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GENERALIZED RESISTANCE TO THYROID HORMONE: IDENTIFICATION OF A NOVEL c-erbAβ THYROID HORMONE RECEPTOR VARIANT (Leu^{450}) IN A JAPANESE FAMILY AND ANALYSIS OF ITS SECONDARY STRUCTURE BY THE CHOU AND FASMAN METHOD*

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Summary Generalized resistance to thyroid hormone (GRTH) is characterized by elevated circulating levels of thyroid hormone in the presence of a eumetabolic state and failure to respond to triiodothyronine. Various point mutations in the c-erbAβ thyroid hormone receptor gene are known to be responsible for different phenotypes of GRTH. We herein report a new c-erbAβ variant in a Japanese family. The variant consisting of a cytosine to adenine base substitution at nucleotide position 1650 altered phenylalanine to leucine in codon 450 in the T_3-binding domain of c-erbAβ. This base substitution was found in one allele of the 2 affected members of the family. The *in vitro* translation products of this mutant c-erbAβ gene demonstrated a significantly reduced T_3-binding affinity. The secondary structure of this mutant thyroid hormone receptor predicted by the Chou and Fasman method included a new turn in the α helix structure in the T_3-binding domain. We also discuss the secondary structures of the previously reported mutant receptors.

Key Words generalized resistance to thyroid hormone, thyroid hormone receptor, c-erbAβ, gene analysis, Chou and Fasman analysis

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INTRODUCTION

Generalized resistance to thyroid hormone (GRTH) is a genetic disorder characterized by a clinically euthyroid state, an elevated level of free thyroid hormones, and an inappropriately normal or elevated level of thyroid-stimulating hormone (TSH) (Reffetoff, 1982). The majority of patients with this disorder have been found to have mutations in one allele of the c-erbAβ thyroid hormone receptor gene on chromosome 3 (Usala and Weintraub, 1991; Usala, 1991). These mutations are located either in the thyroid hormone-binding domain (T_3-binding domain) or in the hinge domain of the c-erbAβ receptor (Behr and Loos, 1992). The variant receptors are supposed to reduce their T_3-binding affinity probably by changing their protein structure. However, the relationship between the structure and the functions of the variant receptors have not been studied previously.

We herein report a new point mutation found in a Japanese family, *i.e.* a cytosine to adenine base change, which causes an amino acid substitution from phenylalanine to leucine in the T_3-binding domain. In addition, we also discuss the secondary structure of the present as well as other mutant thyroid hormone receptors as predicted by the Chou and Fasman method.

CASE REPORT AND METHODS

Clinical studies. The proband was a 14-yr-old girl, who presented with a soft, symmetrically enlarged thyroid gland. She was born at fullterm and gained weight normally in the neonatal period. Her height and weight had never been more than 2 SD below the mean. No history of delayed speech development, hyperactivity, or learning disability was demonstrated. She was within the average range in general performance and intelligence at school. Her physical examination revealed that her height was 146 cm, which was between 1–2 SD below the mean, and she weighed 43 kg. Her resting pulse was 80 beats/min and blood pressure was 110/70 mm Hg. No tremor or ocular proptosis was found. Neither her liver nor spleen was enlarged. Therelaxation phase of deep tendon reflexes was not prolonged. Serum total cholesterol was within the normal range. No pituitary tumor was visualized by magnetic resonance imaging of the brain.

Her mother had small diffuse goiter, but she had no stigmata of thyrotoxicosis. She was 40 years old and her height was 155 cm, which was close to the mean. The pulse rate was 70/min. Neither the liver nor spleen was enlarged. Serum total cholesterol was within the normal range. She had always been in good health. She had no history of hyperactivity. The grandmother of the proband was also pointed out to have earlier had a thyroid disorder, but the details were not known.

