Tight Linkage between the Syndrome of Generalized Thyroid Hormone Resistance and the Human c-erbAβ Gene

S. J. Usala, A. E. Bale, N. Gesundheit, C. Weinberger, R. W. Lash, F. E. Wondisford, O. W. McBride, and B. D. Weintraub

National Institute of Diabetes and Digestive and Kidney Diseases
(S.J.U., N.G., R.W.L., F.E.W., B.D.W.)
National Cancer Institute (A.E.B., O.W.M.)
National Institute of Mental Health (C.W.)
National Institutes of Health
Bethesda, Maryland 20892

Multiple cDNAs belonging to the c-erbA gene family encode proteins that bind T_3 with high affinity. However, the biological functions of these multiple thyroid hormone receptors have not yet been clarified. Generalized thyroid hormone resistance (GTHR) refers to a human syndrome characterized by tissue refractoriness to the action of thyroid hormones; several studies have suggested quantitative or qualitative defects in T_3 binding to nuclear receptors in certain kindreds. To investigate the biological functions of the c-erbA genes, c-erbAα and c-erbAβ, we tested the hypothesis that an abnormal c-erbA gene product is present in GTHR by examining these genes in members of one kindred. Restriction enzyme analysis failed to identify an abnormal pattern in affected individuals suggesting no rearrangements or large deletions. However, we demonstrated that the gene conferring the GTHR phenotype is tightly linked to the c-erbAβ locus on chromosome 3. This linkage strongly suggests that the c-erbAβ gene is important in man as a thyroid hormone receptor and identifies a putative c-erbAβ mutant phenotype with central nervous system, pituitary, liver, metabolic, and growth abnormalities. (Molecular Endocrinology 2: 1217–1220, 1988)

INTRODUCTION

Thyroid hormone action is mediated through the binding of hormone-nuclear receptor complexes to promoter regions of specific genes (1). Recently, two distinct c-erbA cDNAs, isolated from human placental and chicken embryonal libraries, were shown in *in vitro* translation studies to code for proteins that bound T_3 with high affinity and with the specificity expected for a nuclear T_3 receptor (2, 3). The corresponding genes, c-erbAβ and c-erbAα, have been localized to chromosome 3p21-25 (4) and chromosome 17q11.2-21 (5), respectively. Subsequently, a c-erbA cDNA, type α1, was isolated from a rat brain library and found to be highly homologous to the chicken c-erbAα (6). A second c-erbAα cDNA, type α2, was cloned from human testis (7) and kidney (8) libraries, mapped to chromosome 17 (8), and may result from alternative splicing of the same c-erbAα gene (8). However, the particular biological functions of these multiple thyroid hormone receptors have not yet been established. Multiply-sized c-erbAβ and c-erbAα mRNAs have been reported in several tissues and cell lines, and their relative abundance in various tissues has not been clearly elucidated (2, 6–9). Moreover, one recent study showing differences in antibody recognition of *in vivo* T_3 nuclear receptors and *in vitro* translated products of human c-erbAβ suggested that the c-erbAβ protein may not function as an *in vivo* nuclear T_3 receptor (10). Controversy exists, therefore, as to which, if any, of these c-erbA proteins function as physiological thyroid hormone receptors.

Generalized thyroid hormone resistance (GTHR) 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-stimulating hormone (11, 12). Although in the first reported cases the mode of transmission was unclear (13), it now appears that in the majority of cases the syndrome segregates as an autosomal dominant disorder (12). In a less common variant of this syndrome, selective pituitary thyroid hormone resistance, the pituitary gland is more refractory than peripheral tissues to thyroid hormones, and hyperthyroidism results from impaired feedback on TSH secretion (14); an autosomal dominant inheritance pattern has been observed in one kindred (15). The molecular basis for these disorders is heterogeneous and in some patients

0888-8809/88/1217-1220$02.00/0
Molecular Endocrinology

probably secondary to receptor abnormalities (16). Defects in nuclear receptor number or affinity as well as impaired nuclear uptake of T_3 have been demonstrated in certain cases (17, 18).

In order to explore more fully the biological functions of the c-erbAα and c-erbAβ genes we have studied their role in a kindred with generalized thyroid hormone resistance where defective thyroid hormone action is known. It was postulated that if a c-erbA gene product is a functional thyroid hormone receptor, then a relationship might exist between the gene and the GTHR syndrome. We used the candidate gene approach (19) and show that restriction fragment length polymorphisms (RFLPs) of the c-erbAβ gene are tightly linked to the gene for the clinical phenotype of GTHR.

