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Cyclic GMP-Dependent Protein Kinase II Plays a Critical Role in C-Type Natriuretic Peptide-Mediated Endochondral Ossification

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Longitudinal bone growth is determined by endochondral ossification at the growth plate, which is located at both ends of long bones and vertebrae, and involves many systemic hormones and local regulators. C-type natriuretic peptide (CNP), a third member of the natriuretic peptide family, occurs at the growth plate and acts locally as a positive regulator of endochondral ossification through the intracellular accumulation of cyclic GMP (cGMP). The increase in cGMP concentrations is known to activate different signaling mediators, such as cyclic nucleotide phosphodiesterases, cGMP-regulated ion channels, and cGMP-dependent protein kinases (cGKs). The type II cGK (cGKII)-deficient mice (*Prkg2*$^{-/-}$ mice) develop dwarfism as a result of impaired endochondral ossification, suggesting that cGKII is important for the CNP-mediated endochondral ossification. However, given that *Prkg2*$^{-/-}$ mice differ from CNP-deficient mice (*Nppc*$^{-/-}$ mice) in the growth plate histology, which downstream mediator(s) of cGMP play key roles in the process is still an enigma. Here we show that targeted expression of CNP in the growth plate chondrocytes fails to rescue the skeletal defect of *Prkg2*$^{-/-}$ mice. Using cultured fetal mouse tibias, an *in vitro* model system of endochondral ossification, we also demonstrated that CNP cannot increase the longitudinal bone growth, and chondrocytic proliferation and hypertrophy, and cartilage matrix synthesis in *Prkg2*$^{-/-}$ mice. This study provides *in vivo* and *in vitro* genetic evidence that cGKII plays a critical role in CNP-mediated endochondral ossification. (*Endocrinology* 143: 3604–3610, 2002)

BONE FORMATION OCCURS through two different mechanisms: membranous and endochondral ossification (1). Most of the craniofacial bones develop through membranous ossification, in which mesenchymal precursor cells directly differentiate into bone-forming osteoblasts. On the other hand, longitudinal growth of long bones and vertebrae is determined by the process of endochondral ossification in the cartilaginous growth plate, which consists of the resting, proliferative, and hypertrophic zones of chondrocytes, typically in orderly columnar arrays. In this process, chondrocytes that arise from the undifferentiated mesenchymal cells undergo proliferation, hypertrophy, and cartilage matrix synthesis. Finally, the matrix around hypertrophic chondrocytes calcifies; they then undergo apoptosis and are replaced by bone at the adjacent metaphysis. During the past years, various growth factors and hormones have been implicated in the regulation of chondrocytic proliferation and differentiation. They include fibroblast growth factor, PTHrP, TGFβ, and bone morphogenetic proteins (2–5).

The natriuretic peptide family consists of three structurally related peptides: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) (6). These peptides can influence a variety of homeostatic processes by the intracellular accumulation of cyclic GMP (cGMP) through two different membrane-bound guanylyl cyclase (GC)-coupled receptors (GC-A and GC-B) (7, 8). ANP and BNP are cardiac hormones that are produced predominantly by the atrium and ventricle, respectively (9–11), and are thought to play important roles in the regulation of cardiovascular homeostasis, primarily through GC-A (12, 13). On the other hand, CNP occurs in a wide variety of tissues (7, 14–16), where it may act locally as an autocrine/paracrine regulator through GC-B (12, 13). Recently, we have generated CNP-deficient mice (*Nppc*$^{-/-}$ mice) and reported that they exhibit dwarfism as a result of impaired endochondral ossification (17). Targeted expression of CNP in the growth plate chondrocytes has rescued the skeletal defect of *Nppc*$^{-/-}$ mice (17). These observations indicate that CNP is a local positive regulator of endochondral ossification.

The increase in intracellular cGMP concentrations leads to the activation of several downstream mediators such as cyclic nucleotide phosphodiesterases (PDEs), cGMP-regulated ion channels, and cGMP-dependent protein kinases (cGKs) (18–20). For instance, at least two PDE isoforms (PDE1 and PDE5), which hydrolyze cGMP, are expressed during chondrogenesis in a mouse embryonal carcinoma-derived ATDC5 cell line (21). Two known cGK isoforms (cGKI and cGKII) are also present, but they are distributed differently in the mouse growth plate chondrocytes *in vivo* (22). We

Abbreviations: ANP, Atrial natriuretic peptide; BNP, brain natriuretic peptide; BrdU, bromodeoxyuridine; cAK, cAMP-dependent protein kinase A; cGMP, cyclic GMP; CNP, C-type natriuretic peptide; cGKs, cGMP-dependent protein kinases; GC, guanylyl cyclase; Ihh, Indian hedgehog; *Nppc*, CNP gene; *Nppc*$^{-/-}$ mice, CNP-deficient mice; *Npr2*, GC-B gene; PDE, cyclic nucleotide phosphodiesterases; *Prkg*, cGK gene; *Prkg2*$^{-/-}$ mice, cGKII-deficient mice; Tg, transgenic.

previously reported that cGKII-deficient mice ($Prkg2^{-/-}$ mice) show dwarfism because of impaired endochondral ossification (22). However, $Nppc^{-/-}$ mice differ remarkably from $Prkg2^{-/-}$ mice in the histology of the growth plate (17, 22). The $Nppc^{-/-}$ mice show a reduction of the growth plate in height with its chondrocytes arranged in regularly columnar array, whereas the growth plate of $Prkg2^{-/-}$ mice is characterized by increased height of the growth plate with proliferative chondrocytes intermingled in the hypertrophic zone. Which downstream mediator(s) of cGMP play key roles in CNP-mediated endochondral ossification is still an enigma.

We, therefore, postulate that the CNP/cGMP/cGKII pathway is important for endochondral ossification. In this study, we use both *in vivo* and *in vitro* genetic strategies to establish that cGKII plays a critical role in CNP-mediated longitudinal bone growth, chondrocytic proliferation and hypertrophy, and cartilage matrix synthesis during endochondral ossification.

Materials and Methods

Animals

Generation of transgenic (Tg) mice with targeted expression of CNP in the growth plate chondrocytes under the control of the mouse pro-α1 (II) collagen (*Col2a1*) promoter was reported previously (17). The CNP-Tg mice were crossed with heterozygous cGKII-deficient mice ($Prkg2^{+/-}$ mice) (22) to produce CNP-Tg mice with the disrupted cGKII allele (CNP-Tg/$Prkg2^{+/-}$ mice). Among the F1 generation, $Prkg2^{+/-}$ mice were intercrossed with CNP-Tg/$Prkg2^{+/-}$ mice to obtain nontransgenic $Prkg2^{+/+}$ mice (wild-type mice), CNP-Tg $Prkg2^{+/+}$ mice (CNP-Tg mice), nontransgenic $Prkg2^{-/-}$ mice ($Prkg2^{-/-}$ mice), and CNP-Tg $Prkg2^{-/-}$ mice (CNP-Tg/$Prkg2^{-/-}$ mice). In this study, we analyzed the skeletal phenotypes of wild-type, CNP-Tg, $Prkg2^{-/-}$, and CNP-Tg/$Prkg2^{-/-}$ mice. Genotypes for the CNP transgene and disrupted cGKII allele were determined by Southern blot analysis using mouse tail DNAs. All the experimental procedures were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research.

Skeletal preparation and histology

Skeletal preparation and histological analysis were performed as described (23). Briefly, mice were killed, skinned, eviscerated, and subjected to soft x-ray analysis. Tibias of 4-wk-old mice were fixed in 70% ethanol, decalcified in 5% formic acid and 5% formalin for 7 d, and embedded in paraffin. Five-micrometer-thick sections were cut and stained with alcian blue (pH 2.5) and hematoxylin-eosin (23). Immunohistochemical staining for Indian hedgehog (Ihh) and type X collagen was performed using a polyclonal goat anti-Ihh(C) antibody (1:10; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and a polyclonal rabbit anti-type X collagen antibody (1:400; LSL, Tokyo, Japan) as a primary antibody (24). Immunoreactions were visualized by a biotinylated antipolyvalent antibody, a streptavidin-biotin-horseradish peroxidase complex, and diaminobenzidine (Vector Laboratories, Inc., Burlingame, CA). The specificity of immunoreactions was controlled by omitting the primary antibody.

cGMP concentration measurements

The tail bone cGMP concentrations in 7-d-old neonates were determined by a RIA for cGMP as described (25).

Organ culture study

Tibial explants from wild-type and $Prkg2^{-/-}$ fetuses at 16.5 d post coitus were cultured for 4 d with vehicle or 10^{-7} M CNP as described (25). Before and after the culture, total longitudinal length of explants was measured by a light microscope with a linear ocular scale.

For histological analysis, cultured tibias were fixed in 10% formalin neutral buffer solution for 24 h, embedded in paraffin, and stained with alcian blue and hematoxylin-eosin. Immunohistochemical staining of type X collagen were performed as described above. The size of hypertrophic cells was measured on 5-μm-thick sections of cultured tibias for 4 d with or without 10^{-7} M CNP by computerized measurement system (KS400 Imaging System; Carl Zeiss, Eching, Germany).

Cell proliferation was assessed in the cultured tibias by measuring the incorporation of bromodeoxyuridine (BrdU) as previously described (25). After 3 d of culture with or without 10^{-6} M CNP, BrdU was added to the culture medium at a concentration of 10^{-5} M, and the tibias were incubated for an additional 3 h. Immunohistochemical staining of incorporated BrdU was performed using the 5-bromo-2'-deoxy-uridine labeling and detection kit II (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. The labeling index was calculated as the ratio of BrdU-labeled cell number to total cell number at the proliferative zones of growth plates.

Glycosaminoglycan synthesis was assessed in cultured tibias by measuring the incorporation of $^{35}SO_4$ as previously described (26). After 3 d of culture with or without 10^{-6} M CNP, $Na_2{}^{35}SO_4$ (Amersham Pharmacia Biotech, Buckinghamshire, UK) was added to the culture medium at a concentration of 5 μCi/ml for an additional 6 h. The tibias were then rinsed three times for 10 min in Pucks saline solution (Sigma, St. Louis, MO) and digested in 0.8 ml of 0.3% papain (Sigma) at 60 C for 24 h. To this digest, 0.8 ml of 10% cetylpyridinium chloride (Sigma) in 0.2 M NaCl was added. After incubation at 22 C for 18 h, the precipitate was washed three times with 0.1% CPC in 0.2 M NaCl, and dissolved in 0.8 ml of 23 N formic acid. Total $^{35}SO_4$ incorporation was measured by liquid scintillation counter.

Northern blot analysis

Total RNA was extracted from 1-wk-old mouse tibial epiphysis by the acid guanidinium phenol chloroform method (13). Northern blot analysis was performed using the mouse cetylpyridinium chloride (27), rat GC-B (13), mouse PDE1A, and mouse PDE5A (28) cDNA fragments.

Statistical analysis

Data were expressed as the mean ± SE. The statistical significance of differences in mean value was assessed by two-way ANOVA or Fisher's test. Differences among mean values were considered significant as values of $P < 0.05$ and $P < 0.01$.

Results

Skeletal phenotypes

Figure 1A shows the gross appearance of 25-wk-old female wild-type, CNP-Tg, $Prkg2^{-/-}$, and CNP-Tg/$Prkg2^{-/-}$ mice. The CNP-Tg mice exhibit kyphosis and elongated limbs, paws, and tails, which are similar to that found in BNP-Tg mice (23, 29). The $Prkg2^{-/-}$ mice develop dwarfism and short limbs, paws, and tails as reported (22). The CNP-Tg/$Prkg2^{-/-}$ mice are phenotypically indistinguishable from $Prkg2^{-/-}$ mice; they both show severe dwarfism characterized by short tails and extremities. At birth, there are no significant differences in the naso-anal length among genotypes (Fig. 1B). At 5 wk of age, CNP-Tg mice grow larger, whereas $Prkg2^{-/-}$ mice smaller, than wild-type mice. The naso-anal length differences among these animals increase progressively until 10 wk of age, which are unchanged thereafter. However, no significant difference in the naso-anal length is noted between $Prkg2^{-/-}$ and CNP-Tg/$Prkg2^{-/-}$ mice at any time points examined.

