Mapping of a potent transcriptional repression region of the human homeodomain protein EVX1

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Abstract The human homeodomain protein EVX1 is a transcriptional repressor in transfected mammalian cells and this function depends on a region carboxyl-terminal to the homeodomain. In this study, we transiently expressed several deletions of the EVX1 C-terminal region in mammalian cells and investigated their effect on the transcription of a reporter gene directed by different promoters. We show that the repressor activity maps to a region of 51 amino acids with a high abundance of alanine and proline residues. This region is able to transfer the repressor function to either the entire HOXC6 or CREB transcription factors, or to the GAL4 DNA binding domain.

Key words: EVX1; Gene transcription; Repression domain

1. Introduction

Homeodomain (HD) proteins regulate transcription during development [1,2]. The human HD protein EVX1 [3] is closely related to the product of the *Drosophila* gene *even-skipped* (*eve*) which is required for the proper development of the metameric body plan of the fruit fly [4]. In mammals, the early murine *Evx 1* expression pattern is compatible with a role in specifying posterior positional information along the embryonic axis while the late *Evx 1* expression pattern is compatible with a role in specifying neuronal cell fates within the differentiating neural tube [5]. The targeted disruption of the murine *Evx 1* gene, has been shown to cause early post implantation lethality of the conceptus [6]. All these data suggest a relevant role also for the human EVX1 protein during development.

Several authors have reported that the *eve* gene product (Eve) acts as a transcriptional repressor both in vitro [7–9] and in transfected cells [10,11]. Recently, we demonstrated that the human EVX1 expression represses the transcription of a reporter gene directed by either cell-specific or viral promoter/enhancer sequences in a variety of mammalian cell lines [12]. The repressor function of EVX1 is independent of the presence in the promoter of DNA-binding sites for the protein and the region carboxyl-terminal to the HD is responsible for this activity [12].

The aims of our work were (i) to identify and map the C-terminal repression domain involved in the transcriptional repressor function of EVX1 and (ii) to investigate whether its repressor function is transferable either to entire transcription factors or to a heterologous DNA binding domain.

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2. Materials and methods

2.1. Plasmids

p[-1.1]GLU-CAT, pCMV-EVX1, and pCMV-ΔEVX1 were previously described and characterized ([12] and references cited therein). pCMV-Δ1EVX1, pCMV-Δ2EVX1, pCMV-Δ3EVX1, and pCMV-Δ4EVX expression constructs were generated by cloning PCR-amplified fragments containing the 5′ 1122 bp, 939 bp, 858 bp, and 786 bp, respectively, of the open reading frame of the human EVX1 cDNA [3] in the *Hin*dIII-*Xba*I sites of the pRC-CMV expression vector (Invitrogen, San Diego, CA). To obtain the Δ3mutEVX1, the Δ3EVX1 cDNA was mutagenized by a PCR-mediated strategy [13]. Four point mutations (C→A) in the first position of codons corresponding to amino acids 274, 278, 280, and 284 were introduced and the mutated cDNA was cloned into the *Hin*dIII-*Xba*I sites of pRC-CMV. As a consequence of the mutations, four Leu residues were present in the protein instead of Pro residues at the above-mentioned positions. The chimeric construct pCMV-HOXC6N/rEVX1 was created by ligating the 5′ 612 bp fragment of HOXC6 cDNA ([14] and references cited therein) (indicated as pCMV-HOXC6N when cloned into pRC-CMV) in frame with 153 bp of the coding region of EVX1 cDNA (aa 263–313). The chimeric construct pCMV-CREB/rEVX1 was created by ligating the complete open reading frame of the human CREB cDNA ([15] and references cited therein) (a gift from Dr. R. Goodman, Portland, OR, indicated as pCMV-CREB when cloned into pRC-CMV) in frame with 153 bp of the coding region of EVX1 cDNA (see above). The chimeric construct pCMV-GAL4 1–147/rEVX1 was created by ligating the 5′ 441 bp of the open reading frame of yeast GAL4 cDNA [16] (Clontech, aa 1–147, indicated as pCMV-GAL4 1–147 when cloned into pRC-CMV) in frame with 153 bp of the coding region of EVX1 cDNA (see above). The GAL-TK-CAT and the TK-CAT vectors ([17] and references cited therein) were gifts from Dr. F.J. Rauscher III, Philadelphia, PA. For PCR reactions, ULTma DNA polymerase from Perkin Elmer was used and each construct was checked by sequencing.

