# Characterization of Seven Novel Mutations of the c-erbAβ Gene in Unrelated Kindreds with Generalized Thyroid Hormone Resistance

## Evidence for Two "Hot Spot" Regions of the Ligand Binding Domain

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## Abstract

**Genetic analysis in our laboratory of families with generalized thyroid hormone resistance (GTHR) has demonstrated tight linkage with a locus, c-erbAβ, encoding a nuclear T3 receptor. Three point mutations and two deletions in this locus have previously been reported in affected individuals in unrelated families as potential molecular bases for this disorder. In the present study, we have used direct sequencing of polymerase chain reaction-amplified exons of the c-erbAβ gene to rapidly identify novel point mutations from seven previously uncharacterized kindreds with GTHR. Six single base substitutions and one single base insertion were identified and found to be clustered in two regions of exons 9 and 10 in the ligand binding domain of the receptor: in the distal ligand-binding subdomain L2 and across the juncture of the $tau_i$ and dimerization subdomains. Reduction of T3-binding affinity in each of four mutations tested as well as segregation of all mutations to clinically affected individuals strongly supports the hypothesis that these changes are the cause of GTHR in these kindreds. In view of the diversity of clinical phenotypes manifested, the distinct topographic clustering of the mutations provides an invaluable genetic tool for the molecular dissection of thyroid receptor function. (*J. Clin. Invest.* 1991. 88:2123–2130.) Key words: mutations • c-erbA • thyroid resistance gene**

## Introduction

The c-erbA genes encode thyroid hormone-activated transcriptional regulatory proteins, the triiodothyronine (T3) receptors (1, 2). These proteins belong to a superfamily of nuclear receptors including those for steroid hormones, vitamin D, and retinoic acid, whose action is to modulate the transcriptional activity of certain genes in response to hormonal signals. Thus far, two different subfamilies of T3 receptors, α and β, have been characterized (3–6). The c-erbAα gene has been located on chromosome 17q11.2-21 (7) and the same gene encodes for two alternatively spliced forms of proteins, α1 and α2 (4). The c-erbAβ gene, encoding the two forms of the β receptor, β1 and β2, has been mapped to chromosome 3p21-25 (8). The β2 subtype, a pituitary-specific T3 receptor, has been found in rats (9) but not yet in humans. The $\alpha_1$, $\beta_1$, and $\beta_2$ forms all bind thyroid hormone, while the $\alpha_2$ does not; however, the latter may also play a physiological role in hormone action since it inhibits the transcriptional effects of the $\alpha_1$ and β receptors (10). The diversity of T3 receptors suggests that specific cell responses to thyroid hormone may be the result of different levels of expression of each one of these genes. However, the relative importance of each of these receptor subtypes in mediating thyroid hormone action in different tissues is not established (11).

The syndrome of generalized thyroid hormone resistance (GTHR)[1] is characterized by elevated serum levels of free thyroid hormones with inappropriately elevated thyroid-stimulating hormone (TSH) as well as clinical and biochemical evidence of decreased thyroid hormone action (12, 13). These patients also show unresponsiveness to large doses of exogenous thyroid hormones. Recent studies have demonstrated a genetic linkage between the c-erbAβ gene and thyroid hormone resistance in several families (14, 15). Subsequently, in three unrelated families three different point mutations were found in different areas of the ligand binding domain resulting in nonconservative amino acid substitutions (15–17). In addition, two families have been reported with large and small deletions, respectively, in the c-erbAβ gene (18, 19). Two of these mutations as well as the small deletion resulted in decreased T3 affinity for the receptor (16, 19, 20).

In the present studies we have attempted to elucidate the molecular basis of GTHR in several additional kindreds evaluated at the National Institutes of Health. Direct sequencing of polymerase chain reaction (PCR)-amplified genomic DNA has permitted rapid and accurate detection of novel point mutations in exons 9 and 10 of c-erbAβ gene in seven of nine additional GTHR kindreds studied. In both familial (autosomal dominant) and sporadic cases, as well as one with unknown inheritance, affected members were all heterozygous. The finding of common biochemical features of thyroid hormone resistance consistently associated with mutation in the ligand binding domain of the T3 receptor contrasts with the absence of a distinct clinical phenotype in this genetic condition. Nonetheless, the mutations found are clearly grouped in two clusters: one in the center of the ligand-binding domain involving the 5′ end of the dimerization subdomain and the 3′ end of the $tau_i$ transcriptional subdomain (21), and the other in the distal 3′ region of the ligand-binding domain which is thought to be involved in direct contact with T3 (22, 23). Indeed, each of four mutations studied showed moderate to severe reduction in T3-binding affinity to the in vitro translated receptors.

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1. *Abbreviations used in this paper:* ASPCR, allele-specific PCR; GTHR, generalized thyroid hormone resistance; PCR, polymerase chain reaction.