RESULTS AND DISCUSSION

We examined the c-erbAβ and c-erbAα genes in kindred A (12) with GTHR where a significant decrease in T_3-binding affinity of salt-extracted fibroblast nuclear receptors was demonstrated in an affected member compared to that in normal subjects (0.9×10^{10} M^{-1} compared to normal range of $2.5–8.0 \times 10^{10}$, Weintraub, B. D., *et al.*, manuscript submitted). Restriction analysis with *Bgl*I, *Bgl*II, *Eco*RI, *Bam*HI, *Pst*I, *Hinf*I, and *Eco*RV, using the human c-erbAβ cDNA probe (*Materials and Methods*), and *Hind*III, *Eco*RI, and *Pst*I, using a full-length human c-erbAα1 cDNA probe (Weinberger, C., unpublished data), failed to identify any abnormal bands on Southern blots. This indicated there were no detectable chromosomal rearrangements or large deletions in these c-erbA genes in this kindred (data not shown).

As an alternative method of establishing a relationship between the c-erbA genes and the GTHR trait we performed linkage analysis. Because there existed *in vitro* evidence for a c-erbAβ receptor DNA binding site which mediated thyroid hormone action (20) we first studied linkage between GTHR and c-erbAβ. Seventeen family members of kindred A were typed for polymorphisms of the c-erbAβ gene detected with *Bam*HI and *Eco*RV [Table 1; (21)]. Both RFLPs cosegregated with the GTHR trait (Fig. 1), and sixteen members were informative when haplotyped using the combination of the two RFLPs (fig. 2). Linkage analysis was performed using the program LIPED (22), with the assumptions of complete penetrance, a gene frequency of 10^{-5}, and equal male and female recombination rates. Table 2 shows the maximum lod score (logarithm of the odds) between the GTHR and c-erbAβ loci was 3.91 at a recombination fraction of 0 (absolute linkage). This

Table 1. Haplotype Frequencies for RFLPs at the c-erbAβ Locus

Haplotype	*Bam*HI Fragment (Kb)	*Eco*RV Fragment (Kb)	Frequency (n = 68)
A	5.3	1.6	0.26
B	2.8	3.3	0.21
C	2.8	1.6	0.53
D	5.3	3.3	Not observed

Kb, Kilobase.

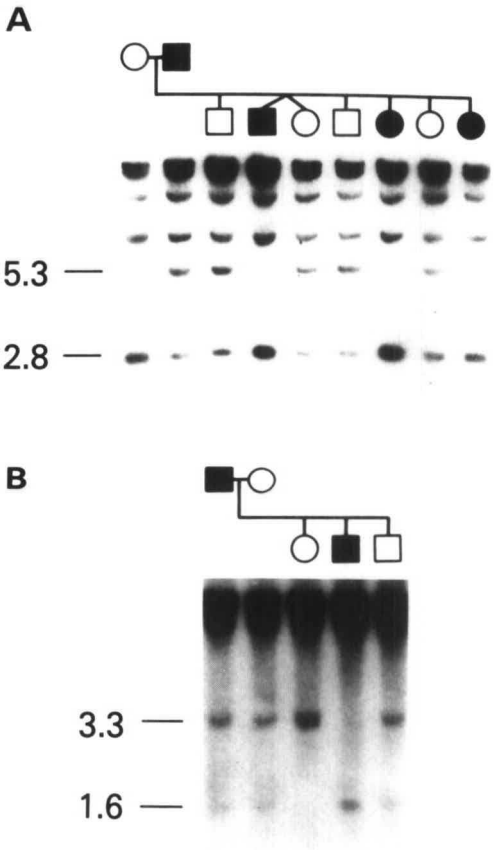

Fig. 1. Segregation of *Bam*HI and *Eco*RV RFLPs with GTHR in Families from Kindred A

