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Expression of human pituitary adenylate cyclase activating polypeptide (PACAP) cDNA in CHO cells and characterization of the products

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cDNA encoding human PACAP precursor was expressed in non-neuroendocrine Chinese hamster ovary cells, CHO-K1, The cells were transfected with expression vector (pTS705) containing the human PACAP cDNA by electroporation A cell line which produced more than 80 ng/ml of immunoreactive PACAP (ir-PACAP) into the conditioned medium was established RP-HPLC analysis of culture medium of this established cell line exhibited the presence of two types of PACAP, i e. PACAP38 and PACAP27 At the same time, it was also revealed that immunoreactive PACAP-related peptide (ir-PRP) was secreted into the cultured medium The ir-PACAPs were confirmed to have biological activities such as induction of cAMP and neurite outgrowth in rat pheochromocytoma PC12h cells

Human PACAP, PRP, Gene expression system (CHO-K1), PC12h, Neurite extension, cAMP accumulation

1 INTRODUCTION

A novel neuropeptide with 38 amino acid residues and its shorter form (the N-terminal 27 residues), which stimulate adenylate cyclase in a rat anterior pituitary cell culture, has been isolated and purified by Miyata et al. [1,2]. These peptides named PACAP38 and PACAP27 have amidated C-termini. The N-terminal amino acid sequence of PACAP38 shows similarity to vasoactive intestinal polypeptide (VIP); however, adenylate cyclase-stimulating activity of PACAP in cultured rat pituitary cells is at least 1,000-times more than that of VIP [1]. In addition, the rat pheochromocytoma PC12h responds to PACAP by the extension of neurites and the secretion of cAMP [3].

Recently we have cloned and determined the nucleotide sequence of cDNAs ecoding the PACAP precursor from ovine hypothalamus, human testis and rat brain [4–6]. The mature form of PACAP38 was found to be identical in these animals. An amino acid sequence which has homology to peptide histidine methionine (PHM) and growth hormone-releasing hormone (GRH) was identified in the PACAP precursor protein and named PACAP-related peptide (PRP) [6]. To decipher processing and secretion of PACAP and PRP, we attempted to express the human PACAP cDNA in cultured mammalian cells.

The present report deals with the successful expression of cDNA encoding the human prepro-PACAP38 in non-neuroendocrine Chinese hamster ovary cells CHO-K1 The transfected CHO-K1 cells produced more than 80 ng/ml of immunoreactive PACAP (ir-PACAP) which exhibited biological activities in PC12h cells similar to chemically synthesized PACAPs. Partial characterization of PACAP precursor protein from the transformed cells and secreted form of PRP are also reported.

Abbreviations PACAP, pituitary adenylate cyclase activating polypeptide, PRP, PACAP-related peptide; ir-PACAP, immunoreactive PACAP, FBS, fetal bovine serum, HRP, horseradish peroxidase, EIA, enzyme immunoassay, FITC, fluorescein isothiocyanate, PBS, phosphate-buffered saline, HBSS, Hanks' balanced salt solution, TFA, trifluoroacetic acid, RP-HPLC, reverse-phase high performance liquid chromatography

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2. MATERIALS AND METHODS

2 1. *Construction of expression vector*

Restriction enzymes, T4 DNA ligase and large fragment of *E coli* DNA polymerase I (Klenow fragment) were purchased from Takara Shuzo Co (Kyoto) An *Eco*RI 1 4 kb fragment from plasmid pHT38P8 [4] containing the entire coding region of human prepro-PACAP was inserted into the *Bgl*II site located downstream of SV40 early promoter of pTB551 to obtain pTS705 The details of construction of pTS705 are summarized in Fig 1

2 2 *Cell culture and transfection of DNAs*

CHO-K1 cells (ICN Biomedicals Inc., Costa Mesa, CA) were grown in Ham's F12 medium (Nissui Seiyaku, Tokyo) with 10% bovine serum (FBS, from M.A Bioproducts, MD), penicillin G (Ban-yu Seiyaku, Tokyo) and streptomycin (Meiji Seika, Tokyo) under 5% CO_2/95% air at 37°C

PC12h cells, kindly provided by Dr H Hatanaka (Institute for Protein Research, Osaka University), were grown in Dulbecco's modified Eagle's medium (DMEM, Nissui Seiyaku) with 10%

newborn calf serum (Mitsubishi Kasei Corp., Tokyo), penicillin G and streptomycin.

Transformation of CHO-K1 cells was performed by electroporation method [7]. CHO-K1 cells were co-transfected with pTS705 and pSV2neo (Pharmacia) in mannitol buffer (0.25 M mannitol, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.2 mM Tris-HCl, pH 7.4) by three rectangle pulses of 3.0 kV/cm, 50 μsec using Shimadzu Somatic Hybridizer SSH-1 (Shimadzu, Kyoto). Transformed cells were selected with 500 μg/ml of G418 (GIBCO, Life Technologies Inc.) [8], and cloned by colony formation and limiting dilution method.

