New Mutation and Prenatal Diagnosis in Ornithine Transcarbamylase Deficiency

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SUMMARY

Ornithine transcarbamylase (OTC) (E.C.2.1.3.3) is an X-linked hepatic enzyme in the urea cycle necessary for ammonia detoxification. Deficiency of OTC results in neonatal hyperammonemia, coma, and death in childhood. Because fibroblasts do not express OTC, prenatal diagnosis in the past has required fetal liver biopsy. Using a complementary DNA (cDNA) for OTC for Southern blot analysis of genomic DNA, we have found probands with complete OTC deficiency from two unrelated families in whom the same TaqI restriction endonuclease site has been altered because of independent, but not necessarily identical, mutations in the OTC gene, suggesting that this site may be a relative hotspot for mutation at a location that is critical for normal gene function. This TaqI alteration has allowed the identification of the individual in each family in whom the mutation originated as well as the exclusion of a recurrence of OTC deficiency in a male fetus at risk for the disease. OTC deficiency joins the growing list of genetic disorders for which Southern blot analysis allows accurate heterozygote detection and prenatal diagnosis in conditions for which they were not previously available.

INTRODUCTION

Ornithine transcarbamylase (E.C.2.1.3.3) (OTC), which catalyzes the condensation of ornithine with carbamyl phosphate, is an important enzyme in the
metabolic pathway for the conversion of ammonia to urea [1, 2]. Its activity is located in mitochondria and restricted to the hepatocyte and intestinal epithelium [2]. Deficiency of OTC is the most common inborn error of ureagenesis in man, causing neonatal or infantile hyperammonemia, which frequently leads to mental retardation and death [2]. In both humans and mice, the gene encoding OTC is located on the X chromosome and undergoes random X-inactivation [3]. Females heterozygous for OTC deficiency can be asymptomatic or may be protein intolerant and suffer from episodes of hyperammonemia, mental retardation, and, occasionally, death in hyperammonemic coma [2].

We have isolated a complementary DNA (cDNA) sequence for human OTC and are using it to study the molecular basis for OTC deficiency in patients with the disorder. In two patients from unrelated families, Southern analysis has revealed the identical abnormality in the pattern of DNA fragments generated by the restriction endonuclease TaqI. We used this Southern blot alteration to follow the inheritance of OTC deficiency in both families and have identified the individual in each family in which the OTC mutation first occurred. We also have monitored a pregnancy in the mother of one of the probands by Southern analysis of amniocyte DNA and have successfully excluded a recurrence of OTC deficiency in a male fetus.

CASE REPORTS

The proband in family A (individual IV-1 in fig. 1) is a 26-month-old male of Mexican-American background originally admitted to an intensive-care unit on the fourth day of life in coma with a blood ammonia level of 1,250 µg/dl and a markedly elevated urinary orotic acid level. OTC deficiency was diagnosed on the basis of these biochemical data and a family history demonstrating X-linked inheritance of protein intolerance. The patient survived the neonatal period but was severely neurologically damaged, with marked spasticity and cortical blindness, and has attained essentially no milestones. His mother (III-4), maternal aunt (III-1), and maternal grandmother (II-3) all had a lifelong history of dietary protein intolerance. Another maternal aunt (III-2) and maternal great-grandmother (I-2) were not protein intolerant. Another maternal aunt (III-5) died in an unexplained coma during infancy; although she was thought to have Reyes syndrome, she probably had undiagnosed heterozygous OTC deficiency.

At the time of these studies, the proband’s mother became pregnant. She underwent amniocentesis for fetal sex determination and Southern blot analysis of fetal DNA. She subsequently decided to continue the pregnancy regardless of the outcome of the sex determination, which showed a 46,XY fetus. She has since given birth to a male infant unaffected with OTC deficiency.

The proband in family B (individual III-1 in fig. 2) is a now-deceased Caucasian infant who presented on the second day of life in a coma with a serum ammonia level of 1,012 µg/dl, markedly elevated serum glutamine and alanine, and a urinary orotic acid level that was 20-fold elevated. He expired on the eighth day of life. OTC activity was absent in a post-mortem liver specimen, whereas other urea-cycle enzymes were normal. Family history was unremarkable for infant deaths. Although the proband’s mother, II-2, gave a lifelong history of protein intolerance, the maternal grandmother (I-2) and the three maternal aunts (II-3, II-4, II-5) differed from II-2 in their ability to eat protein normally; the three sisters, in addition, had normal serum ammonia and urine orotic acid levels following oral protein loading (1 gm/kg body weight) [4].
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Fig. 1.—Pedigree and Southern blot analysis of family A. Top: Pedigree of family A with proband shown as ■, carrier females as ○, normal females as ◦. Females were identified as carriers based on dietary protein intolerance and the presence of the 3.4-kb TaqI fragment, except for individual III-5 whose carrier status is inferred by history. Bottom: Southern blot analysis of TaqI-digested DNA probed with OTC cDNA. Each lane in the Southern blot is labeled with the pedigree designation of the family member whose DNA was analyzed in that lane.

