EXPERIMENTAL STUDY

Genomic organization of mouse *ZAKI-4* gene that encodes *ZAKI-4* α and β isoforms, endogenous calcineurin inhibitors, and changes in the expression of these isoforms by thyroid hormone in adult mouse brain and heart

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Abstract

Objective: *ZAKI-4* was identified as a thyroid hormone-responsive gene in cultured human fibroblasts. A single *ZAKI-4* gene encodes two isoforms, *ZAKI-4* α and β , both inhibiting calcineurin activity. *ZAKI-4* α and β differ at their N termini, and show distinct distribution profiles in human tissues. The aim of this study was to elucidate the organization of the mouse *ZAKI-4* gene and to determine the effect of thyroid hormone on the expression of *ZAKI-4* isoforms *in vivo*.

Design: We cloned mouse homologues of human *ZAKI-4* α and β cDNA. Fluorescence *in situ* hybridization and bioinformatics analysis were employed to determine the gene organization. The effect of thyroid hormone on the expression of *ZAKI-4* isoforms in mouse brain and heart was also studied.

Methods: Total RNA extracted from mouse cerebellum was used to clone *ZAKI-4* α and β cDNAs by RT-PCR followed by rapid amplification of cDNA ends. Mice were rendered hypothyroid by feeding a low iodine diet supplemented with propylthiouracil for 2 weeks. In one group (hyperthyroid) L-T₃ was injected i.p. for the last 4 days whereas another group (hypothyroid) received vehicle only. Non-treated mice were controls.

Results and conclusion: Mouse *ZAKI-4* α and β cDNAs were highly homologous to the human isoforms. The gene was mapped on chromosome 17qC, syntenic to human chromosome 6 where the human *ZAKI-4* gene is located. As observed in human, *ZAKI-4* α mRNA was expressed only in brain whereas β mRNA was distributed in other tissues as well, such as heart and skeletal muscle. *ZAKI-4* α mRNA was lower in the cerebral cortex of hypothyroid mice. Injection of L-T₃ caused an increase in *ZAKI-4* β mRNA in heart; however, expression of neither *ZAKI-4* α nor β mRNA was influenced by thyroid status in other tissues. These results indicate that expression of *ZAKI-4* α and β isoforms is regulated by thyroid hormone *in vivo*, and the regulation is isoform- and tissue-specific.

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Introduction

ZAKI-4 (*DSCR1L1*, according to the Human Gene Nomenclature Committee) was identified as a thyroid hormone-responsive gene in cultured human skin fibroblasts (1). In fibroblasts the expression of *ZAKI-4* mRNA was enhanced transcriptionally by physiological concentrations of 3,3',5-triiodothyronine (T₃), the active form of thyroid hormone. This effect was indirect, requiring *de novo* protein synthesis. A recent study (2) demonstrated that a product of *ZAKI-4* belongs to a family of proteins that inhibit calcineurin activity.

This family includes DSCR1 (Down syndrome candidate region 1) (3) (also termed as MCIP1 (4) or Adapt 78 (5)) and DSCR1L2 (DSCR1 like protein 2) (6) in mammals, Rcnp1 in *Saccharomyces cerevisiae* (2) and *Caenorhabditis elegans* (7), and CBP1 (8) in *Cryptococcus neoformans*.

Calcineurin is a calcium/calmodulin-dependent serine/threonine protein phosphatase that exerts a variety of functions by dephosphorylating a family of transcription factors such as nuclear factor of activated T cells (NF-AT) (9) and myocyte-specific enhancer factor 2 (10). In brain, for example, calcineurin is involved in

synaptic plasticity, neuronal apoptosis, amyloid β -peptide formation and regulation of ion channels (reviewed in (11)). It has also been demonstrated that calcineurin plays a role in cardiac hypertrophy in mice (12, 13) and human (14). These recent reports suggest that a family of proteins including ZAKI-4/DSCR1L1 regulate neuronal and cardiac functions by inhibiting calcineurin function.

