Promoter-specific regulation of gene expression by an exogenously added homeodomain that promotes neurite growth

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Abstract pAntp, a 60 amino acid long peptide corresponding to the homeodomain of the *Drosophila Antennapedia* protein, translocates through neuronal membranes when added exogenously to neurons in culture, where it accumulates in the nucleus and promotes neurite outgrowth. We proposed that the peptide, once internalized, may compete for homeoprotein DNA binding sites. To investigate this point, we have produced a permanent fibroblast cell line which carries a luciferase reporter gene under the control of a 93 bp genomic region of the *HOXD9* promoter with binding sites for homeoproteins. Externally added pAntp specifically down-regulates the expression of the reporter gene, suggesting that the neurotrophic effects observed previously are mediated by direct binding of pAntp to homeoprotein target sites.

Key words: Homeoprotein; Retinoic acid; Fibroblast cell line; Luciferase assay; Transcription

1. Introduction

In previous reports [1–4], we demonstrated that pAntp translocates through neuronal membranes, accumulates in the nucleus and promotes neurite outgrowth in vitro. To analyse the specificity of pAntp neurotrophic effect we generated several mutant peptides. One of these mutants, pAntp50A, carries a Glutamine to Alanine mutation in position 9 of the third helix [4]. pAntp50A can still translocate through membranes, accumulates in the nucleus but does not bind with high affinity to the homeodomain target sequences recognized by pAntp [4].

Only pAntp enhanced neurite outgrowth; pAntp50A as well as other variants devoid of DNA-binding activity and/or not internalized by the cells were devoid of neurotrophic effect [4]. These results suggested that the mode of action of pAntp involves a direct competition for sequences present in the promoters of genes normally regulated by homeoproteins, consequently affecting their transcription.

In order to test this hypothesis we have now produced an LTK fibroblast cell line (LTK-HCRluc) permanently transformed with a luciferase reporter gene under the control of a weak thymidine kinase (TK) promoter adjacent to a 93bp genomic fragment (HCR, for Hox Crosstalk Region), characterized by Zappavigna et al. [5], containing multiple binding sites for HOXD proteins. In the presence of pAntp, the expression of the reporter gene in this cell line was reduced, suggesting that pAntp neurotrophic effects are mediated by direct binding of pAntp to genomic homeoprotein target sites.

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2. Materials and methods

2.1. Plasmids

The reporter plasmids pTHCR and pTHCR* (a kind gift from V. Zappavigna, Milano) have a luciferase gene driven by a weak Thymidine kinase promoter. In pTHCR, a 93 bp genomic fragment containing multiple binding sites for HOXD proteins has been inserted upstream the TK promoter. In pTHCR*, all the specific binding sites were mutated [5]. pSGH4C [5], a derivative of pSG5 expressing HOXD9 through the SV40 promoter, was a kind gift from D. Duboule (Genève).

2.2. Homeodomains

pAntp and pAntp50A ($Gln^{50} \rightarrow Ala^{50}$) were prepared in *E. coli* as described previously [4]. Their internalization was tested as described in Joliot et al. [1].

2.3. Gel retardation

Gel retardation experiments were as described in [4]. The *Hin*dIII–*Bam*HI fragment of pTHCR was gel-purified and end-labelled with the Klenow DNA polymerase in the presence of [^{32}P]dCTP. This probe was incubated with 100 ng of pAntp or pAntp50A in binding buffer [1] in the presence of poly (dI–dC). The reaction products were electrophoresed in 0.25 × TBE through a 6% polyacrylamide gel and autoradiographed.

2.4. Cell culture, transfections and reporter assays

The fibroblast LTK$^-$ cells were maintained in DMEM/F12 medium supplemented with 10% fetal calf serum, glutamine 2 mM, HEPES 10 mM pH: 7.0, D-glucose 33 mM, penicillin 5 U/ml, streptomycine 5 μg/ml and $NaHCO_3$ 7.5 mM. Transfections were performed using the calcium phosphate precipitation method. Permanent LTK-HCRluc and LTK-HCR*luc cell lines were produced by cotransfecting pTHCR (or alternatively pTHCR*) with pSV2neo, selecting for neomycine resistance, then screening for pTHCR or pTHCR* integration. For transient expression experiments, pKRSV-βgal was included in the transfection for calibration.

To assay for reporter activity, cells were rinsed then lysed for 1h at 4°C in 150 μl of buffer BLUC (Tris-H_3PO_4 25 mM pH: 7.8, $MgCl_2$ 10 mM, Triton X-100 1%, glycerol 15%, EDTA 1 mM, DTT 1 mM). Luciferase activity was subsequently measured in a LUMAC luminometer, using 100 μl of the lysate in the presence of 150 μl of substrate (ATP 1.25 mM, D-luciferin 85 μg/ml dissolved in BLUC buffer).