MATERIALS AND METHODS

A synthetic oligonucleotide was constructed to contain 21 bases from the coding region of rat OTC cDNA, corresponding to the peptide sequence trp-thr-ile-met-ala-val-met. The oligonucleotide sequence was based on the data of Horwich et al. [5] and was synthesized by Dr. J. Habener in the Howard Hughes Medical Institute oligonucleotide synthesis facility. The oligonucleotide was end-labeled with T4 kinase and hybridized at 47°C (15° below its theoretical melting temperature of 62°C) [6] to arrays of lysed colonies from a rat liver cDNA library constructed in pBR322. One colony out of 3,000 was found to hybridize consistently to the oligonucleotide probe and contained a 700-base pair (bp) insert that, by restriction endonuclease mapping, was found to correspond to the known rat OTC sequence [5]. A 250-bp PstI/HindIII fragment from the 5’ end of the rat OTC insert was then used as probe to screen 20,000 colonies from a human liver cDNA library [6]. One colony was identified that contained a ~1,150 bp insert. The identity of this clone as OTC was confirmed in two independent ways. First, the cDNA was mapped to the short arm of the human X chromosome, where OTC maps [7], by Southern analysis of DNA from cells containing one, two, or four copies of the short arm of the X. Single-copy intensity was seen with 46,XY male cells and cells containing a single normal X and an isochromosome consisting of two copies of the X long arm (GM88), a two-copy intensity with DNA from a 46,XX female, and four-copy intensity in DNA from a 49,XXXXY cell line (GM1416) (cell lines GM88 and GM1416 were obtained from the Human Genetic Mutant Cell Repository, Camden, N.J.). Second,
FIG. 2.—Pedigree and Southern blot analysis of family B. Top: Pedigree of family B with proband shown as ■, carrier females as ○, normal females as O. Females were identified as carriers based on the presence of the 3.4-kb TaqI fragment or as noncarriers based on normal protein-loading tests and the absence of the 3.4-kb TaqI fragment. Bottom: Southern blot analysis of TaqI-digested DNA probed with OTC cDNA. Each lane in the Southern blot is labeled with the pedigree designation of the family member whose DNA was analyzed in that lane.

restriction enzyme digestion of the cloned insert provided a restriction map at the XhoI, PvuII, KpnI, BstEII, EcoRV, HindII, and TaqI sites compatible with the published sites for these enzymes in the human sequence [8]. The insert contains all of the 3' untranslated region and two-thirds of the coding region and extends to just 5' of the Xho site at position 345 [8].

High molecular weight DNA was isolated from peripheral blood leukocytes, cultured cells, or liver biopsy specimens by published procedures [9]. For Southern blot analysis, 5–10 μg of DNA was digested with restriction endonucleases at 37°C for 4–6 hrs, at a ratio of 4 U of enzyme per microgram DNA, except for TaqI digests, which were carried out at 65°C under paraffin oil at 8–10 U of enzyme per microgram DNA. The DNA underwent agarose gel electrophoresis overnight, was denatured, neutralized, and transferred to nitrocellulose by published methods [10]. Prehybridization and hybridization were carried out at 42°C in 50% formamide by published procedures using purified human OTC insert nick-translated with [α³²P]deoxyctydine triphosphate to 10⁸ counts per minute per microgram as probe [10].