We have recently shown that at least three transcripts (ZAKI-4 α , β 1 and β 2 mRNAs) are generated from a single human ZAKI-4 gene by both alternative initiation and splicing (15). ZAKI-4 β 1 and β 2 mRNAs encode an identical protein, ZAKI-4 β , which differs at the N-terminal region from ZAKI-4 α , whereas the rest of the amino acid sequences of these isoforms are identical. This common region contains the calcineurin-interacting domain, so that both isoforms inhibit calcineurin activity (15). Human ZAKI-4 α and β display a unique tissue distribution. ZAKI-4 α mRNA is expressed predominantly in brain whereas ZAKI-4 β mRNA is distributed not only in the brain but also in other tissues such as heart, skeletal muscle and kidney (15). Regulation of ZAKI-4 isoforms expression by thyroid hormone is also unique. In cultured human skin fibroblasts thyroid hormone enhanced the expression of ZAKI-4 α mRNA but did not affect that of ZAKI-4 β mRNA (15). However, it is not known how thyroid hormone regulates the expression of ZAKI-4 isoforms *in vivo*.

In order to determine the mechanism of thyroid hormone action on the expressions of ZAKI-4 isoforms *in vivo*, we first cloned the cDNAs of mouse ZAKI-4 isoforms. Then, the genomic organization and chromosomal localization of ZAKI-4 gene was determined. In addition, the tissue distribution of ZAKI-4 α and β isoforms and the effect of thyroid hormone on the expression of these isoforms were studied. The present study demonstrates, for the first time, that mouse ZAKI-4 gene encodes two isoforms, ZAKI-4 α and β . Genomic organization is similar to that of the human counterpart and is localized in a region of chromosome 17 syntenic to the human chromosome 6. We also show that thyroid status affects the expression of ZAKI-4 in an isoform- and tissue-specific manner.

Materials and methods

Cloning of mouse ZAKI-4 cDNA isoforms

Total RNA was extracted from the cerebellum of an adult 129/SvJ mouse by the method of Chomczynski & Sacchi (16). Specific primers for ZAKI-4 α (ZAK- α ; 5'-AATGCCCTAGCATGGACT-3') and ZAKI-4 β (ZAK- β ; 5'-ATGAGGGGAGACGCCTACTTCA-3'), and a common primer between α and β (1059M; 5'-GATATATCACCTTTTCCTAGCCC-3') were designed according to the sequence of human ZAKI-4 α (GenBank Accession No. D83407) and β (GenBank Accession

No. AY034086 and AY034085) cDNAs. Amplified cDNA fragments were cloned into pGEM-T Easy vector (Promega Corp., Madison, WI, USA). To amplify either 5' or 3' termini of ZAKI-4 α and β cDNAs, 5' or 3' RACE (rapid amplification of cDNA ends) was performed respectively. Specific primers for 5' RACE were as follows: mZ-1, 5'-TTGAACTGAGTCTCATGAAGC-3'; mZ-2 (a nested primer to amplify the 5' end of α), 5'-CCTCCTGATTGGTAAAGACC-3'; and mZ-3 (a nested primer to amplify the 5' end of β) and 5'-TGGTGAACA-TTGCAGGCAAAC-3'. Specific primers for 3' RACE were designed as: mZ-10, 5'-CCTTTAAGTCCTGGGTGTT-3'; and mZ-11 (a nested primer), 5'-GGCTAGGCAAAGGTG-ATATG-3'. Amplified fragments of 5' and 3' RACE were cloned into pGEM-T Easy vector and were sequenced. The mouse ZAKI-4 α and β cDNA sequences were deposited in GenBank; Accession Nos AB61524 and AB61525 respectively.

Mouse genome database search

Mouse Genome Assembly (NCBI build 30) constructed with the Mouse Genome Sequence Consortium Whole Shotgun Assembly and High Throughput Genome Sequence was searched with MegaBlast using the Ensemble Mouse Genome Server (http://www.ensembl.org/Mus_musculus/). The search was carried out between July and August 2003.

