Demonstration of thyrotropin receptor mRNA in orbital fat and eye muscle tissues from patients with Graves' ophthalmopathy by *in situ* hybridization

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ABSTRACT. There is a controversy regarding whether there are thyrotropin (TSH) receptors in orbital fat and eye muscle tissues that may play a role in the pathogenesis of Graves' ophthalmopathy. To elucidate whether there are TSH receptors in orbital fat and eye muscle tissues in patients with Graves' ophthalmopathy, we applied the method of *in situ* hybridization in orbital fat and eye muscle tissues obtained during the operation for patients with Graves' ophthalmopathy, to directly detect TSH receptor mRNA. To identify whether the cells with positive TSH receptor mRNA are fibroblasts, we also did vimentin immunoreactivity study. To further prove the transcript does have a full length of TSH receptor, the samples of total RNA preparations, extracted from orbital fat and eye muscle tissues, were used as a template for reverse transcriptase polymerase chain reaction (RT-PCR) using three primer sets to generate cDNA fragments and cloned for sequencing. The results showed that the expression of TSH receptor mRNA was demonstrated in adipocytes and fibroblasts of orbital fat, and perimysial fibroblasts within eye muscle tissues by *in situ* hybridization and vimentin immunoreactivity study. Also, by using the RT-PCR, cloning and sequencing, we further proved that the transcript does have a full length of TSH receptor. The present study suggested that there are TSH receptors expressed in orbital fat and eye muscle tissues.

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INTRODUCTION

Graves' disease is an autoimmune disease. Typical clinical features include diffuse toxic goiter, ophthalmopathy and pretibial myxedema. Hyperthyroidism is related to the overproduction of thyroid hormones by the stimulation of thyrotropin (TSH) receptor antibodies on thyroid follicular cells through TSH receptors (1). However, the pathogenesis of Graves' ophthalmopathy and pretibial myxedema is not clear yet.

Orbital and pretibial fibroblasts are considered to be targets of autoimmune attack in Graves' ophthalmopathy and pretibial myxedema (2). It is possible that T cells, which react with the TSH receptor, will be targeting to the orbital and pretibial fibroblasts where in the presence of antigen (extracellular domain of TSH receptor); they will secrete various cytokines, such as glycosaminoglycan-stimulatory lymphokine (3). These cytokines may stimulate fibroblasts to secrete glycosaminoglycans, to proliferate, to express MHC class II antigens (HLA-DR), and to synthesize collagen and connective tissue (4).

However, there is a controversy regarding whether there are TSH receptors on fibroblasts, which may play a role in the pathogenesis of Graves' ophthalmopathy and pretibial myxedema. RNA encoding the extracellular domain of the TSH receptor has been demonstrated in orbital (5-7) and pretibial fibroblasts (5, 8), but these were only limited in the cultured fibroblasts. Recently, Hiromatsu *et al.* (7) demonstrated the presence of TSH receptor mRNA in cultured orbital and subcutaneous fibroblasts,

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but failed to directly detect that in orbital fat and eye muscle tissues by *in situ* hybridization (7). However, Feliciello *et al.* (9) demonstrated TSH receptor mRNA in retro-orbital tissue but not in fibroblasts. Paschke *et al.* (10) were also unable to demonstrate transcripts of the extracellular domain of the TSH receptor in extraocular muscle.

We have demonstrated that TSH and TSH receptor antibody can bind to fibroblasts (8) and also identified the presence of complete TSH receptor transcripts in pretibial fibroblast culture cells (11). The aim of the present study was to elucidate whether there are TSH receptors in orbital fat and eye muscle tissues in patients with Graves' ophthalmopathy by *in situ* hybridization, and to prove the transcript does have a full length of TSH receptor by using the technique of reverse transcriptase polymerase chain reaction (RT-PCR) using three primer sets to generate cDNA fragments and cloned for sequencing.