Soft x-ray analysis revealed that CNP-Tg mice have longer vertebral bodies, tails, and extremities compared with wild-type mice at 25 wk of age (Fig. 1C). Both $Prkg2^{-/-}$ and CNP-Tg/$Prkg2^{-/-}$ mice show a reduction in the length of

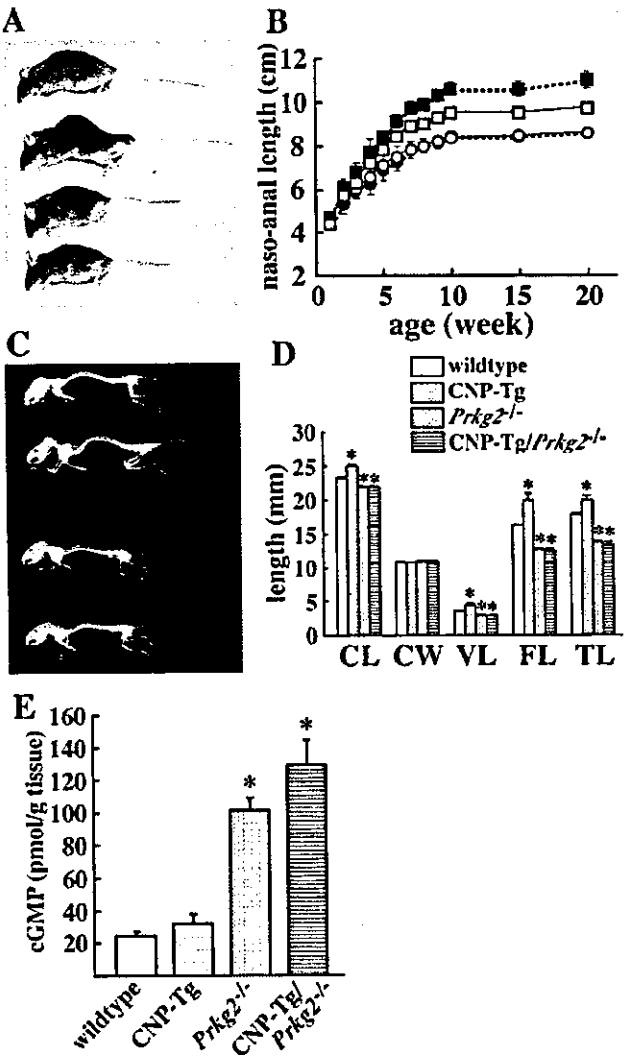

FIG. 1. Skeletal phenotypes of wild-type, CNP-Tg, *Prkg2*$^{-/-}$, and CNP-Tg/*Prkg2*$^{-/-}$ mice. A, Gross appearance (25-wk-old females). From *top* to *bottom*, wild-type, CNP-Tg, *Prkg2*$^{-/-}$, and CNP-Tg/*Prkg2*$^{-/-}$ mice. B, Growth curves of wild-type (*open square*), CNP-Tg (*closed square*), *Prkg2*$^{-/-}$ (*open circle*), and CNP-Tg/*Prkg2*$^{-/-}$ (*closed circle*) mice. The symbol of CNP-Tg/*Prkg2*$^{-/-}$ mice is behind that of *Prkg2*$^{-/-}$ mice (n = 6). C, Soft x-ray analysis (25-wk-old females). From *top* to *bottom*, wild-type, CNP-Tg, *Prkg2*$^{-/-}$, and CNP-Tg/*Prkg2*$^{-/-}$ mice. D, Bone lengths of wild-type, CNP-Tg, *Prkg2*$^{-/-}$, and CNP-Tg/*Prkg2*$^{-/-}$ mice (n = 4, 25-wk-old females). CL, Naso-occipital length of the calvarium; CW, maximal interparietal distance of the calvarium; VL, fifth lumbar vertebral length; FL, femoral length; TL, tibial length. *, $P < 0.01$ *vs.* wild-type mice. E, Bone cGMP concentrations in wild-type, CNP-Tg, *Prkg2*$^{-/-}$, and CNP-Tg/*Prkg2*$^{-/-}$ mice (n = 3–7, 1-wk-old). *, $P < 0.01$ *vs.* wild-type.

vertebral bodies, tails, and extremities relative to wild-type mice. The longitudinal lengths of femurs, tibias, vertebrae, and calvarium formed through endochondral ossification are 10–25% longer in CNP-Tg mice, whereas approximately 20% shorter in *Prkg2*$^{-/-}$ and CNP-Tg/*Prkg2*$^{-/-}$ mice, than wild-type mice (Fig. 1D). No significant difference in the longitudinal bone length is noted between *Prkg2*$^{-/-}$ and CNP-Tg/*Prkg2*$^{-/-}$ mice. There are no appreciable changes in the width of calvarium, which is determined by membranous ossification among genotypes.

Histological analysis

Histological examination of the tibial growth plate revealed no significant differences among genotypes at perinatal stage (data not shown). At 4 wk of age, the height of the proliferative and hypertrophic chondrocyte zones in tibias from CNP-Tg mice increases prominently (Fig. 2A). The tibial growth plate from 4-wk-old *Prkg2*$^{-/-}$ mice is characterized by irregular and broadened hypertrophic zones with nonhypertrophic cells intermingled with hypertrophic chondrocytes as reported (22). The growth plate histology of CNP-Tg/*Prkg2*$^{-/-}$ mice is similar to that of *Prkg2*$^{-/-}$ mice. The disorganized growth plate is not rescued by targeted expression of CNP in the growth plate chondrocytes in CNP-Tg/*Prkg2*$^{-/-}$ mice.

Immunohistochemical analysis revealed that the extracellular space around hypertrophic chondrocytes stained positively with type X collagen, increases in 4-wk-old CNP-Tg mice (Fig. 2B). By contrast, type X collagen-positive extracellular space is not detected in either *Prkg2*$^{-/-}$ or CNP-Tg/*Prkg2*$^{-/-}$ mice. Furthermore, we examined the expression of Ihh. In the growth plate of wild-type and CNP-Tg mice, the expression of Ihh was limited to the prehypertrophic chondrocytes (Fig. 2C). On the other hand, in the growth plate of *Prkg2*$^{-/-}$ or CNP-Tg/*Prkg2*$^{-/-}$ mice, the expression of Ihh was detected in the prehypertrophic chondrocytes and disorganized hypertrophic zones.

Bone cGMP concentrations

To examine the cGMP production in the bone, we measured tail bone cGMP concentrations in 7-d-old neonates (Fig. 1E). Bone cGMP concentrations in CNP-Tg mice (32.1 ± 16.2 pmol/g tissue) are roughly equivalent to those in wild-type mice (24.4 ± 4.1 pmol/g tissue) (n = 3–7). Bone cGMP concentrations are elevated significantly in *Prkg2*$^{-/-}$ and CNP-Tg/*Prkg2*$^{-/-}$ mice (101.0 ± 19.9 and 251.1 ± 211.6 pmol/g tissue, respectively) relative to wild-type and CNP-Tg mice (n = 3–7; $P < 0.01$).

Organ culture

The organ culture of fetal mouse tibias provides an *in vitro* unique experimental model system with which to assess the process of endochondral ossification (25). In this study, using cultured tibias prepared from wild-type and *Prkg2*$^{-/-}$ mice, we examined the effect of CNP on the longitudinal bone growth in the absence of cGKII (Fig. 3A). Before the culture, there is no significant difference in the length of fetal tibias between wild-type and *Prkg2*$^{-/-}$ mice explants. Treatment with 10^{-7} M CNP for 4 d produces an approximately 40% increase in the total length of wild-type mice explants (25). On the other hand, *Prkg2*$^{-/-}$ mice tibias treated with 10^{-7} M CNP show only a 20% increase in the total length, which is roughly comparable to that found in vehicle-treated groups. Histological examination revealed a significant increase in the height of the proliferative and hypertrophic zones in the growth plate of wild-type mice tibias treated with 10^{-7} M

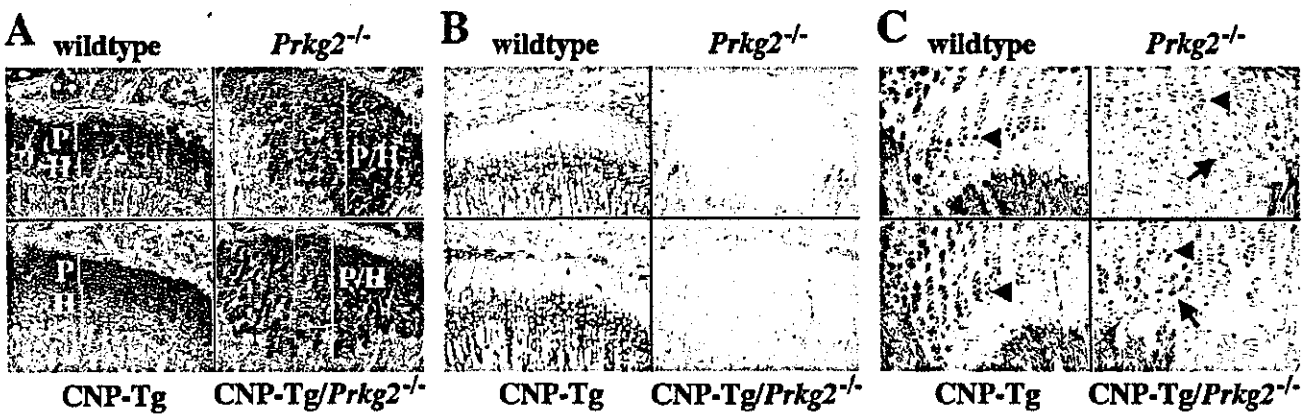

FIG. 2. Histological analysis of the tibial growth plate from 4-wk-old wild-type, CNP-Tg, *Prkg2*$^{-/-}$, and CNP-Tg/*Prkg2*$^{-/-}$ mice. A, Alcian blue and hematoxylin-eosin staining of tibias (magnification, ×30). P, Proliferative zone of the growth plate. H, Hypertrophic zone of the growth plate. P/H, Hypertrophic zone intermingled with proliferative chondrocytes. B, Type X collagen immunohistochemical staining of tibias (magnification, ×30). C, Indian hedgehog immunohistochemical staining of tibias (magnification, ×30). Ihh is expressed in the prehypertrophic chondrocytes (*arrowhead*), and also in the disorganized hypertrophic zone (*arrow*) of the growth plate from *Prkg2*$^{-/-}$ and CNP-Tg/*Prkg2*$^{-/-}$ mice.

CNP (Fig. 3B). No significant difference in the height of the growth plate of *Prkg2*$^{-/-}$ mice is noted between vehicle- and CNP-treated groups.

Cell proliferation and hypertrophy, and matrix synthesis

The longitudinal bone growth is determined by a function of chondrocytic proliferation and hypertrophy, and cartilage matrix synthesis (30). Using an *in vitro* organ culture system, we also examined the involvement of CNP/cGMP/cGKII pathway in each process.

Cell proliferation was assessed by BrdU incorporation into cultured tibias from wild-type and *Prkg2*$^{-/-}$ fetuses with or without 10^{-6} M CNP for 3 d (Fig. 4A). In wild-type fetal tibias, 10^{-6} M CNP increases significantly the labeling index ($n = 5$; $P < 0.01$), which is consistent with previous reports (25, 26). However, CNP is unable to increase the labeling index in *Prkg2*$^{-/-}$ fetal tibias (Fig. 4B). Next, we measured the size of hypertrophic chondrocytes in cultured tibias treated with or without 10^{-7} M CNP (Fig. 4C). In wild-type fetal tibias, CNP causes a significant increase in the size of hypertrophic chondrocytes ($n = 10$; $P < 0.01$). By contrast, in *Prkg2*$^{-/-}$ fetal tibias, CNP does not increase the size of hypertrophic chondrocytes (Fig. 4D). Third, we assessed cartilage matrix synthesis by immunohistochemical staining of type X collagen and measuring newly-synthesized glycosaminoglycans (Fig. 4, E and F). There is an appreciable increase in the extracellular space positive for type X collagen in wild-type fetal tibias treated with 10^{-7} M CNP, whereas no significant increase is noted in CNP-treated *Prkg2*$^{-/-}$ fetal tibias (Fig. 4E). Furthermore, wild-type fetal tibias treated with 10^{-6} M CNP exhibit an approximately 30% increase in $^{35}SO_4$ incorporation relative to vehicle-treated groups ($n = 4$–5; $P < 0.05$). By contrast, in *Prkg2*$^{-/-}$ fetal tibias, CNP does not increase $^{35}SO_4$ incorporation (Fig. 4F).

Discussion

Evidence has accumulated indicating that CNP is an endogenous natriuretic peptide that activates endochondral ossification through the intracellular accumulation of cGMP. We have reported that both *Nppc*$^{-/-}$ and *Prkg2*$^{-/-}$ mice exhibit dwarfism as a result of impaired endochondral ossification (17, 22), and hypothesized that the CNP/cGMP/cGKII pathway is important for endochondral ossification. Genetic models can provide a strong means to define signaling pathways. This study was designed to elucidate the role of cGKII in CNP-mediated endochondral ossification by use of CNP-Tg mice (17) and *Prkg2*$^{-/-}$ mice (22).