Chromosomal mapping by fluorescence in situ hybridization (FISH)

FISH was carried out essentially as described by Inazawa *et al.* (17). In brief, mouse metaphase chromosomes were prepared from spleen lymphocyte culture as described by Matsuda *et al.* (18), following the same thymidine-bromodeoxyuridine treatment. To produce R- and Q-banding, the chromosomes were stained with 1 μ g/ml Hoechst 33258 (Sigma, St Louis, MO, USA) and then irradiated with UV light (wave length 302 nm). Chromosomes on slides were heated at 65 °C for 1.5 h and treated with ribonuclease A (Roche Diagnostics GmbH, Mannheim, Germany) in 2 $\times$ SSC (1 $\times$ SSC, 150 mmol/l NaCl, 15 mmol/l trisodium citrate), pH 7.0 at 37 °C for 1 h, then dehydrated with increasing concentrations of ethanol (70, 80, 90 and 100% for 5 min each) and dried in air. Just before FISH, chromosomes were denatured for 1–2 min at 70 °C in 70% formamide, 2 $\times$ SSC, pH 7.0, then again dehydrated and air-dried as described above. Full-length mouse ZAKI-4 α cDNA (3.4 kb) was used as a probe. The cDNA (about 400 ng) was labeled with biotin-16-dUTP using a Biotin-Nick Translation Kit (Roche) and co-precipitated with 50–100 μ g mouse COT-1 DNA (Roche), 20 μ g salmon sperm (ss) DNA and 20 μ g *Escherichia coli* tRNA. After overnight precipitation the dry pellet was dissolved in 10 μ l 100% formamide. The hybridization mixture

containing the labeled probe at a final concentration of 20 ng/ μ l in solution (50% formamide, 10% dextran sulfate, 2 $\times$ SSC, ssDNA (1 μ g/ μ l), *E. coli* tRNA (1 μ g/ μ l)) was denatured for 10 min at 80 °C. Pre-annealing of repetitive sequences with mouse COT-1 DNA was carried out for 1 h at 37 °C. Then, the hybridization mixture (20 μ l) was applied to the denatured chromosomes on slides. The slides were sealed with a cover slip and incubated in a moist chamber at 37 °C for 16 h. After hybridization the slides were washed at 42 °C consecutively in 2 $\times$ SSC, twice in 50% formamide with 2 $\times$ SSC, twice with 2 $\times$ SSC and once with 0.1 $\times$ SSC for 5 min each. Hybridization signals were detected with fluorescein isothiocyanate-conjugated avidin and biotinylated anti-avidin goat IgG, and chromosomes were counterstained with propidium iodide (1 μ g/ml). Photographs were taken on Fujicolor 400 ASA film (Fuji Photo Film, Tokyo, Japan).

Animal experiments

All mice were treated in accordance with the principles and procedures outlined by the Committee for Animal Experiments of Nagoya University School of Medicine and Research Institute of Environmental Medicine. Male 60-day-old ICR mice were used to study the effect of thyroid hormone on the expression of ZAKI-4 α and β mRNAs in brain and heart. Mice were rendered hypothyroid by feeding a low iodine (LoI) diet supplemented with 0.15% propylthiouracil (PTU) (Harlan Teklad Co., Madison, WI, USA) for 2 weeks. The hyperthyroid group received daily i.p. injections of L-T₃ (1 μ g/mouse per day) (Sigma) during the last 4 days of the 2-week treatment with LoI/PTU diet. The hypothyroid group received injection of vehicle (0.5 mmol/l NaOH and 0.1% BSA in 0.01 mol/l PBS, pH 7.0) only. Mice fed normal chow diet and receiving vehicle injections are referred as the control group. Mice were killed by exsanguination under deep ether anesthesia, and then the brain and heart were dissected out. Three parts were separated from the brain: cerebral cortex, cerebellum and the rest of the brain. These tissues were quickly frozen under liquid nitrogen or placed on dry ice and kept at -80 °C until use.

