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**C-type Natriuretic Peptide:  
Cell-type Specific Promoter Function and Regulation by a Transcriptional  
Regulation Factor for Transforming Growth Factor- $\beta$ -Induced Gene  
Expression**

**Shigeki Ohta**

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## List of Abbreviations

ANP	atrial natriuretic peptide
AtT20/D16V-F2	mouse anterior pituitary-derived cancer cell line
BNP	brain natriuretic peptide
BSA	bovine serum albumin
CAT	chloramphenicol acyl transferase
CDM	chemically defined medium
CNP	C-type natriuretic peptide
CRE	cAMP responsive element
CREB	CRE binding factor
DMEM	Dulbecco's modified minimum essential medium
DTT	dithiothreitol
FCS	fetal calf serum
FR	rat fibroblast cell line
G3PDH	glyceraldehyde-3-phosphate dehydrogenase
GAL	$\beta$ -galactosidase
GH1	rat anterior pituitary-derived cancer cell line
GH3	rat anterior pituitary-derived cancer cell line
HA	influenza virus hemagglutinin
HAEC	human aortic endothelial cells
HSC-39	human gastric carcinoma cell line
IFN- $\gamma$	interferon- $\gamma$
IL-1 $\beta$	interleukin-1 $\beta$
LPS	lipopolysaccharide
LUC	luciferase
$\alpha$ -MEM	$\alpha$ -minimal essential medium
PAEC	porcine aortic endothelial cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RACE	rapid amplification of cDNA ends
RASMC	rat aortic smooth muscle cells
TGF- $\beta$	transforming growth factor- $\beta$
TNF- $\alpha$	tumor necrosis factor- $\alpha$
TSC-22	TGF- $\beta$ stimulated clone 22

## Summary

The promoter function of human C-type natriuretic peptide (CNP) gene was examined in various cultured cells by transient transfection assays. The CNP promoter functioned very effectively in GH3 cells which originated from the growth hormone-producing tumor of rat anterior pituitary and somatomammotroph phenotype, and much less effectively in GH1 cells, another type of rat pituitary-derived cells with somatotroph phenotype, and rat primary cardiocytes. The CNP promoter did not function at all in other cells, including AtT20 cells of murine pituitary corticotroph origin. Functional analyses of the deleted promoters with various 5' deletion breakpoints revealed the existence of at least two negative and one positive regulatory regions. Within the positive regulatory region (from -54 to -19) which conferred 90% of the promoter activity in GH3 cells, two equipotent GC-rich cis elements (from -49 to -45 and -40 to -35) were identified. Both sites shared half the promoter activity and binding properties to the nuclear protein in GH3 cells. Rat anterior pituitary tissue contained the binding protein of the identified cis element, which was identical or similar to that of GH3 cells (Chapter 2).

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The GC-rich cis elements essential for the constitutive gene transcription in rat GH3 cells were responsible for the stimulation of the CNP promoter activity by transforming growth factor  $\beta$  (TGF- $\beta$ ). To isolate transcription factors that bind to those GC-rich elements, a  $\lambda$ -ZAP cDNA library derived from GH3 cells was screened by Southwestern screening technique. Several positive clones with specific binding ability were obtained, and one was identical to TSC-22, a speculated transcriptional modulator stimulated by TGF- $\beta$  of unknown function. TSC-22 significantly enhanced CNP promoter activity in GH3 cells. A 1.8-kb full-length human TSC-22 cDNA was further cloned from a fetal kidney cDNA library by a combination of polymerase chain reaction and "rapid amplification of the cDNA ends" technique. In adults, human TSC-22 mRNA was

expressed highly in brain, lung, and heart. TSC-22 gene expression in GH3 and human aortic endothelial cells was stimulated by cytokines including TGF- $\beta$ , in correlation with the CNP mRNA increase. These results suggest that TSC-22 is a transcriptional regulator of the CNP gene and transmits signals from cytokines, such as TGF- $\beta$ , for CNP gene expression (Chapter 3).

Human gastric carcinoma cell line, HSC-39, has been shown to undergo apoptotic cell death in response to treatment of TGF- $\beta$ 1. The effect of the expression of TSC-22 on the apoptosis of this cell line was investigated as one of other TGF- $\beta$ -mediated cell events. TGF- $\beta$ 1 induced TSC-22 gene expression in HSC-39 cells only when the cells were previously adapted to the serum-free culture conditions required for undergoing TGF- $\beta$ 1-mediated apoptosis. HSC-39 cells transfected with a TSC-22 expression vector showed significant decrease in cell viability compared with those transfected with a control empty vector. The cellular events characteristic of apoptosis, chromatin condensation and DNA fragmentation, were observed only in cells transfected with a TSC-22 expression vector. In immunostaining of transfected cells, almost every cell which expressed TSC-22 tagged with influenza virus hemagglutinin exhibited the morphology of apoptotic cell. Thus, TSC-22 elicited apoptotic cell death of human gastric carcinoma cells probably mediating TGF- $\beta$ 1 signaling pathway to apoptosis (Chapter 4).

## Chapter 1. Introduction

Identification of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) in mammalian heart and brain has opened the possible presence of a natriuretic peptide family that is involved in the control of cardiovascular homeostasis [1-3]. C-Type natriuretic peptide (CNP) is the third member of the mammalian natriuretic peptide family. CNP was first isolated from porcine brain as two peptide forms, CNP-22 with 22 amino acid residues and CNP-53 with 53 residues [4, 5], and then identified in rat and human brains [6, 7]. According to the cloned cDNA and genes for CNP from various species [6-10], the structure of CNP is highly conserved among species, and rat, porcine and human CNP-22 are actually identical. Human CNP is synthesized as a precursor form (126-residues) and processed further to secreted form CNP-53 and CNP-22. It is likely that CNP-22 is generated from CNP-53 by Kex2/furin-type processing enzyme as in many processing pathways of hormones because of the existence of double basic residues (K-K) at the processing site. The actual processing mechanism, however, has not been investigated yet.

CNP seems to exert pharmacological actions similar to atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), including natriuretic, diuretic and vasorelaxant effects [9], albeit with a lesser potency. While ANP and BNP are mainly cardiac hormones [11], CNP is distributed throughout the human brain in concentrations at least one order of magnitude higher than the ANP and BNP concentrations [7]. At the very beginning of CNP research, it was demonstrated that CNP localized in the brain and very little was detected in other peripheral tissues including the heart [6, 7, 12]. The receptor specific to CNP (ANP-B receptor) is also shown to exist in the brain [13]. Therefore, CNP is considered as the major natriuretic peptide in the central nervous system with functions distinct from ANP and BNP, for example, as a neuromodulator.



The actual physiological function of CNP, however, was unclear, when I started this study.

To understand the actual physiological functions of CNP, it is very important to clarify which organs produce CNP molecules and how the CNP gene expression is regulated in those organs, for example, tissue or cell-type specific expression, inducing or repressing mechanism, and developmental regulation. The main purpose of this study is to understand the mechanism of the CNP gene expression. To achieve this purpose, the cell-type specific promoter function of human CNP gene and its stimulation by cytokines, such as transforming growth factor- $\beta$  (TGF- $\beta$ ) or interferon- $\gamma$  (IFN- $\gamma$ ), were extensively investigated. Clarification of the gene expression mechanism must be of great help to elucidate the physiological roles of CNP and its relation to some diseases. The novel findings that the constitutive expression of the CNP promoter activity exhibits cell-type specificity and the highest promoter activity is achieved in the anterior pituitary-derived cell line (GH3) of a somatomammotroph origin are described in Chapter 2. The results of detailed analyses of the CNP promoter including the identification of cis regulatory elements required in the constitutive promoter activity in GH3 cells and the analysis of a nuclear protein that may be responsible for the constitutive expression of CNP gene are also presented in Chapter 2. These findings suggest the production of CNP molecules in the anterior pituitary gland and its possible role in the regulation of the pituitary function. According to this suggestion, one of my colleagues succeeded in demonstrating that CNP stimulated the growth hormone release from GH3 cells as well as primary cultured cells prepared from anterior lobes of rat pituitary gland through the cGMP-mediated pathway [14].

In addition to the function in the pituitary system, recent studies demonstrated the possible role of CNP in the vascular systems. CNP was reported to be secreted from cultured vascular endothelial cells in response to some stimulants, such as TGF- $\beta$  and lipopolysaccharide (LPS)[15, 16]. TGF- $\beta$  has an important role in the vascular systems, such as atherosclerosis and angiogenesis [17- 19]. Therefore, clarification of the

mechanism of the CNP gene expression stimulated by TGF- $\beta$  may lead to understanding the role of CNP in vascular diseases. For this purpose, I tried to identify a factor responsible for the induced expression of the CNP gene caused by TGF- $\beta$  treatment. In chapter 3, I refer the molecular cloning and the characterization of a transcriptional regulatory factor that is involved in the TGF- $\beta$ -induced expression of the CNP gene. The cloned factor is identical to TSC-22 (TGF- $\beta$  stimulated clone 22) of unknown function. Therefore, this is the first evidence on the actual function of TSC-22 as a transcriptional regulatory factor. In addition to the role as a transcriptional regulatory factor for the CNP gene expression, TSC-22 may participate in other function of TGF- $\beta$  because of the rather wide tissue distribution of its mRNA. To elucidate this point, the involvement of TSC-22 in the TGF- $\beta$ -mediated apoptosis in human gastric carcinoma cell line HSC-39 is described in Chapter 4. TSC-22 gene expression in HSC-39 cells was induced by TGF- $\beta$ 1, and there was a good correlation between the ectopic expression of TSC-22 in HSC-39 cells and the exhibition of apoptotic cell events. Thus, the evidence supporting that TSC-22 may mediate the cell death signal caused by TGF- $\beta$  is presented for the first time.

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## **Chapter 2. Cell-type specific function of C-type natriuretic peptide gene promoter and identification of its regulatory region**

### **2-1. Introduction**

CNP was first isolated from porcine brain as two peptide forms, CNP-22 with 22 amino acid residues and CNP-53 with 53 residues [1, 2], and then identified in rat and human brains [3, 4]. CNP shares considerable sequence homology with ANP and BNP especially within the 17-residue ring portion formed by a disulfide bond between two cysteine residues. According to the cloned cDNA and genes for CNP from various species [3-7], the structure of CNP is highly conserved among species, and rat, porcine and human CNP-22 are actually identical.

CNP seems to exert pharmacological actions similar to ANP and BNP, including natriuretic, diuretic and vasorelaxant effects [6], albeit with a lesser potency. Though the vasorelaxant activity of CNP in isolated rat aorta is 16-fold lower than that of ANP and the natriuretic activity of CNP in rat is 100-fold less potent than other peptides [1], CNP is a more effective stimulator of cGMP generation in cultured vascular smooth muscle cells [8] and may be a more potent growth inhibitor of these cells [9]. From that point, the growth inhibition of vascular smooth muscle cells was proposed as a pharmacological function of CNP, but CNP at 1  $\mu\text{M}$  (much higher concentration than the physiological CNP concentration) inhibited cell growth only by 25% and  $\text{EC}_{50}$  could not be calculated [9]. Therefore, actual biological functions of CNP are still unclear. ANP- and BNP-like immunoreactivities were observed mainly in heart [10], and ANP is secreted from atrium and BNP is secreted from ventricle [11], therefore ANP and BNP are cardiac hormone. CNP is localized mainly in the brain [3, 4, 12]. Tissue distribution pattern of CNP suggests that it may exert unique function(s) distinct from ANP and BNP.

Most of the biological actions of natriuretic peptides are thought to be mediated by two guanylyl cyclase-linked receptor subtypes with different ligand selectivities (ANP-A and ANP-B receptors) [13, 14]. The ANP-A receptor is selectively activated by ANP and BNP, while it is activated by CNP less effectively. A receptor specific for CNP has been designated the ANP-B receptor [15] and shown to exist in the brain and pituitary gland [16, 17]. Therefore, it is possible that CNP functions in a manner distinct from ANP and BNP, for example, as a neuromodulator. In fact, CNP is suggested to have some actions on the rat central nervous system [18-20]. In rat, CNP exists in pituitary gland at the highest level in the anterior lobe of all tissues tested [3], although the origin of CNP in the pituitary is still unknown. ANP-B receptor specific to CNP was also demonstrated to exist in pituitary gland [17]. These findings suggest that CNP may play an important role in the anterior pituitary in addition to the central nervous system. When I started this study, detection of CNP mRNA in rat hypophysis and hypothalamus by Northern analysis or by polymerase chain reaction (PCR) were unsuccessful, probably because rat CNP mRNA contains four copies of the AUUUA sequence, the signal for the destabilization of mRNA molecules [21], in the 3'-untranslated region. This makes it difficult to find exactly where the CNP gene expression occurs, for example, by *in situ* hybridization. To understand the actual physiological functions of CNP, clarification of the mechanism regulating its gene expression, for example, tissue or cell-type specific expression, inducing or repressing mechanism, and developmental regulation should be of great help. In this chapter, the cell-type specific function of human CNP gene promoter and identification of its regulatory region are presented.

## 2-2. Materials and methods

### 2-2-1. Genomic Southern analysis

Human genomic DNA (Clontech Laboratories) completely digested with restriction enzymes (NotI, NcoI or PvuII) was analyzed by 0.7% agarose gel electrophoresis (20 µg per lane), transferred onto a Biotrans nylon filter (Pall Biosupport), and then hybridized overnight at 65 °C in 0.5 M sodium phosphate (pH 7.5), 10 mg/ml bovine serum albumin (BSA), 1 mM EDTA and 7% SDS with a <sup>32</sup>P-labeled probe of 61 bp BamHI-NotI fragment from human CNP gene [4, 7]. The filter was washed three times in 1xSSPE (0.15M NaCl, 0.01 M sodium phosphate and 1 mM EDTA, pH 7.7) plus 0.1% SDS at 50 °C.

### 2-2-2. Cloning of 5'-flanking (FK) region of human CNP gene

The 5'-FK region up to the BamHI site 309 nt upstream of the ATG codon was obtained as in [4]. To clone a region further upstream, 40 µg of human genomic DNA was completely digested with NotI, separated by 1.0% low melting agarose gel electrophoresis. Digested DNA at the position of 1.3 - 1.5 kb was recovered from the gel slice, and ligated with 100 ng Bluescript SK(-) (Stratagene) previously digested with NotI and treated with bacterial alkaline phosphatase. *E. coli* strain DH5α was electrically transformed with the ligated DNA in a Gene Pulser apparatus (Bio Rad Laboratories) to make a plasmid mini library. Positive colonies were selected by colony hybridization [22] under similar conditions to Southern analysis. The DNA sequence of the cloned fragment (1.4 kb NotI-NotI) was determined by dideoxy chain termination method [23] with a Sequenase sequencing kit (USB).

### *2-2-3. Construction of plasmids with chloramphenicol acyl transferase (CAT) and $\beta$ -galactosidase (GAL) genes*

The 212 bp BamHI-PstI fragment (PstI site 97 nt upstream of ATG codon) was subcloned in Bluescript SK (pBSBP1). Then, pBSBP1 was cut with NotI to excise the 61 bp BamHI-NotI fragment at the 5' end, and ligated with 1.4 kb NotI-NotI fragment to yield pBSNP1. The 1,510 bp NotI-PstI fragment of pBSNP1 and the 212 bp BamHI-PstI fragment of pBSBP1 were put into the cloning site of pUCOCAT [Fig. 2-2, a promoterless CAT plasmid with a multicloning site and an additional SV40 poly(A) signal just upstream of CAT gene, a kind gift from Dr. M. Takeichi, Kyoto University] to make reporter plasmids CNP12CAT and CNP6CAT, respectively (Fig. 2-2). Other reporter plasmids with various 5' deletion breakpoints (Fig. 2-6A) were made by exonuclease III limited digestion (CNP11CAT, CNP10CAT and CNP9CAT), by excision of fragments using BamHI, ApaLI, NotI, SmaI sites (CNP8CAT, CNP7CAT, CNP5CAT and CNP4CAT, respectively), or by PCR with synthetic primers and CNP6CAT as a template (CNP3CAT, CNP2CAT and CNP1CAT). Reporter plasmids with substituted mutations (CNPM1CAT - CNPM6CAT in Fig. 2-7) were also made by PCR. To make ANPCAT (Fig. 2-7), human ANP gene fragment (from -465 to +100 [24]) was amplified from genomic DNA by PCR and subcloned in pUCOCAT. For C/ACAT, the chimeric promoter between CNP and ANP was first prepared by ligation of 14 synthetic single-strand oligonucleotides (30- to 40-mer) to Bluescript, and then subcloned in pUCOCAT. 2C/ACAT was prepared by insertion of another synthetic double-strand oligonucleotide at the 5' end of the chimeric promoter in C/ACAT with a linking sequence of 5'-GATC-3' to make an additional BamHI site. Construction of BNPCAT with 1.8 kb human BNP promoter (Fig. 2-7) was as shown in [25]. All CAT reporter plasmids were verified by sequencing. Raus sarcoma virus long terminal repeat (RSV LTR)-driven GAL reporter plasmid, pRSVGAL, was constructed by replacing CAT gene of pRSVCAT (American Type Culture Collection No.37152) with bacterial GAL gene. Human elongation factor 1 $\alpha$  promoter-driven GAL reporter plasmid, pEFGAL, was



obtained by replacing stuffer sequence of pEF-BOS (a kind gift from Dr. S. Nagata, Osaka Bioscience Institute [26] ) with GAL gene.

#### *2-2-4. Cell cultures*

All established cell lines were from the American Type Culture Collection. Rat pituitary-derived cells, GH3 and GH1, were cultured in Ham's F10 medium (Flow Laboratories) supplemented with 15% horse serum and 2.5% fetal calf serum (FCS). Mouse pituitary-derived AtT20/D16V-F2 and rat fibroblast FR were cultured in Dulbecco's modified minimum essential medium (DMEM, Gibco Laboratories) plus 10% FCS. Rat aortic vascular smooth muscle cells (RASMC) were prepared by explant method as in [27] and grown in DMEM with 10% FCS. Porcine aortic endothelial cells (PAEC) were prepared as in [28] and cultured in medium 199 (Gibco Laboratories) with 20% FCS. Primary cardiocytes were enzymatically dispersed from ventricle-rich portions of hearts of neonatal (1-2 day old) Sprague-Dawley rats, purified with a Percoll (Pharmacia) density gradient system according to Jones *et al.* [29], and cultured in DMEM with 10% FCS without L-glutamine. All cultures were at 37 °C under 5% CO<sub>2</sub> atmosphere.

#### *2-2-5. Transient cell transfection*

Cells were seeded in a 10-cm dish at a density that gave 10-20% confluency on the next day. After 18-24 h, cells were transfected with 25 µg of each test plasmid and 5 µg of GAL expression plasmid (pRSVGAL in a series of experiments shown in Table 2-1 or pEFGAL in other experiments) according to the calcium phosphate protocol of a Mammalian Transfection kit (Stratagene). For cardiocytes, freshly prepared  $5 \times 10^6$  cells were immediately incubated with DNA-CaPO<sub>4</sub> co-precipitates in a 10-cm dish. After 18-22 h, the cells were gently washed twice with phosphate-buffered saline (PBS) and cultured again in the fresh medium. After 48 h, the cells were washed twice with PBS, collected in a microcentrifuge tube with a rubber policeman, and disrupted by four freeze-

thaw cycles in 0.1 ml 0.25 M Tris-Cl (pH 7.5). After centrifugation for 10 min, the supernatant was pooled to measure CAT and GAL activities.

#### *2-2-6. Assays for CAT and GAL activities*

CAT activity was measured according to Amersham's protocol using D-threo-[dichloroacetyl-1-<sup>14</sup>C] chloramphenicol (Amersham) as a substrate in 0.25 M Tris-Cl (pH 7.5) buffer with a 10-50  $\mu$ l cell extract. Thin-layer chromatography was performed on a Chromagram sheet (Kodak) in chloroform/methanol (94/6). After exposure to X-ray film, areas for non-acetylated and acetylated chloramphenicol were cut out of the sheet for liquid scintillation counting to calculate % acetylation. Linearity existed at least up to 20% acetylation in this assay. GAL activity was measured according to the method in [30] using *o*-nitrophenol- $\beta$ -D-galactopyranoside as a substrate in sodium phosphate buffer (0.1 M, pH 7.5) with 10-30  $\mu$ l cell extract. After incubation for an appropriate period, 1 M Na<sub>2</sub>CO<sub>3</sub> was added to stop the reaction and the optical density at 420 nm was measured. The amount of CAT activity giving 1% acetylation in 1 h and that of GAL activity causing 1.0 OD unit increase in 1 h under these conditions are defined as one unit (U) in this study. All values were presented after the subtraction of back ground values obtained with extracts from mock transfected cells.

#### *2-2-7. Determination of transcription start site*

To determine the transcription start site within the CNP promoter, primer extension and riboprobe mapping were carried out using total RNA recovered from GH3 cells at 24 h after transfection with CNP6CAT reporter DNA. Primer extension was done with a kit from Promega. The <sup>32</sup>P-labeled primer 1 (5'-CTCAAATGTTCTTTACGAT-3'; 500,000 cpm) was hybridized with 50  $\mu$ g of total RNA, and extended with reverse transcriptase. The primer extension product was analyzed by 7 M urea/5% polyacrylamide gel electrophoresis together with dideoxy sequencing ladders of CNP6CAT DNA with the same primer to determine the position of the product. Riboprobe mapping was done with

an RPA II kit from Ambion. BamHI-PvuII fragment (375 bp) from CNP6CAT which covered 212 bp of the 5'-FK region of the CNP gene, 14 bp of Bluescript multicloning site and 149 bp of the CAT gene was subcloned in the NotI-XhoI site of Bluescript (the XhoI site was blunted by a fill-in reaction with Klenow fragment), cut with BamHI and transcribed *in vitro* with T7 RNA polymerase to yield a 404 nt <sup>32</sup>P-labeled riboprobe (Fig. 2-5). Total RNA (10 µg) was hybridized with the riboprobe (400,000 cpm) and digested with 100-fold diluted RNase solution. The protected product was analyzed by 7 M urea/5% polyacrylamide gel electrophoresis with sequencing ladders of CNP6CAT DNA with primer 2 (5'-CCGTCTTTCATTGCCATACG-3') which starts at 125 nt downstream of the PvuII site (the expected starting position of the RNA-RNA hybrid) to determine the length of the product.

