1990

Identification of RNA splicing errors resulting in human ornithine transcarbamylase deficiency

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Identification of RNA Splicing Errors Resulting in Human Ornithine Transcarbamylase Deficiency

A Thesis Submitted to the Yale University School of Medicine

in Partial Fulfillment of the Requirements for the

Degree of Doctor of Medicine

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1990

ACKNOWLEDGEMENTS

There are several people to whom I am deeply indebted for helping me pursue and complete this project. Certainly, I must start by thanking Dr. Leon Rosenberg for providing the opportunity, direction, and encouragement to undertake a study such as this. Throughout my progress with this project he provided a model for how a critical scientist must pursue knowledge, while at the same time demonstrating the patience needed to remain on target. Dr. Wayne Fenton provided essential help along the way, without which it would have been immensely more difficult, if not impossible, to achieve my results. Wayne was always able to help at those critical moments where one feels he has reached a dead end, and was extremely generous with his time when I was in need of help. I only hope I can incorporate into myself the qualities Drs. Rosenberg and Fenton use in performing and critiquing science in my own future scientific endeavors. The rest of the people in the lab, Jan Kraus, Franta Kalousek, Peter Hodges, Grazia Isaya, Franco Taroni, Adelle Hack, Jörg Kleiber, Tak Tahara, Joe Hendrick, Manju Swaroop, Michelle Browner, Elizabeth Stzul, and Evelyn Traeger were likewise a necessary ingredient in my ability to proceed on a day-to-day basis. Thanks also to Connie Woznick for help in preparing the thesis and a manuscript, and to Dr. Margretta Seashore and Dr. Arthur Horwich for reviewing the final copy. In addition, I thank Saul Brusilow at Johns Hopkins for providing some of the specimens used in this study and for helpful discussion. Finally, I would like to thank my parents for providing continued support in all of my endeavors.

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ABSTRACT

IDENTIFICATION OF RNA SPLICING ERRORS RESULTING IN HUMAN ORNITHINE TRANSCARBAMYLASE DEFICIENCY. Russ P. Carstens. Department of Human Genetics, Yale University School of Medicine. New Haven, Connecticut.

Ornithine transcarbamylase (OTC) is an X-linked, liver specific enzyme which catalyzes the second step of the urea cycle. In humans, inherited deficiency of OTC in hemizygous affected males usually results in severe ammonia intoxication and early death. In order to characterize mutations responsible for OTC deficiency, we used the polymerase chain reaction to amplify cDNAs prepared from patient livers, obtained at autopsy, which demonstrated no OTC enzyme activity. Of eleven livers tested, it was possible to produce amplifiable cDNA from seven. In three of these seven cases, smaller than normal products were observed. Sequencing of the cDNAs revealed that two were missing exon 7 of the OTC gene and that the other was missing the first 12 base pairs of exon 5. Sequencing of genomic DNA from these patients revealed that one mutant missing exon 7 had a point mutation causing a T to C substitution in the 5' splice donor site of intron 7. The other mutant missing exon 7 had an A to G change in the third position of intron 7. Interestingly, both of these mutations resulted in skipping the preceeding exon, rather than inclusion of some or all of the affected intron. In the third mutant, an A to T substitution was found in the 3' splice acceptor site at the end of intron 4. Here,

a cryptic splice acceptor site within exon 5 was used. Although each of the deletions was in frame, Western blotting of liver homogenates from these patients with anti-OTC antiserum showed no immunoreactive material. Northern blotting of liver RNA from these patients demonstrated reduced, but significant amounts of OTC mRNA in one of the patients with a deleted exon, but dramatic reduction in OTC mRNA in the other two. We propose that these point mutations, which result in abberant splicing of the OTC pre-mRNAs, lead to OTC deficiency through either decreased efficiency of mRNA export from the nucleus to the cytosol or synthesis of enzyme subunits that are unstable and rapidly degraded. These are the first examples of human OTC deficiency resulting from mutations that cause production of abnormally spliced mRNAs and may represent a common mechanism involved in the pathogenesis of this disease.

THE BIOCHEMISTRY AND MOLECULAR BIOLOGY OF ORNITHINE TRANSCARBAMYLASE

Biochemistry

Ornithine transcarbamylase (OTC; carbamoyl-phosphate: L-ornithine carbamoyltransferase, EC 2.1.3.3) is the enzyme that catalyzes the second step of the urea cycle, the condensation of carbamyl phosphate with ornithine to form citrulline, a reversible reaction whose equilibrium favors the formation of citrulline. This cycle is the predominant pathway through which ureotelic organisms are able to rid the body of excess nitrogenous wastes that would otherwise have a detrimental effect, especially upon the nervous system. The net effect of this cycle is the incorporation of ammonia nitrogen into urea, the main nitrogenous waste product in mammals. The only other known significant pathway through which the body can eliminate excess nitrogen is through the transamination of alpha-ketoglutarate to glutamate or of glutamate to glutamine. Five enzymes, carbamyl phosphate synthetase (CPS), OTC, argininosuccinic acid synthetase, arginosuccinate lyase and arginase, function in this cycle and are predominantly expressed in liver. CPS and OTC are located subcellularly to the mitochondrial matrix; the other three enzymes are cytosolic.

The pathways of biogenesis and assembly of OTC have been well studied in human and rat tissue (Isaya *et al.*, 1988; Sztul *et al.*, 1987; Horwich *et al.*, 1986; Kraus *et al.*, 1988). OTC is translated on free cytosolic ribosomes as a 40 kDa precursor protein bearing a 32 amino acid NH_2-terminal leader peptide which directs uptake of the protein into mitochondria. Like most such leader peptides, this region of the OTC precursor contains several basic amino acid

residues and an absence of acidic residues. After the OTC precursor is translocated across both mitochondrial membranes, the 32 amino acid leader is cleaved in a two step process. Several studies have generated mutations in the leader sequence and have defined critical residues required for proper mitochondrial import and processing (Horwich *et al.*, 1986; Isaya *et al.*, 1988). In particular, it has been found that the arginine residue at position 23 of the leader is especially critical for normal processing. Following processing, mature 36 kDa subunits assemble into homotrimers which comprise the catalytic enzyme.

The complete amino acid sequence of human OTC, as well as that of several other species, is known, as deduced from cloned cDNAs and consists of 354 amino acids (Horwich *et al.*, 1984). Through comparisons of homologies, chemical modification studies, and site directed mutagenesis, it has been possible to identify regions of the protein which are involved in different functions of the enzyme. In addition, the significant homology between OTC and the catalytic moiety of aspartate transcarbamylase (ATC) has allowed further speculation regarding regions involved in enzyme function, as *E. coli* ATC has been cloned, crystallized, and characterized much more extensively than OTC (Hoover *et al.*, 1983; Kantrowitz and Lipscomb, 1988; Allewell, 1989). When regions of homology are aligned, there are 46 residues which are completely conserved for OTC across species, of which 30 are conserved in *E. coli* ATC (Kraus *et al.*, 1988). A conserved sequence of phe-leu-his-cys-leu-pro is seen at amino acids 300-305 of the human OTC precursor, in which the cysteine is believed to be essential to ornithine binding (Marshall and Cohen,

1980). A sequence of ser-thr-arg-thr-arg at amino acids 90 to 94 is also highly conserved, not only in OTC, but in ATC as well, and is believed to be the carbamyl phosphate binding site (Marshall and Cohen, 1980; Kraus *et al.* 1988). The arginine residue at position 92 may be homologous to the arginine at position 57 in *E. coli* OTC which has been determined by X-ray crystallography to be involved in isomerization following binding of carbamyl phosphate (Kuo and Seaton, 1990). This isomerization is believed to be crucial for the subsequent orderly binding of ornithine to occur. Current efforts to further characterize crystallized *E. coli* OTC are under way and it is hoped that similar analyses will also be possible for human OTC as well. By elucidating functions of other conserved residues in OTC, it is hoped that a better appreciation of critical regions of OTC and their functions can be gained.

Molecular Biology

The hypothesis that the gene for OTC was on the X-chromosome, which was suggested by pedigree analysis of families of patients with OTC deficiency in the early 1970's (Campbell *et al.*, 1971; Short *et al.*, 1973; Palmer *et al.*, 1974) received strong support by subsequent demonstration of two populations of cells in a liver biopsy from a female heterozygous for OTC deficiency: those with OTC activity and those without (Ricciuti *et al.*, 1976). This finding was consistent with X-linkage of OTC according to the Lyon hypothesis (Lyon, 1961), which states that, in females, random inactivation of one of the X-chromosomes occurs in various tisues such that they are mosaics of tissue expressing either the maternal or the paternal X-chromosome. The further

localization of OTC to Xp21.1 using somatic cell hybrids and in situ hybridization was performed by Lindgren *et al.* (1984).

Rat OTC mRNA was isolated by polysome immunoabsorption, and this material was used to prepare rat OTC cDNA clones (Kraus and Rosenberg, 1982). These rat OTC clones were then used to isolate human OTC cDNA (Horwich *et al.*, 1984). Although initially believed to consist of 8 exons covering approximately 85 kbp (Horwich and Brusilow, 1989; Hata *et al.*, 1986), recent evidence indicates that the human OTC gene consists of 10 exons spanning about 73 kbp (Hata *et al.*, 1989). The smallest exon is 54 bp and the largest exon (the last one) is believed to be at least 764 bp. Intron sizes range from 80 bp to 21.7 kbp. The gene produces an mRNA transcript which contains a 1062 base coding region which is translated into the 354 amino acid OTC precursor protein.

Although Hata *et al.* (1989) reported a cDNA of 1849 bases, it may be that mRNA transcripts of differing lengths are produced, at least in humans. Northern blot analysis performed for this thesis, as well as those reported by other authors (Grompe, *et al.*; 1989, Hodges, P.E. unpub. obs.), shows three predominant sizes of mRNAs: ~3200 bases, ~1800 bases, and ~1300 bases. Available sequence data for the 3' end of the OTC gene shows several putative AATAAA polyadenylation sequences. Relative to the translation start codon, these sequences begin at bases 1151, 1408, and 1934. Because they isolated a cDNA clone consisting of 1769 bases from the initiation codon, Hata *et al.* (1989) hypothesized that the third polyadenylation sequence was the functional signal. However, given the different sizes of transcripts seen on Northern

blotting, it may be that at least one of the upstream sequences also functions as a polyadenylation signal for some transcripts, in addition to an as yet unidentified sequence further downstream.

The 5' end of the human OTC gene has been sequenced 665 bp upstream from the translation initiation codon (Hata, 1986) in an attempt to elucidate sequences in the 5' flanking sequence which regulate expression. Within this region there are two candidates for the CAAT and TATA boxes believed to be involved in gene expression. There are CTCAAT and TATAAAT at positions -329 to -324 and -285 to -279, respectively, and CAAT and GCATAA at positions -252 to -249 and -200 to -195, respectively. In addition, there is the well-known viral core enhancer sequence GTGGAAAG at bases -501 to -494. Attempts to define the transcription start site have shown that, in fact, there are multiple start sites. Using the technique of S1 nuclease mapping with primer extension, start sites have been identified at nucleotide positions -95, -120, -150, -161, and -166 with respect to the translation start codon (West, A., unpub. obs.). Given the variable sites at which transcription is initiated, it is difficult to ascertain the role played by any specific sequences in the 5' untranslated region in regulation of OTC expression.

ORNITHINE TRANSCARBAMYLASE DEFICIENCY

History

The first clinical description of inherited disease due to deficiency of OTC was that of Russell *et al.* (1962) who documented two female first cousins who presented with vomiting, screaming, lethargy, stupor, and failure to thrive. Both patients were found to have high ammonia levels, both in the blood and cerebrospinal fluid, as well as cortical atrophy as demonstrated on pneumoencephalography. A liver biopsy was obtained from one of these patients, and enzyme assay found only 10% of the normal level of OTC, whereas the level of CPS was within normal limits. In the years following this report, similar descriptions of this disease appeared, in which ammonia intoxication due to OTC deficiency explained the patient's clinical course. (Campbell *et al.* 1973; Levin *et al.*, 1969a; Levin *et al.*, 1969b; Levin *et al*, 1969c; Matsuda *et al.*, 1971; Sunshine *et al.*, 1972). In these patients, however, both the character of their illness, as well as the degree of enzyme deficiency, varied greatly. It was also noted that almost all of these patients were female. Subsequent studies of two different pedigrees revealed that mothers of female patients also had reduced activity of OTC, whereas their fathers had normal OTC activity (Short *et al.*, 1973; Levin *et al.*, 1969b). These observations of mother-to-daughter transmission and mostly female patients led to the original proposal that this disease was transmitted as an autosomal dominant, sex-limited trait (Levin *et al.*, 1969a). Campbell *et al.* (1971) subsequently described two male siblings who presented with striking hyperammonemia, coma, and

death within 3 days of birth. Assay for OTC activity in these patients revealed complete absence of the enzyme, whereas activity of the other urea cycle enzymes were all within normal range. At that time these were the first reported cases of complete OTC deficiency; all previously reported cases had shown only partial deficiency. These descriptions of disease led these authors to propose that OTC was encoded on the X-chromosome and that mutations in this gene could lead to partial deficiency in affected females through disadvantageous Lyonization and complete deficiency in hemizygous males. This hypothesis was strengthened by study of four kindreds with complete OTC deficiency in hemizygous males and partial deficiency in some female probands (Short *et al.*, 1973). The hypothesis of X-linked transmission has since been borne out by direct demonstration of Lyonization in heterozygotes for OTC deficiency (Ricciuti, *et al.*, 1976) and by localization of the OTC gene specifically to band Xp21.1 on the X-chromosome (Lindgren, *et al.*, 1984). Since the earliest description of OTC deficiency many more cases have now been reported, although the true prevalence of the disease in the population is difficult to determine because many cases are probably not diagnosed (Walser, 1983).