MATERIALS AND METHODS

Tissue preparation

Orbital fat tissues were obtained at surgery for orbital decompression from 6 patients with Graves' ophthalmopathy. Eye muscle tissues were obtained at eye muscle surgery from 8 patients with Graves' ophthalmopathy. All the patients had past history of hyperthyroidism with elevated TSH receptor antibodies in the sera. However, the patients were in euthyroid state when orbital decompression or eye muscle surgery were done. Orbital decompression was performed for patients with exophthalmos. Eye muscle surgery was performed for patients with limitation of eye muscle movement and diplopia. Normal thyroid tissue was obtained at surgery from normal tissue of one patient with papillary thyroid carcinoma. Normal abdominal fat and muscle tissues were obtained at surgery from normal tissues of one patient with gastric cancer. Fresh tissue specimens were quickly frozen in liquid nitrogen, then stored at -80 C until use.

In situ hybridization

Tissue preparation

Forty-micron thick of sections of thyroid tissue, orbital fat and eye muscle tissues were cut in a cryostat and thaw-mounted onto gelatin-coated slides.

Probe preparation

^{35}S-Labeled single-strand RNA was prepared and used as a probe for *in situ* hybridization. Antisense RNA probe was generated with SP6 RNA polymerase from a *Eco*RI-linearized pGEM-11Zf(+) phagemid carrying a 183 bp fragment (-11/172) of the human TSH receptor cDNA constructed by Wu *et al.* (11). The sense RNA generated from the same phagemid, linearized by *Hin*dIII and transcribed with T7 RNA polymerase, was used as a negative control. The *in vitro* transcription reaction mixture contained 250 µCi [^{35}S]α-thio-UTP (Amersham, UK) and 500 µmol/l each of ATP, CTP, and GTP in 1 x transcription buffer (40 mmol/l Tris, pH 7.5; 6 mmol/l $MgCl_2$; 2 mmol/l spermidine; 10 mmol/l NaCl; and 9 mmol/l dithiothreitol), and 2 U/µl placenta RNAse inhibitor. The transcription reaction was stopped by treating the mixture with 0.1 mg/ml DNAse-I. cRNA probes were purified on Sephadex G-50 columns.

The labeled riboprobes were mixed with 2 mg/ml total yeast RNA and heat denatured before addition to the hybridization buffer, which contained 50% deionized formamide, 20% dextran sulfate, 1xDenhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA), 300 mmol/l NaCl, 8 mmol/l Tris (pH 8.0), 0.8 mmol/l EDTA, and 8 mmol/l dithiothreitol. The final concentration of the probes was $2.6x10^8$ cpm/ml/Kb (*i.e.* $4.8x10^7$ cpm/ml for our 183 bp probes).

Hybridization assay

The tissue sections were fixed in 4% paraformaldehyde in 0.1 mol/l Na phosphate buffer (20 mmol/l Na_2HPO_4 and 80 mmol/l NaH_2PO_4) for 5 min. After rinsing in Na phosphate buffer, the slides were treated with 0.25% acetic anhydride in triethanolamine (0.1 mol/l; pH 8.0) for 10 min, rinsed in 2xSSC, dehydrated through ethanol solutions, and then delipidated in chloroform. One hundred microliters of hybridization mixture containing cRNA probes was added to each slide. The slides were coverslipped, placed into moist chambers and incubated at 55 C for 8 h. After hybridization, the slides were rinsed in 4xSSC, treated with RNAse (30 µg/ml at 37 C, and then washed through 2xSSC at room temperature and 0.1xSCC at 60 C. The slides were dehydrated in an alcohol series containing ammonium acetate

