Antennapedia Homeobox Peptide Enhances Growth and Branching of Embryonic Chicken Motoneurons In Vitro

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Abstract. Spinal motoneuron development is regulated by a variety of intrinsic and extrinsic factors. Among these, a possible role for homeoproteins is suggested by their expression in the motoneuron at relatively late stages. To investigate their possible involvement in motoneuron growth, we adapted a novel technique recently developed in this laboratory, based on the ability of the 60 amino acid-long homeobox of *Antennapedia* (pAntp) to translocate through the neuronal membrane and to accumulate in the nucleus (Joliot, A. H., C. Pernelle, H. Deagostini-Bazin, and A. Prochiantz. 1991. *Proc. Natl. Acad. Sci. USA.* 88:1864–1868; Joliot, A. H., A. Triller, M. Volovitch, C. Pernelle, and A. Prochiantz. 1991. *New Biol.* 3:1121–1134). Motoneurons from E5 chicken spinal cord were incubated with pAntp, purified by panning on SC1 antibody and plated on polyornithine/laminin substrata without further addition of pAntp. After 24 h, neurite outgrowth was already extensive in controls. In cultures of motoneurons that had been preincubated with 10^{-7} M pAntp, neurite length was doubled; a similar effect was obtained using postnatal muscle extracts. Morphological analysis using a neurofilament marker specific for axons indicated that the homeobox peptide enhances primarily axonal elongation and branching. To test the hypothesis that the biological activity of pAntp involves its specific attachment to cognate homeobox binding sites present in the genome, we generated a mutant of pAntp called pAntp40P2, that was still able to translocate through the motoneuron membrane and to reach the nucleus, but had lost the specific DNA-binding properties of the wild-type peptide. Preincubation of pAntp40P2 with purified motoneurons failed to increase neurite outgrowth. This finding raises the possibility that motoneuron growth is controlled by homeobox proteins.

THE spinal motoneuron differentiates early within the embryonic neural tube (Hamburger, 1977). By day 5 in ovo in the chicken, the post-mitotic motoneuron has an axon that has already grown through the anterior half of a somite and reached the developing muscles, where the first synaptic connections are being formed. At approximately the same time, its dendrites ramify within the spinal cord. It is likely that the development and survival of the motoneuron over this crucial period are regulated by the interplay of several different classes of molecules: soluble neurotrophic factors (mostly unknown, but possibly including fibroblast growth factors) (Arakawa et al., 1990), cell surface molecules (for instance, those involved in the growth-inhibitory properties of the posterior somite) (Keynes et al., 1984) and perhaps endogenous regulatory proteins. Potential members of the last class of homeobox proteins: the motoneuron expresses Hox 3.1 (Le Mouellic et al., 1988; 1992) and Islet-1 (Ericson et al., 1992) until relatively late stages.

A role for homeoproteins in neurite growth and terminal neuronal differentiation is suggested by the pattern of homeogene expression in other regions of the nervous system. This expression is not restricted to the early periods of development but is also observed around birth and in some cases throughout adulthood (Awgulewitsch and Jacobs, 1990; Porteus et al., 1991; Thor et al., 1991; Price et al., 1992). The physiological significance of this late expression of homeogenes is not established. However, the finding that unc-4, a nematode homeogene mutation, alters the synaptic input to ventral cord motor neurons suggests that homeogenes play a role in the establishment of neuronal networks (Miller et al., 1992; White et al., 1992).

We recently developed a new technology that allows for the perturbation of homeoprotein action within individual cells in culture. We showed that pAntp, the 60 amino-acid long region corresponding to the homeodomain of *Antennapedia*, a *Drosophila* homeotic gene, is able to cross the membrane of embryonic rat neurons in culture and to reach their nuclei. This phenomenon, which is energy-independent, is accompanied by enhanced differentiation of the nerve cells (Joliot et al., 1991*a*,*b*). *Antennapedia* homeodomain (pAntp) translocation was observed in all cell types tested, but was particularly high in neurons and other cells expressing α,2-8 polysialic acid, a complex sugar carried by the neural cell adhesion molecule and expressed strongly at embryonic and early postnatal ages. On the basis of structural comparisons, we speculated that α,2-8 polysialic acid

(PSA)[1] might mimic a molecular motif of the dsDNA large groove recognized by the third helix of homeodomains and thereby concentrate the DNA-binding peptide at the neuronal surface (Joliot et al., 1991*b*).

We used this experimental system to look for a potential role for homeoproteins in the differentiation of chick motoneurons in culture. We demonstrate here that motoneurons incubated with low amounts of pAntp take up the peptide and transport it to their nuclei. This phenomenon is followed by enhanced growth of motoneurons comparable to that observed with muscle extracts. In contrast, a mutant of pAntp capable of penetrating into the cells and of accumulating in their nuclei, but unable to bind specifically to homeoprotein cognate binding sites does not promote motoneuron differentiation.

Materials and Methods

Culture Conditions

Spinal cords were dissected from 5-d old White Leghorn chick embryos (Hamburger-Hamilton stage 24–25, incubation at 37.6°C), treated with trypsin and dissociated as described elsewhere (Henderson et al., 1984). Ventral spinal cord cells or purified motoneurons were plated at the density of 4,000 cells/cm^2 on 16-mm glass coverslips (immunocytochemistry) or 16-mm plastic culture dishes (morphological analysis) precoated with polyornithine (1.5 μg/ml; M_r 40,000, Sigma Chemical Co., St. Louis, MO) and laminin (3 μg/ml) (Bloch-Gallego et al., 1991).