DNA preparation and amplification of the c-erbAβ gene using the polymerase

chain reaction (PCR). Blood samples were obtained from individuals, and genomic DNA was isolated from leukocytes by standard procedures (Sambrook *et al.*, 1989). Exon 5 through 8 of the c-erbAβ were amplified by PCR (Sakurai *et al.*, 1990) (Exons were designated numerically). The amplification was carried out using *Taq* polymerase (Promega, Madison, WI) and a supplemented buffer. Routinely 0.5 μg portion of genomic DNA was exposed to 24 cycles of amplification, each comprising 1 min of denaturing at 94°C, 1 min at 55°C for annealing, and 1 min at 72°C for extension. Primers were synthesized based on the sequences reported by Sakurai *et al.* (1990) (Table 1). Fifty picomoles of the sense and the antisense primers were mixed in each reaction.

Direct sequencing of PCR products. The PCR products of each exon were amplified asymmetrically with 50 pmol of the single-sided sense or antisense primers, and then were sequenced in both directions with the opposite primer that was used in the asymmetrical PCR reaction. The asymmetrical PCR products were then purified with the PCR Purification Spin Kit (Qiagen, Chatsworth, CA). Sequencing was performed by the dideoxy method (Tabor and Richardson, 1987) using Sequenase, version 2.0 (United States Biochemical Corp., Cleveland, OH). High specific activity dCTP-5′-[α-^{32}P] (sp. act. 3,000 Ci/mmol) was obtained from New England Nuclear, Wilmington, DE. The samples were run for 1.5–3 h in a 6% polyacrylamide denaturing gel in 0.5×TBE buffer (Tris/borate/EDTA), pH 8.0, and processed for fluorography.

In vitro expression of normal and mutant c-erbAβ. Exon 8 which contained the mutation site was amplified by the PCR of 35 cycles under the conditions described above for the standard PCR. The *Hin*dIII restriction site was introduced to the antisense primer; antisense strand, 5′-GGAAGCTTAAAGAGCTAGGCAATGGAAT-3′. The PCR products were digested with *Bgl*II (the restriction site of which was present only in exon 8) and *Hin*dIII, and purified with Geneclean II. peA101 (American Type Culture Collection, Rockville, MD), containing the entire coding region of the normal c-erbAβ gene, was also digested with *Bgl*II and *Hin*dIII. The resulting fragment of peA101 lacking *Bgl*II/*Hin*dIII fragment was ligated

Table 1. Sequences of oligonucleotide primers.

Exon	Orientation	Sequence
5	Sense	5′-AGTGGTGCTGGATGACAGCAAG-3′
	Antisense	5′-GCCTTACCAGGAATTTCGCTTT-3′
6	Sense	5′-TCCTCCTTAGCCAGAAGACATT-3′
	Antisense	5′-TCCTCACCTCACAAAACATAGG-3′
7	Sense	5′-GGAATTCTGCTGACATGAACTGGTTCT-3′
	Antisense	5′-GCGTACTCACCTGAAGACATCAG-3′
8	Sense	5′-GGCTTGCCTGTGTTGAGAGA-3′
	Antisense	5′-AAAGAGCTAGGCAATGGAATGAAA-3′

with the PCR products digested with *Bgl*II/*Hin*dIII using T4 DNA ligase (Takara, Kyoto). Competent cells, HB101 (Nippongene, Tokyo) were transformed with the ligated plasmid. The sequences of the normal and mutant clone were confirmed by the dideoxy method. They were linearized with *Hin*dIII, transcribed with T7 RNA polymerase (Stratagene, La Jolla, CA), and translated in rabbit reticulocyte lysate (Boehringer Mannheim Biochemica, Mannheim, Germany) with [^{35}S]methionine (sp. act.: 1,000 Ci/mmol, New England Nuclear).

