A Base Mutation of the C-erbAβ Thyroid Hormone Receptor in a Kindred with Generalized Thyroid Hormone Resistance
Molecular Heterogeneity in Two Other Kindreds

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Abstract

Generalized thyroid hormone resistance (GTHR) is a disorder of thyroid hormone action that we have previously shown to be tightly linked to one of the two thyroid hormone receptor genes, c-erbAβ, in a single kindred, A. We now show that in two other kindreds, B and D, with differing phenotypes, there is also linkage between c-erbAβ and GTHR. The combined maximum logarithm of the odds score for all three kindreds at a recombination fraction of 0 was 5.77. In vivo studies had shown a triiodothyronine (T₃)-binding affinity abnormality in nuclear receptors of kindred A, and we therefore investigated the defect in c-erbAβ in this kindred by sequencing a major portion of the T₃-binding domain in the 3'-region of fibroblast c-erbAβ cDNA and leukocyte c-erbAβ genomic DNA. A base substitution, cytosine to adenine, was found at cDNA position 1643 which altered the proline codon at position 448 to a histidine. By allelic-specific hybridization, this base substitution was found in only one allele of seven affected members, and not found in 10 unaffected members of kindred A, as expected for a dominant disease. Also, this altered base was not found in kindreds B or D, or in 92 random c-erbAβ alleles. These results and the fact that the mutation is predicted to alter the secondary structure of the crucial T₃-binding domain of the c-erbAβ receptor suggest this mutation is an excellent candidate for the genetic cause of GTHR in kindred A. Different mutations in the c-erbAβ gene are likely responsible for the variant phenotypes of thyroid hormone resistance in kindreds B and D. (*J. Clin. Invest.* 1990. 85:93–100.) linkage • candidate mutation • proline codon 448 • histidine • triiodothyronine-binding domain

Introduction

Generalized thyroid hormone resistance (GTHR)[1] refers to a syndrome characterized by elevated circulating levels of free thyroid hormones, resistance to thyroid hormone action, and inappropriately normal or elevated levels of thyroid-stimulat-

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1. *Abbreviations used in this paper:* GTHR, generalized thyroid hormone resistance; RFLP, restriction fragment length polymorphism.

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ing hormone (1). Refractoriness to thyroid hormones was first noted by Refetoff et al. (2) in two siblings with stippling of the epiphyses characteristic of juvenile hypothyroidism, short stature, and multiple somatic abnormalities. The mode of transmission in the original kindred was unclear; however, it now appears that, in most patients with GTHR, the syndrome segregates as an autosomal dominant disorder, the patients have no somatic abnormalities, and the degree of tissue refractoriness to thyroid hormone action is variable among different kindreds (3). In one variant of thyroid hormone resistance, selective pituitary resistance (4, 5) the pituitary gland is more refractory than peripheral tissues to thyroid hormones, and hyperthyroidism results from abnormal control of thyrotropin-secreting hormone (TSH) secretion. A single case of selective peripheral but not pituitary resistance has been found (6).

It is generally accepted that thyroid hormones regulate gene function through interactions with a nuclear receptor (7, 8). Thus, several workers have performed studies of labeled triiodothyronine (T₃) binding to nuclei from fibroblasts or lymphocytes in patients with GTHR (9). Although certain workers using these methods have reported small abnormalities of T₃-binding affinity or number, most of these reports showed no binding abnormalities (9). Subsequently, it was shown that kinetic methods of analysis might be more sensitive than equilibrium methods in defining T₃-binding abnormalities (9). Recently, new methods of salt extraction of fibroblast nuclear receptors have suggested abnormal T₃-binding affinities or capacities in certain patients with thyroid hormone resistance (10, 11). However, these extraction methods are relatively cumbersome and imprecise, and it has not been possible with such technology to establish unequivocally that any patients with GTHR have abnormalities of the nuclear receptor for T₃.

A major advance enabling further insights into thyroid hormone action was made when two genes, c-erbAα (chromosome 17) and c-erbAβ (chromosome 3), were identified that encode proteins with properties of a T₃ receptor (12, 13). Although the native T₃ receptor has not yet been fully characterized, the c-erbAβ cDNA cloned human placenta (c-erbAβ1) and the c-erbAβα cDNA (α1) cloned from embryonal chicken tissue have been used to synthesize proteins that bind T₃ with high affinity, and with the specificity of a T₃ receptor. There are multiple-receptor forms or isotypes encoded by these α and β genes. The c-erbAα1 mRNA has been shown in vitro to encode a protein which binds T₃ with high affinity and is expressed in a wide range of tissues (14, 15). In particular, it is found in high abundance in specific regions of the rat central nervous system (16). An alternatively spliced form, c-erbAα2, is not a T₃ receptor, but can bind to thyroid hormone–respon-

sive elements in genes in vitro and may have a role in modulating T_3-regulated expression (17–20). The $\alpha 2$ message is more abundant than $\alpha 1$ in the rat brain (16). In addition to the original c-erbAβ cDNA cloned from human placenta, another β-isoform, c-erbA$\beta 2$, has been identified which appears to be expressed only in the rat anterior pituitary gland (21). However, no specific in vivo function has yet been determined for any of these receptor forms and their relative physiologic role in humans is not known.

