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EXPRESSION OF HUMAN ATRIAL NATRIURETIC POLYPEPTIDE GENE IN COS 7 CELLS

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Summary : Cos 7 cells transfected with human atrial natriuretic polypeptide (hANP) gene with SV40 enhancer and replication origin sequences expressed hANP gene. The expressed RNA was indistinguishable from native hANP mRNA and the transcribed protein seemed to be properly processed to α-hANP and β-hANP. This system provides a useful approach to investigate the processing of hANPs and the structure-function relationship of amino acid sequences of hANPs.

Atrial natriuretic polypeptides (ANP), recently purified from several mammalian atria, are potent hormonal modulator in cardiovascular, body fluid, and electrolyte homeostasis(1-4). They are synthesized not only in atria but also in many other tissues including hypothalamus(5,6), pituitary, lung(7), and so on. The cDNAs and genes for ANPs of several mammalian species have been cloned and sequenced(8-13), and the structures of the prepro-ANP have been deduced from them.

Human ANPs (hANP) consist of three distinct components, namely α-, β-, and γ types. The γ-hANP is considered to be a signal-peptide cleaved pre-ANP. The β-hANP is considered to be a circulating form. The β-hANP is an anti-parallel dimer of α-hANP(14,15). However, the precise mechanism of the processing of hANPs remains to be determined. The amino acid sequences of γ-type ANPs are highly homologous among the mammalian species examined, suggesting that some important functions may reside in the amino terminal segment of γ-ANP(16).

To clarify these aspects of hANPs, it would be useful to express hANP gene in mammalian cells. In such a mammalian cell expression system, the directed mutagenesis could elucidate the structure-function relationship of amino acid sequences of a protein and the mechanism of the directed transport and the processing of a protein.

This paper describes a model system of the introduced hANP genes being expressed in mammalian cells.

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Materials and Methods

Human ANP gene

Charon 4A human genomic library was screened with 581-nucleotide SacI-PstI fragment derived from hANP cDNA, which were kindly provided by Nakayama et al(8). A 3.5 kb BamHI-BamHI fragment hybridizing to the cDNA probe was subcloned to pBR322 and identified as the human ANP gene by partial sequencing.

Construction of an expression vector (Fig. 1)

Simian Virus 40 Hind III C fragment and the BamHI-BamHI fragment derived from hANP gene were subcloned to pUC18 in a way that hANP gene sequences were under the control of SV40 early promoter (designated as pSVE-hANP).

Cell culture and transfection

Cos 7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal bovine serum. Transfection was performed according to the procedures described by Maniatis et al(17). Cells were incubated for 48 hr from the transfection.

RNA purification and analysis

Total RNA was extracted as described by Chirgwin et al(18). S_1 nuclease mapping was performed according to Berk et al(19). Briefly, with T_4 polynucleokinase end labelled 316-nucleotide DraI-RsaI fragment derived from hANP gene was incubated with 20 µg sample RNA in 80% formamide, 0.5M NaCl, 1mM EDTA, and 50mM PIPES, pH 6.4 at 42 °C for 3 hr. S_1 nuclease digestion was performed at 40°C for 30 min. with 0.5 unit S_1 nuclease (BRL) per 1 µg total RNA. S_1 resistant DNA-RNA hybrids were denatured and resolved on 7M urea/5% polyacrylamide gel.

RNA blot-hybridization analysis (Northern Blotting)

RNA was denatured with 1M glyoxal/50%(V/V) dimethyl sulfoxide, electrophoresed on a 1.2% agarose gel, and transferred to a Biodyne A membrane (Pall). Hybridization and washing were carried out according to the procedures described Thomas et al(20).

Other DNA manipulations were by standard techniques as described by Maniatis et al(Molecular Cloning, A LABORATORY MANUAL, T. Maniatis, E.F. Fritsch, and J. Sambrook, Cold Spring Harbor Laboratory, 1982).

