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Antennapedia homeobox peptide regulates neural morphogenesis

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ABSTRACT **We synthesized the 60-amino acid polypeptide corresponding to the sequence of the *Drosophila* antennapedia gene homeobox. This peptide (pAntp) recognized the consensus motif for binding to the promoter region of *Hox-1.3*. pAntp mechanically introduced into mammalian nerve cells provoked a dramatic morphological differentiation of the neuronal cultures. Moreover, pAntp directly added to already differentiated neuronal cultures penetrated the cells and further augmented their morphological differentiation. Examination of live and fixed neurons in classical and confocal fluorescence microscopy demonstrated that pAntp was captured at all regions of the nerve cells and accumulated in the nuclei. In addition, the effect of pAntp on neurite extension was blocked in the presence of the protein synthesis inhibitor cycloheximide. Thus, our results demonstrate that neurons possess an efficient uptake system for the antennapedia homeobox peptide and suggest that binding of pAntp to consensus motifs present in nerve cell nuclei influences neuronal morphogenetic programs.**

Studies on developmental mutants in *Drosophila* have demonstrated that several DNA binding proteins encoded by homeotic genes are endowed with morphogenetic functions (1, 2). In *Drosophila* at least, the expression of specific homeotic genes is responsible for the formation of cell assemblies exhibiting precise and defined morphologies. The DNA binding properties of these proteins is due to a sequence of about 60 amino acids called the homeobox.

The homeobox sequences have been highly conserved during evolution and genes containing homeobox sequences are present in all vertebrates, including mammals (3, 4). In vertebrates, as in *Drosophila*, homeobox gene expression is not limited to the period during which the general features of body organization are established. In particular, a number of these genes are expressed in the nervous system rather late during development and, in some cases, through adulthood (5–7).

In vitro studies have shown that the regulatory effect of homeotic proteins on gene expression depends on the specific binding of the homeobox domain to consensus DNA sequences found in the promotors or enhancers of several reporter genes and homeobox-containing genes (8–10). It has also been clearly demonstrated that the 60-amino acid homeobox polypeptide alone, isolated from the flanking regions, which are necessary for the activation or repression of transcription, has *per se* a high affinity for such consensus motifs (11).

Thus, one possible way to study directly, within the live cell, the role of a homeotic protein family, as defined by the primary structure of the homeobox (e.g., antennapedia- or engrailed-like) and by the recognition of identical binding sites, on neural development would be to synthesize and inject the 60-amino acid homeotic polypeptides into the cells. In fact, such peptides could act as competitive inhibitors of endogeneous homeotic proteins with similar binding specificities.

In the experiments presented herein we have used this strategy and introduced the peptide corresponding to the *Drosophila* antennapedia homeobox sequence (pAntp) into nerve cells during the dissociation procedure just before plating. We demonstrate that the presence of pAntp produces dramatic and rapid morphological modifications of the cultures. In addition, we show that pAntp added to already differentiated neurons enters into nerve cells, accumulates in their nuclei, and further enhances their morphological differentiation.

MATERIALS AND METHODS

Synthesis of pAntp. The homeodomain coding sequence was synthesized by the PCR from the p903G plasmid, which contains a 600-base-pair Antp cDNA sequence between its *Bam*HI and *Pvu* II sites (12). The two primers used corresponded to the first and last 21 nucleotides of the Antp homeodomain. The first primer (5′-GGGGGAATTC-CATATGCGCAAACGCGCAAG-3′) contains an *Nde* I restriction site upstream of the initiation codon and the second primer (5′-GGGGAAGCTTGGATCCTCAGTTCTCCT-TCTTCCACTTCAT-3′) contains a stop codon followed by a *Bam*HI site. The plasmid pAH1 was formed by ligating the *Nde* I–*Bam*HI 220-base-pair PCR product to the plasmid pET3a (13). The polypeptide was then expressed in *Escherichia coli* BL21 (Lys S). Freshly transformed cells were grown to an OD_{600} value of 1.2 at 37°C in LB medium with ampicyllin (100 μg/ml) and chloramphenicol (100 μg/ml). After a 5-hr induction with 1 mM isopropyl β-D-thiogalactoside (final concentration), cells were harvested by centrifugation (16,000 × *g*, 15 min), washed three times with 50 mM sodium phosphate, pH 7.5/0.4 M NaCl/2 mM EDTA/1 mM phenylmethylsulfonyl fluoride/1 mM dithiothreitol (buffer A), and sonicated.