T_3 binding studies. The protein product (10 μl) of the translation reaction was incubated overnight with various amounts (0.05–0.5 nmol/liter) of [^{125}I]T_3 (sp. act.: 2,200 Ci/mmol, New England Nuclear) at 4°C. The binding was carried out in a total volume of 0.5 ml containing 20 mM Tris-HCl (pH 8.0), 0.3 M KCl, 1 mM $MgCl_2$, and 1 mM dithiothreitol. Nonspecific binding was determined by the addition of a 1,000-fold excess of non-radioactive T_3. Protein bound T_3 was separated from unbound T_3 with Centrifree tubes (Amicon, Danvers, MA). Interference by the radioactivity of [^{35}S]methionine in the filtrate was determined in the absence of [^{125}I]T_3, and the radioactivity was subtracted from all counts. All assays were done in duplicate.

The secondary structure analysis of the thyroid hormone receptor. The secondary structure prediction was performed by the method of Chou and Fasman (Chou and Fasman, 1978) using DNAsis, version 7.00 (Hitachi Software Engineering Co., Yokohama).

RESULTS

Thyroid function tests

The results of the thyroid function tests are shown in Table 2. The proband demonstrated high levels of thyroid hormones, with an inappropriate secretion of TSH. No circulating antibodies to T_3 and T_4, TSH-binding inhibitory immunoglobulins, or antithyroglobulin antibodies were detected, but the antimicrosomal

Table 2. Hormonal and serological studies of the patient's family.

	Proband	Father	Mother	Sister	Normal range
Free T_3 (pg/ml)	9.6	3.6	7.2	5.3	2.7–5.9
Free T_4 (ng/ml)	2.4	1.0	2.5	1.3	0.7–1.8
TSH (μU/ml)	11.5	0.44	1.5	0.9	0.3–3.5
TBG[a] (μg/ml)	N.S.	25	25.7	23.2	17–29
TGHA[b]	(−)	(−)	(−)	(−)	(−)
MCHA[c]	10×2^3	(−)	(−)	(−)	(−)

[a] Thyroxine binding globulin. [b] Antithyroglobulin antibody. [c] Antimicrosomal antibody. N.S., not studied.

antibodies were positive. The TSH response to thyrotropin-releasing hormone (TRH) was inhibited by T_3, but it was still clearly observed even when a supraphysiological dose of T_3 was administered (Fig. 1). Thyroid function tests for her other 3 family members were also performed (Table 2). Her mother's TSH level was normal in spite of her high thyroid hormone levels, which indicated an inappropriate secretion of TSH. The thyroid hormones and TSH levels in both the patient's father and sister were normal.

Nucleotide sequence of amplified exons of c-erbAβ gene

Asymmetrically amplified exons 5–8 from c-erbAβ gene were sequenced directly by the dideoxy method. These portions of the gene contained the hinge domain and T_3-binding domain. Figure 2 shows a fragment of the sequencing ladder of

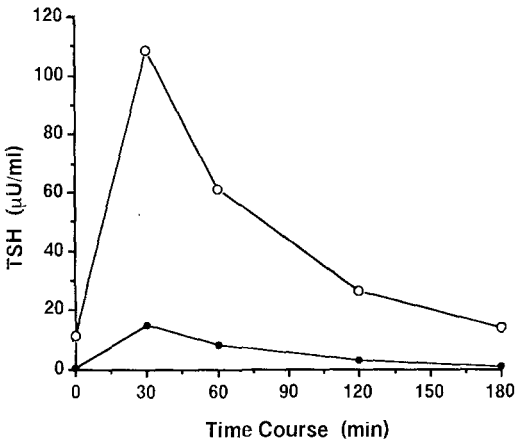


Fig. 1. TSH responses to TRH injection in the proband. Five hundred μg bolus of TRH was injected before (open circle) and after (closed circle) the ingestion of 75 μg/day T_3 for 8 days in the proband.