We have shown in one kindred, A, that the gene for GTHR maps to the same region as c-erbAβ (22). Recent studies have shown that there is a single human c-erbAβ gene (Bradley, W. E. C., Institut du Cancer, Montreal, Canada, personal communication). The tight linkage between a gene that codes for a thyroid hormone receptor and a syndrome with abnormal thyroid hormone action strongly suggests that the syndrome of GTHR in kindred A results from a mutation in c-erbAβ, and that c-erbAβ has important in vivo functions in diverse human tissues.

In the present study, we present partial cDNA and genomic sequence data from kindred A and identify a base substitution at the COOH terminus of c-erbAβ which is not a polymorphism. This variant sequence can be used to identify unequivocally future affected members of kindred A, thereby enabling rapid diagnosis. Although it has not been proved to be the cause of the syndrome in kindred A, it is an excellent candidate mutation for the genetic etiology of abnormal c-erbAβ function. We have also shown linkage of c-erbAβ to GTHR in two additional kindreds. These kindreds, although similar in having features of pituitary and peripheral resistance to thyroid hormones, have distinct features presumably related to differences in tissue regulation by thyroid hormones. Both kindreds lack the mutation seen in kindred A. These new linkage data underscore that there is a crucial role for c-erbAβ in humans and suggest that differing mutations of c-erbAβ may be responsible for differing patterns of resistance to thyroid hormone action.

Methods

Clinical studies. Clinical data on members of kindreds A, B, and D were obtained during hospitalizations at the Clinical Center of the National Institutes of Health. All patients were entered into Clinical Center protocols and gave informed consent. Serum thyroxine (T_4), free thyroxine (FT_4), T_3, and TSH were measured at the Clinical Center and corroborated by Hazelton Biotechnologies (Vienna, VA). The clinical criteria used to assess thyroid hormone action in target tissues in kindreds A, B, and D have been previously defined (3). To summarize, the following scale is used: SR = severe resistance; R = resistance to thyroid hormones. Bone, R = adult height < 5 percentile or bone age retarded > 2 SD, and different from unaffected members; liver, SR = sex hormone-binding globulin (SHBG) within the lower third of the normal range (male < 0.35 μg/dl, female < 0.70 μg/dl) or cholesterol > 300 mg/dl, R = SHBG within the upper two-thirds of the normal range, or cholesterol > 240 mg/dl but < 300 mg/dl; brain, SR = verbal or full-scale IQ, or Kaufman Assessment/Wechsler Intelligence scales in children < 80, R = history of inadequate school performance that necessitated formal special education, or verbal or full-scale IQ < 86 but > 80, or presence of subtle abnormalities of spatial relationships; heart, SR = sleeping pulse < 80, R = sleeping pulse > 80 but < 90. Pulse wave arrival time (QK_d) measurements were also performed at the Clinical Center to assess cardiac resistance and correlated with the sleeping pulses (hypothyroid range was > 250 ms, hyperthyroid range < 150 ms [23]); body metabolism, R = BMR < 120%; Pituitary, R = basal TSH inappropriately normal

and maximal value > 15 mU/liter after thyrotropin-releasing hormone stimulation.

Genetic studies. DNA samples from kindreds B and D were prepared from leukocytes as described for the original kindred A (22). The c-erbAβ cDNA probe (Phe A12) was used to identify restriction fragment length polymorphisms (RFLPs) with BamHI and EcoRV at the c-erbAβ locus as previously described (22). Linkage analysis in kindreds B and D were performed using the program LIPED with the assumptions of complete penetrance, a gene frequency of 10^{-5}, and equal male and female recombination rates (24).