After centrifugation (16,000 × *g*, 15 min), the supernatant was precipitated with streptomycin sulfate (20 mg/ml) with gentle agitation for 15 min at room temperature and centrifuged again (16,000 × *g*, 15 min). The supernatant was directly loaded on a fast flow column (Pharmacia) preequilibrated in buffer A. The column was extensively washed with 50 mM sodium phosphate, pH 7.5/0.5 M NaCl/5 mM dithiothreitol, and pAntp was eluted by application of a 0.5–1 M NaCl gradient. The eluted peptide (3 mg/liter of culture) was then dialyzed for 24 hr against 50 mM sodium phosphate, pH 7.5/150 mM NaCl. The amplified DNA segment and peptide were sequenced (Applied Biosystems, model 477) and the sequences corresponded to those of the antennapedia ho-

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Abbreviation: E, embryonic day.

meobox. Analysis by SDS/gel electrophoresis of the peptide demonstrated the presence of a single band of the appropriate molecular weight.

Electrophoretic Mobility-Shift Assay. pAntp (80 ng) was preincubated 15 min at 4°C in a total volume of 24 μl containing 4 μl of 5× binding buffer (14), 12% (vol/vol) glycerol, 0.5 μg of poly(dI-dC), 4 μl of extraction buffer (15), 80 ng of pAntp, and various amounts (0, 20, or 200 ng) of the 26-mer oligonucleotide duplex corresponding to an antennapedia-type homeobox protein binding site from the *Hox-1.3* promotor (Hox-1.3p: 3′-CAGAGCACGTGATTACCCCCT-CAACC-5′/5′-GTCTCGTGCACTAATGGGGGAGT-TGG-3′) (16). Samples were incubated for 30 min at 4°C in the presence of ^{32}P-labeled (0.2 ng in 2 μl) *Hox-1.3* promotor (Hox-1.3p), which was labeled using T4 polynucleotide kinase (Biolabs, Northbrook, IL) and [γ-^{32}P]ATP (3000 Ci/mmol, 1 Ci = 37 GBq; Amersham). A 12-μl sample of the incubation mixture was separated for 4.5 hr (35 mA, 4°C) on a 10% nondenaturing polyacrylamide gel (13.4 mM Tris·HCl, pH 7.5/6.6 mM sodium acetate/1 mM EDTA), which was exposed for 1 hr.

Fluorescein Labeling of pAntp. pAntp (200 μl, 52 μg) was dialyzed against two 100-ml changes of 150 mM NaCl (overnight), 100 ml of 50 mM bicarbonate-buffered saline (pH 8.5, 4 hr), and 100 ml of 50 mM bicarbonate-buffered saline (pH 9.2, 2 hr). pAntp was then dialyzed overnight in the dark against 50 ml of bicarbonate-buffered saline (pH 9.2) with fluorescein isothiocyanate (Sigma; 100 μg/ml). The reaction was stopped by changing the dialysis buffer to phosphate-buffered saline (PBS; pH 7.2, 200 ml, overnight). All steps were carried out at 4°C under aseptic conditions.

Cell Cultures. General cell culture conditions were as described (17). In brief, cells from rat embryonic tissues were mechanically dissociated and plated at the indicated concentrations in plastic wells (1.6- or 16-mm diameter) or on DL-polyornithine (Sigma, 40 kDa, 5 μg/ml)-coated glass coverslips in a chemically defined medium. Under these conditions, nonneuronal growth was inhibited and 95% pure neuronal cultures were routinely obtained. Prior to morphological analysis, cells were fixed with glutaraldehyde [2.5% (vol/vol) in PBS] and stained with toluidine blue [0.05% in 2% (wt/vol) Na_2CO_3]. For the examination of fluorescent pAntp, cells were examined directly without fixation or after fixation for 5 min at −20°C in ethanol/acetic acid [95:5 (vol/vol)], air-dried, and mounted in Mowiol.

