

Differential and opposed transcriptional effects of protein fusions containing the VP16 activation domain

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Abstract Overexpression of strong transcriptional activators like herpes simplex virion protein 16 (VP16) may lead to non-specific inhibition of gene expression as a result of the titration of transcription factors. Here we report that a fusion between the homeoprotein Hoxa2 and the VP16 activation domain inhibits transcription from the strong promoter/enhancers of cytomegalovirus (CMV) and Rous sarcoma virus (RSV). A similar fusion involving a Hoxa2 mutant protein that is defective in DNA binding has no effect on the CMV promoter but increases, rather than inhibits, the RSV promoter activity. This suggests that depending on its ability to bind DNA, the VP16 activator can interact with different sets of cofactors, giving rise to distinct transcriptional effects. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: VP16 activation domain; Hoxa2 homeoprotein; Cytomegalovirus; Rous sarcoma virus; Transcriptional activation; Squelching effect

1. Introduction

Fusions between a regulatory protein and a potent transcriptional activation domain like those of the yeast GAL4 protein or the virion protein 16 of the herpes simplex virus type I (VP16) are commonly used to identify targets for the regulator or to boost the transcriptional activation of a reporter gene in transient transfection assays [1]. However, when expressed at a high level, strong transcriptional activators have been reported to non-specifically inhibit transcription [2–4].

The VP16 activation domain interacts with multiple targets within the RNA polymerase II transcription initiation complex [5–9], as well as with adapter proteins that make the link between activation domains and the transcription machinery [10]. Therefore, it is believed that at a high concentration, the activator sequesters and titrates out transcription factors like

TBP or TAFs, thereby blocking transcription initiation. This phenomenon is referred to as a squelching effect.

In the course of the characterization of a target gene for the murine homeoprotein Hoxa2, we made use of different fusions between the VP16 activation domain and Hoxa2 derivatives (Remacle et al., in preparation). Hoxa2 belongs to the HOM-C/Hox family of homeoproteins, and contains a well-conserved 60-amino acid (aa) homeodomain involved in DNA binding [11]. Here we report that fusion proteins between the VP16 activation domain and the wild-type Hoxa2 or a DNA binding-defective mutant differently affect the activity of the cytomegalovirus (CMV) and the Rous sarcoma virus (RSV) strong promoters/enhancers, leading to the inhibition, but in some cases to the activation, of transcription. As neither of the two proteins alters the CMV or RSV promoter activity in the absence of the VP16 activation domain, we conclude that the transcriptional effects reported here account for new distinct consequences of VP16-mediated squelching.

2. Materials and methods

2.1. Reporter plasmids and expression vectors

Reporter plasmids and expression vectors used in this study are listed in Table 1. Details of the constructs are available upon request.

Plasmids encoding fusion proteins with the 77-aa activation domain of VP16 (first residue of the 78-aa sequence omitted) have been obtained by removing the *hoxa2* stop codon. The VP16 sequence encoding the activation domain was further amplified by PCR from pPGKHoxa1VP16 [17], and inserted as a *Clal*-*HindIII* fragment in frame with the Hoxa2 or Hoxa2(QN-AA) coding sequence.

2.2. Cell culture and transient transfection

COS7 cells were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum (PAA Laboratories GmbH), 100 IU/ml of penicillin and 100 µg/ml streptomycin (Sigma), at 37°C in a humidified, 5% CO₂ atmosphere. Before transfection, exponentially proliferating cells were trypsinized and 10⁵ cells were plated per 35-mm culture dish. Cells were transfected by standard calcium phosphate precipitation procedure, 24 h after plating [18].

Each cotransfection experiment was performed with a constant amount of 10.2 µg of DNA containing, when appropriate, 4 µg of the *luc*-based reporter (pTKluc or pAdMLluc), 2 µg of the Hoxa2 derivative expression vector, 0.2 µg of the *lacZ* internal standard plasmid (pCMVlacZ, pRSVlacZ, pSVK3lacZ or pCD-SRαlacZ), and carrier DNA to complement.