#### 2-2-8. Gel retardation assays

Oligonucleotides were synthesized with a DNA synthesizer model 391 (Applied Biosystems Inc.) and purified by high performance liquid chromatography with a TSK-Gel DEAE-2SW column (Toyo Soda). Purified oligonucleotides were annealed to make double-strand probes. Synthetic double stranded DNA probes were A (from -55 to -18 in Fig. 2-6B, 5'-CCGGGCGGCCCGGTGGGCGGGAGGATGACATCAGCGGC-3'), A1 (from -55 to -39, 5'-CCGGGCGGCCCGGTGGG-3'), A1M (mutated A1, 5'-CCGGCGAAACGGTGGG-3'), A2 (from -41 to -18, 5'-GGGCGGGAGGATGACATCAGCGGC-3'), A2' (from -41 to -27, 5'-GGGCGGGAGGATGAC-3'), A2'M (mutated A2', 5'-GGAAGAAAGGATGAC-3') and B (from -17 to +7, 5'-AGGTTGGATTATAAAGGCGCGAGC-3'). Consensus oligonucleotides for SP-1 (5'-ATTCGATCGGGGCGGGGCGAGC-3'), AP-2 (5'-GATCGAACTGACCGCCCGCGGCCCGT-3') and cAMP-responsive element (CRE) (5'-AGAGATTGCCTGACGTCAGAGAGCTAG-3') were from Promega. Oligonucleotides for probes were labeled with  $\gamma$ -<sup>32</sup>P ATP and T4 polynucleotide kinase. Crude nuclear extracts from cultured cells and from pituitary anterior lobes of adult Sprague-Dawley rats were prepared essentially following Dignam

*et al.* [31] and Schreiber *et al.* [32], respectively. Finally, extracts were dialyzed against 20 mM HEPES, pH 7.9, 20% glycerol, 20 mM KCl, 2 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethane sulfonyl fluoride for 5 h, and were frozen in aliquots at -80 °C. The optimized conditions for the binding reaction were 10 mM Tris-Cl (pH 8.0), 10% glycerol, 0.05% Nonidet P-40, 1.25 mM DTT, 50 mM NaCl, 1 mM EDTA and each reaction mixture (20 µl) contained 2.5 µg poly(dI-dC)•poly(dI-dC) (Pharmacia), <sup>32</sup>P-labeled probe (ca. 10,000 cpm) and 1-10 µg protein of crude nuclear extract. DNA-protein interaction was analyzed in 5% polyacrylamide gel running in 7 mM Tris-Cl (pH 7.5), 1 mM EDTA, 3 mM sodium acetate.

#### 2-2-9. Southwestern analysis

Crude nuclear extracts (20 µg protein each) pretreated with 1% SDS and 2.5% 2-mercaptoethanol for 10 min at room temperature was separated by SDS-10% polyacrylamide gel electrophoresis and blotted onto a nitrocellulose filter. The filter was incubated in the binding buffer (20 mM Tris-Cl, pH 8.0, 50 mM NaCl, 0.05% Nonidet P-40, 1.25 mM DTT, 1 mM EDTA, 10% glycerol) containing 5% nonfat dried milk for 1 h at 4 °C, then in the same buffer containing sonicated salmon sperm DNA (10 µg/ml) and <sup>32</sup>P-labeled probe (2 x 10<sup>6</sup> cpm in 10 ml) for 2 h at 4 °C. After washing three times for 5 min at 4 °C in the binding buffer, the filter was allowed to expose an X-ray film. Rainbow marker (Amersham) was used as a size marker.

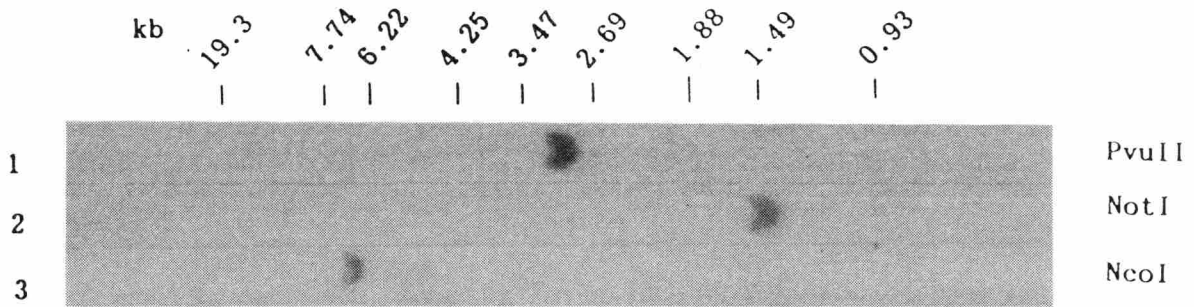
## 2-3. Results

### 2-3-1. Cloning of 5'-FK region of human CNP gene

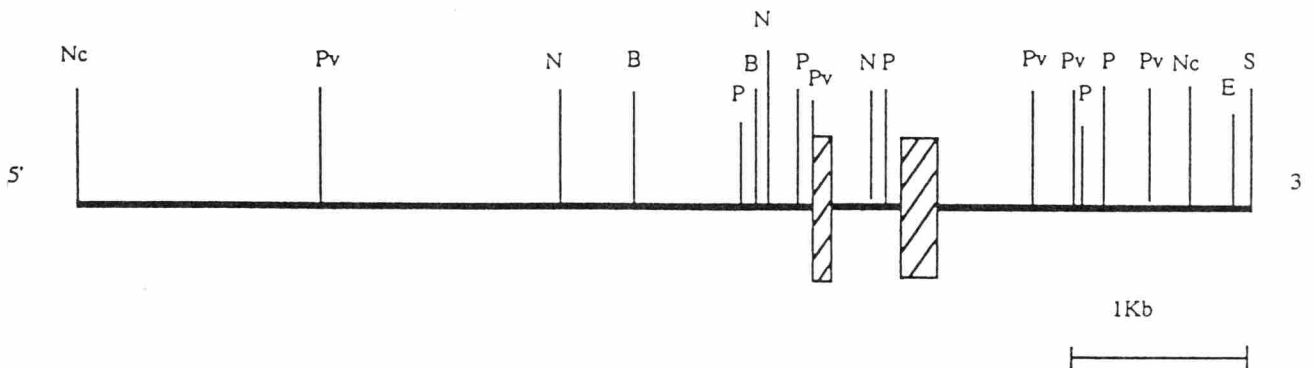
Cloning of the 5'-FK region up to the BamHI site 309 nt upstream of the ATG codon has already been reported [4]. Genomic Southern analysis with 61 bp BamHI-NotI probe showed single bands at 6.7 kb, 3.0 kb and 1.4 kb for NcoI, PvuII and NotI digestions, respectively (Fig. 2-1A). As the nearest sites from the reported 5' end (the BamHI site) are 2,401 nt for NcoI, 322 nt for PvuII and 61 nt for NotI, bands which appeared in Southern analysis indicate the existence of NcoI, PvuII and NotI sites 4.3 kb, 2.7 kb and 1.4 kb upstream of the BamHI site, respectively (Fig. 2-1B).

Therefore, to clone a region further upstream, a plasmid mini library consisting of 1.3-1.5 kb NotI-NotI fragments of genomic DNA was constructed. Two positive clones were selected from approximately 20,000 clones by colony hybridization. The cloned 1.4 kb NotI-NotI fragment was then linked to 151 bp NotI-PstI fragment in Bluescript to make pBSNP1 which contained 1,510 bp NotI-PstI fragment of the 5'-FK region of human CNP gene.

(A)



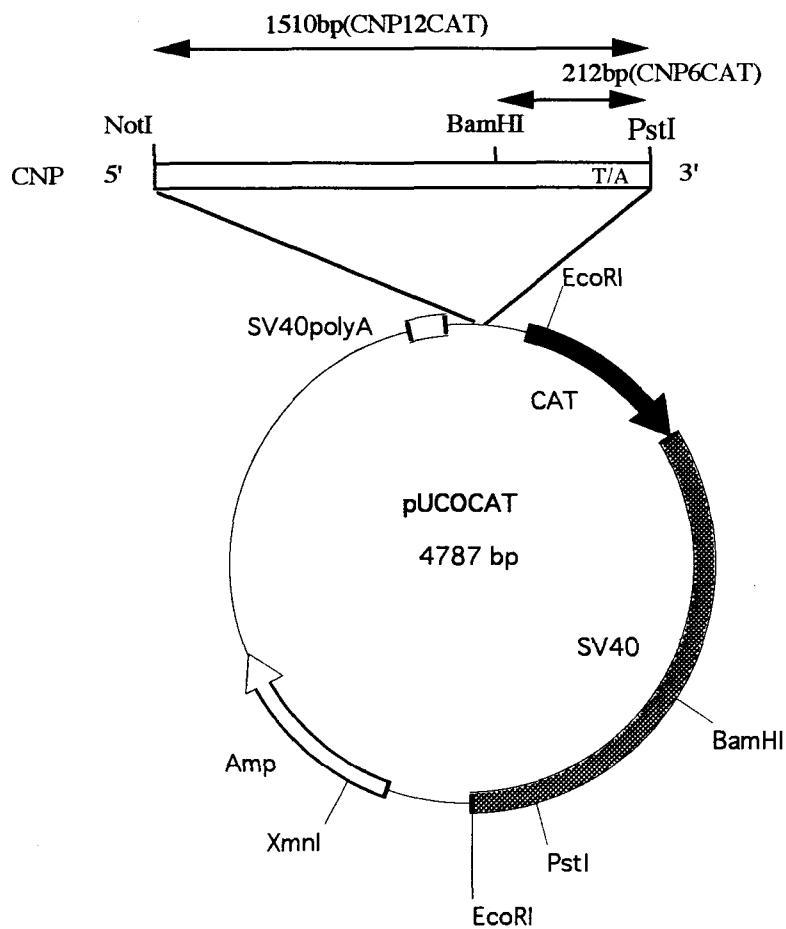
(B)



**Fig. 2-1. Genomic Southern analysis of human DNA with a CNP probe.** (A) Human genomic DNA (20  $\mu$ g per lane) completely digested with PvuII (lane 1), NotI (lane 2) or NcoI (lane 3) was separated with 0.7% agarose electrophoresis, blotted onto a nylon filter and then hybridized with a  $^{32}$ P-labeled 61 bp BamHI-NotI probe of human CNP gene. (B) Restriction map of the human genomic DNA region containing CNP gene. Hatched boxes represent two coding regions for CNP. B, BamHI; E, EcoRI; N, NotI; Nc, NcoI; P, PstI; Pv, PvuII; S, Sall.

### 2-3-2. Cell-specific expression of CNP promoter-driven CAT reporter gene

The cloned 1,510 bp 5'-FK region contained a TATAA box-like sequence at 80 nt upstream of the PstI site (Fig. 2-2). Although the transcription start site had not been identified, 1,510 bp NotI-PstI and 212 bp BamHI-PstI fragments were subcloned in pUCOCAT (CNP12CAT and CNP6CAT, respectively).



**Fig. 2-2. Structure of CNP promoter-CAT reporter construct.** A 1,510 bp NotI-PstI fragment (CNP12CAT) or a 212 bp BamHI-PstI fragment (CNP6CAT) was subcloned in the multicloning site of the parent plasmid, pUCOCAT, in the right orientation.

Three pituitary cell lines (GH3, GH1 and AtT20/D16V-F2), three cultured cells of cardiovascular origins (rat cardiocytes, RASMC and PAEC) and a rat fibroblast cell line (FR) were transfected with these reporter plasmids to examine the cell-specific activity of the CNP promoter. Table 2-1 shows the quantitative comparison of CNP promoter activities in the respective cells. The results of the CAT assay showed that the CNP promoter worked more effectively in GH3 cells than in any other cells tested. The CNP promoter also worked in cardiocytes and GH1 cells, but less effectively than in GH3 cells. The values for GAL activity derived from the co-transfected reference plasmid, pRSVGAL, showed wide variation between cell lines, indicating that transfection efficiency or RSV LTR promoter activity or both differed among the respective cell lines. Therefore, absolute CAT activity values (U/ml) as well as values after normalization by GAL activity (CAT/GAL) were taken into account. GH3 cells gave 15-25 times higher absolute CAT activity than GH1 cells and cardiocytes, and at least 200 times higher values than other cells. For CAT/GAL, GH3 gave 15-30 times higher values than GH1 and cardiocytes, and at least 300 times higher values than others. In another pituitary-derived cell line, AtT20/D16V-F2 (corticotroph origin), CNP6CAT showed 20 times higher CAT activity than promoterless pUCOCAT. This suggests that the CNP promoter may also be active in AtT20 cells, but the efficiency is much lower than that in GH3 cells. These results indicate that the CNP promoter can be active in GH3 cells which are of a somatomammotroph or somatotroph origin in a cell-specific manner. On the other hand, BNP and ANP promoters worked well in primary cardiocytes but did not in GH3 cells, showing consistency with the tissue localization of their mRNAs.



**Table2-1. Cell-type specific function of the CNP promoter**

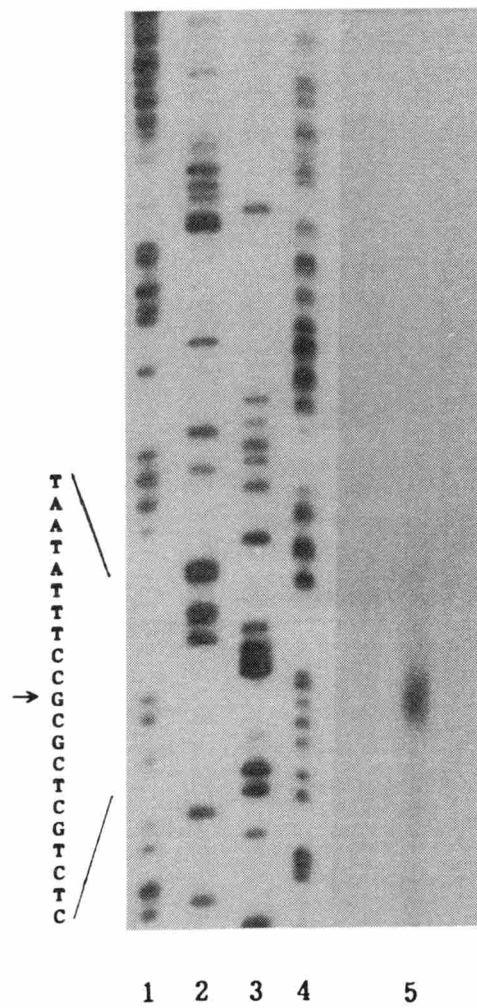
Cell type	Cell-type specific fuction (U/ml) of CNP promoter with reporter construct <sup>a</sup> :				
	pUCOCAT	CNP6CAT	CNP12CAT	BNPCAT	ANPCAT
GH3					
CAT	2.08±0.05	745±137	225±63	8.00±0.71	2.32±0.17
GAL	0.46±0.10	0.78±0.17	1.31±0.65	2.24±0.03	1.80±0.38
CAT/GAL	1.30±0.08	1100±40	188±50	3.58±0.27	1.33±0.38
GH1					
CAT	0.28±0.04	40.3±2.8	15.4±1.4	ND <sup>b</sup>	ND
GAL	0.37±0.01	1.42±0.01	1.71±0.03	ND	ND
CAT/GAL	0.22±0.33	28.5±1.8	9.00±0.70		
AtT20/D16V-F2					
CAT	0.18±0.04	3.74±0.14	0.60±0.07	ND	ND
GAL	5.00±0.02	4.55±0.08	4.91±0.02	ND	ND
CAT/GAL	0.04±0.01	0.82±0.2	0.12±0.02		
Cardiocytes					
CAT	0.62±0.03	31.4±4.2	10.0±1.4	344±28	44.0±5.7
GAL	0.21±0.06	0.42±0.07	0.66±0.13	0.45±0.06	0.86±0.08
CAT/GAL	0.78±0.30	74.3±5.0	15.2±0.8	767±38	50.8±1.5
RASMCs					
CAT	2.90±0.42	1.80±0.28	0.60±0.14	ND	ND
GAL	0.90±0.10	1.09±0.03	1.21±0.07	ND	ND
CAT/GAL	3.12±0.12	1.65±0.0	0.50±0.08		
PAECs					
CAT	0.25±0.06	0.28±0.02	0.35±0.06	ND	ND
GAL	0.31±0.01	0.43±0.04	0.58±0.02	ND	ND
CAT/GAL	0.80±0.8	0.63±0.1	0.53±0.10		
FR					
CAT	1.20±0.14	1.11±0.16	1.08±0.14	ND	ND
GAL	2.56±0.08	2.88±0.17	3.04±0.14	ND	ND
CAT/GAL	0.47±0.05	0.39±0.04	0.35±0.02		

<sup>a</sup>Values are means ± standard deviation(n=2 to ~5).

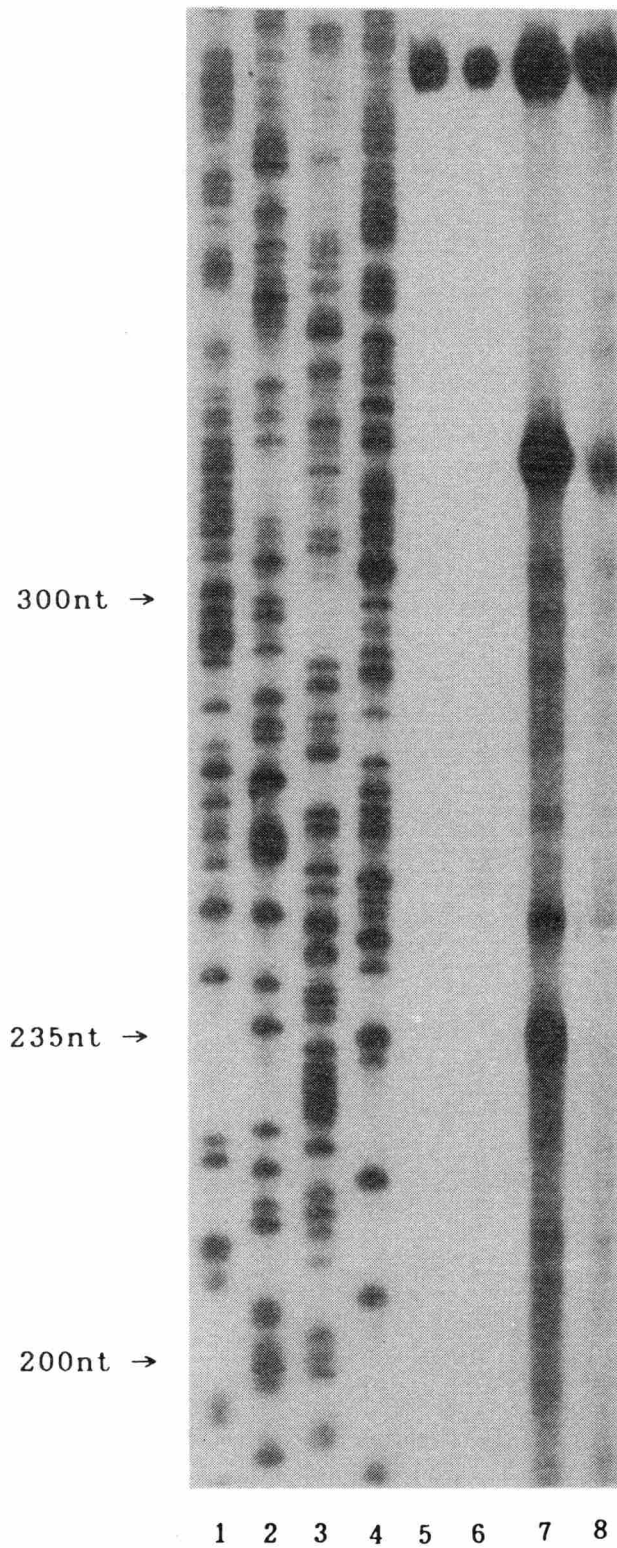
<sup>b</sup>ND, not done.

### *2-3-3. Determination of transcription start site of CNP gene*

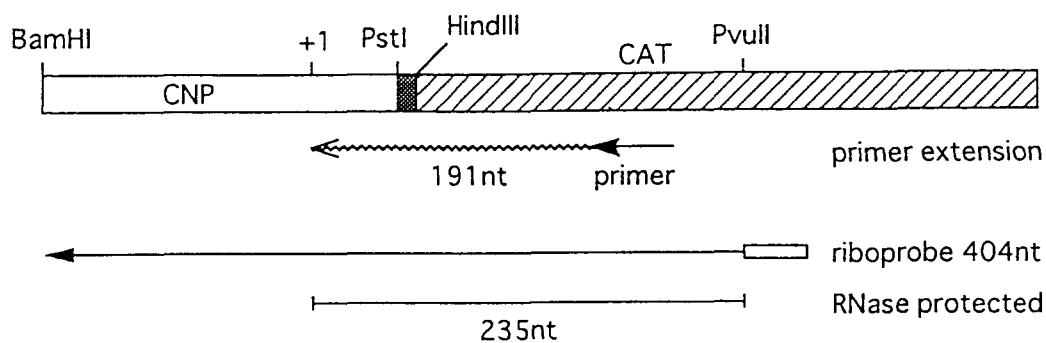
As the numbering of the 5'-FK sequence is conventionally from the transcription start site (+1), the +1 position in human CNP promoter was examined. Because of the difficulty in detecting CNP mRNA in human tissues, the chimeric gene (CNP6CAT) that contained human CNP promoter region and CAT structural gene was transfected into GH3 cells for RNA analysis. The primer extension experiment showed a single position 72 nt upstream of the PstI site of the 3' end of the CNP gene portion (Fig. 2-3, lane 5). This result was further confirmed by riboprobe mapping (Fig. 2-4). Total RNA from transfected cells showed a protected band of 235 nt long (lane 7) which was not seen in the control experiment with RNA from mock transfected cells (lane 8), coinciding with the position shown by the primer extension experiment (Fig. 2-5). Therefore, the transcription start site was determined as shown in Fig. 2-6B. The TATAA box-like sequence of human CNP promoter starts at -8 position in this case, while TATAA box usually locates at around -30 position in many eukaryotic promoters. This result may suggest that the TATAA-like sequence of the CNP promoter does not work as a typical TATAA box at least in GH3 cells.