Peptide Internalization by Trituration. Small pieces (1 mm^3) of embryonic brain were dissociated mechanically in 20 μl of PBS supplemented with 33 mM D-glucose, 10 mM Mg^{2+}, and 1 mM dithiothreitol containing pAntp (160 μg/ml) and incubated for 1 hr in the same medium at room temperature. The cells were then washed once in PBS containing 33 mM D-glucose, twice in the chemically defined medium (17), and plated at the indicated densities.

Confocal Microscopy. Data were obtained with a confocal scanning laser microscope (CSLM Phoibos 1000) with the acknowledged help of A. Triller (Pasteur Institute). Excitation was obtained with an argon ion laser set at 488 nm and the fluorescent light was filtered with a long-pass (515 nm) filter. The size of the pixel was 0.15 μm. The actual depth of focus was closed to 0.7 μm with those optics at the considered wavelength (18).

RESULTS

The 60-amino acid pAntp polypeptide was expressed in *E. coli*, purified as described above, and sequenced. The sequence of the purified protein corresponded exactly to the expected primary structure. In addition, as demonstrated in the gel-shift experiment of Fig. 1, pAntp recognized the consensus sequence motif for binding of homeobox proteins

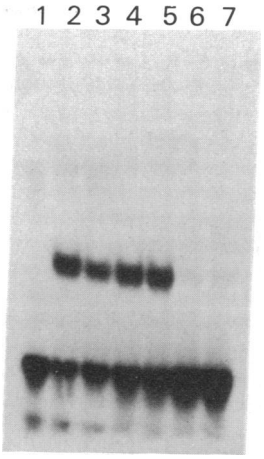

FIG. 1. Electrophoretic mobility-shift assay. pAntp (80 ng, 13 pmol, lanes 2–7) was preincubated for 15 min at 4°C with 0.5 μg of poly(dI-dC) (lanes 1–7) and 2.5 pmol (lanes 4 and 5) or 25 pmol (lanes 6 and 7) of nonradioactive *Hox-1.3* promotor (Hox-1.3p) as nonradioactive competitor. Samples were incubated for 30 min at 4°C in the presence of 0.2 ng (2 fmol) of ^{32}P-labeled Hox-1.3p and the association was analyzed by gel electrophoresis. As expected no competition was seen in the presence of 2.5 pmol of *Hox-1.3p* (still less than the molar amount of pAntp, but the competition for the binding of pAntp to the labeled promotor was complete at 25 pmol).

present in the promotor region of *Hox-1.3* (16). This binding was specific since it could be competed by an excess of the unlabeled binding sequence.

In a first series of experiments, pAntp was introduced by trituration (19, 20). Briefly, cells taken from ventral mesencephalon, tectum, or spinal cord of embryonic day (E) 14 or E15 rat embryos were dissociated directly in 20 μl of a pAntp solution (160 μg/ml in PBS). After a 30-min incubation at room temperature, the cells were washed several times in serum-free medium and plated on Terasaki culture wells at 1 × 10^4 cells per well in chemically defined medium.

Cultured neurons triturated in the presence of pAntp showed significant morphological changes. At 24 hr after plating and in the absence of polyornithine-coating, control cells formed large aggregates that were loosely attached to the substrate. In the presence of pAntp, however, aggregates spread out along the substrate and neurites emanating from the clustered neurons were clearly visible. At 48 hr after plating cells triturated in the presence of pAntp had continued differentiating with large fascicles linking the neuronal aggregates whereas, in control cultures, cells manifested no overt sign of differentiation and the aggregates were enlarged, presumably by the addition of cells (Fig. 2 *A* and *B*).