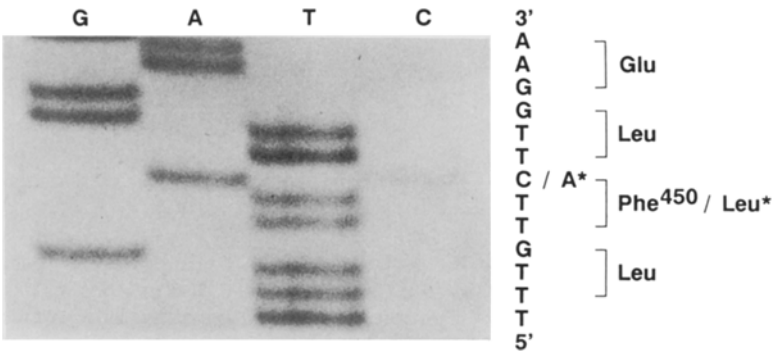


Fig. 2. The partial nucleotide sequence of c-erbAβ exon 8. C→A transversion at nucleotide position 1650 in one allele of the proband is shown. *, mutation.

amplified c-erbAβ exon 8 from the proband. A C→A transversion mutation is shown as C and A bands at the same position at nucleotide position 1650. This mutation caused phenylalanine to leucine substitution in codon 450. As expected, sequencing of the antisense strand showed G and T bands at the same position (data not shown). The sequencing of amplified c-erbAβ exon 8 from her parents was also studied. The same point mutation was detected in her mother, while her father had a normal sequence (data not shown). Amplified exons 5–7 from the proband were sequenced, but no other mutations were detected.

T_3 binding studies

Mutant and normal receptors were translated *in vitro*. The sequences of the normal and mutant clone were confirmed by the dideoxy method. When the ^{35}S-labeled *in vitro* translation products were analyzed by SDS-polyacrylamide gel electrophoresis, they displayed the expected 52 and 55 kDa products (data not shown). The binding affinities for T_3 were calculated by the Scatchard analysis. The wild-type receptor T_3 binding affinity was 8.3×10^{10} M^{-1} and the mutant receptor failed to bind T_3 (Fig. 3).

The secondary structure analysis of the thyroid hormone receptor

Figures 4A and B show the predicted secondary structure of the normal and mutant receptors, respectively. The mutant receptor was predicted to have a new turn structure in the α helix structure in the T_3-binding domain.

The secondary structure analysis of the previously reported mutant thyroid hormone

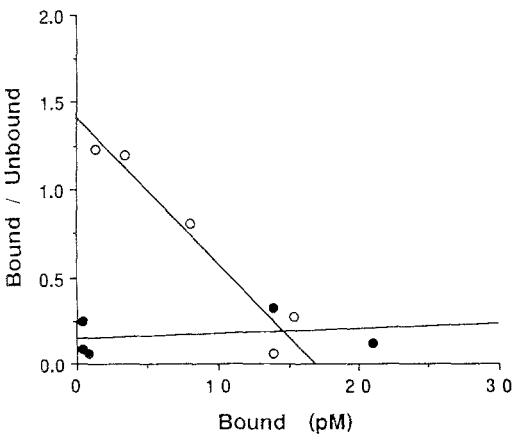


Fig. 3. A Scatchard analysis of [^{125}I]T_3 binding to the *in vitro* translated c-erbAβ receptor protein wild type (open circle) and mutant (closed circle). T_3 binding affinity of the wild type receptor is 8.3×10^{10} M^{-1}. The mutant receptor failed to bind T_3.

receptors

The secondary structure of the previously reported mutant thyroid hormone receptors were predicted by the Chou and Fasman method (Table 3). Seventeen of the 26 reported mutant receptors (65.4%) were predicted to result in some changes of the secondary structure. These changes included 1) α helix to β sheet, turn, or

Table 3. Predicted changes of the secondary structure of the mutant c-erbAβ thyroid hormone receptor proteins reported previously.

