Am. J. Hum. Genet. 48:1105–1114, 1991

Identification of RNA Splicing Errors Resulting in Human Ornithine Transcarbamylase Deficiency

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Summary

Ornithine transcarbamylase (OTC) is an X-linked, liver-specific enzyme that 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. To characterize mutations responsible for OTC deficiency, we used the PCR to amplify cDNAs prepared from patient livers which demonstrated no OTC enzyme activity and no OTC cross-reacting material on western blots. In three of seven cases, smaller than normal products were observed. Sequencing of these cDNAs revealed that two were missing exon 7 of the OTC gene and that the other was missing the first 12 bp of exon 5. Sequencing of genomic DNA from these three patients revealed that one mutant missing exon 7 had 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. It is interesting that both of these mutations resulted in skipping the preceding exon rather than in 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. Northern blotting of liver RNA from these patients demonstrated (*a*) reduced, but significant, amounts of OTC mRNA in one of the patients who had a deleted exon 7 but (*b*) very little OTC mRNA in the other two patients. We propose that these point mutations, which result in aberrant 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. We speculate that abnormal mRNA splicing may represent a relatively common mechanism in the pathogenesis of this disease.

Introduction

Ornithine transcarbamylase (OTC; carbamoyl-phosphate:L-ornithine carbamoyltransferase, E.C.2.1.3.3) is a mitochondrial matrix enzyme that is encoded by a gene on the X chromosome, translated on free cytosolic ribosomes as a larger precursor, translocated across both mitochondrial membranes, cleaved to its mature size, and assembled into an active homotrimer (Rosenberg et al. 1987). Within mitochondria, OTC catalyzes the condensation of ornithine and carbamyl phosphate to form citrulline, the second step of the urea cycle. Human OTC cDNAs and genomic DNAs have been cloned and characterized (Horwich et al. 1984; Hata et al. 1986, 1988). The gene consists of 10 exons, spread over ~73 kbp, which form a ~1,700-base mRNA with a coding region of 1,062 bases. This mRNA is translated into the 40-kDa OTC precursor consisting of 354 amino acids; the amino-terminal 32 residues constitute the leader peptide that is proteolytically removed following translocation (Horwich et al. 1984; Rosenberg et al. 1987).

Deficiency of OTC activity is the most common hereditary urea-cycle enzyme defect. As expected for an X-linked disorder, clinical manifestations are generally most severe in hemizygous males, who present with protein intolerance and marked hyperammonemia, often leading to coma and death in the neonatal period (Campbell et al. 1973; Short et al. 1973; Bru-

Received September 19, 1990; revision received January 15, 1991.

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0002-9297/91/4806-000011$02.00

silow and Horwich 1989). Heterozygous (carrier) females display a wide variation in the clinical expression of their disease, depending on random X-chromosome inactivation. As expected for an X-linked lethal disease (Haldane 1935), the frequency of new mutations in OTC deficiency appears to be high, accounting for as many as one-third of new cases. In most patients, the precise molecular defect which causes the disease has not been determined: fewer than 10% display gross deletions or rearrangements of the OTC gene (Rozen et al. 1985); a few others have an alteration of a restriction site within the coding sequence which reflects the mutation (Nussbaum et al. 1986; Maddalena et al. 1988; Hata et al. 1989), but the majority have mutations which remain to be defined (Grompe et al. 1989).

We have used the PCR to amplify cDNAs from livers of 11 male patients with lethal OTC deficiency to identify, at the molecular level, the mutations responsible for their disease. We anticipate that these data will allow us to define further the molecular mechanisms involved in the pathogenesis of OTC deficiency and to understand the effects that these molecular defects have on the pathway of OTC biogenesis.

Material and Methods

RNA and DNA Preparation

Total human liver RNA was prepared by the guanidine thiocyanate extraction procedure, as described by Chirgwin et al. (1979). The total RNA recovered was dissolved in diethylpyrocarbonate-treated water. Genomic DNA was prepared from frozen liver by the method of Fox et al. (1986); the final solution was dialyzed overnight at 4°C against 10 mM Tris-Cl pH 8.0, 1 mM EDTA before use.