Clinical Features of OTC Deficiency

The clinical severity of ornithine transcarbamylase deficiency is influenced by several factors, some of which are still poorly understood. It is believed that the severity of disease is primarily related to the degree of hyperammonemia. Severe hyperammonemia is also seen in deficiency of some of the other urea cycle enzymes, such as CPS deficiency, arginosuccinic acid sythetase

deficiency, and arginosuccinase deficiency. Thus, the syndromes in each of these diseases are similar. Various authors have divided OTC deficiency into different syndromes depending upon period of onset and sex of the patient, although it appears most likely that the degree of severity is along a continuum. It is presumed that the degree of hyperammonemia is primarily related to the degree to which enzyme activity is preserved in vivo, which would most likely depend upon presence of a normal allele (in females) and upon the severity of the different mutations.

Clearly, the most important distinction which defines the degree of hyperammonemia in OTC deficiency is the sex of the patient. With some exceptions, most hemizygous affected male patients experience onset of symptoms in the neonatal period with a progressive downhill course, whereas heterozygous (carrier) females display a wider variation in onset and severity. Male patients with neonatal onset of the disease are usually the term products of normal labor and delivery with normal Apgar scores, but within 24 hours to several days after birth are noted to have increasing irritability, lethargy, and poor feeding. Soon thereafter further symptoms, including vomiting, convulsions, grunting respirations, hypotonia, apnea, seizures, and hyperventilation may develop, which can rapidly progress to coma and death if left untreated. Because of their clinical picture, these infants are often misdiagnosed with either sepsis or pulmonary disease, although further workup for these causes is usually non-contributory. In most of these cases, therapeutic attempts at lowering serum ammonia levels are unsuccessful and

the patient's demise ensues within a short period after the appearance of symptoms.

A number of male patients affected by OTC deficiency have been found either to have a more benign course of disease or to be more responsive to efforts at therapy. Some of these patients have a normal neonatal period, but experience increasingly severe hyperammonemic crises later in infancy or during childhood. Likewise, some patients who are aggressively treated following birth (usually because of prior information suggesting the diagnosis) are able to live beyond the neonatal period, but are subject to periods of hyperammonia later in childhood. Often these periods of hyperammonemic crisis are precipitated by increases in protein intake, immunizations, infection, or surgery. It is believed that these less severe forms of the disease could be due to incomplete deficiency of enzyme. Some of these patients have demonstrated no OTC activity in vitro, although it may be that the in vitro assay for OTC does not completely represent in vivo OTC activity (Krieger *et al.*, 1979). Presuming this to be the case, the mutations responsible for these cases may be small defects, such as in-frame point mutations, which may alter the enzyme enough to reduce activity to levels well below normal, yet not enough to completely abolish activity. One interesting case in which OTC deficiency was diagnosed in an otherwise normally developing male at age three turned out to be due to mosaicism for OTC deficiency, with one population of cells exhibiting a large deletion of the OTC gene (Maddalena *et al.* 1988). Whether such mocaicism could account for other male cases with later onset of symptoms remains to be seen.

Among females who carry a defective allele for OTC, the clinical course can range from the severe neonatal course seen in males to absence of symptoms (asymptomatic carriers). As for affected males, it appears that the severity of disease is related to residual OTC enzyme activity. The amount of OTC activity, and hence the severity of disease, is much more variable in females due to the random inactivation of the X-chromosome. Females with a favorable pattern of inactivation of defective alleles in the liver are less likely to experience severe disease and more likely to be asymptomatic carriers. It is in females that more of the features of the disease in childhood have been described. These include vomiting, screaming, lethargy, headache, slurred speech, irritability, agitation, ataxia, muscular rigidity, delayed growth, abnormal development, and aversion to protein rich foods. Again, those who have severe forms of the disease can progress to coma and death if hyperammonemia cannot be controlled.

Asymptomatic carriers, although seemingly symptom-free, have often been suspected of having some of the sequelae of mild OTC deficiency. It has been suggested that minor, undetected hyperammonemic episodes may progressively impair normal brain development. Some authors have suggested that some of these carriers may have lower IQs as a result of these impairments (Batshaw *et al.*, 1980). It has also been suggested that undetected carriers may experience frequent "migraine" headaches, and that they may unconsciously self-regulate their diets to avoid protein rich foods (Brusilow, 1985).

Diagnosis

Correct diagnosis of OTC deficiency usually begins with the finding of an elevated serum ammonia level. The differential diagnosis of hyperammonemia includes deficiency of other urea cycle enzymes, transient hyperammonemia of the newborn, Reye's syndrome, and certain organic acidemias. A finding more specific for OTC deficiency is an elevated urine orotate, which is believed to result from the shunting of unused carbamyl phosphate into the cytosolic pyrimidine biosynthetic pathway. A definitive diagnosis of OTC deficiency can only be made by assay of OTC activity from biopsied liver, or possibly via small intestinal biopsy (Holzgreve and Golbus, 1984).

With a better understanding of the genetics and biochemistry associated with OTC deficiency have come improved methods for establishing carrier status and diagnosing OTC deficiency prenatally. Approximately 10% of patients affected by OTC deficiency show large deletions at the OTC locus (Rozen *et al.*, 1985). Additionally, several pedigrees have shown alteration of a Taq I restriction endonuclease site in the OTC coding sequence which represents the OTC defect (Maddalena *et al.*, 1988; Hata *et al.*, 1989). For families with either of these defects, prenatal diagnosis can be made by direct DNA analysis for the defect, using tissue obtained by amniocentesis or chorionic villus sampling. For cases of OTC deficiency where such defects are not present, prenatal diagnosis rests upon use of restriction fragment length polymorphisms (RFLPs). RFLPs have been demonstrated at the OTC locus using the restriction endonucleases Msp I, Bam HI, and Taq I (Fox *et al.*, 1986; Nussbaum *et al.*, 1988; Rozen *et al.*, 1985; Spence *et al.*, 1989). Combining all

of these RFLPs, it has been shown that approximately 80% of female carriers are heterozygous for at least one of them. The main limitation of RFLP diagnosis is that it does not identify actual mutations in OTC and so is unable to account for cases due to spontaneous mutation. In addition, recombination at the OTC locus could affect the results of linkage with RFLPs, although the frequency of this occurence should be less than 0.1%.

Crucial to the process of prenatal diagnosis is the establishment of carrier status in mothers. This can be done either through pedigree analysis, a protein tolerance test, or the recently described allopurinol test (Brusilow and Valle, 1987). The protein tolerance test involves a nitrogen challenge given as a high protein load, followed by measurements of urinary orotate. Orotate levels in carriers are often elevated due to the presence of a population of OTC deficient hepatocytes in these women. This test is believed to have a false negative rate of about 10% (Brusilow and Horwich, 1989). One of the disadvantages of this test is the fact that it also is capable of inducing hyperammonemia, and so carries the risks associated with periods of hyperammonemia. Recently, the allopurinol test has been shown to as reliable as the protein tolerance test, but safer because of the reduced risk of hyperammonemia. This test consists of the administration of a dose of allopurinol followed by measurements of urine orotidine. In this test, oxypurinol monophosphate, a byproduct of allopurinol, inhibits orotidine monophosphate decarboxylase, thus reducing the amount of orotate which can be metabolized to uridine 5'-phosphate. This results in shunting of the increased orotate seen in carriers into orotidine, which can then be detected in the urine.

In cases where a woman can be established as a carrier of OTC deficiency, but no detectable mutation or RFLP linkage can be found, only two options remain. First, fetal sex determination can be carried out with selective termination of male fetuses. Alternatively, if the fetus is found to be male, biopsy of fetal liver can be performed to assay for OTC activity (Holzgreve and Golbus, 1984). Unfortunately, this procedure is associated with a higher risk of complications, including fetal loss. It is hoped that in the future techniques will be introduced which can allow prenatal diagnosis through direct detection of mutations in the OTC gene when other methods are uninformative (Cotton *et al.*, 1988; Grompe *et al.*, 1989).

The establishment of carrier status and prenatal diagnosis are of utility for affected families for several reasons. First, in those families in which carrier status can be established, the information can be used to make informed decisions regarding future pregnancies. In cases where women proceed with pregnancy, prenatal diagnosis of affected males can allow these families to choose whether they wish to carry an affected child to term or elect to terminate pregnancy. The option of pregnancy termination, though controversial, is certainly one which has been beneficial to families hoping to avoid many of the unfortunate sequelae of bearing a severely OTC deficient child. Even in cases where families choose to continue the pregnancy, the information gained from prenatal diagnosis can be used to prepare therapeutic interventions as early as possible following birth. This information can be especially relevant if the diagnosis of a female carrier can be made prenatally. Because many or most carriers will be asymptomatic, prompt recognition of carriers can again allow

early efforts at treatment which may avoid periods of hyperammonemia which could be detrimental to these females during later development.

Therapy

Because the severe clinical effects of OTC deficiency are primarily due to profound hyperammonemia, effective therapies have been designed with reduction of body ammonia as a main goal. Simplistically, this approach takes on two forms: aiding excretion of existing stores of ammonia and reducing the load of nitrogen taken in. One other goal of treatment is to maintain glutamine levels within the normal range, as it has been suggested that hyperglutaminemia may signal impending hyperammonemia (Brusilow and Horwich, 1989). The simplest efforts at reducing serum ammonia levels consist of restricting dietary nitrogen intake. Thus, protein intake is kept at minimal levels (0.7g/kg per day), and essential amino acids are added to the diet. Arginine becomes an essential amino acid when the urea cycle is not functioning, therefore dietary supplementation of arginine is often also necessary. In order to suppress endogenous protein breakdown, intravenous glucose can be added to provide nitrogen-free nutrition.

A therapy which exploits alternate pathways of ammonia excretion is administration of sodium benzoate and sodium phenylacetate. Both of these compounds divert nitrogen from the usual excretion route via urea synthesis. The two known alternate pathways of excretion of ammonia involve transamination of alpha-ketoglutarate to glutamate and of glutamate to glutamine. Benzoate functions by conjugating with glycine (a product of glutamine

metabolism) to form hippurate, which can be excreted in the urine. Similarly, phenylacetate conjugates directly with glutamine to form phenylacetylglutamine, which can also be excreted in the urine. Because phenylacetylglutamine contains two atoms of nitrogen to hippurate's one, it has been suggested that phenylacetate may be twice as efficient as benzoate, on a molar basis, at disposing nitrogen (Brusilow *et al.*, 1984). Severe hyperammonemia and hyperammonemic coma require more dramatic treatment. Historically, peritoneal dialysis and exchange transfusions have been used in attempts to rid the body of excess ammonia. Because exchange transfusions are able to remove ammonia only from the vasular space and excess ammonia is distributed in the total body water, this method is in fact of limited usefulness. More recently, hemodialysis has also been used and has been found to be more effective than either exchange transfusion or peritoneal dialysis. In addition, peritoneal dialysis takes a longer period of time to remove a given quantity of ammonia than hemodialysis. More dramatic yet is the use of liver transplantation for patients with severe OTC deficiency; this therapy has only been reported for one patient, who has survived for at least one year (Brusilow and Horwich, 1989). The recent use of partial liver transplantation from a parent to child may also be a therapy of use in the future. Although more aggressive attempts at treatment are required to control hyperammonemia in hemizygous males and some severely affected females, certain therapies may also be useful in preventing minor hyperammonemic episodes in asymptomatic carriers as well. Because such episodes in carriers (as well as in affected males) may contribute to intellectual deterioration, treatments such as dietary restrictions

and benzoate or phenylacetate administration may be appropriate to prevent these deleterious effects.

Pathology

Although pathologic changes are sometimes seen in livers from females heterozygous for OTC deficiency, most severely affected males show no characteristic pathologic changes in liver. In females, the most common finding is mild portal fibrosis. Other changes seen in these livers include steatosis, focal inflammation, and distorted endoplasmic reticulum (Labrecque *et al.*, 1979). Changes in pathologic brain specimens from patients with OTC deficiency are more common. Because these changes are believed to be due to hyperammonemia, similar pathology is seen in other hyperammonemic disorders such as CPS deficiency. Patients with acute, severe hyperammonemia typically develop brain swelling, dilated ventricles, cortical atrophy, metabolic gliosis, and presence of Alzheimer type II astrocytes (Kornfeld *et al.*, 1985; Walser, 1983). The brains of longer term survivors of such diseases have more variable changes, but usually show some generalized gliosis.

THE MOLECULAR PATHOLOGY OF OTC DEFICIENCY

Biochemical Variants

Much of the initial work carried out towards investigation of the enzyme defect in OTC deficiency characterized different mutant enzyme variants biochemically. It has been observed that some patients with reduced or absent OTC activity have mutant enzymes that display abnormal kinetics that may account for their deficiency. Several mutant enzymes have displayed abnormal pH dependency, whereas others have shown decreased affinity for carbamyl phosphate or ornithine (Levin *et al.*, 1969a; Qureshi *et al.*, 1978; Walser, 1983) In addition to these variants, other mutant enzymes have been described with increased affinity for ornithine, decreased inhibition by ornithine, and abnormal temperature dependence (Walser, 1983). It may be that the types of mutations responsible for the different enzyme defects seen are likewise diverse. In cases where little or no OTC activity is present, as well as a lack of OTC immunoreactive protein (Saheki *et al.*, 1984), the defects involved may be mutations affecting larger portions of the coding regions, for example, deletions, splicing errors, and nonsense mutations, or mutations which affect sequences necessary for efficient initiation of transcription or translation or for post-translational transport and processing of the precursor protein. The biochemical variants in which the enzyme has altered kinetics may be due to smaller mutations, such as point mutations which affect individual protein domains and cause the specific changes seen. Identifying the mutations responsible for

these defective enzymes may aid in further characterizing the functions associated with given residues and domains in the mature enzyme.