2.3. *Chemical synthesis of PACAP and PRP*

PACAP38, PACAP27 and PRP (Ser^{82}–Asp^{110}) were chemically synthesized by an automatic synthesizer (ABI model 430A, Applied Biosystems Inc., Foster City, CA) in our laboratory.

2.4. *Enzyme immunoassay of PACAP and PRP*

Immunoreactivity of PACAP and PRP was tested by the competitive enzyme immunoassay method [9]. Briefly, 50 μl of samples and 50 μl of 1–1.3 × 10^5 diluted rabbit anti-PACAP27 antiserum [10] or 1–1.25 × 10^4 diluted rabbit anti-PRP antiserum were mixed on an

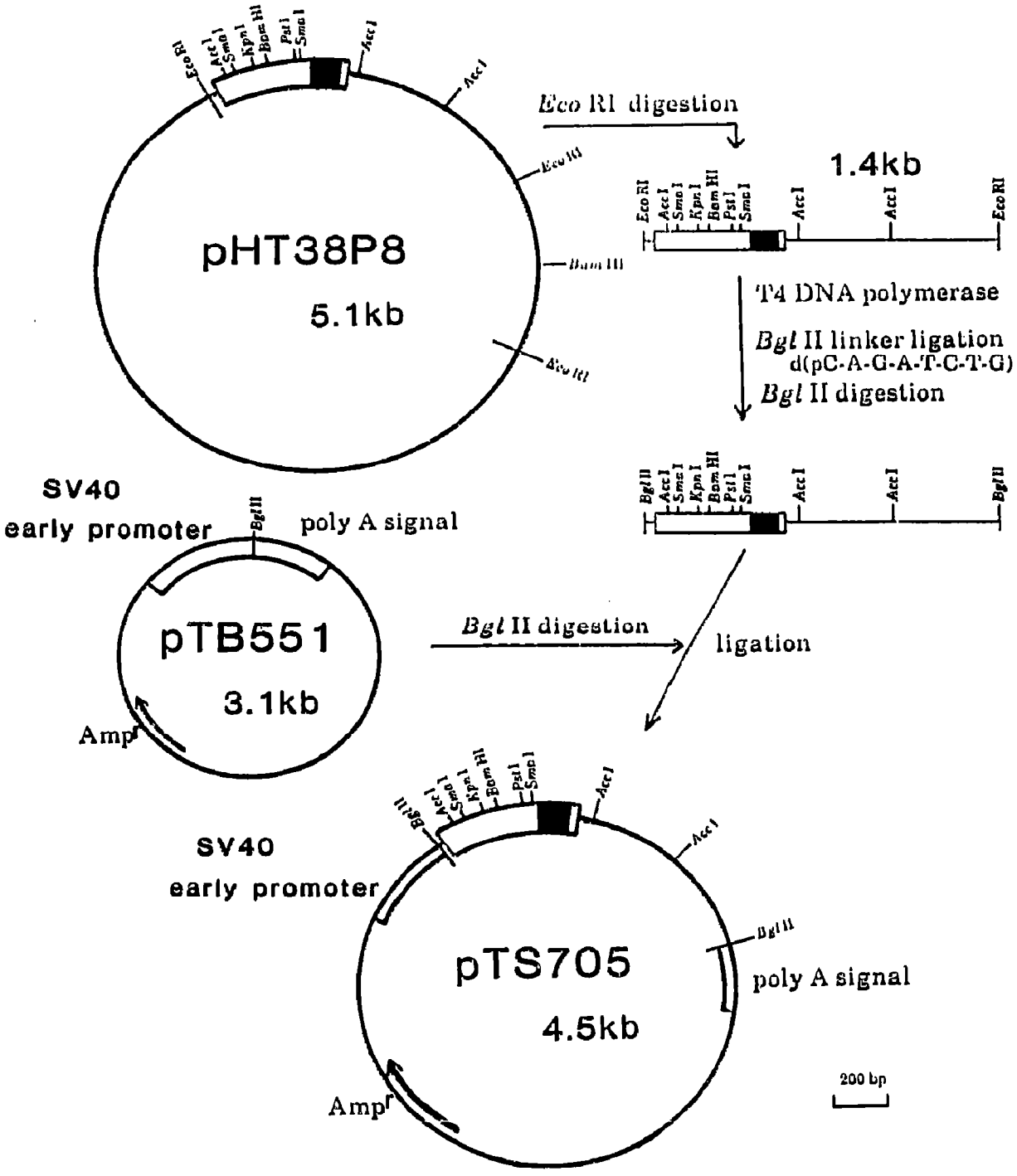

Fig. 1. Construction of human PACAP expression vector pTS705.