To verify the effectiveness of the trituration protocol in allowing the entry of pAntp, some cells were triturated as above in the presence of fluorescent pAntp (160 μg/ml), plated on polyornithine-coated glass coverslips, and cultured for 24 hr. As shown in Fig. 2 *C* and *D*, all cells were strongly fluorescent. It should be noted that at this stage of differentiation, as clearly seen under phase-contrast microscopy, the nucleus occupies nearly the entire volume of the neuronal soma. For this reason, although the nuclei seemed strongly fluorescent, it was difficult to ascertain the nuclear localization of the antigen. However, confocal microscopy was useful in resolving this point (see below).

The strong fluorescence due to fluorescein isothiocyanate-labeled pAntp suggested that the peptide may enter the cells not only by nonspecific diffusion after dissociation-induced neurite damage but also, perhaps, by an uptake mechanism. We examined this by adding low concentrations (16 μg/ml, 1 hr) of fluorescent pAntp 24 hr after plating on polyornithine-coated glass coverslips. At this time the cells had elaborated

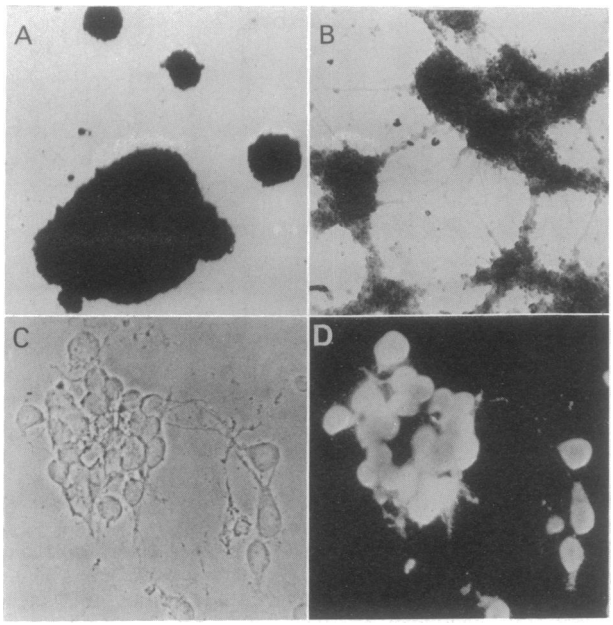

FIG. 2. pAntp-induced morphological differentiation. (*A* and *B*) Tectal cells from E15 embryos were dissociated in control buffer (*A*) or in pAntp (160 μg/ml) (*B*), cultured for 48 hr in Terasaki wells, fixed with glutaraldehyde, and stained with toluidine blue. At high cellular concentration (10^4 cells in 10 μl) and in the absence of a substrate coating, control cells formed large aggregates (*A*). However, the presence of pAntp provoked a dramatic effect on the overall differentiation of the culture (*B*). (×65.) (*C* and *D*) Tectal cells from E15 embryos were dissociated in the presence of fluorescent pAntp, plated on polyornithine-coated coverslips, cultured for 24 hr, fixed in ethanol/acetic acid, and examined in phase-contrast (*C*) or epifluorescence (*D*) microscopy. (×275.)

long processes. As expected, fluorescent ovalbumin fragments of the size of pAntp could not cross the cell membrane (Fig. 3 *A* and *B*). However, fluorescent pAntp did penetrate the cells and was targeted to the nucleus as demonstrated in