A, Segregation of the *Bam*HI RFLP with GTHR in a nuclear family of kindred A. Affected members are indicated with *solid symbols*. *Squares* represent males; *circles* represent females. The *Bam*HI RFLP consists of a band at 5.3 kilobases or 2.8 kilobases. Note that in the affected father one c-erbAβ allele is marked by a 5.3 kilobase *Bam*HI band (RFLP) and one c-erbAβ allele is marked by a 2.8 kilobase *Bam*HI band (RFLP), whereas in the mother both c-erbAβ alleles are marked by the 2.8 kilobase *Bam*HI band. All the children are informative in that paternal c-erbAβ alleles, using *Bam*HI RFLPs, can be identified. All unaffected children carry the father's 5.3 kilobase *Bam*HI RFLP and all affected children carry his 2.8 kilobase *Bam*HI RFLP. The father's c-erbAβ allele marked by the 2.8 kilobase *Bam*HI RFLP segregates with GTHR in this nuclear family. Constant bands occur at 23, 21, 13, and 7.0 kilobases. B, Segregation of the *Eco*RV RFLP with GTHR in a portion of kindred A. The *Eco*RV RFLP consists of a band at 3.3 or 1.6 kilobases. Note the affected father's c-erbAβ allele marked by the 1.6 kilobase *Eco*RV band segregates with GTHR and the 3.3 kilobase *Eco*RV RFLP segregates with the normal phenotype. The child in lane 5 is not informative since it is uncertain which paternal RFLP he received. Constant bands appear at 15 and 8.9 kilobases. An additional polymorphism with a band at 13.5 kb or 10.5 kb was uninformative in kindred A. The segregation of the *Bam*HI or *Eco*RV RFLPs with GTHR in the other members of kindred A that were haplotyped can be followed in Fig. 2.

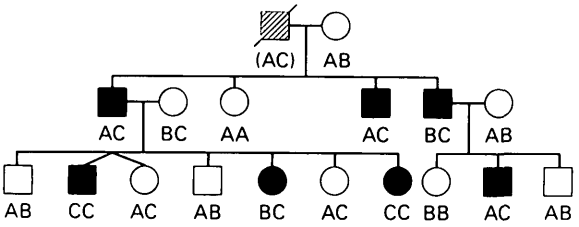

Fig. 2. Haplotyping of kindred A using the *Bam*HI and *Eco*RV restriction fragment length polymorphisms at the c-erbAβ locus

All individuals were screened for thyroid hormone resistance by measurement of serum T_4, free T_4, T_3, TSH, and free α-subunit, and through metabolic testing (12). The first generation affected male was identified by history. The daughter in lane 8, Fig. 1A, was erroneously assigned as affected in Ref. 12 because of limited data showing borderline elevated serum T_4 and T_3. She was reclassified as unaffected before this study based on several additional studies showing normal thyroid hormone and metabolic parameters.

Table 2. Pairwise LOD Scores for Linkage between GTHR and c-erbAβ

	Recombination Fraction				
	0.0	0.1	0.2	0.3	0.4
Kindred A	3.91	3.27	2.59	1.76	0.89

score means there is an approximately 1/10,000 probability of the segregation we observed occurring through chance. A lod score of 3.0 or greater is accepted as significant evidence for linkage (23). These data indicate that the gene for GTHR is located close to or is identical with c-erbAβ on chromosome 3. Although this type of study lacks the resolution necessary to prove identity between the GTHR gene and c-erbAβ, the close relationship between a gene that codes for a high affinity thyroid hormone binding protein and a syndrome with abnormal thyroid hormone action strongly suggests: 1) the c-erbAβ gene codes for a physiological thyroid hormone receptor; 2) the syndrome of GTHR in kindred A results from a mutation in the c-erbAβ gene.

A mutation in the c-erbAβ gene in kindred A appears to result in abnormalities of thyroid hormone action in many tissues (12). In addition to inappropriate secretion of TSH from the pituitary, affected members of kindred A demonstrated delayed bone age and short stature. Most also displayed mild mental retardation, diminished or inappropriately normal basal metabolic rates, and hepatic resistance to thyroid hormone (relatively low sex hormone binding globulin and relatively high cholesterol).

Thyroid hormone resistance is clinically heterogeneous, and the disorder in some kindreds may be caused by mutations in the c-erbAα gene. It will be interesting to examine whether the gene for selective pituitary resistance to thyroid hormones, in which defective thyroid hormone action is restricted to the pituitary (14), is linked to the c-erbAα gene, which is expressed in relatively high concentrations in the pituitary gland (6). In addition, since a single patient with GTHR can show subtle variability in the degree of resistance from tissue to tissue (12), it is possible that there are tissue-specific distributions of different thyroid hormone receptors.