anti-rabbit IgG-coated 96-well plate. After incubation for 1 h at room temperature, 50 μl of 1:100 diluted PACAP38-horseradish peroxidase (HRP) conjugate or 1:2,000 diluted PRP-HRP conjugate were added. These conjugates were made according to the methods of Ishikawa et al. [11]. After an overnight incubation at 4°C, the plate was washed with phosphate-buffered saline (PBS) and then 100 μl of HRP substrate solution (TMB Microwell Peroxidase Substrate System, Kirkegaard & Perry Labs. Inc., MD) was added to each well. The plate was incubated for 20 min at room temperature and 100 μl of 1 M H_3PO_4 was added for terminating the reaction. Optical density of each well was measured at 450 nm.

2.5. *Northern blot analysis*

Total RNA extraction, purification of poly(A)$^+$ RNA, size fractionation of poly(A)$^+$ RNA, transfer to nylon membrane filter (Biodyne A, Pall Biosupport, Glen Cove, NY) [12] and hybridization were done as previously described [5]. The probe used was [^{32}P]-labeled 1.4 kb *Eco*RI fragment of pHT38P8 which encodes PACAP38 precursor protein. The specific activity of the probe was 4×10^8 cpm/μg DNA.

2.6. *Immunofluorescent staining of cells*

CHO-K1 cells and their transformants cultured on Slide Chambers (Nunc) were fixed with 3% paraformaldehyde, made permeable with 0.1% Triton X-100 and blocked with 2% bovine serum albumin. These were treated with 1:1,000 diluted mouse anti-PACAP38 monoclonal antibody PA-1N (Suzuki, N. et al., in preparation) and 1:2,000 diluted fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG antibody (Wako Pure Chemical Ind., Osaka). Cells were observed under a Nikon fluorescence microscope as previously described [13].

2.7. *Immunoblotting analysis*

Total cellular protein of CHO-K1 cells and their transformants was extracted by lysis with the SDS-PAGE sample loading buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.02% Bromophenol blue and 62,5 mM Tris-HCl, pH 6.8). After centrifugation, the cell lysates were separated on SDS-polyacrylamide gel (16%) [14] and blotted onto a nitrocellulose filter (Sartorius, Göttingen) by the electroblotting method [15]. The immunoreactive gene products were detected with PA-1N and colloidal gold particle-labeled goat anti-mouse IgG antibody (AuroProbe BLplus GAM IgG, Amersham). IntenSE BL silver enhancement kit (Amersham) was used for intensification of the positive bands.

2.8. *High performance liquid chromatography*

The conditioned medium of transformed CHO-K1 cells was concentrated with Amprep C2 columns (Amersham) and applied on TSKgel ODS-80T$_M$ column (4.6 mm i.d. × 15 cm, TOSOH, Tokyo). Samples were eluted from the column with a linear 50 min-gradient of acetonitrile (0–50%) containing 0.1% TFA at a flow rate of 1.0 ml/min at room temperature. Fractions (1.0 ml each) were collected from 30–45 min retention time.

Collected samples were lyophilized and dissolved in 0.5 ml of Hanks' balanced salt solution (HBSS) and were used for immunoreactivity and biological activity tests.

2.9. *Detection of biological activity*

CHO-K1 cells and their transformants were seeded into 150-cm^2 flasks at the density of 4×10^4 per ml and cultured in normal medium for two days after which serum-free medium was used. This serum-free conditioned medium was harvested on the next day and used to examine the biological activity of secreted PACAPs.

PC12h cells were seeded into a SUMILON collagen-coated 24- well plate (Sumitomo Bakelite Co., Tokyo) at the density of 2×10^4 per well. Three days after seeding, cells were washed twice with serum- free DMEM, and then 1 ml of serum-free DMEM containing 50, 25 and 10% of serum-free conditioned medium of CHO-K1 cells or their transformants was added to the PC12h cells. The morphological change of the treated cells were observed three days after treatment.

To examine the cAMP induction, PC12h cells were seeded in a SUMILON collagen-coated 48-well plate at the density of 5×10^4 per well and cultured for three days. Cells were washed twice with HBSS, and 500 μl of sample prepared with RP-HPLC was applied into the well. Cells were then incubated for 2 h at 37°C and the conditioned media were collected. cAMP secreted into the conditioned media was assayed with [^{125}I]cAMP Assay Kit (Amersham).

3. RESULTS AND DISCUSSION

3.1. *Establishment of the cell line which expresses ir-PACAP*

In recent years, the cDNAs encoding neuropeptides have been cloned and expressed in cultured cells, such as neuropeptide Y (NPY) in CHO [16], enkephalin in BSC-40 cells [17], proopiomelanocortin (POMC) in CV-1 (green monkey kidney) [18], and somatostatin in 3T3 cells [19]. We have presently succeeded in establishing a novel CHO transformant which produces a large amount of ir-PACAP into the culture medium.