RESULTS

A series of Southern blots was performed using the restriction endonucleases MspI, TaqI, HindIII, PstI, and EcoRI to digest DNA from the probands in these two families and their relatives and probing with the human OTC cDNA. Only with the enzyme TaqI was a difference seen in the Southern blot restriction pattern between the probands and normals. As shown in the Southern blot in figure 1 (lane labeled IV-1), DNA from the proband in family A demonstrated a new 3.4-kilobase (kb) TaqI fragment and the loss of the usual 1.8-kb fragment
when compared with normal males such as his father (lane III-3). This variation with TaqI was seen in DNA obtained from peripheral blood leukocytes as well as in two DNA samples prepared independently from a transformed lymphoblastoid line. His mother (III-4) and maternal aunt (III-1), who were both intolerant of protein by history, had both the aberrant 3.4-kb band and the normal 1.8-kb band, as did his maternal grandmother (II-3). We interpreted the 1.8-kb and 3.4-kb bands as different alleles for which the carrier females were heterozygotes. As shown in figure 1, his father (III-3) and maternal aunt without clinical symptoms of protein intolerance (III-2) lacked the 3.4-kb band, as did his maternal grandfather (II-2) and great aunt (II-1). In family B, shown in figure 2, the proband (III-1) had exactly the same alteration in the TaqI pattern as that seen in the proband in family A. His mother (II-2) and sister (III-2) also had the 3.4-kb band, while three maternal aunts (II-3, II-4, II-5) and the maternal grandmother (I-2) all lacked the 3.4-kb band.

Evidence that the TaqI site alteration reflects the actual mutations in these families, rather than a polymorphism, was obtained in two ways. First, the 3.4-kb TaqI fragment was looked for and not found in 84 additional X chromosomes from control individuals of Anglo and Mexican-American backgrounds. Second, and more important, our studies of the relatives of both probands for the presence of the TaqI alteration and the OTC mutation demonstrated that the OTC deficiency and the Southern blot alteration appeared simultaneously for the first time in these families in a single female antecedent of each proband. In family A (fig. 1), the maternal great-grandmother (I-2) was not clinically protein intolerant and did not carry the TaqI alteration. The maternal great-grandfather (I-1) was unavailable for study but clinically did not have OTC deficiency. In addition, he could not have been carrying the TaqI alteration as a benign polymorphism since his daughter II-1 must inherit his X chromosome and yet does not have the aberrant 3.4-kb TaqI fragment, assuming paternity is correct. Likewise, in family B (fig. 2), the maternal grandmother (I-2) was not protein intolerant and showed a normal Southern blot pattern in TaqI-digested DNA; her husband (I-1), although unavailable for study, was not OTC-deficient clinically. He also could not be carrying the TaqI alteration as a benign polymorphism since none of his three daughters (II-3, II-4, or II-5) had the aberrant 3.4-kb band, if paternity is again assumed to be correct. Thus, if the TaqI alteration were a benign polymorphism unrelated to the OTC mutations in both of these families, then a pair of independent de novo mutations, one causing the polymorphism and one the disease mutation, would have to have occurred simultaneously in the same individual in two different families. The probability of one such pair of independent mutations is small; the chance of two such pairs of independent mutations in two families is essentially zero. Therefore, we can conclude that the maternal grandmother (II-3) in family A and the mother (II-2) of the proband in family B must both be carriers of OTC deficiency by virtue of new mutations that have altered the same TaqI site in the OTC gene.

In addition to following the inheritance of OTC deficiency retrospectively to its origin in these families, we also were able to follow its inheritance prospectively in prenatal diagnosis. DNA was isolated from amniocytes grown for 3
weeks after a routine 16-week amniocentesis. Southern blot analysis of fetal DNA from the pregnancy at risk for recurrence in family A readily provided prenatal exclusion of OTC deficiency. As shown in figure 3, the fetus (lane 1) lacked the aberrant 3.4-kb band as did his father, a normal male control (lane 3). The 3.4-kb band was, however, present in the proband (lane 2). The pregnancy was continued, and, based on these results, the mother was delivered at her local hospital rather than at a tertiary care facility. The newborn’s serum ammonia after 12 hrs of a normal protein-containing formula was 30 μg/dl. The infant is currently 5 months old and has remained clinically normal on regular formula.

**DISCUSSION**

Using a cDNA sequence corresponding to most of the human OTC gene, we have undertaken a survey of patients with OTC deficiency in order to identify those with molecular abnormalities detectable by Southern blotting. In the first few patients analyzed, we found two unrelated individuals of different ethnic backgrounds with clinically similar severe disease, who demonstrated an identical rare alteration in the Southern blot pattern of their OTC gene. By studying other family members and controls, we obtained convincing data that this alteration is the result of the mutations responsible for the disease itself rather than to a causally unrelated but linked polymorphism within the OTC gene. In both families, we found a heterozygote for OTC deficiency who demonstrated a Southern blot alteration of TaqI-digested DNA that was not present in her clinically noncarrier mother nor, by examining her sister(s), in her clinically unaffected father. If the TaqI alteration did not represent the new mutational events in these families, then what alternative explanations would need to be considered? If the OTC deficiency were a new mutation, but the TaqI alteration were inherited as an unrelated polymorphism, then the polymorphism would have to come from the new mutation heterozygote’s father, in which case the paternity in both these families would have to be incorrect and both biological fathers would have to be hemizygotes for what appears to be a rare polymor-