This study demonstrated that targeted expression of CNP in the growth plate chondrocytes does not rescue the skeletal defect of *Prkg2*$^{-/-}$ mice (CNP-Tg/*Prkg2*$^{-/-}$ mice). Using an *in vitro* organ culture of mouse tibias, we also found that CNP does not increase longitudinal bone growth in the absence of cGKII. Recently, we have reported both *in vivo* and *in vitro* that natriuretic peptides can affect endochondral ossification through a GC-coupled receptor other than GC-A (25, 31). It is likely that CNP acts as a positive regulator of endochondral ossification via cGMP mechanism; CNP may lead to the intracellular accumulation of cGMP via its cognate receptor (probably GC-B), which, in turn, activates cGKII in the growth plate chondrocytes. This notion is supported by the previous *in situ* hybridization analysis that *Nppc*, GC-B (*Npr2*), and *Prkg2* mRNAs are all expressed in the proliferative and prehypertrophic zones of the growth plate (17, 22).

From 1 wk of age, *Prkg2*$^{-/-}$ and CNP-Tg/*Prkg2*$^{-/-}$ mice start to show histological changes in the growth plate. This histological change may affect cGMP generation, and thereby affect the process of endochondral ossification. In this study, we confirmed no significant difference in *Nppc* and *Npr2* mRNA expression in the growth plate from 1-wk-old *Prkg2*$^{-/-}$ and CNP-Tg/*Prkg2*$^{-/-}$ mice relative to wild-type mice (data not shown). Furthermore, there was no impairment of bone cGMP generation in *Prkg2*$^{-/-}$ and CNP-Tg/*Prkg2*$^{-/-}$ mice (Fig. 1E). These findings suggest that the defect in endochondral ossification in *Prkg2*$^{-/-}$ and CNP-Tg/*Prkg2*$^{-/-}$ mice lies downstream of cGMP; it should be due to the disruption of cGKII *per se*.

It is generally understood that longitudinal bone growth is determined by a function of chondrocytic proliferation and

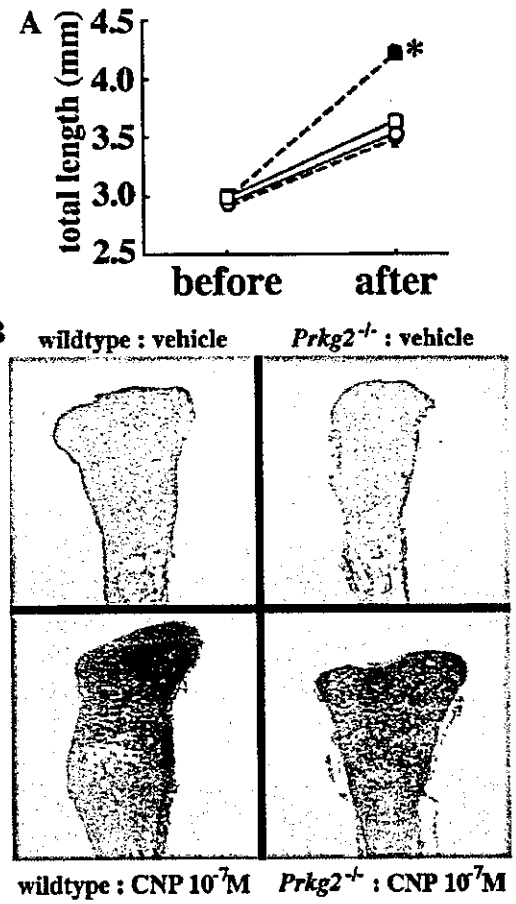

FIG. 3. Effect of CNP on longitudinal bone growth in cultured tibias. A, Total length of cultured tibias from wild-type and *Prkg2*$^{-/-}$ 10^{-7} M CNP for 4 d (n = 8–9). Wild-type fetal tibias treated with vehicle (*open square*), wild-type fetal tibias treated with 10^{-7} M CNP (*closed square*), *Prkg2*$^{-/-}$ fetal tibias treated with vehicle (*open circle*), *Prkg2*$^{-/-}$ fetal tibias treated with 10^{-7} M CNP (*closed circle*). The symbol of *Prkg2*$^{-/-}$ fetal tibias treated with CNP is behind those treated with vehicle. *, $P < 0.01$ *vs.* wild-type fetal tibias treated with vehicle. B, Alcian blue and hematoxylin-eosin staining of cultured fetal tibias treated with vehicle or 10^{-7} M CNP for 4 d (magnification, ×50).

hypertrophy, and cartilage matrix synthesis during the process of endochondral ossification (30). Because of the marked change in the growth plate histology in *Prkg2*$^{-/-}$ and CNP-Tg/*Prkg2*$^{-/-}$ mice, it is difficult to investigate *in vivo* the cellular mechanisms underlying the CNP-mediated endochondral ossification. We, therefore, examined the role of cGKII in CNP-mediated chondrocytic proliferation and hypertrophy, and cartilage matrix synthesis using an organ culture of fetal mouse tibias. The data of this study demonstrate that CNP can stimulate chondrocytic proliferation and hypertrophy, and cartilage matrix synthesis, which is consistent with previous reports (17, 25, 26). By contrast, in the absence of cGKII (or in *Prkg2*$^{-/-}$ tibias), CNP does not stimulate the above cellular functions compared with vehicle-treated groups. These observations indicate that cGKII is important for CNP-mediated chondrocytic proliferation and hypertrophy, and cartilage matrix synthesis, thus suggesting the notion that CNP signals through cGKII during the process of endochondral ossification.

The gross skeletal phenotype of *Nppc*$^{-/-}$ mice is similar to that of *Prkg2*$^{-/-}$ mice; they both develop severe dwarfism as a result of endochondral ossification. However, they differ remarkably in the growth plate histology. The *Nppc*$^{-/-}$ mice show a reduction of the growth plate in height with its chondrocytes arranged in regularly columnar array (17), whereas the growth plate of *Prkg2*$^{-/-}$ mice is characterized by increased height of the growth plate with BrdU-positive proliferative chondrocytes intermingled in the hypertrophic zone. These observations suggest the involvement of other mediator(s) in the CNP-mediated endochondral ossification. In this regard, there are no skeletal abnormalities reported in mice deficient in cGKI (32). Furthermore, we observed no significant difference in PDE1 (*Pde1*) and PDE5 (*Pde5*) mRNA expression among wild-type, CNP-Tg, *Prkg2*$^{-/-}$, CNP-Tg/*Prkg2*$^{-/-}$ mice (data not shown). On the other hand, we have demonstrated that cGMP is produced, although reduced (approximately 40% of the wild-type littermates), in *Nppc*$^{-/-}$ mice tibias (17). We speculate that non-CNP-derived cGMP is capable of activating cGKII, thereby producing regularly columnar array of hypertrophic chondrocytes in *Nppc*$^{-/-}$ mice. However, in *Prkg2*$^{-/-}$ mice, cGMP, although overproduced in the bone, is unable to signal through cGKII, thus leading to the marked histological abnormality of the growth plate. The above discussion supports the notion that cGKII is a major mediator of the CNP-mediated endochondral ossification, but dose not rule out the possibility of other mediator(s), such as cGKI. To address this issue, it would be helpful to examine closely the growth plate of mice deficient in cGKI and to investigate whether double homozygous mutant mice for cGKI and cGKII would mimic the phenotype of *Nppc*$^{-/-}$ mice.

To obtain further insight into how the CNP/cGMP/cGKII pathway is involved in endochondral ossification, we examined the expression of Ihh and type X collagen immunohistochemically in the growth plate chondrocytes from 4-wk-old *Prkg2*$^{-/-}$ and CNP-Tg/*Prkg2*$^{-/-}$ mice. In this study, Ihh, a marker of prehypertrophic chondrocytes (33–36), is expressed in the disorganized hypertrophic zone of the growth plate from *Prkg2*$^{-/-}$ and CNP-Tg/*Prkg2*$^{-/-}$ mice, where type X collagen, a marker of hypertrophic chondrocytes (37), is not detected. We have also found by Northern blot analysis and *in situ* hybridization analysis that type X collagen expression is markedly reduced in the growth plate from *Prkg2*$^{-/-}$ and CNP-Tg/*Prkg2*$^{-/-}$ mice relative to wild-type mice at 1 wk of age (Miyazawa, T., *et al.*, unpublished data). These observations suggest the delay of chondrocytic differentiation (*i.e.* conversion from prehypertrophic to hypertrophic chondrocytes) in *Prkg2*$^{-/-}$ and CNP-Tg/*Prkg2*$^{-/-}$ mice. In this context, we have observed that the band width of Ihh- and type X collagen-expressing cells is narrowed in the growth plate from *Nppc*$^{-/-}$ mice relative to wild-type mice (17). Collectively, we postulate that CNP controls the rate of chondrocytic differentiation through cGKII during the process of endochondral ossification.

Targeted expression of a constitutively active receptor for PTH and PTHrP (PTH/PTHrP receptor) delays endochondral ossification through ligand-independent constitutive

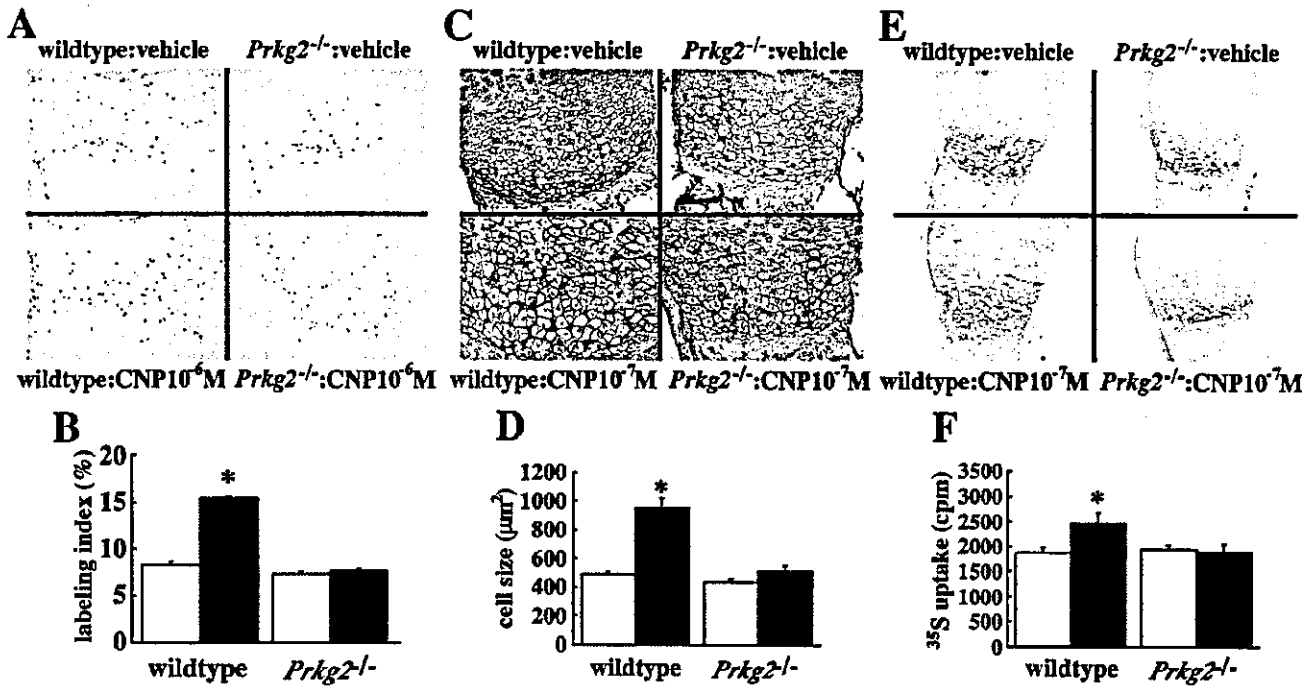

FIG. 4. Effects of CNP on chondrocytic proliferation and hypertrophy, and cartilage matrix synthesis in cultured tibias. A, BrdU immunohistochemical staining of cultured tibias from wild-type and *Prkg2*$^{-/-}$ fetuses treated with vehicle or 10^{-6} M CNP (magnification, ×30). B, Effect of CNP on BrdU-labeling index at proliferative zone (n = 5). Vehicle-treated (*open bar*), 10^{-6} M CNP-treated (*closed bar*). *, $P < 0.01$ *vs.* wild-type fetal tibias treated with vehicle. C, Alcian blue and hematoxylin-eosin staining of cultured tibias from wild-type and *Prkg2*$^{-/-}$ fetuses treated with vehicle or 10^{-7} M CNP (magnification, ×50). D, Effect of CNP on the size of hypertrophic chondrocytes (n = 10). Vehicle-treated (*open bar*), 10^{-7} M CNP-treated (*closed bar*). *, $P < 0.01$ *vs.* wild-type fetal tibias treated with vehicle. E, Type X collagen immunohistochemical staining of cultured tibias from wild-type and *Prkg2*$^{-/-}$ fetuses treated with vehicle or 10^{-7} M CNP (magnification, ×30). F, Effect of CNP on glycosaminoglycan synthesis (n = 4–5). Vehicle-treated (*open bar*), 10^{-6} M CNP-treated (*closed bar*). *, $P < 0.05$ *vs.* wild-type fetal tibias treated with vehicle.

cAMP accumulation (38). Increased accumulation of cAMP may lead to the activation of cAMP-dependent protein kinase A (cAK). The growth plate histology of Tg mice expressing a constitutively active PTH/PTHrP receptor in the growth plate is characterized by the absence of *Col10a1* mRNA expression and irregular and broadened hypertrophic zone, which is similar to that of *Prkg2*$^{-/-}$ mice. Furthermore, unlike cGMP, cAMP suppresses terminal differentiation of chondrocytes and cartilage matrix calcification *in vitro* (39). These findings suggest that the CNP/cGMP/cGKII and PTHrP/cAMP/cAK signaling pathways coordinate to control the rate of chondrocytic differentiation; it is accelerated by CNP via cGKII and decelerated by PTHrP via cAK. Further studies are needed to elucidate the mechanism of a delay in chondrocytic differentiation by the ablation of cGKII.