Primers

Primer sequences were as follows: IL, 5′-GGGCATAGAATCGTCCTTTA-3′; IXaR, 5′-CAATGGCAAAGCATATCATA-3′; VL, 5′-TCTTTTTCTTGGTTTACCAC-3′; VR, 5′-GTAAGACAAATAAATAAACC-3′; VIIL, 5′-TTTAAATTCCTTCCTCCTTT-3′; and VIIR, 5′-CCTGAGAGAGCATCAATTTG-3′. Primers IL and IXaR are based on the 5′- and 3′-untranslated regions of human OTC cDNA, respectively (Horwich et al. 1984). They encompass a 1,242-bp fragment that contains the entire coding region of the OTC cDNA. Primers VL, VR, VIIL, and VIIR are intron sequences based on the OTC genomic sequence reported by Hata et al. (1988). VL and VR flank exon 5 (154 bp) and generate a 199-bp fragment containing both this exon and adjacent intron sequences. VIIL and VIIR flank the exon 7–exon 8 region and generate a 329-bp fragment containing exon 7 (54 bp), intron 7 (80 bp), exon 8 (150 bp), and adjacent intron sequences.

Reverse Transcription

Five micrograms of total RNA in a volume of 22.5 μl were heated to 80°C for 3 min and then were added to 27.5 μl of a reaction mixture containing (final concentrations) 50 mM Tris-Cl pH 8.3, 75 mM KCl, 10 mM $MgCl_2$, 4.35 mM DTT, 0.175 mg BSA/ml, 0.5 mM of each deoxyribonucleoside triphosphate, 1.25 Units RNasin (Boehringer Mannheim or Promega), 70 Units avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim), and 70 ng IXaR reverse primer in a total volume of 50 μl. Each reaction was incubated at 44°C for 1 h; an additional 40 Units of 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 according to a method described by Saiki et al. (1988). To a 5-μl aliquot of each reverse transcription mixture was added 10 μl 10 × reaction buffer (0.5 M KCl, 100 mM Tris-Cl pH 8.3, 15 mM $MgCl_2$, and 200 μg gelatin/ml), 16 μl dNTP mixture (1.25 mM each), 5 μl each of IL and IXaR primers (20 μM), 2.5 Units *Taq* DNA polymerase (United States Biochemical), and H_2O. Amplification conditions consisted of an initial denaturation at 94°C for 5 min, followed by 40–50 cycles of denaturation at 94°C for 2 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min. Aliquots were analyzed on 1% SeaKem or SeaPlaque agarose (FMC Bioproducts). Genomic amplifications were performed similarly, except that 1 μg total genomic DNA was used as template. Products were analyzed on 3% NuSieve agarose (FMC Bioproducts).

Restriction-Endonuclease Digestion, Cloning, and Sequencing of PCR Products

After each amplification reaction, samples were extracted and ethanol precipitated as above and were 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. Minipreparations of DNA of selected colonies (Birnboim and Doly 1979) were analyzed by restriction digestion and were subjected to dideoxynucleotide sequencing by using Sequenase (United States Biochemical) according to the supplier's specifications.

Northern Blotting

Ten micrograms total liver RNA was electrophoresed on a 1.1% agarose gel in formaldehyde according to a method described elsewhere (Maniatis et al. 1982). After electrophoresis, the RNA was transferred to a nitrocellulose filter and hybridized with [^{32}P]-labeled cDNA probe prepared by the oligolabeling method (Feinberg and Vogelstein 1984) by using a kit (Boehringer Mannheim) according to the supplier's recommendations and a template (100–150 ng) prepared by PCR amplification of a plasmid containing either an OTC or a methylmalonyl CoA mutase cDNA. Filters were washed at high stringency and then were exposed to XAR-5 film with an intensifying screen at −70°C for 1d–1 wk. Prior to the reprobing, filters were washed in 2 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1% SDS for 20–30 min at 60°C.