Culture medium consisted of L15 medium (Gibco Laboratories, Grand Island, NY) supplemented with penicillin (5 IU/ml), streptomycin (5 μg/ml), L-glutamine (2 mM), glucose (33 mM), insulin (25 μg/ml), transferrin (100 μg/ml), putrescine (6.10^{-5} M), progesterone (2.10^{-8} M), sodium selenite (3.10^{-8} M), and sodium bicarbonate (90 mM). Cultures were maintained for the time indicated in the text without medium change at 37°C in 5% CO_2/95% air and saturating humidity. When indicated, survival and differentiation factors were provided by the addition of extracts of neonatal chick muscle prepared as previously described (Henderson et al., 1983), centrifuged, and stored at −20°C.

Purification of Motoneurons

Polystyrene Petri dishes (90 mm) were coated overnight at 4°C with 8 μg of secondary antibody (affinity-purified goat anti-mouse IgG, Cappel Laboratories, Malvern, PA) in 10 ml Tris buffer, pH 9.5. After three washes with PBS pH 7.4, SC1 hybridoma supernatant diluted fivefold in PBS was added to the dishes for 1 h at room temperature. Dishes were then washed twice with PBS and twice with L15 medium.

Spinal neurons dissociated and resuspended in culture medium were added to the dishes. After 35 min at room temperature, the plates were washed eight times with PBS and the remaining adherent cells were eluted with SC1 hybridoma undiluted supernatant. Detached purified motoneurons were washed once by centrifugation and seeded.

Immunocytochemistry

Immunostaining of tubulin or axon-specific neurofilament isoforms (P-NFH) was performed on cells fixed with paraformaldehyde (4% in PBS) for 15 min at room temperature, and further permeabilized in methanol for 5 min at −20°C. All other steps were as indicated in Lafont et al. (1992). The monoclonal antibody against P-NFH, a kind gift of Pat Levitt (Medical College of Pennsylvania, Philadelphia, PA) (Pennypacker et al., 1991), and the polyclonal anti-tubulin antibody (Amersham Corp., Arlington Heights, IL) were diluted 50- and 250-fold, respectively. Secondary antibodies and streptavidin-fluorescein were from Amersham Corp. All dilutions and washes were done in PBS supplemented with 5% FCS. Immunostaining with the motoneuron-specific SC1 hybridoma supernatant (a kind gift of Dr. H. Tanaka, Gunma University, Maebashi, Japan) was done as previously described (Bloch-Gallego et al., 1991).

1. *Abbreviation used in this paper*: PSA, α,2-8 polysialic acid.

Synthesis of pAntp and of the pAntp40P2 Mutant

pAntp was synthesized as described by Joliot et al. (1991*a*). pAntp40P2 was obtained by site-directed mutagenesis of the sequence coding for pAntp within plasmid pAH1 (Joliot et al., 1991*a*). To this end, we constructed a plasmid derived from pAH1 and including single SphI and BglII sites in positions 105 and 137 of the original sequence. Between these two sites, we inserted two oligonucleotides allowing for the replacement of leucine 40 and threonine 41, located in the turn between helices 2 and 3, by two proline residues. The new construction was transfected into BL21(DE3)pLysS bacteria. IPTG induction, peptide purification and gel shift experiments were performed as in Joliot et al. (1991*a*).

Metabolic labeling was performed by transferring the cells (A600:0.3) to M9 medium supplemented with 0.2% glycerol and 10% Methionine-free MEM (Gibco-BRL, Gaithersburg, MD). After 1 h in culture the cells were induced with IPTG (1 mM), incubated for 15 min with rifampicin (200 μg/ml) and 5 min in the presence of ^{35}S-Methionine (Amersham Corp., 15 μCi/ml). After a 10 min chase with 5 μM unlabeled methionine, proteins were extracted and purified as in Joliot et al. (1991*a*).

Direct Labeling of Polypeptides with Fluorescein Isothiocyanate

After dialysis (5 kD exclusion limit) against two 100 ml changes of 150 mM NaCl, 100 ml of 50 mM bicarbonate-buffered saline (pH 8.5, 4 h) and 100 ml of 50 mM bicarbonate-buffered saline (pH 9.2, 2 h), peptides (1 ml, variable concentrations) were dialyzed overnight in the dark against fluorescein isothiocyanate (Sigma Chemical Co., 100 μg/ml) diluted in bicarbonate-buffered saline (pH 9.2). The reaction was stopped by changing the dialysis buffer to PBS (pH 7.2) overnight. All steps were performed at 4°C. The localization of fluorescent peptides was determined by classical and confocal microscopy as described in Joliot et al. (1991*b*).

Peptide Internalization

Dissociated ventral spinal cord cells (10^5 to 10^6) were resuspended in 500 μl PBS supplemented with glucose (33 mM), BSA (1 mg/ml) and DNase I (30 μg/ml) and incubated at 37°C with or without the indicated concentrations of peptide. Every 15 min, the cells were gently agitated in order to avoid reaggregation. After 2 h, the cells were washed with a 20-fold excess of PBS-glucose, centrifuged, resuspended in culture medium and plated either on polyornithine/laminin coated dishes (ventral spinal cord cultures) or on SC1-coated panning dishes for further purification of motoneurons.