2.2. Cells and DNA transfections

InR1-G9 hamster glucagonoma cells, β-TC1 mouse insulinoma cells, and NIH-3T3 mouse fibroblast cells were cultured as described [12]. BHK-21C13 hamster kidney cells were obtained from ATCC. The DEAE-dextran technique, calcium phosphate technique, and CAT assays were carried out as reported in [12].

2.3. Immunofluorescence and RT-PCR experiments

Immunofluorescence experiments were performed as described in [12] using the monoclonal antibody anti-HOXC6 [18]. RT-PCR analysis was performed essentially as described in [19]. Briefly, total RNA was prepared from InR1-G9 cells transiently transfected with pCMV-CREB/rEVX1 and from BHK-21C13 cells transfected with either pCMV-GAL4 1–147 or pCMV-GAL4 1–147/rEVX1 by the guanidine/cesium chloride method [13].

3. Results and discussion

We have previously shown that EVX1 represses the transcription of several promoters (~10-fold) in transiently transfected mammalian cells [12]. The deletion mutant ΔEVX1, which lacks the entire region carboxyl-terminal to the HD (Fig. 1B), loses the ability to inhibit transcription [12]. In this report we first investigated which region(s) of the EVX1

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C-terminus are necessary for the transcriptional repression activity. To this purpose, we performed the deletion analysis described under Section 2 and schematically illustrated in Fig. 1. Truncated EVX1 proteins were all expressed in mammalian cells and their correct processing and transport to the nucleus was checked by Western blot utilizing the previously characterized mAb B6-41 anti-EVX1 monoclonal antibody ([12] and data not shown). To assay the transcriptional repression activity of the deletion mutants in transiently transfected cells, the well characterized rat proglucagon gene promoter was used [12]. Glucagonoma InR1-G9 cells were cotransfected with each expression construct together with the plasmid p[1.1]GLU-CAT in which the transcription of the reporter gene is directed by 1.1 kilobases of the rat proglucagon gene promoter [12]. As shown in Fig. 1B, Δ1EVX1 and Δ2EVX1 deletion mutants are able to repress CAT activity at levels comparable to wild-type EVX1. Also, the Δ3EVX1 protein is able to repress gene transcription although it functions as a less effective repressor when compared to Δ1EVX1 and