Isolation and characterization of c-erbAβ sequences from kindred A. Total RNA specimens from pituitary fibroblast cultures of members of kindred A were prepared by lysis in guanidine isothiocyanate and ultracentrifugation through a cesium chloride gradient (25). Reverse transcription of these specimens was according to a previously published method (26). The antisense primer (AS1) used for first-stand synthesis was specific for the noncoding region of c-erbAβ cDNA and had a thymine substitution to produce an EcoRI site for subcloning purposes: 5'-GGAATTATAGGAAGGAATTCAGTCAGT-3' (nucleotides 1672–1698, Fig. 1). The cDNAs were phenol/chloroform extracted, precipitated in ethanol, resuspended in 10 mM Tris, 1 mM EDTA, and the 335-bp sequence in c-erbAβ shown in Fig. 1 amplified. The sense primer (S1) for cDNA amplification had adenine and cytosine substitutions to create a BamHI site: 5'-AAAAA-TGGGGATCCTGGGGTGGTGTCA-3' (nucleotides 1309–1336, Fig. 1). The polymerase chain reaction mixture was according to specifications in the GeneAmp DNA amplification reagent kit (Perkin Elmer Cetus, Norwalk, CT) and amplification was done with the Perkin Elmer thermal cycler. The cycle parameters were as follows: denaturation 94°C for 30 s, annealing 55°C for 30 s, and extension 72°C for 2 min, 30–40 cycles. The amplified c-erbAβ cDNAs were not visible on ethidium bromide staining, but easily detectable on Southern blots probed with phe A12. Amplified cDNAs were run on a 3% NuSieve GTG gel (FMC, Rockland, ME), the appropriate region of the gel was excised, and the cDNAs were subcloned into the BamHI/EcoRI site of pGEM3Z (Promega Biotec, Madison, WI). Six independent cDNA clones from an affected member of kindred A (J.H.) containing cDNA homologous to c-erbAβ were isolated. DNA sequencing was per-
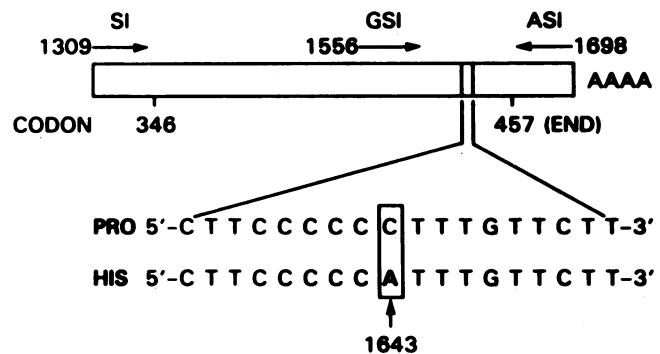

Figure 1. Schematic representation of the 3'-end of c-erbAβ cDNA with the locations of the primers used for cDNA and genomic amplification and of the Pro and His sequences in kindred A. The c-erbAβ cDNA sequence represented is from nucleotides 1309–1698 according to coordinates for human placental c-erbAβ cDNA (3). Sequence bounded by codons 346–457 was amplified in kindred A by reverse transcription-polymerase chain reaction methodology using primers S1 and AS1 (see Methods). Primers GS1 and AS1 were used in polymerase chain reactions to amplify genomic sequences in kindreds A, B, and D and in random subjects. Pro and His are oligomers used in allelic-specific hybridization (Methods). The Pro sequence encompassing codons 445–451 is the wild-type sequence for c-erbAβ. The His sequence contains a single nucleotide substitution, C → A, at position 1643 found in kindred A c-erbAβ (see Results).

formed on one clone using the K/RT Universal Sequencing System (Promega Biotec), and the adenine sequence of the other cDNA clones was rapidly determined by single-lane sequence analysis (26).

Amplification of the kindred A genomic sequence was done in the c-erbAβ region shown in Fig. 1. The 5' primer (GS1) was chosen to avoid an intron and had a PstI site constructed: 5'-AGGTGACAGAT-CTGCAGATGATAGGAG-3' (nucleotides 1556–1582, Fig. 1). Several genomic clones from affected members J.H. and B.H. were isolated and sequenced.

Protein modeling of the human placental and the kindred A c-erbAβ forms was done by Garnier structural analysis (PCGENE Software, Intelligenetics, Inc., Mountainview, CA).

Allelic-specific hybridization. The presence of a single nucleotide change, C to A, at position 1643 (coordinates according to reference 3) in genomic sequences of kindreds A, B, and D, and of random subjects was done using an 18mer, Pro, as probe for the wild-type sequence and another 18mer, His, as probe for the C to A substitution (Fig. 1). Samples of 0.5–1.0 μg of genomic DNA from affected members of all three kindreds and from 46 random individuals (i.e., 92 random c-erbAβ alleles) were used in the polymerase chain reaction to amplify the genomic sequence shown in Fig. 1. All amplifications were verified by visualization of the 143-bp band on ethidium bromide-stained gels. The amplified gemonic DNA samples were separated on 2% NuSieve/1% agarose minigels and transferred under alkaline conditions to Nytran membranes (27). These Southern blots were prehybridized at 37°C in 5× SSPE, 5× Denhardt's, 0.5% SDS, for 1 h, and hybridized with 1×10^6 cpm/ml of end-labeled His or Pro oligomers. The blots were washed twice for 5 min each at room temperature in 2× SSPE, 0.5% SDS, and then for 10 min at 55°C in 5× SSPE, 0.5% SDS. Most of the random allelic sequences were additionally checked for hybridization to the His oligomer using a slot-blot method that has been previously described for allelic specific hybridization (28).

Results

Phenotype of thyroid hormone resistance kindreds. The levels of thyroid hormones and TSH for kindreds A, B, and D are shown in Table I. These kindreds all demonstrated inappropriately normal or elevated TSH with high levels of thyroid hormones. In addition to pituitary resistance to thyroid hormones, these kindreds also displayed varying patterns of target organ resistance to the action of thyroid hormones. Table I compares thyroid hormone action in selected target tissues of members from kindred D with that in kindreds A and B which have been reported previously (3). A striking feature of kindred A, which contrasts with the other two kindreds, is the association of short stature and the resistant state (29). Affected members of kindred D showed marked cognitive deficits on IQ testing (average full-scale IQ = 75±6, $n = 5$) compared with an unaffected sibling and other unaffected member (average IQ = 91±0, $n = 2$). Affected members of kindred A also have modest cognitive deficits (3) as well as a "hyperactivity" syndrome that has greatly impaired school performance. Such a hyperactivity state has also been observed in members of the B and D kindreds to a lesser extent. To summarize the clinical features, all three kindreds had similar levels of elevated thyroid hormones and TSH, but were heterogeneous in terms of abnormalities of thyroid hormone action in peripheral tissues.

