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The Existence of Thyroid Hormone Responsive Element, TRE, Was Confirmed in the First Intron of Rat α_1-Acid Glycoprotein Gene[1]

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A set of acute-phase proteins was found to have been induced in rat after triiodothyronine (T_3) treatment. To study the mechanism of gene activation by T_3, the rat gene of α_1-acid glycoprotein (AGP), one of the major acute-phase proteins, was cloned and analyzed. CAT assay identified a thyroid hormone responsive element (TRE) in the first intron of the AGP gene and showed that mutations introduced into the TRE region destroyed the TRE activity. Gel shift assay and methylation interference assay showed that the thyroid hormone receptor bound to the TRE. The TRE identified here had a palindromic structure and was highly homologous to other known TREs.

Key words: α_1-acid glycoprotein, acute-phase proteins, gene expression, thyroid hormone, TRE.

Acute-phase proteins are a number of specific plasma proteins whose concentration increases or decreases following inflammatory stimuli in vertebrates. These changes result largely from transcriptional activation (or inactivation) of these protein genes in hepatocytes. Many reports have shown that cytokines, such as IL-1 and IL-6, as well as glucocorticoids are involved in these activations (*1-3*). Until recently, however, little attention has been paid to the regulation of acute-phase proteins by the thyroid hormone. Investigating genes whose transcriptional level was affected by triiodothyronine (T_3) in rat liver, we found the mRNA and protein levels of a set of acute-phase protein genes to increase as a result of T_3 administration in rats (*4, 5*). Kobayashi *et al.* discovered that T_3 stimulates synthesis of α_1-acid glycoprotein (AGP), an acute-phase protein, in a human hepatoblastoma cell line, HepG2 (*6*). The question we tried to answer was how T_3 regulates these acute-phase protein genes.

We chose the AGP gene as a model system from among other acute-phase protein genes, because AGP is one of the major acute-phase reactants in rat, mouse, and human. In addition, we have been interested for some time in its multi-hormone-responsive and tissue- and development-specific expression pattern (*7*). IL-1, IL-6, TNFα, and glucocorticoids have been recognized as the principal factors which stimulate transcription of the AGP gene.

T_3 has marked effects on the growth, development, and metabolism of essentially all tissues (*8*). It exerts these effects primarily through interaction with a nuclear thyroid hormone receptor (T_3R). T_3-bound T_3R then binds to a specific DNA region, designated as the thyroid hormone responsive element (TRE), and regulates expression of target genes (for reviews, see Refs. *9* and *10*). IL-1, IL-6, TNFα, and glucocorticoids also control gene expression *via* specific DNA elements and *trans*-activators. Our results show that a TRE is identified in the rat AGP gene and that T_3 directly regulates the AGP gene.

MATERIALS AND METHODS

Materials—Wister rats were purchased from Sankyo Lab. Service, Toyama. Restriction enzymes, Klenow fragment, and T4 polynucleotide kinase were purchased from Toyobo, Tokyo, or Takara Shuzo, Ohtsu. Radioisotopes were from DuPont/NEN Research Products, Boston, USA. All other chemicals were provided by Wako Pure Chemicals Industries, Osaka, or Nacalai Tesque, Kyoto. An African green monkey kidney-derived cell line, CV-1, was obtained from the Japan Cancer Research Resources Bank, Tokyo.

Methods—Preparation of mRNA, cDNA library, and colony hybridization: mRNA from rat liver and cDNA were prepared according to previously described methods (*4*). PolyA(+) mRNA for the cDNA library was prepared from rat liver removed 8 h after T_3 injection.

T_3(+)-polyA(+) mRNA for probe was prepared from livers removed from thyroidectomized rats 3 h after T_3 injection, and T_3(−)-polyA(+) mRNA from livers without T_3 treatment. They were labeled with [γ-^{32}P]ATP and used as probes. Then colony hybridization was performed as described (*11*).