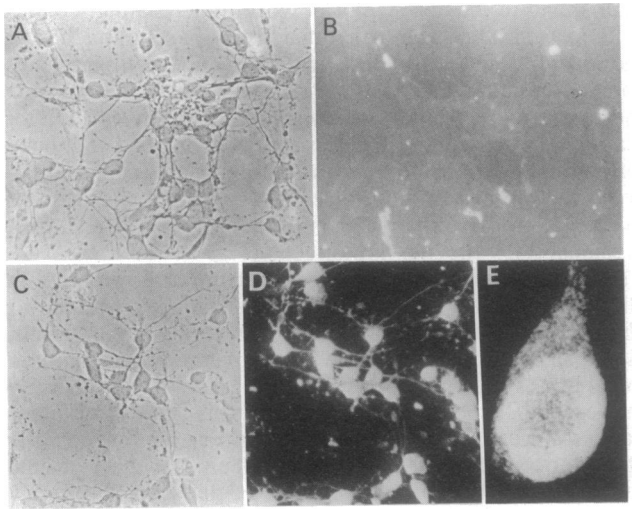

FIG. 3. Fluorescent pAntp added 24 hr after the onset of the culture penetrates into neurons. Cells from E14 embryonic brain stem were dissociated and cultured for 24 hr on polyornithine-coated glass coverslips before the addition of fluorescent ovalbumin tryptic fragments (*A* and *B*) or pAntp (*C–E*) at 16 μg/ml. The cells were further cultured for 1 hr, fixed, and examined under phase-contrast and epifluorescence microscopy. Compare the absence of ovalbumin entry into the cells (*A* and *B*) with the significant cellular and nuclear concentration of pAntp (*C–E*). (*A–D*, ×220.) (*E*) Confocal section demonstrating the nuclear localization of pAntp. (×1500.)

classical and confocal fluorescence microscopy (Fig. 3 *C–E*). We also verified that nonfluorescent pAntp was captured and addressed to the nucleus. Indeed, 3 hr after its addition to the medium >50% of the original peptide was reextracted, nondegraded, from pure nuclei fractions (data not shown).

These results on pAntp penetration prompted us to analyze the morphological effects of adding pAntp 24 hr after cells were plated. Embryonic cells from mesencephalon, spinal cord, and cortex were dissociated, plated on noncoated plastic substrate at two densities (2×10^4 and 5×10^3 cells per Terasaki well), and cultured for 24 hr. pAntp was then added to the medium (final concentration, 8 or 16 μg/ml) and cultures were incubated for another 24 hr. Morphological effects were observed at the two cell concentrations and in cells from three brain regions. The effect of pAntp addition on spinal cord (Fig. 4 *A* and *B*) and cortical neurons (Fig. 4 *C* and *D*) is shown. The presence of pAntp decreased the number and the size of aggregates, lessened the number of fasciculated neurites, and increased that of fine highly branched neurites.

Since pAntp is a very basic peptide (pK = 11.35), it is possible that it could bind to the cell surface and enter the cells only during the course of fixation. Thus, we examined the pattern of fluorescence in nonfixed living cells. Confocal microscopy (Fig. 5 *A* and *B*) shows that fluorescent pAntp was internalized by live cells, including the growth cone, and accumulated in the nucleus. Moreover, when live neurons preincubated with pAntp were treated by proteinase K in conditions (1 mg/ml, 15 min, 4°C) that completely degrade the cell surface molecule neural cell adhesion molecule (NCAM) (Fig. 5 *C* and *D*) and fixed, the fluorescent peptide