2.3. β-Galactosidase and luciferase assays

Cells were harvested 48 h after transfection for enzymatic assays. Lysis and enzymatic activity dosages were performed with the β-gal reporter gene assay (chemiluminescent) kit (Roche) and the luciferase reporter gene assay (high sensitivity) kit (Roche).

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Abbreviations: AdML, adenovirus-2 major late promoter; CMV, cytomegalovirus; *lacZ*, β-galactosidase gene from *Escherichia coli*; *luc*, firefly luciferase gene; RSV, Rous sarcoma virus; SV40, simian virus 40; TK, thymidine kinase; VP16, herpes simplex virion protein 16

Table 1
Reporter plasmids and expression vectors used in this study

Reporter plasmid	Promoter	Reporter gene	Origin
pTKluc	TK ^a	<i>luc</i> ^b	pXP2 [12]
pAdMLluc	AdML ^c	<i>luc</i>	[13]
pCMVlacZ	CMV ^d	<i>lacZ</i> ^e	pCMX-PL1 [14]
pRSVlacZ	RSV ^f	<i>lacZ</i>	[15]
pSVK3lacZ	SV40 ^g	<i>lacZ</i>	pSVK3; Pharmacia
pCD-SR α lacZ	HTLVILTR-SV40 ^h	<i>lacZ</i>	pCD-SR α [16]
Expression vector	Promoter	Encoded Hoxa2 derivative	Origin
pCMVHoxa2	CMV	Hoxa2 wild type	pCMX-PL1 [14]
pCMVHoxa2(QN-AA)	CMV	Hoxa2(QN-AA) ⁱ	pCMX-PL1 [14]
pCMVHoxa2VP16	CMV	Hoxa2::VP16 ^j	pCMX-PL1 [14,17]
pCMVHoxa2(QN-AA)VP16	CMV	Hoxa2(QN-AA)::VP16 ^k	pCMX-PL1 [14,17]

^aFrom herpes simplex I thymidine kinase (from nt –105 to +51).

^bFirefly luciferase gene.

^cFrom adenovirus-2 major late promoter (from nt –33 to +33).

^dCytomegalovirus major immediate-early promoter.

^e*Escherichia coli lacZ* gene.

^fRous sarcoma virus long terminal repeat promoter.

^gSV40 early promoter.

^hR-U5 segment of the long terminal repeat of human T-cell leukemia virus type I and SV40 early promoter.

ⁱHoxa2 with homeodomain residues Q50 and N51 converted into A50 and A51.

^jFusion protein between Hoxa2 and the VP16 activation domain.

^kFusion protein between the Hoxa2(QN-AA) mutant and the VP16 activation domain.

3. Results

3.1. Fusion protein Hoxa2VP16 modifies the transcriptional activity of the CMV and RSV strong promoters in transient transfection assays

In the context of analyzing a Hoxa2-responsive enhancer (Remacle et al., in preparation), the effect of the Hoxa2 and Hoxa2VP16 fusion proteins was examined in cotransfection with a control reporter plasmid in which the firefly *luc* gene is under the control of the thymidine kinase (TK) promoter (pTKluc) or the adenovirus-2 major late (AdML) promoter (pAdMLluc). To avoid experimental variations due to variable transfection efficiencies, an internal standard reporter containing the *lacZ* gene under the control of the CMV (pCMVlacZ) or RSV (pRSVlacZ) promoter was also added in the cotransfection experiments. Luciferase activity was then normalized to that of β -galactosidase.

Transfection of the Hoxa2 expression vector in combination with pTKluc or pAdMLluc did not modify the level of normalized luciferase activity as compared to the experiments in which the Hoxa2 plasmid was omitted (reference value of 1; Fig. 1A). In contrast, transfection of the vector expressing the Hoxa2VP16 fusion protein resulted in a dramatic increase in the ratio between the luciferase and β -galactosidase activities, whatever the reporter (pAdMLluc or pTKluc) and the standard (pCMVlacZ or pRSVlacZ) plasmids used (Fig. 1B). This indicates that the VP16 moiety of the Hoxa2VP16 protein either activates *luc* expression or represses that of *lacZ*, or both.