## Methods

*Patients.* Data were obtained on these patients during hospitalizations at the Clinical Center at the National Institutes of Health (NIH). After signing appropriate consent forms, patients were entered into a peer-reviewed Clinical Center protocol. All laboratory thyroid function tests were carried out by the NIH Clinical Pathology Department or by Hazleton Laboratories (Vienna, VA). To assess tissue responsiveness to thyroid hormone, we selected cases representative of other members of the family. The criteria have been previously defined in our laboratory (13, 15). The following scale was employed: ++ = severe resistance; + = resistance, and − = minimal or no signs of resistance; bone, + = height < 5% with no family history of short stature or bone age retarded > 2 SD; liver, ++ = sex hormone-binding globulin or ferritin within the lower ⅓ of the normal range, or a cholesterol level > 300; + = sex hormone-binding globulin or ferritin within the upper ⅔ of the normal range, or a cholesterol > 240 mg/dl but < 300 mg/dl; brain, ++ = verbal or full scale IQ, or the Kaufman Assessment/Wechsler Intelligent scales in children < 80; + = history of abnormalities of spatial relationship, inadequate school performance necessitating formal special education, or a verbal or full scale IQ between 80 and 86; heart, ++ = sleeping pulse of < 80; + = sleeping pulse > 80 but < 90; body metabolism, + basal metabolic rate < 120%; pituitary, + = basal TSH inappropriately elevated, in the presence of elevated free T4 and/or free T3.

*Genomic DNA amplification.* Genomic DNA was obtained from white blood cells according to previously described protocols (15). Exons 9 and 10 of the c-erbAβ gene were amplified by PCR (24). The amplification was carried out with Taq polymerase according to the protocol recommended by Perkin-Elmer Cetus, Norwalk, CT. Routinely 1-μg portions of genomic DNA were exposed to 25 cycles of amplification, each comprising 0.5 min of denaturing at 94°C, 0.5 min at 55°C for annealing, and 2 min at 72°C for extension. Primers for exon 9: sense strand, 5′-TGGAATTCTGCATTTGTTCTTTGCTGACA-3′ (intron 8, −47 → −18); antisense strand, 5′CTTTGGATCCCCACTAACGAGTCTAGTGTACTCAC-3′ (intron 9, +35 → 0). Primers for exon 10: sense strand, 5′-TTCCGAATTCCATCTCTGAATCAATGTCCATC-3′ (intron 9, −49 → −17); antisense strand, 5′-GCAATGGATCCAAATGACACCCAGTAGTGCTGT-3′ (intron 10, +34 → +1) (17, 25). EcoRI and BamHI restriction sites were introduced to permit the eventual insertion of the amplified exons into cloning vectors. The nucleotide and amino acid sequences have been numbered according to Weinberger et al. (2), rather than to the revision suggested by Sakurai et al. (25), who assumed a different translational initiation site. Exons 9 and 10 correspond to exons G and H in the genomic structure described by Sakurai et al. (25).

*Direct sequencing of PCR products.* The template for sequencing was obtained by the asymmetric PCR amplification method (26) modified according to Kadowaki et al. (27). Each exon was amplified asymmetrically with 60 pmol of the sense or with the antisense primers, and sequenced in both directions with the primer opposite that used in the PCR reaction. Routinely, PCR products were chloroform extracted and 10 μl was removed for agarose gel analysis. Primers and free nucleotides were removed by using centrifuge-driven dialysis in a microconcentrator (Ultrafree-MC; Millipore Corp., Bedford, MA). The retentate was vacuum dried and resuspended in an adequate volume (~ 6 μl) of water to be used as a template for sequencing. Sequencing was performed by the dideoxy method (28) using Sequenase (United States Biochemical Corp., Cleveland, OH). High specific activity dATP-5′-α[$^{35}$S] (sp act 1,000 Ci/mmol) was obtained from Amersham Corp., Arlington Heights, IL. The samples were run for 4–6 h in a 6% polyacrylamide denaturing gel (Bethesda Research Laboratories, Gaithersburg, MD) in 1× TBE buffer (Tris/borate/EDTA), pH 8.0.

*Restriction enzyme digestion.* PCR-amplified exons were chloroform extracted and then purified and concentrated by passage through Ultrafree MC-30 (Millipore). After restriction enzyme digestion in the appropriate buffer, digestion products were analyzed in 3% Nusieve agarose (FMC Bioproducts, Rockland, ME)/1% agarose containing ethidium bromide and visualized by UV illumination. Restriction mapping and sequence analysis were carried out according to Marck (29).

*Allele-specific amplification.* In those cases in which a mutation did not result in gain or loss of a restriction site, allele-specific polymerase chain reaction (ASPCR) amplification of the exon was used to confirm and/or detect other affected members of a kindred. The principle of the method is that a PCR amplification would not proceed if the 3′ end of one of the primers did not match the complementary base of the template (30). Oligonucleotides of 14 bases were prepared in which the 3′ (sense) or 5′ end (antisense) base matched either the normal or the mutant. Products of amplification are detected only in samples from affected persons when the "mutant" primer is used. In certain instances the efficiency of the reaction with the mutant primer was low and hybridization with a labeled probe (random primer-labeled, linearized plasmid containing the c-erbAβ insert) was used to visualize the amplification products.

*Chemical mismatch.* The method of Cotton et al. (31) as modified by Grompe et al. (32) of chemical mismatch cleavage has also been used in certain cases for the mapping of mutations in DNA heteroduplexes formed with amplified c-erbAβ exons 9 or 10 from control subjects and patients with GTHR.

