expected, the yeast ADH promoter was found to be ∼100-fold weaker than the canonical CMV and SV40 promoters/enhancers in cultured mammalian cells. Using the pADH-based gene expression system, we re-examined the transcriptional properties of three transcription factors, YY1, SRF and Msx-1, in the context of the GAL4 DNA binding domain. Our results show that transrepression by all three transcription factors, as reported previously (9–12), is caused by the use of high level gene expression systems such as those mediated by pCMV and pSV. Transcriptional activation or relief of transrepression, on the other hand, could be readily observed when the GAL4 fusion proteins were expressed from the much weaker
ADH promoter. This study highlights the pitfall of high level gene expression and the need to select a suitable expression vector for transcriptional analysis.

MATERIALS AND METHODS

Cell culture and transient transfection

Cultured mouse NIH 3T3 cells, 10T1/2 cells and SOl8 myoblasts were maintained in minimum essential medium supplemented with 10% foetal calf serum (FCS) and 50 µg/ml gentamycin at 37°C in a humidified 5% CO2 atmosphere. Exponentially proliferating cells were trypsinized and 1–2 × 10^6 cells were plated onto 35 mm dish as described in the figures. Cells were transfected 20 h after plating using the calcium phosphate method detailed previously, except that DNA–phosphate crystals were washed off within 2.5–6 h after addition. For gel shift assays, SOl8 myoblasts were transfected with DOTAP (Boehringer Mannheim) following the manufacturer’s protocol. Cells were incubated with the DOTAP/DNA mixture for 6 h and lysates were prepared 30 h after transfection as described.

β-Galactosidase and luciferase assays

Cells were harvested 16–20 h after transfection for β-galactosidase and luciferase assays as detailed. Where indicated, 5 µg internal control reporter plasmid pRSV-lacZ were co-transfected for normalization. Luciferase activity was either normalized against β-galactosidase activity or protein concentration as determined by the Bradford protein-dye binding method using bovine serum albumin as standard. Data presented are representative of at least two separate transfection assays.

Gel shift assays

The methods for lystate preparation and gel shift assays were documented previously. Oligonucleotides corresponding to the GAL4 binding site were synthesized (top, CGGAAGACTCCTCCTCCG; bottom, TCGGAGAGAGTGCTTCCG), annealed and labeled with 32PdATP using Klenow. SRF3 oligonucleotides (for serum response factor) were described previously. DNA–protein complexes were resolved on 6% polyacrylamide gels cast in 0.5x Tris–borate buffer.

RESULTS

The well-characterized yeast GAL4 transactivation assay system has facilitated the analysis of eukaryotic transcription factors. We and others have previously found that YY1 and SRF when fused to transcription factors have also been found to exhibit a dominant transcriptional repression function when examined using the GAL4 assay system. To explore the possibility that ‘squelching’ caused by the use of potent viral promoters in these studies may have led to the apparent paradox, we sought to engineer an expression vector mediated by a much weaker eukaryotic promoter. The rationale behind this approach is that promoter sequences contained in a less potent promoter are less likely to draw essential or limiting transcription cofactors away, causing potentially artifactual repression in transient transfection analysis, as demonstrated recently for the potent cytomegalovirus (CMV) promoter/enhancer (7). The yeast alcohol dehydrogenase promoter (pADH) was chosen for the purpose and was compared in parallel with the widely used CMV and SV40 promoters/enhancers.

Table 1. Comparison of pADH, pSV and pCMV potency by transient transfection

<table>
<thead>
<tr>
<th>Promoter/enhancer</th>
<th>Normalized luciferase activity</th>
<th>Fold activation (versus no promoter)</th>
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<tr>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pADH</td>
<td>65 ± 267</td>
<td>15</td>
</tr>
<tr>
<td>pSV</td>
<td>20 093 ± 482 ± 1 393 279</td>
<td>4643</td>
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<tr>
<td>pCMV</td>
<td>7 687 593 ± 725 535</td>
<td>1776</td>
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</table>

Five micrograms of each of the luciferase reporter vectors and 5 µg pRSV-lacZ were co-transfected into Sol8 cells as described in the text. Cells were harvested 20 h after transfections for luciferase and β-galactosidase assays. Luciferase activities presented were normalized against β-galactosidase activities. Data are averages ± SE (n = 3).

To estimate the relative promoter strengths in mammalian cells, the firefly luciferase gene was expressed from the three promoters as reporter enzyme. Table 1 shows that while pSV was somewhat less potent than pADH, the β-galactosidase activity in transfections with pCMV was less than that with pADH.