Northern blot analysis

Total RNA was extracted by the method of Chomczynski & Sacchi (16) and aliquots of 15 μ g RNA were used to determine ZAKI-4 α and β mRNAs by Northern blot analysis. Procedures for RNA denaturation, electrophoresis and hybridization were described before (19). Two cDNA fragments specific for either ZAKI-4 α (exon 3) or β (exons 1 and 2) were labeled with [α -³²P]dCTP (specific activity 111 TBq/mmol) (Perkin Elmer Life and Analytical Sciences, Boston, MA, USA) using a Random Primer DNA Labeling Kit

Ver. 2 (Takara Bio, Inc., Osaka, Japan). After the hybridization, the radioactivity of bands was measured using a Fuji Bioimage Analyzer (BAS 2000; Fuji Photo Film). The hybridized membranes were reprobated with cDNA for 18S ribosomal RNA and data were used to correct the amount of ZAKI-4 α and β mRNAs expressed in arbitrary units (AUs).

Statistical analysis was carried out using one-way ANOVA followed by Scheffe's F analysis. *P* values less than 0.05 were considered as significant differences.

Results

Cloning of mouse ZAKI-4 α and β cDNAs

Using RT-PCR coupled with 5' and 3' RACE, the entire open reading frames of both ZAKI-4 α and β cDNAs were identified and the deduced amino acid sequences are shown in Fig. 1. Mouse ZAKI-4 α consists of 192 amino acids and its estimated molecular mass is 21.5 kDa whereas mouse ZAKI-4 β consists of 243 amino acids, being 27.3 kDa. Primary structures of these two isoforms are distinct at their N termini; 1–23 in α and 1–74 in β . The rest of the amino acid sequence is identical and contains the calcineurin-binding region (Fig. 1). The sequence of mouse ZAKI-4 α cDNA was identical to the one reported previously: MCIP2 (GenBank Accession No. AU035927) (4) or DCR1L1 (GenBank Accession No. AF237887) (6). However, mouse ZAKI-4 β has not been reported previously.

Mouse and human ZAKI-4 α and β have similar amino acid sequences. The α -specific region is 100% identical and the identities between human and mouse β -specific and the common regions are 88 and 98.5% respectively.

Genomic organization and chromosomal mapping of mouse ZAKI-4 gene

A database search of the mouse genome demonstrated that ZAKI-4 gene consists of seven exons, as illustrated in Fig. 2. The first and second exons encode β -specific regions of ZAKI-4 mRNA whereas the third exon encodes an α -specific region. The rest of the four exons encode the regions common to α and β . This genomic organization of the mouse ZAKI-4 gene is similar to that of the human ZAKI-4 gene except that a sequence corresponding to the first exon of human ZAKI-4 gene is missing in the mouse genome. It also should be noted that a sequence corresponding to exon 7 of human ZAKI-4 gene is separated into two exons in the mouse genome by an intronic sequence, 12 bp in size (Fig. 2). Consequently, there are seven exons in both human and mouse ZAKI-4 genes. Two polyadenylation signals were located in exon 6 and 7, generating transcripts that differ in their 3' non-coding sequences. There are three NF-AT-binding sites

Figure 1 Amino acid sequences of mouse and human ZAKI-4 isoforms: *, amino acids common to all four isoforms; +, identity between mouse and human ZAKI-4 α or β . A putative calcineurin-binding site is marked in the shaded box. Sequences common between ZAKI-4 α and β are boxed in.