*Preparation of mutant receptors cDNA.* cDNA clones of exon-10 c-erbAβ mutants (cases Q-W, O-K, and P-V) were prepared as follows: exon-10 was amplified by PCR from genomic DNA as described above and the fragment cloned into pGEM4Z vector. The DNA fragment from BglII to HindIII was subcloned into the wild type human placental c-erbAβ clone, pea101 (2). cDNA for the exon 9 mutation E-D was prepared from the template pea101 by the splicing overlap method of PCR (33) using complementary internal oligonucleotide primers containing the E-D mutation and 3′ and 5′ primers overlapping StyI and BglII sites of the wild type sequence. After StyI and BglII restriction digest, the fragment was substituted for the corresponding region of pea101.

*In vitro translation.* In vitro translation of the receptors was carried out with a reticulocyte lysate L-[$^{35}$S]-methionine translation kit from Dupont-NEN, Wilmington, DE, following the protocol described by Usala et al. (20). Products of the translation were analyzed by 10% SDS PAGE.

*T3-binding assay.* A portion of the in vitro translation product was used for studying T3-receptor binding by the nitrocellulose filter assay as described in detail by Inou et al. (34) and by Usala et al. (20). [$^{125}$I]-triiodothyronine at sp act 2,200 Ci/mmol was obtained from Dupont-NEN. The binding of T3 was measured in triplicate filters. Nonspecific binding was determined in the presence of 1,000-fold excess of unlabeled triiodothyronine.

## Results

*Thyroid function tests.* Table I summarizes the thyroid function tests of representative individuals from each kindred whose genomic DNA was used for amplification and sequencing of the c-erbAβ gene. In all cases, the TSH was inappropriately high and would be expected to be < 0.1 μU/ml in the face of elevated levels of both total and free thyroid hormones. Attention deficit-hyperactivity disorder was the most common clinical feature in these patients and growth retardation was present in two families (13, 15). Perhaps the most striking finding was the absence of any other consistent phenotypic characteristics associated with this clinical syndrome.

*Nucleotide sequence of amplified exons of c-erbAβ gene.* Asymmetrically amplified exons 9 and 10 from c-erbAβ gene have been sequenced directly by the dideoxy nucleotide method of Sanger. This procedure is not only simpler and faster than sequencing clones of the amplified DNA fragments, but also offers other advantages. Simultaneous amplification of both alleles permits detection of the heterozygous states and

*Table I. Representative Thyroid Function Tests in Selected Patients from Seven Unrelated Kindreds with Generalized Thyroid Hormone Resistance*

| Kindred (case) | Age (sex) | TSH | T4 | T3 | FT4 | Tissue Responsiveness* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Bone | Brain‡ | Metabolism | Liver | Heart | Pituitary |
| | | μIU/ml | μg/dl | ng/dl | ng/dl | | | | | | |
| E-D (4)§ | 11 (M) | 4.7 | 1.2 | 641 | 1.2 | − | + | + | − | ++ | + |
| F-W (1) | 10 (M) | 5.5 | 21.6 | 401 | 4.1 | + | ++ | + | N.D. | − | + |
| G-S (1) | 16 (M) | 2.0 | 17.8 | 268 | 4.5 | − | + | + | ++ | + | + |
| N-N (1)§ | 36 (M) | 0.3 | <1.0 | 328 | 0.2 | − | + | + | + | + | + |
| O-K (1) | 4 (M) | 0.7 | 21.2 | 268 | 3.9 | − | − | + | ++ | + | + |
| P-V (1) | 7 (M) | 4.2 | 21.9 | 369 | 4.6 | + | + | N.D. | + | + | + |
| Q-W (3) | 37 (M) | 1.9 | 15.2 | 230 | 3.1 | − | − | + | ++ | + | + |

Normal values: T4: 4.5 to 12.5; T3: 88 to 162; FT4: 1.0 to 1.9; TSH: 0.5 to 4.0. * Symbols: −, no or minimal resistance; +, resistance; ++, severe resistance; ND, not determined; see Methods for description of tests employed to determine tissue responsiveness. ‡ Refers to cognitive or motor function, in addition to the common attention deficit-hyperactivity disorder. § Previous thyroidectomy or $^{131}I$ ablation, on T3 therapy when thyroid function tests were performed.

obviates the need to sequence multiple samples, as with conventional cloning techniques. Fig. 1 *A* illustrates an example showing a fragment of the sequencing ladder of amplified c-erbAβ exon 9 from patient N-N1. A G → A transition mutation is shown as distinct G and A bands at the same position in the sequencing ladder. The intensity of the bands is less than if only a single allele were expressed. As expected, sequencing of the antisense strand (Fig. 1 *A*) shows T and C bands. In all cases both DNA strands were asymmetrically amplified and the products used to sequence in the sense and antisense directions. Sequencing of both strands has been essential in resolving all the possible ambiguities intrinsic to the sequencing method.