Work is in progress to clone and sequence the c-erbAβ genes of kindred A. It is possible that the putative c-erbAβ mutant allele is not expressed, and that the resistance to thyroid hormones results solely from a diminished number of c-erbAβ receptors. However, there is a 3- to 5-fold reduction in the T_3-binding affinity of the mutant receptor from salt-extracted fibroblasts in kindred A, and the Scatchard plot appeared linear rather than curvilinear as expected for two markedly different affinity components (Weintraub, B. D., *et al.*, manuscript submitted). These observations may mean that the abnormal c-erbAβ gene is expressed and inhibits the function of the wild type gene product, thus acting as a dominant negative mutation (24). Studies with the estrogen receptor, another c-erbA related protein, have demonstrated that estradiol binding induces a homodimer that shows increased affinity for estradiol (25, 26). If a similar structure exists for the thyroid hormone receptor, the mutant c-erbA gene may produce an inhibitory subunit that in a heterodimer diminishes T_3-binding affinity and impairs receptor activation. Further characterization of the kindred A mutant c-erbAβ gene should provide a novel way to study mechanisms of thyroid hormone action in man.

MATERIALS AND METHODS

DNA was prepared from human leukocytes as follows: 10–20 ml blood were centrifuged at 400 × *g* for 5 min and the supernatant was gently removed. The cell pellet was resuspended in 5 vol lysis buffer (0.15 M ammonium chloride, 0.01 M potassium bicarbonate, and 0.1 mM EDTA) and incubated 10–15 min on ice until the solution appeared black. The lysate was centrifuged at 400 × *g* for 5 min, the supernatant was removed, and the pellet was resuspended in 20 ml lysis buffer. After 5 min on ice the suspension was centrifuged at 400 × *g* for 5 min and the supernatant was removed. The pellet was quickly resuspended in 2–4 ml 10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$; and an equal volume of 1% sodium dodecyl sulfate (SDS), 0.6 M NaCl, 20 mM Tris, pH 7.4, 10 mM EDTA, 400 μg/ml proteinase K, was immediately added. This proteinase K digestion was incubated at room temperature overnight, and then extracted twice with phenol, twice with phenol-chloroform-isoamyl alcohol and twice with chloroform-isoamyl alcohol (24:1). High molecular weight DNA was then obtained by ethanol precipitation and dissolved over 3–4 days in TE at 4 C to a concentration of 0.5–1 μg/μl.

DNA (4–6 μg) was digested with either *Bam*HI or *Eco*RV at 37 C for 3 h and fractionated by electrophoresis on 0.8% agarose. Gels were soaked for 30 min in 0.4 M NaOH, 0.6 M NaCl and the DNA was transferred overnight to GeneScreenPlus (New England Nuclear, Boston, MA) using the solution in a capillary blot procedure (27). Blots were neutralized in 0.5 M Tris, pH 7, 1 M NaCl for 15 min. The c-erbAβ 1.5 kb cDNA probe (phe A12, Ref. 2) was labeled by random priming (Pharmacia oligolabeling kit, Piscataway, NJ) to a specific activity of 1×10^9 cpm/μg using 5000 Ci/mmol (α-^{32}P) dCTP (New England Nuclear) and hybridized to the blots overnight at a concentration of 1 ng/ml at 42 C in 50% formamide, 10% dextran sulfate, 1% SDS, and 1 M NaCl. The blots were washed twice in 2× SSC (0.3 M sodium chloride,

0.03 M sodium citrate) for 5 min at 25 C, twice in 2× SSC with 1% SDS for 30 min at 65 C, and twice in 0.1× SSC for 30 min at 25 C.

Acknowledgments

Received August 9, 1988. Accepted August 17, 1988.

Address requests for reprints to: Stephen J. Usala, National Institutes of Health, Building 10, Room 8D14, Bethesda, Maryland 20892.

