Molecular Cloning of a Novel Thyroid Hormone-responsive Gene, ZAKI-4, in Human Skin Fibroblasts*

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Thyroid hormones (thyroxine and triiodothyronine (T₃)) play a vital role in fetal development and throughout life in humans. T₃, an active form of the thyroid hormones, exerts its effect through binding to its nuclear receptor and regulating expression of target genes. Thus, identification of T₃-responsive genes in various tissues is important to elucidate T₃ action at the molecular and cellular levels in humans. However, the search for T₃-responsive genes in human tissues is hampered by the difficulty of obtaining tissues from subjects in various thyroid states. Clonal cell lines established from malignant tissues could be used to identify T₃-responsive genes. However, they might aberrantly respond to hormones (1, 2). Identification of T₃-responsive genes from the tissues that maintain differentiated function is thus preferable. Human skin fibroblasts fulfill this requirement since they express T₃-receptors (3–6) and are responsive to T₃. In cultured skin fibroblasts, we have shown that T₃ inhibits the synthesis of glycosaminoglycan (7, 8) and fibronectin (9), and Chait et al. (10) demonstrated that it enhances low density lipoprotein degradation. These effects of T₃ were used for the tissue diagnosis of generalized resistance to thyroid hormone (11).

Despite the fact that cultured human skin fibroblasts are responsive to T₃, few T₃-responsive genes have been cloned. Recently, Liang and Pardee (12, 13) developed a method called “differential display of mRNA by means of the polymerase chain reaction (PCR)” to identify and analyze altered gene expression at the mRNA level in any eukaryotic cells. A similar method was developed by Welsh et al. (14, 15). We have chosen the method to clone T₃-responsive genes in cultured skin fibroblasts, since it has been successfully employed to identify transcripts specific for human tumors (13).

We identified a T₃-responsive gene expressed in cultured human skin fibroblasts. The expression of the gene termed ZAKI-4 is positively regulated by a physiological concentration of T₃. Complete cDNA sequence obtained by rapid amplification of cDNA ends (16) revealed that the gene is transcribed into two mRNA species, both of which code a single peptide of 192 amino acids.

MATERIALS AND METHODS

Cell Culture and Treatment—Skin fibroblasts were obtained by punch biopsies from the deltoid region of four healthy subjects (two men and two women, ranging in age from 9 to 37 years old (males 9 and 29 years, females 25 and 37 years)). The fibroblasts were used between the fourth and the ninth passages after the initial plating and were grown to confluence in 60-mm-diameter plastic culture dishes (Becton Dickinson Labware, Lincoln Park, N.J.) with 4 ml of Dulbecco’s modified Eagle's medium (DMEM, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal bovine serum (Bio serum, Victoria, Australia) and 50 units/ml penicillin G (Life Technologies, Inc., Grand Island, N.Y.) and 50 mg/ml streptomycin (Life Technologies, Inc.) at 37 °C in an atmosphere of air, 5% CO₂, and 100% relative humidity. To study the effect of T₃ on ZAKI-4 mRNA, the cells were exposed to 4 ml of the same medium in which fetal bovine serum was replaced with 10% bovine serum from a thyroidectomized calf (TxBS; Rodland Farms, Gilbertsville, PA) (7). After three days of culture, the medium was replaced with DMEM containing either 10% TxBS alone or TxBS plus T₃ (10⁻¹⁰ to 10⁻⁸ M; Sigma). The cells were further incubated for the indicated periods of time. Total RNA was extracted from cells by methods of Chomczynski and Sacchi (17).

To examine whether the T₃-mediated increase in ZAKI-4 mRNA
subsequently amplified by PCR using five different arbitrary primers (TTTTTTTTTTTTMA-3, where M represents degenerate bases) and 9m25m de novo. Incubated with or without T3 for 24 h. Differential display was carried out using RNAmap Kit (GenHunter Corp., Brookline, MA). Briefly, 0.2 µg of the total RNA was reverse transcribed with T12MA (5'-TTTTTTTTTTTTTTMA-3', where M represents degenerate bases) and subsequently amplified by PCR using five different arbitrary primers (AP-16, AP-17, AP-18, AP-19, AP-20) and 9m25m.

**RESULTS**

**Identification of T3-responsive Genes by Differential Display of mRNAs**—Fig. 1A illustrates the differential display of mRNAs in human skin fibroblasts cultured in the presence or absence of T3. The complete cDNAs coding ZAKI-4 mRNA expression, ZAKI-4 mRNA fragments corresponding to the ZAKI-4 band in Fig. 1B was excised from the gel and reamplified using T12MA and AP-16 and subsequently cloned into pGEM-T vector. The upper panel shows the autoradiograph, and the lower panel shows 28 and 18 S ribosomal RNAs.

**Figure 2** illustrates the dose-dependent increase in ZAKI-4 mRNA by T3. Total RNA was extracted from the cultured fibroblasts from "subject a" 24 h after the addition of T3. Ten micrograms per lane was electrophoresed and hybridized with ZAKI-4 DNA probe. To prepare the probe, a cDNA fragment corresponding to the ZAKI-4 band was amplified using the PCR [33P]dATP (specific activity 70 TBq/mmol; DuPont NEN) was used instead of [35S]dATP as recommended by Trentmann et al. (18).