**Fig. 2-3.** Determination of the transcription start site of CNP promoter by primer extension of total RNA from GH3 cells transfected with CNP6CAT. Lanes 1-4 show dideoxy sequencing ladders (G, A, T and C, respectively) with CNP6CAT DNA as a template and the same primer as extension. Lane 5 shows the extended pattern and the position assigned is shown in the left with an arrow.



**Fig. 2-4. Determination of the transcription start site of CNP promoter by riboprobe mapping of total RNA from GH3 cells transfected with CNP6CAT.** Lanes 1-4 show sequencing ladders (G, A, T and C, respectively) of CNP6CAT with primer 2. Lanes 5 and 6, untreated riboprobe. Lane 7, RNase-protected pattern with total RNA from transfected cells. Lane 8, pattern with total RNA from mock transfected cells as a control. The position assigned (235 nt long) is shown in the left with an arrow.

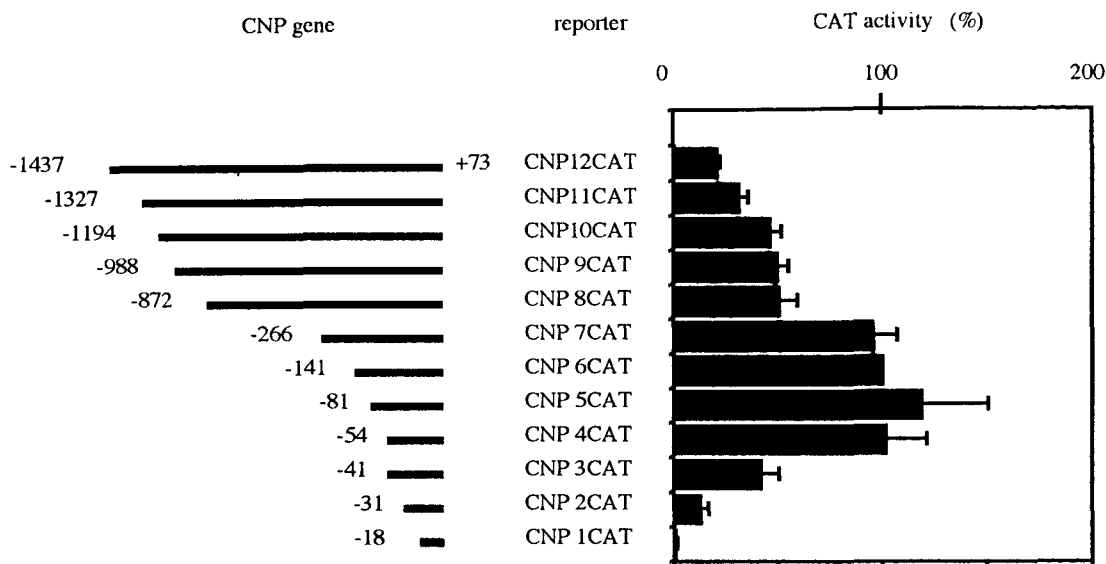


**Fig. 2-5.** Structure of chimeric reporter gene and positions of extended and protected sequences shown in Figs. 2-3 and 2-4.

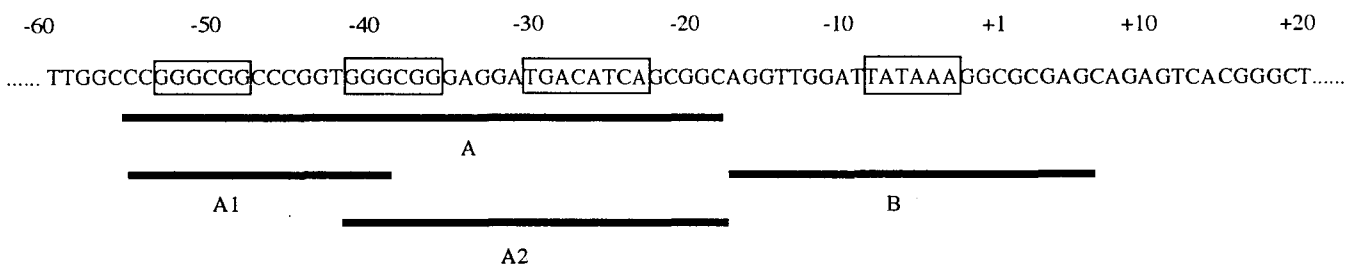
#### *2-3-4. Identification of negative and positive regulatory regions*

CNP promoter CAT constructs with different 5' deletion breakpoints were transfected into GH3 cells for functional analyses. The results (Fig. 2-6A) showed the existence of at least two negative regulatory regions (from -872 to -267 and from -1,437 to -1,195) and one positive regulatory region (from -54 to -19). Among those regions, characterization of the positive regulatory region was our priority. As shown in Fig. 2-6B, the positive regulatory region contained two GC box sequences (5'-GGGCGG-3', a core sequence of SP-1 binding site) and a CRE-like sequence (consensus CRE core sequence is 5'-TGACGTCA-3'). Deletion of one GC box (CNP3CAT) and two GC boxes (CNP2CAT) caused 60% and 90% decrease of activity, respectively, and deletion of GC boxes and the CRE-like sequence led to a 99% decrease. CRE sequences have been identified in the promoter regions of several genes expressed in a pituitary-specific manner including a transcription factor Pit-1/GHF1 [33-35]. No significant increase of CAT activity was observed when GH3 cells transfected with CNP6CAT or CNP12CAT were treated with either forskolin or 8-bromo cAMP (data not shown), indicating that the CRE-like sequence in the CNP promoter does not potently affect the response to cAMP.

[A]



[B]

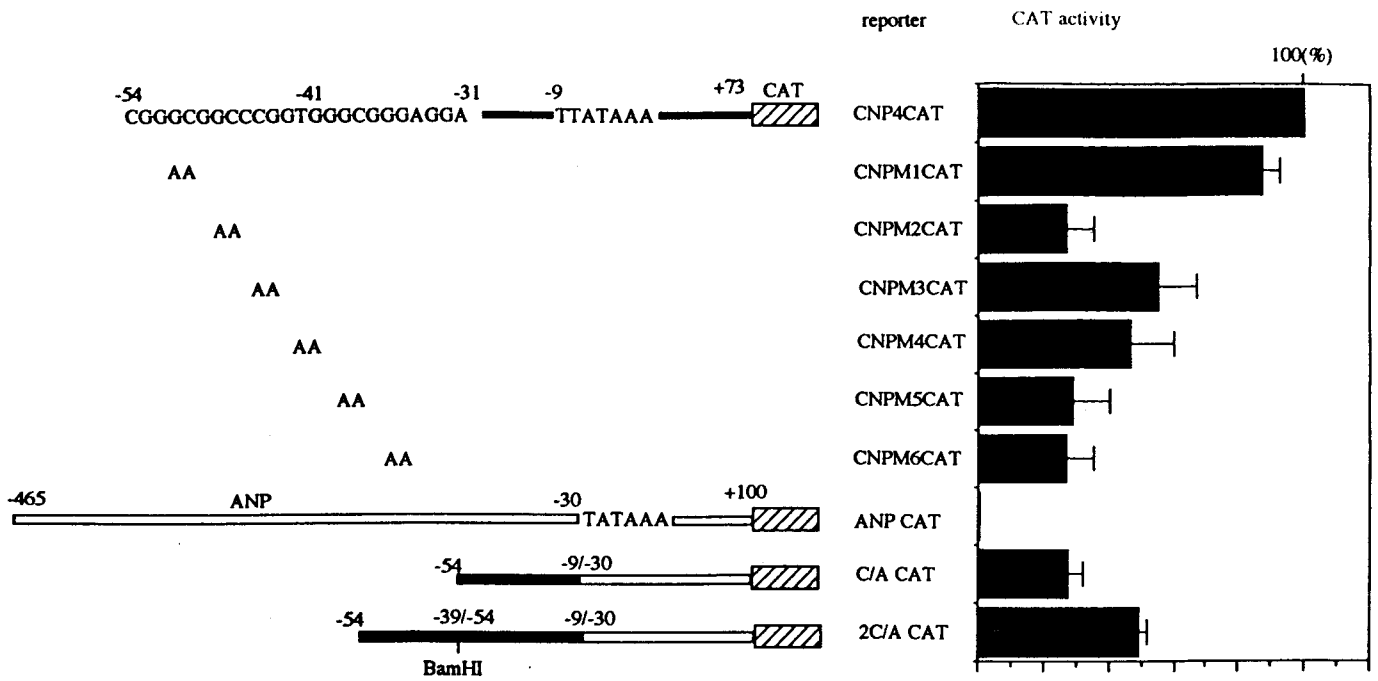


**Fig. 2-6. Functional analyses of CNP promoters with different 5' deletion**

**breakpoints in GH3 cells.** (A) 5' breakpoints of reporter constructs are shown on the left and CAT activities relative to the activity of CNP6CAT as 100% are shown on the right. Values are expressed as mean±SD (N=3~5). (B) Nucleotide sequence around the identified positive regulatory region of CNP promoter with numbering based on the determined +1 position. Boxes show the two GC-box core sequences, CRE-like sequence and TATAA box-like sequence. Positions of synthetic oligonucleotide probes for gel retardation assays are also shown with bars.

*2-3-5. Further characterization of the positive regulatory region by substitution mutant constructs*

A series of substitution mutant reporter plasmids were constructed and subjected to functional analyses in GH3 cells (Fig. 2-7). The results showed that two sites (from -49 to -45 and -40 to -35) were particularly crucial for the promoter activity. The ANP promoter-driven CAT construct (ANPCAT) that also had a TATAA box-like sequence exhibited positive function in cardiocytes but not at all in GH3 cells. When the sequence upstream of the TATAA box in the ANP promoter was replaced by the positive regulatory region of CNP promoter (from -54 to -9), the resultant chimeric promoter construct (C/ACAT) showed about 60-fold higher activity than ANPCAT. This value was comparable to an 80-fold increase in activity of CNP4CAT over CNP1CAT in which the positive regulatory region A was completely deleted, indicating that the sequence from -54 to -9 of the CNP promoter was sufficient to confer the cell-type specific function. Another chimeric construct, 2C/ACAT, in which the additional A1 region (from -54 to -39) was inserted just upstream of the promoter region of C/ACAT, exhibited 50% increase of activity over C/ACAT, suggesting that the A1 region alone was capable of acting as an activator for the promoter function.



**Fig. 2-7. Functional analyses of CNP promoters with substitution mutations in GH3 cells.** Positions of substitution of reporter constructs are shown on the left and CAT activities relative to the activity of CNP4CAT as 100% are shown on the right. Values are expressed as mean $\pm$ SD (N=2~4).



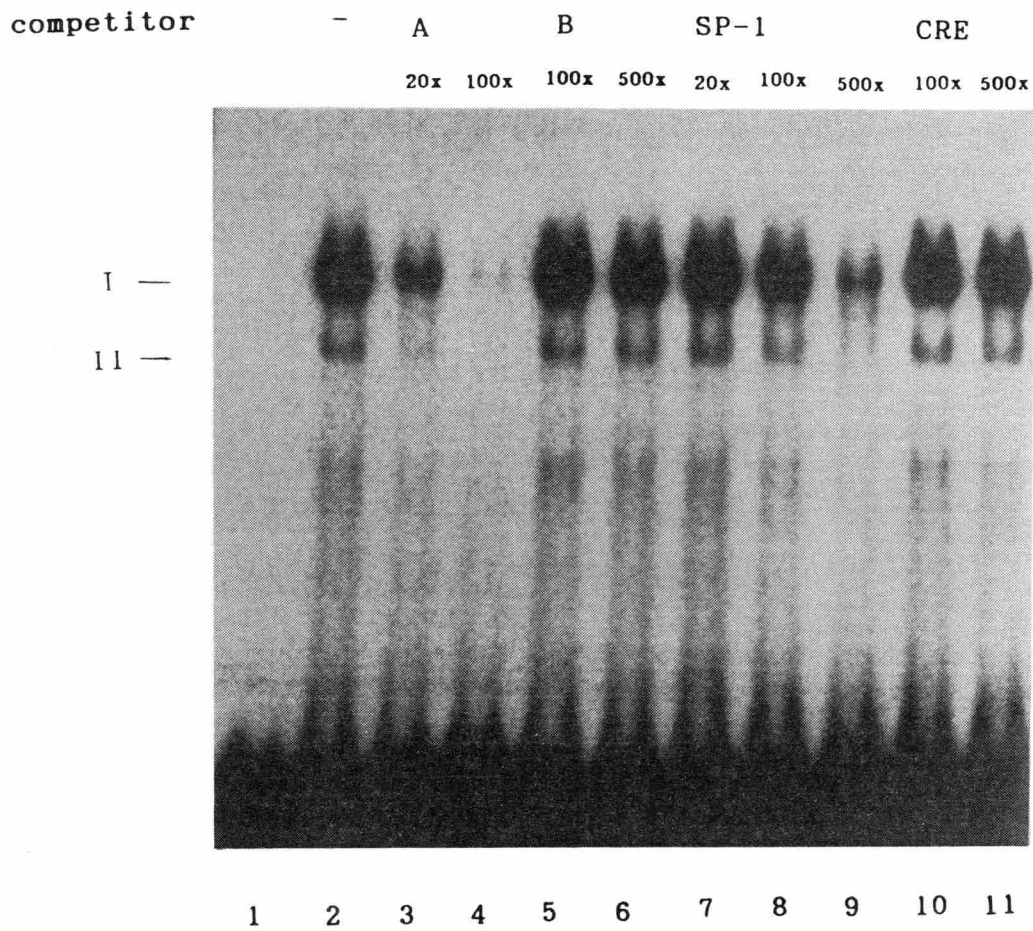
### 2-3-6. Gel retardation assays

To characterize the positive regulatory region of the CNP promoter, gel retardation experiments were carried out with synthetic oligonucleotide probes within the target region and several synthetic oligonucleotide competitors. With probe A that covered the whole positive regulatory region (from -55 to -18), one major shifted band (band I in Fig. 2-8) and another minor band of faster mobility (band II in Fig. 2-8) were observed. Both bands showed competition on addition of cold oligonucleotide A but not on addition of up to 500-fold molar excess of oligonucleotide B which was adjacent to oligonucleotide A in the CNP promoter sequence (Fig. 2-6B). A CRE consensus oligonucleotide was not an effective competitor. Probe A contained two GC box sequences, but shifted bands did not compete effectively with an SP-1 consensus oligonucleotide (about 10-fold less effective than oligonucleotide A), suggesting the binding protein specific for probe A could be different from the ubiquitous SP-1 factor. According to functional analyses with 5'-deleted promoter constructs (Fig. 2-6A), the region from -54 to -41 which contained one of the two GC boxes mediated 60% of the promoter activity.

A single shifted band was observed with the A1 probe (from -55 to -39), and the shifted band competed with the A2 probe (from -41 to -18), which mediated the rest of the whole promoter activity, as effectively as the A1 probe itself (Fig. 2-9). An SP-1 consensus oligonucleotide was again a less effective competitor (at least 20 times less effective than the A1 probe). Another GC-rich oligonucleotide, AP-2 consensus oligonucleotide, was at least 10 times less effective than the A1 probe. With the <sup>32</sup>P-labeled A2 probe, two shifted bands were observed (Fig. 2-10). Both bands competed with the cold A2 probe, but only the lower band II competed with the A1 probe as effectively as A2 itself. An SP-1 consensus oligonucleotide again showed very weak competition with both bands. The upper band I effectively competed with a CRE consensus oligonucleotide, suggesting some factor binding to the CRE-like sequence in the A2 region. The shifted band with the <sup>32</sup>P-labeled A1 probe did not compete with

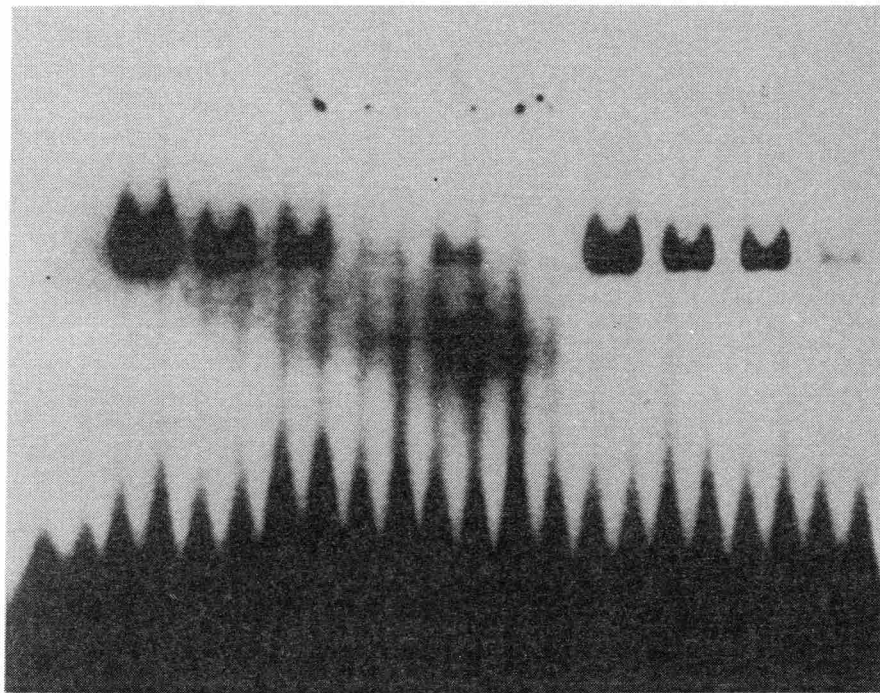
mutated A1 (A1M) or mutated A2' (A2'M) oligonucleotides (Fig. 2-11), both of which were designed according to the results in Fig. 2-7, suggesting that the nucleotide sequence required for the promoter function was also essential for the band shift.

These results together with those of functional analyses indicate that two equipotent GC-rich binding sites exist within the positive regulatory region of the CNP promoter, which confers 90% of the promoter function, and the binding protein is different from other GC-rich region binding factors, SP-1 and AP-2, and that the CRE-like sequence adjacent to the GC-rich region is also a binding site for a factor distinct from the CRE binding protein (CREB) which exerts the response to cAMP because the CNP promoter is not cAMP-responsive according to function analysis. GH3 cells originate from the growth hormone-producing tumor of rat pituitary gland, and therefore have somatomammotroph or somatotroph-like phenotype. The nuclear extract of rat pituitary anterior lobe showed the same shifted pattern as the GH3 nuclear extract with an A1 probe (Fig. 2-12), suggesting the existence of an identical or very similar binding factor in both GH3 cells and rat hypophysical tissue.