Fig. 1 *B* shows the wild-type and case E-D4 sequencing ladders of a fragment of exon 9 in which two mutations were found within a narrow range of bases. The G to A transition at nucleotide position 1234 is nonconservative, whereas the G to C transversion at nucleotide 1242 does not change the coded amino acid and is therefore neutral. Another advantage of the simultaneous sequencing of two or more alleles is that, in heterozygous individuals, the overlapping of the sequencing ladders facilitates the detection of base insertions and/or deletions that otherwise might have been missed if a cloned allele was sequenced. Fig. 1 *C* illustrates a case of a single base insertion, cytosine in nucleotide position 1627, leading to a frame-shift

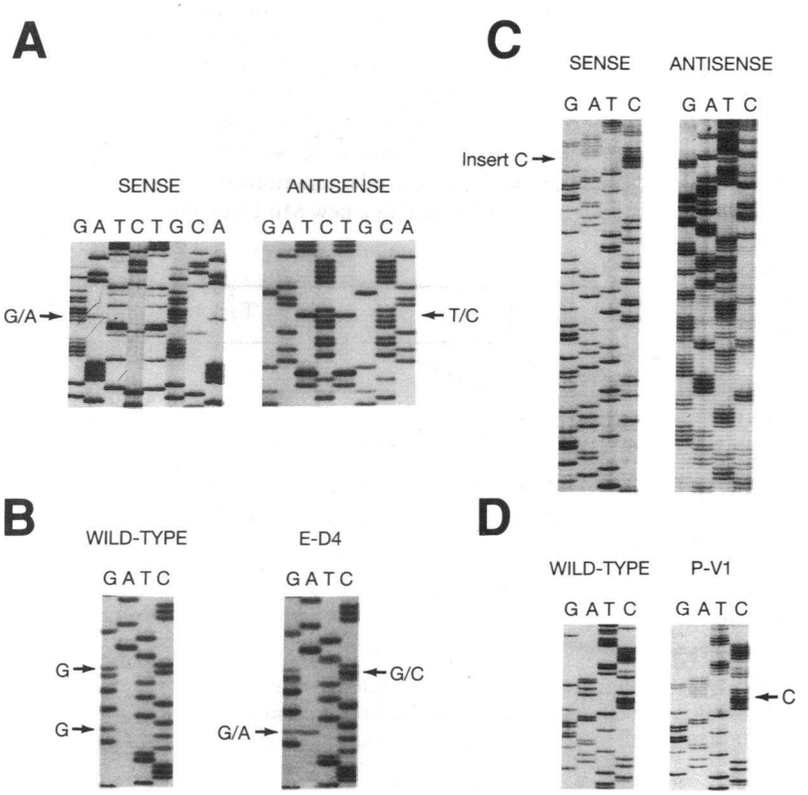


*Figure 1.* (*A*) Partial nucleotide sequence of the sense and antisense strands of exon-9 of c-erbAβ gene. The G to A transition at nucleotide 1325 changes $Gly^{342}$ to $Glu^{342}$. (*B*) Partial nucleotide sequences of wild type and case E-D4 exon-9 of c-erbAβ gene. The G to A transition at nucleotide position 1234 changes $Ala^{312}$ to $Thr^{312}$. The G to C transversion at nucleotide 1242 is a conservative mutation. (*C*) Partial nucleotide sequences of the sense and antisense strands of exon-10 of c-erbA-β gene from case P-V1. A cytosine insertion at nucleotide 1627 results in a frame-shift 443 → 458 changing most amino acids from 443 to 456 and adding two new codons. The indistinct appearance of the antisense sequence is due to the overlapping of the wild-type and the mutant ladders. (*D*) Partial nucleotide sequence of wild-type and case P-V1 sense strands of exon-10 of c-erbA-β gene. A C insertion at nucleotide 1627 changes the wild-type sequence: $Pro^{442}$-$Thr^{443}$-$Glu^{444}$-$Leu^{445}$-$Phe^{446}$-$Pro^{447}$-$Pro^{448}$-$Leu^{449}$-$Phe^{450}$-$Leu^{451}$-$Glu^{452}$-$Val^{453}$-$Phe^{454}$-$Glu^{455}$-$Asp^{456}$ to the following which is two amino acids longer: $Pro^{442}$-$His^{443}$-$Aro^{444}$-$Thr^{445}$-$Leu^{446}$-$Pro^{447}$-$Pro^{448}$-$Phe^{449}$-$Val^{450}$-$Leu^{451}$-$Gly^{452}$-$Ser^{453}$-$Val^{454}$-$Arg^{455}$-$Gly^{456}$-$Leu^{457}$-$Asp^{458}$.

*Table II. Mutations in c-erbA-β Gene in Patients with Generalized Thyroid Hormone Resistance*

| Kindred | Mutation | AA substitution | Nucleotide/Codon* | Exon* | Inheritance |
|---|---|---|---|---|---|
| E-D | GCT → ACT | ALA → THR | 1234/312 | 9 | Sporadic‡ |
| F-W | GGG → AGG | GLY → ARG | 1279/327 | 9 | Unknown |
| G-S | GGT → GTT | GLY → VAL | 1319/340 | 9 | Sporadic‡ |
| N-N | GGG → GAG | GLY → GLU | 1325/342 | 9 | Familial |
| O-K | AGT → GTG | MET → VAL | 1609/437 | 10 | Familial |
| P-V | C → Insertion | Frame shift (443 → 458) | 1627/443 | 10 | Sporadic‡ |
| Q-W | CCT → ACT | PRO → THR | 1642/448 | 10 | Familial |

* Nucleotide and codon numbering is as originally described by Weinberger et al. (2) and differs from the revised system proposed by Sakurai et al. (25). Exon numbering refers to total (untranslated and translated) exons and corresponds to exons G and H described by Sakurai et al.
‡ Sporadic cases are defined as those in which both parents were tested and each found to have two normal alleles.