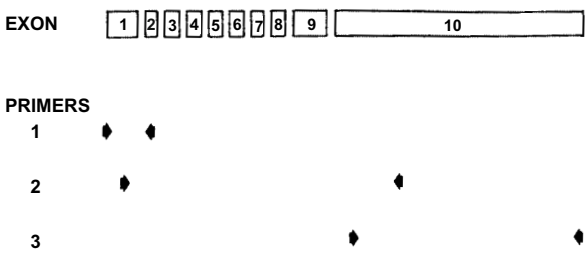

Fig. 1 - *Exonic representation of the human TSH receptor gene showing the positions of the three primer pairs used for PCR amplification of TSH receptor cDNA fragments.*

(300 mmol/l) and air-dried. All slides were then coated with emulsion (Kodak NTB2, Eastman Kodak, Rochester, NY; diluted 1:1 in 600 mM ammonium acetate) and exposed at 4 C in the dark. The slides were developed at 14 C for 4 min in developer (Kodak D-19; diluted 1:1 in distilled water), fixed, and counterstained with cresyl violet.

Immunohistochemistry study of vimentin

To identify fibroblasts on the tissue, vimentin immunoreactivity was localized in sections adjacent to those processed by *in situ* hybridization of TSH receptor mRNA.

Immunohistochemistry study of vimentin was performed by an avidin-biotin complex (ABC) method. Briefly, tissues were fixed in 4% paraformaldehyde and incubated overnight at 4 C with a monoclonal antiserum against vimentin at the concentration of 1:5,000 (Chemicon, CA, USA). After rinsing in potassium phosphate-buffered saline, tissues were reacted with biotinylated anti-mouse IgG (1:600; Jackson Immunoresearch Laboratories, PA, USA) for 1 hr at room temperature followed by incubation with a horse radish peroxidase-conjugated ABC kit (Biomeda, CA, USA). Color reaction was performed by incubating with 0.2 mg/ml diaminobenzidine plus 0.0025% H_2O_2. Sections were then counterstained by hematoxylin, dehydrated and coverslipped.

Cloning and sequencing of TSH receptor mRNA

The RNA used in this experiment was prepared from orbital fat and eye muscle tissues of patients with Graves' ophthalmopathy by the method of Chomczynski and Sacchi (12). Briefly, the tissues were ho-