CAT reporter plasmid construction and CAT assay: A genomic DNA fragment of the AGP gene, *Sal*I-*Bam*HI (located about −4 kb and +375 b from one of the transcriptional initiation sites (*12*), respectively) fragment,

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Abbreviations: AGP, α_1-acid glycoprotein; T_3, triiodothyronine; TRE, thyroid hormone responsive element; T_3R, thyroid hormone receptor.

was subcloned into the *Sal*I-*Bam*HI site of pBLCAT2 (*13*) (4.2KCAT was obtained, see Fig. 2 for CAT reporter construction). The *Pst*I-*Bam*HI fragment (−70 b to +375 b) was inserted into the *Hin*dIII-*Bam*HI site of pBLCAT2 (0.4KCAT was obtained). The 4.2KCAT was digested with *Sal*I and *Sac*I at −737 b, the 3.5-kbp fragment (*Sal*I-*Sac*I) was removed, and the resulting *Sac*I-*Bam*HI (−737 b to 375 b)-pBLCAT2 fragment was self-ligated (1.1KCAT was obtained). *Ava*II (+185 b)-*Bam*HI fragment was inserted into the *Hinc*II-*Bam*HI site of pBLCAT2. Synthetic oligonucleotide (from +169 b to +198 b) and mutant fragments were inserted into the *Hin*dIII-*Bam*HI site of pBLCAT2. These CAT reporter plasmids and an activator plasmid, either pRShTRβ (*14*) or pRSrTRα (*15*) (both plasmids were kindly provided by Dr. R. Evans, the Howard Hughes Medical Institute, Salk Institute for Biological Studies, La Jolla, USA), which contained respectively human $T_3R\beta$ or rat $T_3R\alpha$ cDNA, were co-transfected into CV-1 cells by the DEAE dextran method, and CAT assays were performed (*11*).

T_3 receptor preparation: Nuclei were isolated from rat liver as described (*16*). Purified nuclei were mixed with about 5×10^{-10} M [^{125}I]T_3 in 0.25 M sucrose, 20 mM Tris/HCl (pH 7.8), 1.1 mM $MgCl_2$, 1 mM PMSF, 1 mM DTT, and incubated for 30 min on ice. Most T_3R molecules are T_3 free at this T_3 concentration. After washing, DNA-binding proteins were extracted in 0.4 M KCl, 20 mM Tris/HCl (pH 8.0), 2 mM EDTA, 10% (v/v) glycerol, 1 mM PMSF, 5 mM 2-mercaptoethanol at 4°C. After centrifugation at $3.6\times10^4\times g$ for 30 min, the extracts were dialyzed against 20 mM Tris/HCl (pH 8.0), 2 mM EDTA, 10% (v/v) glycerol. Following further centrifugation at $8\times10^4\times g$ for 20 min, the supernatant was loaded onto a MonoQ FPLC column (Pharmacia LKB, Uppsala, Sweden) and eluted with 0.05–0.35 M NaCl gradient (*17*). Each fraction was counted in a gamma counter. Two peaks were found, at 0.1 M NaCl and 0.15 M NaCl. The elution profiles of T_3-free T_3R and T_3-bound T_3R were the same on this column chromatography. The fractions of each peak were pooled and used for the gel shift assay.

Gel shift assay: The 60-bp DNA fragment used as a probe was synthesized by PCR. The primers used, one of which was 5′ end-labeled with [^{32}P]ATP, are described in Fig. 4. After polyacrylamide gel electrophoresis, the labeled probes were isolated from the gel as described (*11*). Each of these probes was mixed with poly(dI-dC), T_3R fraction (0 to 5 μl), and $MgCl_2$ at a final concentration 2 mM. A dialyzing buffer was then added in order to adjust final volume to 8 μl, and the mixture was incubated at 25°C for 20 min. Finally, the mixtures were loaded on 5% acrylamide, 25 mM Tris, 190 mM glycine, 1 mM EDTA gels, and run at 70 V in a 4°C chamber.

RESULTS

Acute-Phase Proteins Were Induced in Rat Liver after T_3 Administration—To obtain clones whose mRNA content increased or decreased in rat liver after T_3 administration, the cDNA library was screened. Four inducible cDNA clones and two suppressive ones were obtained, and all were sequenced. The two inducible clones were identified as AGP and β-fibrinogen and the suppressive clones as α_1-inhibitor-3 and serum albumin. They were all included in acute-phase proteins.

