Partial deficiency of Thyroid transcription factor 1 produces predominantly neurological defects in humans and mice

Joachim Pohlenz,1,2 Alexandra Dumitrescu,2,3 Dorothee Zundel,4 Ursula Martiné,1 Winfried Schönberger,1 Eugene Koo,2 Roy E. Weiss,2 Ronald N. Cohen,2 Shioko Kimura,5 and Samuel Refetoff2,3,6

1Children’s Hospital of Johannes Gutenberg University of Mainz, Mainz, Germany
2Department of Medicine, and
3Committee on Genetics, The University of Chicago, Chicago, Illinois, USA
4Sozialpädiatrisches Zentrum, Bad Kreuznach, Germany
5Laboratory of Metabolism, National Cancer Institute, Bethesda, Maryland, USA
6Department of Pediatrics, The University of Chicago, Chicago, Illinois, USA

Address correspondence to: Samuel Refetoff, University of Chicago, MC3090, 5841 S. Maryland Avenue, Chicago, Illinois 60637, USA.
Phone: (773) 702-6939; Fax: (773) 702-6940; E-mail: refetoff@medicine.bsd.uchicago.edu.

Received for publication September 11, 2001, and accepted in revised form January 8, 2002.

Three genes, TTF1, TTF2, and PAX8, involved in thyroid gland development and migration have been identified. Yet systematic screening for defects in these genes in thyroid dysgenesis gave essentially negative results. In particular, no TTF1 gene defects were found in 76 individuals with thyroid dysgenesis even though a deletion of this gene in the mouse results in thyroid and lung agenesis and defective diencephalon. We report a 6-year-old boy with predominant dyskinesia, neonatal respiratory distress, and mild hyperthyrotropinemia. One allele of his TTF1 gene had a guanidine inserted into codon 86 producing a nonsense protein of 407, rather than 371, amino acids. The mutant TTF1 did not bind to its canonical cis-element or transactivate a reporter gene driven by the thyroglobulin promoter, a natural target of TTF1. Failure of the mutant TTF1 to interfere with binding and transactivation functions of the wild-type TTF1 suggested that the syndrome was caused by haploinsufficiency. This was confirmed in mice heterozygous for Ttf1 gene deletion, heretofore considered to be normal. Compared with wild-type littermates, Ttf1+/– mice had poor coordination and a significant elevation of serum thyrotropin. Therefore, haploinsufficiency of the TTF1 gene results in a predominantly neurologically phenotype and secondary hyperthyrotropinemia.

This article was published online in advance of the print edition.
The date of publication is available from the JCI website, http://www.jci.org.

Introduction
Congenital hypothyroidism occurs in 1:3,500–1:4,000 newborns (1). Serum thyrotropin (TSH) levels are elevated in the majority of cases, and, therefore, congenital hypothyroidism can be detected by neonatal screening programs. This allows for early initiation of thyroid hormone replacement to avert irreversible neurological and psychomotor damage. In 80–85% of the cases, congenital hypothyroidism is due to developmental abnormalities of the thyroid gland leading to thyroid dysgenesis (22–42% agenesis, 35–42% ectopia, and 24–36% hypoplasia) (2). In the remaining 15–20%, congenital hypothyroidism is associated with a goiter, is often familial, and is usually due to dyshormonogenesis, a defect in one of the well-characterized biochemical processes involved in thyroid hormone synthesis (1). While the genetic causes of most forms of thyroid dyshormonogenesis have been identified, little is known regarding the cause of thyroid dysgenesis.

Three genes encoding transcription factors have a major role in the development of the thyroid gland. These are PAX8, TTF1, and TTF2, whose functions have been recognized mainly through observations made in knockout mice.

PAX8 is a transcription factor belonging to a protein family that binds DNA via the paired domain (3). Expressed in the kidney and thyroid, it is implicated together with TTF1 in the tissue-specific expression of thyroglobulin and thyroperoxidase. In humans, PAX8 maps to 2q12–q14 and consists of 11 exons. Mice lacking the Pax8 locus have no thyroid follicular cells and, if left untreated, die before 3 weeks of age (4). Two families and two isolated cases were found to have nonsense and missense mutations in one allele of the PAX8 gene, resulting in hypothyroidism associated with thyroid hypoplasia and, in one case, ectopy (5, 6). TTF2, also known as TITF2 or FKHL15, belongs to the family of proteins that bind DNA through a “forkhead domain” (7). It is encoded by a single exon in human 9q22 (8). Mice lacking the Ttf2 locus (9) and two brothers homozygous for a missense mutation (A65V) within the forkhead domain (10) show similar findings: severe hypothyroidism due to completely absent or sublingual thyroid gland and cleft palate.