The methods for lystate preparation and gel shift assays were documented previously. Oligonucleotides corresponding to the GAL4 binding site were synthesized (top, CGGAAGACTCCTCCTCCG; bottom, TCGGAGAGAGTGCTTCCG), annealed and labeled with 32PdATP using Klenow. SRF3 oligonucleotides (for serum response factor) were described previously. DNA–protein complexes were resolved on 6% polyacrylamide gels cast in 0.5x Tris–borate buffer.
Figure 1. Promoter potency modulates the transcriptional effects of GAL–YY1. Two micrograms of p(G)5-Luc reporter DNA were co-transfected into Sol8 cells (1.5 × 10^5 per 35 mm dish) with 5 µg GAL (open bar) or GAL–YY1 (filled bar) expression vectors, which were controlled by pCMV, pSV or pADH as indicated below the bars. Since the internal control plasmid pRSV-lacZ was found to interfere with the activity of p(G)5-Luc, luciferase activity was normalized against protein concentrations. Data (n = 3) are averages ± SE. Some error bars were too small to appear above the graph.

Figure 2. pSV and pADH differentially modulate the effects of GAL–YY1 in 3T3 cells. Transfection was performed as described in Figure 1 using cultured 3T3 cells. Data (n = 3) are averages ± SE. Some error bars were too small to appear above the graph.

Figure 3. pSV and pCMV produce high levels of GAL–YY1 in transfected cells. Sol8 cells were transfected with 50 µg DOTAP and 10 µg pSV-GAL-YY1, pCMV-GAL-YY1 or pADH-GAL-YY1. Lysates were prepared 30 h after transfection. Twenty micrograms of each lysate were assayed by electrophoretic mobility shift assay (A and B) and examined by SDS–PAGE (C). The DNA probes used in (A) and (B) were oligonucleotides containing binding sites for GAL4 and SRF respectively.

Figure 4 shows that deletion of nearly two thirds of the YY1 C-terminal sequence (see N395, N271, N200 and N152) had no effect on transrepression caused by high level expression. Removal of 15 amino acid residues from N152 (thus generating N138) relieved the transrepression and further allowed the intrinsic transactivation domain of YY1 to be functionally expressed from the N138 and N90 constructs in spite of the fact that the two YY1 deletion mutants were expressed from pSV. This finding thus substantiates the notion that high levels of GAL–YY1 in transfected cells cause transcriptional repression through protein–protein interactions, which may involve the internal 15 amino acid region of YY1 identified here.

To strengthen the conclusion that the observed transrepression was caused by the promoter potency of pCMV and pSV, a competition assay was performed as shown in Figure 5, where transactivation was mediated by pADH-GAL-YY1 (top) or pSV-GAL-YY(N90) (bottom). This competition assay showed that...
Figure 4. Transrepression mediated by high levels of GAL–YY1 fusion protein is associated with an internal 15 amino acid domain of YY1. Expression of GAL and GAL–YY1 was controlled by pSV in Sol8 cells. YY1 C-terminal deletion mutants were described previously (9, 21). DNA dosages and transfection conditions were as above. Data (n = 2–4) are averages ± SE. Some error bars were too small to appear above the graph.

Figure 5. Competition assays confirm that transrepression is caused by high levels of YY1. (Top) Two micrograms of p(G)5-Luc and 5 µg pADH-GAL-YY1 were co-transfected into Sol8 cells with 3 µg pADH-YY1, pADH, pCMV-YY1 or pCMV. (Bottom) Two micrograms of p(G)5-Luc and 5 µg pSV-GAL-YY(N90) were co-transfected with 3 µg pCMV-YY1 or pCMV. Data (n = 3) are averages ± SE. Some error bars were too small to appear above the graph.

pCMV-YY1 (but not pCMV alone) suppressed transactivation mediated by pADH-GAL-YY1. As expected, the transactivation function was unaffected by either pADH or pADH-YY1. To unequivocally rule out the possibility that the CMV promoter might directly or indirectly interfere with the ADH promoter, transactivation was also achieved using the N90 derivative of YY1 expressed from pSV [see construct pSV-GAL-YY(N90) in Fig. 4]. Figure 5 (bottom) shows that a high level of YY1 produced from pCMV-YY1 also interfered with the transactivation function mediated by pSV-GAL-YY(N90). These results indicate that only a low level of YY1 expression in transfected cells is permissive for transactivation from the GAL4-responsive promoter.

We went on to re-examine the transcriptional properties of the myogenic transcriptional activator SRF (25) and the homeodomain protein Msx-1 (26), again using the GAL4 target vector system. Both the full-length SRF and Msx-1 proteins (expressed from a potent viral promoter) have previously been found to exhibit a dominant transrepression function in the context of GAL4 (11, 12). Indeed, we confirmed that the GAL–SRF fusion protein repressed luciferase expression when the potent pSV vector was used for overexpression in transient transfection assays (Fig. 6). It is evident from the analysis that GAL–SRF expressed from the low level pADH expression vector activated rather than repressed transcription (Fig. 6), which is a finding consistent with the demonstrated transactivator roles of SRF in myogenic control and serum response regulation (19, 25, 27–29). Figure 7 further confirms that the GAL–Msx-1 fusion protein repressed transcription when pSV was used for overexpression. Again, the potent transrepression mediated by the pSV expression system was not observed using the pADH expression system. However, Msx-1 appeared to differ from YY1 and SRF in that a transcriptional activation function could not be demonstrated even with the pADH expression system, which may not be unexpected, given that the transactivation domain or function of Msx-1 was not observed in a previous study (12).