Mutations responsible for OTC deficiency

To date, few of the mutations responsible for OTC deficiency have been well characterized. Unlike autosomal recessively inherited conditions, OTC deficiency, as an X-linked lethal disease, may result from a larger variety of different mutations (Haldane, 1935). In some patients studied, the OTC gene has demonstrated gross deletion or rearrangement. Most of these mutations are partial deletions of the OTC gene, but cases of complete deletion of the entire OTC gene have been seen (Levy, 1987; Rozen *et al.*, 1985; Brusilow, pers. comm.). Such patients are not believed to be responsible for more than 10% of OTC patients, however.

Several point mutations have been identified in unrelated patients which alter a Taq I restriction endonuclease site in exon 5 of the OTC coding region (Maddalena *et al.*, 1988; Hata *et al.*, 1989). Two of these patients display an A to G missense mutation in which a glutamine is substituted for the arginine normally present at amino acid position 141 of the OTC precursor. The other mutation, also seen in two unrelated patients, is a C to T transition at the same codon which generates a stop codon. This area may be susceptible to mutation because of the presence of a CpG dinucleotide, for which a higher rate of mutation has been observed. This higher susceptibility to mutation is believed to be due to methylation of cytosine, with subsequent deamination to form thymidine (Youssoufian *et al.*, 1988). Both of these mutations may involve

C to T transitions, on either the sense or antisense strand. Grompe et al. (1989) used the newly described method of chemical mismatch cleavage (Cotton *et al.*, 1988) to analyze amplified cDNAs prepared from patients affected by OTC deficiency. This technique detected single base changes in four patients which are believed to represent the basis of the observed enzyme deficiency. Of the patients described, two had single missense mutations, one had a nonsense mutation, and one displayed two missense mutations. Interestingly, one of the missense mutations involved the 5' splice donor consensus sequence at the end of exon 1, possibly making it less suitable for normal splicing. Whether the enzyme deficiency in this patient was due more to effects of the amino acid change on the protein or to decreased mRNA levels as a consequence of reduced normal splicing is uncertain.

Mutations have also been described which account for two murine models of OTC deficiency: sparse fur, and sparse fur-ash. Sparse fur mice exhibit decreased OTC activity at physiologic pH, but actually have 150% normal OTC activity at alkaline pH. In addition, these mice have greater than normal amounts of OTC present in liver. The mutation causing this phenotype has been characterized and involves a C to A transversion resulting in substitution of an asparagine residue for a histidine at amino acid position 117 (Veres *et al.*, 1987). The other mouse model, sparse fur-ash, which also exhibits abnormal skin and hair, displays a reduced level of enzyme activity and cross -reactive material. The mutation identified in these animals is a G to A missense mutation in the last position of exon 4, which is similar to the defect in human OTC described above (in which the splice donor sequence at the end of exon 1 is

altered) in that the defect also involves a 5' splice donor sequence (Hodges and Rosenberg, 1989). In addition to causing an amino acid substitution and reducing levels of mRNA, this mutation also activates a cryptic 5' splice donor sequence in intron 4. This results in the production of an elongated mRNA containing some sequence from intron 4, in addition to normal length mRNA, both of which are present at much reduced levels compared to normal.

With the advent of the polymerase chain reaction (Mullis and Faloona, 1987; Saiki *et al.*, 1988), which has greatly simplified many aspects of molecular analysis, it can be expected that molecular defects in a broad range of diseases will be revealed at an increasing pace. Much as the method of site directed mutagenesis has been employed to define critical regions of proteins required for proper synthesis and function, analysis of mutations causing disease can aid in the process of characterizing structure-function relationships for different proteins. In addition, by identifying regions which are especially prone to mutation, such analyses may facilitate improved methods of diagnosis through direct mutational analysis. Because diagnosis of inherited disease often relies upon linkage, which may be difficult to establish, and because (especially for X-linked diseases) new mutations may account for a substantial number of cases, improved methods of direct analysis of mutations are desired. Recently developed techniques, such as chemical mismatch cleavage (Cotton *et al.*, 1988) may facilitate easier direct mutational analysis in the future.

CASE HISTORIES

In the following section case histories are provided for those patients in whom mutations believed to represent the cause of OTC deficiency were identified. Each of these patients succumbed to hyperammonemia that was subsequently attributed to OTC deficiency on the basis of a liver assay for OTC activity.

Case 1

Patient OW was the male product of a pregnancy which was believed to be at or near term, although the mother's LMP was uncertain. Gestation was remarkable for maternal use of alcohol, tobacco, marijuana, and cocaine, but was without complications. Birth was by spontaneous vertex delivery and Apgar scores were 7 and 8 at 1 and 5 minutes, respectively. Shortly following birth he developed tachypnea and grunting which improved on 30% oxygen. The next day tachypnea recurred at a rate of 80 respirations per minute and at 40 hours he had a 6 minute generalized seizure associated with a temperature of 102.6°F. Chest X-ray was unremarkable. Ampicillin and gentamicin were started for presumed sepsis. At 43 hours, he was transferred to the neonatal unit and intubated due to prolonged apnea and repeated seizures. An ammonia level which was drawn at 24 hours was significantly elevated to 990 μg/dl (normal serum ammonia values in newborns are 110-180 μg/dl). A metabolic error was considered at this time. Peritoneal dialysis was started with some improvement of hyperammonemia to 330 on day 3. He continued to be unresponsive, however, and was maintained on a respirator. On day five he was extubated

after he had some improvement in level of consciousness, but his ammonia level, though improved, was still elevated to 160 one week after delivery. Further laboratory tests revealed elevated serum glutamine and elevated urine orotate. He was started on a protocol to reduce serum ammonia using benzoate and also continued to receive peritoneal dialysis, but after 4 weeks his hyperammonemia continued and he again required use of a respirator. At this point he was taken off the protocol and dialysis at the parents' request, and, interestingly, his ammonia then fell to 100. Unfortunately, he continued to do poorly and 10 weeks after birth he developed tachycardia and died. The patient's mother was of Asian descent and his father was black. Family history was remarkable for a maternal cousin who carried a diagnosis of multiple carboxylase deficiency. A subsequent pregnancy by the mother ended in miscarriage.

Case 2

RD was a white male product of a full term pregnancy with minor complications including headaches, first trimester spotting, and iron deficiency anemia. He was the first pregnancy of the mother and was delivered vaginally without complications. At 24 hours he was noted to feed poorly, and at 48 hours was in hyperammonemic coma with an ammonia of 4000 μg/dl. After transfer to a tertiary care hospital, he was treated with peritoneal dialysis. On the basis of elevated urine orotate and other metabolic screening, he was given a diagnosis of ornithine transcarbamylase deficiency, although liver assay was not performed. Management with dialysis and nitrogen-free essential amino

acids reduced his ammonia to less than 100. He was able to be discharged at 3 months with the ability to cry and suck and was maintained on a restricted protein diet of 1 to 1.5 gm of protein per day. He was developmentally retarded following discharge, being minimally responsive, and had daily myoclonic seizure activity which could not be controlled by anticonvulsants. Although he was maintained predominantly by dietary management only, he required almost monthly admission for hyperammonemia, which was usually heralded by vomiting and protein intolerance requiring cessation of all dietary protein. At 15 months of age the patient was admitted for a trial of phenylacetic acid and benzoate management, which was of some aid in reducing serum ammonia. His subsequent course continued to be downhill, and at 18 months of age he became hypotensive in the intensive care unit and succumbed to cardiopulmonary arrest. Family history was unremarkable for neonatal death or miscarriage. The patient's mother reported a history of recurrent headaches. Upon protein loading she experienced a headache and had increased urinary orotate, consistent with carrier status for OTC deficiency. A subsequent pregnancy by the mother yielded an affected hemizygous male who was treated starting at birth with reasonable control of hyperammonemia; the child is reportedly growing and developing reasonably well at 5 months.

Case 3

Unfortunately, the clinical information regarding this patient is extremely limited due to difficulties in obtaining relevant data from the original referring physician. What is known is that he died in the newborn period, most likely at

around 48 hours of age, with very high plasma ammonium levels, and had an elevated urinary orotate.

MATERIALS AND METHODS

RNA Preparation

Total human liver RNA, preserved at -80°C prior to use, was prepared by the guanidine thiocyanate extraction procedure as described (Chirgwin *et al.*, 1979). Guanidine thiocyanate was purchased from Eastman Kodak Company; guanidine hydrochloride and 30% Antifoam A were obtained from Sigma. The total RNA recovered was dissolved in diethylpyrocarbonate-treated water.

DNA Preparation

Genomic DNA was prepared by homogenizing liver in lysis buffer (200 mM Tris, pH 8.0, 100 mM EDTA, 1% SDS, 100 μg/ml proteinase K)(20 ml/g liver) and incubating overnight at 50°C with shaking. This solution was extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), followed by extraction with chloroform: isoamyl alcohol (24:1) alone. DNA was precipitated with 1/10 volume sodium acetate and 2 volumes of ethanol, washed with 70% ethanol, lyophilized, and resuspended in RNase digestion buffer (50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 10 mM NaCl, 100 μg/ml RNase A)(0.5 ml/g liver). After 3-4 hours digestion at 37°C, the solution was extracted as described above and dialyzed overnight at 4°C vs. 10 mM Tris-Cl, pH 8.0, 1 mM EDTA.

Reverse Transcription

Five micrograms of total RNA in a volume of 22.5 μl were heated to 80°C for 3 min, then reverse transcribed in a reaction mixture containing 50 mM Tris-

Cl, pH 8.3, 75 mM KCl, 10mM $MgCl_2$, 4.35 mM dithiothreitol, bovine serum albumin at 0.175 mg/ml, 0.5 mM of each deoxyribonucleoside triphosphate, 1.25 Units RNasin (Boehringer Mannheim or Promega), 70 Units AMV reverse transcriptase (Boehringer Mannheim), and 70 ng IXaR reverse primer (5'-CAAT-GGCAAAGCATATCATA-3') in a total volume of 50 μl. A similar reaction was carried out using P2, a reverse primer for ß-propionyl CoA carboxylase (ß-PCC) whose sequence was 5'-AGGCCTTCCTGGTGATGACTGTGAC-3'. Each reaction was incubated at 44°C for one hour; an additional 40 Units RNasin and 20 Units of reverse transcriptase were added, and the incubations were continued at 44°C for another 40 min. The cDNA samples were frozen at -20°C until used in PCR amplification.

PCR amplification of cDNA and genomic DNA

Reactions were done in a total volume of 100 μl, essentially as described (Saiki *et al.*, 1988). To a 5 μl aliquot of each reverse transcription mixture was added 10 μl 10X reaction buffer (0.5M KCl, 100 mM Tris-Cl, pH 8.3, 15 mM $MgCl_2$, gelatin at 200 μg/ml), 16 μl dNTP mixture (1.25 mM each), 5 μl each of IL and IXaR (or P1 and P2 when amplifying a region of ß-PCC cDNA) primers (20 μM), 2.5 Units *Taq* DNA polymerase (United States Biochemical Corp.), and H_2O. The sequence of IL primer is 5'-GGGCATAGAATCGTCCTTTA-3' and that of P1 is 5'-TCCAATATTCCACTCATCACTTTTG-3'. Each sample was overlaid with 100 μl mineral oil to prevent evaporation. The IL and IXaR primers for OTC were selected because they yielded a 1242 bp product which covered the entire 1062 bp coding region of OTC. The P1 and P2 primers amplified a 150 bp

fragment within the coding region of ß-PCC. Amplification conditions consisted of an initial denaturation at 94°C for 5 min., followed by 40 to 50 cycles of denaturation at 94° for 2 min., annealing at 55° for 2 min., and extension at 72° for 3 min. Aliquots were analyzed on 1% SeaKem or SeaPlaque agarose (FMC Bioproducts).

Genomic amplifications were performed similarly, except that 1 μg of total genomic DNA was used as template. Primers used were VL (5'-TCTTTTTCT-TGGTTTACCAC-3'), VR (5'-GTAAGACAAATAAATAAACC-3'), VIIL (5'-TTTAAA-TTCCTTCCTCCTTT-3'), and VIIR (5'-CCTGAGAGAGCATCAATTTG-3'). VL and VR amplified all of exon 5 as well as the flanking portions of intron 4 and intron 5. VIIL and VIIR yielded amplified exons 7 and 8, all of intron 7, and flanking portions of intron 6 and intron 8. Products were analyzed on 3% NuSieve agarose (FMC Bioproducts).

Agarose Gel Electrophoresis

Electrophoresis of DNA samples on agarose gels was done in either TAE (0.04 M Tris acetate, 0.001 M EDTA) or TBE (0.089 M Tris-borate, 0.089 boric acid, 0.002 M EDTA) buffer containing 0.35 ug/ml of ethidium bromide. DNA in the gels was then visualized and photographed under UV light. Samples were added to loading buffer (0.025% bromophenol blue, 0.025 xylene cyanol, and 5% glycerol) and electrophoresed at constant voltage.

Restriction Endonuclease Digestion, Cloning, and Sequencing of PCR products

Following each amplification reaction, samples were extracted and ethanol precipitated as above and resuspended in an appropriate volume of 10 mM Tris-Cl, pH 7.5, 1 mM EDTA. Aliquots were taken for restriction endonuclease digestion under conditions described by the manufacturer (Boehringer Mannheim or New England Biolabs).

Subcloning of PCR products was carried out by standard methods (Maniatis, *et al.*, 1982) using pBluescript KS (Stratagene) as a vector. PCR products were either phosphorylated with T4 polynucleotide kinase (Promega) (for direct blunt-ended ligation) or digested with restriction enzymes (for cohesive end ligation) prior to incubation with T4 DNA ligase (New England Biolabs) and the appropriately digested vector. Digested vectors with blunt ends were treated with calf intestinal phosphatase (Boehringer Mannheim) before ligating. Both DNA inserts and vector were purified by agarose electrophoresis, followed by recovery by electroelution as described (Maniatis *et al.*, 1982). Ligations were carried out at 16°C (cohesive end ligation) or room temperature (blunt-ended ligation) for 12-20 hours using 200 ng of vector DNA and a several-fold molar excess of insert. Aliquots were taken from ligation mixtures and used to transform DH5-α cells (Bethesda Research Labs) according to the supplier's protocol. Cells were plated on LB (Luria Broth) plates containing 50 ug/ml ampicillin, 0.01 M $MgSO_4$, 0.2 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) and 0.04 ug/ml 5-bromo-4-chloro-3-indoyl ß-D-galactopyranoside (X-gal) for blue-white color selection of recombinant colonies.