TTF1, also called TITF1, T/EBP, and NKX2.1, is the prototype of a subfamily of transcription factors containing a homeobox domain. Ttf1-null mice lack thyroid glands, lungs, and pituitary glands and have defects in the
ventral forebrain (11). The human gene, located in 14q13, is encoded by three exons and is transcribed from several start sites (12). The most abundant gene product, also found in the thyroid, is generated from an open reading frame contained in exons 2 and 3 and encoding 371 amino acids (12–14). TTF1 has multiple binding sites in the promoters and distant enhancers of the thyroglobulin and thyroid peroxidase genes and controls their expression in the adult thyroid gland (15). Two systematic surveys for defects in the TTF1 gene in subjects with thyroid dysgenesis yielded negative results (16, 17). However, one case with “compensated hypothyroidism” (high TSH but normal thyroxine [T4]), hypoponita, truncal ataxia, and respiratory failure had a 13–centi-Morgan deletion of chromosome 14, which included the TTF1 gene locus (18). In two siblings of another family, a large deletion of 14q13–q13.3 was associated with hyperthyrotopinemia, microcephaly, major feeding difficulties, and respiratory distress leading to the death of one of the sibs (19). In all three cases the deletion was present in only one allele but involved other genes, such as PAX9. Thus it is not clear to what extent deletion of genes other than TTF1 contributed to the observed abnormalities. This and the accompanying contribution from Grütters’s laboratory (20) describe heterozygous mutations within the TTF1 gene that cause predominantly neurological abnormalities.

Methods

Patient and other human subjects. The propositus, a 6.5-year-old boy, is the second child of a nonconsanguineous marriage. He was born at term after a normal pregnancy but required mechanical ventilation for the first 2 weeks of life. Newborn screening revealed a TSH of 20 mU/l (upper limit of normal for age - 15 mU/l) with normal free T4 and 3,3′,5-triodothyronine concentrations, which remained in the mid-normal range when measured at 1, 1.5, 8, 10, and 48 months of age. During this period of time, serum TSH levels remained elevated, ranging from 7 to 21 mU/l (normal 0.3–4.5). He had delayed development, was hypotonic, and started walking at the age of 2 years with staggering gait. Height, weight, and head circumference ranged in the 3rd to 10th percentiles. The thyroid gland was normal in size and location by ultrasound, and brain magnetic resonance imaging showed no defects. Because of persistent elevation of TSH and an increase to 33 mU/l after administration of thyrotropin releasing hormone, treatment with 50 µg L-T4 was begun at age 2.5 years. However, hypotonia worsened, and by age 4.5 years, the extrapyramidal symptoms became more apparent, manifesting choreiform hyperkinesia. Height, weight, and head circumference were at the 25th percentile. A brother, older by 2 years, and both parents are healthy and do not exhibit any of the abnormalities observed in the propositus.

Blood samples were obtained from the propositus, his 8.5-year-old brother, and their parents. DNA was collected from 50 unrelated individuals from the same ethnic background as controls. Studies carried out in humans were approved by the Institutional Review Boards of the Universities of Mainz and Chicago, and all individuals, or parents of minors, gave informed consent.

Mice. Animals were wild-type (+/+, 12 mice) and heterozygotes (+/−, 22 mice) for inactivated TfⅠ gene by targeted gene disruption, as described (11). They were of both sexes (19 female and 15 male), ranged in age from 8 to 14 weeks, and were littermates of six litters of heterozygous parents back-crossed 11 times into the 129/Sv strain. All animal manipulations were performed according to protocols approved by the Institutional Animal Care and Use Committees of the National Cancer Institute and the University of Chicago.

Coordination was evaluated by the Rotarod test used in mice (21), modified as follows. A motorized 36-mm-diameter wood rod was rotated at five incremental speeds of 5, 10, 15, 20, and 25 rpm. Starting at the lower speed, if the mouse could remain on top of the rod for 10 seconds by walking against the direction of the rotation, the speed was increased to the next incremental, until the mouse fell off the rod or spun around by gripping the rod. The time the mouse spent on the rod was recorded, and the mouse was given a second chance at the same rod speed. If the mouse failed again to remain on the rod for 10 seconds, the time was recorded and the average time of the two trials was retained. Mice that succeeded on the second trial were tested at the next speed as were mice that succeeded on the first trial. Scores of arbitrary relative distance were calculated, with maxima of 1, 2, 3, 4, and 5 at each of the five speeds if the mouse remained on the rod for 10 seconds. Distances at each speed were summed. All mice were tested five times over the period of 3 days. The person performing the test had no knowledge of the mouse genotypes, and it was impossible to predict their genotypes from external appearances or behavior.