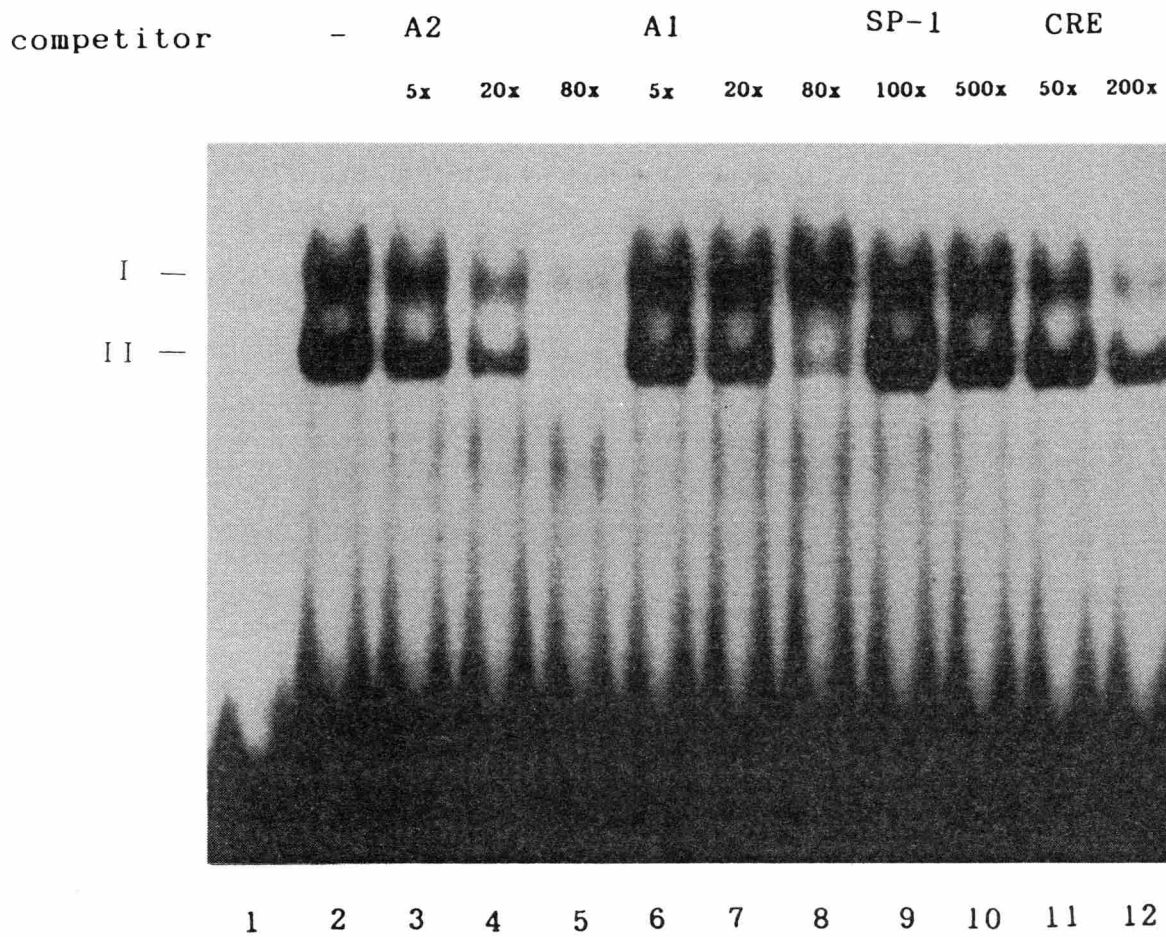
**Fig. 2-8.** Gel retardation patterns with a  $^{32}\text{P}$ -labeled probe A and GH3 nuclear extract. Lane 1, free probe. Lanes 2, with nuclear extract without competitor. Lanes 3-11, with cold competitors (20- and 100-fold molar excess of probe A, lanes 3 and 4; 100- and 500-fold molar excess of probe B, lanes 5 and 6; 20-, 100- and 500-fold molar excess of SP-1 consensus oligonucleotide, lanes 7-9; 100- and 500-fold molar excess of CRE consensus oligonucleotide, lanes 10 and 11).

competitor	-	A1			A2		SP-1		AP-2	
		5x	25x	100x	50x	100x	100x	500x	100x	500x



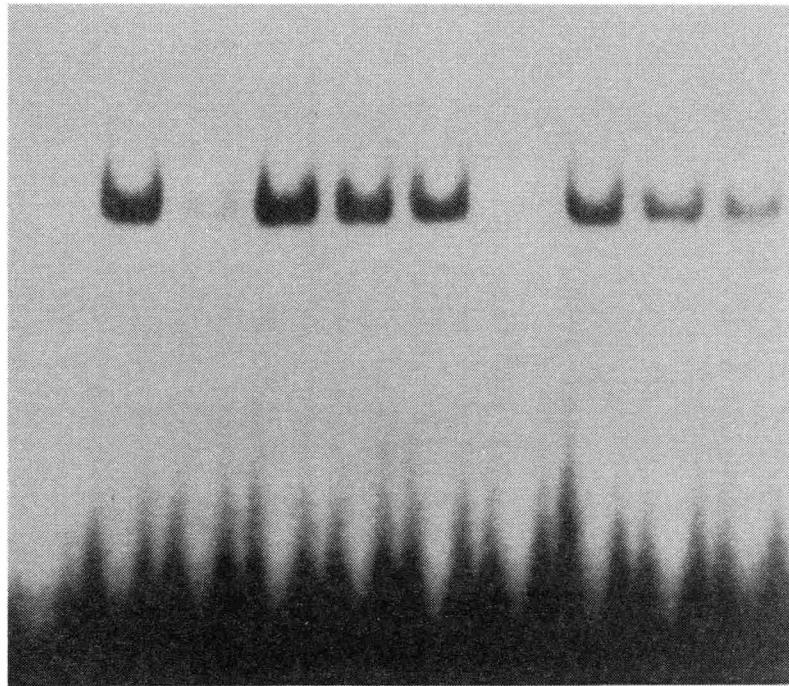
1 2 3 4 5 6 7 8 9 10 11

**Fig. 2-9.** Gel retardation patterns with a  $^{32}\text{P}$ -labeled probe A1 and GH3 nuclear extract. Lane 1, free probe. Lanes 2, with nuclear extract without competitor. Lanes 3-11, with competitors (5-, 25- and 100-fold molar excess of A1, lanes 3-5; 50- and 100-fold molar excess of A2, lanes 6 and 7; 100- and 500-fold molar excess of SP-1 oligonucleotide, lanes 8 and 9; 100- and 500-fold molar excess of AP-2 oligonucleotide, lanes 10 and 11).



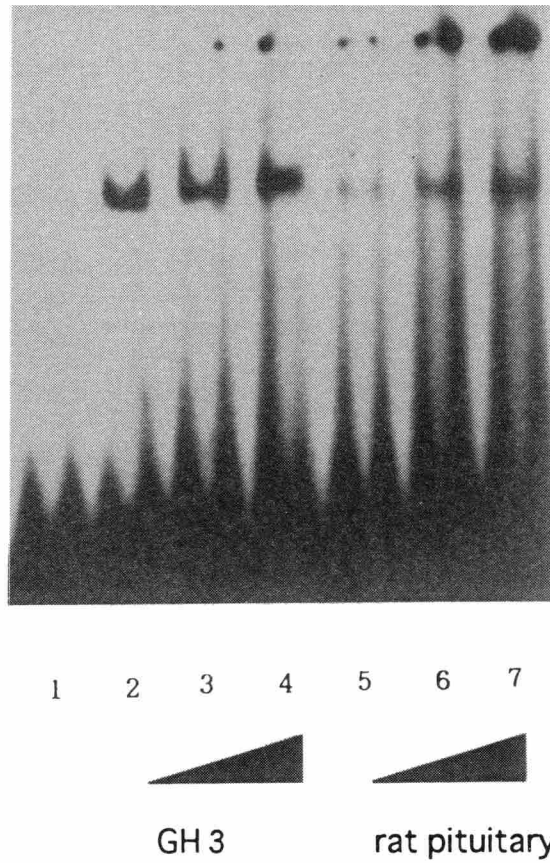
**Fig. 2-10.** Gel retardation patterns with a  $^{32}\text{P}$ -labeled probe A2 and GH3 nuclear extract. Lane 1, free probe. Lane 2, with nuclear extract without competitor. Lanes 3-12, with competitors (5-, 20- and 80-fold molar excess of A2, lanes 3-5; 5-, 20- and 80-fold molar excess of A1, lanes 6-8; 100- and 500-fold molar excess of SP-1 oligonucleotide, lanes 9 and 10; 50- and 200-fold molar excess of CRE oligonucleotide, lanes 11 and 12).

competitor	-	A1	A1M			A2' A2'M			
		50x	125x	250x	500x	50x	125x	250x	500x



1 2 3 4 5 6 7 8 9 10

**Fig. 2-11.** Gel retardation patterns with a  $^{32}\text{P}$ -labeled probe A1 and GH3 nuclear extract. Lane 1, free probe. Lane 2, with nuclear extract without competitor. Lanes 3-10, with competitors (50-fold molar excess of A1, lane 3; 125-, 250- and 500-fold molar excess of A1M, lanes 4-6; 50-fold molar excess of A2', lane 7; 125-, 250- and 500-fold molar excess of A2'M, lanes 8-10).

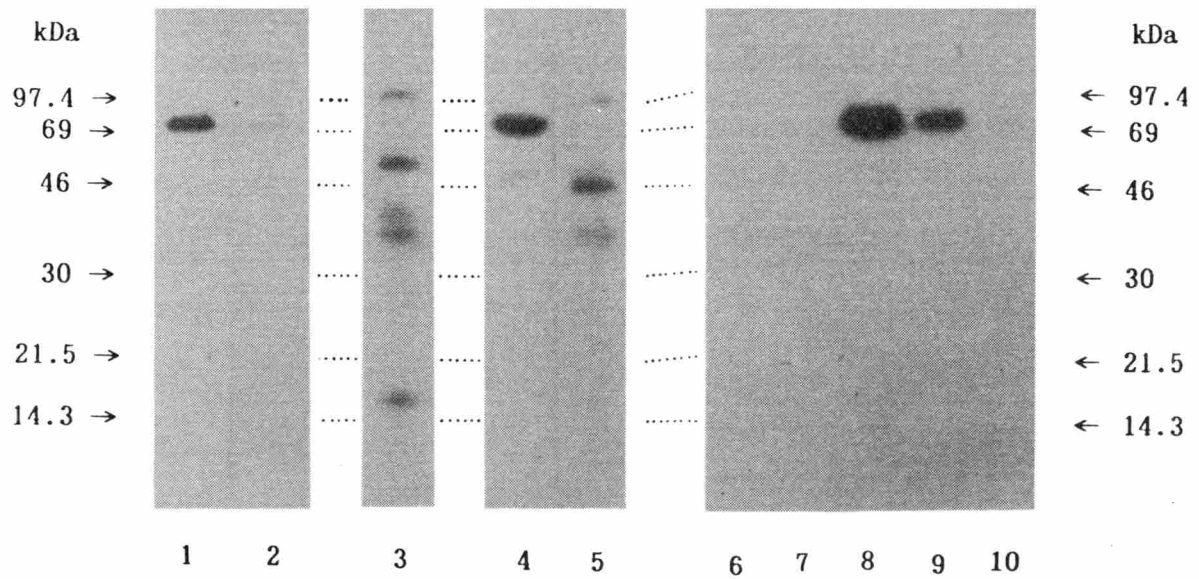


**Fig. 2-12. Gel retardation patterns with a  $^{32}\text{P}$ -labeled probe A1 and rat anterior pituitary nuclear extract. Lane 1, free probe. Lanes 2-4, with 2, 4 and 5  $\mu\text{g}$  protein of GH3 nuclear extract, respectively. Lanes 5-7, with 2, 4 and 8  $\mu\text{g}$  of rat pituitary nuclear extract.**

### *2-3-7. Southwestern analysis of the binding factor in GH3 and other cells*

To characterize the binding factor, nuclear extracts of GH3 and other cells were subjected to SDS-polyacrylamide gel electrophoresis and further analysis by Southwestern blotting with  $^{32}\text{P}$ -labeled probes (Fig. 2-13). A single band of 70 kDa protein was detected in the GH3 nuclear extract with the  $^{32}\text{P}$ -labeled A1 probe (lane 1), which weakened in the presence of 20-fold molar excess of cold A1 probe (lane 2). With the  $^{32}\text{P}$ -labeled A1M probe, several bands were observed but the 70 kDa band almost disappeared (lane 3). With the  $^{32}\text{P}$ -labeled A2' probe (nine nucleotides at the 3' end of A2 were deleted to disrupt the CRE-like sequence), the strong band at 70 kDa was again detected with a faint band at 50 kDa (lane 4). The 70 kDa band was not detected with the  $^{32}\text{P}$ -labeled A2'M probe (lane 5). A very faint band at 50 kDa was also visible with the  $^{32}\text{P}$ -labeled A1 probe on much longer exposure (lane 8), but the sequence specificity seemed to be lower than the 70 kDa protein because the 50 kDa band was seen with the mutated probe, A1M or A2'M. These results suggest that the 70 kDa band is specific for the A1 and A2' sequences. This protein was hardly detected in nuclear extracts from non-pituitary cells such as RASMC (lane 6) and FR (lane 7). The somatotroph-derived GH1 nuclear extract showed the existence of this protein (lane 9), but its proportion to the whole extract proteins in GH1 cells was several times less than that in GH3 cells. Another pituitary-derived cell line, AtT20/D16V-F2 (corticotroph origin), also contained a detectable amount of this 70 kDa protein (lane 10), although the concentration seemed to be less than a few percent of that in GH3 cells. These results suggest the 70 kDa protein detected with the A1 and A2' probes is specific for pituitary cells, especially for somatomammotrophs or somatotrophs rather than corticotrophs. The molecular weight of 70 kDa apparently differed from those of Pit-1, SP-1 or AP-2 [36-38].





**Fig. 2-13. Southwestern analysis.** Nuclear extracts (20  $\mu$ g protein per lane) were separated by SDS-polyacrylamide gel electrophoresis, blotted onto a nitrocellulose membrane and then detected with  $^{32}$ P-labeled probes. Extracts used were from GH3 (lanes 1-5 and 8), RASMC (lane 6), FR (lane 7), GH1 (lane 9) and AtT20/D16V-F2 (lane 10).  $^{32}$ P-labeled probes were A1 (lanes 1, 2, 6, 7, 8, 9 and 10), A1M (lane 3), A2' (lane 4) and A2'M (lane 5). Cold probe A1 (20-fold molar excess) was added in lane 2. Films were exposed for 20 h (lanes 1-5) or 70 h (lanes 6-10) at  $-80^{\circ}\text{C}$  with a pair of intensify screens. Positions of molecular weight marker proteins are shown in arrows and dotted lines.

## 2-4. Discussion

Detection of the CNP-like immunoreactivity was restricted within the brain and hypophysis [3, 4, 12]. On the other hand, detection of CNP mRNA or gene expression in certain tissues was unsuccessful except for Northern blot of rat whole brain RNA [5], suggesting the difficulty of study with an experiment like *in situ* hybridization that is useful for knowing exactly where the specific gene expression occurs. In the study of this chapter, CNP promoter worked very effectively in GH3 cells that have an origin of rat anterior pituitary and somatomammotroph or somatotroph-like phenotype, but did not work so efficiently in AtT20/D16V-F2 cells of corticotroph origin, or not at all in several other cell lines of different origins. The identified region of human CNP promoter (from -54 to -19) which conferred this cell-type specific promoter function was highly conserved in the corresponding region of the porcine gene (only two bases at -45 and -44 were substituted [6] ), but there is no homologous sequence in ANP or BNP promoter. This region was separable into two equipotent sites, and both mediated about half the promoter activity and shared the binding properties to protein factors. These two binding sites had GC-rich sequences, not identical to SP-1 or AP-2 consensus binding sequences. The identified sequence was also quite different from the binding sequence of Pit-1/GHF1 (consensus sequence, 5'-A/TTATC/TCAT-3') which activates the growth hormone and the prolactin genes in adult pituitary gland (somatotrophs, thyrotrophs and lactotrophs) [36], or steroid hormone responsive elements (consensus sequence, 5'-AGAACANNNTGTTCT-3') which are also found in pituitary hormone genes [39, 40]. Southwestern analysis suggested the involvement of a 70 kDa protein factor in the CNP promoter function in GH3 cells. This protein seemed to be specific for pituitary cells, and its concentration in nuclear extract coincided with the efficiency of the CNP promoter function (GH3>GH1>>AtT20/D16V-F2>>RASM, FR). The molecular weight of 70 kDa apparently differs from those of SP-1 (95-105 kDa [37] ), AP-2 (52 kDa [38] ) and

Pit-1 (33 kDa [36] ). Together, the 70 kDa protein is distinct from other known factors responsible for the pituitary-specific gene expression such as Pit-1, CREB, AP-1, AP-2, SP-1 and steroid hormone receptors in the binding sequence or molecular size, and seems to be a novel binding factor which is responsible for the CNP promoter function.

Recent reports show that CNP alters the secretion of luteinizing hormone and prolactin through its hypothalamic actions [18, 19]. However, it is still unclear whether CNP has direct effects on hypophysis. The results in gel retardation assays in this chapter show the existence of the identical or very similar factor in rat anterior pituitary, suggesting that CNP might be constitutively produced in somatomammotrophs or somatotrophs. Recently Shimekake and myself detected a significant amount of immunoreactive CNP in the culture media of GH3 cells and CNP mRNA in total RNA from the same cells under normal culture conditions [41]. As CNP-specific ANP-B receptor has been shown to exist in the anterior pituitary as well as the brain [16, 17] , it is possible that CNP may act locally in the anterior pituitary as an autocrine or paracrine regulator. My observation that CNP promoter functioned more effectively in GH3 cells than in GH1 cells provides another speculation that CNP might have significant physiological roles in somatomammotrophs, proposed progenitor cells of somatotrophs and lactotrophs [42], for example, in their differentiation process.

Surprisingly, the CNP promoter also functioned in primary cardiocytes, albeit the activity was much lower than in GH3 cells. While the 5'-FK sequence of the CNP gene shows little similarity to those of ANP [24] and BNP [25, 43] genes, cardiocytes like GH3 cells seem to have the basic transcriptional machinery necessary for the constitutive expression of the CNP gene. Inability to detect the CNP-like immunoreactivity in the heart was essentially due to the cross-reactivity of the anti-CNP antibody to ANP molecule that should hide the low amount of immunoreactive CNP, if any, within the immunoreactivity attributed to ANP co-existed abundantly especially in the heart [12] . Recently CNP has been isolated from dogfish heart in a high-molecular weight form [44], but not from mammal hearts. My observation suggests the possibility of CNP gene

expression also in mammalian heart. CNP gene expression is greatly induced by certain stimulants [45, 46] as in the case of ANP gene [47, 48]. Stimulants that could induce the CNP gene expression must be important for understanding the physiological roles of CNP in hypophysis as well as in other tissues. One of the transcriptional regulatory factors involved in the stimulated expression of CNP gene is described in Chapter 3.

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## **Chapter 3. Molecular cloning of a transcription factor for transforming growth factor- $\beta$ -induced CNP gene expression**

### **3-1. Introduction**

In Chapter 2, I described that the CNP gene promoter functions in the rat pituitary-derived cell line (GH3) and two GC-rich cis elements are essential for its transcription. The findings suggest the production of CNP molecules in the anterior pituitary gland and its possible role in the regulation of the pituitary function. According to this suggestion, my colleague examined the action of CNP on GH3 cells and primary cultured cells from rat anterior pituitary, and found that GH3 cells constitutively expressed the CNP gene at a significant level and the addition of CNP to the cells stimulated the release of growth hormone from pituitary cells through a cGMP-mediated pathway, independent of the cAMP or phospholipase C-mediated pathway [1]. This finding was recently confirmed by studies by Hartt *et al.* [2]. Therefore, CNP seems to act as a local modulator in the pituitary system in a paracrine/autocrine manner.

In addition to the function in the pituitary system, several reports suggest other functions of CNP. Recent studies demonstrated that CNP exists in normal plasma [3] and that its plasma level is elevated in sepsis patients [4], suggesting the possible involvement of CNP in septic or other hypovolemic shock that is induced by some cytokines. CNP was also reported to be secreted from cultured vascular endothelial cells in response to some stimulants, such as TGF- $\beta$  [5, 6]. On the other hand, CNP was reported to inhibit the proliferation of cultured vascular smooth muscle cells [7], and therefore suggested to be an endothelium-derived paracrine growth modulator of vascular smooth muscle cells, i. e. a hypothesized 'vascular natriuretic peptide system'. From these observations, understanding of the regulatory mechanism of the stimulated expression of the CNP gene became more important to elucidate its physiological



function(s) in such as vascular system . In Chapter 2, the existence of a putative regulatory factor with a molecular weight around 70 kDa that binds to the GC-rich elements of the CNP promoter was shown. The factor was expected to play an important role in the constitutive cell-specific expression of the CNP gene. In this chapter, I tried to identify and characterize transcriptional regulatory factors for the CNP gene. Several positive clones were isolated by Southwestern screening, but no clone corresponded to the 70 kDa factor. Among cloned factors, there was a clone homologous to mouse TSC-22 (TGF- $\beta$  stimulated clone 22). TSC-22 was originally identified in mouse osteoblastoma cell line MC3T3 E1 as a factor induced with TGF- $\beta$  treatment, but with unknown function [8]. As described before, the expression of CNP is enhanced with TGF- $\beta$  treatment in vascular endothelial cells, and so I focused on the analysis of biological function of TSC-22 as a transcriptional factor of the CNP gene.

## 3-2. Materials and methods

### 3-2-1. Construction of cDNA library and Southwestern screening

Total cellular RNA was purified from GH3 cells by acid-guanidinium thiocyanate-phenol-chloroform (AGPC) method as described by Chomczynski and Sacchi [9], and poly(A)<sup>+</sup> RNA was isolated with oligo(dT) spun column (Pharmacia). cDNA was synthesized from 5 µg of poly(A)<sup>+</sup> RNA with random and oligo(dT) primers using Pharmacia Time-saver cDNA construction kit. After ligation of the EcoRI adapter with an internal NotI site to the cDNA, it was then ligated to the right and left arms of EcoRI-digested λ-ZAPII phage vector (Stratagene). The ligation mixture was packaged *in vitro* with Gigapack II Gold lambda packaging extracts (Stratagene). The constructed expression library ( $5 \times 10^5$  p.f.u. with an average insert size of 1.2 kb) was then subjected to Southwestern screening according to Singh *et al.* [10] with some modifications by Vinson *et al.* [11]. Briefly, phage plaques were incubated for 4 h at 42°C, and then contacted with Hybond-C nitrocellulose membranes (Amersham) previously soaked in 20 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37°C. Next, the nitrocellulose membranes with lifted-up plaques were soaked in the binding buffer [10 mM Tris-HCl (pH 8.0), 10% glycerol, 0.05% Nonidet P-40, 1.25 mM DTT, 50 mM NaCl, and 1 mM EDTA] containing 6 M guanidine hydrochloride for 30 min at 4°C. The concentration of guanidine hydrochloride was gradually lowered by two-fold dilution with binding buffer every 15 min until it became 0.375 M, and finally the membranes were soaked in the binding buffer without guanidine. Next, the membranes were treated for 60 min at room temperature with binding buffer containing 5% nonfat dried milk for blocking. After washing twice with the binding buffer, the membranes were probed for 2 h at 4°C in the same buffer containing 10 µg/ml calf thymus DNA with a multimer of the <sup>32</sup>P-labeled ( $10^6$  cpm/ml) double-stranded A1 oligonucleotide which contains one of two GC-rich cis elements of the CNP promoter as shown in Chapter 2 and linker sequence (sense, 5'-gatcCCGGGCGGCCCGGTGGG-3', and antisense, 5'-

gatcCCCACCGGG-CCGCCCCGG-3'; linker sequence in small letters). After washing twice with the binding buffer for 5 min at 4°C, the membranes were subjected to autoradiography. Positive plaques were picked up and processed to the second and third screening with <sup>32</sup>P-labeled double stranded A1 (5'-CCGGGCGGCCCGGTGGG-3') and mutated A1 (A1M, 5'-CCGGGCGAAACGGTGGG-3') oligonucleotide probes. Clones which were positive to A1 probe and negative to A1M probe were finally selected.