After an initial 2-min denaturation at 94°C, PCR was carried out using AmpliTaq DNA polymerase (Perkin-Elmer) for 40 cycles with denaturation at 94°C for 30 s, annealing at 40°C for 2 min, and extension at 72°C for 30 s. An aliquot of each PCR product (5 µl) was analyzed on a 1% DNA sequencing gel, which was subsequently exposed to X-AR film (Eastman Kodak Co.) for autoradiography. The cDNA fragments of interest were recovered from the gel and subsequently reamplified with the same primer set and PCR conditions except that no isotopes were added. The reamplified cDNA fragments were cloned into pGEM-T vector from Promega (Madison, WI).

**Northern Blot Analysis**—Total RNA samples isolated from the four fibroblast cell lines cultured in the presence or absence of T3 were subjected to Northern blot analysis as described previously (19).

For the analysis of tissue distribution of ZAKI-4 mRNA expression, human multiple tissue Northern blot (Clontech, Palo Alto, CA), pre-made Northern blot of 2.5 µg of poly(A) RNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas, was used. Rapid Amplification of cDNA Ends (RACE)—RACE (13), a procedure to amplify nucleic acid sequences from an mRNA template between a defined internal site and unknown sequences at either the 3' or 5' end of mRNA, was performed by utilizing a kit (Life Technologies Inc.) according to the manufacturer’s instruction. The synthesized proteins were analyzed by 17.5% SDS-polyacrylamide gel electrophoresis, followed by autoradiography.

**Identification of T3-responsive Genes by Differential Display of mRNAs**—Fig. 1A illustrates the differential display of mRNAs...
absence of T3. At a glance, the patterns of bands were similar between T3-treated and nontreated fibroblasts. The patterns between the two fibroblasts from different individuals were similar when the same set of primers was used. However, a closer look identified some bands whose densities were either increased or decreased in the fibroblasts cultured with or without T3 (Fig. 1B). When the changes in the densities by T3 were reproduced in the two fibroblasts from different individuals, the bands were selected as candidates of T3-responsive genes. Nine cDNA fragments were extracted from the gel, reamplified by PCR with the same set of primers, and subcloned into pGEM-T vector. The cloned cDNA fragments were used as the probes for Northern blot analysis to confirm that the expression of the putative T3-responsive genes was indeed regulated by T3. It was demonstrated that the expression of only one gene (ZAKI-4) was up-regulated by T3. The mRNA for the rest of the genes were either undetectable or unaltered by the addition of T3 (data not shown). As shown in Fig. 2, a significant increase was observed 24 h after the addition of $10^{-10} M$ T3, a physiological concentration in the presence of 10% TxBS (7). The mRNA was further increased by the addition of $10^{-8} M$ T3. Also shown in the figures was the existence of two species of ZAKI-4 mRNA. In addition to a dominant band of 3.4 kb, a faint band of 1.4 kb was observed. The two mRNA species were increased proportionately by T3. As illustrated in Fig. 3, ZAKI-4 mRNA levels increased 12 h after the addition of T3 ($10^{-8} M$). These results were reproduced with four different human skin fibroblasts obtained from different individuals (data not shown).

Mechanism Involved in the Induction of ZAKI-4 mRNA by T3—As shown in Fig. 4, the increase in ZAKI-4 mRNA was completely blocked by the treatment with cycloheximide. By using actinomycin D the half-life of ZAKI-4 mRNA was studied (Fig. 5). It was demonstrated that T3 did not affect the stability (22 h in the absence and 20 h in the presence of T3). These results indicate that T3 induces ZAKI-4 mRNA at the transcriptional level, but de novo protein synthesis is required for the induction.

Tissue Distribution of ZAKI-4 mRNA—The expression of ZAKI-4 mRNA was evident in the poly(A) RNAs from heart, brain, lung, liver, skeletal muscle, kidney, and pancreas. Thus, ZAKI-4 mRNA is not only expressed in the fibroblasts but also in various T3-responsive organs.

Note that dominant ZAKI-4 mRNA in skeletal muscle as well as in fibroblasts is 3.4 kb in size, whereas it is 1.4 kb in the other organs.