Linkage between GTHR and c-erbAβ. We have previously reported that the gene for the syndrome of GTHR in kindred A was tightly linked to c-erbAβ (22). RFLPs observed with BamHI and EcoRV restriction endonucleases and a c-erbAβ cDNA probe cosegregated with the GTHR trait. The relationship between c-erbAβ and the thyroid hormone resistance syndromes of kindreds B and D was also tested using linkage analysis. Linkage studies with the c-erbAα locus could not be readily accomplished since no high-frequency RFLPs were found using a c-erbAα cDNA probe and screening 10 normal subjects with 35 different restriction endonucleases. However, c-erbAβ was linked to GTHR in both kindred B and kindred D. Nine members of kindred B were fully informative with the BamHI RFLP alone (Fig. 2). Eight members of kindred D were informative when haplotyped with the combination of

Table I. Laboratory and Clinical Features of Kindreds with Generalized Thyroid Hormone Resistance*

Kindred[‡]	Status	T₄	Free T₄	T₃	TSH	Tissue Responsiveness[§]					
						Bone	Brain	Liver	Heart	Metabolism	Pituitary
		μg/dl	ng/dl	ng/dl	mU/liter						
A	Affected (n = 6)	20.6±3.1	4.6±0.7	247±46	3.8±2.3	R	SR	R	v	R	R
	Unaffect. (n = 7)	7.2±2.0	1.1±0.3	143±33	2.6±0.9						
B	Affected (n = 3)	18.6±4.0	2.7±0.2	247±45	3.4±1.2	−	v	R	SR	R	R
	Unaffect. (n = 6)	6.8±1.1	1.1±0.2	150±33	2.3±1.6						
D	Affected (n = 5)	17.2±2.0	3.3±0.5	234±52	3.4±1.4	−	SR	R	SR	R	R
	Unaffect. (n = 4)	8.2±1.7	1.3±0.1	156±26	3.2±1.4						
Normal range:		5.0–10.0	1.0–1.9	89–166	0.5–4.6						

* Values are mean±SD. Patients with prior thyroidectomy, on thyroid hormone supplementation, or with biochemical evidence of autoimmune thyroiditis have been excluded from this table. ‡ Partial clinical data from certain members of kindreds A and B have been reported previously (3). § Symbols used in this table (see Methods for a description of the rating system): −, minimal or no resistance or abnormality; R, moderate resistance or abnormality; SR, severe resistance or abnormality; v, variable resistance.
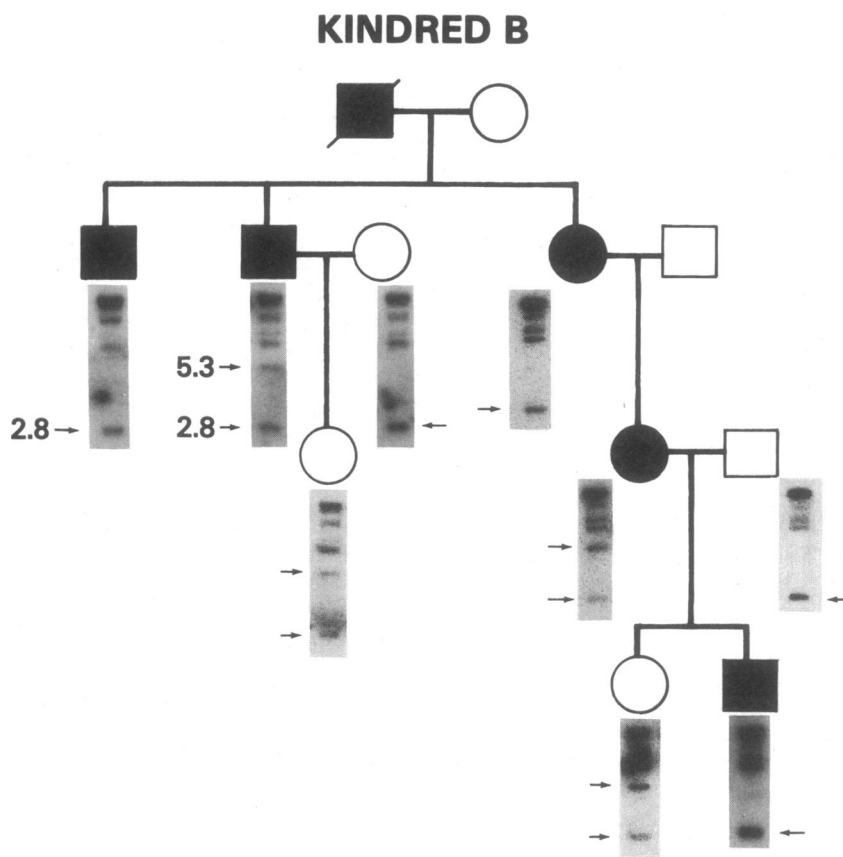
KINDRED B