CHO-K1 cells were transfected with the expression

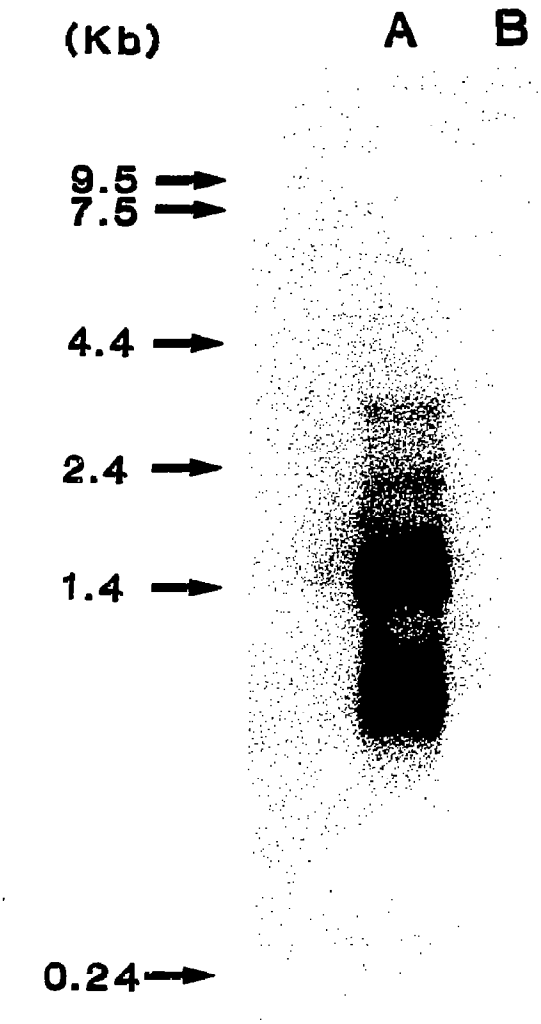

Fig. 2. Northern blot analysis of poly(A)$^+$ RNA extracted from CHO cells. (Lane A) CHOtf705.5; (lane B) CHO-K1.