Immunoprecipitation and Western Blotting

Liver tissue was homogenized in NETS buffer (150 mM NaCl, 0.5% Triton X-100, 10 mM EDTA, 0.25% SDS) 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 microliters 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), followed by centrifugation. The *S. aureus* cells were washed, and the immunoprecipitated proteins were recovered according to a method described elsewhere (Kessler 1981). Samples were loaded onto an SDS–polyacrylamide gel according to the method of Laemmli (1970). The stacking gel consisted of 3.8% acrylamide/0.16% bis-acrylamide, and the running gel consisted of 8% acrylamide/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 min and then was electroblotted onto nitrocellulose at 100 mA overnight according to a method described by 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_2HPO_4, 0.02% KH_2PO_4) (pH 7.3) for 2–3 h and then was transferred to 50 ml of a solution of 13% bovine hemoglobin, 0.02% sodium azide in PBS to which 500 μl each of anti-OTC and anti-mutase antiserum had been added. The filter was washed twice in PBS for 10 min and then was washed twice in PBS with 0.5% Nonidet P-40 for 10 min. The filter was soaked in 50 ml of 5% Carnation dry milk in PBS containing 50 μl [^{125}I]–protein A (Amersham). 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 1,062-base coding sequence for OTC, RNA was isolated from 11 livers obtained at autopsy from male patients found, by assay of OTC activity, to have OTC deficiency. The isolated RNA was used to synthesize single-stranded cDNAs. This material was subjected to PCR amplification using a set of primers that yielded a 1,242-bp product, which included the entire coding region of the OTC cDNA, from normal subjects. In seven of the 11 patients studied, cDNA products were observed after PCR amplification and agarose gel electrophoresis. After several independent amplification reactions, three of these seven cDNAs were found to be shorter than the normal cDNA. The other four appeared to be of normal length (not shown).

Localization of Deletions in cDNAs by Restriction-Enzyme Analysis

Amplified cDNAs were subjected to digestion with restriction endonucleases to localize the coding-sequence regions from which sequences were missing, as well as to look for smaller deletions which may

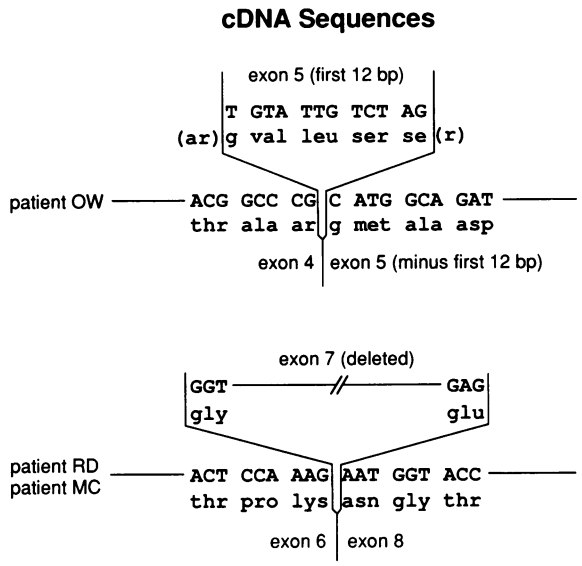

Figure 1 Diagram showing deletions in cDNAs from three OTC-deficient patients, with normal sequences above.

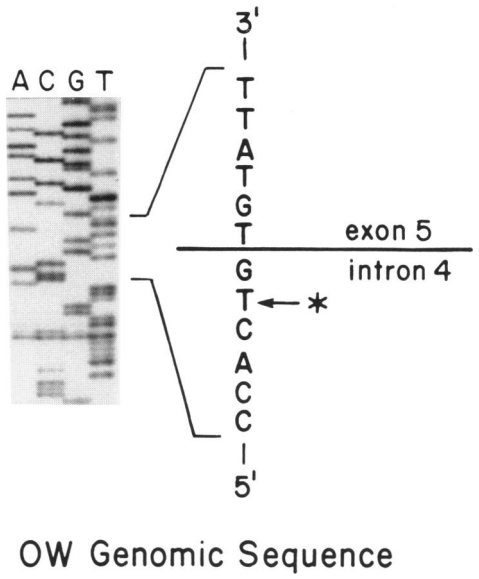

Figure 2 Sequence of genomic DNA from patient OW. Genomic DNA was amplified as described, was subcloned into the *Sma*I site of pBluescript, and was sequenced. A portion of the sequencing gel is shown with its interpretation. The asterisk-marked T (*arrow*) is an A in normal DNA.

not have been apparent in the full-length products. Separation of restriction fragments on NuSieve agarose revealed that, in one patient, there was in the cDNA a deletion of ~10–15 bp located between the unique *Acc*I and *Xho*I sites. 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 ~50 bp of the cDNA. The apparently full-length cDNAs from the other patients showed no evidence of deletions (not shown).

Identification of Mutations at the cDNA Level by DNA Sequencing

To characterize the mutations in those three patients in whom deletions could be localized, appropriate regions of PCR-amplified cDNA were subcloned and sequenced. As shown in figure 1, the mutation between the *Acc*I and *Xho*I site was found to be a 12-bp deletion corresponding to the first 12 bp 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 four amino acids in the mature protein. In the other two patients, sequencing of the relevant portion of the cDNAs demonstrated that both were missing the 54 bp constituting exon 7 (fig. 1). Again the mutations observed were in-frame; no other changes were found. In each of these three cases, the sequencing results suggested that RNA splicing errors were responsible for the findings.