Minipreparations of DNA of selected colonies (Birnboim and Doly, 1979) were analyzed by restriction digestion and subjected to dideoxynucleotide sequencing using Sequenase (United States Biochemical) according to the supplier's specifications. Samples from sequencing reactions were boiled 3-5 minutes and electrophoresed on 6-8% denaturing acrylamide gels in TBE buffer using 60-70 Watts at constant power. Gels were fixed in 10% methanol, 10% acetic acid for 60-90 minutes, dried, and exposed to XAR-5 film at room temperature.

Northern Blotting

10 μg of total liver RNA was electrophoresed on a 1.1% agarose gel in formaldehyde as described (Maniatis, *et al.*, 1982). Following electrophoresis the gel was soaked 30 min. in 10X SSC (1X SSC is 150 mM NaCl, 167 mM Na-Citrate, pH 7.0) and then directly transferred to nitrocellulose as described. The filter was baked 2 hours under vacuum at 80°C. Filters were prehybridized in 50% formamide, 5X SSC, 5X Denhardt's solution (1X Denhardt's solution is 0.02% each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin in H_2O), 50 mM Na_2PO_4 (pH 7.0), and 250 ug/ml sheared salmon sperm DNA for 3-6 hours at 42°C. Filters were hybridized with ^{32}P labeled cDNA probes prepared by the oligolabelling method (Feinberg and Vogelstein, 1984) using a priming kit (Boehringer Mannheim) according to the supplier's recommendations. Material for oligolabelling was prepared by PCR amplification of cDNAs from plasmids. Generally, 100-150 ng of this material to be used as a probe was used in the oligolabelling reaction. Using this method, 1 to 2 x 10^8 cpm/ug probe was obtained. Unincorporated nucleotides were removed by spun-column

chromatography, and probes were boiled 5 minutes before adding to hybridizations. Hybridization was carried out in 50% formamide, 5X SSC, 5X Denhardt's solution, 20 mM Na_2PO_4 (pH 7.0), 10% dextran sulfate, and 75 ug/ml sheared salmon sperm DNA at 42°C for 12-20 hours. Filters were washed 10 minutes with 2X SSC, 0.1% SDS at room temperature, then 30-60 minutes in 0.1X SSC, 0.1% SDS at 50-55°C. Filters were exposed to XAR-5 film with an intensifying screen at -70°C for one day to one week. Following hybridization, filters were washed in 2 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.1% SDS for 20-30 minutes at 60°C, after which they could be reused.

Immunoprecipitation and Western Blotting

Liver tissue was homogenized in NETS buffer (150 mM NaCl, 0.5% Triton X-100, 10 mM EDTA, 0.25% sodium dodecyl sulfate) as a 10% (w/v) solution. Homogenates were centrifuged at 8,000 rpm in an SS-34 rotor (Sorvall) to remove insoluble materials, and 100 μl of this homogenate (10 mg liver) was used for immunoprecipitation. Five μl each of anti-OTC and anti-methylmalonyl-CoA mutase ("mutase") antiserum were added to the homogenates, and the solutions were rocked overnight at 4°C. Immune complexes were recovered by incubation with 50 μl 10% (w/v) *Staphylococcus aureus* cells (Bethesda Research Labs) and centrifugation (Kessler, 1981). The *S. aureus* cells were washed five times with 1.0 ml of RIPA buffer (10mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1.0% Triton X-100, 1.0% sodium deoxycholate), and the immunoprecipitated proteins were vortexed into 40 ul loading buffer (62.5 mM Tris-Cl, p 6.8, 2.0% SDS, 10% glycerol, 5.0% ß-mercaptoethanol, 0.001%

bromophenol blue) and boiled for 5 min. (Kessler, 1981). Supernatants were loaded onto an SDS-polyacrylamide gel according to Laemmli (1970). The stacking gel consisted of 3.8% acrylamide/0.16% bis-acrylamide and the running gel, 8% polyacrylamide/0.34% bis-acrylamide. After electrophoresis, the gel was placed in electroblotting transfer buffer (20 mM Tris, 150 mM glycine, 20% methanol) for about 10 minutes, and then electroblotted onto nitrocellulose at 100 milliamperes overnight as described (Towbin *et al.*, 1979). The filter was soaked in a solution of 5% Carnation dry milk in PBS buffer (0.8% NaCl, 0.02% KCl, 0.115% Na_2HPO4, 0.02% KH_2PO4) for 2-3 hours, then transferred to 50 ml of a solution of 13% bovine hemoglobin and 0.02% sodium azide in PBS to which 500 μl of both anti-OTC and anti-mutase antiserum had been added. The filter was washed in PBS for ten minutes twice, then washed in PBS with 0.5% Nonidet P-40 for ten minutes two more times. The filter was soaked in 50 ml of 5% Carnation dry milk in PBS containing 50 μl [^{125} I]-protein A. Washes in PBS and Nonidet P-40 were repeated as above, after which the filter was exposed to Kodak XAR-5 film with intensifying screens at -70°C.

RESULTS

Amplification of cDNAs prepared from patient livers

To detect mutations within the 1062 base coding sequence of OTC, RNA was isolated from eleven livers taken at autopsy from male patients found to have OTC deficiency by assay of OTC activity and was used to synthesize single stranded cDNAs. This material was subjected to PCR amplification using a set of primers which yielded a 1242 bp product from normal subjects that included the entire coding region of the OTC cDNA (Figure 1). In seven of the patients studied, cDNA products were observed following PCR amplification and agarose gel electrophoresis. A control cDNA template, pHO731 (Horwich *et al.*, 1984), was used for comparison. Three of these seven cDNAs were found to be shorter than the normal cDNA (Figure 2). The other four appeared to be of normal length (not shown). In order to determine whether the inability to amplify cDNA from the other four patients was due to lack of OTC expression or overly degraded RNA, a second amplification was performed using primers for a 150 bp segment of ß-propionyl CoA carboxylase (ß-PCC) cDNA. As seen in Figure 3, two of the patients in whom no OTC cDNA could be demonstrated yielded clear bands corresponding to amplified ß-PCC cDNA. Thus, it appeared that these patients did not express sufficient OTC mRNA to be amplified, while in the other two samples it seemed that degradation of the RNA made them uninformative in this analysis.

Localization of deletions in cDNAs by restriction enzyme analysis

Amplified cDNAs were subjected to digestion with restriction endonucleases in order to localize the regions in the coding sequence from which sequences were missing, as well as to look for smaller deletions which may not have been apparent in the full length products. Separation of restriction fragments on NuSieve agarose revealed that, in one patient, there was a deletion in the cDNA of approximately 10-15 bp located between the unique Acc I and Xho I sites (Figure 4). In the other two patients, similar analyses showed deletions 5' to the Asp 718 (Kpn I) site and 3' to the most distal Hae III site which removed an Msp I site and approximately 50 bp of the cDNA (Figures 5 and 6). Figure 7 shows cleavage sites of restriction enzymes used to localize cDNA deletions and the normal expected band sizes for the digests performed. The apparently full length cDNAs from the other four patients from whom these amplified cDNAs could be produced showed no evidence of deletions (data not shown).

Identification of mutations at the cDNA level by DNA sequencing

In order to characterize the mutations in those three patients in which they could be localized, appropriate regions of PCR amplified cDNA were subcloned and sequenced. As shown in Figure 8, the mutation in patient OW between the Acc I and Xho I site was found to be a 12 bp deletion corresponding to the first 12 base pairs of exon 5. No other changes in sequence were found. Thus, the net effect of the mutation was an in-frame excision of nucleotides coding for 4 amino acids (val-leu-ser-ser) in the mature

protein. In the other two patients, sequencing of the relevant portion of the cDNAs demonstrated that both were missing the 54 base pairs which correspond to exon 7 (Figure 9). Again, the mutation observed was an in-frame cDNA deletion with no evidence of other changes. These cDNA deletions are shown schematically in Figure 10. In all three cases, the sequencing results suggested that RNA splicing errors were responsible for the enzyme defects.

Identification of mutations in genomic DNA by DNA sequencing

In order to characterize mutations in the genomic DNA which could have led to the presumed abberant splicing, two sets of primers were chosen which corresponded to intron sequences flanking the exons involved. These primers were used to amplify the regions of interest from genomic DNAs isolated from patient livers, and the fragments were subcloned and sequenced using a protocol similar to that used to sequence the cDNAs.

In patient OW, whose cDNA was missing the first 12 bp of exon 5, sequencing of genomic DNA revealed that the flanking sequence of intron 4 contained a point mutation substituting a T for the A in the AG dinucleotide that normally ends introns (Figure 11). Because the splicing system in primates requires introns to begin with GT and end with AG for proper splicing to occur (Breathnach and Chambon, 1981; Mount, 1982), it was likely unable to recognize the normal intron 4/exon 5 splice junction in this patient's precursor RNA and instead utilized a nearby cryptic splice site within exon 5 which conformed to the "GT-AG rule" for correct RNA splicing. Thus, the deletion seen

at the cDNA level resulted from a point mutation within the 3' splice acceptor site which led to abberant splicing.

In the two patients missing exon 7, two different point mutations were found in genomic DNA which may have caused abnormal splicing. In both patients, no sequence abnormalities were observed in the 3' splice acceptor region at the intron 6/ exon 7 junction. On the other hand, point mutations were seen in the 5' splice donor region around the exon 7/intron 7 junction. In one patient, the mutation was a substitution of a C for the T in the initial dinucleotide of intron 7, changing GT to GC (Figure 12). This mutation also violated the GT-AG rule referred to above and likely made the normal 5' splice donor region unsuitable for use in joining exon 7 to exon 8. The other patient had an A to G substitution in the third position of intron 7 (Figure 13). Although this point mutation does not involve the invariant GT of the splice donor, it does affect the established consensus sequence for 5' splice donors. It should be noted that both of these mutations cause the preceding exon to be skipped, even though the 3' splice acceptor which would function to join exon 6 to exon 7 is unchanged. No evidence was seen in the cDNA sequences to suggest either that normal splicing occurred or that a cryptic 5' splice donor site within exon or intron 7 was activated in these patients.

Quantitation of OTC mRNA by Northern Analysis

Analysis of patient livers to determine the amount of OTC mRNA was carried out to determine whether any of these OTC deficiencies were associated with reduced amounts of message. This was of particular interest for those

patients with splicing defects, as it was desirable to know if the abberant splicing was also carried out at reduced efficiency. RNA samples prepared from patient and control liver were probed with a nearly full length OTC cDNA obtained by amplifying an OTC cDNA template with the IL and IXaR primers described previously. After exposure, the filter was washed and rehybridized with a probe containing about 300 bases of coding sequence for methylmalonyl-CoA mutase as an internal control. Figure 14 shows Northern blots obtained after probing for both OTC and mutase. Among the patients with splicing errors, patient MC contains a significant, although decreased, amount of OTC mRNA compared to normal, whereas patients RD and OW have minimally detectable levels of OTC mRNA. Of the remaining patients, one actually appears to have normal to increased amounts of OTC message, whereas two others have reduced levels of expression. One of these samples contained no bands for either OTC or mutase (presumably because of RNA degradation) and thus was uninformative.

Analysis of liver homogenates for OTC cross-reacting material

In order to assess the effects of these mutations at the protein level, we assayed immunologically for OTC cross-reacting material in patient liver tissue. Homogenates were prepared from liver and immunoprecipitated with a polyclonal anti-OTC antiserum, as well as with an antiserum against methylmalonyl-CoA mutase as a control. Immunoprecipitated material was then electrophoresed on an SDS-polyacrylamide gel, blotted onto nitrocellulose, and probed with anti-OTC and anti-mutase antisera. As shown in Figure 15, a homogenate prepared from liver of a patient unaffected by OTC deficiency

(lanes 1 and 2) yielded clear bands corresponding to both the 36 kDa mature OTC subunit and the 77.5 kDa mutase protein. Liver from each of the patients with OTC deficiency also showed clear evidence of material cross-reacting with mutase, but none of the samples for which the defect is described demonstrated a band corresponding to mature OTC or to the shorter proteins predicted from the deletions, even when the filter was exposed for as long as 10 days. Of the other four patients from whom full length cDNA could be amplified, one displayed a minimal amount of OTC cross reacting material, the others had none. Thus, although these splicing mutations produced in-frame alterations in the OTC mRNA, no cross-reactive OTC protein could be identified in the livers from these patients. The molecular defects in the other four patients remain to be defined.

DISCUSSION

We were able to amplify cDNAs containing the coding region of the OTC mRNA from liver tissue of seven of eleven 11 male patients with OTC deficiency. Among these seven patients, we detected and identified RNA splicing errors believed to be responsible for clinical OTC deficiency in three. These mutations are single base changes altering the highly conserved sequences seen at intron-exon boundaries and prevent the splicing apparatus from properly recognizing and acting at the normal splice junctions. One of these mutations is a single base alteration (A→T) in the 3' splice acceptor AG dinucleotide at the end of intron 4, making this region an unacceptable splice junction. As a result, a cryptic 3' splice acceptor within exon 5 is used, producing a deletion of the first 12 base pairs of exon 5 in the resulting mRNA. The other two mutations both involve the deletion of exon 7 from the mRNA as a result of point mutations within the 5' splice donor sequence at the junction between exon 7 and intron 7. One of these mutations changes the second base of intron 7 such that the GT dinucleotide becomes GC, whereas the other one changes the A in the third position of the intron to a G. Both of these mutations result in skipping the preceding exon, even though the preceding 5' and 3' splice consensus sequences are apparently intact.