Although the pADH expression system allows the transactivation functions of YY1 and SRF to be demonstrated, the amount of
DNA transfected can profoundly influence transfection results (19). As shown in Figure 8, the transactivation function of GAL–SRF was greatly diminished when progressively more pADH-GAL-SRF vector DNA was used in transfection. Thus, even with the weak promoter system, we could only observe maximal transactivation with <5 µg GAL4 fusion construct DNA.

**DISCUSSION**

A recent study has highlighted a major pitfall in the use of co-transfection experiments to study transcriptional regulation (7). This study showed that the transcriptional property of the WT1 Wilms’ tumor suppressor protein was differentially affected by the use of different expression vectors. It was concluded that the transfected CMV promoter competes with the Egr promoter, which is regulated by WT1, for transcription factors or cofactors required for the transactivation function of WT1. The pitfall unveiled by this previous study is thus caused by DNA–protein interactions. Our study presented here highlights another pitfall associated with protein–protein interactions among transcription factors and co-activators. Transfections of potent expression vectors such as those based on pCMV and pSV apparently result in abnormally high levels of the expressed protein in transfected cells, which presumably causes transcriptional repression through ‘squelching’. We show that this problem can be corrected by the use of a pADH-based expression vector, allowing the intrinsic transcription activation function to be readily demonstrated in transient transfection analysis. This finding may explain why the activity of the potent proline-rich activation domain of AP-2 was not observed previously using a similar GAL4 transcription assay system (30) and is certainly consistent with the documented concentration-dependent transcriptional activation or repression mediated by transfected Krippel, a YY1-related zinc finger protein (31).

YY1 has been shown to interact with a wide variety of transcriptional regulators (21,32–39). Thus, the identity of the protein factor(s) ‘squelched’ by high levels of YY1 causing transrepression in the GAL4 assay system remains to be determined. Our domain mapping analysis indicates that an internal 15 amino acid domain of YY1 is involved in mediating transrepression, which essentially rules out protein factors such as Sp1, YAF2 and E1A-like cellular factors, since these proteins have been found to interact with YY1 through its C-terminal zinc finger region (10,21,35). Our preliminary results reveal that transrepression caused by high levels of GAL–YY1 cannot be relieved by co-transfections with TFIIB, TBP or CBP/p300 expression vectors (data not shown). It is also possible that the function or availability of one or more components of the basal transcription machinery, such as TAFq55 and TFIIF, which have been shown to interact with YY1 and SRF respectively (34,40), may be affected by high levels of YY1 or SRF proteins. Alternatively, high levels of an exogenously derived transcription factor may aberrantly influence protein contacts with the basal transcription machinery, as has been demonstrated for YY1 and c-Myc (41).

Aside from YY1, SRF and Msx-1, many other transcription factors, including but not limited to c-Fos (13), adenovirus E1A (4), growth suppressor proteins Rb and p107 (14,15), the heat shock factor HSFI (16) and the bHLH factor Mad1 (17), were shown to exhibit a dominant repressor function using the GAL4 assay system, which invariably involved the use of a potent viral promoter/enhancer. The yeast ADH expression system employed here clearly unveils the transactivation function of YY1 and SRF even in the context of GAL4, suggesting that the bifunctional transcriptional property may be a general feature of many transcription factors. In this respect, the homeodomain regulator Msx-1 may have evolved as a specialized repressor, as speculated (12), since no transcription activation function can be demonstrated even with the yeast ADH expression vector. A distinct class of transcription factors represented at least by CBP/p300 and members of the MyoD family appears to exist that is capable of exhibiting their transactivation functions even when fused to GAL4 and expressed at high levels in transfected cells (42–44). We speculate that intrinsic differences in utilization or targeting of components of the core transcription complex may constitute the basis for the observed differential transcriptional effects. Aside from transcriptional regulation, protein–protein interaction also plays a central role in many other biological systems. The use of a potent protein expression vector may similarly disrupt important regulatory circuits and inadvertently mask an activating effect. Taken together, our studies show that the use of different expression vectors may lead to different transcriptional effects in transient transfection analysis. A more comprehensive comparison of strong versus weak promoters will be necessary to determine whether our observation reported here is indeed due to intrinsic differences in promoter potency.

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**REFERENCES**