Determination of Full-length Nucleotide Sequence of ZAKI-4 cDNA—The entire nucleotide sequence of ZAKI-4 cDNA is shown in Fig. 7. As illustrated at the top of the figure, the original cDNA isolated from the differential display was ap-
proximately 180 base pairs long. To isolate the full-length cDNA, RACE was employed (primer design was indicated in the figure). 5’-RACE produced a product extending 680 base pairs from the 5’-end of the original clone. On the other hand, 3’-RACE produced two products, one extending approximately 200 base pairs from the 3’-end of the original clone and the other extending 2,300 base pairs. Two products produced by 3’-RACE suggested the presence of two mRNA species with different 3’-ends. Alignment of the sequences enabled us to delineate the entire cDNA sequence. Putative polyadenylation signals could be assigned at nucleotide positions 1023AT-TAAA1028 and 3168AATATA3173, giving rise to two mRNA species. The search for an open reading frame revealed that both short and long species of ZAKI-4 mRNAs code a single polypeptide. In frame termination codon 160TAA162 was followed by two initiation codons. The presence of two ATG codons for methionine at N-terminal ends suggested that ZAKI-4 mRNAs may code a protein of either 192 or 197 amino acids. Since Kozak’s sequence (22) is present 5’-upstream of the second ATG codon, translation initiation likely occurs at the second ATG, giving rise to a protein of 192 amino acids. Proline and valine residues were abundant in the molecule. From the cDNA sequence, it was also deduced that the original clone isolated from the differential display was amplified from the sequence 5’-684CGACAGTGAC693-3’ to 5’-848TCAA-AAAAAA360-3’ with the corresponding primer AP-16 (5’-CGACAGTGAC-3’). Homology search of the ZAKI-4 sequence revealed that there is only one mismatch between 5’-end sequence of the arbitrary primer, while 3’-primer T12MA did not hybridize with the actual cDNA ends but recognized eight adenylate sequences present in the nucleotide from 848 to 857.
Cloning of a T₃-responsive Gene, ZAKI-4, in Human Fibroblasts

![Fig. 8. In vitro translation of ZAKI-4 cDNAs. ZAKI-4 cDNAs corresponding to short (1.4-kb) and long (3.4-kb) mRNAs were synthesized by reverse transcription-PCR. Double-stranded cDNAs prepared by PCR were cloned into pGEM-T vector and used for translation. Lane 1, molecular weight marker; lane 2, translation product from the short ZAKI-4 cDNA; lane 3, translation product from the long cDNA; lane 4, no vector.](image)

are at least two related sequences. Both of them are short sequences reported as expressed sequence tag (EST). The 5′-end (277–614) of ZAKI-4 cDNA is identical to the EST (NCBI accession number T09144) from 73 days postnatal female human brain (24). Another EST (NCBI accession T23571) from 3-month human infant brain is identical to the 3′-end (2939–3187). Since the present study revealed that ZAKI-4 gene is also expressed in human brain, these two ESTs are likely to be a part of ZAKI-4 cDNA.

In Vitro Translation of ZAKI-4 mRNA—Both short and long species of mRNAs for ZAKI-4 contained an open reading frame as described above. To confirm that both mRNAs code a single peptide, each cDNA was synthesized by reverse transcription-PCR using RNA obtained from the fibroblasts cultured in the presence of T₃ and cloned into pGEM-T vector. The corresponding mRNAs were transcribed by T7 RNA polymerase and translated in rabbit reticulocyte lysate. As shown in Fig. 8, both mRNAs programmed the synthesis of a peptide with a molecular mass of 26 kDa, which roughly corresponds to the molecular weight estimated from the amino acid sequence.

**DISCUSSION**

The cloning of a T₃-responsive gene ZAKI-4 in human fibroblasts was described. It was demonstrated that the increase of ZAKI-4 mRNA by T₃ is regulated at the transcriptional level, since the stability of the mRNA was not affected by T₃. However, the T₃ effect requires de novo protein synthesis, suggesting that regulation of ZAKI-4 gene expression by T₃ is indirect. To date, very few T₃-responsive genes have been cloned in human skin fibroblasts. Expression of fibronectin mRNA was suppressed by T₃ (23). The decrease in glycosaminoglycan (7, 8) synthesis by T₃ suggested that expression of some genes involved in the synthesis could be repressed by the hormone. To our knowledge, ZAKI-4 gene is the only gene that is up-regulated by T₃ in human skin fibroblasts.

Northern blot analysis revealed the presence of two mRNA transcripts (3.4 and 1.4 kb) for ZAKI-4 gene. Since both mRNA species were proportionately increased by T₃, it was suggested that both mRNAs were transcribed from the same gene. The data from 3′-RACE also suggested the presence of two mRNA species with different 3′-ends. From the sequence of the entire cDNA it is suggested that the two mRNA species originate from alternative polyadenylation, since putative polyadenylation signals could be localized at two sites with a distance of 2 kb, accounting for the size difference.

No homologous protein sequence to ZAKI-4 was found in the SWISSPROT data base. It is thus impossible to speculate about the function of ZAKI-4 gene product based on the sequence data. However, ZAKI-4 mRNA is expressed not only in fibroblasts but also in brain, heart, liver, and skeletal muscle, suggesting that thyroid hormone exerts its effect by up-regulating its expression in these organs.

There was a size difference in the predominant ZAKI-4 mRNA species in different organs. In skin fibroblasts and skeletal muscles, the mRNA of 3.4 kb in size was the major species, whereas 1.4 kb mRNA was dominant in brain, heart, and liver. Specific distribution of ZAKI-4 mRNA species with differing 3′-ends raises a possibility that there is a mechanism for tissue-specific regulation of alternative polyadenylation.

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