When radioactive peptides were used, cells incubated in suspension with ^{35}S-labeled peptides (50,000 cpm) were carefully washed, lysed for 15 min on ice in PBS containing 0.5% NP-40, 1 mM Ca^{++} and Mg^{++}, and the following protease inhibitors: Pefablock (0.5 mM), α2-macroglobulin (1 μg/ml), leupeptin (10 μg/ml) and pepstatin (1 μg/ml). The nuclear and postnuclear fractions were separated by centrifugation at 2,000 *g* over a 0.32 M sucrose layer for 10 min at 4°C. After resuspension of the nuclear pellets, the radioactivity contained in aliquots of the different fractions was determined and the integrity of the recovered peptides was analyzed by gel electrophoresis.

Cell Survival and Morphological Quantification

The degree of cell survival was estimated by Trypan blue exclusion. The number of live cells present 3 h after plating was taken as 100%. Cells from six fields were counted directly under the microscope. To quantify neurite length, cells were fixed with glutaraldehyde (2.5% in PBS) for 20 min at room temperature, washed twice with PBS and stained with toluidine blue (0.2% in 2% Na_2CO_3). Stained cells were air dried and observed with an optical microscope (E. Leitz, Inc., Rockleigh, NJ). For each experiment, 50–100 neurons were digitalized and analyzed with a morphological analysis software (IMSTAR, Inc., Paris, France).

Results

Effects of pAntp on Motoneuron Neurite Length

In all the experiments described, to avoid the possibility that the pAntp homeobox peptide might coat the culture dish and induce neurite outgrowth solely as a result of its highly basic nature, the peptide was incubated with cells in suspension,

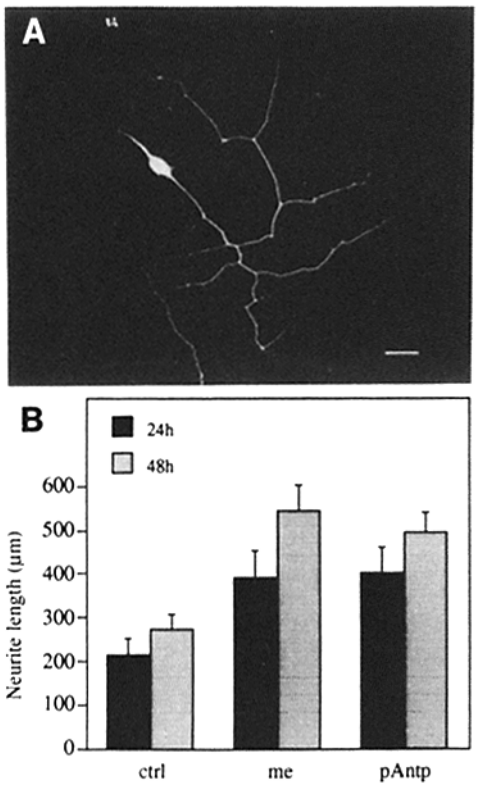

Figure 1. pAntp-induced motoneuron differentiation. (*A*) Cells preincubated with 10^{-7} M pAntp were fixed after one day in culture in serum-free medium and immunostained with an antitubulin antibody. Bar, 10 µm. (*B*) Quantification of total neurite length after 24 or 48 h in culture on polyornithine/laminin. One hundred neurons were analyzed in each condition and three independent experiments gave similar results. Preincubation with pAntp (10^{-7} M) or muscle extract (*me* 54 µg/ml) increased neurite length significantly ($p < 0.02$, Student's *t* test).

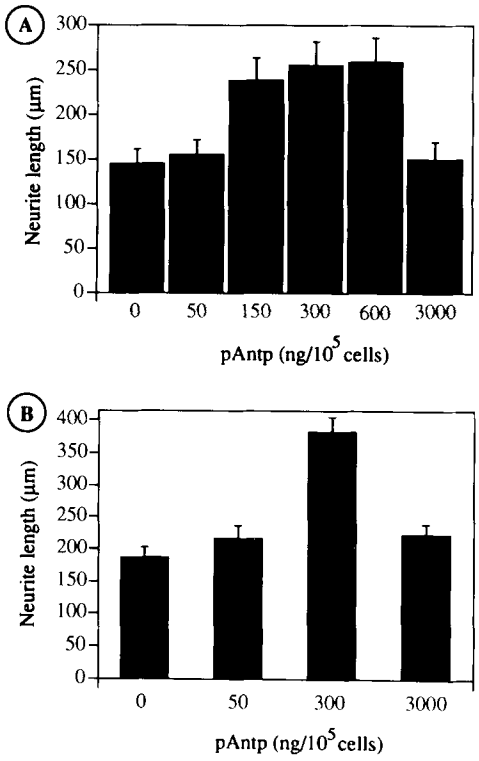

Figure 2. Dose-response curves for the neurite outgrowth-promoting activity of pAntp on ventral spinal neurons (*A*) or purified motoneurons (*B*). Freshly dissociated neurons were incubated in suspension with the indicated amounts of peptide, and washed thoroughly before direct plating or further motoneuron purification. All cultures were on polyornithine-laminin substrata for 24 h. Total neurite length of 70 neurons was analyzed for each peptide concentration. Similar results were obtained in two independent experiments. Means calculated for 150, 300, and 600 ng of peptide were significantly different from control values. (Student's *t* test, $p < 0.001$).

followed by washing. Suspensions of dissociated ventral spinal cord cells from 5-d old chicken embryos were incubated for 2 h with or without pAntp (10^{-7} M), or in the presence of 54 µg/ml neonatal muscle extract, found to be optimal in a preliminary experiment. The incubation was performed in PBS containing DNase and BSA. DNase was used to prevent pAntp binding to dsDNA that leaked from damaged cells; the function of BSA was to inhibit cell and peptide adsorption to the tube walls.