To discriminate between these two possibilities, cotransfection experiments were performed with two other reporter plasmids, in which the *lacZ* reporter gene is under the control of either the SV40 early promoter (pSVK3lacZ) or the SV40 early promoter combined with the R-U5 enhancer of HTLV-1 (pCD-SR α lacZ). With these two *lacZ* reporter plasmids, the addition of either Hoxa2 or Hoxa2VP16 resulted in only a slight change in the luciferase/ β -galactosidase ratio (Fig. 1A,B). This implies that Hoxa2VP16 has no effect on

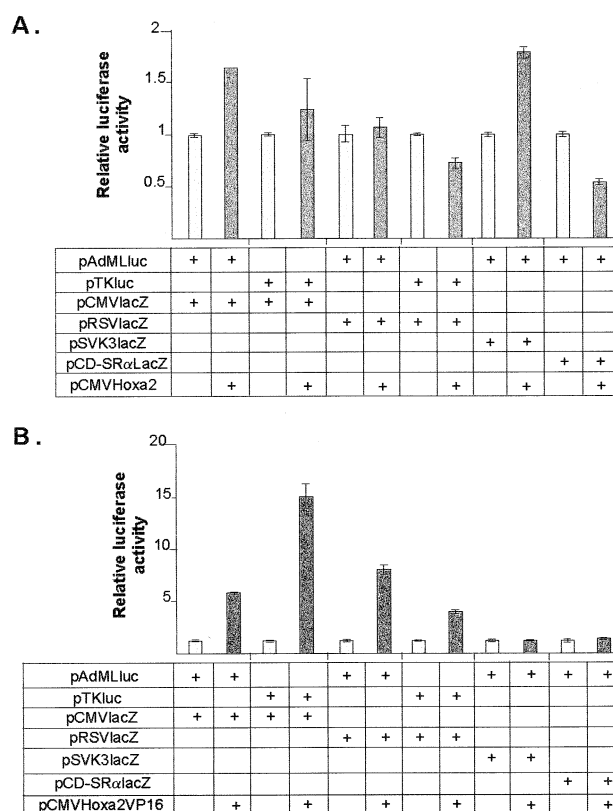


Fig. 1. The Hoxa2VP16 protein induces changes in relative reporter activities. Different *luc* and *lacZ* reporter constructs were transfected in COS7 cells in combination with an expression vector for (A) Hoxa2 and (B) Hoxa2VP16. For each combination of reporter plasmids, the relative luciferase activity obtained in the absence of expression vector is set at 1. Each series of experiments has been repeated more than twice, in each experiment sample size is 2 for each plasmid combination.

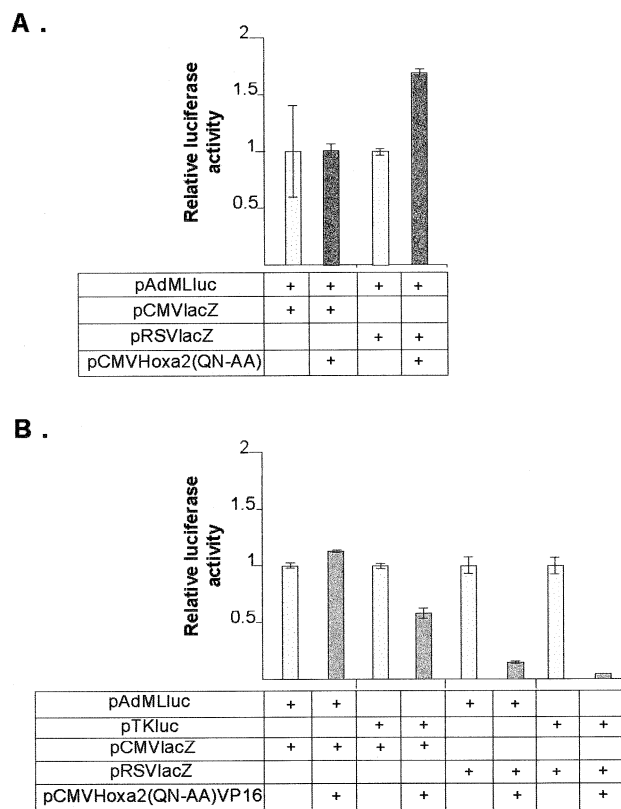


Fig. 2. The Hoxa2(QN-AA)VP16 fusion protein induces an increase in *lacZ* expression mediated by the RSV promoter. Different *luc* and *lacZ* reporter constructs were transfected in COS7 cells in combination with an expression vector for (A) Hoxa2(QN-AA) and (B) Hoxa2(QN-AA)VP16. For each combination of reporter plasmids, the relative luciferase activity obtained in the absence of expression vector is set at 1. Sample size is 2 for each plasmid combination.