REFERENCES

1. Samuels HH, Forman BM, Horowitz ZD, Ye ZS 1988 Regulation of gene expression by thyroid hormone. J Clin Invest 81:957–967
2. Weinberger C, Thompson CC, Ong ES, Lebo R, Gruol DJ, Evans RM 1986 The c-erbA gene encodes a thyroid hormone receptor. Nature 324:641–646
3. Sap J, Munoz A, Damm K, Goldberg Y, Ghysdael J, Leutz A, Beug H, Vennstrom B 1986 The c-erbA protein is a high-affinity receptor for thyroid hormone. Nature 324:635–640
4. Gareau JL, Houle B, Leduc F, Bradley WEC, Dobrovic A 1988 A frequent *Hind*III RFLP on chromosome 3p21-25 detected by a genomic c-erbAβ sequence. Nucleic Acids Res 16:1223
5. Sheer D, Sheppard DM, LeBeau M, Rowley JD, San Roman C, Solomon E 1985 Localization of the oncogene c-erbA1 immediately proximal to the acute promyelocytic leukemia breakpoint on chromosome 17. Ann Hum Genet 49:167–171
6. Thompson CC, Weinberger C, Lebo R, Evans RM 1987 Identification of a novel thyroid hormone receptor expressed in the mammalian central nervous system. Science 237:1610–1614
7. Benbrook D, Pfahl M 1987 A novel thyroid hormone receptor encoded by a cDNA clone from a human testis library. Science 238:788–791
8. Nakai A, Seino S, Akihiro S, Szilak I, Bell GI, DeGroot LJ 1988 Characterization of a thyroid hormone receptor expressed in human kidney and other tissues. Proc Natl Acad Sci USA 85:2781–2785
9. Lazar MA, Chin WW 1988 Regulation of two c-erbA messenger ribonucleic acids in rat GH_3 cells by thyroid hormone. Mol Endocrinol 2:479–484
10. Freake HC, Santos A, Goldberg Y, Ghysdael J, Oppenheimer JH 1988 Differences in antibody recognition of T_3 nuclear receptors and *in vitro* translated products of c-erbA indicate these proteins are not identical. Clin Res 336:552A (Abstract)
11. Refetoff S 1982 Syndromes of thyroid hormone resistance. Am J Physiol 243:E88–E98
12. Magner JA, Petrick P, Menezes-Ferreira MM, Stelling M, Weintraub BD 1986 Familial generalized resistance to thyroid hormones: report of three kindreds and correlation of patterns of affected tissues with the binding of [^{125}I] triiodothyronine to fibroblast nuclei. J Endocrinol Invest 9:459–469
13. Refetoff S, DeWind LT, DeGroot LJ 1967 Familial syndrome combining deaf-mutism, shippled epiphyses, goiter and abnormally high PBI: possible target organ refractoriness to thyroid hormone. J Clin Endocrinol Metab 27:279–294
14. Gershengorn MC, Weintraub BD 1975 Thyrotropin-induced hyperthyroidism caused by selective pituitary resistance to thyroid hormone: a new syndrome of "inappropriate secretion of TSH." J Clin Invest 56:633–642
15. Rosler A, Litvin Y, Hage C, Gross J, Cerasi E 1982 Familial hyperthyroidism due to inappropriate thyrotropin secretion successfully treated with triiodothyronine. J Clin Endocrinol Metab 54:76–82
16. Eil C, Fein HG, Smith TJ, Furlanetto RW, Bourgeois M, Stelling MW, Weintraub BD 1982 Nuclear binding of [^{125}I] triiodothyronine in dispersed cultured skin fibroblasts from patients with resistance to thyroid hormone. J Clin Endocrinol Metab 55:502–510
17. Menezes-Ferreira MM, Eil C, Wortsman J, Weintraub BD 1984 Decreased nuclear uptake of [^{125}I]-triiodo-L-thyronine in fibroblasts from patients with peripheral thyroid hormone resistance. J Clin Endocrinol Metab 59:1081–1087
18. Ichikawa KI, Hughes IA, Horwitz AL, DeGroot LJ 1987 Characterization of nuclear thyroid hormone receptors of cultured skin fibroblasts from patients with resistance to thyroid hormone. Metabolism 36:392–399
19. McKusick VA 1988 The Morbid Anatomy of the Human Genome. Howard Hughes Medical Institute, Bethesda, MD, p 160
20. Glass CK, Franco R, Weinberger C, Albert VR, Evans RM, Rosenfeld MG 1987 A c-erbA binding site in rat growth hormone gene mediates trans-activation by thyroid hormone. Nature 329:738–741
21. Bale AE, Usala SJ, Weinberger C, Weintraub BD, McBride OW, A cDNA probe from the hc-erbAc-erbAβ gene on chromosome 3 detects a high frequency RFLP. Nucleic Acids Res, in press
22. Ott J 1976 A computer program for linkage analysis of general human pedigrees. Am J Hum Genet 28:528–529
23. Morton NE 1955 Sequential tests for the detection of linkage. Am J Hum Genet 7:277–318
24. Herskowitz I 1987 Functional inactivation of gene by dominant negative mutations. Nature 329:219–222
25. Notides AC, Lerner N, Hamilton DE 1981 Positive cooperativity of the estrogen receptor. Proc Natl Acad Sci USA 78:4926–4930
26. Gordon MS, Notides AC 1986 Computer modeling of estradiol interactions with the estrogen receptor. J Steroid Biochem 25:177–181
27. Chomchynski P, Qasba PK 1984 Alkaline transfer of DNA to plastic membrane. Biochem Biophys Res Commun 122:340–344