At the end of the incubation, motoneurons were purified by panning on Petri dishes coated with the SC1 antibody (see Methods). They were cultured for different periods on coverslips coated with concentrations of polyornithine and laminin that had been found to give the greatest levels of neurite outgrowth in control motoneuron cultures (Bloch-Gallego et al., 1991). After 24 h, neurites were visualized by immunostaining with an antitubulin antibody that stained all cell compartments (Fig. 1 *A*). Neurite length was measured by morphometric analysis (see Methods).

Motoneurons were fixed and stained after 24 or 48 h in culture; the histograms of Fig. 1 *B* correspond to the analysis of 100 neurons in each category. Compared to controls that had been preincubated without peptide, which showed already considerable neurite outgrowth, pAntp caused a doubling in total neurite length at both culture times. It is striking that this concentration of pAntp was as efficient as muscle extract in enhancing neurite outgrowth.

To determine the potency of the observed effect of pAntp on neurite outgrowth, neurons were preincubated with increasing concentrations of homeobox peptide. Because it has already been reported that neurons other than motoneurons can respond to pAntp when it is present in the culture medium (Joliot et al., 1991), we wished first to confirm that all ventral spinal neurons showed an increase in neurite outgrowth when the peptide was only present during a short preincubation.

Freshly-dissociated cells from the ventral half of E5 chicken spinal cord were, therefore, incubated for 2 h with pAntp at concentrations ranging from 0 to 10^{-6} M, washed twice by centrifugation and plated without further purification. One day later, neurite length was quantified by morphological analysis (Fig. 2 *A*). No effect of pAntp was detected below 50 ng in 0.5 ml, and maximal enhancement of neurite outgrowth was already apparent after incubation for 2 h in 0.5 ml with 150 ng of peptide, corresponding to a concentration of 4.5×10^{-8} M pAntp. No further increase in neurite length was observed between 150 and 600 ng/10^5 cells. Owing to cytotoxic effects, pAntp-induced differentiation was lost at high doses (in the µg range).

To exclude a nonspecific effect of the highly basic homeo-

Table I. Percentage of Surviving Motoneurons after 24, 48, 72, and 96 h

Conditions	Time in culture (h) 24	48	72	96
ctrl	83 ± 14	68 ± 16	34 ± 15	19 ± 4
me	95 ± 6	85 ± 8	52 ± 13	27 ± 7
pAntp	86 ± 13	86 ± 10	45 ± 2	24 ± 2
pAntp40P2	85 ± 19	74 ± 16	42 ± 9	21 ± 3

The survival promoting effects of preincubation with pAntp, muscle extract or mutant peptide were tested on purified motoneurons. Cells from five independent fields were counted directly under the microscope. Values are the results of three independent experiments. The survival response is the same in each condition during the first 24 h in culture. In these preincubation conditions, even muscle extract has only a low effect on motoneuron survival. (*ctrl*) Preincubation with medium alone. (*me*) Preincubation with 54 μg/ml muscle extract. The two homeopeptides were used at 10^{-7} M.

box peptide, a similar experiment was performed using increasing concentrations (0–500 ng/500 μl) of histone H1 or poly-DL-ornithine (MW 33000) in the place of pAntp. The basic polypeptides had no effect on neurite growth at any concentration. In a typical experiment with 300 ng/500 μl of histone H1 or polyornithine, total neurite length was 120 ± 32 μm (n = 24) in controls without peptide, 106 ± 29 μm (n = 24) in cultures preincubated with histones, and 130 ± 34 μm (n = 24) in those preincubated with polyornithine.

A dose-response curve was performed using motoneurons that were purified by panning from suspensions of ventral spinal neurons that had been preincubated with pAntp at concentrations in the same range (Fig. 2 *B*). The morphological effect on motoneurons occurred at concentrations very similar to those required to enhance neurite outgrowth from the total population of ventral spinal neurons. Thus, in all subsequent experiments we used the concentration of 300 ng for 10^5 cells in 0.5 ml (10^{-7} M).

Effects of pAntp on Motoneuron Survival

Motoneurons plated at low density do not survive well in the absence of external trophic supply. To ensure that morphological analyses were performed on comparable and representative populations, motoneurons incubated for 2 h with or without pAntp (300 ng/10^5 cells) or muscle extracts (27 μg/500 μl) were purified by panning, plated and cultured for various periods of time at the density of 8,000 cells/cm².