Identification of Mutations in Genomic DNA by DNA Sequencing

To characterize genomic-DNA mutations which could have led to the presumed aberrant splicing, two sets of primers were chosen which corresponded to intron sequences flanking the exons involved. These primers were used to amplify, from genomic DNAs isolated from patient livers, the regions of interest; and the fragments were subcloned and sequenced by 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 this intron (fig. 2). Because, for proper splicing to occur, the splicing system in primates requires introns to begin with GT and to end with AG (Breathnach and Chambon 1981; Mount 1982), the splicing apparatus 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, a splice site which conformed to the "GT-AG rule." Thus, the deletion seen at the cDNA level resulted from a point mutation within the 3′ splice acceptor site, a point mutation which led to aberrant splicing.

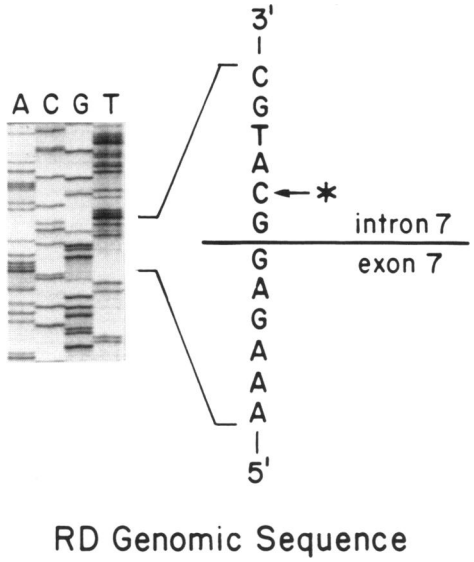

Figure 3 Sequence of genomic DNA from patient RD. DNA was amplified, subcloned, and sequenced as above. The asterisk-marked C (*arrow*) is normally a T.

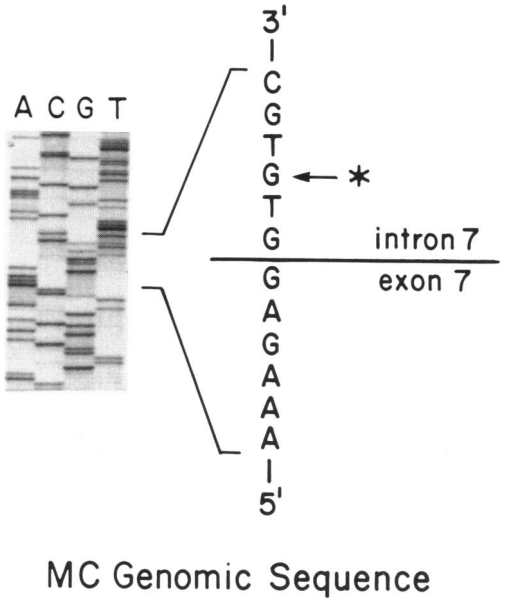

Figure 4 Sequence of genomic DNA from patient MC. DNA was amplified, subcloned, and sequenced as above. The asterisk-marked G (*arrow*) is normally an A.

In the genomic DNA of the two patients missing exon 7, two different point mutations were found 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, RD, the mutation was a substitution of a C for the T in the initial dinucleotide of intron 7, changing GT to GC (fig. 3). 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, MC, had an A-to-G substitution in the third position of intron 7 (fig. 4). 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 (exon 7) to be skipped, even though the 3′ splice acceptor which would function to join exon 6 to exon 7 is unchanged. There was nothing 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.