3. Results

The structure of the 2 reporter constructs integrated in the permanent cell lines is schematized in Fig. 1A. We verified (Fig. 1B) that pAntp binds the HOX protein target sites present in the HCR region, while pAntp50A has only a very weak affinity for this sequence. The two peptides do not bind to the mutated DNA sites (data not shown). By using FITC-tagged pAntp and pAntp50A we also confirmed that both peptides can translocate through LTK cell membranes and accumulate in the nucleus (shown for pAntp in Fig. 1C and D).

In cell lines harbouring the HCR region, basal luciferase activity was quite high (Fig. 2A) and could be slightly but reproducibly reduced by the addition to the culture medium of pAntp at a concentration of 9 μg/10^5 cells. The mutated peptide

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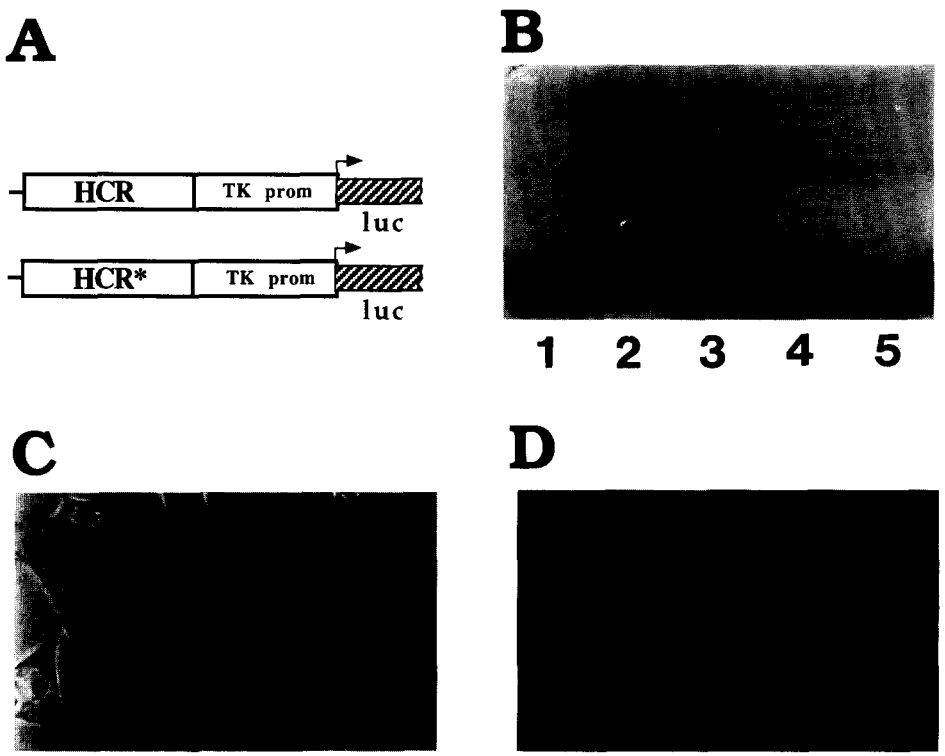


Fig. 1. (A) pTHCR and pTHCR* constructs [5]. HCR and HCR*: wild type and mutated homeoprotein cross-talk region; TK prom: basal thymidine kinase promoter; luc: luciferase reporter gene; arrowhead: transcription start site. (B) Specific DNA recognition by the wild type and mutant Antennapedia homeodomains. The HCR target sequence [5] was end-labeled with ^{32}P and incubated with 100 ng of purified pAntp (lanes 2 and 3) or pAntp50A (lanes 4 and 5), in the presence of 10 ng (lanes 2 and 4) or 1 μg (lanes 3 and 5) of poly (dI–dC). Lane 1, free probe. (C and D) Distribution of fluoresceinated homeodomain peptides in LTK$^-$ cells.After trypsinization, cells were rinsed and incubated for 2h at 37°C with fluoresceinated peptides [1]. After two hours, cells were fixed in ethanol/acetic acid, mounted in moviol and visualised by optical microscopy. C, phase contrast; D, epifluorescence.

pAntp50A at an identical concentration (Fig. 2A) or at higher concentrations (up to 18 μg/10^5 cells, not shown) had no effect on HCR basal activity. As reported by Zappavigna et al. [5] for NIH 3T3 cells, basal activity of the HCR* region was always very low (under 500, see Fig. 3). This suggests that LTK$^-$ fibroblasts express endogenous homeoproteins that bind to and activate the integrated HCR region.