Figure 1 shows the changes in the mRNA contents of these genes after T_3 administration. Another acute-phase protein, α_2-macroglobulin, was also tested [plasmid pα_2 M32, which contains α_2-macroglobulin cDNA (*18*), was kindly provided by Dr. S. Hattori, Kyushu University, Kitakyushu]. After T_3 injection in rats, livers were removed at the time indicated, and the mRNA content of each acute-phase protein was estimated by dot-blot hybridization. These contents increased for AGP, α_2-macroglobulin, and β-fibrinogen and decreased for serum albumin (Fig. 1) and α_1-inhibitor-3 (data not shown) after T_3 administration. For a positive control, experimental hepatitis was induced in rats by turpentine oil injection. The mRNA levels of AGP, α_2-macroglobulin, and β-fibrinogen increased in close correlation with the time courses for induction by T_3. In addition, the plasma protein levels were determined by Western blotting. The protein concentrations of AGP, α_2-macroglobulin, and β-fibrinogen were found to have increased, and α_1-inhibitor-3 and albumin to

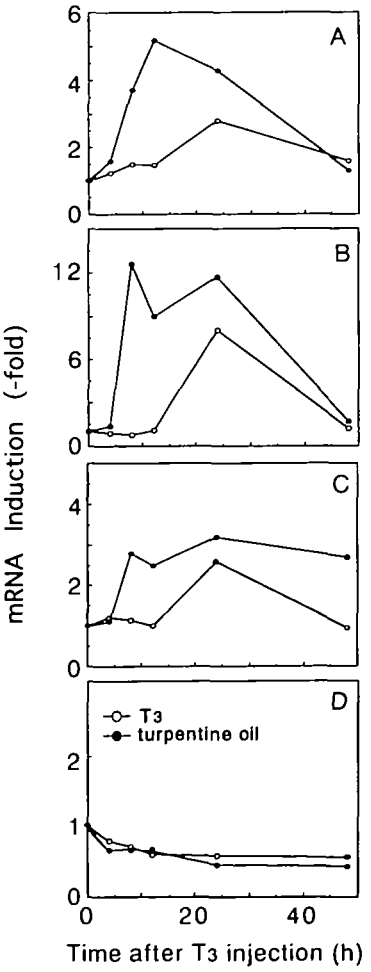


Fig. 1. mRNA contents of three acute-phase protein genes after T_3 treatment in rat liver. T_3 (open circles) was injected intraperitoneally into rats. Turpentine oil (closed circles) was injected under back skin of rats. Livers were excised from rats at the times indicated. mRNAs were evaluated by dot blot hybridization. Probes: A, β-fibrinogen; B, α_2-macroglobulin; C, α_1-acid glycoprotein; D, albumin.

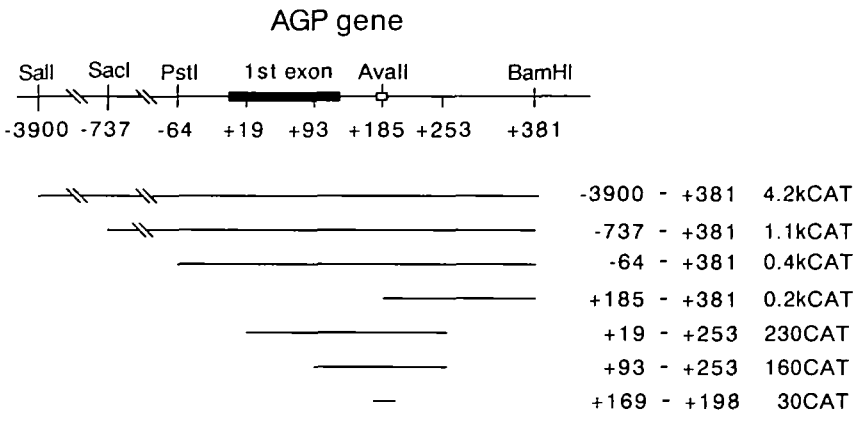


Fig. 2. **The construction of CAT reporter plasmids.** The closed and open boxes indicate an exon and the TRE described here, respectively, and the numbers are distances from one of the transcriptional initiation sites (*12*). Several fragments of the AGP gene were subcloned and inserted into the *Sal*I-*Bam*HI sites of pBLCAT2 (*13*), just upstream from the HSV tk promoter.