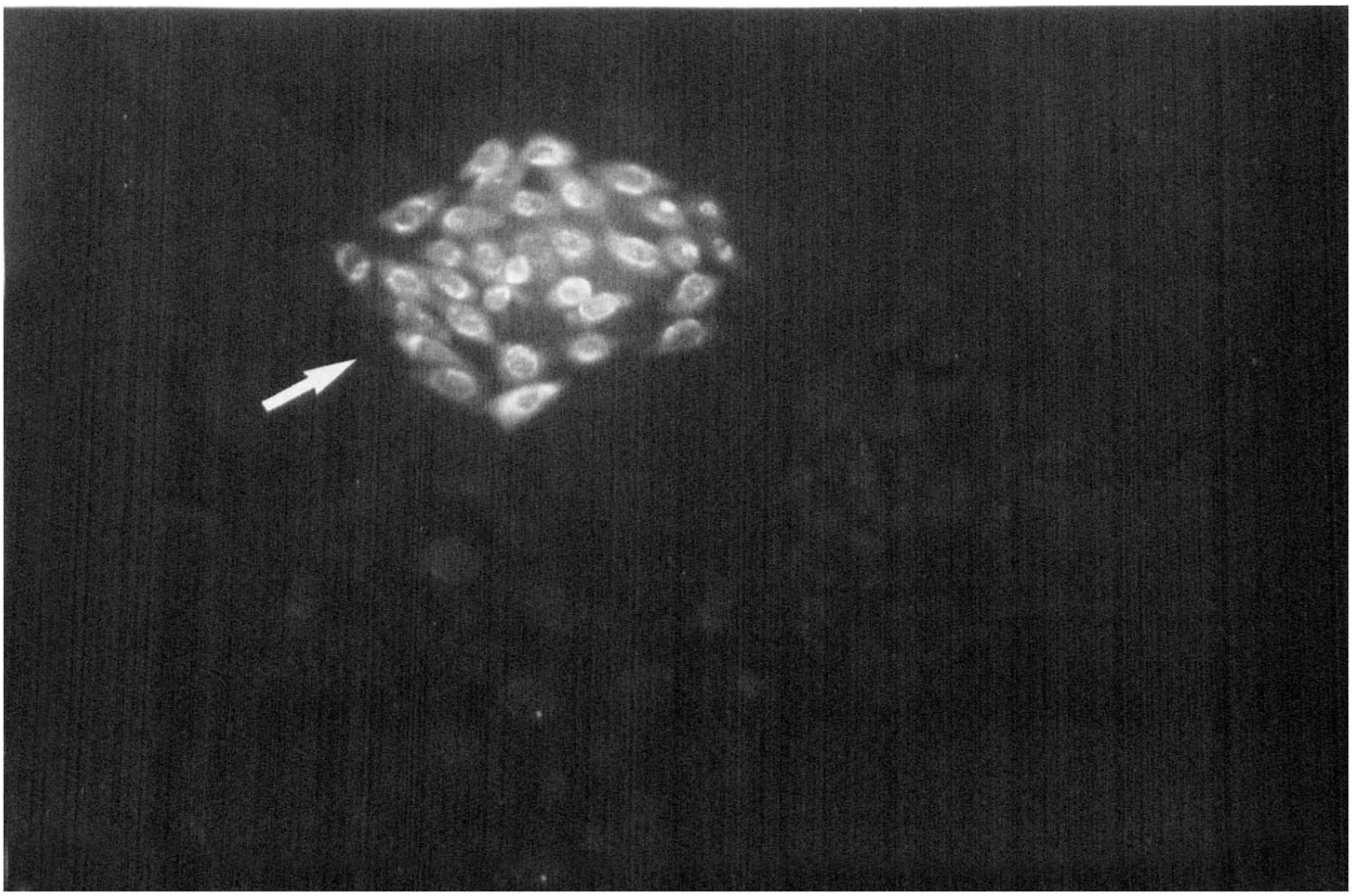

Fig. 3. Immunofluorescent staining of PACAP in intact cells. CHOtf705.5 and CHO-K1 cells were mix-cultured and stained with mouse anti-PACAP monoclonal antibody PA-1N and FITC-conjugated rabbit anti-mouse IgG antibody after fixation (× 280). Arrow indicates a colony of CHOtf705.5 cells.

vector pTS705 containing human PACAP cDNA and pSV2neo, and the transformants were selected by G418. Of the isolated 22 transformants, 20 transformants were revealed to produce ir-PACAP. Especially, clone No. 5 (named CHOtf705.5) synthesized more than 80 ng/ml of ir-PACAP. The cell number of CHOtf705.5 increased until day 3 after seeding and reached a plateau, but ir-PACAP production decreased after the peak on day 3. These facts suggest that the sequence of the PACAP cDNA in pTS705 is functional for translation, although it has only one base before the translation initiation codon ATG [4], and that PACAP has a very short lifetime because PACAP has three dibasic pairs of amino acid residues, Arg^{145}–Lys^{146}, Lys^{151}–Lys^{152} and Lys^{160}–Arg^{161} in a molecule and is processed easily by proteases. This established cell line was used for further experiments.

3.2. *Size of PACAP mRNA expressed in transformed CHO cells*

To clarify that the production of ir-PACAP by CHOtf705.5 was the result of transcription and transla-

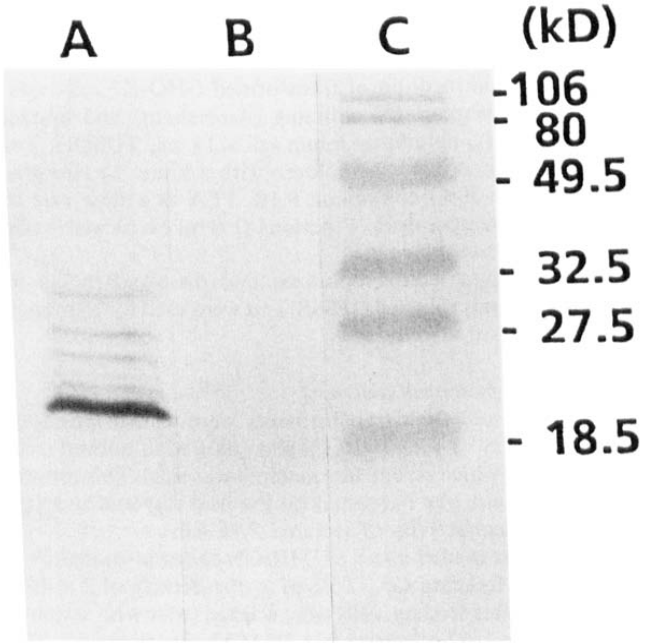

Fig. 4. Immunoblotting analysis of cell extracts. (Lane A) CHOtf705.5; (lane B) CHO-K1; (lane C) molecular weight markers.