In conclusion, we have demonstrated that cGKII plays a critical role in longitudinal bone growth, chondrocytic proliferation and hypertrophy, and cartilage matrix synthesis during the CNP-mediated endochondral ossification. This study will provide further insight into the molecular mechanism of the CNP-mediated endochondral ossification.

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Original articles

C-type natriuretic peptide/guanylate cyclase B system in ATDC5 cells, a chondrogenic cell line

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Abstract Natriuretic peptides constitute a family of three structurally related peptides: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). Particulate guanylate cyclases, GC-A, and GC-B, are the receptors for these peptides to mediate their action. ANP and BNP possess high affinities for GC-A, and CNP is the preferred ligand for GC-B. In this article, we report our study of the expression and possible role(s) of natriuretic peptides in ATDC5 cells, which represent a chondrogenic cell line. ATDC5 cells produced cyclic guanosine monophosphate (cGMP) in response to natriuretic peptides. CNP was far more potent than ANP in terms of cGMP production. The messages for GC-A and GC-B were demonstrated by means of Northern blot analysis, and the presence of CNP was shown by Southern blotting coupled with reverse transcription-polymerase chain reaction (RT-PCR). These results suggest that the CNP/GC-B system is preferentially expressed in ATDC5 cells. GC-B mRNA expression was higher at 14 days after confluency than that at confluency. CNP or 8-bromo cGMP reduced [^{3}H] thymidine uptake and slightly increased the message for collagen type X, which is a marker of hypertrophic chondrocytes. These data suggest that the CNP/GC-B system is likely to be an autocrine/paracrine regulator of ATDC5 cells, thus affecting both their growth and differentiation.

Key words C-type natriuretic peptide · guanylate cyclase-B · chondrocyte · ATDC5

Introduction

The natriuretic peptide family consists of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). ANP and BNP are mainly secreted from the atrium and ventricle, respectively, and cause natriuresis and hypotension [1]. CNP, in contrast, is thought to be more engaged in local regulation in the central nervous system and blood vessels [2–4]. There are two biologically active receptors for natriuretic peptides; namely, the membrane-bound guanylate cyclases, GC-A, and GC-B. ANP and BNP are high-affinity ligands for GC-A, and CNP is the preferred ligand for GC-B [5]. Recently, accumulating evidence has indicated that natriuretic peptides are also functional outside the cardiovascular system [6–8]. Others and we have demonstrated that natriuretic peptides are likely to be important autocrine/paracrine regulators of osteoblasts [6–9]. In all the studies reported so far, CNP was found to be far more potent than ANP in terms of cyclic guanosine monophosphate (cGMP) production in osteogenic cells, including primary osteoblasts, MC3T3-E1, and ROB-C26 [8–10]. In practically all the systems studied, CNP was inhibitory for cell growth and stimulatory for cell differentiation in these cells [8–10]. These results suggest that the CNP/GC-B system is preferentially expressed in osteogenic lineage cells and regulates cell growth and differentiation in an autocrine/paracrine manner.

Bone is formed through two distinct mechanisms: membranous ossification and endochondral ossification. Some flat bones are formed by the former mechanism, whereas most other bones, such as the long bones and vertebrae, are formed by the latter. Our recent reports suggested the possibility that natriuretic peptides are important regulators of growth plate cartilage. We showed that transgenic mice that overexpressed BNP (BNP-Tg) exhibited body elongation accompanied by overgrowth of the growth plate. In contrast, no remarkable changes were seen in permanent cartilage, such as the xyphoid process or nasal septum, and bones formed through membranous ossification were little affected [11].

We further showed that natriuretic peptides markedly enhanced the longitudinal growth of fetal mouse

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tibia in an ex-vivo organ culture [12]. CNP was far more potent than ANP and BNP in terms of bone elongation. We then hypothesized that the CNP/GC-B system may represent the major natriuretic peptide system in bone and cartilage, and that extremely excessive BNP cross-reacted with GC-B in BNP-Tg [11–13].

However, there have been no reports regarding the cellular basis of the effect of natriuretic peptides on growth plate cartilage. The only report on this effect on chondrocytes dealt with cells from the xyphoid process, which is a permanent cartilage [14].

In this study, in order to gain insight into the cellular basis of natriuretic peptides in endochondral bone formation, we have chosen mouse chondrogenic ATDC5 cells. ATDC5 cells initially have a fibroblastic-like appearance, but undergo efficient chondrogenic differentiation in the presence of insulin, serially expressing the differentiation markers of growth-plate chondrocytes, such as collagen type II, parathyroid hormone (PTH)/PTH related peptide (rP) receptor, and collagen type X [15].

Materials and methods

Reagents

ANP and CNP were purchased from the Peptide Institute (Osaka, Japan), and 8-bromo cGMP was from Sigma (St. Louis, MO, USA).

Cell culture

ATDC5 cells were purchased from RIKEN Gene Bank (Tsukuba, Japan). Cells (7000 cells/cm^2) were cultured in culture dishes with a 1:1 mixture of Dulbecco's modified Eagle's and Ham-F 12 (DME/F12) medium (Flow Laboratories, Irvine, UK) containing 5% fetal calf serum (FCS; Sankou Junyaku, Tokyo, Japan) and bovine insulin (10 μg/ml; Wako Pure Chemical, Osaka, Japan) at 37°C in a humidified atmosphere of 5% CO_2. The medium was replaced every other day.

cGMP response to natriuretic peptides

Cells became confluent 3 days after seeding. At confluency, cells were washed with DME/F12 medium containing 0.1% FCS and 10 μg/ml bovine insulin (starvation medium) and exposed to various concentrations of mouse ANP, BNP, or CNP at 37°C for 30 min in the presence of 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX). The cGMP content was measured by means of a radioimmunoassay after extraction with 6% trichloroacetic acid, as described previously [9]. cGMP production induced by natriuretic peptides was corrected by the number of cells.

RNA extraction and Northern blot analysis

Total RNA was extracted by the acid guanidinium phenolchloroform method (AGPC) method from ATDC5 cells cultured for 3 weeks. Total RNA was similarly obtained from PC12, which is a cell line from pheochromocytoma, and from rat smooth muscle cells (SMC), as the positive controls for GC-A and GC-B, respectively. Twenty μg of total RNA from these sources was electrophoresed on an agarose gel and transferred onto a Biodyne B nylon membrane (Pall Biosupport, East Hills, NY, USA). [^{32}P] dCTP-labeled (3000 Ci/mmol) probes for GC-A and GC-B were hybridized as described previously [11,12]. With the process of cell culture, ATDC5 cells undergo efficient chondrogenic differentiation through a cellular condensation stage, as reported by Shukunami et al. [15]. GC-A and GC-B expression was analyzed at different stages of cell differentiation. At 1, 7, 14, 21, and 28 days after confluency, total RNA was extracted, by the AGPC method, from ATDC5 cells, and 15 μg of total RNA from these sources was electrophoresed on an agarose gel and transferred onto a Biodyne B nylon membrane. [^{32}P] dCTP-labeled (3000 Ci/mmol) probes for GC-A, GC-B, PTH/PTHrP, collagen type II, collagen type X, and genomic β-actin were hybridized. The rat PTH/PTHrP receptor cDNA probe was synthethized by reverse transcription-polymerase chain reaction (RT-PCR). The collagen type II cDNA was a generous gift from Dr. Y. Yamada (NIH, Bethesda, MD, USA). The collagen type X cDNA probe was a generous gift from Dr. Bjørn R. Olsen (Department of Anatomy and Cellular Biology, Harvard Medical School at Boston). To examine collagen type II and collagen type X expression by cGMP, cells were cultured with various concentrations of 8-bromo cGMP for 10 and 21 days from confluency. cGMP was replaced every 3 days. To examine collagen type X expression by CNP, cells were cultured with 10^{-8}M and 10^{-7}M of CNP for 21 days from confluency. CNP was replaced every other day. After the culture with cGMP or CNP, 25 μg or 15 μg of total RNA was electrophoresed on an agarose gel and transferred onto a nylon membrane and hybridized with the collagen type II/collagen type X and the genomic β-actin probes, respectively.

Reverse transcription-polymerase chain reaction (RT-PCR) coupled with Southern blotting

First-strand cDNA was synthesized from 2.5 μg of total RNA by reverse transcriptase (Superscript Preamplification System, Life Technologies, Grand Island, NY, USA) with oligo (dT) primer. The primers for CNP and the reaction conditions for PCR were described previously [9]. The PCR products were

transferred onto a nylon membrane and hybridized with ^{32}P-labeled oligo probes for CNP as described previously.

[^{3}H] Thymidine uptake

Cells became confluent 3 days after seeding, and the cells in a 24-well plate were then incubated with the starvation medium for 24 h, followed by incubation in a medium containing various concentrations of 8-bromo cGMP, ANP, or CNP for 36 h. Cells were then labeled with 0.7 μCi/well of [methyl-^{3}H] thymidine for 4 h. The acid-insoluble precipitates were dissolved in 1 N NaOH, neutralized by 1 N HCl, and counted for radioactivity. Data were expressed as percentages of the control values.

Statistical analysis

Statistical analysis was done with the Student's *t*-test. Data values are expressed as means ± SE, and a significant difference was defined as $P < 0.05$.

Results

Natriuretic peptides stimulated intracellular cGMP production in ATDC5 cells at confluency (Fig. 1A,B). Average basal cGMP content was 5–10 fmol/10^5 cells. CNP was 20 times more potent than ANP and BNP in terms of cGMP production (Fig. 1A,B). We then studied the expression of receptors for natriuretic peptides. The presence of GC-A and GC-B mRNAs was demonstrated in ATDC5 cells (Fig. 2A).

We next studied the expression of CNP mRNA by means of RT-PCR coupled with Southern blotting. The message for CNP was demonstrated with the predicted size of 384 bp in ATDC5 cells (Fig. 2B). ATDC5 cells underwent efficient chondrogenic differentiation through a cellular condensation stage. GC-A and GC-B mRNA expressions were analyzed in relation to cell differentiation. The PTH/PTHrP receptor was highly expressed from day 14 after confluency. Collagen type II was expressed throughout the culture period. The expression of collagen type X, which is a marker of hypertrophic chondrocytes, gradually became evident from 21 days after confluency. GC-A was slightly, but stably, expressed from confluency. A higher level of GC-B was expressed at 14 days after confluency than at confluency, and the level gradually decreased (Fig. 3).