Blood samples (150–200 µl) were obtained from the tail vein, under light methoxyflurane (Pitman Moore Inc., Mundelein, Illinois, USA) anesthesia. Serum was separated and used for the measurement of TSH and T4 concentrations (22). No samples were obtained during Rotarod testing.

DNA amplification and sequencing. Genomic DNA from all available family members and from 50 normal individuals was isolated from peripheral blood (QUIGEN Inc., Valencia, California, USA) after informed consent was given to participate in the study. The TTF1 gene was then amplified with primers flanking the three exons of the gene (PCR conditions and sequences of the primers will be supplied upon request). The PCR products were purified and sequenced using an automated sequencing system (A377; Applied Biosystems, Weiterstadt, Germany).

Construction of the vectors. The cDNA clone 12A2 of the wild-type TTF1, in pRC/CMV (Invitrogen Corp., Carlsbad, California, USA), was obtained from Parviz Minoo (University of Southern California, Los Angeles, California, USA) (12). It was recloned into EcoRI site of pSG5 (Stratagene, La Jolla, California, USA) after removal of 171 bp of the sequence preceding the translation start codon. The plasmid harboring
The Journal of Clinical Investigation | February 2002 | Volume 109 | Number 4

Figure 1
Identification of the mutation in the TTF1 gene and synthesis of the mutTTF1. (a) Fragments of sequence chromatograms of PCR-amplified genomic DNA from a normal individual and the patient. Sense and the corresponding antisense sequences show the insertion of a G and a C, respectively (arrows), at position 255 of the patient’s TTF1 gene (numbering beginning with the first coding nucleotide in exon 2). The mutation involves one of the two alleles producing a “double” sequence beyond the insertion site. The encoded amino acids of wild-type and mutant TTF1 genes are indicated. (b) The deduced amino acid sequence of the mutTTF1 (divergent sequence in red) compared with the wtTTF1 (in black). The homeobox and the glutamine/alanine-rich regions of the wtTTF1 are boxed and underlined, respectively. Note that these sequences are lost in the mutTTF1, which has 36 additional amino acids. (c) The products of in vitro translation, analyzed by SDS-PAGE. One microgram of plasmid DNA containing the wild-type (dog and human) TTF1 and mutTTF1 cDNAs served as template in a coupled transcription/translation reaction in rabbit reticulocyte lysate in the presence of 35S-Met. The dried gel was then radioautographed. The larger size of the mutTTF1 was confirmed. A smaller amount of a lower–molecular weight product represents a product translated from a downstream ATG, as previously described (13). Molecular weights were deduced from the amino acid sequences. Wt, wild-type.

Results and Discussion
The combination of thyroid, lung, and brain involvement in the child prompted the analysis of the TTF1 gene. The rationale for this approach was admittedly weak considering that heterozygous Ttf1 knockout mice have been reported to have no apparent defects and homozygotes are born dead (11). Our patient had only a mild increase in serum TSH ranging from 7 to 21 mU/l (normal 0.3–4.5) with normal thyroid hormone levels and respiratory distress during the first 2 weeks of life. The most prominent clinical findings were neurological, consisting of hypotonia and dyskinesia that did not respond to thyroid hormone.

Sequencing of genomic DNA, from peripheral blood leukocytes of the child, revealed a heterozygous insertion of a guanine in codon 86, producing a frameshift and a nonsense, abnormally long protein (a). The mutTTF1 contained 407 rather than the normal 371 amino acids, when translated from the first ATG in exon 2 (Figure 1b). This was confirmed by in vitro transcription and translation of the mutTTF1 (Figure 1c). His
brother and parents, who were clinically normal and had normal serum TSH concentrations, as well as 50 unrelated normal individuals, did not harbor the mutation, indicating that the observed nucleotide insertion was not polymorphic and resulted from a de novo mutation event.

In order to characterize the mechanism by which this mutation causes the disease, we examined the mutant protein for its ability to bind to a canonical TTF1 cis-element and to activate the expression of TTF1-regulated genes in vitro. As shown in Figure 2a, the mutTTF1 did not bind to the DNA sequence representing the full sequence of the high-affinity TTF1 binding site located in the rat thyroglobulin gene promoter (oligonucleotide C) (24) and containing the octamer consensus sequence for the TTF1 binding sites of rat, human, dog, and cow thyroglobulin and thyroperoxidase gene promoters (15). In addition, the mutTTF1 molecule did not interfere with the binding of the wtTTF1, when added in equal amounts (Figure 2a) and up to fourfold excess relative to the wild-type protein. The radioactivity in the shifted band produced by the wtTTF1 in the presence of an equal amount, and twice and four times excess of the mutTTF1, was, respectively, 94%, 123%, and 109%. These are expressed as percentages of the radioactivity generated with the wtTTF1 in the presence of the corresponding amounts of lysate reacted with unprogrammed vector. Furthermore, the mutTTF1 failed to transactivate the reporter driven by the thyroglobulin enhancer/promoter sequence or to interfere with the function of the wtTTF1 (Figure 2b). The lack of function of the mutTTF1 is not surprising considering the complete absence of the homeobox amino acid sequence involved in DNA binding.