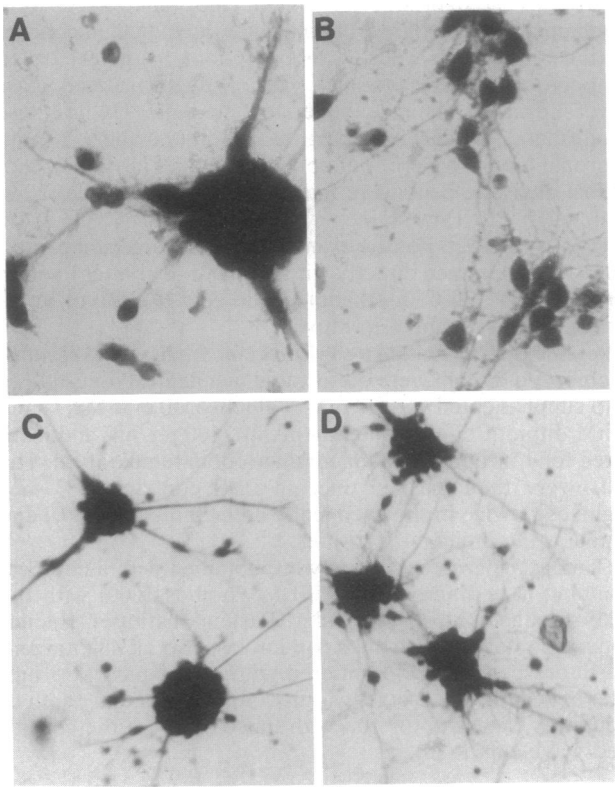

FIG. 4. pAntp added 24 hr after the onset of the culture enhances the differentiation of spinal cord and cortical neurons. Spinal cord (*A* and *B*) and cortex (*C* and *D*) neurons from E15 embryos were dissociated and cultured for 24 hr on polyornithine-coated Terasaki wells (5×10^3 cells per well) before the addition of medium alone (*A* and *C*) or pAntp (8 μg/ml) (*B* and *D*). The cells were fixed and stained with toluidine blue 24 hr later. Note the effect of pAntp on neuronal differentiation. (*A* and *B*, ×260; *C* and *D*, ×120.)

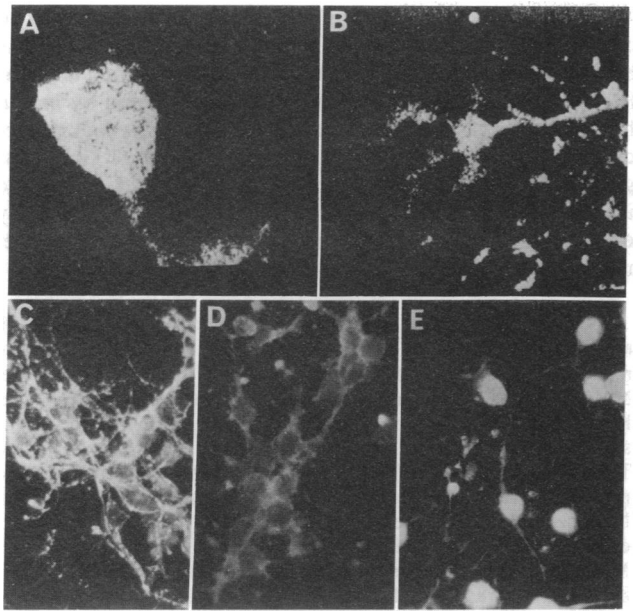

FIG. 5. Fluorescent pAntp penetrates live nerve cells. Confocal sections of living neurons at the soma (*A*) and the growth cone (*B*). Neural cell adhesion molecule immunostaining of live cells untreated (*C*) or treated with proteinase K (*D*). (×500.) (*E*) Intracellular and nuclear localization of pAntp in cells incubated for 1 hr with the fluorescent peptide, treated with proteinase K as in *D*, and fixed. (×260.)

was still seen in the nucleus (Fig. 5*E*). This series of experiments demonstrated that living cells internalize pAntp and that the nuclear localization is not a fixation-related artifact.

Finally, it could still be speculated that small amounts of pAntp bind to the cell surface and enhance neuronal adhesion nonspecifically, as does polyornithine, thus provoking neuronal differentiation independently of its localization to the nucleus. To examine this possibility directly, we plated the cells at high density (5×10^4 cells per Terasaki well) on noncoated plastic and after 24 hr, cell aggregates interconnected with long fascicles had formed. Increasing concentrations of soluble polyornithine (10 kDa) or pAntp (7 kDa) were then added in the presence or absence of cycloheximide at 5 μM and the cells were cultured for another 24 hr before fixation and staining. Cells in the control wells (no addition) had continued to aggregate, forming large clumps loosely attached to the substrate (Fig. 6*A*). Cycloheximide alone prevented further aggregation (compare Fig. 6 *A* and *B*, which are taken at the same magnification) and the morphological aspect of the 48-hr cultures was virtually identical to that observed after 24 hr with large interfasciculated aggregates loosely attached to the substrate (Fig. 6 *B* and *C*). The addition of soluble polyornithine at concentrations higher than 5 μg/ml was highly toxic for the cells. However, at 5 μg/ml, the polycation added during the last 24 hr slightly increased neuronal adhesion, as demonstrated by the flattening of the aggregates (Fig. 6*D*), and this effect was not inhibited by cycloheximide (compare Fig. 6 *E* and *D*), demonstrating that it was a pure "adhesion effect." In contrast, the strong effect of pAntp in inducing differentiation and elaboration of neurites (Fig. 6*F*) was blocked by the protein synthesis inhibitor (Fig. 6*G*). A residual effect on spreading was, however, still visible in the presence of cycloheximide (Fig. 6*G*). Thus we conclude that although pAntp at a fairly high concentration (90 μg/ml) has a slight adhesion effect not inhibited by cycloheximide, its capacity to induce neuronal differentiation requires intact protein synthesis machinery.