The genomic organization of mouse *ZAKI-4* gene was confirmed by the analysis on bacterial artificial chromosomes and yeast artificial chromosomes for

Chromosomal mapping of the mouse *ZAKI-4* gene by FISH is shown in Fig. 3. A twin-spot signal was consistently observed on chromosome 17q (Fig. 3A). By comparison with G-like banding pattern (Fig. 3B), the mouse *ZAKI-4* gene was mapped to mouse

Figure 2 Gene organization of *ZAKI-4* and its products assessed by the alignment of 26 contigs found in Mouse Genome Assembly. They are: CAAA01134396 (include exon 1), CAAA0115563, CAAA01049523, CAAA01049520, CAAA01049517, CAAA01125345 (include exon 2), CAAA01049514, CAAA01049511, CAAA01049508, CAAA01088995, CAAA01157047, CAAA01182315, CAAA01049505, CAAA01049503, CAAA01140717, CAAA01158361, CAAA01131153 (include exon 3), CAAA 01162914, CAAA 01162918, CAAA 01162923, CAAA 01143409 (include exon 4), CAAA 01090350 (include exon 5), CAAA 01049500, CAAA 01049497, CAAA 01049494 (include exon 6) and CAAA 01049491 (includes exon 7). (Upper panel) The *ZAKI-4* gene. β -specific exons are indicated in filled boxes; α -specific are in open boxes and common exons are in shaded boxes. Numbers of nucleotides in exons and introns are shown. (Middle panel) Two transcripts are generated by alternative initiation and splicing. ATG: the translation start site, TGA: the stop codon. Note that one polyadenylation site is present on exon 6 and another on exon 7. (Lower panel) Schematic structures of *ZAKI-4* α and β proteins. SP: a putative calcineurin-binding site.

Figure 3 Chromosomal localization of *ZAKI-4* gene analyzed by FISH. (A) The white arrow indicates a twin-spots detected by FISH. (B) R- and Q-banding stained with Hoechst 33258. (C) A cartoon indicating chromosomal localization of the *ZAKI-4* gene (black arrow) on chromosome 17.

Figure 4 Tissue distribution of ZAKI-4 isoforms. Tissues were obtained from two C57BL/6J adult mice and 15 µg total RNA were subjected to Northern blot analysis. Tissue distribution profiles of ZAKI-4 α mRNA (A) and ZAKI-4 β mRNA (B) are shown. Membranes were reprobed with rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA to monitor the integrity of mRNA.

chromosome 17qC (Fig. 3C). Under the conditions used in this study, we could not detect any additional twin-spot.

Tissue distribution of mouse ZAKI-4 α and β mRNAs

To study tissue distribution of ZAKI-4 isoforms in adult mice, total RNA was extracted from brain, thymus, heart, liver, kidney, skeletal muscle and testis of adult C57BL/6J mice and the RNA was subjected to Northern blot analysis. As shown in Fig. 4A, ZAKI-4 α mRNA was identified as a band at about 3.4 kb in all parts of brain examined. An additional 'faint' band at about 1.4 kb was also detected in the brain. This band was probably produced by use of a polyadenylation signal

in exon 6. In contrast to the restricted tissue distribution of ZAKI-4 α mRNA, the β isoform was expressed in various tissues. As shown in Fig. 4B, ZAKI-4 β mRNA was also identified as two bands at 3.5 and 1.5 kb. The expression of ZAKI-4 β mRNA was most abundant in the heart. In addition, a robust expression was demonstrated in the three parts of the brain and skeletal muscle. Although faint, the β isoform was also found in liver, kidney and testis.

Effect of thyroid hormone on the expression of ZAKI-4 α and β isoforms

Since tissue distribution of ZAKI-4 isoforms revealed robust expression in brain and heart (β isoform only), we studied the effect of thyroid hormone on the

	Control	Hypothyroid	Hyperthyroid
TSH (mU/l)	161±37	>9,000	<20
T ₄ (µg/dl)	3.90±0.72	<0.25	<0.25
T ₃ (ng/dl)	153±20	51±5	229±22
Hepatic 5'DI mRNA (in A.U.)	(225±110)	(14±4)	(3760±391)

Figure 5 Serum concentrations of mouse TSH, T₄ and T₃ after treatment with Lol/PTU with or without L-T₃ injection. Expression of hepatic 5'DI mRNA analyzed by Northern blotting is also presented. Values are means±s.d. of quadruplicate samples.