Because the in vitro experiments excluded a dominant negative effect as a mechanism for expression of the phenotype in the heterozygous patient, we sought to demonstrate haploinsufficiency by examining mice heterozygous for Tfl gene deletion. Based on anatomical and morphological studies, these mice were formerly considered to be normal (11). Upon examination by a standard test for motor coordination, Tfl+/− mice had a mean ± SEM Rotarod score of 1.87 ± 0.23, compared with that of Tfl+/+ mice, 30.55 (P < 0.01) (Figure 3a). Similarly, as in the patient, the mean serum TSH concentration in Tfl+/− mice was higher (37 ± 8 mU/l) than in Tfl+/+ mice (7 ± 3 mU/l; P < 0.001) (Figure 3b), whereas the serum T4 concentration was not different (3.08 ± 0.16 vs. 3.42 ± 0.45 µg/dl, respectively) (Figure 3c). Furthermore, despite elevated serum TSH concentration, the thyroid gland size of Tfl+/− mice was not significantly different from that of Tfl+/+ mice, in absolute weight (3.71 ± 0.29 vs. 4.30 ± 0.87 g) or corrected for body weight (3.98 ± 0.17 vs. 4.62 ± 0.58 g/25g). Thus, heterozygous Tfl knockout mice exhibit hyperthyrotopinemia with normal thyroid hormone levels and no goiter, but decreased coordination, findings similar to those observed in our patient.

While we cannot exclude a mild subclinical hypothyroidism as the cause for modest elevation of serum TSH concentrations despite normal
T₁ levels, resetting of the feedback regulation of TSH due to a primary brain defect is a viable alternative explanation. The absence of goiter despite high serum TSH levels could be due to a reduced amount of TSH receptor, the expression of which is partially regulated by TTF1 (15). On the other hand, decreased stimulation by TSH-releasing hormone can produce a TSH of reduced biological activity (25). This could be caused by a hypothalamic defect and is compatible with the observation that TTF1 is directly involved in the neuroendocrine regulation of sexual maturation (26). Unpublished work shows that various regions of the brains of Ttf1<sup>−/−</sup> embryos at embryonic days 12.5–18.5 are morphologically normal at the gross level. These include the medial ganglionic eminence (anlage of the globus pallidus), hypothalamus (H. Kawano, L. Sussel, and J. Rubenstein); amygdala, hippocampus (S. Tole); cerebral cortex, thalamus (H. Kawano, L. Sussel, and J. Rubenstein); and midbrain (H. Kawano); corpus callosum, neocortex, piriform cortex, and hippocampal commissure, hippocampal commissure, callosum, hippocampal commissure, and midbrain (H. Kawano); corpus callosum, neocortex, piriform cortex, and basal ganglia (K. Ohyama). Collectively, our data show that haploinsufficiency of TTF1 causes more severe neurological disturbance than thyroid dysfunction.

Acknowledgments
We thank all family members for their participation in this study; Daniel Christophe for provision of the dog TTF1 expression vector, the original CAT reporter construct, and oligonucleotide C; Parvis Minoo and Quiping Pan (University of Southern California, Los Angeles, California, USA) for the provision of the 12A2 clone of TTF1 CDNA; Rusheng Zhang for helpful advice; and Sergio Alocer for assistance with sequencing. We are also grateful to the following investigators for providing unpublished information regarding the neuroanatomy of Ttf1<sup>−/−</sup> mouse embryos: Hitoshi Kawano (Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan), Lori Sussel (University of Colorado, Health Science Center, Denver, Colorado, USA), John Rubenstein (University of California–San Francisco, San Francisco, California, USA), Shubha Tole (Tata Institute of Fundamental Research, Mumbai, India), and Kyoji Ohyama (Krebs Institute, Sheffield, United Kingdom). This work was supported by grants from NIH (DK-15070 to S. Refetoff and DK-58258 to R.E. Weiss) and the US Public Health Service (RR-00055) and by the Tivoli Wien Katz fund.