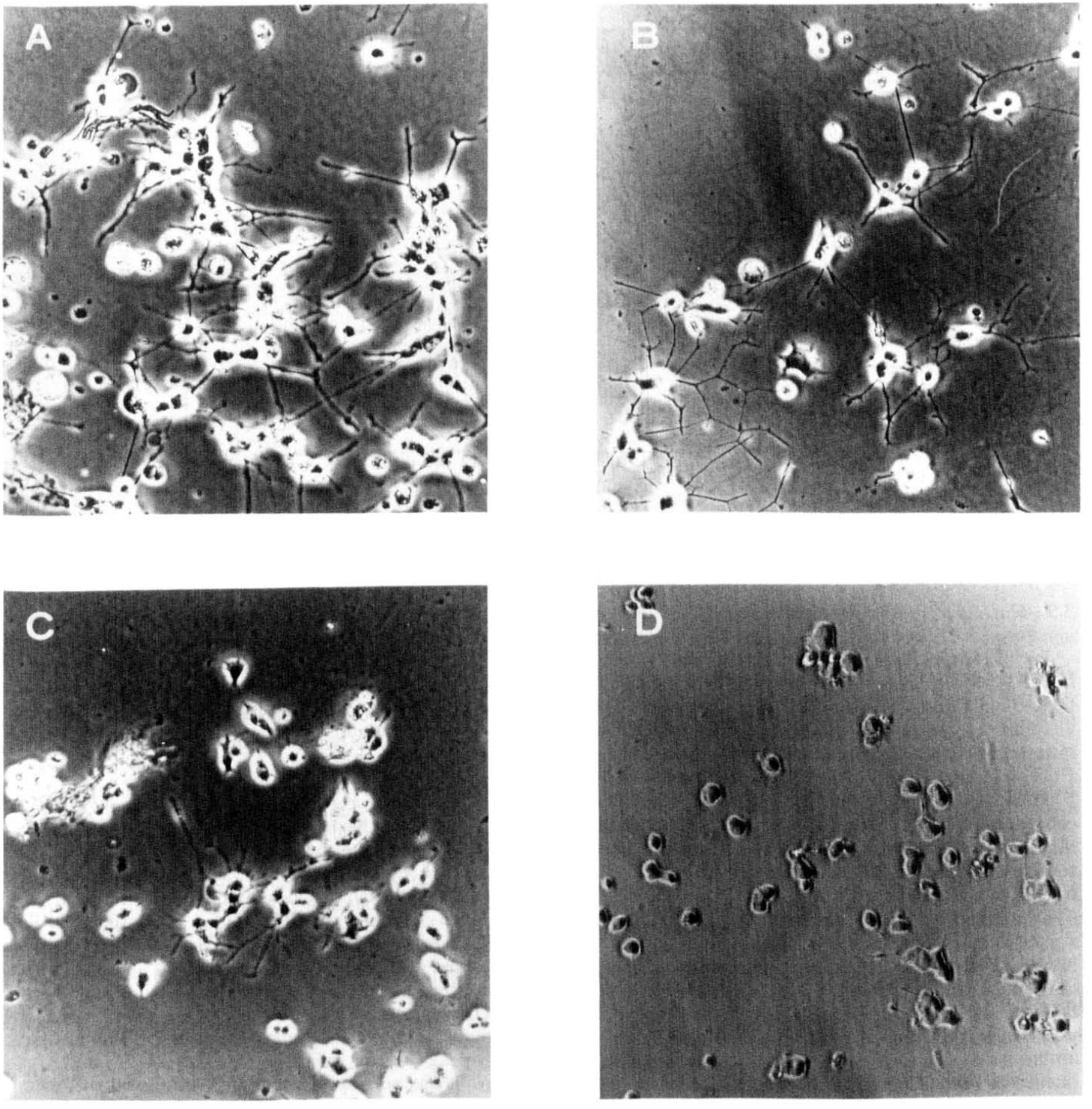

Fig. 5. Neurite outgrowth of PC12h cells after treatment with the serum-free conditioned medium of CHOtf705.5 (× 180). (A–C) CHOtf705.5 conditioned medium, 50, 25 and 10%, respectively; (D) CHO-K1 conditioned medium, 50%.

tion of inserted human PACAP cDNA, Northern blotting analysis was performed. The mRNA hybridized to the human PACAP cDNA probe was detected in the poly(A)$^+$ RNA extracted from CHOtf705.5 cells but not from CHO-K1 cells (Fig. 2). The main band of 1.4 kb was almost the same size of the cDNA in the expression vector pTS705. This fact indicates that the transcription of the gene was generated under the control of the SV40 early promoter. However, the second band of 0.8 kb remains unknown.