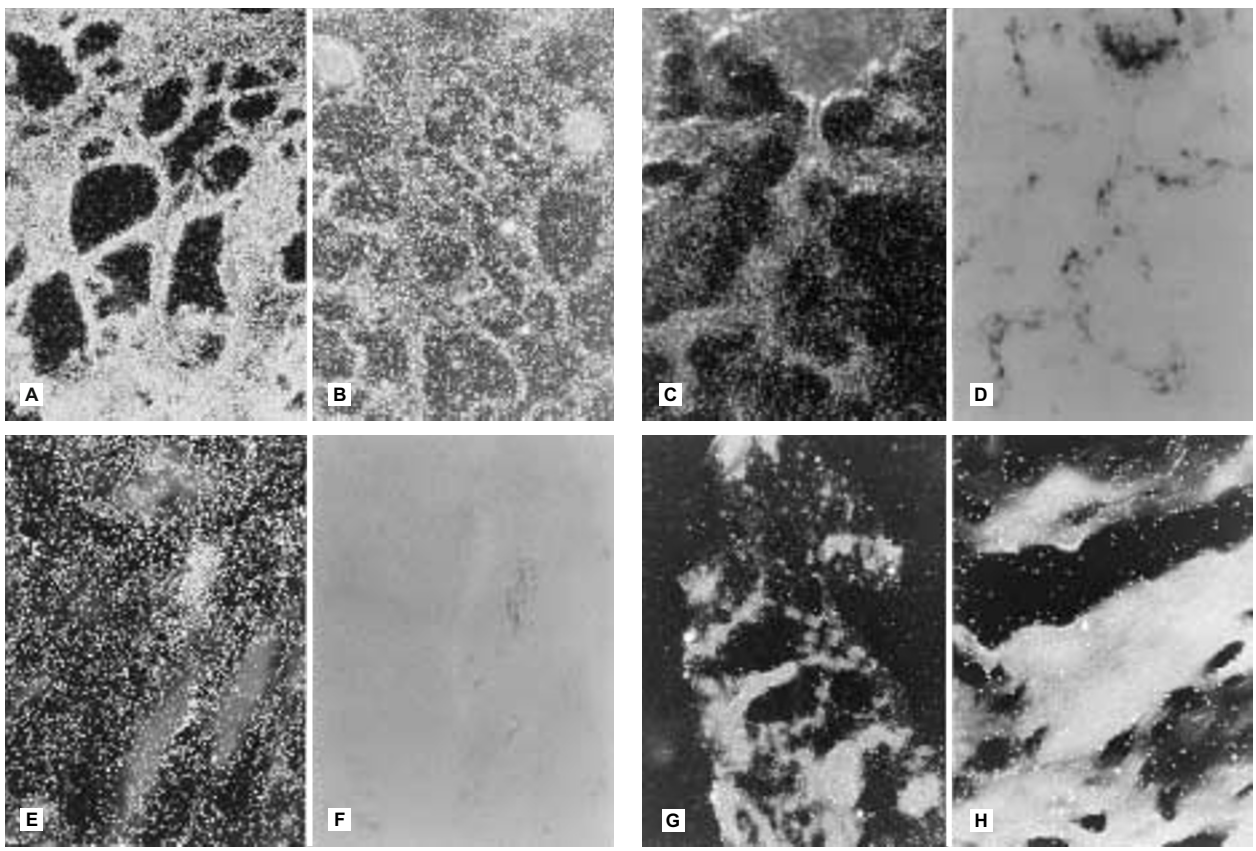

Fig. 2 - *Photomicrographs of the results of* in situ *hybridization of TSH receptor mRNA in the thyroid tissue (A, B), orbital fat (C, D) and eye muscle tissues (E, F). A. Silver grains in the dark field optics showing the distribution of TSH receptor mRNA in the follicular cells (X20). B. Dark field photomicrograph of the thyroid tissue hybridized with sense control probes (X20). C. TSH receptor mRNA was only expressed in certain cells in the fat tissue (X50). D. Bright field photomicrograph of the same field as in C showing the morphology of the fat tissue (nuclei were stained with cresyl violet) (X50). Similar results were observed in the eye muscle tissue (E: dark field; F: bright field) (X50). G. Dark field photomicrograph of orbital fat hybridized with sense control probes (X50). H. Dark field photomicrograph of the eye muscle hybridized with sense control probes (X50).*

mogenized in a denaturing solution (4 M guanidinium thiocyanate, 25 mmol/l sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol) and then extracted with a phenol/chloroform/isoamyl alcohol solution followed by isopropanol precipitation. The RNA precipitate was rinsed with 75% ethanol, vacuum dried, and stored at -70 C until use. The first-strand cDNA synthesis, PCR amplification, cloning and sequencing of TSH receptor cDNA were performed as described previously (11).

In order to determine whether there are complete TSH receptor transcripts in orbital fat and eye muscle tissues obtained from patients with Graves' ophthalmopathy, three sets of primers (-11/8 and 199/219, 58/75 and 1225/1242, 1000/1017 and 2284/2301; 1 refers to the first nucleotide of start codon) were selected and used in this study. The positions of these primers sets in regard to the corresponding exons of TSH receptor gene are demonstrated in Figure 1. The overlapped 2312 bp cDNA sequence was expected to contain the genetic sequences of the signal peptide (1/60), extracellular domain (61/1254), transmembrane domain (1255/2046), and cytoplasmic domain (2047/2292) of the TSH receptor (13). The nucleotide sequences of the three primer sets were (1) 5'TCCCGTGGAAAATGAGGCC3' and 5'AGAAAATGCATGACTTGGAAT3', (2) 5'GGCGGAATGGGGTGTTCG3' and 5'GTAGCCCATTATGTCTTC3', and (3) 5'ATTGTTGGGTACAAGGAA3' and 5'GTTAACTTACAAAACCGT3'. The sequences of the first and the latter two primer sets were according to Bahn *et al*. (14) and Paschke *et al*. (10), respectively.