expression of ZAKI-4 isoforms in these tissues. As shown in Fig. 5, treatment with Lol/PTU diet for 2 weeks lowered the serum thyroxine (T₄) and T₃ concentrations and elevated the thyrotropin (TSH) levels, compatible with hypothyroidism. T₃ injection in hypothyroid mice resulted in the suppression of serum TSH levels, indicating that these mice were hyperthyroid. Effectiveness of T₃ treatment was also confirmed by the changes in the expression of type I 5'-deiodinase (5'DI) mRNA in the liver.

Brains were dissected into three parts: cerebral cortex, cerebellum and the rest of the brain. As shown in Fig. 6A, expression of ZAKI-4 α mRNA was decreased significantly in the cerebral cortex of hypothyroid mice. But injection of L-T₃ to the hypothyroid mice failed to increase the expression significantly and it remained low compared with the level in control mice. It is likely that up-regulation requires longer exposure to thyroid hormone. On the other hand, thyroid hormone treatment did not change expression of ZAKI-4 β mRNA in the cerebral cortex (Fig. 6B). In contrast, in the cerebellum or the rest of the brain neither ZAKI-4 α nor β mRNA changed significantly among three groups (Fig. 6A and B).

In heart, where only ZAKI-4 β mRNA is expressed, hypothyroidism failed to reduce expression of ZAKI-4 β mRNA but a supraphysiological dose of L-T₃ markedly increased the expression (Fig. 7). In liver, thyroid status did not influence the expression of ZAKI-4 β (data not shown).

Discussion

This is the first report on the genomic organization of the mouse ZAKI-4 gene. The results demonstrated that the structure and expression pattern of ZAKI-4 gene is well preserved among mammalian species. Similar to the human, two isoforms, ZAKI-4 α and β , are generated from the single mouse ZAKI-4 gene, mapped on chromosome 17 that is syntenic to human chromosome 6. Genomic organization of the ZAKI-4 gene is also similar in mice and humans. Both consist of seven exons and the lengths of

intervening sequences are comparable (Fig. 2) (15). However, there are small differences in gene structures. The mouse ZAKI-4 gene sequence corresponding to exon 1 of the human ZAKI-4 gene is missing. Thus, a transcript homologous to human ZAKI-4 β 1 would not be generated from mouse ZAKI-4 gene. Indeed, we could identify only one, the ZAKI-4 β , isoform in mouse tissues. Note that the difference in human ZAKI-4 β 1 and β 2 exists outside the open reading frame so that both transcripts encode an identical ZAKI-4 β protein (15). At the amino acid level, ZAKI-4 α and β isoforms are well conserved in human and mouse, suggesting strongly that, as in man, both mouse isoforms inhibit calcineurin (15).

Mouse and human ZAKI-4 α and β also showed similar tissue distribution. Expression of ZAKI-4 α was limited to brain whereas ZAKI-4 β was expressed also in other tissues. The β isoform mRNA was abundantly expressed in heart and skeletal muscle as well as in brain. There are distinct tissue distribution profiles among the DSCR1 family. Whereas ZAKI-4 β (DSCR1L1 β) mRNA was highly expressed in brain, heart and skeletal muscle in human and mice, expression of DSCR1 mRNA was abundant in heart and skeletal muscle but much less in brain of the adult human (20). As for expression of DSCR1L2 mRNA, high expression was observed in liver and heart but it was low in brain and skeletal muscle in mice (21). Therefore, it is of note that among the DSCR1 family ZAKI-4 is the only gene that is abundantly expressed in adult brain where calcineurin has a variety of crucial roles (11).

As observed in cultured human skin fibroblasts (15), expression of ZAKI-4 α but not β mRNA was regulated by thyroid hormone in the cerebral cortex of adult mice. However, the regulation was region-specific as it was not observed in the cerebellum or the rest of the brain. Previously we also found that expression of ZAKI-4 mRNA (sum of ZAKI-4 α and β because a cDNA probe used in the study recognizes both ZAKI-4 α and β mRNAs) was reduced in hypothyroid neonatal rats (22).