the promoters carried by the pTKluc, pAdMLluc, pSVK3lacZ or pCD-RS ϕ lacZ reporter plasmids, and that the significant increase in normalized activities observed with pCMVlacZ and pRSVlacZ arises from a strong inhibition of the CMV and RSV promoters, rather than the activation of the AdML or TK105 promoters.

3.2. The DNA binding-defective Hoxa2(QN-AA)VP16 fusion protein activates rather than inhibits RSV promoter activity

The proteins of the Hox family share a very well conserved homeodomain. Contacts between the homeodomain and DNA involve several amino acids, among which a glutamine at position 50 and an asparagine at position 51 are perfectly conserved within the Hox family. These residues have been shown to make critical contacts with bases within the TNATNN sites recognized by homeoproteins [11,19]. Both residues have pivotal roles in the binding specificity of the proteins, and contribute to their affinity for the DNA [20–22].

Cotransfection experiments involving combinations of pTKluc, pAdMLluc, pCMVlacZ and pRSVlacZ reporters were performed with vectors encoding the Hoxa2(QN-AA) and Hoxa2(QN-AA)VP16 mutant proteins (Fig. 2). In these proteins residues 50 and 51 of the homeodomain have been converted to alanines.

Similarly to wild-type Hoxa2, the Hoxa2(QN-AA) mutant

protein alone did not change or only slightly altered the luciferase/ β -galactosidase ratio when combined with the different reporters (Fig. 2A). By contrast, cotransfections performed with the Hoxa2(QN-AA)VP16 fusion protein yielded different results than those reported above for Hoxa2VP16 (Fig. 2B). Hoxa2(QN-AA)VP16 only weakly affected the luciferase/ β -galactosidase ratio when the *lacZ* gene expression was driven by the CMV promoter, whereas it caused a severe drop in this ratio when the pRSVlacZ standard vector was used (Fig. 2B). This indicates that, unlike Hoxa2VP16, the mutant Hoxa2(QN-AA)VP16 fusion protein has no or little effect on the CMV promoter, and that it activates rather than inhibits transcription from the RSV promoter. Since the two proteins differ by only two amino acid residues in the Hoxa2 homeodomain, it is likely that their different effects on transcription are direct or indirect consequences of their DNA binding properties.