RESULTS

The results of *in situ* hybridization of TSH receptor mRNA in the thyroid tissue, orbital fat and eye muscle tissues are shown in Figure 2. The thyroid tissue hybridized with antisense probes showed strong signals over the follicular cells (Fig. 2A), whereas there was no apparent binding over the background in the sense control sections (Fig. 2B). This confirmed the specificity of our probe. In the orbital fat and eye muscle tissues, positive signals were also found in tissues hybridized with the antisense probe (Fig. 2C and 2E, respectively). However, TSH receptor mRNA was expressed only in certain cells. The signal intensity was usually higher in the orbital fat than eye muscle tissues. In the sense control tissues, silver grain levels were similar to that of the background (Fig. 2G and 2H).

Clusters of vimentin-postive cells were present in both orbital fat and eye muscle tissues (Fig. 3B and 3D). When compared with adjacent sections in which TSH receper mRNA was visualized by *in situ* hybridization, vimentin immunoreactivity was localized in the same area as TSH receptor mRNA in eye muscle tissues, suggesting that TSH receptor gene is expressed in perimysial fibroblasts. However, not all TSH receptor mRNA-containing cells coexpressed vimentin in the orbital fat. The expression of TSH receptor mRNA was demonstrated in fibroblasts and also in adipocytes of orbital fat.

To clone the amplified TSH receptor cDNA sequences, three sets of primers for RT-PCR amplification were chosen to amplify overlapped fragments of cDNA encompassing the entire open reading frame of the TSH receptor. The samples of total RNA preparations, extracted from orbital fat and eye muscle tissues, were used as a template for RT-PCR to generate cDNA fragments and cloned for sequencing. All samples had amplified products of 230 bp, 1185 bp, and 1302 bp in length generated (Fig. 4).

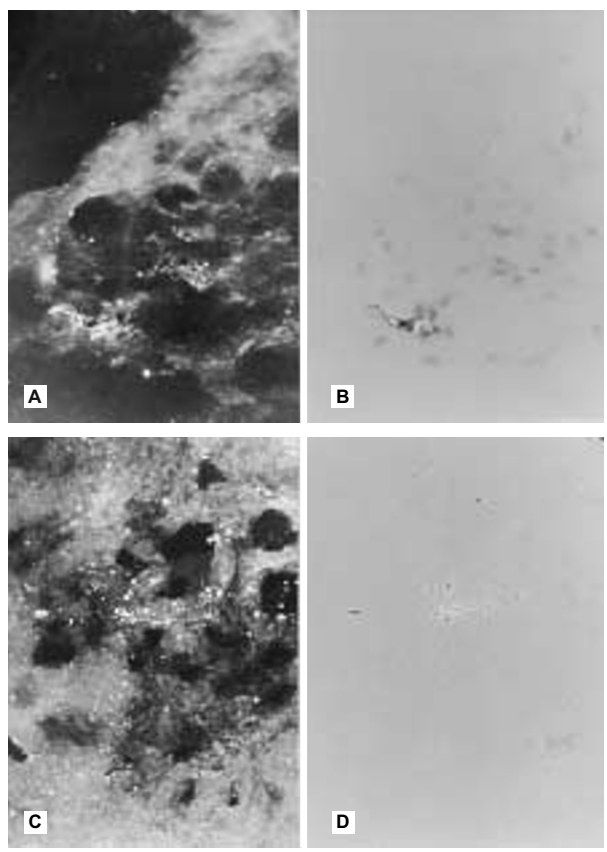

Fig. 3 - *Photomicrographs of immunohistochemical staining of vimentin in orbital fat (A: dark field; B: bright field) (X50) and eye muscle tissues (C: dark field; D: bright field) (X50) from patients with Graves' ophthalmopathy. Brown staining product shows the presence of vimentin immunoreactivity.*