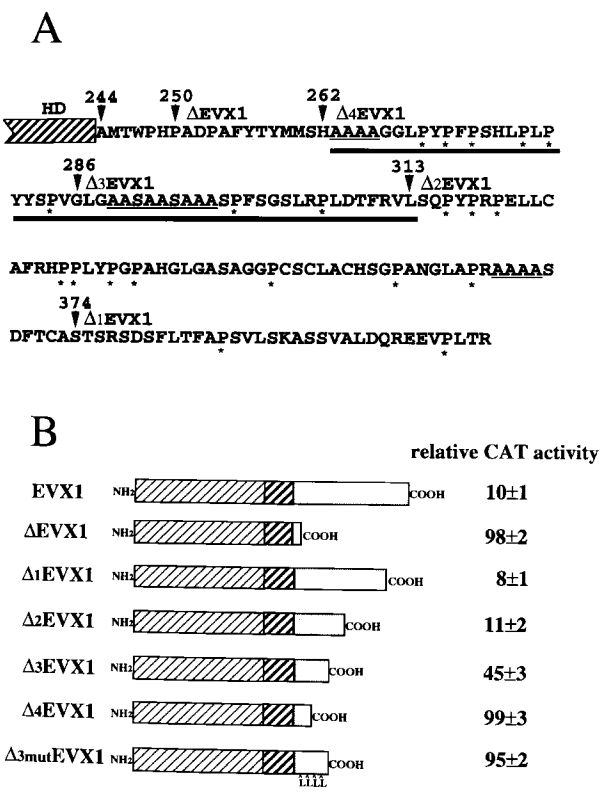

Fig. 1. Mapping of the transcriptional repression region of EVX1 (rEVX1) using deletion mutants. (A) Carboxyl-terminal region of EVX1. Arrowheads point to the position either of the first residue of this region or of the last residue of each truncated protein. Alanine stretches are underlined and proline residues are indicated with an asterisk. rEVX1 region is underlined with a bar. (B) Left: schematic representation of EVX1, the deletion mutants, and the deletion mutant in which four proline residues are substituted by four leucine residues. Details on the construction of the mutant expression vectors are reported under Section 2. Right: relative CAT activities exerted by wild-type EVX1 and by each mutant protein in transiently transfected InR1-G9 cells. The control (relative CAT activity = 100) is represented by the CAT activity measured in cells cotransfected with the reporter p[-1.1]GLU-CAT together with the pRC-CMV expression vector (from which all our EVX1 constructs were generated). The average (±S.E.) of four independent experiments performed in duplicate is presented.

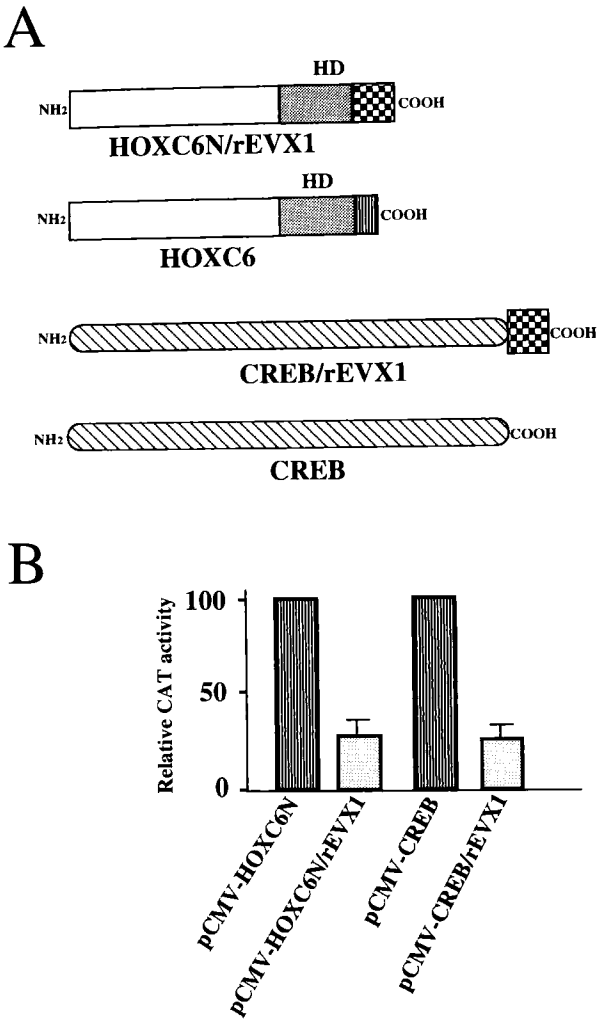

Fig. 2. Transcriptional repression mediated by HOXC6N/rEVX1 and CREB/rEVX1 fusion proteins. (A) Schematic representation of HOXC6, HOXC6N/rEVX1, CREB, and CREB/rEVX1 proteins. The checked box indicates the rEVX1 region. Details on the construction of the expression vectors are reported under Section 2. (B) InR1-G9 cells were transiently transfected using the DEAE-Dextran technique with 10 μg of p[-1.1]GLU-CAT reporter plasmid and 10 μg of the indicated expression plasmids. 46 h after transfection, cells were harvested, and aliquots of lysates were assayed for CAT activity as described under Section 2. The results are the average (±S.E.) of four independent experiments performed in duplicate.