In order to study the possible functional role(s) of cGMP and natriuretic peptide in ATDC5 cells, the effects of 8-bromo-cGMP, ANP, and CNP on cellular function were studied. The cellular growth, evaluated by [^{3}H] thymidine uptake, was dose-dependently suppressed by 8-bromo-cGMP. Approximately 40% inhibition was observed by 10^{-3} M of 8-bromo-cGMP (Fig. 4A). To determine which subtype of natriuretic peptide

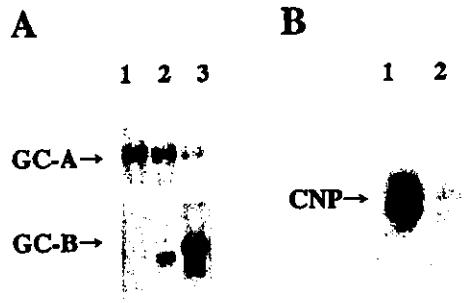

Fig. 2. A Northern blot analysis of particulate guanylate cylase (*GC*)-A and GC-B mRNA expression in PC12 cells (*lane 1*), rat smooth muscle cells (SMC; *lane 2*), and ATDC5 cells (*lane 3*). The pheochromocytoma cell line (PC12) and SMC acted as positive controls for GC-A and GC-B, respectively. **B** Reverse transcription-polymerase chain reaction (RT-PCR) coupled with Southern blot analysis for CNP in brain (*lane 1*) and ATDC5 cells (*lane 2*). CNP mRNA expression in rat brain acted as a positive control [3]

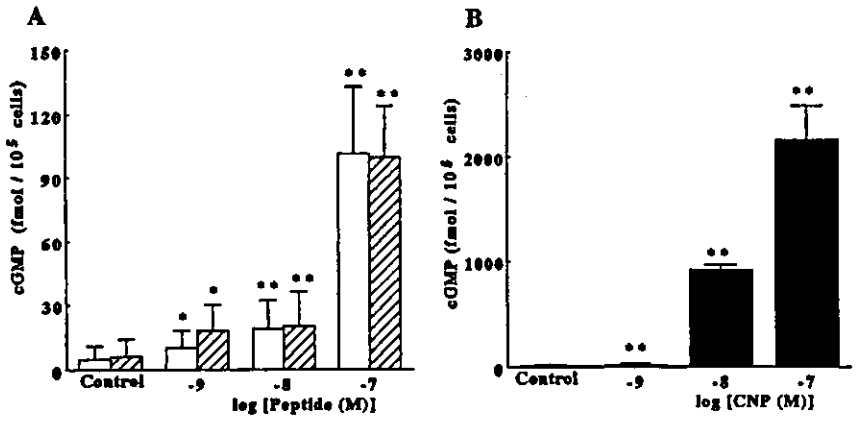

Fig. 1. A Natriuretic peptides stimulated intracellular cyclic guanosine monophosphate (*cGMP*) production. ATDC5 cells were exposed to various concentrations of atrial natricuretic peptide (ANP; *open bars*) or brain NP (BNP; *shaded bars*). **B** ATDC5 cells were exposed to various concentrations of C-type NP (CNP; *closed bars*). Data show mean ± SE values from quadruplicate determinations. $^{**}P < 0.01$ and $^{*}P < 0.05$ vs vehicle-treated groups

receptor was mainly engaged in this function, ANP, which is a specific ligand for GC-A, and CNP, which is a specific ligand for GC-B, were studied, in terms of [^{3}H] thymidine uptake, in ATDC5 cells. CNP was found to be more potent than ANP, causing approximately 30% inhibition in [^{3}H]thymidine uptake at 10^{-7}M (Fig. 4B).

We then studied the effects of 8-bromo cGMP and CNP on collagen type II and collagen type X mRNA expression in ATDC5 cells. Collagen type X mRNA expression was increased after culture with 10^{-4}M of 8-bromo cGMP for 10 days and 21 days (Figs. 5, 6). Collagen type II mRNA expression was little affected by cGMP. Collagen type X mRNA expression was also increased after culture with CNP (Fig. 7).

Discussion

We first studied the possible presence of the natriuretic peptide system in ATDC5 cells. Northern blot analysis revealed that GC-A and GC-B mRNAs were present in ATDC5. cGMP production was more than 20 times more potently stimulated by CNP than by ANP or BNP. These results suggest that GC-B mainly mediates the natriuretic action in ATDC5 cells, because CNP is the specific ligand for GC-B. The presence of CNP mRNA was also confirmed by RT-PCR. These data indicate

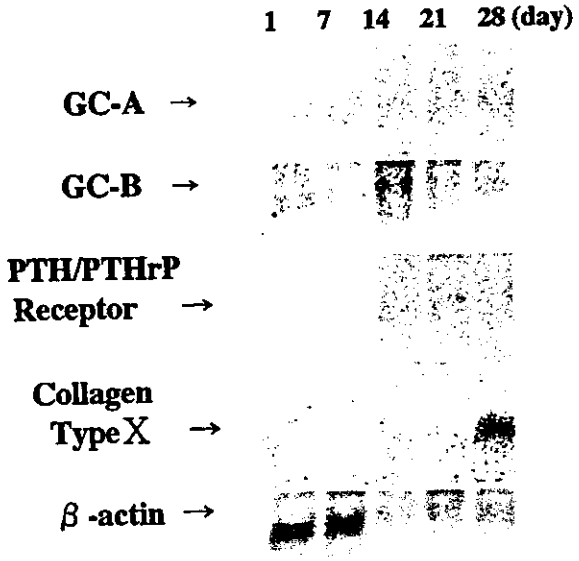

Fig. 3. The expression of biologically active receptors changed in relation to the differentiation of ATDC5 cells. Total RNAs were extracted from ATDC5 cells cultured for 1, 7, 14, 21, and 28 days after confluency and analyzed by Northern blot analysis for the expression of GC-A, GC-B, parathyroid hormone (*PTH*)/*PTH* related peptide (*rP*) receptor, collagen type II, collagen type X, and β-actin mRNA

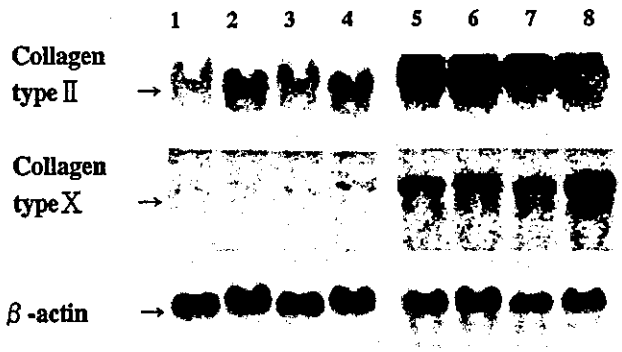

Fig. 5. Effects of 8-bromo cGMP treatment on the differentiation markers of collagen type II and collagen type X. Shown are the expressions of collagen type II and collagen type X mRNA after treatment with 8-bromo cGMP for 10 days (*lane 1*, vehicle; *lane 2*, 10^{-8}M; *lane 3*, 10^{-6}M; *lane 4*, 10^{-4}M) and 21 days (*lane 5*, vehicle; *lane 6*, 10^{-8}M; *lane 7*, 10^{-6}M; *lane 8*, 10^{-4}M)

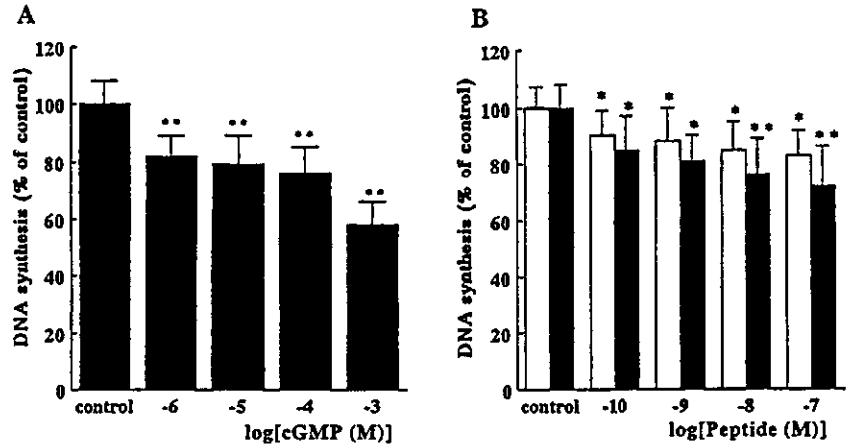

Fig. 4. A DNA synthesis evaluated by [^{3}H] thymidine uptake. Effects of various concentrations of 8-bromo cGMP on ATDC5 cells. B Effects of various concentrations of natriuretic peptides, ANP (*open bars*) and CNP (*closed bars*), on ATDC5 cells

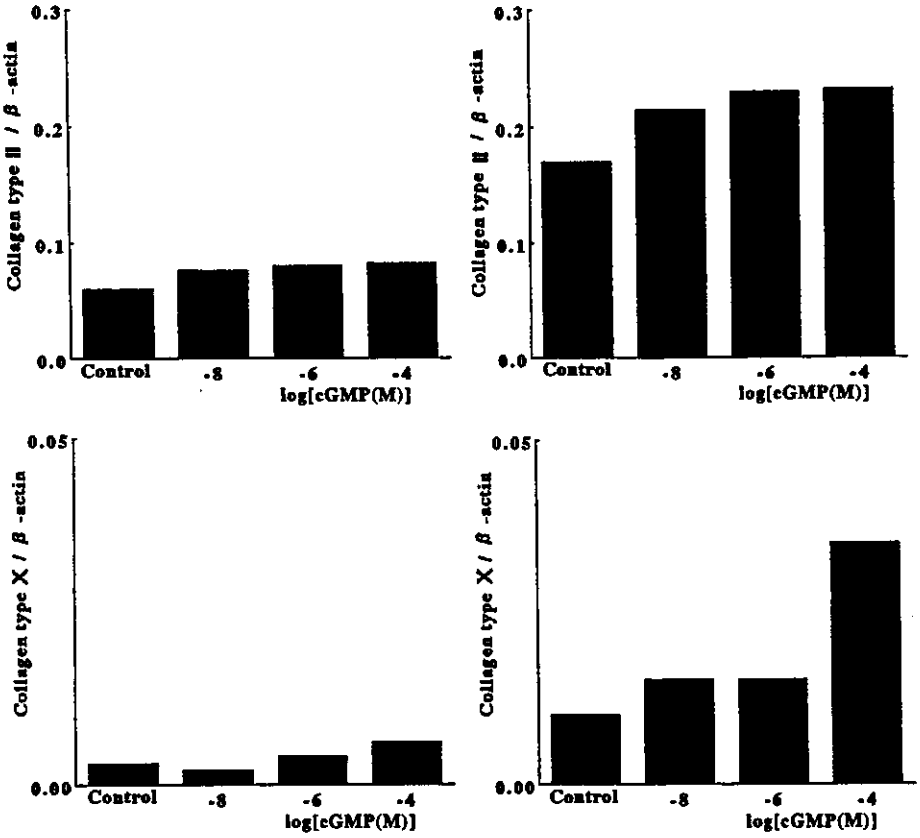

Fig. 6. Effects of 8-bromo cGMP on the expression levels of the differentiation markers (collagen type II, collagen type X) normalized by β-actin. *Left panels* show data from cells cultured for 10 days and *right panels* show data from cells cultured for 21 days

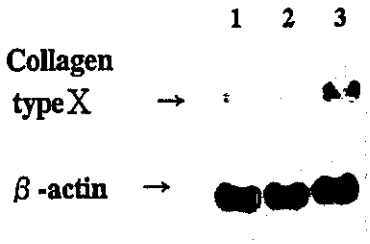

Fig. 7. The expression of collagen type X mRNA after treatment with CNP for 21 days (*lane 1*, vehicle; *lane 2*, 10^{-8} M; *lane 3*, 10^{-7} M)

that CNP/GC-B is an autocrine/paracrine regulator of ATDC5 cells. When these data are compared with our former result, cGMP production induced by CNP, corrected for cell numbers, was higher in ATDC5 cells than in ROB-C26 and MC3T3-E1 cells [9,10]. Our data suggest that the CNP/GC-B system is highly present in ATDC5 cells.

We then studied the expression profile of GC-B to gain an insight into the action site of CNP, and we also studied GC-B expression in ATDC5 cells and compared it with the expression pattern of known differentiation markers of chondrocytes. The PTH/PTHrP receptor was expressed at 14 and 21 days post-confluency. Although GC-A mRNA was slightly, but stably, expressed throughout the culture period, GC-B mRNA expression peaked at 14 days after confluency. cGMP and CNP increased the mRNA expression for collagen type X, without decreasing the mRNA for collagen type II. Because PTH/PTHrP receptor and collagen type X are expressed highly in prehypertrophic and hypertrophic zones, respectively, our data raise the possibility that the prehypertrophic zone is the major site of action of CNP.