The conserved sequences present at 5' and 3' splice junctions that are required for correct splicing have been characterized by several authors (Breathnach and Chambon, 1981; Mount, 1982; Ohshima and Gotoh, 1987; Shapiro and Senepathy, 1987; Aebi *et al.*, 1986). By analyzing a large number

of eukaryotic splice junction regions, consensus sequences have been established which determine the suitability of these regions to function in normal splicing. For 5' splice donors, a 9 base consensus sequence of $^{C}_{A}$AG:GT$^{A}_{G}$AGT has been established (Mount, 1982), although some authors do not include the first nucleotide in this consensus (Shapiro and Senepathy, 1987). This sequence is notable for its ability to form base pairs with the sequence AC$\psi\psi$ACCUG of U1-snRNA that binds to 5' splice donors during the splicing process (Mount *et al.*, 1983). Similarly, a consensus sequence of $(Py)_{n>10}$ N^{C}_{T}AG:G has been given for 3' splice acceptor sites. Within these consensus regions, the dinucleotides GT for 5' donors and AG for 3' acceptors have been shown, with few exceptions, to be invariant and required for correct splicing (the "GT-AG rule"; Breathnach and Chambon, 1981; Mount, 1982). A scoring system has been descibed (Shapiro and Senepathy, 1987) which gives a numerical value for any proposed 5' or 3' consensus sequence, based on the frequency of each nucleotide in normal splice sequences. Using this system, the consensus sequences AG:GTAAGT and PyPyTTPyPyPyPyPyPyNCAG:G yield a score of 100, and most splice sequences normally used have a score greater than 70.

Figure 16 shows the scores for the normal splice sites and the mutant ones detected in this group of patients, as well as for the proposed cryptic site within exon 5. The mutations in patients OW and RD, which involve the highly conserved AG and GT dinucleotides, respectively, significantly reduce the scores for these sequences. In patient MC, on the other hand, the A→G mutation in the third position of intron 7 produces only a slight reduction in the

score. It is interesting that such a mutation leads to abnormal splicing, as demonstrated in this patient, even though it might be predicted that normal splicing could still occur at this site. In some β-thalassemia patients, for example, point mutations in the consensus sequence outside of the terminal dinucleotides give rise to aberrantly spliced products, as well as to some normally spliced ones (Kazazian and Boehm, 1988; Treisman *et al.*, 1983; Atweh, *et al.*, 1987). We see no evidence, however, of any normally-spliced OTC mRNA from patient MC upon agarose gel analysis of PCR amplified cDNA (see Figure 2 right, lane 3).

Several papers and reviews have described the current model of a spliceosomal complex which is involved in the process of splicing primary transcripts into mRNAs that are then exported to the cytoplasm (Sharp, 1987; Green, 1986; Padgett *et al.*, 1986; Steitz, 1988; Maniatis and Reed, 1987;). Although the understanding of the pathway of RNA splicing is still in evolution, the general mechanism which has been proposed suggests the formation of a spliceosomal complex containing the small nuclear ribonucleoproteins (snRNPs), U1, U2, U4, U5, and U6, and newly-transcribed RNA, which is involved in recognition and splicing at appropriate 5' and 3' sites. An early step in the splicing process is believed to involve recognition of a 5' splice donor by U1 snRNP via base pairing with the homologous region of U1 (Lerner *et al.*, 1980; Zhuang and Weiner, 1986; Mount *et al.*, 1983). U2 and U5 snRNPs are believed to participate in recognition at the 3' end of an intron, followed by the addition of U4 and U6 (usually associated as one particle, U4+U6) and final spliceosome assembly (Maniatis and Reed, 1987; Sharp, 1987). The U5 particle

is postulated to be involved in recognition at the 3' splice site, and U2 recognizes a weakly conserved branch sequence located about 20 to 40 bases upstream of the 3' splice site. Once assembly of the spliceosome has occurred, the excision of intronic sequence and joining of the exons occurs as a two step process. First, the 5' splice site is cut, followed by formation of a lariat structure in which the branch site is joined to the excised upstream end of the intron (Sharp, 1987; Reed and Maniatis, 1985). Then, in the second step, the exons are joined as the intron is cut at the 3' splice site and released as a lariat.

How this mechanism functions to appropriately excise all introns from genes containing multiple introns has not been established. Initially, a 5' to 3' directional scanning model was proposed (Lang and Spritz, 1983) in which the spliceosomal complex moved linearly along the pre-mRNA, selecting 5' and 3' splice sites as it proceeded. Such a model is unable to explain the exon skipping described in two patients here, however, in which mutations in 5' splice donors cause skipping of the previous exon. Similar effects have also been seen in certain patients with phenylketonuria (Marvit *et al.*, 1987), Ehlers-Danlos syndrome (Weil *et al.*, 1988), leukocyte adhesion deficiency (Kishimoto, *et al.*, 1989), APRT deficiency (Hidaka *et al.*, 1987), and acute intermittent porphyria (Grandchamp *et al.*, 1989), as well as in the hamster dihydrofolate reductase gene (Mitchell *et al.*, 1986). A similar mutation was also seen in one of the ß-thalassemias, but in this case another RNA species was also produced in which a cryptic 5' splice site in the intron was used (Treisman *et al.*, 1982). Whatever model of splicing is proposed would need to show how, in such mutants, the preceding exon could be spliced out, even though the preceding 5' and 3'

splice sequences are intact. Marvit *et al.* (1987) have proposed that cooperative interactions between snRNPs may be at work, such that ribonucleoproteins are unable to bind efficaciously at a 3' acceptor site unless snRNPs are also present at the downstream splice donor. Consistent with this suggestion is a recent model which describes exon definition as an early step required for efficient splicing (Robberson *et al.*, 1990). According to this scheme, U1 also functions at 3' splice sites, and it is proposed that this interaction at the 3' splice site dictates sliceosome assembly. The authors suggest that this interaction at the 3' splice site includes a mechanism for scanning downstream for an acceptable 5' splice donor and that recognition of a suitable donor sequence within approximately 300 bases is necessary for efficient spliceosome complex formation at the preceding 3' acceptor site. This step of "exon definition" would thus be a necessary element of the splicing pathway, required in order for the preceding intron to be excised. If no acceptable 5' splice site can be found within ~300 bases of the 3' splice site, exon definition does not occur, stable assembly intermediates do not form, and exon skipping can result.

It is also of interest that, although the mutation in patient MC alters the splice consensus sequence, the mutated sequence still appears to be a reasonably good donor. It has been shown that the lower limit of intron size allowable for proper splicing to occur is about 65-80 nucleotides (Steitz, 1988; Weiringa *et al.*, 1984), and, in fact, the intron separating exon 7 from exon 8 consists of only 80 bases. This suggests that splicing of exon 7 to exon 8 may already be an inefficient process and that, when the splice site at the end of exon 7 becomes even slightly less effective, correct splicing may become even

more untenable. This argument, however, is not entirely consistent with the model of exon definition given above, because stable exon definition should still occur to allow splicing of intron 6, irrespective of the efficiency of the downstream splicing event. Interactions between snRNPs at neighboring splice sites may possibly dictate the most likely splicing pattern to occur. It is hoped that a further analysis of mutations such as these will contribute to better understanding of the mechanisms by which the spliceosomal machinery processes RNA transcripts into mature mRNAs.

Another question raised by these defects is what effect these mutations have at the level of the mature OTC protein. Because the deletions in the mRNA are in-frame, it is possible that the products of translation could be at least somewhat functional in catalysis. When homogenates from these patients were assayed for OTC cross-reacting material, however, none was observed. In addition, OTC assays showed no evidence for any OTC activity in these patients (data not shown). The step (or steps) in the complex pathway from RNA transcript to mature protein responsible for the enzyme deficiency in these patients remains to be defined. Export to the cytoplasm of the mRNAs or their stability could be affected. The Northern blot data demonstrate that patient MC has a reduced, but significant amount of OTC message present, whereas patients RD and OW have greatly reduced amounts compared with normal. While this suggests that reduced levels of processed mRNA in the cytoplasm may be one consequence of these mutations, the fact that one patient had nearly normal levels of OTC mRNA yet still produced no OTC cross-reacting material, emphasizes the observation that decreased amounts of message

cannot fully explain the deficiency observed. It could be that the deletions in the protein omit important regions which direct mitochondrial import, mediate trimer assembly, or confer stability. In all of these cases, the mutant protein would be likely to be rapidly degraded. Further studies may determine more precisely the relationship between the mutations described here and the observed enzyme deficiency.

Further study is also necessary to determine the nature of the molecular lesions producing disease in the other patients studied. Investigation of other single gene disorders such as the thalassemias and hemophilias (Kazazian and Boehm, 1988; Youssoufian, *et al.*, 1988; Weatherall, 1987) have demonstrated that common types of mutations leading to disease are deletions, single base nonsense and missense changes, splicing errors, and frameshift errors. Less commonly observed are such changes as alterations in promoter regions affecting transcription, inversions, and insertions. In the four additional patients in whom amplifiable cDNAs could be produced, it is likely that small mutations such as point mutations or single to several base insertions or deletions resulting in frameshifts have occurred. These cDNAs were digested with several combinations of restriction endonucleases in an attempt to locate regions containing deletions or insertions without success. They were also digested with Taq I to determine whether any of these patients had an alteration in a site for this enzyme as has been seen in unrelated patients by other workers, but none exhibited any alterations in cDNA Taq I sites. Although these mutations are most likely small ones, only one of them demonstrated any OTC cross reacting material, and, even then, the amount was minimal. In those four

patients in whom no amplified cDNAs were produced, it is difficult to determine what mechanism of mutation is most likely to account for disease. In two of these patients a control amplification with primers for ß-PCC yielded cDNA products suggesting that mRNA was present. OTC mRNA may be more unstable than ß-PCC message, however, and it may simply be that the liver samples used were too degraded for obtaining OTC mRNAs in all four. Therefore, another approach may be needed to detect and characterize mutations in these patients.

In summary, we were able to identify 3 cases of aberrant RNA splicing out of seven OTC deficient patients from whom we could obtain sufficient quantities of cDNA for study using PCR. These are the first cases of human OTC deficiency for which aberrantly spliced products have been observed. Given the number of these errors found among these patients, it may be that abnormal RNA splicing is a common mechanism of disease in OTC deficiency, just as many of the thalassemias have been found to be due to errors of RNA splicing. To date, only a limited number of inherited diseases have been found to be due to splicing errors. The use of techniques which simplify gene analysis, such as PCR, may reveal that splicing errors are a more common mechanism of mutation than has previously been believed. Better understanding of the mechanisms of these diseases will be crucial not only to simplify diagnoses, but also to develop strategies for their treatment.

Figure 1:

Structure of OTC genomic DNA and its representation in the OTC cDNA. Primers IL and IXaR, which correspond to 5' and 3' non-coding sequence, were used to amplify a region of the cDNA containing the entire coding sequence of the cDNA, producing a 1242 bp product in control samples. The numbers below the representation of the cDNA indicate exon lengths in nucleotides.

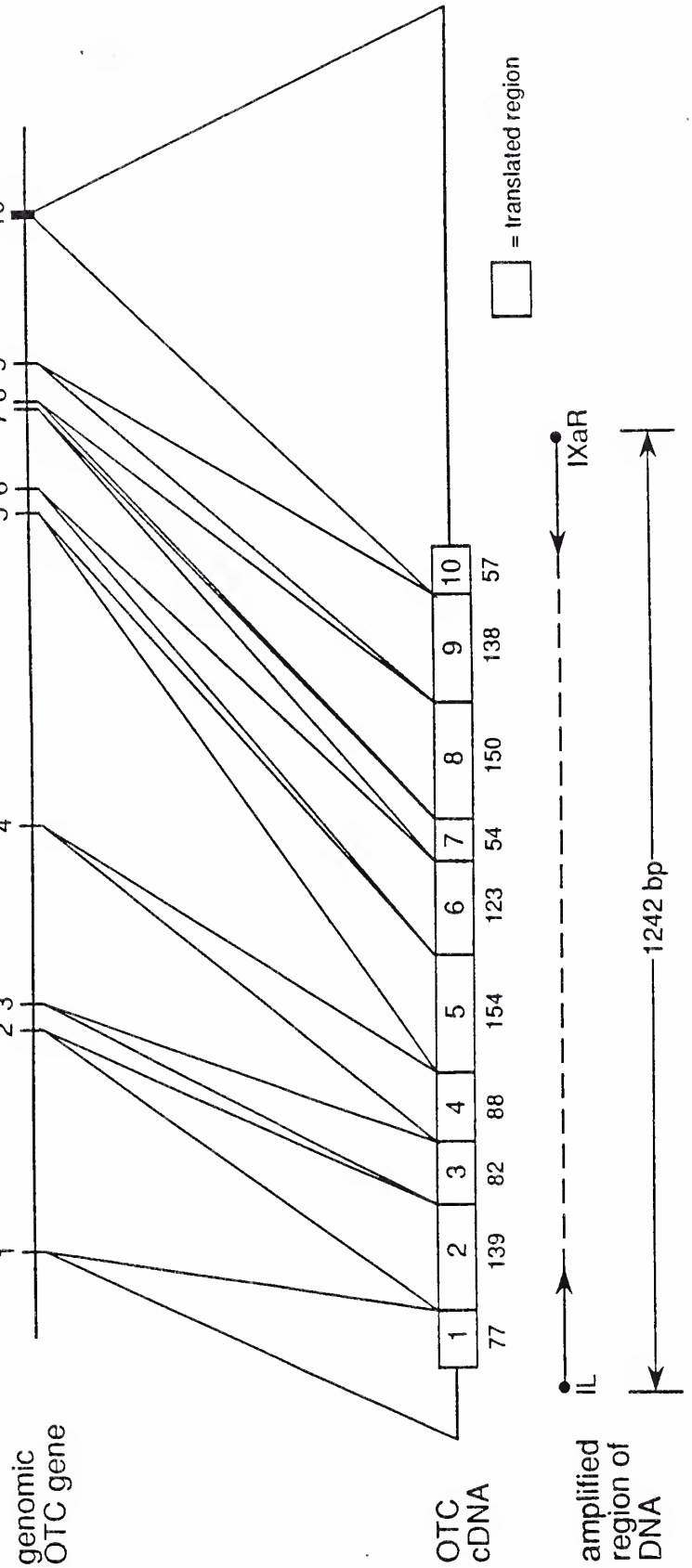

genomic OTC gene
1
2 3
4
5 6
7 8 9
10
OTC cDNA
1
2
3
4
5
6
7
8
9
10
77
139
82
88
154
123
54
150
138
57
= translated region
amplified region of DNA
IL
IXaR
1242 bp

Figure 2:

Agarose gel electrophoresis of amplified cDNAs from patients exhibiting less than full length products. 10 μl aliquots were taken from each 100 μl PCR amplification mixture and electrophoresed on 1% agarose. A control OTC cDNA template (pHO731) was used for comparison. Left: Lane 1: Patient RD. Lane 2: pHO731. Lane 3: *Hae* III-digested ØX174 size markers. Right: Lane 1: Patient OW. Lane 2: Patient MC. Lane 3: pHO731. Lane 4: *Hae* III-digested ØX174 size markers.