Δ2EVX1 (Fig. 1B). In contrast, Δ4EVX1 does not significantly repress CAT activity (Fig. 1B). These findings suggest that the region of the EVX1 protein required for full transcriptional repression activity in mammalian cells is located between amino acids 263 and 313 (Fig. 1A). The prominent feature of this region, called in this report rEVX1 (r denotes repressor), is that it is particularly rich in alanine and proline residues (37.2% compared to 23.3% in the entire protein, Fig. 1A). Regions rich in alanine/proline/glutamine residues have been found in other well characterized transcriptional repressors such as Eve, Dr1, engrailed, Krüppel, Msx-1, and RGM1 [20]. It is noteworthy that, although EVX1 and the *Drosophila* Eve do not show a very high degree of homology outside the homeodomain [3], they both possess regions abundant in alanine and proline residues. Furthermore, similarly to the Eve repressor domain [11], rEVX1 contains only four charged res-

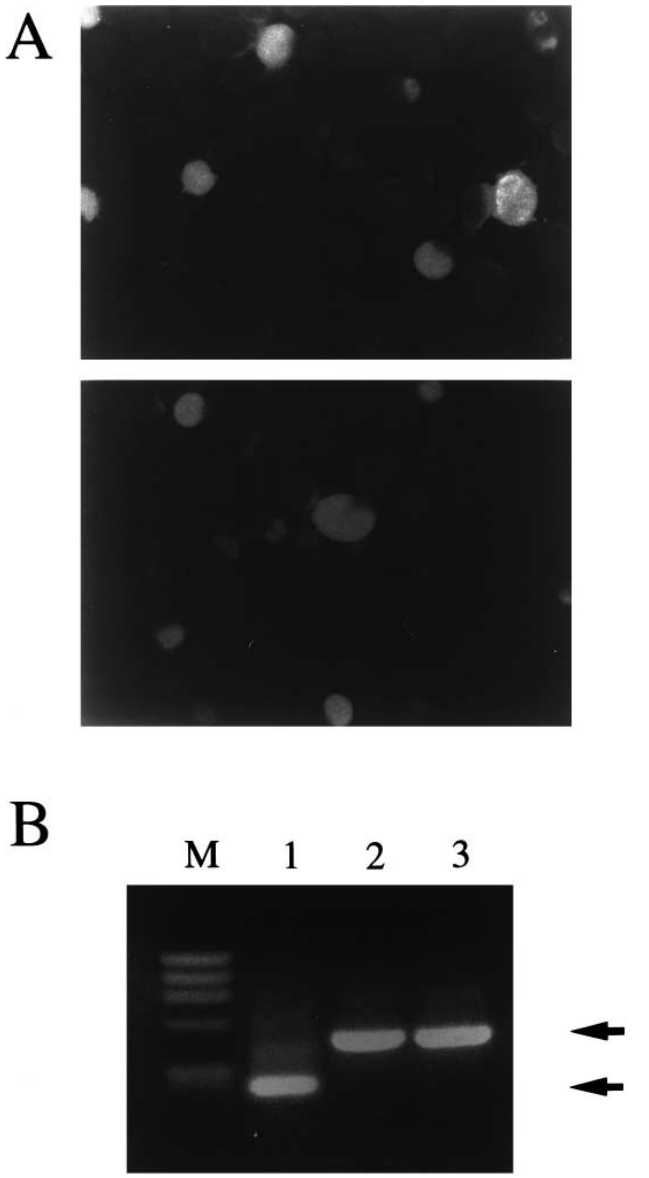

Fig. 3. Expression of the chimeric constructs in transfected cells. (A) Immunofluorescence staining with the anti-HOXC6 monoclonal antibody of InR1-G9 cells transiently transfected with pCMV-HOXC6N (top) and pCMV-HOXC6N/rEVX1 (bottom). (B) RT/PCR analysis of the EVX1 transcript in InR1-G9 cells transiently transfected with pCMV-CREB/rEVX1 (lane 1) and the GAL4 transcript in BHK-21C13 transiently transfected with either pCMV-GAL4 1–147 (lane 2) or pCMV-GAL4 1–147/rEVX1 (lane 3). The EVX1 amplification product is 177 bp long and the GAL4 amplification product is 425 bp long.

idues. In summary, rEVX1 is a small, hydrophobic and not highly charged domain.