### *3-2-2. Plasmid rescue and DNA sequencing*

Positive phagemid clones in Southwestern screening were isolated, and treated with a helper phage R-408 (Stratagene) to recover cDNA clones as Bluescript plasmids. DNA sequencing was performed on both strands by the dideoxy chain-termination method with a Bcabest sequencing kit (Takara) in an ALFred DNA sequencer (Pharmacia).

### *3-2-3. Molecular cloning of Human TSC-22 cDNA*

Human TSC-22 coding region was cloned from human fetal kidney cDNA library (Clontech) by PCR [12] with 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min using primers designed from rat and mouse TSC-22 sequences [sense primer 1, 5'-TATTTGGCTGCAATTGCATG-3', and antisense primer 2, 5'-TTGGGGC(A/C)TAG-(C/G)AGGCTA-3']. To obtain the 5' portion of human TSC-22 cDNA, the "rapid amplification of cDNA ends" (RACE) technique [13] was performed with the same human library using a TSC-22 gene-specific primer (antisense primer 3, 5'-ACACTT-GCACCAGAGGAGCTATTA-3') and a sense primer for the vector arm (5'-CTTATG-AGTATTTCTTCCAGGGTA-3'). The 3' portion was also obtained by RACE using a gene-specific primer (sense primer 4, 5'-ACTGGCCAGTCCTGAGCAGCTT-3') and a poly(dT)-adapter primer (5'-GAGTCGACTCGAGAATTCCTTTTTTTTTTTTTTTTTT-3'). Amplification for both 5' and 3' RACE was performed with 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. Amplified fragments were isolated by agarose gel

electrophoresis and subcloned to Bluescript SK(-) vector (Stratagene). Several clones were sequenced to fix the cDNA sequence.

#### 3-2-4. *In vitro* transcription-translation

Bluescript plasmid containing rat TSC-22 cDNA was linearized by XhoI digestion at its 3' end, transcribed *in vitro* by T3 RNA polymerase (Stratagene), and then translated *in vitro* in *E. coli* S-30 extract (Promega) with unlabeled amino acids. The non-radioactive product was used for electrophoretic mobility shift assay. On the other hand, the *in vitro* transcription-translation product labeled with [<sup>35</sup>S]methionine was evaluated by SDS-polyacrylamide gel electrophoresis.

#### 3-2-5. Gel retardation assay

Gel shift assay was essentially performed as described in Chapter 2. Briefly, *E. coli* S-30 extract containing *in vitro*-translated rat TSC-22 was incubated with poly(dI-dC)·poly(dI-dC) (2.5 µg) in 10 mM Tris-HCl (pH 8.0), 10% glycerol, 0.05% Nonidet P-40, 1.25 mM DTT, 50 mM NaCl, and 1 mM EDTA for 20 min on ice. A <sup>32</sup>P-labeled double-stranded A1 oligonucleotide (10000 cpm) was added to the mixture (final volume of 20 µl). After incubation for another 20 min at room temperature, the mixture was analyzed in a 5% polyacrylamide gel run in 7 mM Tris-Cl (pH 7.5), 1 mM EDTA, and 3 mM sodium acetate. For the competition experiment, 100- or 200-fold excess amount of unlabeled A1 oligonucleotide was added to the incubation mixture.

#### 3-2-6. Cell culture and chemicals

GH3 cells (American Type Culture Collection) were cultured in Ham's F10 medium (Flow Laboratories) supplemented with 15% horse serum and 2.5% FCS as described in Chapter 2. Endothelial cells from human aorta (HAEC, Sanko Junyaku) were cultured in E-GM-UV defined medium (Sanko Junyaku). All cultures were kept at 37 °C under 5% CO<sub>2</sub> atmosphere. To study the effects of cytokines and other reagents on gene

expression, recombinant proteins [human TGF- $\beta$  (Wako Pure Chemicals), human tumor necrosis factor (TNF)- $\alpha$  (Genzyme), human interleukin (IL)-1 $\beta$  (Becton Dickinson), mouse interferon (IFN)- $\gamma$  (home-made [14]), and human IFN- $\gamma$  (Shionogi & Co., Ltd.)] and bacterial LPS (Diffco) were used.

### *3-2-7. Co-transfection studies*

Rat TSC-22 full-length cDNA (1.75 kb NotI-NotI fragment) was subcloned at the NotI site of the pEF-BOS expression vector [15] (the stuffer fragment was excised by XbaI digestion and replaced by NotI linker in advance) to obtain pEF-TSC22. For luciferase (LUC) assay, CNP promoter fragment in CNP4CAT in Chapter 2, which has the shortest human CNP promoter fragment with full activity in GH3 cells, was excised by BamHI and HindIII digestion, and then ligated to the BamHI-HindIII site of pGV-B vector (Promega) to obtain CNP4LUC. The mutated CNP promoter fragment from CNPM2CAT was also recloned in pGV-B vector to make CNPM2LUC. GH3 cells were seeded in a 6-cm dish at a density that gave 10 to 20% confluency on the next day. After 18 to 24 h, cells were transfected according to the calcium phosphate protocol of the Stratagene mammalian transfection kit with 0.5  $\mu$ g CNP4LUC reporter plasmid, 1  $\mu$ g pEF-TSC22 (or pEF-BOS for control), and 1  $\mu$ g of reference plasmid [SR $\alpha$ -GAL, SR $\alpha$  promoter-driven  $\beta$ -galactosidase (GAL)] to normalize transfection efficiency. At 48 h after transfection, cells were lysed in the lysis buffer compatible to GAL and LUC (Promega), and subjected to assays for the respective reporter molecules. GAL assay was carried out as described in Chapter 2, and LUC assay was carried out in a Berthold Lumat LB9501 luminometer using a Pikka Gene Assay kit (Toyo Ink). To examine the effects of TGF- $\beta$  and murine IFN- $\gamma$ , they were added to the culture medium at 24 h after transfection, and incubated for a further 24 h before cell lysis.

### *3-2-8. Northern blot and PCR analyses*

Cells were incubated in respective media containing 0.5% FCS for 24 h, and further incubated with the respective cytokines in the low serum medium. After incubation for 2-

4 h, cells were harvested and total cellular RNA was obtained by the AGPC method, and then treated with RNase-free DNase I (2 µg/ml, Pharmacia) for 60 min at 37 °C followed by phenol/chloroform extraction. Total cellular RNA (10 µg) was subjected to electrophoresis in a denatured agarose gel, and transferred to a Hybond-N nylon membrane (Amersham). The membrane was hybridized overnight at 65°C with a <sup>32</sup>P-labeled full-length TSC-22 cDNA probe in the GMC buffer [0.5 M sodium phosphate (pH 7.5), 10 mg/ml bovine serum albumin, 1 mM EDTA, 7% SDS], and washed for 30 min at 65°C in 0.1xSSC (1xSSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) plus 0.1% SDS. The filters were rehybridized with <sup>32</sup>P-labeled glyceraldehyde-3-phosphate dehydrogenase (G3PDH) or β-actin probe (Toyobo) for control. Human multi-tissue blot was purchased from Clontech. For detecting CNP signals, equal amounts of total cellular RNA (10 µg each) were reverse transcribed into cDNA with oligo(dT) primer, and then amplified by PCR with CNP-specific primers (sense, 5'-CAGAGGAGCTGGCCGAGCCGCAG-3' and antisense, 5'-CCGCACTAACATCCC-AGGCCGCTC-3', respectively corresponding to 863-885 and 1139-1116 of the human CNP sequence described in [16]; 25 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min). Amplified products were electrophoresed in a 2% agarose gel and transferred to a nylon membrane. The membrane was subsequently hybridized with <sup>32</sup>P-labeled CNP cDNA probe in the GMC buffer overnight at 65°C, and then washed for 30 min at 50°C in 2xSSC plus 0.1% SDS.

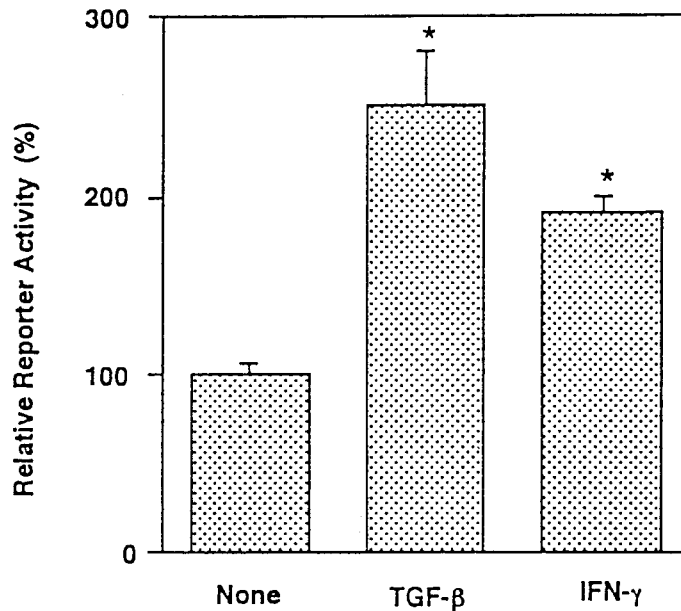
### 3-3. Results

#### 3-3-1. *Enhancement of CNP promoter function by TGF- $\beta$*

It was reported that the expression of CNP gene in endothelial cells from bovine carotid artery was induced by the treatment of TGF- $\beta$  [5]. Therefore, I first examined the effect of TGF- $\beta$  on the transient transfection assay with CNP4LUC reporter plasmid that has the shortest promoter region (-54 to +73) of the human CNP gene essential for full promoter activity in GH3 cells as shown in Chapter 2. This promoter region contains the GC-rich sequences and a single CRE-like sequence just upstream of the TATAA box. TGF- $\beta$  were added to the culture media at 24 h after transfection, and reporter assay was done after another 24-h incubation. As shown in Fig. 3-1, TGF- $\beta$  significantly enhanced the transcription of CNP4LUC reporter in GH3 cells. The result suggests the existence of transcription factor(s) involved in the enhancement of CNP promoter activity by TGF- $\beta$ . It is also suggested that the shortest promoter region for expression the full promoter activity exhibited significant induction by TGF- $\beta$ . In addition to TGF- $\beta$ , IFN- $\gamma$  also stimulated the CNP promoter activity weakly but significantly.

#### 3-3-2. *Isolation of rat TSC-22 cDNA clone as a binding factor to the GC-rich element of CNP promoter*

To isolate transcription factors responsible for the enhancement of CNP promoter activity by TGF- $\beta$  treatment, I constructed a rat GH3 cDNA library in  $\lambda$ -ZAPII expression vector. About  $5 \times 10^5$  plaques were subjected to Southwestern screening with  $^{32}\text{P}$ -labeled A1 probe. Several positive clones were obtained and subjected to sequencing analysis. One clone among them ( $\lambda$ 35) was identical to rat TSC-22 [17] by homology search in the GenBank and EMBL DNA databases. The insert of this clone was 1.1 kb containing the open reading frame of 143 amino acid residues and no poly(A)<sup>+</sup> tail. The deduced amino acid sequence of rat TSC-22 was the same as that of mouse TSC-22 (Fig. 3-7).



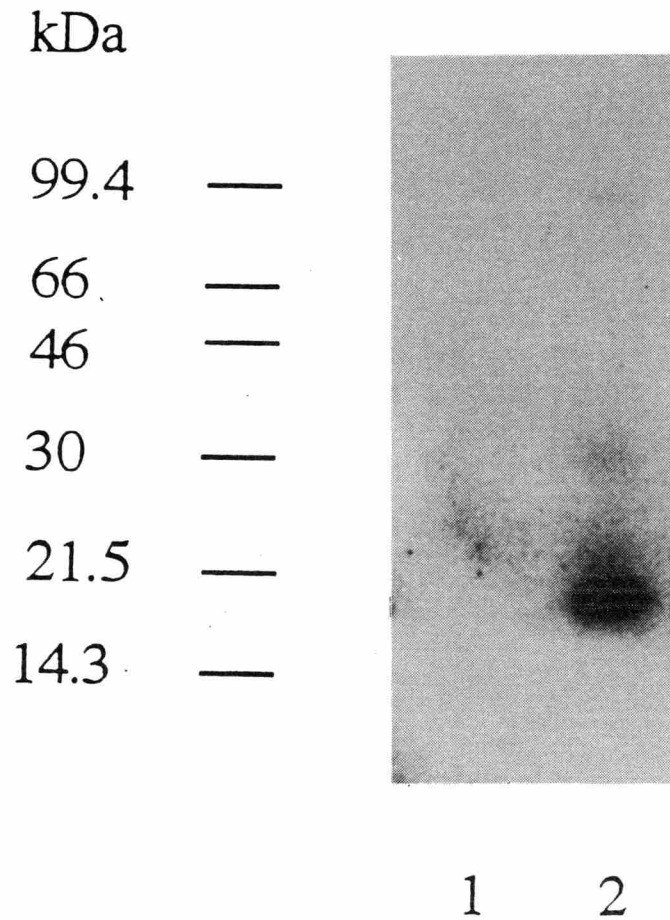
**Fig. 3-1. Effect of TGF- $\beta$  on the CNP promoter activity in GH3 cells.**

GH3 cells were co-transfected with CNP4LUC reporter plasmid and SR $\alpha$ -GAL reference plasmid. After 24 h, 10 ng/ml human TGF- $\beta$ 1 or 1000 U/ml mouse IFN- $\gamma$  was added to the culture medium, and cells were incubated for another 24 h before the reporter assay. Normalized LUC activity was expressed as relative value to activity of control transfection (without cytokine treatment) as 100%. Results from 3 independent experiments are expressed as means  $\pm$  SD. \*, significantly different from control (P < 0.01) by Student's *t* test.

### *3-3-3. Specific binding of TSC-22 to the GC-rich sequence of CNP promoter and regulation of its promoter function*

To confirm the DNA binding ability of rat TSC-22, it was transcribed and translated *in vitro* in the *E. coli* S-30 extract system. The product of *in vitro* transcription-translation labeled with [<sup>35</sup>S]methionine showed a single band of 18 kDa in SDS-polyacrylamide gel electrophoresis (Fig. 3-2).



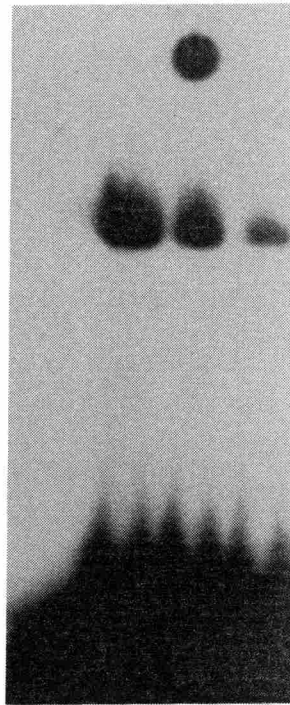


**Fig. 3-2. *In vitro* transcription and translation of rat TSC-22 cDNA.**

Bluescript plasmid containing rat TSC-22 cDNA was linearized by XhoI digestion at its 3' end, transcribed *in vitro* by T3 RNA polymerase, and then translated *in vitro* in *E. coli* S-30 extract with unlabeled amino acids and [<sup>35</sup>S]methionine. Lane 1, empty vector; lane 2, TSC-22 cDNA.

The non-radioactive translated crude extract was subjected to gel shift assay with <sup>32</sup>P-labeled A1 oligonucleotide. As shown in Fig. 3-3, a shifted band was observed and that band competed with an excess amount of non-labeled A1 oligonucleotide in a dose-dependent manner. These results show that TSC-22 is actually capable of binding to the GC-rich sequence of CNP promoter which is essential for promoter function.

1 2 3 4

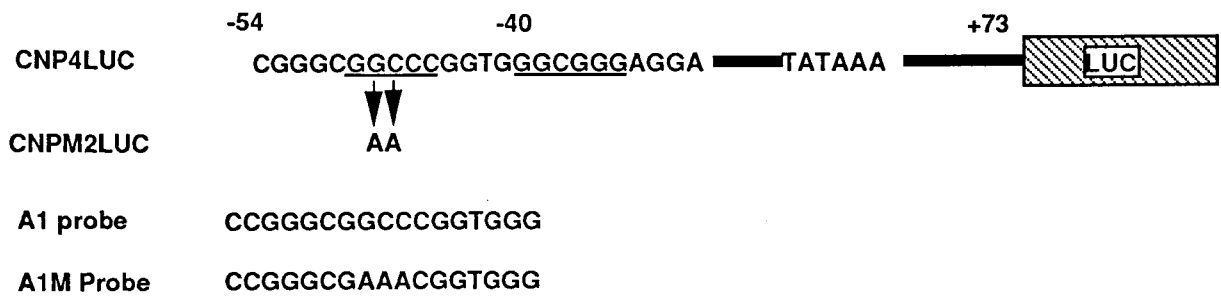


**Fig. 3-3. Binding of *in vitro* translated rat TSC-22 to the GC-rich sequence element of CNP promoter analyzed by electrophoretic mobility shift assay.**

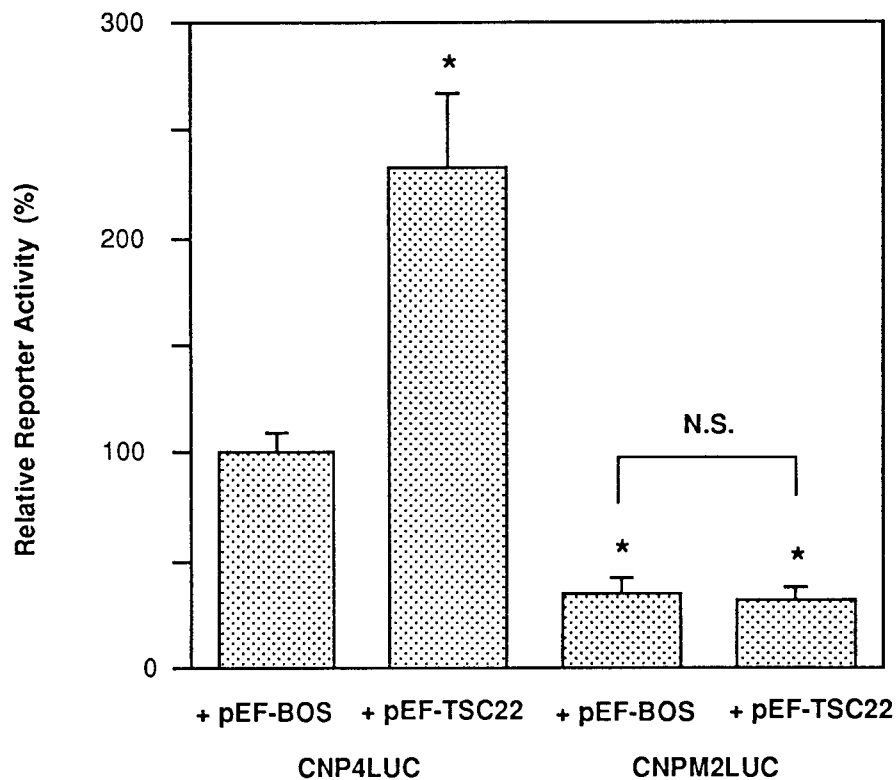
S-30 *E. coli* extract containing translated TSC-22 protein was incubated with 20,000 cpm of the A1 probe, and the DNA-protein complexes were analyzed in 5% polyacrylamide gel. Lane 1, without extract; lane 2, with extract; lane 3, with extract and 100-fold excess unlabeled A1 oligonucleotide; lane 4, with extract and 200-fold excess unlabeled A1 oligonucleotide.

The function of TSC-22 was also examined by co-transfection experiments using TSC-22 expression vector (pEF-TSC22) and a reporter plasmid, CNP4LUC. SR $\alpha$ -GAL vector was co-transfected for normalization of transfection efficiency. The results of functional analysis revealed that TSC-22 activated the CNP promoter function in GH3 cells (Fig. 3-4). TSC-22 did not activate the mutated promoter in CNPM2LUC, which has two-base substitution within the upstream GC-rich element (-49 to -45) of CNP promoter (as in Fig. 2-7 in Chapter 2). This suggests that TSC-22 may not give an effect on the second GC-rich element (-40 to -35) or that both GC-rich elements are required for activation by TSC-22 although one element is enough for binding. Thus, TSC-22 actually regulated the CNP promoter function through at least one of the GC-rich elements in the CNP promoter.