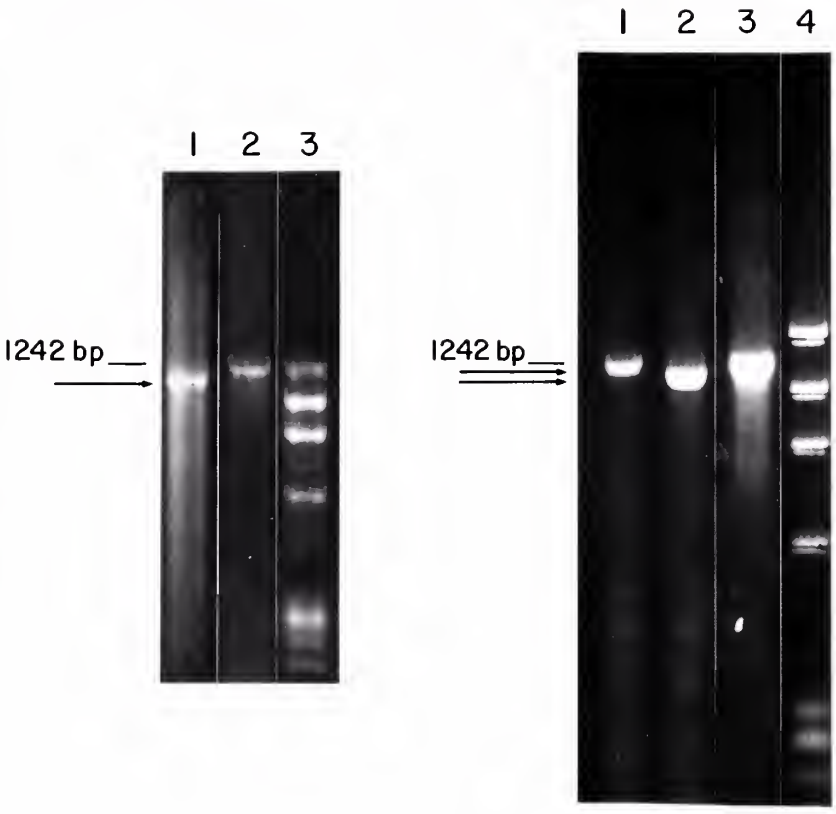

1 2 3
1242 bp
1 2 3 4
1242 bp

Figure 3:

Agarose gel electrophoresis of a partial amplified cDNA for ß-propionyl CoA carboxylase. 50 μl aliquots from each PCR reaction mixture were electrophoresed on 1% agarose. Lane 1: Patient DG. Lane 2: Patient BM. Lane 3: Patient BC. Lane 4: Patient PF. Lane 5: Control. Lane 6: Size markers.

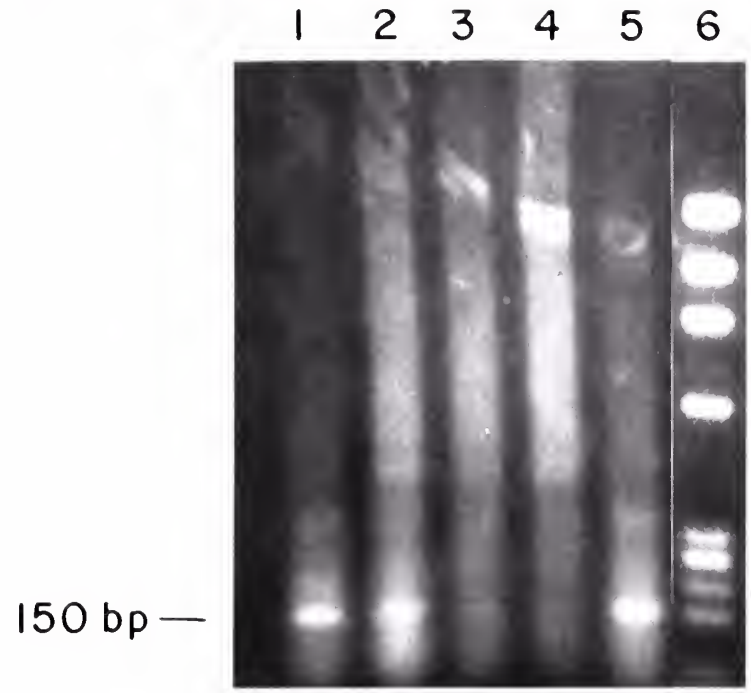

1 2 3 4 5 6
150 bp

Figure 4:

Agarose gel electrophoresis of restriction endonuclease-digested cDNA from patient OW. Aliquots were taken from cDNA PCR reactions, subjected to digestion with *Acc* I and *Xho* I, and electrophoresed on 3% NuSieve agarose. Lane 1: Patient OW. Lane 2: pHO731. Lane 3: Size markers. The arrow designates the shortened fragment seen in patient OW.

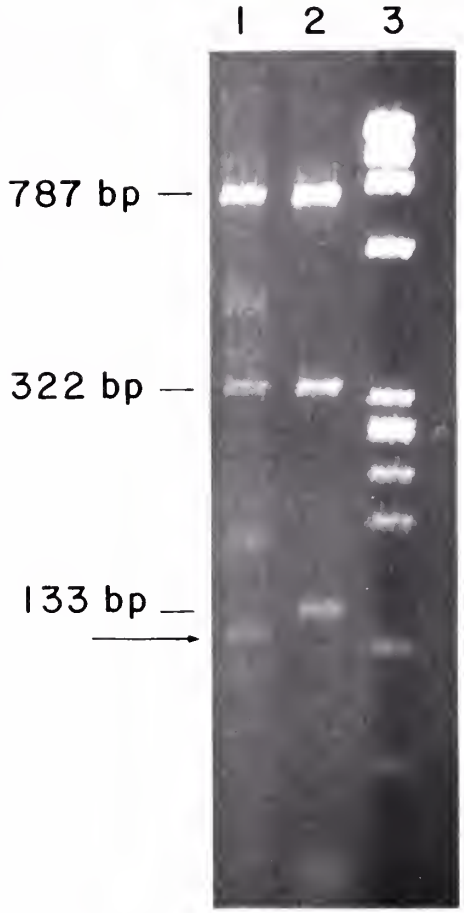

1
2
3
787 bp
322 bp
133 bp

Figure 5:

Agarose gel electrophoresis of restriction endonculease-digested cDNAs from patients RD and MC. Aliquots were taken from PCR reactions, subjected to digestion with *Asp* 718 (*Kpn* I) and *Hae* III, and electrophoresed on 3% NuSieve agarose. The arrows designate the shortened fragments observed in these patients. Left: Lane 1: pHO731. Lane 2: Patient MC. Lane 3: Size markers. Right: Lane 1: Size markers. Lane 2: Patient RD. Lane 3: pHO731. The extra band of 200 bp seen in lane 2 on the right is a non-specific product carried over from the amplification step.

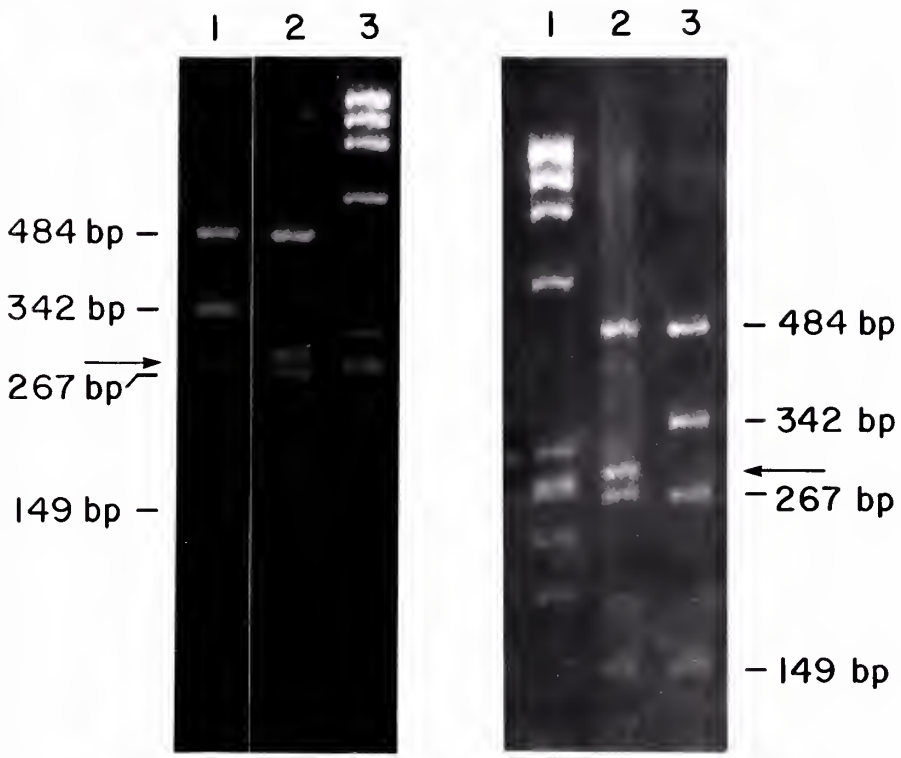

1
2
3
484 bp –
342 bp –
267 bp
149 bp –
1
2
3
– 484 bp
– 342 bp
– 267 bp
– 149 bp

Figure 6:

Agarose gel electrophoresis of restriction endonuclease-digested cDNAs from patients RD and MC. Aliquots were taken from PCR reactions, subjected to digestion with Msp I and electrophoresed on 3% NuSieve agarose. The arrows designated the approximately 1000 base fragment observed in the absence of the second Msp I site of the cDNA, which when present yields the 527 and 533 base fragments shown. Left: Lane 1: Patient RD. Lane 2: pHO731. Lane 3: Size markers. Right: Lane 1: Patient MC. Lane 2: pHO731. Lane 3: Size markers.

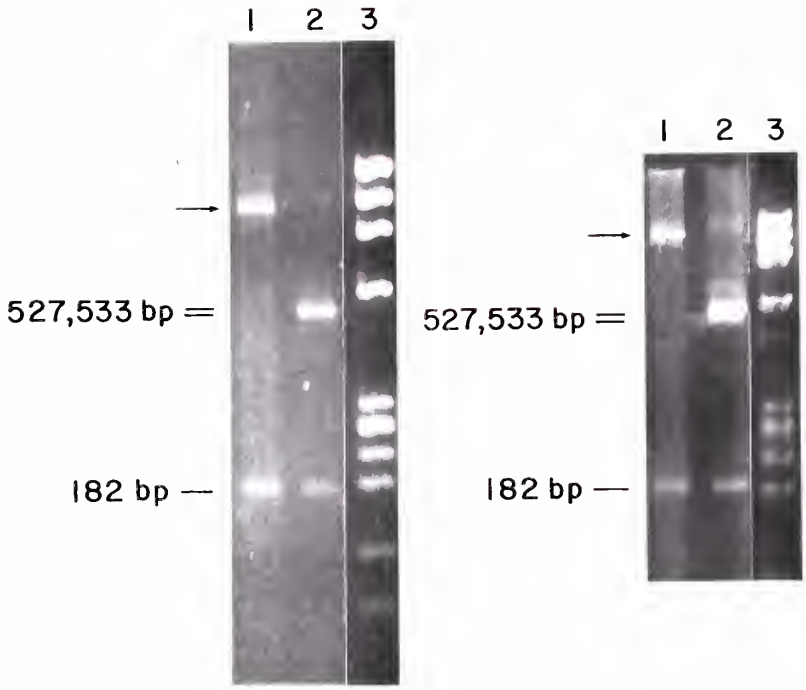

1 2 3
527,533 bp
182 bp
1 2 3
527,533 bp
182 bp

Figure 7:

Restriction maps of the normal 1242 base amplified cDNA when subjected to digestion with the enzymes shown. Also shown are the normal expected band sizes.