Most T₃-responsive genes in brain are regulated by thyroid hormone in a temporally and spatially specific manner (23). For example, expressions of myelin basic protein, calbindin, myo-inositol-1,4,5-triphosphate receptor and Purkinje cell protein-2 are reduced in the hypothyroid rat 0–15 days after birth but this reduced expression recovers by 7 or 9 postnatal weeks (24). On the other hand, expression of rat cerlex cloué 3 (RC3)/neurogranin is up-regulated by thyroid hormone throughout the rat's life, but there is regional selectivity in RC3 expression: up-regulation in the cerebral cortex was observed only in layer VI (25). Interestingly, the reduced expression of ZAKI-4 mRNA in rat cerebrum caused by hypothyroidism was also limited in layer VI (22). Thus, regulation of ZAKI-4 α mRNA by thyroid hormone is similar to

Figure 6 Effect of thyroid hormone on the expression of ZAKI-4 α (A) and β (B) mRNAs in mouse brain. Mouse brain was divided into three parts, cerebral cortex, cerebellum and the rest of the brain, and total RNA was extracted. Total RNA (15 μ g) was subjected to Northern blot analysis for ZAKI-4 α and β mRNAs. The experiment was done in quadruplicate and representative autoradiographs are shown in the upper panel in duplicate. In bar graphs amounts of ZAKI-4 α and β mRNAs corrected with respect to rRNA are shown as means $\pm$ S.E.M. ($n = 4$). * $P < 0.05$.

Figure 7 Effect of thyroid hormone on the expression of ZAKI-4 β mRNA in mouse heart. Total RNA (15 μ g) extracted from mouse hearts was analyzed by Northern blotting for ZAKI-4 β mRNA. The experiment was done in quadruplicate and representative autoradiographs are shown in the upper panel in duplicate. In the lower panel, amounts of ZAKI-4 β mRNA corrected for rRNA are shown in bar graphs. Data are means $\pm$ S.E.M. ($n = 4$). ** $P < 0.005$.

RC3 because the increased expression was also demonstrated in adulthood in the present study.

In contrast to cultured fibroblasts and cerebral cortex, only ZAKI-4 β mRNA was increased by thyroid hormone in heart. To induce ZAKI-4 β mRNA a supra-physiological dose of T₃ was required whereas a physiological dose of T₃ could increase ZAKI-4 α mRNA from levels measured in the hypothyroid state in cultured human skin fibroblasts (1, 15) or mouse cerebral cortex (this report). Thus, the mode of T₃ action on the expression of ZAKI-4 α and β seems to be different. In our previous report (1) it was demonstrated that T₃ increased the expression of ZAKI-4 α mRNA at the level of gene transcription but *de novo* protein synthesis was required to enhance transcription. Furthermore, in the present study, no typical TRE was found in both regulatory regions of ZAKI-4 α and β . Therefore, we speculated that T₃ induces different ZAKI-4-regulatory factors in the cerebral cortex and heart of mice. It is noteworthy that increased expression of MCIP2 mRNA in mouse heart by hyperthyroidism was reported previously (26). An earlier publication from the same laboratory (4) states that MCIP2 is identical to ZAKI-4 α . However, since the present study demonstrated that ZAKI-4 β but not α is expressed in mouse heart, it is likely that the increased transcript by thyroid hormone in their report is ZAKI-4 β . Therefore, the

result of our present study is consistent with that presented by Yang *et al.* (26).

Brain and heart are the main target organs of thyroid hormone. In these organs, calcineurin is also known to play critical roles. Since the present study reveals that thyroid hormone increases the expression of both ZAKI-4 α and β mRNAs in adult mice, it suggests that thyroid hormone affects the function of these organs by regulating calcineurin activity *in vivo*.

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