Northern Analysis of OTC mRNA

Analysis of patient livers was carried out to determine whether any of these OTC deficiencies were associated with reduced amounts of OTC message, particularly in the case of those patients with splicing defects. RNA samples prepared from patient and control livers were probed with an OTC cDNA obtained by amplifying a normal OTC cDNA template with the IL and IXaR primers described above. After exposure, the filter was washed and rehybridized with a probe containing ~300 bases of coding sequence for methylmalonyl–CoA mutase as an internal control. Figure 5 shows northern blots obtained after probing for both OTC and mutase. Of the three patients with splicing errors, the liver of MC contains a significant, although decreased, amount of OTC mRNA as compared with normal levels, 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 the 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

To assess the effects that these mutations have at the protein level, we assayed for OTC cross-reacting material in patient livers. Homogenates were prepared from liver and immunoprecipitated with a polyclonal

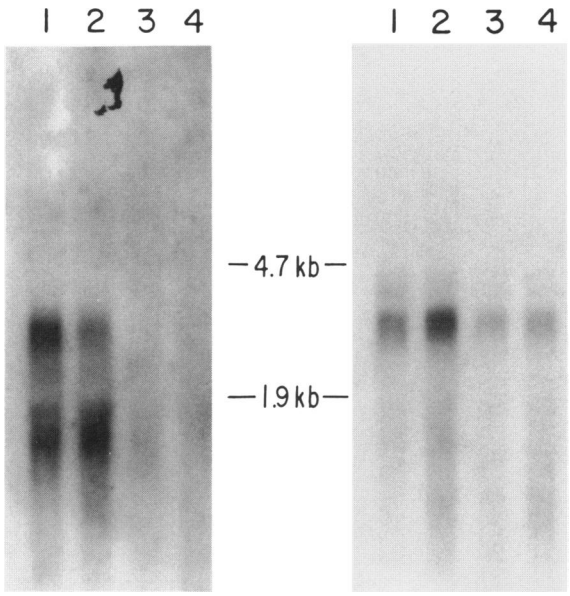

Figure 5 Northern blot of liver RNA probed for OTC, washed, and reprobed with methylmalonyl CoA mutase probe as internal control. *Left,* Probed for OTC. Lane 1, Normal liver. Lane 2, Patient MC. Lane 3, Patient RD. Lane 4, Patient OW. *Right,* Same filter washed and probed with mutase.

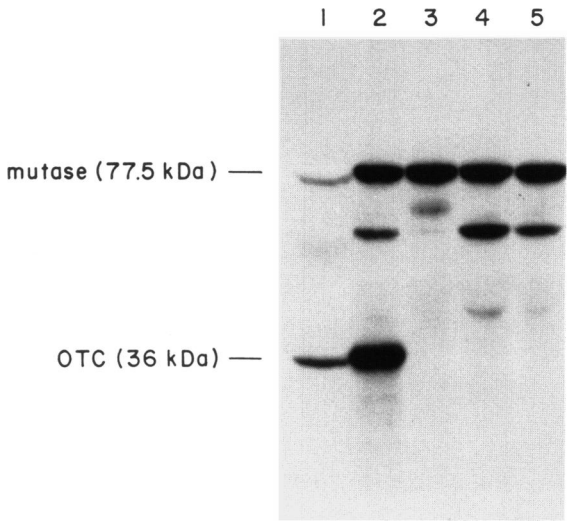

Figure 6 Western blot of liver extracts analyzed for cross-reactive material to anti-OTC and anti–methylmalonyl–CoA mutase antisera. The first lane contains a homogenate analyzed directly, whereas the other lanes are immunoprecipitated material (see Material and Methods). Lanes 1 and 2, Normal liver. Lane 3, Patient MC. Lane 4, Patient RD. Lane 5, Patient OW. (The variable bands between mutase and OTC are due to nonspecific reactions with anti–methylmalonyl–CoA mutase.)

anti-OTC antiserum, as well as with anti-methylmalonyl–CoA mutase used as a control. Immunoprecipitated material was then electrophoresed on an SDS–polyacrylamide gel, was blotted onto nitrocellulose, and was probed with anti-OTC and anti–methylmalonyl–CoA mutase. As shown in figure 6, a homogenate prepared from liver of a patient unaffected by OTC deficiency 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 them demonstrated a band corresponding either to mature OTC or to the shorter proteins predicted from the deletions, even when the filter was exposed for as long as 10 d. Thus, although these mutations produced in-frame alterations in OTC mRNA, no cross-reactive OTC protein could be identified in the livers from any of these three patients.