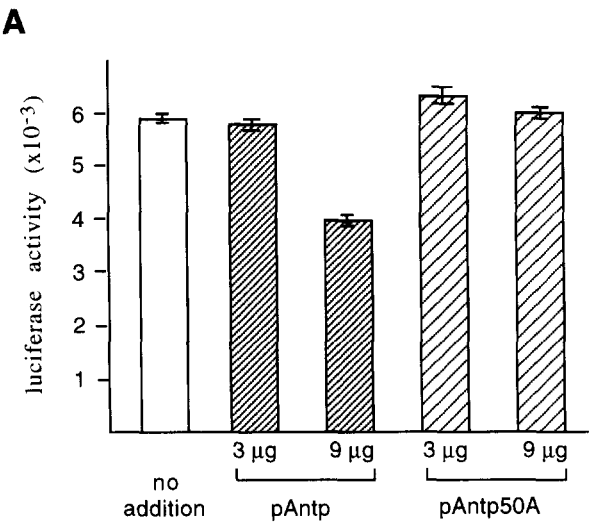
Retinoic acid (RA) is known to enhance the transcription of *HOX* genes [6]. We therefore decided to test the activity of pAntp in LTK-HCRluc cells treated or not with this agent. We used a protocol developed by Boncinelli and colleagues who showed that, in a human carcinoma cell line, *HOX* genes are sequentially activated by increasing concentrations of RA (10^{-8} to 10^{-5} M) [6,7]. We selected the highest concentration which did not produce toxic effects (10^{-6} M RA) and incubated the cells for 3 days. The presence of RA increased luciferase activity by 83% (Fig. 2B) with no effect of the diluant (DMSO). The addition of increasing concentrations of pAntp in the culture medium decreased luciferase activity, reaching levels slightly below basal activity, at a concentration of 6 μg of pAntp per 10^5 cells (Fig. 2B). We indeed verified that, after 3 days, pAntp was still intact in the nuclei and capable of binding specifically to a *Hox* protein target sequence [8].

Using cotransfection experiments, Zappavigna et al. [5] demonstrated that HOXD9 binds and activates the HCR region. We therefore tested whether transactivation of the integrated reporter construct by HOXD9 was modified by the addition of pAntp in the medium. We chose to add pAntp 42 h after transfection of pSGH4C, a plasmid expressing HOXD9, and to harvest 6 h later. As shown in Fig. 3, and as reported by Zappavigna et al. [5], a twofold increase of luciferase activity could be detected after transfection of 5 μg pSGH4C. Addition of pAntp (9 μg per 10^5 cells) led to a significant decrease in both basal (not shown) and HOXD9 driven activities (Fig. 3). The observed reduction in luciferase activity was specific to pAntp since the addition of identical concentrations of pAntp50A did not lead to any detectable change in reporter gene activity. Furthermore, as expected, we detected no luciferase activity in extracts from the LTK-HCR*luc cell lines (Fig. 3).

4. Discussion

The present results demonstrate that pAntp, when added exogenously to the cell culture medium, can repress an integrated promoter carrying homeoprotein binding sites, even in the presence of relatively high concentrations of its naturally occurring *trans*-activating factor. The latter repression is specific since the mutated pAntp50A peptide is unable to activate or repress the reporter gene from the same sites. Furthermore, the control LTK-HCR*luc cell line, whose reporter gene carries a promoter mutated for all of the target binding sites, never responds to pAntp treatment.

The addition of RA induces a significant increase in luciferase activity in our LTK-HCRluc cell line, which is probably the result of the activation of endogenous *Hox* genes. The expression of endogenous homeoproteins in mouse NIH 3T3 fibroblasts has already been reported by Odenwald et al. [9]. Our experiments confirm this finding on the LTK$^-$ cell line, where we detected, by PCR assay and gel shift, the expression of *Hoxc-8* and several other *Hox* genes that we have not fully identified (unpublished results).