Region	Mutation	Change of the secondory structure	Ka mutant/ Ka wild type	Reference
Hinge domain	A[a]229[b]T	+ (3, 4, 9)[c]	0.34	Behr & Loos, 1992
The first "hot spot" in T_3-binding doman	M305T	+ (1)	N.R.	Takeda *et al.*, 1992
	R311H	−	0.024	Geffner *et al.*, 1993
	A312T	+ (1, 8)	0.20	Parrilla *et al.*, 1991
	R315C	+ (9)	0.5	Burman *et al.*, 1992
	R315H	+ (9)	0.46	Cugini *et al.*, 1992
	D317H	+ (7)	0.39	Mixon *et al.*, 1992
	G327R	−	N.R.	Parrilla *et al.*, 1991
	T332@	+ (1, 8, 9)	0.125	Usala *et al.*, 1991b
	R333W	+ (1, 8, 9)	0.21	Mixon *et al.*, 1992
	E335H	−	N.R.	Usala *et al.*, 1991a
	G340S	+ (5)	~0	Adams *et al.*, 1992
	G340V	+ (4, 8 ,9)	N.R.	Parrilla *et al.*, 1991
	G340R	−	~0	Sakurai *et al.*, 1989
	G340D	−	N.R.	Takeda *et al.*, 1992
	G342E	+ (4)	N.R.	Parrilla *et al.*, 1991
The second "hot spot" in T_3-binding domain	R433H	−	1.06	Boothroyd *et al.*, 1991
	M437V	−	0.17	Parrilla *et al.*, 1991
	K438E	+ (4)	0.09	Sasaki *et al.*, 1992
	433@@	+	<0.05	Parrilla *et al.*, 1991
	L445H	+ (5)	0.39	Mixon *et al.*, 1992
	P448T	+ (1)	0.41	Parrilla *et al.*, 1991
	P448S	+ (2)	N.R.	Takeda *et al.*, 1992
	P448H	−	0.17	Usala *et al.*, 1990
	F450L	+ (2)	~0	♯
	F454C	−	0.33	Mixon *et al.*, 1992

[a] Amino acid residue expressed in a one-letter code. [b] Codon numbering is as described by Weinberger *et al.* (1986). [c] Numbers in parenthesis mean the type of the secondary structure change: 1, α helix to β sheet; 2, α helix to turn; 3, α helix to coil; 4, β sheet to α helix; 5, β sheet to coil; 6, β sheet to turn; 7, coil to α helix; 8, coil to β sheet; 9, shift of turn; 10, turn to α helix; 11, turn to β sheet (6, 10, and 11 were not recognized in these mutants). Ka, binding affinity; +, presence of the secondary structure change; −, absence of the secondary structure change; @, deletion; @@, frame shift; N.R., not reported; ♯, present case.