TABLE I. **Summary of CAT assay.**

CAT reporters	Induction (T_3+/T_3-)[b]
pBLCAT2	1.0
−3900-+381	2.9
−737-+381	5.6
−64-+381	2.1
+185-+381	1.0
+19-+253	5.7
+93-+253	20.3
+170-+198	4.8
+93-+253 (−T_3R)[a]	1.0

[a]The activator plasmid was not transfected. [b]All values are the averages of 2-4 independent experiments.

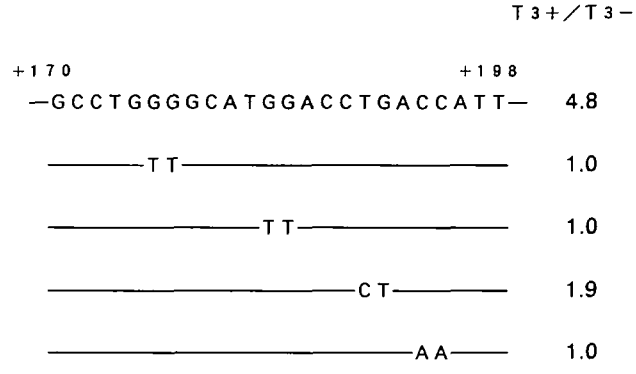


Fig. 3. **CAT assay of mutant fragments.** Synthetic DNA fragments with mutations in the motif were inserted into pBLCAT2 and assayed as described above. The substituted residues are shown, and bars represent the same residues as those of the wild type, which is shown in the upper part.

have decreased after T_3 treatment (data not shown).

A TRE Identified in the First Intron of the AGP Gene—The AGP gene was selected from among a series of genes of acute-phase proteins induced by T_3. We wanted to know if the AGP gene had a TRE and if T_3 directly activated the AGP gene. To answer this question, a genomic DNA fragment of the AGP gene was cloned from a rat genomic library. The sequence of the rat AGP gene was previously reported (*12, 19*).

When checking the DNA sequence, we found a TRE-like stretch, that is (+178) ATGGACCTGACCAT (+191), in the first intron (arrows indicate a palindromic structure). Two DNA fragments, +19-+253 and +93-+253, which contained this stretch as shown in Fig. 2, were inserted just upstream of the tk promoter of the CAT reporter plasmid, pBLCAT2. Both of these fragments showed TRE activity (Table I). A synthesized oligonucleotide, (+169) TGCCTGGGCATGGACCTGACCATTGTAGG (+198) (the underlined sequence shows the palindromic structure), which included this TRE-like motif, retained the TRE activity. We concluded that this stretch represented the TRE of the AGP gene. A series of deletion fragments was inserted into the CAT plasmid to examine if the AGP gene had other TREs elsewhere (Fig. 2). The 4.2-k, 1.1-k, and 0.4-k bp fragments activated CAT synthesis to more or less the same degree in the presence of T_3 (Table I). Deletion between −64 and +184 bp, representing removal of half of the stretch from this fragment, led to a drop in the T_3-responsive CAT activation. We concluded that the stretch was the only TRE in the −3900 to +381 bp region of the AGP gene.

This stretch was thus important for the activation. To confirm this, four mutant fragments were synthesized (Fig. 3) and fused to the CAT reporter plasmids. The introduction of mutations into the palindromic motif resulted in failure of activation. Furthermore, the mutation of the 5′ upstream region from the palindrome also destroyed the T_3 responsiveness. In addition to the palindromic region, the 5′-upstream flanking region was required for activation. These findings led us to conclude that this DNA stretch, between +169 and +198 bp, was important for T_3 activation.

T_3R Bound to a TRE Fragment—Gel shift assay was used to investigate if T_3R could bind to this TRE region of the AGP gene. T_3R prepared from rat liver and labeled with ^{125}I was partially purified on FPLC as described in "MATERIALS AND METHODS." After FPLC, T_3R was fractionated into two fractions, peak 1 and 2 (in order of elution, data not shown). Both of the MonoQ fractions were used as T_3R sources.

A 60-bp fragment used as a probe was synthesized by PCR, and the two primers used are shown in Fig. 4. A major band shift and minor ones were observed on the autoradiogram for both peak 1 and 2 (Fig. 4, lanes 1 and 3, arrows, marked S and F). Minor ones were not always observed (see Fig. 5 for an example). Peak 1 formed band S and peak 2, band F. These two shifted bands disappeared in the presence of a 50-fold amount of the specific competitor.