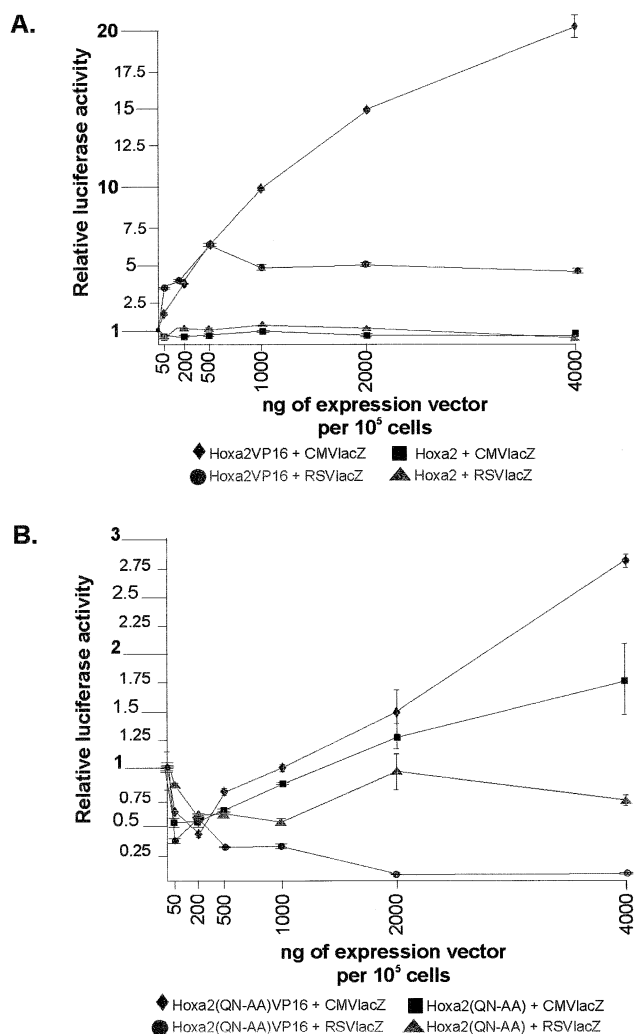


Fig. 3. The level of transcriptional activation/repression mediated by VP16 fusion proteins varies according to the amount of expression vector transfected. Cotransfection experiments (COS7 cells) have been performed with pAdMLluc, pCMVlacZ, pRSVlacZ, and increasing amounts of pCMVHoxa2 (A), pCMVHoxa2VP16 (A), pCMVHoxa2(QN-AA) (B), or pCMVHoxa2(QN-AA)VP16 (B), ranging from 0.05 to 4 μ g of DNA for 10^5 cells. For each combination of reporter plasmids, the relative luciferase activity obtained in the absence of expression vector is set at 1. Sample size is 2 for each combination of plasmids tested.

3.3. The level of transcriptional activation/repression mediated by VP16 fusion proteins increases with the amount of transfected expression vector

The squelching effects caused by strong transcriptional activators may depend on the abundance of the activator within the cell. To test this, cotransfection experiments were performed with increasing amounts of plasmids encoding the Hoxa2, Hoxa2(QN-AA), Hoxa2VP16 or Hoxa2(QN-AA)-VP16 proteins (Fig. 3). As expected, adding up to 4 µg of the Hoxa2 and Hoxa2(QN-AA) expression vectors only led to minor fluctuations in the ratio between reporter activities (Fig. 3A,B).

For the Hoxa2VP16 fusion protein, the luciferase/β-galactosidase ratio measured in the presence of the CMVlacZ reporter progressively increased in relation to the amount of expression vector, to reach a value up to 20 times higher than the background level (Fig. 3A). With the RSVlacZ reporter, addition of up to 0.5 µg of Hoxa2VP16 vector DNA induced a 5–7-fold increase in the luciferase/β-galactosidase ratio, with no further increase when more expression vector was added.

Transfection of increasing amounts of expression vector for the Hoxa2(QN-AA)VP16 mutant did not strongly affect the relative luciferase activity when *lacZ* was expressed under the control of the CMV promoter (Fig. 3B). Inversely, with the RSVlacZ reporter, increasing amounts of Hoxa2(QN-AA)VP16 induced a progressive decrease of the luciferase/β-galactosidase ratio. Upon transfection of 2 µg of the Hoxa2-(QN-AA)VP16 expression vector, the ratio was 10 times lower than in the absence of Hoxa2(QN-AA)VP16.

Altogether these results confirm that the activity of different promoters can be either enhanced, inhibited or unaffected as a consequence of the expression of the VP16 activator. Furthermore, variations in promoter activity can be different, or even opposite, depending on the DNA binding properties of the activator. Finally, the relationship between the amount of expression vector and the change in promoter activity also appears to be different according to the promoter and probably to the VP16 fusion protein. Indeed, the ratio between the measured enzymatic activities can either reach a plateau or continuously increase, or decrease, upon addition of increasing amounts of expression vector.

4. Discussion

The data presented here provide new cases and new consequences of VP16-mediated squelching effects. Using different combinations of luciferase and β-galactosidase reporter plasmids, we found that the reporters' relative activities changed upon addition of VP16 fusion proteins when the *lacZ* gene was transcribed from either the CMV or the RSV promoter. Furthermore, whereas the Hoxa2VP16 protein caused a decrease in CMV- and RSV-driven *lacZ* expression, the DNA binding-defective mutant, Hoxa2(QN-AA)VP16, had no or a slight effect on the CMV promoter, but induced an increase in RSV promoter activity. In similar conditions, Hoxa2 and Hoxa2(QN-AA) proteins did not affect the relative reporter activities.