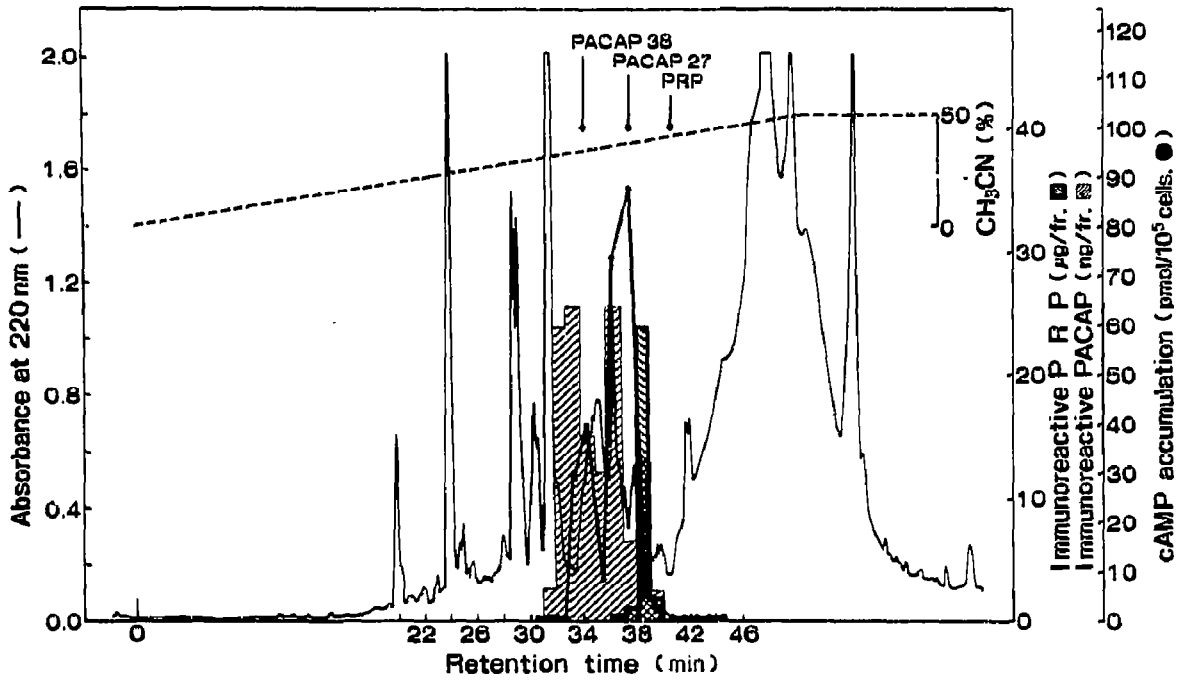

Fig. 6. RP-HPLC analysis of the concentrated cultured medium of CHOtf705.5 cells. 100 ml of the conditioned medium was concentrated and applied on RP-HPLC.

3.3. *Molecular profile of human ir-PACAP in transformed CHO cells*

To determine the localization of the produced PACAP in the cells, immunofluorescent staining of CHOtf705.5 and CHO-K1 cells was performed. When CHOtf705.5 and CHO-K1 cells were treated with mouse anti-PACAP monoclonal antibody PA-1N and FITC-conjugated anti-mouse IgG, the fluorescence of FITC was detected in the cytoplasm of CHOtf705.5 cells, but not in the CHO-K1 cells (Fig. 3). This suggests that the PACAP precursor was synthesized in the cytoplasm of CHOtf705.5 cells and the mature PACAP was secreted after processing.

To determine the molecular size of the PACAP precursor in cytoplasm, immunoblotting analysis was performed. Fig. 4 shows that immunoreactive materials in the cell extract of CHOtf705.5 were detected as a main band of 21.5 kDa, which was nearly equal to the molecular weight of prepro-PACAP calculated from the amino acid sequence deduced from the nucleotide sequence of human PACAP [5].

These results confirmed that we had cloned the PACAP cDNA which could reasonably be expressed in the CHOtf705.5 cells.

3.4. *Neurite extension with PACAP*

The PC12h cell is known to respond to nerve growth factor, basic fibroblast growth factor (bFGF), cAMP and forskolin by the extension of neurites [20,21]. We observed that PC12h cells extended neurites when stimulated with synthetic PACAP38 and PACAP27 (data not shown). To know whether the ir-PACAP produced by CHOtf705.5 is biologically active, we treated the PC12h cells with the serum-free conditioned medium of CHOtf705.5. As expected, neurite outgrowth of PC12h was observed, and its frequency depended on the concentration of conditioned medium added to PC12h cells (Fig. 5A–D).

3.5. *Analysis of ir-PACAP and ir-PRP by RP-HPLC*

As it has been reported that PACAP38 and PACAP27 activate the adenylate cyclase in PC12h cells [3], the cAMP production in PC12h cells was tested to determine the biological activity of molecules in conditioned medium of CHOtf705.5 separated by RP-HPLC. As shown in Fig. 6, the peak of ir-PACAP was observed at the retention time of 33–34 and 36–37 min, respectively, and that of ir-PRP was detected at the retention time of 38–39 min. On the other hand, the activity against cAMP production in PC12h cells gave two peaks at the retention times of 34–35 and 37–38 min. These correspond to the positions where chemically synthesized PACAP38 and PACAP27, respectively, were eluted.