Initial counts of attached cells were performed at 3 h after seeding. Subsequently, neuronal survival was virtually quantitative in all conditions until 48 h (Table I). Cell death became apparent after 72 and at 96 h only, 20 to 27% of the motoneurons were still able to exclude Trypan blue. Prein-

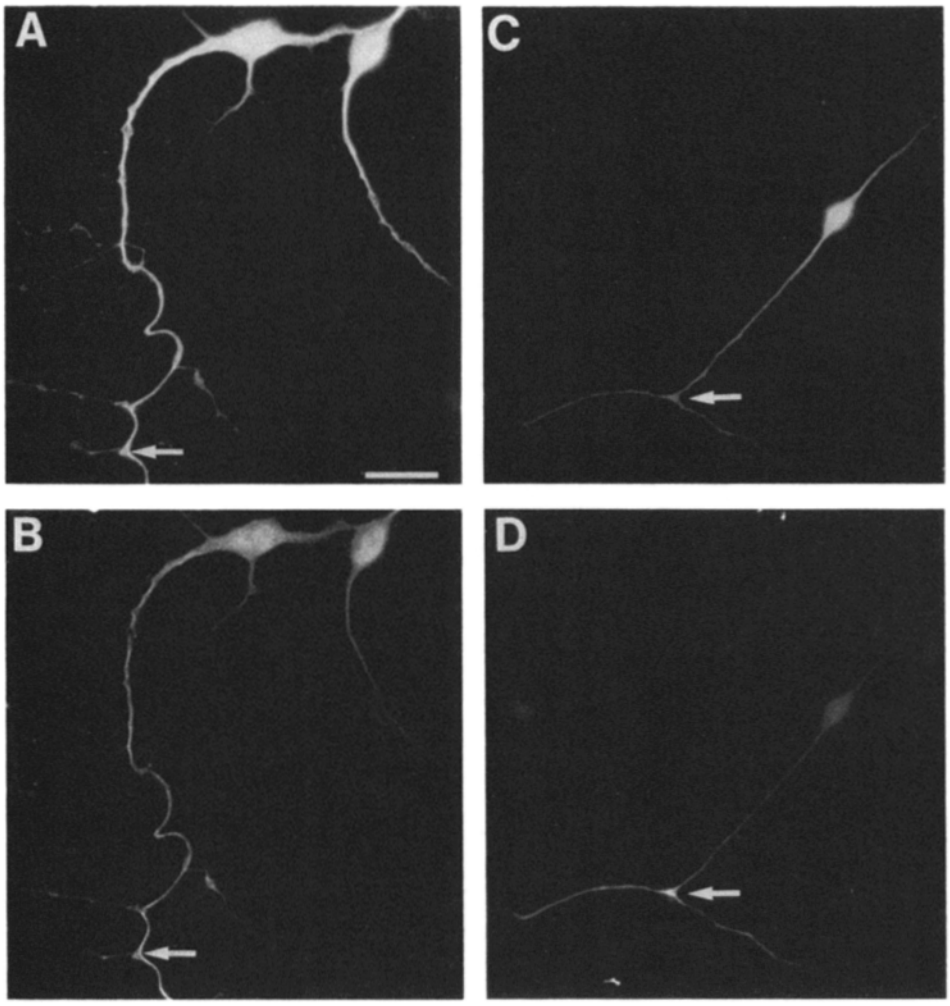

Figure 3. Identification of motoneuron axons by double immunostaining. Antitubulin stains all neurites (*A*, *C*) whereas the axon-specific anti-NFH antibody stains the longest neurite (*B*, *D*). Note that, in the longest neurite (the axon), anti-NFH staining remains constant or even increases with the distance from the cell body. Bar, 10 μm.

Table II. Average Number of Primary and Secondary Neurites for Neurons Grown for 24 h With or Without pAntp

	Primary neurites	Secondary neurites
Control	1.9 ± 0.1	1.3 ± 0.4
pAntp	2.5 ± 0.4	3.0 ± 0.3

From a morphometric analysis, the number of primary and seconddary neurites was determined for each neuron. 60 neurons were analyzed in each condition. Values are the results of three independent experiments. Differences in the number of primary neurons are not significant. Differences between the number of secondary neurites are significant ($p < 0.01$, distribution analysis, Statworks).

cubation, before plating, with pAntp or muscle extract was without effect on survival. Similar results were obtained with the mutant peptide pAntp40P2 (see below). Thus, our morphological analyses of motoneurons performed after 24 or 48 h in vitro took into account ~90% of the motoneurons initially obtained by panning.

Neurite-promoting Effect of pAntp Is Primarily on Axons

Visual inspections of motoneuron morphology after incubation with peptide suggested that pAntp might increase axonal elongation (Fig. 1 *A*). We therefore analyzed the effect of pAntp on neurite initiation and branching and quantified separately the length of all neurites and that of the longest neurite. Identification of the longest neurite as the axon was provided by double immunolabeling experiments using antitubulin and an antibody directed against neurofilament isoforms specifically enriched in the axon (Pennypacker et al., 1991). The longest neurite was always stained with the axon-specific antibody (Fig. 3).