(A)



(B)

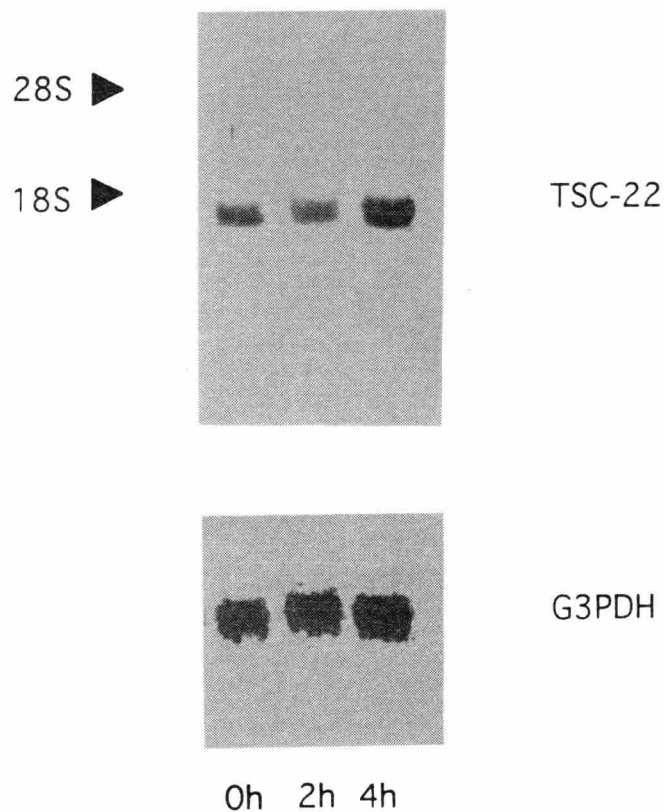


**Fig. 3-4. Co-transfection of TSC-22 expression vector and CNP4LUC reporter**

**plasmid in GH3 cells.** (A) Partial nucleotide sequence of CNP promoter region cloned in reporter plasmids, CNP4LUC and CNPM2LUC. Position of A1 probe for Southwestern screening and mobility shift assay is shown with a bar and two GC-rich sequences crucial for promoter activity are underlined. (B) A TSC-22 expression vector (pEF-TSC22) or control empty vector (pEF-BOS) was co-transfected into GH3 cells with a reporter plasmid and SR $\alpha$ -LUC reference plasmid. After 48 h, cells were lysed for assays of reporter molecules. LUC activity was normalized with GAL activity in each transfection, and expressed as relative value to activity of control transfection (CNP4LUC with pEF-BOS) as 100%. Results from 3 independent experiments are expressed as means  $\pm$  SD. \*, significantly different from control ( $P < 0.01$ ) by Student's  $t$  test.

3-3-4. Enhancement of TSC-22 expression in GH3 cells by TGF- $\beta$

TSC-22 was originally identified as a cDNA clone stimulated by TGF- $\beta$  in mouse osteoblastoma cells [8]. Expression level of TSC-22 in GH3 cells also increased up to three-fold after 4-h stimulation by TGF- $\beta$  (Fig. 3-5). The reason for the lower induction ratio than that observed in osteoblastoma cells probably is the relatively high basal expression of TSC-22 (with no stimulation) compared with that in osteoblastoma cells.



**Fig. 3-5. Effect of TGF- $\beta$  on TSC-22 gene expression in GH3 cells.**

After serum depletion, GH3 cells were treated with 10 ng/ml human TGF- $\beta$ 1 for 2 h or 4 h. DNA-free total cellular RNA (10  $\mu$ g each) was analyzed for TSC-22 expression by Northern blot. The filter was rehybridized with G3PDH probe (lower) for control.

*3-3-5. Isolation of human TSC-22 cDNA clone by PCR and expression pattern in human tissues*

To understand the involvement of TSC-22 in human TGF- $\beta$  systems, such as TGF- $\beta$ -related diseases and cell signaling, human counterpart should be identified. Therefore, human TSC-22 cDNA was isolated from the human fetal kidney cDNA library by PCR according to the coding sequences of rat and mouse TSC-22. Full sequences of both 5' and 3' regions were obtained by RACE. The full length of human TSC-22 cDNA shown in Fig. 3-6 was 1.8 kb long, and contained three copies of ATTTA sequence and polyadenylation site in its 3' untranslated region. The deduced amino acid sequence revealed the insertion of one residue and one substitution in human TSC-22 compared with the rat sequence, and a putative leucine zipper motif, but no characteristic basic amino acid domain (Fig. 3-7). In addition, a putative protein kinase-C consensus phosphorylation site and casein kinase-II phosphorylation site [18] were also observed. Northern blot analysis of poly(A)<sup>+</sup> RNA from adult and fetal human tissues with a human TSC-22 cDNA probe with a full coding region revealed a single transcript of approximately 1.8 kb in adult lung, heart, brain, kidney, placenta, thymus, prostate, ovary, small intestine and colon (Fig. 3-8). The expression in adult kidney and testis was slight, and very little was observed in liver, pancreas, spleen and peripheral blood leukocytes. Compared with the adult tissue, fetal kidney showed higher TSC-22 expression. A different size of transcript (4.8 kb) was observed in skeletal muscle. The wide tissue distribution of TSC-22 mRNA in human suggests the important role of TSC-22 in human systems.

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1   CGC CTC TTC ACG GCA CTG GGA TCC GCA TCT GCC TGG GAT CAT CAA GCC CTA GAA GCT GGG
61  TTT CTT TAA ATT AGG GCT GCC GTT TTC TGT TTC TCC CTG GGC TGC GGA AAG CCA GAA GAT
121 TTT ATC TAG CTT ATA CAA GGG CTG CGG AAA GCC AGA AGA TTT TAT CTA GCT TAT ACA AGA
181 AGA TTT TAT CTA GCT TAT ACA AGG CTG CTG GTG TTC CCT CTT TTT TTC CAC GAG GGT GTT
    primer 1
241 TTT GGC TGC AAT TGC ATG AAA TCC CAA TGG TGT AGA CCA GTG GCG ATG GAT CTA GGA GTT
    1   M   K   S   Q   W   C   R   P   V   A   M   D   L   G   V

301  TAC CAA CTG AGA CAT TTT TCA ATT TCT TTC TTG TCA TCC TTG CTG GGG ACT GAA AAC GCT
    16  Y   Q   L   R   H   F   S   I   S   F   L   S   S   L   L   G   T   E   N   A

361  TCT GTG AGA CTT GAT AAT AGC TCC TCT GGT GCA AGT GTG GTA GCT ATT GAC AAC AAA ATC
    36  S   V   R   L   D   N   S   S   S   G   A   S   V   V   A   -   I   D   N   K   I

421  GAG CAA GCT ATG GAT CTA GTG AAA AGC CAT TTG ATG TAT GCG GTC AGA GAA GAA GTG GAG
    56  E   Q   A   M   D   L   V   K   S   H   L   M   Y   A   V   R   E   E   V   E

481  GTC CTC AAA GAG CAA ATC AAA GAA CTA ATA GAG AAA AAT TCC CAG CTG GAG CAG GAG AAC
    76  V   L   K   E   Q   I   K   E   L   I   E   K   N   S   Q   L   E   Q   E   N

541  AAT CTG CTG AAG ACA CTG GCC AGT CCT GAG CAG CTT GCC CAG TTT CAG GCC CAG CTG CAG
    96  N   L   L   K   T   L   A   S   P   E   Q   L   A   Q   F   Q   A   Q   L   Q

601  ACT GGC TCC CCC CCT GCC ACC ACC CAG CCA CAG GGC ACC ACA CAG CCC CCC GCC CAG CCA
    116 T   G   S   P   P   A   T   T   Q   P   Q   G   T   T   Q   P   P   A   Q   P

661  GCA TCG CAG GGC TCA GGA CCA ACC GCA TAG CTG CCT ATG CCC CCG CAG AAC TGG CTG CTG
    136 A   S   Q   G   S   G   P   T   A   *

721  CGT GTG AAC TGA ACA GAC GGA GAA GAT GTG CTA GGG AGA ATC TGC CTC CAC AGT CAC CCA
781  TTT CAT TGC TCG CTG CGA AAG AGA CGT GAG ACT GAC ATA TGC CAT TAT CTC TTT TCC AGT
841  ATT AAA CAC TCA TAT GCT TAT GGC TTG GAG AAA TTT CTT AGT TGG GTG AAT TAA AGG TTA
901  ATC CGA GAA TTA GCA TGG ATA TAC CGG GAC CTC ATG CAG CTT GGC AGA TAT CTG AGA AAT
961  GGT TTA ATT CAT GCT CAG GAG CTG TGT GCC TTT CCA TCC CTT CCG GCT CCC TAC CCC TCA
1021 CTT CCA AGG GTT CTC TCT CCT GCT TGC CCT TAG TGT CCT ACA TGG GGT TGT GAA GCG ATG
1081 GAG CTC CTC ACT GGA CTC GCC TCT CTC CTC TCC TCC CCC CAG GAG GAA CTT GAA AGG AGG
1141 GTA AAA AGA CTA AAA TGA GGG GGA ACA GAG TTC ACT GTA CAA ATT TGA CAA CTG TCA CCA
1201 AAA TTC ATA AAA AAC AAT AGT ACT GTG CCT CTT TCT TCT CAA ACA ATG GAT GAC ACA AAA
1261 CTA TGA GAG TGA CAA AAT GGT GAC AGG TAG CTG GGA CCT AGG CTA TCT TAC CAT GAA GGT
1321 TGT TTT GCT TAT TGT ATA TTT GTG TAT GTA GTG TAA CTA TTT TGT ACA ATA GAG GAC TGT
1381 AAC TAC TAT TTA GGT TGT ACA GAT TGA AAT TTA GTT GTT TCA TTG GCT GTC TGA GGA GGT
1441 GTG GAC TTT TAT ATA TAG ATC TAC ATA AAA ACT GCT ACA TGA CAA AAA CCA CAC CTA AAG
1501 AAA TTT TAA GAA TTT GGC ACA GTT ACT CAC TTT GTG TAA TCT GAA ATC TAG CTG CTG AAT
1561 ACG CTG AAG TAA ATC CTT GTT CAC TGA AGT CTT TCA ATT GAG CTG GTT GAA TAC TTT GAA
1621 AAA TGC TCA GTT CTA ACT AAT GAA ATG GAT TTC CCA GTA GGG GTT TCT GCA TAT CAC CTG
1681 TAT AGT AGT TAT ATG CAT ATG TTT CTG TGC ATG TTC TCT ACA CAA TTG TAA GAT GTC ACT
1741 GTA TTT AAC TGT TGC ACT TGT CAA CTT TCA ATA AAG CAT ATA AAT GTT GAT AAA AAA AAA
1801 AAA AAA AAA A

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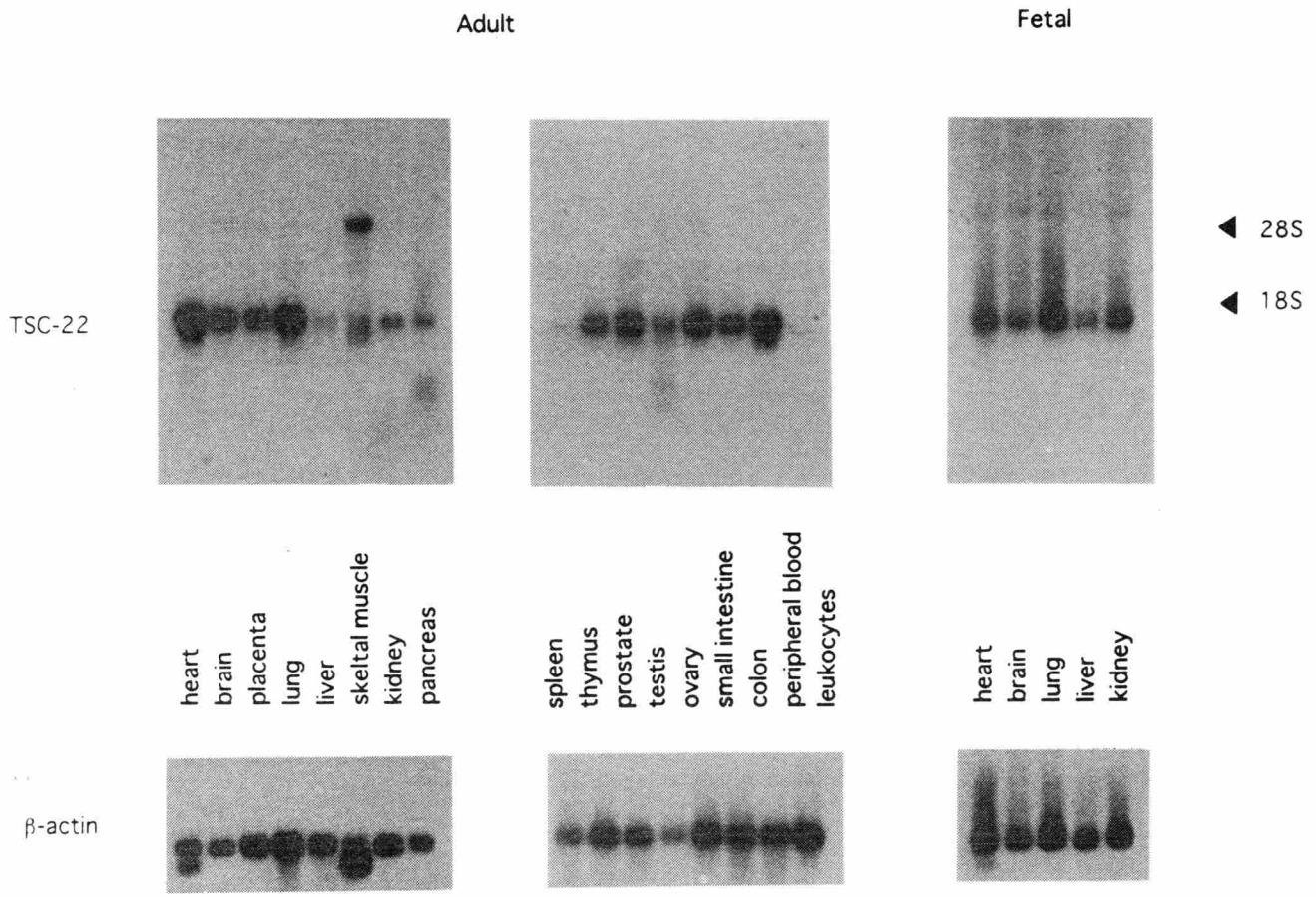
Fig. 3-6. Nucleotide and amino acid sequences of human TSC-22.

The coding region was cloned from human fetal kidney cDNA library by PCR, and 5' and 3' portions including noncoding regions were cloned by RACE technique. The positions of PCR primers (primers 1 and 2) and gene-specific primers for RACE are indicated with arrows. Leucine residues in the putative leucine zipper motif are presented in bold type. AATAAA, a possible polyadenylation signal is double-underlined. ATTTA sequences, which appear to be responsible for the mRNA instability, are underlined. This sequence is registered in the GenBank database under the accession number of D38585.

rat	1	MKSQWCRPVAMDLGVYQLRHFSISFLSLLGTENAS <u>SVRL</u> DNSS-GASVVA	
human	1	MKSQWCRPVAMDLGVYQLRHFSISFLSLLGTENAS <u>SVRL</u> DNSSSGASVVA	*
rat		IDNKIEQAMDLVKSHLMYAVREEVEV <u>L</u> KEQIKEL <u>I</u> EKNS <u>Q</u> LEQENNL <u>L</u> KT	
human		IDNKIEQAMDLVKSHLMYAVREEVEV <u>L</u> KEQIKEL <u>I</u> EKNS <u>Q</u> LEQENNL <u>L</u> KT	
rat		LASPEQLAQFQAQLQTGSPATTQPQGTTPPAQPASQSGSTA	143
human		LASPEQLAQFQAQLQTGSPATTQPQGTTPPAQPASQSGPTA	* 144

**Fig. 3-7. Comparison of deduced amino acid sequences of human and rat TSC-22.**

The consensus sequence of protein kinase C phosphorylation site is underlined. The casein kinase-II consensus phosphorylation site is double-underlined. Leucine residues in the putative leucine zipper motif are boxed. Residues that differ in human and rat are indicated with asterisks.

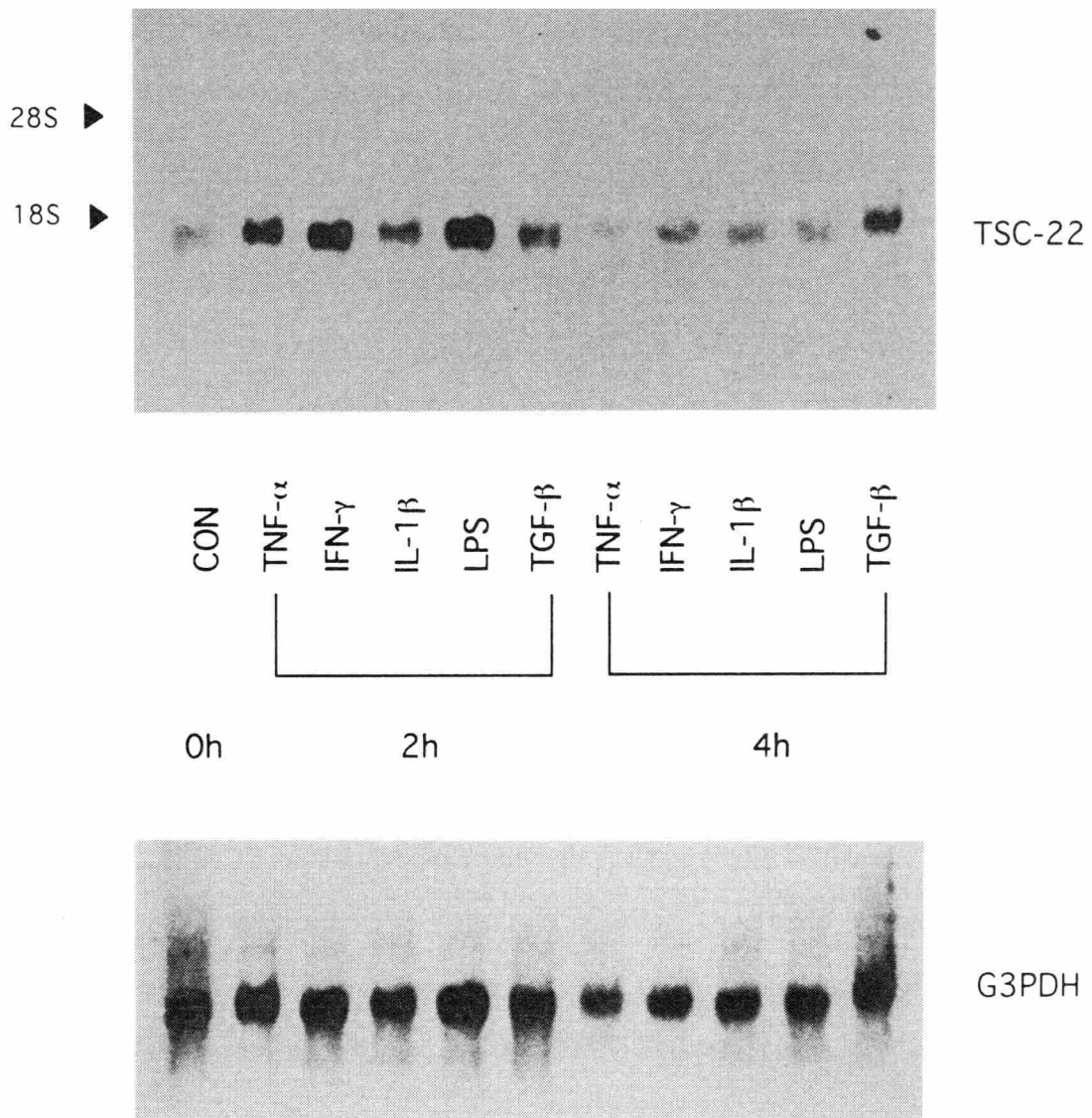


**Fig. 3-8. Distribution of TSC-22 transcript in human adult and fetal tissues.**  
 Northern filters were prepared by Clontech with poly(A)<sup>+</sup> RNA (2  $\mu$ g each) from the indicated human organs. Filters were hybridized with a human TSC-22 cDNA probe (upper). The same filters were rehybridized with a  $\beta$ -actin probe (lower). Positions of 18S and 28S RNA are indicated.

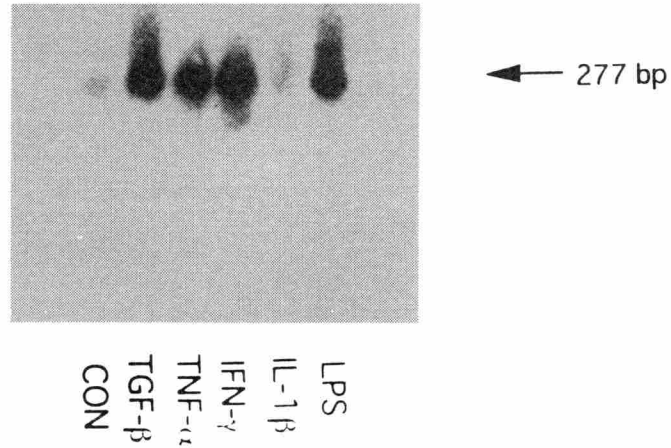


### *3-3-6. Induction of TSC-22 and CNP mRNA by cytokines*

The expression of CNP in cultured endothelial cells from bovine carotid artery was reported to be induced by TGF- $\beta$  or TNF- $\alpha$  [5, 6]. So, I next examined the effect of some cytokines on the expression patterns of TSC-22 and CNP in HAEC. As shown in Fig. 3-9, TSC-22 gene expression was induced by TNF- $\alpha$ , TGF- $\beta$ , IFN- $\gamma$  and LPS, not by IL-1 $\beta$ , in HAEC. There seemed to be some difference in the induction mode by TGF- $\beta$  and other stimulants because induction by TGF- $\beta$  was almost at the same level in 2-h and 4-h stimulation, while the induction of TSC-22 mRNA by other stimulants was more quickly extinguished. Expression of CNP mRNA was examined by reverse transcription and PCR with CNP-specific primers. By amplification of 25 cycles, the induction of CNP mRNA was observed in 4 h with all stimulants except IL-1 $\beta$  (Fig. 3-10), in good correlation with the induction of TSC-22 mRNA. PCR of 30 cycles did not show any significant difference among all RNA samples (data not shown), probably because the amplification reaction of CNP fragment reached plateau. TSC-22 was induced in all cases within 2 to 4 h of stimulation, while induction of CNP was observed significantly with 4-h stimulation, but very little with 2 h (data not shown). Therefore, TSC-22 expression seemed to precede CNP expression. These results suggest that the induction of TSC-22 correlated well with the stimulation of CNP gene expression by cytokines.