(codons 443 → 458) that changes the carboxy-terminal amino acids from 443 to 456 and increases the polypeptide chain length by two amino acids. The ladders in Fig. 1 *C* are sense and antisense from case P-V1. The antisense ladder has unusual appearance with duplicated bands at multiple positions. In contrast, the sense ladder shows a normal appearance except for the first few bands at the top of the sequence. The sequences shown in Fig. 1 *C* could only be compatible with a base insertion and/or deletion in only one allele and in a position close to the 3′ end of the template. Fig. 1 *D* clearly shows, by comparison with the wild-type sequencing ladder, that a single base insertion is the actual genetic lesion. As this mutation did not result in gain or loss of restriction sites, its existence was verified by ASPCR. Amplification of exon 10 was obtained from genomic DNA of this patient but not the DNA of unaffected members when an antisense primer in which the 3′ end base matching the mutant allele was used. Conversely, no amplification was obtained when the same set of primers was used to amplify wild-type DNA (results not shown).

*Overview of the mutations found in exons 9 and 10.* Table II summarizes the characteristics of the mutations found in exons 9 and 10 of c-erbAβ gene from thyroid hormone–resistant patients. The frequency of mutations in exons 9 and 10, four and three, respectively, suggests that there is not a preference for either one. Similar frequency distribution was also obtained among sporadic and familial cases. The mutations found were one single base insertion and six base substitutions, four of them transitions and two transversions. Moreover, the finding that 50% of the base substitutions were G → A transitions suggests that spontaneous deamination of methylated cytosine (35) is the most common mutagenic mechanism in this gene. All these mutations are different from the three previously described, although in the same two regions of the ligand binding domain (15–17). The mutation in kindred Q-W, in nucleotide 1642, affects codon 448 in which a mutation has been reported (14); however, the substituted nucleotide is not the same and therefore the encoded amino acid is also different, $Pro^{448}$ → $Thr^{448}$ instead of $Pro^{448}$ → $Gln^{448}$. Similarly, the mutation in G-S affects the same codon (340) as did the other previously reported mutation (16), but also differs in the base changed and the replacement amino acid. It is interesting that of the mutations detected to date, two pairs are in the same codons, one in exon 9 and one in 10.

*Topographical distribution of the mutations in the c-erbAβ gene.* Fig. 2 is a schematic representation of the c-erbAβ gene structure. The ligand binding domain spans the major part of the last four exons. The boundaries for the different functional subdomains are drawn to scale using data from Forman and Samuels (23). The mutations appear not to be distributed at random but rather in two distinct clusters, one in the 5′ end of the dimerization subdomain and the 3′ end of the $tau_i$ transcriptional subdomain, and the other in the L2 ligand-binding subdomain. The latter is presumably one of the two major ligand contact zones (22), located close to the 3′ end of the gene (Fig. 2).

*Strategies to verify each mutation and for rapid screening of family members.* In all instances alternative procedures have been used to verify the sequencing results and to simplify the screening for carriers of mutant alleles. Whenever a gain or loss of a restriction site was caused by a mutation, restriction enzyme analysis of PCR-amplified exons was the method of choice for confirmation and screening of other members of the same kindred. This method is convenient and accurate, and does not involve the preparation of probes and/or the use of radioactivity. Fig. 3, *A* and *B*, shows the results obtained in cases of familial and sporadic inheritance. The mutation N-N should result in the gain of a new MnlI site. Fig. 3 *A* shows that

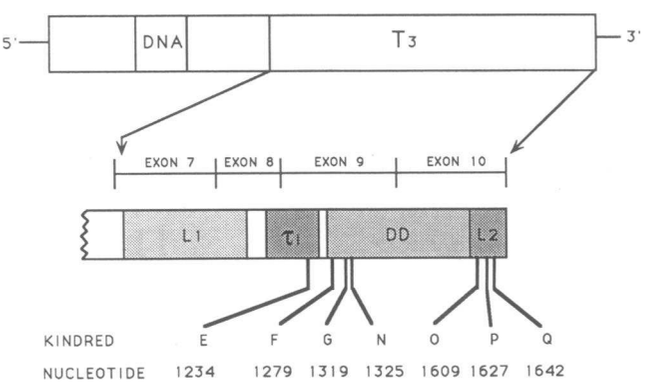


*Figure 2.* Schematic representation of the c-erbAβ gene. The T3 domain and its corresponding subdomains have been drawn according to scale to represent the topographical distribution of mutations (see Table II and Discussion).