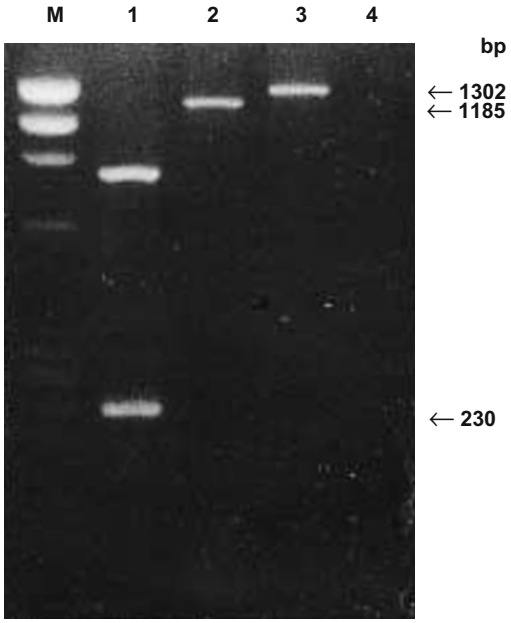

Fig. 4 - *The RT-PCR amplified cDNA products of RNA extracted from orbital fat and eye muscle tissues obtained from patients with Graves' ophthalmopathy. The products of RT-PCR were electrophoresed in a 2.5% agarose gel and stained with ethidium bromide. Lane 1 shows the cDNA products of 230 bp resulted from RT-PCR using primers spanning exons 1-2 (-11/219). Lane 2 shows the cDNA products of 1185 bp resulted from RT-PCR using primers spanning exons 1-10 (58/1242). Lane 3 shows the cDNA products of 1302 bp resulted from RT-PCR using primers spanning exon 10 (1000/2301). Lane 4 shows the RT-PCR product resulted from RT-PCR using primers spanning sequences of 1000/2301 in the absence of reverse transcriptase. Lane M indicates the molecular weight markers ϕX174/HaeIII (1353, 1078, 872, 603, 310, 281, 234, 194, 118, and 72 bp).*

The full length 2312 bp sequences of the TSH receptor coding region were determined by matching those three overlapped RT-PCR products sequences. All of the 2312 nucleotide sequences in orbital fat and eye muscle tissue were consistent with the TSH receptor sequence reported by Nagayama *et al.* (13) except the nucleotide at 1801 was T instead of C, same as reported by Frazier *et al.* (15), Libert *et al.* (16), and Misrahi *et al.* (17). Abdominal fat and muscle tissues had amplified products of 230 bp in length generated, but without 1185 bp and 1302 bp products.

DISCUSSION

The present study showed that the expression of TSH receptor mRNA was demonstrated in orbital fat and eye muscle tissues by *in situ* hybridization. Also, by using the RT-PCR to generate cDNA fragments and cloned for sequencing, we further proved that the transcript does have a full length of TSH receptor.

Hiromatsu *et al.* (7) could demonstrate TSH receptor mRNA in cultured orbital and skin fibroblasts, but not in orbital fat and eye muscle tissues by *in situ* hybridization. In the present study, we demonstrated the presence of TSH receptor mRNA in orbital fat and eye muscle tissues by *in situ* hybridization. The use of a ^{35}S-labeled cRNA probe may account for our success in detecting low transcript levels of TSH receptor gene. Although recently digoxigenin-labeled probes are widely applied to the biomedical research, our experience indicates that those probes are often not sensitive enough to detect low levels of mRNA. This could explain why previous attempt of Hiromatsu *et al.* (7) failed to obtain significant binding in the tissue sections of orbital fat and eye muscle.