Discussion

We have detected and identified RNA splicing errors believed to be responsible for clinical OTC deficiency in three affected male patients. 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 to 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 bp of exon 5 in the resulting mRNA. The other two mutations both involve 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 mutation 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 which are required for correct splicing have been characterized by several authors (Breathnach and Chambon 1981; Mount 1982; Aebi et al. 1986; Ohshima and Gotoh 1987; Shapiro and Senepathy 1987). By analyzing a large number of eukaryotic splice junction regions, consensus sequences have been estab-

lished 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$^{A}_{C}$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 basepairs with the sequence ACψψACCUG of U1-snRNA that binds to 5′ splice donors during the splicing process (Mount et al. 1983; Zhuang and Weiner 1986). Similarly, a consensus sequence of $(^{T}_{C})_{n>10}N^{C}_{T}$ 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 described by Shapiro and Senepathy (1987) gives a numerical value for any proposed 5′ or 3′ consensus sequence, on the basis of the frequency of each nucleotide in normal splice sequences. When this system is used, the consensus sequences AG:GTAAGT and $^{TT}_{CC}$TT$^{TTTTTT}_{CCCCCC}$ NCAG:G yield a score of 100, and most splice sequences normally used have a score >70.

Figure 7 shows the scores both for the normal splice sites and for the mutant ones detected in this group of patients, as well as for the proposed cryptic site within exon 5. Both the mutations in patient OW and that in 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-to-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 the terminal dinucleotides give rise to aberrantly spliced products, as well as to some normally spliced ones (Treisman et al. 1983; Atweh et al. 1987; Kazazian and Boehm 1988). We see no evidence, however, of any normally spliced OTC mRNA in patient MC on the basis of agarose-gel analysis of PCR-amplified cDNA (data not shown).

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 (Green 1986; Padgett et al. 1986; Sharp 1987; Steitz 1988). The general mechanism which is proposed

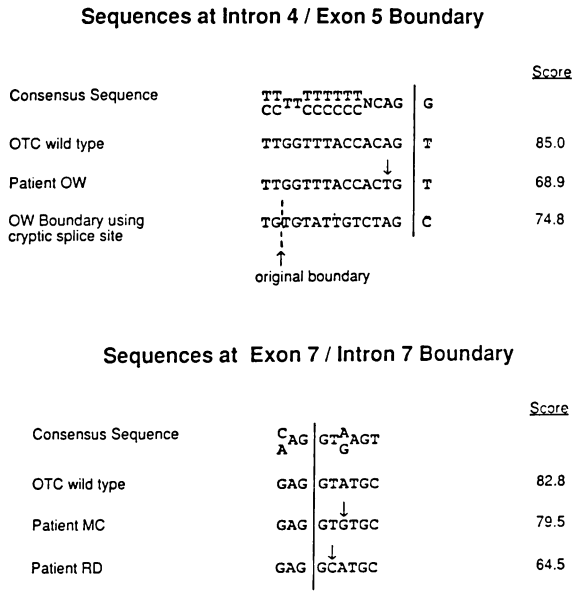

Figure 7 Splice-site sequences changed in patients studied, compared with consensus and wild-type sequences. Scoring is according to the method of Shapiro and Senepathy (1987).

suggests the formation of a spliceosomal complex—containing the small nuclear ribonucleoproteins (snRNPs), U1, U2, U4, U5, and U6, as well as newly transcribed RNA—which is involved in recognition and splicing at appropriate 5′ and 3′ sites. How this complex 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 cannot explain the exon skipping described in two of our patients, however, in whom mutations in 5′ splice donors cause skipping of the previous exon. Similar results have 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). Whatever modified model of splicing is proposed will 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