Figure 2. Segregation of BamHI RFLP with GTHR in families from kindred B. Affected members are indicated with solid symbols. Squares represent males and circles represent females. The BamHI RFLP consists of a band at 5.3 or 2.8 kb denoted with arrows. Constant bands occur at 23, 21, 13, and 7.0 kb. In this kindred, GTHR segregates with the 2.8-kb band and the BamHI haplotyping is fully informative. This segregation can be seen as follows. If the c-erbAβ RFLP and GTHR are linked, the second generation demonstrates that the mutant or disease allele must be marked by a 2.8-kb band, since there are affected members in this generation with both alleles marked by the 2.8-kb band. This hypothesis is tested in the subsequent generations. The unaffected female in generation III could only have received the 5.3-kb RFLP from her father and this is consistent with linkage. The affected daughter in generation III has 5.3- and 2.8-kb RFLPs; her allele marked by the 2.8-kb RFLP segregates with the affected male in generation IV (consistent with linkage), and her allele marked by the 5.3-kb RFLP segregates with the unaffected female (consistent with linkage). The probability of this segregation of the BamHI RFLP of c-erbAβ with GTHR by chance alone (i.e., without linkage between GTHR and c-erbAβ) is 1/14.29. The probability of the segregation of the c-erbAβ RFLPs with GTHR in all three kindreds (A, B, D) by chance alone is $\sim 1/600,000$ (see Fig. 3 and Table II).

the two RFLPs (Fig. 3). Table II shows that the maximum logarithm of the odds scores between the GTHR and c-erbAβ loci were 3.91, 1.15, and 0.71 at a recombination fraction of 0 for kindreds A, B, and D, respectively. The summed logarithm of the odds score between GTHR and the c-erbAβ gene was 5.77, which means there is an $\sim 1/600,000$ probability that this cosegregation of c-erbAβ and GTHR occurred through chance. The summed logarithm of the odds score tests the hypothesis that GTHR is caused in general by defects in c-erbAβ. Other recent linkage data also support this hypothesis (30). The linkage of c-erbAβ and GTHR in these three kindreds strongly suggests that c-erbAβ is an important thyroid hormone receptor in vivo and that genetic defects of c-erbAβ are responsible for thyroid hormone resistance in various tissues. Furthermore, the phenotypic variation among these kindreds raises the possibility that there may be differing genetic abnormalities of c-erbAβ, qualitative or quantitative, resulting in GTHR.

Variant sequence of c-erbAβ in kindred A. The genetic abnormality of c-erbAβ in kindred A was investigated using a modification of the polymerase chain reaction (26, 31). We focused on the COOH terminus sequence because it is crucial for the T_3-binding property of the c-erbA proteins (32, 33) and previous Scatchard analysis of T_3 binding from salt-extracted fibroblast nuclear receptors showed a significant reduction in T_3-binding affinity in an affected member (B.H.) compared with that in normal subjects (11). B.H. had nuclear receptors with a T_3-affinity of 0.91×10^{10} M^{-1}, capacity of 33 fmol of T_3/mg of protein compared to controls with T_3 affinities of
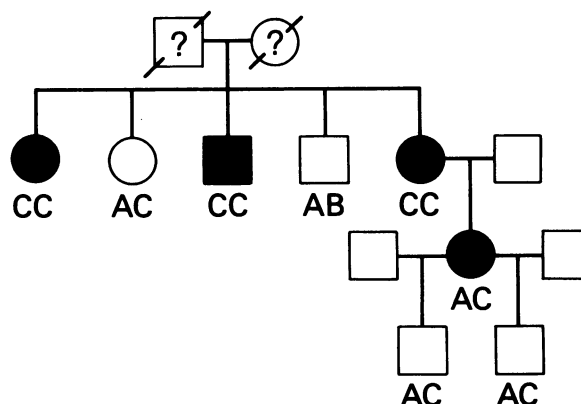
KINDRED D

Figure 3. Haplotyping of kindred D using BamHI and EcoRV RFLPs at the c-erbAβ locus, showing linkage between GTHR and c-erbAβ. Symbols represented are as described in Fig. 2. The first generation parents are dead and the affected one is unknown. Both BamHI and EcoRV haplotypes were required to be informative and demonstrate linkage between GTHR and c-erbAβ. The EcoRV RFLP is a band at 3.3 or 1.6 kb and examples have been previously published (22). The haplotype nomenclature is A (BamHI 5.3-kb, EcoRV 1.6-kb bands), B (BamHI 2.8-kb, EcoRV 3.3-kb bands), and C (BamHI 2.8-kb, EcoRV 1.6-kb bands) (18). The D haplotype (BamHI 5.3-kb, EcoRV 3.3-kb bands) was not seen in kindreds A, B, and D.