Comparison of adjacent tissue sections subjected to *in situ* hybridization of TSH receptor mRNA and vimentin immunohistochemistry, respectively, indicates that fibroblasts in both orbital fat and eye muscle tissues are able to express TSH receptor in our study. The presence of TSH receptor in cultured orbital fibroblasts has been demonstrated by the immunohistochemical staining using polyclonal antibody against a synthetic oligopeptide of TSH receptor (18, 19). Recently, Spitzweg *et al.* (20), using polyclonal or monoclonal human TSH receptor antibody to do immunocytochemical staining of paraffin-embedded orbital connective tissue and extraocular muscle derived from patients with active Graves' ophthalmopathy, also revealed distinct human TSH receptor-like immunoreactivity in orbital connective tissue and perimysial fibroblasts within extraocular muscle, but not extraocular muscle fibers. Therefore, perimysial fibroblasts, but not muscle fibers, may be the major cells to express TSH receptor in the eye muscle tissues.

Guinea pig fat cell membranes have long been known to contain TSH-binding sites (21, 22). In addition, radiolabeled bovine TSH was found to bind to porcine orbital connective tissues (23). Recently, Bahn *et al.* (24) also showed the presence of TSH receptor-like immunoreactivity in frozen section of uncultured orbital adipose/connective tissue from patients with Graves' ophthalmopathy, and in early passage Graves' ophthalmopathy preadipocyte orbital fibroblasts culture, by the immunohistochemical staining using monoclonal antibody directed against TSH receptor C-terminal amino acids 604-764. Liquid hybridization analysis also confirms the expression of TSH receptor mRNA in orbital adipose/connective tissue specimens and the ear-

ly passage Graves' ophthalmopathy preadipocyte orbital fibroblasts culture (24). In our study, not all TSH receptor mRNA-containing cells coexpressed vimentin in the orbital fat. The expression of TSH receptor mRNA was demonstrated in fibroblasts, and also in adipocytes of orbital fat. This may explain why the signal intensity was usually higher in the orbital fat than eye muscle tissues in our study. To further prove the transcript does have a full length of TSH receptor, we performed the RT-PCR using primer sets different from our previous experiment (11) and obtained the 230 bp (-11/219),1185 bp (58/1242) and 1302 bp (1000/2301) cDNA products (Fig. 4). Both of the 230 bp and 1185 bp cDNAs were amplified by using primers spanning exons 1 to 2 and exons 1 to 10, respectively (25). The 1302 bp cDNA was amplified by using primers spanning exon 10. The possibility of PCR amplification of genomic DNA in exon 10 was ruled out by the absence of a 1302 bp product in a control reaction without reverse transcriptase (Fig. 4). As the overlapped 2312 bp cDNA corresponding to the RNA transcript containing the signal peptide (1/60), extracellular domain (61/1254), transmembrane domain (1255/2046), and cytoplasmic domain (2047/2292) of the TSH receptor was detected by RT-PCR in the present study, the complete TSH receptor transcripts are very possible to be present in orbital fat and eye muscle tissues.

Spitzweg *et al.* (20) have demonstrated human TSH receptor-like immunoreactivity in orbital connective tissue and perimysial fibroblasts of extraocular muscle in two control individuals, but less prominent than in the tissues from patients with Graves' ophthalmopathy. Although we did not perform studies in normal orbital fat and eye muscle tissues due to the limitation of available tissues, the RT-PCR amplified cDNA products of RNA extracted from the abdomial fat and muscle tissues in our study showed the transcripts of these tissues do not have a full length of TSH receptor. Spitzweg *et al.* (20) also could not detect human TSH receptor immunoreactivity in abdominal fibroblasts. Using RT-PCR and direct sequencing of PCR products, Major *et al.* (26) demonstrated that the full length TSH receptor is expressed in extraocular muscle but not in abdominal muscle as shown in our study. Taken together, these findings may possibly explain why the extrathyroidal manifestations appear in the orbit but not in the abdomen.

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