Han and Manley [11] analysing a large number of Eve deletion mutants and chimeric constructs concluded that proline richness is the major feature of a strong repression domain. In order to check the importance of the proline residues located within the rEVX1 region, we mutated in the Δ3EVX1 protein four of the proline residues to leucine (an amino acid similar to proline in terms of hydrophobicity) and obtained Δ3mutEVX1. As shown in Fig. 1B, the pCMV-Δ3mutEVX1 protein completely lacks the ability to repress the activity of the reporter plasmid. Thus, our results show that the presence of a random hydrophobic amino acid instead of proline is not sufficient to maintain the transcriptional repressor activity. These results were repeated using several cell lines (HeLa, human cervix carcinoma cells, NIH-3T3, mouse fibroblast cells, and U87-MG7, human glioblastoma cells) and different mammalian and viral promoters (the Rous sarcoma virus LTR, the SV40 promoter, and the human tenascin C promoter) ([12] and not shown).

Next, we investigated whether the rEVX1 region per se functions as a transcriptional repressor when it is transferred to either a distinct HD protein or to an unrelated transcription factor. The pCMV-HOXC6N/rEVX1 plasmid which expresses the HOXC6 HD protein lacking the region carboxyl-

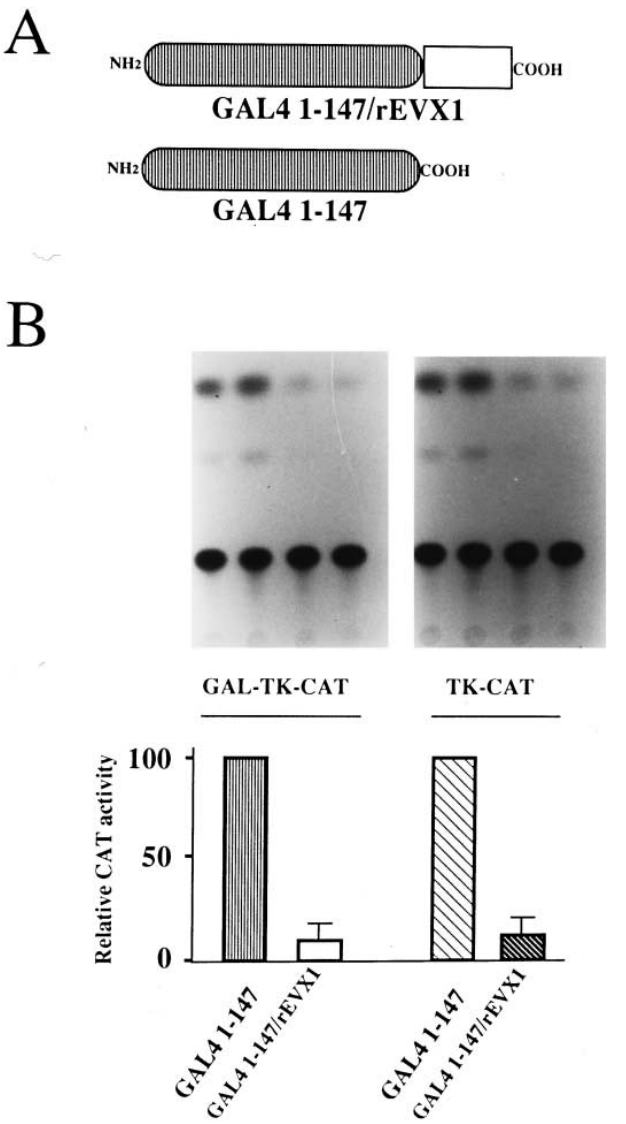

Fig. 4. Transcriptional repression mediated by the GAL4 1–147/rEVX1 fusion protein. (A) Schematic representation of GAL4 1–147 and GAL4 1–147/rEVX1 proteins. Details on the construction of the expression vectors are reported under Section 2. (B) BHK-21C13 cells were transiently transfected in duplicate using the calcium phosphate technique with 10 μg of the indicated reporter plasmids and 10 μg of the indicated expression plasmids. 46 h after transfection, cells were harvested, and aliquots of lysates were assayed for CAT activity as described under Section 2. The results are the average (±S.E.) of four independent experiments performed in duplicate.

terminal to the HD fused with rEVX1 (Fig. 2A) was cotransfected together with the p[-1.1]GLU-CAT reporter plasmid in InR1-G9 cells. As shown in Fig. 2B, the CAT activity was 65% inhibited in cells transfected with pCMV-HOXC6N/rEVX1 in comparison to those transfected with pCMV-HOXC6N as the control. The HOXC6N/rEVX1 fusion protein is correctly expressed, processed and transported to the nucleus, as demonstrated by immunofluorescence experiments performed with an anti-HOXC6 monoclonal antibody [18] (Fig. 3A). Thus, the substitution of the C-terminal region of a HD protein per se unable to influence the transcription directed by the proglucagon gene promoter with the rEVX1 domain confers to this protein the ability to repress the transcription of the reporter gene.