Quite recently, we developed BNP-transgenic mice lacking GC-A (BNP-Tg/GC-A-/-mice) and examined their skeletal phenotypes. BNP-Tg/GC-A-/-mice exhibited longitudinal growth of vertebra and long bones similarly to BNP-Tg mice [13]. Furthermore, there were no skeletal abnormalities in GC-A-lacking mice. These data suggest that GC-A does not mediate the BNP action to cause skeletal overgrowth in BNP-Tg, and are compatible with the present results, indicating that GC-B, rather than GC-A, is functional in ATDC5 cells.

One of the aims of the current study was to reveal the cellular basis for the marked skeletal overgrowth in BNP-Tg and the enhanced longitudinal bone growth by CNP in organ cultures of long bone [11,12]. Because

cells from different differentiation stages coexist in these in-vivo and ex-vivo models, it is difficult to identify the underlying cellular mechanism in these systems. Unlike these models, ATDC5 cells consist of cells of rather uniform differentiation stages, because ATDC5 is a cell line.

In our previous study, CNP increased the height of the growth plate of fetal mouse tibias in organ cultures [12]. The mechanism of overgrowth of the growth plate by CNP was considered to be the increased proliferation of chondrocytes. However, natriuretic peptides inhibited the cell growth, as evaluated by [^{3}H] thymidine in ATDC5 cells. CNP dose-dependently suppressed DNA synthesis, and 8-bromo cGMP mimicked the action of CNP. With regard to their effects on cell differentiation, collagen type X mRNA expression, which is a late differentiation marker of chondrocytes, was slightly increased by 8-bromo cGMP and CNP. These results suggest the possibility that CNP directly decreases the proliferation, and promotes the differentiation, of chondrocytes. Therefore, it is likely that the stimulation of cellular differentiation is the cellular basis for the enhancement of longitudinal bone growth in vivo. The apparent discrepancy with regard to the growth between the in-vivo and in-vitro findings is most likely due to the experimental design. In the in-vivo model, cells from different differentiation stages coexist, and more primitive cells would be continuously supplied from the resting zone. Therefore, even if the major effect of CNP is the promotion of differentiation, it is possible that the overall changes result in the marked overgrowth of the growth plate. In contrast, because cultured cells are at the same differentiation stages, it is likely that enhanced differentiation by CNP alone was observed without secondary consequences. We previously found that CNP increased cell numbers in the growth plate in an organ culture system, which is likely to have been caused secondarily by increased differentiation [12].

In conclusion, the present study revealed the presence of the CNP/GC-B system in mouse chondrogenic ATDC5 cells and suggested that CNP is likely to be an autocrine/paracrine factor that affects chondrogenic cell growth and differentiation. Our data could be the cellular basis for the recently discovered action of natriuretic peptides in enhancing the growth of the growth plate.

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Brief Genetics Report

A Novel Homozygous Missense Mutation of Melanocortin-4 Receptor (*MC4R*) in a Japanese Woman With Severe Obesity

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The melanocortin-4 receptor (MC4R) is a member of the seven membrane-spanning G protein–coupled receptor superfamily and signals through the activation of adenylyl cyclase. The *MC4R* mutations are the most common known monogenic cause of human obesity. However, no such mutations have been found in Japanese obese subjects. Here we report a novel homozygous missense mutation of *MC4R* (G98R) in a nondiabetic Japanese woman with severe early-onset obesity, which is located in its second transmembrane domain. Her birth weight was 3,360 g, and she gained weight progressively from 10 months of age. At 40 years of age, her weight reached 160 kg and a BMI of 62 kg/m^2. Her parents, who are heterozygous for the mutation, have BMIs of 26 and 27 kg/m^2. In vitro transient transfection assays revealed no discernable agonist ligand binding and cAMP production in HEK293 cells expressing the mutant receptor, indicating a severe loss-of-function mutation. This study represents the first demonstration of a pathogenic mutation of *MC4R* in Japan and will provide further insight into the pathophysiologic role of the hypothalamic melanocortin system in human obesity.
***Diabetes* 51:243–246, 2002**

Obesity is a multifactorial disease that arises from complex interactions between genetic predisposition and environmental factors (1). It increases the risk of cardiovascular and metabolic diseases such as diabetes, hypertension, and hyperlipidemia, thus contributing to a marked increase in atherosclerotic disorders in Westernized countries. Recent molecular genetic studies have disclosed at least five monogenic forms of obesity in humans. They include leptin (*LEP*) (2,3), leptin receptor (*LEPR*) (4), prohormone convertase 1 (*PC1*) (5), proopiomelanocortin (*POMC*) (6), and melanocortin-4 receptor (*MC4R*) (7–13). The MC4R is a seven-transmembrane G protein–coupled receptor that is expressed in the hypothalamic nuclei implicated in the regulation of food intake and body weight (14,15). It signals through the activation of adenylyl cyclase in response to its endogenous agonist ligand, α-melanocyte-stimulating hormone (α-MSH), a well-established satiety neuropeptide produced upon cleavage from POMC (14,16). The *MC4R* mutations are remarkable in that they have been identified at a relatively high frequency of 3–4% in severe early-onset obesity in France and the U.K. (12,13). All the mutations reported to date have occurred in an autosomal-dominant fashion, except for a single unique pedigree in the U.K. (13). In this context, Ohshiro et al. (17) found no obesity-causing mutations of *MC4R* in 50 Japanese patients with obesity/diabetes. Here, we report a novel homozygous missense mutation of *MC4R* in a nondiabetic Japanese woman with severe early-onset obesity. The mutant receptor does not bind to and respond to α-MSH, indicating a loss-of-function mutation. This study represents the first demonstration of a pathogenic mutation of *MC4R* in Japan.

The proband is a 40-year-old obese Japanese woman who is the second child of two siblings born of nonconsanguineous parents. The pedigree is illustrated in Fig. 1. Her weight is 160 kg, her height is 161 cm, and her BMI is 62 kg/m^2. Her birth weight was 3,360 g, and she began to gain weight progressively at 10 months of age. She weighed 15 kg at 1 year of age. Figure 2 shows her photographs at 2, 3, and 23 years of age. She had normal mammary glands and pubic and axillary hair. There was a history of continuous nocturnal carbohydrate hyperphagia with food seeking and distress when food was not provided. She passed through puberty normally, with the onset of menstruation at 15 years of age. Her menstrual cycles had been irregular, and she had never been pregnant. Dual-energy X-ray absorptiometry scanning showed that her bone mineral density of lumbar vertebrae is greater than that expected from the age-adjusted popula-

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MC4R, melanocortin-4 receptor; α-MSH, α-melanocyte-stimulating hormone; PCR, polymerase chain reaction; POMC, proopiomelanocortin; RFLP, restriction fragment–length polymorphism; TCA, trichloroacetic acid.

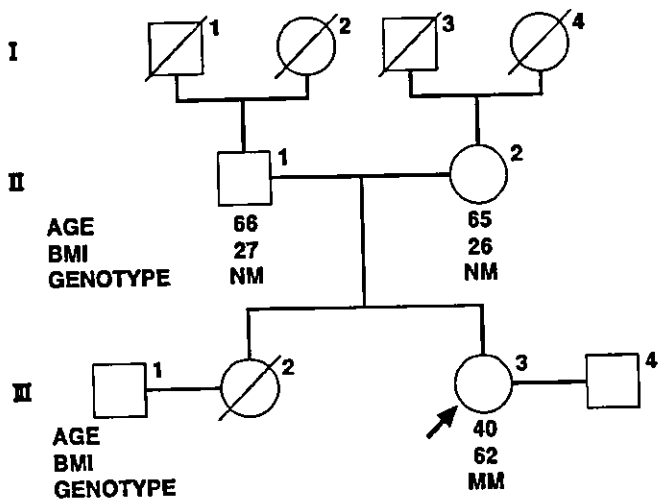

FIG. 1. Pedigree of the family. The arrow indicates the proband. The age, BMI, and *MC4R* genotype of the family members, if available, are shown below each symbol. M, mutant allele (G98R); N, normal allele.

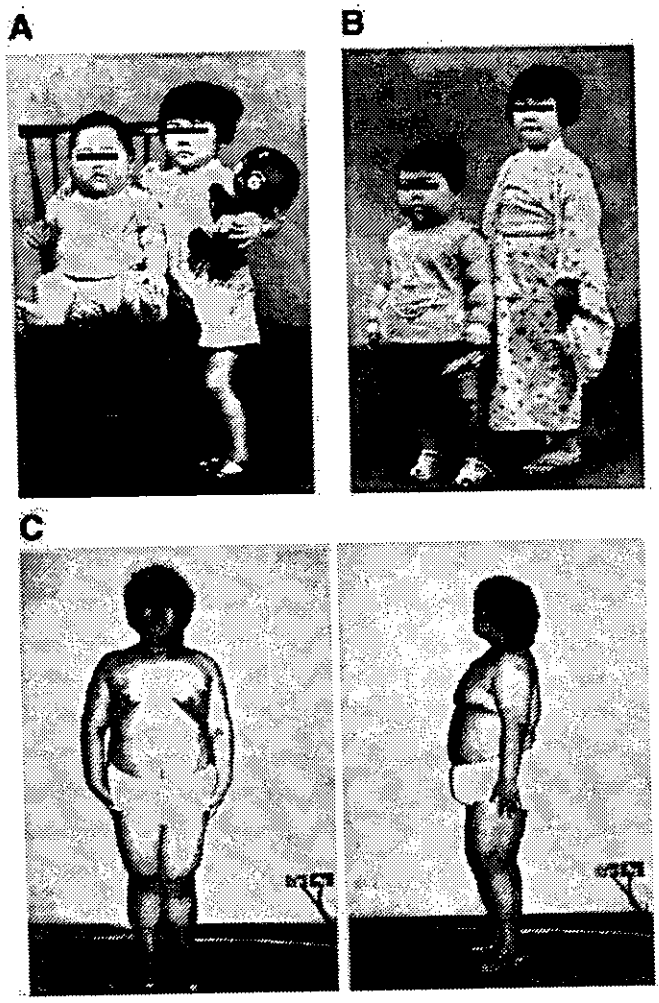

FIG. 2. Photographs of the proband at 2 (*A*) and 3 (*B*) years of age (with her elder sister at 4 and 5 years of age, respectively) and 23 years of age (*C*) (she weighed 115 kg). The photographs are reproduced with the informed consent of the proband and her mother.

tion range (Z score: 2.02). The proband had an average record during her school days, but she was slightly mentally retarded at 22 years of age as judged by the Weschler Adult Intelligence Scale (WAIS) test. Table 1 summarizes her metabolic and endocrine measurements on admission. Except for slightly decreased levels of cortisol, the values of all other anterior pituitary–derived hormones were within normal limits. The serum leptin concentration was in proportion to the degree of adiposity and appeared to reflect body fat mass. She was normoglycemic, with high levels of fasting serum insulin, consistent with hyperinsulinemia and insulin resistance seen in *Mc4r*-deficient mice (15) and humans with *MC4R* mutations (12,13). Further analysis revealed a normal karyotype (46, XX), normal computerized tomography of the brain, and no clinical features of Prader-Willi syndrome.

Her parents and her elder sister (who died of liver cirrhosis at 38 years of age) did not exhibit such severe obese phenotype, but they all had been overweight or even obese according to the criteria of the Japan Society for the Study of Obesity (the cutoff for obesity is BMI >25 kg/m^2) (Fig. 1). Her paternal grandparents (I-1, I-2) had a history of being overweight or obese, whereas her maternal grandfather (I-3) did not.

To examine whether a *MC4R* mutation might be involved in the proband's morbid obesity, the coding sequences of *MC4R* were amplified by polymerase chain reaction (PCR) using genomic DNA extracted from peripheral leukocytes and subjected to direct sequencing. We identified a novel homozygous missense mutation in the second transmembrane domain of MC4R (G98R [*G*GA → *A*GA transition]) (Fig. 3*A*). The G98R mutation was not detected in 100 healthy Japanese volunteers using PCR analysis combined with *Alw* I restriction fragment–length polymorphism (RFLP) (data not shown). Her parents proved to be heterozygous for the mutation (Fig. 3*B*).

To explore the pathogenic implication of the G98R mutation, the wild-type and mutant receptors were expressed in HEK 293 cells and assayed for their ability to bind and respond to α-MSH (Fig. 4*A*). Cells expressing the wild-type receptor showed a sigmoidal dose response to α-MSH. By contrast, cells expressing the G98R mutant receptor showed no response. They were unable to bind to α-MSH in a competitive-binding assay (Fig. 4*B*), indicating a loss-of-function mutation. We could not examine whether the mutant receptor is expressed on the cell surface by Western blot analysis because an antibody specific for human MC4R is currently unavailable. In this context, a previous study showed that rat prostaglandin $F_{2\alpha}$ receptor with amino acid substitutions of several kinds in its second transmembrane domain, when transfected, is expressed on the cell surface, although the level is slightly reduced relative to the wild-type receptor (18). Given that the basic Arg residue at position 8 of α-MSH may interact with the acidic residues in the second and third transmembrane domains of MC4R (19), it is conceivable that α-MSH does not bind to the G98R mutant receptor because of the alteration from the neutral Gly to basic Arg residues in its second transmembrane domain.