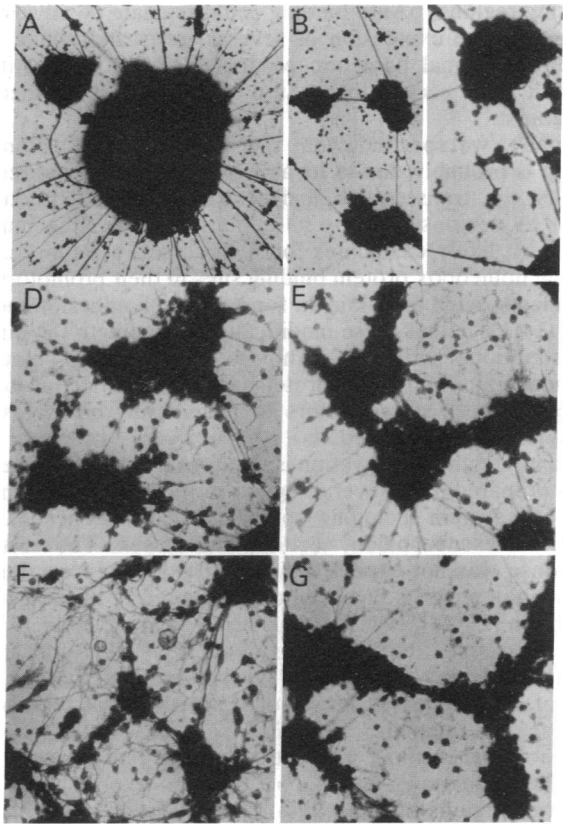

FIG. 6. pAntp-induced morphological differentiation requires an intact protein synthesis machinery. All cells were cultured 24 hr at the density of 5×10^4 cells per 10 μl in noncoated Terasaki wells, under the following addition conditions, fixed, stained, and examined 24 hr later. (*A*) Medium alone. (*B* and *C*) Cycloheximide (5 μm). (*D*) Polyornithine alone. (*E*) Polyornithine plus cycloheximide. (*F*) pAntp alone. (*G*) pAntp plus cycloheximide. (*A* and *B*, ×35; *C–G*, ×70.)

DISCUSSION

We demonstrate herein that a polypeptide of 60 amino acids corresponding to the primary structure of the antennapedia homeobox enters nerve cells, reaches the nucleus, and dramatically modifies the morphology of the neurons. These experiments strongly suggest that homeobox proteins participate in neural differentiation and open the way to study their mode of action at the cellular level.

Indeed, we have not demonstrated a causal link between pAntp penetration and the increse of neuronal differentiation. In fact, whether the effect of pAntp is due to the binding to specific canonical sequences on the cell chromosome will require the injection of mutated peptides. However, the results of control experiments favor an action at the gene level. (*i*) pAntp was shown to bind specifically to the *Hox-1.3* promotor in a gel-shift experiment. (*ii*) Fluorescent pAntp accumulates in the nucleus. (*iii*) pAntp entry is specific since other polypeptides of the same size and/or charge content (ovalbumin fragments or 10-kDa polyornithine) do not reach the nucleus and do not have the same effects. (*iv*) In contrast to that of soluble polyornithine the effect of pAntp added to 24-hr-old cultures is blocked by cycloheximide.