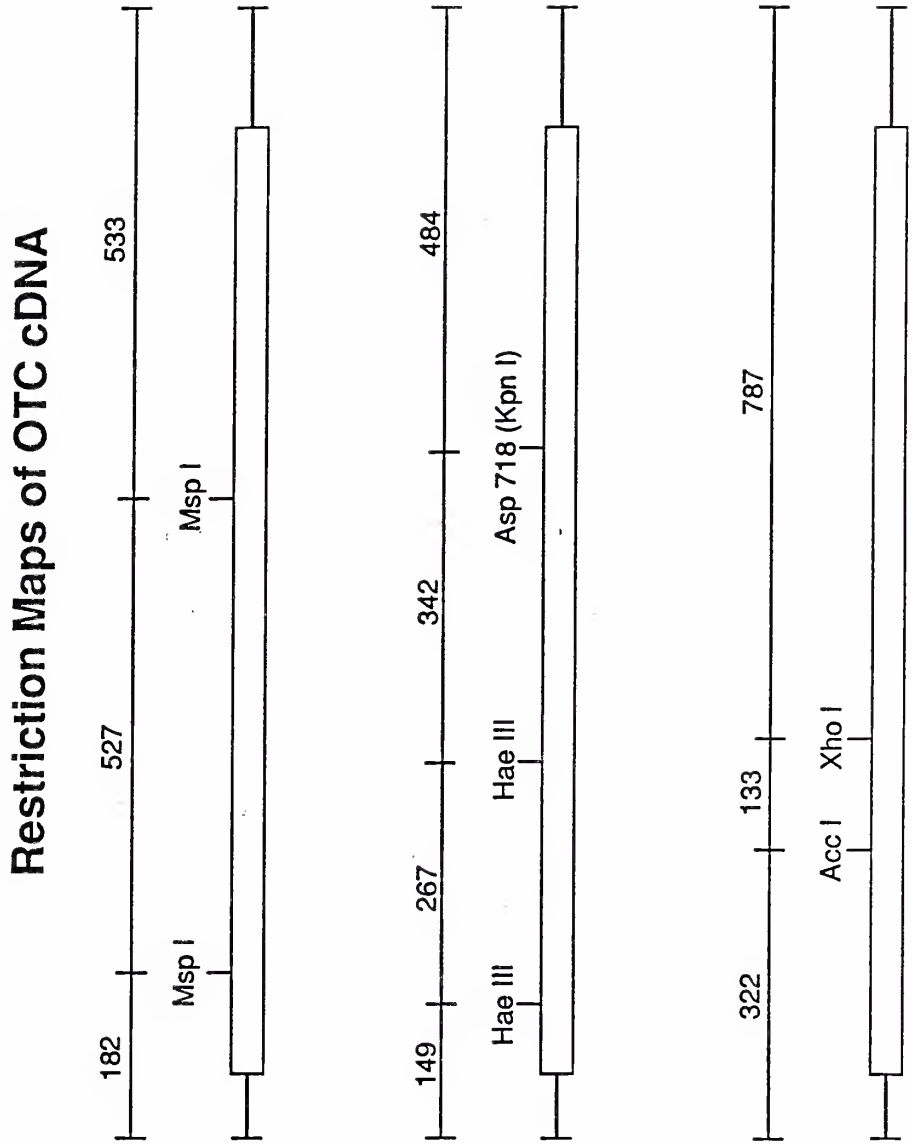

Restriction Maps of OTC cDNA
182
Msp I
527
Msp I
533
149
Hae III
267
Hae III
342
Asp 718 (Kpn I)
484
322
Acc I
133
Xho I
787

Figure 8:

Sequence of OW cDNA. Amplified cDNA from patient OW was digested with *Xho* I and *Sca* I, and the fragment between these sites was subcloned into the *Xho* I and *Eco R*V sites of pBluescript KS and sequenced using Sequenase. The relevant portion of the gel and its interpretation are shown.

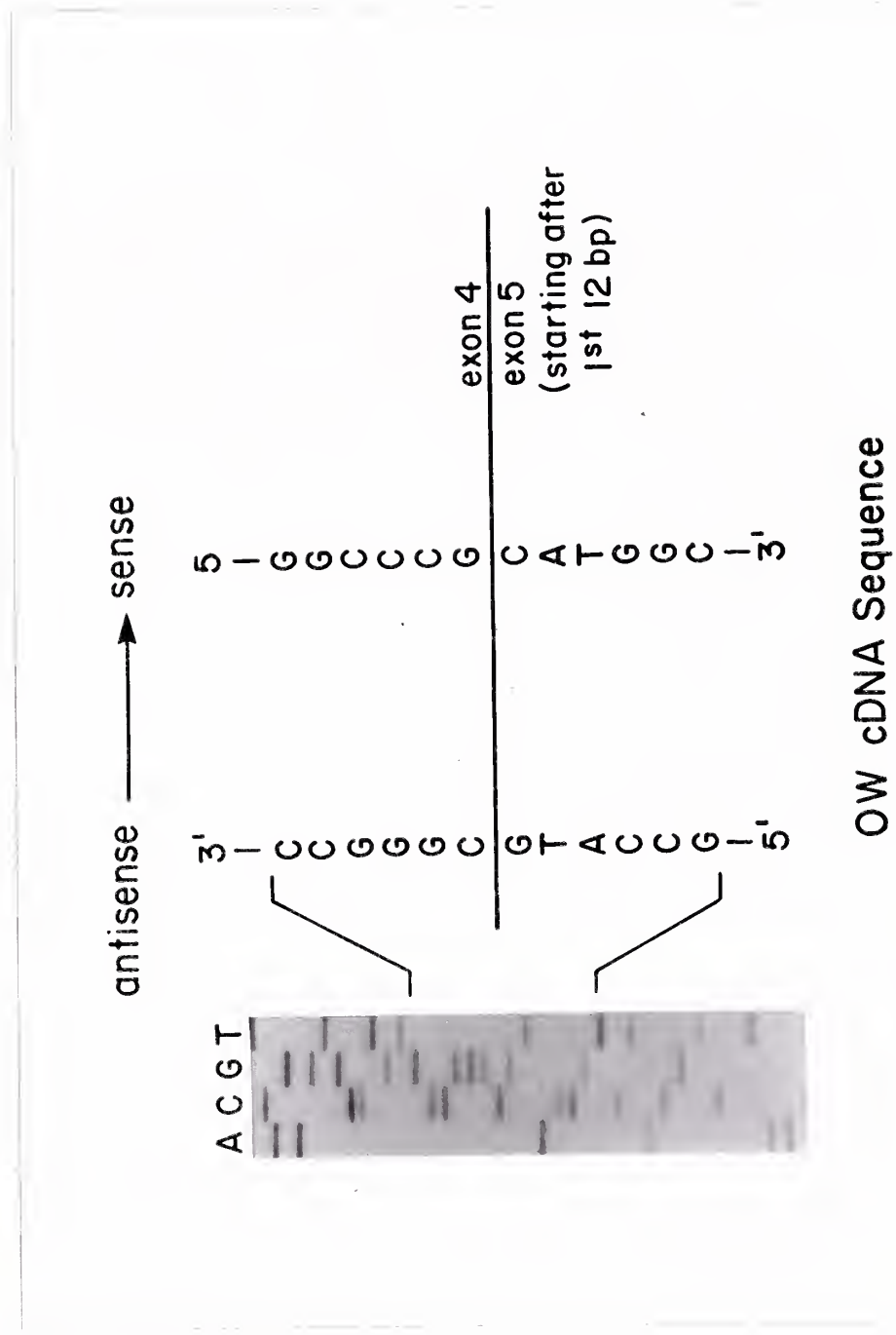
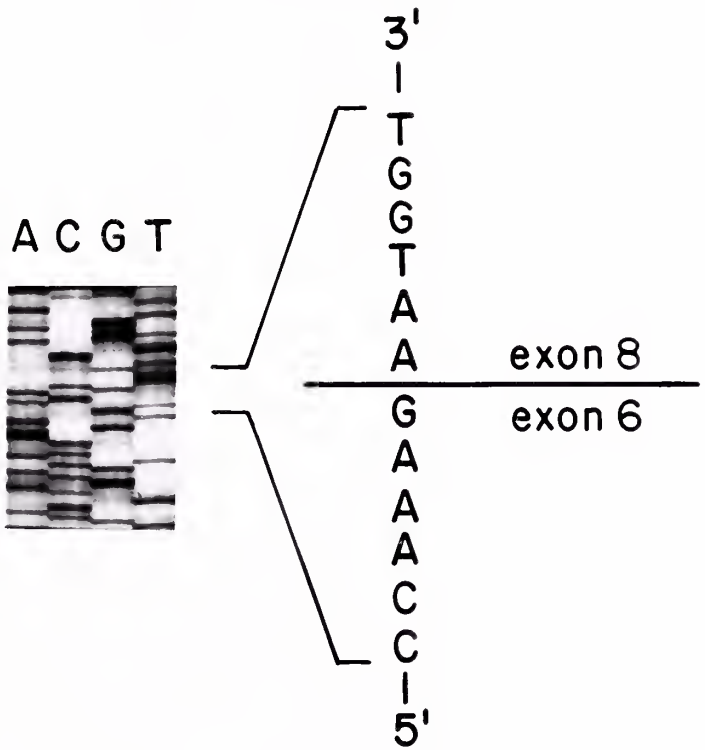
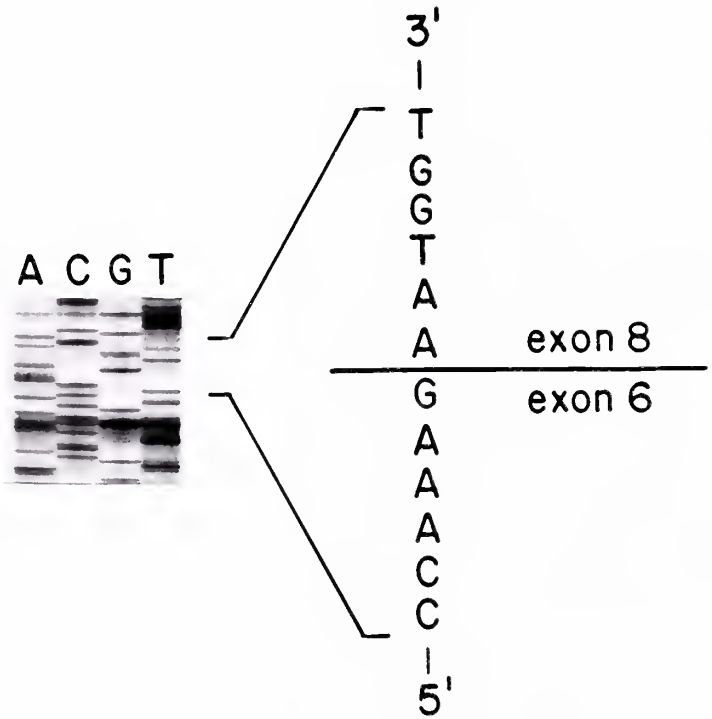

antisense
sense
3'
5'
5'
3'
exon 4
exon 5
(starting after
1st 12 bp)
A C G T
OW cDNA Sequence

Figure 9:

Sequences of MC and RD cDNAs. Amplified cDNA from patients RD and MC were digested with *Asp* 718 and *Bst Y*1, and fragments between the third *Bst Y*1 site and the *Asp* 718 site of the cDNA were subcloned into the *Asp* 718 and *Bam H*1 sites of pBluescript and sequenced. Left: Sequencing gel of patient MC. Right: Sequencing gel from patient RD. Relevant portions of the gels and their interpretations are shown.



MC cDNA Sequence

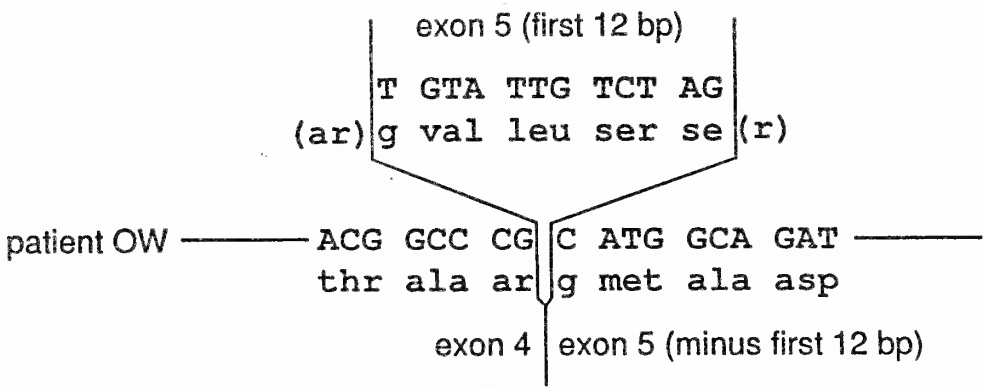


RD cDNA Sequence

Figure 10:

Diagram showing the deletions in the cDNAs from these patients, with the normal sequences above.