Immunoprecipitation

Transfected Cos 7 cells (5×10^6) were scraped into 0.5M acetic acid and sonicated for 2 min. (continuous power 4 with Model W-225 Heat System Ultrasonics Inc.) and were centrifuged at 15000 rpm for 30 min. The supernatant was lyophylized and dissolved in 0.05M phosphate buffer saline (PBS) pH 7.4. The extracts were radioiodinated with ^{125}I by the chloramine-T technique(21), and purified by reverse-phase extraction with Sep-Pak C18

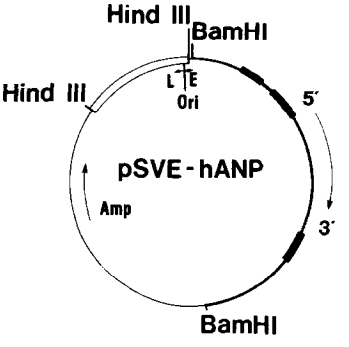


Fig. 1. The construct of pSVE-hANP
A 3.5 kb BamHI-BamHI fragment of hANP gene is oriented under the control of SV40 early promotor. E, Ori, L denote the SV40 early promoter, replication origin, and SV40 late promoter, respectively.

cartridges (Waters Associates). The radioiodinated extracts were recovered from the cartridge with 3 ml of 60% acetonitrile 0.1% fluoroacetic acid, and lyophylized and desolved in 0.5 ml of 0.1% tween 20-0.05 M PBS pH 7.4. One microliter of anti α-hANP sheep serum was added to 200 µl of the resupended extracts with and without 7.5 µg of synthetic α-hANP. After the incubation at 4°C for 18 hr, an excess of anti-sheep IgG IgG (Cappel) was added and incubated at 4°C for 18hr. The immunopreciptitates were centrifuged and extensively washed with 0.1% tween 20-0.05 M PBS pH 7.4. The final pellets were resuspended in sample buffer (50 mM Tris-HCl pH 6.8 2% sodium dodecyl sulfate, 100mM β-mercaptoethanol, 10% glyceol), boiled, and electrophoresed on a $NaDodSO_4$/20% polyacrylamide gel. The antibody against α-hANP was supplied by Professor Colin I. Johnston, University of Melburne, Department of Medicine, Austin Hospital. This antibody mainly recognizes C-terminal of α-hANP and also crossreacts with β- and γ-hANP. As molecular markers, soybean trypsin inhibitor (Sigma 21.5 K), horse cytochrome C (Sigma 12.3 K), pocine insulin (Sigma 3.4 K, 2.3 K) α-hANP (Peptide Institute 3.1 K) were used.

Results and Discussion

Cos cells were reported to have some proteolytic processing enzymes and two separate secretory pathways, namely a constitutive pathway and a pathway regulated by secretagogues(22,23). Thus we used Cos 7 cells as recipient cells.

Fig. 2 shows the RNA blot-hybridization analysis of the transcripts of the transfected Cos 7 cells. Human ANP specific RNA was detected, and its length was almost or completely identical to that of hANP mRNA in human atria. To exclude the possibility that this expressed RNA was cryptic, S_1 nuclease mapping analysis in the promoter region of hANP was performed (Fig. 3). The expressed RNA protected a fragment of 185 nucleotides, mapping in a region completely identical to the initiation site of hANP mRNA in human atria.

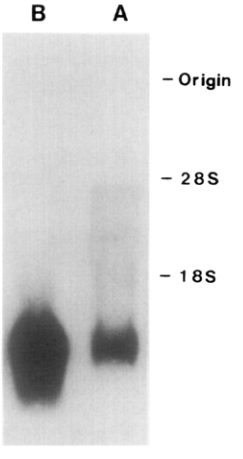
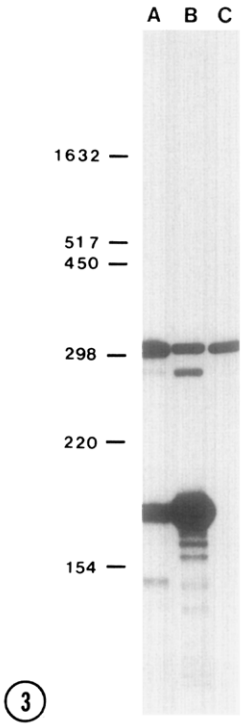


Fig. 2. RNA Blot-hybridization analysis
Ten micrograms of total RNA from the transfected Cos 7 cells (lane A) and human atria (lane B) was size-fractionated on a 1.2% agarose gel, blotted to a Biodyne A membrane, and hybridized to the same probe used in S_1 nuclease mapping analysis. Human ribosomal RNAs were used as size markers.