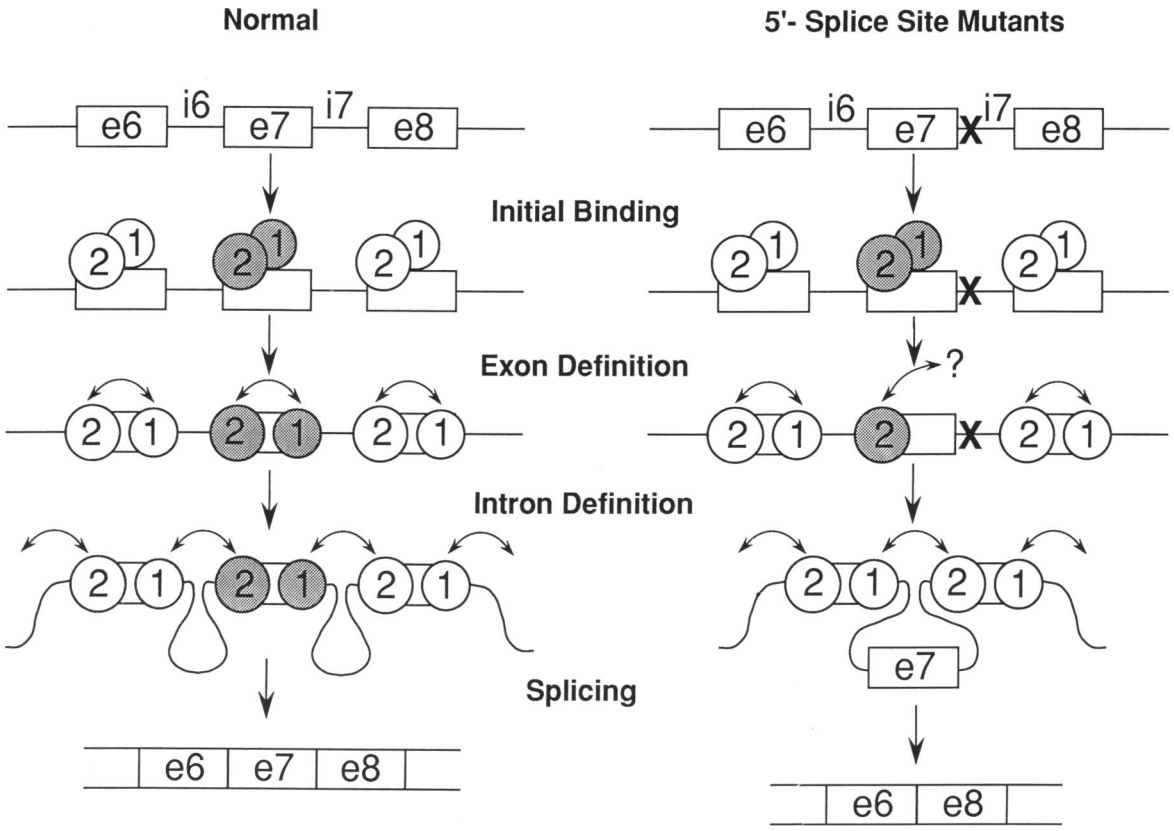

Figure 8 Scheme depicting splicing pathway in normal RNA (*left*) and in RNA from patients MC and RD (*right*). Exons and introns are labeled e5 and i5, respectively (not to scale). For simplicity, the complex recognizing the 3′-splice site is depicted as a U2 snRNP (②); the U1 snRNP is also shown (①). One pair of snRNPs is shaded for clarity. In the right-hand panel, the X in intron 7 identifies the sites of the mutations in MC and RD that lead to the skipping of exon 7 (modified from the model of Robberson et al. [1990], with permission).

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). Figure 8 is derived from this model. According to this mechanism, the spliceosome recognizes a 3′ splice site and then scans downstream for an acceptable 5′ splice site, thereby defining an exon. If no acceptable 5′ splice site can be found within ~300 bases of the 3′ splice site, no exon appears to be present. Thus, stable spliceosome assembly intermediates do not form, and, even if a potential exon is contained in this region, it is skipped.

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. With regard to intron size, it has been shown that the lower limit that will allow proper splicing to occur is ~65–80 nucleotides (Wieringa et al. 1984; Steitz 1988). It is interesting that in OTC 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 untenable. In support of this hypothesis, Akeson et al. (1988) demonstrated in normal cell lines the presence of a minor adenosine deaminase mRNA product in which this gene's exon 7 was deleted. In this case, the intron between exon 7 and exon 8 is only 76 bases. 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 splicing pattern most likely 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.

Finally, it must be asked 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' livers 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). In the complex pathway from RNA transcript to mature protein, the step(s) responsible for the enzyme deficiency in these patients remains to be defined. 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 levels. While this suggests that reduced levels of processed mRNA in the cytoplasm may be one consequence of these mutations, it does not fully explain the deficiency observed in all of the patients. It could be that the deletions in the protein omit critical regions which direct mitochondrial import, mediate trimer assembly, or confer stability. If any of these steps were interfered with, the mutant protein would likely be rapidly degraded. Studies aimed at defining the three-dimensional structure of OTC will be necessary before we will be able to determine more precisely the relationship between the mutations described here and the observed enzyme deficiency.

Acknowledgments

We thank Drs. Saul Brusilow and Y. Edward Hsia for providing patient samples, and we thank Connie Woznick for secretarial assistance. This work was supported by National Institutes of Health grant DK09527 and by National Institutes of Health Training grant T35 HL07649 to R.P.C.

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