Fig. 4 shows the cumulative distributions of neurons according to total neurite length (Fig. 4 *A*), to the length of the longest neurite (Fig. 4 *B*), to the length of the other neurites (Fig. 4 *C*) and to the surface area of the cell soma (Fig. 4 *D*). This cumulative mode of representation indicates the percentage of neurons of total neurite length, axonal length or soma surface area superior to a given value. For example, in *B*, the length of 300 μm is reached by 65% (pAntp) or 30% (control) of the neurons. Such distribution curves can be statistically analyzed (Statworks, Cricket Software, Inc., Philadelphia, PA). They demonstrate that the differences observed in *A* and *B* (total neurite length and axonal length) between pAntp and controls are highly significant ($p < 0.001$, $n = 150$), whereas pAntp has no significant effect on dendrite length or soma surface area.

These results fit well with the observation that pAntp had no significant effect on the number of primary neurites but more than doubled the number of branching points (Table II). The homeobox peptide thus has clear effects on both axonal elongation and branching.

A Mutant of pAntp Lacking Growth-promoting Activity

pAntp is a DNA-binding peptide that recognizes cognate binding sites for homeoproteins (Affolter et al., 1990; Joliot et al., 1991*a*). This is illustrated by the binding of pAntp to labeled Hox-1.3 promoter in the gel retardation assay of Fig. 5. In addition to being able to bind specific DNA sequences, this peptide has the unexpected property to translocate through the plasma membrane and then of being able to reach the cell nucleus (Joliot et al., 1991*a*,*b*; Perez et al., 1992; Fig. 5 *A*).

To study whether the neurotrophic effects of pAntp were associated with its specific DNA-binding properties, we designed a mutant, pAntp40P2, that was still able to translocate through the membrane but with no DNA-binding capacities. To this end, we replaced the serine and threonine residues in positions 40 and 41 by two proline residues. This modification left all helices intact but rigidified and modified the turn between helices 2 and 3 (computational simulation, not shown).

This mutation did not affect the capacity of the peptide to translocate through the neuronal membrane and to accumu-

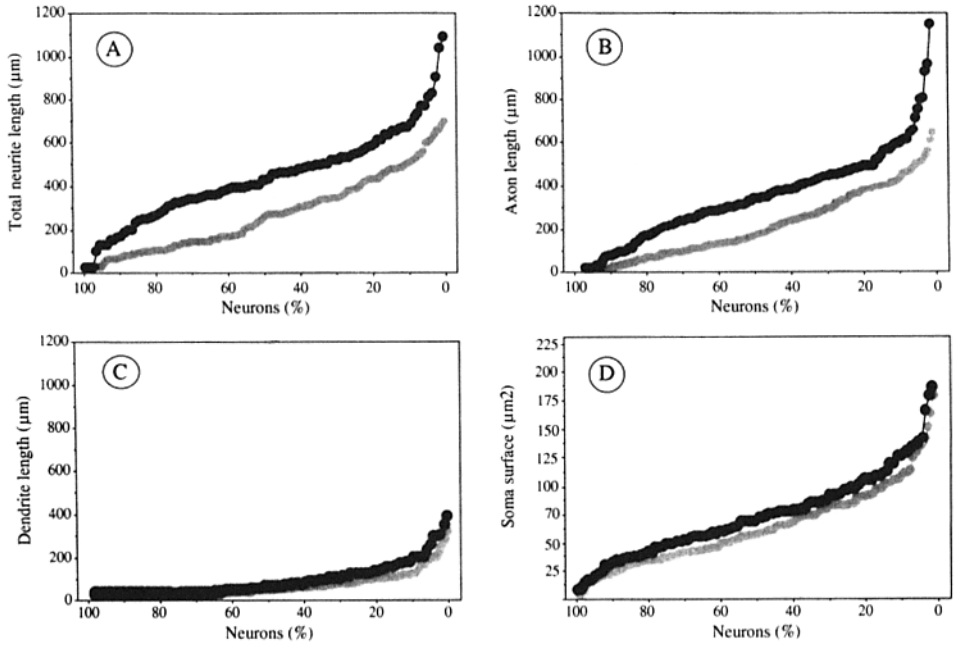

Figure 4. Selective effect of pAntp on motoneuron axonal elongation. The cumulative distributions of motoneurons according to total neurite length. (*A*), length of the longest neurite (*B*), length of all neurites but the longest (*C*) or soma surface (*D*) are plotted. This figure pools the results of three independent experiments and corresponds to the analysis of 150 neurons per condition. (*Filled circles*) motoneurons preincubated with 10^{-7} M pAntp. (*Hatched circles*) no added peptide. Differences observed in *A* and *B* between pAntp and controls are highly significant ($p < 0.001$).