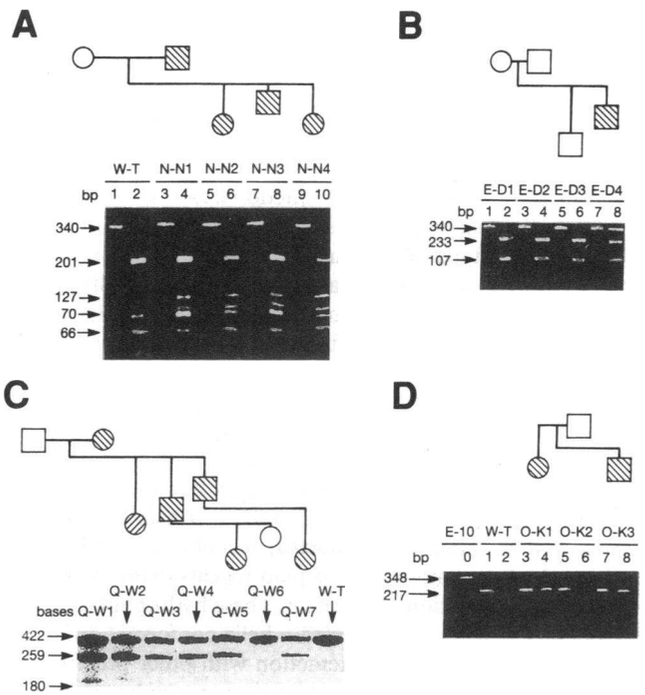


*Figure 3.* (*A*) MnlI digestion of PCR-amplified exon-9 of c-erbA$\beta$ gene. Experimental; details are described in Methods. W-T stands for wild-type allele, N-N1 to N-N4 (*striped symbols*) are all affected members of the same kindred showing a G to A transition at nucleotide 1325 leading to the gain of a MnlI restriction site. Lanes with odd numbers are the undigested controls. In the mutants the 201-bp fragment is split in two fragments of 127 and 74 bp. For the sake of clarity, only the 127-bp fragment is indicated with an arrow. (*B*) BstUI digestion of exon-9 of c-erbA$\beta$. A G to A transition at nucleotide 1234 results in the lost of a BstUI restriction site. Due to the heterozygosity of all these patients they show both wild-type and mutant patterns of restriction fragments. The striped symbols correspond to the affected members. (*C*) Hydroxylamine treatment of heteroduplexes formed with PCR-amplified wild-type and kindred Q-W (C to A transversion at nucleotide 1642) exon-10 of c-erbA$\beta$ gene. Cleavage products are observed only in the affected members of the kindred (*striped symbols*) but not in one unaffected member (Q-W6) or the wild-type template (W-T). (*D*) Allele-specific amplification of a fragment of exon-10 of c-erbA$\beta$ gene. Genomic DNA ($\sim$ 1 $\mu$g) from each affected member of kindred O-K, who showed an A to G transition at nucleotide 1609, were amplified using the sense primer described in Methods for exon-10. The antisense primer which 3′ base is complementary to the mutant was 5′-GGGGCATTCCACCTTCAC-3′. As expected, amplification is shown only in the clinically affected members, O-K1 and O-K3 (*striped symbols*), but not in the normal O-K2, or in the wild-type control. E-10 is an internal reference of the full size exon-10.

the presence of the new restriction site was consistently detected in all the kindred members clinically affected. A case showing the opposite conditions, sporadic inheritance and loss of a unique restriction site, is shown in Fig. 3 *B*. The pattern of fragments as in the case in Fig. 3 *A*, is the result of overlapping the ladders obtained by the enzymatic digestion of both the wild-type and the mutant alleles. These observations thus provide further evidence of the heterozygosity of these cases.

ASPCR amplification (30) and/or chemical cleavage of mismatched heteroduplexes (31) were used successfully in those cases in which a mutation did not result in gain or losses of restriction sites. The chemical cleavage method proved to be more cumbersome and less reliable than ASPCR, and its use is probably more appropriate for screening purposes and gross topographical location of mutations. Fig. 3 *C* shows the pedigree of kindred Q-W and the result of the hydroxylamine action on the heteroduplexes formed with amplified exon-10 DNA from wild type and from each one of the kindred components. The heteroduplex cleaved only in the affected members (Fig. 3 *C*), indicating once again a definite correlation between the clinical syndrome of thyroid hormone resistance and the presence of a mutation in the ligand-binding domain of the c-erbA$\beta$ gene. However, we found ASPCR to be the method of choice due to its speed and technical simplicity. Fig. 3 *D* exemplifies the results obtained with this method in a kindred with familial inheritance of a mutation in exon-10 of c-erbA$\beta$. Only DNA from the clinically affected member was amplified when the 3′ end base of the antisense primer matched the mutation previously detected by sequencing.

*Table III. $T_3$-Binding Affinities of Wild-Type and Mutant c-erbA$\beta$ Receptors*

| Kindred | Ka ($M^{-1}$) | $Ka_{mutant}/Ka_{wildtype}$ |
|---|---|---|
| Wild type | $3.39 \times 10^{10}$ | 1.00 |
| E-D | $0.70 \times 10^{10}$ | 0.20 |
| O-K | $0.58 \times 10^{10}$ | 0.17 |
| P-V | $<0.17 \times 10^{10}$ | <0.05 |
| Q-W | $1.40 \times 10^{10}$ | 0.41 |

c-erbA$\beta$ receptors were prepared by in vitro cRNA translation in a reticulocyte lysate system as described in Methods. T3-binding was carried out by the nitrocellulose filter assay of Inou et al. (34). Binding affinity was determined by Scatchard plot as the mean of two experiments (WT), four experiments (E-D), two experiments (O-K and Q-W), and three experiments (P-V).