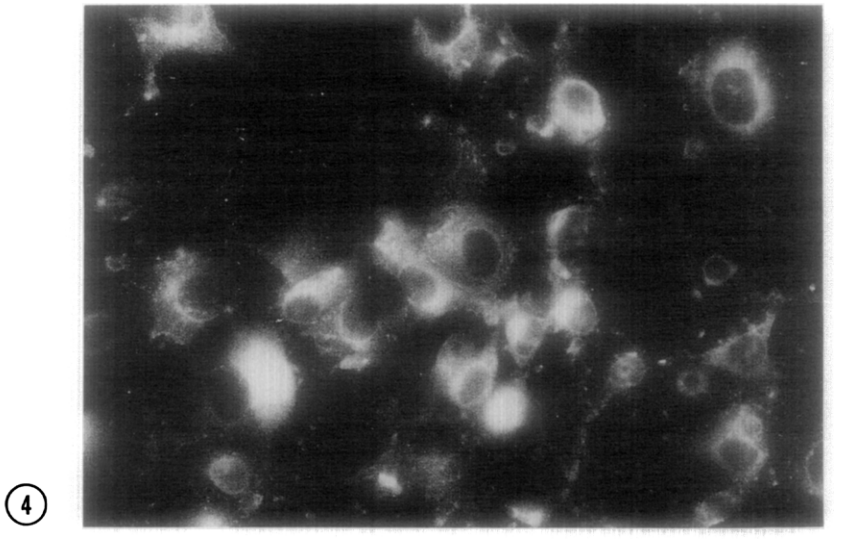


Fig. 3. S_1 nuclease mapping analysis
S_1 nuclease mapping analysis with the use of DraI-RsaI 316 nucleotide fragment derived from human ANP gene. The size markers are given in nucleotide on the side of the autoradiogram. RNAs analyzed are as follows, lane A; total RNA from the transfected Cos 7 cells, lane B; total RNA from human atria, lane C; total RNA from untransfected Cos 7 cells.

Fig. 4. Immunofluoresence study of the transfected Cos 7 cells
The transfected Cos 7 cells were fixed in 10% formalin solution, washed with 0.05 M phosphate buffer saline (PBS) pH 7.4, and then incubated with 1:50 dilution of anti α-hANP sheep sera at 4 °C for 72 hr. Washed with 0.05 M PBS and then stained with 1:50 dilution of FITC(fluorescein isothiocyanate)-labelled goat anti-sheep IgG (Cappel) at room temperature for 30 min.

A fragment of about 140 nucleotides was also protected, though at a low level, by the expressed RNA and the hANP mRNA. This may represent the existence of another physiological initiation site.

Fig. 4 shows the immunofluoresence study of the transfected Cos 7 cells. Human ANP-like immunoreactive material was detected in the cytoplasm with a granular pattern. To clarify the molecular basis of this immunoreactive material, the extracts of the transfected Cos 7 cells were labelled with ^{125}I, treated with anti-hANP antisera with and without cold synthetic α-hANP, and the imunoprecipitates were resolved by $NaDodSO_4$ /20% polycrylamide gel.

Though the competition was not complete, two polypeptides migrating with a Mr 6000 and a Mr 3000 were identified. The latter polypeptide

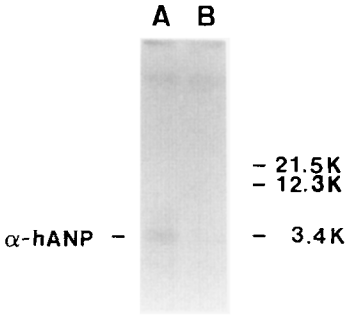


Fig. 5. Immunoprecipitation of hANP-like immunoreactive material
The arrow refers to the position at which synthetic α-hANP migrates in this gel system. Numbers on the left refer to molecular size markers (Mr x 10^{-3}), lane A; immunoprecipitate without synthetic α-hANP, lane B; immunoprecipitate with synthetic α-hANP.

comigrated exactly with the synthetic α-hANP. As β-hANP is an anti-parallel dimer of α-hANP and has a molecular weight of about 6000, the former may be β-hANP(15)(Fig.5).

In conclusion, the Cos 7 cells transfected with pSVE-hANP expressed hANP specific RNA, which was indistinguishable from the native hANP mRNA in human atria, and also expressed hANP-like immunoreactive material that seemed to be properly processed to α-hANP and β-hANP. However, γ-hANP was not detected.

It was reported that the relative amount of γ-ANP to α-, and β-ANP varied among individuals and also amomg mammalian species(24,25). And the high homology of the amino acid sequences of γ-ANPs among mammalian species suggest that some important role may reside in the amino terminal segment of γ-ANPs. By the use of directed mutagenesis and the administration of some biological active substances, our system will provide a useful approach to identify the functions of γ-hANP and the processing mechanism of hANPs.

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