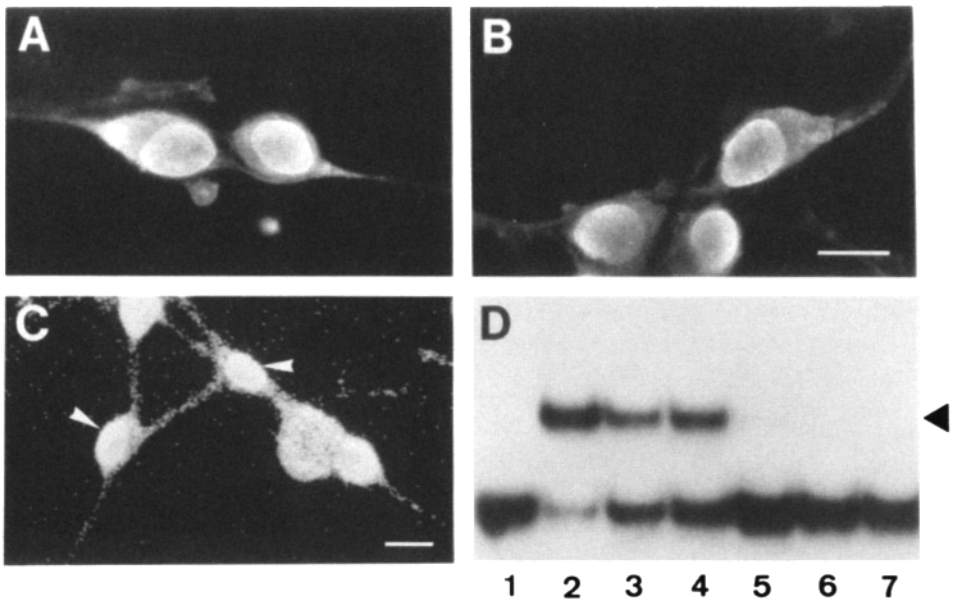

Figure 5. pAntp40P2 is internalized by motoneurons but does not have the specific DNA-binding properties of pAntp. Fluoresceinated pAntp (*A*, *C*) or fluoresceinated pAntp40P2 (*B*) were incubated at 10^{-7} M with suspensions of ventral spinal neurons. Motoneurons were subsequently purified by panning and cultured for 24 h before fixation. Internalized fluorescent peptides clearly present in the cytoplasm and the nucleus of motoneurons were visualized using optical microscopy (*A*, *B*). Confocal sections (shown here for pAntp) further demonstrated the intracellular localization of the peptides (*C*). The two arrowheads indicate the edge of nuclei through which sections were made. Scale bar, 10 μm. (*D*) Electrophoretic mobility-shift assay to monitor the specific interaction of homeopeptides with the Hox-1.3 promoter. Each peptide (100 ng) was incubated with different concentrations of poly (dI-dC) to block nonspecific binding to DNA before incubation with ^{32}P-labeled Hox-1.3 promoter (pHox-1.3). Gel electrophoresis followed by autoradiography was used to analyze radioactive species. Only pAntp was able to retard (*arrowhead*) the homeobox cognate binding site present in pHox-1.3. (Lane *1*) pHox-1.3 alone. (Lanes *2–5*) incubation of 100 ng pAntp with labeled pHox-1.3 was preceded by incubation with 15 ng (lane *2*) or 500 ng (lane *3*) of poly (dI-dC) for 15 min at 4°C, or with 15 ng (lane *4*) or 500 ng (lane *5*) of pHox-1.3 in the same conditions. (Lanes *6–7*) incubation of 100 ng pAntp40P2 with labeled pHox-1.3 was preceded by incubation with 15 ng (lane *6*) or 500 ng (lane *7*) of poly (dI-dC).

late in the nuclei. This latter point is demonstrated by the clear accumulation of fluorescent peptides in the nuclei (Fig. 5, *A–C*) and by the quantification of radioactive peptides internalized within 2 h of incubation: the percentages of internalized radioactive peptide recovered intact from the cells were 11 ± 6% (n = 3) and 12 ± 4% (n = 3) for pAntp and pAntp40P2, respectively. Similar percentages of internalization were obtained in six experiments with neurons from different origins and species (Le Roux, I., E. Bloch-Gallego, A. H. Joliot, A. Prochiantz, and M. Volovitch, submitted for publication). However, and as opposed to pAntp (Fig. 5 *D*, lanes *2–5*), pAntp40P2 failed to retard the homeobox binding site present in the Hox-1.3 promoter (Fig. 5 *D*, lanes *6*, *7*).

The biological effects of pAntp and pAntp40P2 on purified motoneurons were compared. As already seen in Table I, pAntp40P2 did not modify neuronal survival. Strikingly, the mutant peptide was completely devoid of neurite promoting activity at all concentrations tested (up to 6 × 10^{-7} M) (Fig. 6), indicating a direct relationship between the ability to bind homeodomain target sequences and growth-regulatory properties.

Discussion

We demonstrate here that a 2-h incubation of E5 chicken spinal cord neurons with low concentrations (10^{-7} M) of the *Antennapedia* homeodomain is sufficient to allow peptide internalization and accumulation within neuronal nuclei. This internalization process is followed by clear biological changes such as enhanced motoneuron differentiation. In contrast, a mutant peptide still able to be internalized and to accumulate in nuclei, but devoid of the capacity to bind a specific homeodomain site present in the Hox-1.3 promoter, has no neurite-promoting activity. These results confirm and extend our previous conclusion that pAntp can induce neuronal differentiation in the absence of other cell types, and that its biological activity requires association with promoters normally recognized by homeoproteins.