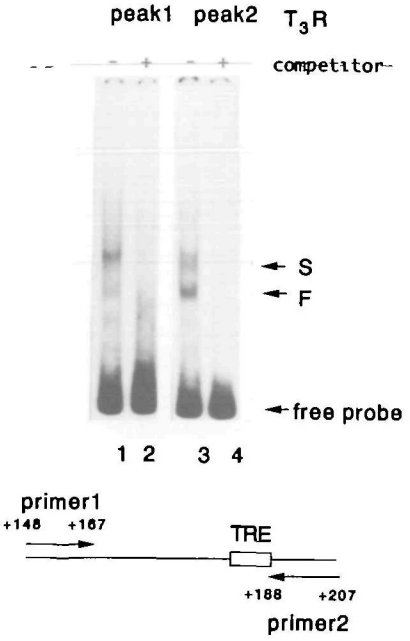


Fig. 4. **Gel-shift assay of a TRE fragment.** A 60-bp TRE fragment was 5′-end labeled and mixed with T_3R fractions and poly (dI-dC). Lane 1: probe+T_3R fraction peak 1, lane 2. lane 1+50-fold amount of specific competitor, lane 3: probe+T_3R fraction peak 2, lane 4 lane 3+50-fold amount of specific competitor. Lower panel shows a schematic drawing of the two primers used in PCR and of the position of the TRE

These two bands thus showed specific binding.

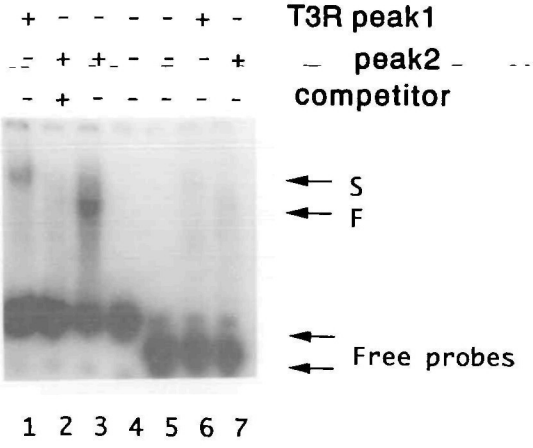
To determine if the determined TRE sequence was important for T_3R binding, the probe was digested with *Ava*II nuclease, which cut the probe at the center of the motif. Both ends of the probe were labeled and then digested with *Ava*II. As shown in Fig. 5, no band shift was observed after digestion, while band shifts were usually observed when T_3Rs were mixed with nondigested probes. This result indicated that the motif was important for T_3R binding.

T_3R Bound to the Motif—To define T_3R binding sites more precisely, a methylation interference assay was performed. The methylated probe was mixed with a peak 1 or peak 2 fraction, and shifted bands were excised. Isolated DNAs were degraded as described (*11*).

The G residue at +184 b of the anti-sense strand disappeared in the B lane of peak 1 and peak 2 (Fig. 6). Other G residues were shown to behave very similarly. Both T_3Rs were interfered with by the methylation of a G-residue at the center of the palindrome. This result further confirmed that T_3R bound to the motif of probe DNA.

DISCUSSION

Acute-phase proteins are expressed in response to disturbances of homeostasis due to infection, tissue injury, neoplastic growth, or immunological disorders. Although cytokines and glucocorticoids are known to be inducers of these proteins, T_3 has been paid very little attention. As shown in Fig. 1, T_3 induced expression of AGP, α_2-macroglobulin, and β-fibrinogen, and suppressed that of α_1-inhib-



Lane 1-4:undigested probe
Lane 5-7: AvaII digested probe

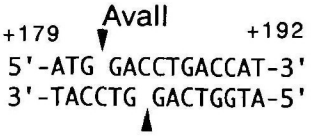


Fig 5. **Failure of T_3R binding to the *Ava*II-digested probes.** The 60-bp probe was labeled at both 5′ ends and digested with *Ava*II Undigested probe (lanes 1, 2, 3, 4) or digested probe (lanes 5, 6, 7) was mixed with T_3R fraction peak 1 (lanes 1, 6), peak 2 (lanes 2, 3, 7) or only the buffer (lanes 4, 5) and electrophoresed. Poly (dI-dC) was added to all mixtures and specific competitor (50-fold excess) was added to mixture 2.