*Functional properties of four selected mutations.* In four of the seven mutations T3-binding studies were performed. These four mutations were chosen on the basis of the novelty of their topographical location (E-D and O-K), the unprecedented structural abnormality (case P-V, base insertion with frame shift in the 3′ end of the gene) or the coincidence with a mutation in the same codon of a previously described case (Q-W). Table III shows the T3-binding affinities of wild-type and mutant receptors E-D, Q-W, O-K, and P-V. All mutant receptors showed impaired binding affinity compared to the wild-type. Case P-V, who has a base insertion at position 1627 with a resulting frame shift, was the most severely affected showing no detectable binding. Although case Q-W and a previously described case A-H (15) had mutations in the same codon but their mutations resulted in a different base change, their T3-binding affinities were markedly different. Case Q-W had a two- to fivefold greater binding affinity compared to A-H.

## Discussion

Recent genetic analysis has demonstrated linkage of the syndrome of GTHR to the same locus as the c-erbA$\beta$ gene on chromosome 3 in three families (14, 15). The tight linkage

between the clinical syndrome and this gene strongly suggested that the molecular basis of this disease could be a mutation leading to quantitative and/or qualitative changes in the products of this gene. This hypothesis has been strongly supported by the elucidation of different nonconservative mutations in the ligand-binding domain (15–19), as well as large and small deletions (18, 19), of the c-erbA$\beta$ gene in unrelated kindreds with generalized thyroid hormone resistance. Our current observations of seven novel point mutations in additional kindreds with thyroid hormone resistance greatly adds to the understanding of the function of the c-erbA$\beta$ receptor. The high percentage of GTHR patients in which a mutation has been found in two specific areas of the ligand-binding domain of the c-erbA$\beta$ gene, seven kindreds described herein as well as in the three previously reported (15, 16, 17) families, is striking. Moreover, the absolute correlation of these mutations with the presence of disease in all of these kindreds supports the inference that they are the cause of the GTHR. Polymorphisms in these genes are rare (15, 16) and all of these nonconservative mutations produce major changes in the amino acids of the ligand binding domain (Table II).

In two of the three previously reported mutants in the same two subdomain regions, hormone binding studies of in vitro translated mutant receptors clearly demonstrated decreased affinity for T3 (16, 20, 36). The study of four of our cases, one from exon-9 (E-D) and three from exon-10, Q-W, O-K, and P-V, showed impairment of T3-binding to the in vitro translated receptors in all of them (Table III). No apparent correlation was observed between topographical location of the mutation and degree of T3-binding impairment. Some of these mutant receptors have been shown to inhibit the ability of a cotransfected wild type receptor to mediate the T3 stimulation of a thyroid hormone responsive promoter (36, 37, 38). These observations give further support to the hypothesis that the observed mutations in the c-erbA$\beta$ gene are the cause of the GTHR syndrome. Moreover, it will ultimately be necessary to sequence the entire coding and adjacent noncoding regions of these mutant genes to exclude entirely the possibility of additional mutations which might affect receptor expression and/or function. This is unlikely in such an autosomal dominant disease, since it has been demonstrated that single amino acid changes in these regions of the ligand-binding domain, alone, are sufficient to account quantitatively for the T3 binding abnormalities observed in patients' fibroblasts (16, 20). Thus the method of casette insertion of the respective mutations into wild-type cDNA excludes the possibility that any other unidentified mutations may have contributed to the decreased T3-binding observed in the present study.

Despite common biochemical features of thyroid hormone resistance observed in all our patients (Table I) and common localizations of mutations to the ligand-binding domain of the c-erbA$\beta$ gene, it is remarkable that there are different patterns of tissue resistance in different families as well as different clinical phenotypes. There are at least two explanations for the apparent heterogeneity of phenotypes. First, variable levels of expression of normal and mutant receptors in each individual and perhaps also in each particular tissue. Second, the particular topographical location of each mutation might lead to distinct perturbations of protein-protein and/or protein-DNA interactions which are manifest as different phenotypes. The latter suggestion is based on recent work showing that hormone-mediated transcriptional activation is more complex than previously thought and presumably involves the binding of other proteins (38–41) as well as the formation of homo- and heterooligomers with other nuclear receptors to form active protein-DNA-T3 complexes (42, 43).

*Structural organization of c-erbA$\beta$ gene.* Fig. 2 shows schematically some of the structural-functional features of this gene as well as the locations of each mutation. The c-erbA$\beta$ gene belongs to a subtype of nuclear receptors (T3, vitamin D, and retinoic acid receptors) that share the sequence Glu-Gly-Gly in the DNA binding discriminatory region located at the base of the first Zn finger (23). Recent evidence suggests the presence of at least three functional subdomains in the ligand binding domain of these receptors (23). The ligand-binding subdomains, L1 and L2 (Fig. 2), are receptor specific and therefore are presumably involved in direct contact with ligand. These subdomains are required for high affinity binding of T3 (23). The dimerization domain (Fig. 2, DD) is a highly conserved region among this group of nuclear receptors (22) with nine repeats of seven amino acids (heptad repeats) with hydrophobic residues in positions 1, 5, and 8. These hydrophobic amino acids would provide in the coiled $\alpha$-helix conformation of this polypeptide the points of interaction with other proteins, including receptors and other transcription regulatory factors (32, 40, 44).