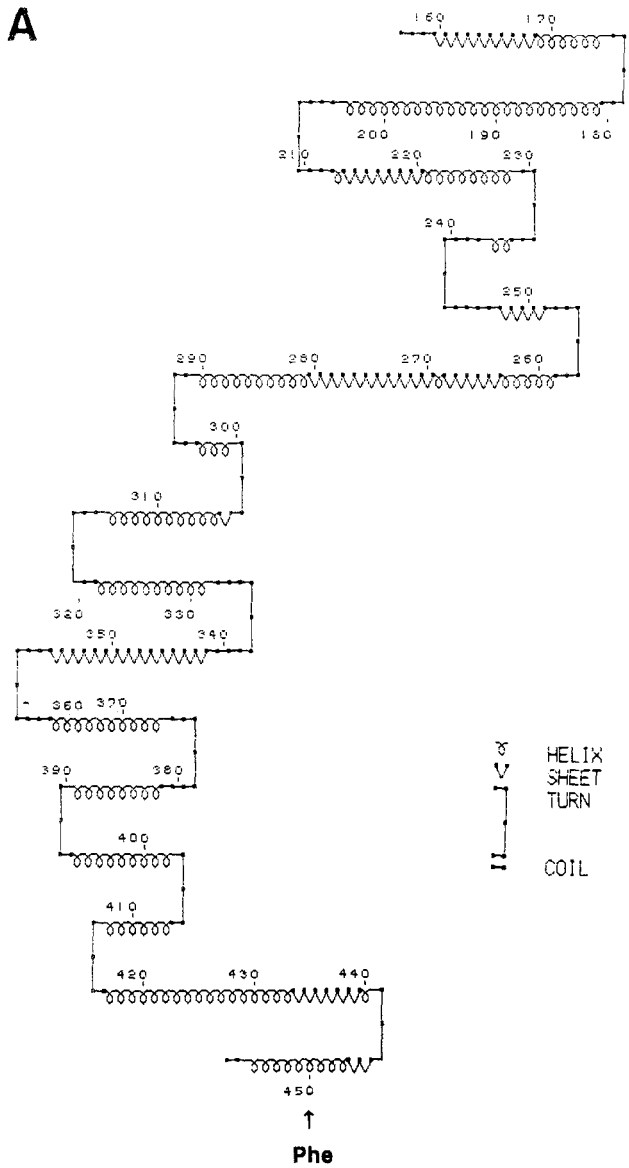
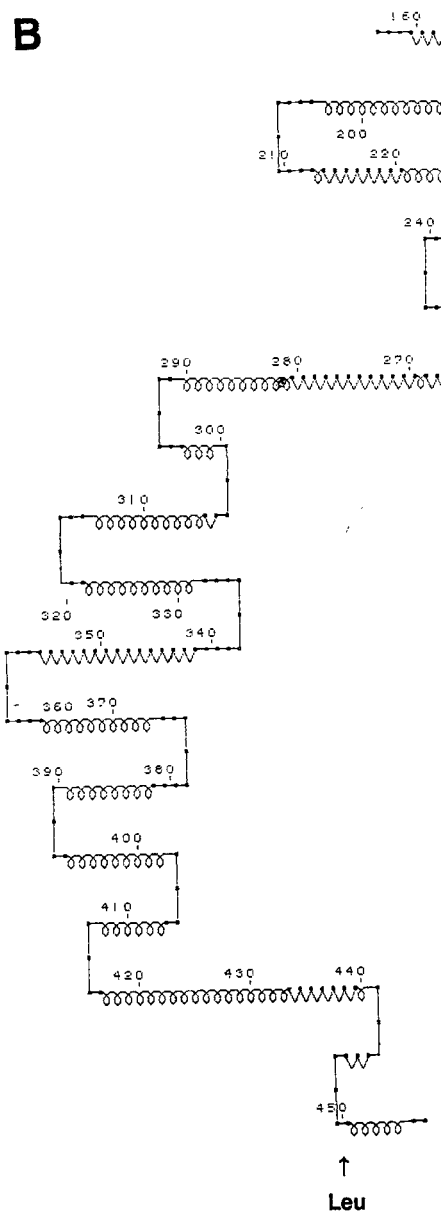


Fig. 4. The secondary structure of the hinge domain and T_3 binding domain of the c-erbAβ receptor protein predicted by the method of Chou and Fasman. A. The normal c-erbAβ receptor. B. The mutant receptor presented in this study. The mutant receptor is predicted to have a new turn structure in the α helix structure in the T_3-binding domain.

coil, 2) β sheet to α helix or coil, 3) coil to α helix or β sheet, 4) shift of turn. The relationship between the types of secondary structure changes and T_3 binding affinity could not be clearly elucidated.



The secondary structure analysis of the posturated thyroid hormone receptor variants in the region between the two hot spots

It has been reported that there are two "hot spot" regions in the T_3-binding domain (Parrilla *et al.*, 1991). We studied the secondary structure of the mutant proteins which were postulated to have the same amino acid substitution in the region

Table 4. Predicted changes of the secondary structure of the thyroid hormone receptor variants which were postulated to have the same amino acid substitution in the region between the two hot spots as that in the previously reported mutants.