We think that these transcriptional effects associated with Hoxa2VP16 and Hoxa2(QN-AA)VP16 result from VP16-associated 'squelching' rather than from the specific interaction of Hoxa2 derivatives with cognate sites that would reside in

the CMV or RSV loci. Supporting this view, (i) the Hoxa2 protein does not appear to affect CMV- and RSV-mediated transcription; (ii) in experiments similar to those presented here, Hoxa2 and Hoxa2VP16 properly activate (rather than inhibit) a reporter gene controlled by Hox-responsive sequence elements (Matis et al., unpublished data); and consistently, (iii) Hoxa2(QN-AA)VP16 which cannot bind to Hoxa2 recognition sites has no effect on a proper specific target gene (Matis et al., unpublished data).

Altogether, our data show, firstly, that transcriptional interference mediated by high amounts of the VP16 activation domain affects the strong CMV and RSV promoters that are often used in internal standard constructs to normalize transfection assays. Conversely, the activity of AdML and TK105 promoters as well as that of the SV40 early promoter are not affected by VP16. This means that, in our assay, the VP16 domain does not interfere with the basal transcription machinery, but rather with accessory factors required for specific promoters/enhancers.

Secondly, the transfection of the Hoxa2VP16 vector caused the ratio between the luciferase and β-galactosidase activities to reach a plateau when the *lacZ* gene is controlled by the RSV promoter. This ratio did not vary further upon transfection of higher amounts of expression vector. Inversely, this enzymatic ratio continuously increased upon addition of increasing amounts of this expression vector to transfected pCMVlacZ reporter plasmid. Therefore, the relationship between the amount of transfected vector encoding the VP16 fusion protein and the change in promoter activity appears to be different according to the promoter.

Thirdly, our results stressed that, depending on the fusion protein, the VP16 activation domain can induce an inhibitory or a stimulatory effect on transcription. The Hoxa2VP16 protein that has an intact homeodomain caused a typical inhibitory squelching effect on both the CMV and RSV promoters. By contrast, the Hoxa2(QN-AA)VP16 mutant, which is impaired for its ability to bind DNA, activated RSV-mediated transcription and had no or little effect on the CMV promoter. The Hoxa2VP16 and Hoxa2(QN-AA)VP16 fusion proteins differ by only two amino acid residues in the Hoxa2 homeodomain. These residues are involved in the binding and the recognition of DNA sites. We cannot exclude the possibility that the substitution of the glutamine and asparagine residues by alanines may impair other properties than the DNA binding of the protein. However, these amino acids that are perfectly conserved among the Hox proteins do not appear to be involved in other activities of these proteins, and in particular they are not involved in the interaction between Hox and their known cofactors [19,23,24]. Therefore, it can be assumed that the different effects caused by the expression of Hoxa2VP16 or Hoxa2(QN-AA)VP16 on CMV and RSV activity are direct or indirect consequences of their distinct DNA binding properties.

Glutamine 50 and asparagine 51 have been shown to contact the DNA base pairs, and to critically contribute to the binding specificity of the Hox proteins [11,20–22]. However, even if converting these residues into alanines may affect the affinity of the protein for the DNA, it is likely that, within a cell, the mutant proteins will still bind to DNA, but in a more relaxed fashion [22]. Therefore, the distinct squelching effects induced by Hoxa2VP16 and Hoxa2(QN-AA)VP16 may be the consequence of the different distribution of these two proteins

on the DNA. Alternatively, the decrease in DNA binding affinity displayed by the mutant may be such that it preferentially interacts with cofactor proteins rather than with DNA, which in turn would lead to the distinct effects observed on the transcriptional activity. Our data do not allow us to favor one of the above hypotheses, and additional experiments are required to sort out how differences in DNA binding may affect VP16-mediated squelching.

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