Deletion of the carboxy-terminal region produced ligand-independent, constitutively active receptors (45, 46). This observation led to the hypothesis that the ligand-binding domain represses transactivation of transcription and the binding of ligand presumably relieves the repression. The subdomain $tau_i$ (Fig. 2) is a highly conserved region among the nuclear receptors (47, 48) that has also been implicated in the ligand-relieved expression (22, 45, 49).

*Functional implications of the topographical distribution of c-erbA$\beta$ mutations.* The mutations of the TR$\beta$ gene we have described are not distributed evenly throughout exons 9 and 10, but rather in two well defined clusters located in the amino-terminal region of the dimerization subdomain and also in the L2 subdomain. Case E-D shows a singular location within the $tau_i$ subdomain. Although the associated clinical features vary, inappropriately elevated TSH levels and indicators of peripheral resistance to exogenous and endogenous hormone are common to all kindred and are evidence of an impaired capacity of all of the mutant receptors in vivo to inhibit expression of the TSH$\beta$ gene and to regulate the expression of other thyroid-hormone responsive genes. Additionally, each of the four mutations tested for T3-binding showed a decreased affinity for its ligand (Table III). For mutations clustered in exon 10, this was not a surprise, particularly in view of the precise changes identified. The mutation in case O-K substitutes $Met^{437}$ with valine. $Met^{437}$ is a highly conserved residue among nuclear receptors of this group and site-directed mutagenesis of this residue has been shown to result in a non-functional T3 receptor (50). The case P-V, a C insertion with frame-shift, produces a change in most of the 14 carboxy-terminal amino acids. Deletions of this region have impaired or abolished T3 binding (22, 36). The previously described mutant receptor (kindred A-H) in which histidine is substituted for $Pro^{448}$ displays a five- to tenfold reduction in the T3 binding affinity (20, 36). Likewise, mutant Q-W, in which threonine replaces $Pro^{448}$, also demonstrates impaired T3 binding although to a lesser degree. The up to

fivefold difference in the T3-binding affinity between A-H and Q-W probably indicates the relative importance of the amino acid substituted as well as its topographical location. Of note is the fivefold decrease in T3 binding affinity of mutant E-D (Table III), located in the $tau_i$ dimerization subdomain. This finding implies that this subdomain contains determinants of ligand-binding affinity as well as for ligand-relieved repression and is consistent with earlier data indicating that amino acids essential for normal T3-receptor interaction (19, 50, 51) lie outside of the subdomains, L1 and L2, required for ligand binding as defined by deletion mutagenesis of chicken c-erbA (21, 22). Similarly, the currently described mutation of $Gly^{340}$ is also likely to decrease T3 binding as previously reported for another mutation of this codon (20).

The absence of mutations among our cases in areas of exon 9 and 10 outside the clusters may indicate that alterations of these regions of the protein produce phenotypes not recognizable as generalized thyroid hormone resistance. Alternatively, such alterations may result in no detectable functional abnormality. If the resistance phenotype were to reflect solely the decreased functionality of receptors acting in isolation, this would suggest that relatively few individual amino acids outside the clusters make a substantial contribution to this functionality. A more interesting possibility is suggested by the observation that the protein products of mutant alleles may inhibit the function of the normal product (or of other proteins) with which they interact in a "dominant negative" fashion (52). This dominant negative effect has been shown in abnormal T3 receptors produced by site-directed mutagenesis (21, 51, 53) and may also underlie the inhibitory action of retroviral or non-ligand binding forms of the T3 receptor (10, 21, 54, 55). Structurally defective monomers, in which the capacity to dimerize is preserved, may form nonfunctional oligomers that inhibit the function of the normal receptor, perhaps through competition for binding to T3 response elements or for interaction with associated proteins. As described above, mutant receptors from patients with thyroid hormone resistance have also been shown to inhibit the transcriptional capacity of wild type receptors (36, 37). In light of these observations, it is possible that alterations in areas of the protein other than the clusters, even if functionally detrimental, do not preserve the dominant negative effect. Such mutations, in allowing unimpaired functioning of the normal alpha and beta receptors from other alleles, may not cause a clinically significant abnormality of the thyroid axis. We note that none of the mutations results in an amino acid change expected to alter known protein–protein interactions; none of the three mutations within the dimerization subdomain disrupts a heptad repeat and no mutation to date has been identified in the region 281-300 which is precisely homologous to a region of rat c-erbA$\beta_1$ (aa 286-305) whose deletion disrupts interaction with certain accessory proteins (56, 57). This does not exclude the possibility of abnormal interactions as a result of these mutations and, indeed, some of the phenotypic diversity among kindreds may be related to specifically altered protein interactions in this domain as a result of subtle conformational changes perturbing dimerization and/or binding of other regulatory nuclear proteins.

In summary, the diversity of mutations described herein offers a unique tool for the study of the mechanisms of thyroid hormone action. The ultimate correlation of functional as well as physicochemical properties of different mutant T3 receptors with clinical and biochemical features in various kindreds should yield new insights into the complex interactions involved in regulation of gene transcription.

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