Table II. Pairwise Logarithm of the Odds Scores for Linkage between GTHR and C-erbAβ

Kindred	Recombination fraction				
	0.0	0.1	0.2	0.3	0.4
A	3.91	3.27	2.59	1.76	0.89
B	1.15	0.92	0.67	0.40	0.15
D	0.71	0.55	0.41	0.28	0.15
Sum	5.77	4.74	3.67	2.44	1.19

$2.5-8.0 \times 10^{10}$ M^{-1} and similar capacity (11). Total RNA from cultured fibroblasts of an affected member (J.H.) was used to synthesize cDNA and amplify a 335-bp segment of the T$_3$-binding domain of c-erbAβ bounded by amino acid codons 346 and the stop codon at position 456 (Fig. 1). A fibroblast c-erbAβ cDNA from J.H. was isolated and showed four nucleotide differences compared with the published human placental c-erbAβ cDNA (12): an adenine at 1380, a thymine at 1636, an adenine at 1643, and a thymine at 1651. The corresponding amino acid codons are CTA (Leu 360), TTC (Phe 446), CAT (His 448), and TTG (Leu 451). The codons for human placental c-erAβ at these positions are Leu, Leu, Pro, and Leu, respectively. The PHE condon at 446 has been reported in rat c-erbAβ cDNA (34, 35) and in the homologous positions in human and rat c-erbAα1 cDNAs (14, 15). However, only a cytosine at position 1643 and a proline codon at position 448 have been reported for these c-erbA cDNAs. Five additional independent c-erbAβ-kindred A partial cDNA clones from the same reverse transcription-polymerase chain reaction were isolated, and all had adenine at position 1643 visualized on single-lane sequencing (data not shown). Although we did not find two populations of cDNA as anticipated, the cDNA that was isolated did have an interesting nonconservative amino acid substitution.

In order to establish the authenticity of the adenine at 1643, and to determine if it was associated with the abnormal c-erbAβ-allele in kindred A, the genomic sequence of c-erbAβ in kindred A was analyzed. The intron-exon structure of c-erbAβ is not known, but we suspected it was similar to that of c-erbAα for which there is information on the 3' end (18). An 89-bp gemonic sequence containing the putative adenine substitution in one allele from two affected members of kindred A (J.H., B.H.) was amplified by the polymerase chain reaction (Fig. 1). Several independent gemonic clones were sequenced and two alleles could be distinguished. There were two populations of genomic clones in approximately 1:1 proportion, one containing cytosine at position 1643 (Pro codon 448) and one containing the adenine substitution (His codon). Thymine was found at positions 1636 and 1651 in both alleles. We next demonstrated that the His allele is the mutant allele in kindred A by testing all 17 members of kindred A for adenine 1643 using allelic-specific hybridization. Fig. 4 demonstrates that the adenine-1643 substitution was found in only one of the two alleles in the genomic sequence of all seven affected members of kindred A, but was not present in 10 unaffected members. This analysis demonstrated that the adenine 1643 marks the mutant allele.

Finally, we examined whether the adenine-1643 substitution was associated with GTHR in kindreds B and D which
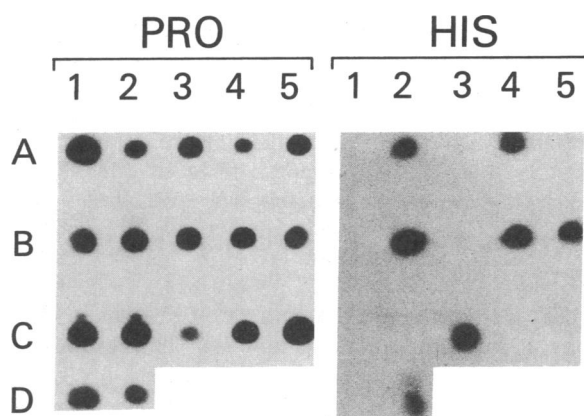

Figure 4. Allelic-specific hybridization of Kindred A genomic sequences with PRO and HIS oligomers. Southern blots are shown of amplified genomic sequence (base pairs 1556–1698, band size 143 bp) from all members of kindred A hybridized to Pro or His oligomers (see Methods) as indicated. Pro probes for the wild-type sequence and HIS probes for the mutant sequence. Affected and unaffected members of kindred A have at least one c-erbAβ allele containing the wild-type (Pro) sequence. Only affected members of kindred A, A2, A4, B2, B4, B5, C3, and D2, carry a c-erbAβ allele with the nucleotide substitution C → A at position 1643. (Here the letters A–D and numbers 1–5 are purely coordinates and do not correspond to kindreds or pedigrees.)

have different phenotypes, and whether it could be considered a variant sequence. Allelic-selective hybridization (Fig. 5) showed that affected members from kindred B and kindred D did not contain this sequence. In addition, 92 random c-erbAβ-alleles were similarly analyzed by amplification/allelic-selective hybridization and none hybridized to the HIS probe (data not shown). These data show that the adenine-1643 substitution, which generates a His codon, is not a polymorphism.