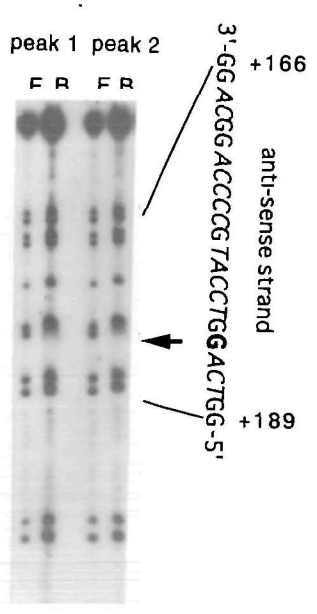


Fig. 6. **Determination of T_3R binding site.** Methylated probe was mixed with T_3R from peak 1 or 2. After electrophoresis, shifted bands and free probes were excised. The DNAs were extracted, treated with piperidine, and then electrophoresed. Arrow and outlined G: the G residue that interfered with T_3R binding; B: bound probe; F free probe.

itor-3 and serum albumin in rat liver. T_3 also induced two other, unidentified, clones which were also expressed in rats by experimental hepatitis. Finally, T_3 was found to stimulate the synthesis of AGP in a human hepatoma cell line, HepG2 (*6*). After thyroidectomy in rats, both AGP mRNA in the liver and protein concentration in plasma had fallen to half of their normal levels 5 days after treatment (manuscript in preparation). These results show that T_3 is a newly identified inducer of the AGP gene, and that T_3 is involved in acute-phase protein expression.

To investigate the mechanism of T_3 induction, the AGP gene was analyzed. We wanted to know whether T_3 controlled the AGP gene directly. CAT assays showed that the AGP gene had a TRE in the first intron (Table I). The region from +169 to +198 bp was then identified as harboring this TRE. This conclusion was confirmed by (1) the fragment between +184 and +381 bp no longer showing T_3-dependent enhancement, (2) the synthetic oligonucleotide of this region showing the enhanced activity (Table I). We found a palindromic structure (+179 to +192 bp) in this region. This palindrome was seen as important because (1) T_3R failed to bind to the probes after digestion by *Ava*II at the center of the palindrome (Fig. 5), and (2) T_3R bound to this palindromic region (Fig. 6). The DNA sequence of this palindrome was highly homologous with that of rat growth hormone gene (*20*), LTR of MoMLV (*21*), and mouse thyrotropin β gene (*22*). In particular, the stem parts of the palindromes were identical in the TREs of the rAGP, rGH gene, MoMLV, and mβ-TSH gene.

Many hormone-responsive elements are located at the 5′ flanking region of genes. In the case of the rat growth hormone gene, a major TRE has been reported in the third intron of the gene (*23*). The TRE of the AGP gene identified here is thus the second example of a gene whose TRE is included in an intron.

The induction folds were very different among reporters (Table I). We found some positive and negative modulator elements in the −730 to −100 bp region of the AGP gene on glucocorticoid induction (manuscript in preparation). These elements may also act as negative and positive modulators of T_3 induction and cause the difference. The induction fold of the +93 to +253 reporter was much higher than that of the +19 to +253 reporter. It is likely that there is a positive modulator element in the +19 to +93 bp region. Because this region is mostly a protein-coding region, it is possible that this element may function incidentally in CV-1 cells *in vitro*, but it is unlikely to do so *in vivo*. However, we did not test this possibility further.

Because one effect of the thyroid hormone is to maintain homeostasis of the body, and acute-phase proteins help to maintain homeostasis, it is very likely that T_3 activates acute-phase proteins for this purpose. It is still unknown whether T_3 plays a role in inflammatory response, since the thyroid hormone concentration in the bloodstream did not undergo any detectable change on induction of experimental hepatitis (data not shown). However, T_3 may maintain a basal level of acute-phase proteins in the bloodstream, and also modulate the effects of cytokines on gene expression in the same manner as glucocorticoids do. On the other hand, acute-phase proteins are also induced at nonacute-phase periods. AGP and α_2-macroglobulin are known to express in the placenta of pregnant rats without any inflammation (*24, 25*). Therefore, T_3 also may work in the case of such nonacute-phase responses.

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