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**Fig. 3.**—Prenatal exclusion of OTC deficiency in family A by Southern analysis. Lane 1 contains TaqI-digested DNA from amniocytes from the pregnancy at risk in family A; lane 2 contains DNA from the proband IV-1; lane 3 contains DNA from his normal father III-3.
phism since 84 control X chromosomes failed to show this TaqI alteration. Conversely, if the OTC deficiency were inherited but the TaqI alteration were a newly arisen polymorphism, then the same very rare polymorphism would have to have occurred by mutation independently in both of the first documented heterozygotes for OTC deficiency in these pedigrees and both their mothers would have to be clinically silent carriers of OTC deficiency, in contrast with the other carriers in these pedigrees who exhibit protein intolerance. If both the OTC deficiency and the TaqI alteration were inherited as independent DNA sequence changes, then the carriers' mothers would again have to be silent carriers, despite their clinically normal status, and the paternity would again have to be incorrect. Finally, if the OTC deficiency and TaqI alteration were both new but causally unrelated mutational events, then a pair of mutations would have to have occurred independently, by chance alone, in both these families. The probability of each of these alternative explanations is extremely small and leaves as the overwhelmingly most likely explanation that the TaqI alteration reflects the new mutational event responsible for OTC deficiency in each family. Proof that the TaqI alteration represents the mutations in the OTC gene in both families will require explicit DNA sequence data from the normal and mutant OTC genes.

Our studies have identified the origin of the mutation in each proband by identifying the female antecedent in whom the mutation initially arose in the family. Since we can only detect the mutations in these families by their effect on a TaqI endonuclease recognition site, the nature of the alteration at the nucleic acid sequence level is not known and could be different in the two families. The alteration was seen only with this one enzyme, and not with any of the other enzymes tested, and therefore most likely resulted from single base changes or small deletions that obliterated the same TaqI site in the OTC gene. By means of genetic markers near the OTC gene, we should be able to determine whether the mutations arose in male or female gametes. Unfortunately, polymorphism analysis did not allow us to determine whether the maternal or paternal gamete in these new mutation carrier females was the origin of the mutation.

Substantial clinical and biochemical evidence exists for heterogeneity in the extent and biochemical mechanisms of enzyme impairment in OTC deficiency [1, 2]. Complete and partial defects are known that differ in clinical severity and prognosis. Although both patients reported here had similarly severe disease, we were surprised to find mutations at the same site in the OTC gene in these two unrelated probands from among the first few families we studied. It is interesting to note that the restriction endonuclease TaqI is known to reveal polymorphic DNA sequence alterations at a high frequency relative to most other restriction enzymes [11]. If TaqI sites are hotspots for benign polymorphic variation, they could also be hotspots for mutations that cause disease if a sequence that is critical for normal gene function is altered. One possible mechanism proposed to explain this observation is that methylated cytosine residues, commonly found in eukaryotic DNA, may undergo spontaneous deamination resulting in replacement of cytosine by thymine. TaqI sites con-
tain a cytosine-guanine dimer in their recognition sequence and would no longer be cut by TaqI if the cytosine underwent transition to thymine. It is interesting to note that all four TaqI sites in the OTC cDNA are in frame to code for arginine with a CGA codon [8]: a C to T transition produces a UGA stop codon, leading to nonsense mutations. Such transition mutations have been found within some altered TaqI sites, although substitution of a guanine or adenine residue for cytosine has also been seen within mutated TaqI sites associated with polymorphic variation [11] and disease [12]. Whether or not deamination of methylcytosine residues is responsible for the high frequency of DNA sequence variation detected with the endonuclease TaqI, TaqI sites do appear to be preferred sites for mutation, which may help explain the findings in the two families reported here. However, in four other OTC-deficient families we have studied with equally severe disease in the male hemizygote and protein intolerance in female carriers, an aberrant 3.4-kb TaqI fragment and loss of a 1.8-kb fragment were not seen, nor was any other Southern blot alteration detectable (our unpublished data, 1984). Thus, the TaqI alteration reported here may be a frequent finding in severe OTC deficiency but is certainly not the exclusive or even a major cause of severe OTC deficiency.