To investigate whether rEVX1 transcriptional repressor activity is also transferable to a transcription factor containing a totally different DNA binding domain, we ligated the cDNA fragment encoding rEVX1 to the 3′ end of the CREB open reading frame [15] and cloned the resulting construct into pRC-CMV (Fig. 2A). CREB, a cAMP-responsive transcription factor, contains a leucine-zipper DNA binding motif [15]. It binds to the cAMP responsive element (CRE) which is present in the rat proglucagon gene promoter. As shown in Fig. 2B, CAT activity was 70% reduced in cells cotransfected with p[-1.1]GLU-CAT together with pCMV-CREB/rEVX1 in comparison to cells cotransfected with p[-1.1]GLU-CAT together with pCMV-CREB. The expression of the pCMV-CREB/rEVX1 construct in transfected cells was demonstrated by RT-PCR experiments shown in Fig. 3B. This finding suggests that the presence of a HD is not necessary for the rEVX1 transcriptional repressor activity.

Finally, we fused rEVX1 to the GAL4 DNA-binding domain. The GAL4 1–147 protein domain contains elements responsible for binding, homodimerization, and nuclear localization [21]. As extensively demonstrated, when bound to GAL4 DNA binding sites in a promoter, the GAL4 1–147 polypeptide is neutral in that it does not significantly activate or repress the transcription of a target gene [17,21]. The fusion protein GAL4 1–147/rEVX1 (Fig. 4A) was cloned into pRC-CMV and the expression construct was transfected in BHK-21C13 cells together with the reporter vector GAL-TK-CAT containing the TK basal promoter element fused to five synthetic GAL4 upstream activator sequences [17]. The transcriptional activity of the reporter vector was not affected by the expression of GAL4 1–147 in comparison to the control (vector alone, not shown). In contrast, the reporter activity was strongly repressed (85%) when cotransfected with the vector expressing the GAL4 1–147/rEVX1 fusion protein in comparison to the vector expressing only the GAL4 DNA binding domain. Remarkably, GAL4 1–147/rEVX1 retained the ability to repress the transcription when CAT expression was directed by a promoter lacking the GAL4 binding sites (TK-CAT, Fig. 4B). A comparable expression of GAL4 1–147 and GAL4 1–147/rEVX1 in transfected cells was demonstrated by RT-PCR experiments shown in Fig. 3B. These results suggest that rEVX1 represses gene transcription also when it is not targeted to the promoter by the specific binding site of a fused transcription factor. We cannot exclude, although it seems unlikely, that this effect arises through non-specific binding of the fusion protein to weak DNA sites located elsewhere in the reporter vector [22,23].

In conclusion, the 51 amino acid long rEVX1 domain: (i) is a strong repressor of gene transcription in transiently transfected mammalian cells; (ii) it is particularly rich in alanine and proline residues; (iii) its activity is completely independent of the presence of the HD and it functions in the context of several transcription factors. For several other transcriptional repressors [20] including Eve [24] the interaction with proteins belonging to the basal machinery of transcription has been demonstrated. Our findings allow us to hypothesize that EVX1 transcription repressor activity could be due to its interaction mediated by the rEVX1 domain with one or more basal transcription factors. The deletion of part of rEVX1 (Δ3EVX1 protein) reduces the repression ability of the resulting protein to 55% in comparison to that of wild-type EVX1, suggesting that rEVX1 could interact with more than one protein. Alternatively, the entire rEVX1 could be required to obtain a stable interaction with a single protein.

Experiments are in progress in order to identify the protein(s) interacting with EVX1 and this in turn will help the analysis of the repression mechanism(s) by which it regulates development.

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