cDNA Sequences



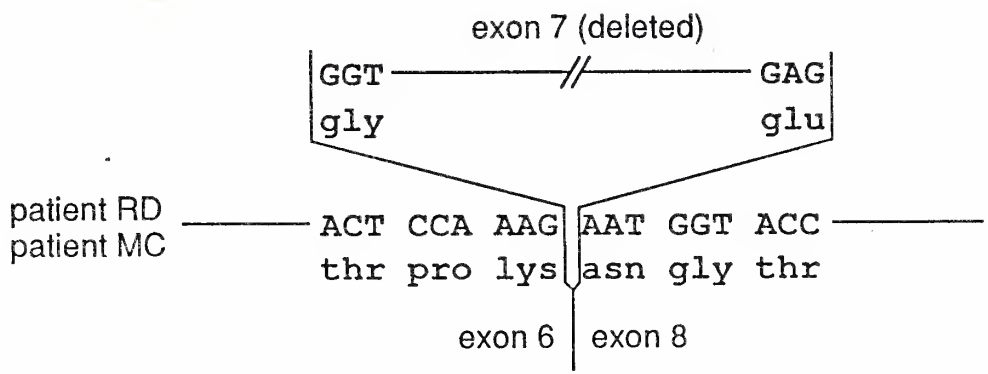
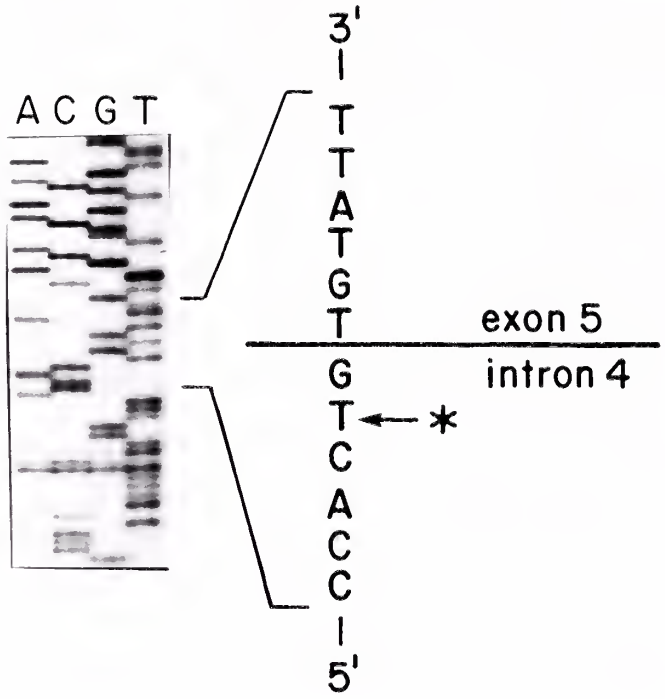


Figure 11:

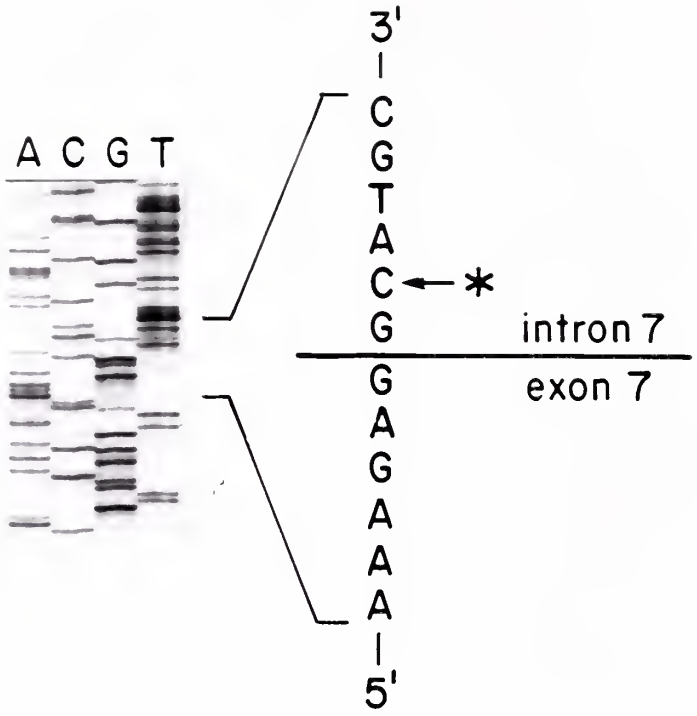
Sequence of genomic DNA from patient OW. Genomic DNA was amplified as described and subcloned into the *Sma* I site of pBluescript and sequenced. A portion of the sequencing gel is shown with its interpretation. The T (arrow) is an A in control.



OW Genomic Sequence

Figure 12:

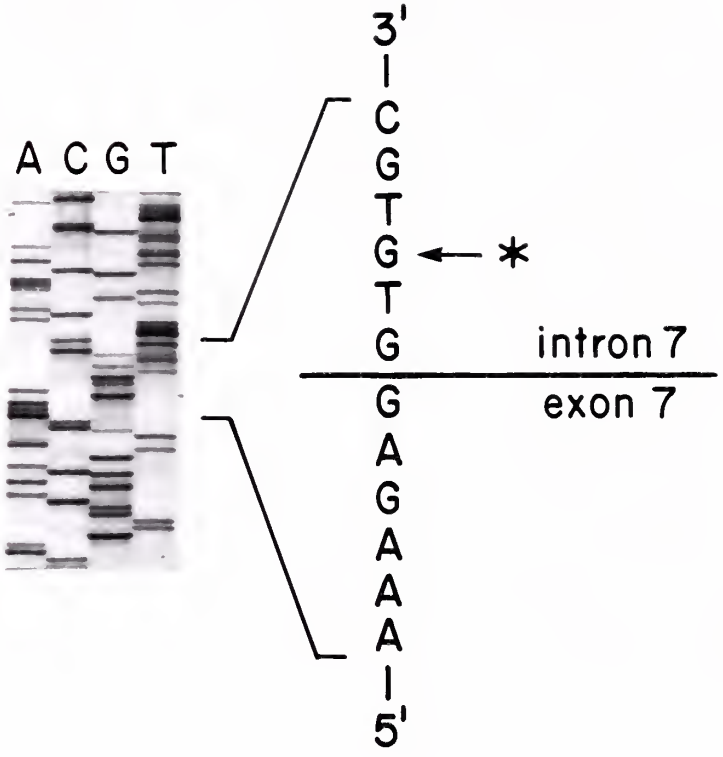
Sequence of genomic DNA from patient RD. DNA was amplified, subcloned, and sequenced as above. The C (arrow) is normally a T.



RD Genomic Sequence

Figure 13:

Sequence of genomic DNA from patient MC. DNA was amplified, subcloned, and sequenced as above. The G (arrow) is normally an A.



MC Genomic Sequence

Figure 14:

Northern blot of liver RNA probed for OTC, washed, and re-probed with a mutase probe as an internal control. Left: Probed for OTC; Lane 1: Normal liver. Lane 2: Patient MC. Lane 3: Patient RD. Lane 4: Patient OW. Lane 5: Patient CC. Lane 6: Patient JF. Lane 7: Patient FN. Lane 8: Patient MT. Right: The same filter washed and probed with mutase.

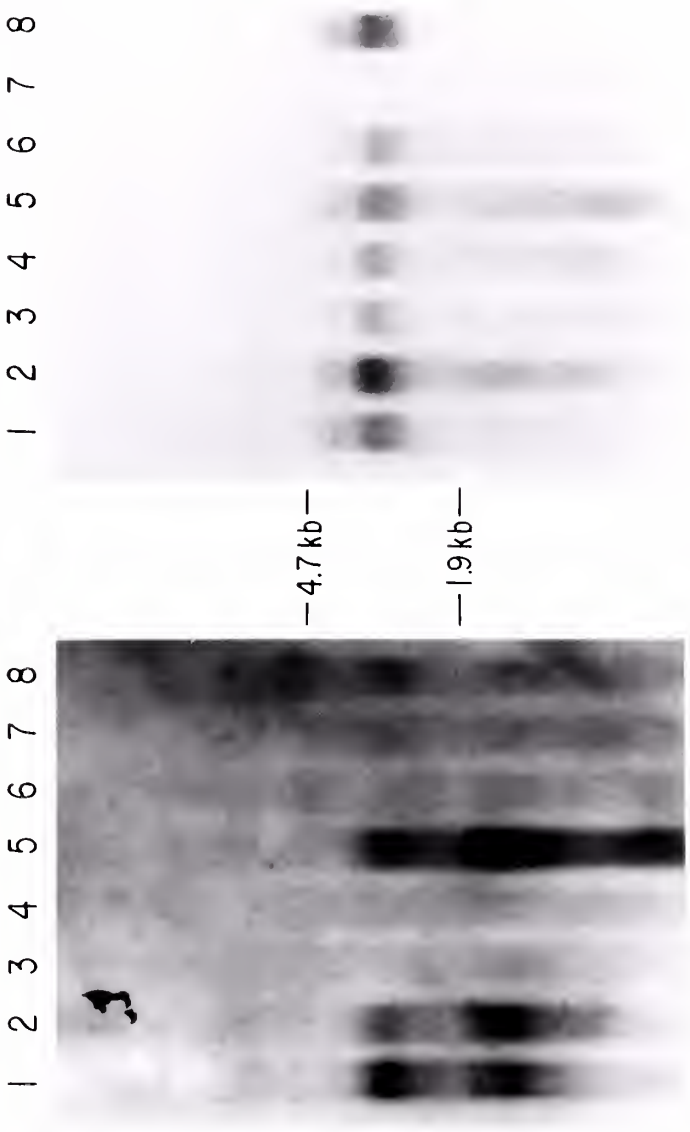

1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
—4.7 kb—
—1.9 kb—

Figure 15:

Western blot of liver extracts analyzed for cross reactive material to anti-OTC and anti-methylmalonyl-CoA mutase antisera. The first lane contains a homogenate analyzed directly, whereas the other lanes are immunoprecipitated material (see Materials and Methods). Lanes 1 and 2: Normal liver. Lane 3: Patient MC. Lane 4: Patient RD. Lane 5: Patient OW. Lane 6: Patient CC. Lane 7: Patient JF. Lane 8: Patient FN. Lane 9: Patient MT.

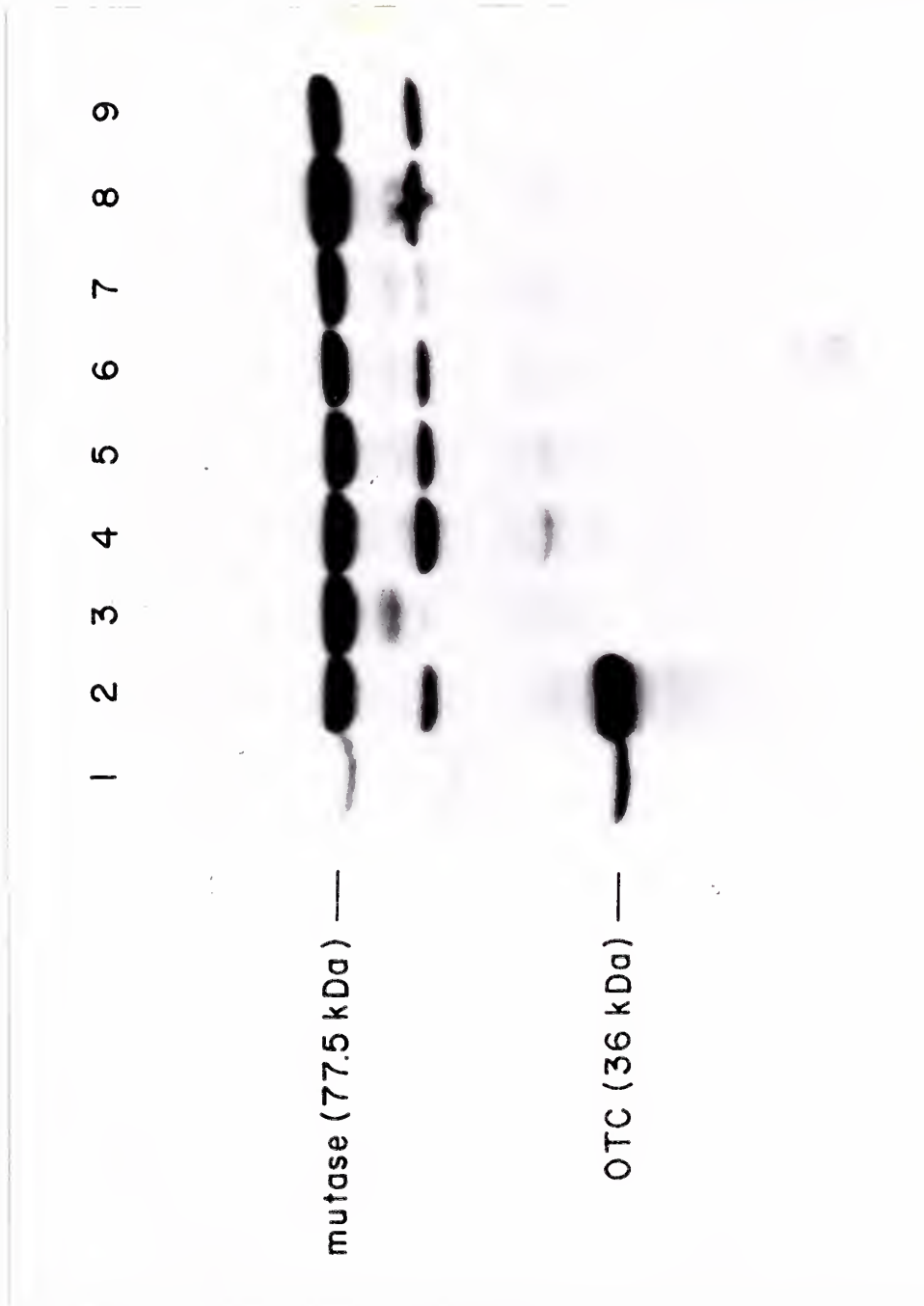

1 2 3 4 5 6 7 8 9
mutase (77.5 kDa) —
OTC (36 kDa) —

Figure 16:

Consensus splice-site sequences changed in these patients compared to wild type sequence and scored according to Shapiro and Senepathy (1987).

Sequences at Intron 4 / Exon 5 Boundary

		Score
Consensus Sequence	$\overset{TT}{CC}\overset{TTTTTT}{TCCCCCC}NCAG \mid G$	
OTC wild type	TTGGTTTACCACAG | T	85.0
Patient OW	TTGGTTTACCACTG | T	68.9
OW Boundary using cryptic splice site	TGTGTATTGTCTAG | C	74.8

original boundary

Sequences at Exon 7 / Intron 7 Boundary

		Score
Consensus Sequence	$\overset{C}{A}AG \mid GT\overset{A}{G}AGT$	
OTC wild type	GAG | GTATGC	82.8
Patient MC	GAG | GTGTGC	79.5
Patient RD	GAG | GCATGC	64.5

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phobilinogen deaminase gene results in exon skipping and is responsible for acute intermittent porphyria. *Nucl. Acids Res.* **15**:6637-6649.

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