**Fig. 3-9. Effects of cytokines on the TSC-22 gene expression in HAEC cells.** After serum depletion, HAEC cells were treated with 500 U/ml TNF- $\alpha$ , 200 U/ml human IFN- $\gamma$ , 10 ng/ml IL-1 $\beta$ , 100 ng/ml LPS, and 10 ng/ml TGF- $\beta$ 1 for 2 h or 4 h. DNA-free total cellular RNA (10  $\mu$ g each) was analyzed for TSC-22 gene expression by Northern blot. The filter was rehybridized with G3PDH probe (lower) for control.



**Fig. 3-10. Effects of cytokines on the CNP gene expression in HAEC cells.**

For detecting CNP transcript, 10 µg each of total RNA prepared from cytokine-treated HAEC cells for Northern analysis was subjected to reverse-transcription and PCR with CNP-specific primers. Amplified products were fractionated in a 2% agarose gel, transferred onto a nylon membrane, and then hybridized with <sup>32</sup>P-labeled CNP cDNA probe. The positions of expected CNP fragment (277 bp) are indicated in arrows.

### 3-4. Discussion

In Chapter 2, the identification of two GC-rich elements (-49 to -45, -40 to -35) in the human CNP promoter region essential for positive regulation of CNP transcription in GH3 cells, and cooperative function of CRE-like sequence with these elements were described. This shortest promoter region with full activity was involved in the enhancement of the promoter activity in response to TGF- $\beta$ . To isolate transcription factor(s) that binds to these GC-rich elements and is involved in the activation by TGF- $\beta$ , the  $\lambda$ -ZAP cDNA library derived from GH3 cells was screened by Southwestern technique. Several positive clones were obtained, and one of them (clone  $\lambda$ 35) was identical to rat TSC-22. TSC-22 was originally identified in mouse osteoblastoma cell line MC3T3 E1 as a cDNA clone that was stimulated by TGF- $\beta$  treatment [8]. Because of the existence of a leucine zipper motif, mouse TSC-22 was speculated to be a transcription regulatory factor, however, its actual biological function had remained unknown. I have been able, for the first time, to clarify the function of TSC-22 as a transcription activator of CNP gene promoter in GH3 cells. These results indicate the possibility of a new class of DNA binding proteins, because no characteristic basic domain upstream of the leucine zipper domain was observed in TSC-22.

Based on the rat TSC-22 DNA sequence, full-length human TSC-22 cDNA was also cloned by PCR and RACE to understand the physiological of TSC-22 in human systems. Rat, mouse and human TSC-22 showed high homology in DNA and protein sequences. All TSC-22 types had the putative leucine zipper motif, but no characteristic basic region. The 3' untranslated region of human TSC-22 gene contained 3 copies of the ATTTA sequence, which appear to be responsible for increasing the instability of mRNA molecules [19], but no TTATTA(T/A)(T/A) or TTATTTATT sequence which was recently identified as an mRNA-destabilizing sequence [20, 21]. In HAEC, TSC-22 gene

was induced immediately by some cytokines and degraded quickly (Fig. 3-9), indicating the possible role of ATTTA motifs in mRNA instability.

In Chapter 2, the CNP function in pituitary-derived GH3 cells was described. Recently, the expression of CNP in bovine carotid vascular endothelial cells was reported to be enhanced by cytokine treatment, such as TGF- $\beta$  and TNF- $\alpha$  [5, 6], suggesting some function of CNP in the vascular system, because the important role of TGF- $\beta$  in the vascular system was reported in atherosclerosis and angiogenesis [22-24]. TGF- $\beta$  is a known inhibitor of vascular smooth muscle cell proliferation [25], and CNP is also reported to inhibit growth of vascular smooth muscle cells [7]. Therefore, the inhibitory effect of TGF- $\beta$  may result, in part, from the action of CNP secreted from endothelial cells. In other words, TGF- $\beta$  enhances the secretion of CNP from endothelial cells, then CNP acts on its receptors on smooth muscle cells, and elevates the cellular cGMP level to repress cell proliferation.

As TSC-22 was originally identified as a cDNA clone stimulated by TGF- $\beta$ , it might be responsible for transmitting a signal from TGF- $\beta$  to CNP gene expression. To help answer this question, I considered the following observations. First, TSC-22 also up-regulated CNP gene expression in GH3 cells (Fig. 3-4). Second, the short promoter region cloned in the reporter construct (-54 to +73) was responsive to both TSC-22 and TGF- $\beta$  (Figs. 3-1 and 3-4). And third, the expression of CNP gene was somewhat delayed in comparison to the expression of TSC-22 when HAEC were treated with TGF- $\beta$  (Figs. 3-9 and 3-10). These observations suggest the participation of TSC-22 in the CNP gene expression stimulated by TGF- $\beta$ . The results in Figs. 3-9 and 3-10 demonstrated the correlation between induction of TSC-22 and CNP gene expression by other stimulants, also suggesting the possible role of TSC-22 in transmitting signals from those stimulants to CNP gene expression. Taken together, TSC-22 seems to be located in the signal transduction pathway from cytokines to CNP gene expression (described in **Concluding Remarks** and Fig. 5-1). Both TGF- $\beta$  and IFN- $\gamma$  are known to repress the proliferation of smooth muscle cells and inhibit intimal thickening after balloon injury

of arterial walls [25, 26]. Therefore, TSC-22 and CNP may play pathophysiologically important roles in vascular diseases.

The existence of a 70 kDa factor that binds to the GC-rich elements of the CNP promoter in GH3 cells was shown in Chapter 2. The 70 kDa protein seemed to be responsible for the cell-type specific constitutive expression of the CNP gene. TSC-22 encodes an 18 kDa protein, so distinct from the 70 kDa factor. In this study, I could not obtain the cDNA clone corresponding to the molecular weight of 70 kDa by the Southwestern screening of a cDNA library constructed from GH3 mRNA. Possible reasons for this inability to obtain clones for 70kDa factor might be as follows. Firstly, the DNA binding ability of certain transcription factors are regulated by post-translational modification such as phosphorylation or proteolytic cleavage. Such transcription factors could not be cloned by bacteriophage-based Southwestern expression cloning method. Secondly, in the procedures of Southwestern cloning method, expressed protein was at first denatured with guanidinium hydrochloride then refolded. Since the efficiency of refolding of protein is critical point in this method, it is likely that the efficiency of correct refolding of higher molecular proteins is lower than that of smaller proteins.

Recently Datto et al. reported the examination of the TGF- $\beta$  responsible elements in the promoter of p21<sup>WAF1</sup> gene [27]. They found the SP-1 binding site in that TGF- $\beta$  responsible elements, and shown that both SP-1 and SP-3, a member of the SP-1 family, were involved in TGF- $\beta$ -mediated transcriptional activation. The region in the CNP promoter that is responsible for TGF- $\beta$ -mediated transcriptional stimulation also contains GC-rich elements. From the gel shift analysis with these GC-rich elements in the CNP promoter, a transcription factor in GH3 cells acting on these elements seems distinct from SP-1 or SP-3. AP-1 is also suggested to be involved in the TGF- $\beta$ -mediated transcriptional regulation [28, 29]. Therefore, it seems that distinct factors may participate in the TGF- $\beta$ -mediated pathway in a cell- or tissue-specific manner. It is also possible that a complex of transcription factors acts on the GC-rich element of the CNP

promoter to elicit the cell- or tissue-specific constitutive expression or the stimulant-dependent induced expression, and TSC-22 is a part of that complex.

TSC-22 was originally isolated in mouse osteoblastoma [8], suggesting its functioning in the bone system. CNP also has been reported to function in bone maturation [30]. Tissue distribution of human TSC-22 showed a wide expression pattern in accordance with the observation in rat tissues [17]. The difference in tissue distribution between rats and humans was observed in liver and spleen which showed TSC-22 expression of high level in rats but of very low level in humans. The expression of TSC-22 gene in kidney decreased from the fetus to the adult, suggesting that the TSC-22 expression may be developmentally regulated. Analysis of TSC-22 tissue distribution also shows its expression in some immune organs. The expression of CNP in the thymus was also reported [31], but its function in the immune system is still unclear. Recently, TSC-22 gene expression was reported in follicle-stimulating-hormone-stimulated Sertoli cells [17]. In those cells, TSC-22 was regulated by factors other than TGF- $\beta$ , but no specific functions were reported. Observations in this chapter indicate the function of TSC-22 as a transcriptional regulatory factor of the CNP gene. Other functions of TSC-22 can be expected because of its wide tissue distribution, and one novel function of TSC-22 is demonstrated in Chapter 4.

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## **Chapter 4. Function of TSC-22 in TGF- $\beta$ -mediated apoptotic cell death of human gastric carcinoma cells**

### **4-1. Introduction**

In Chapter 3, identification of TSC-22 as a transcriptional regulatory factor for the CNP gene and its involvement in the gene expression stimulated by TGF- $\beta$  were described. Because of the wide distribution in human tissues, TSC-22 may participate in other TGF- $\beta$ -mediated physiological cell events. To elucidate this question is the main purpose in this chapter.

As mentioned in Chapter 3, TGF- $\beta$  has a very important role in the vascular systems, and shows variety of biological activities in various organs or cells. TGF- $\beta$  has been shown to be a multifunctional growth regulator that either stimulates proliferation of mesenchymal cells or inhibits the growth of epithelial cells [1, 2], including colon carcinoma [3], breast carcinoma [4], and gastric carcinoma [5]. The inhibitory effect of TGF- $\beta$  is fully reversible [6, 7]. In addition to growth regulation, it has been shown that TGF- $\beta$  can elicit apoptotic cell death in both normal and malignant cells under certain conditions. TGF- $\beta$  has been found to induce apoptosis in endometrial cells [8], normal and transformed hepatocytes [9, 10], acute myelogenous leukemia cells [11], and a number of other cell types. However, it is still unclear why TGF- $\beta$  causes reversible growth arrest of some cells while other cells undergo apoptosis. Apoptosis is a particular cell death process found in eukaryote. All multicellular organisms have death mechanisms to kill their own cells for development, homeostasis, and aging. The death of cells undergoing apoptosis is preceded by morphological changes [12] and the fragmentation of chromatin by a specific calcium regulated nuclease at the linker region between nucleosomes [13]. Human gastric carcinoma cell line HSC-39 was recently established and shown to undergo apoptosis when cells were treated with TGF- $\beta$ 1 under serum-free culture conditions [14]. This cell line required new protein synthesis for

undergoing apoptosis, but further mechanisms underlying this TGF- $\beta$ <sub>1</sub>-mediated apoptosis is still unclear. In the preliminary work, I found that TSC-22 gene expression in HSC-39 cells was also induced by TGF- $\beta$ <sub>1</sub>. Therefore, the possibility of the involvement of TSC-22 in TGF- $\beta$ <sub>1</sub>-mediated apoptosis of HSC-39 cells was further examined. In this chapter, the effect of TSC-22 expression in HSC-39 cells is described, and it is demonstrated that TGF- $\beta$ <sub>1</sub>-mediated apoptosis correlates with the induction of TSC-22 and the ectopic expression of TSC-22 in HSC-39 cells leads to apoptotic cell events.

## 4-2. Material and methods

### 4-2-1. Cell line and culture conditions

HSC-39 human gastric carcinoma cells were grown in  $\alpha$ -minimal essential medium ( $\alpha$ -MEM) supplemented with 10% FCS as previously described [14]. In order to analyze effect of TGF- $\beta$ 1, cells were previously adapted to serum-free chemically defined medium (CDM), which consisted of DMEM: Ham's F12 medium (1:1) supplemented with 0.05% BSA (Sigma), for at least 2 weeks, and then recombinant human TGF- $\beta$ 1 (Wako Pure Chemicals, Osaka, Japan) was added in the same serum-free CDM at the concentration of 2 ng/ml .

### 4-2-2. Northern blot analysis

Total cytoplasmic RNA was prepared from TGF- $\beta$ 1-treated cells by the AGPC method [15] as described in Chapter 3. Cytoplasmic RNA (5  $\mu$ g per lane) was electrophoretically separated in a denatured 1.4% agarose gel, and transferred to Hybond-N nylon membrane (Amersham). The blot was sequentially hybridized to  $^{32}$ P-labeled human TSC-22 cDNA and human G3PDH (Clontech) probes overnight at 65 °C in 0.5 M sodium phosphate (pH 7.5), 10 mg/ml BSA, 1 mM EDTA, and 7% SDS, followed by washing twice for 30 min at 50 °C in 2xSSC and 0.1% SDS.

### 4-2-3. Construction of expression vectors and transfection

Rat TSC-22 full-length cDNA was subcloned into the pEF-BOS expression vector [16] at Xba I site under the control of elongation factor promoter to obtain pEF-TSC22. To construct influenza virus hemagglutinin epitope (HA)-tagged TSC-22 expression vector (pCG-HATSC22), rat TSC-22 cDNA was subcloned into pCG-N-BL vector [17] at SmaI site (the amino acid sequence around fusion region was confirmed by DNA sequencing as HA1 epitope-Ser-Arg-Thr-Ser-Gly-Ser-Pro-Asn-Ser-rat TSC-22). HSC-39 cells ( $1.5 \times 10^6$  cells) were transfected with plasmid DNA (5  $\mu$ g) by

electroporation using CELL-PORATOR™ system (GIBCO BRL) at the setting of 300 V and 880  $\mu$ F.

#### 4-2-4. Apoptosis assay

For the cell viability assay, cell suspensions were scored for numbers of viable and nonviable cells by tripan blue dye exclusion and quantitation was performed by hemocytometer. For the morphological evaluation, cells were fixed on glass slides with 4% formaldehyde in PBS for 5 min at room temperature, and stained with Hoechst dye 33258 (Sigma) at 0.1  $\mu$ g/ml in PBS. Nuclear morphology was observed in a fluorescence microscope. DNA fragmentation analysis was performed according to [18]. Briefly, cell pellets were lysed in 50 mM Tris-Cl (pH 8), 10 mM EDTA, 0.5% SDS, treated with DNase-free RNase A (0.2 mg/ml) for 30 min at 50 °C, and then treated with proteinase K (0.2 mg/ml) for 1 h at 50 °C. DNA was extracted once with phenol and once with phenol/chloroform (1:1), and precipitated with ethanol. Recovered DNA (0.5  $\mu$ g each) was then labeled with [ $\alpha$ -<sup>32</sup>P]-dCTP and Klenow fragment for 10 min at room temperature, and precipitated with ethanol. Labeled DNA (10000 cpm for each lane) was electrophoresed in a 2% agarose gel, and the gel was dried for autoradiography.

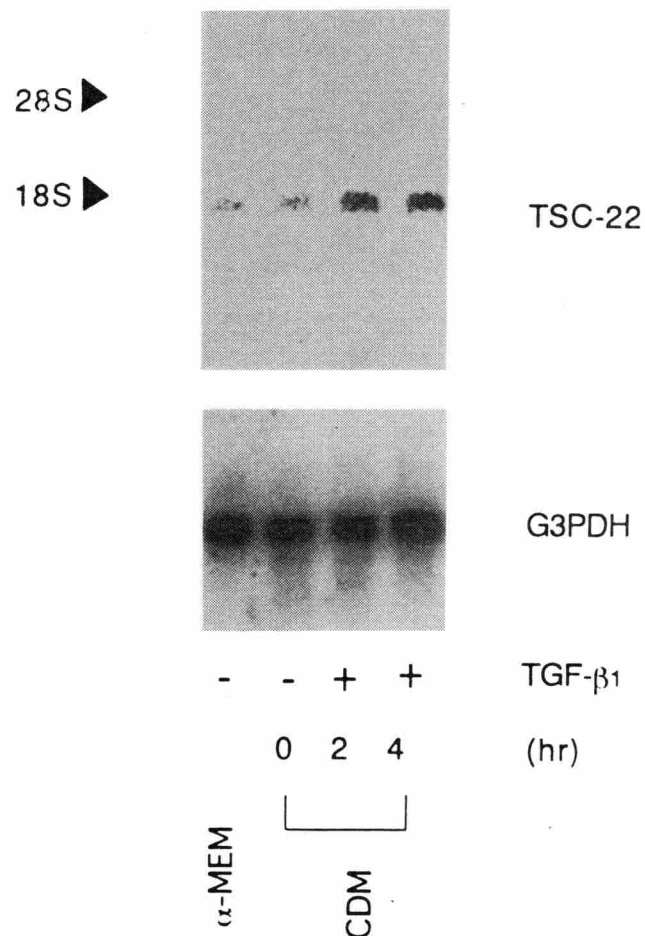
#### 4-2-5. Immunostaining

At 48 h after transfection with pCG-HATSC22 or pCG-N-BL empty vector for control, HSC-39 cells were washed twice with PBS, and collected on glass slides using cytospin system (Tomy Seiko, Tokyo, Japan). Cells were then fixed with acetone for 5 min at -20 °C, and air-dried. After washing twice with PBS, fixed cells were incubated with 500-fold diluted anti HA monoclonal antibody (Boehringer) for 2 h at room temperature, washed extensively and then incubated with 500-fold diluted FluoroLink Cy3-labeled goat anti mouse IgG antibody (Amersham). After washing with PBS, cells were visualized on a fluorescence microscope.

### 4-3. Results

#### 4-3-1. Induction of TSC-22 mRNA in HSC-39 cells by TGF- $\beta$ 1

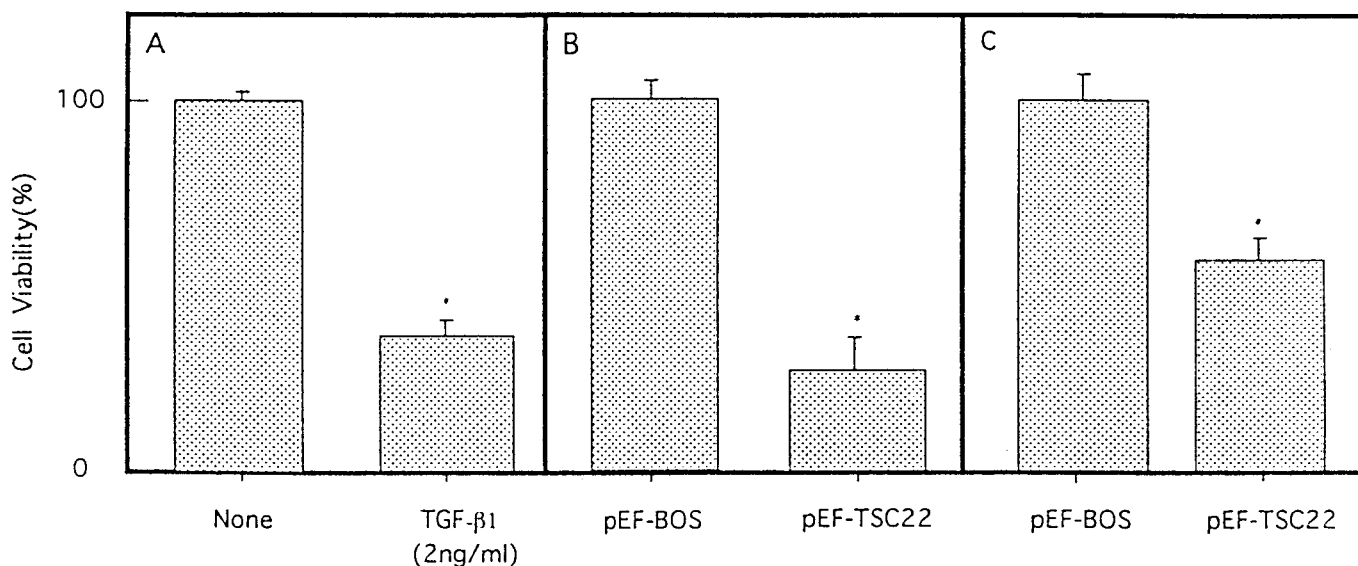
Human gastric carcinoma HSC-39 cells previously adapted to serum-free CDM were treated with TGF- $\beta$ 1, and cytoplasmic RNA were prepared for northern blot analysis of TSC-22 gene expression. After 4-h treatment with TGF- $\beta$ 1, significant induction of TSC-22 mRNA was observed, while G3PDH mRNA did not show any change (Fig. 4-1). TSC-22 gene expression was low in non-treated cells or cells cultured in  $\alpha$ -MEM supplemented with FCS (apoptotic cell death of HSC-39 cells was not induced by TGF- $\beta$ 1 under the serum-containing culture conditions).



**Fig. 4-1. Northern blot analysis of TSC-22 mRNA level in HSC-39 cells after TGF- $\beta$ 1 treatment.** Cells were cultured in  $\alpha$ -MEM plus FCS (lane 1) or adapted to serum-free CDM conditions (lane 2) for 2 weeks. Cells adapted to serum-free CDM conditions were treated with TGF- $\beta$ 1 (2 ng/ml) for 2 h (lane 3) or 4 h (lane 4). Then cytoplasmic RNA was prepared and separated in a denatured gel (5  $\mu$ g per lane) for Northern blot. The blot was sequentially hybridized with TSC-22 and G3PDH probes.