Discussion

Attempts to demonstrate alterations of T$_3$ nuclear receptors in patients with GTHR using binding studies have generated ambiguous results, although more recent experiments with salt-extracted nuclear receptors indicated defects in T$_3$-binding affinity or receptor number among some, but not all, kindreds
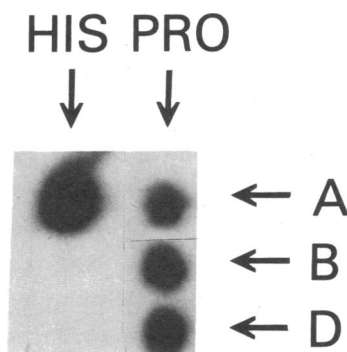

Figure 5. Allelic-specific hybridization using genomic sequences from affected members of kindreds A, B, and D. The Southern blot of genomic sequences was done as described for Fig. 4, except genomic specimens from kindreds A, B, and D were used. Affected members from kindreds B and D have at least one c-erbAβ allele with the wild-type Pro sequence, but do not have the variant His sequence for c-erbAβ.

(9–11). However, recent advances in molecular genetics have provided powerful new tools to address this problem and have enabled us to identify more directly possible receptor abnormalities in patients with GTHR.

There are two putative thyroid hormone receptor genes, c-erbAβ at 3p22 → 3p24.1 (36) and c-erbAα at 17q11.2 → 17q21 (37). Only one β-gene is present when the human placental c-erbAβ cDNA is mapped in human gemonic clones (Bradley, W. E. C., personal communication). Consequently, the finding on linkage analysis of a lod score of 5.77 between GTHR and c-erbAβ in three kindreds and identification of a mutation in c-erbAβ in kindred A show that abnormalities in c-erbAβ cause abnormal thyroid hormone action in diverse human tissues. A c-erbAβ abnormality is linked with impaired thyroid hormone action with regard to general metabolism, liver, and pituitary affects in the three kindreds. The importance of a β-type receptor, c-erbAβ2, in the pituitary has been postulated on the basis of distribution of thyroid hormone receptor mRNAs in rat (21). The His-448 mutation, the likely genetic defect in kindred A, is in a region shared by β1 and β2 isoforms and could theoretically disrupt thyroid hormone regulation mediated by either. The dependency in liver on c-erbAβ for thyroid hormone action is in concordance with the relative abundance in liver of c-erbAβ mRNAs compared with c-erbAα mRNAs (17).

C-erbAβ function in bone, brain, and heart appears to be more complex. In kindreds B and D abnormal β-receptors appear to be responsible for blunting thyroid hormone stimulation of heart rate, but this was not a constant feature in kindred A. Kindreds A and D had remarkable cognitive deficits, and affected members of kindred A had short stature associated with c-erbAβ. Overall, c-erbAβ defects seem to be able to modify thyroid hormone responsiveness and/or result in clinical pathology in brain, heart, and in linear growth. Different c-erbAβ mutations might result in different "dominant negative" effects in tissues (22, 38). The estrogen and glucocorticoid receptors, members of the steroid/thyroid hormone receptor superfamily, undergo ligand-induced dimerization, and the dimers bind cognate DNA sequences (39, 40). One can speculate that if a similar structure exists for the thyroid hormone receptor, a mutant c-erbAβ protein might form a heterodimer with the wild-type β-receptor and impair receptor activation of transcription. This model is implicated by recent studies with genetically engineered c-erbAα mutants with deletions of the DNA-binding domain which act in a dominant negative mode on endogenous and transfected wild-type c-erbA receptors (41). It has also been shown that c-erbA proteins that lack a functional T$_3$-binding domain can inhibit thyroid hormone action. Rat c-erbAα2, which does not bind T$_3$, can inhibit T$_3$ responses generated by rat c-erbAβ1 or c-erbAα1 (19, 20). In addition, v-erbA can behave as a dominant negative inhibitor when coexpressed with its progenitor, the c-erbAα gene (42). It is plausible that a point mutation in the wild-type receptor that disrupts the T$_3$-binding domain would have a dominant negative function through either dimerization or repressor models (41, 42).

The regulatory mechanism could be further convoluted if α- and β-receptors form active heterodimeric complexes. In vitro experiments indicate that both α- and β-receptors can separately activate transcription, to somewhat different degrees, from an identical thyroid hormone-responsive promoter

in a hormone-dependent manner (43). A mutant β-receptor, β_d, might form complexes $\beta_d\beta$, $\beta_d\beta_d$, and $\alpha\beta_d$, and thereby alter steady-state numbers of possible wild-type forms $\beta\beta$, $\alpha\beta$, and $\alpha\alpha$. Specific sets of these receptor dimers might form as a function of tissue, and with a variable β_d dependent on kindred, have different gene-activating capabilities and account for the heterogeneity of tissue resistance to thyroid hormones within a particular kindred and among kindreds. Elucidation of c-erbAβ mutations anticipated in different kindreds with GTHR will enable this hypothesis to be tested.