From RP-HPLC analysis, the components inducing cAMP activity in PC12h cells could be positioned at the same retention time as that of chemically synthesized PACAP38 and PACAP27, and also the peak of ir-PACAP was nearly the same as synthesized PACAPs. Ir-PRP had a different retention time from that of synthesized PRP (Asp^{82}–Ala^{110}) (40–41 min). As the molecular profile, especially the biological activity of this PRP

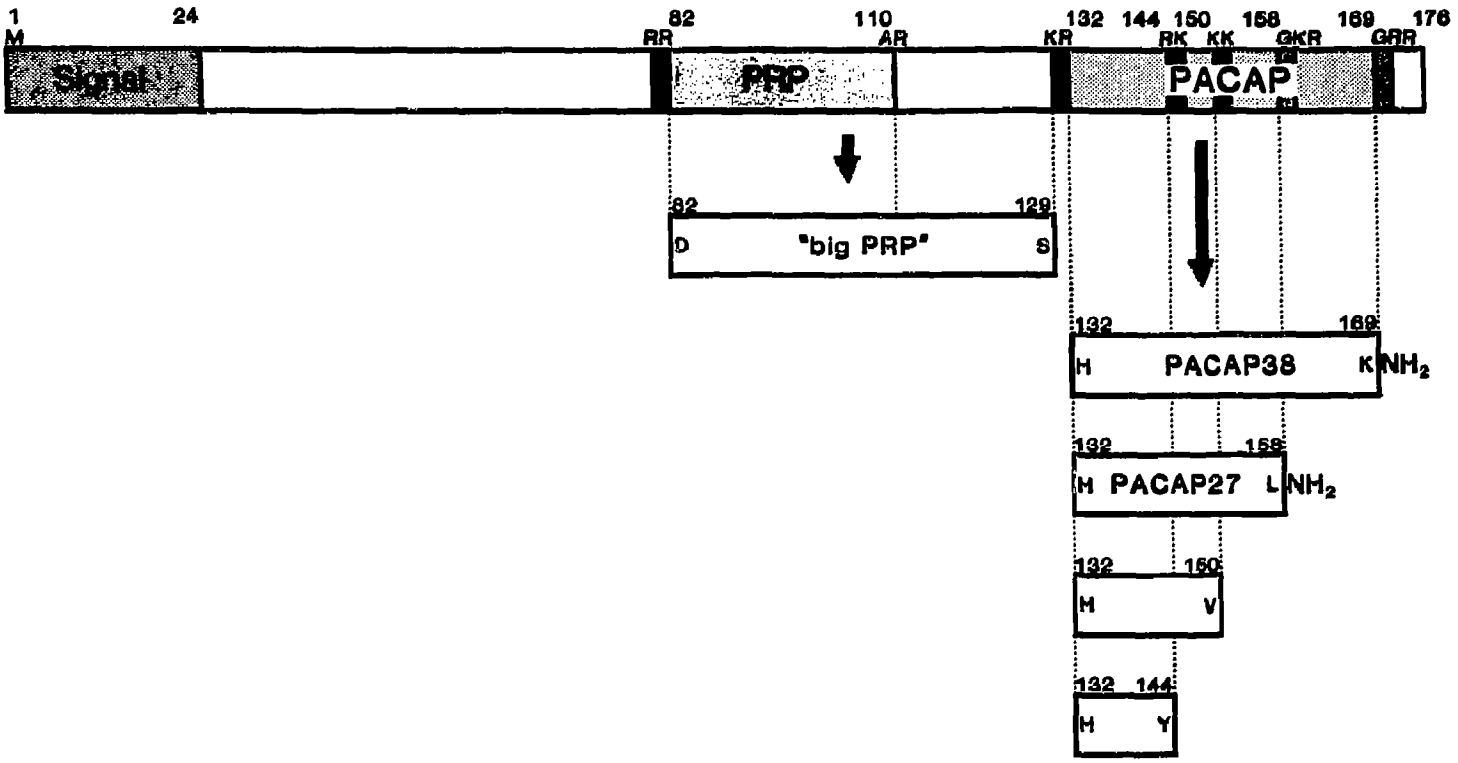

Fig. 7. Schematic model of processing pathway of PACAP precursor.

or PRP-containing peptide, has not been fully examined, further investigations are currently under way. The schematic model of the processing pathway of PACAP precursor is proposed in Fig. 7. PACAP and PRP are thought to be processed from the same precursor.

Although this PACAP has been isolated by monitoring the activity of stimulation of adenylate cyclase, and the neurite outgrowth of PC12h cells may be the result of the cAMP synthesized by the stimulated adenylate cyclase, many other physiological roles of PACAP cannot be ignored. Recently it has been reported that PACAPs stimulate the production of IL-6 in anterior pituitary cells [22] and that they prevent gp120-induced neuronal cell death [23]. In addition, it has been reported that PACAPs stimulate amylase secretion from rat pancreas when injected i.v. [24] It will be intriguing to decipher the multiple in vivo functions of this peptide.

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