The G98R mutation reported herein is the second homozygous mutation of *MC4R* described in human obesity. The homozygous N62S mutation previously described in the U.K. was the first to be associated with a recessive

TABLE 1
Hormonal profile of the proband

	Proband	Normal range
Leptin (ng/ml)	58.4	6.3–10.0
Glucose (mmol/l)	4.8	3.5–5.5
Insulin (pmol/l)	164	12–48
LH (IU/l)	3.58	0.9–15.5 (follicular phase)
FSH (IU/l)	9.61	3.1–23.9 (follicular phase)
Estradiol (pmol/l)	239	51–826
Testosterone (ng/ml)	0.36	0.36–3.1
GH (μg/l)	1.8	<5.0
TSH (mU/l)	1.3	0.5–4.2
FT4 (pmol/l)	16.4	11.5–21.2
ACTH (pmol/l) at 9:00 A.M.	4.2	<13
Cortisol (nmol/l) at 9:00 A.M.	119	138–673
Prolactin (μg/l)	24.6	<30

FSH, follicle-stimulating hormone; FT4, free thyroxine; GH, growth hormone; LH, luteinizing hormone; TSH, thyrotropin-stimulating hormone.

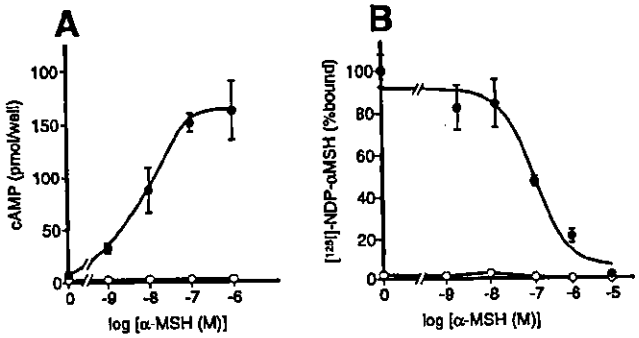

FIG. 4. *A*: Activation of transfected human MC4R by α-MSH. HEK 293 cells transiently transfected with MC4R cDNAs or the vector pcDNA3.1 were stimulated for 1 h with medium only or increasing amounts of α-MSH, after which intracellular cAMP content was measured. All curves are representative of three independent experiments, and each point is the mean triplicate values. Error bars indicate SE. *B*: Competition binding assay. Transiently transfected HEK 293 cells were incubated with [^{125}I]NDP-α-MSH in the presence of an increasing concentration of α-MSH. All curves are representative of three independent experiments, and each point is the mean of triplicate values. Error bars indicate SE. ●, Wild-type allele; ○, G98R mutant allele. The ordinate is expressed as a percentage of total specific binding.

pattern of inheritance and retains some capacity to signal to cAMP generation (13). Thus, this study represents the first description of a homozygous missense mutation of *MC4R* with no signaling capacity, leading to obesity. Her parents, who were heterozygous for the mutation, were overweight. These observations suggest a codominant pattern of inheritance. They all should express one wild-type allele and one functionally null allele, which appears to cause overweight or obesity in this family. It was reported that heterozygous loss-of-function mutations of *MC4R* in humans do not always lead to severe obesity (12,13), as heterozygous *Mc4r*-deficient mice display a broad variety in phenotype, ranging from that of wild-type to that of homozygous *Mc4r*-deficient mice (15). We did not examine the intrafamilial variation in phenotype further because there were no family members available within this pedigree.

In conclusion, we have identified a novel homozygous missense mutation of *MC4R* in a Japanese woman with severe early-onset obesity. This study will provide further

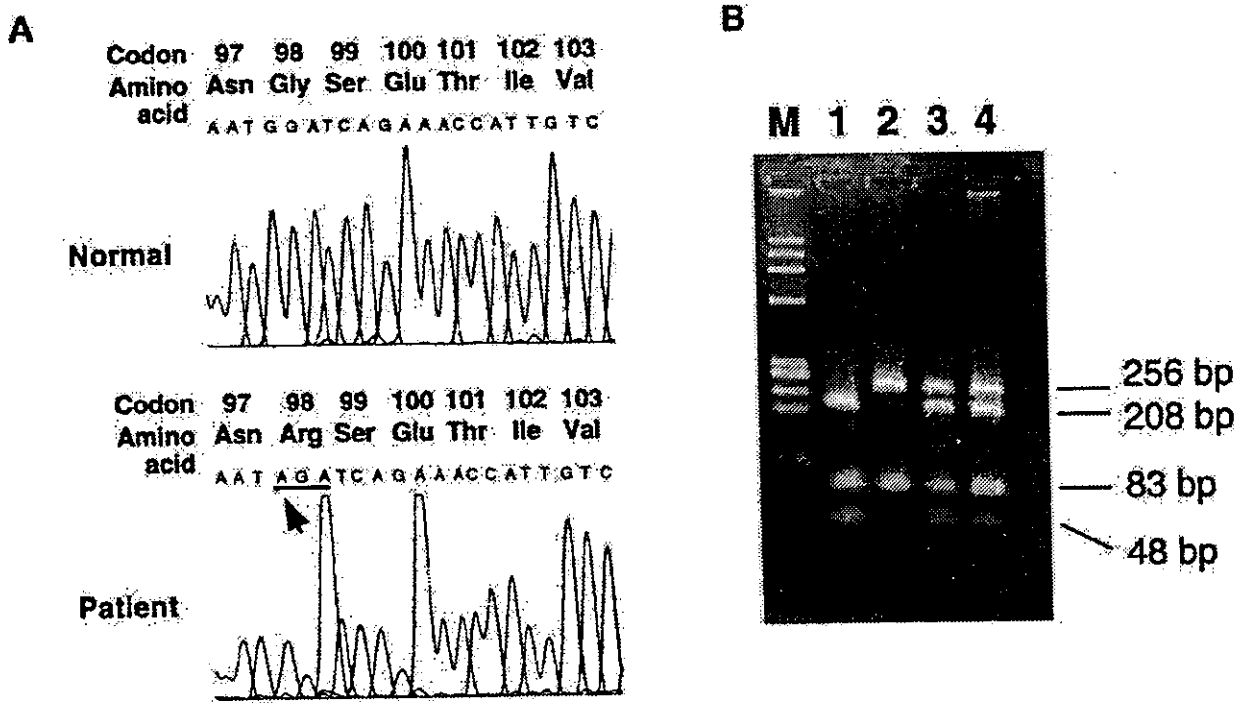

FIG. 3. *A*: PCR direct sequences of *MC4R* from a healthy volunteer and the proband. *B*: Detection of the G98R mutation using PCR analysis combined with *Alw* I RFLP. M, size marker; 1, a healthy volunteer; 2, the proband, with homozygous G98R mutation; 3, the proband's father, with the heterozygous G98R mutation; 4, the proband's mother, with the heterozygous G98R mutation.

insight into the role of the hypothalamic melanocortin system in human obesity.

RESEARCH DESIGN AND METHODS

Patients. This study was conducted with informed consent from the proband and her mother and was approved by the institutional review board of Kobe City General Hospital for genetic analysis and the ethical committee on human research of Kyoto University Graduate School of Medicine.

Genetic studies. Genomic DNA was extracted from peripheral leukocytes by standard techniques. The full-length *MC4R* coding sequences were amplified by PCR using sense and antisense primers (5′-GACTTGGAGGAAATAACTG AGA CG-3′ and 5′-CTACACGGAAGAGAAAGCTGTTGC-3′, respectively). The PCR products thus obtained were subjected to nucleotide sequencing on both strands. The PCR/RFLP analysis of the G98R mutation was performed by PCR-amplified genomic sequences of *MC4R*. An aliquot of the reaction mixture was digested with *Atw* I (Takara, Kusatsu, Japan).

Transfection studies. The wild-type and mutant *MC4R* coding sequences were amplified by PCR using sense and antisense primers (5′-CAGCATGGT-GAACTCCACCCA-3′ and 5′-CTCTGTCCCCATTTAATATCT-3′, respectively) and subcloned into pGEM-T easy vector (Promega, Madison WI). The *Eco*RI-*Spe*I fragment containing MC4R was excised and ligated into the *Eco*RI and *Xba*I restriction sites of pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA). All clones were verified by DNA sequencing. HEK293 cells were maintained as previously described (13), transfected with the wild-type or mutant MC4R expression vectors by LipofectAmine Plus (Life Technologies, Rockville, MD), and assayed 24–48 h after transfection. For both ligand binding and cAMP assays, Renilla luciferase expression vector was cotransfected to monitor the efficiency of transient expression. Protein content was determined in cell extracts to normalize the cell number per well.

Ligand-stimulated receptor activity was measured by increased intracellular cAMP content. Cells cultured in 24-well plates were incubated with α-MSH (Peptide Institute, Minoh, Japan) in the presence of 0.25 nmol/l 3-isobutyl-1-methyl-xanthine (Sigma, St Louis, MO) in the culture media. After 1 h, the media was replaced with 6% trichloroacetic acid (TCA). An aliquot of TCA was used to determine cAMP content by the commercially available radioimmunoassay (Yamasa, Chiba, Japan).

Competitive binding experiments were performed as previously described (20). Cells cultured in 24-well plates were washed with 200 μl of the binding medium (1 mg/ml bovine serum albumin in Ca^{+}/Mg^{2+} phosphate-buffered saline) and incubated in 150 μl binding medium containing 40,000–60,000 cpm of [^{125}I]NDP-MSH (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) per well. Series concentrations of unlabeled α-MSH were used to compete with the labeled NDP-MSH. Controls for nonspecific binding contained 10 μmol/l unlabeled α-MSH. After 1 h of incubation, the binding medium was aspirated, cells were washed with 400 μl of binding medium, and 200 μl of 0.1N NaOH was added. Membrane-bound counts per minute were determined in a gamma counter (Aloka, Mitaka, Japan). Total specific binding and IC_{50} values were determined by nonlinear regression analysis from triplicate data points using Prism software (GraphPad Software for Science, San Diego, CA).

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Missense Mutation of *TRPS1* in a Family of Tricho-Rhino-Phalangeal Syndrome Type III

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We report a new Japanese family with tricho-rhino-phalangeal syndrome type III (TRPS III) who have a missense mutation (Arg908Gln) of the TRPS1 gene (*TRPS1*) in affected individuals of the family. This study supports the notion that TRPS III results from missense mutations in exon 6 of *TRPS1*. © 2001 Wiley-Liss, Inc.

KEY WORDS: missense mutation; tricho-rhino-phalangeal syndrome type III; *TRPS1*

INTRODUCTION

Three types of tricho-rhino-phalangeal syndrome (TRPS) have been described. TRPS type I (TRPS I, MIM 190350) is characterized by sparse hair; a distinctive, bulbous nose; and mild skeletal dysplasia with cone-shaped epiphyses, short stature, and shortening of the metacarpals and metatarsals. TRPS type II (TRPS II, MIM 150230) shares common clinical manifestations of TRPS I with multiple cartilaginous exostoses and mental retardation. TRPS type III (TRPS III, MIM 190351) was first described by Sugio and Kajii [1984]. It differs from TRPS I only by more severe shortening of all phalanges and metacarpals and short stature (< −2SD) [Niikawa and Kamei, 1986] and from TRPS II by normal intelligence and absence of exostoses. There are several clinical reports of TRPS III (chronologically): a sporadic Japanese case [Niikawa and Kamei, 1986], another Japanese family with four affected individuals [Nagai et al., 1994], a Turkish family with seven patients [Itin et al., 1996], and a sporadic case of a European patient [Vilain et al., 1999]. Despite the phenotypic similarity, the allelic relationship of TRPS III with TRPS I has been undefined.

Momeni et al. [2000] isolated a gene that spans the chromosomal breakpoint in two patients with TRPS I, which they termed *TRPS1*. The predicted protein sequence, which may function as a zinc-finger transcription factor, possesses two potential nuclear localization signals (NLSs) and an unusual combination of different zinc-finger motifs, including IKAROS-like and GATA-binding sequences. These authors reported six different nonsense mutations of *TRPS1* in 10 unrelated patients [Momeni et al., 2000]. Recently, Lüdecke et al. [2001] demonstrated that TRPS III is the severe end of the TRPS spectrum and can be caused by a specific class of mutations in *TRPS1*. Here we report a Japanese family of TRPS III in which we identify a missense mutation of *TRPS1*.