The mutant β-allele in kindred A is expressed in fibroblasts as demonstrated by the isolation of a β-type cDNA with a variant sequence. Only the sequence difference, A-1643, is a mutation altering the predicted protein sequence of c-erbAβ in kindred A. The bases A-1380 and T-1651 did not alter the amino acid codons predicted from the human placental c-erbAβ cDNA. The base T-1636 changed the amino acid codon to Phe which has been reported for the homologous positions in rat c-erbAβ1 and chicken and human c-erbAα1 receptors (13, 15, 34, 35). Most importantly, genomic sequences of random alleles contained bases T-1651 and T-1636 (data not shown), indicating they are wild-type bases and that a Phe-446 amino acid is the predicted wild-type residue. The base substitution at position 1643 marks the mutant allele in kindred A since it segregates with all affected members of kindred A, who were also shown to carry a wild-type allele by allelic specific hybridization.

Our data do not definitely prove that this variant sequence, resulting in a Pro-448 to His-448 codon alteration, is the mutation in c-erbAβ responsible for GTHR in kindred A. However, it is certainly a likely candidate since it shows absolute linkage with the abnormal phenotype and has not been observed in 92 random individuals. Moreover, other data on the functional domains on the thyroid hormone receptors show that the COOH terminus is crucial for high-affinity T$_3$ binding (32, 33), and binding studies indicate abnormal T$_3$ affinity for the kindred A receptor (11). Munoz et al. (32) have shown that a recombinant c-erbAα1 receptor with a point mutation and a nine amino acid deletion in the COOH terminus which includes the PRO codon we have identified, diminishes T$_3$ affinity 22-fold compared with the nonmutated α-construct (32). Proline is a residue which cannot hydrogen bond to form an α-helix or β-sheet conformation (44) and the proline-proline sequence at the COOH terminus of the receptor may result in a unique conformation. The predicted secondary structures of wild-type c-erbAβ and c-erbAβ-His-448 by the method of Garnier show that c-erbAβ-His-448 is more likely to form a stretch of α-helices from amino acid positions 412–456 compared to the wild-type receptor with a Pro-Pro sequence at codons 447 and 448 (data not shown). Definitive proof that His 448 is the sole mutation responsible for the syndrome of GTHR in kindred A will require further genomic and cDNA sequence information, as well as functional studies to define the T$_3$-binding properties of the mutant receptor. However, functional studies alone may not be definitive; for example, in vitro studies of T$_3$ binding of the receptor would not reflect possible in vivo steps such as posttransitional processing and association with other transcription factors.

The elucidation of the HIS-448 variant codon in kindred A has immediate clinical applications. It has often been difficult in kindred A and other thyroid hormone-resistant families to

make the diagnosis of thyroid hormone resistance in neonates and young children on the basis of thyroid hormone levels and TSH. Allelic-specific hybridization as shown above can be used to identify affected members in kindred A prenatally or neonatally. This may have therapeutic implications if clinical trials demonstrate amelioration of symptoms such as hyperactivity, mental retardation, and short stature by early treatment with T_3 or T_4. Elucidation of the genetic defect is the prerequisite for considering future gene therapy. Characterization of genomic c-erbAβ and the corresponding cDNA in Kindreds B and D is presently being undertaken to identify similar variant sequences that can be used in diagnosis of the syndrome within these kindreds.

Although a point mutation was demonstrated in the T_3-binding domain of c-erbAβ in kindred A, it is likely that this syndrome will be associated with a variety of molecular defects. Complete androgen insensitivity syndrome, an analogous clinical disorder of peripheral target tissue resistance to hormone action, is caused by deletion of the steroid-binding domain of the androgen receptor gene in one family (45). Regions other than the hormone-binding domain may be mutated in receptors of the steroid/vitamin D/thyroid hormone receptor superfamily and result in insensitivity to hormonal control. Point mutations in the DNA-binding/zinc-finger domain of the vitamin D receptor have been shown to cause hypocalcemic vitamin D-resistance rickets (46). Finally, since abnormalities in receptor number and not T_3 affinity have been characterized in certain thyroid hormone-resistant families, it is possible that mutations in the 5'-flanking regulatory region or in splice junctions as well as those producing premature termination of the coding region may be responsible for certain resistance syndromes.

The selective pituitary form of thyroid hormone resistance (4) may also be caused by a c-erbAβ abnormality. It is interesting to speculate that since the β2-isotype is apparently pituitary-specific and differs from c-erbAβ1 at the NH$_2$ terminus (21), a genetic defect in this domain may be responsible for selective pituitary resistance. C-erbAα has not yet been linked to generalized thyroid hormone resistance; its functions in humans may be different than those for the c-erbAβ receptor. Alternatively, its functions might be similar but so crucial (e.g., for brain development) that mutations in that gene are less commonly observed. It is clear that further study of various patients and families with generalized and selective pituitary thyroid hormone resistance will provide new insights into the complex mechanisms of thyroid hormone action in humans.

Acknowledgments

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