Motoneuron purification was achieved by the panning technique (Bloch-Gallego et al., 1991); the purity of the preparations was very high because in all experiments over 95% of the cells could be stained with an antibody directed against SC1, an antigen which at these stages and within the ventral spinal cord, is highly specific for motoneurons

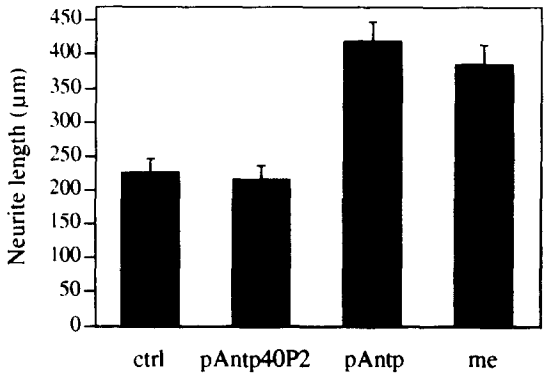

Figure 6. The pAntp40P2 mutant, which lacks specific DNA binding properties, does not enhance motoneuron neurite growth. Compared to control (*Ctrl*), only pAntp and muscle extracts (*me*) enhanced neurite elongation ($p < 0.002$ Student's t test); pAntp40P2 was without effect. Each bar represents the analysis of 100 neurons after 24 h in culture. Identical results were obtained in three independent experiments.

(Tanaka et al., 1991). The fact that 100% of the motoneurons internalized the peptides and that no significant cell death was seen within 24 h precludes the possibility that any of the morphological effects reported here may be attributed to the specific survival of a given neuronal subpopulation.

In the present study, the homeobox peptides were incubated for 2 h with freshly-dissociated spinal cord cells. To eliminate the possibility that homeopeptide internalization could be explained by membrane damage occurring during dissociation of neurons, we added the same concentrations to healthy motoneurons in culture (not shown). In these conditions, as observed using cultures of brain neurons (Joliot et al., 1991*a*,*b*), 100% of motoneurons internalized the peptide, resulting in pronounced increases in neurite outgrowth. However, a potential difficulty in interpretation of results obtained in this way is that the highly basic pAntp peptides might coat the substratum and thus non-specifically enhance motoneuron differentiation.

We used two strategies to avoid potential artefacts of this kind. First, pAntp was only added during the first incubation in suspension. The extensive washing during the subsequent panning procedure should have eliminated molecules not internalized by neurons or strongly attached to their surfaces. We cannot completely exclude that in spite of these washes very small amounts of pAntp managed to coat the substratum. However, the lack of biological effects of pAntp40P2 and of peptides of identical isoelectric point (polyornithine, histone) made it highly unlikely that pAntp was acting in this way. Nevertheless, as a second precaution, we report here only results obtained using substrata that were coated with another highly basic peptide (polyornithine) and concentrations of laminin that had been found optimal in promoting neurite outgrowth from embryonic motoneurons (Bloch-Gallego et al., 1991). This inevitably raised the levels of neurite outgrowth in control cultures (e.g., Figs. 1, 2) but meant that we could be confident that the effects we observed did not simply result from a general enhancement of the cell culture conditions.

Two major classes of mechanism could account for the growth-promoting activity of pAntp. The first is that, although it is subsequently translocated, pAntp mimics the action of a specific growth factor and by acting at the extracellular level initiates a chain of transduction events leading to the neurotrophic effects that we observe. Alternatively, pAntp might act at the level of the genome through specific interactions with homeodomain cognate binding sequences.

The first of these two possibilities cannot be eliminated on the basis of the experiments presented in this study. However, we know from previous studies that removing PSA from the cell surface decreases the rate of peptide internalization and abolishes its biological effects (Joliot et al., 1991*b*). Should a receptor be involved in triggering pAntp activity, PSA is thus, the best candidate. Because computational analysis (Joliot et al., 1991*b*) strongly suggests that pAntp interacts with PSA through its third helix which is entirely conserved in the nonactive peptide pAntp40P2, it is unlikely that pAntp effects be mediated through its interaction with PSA. Rather, all results obtained in the present study and in a separate study (Le Roux, I., E. Bloch-Gallego, A. H. Joliot, A. Prochiantz, and M. Volovitch, submitted for publication) are consistent with the hypothesis that the mode of action of pAntp involves its internalization and its specific binding to cognate sequences present in the promoters of genes normally regulated by homeoproteins. In preliminary experiments we have shown that an excess of pAntp completely inhibits binding of homeoproteins present in spinal cord neurons to the Hox-1.3 promoter. That this inhibition is specific is demonstrated by the observation that only some of the homeoproteins present in cortical neurons are displaced in the same conditions (not shown).

In view of the complexity of the regulatory interactions between homeogenes, it is impossible to speculate on how, exactly, pAntp increases axonal growth. One cannot tell whether pAntp acts by antagonizing the activity of endogenous homeoproteins as suggested earlier (Prochiantz et al., 1992) or by virtue of its own transcriptional activity. The only sensible statement is that it interferes with a role for homeoproteins in the establishment of neuronal circuits. This hypothesis is supported by the existence, in the invertebrate, of homeogene mutations leading to axonal misrouting or aberrant synaptic connections (Doe et al., 1988*a*,*b*; Miller et al., 1992; White et al., 1992). In vivo, motoneurons start to send out their axons soon after having been specified (Lumsden, 1991; Wagner et al., 1990). The presence in the notochord of a high level of retinoic acid, a compound known to have a regulatory effect on homeogene transcription and spinal cord neuron differentiation (Wagner et al., 1991; Wuarin and Sidell, 1991; Hunter et al., 1991; Yamada et al., 1991), suggests a possible physiological involvement of homeoproteins in motoneuron growth, in agreement with the conclusions from our in vitro results.

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