CLINICAL REPORT

The proband (II-6, Fig. 1), a 59-year-old woman, was referred to our hospital because of short stature, thin and slow-growing hair, and brachydactyly. She was born to nonconsanguineous Japanese parents. Her father (I-1) and two elder sisters (II-4 and II-5) exhibited clinical manifestations similar to hers (see below), but her mother (I-2) and three brothers (II-1, II-2, and II-3) are of normal appearance. A total of four affected family members have been recognized. The individual II-5 was examined directly, but I-1 and II-4 were evaluated only by the information gained from the proband.

On admission, her height was 138 cm (−3.8 SD) and her body weight was 38 kg. No apparent endocrinological reasons were noted for growth retardation. Mental development was normal and she was an A student. She had developed secondary sexual characteristics. She had been physically underdeveloped but acted intelligently and worked as a seamstress. Her menstrual cycle was regular until she was 50 years of age. She has never married and has never been pregnant. The scalp hair was very fine and sparse, the ears were protruding, the nose was large and pear-shaped, the upper lip was thin, and the philtrum was long (Fig. 2). Albright's sign was negative and X-ray skeletal

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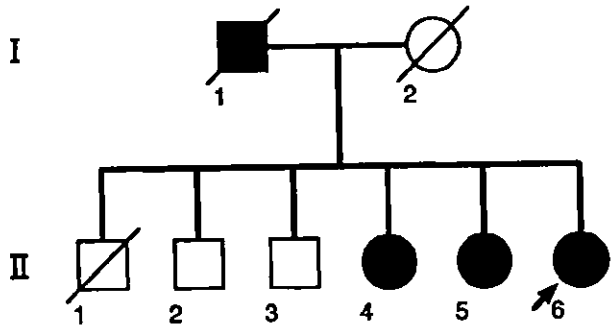

Fig. 1. Pedigree of the family.

investigations showed very severe shortening of metacarpals and phalanges with cone-shaped epiphyses. Metacarpophalangeal pattern profile (MCPP) analysis used to aid in establishing the diagnosis of TRPS [Kajii et al., 1994] revealed severe brachydactyly (– 6.2 SD) with involvement of all metacarpals of the proband.

Her elder sister (II-5) had been followed up at another hospital because of bone malformations and dysplasias. She was 66 years old with normal intelligence. Her height was 130 cm (– 5.4 SD) and her fingers were very short. She also had a typical face for TRPS. We therefore diagnosed both sisters to have TRPS III. This study was conducted with the patients' informed consent.

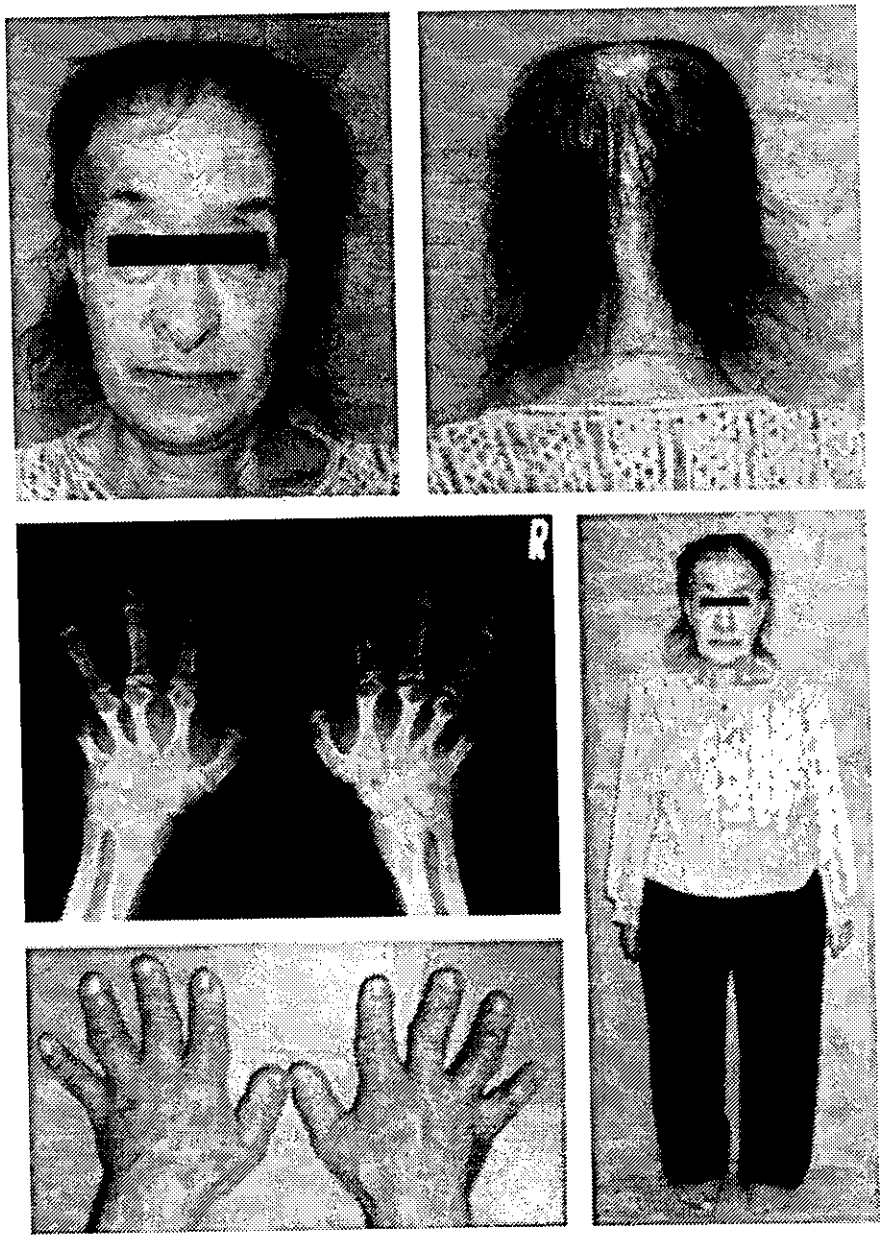

Fig. 2. The proband (II-6). The photographs are reproduced with her informed consent.

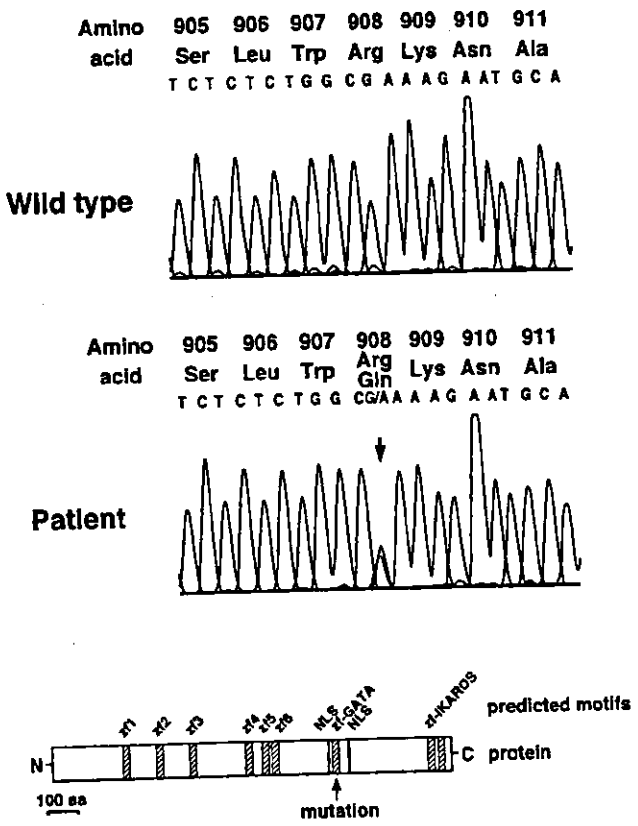

Fig. 3. A heterozygous missense mutation (thick arrow) at codon 908 (Arg908Gln) in exon 6 of *TRPS1* of the patient and the TRPS1 protein structure and location of the mutation (thin arrow). Hatched boxes, zinc-finger motif (zf); vertical bars, putative NLS. The location of the GATA and IKAROS-like zinc-finger motifs are also given.

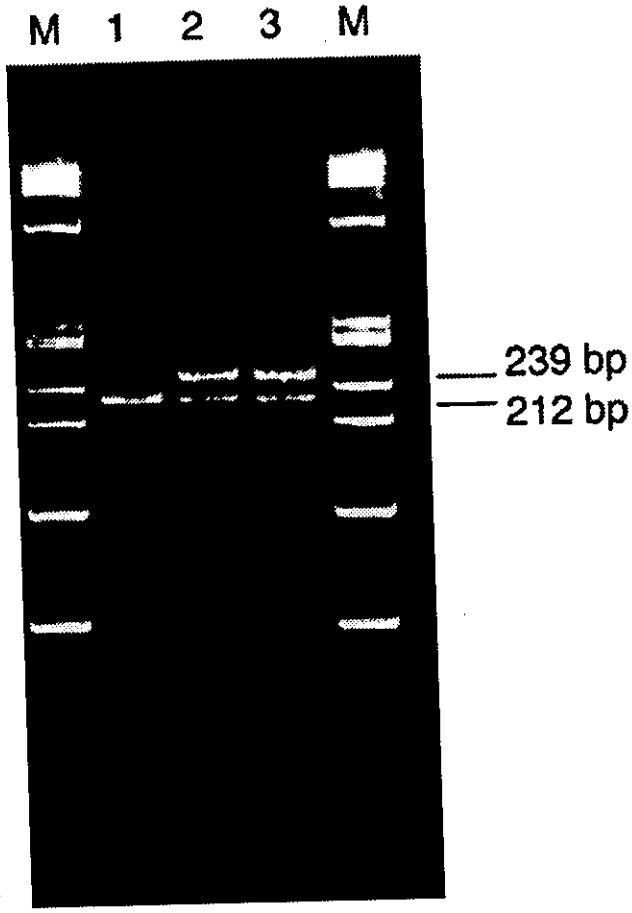

Fig. 4. PCR-RFLP of the coding sequence of exon 6 detected by sense primer (5'-TGCCTGACCACAAAGACCTCTCTCTCGC-3') and antisense primer (5'-CATAAATCACATGTGCACTCAA-3'). Underlined sequence mismatch at the 3' end of the sense primer creates an artificial mutation conditional restriction endonuclease *Nru*I in the PCR product. The smaller DNA fragment (27 bp) is not visualized. Lane 1, control individual; lane 2, the proband (II-6); lane 3, the elder sister (II-5); lane M, ØxHae III digest.

MOLECULAR GENETIC STUDIES

Genomic DNA was extracted from the patients' peripheral blood leukocytes according to the standard technique [Sambrook et al., 1989]. We examined the entire coding sequences of *TRPS1* by polymerase chain reaction (PCR) and direct sequence analysis as described by Momeni et al. [2000]. We identified a missense mutation (a G to A substitution at nucleotide (nt) 2723) that results in a heterogeneous substitution of arginine to glutamine at codon 908 (Arg908Gln). This alteration site is located within the putative GATA DNA-binding zinc-finger flanked by two potential NLSs (Fig. 3). The same mutation was identified in the affected elder sister (II-5) (Fig. 4). No such mutation was found in 100 healthy Japanese subjects by PCR-restriction fragment length polymorphism (RFLP) analysis (data not shown). We did not perform genetic studies for other family members because informed consent was not obtained.

DISCUSSION

We herein report a new Japanese family of TRPS III, in which we identify a missense mutation (Arg908Gln) of *TRPS1*. We have found no such mutation of *TRPS1* in 100 healthy Japanese subjects, thus suggesting that it is not a single nucleotide polymorphism (SNP). Although genetic analysis was not done for other family members, we postulate that the mutation is transmitted in an autosomal dominant manner, because the proband's father exhibited similar clinical manifestations.

The mutation is located in the GATA DNA-binding zinc-finger motif flanked by the two NLSs [Momeni et al., 2000], thus resulting in a basic to neutral amino acid substitution at the tip of the zinc-finger. It is likely that TRPS1 protein with Arg908Gln mutation may enter the cell nucleus and homo- or heterodimerize with other IKAROS transcription factors through the IKAROS-like zinc-finger. The mutant protein may bind to the DNA sequences with much lower affinity or inhibit normal TRPS1 protein binding to it and exert a dominant negative effect. Recently, Lüdecke et al.