Heterozygote detection of OTC deficiency has been successfully carried out using liver biopsy [3] for histochemical evidence of mosaicism and by protein loading to provoke hyperammonemia or orotic aciduria [4]. The less-invasive loading test would clearly be preferable if its sensitivity and specificity could be shown to be nearly 100%. It is interesting that a strong history of protein intolerance was obtained prospectively in all females from both families who were subsequently shown to be heterozygotes by Southern analysis. Conversely, the three maternal aunts of the proband in family B were all thought to be noncarriers both by history and by their normal serum ammonia and urine orotic acid measurements after protein loading and were all proven not to be carriers by Southern analysis of their DNA. Thus, there was complete concordance among the clinical, biochemical, and molecular findings in these heterozygotes. More extensive surveys of females in families with OTC deficiency detectable by Southern blot will allow a more thorough assessment of heterozygote detection, particularly in families with partial OTC deficiency since heterozygotes for partial deficiency would be predictably more difficult to diagnose.

Because OTC is not expressed in fibroblasts, prenatal diagnosis of OTC deficiency has depended until now upon fetal liver biopsy, a difficult procedure that may be associated with substantial morbidity [13, 14]. In pregnancies at risk for OTC deficiency, selective abortion of all male fetuses, as determined by chromosome analysis of amniotic fluid fibroblasts, has been more commonly used to prevent the birth of males affected with this condition. Sex selection has the two great disadvantages of causing the loss of unaffected male fetuses and of failing to detect heterozygous females who may also be at risk for significant morbidity from hyperammonemia. The availability of OTC cDNA greatly increases the potential for prenatal diagnosis of this condition. As we and others [15] have shown, direct analysis of amniocyte DNA can be used to
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predict whether a fetus has inherited a mutant OTC gene with an altered Southern blot pattern. In addition, restriction fragment length polymorphisms (RFLPs) at the OTC locus have been identified and, in informative pedigrees, could be used to predict whether or not a fetus is affected [16]. The prenatal exclusion reported here, by Southern blot analysis of DNA from cells obtained at amniocentesis, is an application of recombinant DNA technology to one of the clinical problems posed by OTC deficiency. This technology, already extensively used in prenatal diagnosis of the hemoglobinopathies, should also have a major impact on the management of families at risk for OTC deficiency.

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REFERENCES

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A RESEARCH SUPPORT PROGRAM ON REPRODUCTIVE HAZARDS IN THE WORKPLACE, HOME, COMMUNITY, AND ENVIRONMENT. The March of Dimes Birth Defects Foundation is pleased to announce the continuation of its small-scale, but broadly based, program designed to recognize and quantify adverse reproductive effects in persons exposed to physical or chemical agents in the workplace, home, community, and environment. Investigations should be designed as a component of an overall model system involving identification of exposed groups at potential risk as well as the quantification and evaluation of risk. Approaches based on the study of exposed groups should emphasize feasibility, detail exposure, investigate dose effects whenever possible, and quantify adverse reproductive effects in a statistically and epidemiologically rigorous manner. Exposed groups at potential risk may be identified by occupational category, place of residence, etc., or by exposure to a specific agent, such as a common drug consumption. Approaches based on the evaluation of risk include: development and refinement of appropriate methodologies for detection of adverse reproductive effects by chemicals in model experimental systems; studies of cellular and molecular mechanisms underlying reproductive damage; development and validation of appropriate methodologies for recognizing “early-warning” effects in body fluids of exposed workers, such as cytogenetic, genetic, biochemical, and molecular techniques; development and application of appropriate methodologies for recognizing adverse prenatal and postnatal effects due to specific exposures; and prospective epidemiological studies of selected high-risk groups.

Requests, in terms of a 300-word abstract, should be received prior to May 1, 1986. The abstracts should contain objectives, hypotheses, and brief methodologies and should be mailed to: Samuel J. Ajl, Ph.D., Vice President for Research, March of Dimes Birth Defects Foundation, 1275 Mamaroneck Ave., White Plains, NY 10605. Initial terms of these modest grants may not exceed 2 years. Also indicate the minimal level of funding per year to be requested. In this first communication, no addenda, appendices, reprints, or such will be accepted. Anything received in addition to the summary will not be reviewed. After receipt of the abstracts, applicants will be advised how to proceed further.