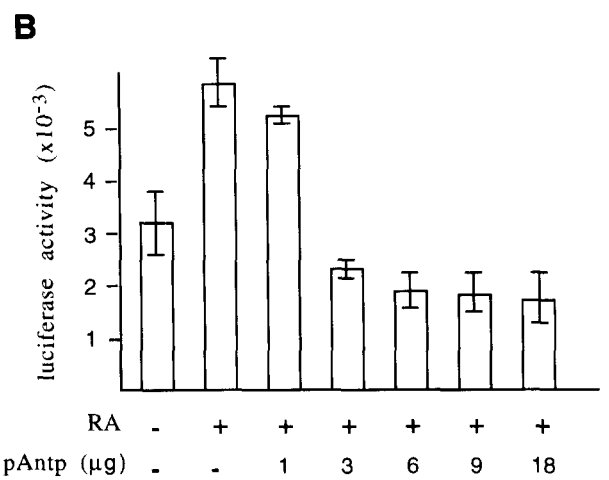


Fig. 2. Luciferase activity in LTK-HCRluc cell line after incubation with Antennapedia homeodomains. (A) pAntp treatment induces a specific decrease in the basal luciferase activity. After trypsinization at day 0, 10^5 cells were plated on dishes coated with polyornithine and laminin. At day 1, various amounts of purified pAntp (3 µg or 9 µg, as indicated) or pAntp50A (3 µg or 9 µg, as indicated) were added to the cells in a fresh medium (left: no addition). Luciferase activity was measured at day 3. The decrease observed upon addition of 9 µg of pAntp is significant ($n = 4$, $P < 0.01$ Student's t-test). (B) pAntp treatment abolishes the stimulation of reporter expression induced by retinoic acid (RA).Using the same protocol as above, various amounts of purified pAntp (0 µg, 1 µg, 3 µg, 6 µg, 9 µg and 18 µg, as indicated) were added in a fresh medium to cells treated or not (as indicated) with 10^{-6} M of RA. Luciferase activity was measured at day 3. The increase observed upon addition of RA is significant ($n = 4$, $P < 0.01$ Student's t-test). The decrease observed upon addition of 3 µg of pAntp is significant ($n = 4$, $P < 0.01$ Student's t-test).

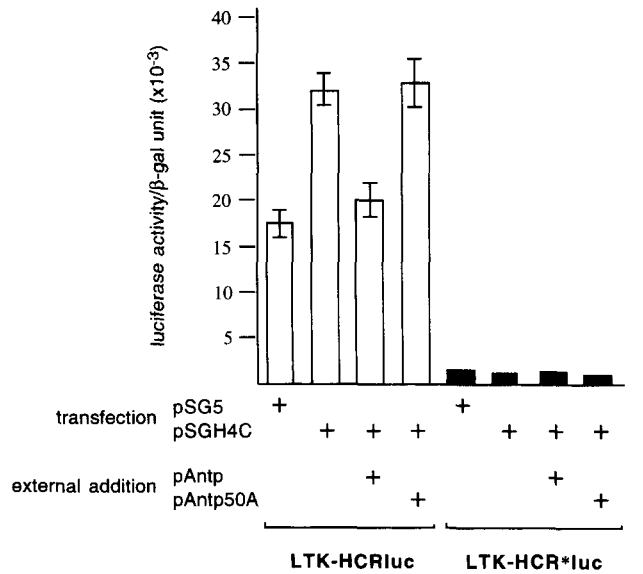


Fig. 3. Effect of external homeodomain addition on transactivation by HOXD9. LTK-HCRluc and LTK-HCR*luc cells, as indicated, were transfected with 5 µg of a plasmid expressing HOXD9 (pSGH4C) or the parental vector (pSG5), together with a β-galactosidase expressing plasmid for calibration. After 42 h, 9 µg of wild type (pAntp) or mutant (pAntp50A) homeodomains were added to the culture medium. Luciferase activity was measured 6 h later. The increase observed upon transfection by pSGH4C is significant ($n = 4$, $P < 0.01$ Student's t test). The decrease observed upon addition of 9 µg of pAntp is significant ($n = 4$, $P < 0.01$ Student's t-test).

The fact that pAntp, but not pAntp50A, acts by repressing the *trans*-activation driven by endogenous homeoproteins is confirmed by experiments in which HOXD9, a natural *trans*-activator of HCR-containing promoter, was overexpressed in the cells. Even in these conditions, pAntp down-regulates the activity of the integrated reporter gene, indicating that it can compete effectively with endogenously-expressed homeoproteins.

The present experiments establish that pAntp added exogenously to cells in culture is internalized and translocated to the nucleus were it specifically down-regulates the expression of target elements for endogenous homeoproteins. Since we have previously reported that pAntp, but not its DNA-binding variants enhances neurite elongation by cortical neurons and purified motoneurons [3,4], the present results lend weight to the suggestion that homeoproteins, in addition to their well-established functions in early embryogenesis, also regulate the morphological differentiation of post-mitotic neurons [1–4].

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