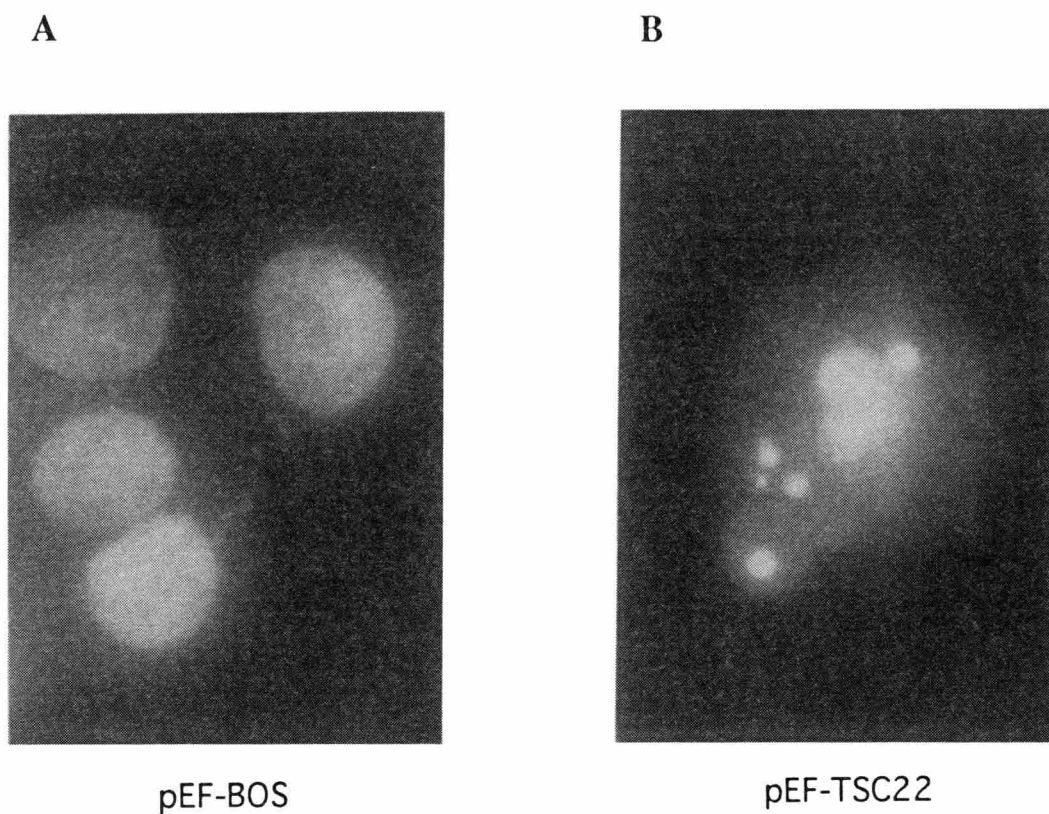
#### 4-3-2. Apoptosis of HSC-39 cells transfected with TSC-22 expression vector

HSC-39 cells were transfected with a TSC-22 expression vector (pEF-TSC22) or a control empty vector (pEF-BOS), and cell viability was examined after 48 h. When HSC-39 cells were previously adapted to serum-free CDM culture conditions to acquire the sensitivity to TGF- $\beta$ 1-mediated apoptosis, pEF-TSC22 transfection significantly reduced cell viability compared with control pEF-BOS transfection to the similar extent to that caused by TGF- $\beta$ 1 (Fig. 4-2A, B). Although HSC-39 cells did not exhibit any apoptotic phenomena under culture conditions of  $\alpha$ -MEM plus FCS, pEF-TSC22 transfection of cells cultured in this serum-containing medium caused significant reduction of cell viability as observed in the serum-free medium (Fig. 4-2C).



**Fig. 4-2. Effects of TGF- $\beta$ 1 treatment and pEF-TSC22 transfection on viability of HSC-39 cells.** (A) Cells were adapted to serum-free CDM culture conditions and then cultured in the absence or presence of TGF- $\beta$ 1 (2 ng/ml). (B and C) HSC-39 cells ( $1.5 \times 10^6$  cells) adapted to serum-free CDM (B) or cultured in  $\alpha$ -MEM plus FCS (C) were transfected with pEF-TSC22 or pEF-BOS control empty vector (5  $\mu$ g for each transfection) by electroporation. After 48 h incubation, cell viability was determined by trypan blue dye staining, and expressed as the percentage relative to untreated or control transfected cells. Results from 3 independent experiments are shown as means  $\pm$  SD. \*, significantly different from control (P < 0.01) by Student's *t* test.

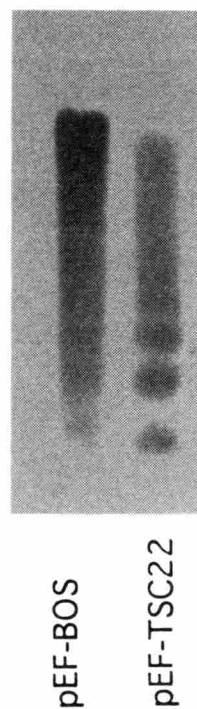
To investigate whether the reduction of cell viability in HSC-39 cells transfected with pEF-TSC22 was due to apoptotic cell death as caused by TGF- $\beta$ 1, I next examined the morphological change of cell nucleus using Hoechst 33258 fluorochrome staining. HSC-39 cells cultured in  $\alpha$ -MEM plus FCS exhibited chromatin condensation, which is characteristic of apoptosis, at 48 h after transfection with pEF-TSC22 (Fig. 4-3B), while cells transfected with pEF-BOS did not show such morphological change of nuclei (Fig. 4-3A). The similar morphological change was observed in cells previously adapted to serum-free CDM and transfected with pEF-TSC22 (data not shown).



**Fig. 4-3. Chromosomal condensation of HSC-39 cells caused by pEF-TSC22 transfection.** HSC-39 cells cultured in  $\alpha$ -MEM plus FCS were transfected with pEF-TSC22 (B) or pEF-BOS control vector (A). After 48 h, cells were fixed and stained with Hoechst 33258 fluorochrome.



Apoptotic cell death of HSC-39 cells by pEF-TSC22 transfection was identified also by DNA fragmentation analysis. Cellular DNA prepared from HSC-39 cells at 48 h after transfection with pEF-TSC22 exhibited ladder pattern, which is also characteristic of apoptosis, while no ladder pattern was observed in DNA preparation from cells transfected with pEF-BOS (Fig. 4-4). These results strongly suggest that the ectopic expression of TSC-22 in HSC-39 cells caused apoptotic cell death even under conditions not sensitive to TGF- $\beta$ 1-mediated apoptosis.



**Fig. 4-4.** DNA fragmentation of HSC-39 cells cultured in  $\alpha$ -MEM plus FCS caused by pEF-TSC22 transfection. After 48h, DNA was prepared from cells transfected as in Fig. 4-3, labeled with [ $\alpha$ - $^{32}$ P] CTP and Klenow enzyme, and then fragmentation was analyzed in a 2% agarose gel.

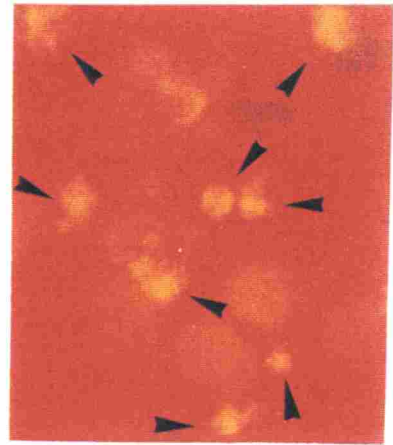
#### 4-3-3. Correlation of TSC-22 expression with apoptosis of HSC-39 cells

To examine the correlation between TSC-22 expression and apoptotic cell morphology, an HA-tagged TSC-22 expression vector (pCG-HATSC22) was constructed and transfected into HSC-39 cells. At 48 h after transfection, cells were subjected to immunostaining with anti HA monoclonal antibody. Fig. 4-5 shows that almost every cell positive to anti HA antibody, therefore expressing TSC-22, exhibited abnormal cell morphology characteristic of apoptosis, indicating one to one correspondence of TSC-22 expression with apoptotic cell death.

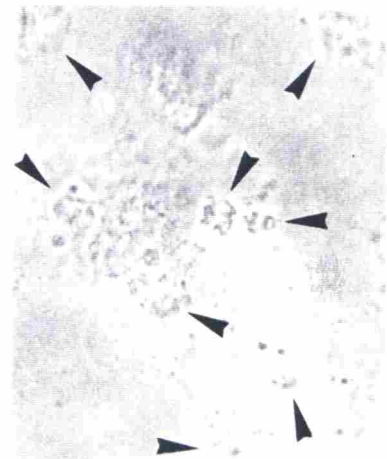
A



B



C



**Fig. 4-5. Correlation of ectopic TSC-22 expression with apoptotic cell morphology.** HSC-39 cells cultured in  $\alpha$ -MEM plus FCS were transfected with an HA-tagged TSC-22 expression vector (pCG-HATSC22). After 48 h, transfected cells were examined by immunostaining with anti HA monoclonal antibody and Cy3-labeled secondary antibody as in "Materials and methods" section. (A) Saline was added instead of anti HA primary antibody. (B) Immunostaining of cells expressing HA-tagged TSC-22. Cells positive to anti HA antibody are indicated by arrow heads. (C) The same visual field as (B) observed in a phase-contrast microscope. Cells corresponding to HA-positives in (B) are also indicated by arrow heads.

#### 4-4. Discussion

TGF- $\beta$ 1 elicits apoptosis in human gastric carcinoma HSC-39 cells under serum-free culture conditions [14]. This TGF- $\beta$ 1-mediated apoptosis is partially prevented by cycloheximide, but further mechanisms have been unclear. My observation that the expression of TSC-22 in HSC-39 cells elicited apoptotic cell events could provide a possible mechanism of TGF- $\beta$ 1-mediated apoptosis of HSC-39 cells by several reasons. First, TSC-22 is involved in TGF- $\beta$ 1 signal transduction pathway in certain cell types [19, 20]. Second, the expression of TSC-22 was greatly stimulated by TGF- $\beta$ 1 also in HSC-39 cells only when the cells were adapted to culture conditions undergoing apoptosis. Third, almost every HSC-39 cell expressing HA-tagged TSC-22 showed apoptotic cell morphology in immunostaining. TSC-22 was originally identified as a clone that was stimulated in mouse osteoblast cells by the treatment of TGF- $\beta$  [19]. It has a putative leucine zipper domain and has been shown to be localized in the cell nucleus. Therefore, it has been speculated as a transcriptional regulator. I firstly demonstrated the actual function of TSC-22 as a transcriptional regulator in the CNP gene expression stimulated by TGF- $\beta$  as in Chapter 3. The results in this chapter indicate a novel biological role of TSC-22 as an apoptosis mediator. Some transcription factors such as p53, c-Myc and AP-1, have been shown to play important roles in apoptotic cell death. However, the true targets of those transcription factors in the apoptotic cell death events are still unknown. Recent studies show that p53 downregulates and upregulates the expression of bcl-2 and bax, respectively [21, 22], but the actual cell death mechanism is still controversial. It is an interesting question whether TSC-22 acts as a transcriptional regulator in the TGF- $\beta$ 1-mediated apoptosis of human gastric carcinoma cell, but further studies are needed to answer this question and to know the cell death mechanism by TSC-22. To understand the cell death mechanism, the status of other apoptosis-related factors, such as bcl-2 and bax, seems important.

The idea that TGF- $\beta$ 1-mediated apoptosis of HSC-39 cells involves the stimulation of TSC-22 coincides with the previous observation of the requirement of new protein synthesis for undergoing apoptosis. Recently, human hepatoma cell line Hep 3B was also reported to undergo apoptosis in response to TGF- $\beta$  [23]. The mechanism of the TGF- $\beta$ 1-mediated cell death of hepatoma, however, seems somewhat different from that of HSC-39 cells, because treatment of cycloheximide had no effect on the TGF- $\beta$ 1-mediated hepatoma cell death and chromatin condensation was not observed in that apoptosis system. Therefore, whether TSC-22 is involved in other TGF- $\beta$ 1-mediated apoptosis systems, such as hepatoma cells and normal hepatocytes, is also an important point to be examined.

In conclusion, the evidence for TSC-22 to elicit apoptotic cell death in human gastric carcinoma cells and possibly to mediate the apoptosis induced by TGF- $\beta$ 1 was firstly presented.

#### 4-5. References

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## Concluding remarks

Transcriptional control plays a fundamental role in a wide variety of biological transitions which result in phenotypic changes and many biological events. It is very important to investigate the transcriptional regulatory mechanism of a certain gene for understanding its physiological role. In this thesis, I focused on the regulatory mechanism of the CNP gene expression including a transcriptional regulatory factor effecting the CNP promoter to understand the actual physiological functions of CNP. First, the cell-type specific function of the CNP promoter was investigated to clarify which organs produce CNP molecules and how the CNP gene expression is regulated. Interesting finding was obtained that the CNP promoter functioned very effectively in GH3 cells of pituitary somatomammotroph-origin and much less effectively in other cell lines. These findings suggest the constitutive production of CNP in the anterior pituitary and therefore, the novel function of CNP as a local modulator within the anterior pituitary in autocrine or paracrine action. This suggestion led my colleague to the finding that CNP acts as a stimulator of the growth hormone release in the pituitary systems. Detailed functional and gel shift analyses of the CNP promoter revealed the existence of one positive regulatory region (from -54 to -19) which conferred 90% of the promoter activity in GH3 cells and contained two equipotent GC-rich cis-elements. Both GC-rich elements shared half the promoter activity and binding properties to the nuclear protein in GH3 cells. This promoter region containing GC-rich elements was also involved in the stimulation of the promoter activity by TGF- $\beta$ . The TGF- $\beta$  responsive sequence element that positively regulates transcription has not been examined so well, and therefore this finding is important to understand the gene expression mechanism stimulated by TGF- $\beta$  as well as the mechanism of the constitutive expression of the CNP gene. Eventually TSC-22 of unknown function was isolated as a specific binding factor to the GC-rich element of the CNP promoter. TSC-22 significantly enhanced the CNP promoter activity in GH3 cells and HAEC. TSC-22 gene expression was stimulated by

cytokines including TGF- $\beta$  in GH3 cells as well as HAEC, in correlation with the CNP mRNA increase. Therefore, TSC-22 seems to be a transcriptional regulator of the CNP gene and to be involved in transmission of signals from TGF- $\beta$  for CNP gene expression. I provided the first evidence to show an actual biological function of TSC-22 as a transcriptional regulatory factor (Chapter 3).

In the vascular systems, biological function of CNP such as a local modulator has been suggested. The overall hypothesis on signal transduction of TGF- $\beta$  in the vascular systems emerges from the above experiments as is summarized in Fig. 5-1. TGF- $\beta$  is activated from the inert form in response to some inflammatory stimulation, and then acts on vascular endothelial cells as well as smooth muscle cells. In vascular endothelial cells, TGF- $\beta$  then enhances TSC-22 expression to stimulate the expression of CNP that may act on smooth muscle cells nearby as a local cell proliferation modulator. Results in this thesis not only support the expression of CNP in vascular endothelial cells and its biological function in the vascular systems, but provide a new strategy to develop compounds that can up-regulate the CNP gene expression in the vascular systems. Those compounds will open the possibility of the novel pharmaceuticals for atherosclerosis or restenosis after percutaneous transluminal coronary angioplasty, because CNP inhibits the proliferation of vascular smooth muscle cells that seems to be the main reason for the intimal thickening after the vascular injury.

Recent studies indicate that heterodimerization between a certain transcription factor and a co-factor is a remarkably effective means to generate diversity of specific gene expression. The complex formed by multiple transcription factors would be needed also for the CNP gene expression. So, identifying such co-factors interacting with TSC-22 may be important for fully understanding of the mechanism of CNP gene regulation as well as signal transduction pathway of TGF- $\beta$ . Efforts to elucidate the signaling pathway from TSC-22 to general initiation factors are also exciting challenge, because the functional relevance of interactions between various activators and general initiation factors has not been well proven. Furthermore, the finding of TSC-22 as a

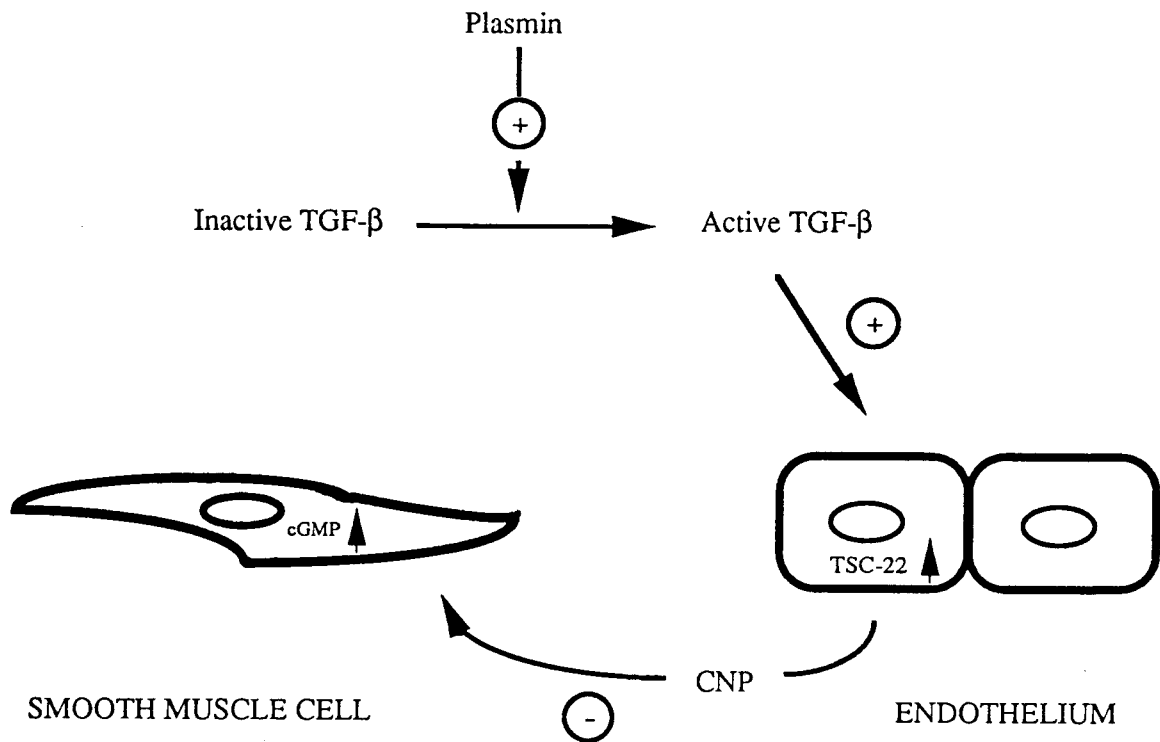
transcriptional regulatory factor of the CNP gene should lead to a deeper understanding of the regulation mechanism of the gene expression of CNP as well as other genes that are affected by TGF- $\beta$ .

TSC-22 participates in not only the stimulation of CNP gene expression by TGF- $\beta$ , but in another TGF- $\beta$ -mediated cell event, apoptosis. Overexpression of TSC-22 in HSC-39 human gastric carcinoma cells caused apoptotic phenomena independently of TGF- $\beta$ . Several transcription factors have been shown to act as a regulator of apoptotic cell death. The study in this thesis proposes that TSC-22 opens a new category of such apoptosis-related factors. Detailed signal transduction pathway of TGF- $\beta$ -mediated apoptosis has not been fully understood. Therefore, the finding in Chapter 4 that TSC-22 is involved in TGF- $\beta$ -mediated apoptosis gives useful information to understand apoptosis induced by TGF- $\beta$ . It is an interesting question whether TSC-22 acts as a transcriptional regulator in the TGF- $\beta$ -mediated apoptosis of human gastric carcinoma cell, but further studies are needed to answer this question. Supposing that transcriptional regulatory function of TSC-22 is required for the induction of apoptosis, identification of the target gene of TSC-22 should be important to know the cell death mechanism by TSC-22, because it seems unlikely that CNP induces apoptosis in HSC-39 cells.

In conclusion, this thesis described the mechanism of the CNP gene regulation focusing on the cis regulatory element in the CNP promoter and transcription factor regulated by TGF- $\beta$ . One of the new findings in this thesis is that the CNP promoter effectively works in cells of somatomammotroph origin, which led to the discovery of a novel function of CNP in the pituitary system. The other finding that TSC-22 of unknown function acts as a transcriptional regulatory factor for the CNP gene gave useful information not only on the mechanism of TGF- $\beta$ -mediated up-regulation of the gene expression but also on the roles of TGF- $\beta$  and CNP in the vascular system. The finding that TSC-22 induces apoptosis in gastric cancer cells reveals the possible involvement of TSC-22 in many TGF- $\beta$ -mediated cell events other than the regulation of the CNP gene expression, supporting the idea that the same transcriptional regulatory factor is capable



of exerting distinct functions in respective biological events. To prove other unknown biological functions of TSC-22, examination of the TSC-22 status in TGF- $\beta$ -related diseases and production of knock-out mice in which the TSC-22 gene is targeted to be deficient must be useful.



**Fig. 5-1. Schematic model of the involvement of TSC-22 in the physiological role of TGF- $\beta$  in vascular system.** Responding to some vascular disorders that cause the proliferation of smooth muscle cells, TGF- $\beta$  is activated by plasmin from the inert form and acts on vascular endothelial cells to stimulate the production of CNP. CNP then acts on smooth muscle cells to inhibit their excessive proliferation and prevent the thickening of the vascular intima that can promote atherosclerotic lesions. TSC-22 participates in the stimulation of the CNP gene expression by TGF- $\beta$  as a transcriptional regulator. (+ means activation, - means repression)

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## List of Publications

### (1) Papers related to this thesis

1. Cell-Type-specific Function of the C-type Natriuretic Peptide Gene Promoter in Rat Anterior Pituitary-Derived Cultured Cell Lines.  
Ohta, S., Shimekake, Y., and Nagata, K.  
*Mol. Cell. Biol.* **13** (1993) 4077-4086.
2. Molecular Cloning and Characterization of a Transcription Factor for C-Type Natriuretic Peptide Gene Promoter.  
Ohta, S., Shimekake, Y., and Nagata, K.  
*Eur. J. Biochem.* **242** (1996) 460-466.
3. Expression of Transforming Growth Factor- $\beta$  Stimulated Clone 22 Induces Apoptotic Cell Death in Human Gastric Carcinoma Cells.  
Ohta, S., Yanagihara, K., and Nagata, K.  
*BIOCHEMICAL JOURNAL* submitted.

### (2) Other published papers

1. C-Type Natriuretic Peptide Stimulates Secretion of Growth Hormone from Rat-Pituitary-Derived GH3 Cells via a Cyclic-GMP-Mediated Pathway.  
Shimekake, Y., Ohta, S., and Nagata, K.  
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