Although we do not know the exact intranuclear concentration of the peptide, if we compare the affinity of pAntp for its cognate binding site estimated between 1 and 0.1 nM (11) and the concentration of pAntp in the culture, it is possible that pAntp acts like a competitive inhibitor for the binding of several endogenous homeobox proteins to their respective binding sites. However, even in the absence of regulatory

flanking regions, pAntp binding could regulate positively or negatively the expression of several genes and mimic the action of normal homeotic products (21, 22). Thus, it is difficult to be certain that pAntp functions as a "homeotic antagonist."

Finally, we speculate that the morphogenetic action of pAntp is probably due to its ability to directly or indirectly modify the transcription and/or translation of structural genes as demonstrated by its inhibition by cycloheximide. The most likely candidates for such control are those containing homeobox protein binding sites in their promotors or enhancers, such as is known for neural cell adhesion molecule (14). Whether these modifications are due to the direct transcriptional activity of pAntp, to the inhibition of binding of endogenous homeobox proteins, or to a modification in the accessibility of other factors to neighboring binding sites remains to be elucidated.

An interesting observation is that pAntp is active on nerve cells prepared from several regions of the embryonic central nervous system including spinal cord, rhombencephalon, ventral mesencephalon, tectum, and cortex. The cortical response was not expected since little or no expression of homeobox proteins has been demonstrated in this region of the brain (23, 24). Indeed, by using an oligonucleotide probe that recognizes the homeobox sequence highly conserved within the *Hox-1.3* gene family, we have verified that, for this gene family at least, the regional specificity of homeobox genes expression described *in vivo* is also valid *in vitro* (unpublished results). Thus, it is possible that pAntp either displaces unknown homeobox proteins expressed in the anterior regions of the brain or, as described above, has a direct modulatory effect on structural genes exhibiting the appropriate binding sites in their promotors or enhancers.

The mode of entry of pAntp into nerve cells is very intriguing. Initially, we used the trituration technique that had allowed us to demonstrate that among several small GTP-binding proteins of the *ras* superfamily, rab2p is the only one to increase neuronal morphological differentiation (20). In this technique, pieces of embryonic brain tissues are directly dissociated in the presence of proteins that diffuse passively into the cells as a consequence of neurite damage. When compared with the effective concentrations of rab2p used in our earlier experiments (1.5 mg/ml), that of pAntp was considerably lower (160 μg/ml), suggesting the existence of a possible facilitated transport. The internalization and nuclear accumulation of fluorescent pAntp added at even lower concentrations (8 and 16 μg/ml) to already developed cells certainly sustains this possibility. Since proteolytic fragments of fluorescent ovalbumin do not enter viable cells in detectable amounts, pAntp internalization is not likely due to cell damage. In addition, we observed that, in contrast to pAntp, the small amounts of fluorescent polyornithine internalized by capping never reach the nucleus (unpublished results).

At this point, we do not eliminate the possibility that pAntp internalization does not relate to any normal physiological phenomenon occurring also *in vivo*. However, the existence of a physiological transport of homeobox peptides through the cell membrane must not be precluded. This later hypothesis is in fact sustained by the finding that the internalization of pAntp as well as its morphogenetic effects are strongly antagonized by the enzymic removal of the polysialic acid residues present at the surface of the nerve cells (A.J., A. Triller, M. Volovitch, C.P., and A.P., unpublished results).

If such a transport exists, we must consider the possibility that DNA binding proteins, devoid of intracellular signal sequences, could be passed into the cellular environment where they would be available to directly affect the programs of cellular differentiation, thus representing a class of neuronal growth factors. Although this possibility remains to be established, we stress that (*i*) *Hox-1.3* immunoreactivity has been reported in regions where no mRNA that could account for the synthesis of this protein could be detected by *in situ* hybridization (24), (*ii*) the secretion of proteins lacking secretion signal peptides, such as interleukin 1 or fibroblast growth factor has been reported (25), and (*iii*) the basic isoform of fibroblast growth factor can be captured by endothelial cells and transported to the nucleolus by a yet unknown mechanism (26).

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