Mutation	Change of the secondary structure
D[a]346[b]H	+ (4)[c]
A347T	+ (6)
F349L	−
D350H	+ (4)
M353T	+ (5, 9)
F358L	+ (9)
D361H	+ (4, 7, 9)
D362H	+ (4, 7, 9)
A366T	+ (1)
L367H	−
L368H	−
A370T	+ (1)
M374T	+ (3, 9)
D377H	+ (7)
R378C	−
R378H	−
P379S	−
P379T	+ (7, 9)
G380E	+ (7, 9)
G380V	+ (7, 9)
L381H	−
A382T	+ (1)
K389E	−
D392H	−
F394L	−
A397T	+ (1)
F398L	−
K406E	+ (1, 11)
F412L	−
P414S	+ (10)
P414T	+ (1, 11)
K415E	+ (3)
L416H	+ (1)
M418T	+ (1)
K419E	−
D422H	+ (1)
R424W	+ (1)
M425T	+ (1)
G427E	−
G427V	+ (1)
A428T	+ (1)
A431T	+ (1, 2, 4)

The symbols are the same as those in Table 3.

between the two hot spots as that in the previously reported variants (Table 4). Any mutant receptors which could not be caused by a one step mutation were excluded. Twenty-nine of the 42 postulated mutations (69.0%) were predicted to change their secondary structures.

DISCUSSION

The cytosine to adenine base substitution at the nucleotide position 1650 of c-erbAβ gene, which alters phenylalanine to leucine in codon 450, is a new mutation of the c-erbAβ gene. This mutation is thus considered to be responsible for GRTH in this patient. Firstly, the major portion of the c-erbAβ gene (exons 5–8, the hinge domain and the T_3-binding domain) was sequenced and it was the only base change found. Secondly, this mutation was found in two affected members of the patient's family (the proband and her mother), but not in an unaffected member (father). Thirdly, this mutation was supposed to alter the secondary structure of the c-erbAβ receptor protein drastically, when predicted by the Chou and Fasman method. Finally, this mutation resulted in the abolishment of the T_3-binding activity when the mutant gene was translated *in vitro*.

The secondary structure of the previously reported mutant thyroid hormone receptors predicted by the Chou and Fasman method revealed that about two-thirds of the reported mutant receptors resulted in some changes of the secondary structure. The remaining mutant receptors might alter their tertiary structure.

The reason why mutations have never been found in the region between the two hot spots seems intriguing. There could be three possible causes for this: 1) This region is an area where DNA mutations are only rarely generated. 2) Allelic mutations are generated in this area, but they do not change their protein structures. 3) The structural changes of the receptors are induced, but they do not interfere with their functions as receptors. To gain an insight to this question, we analyzed the secondary structure of the postulated mutant proteins in the region between the two hot spots. Approximately two-thirds of the postulated mutations were predicted to change their secondary structures. These results thus suggest that most of c-erbAβ receptor protein would change their secondary structure if those mutations were generated in this region. It is therefore speculated that the region between the two hot spots is either an area where mutations are hardly generated or an area where the functions as a receptor are hardly disturbed in spite of the presence of structural changes.

Recently, Weiss *et al.* (1993) reported that the previously reported mutations occurred in CG-rich areas. However, it was also stated that the region between the two hot spots contains CG-rich sequences. They went on to speculate that the failure to detect individuals with mutations in this region can be explained by the inability of the mutant c-erbAβ to exert a "dominant negative effect" through the formation of stable mutant c-erbAβ homodimers, and thus escape clinical recogni-

tion. In order to confirm their hypothesis, it is necessary to investigate whether or not the functions of the thyroid hormone receptor variants in this region might be disturbed when the mutations are actually generated by site-directed mutagenesis. Spanjaard *et al.* (1991) focussed on the region between residues 288–331 of rat c-erbAα-1 (homologous to the region between 337–380 in human c-erbAβ) and studied the T_3 binding affinity of 14 mutant receptors generated by site-directed mutagenesis. Eleven of the 14 mutant receptors showed a decreased binding affinity. Their results suggest that the mutant receptors generated even in this region could have abnormal functions. Consequently, at this moment, we consider that the region between the two hot spots might be an area where mutations are only rarely generated, however, the exact mechanism of this phenomenon thus still remains to be more fully elucidated.

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