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Improved Molecular Diagnostics for Ornithine Transcarbamylase Deficiency

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Summary

Since the cloning of the cDNA for X-linked ornithine transcarbamylase (OTC) in 1984, diagnostic accuracy of OTC deficiency for prenatal and carrier detection has been greatly improved by the use of linkage analysis. However, the use of RFLP-based diagnosis is limited in this and in other new mutation diseases. Here we report both the use of direct mutation detection by new PCR-based techniques and our experience with linkage-based diagnosis in 18 families. We have previously reported the use of chemical mismatch cleavage to detect mutations first in amplified mRNA and then in genomic DNA of patients. This technique has now been utilized for prenatal diagnosis. Primers for specific amplification of OTC exons 1, 3, 5, 9, and 10 have been developed and been employed to map deletions of the OTC gene in two families. These primers also have been used to detect alterations in the *Taq*I sites found in exons 1, 3, 5, and 9. Four novel mutations of the OTC gene leading to abolition of a *Taq*I site in the OTC cDNA were discovered. One of these mutations is in exon 1; two lie in exon 3; and one is in exon 9. In addition, we have used the PCR products as probes to identify the exon-specific bands seen on Southern blots and to map the polymorphic *Bam*HI and *Msp*I sites, which are commonly used for linkage analysis. This information will facilitate the interpretation of altered band patterns seen in deletion cases and in cases of point mutations affecting restriction sites. Utilization of the appropriate combination of these molecular techniques permitted accurate diagnostic evaluations in 17 of 18 families.

Introduction

Ornithine transcarbamylase (OTC; E.C.2.1.3.3) is a mitochondrial matrix enzyme which catalyzes the second step of the urea cycle, the conversion of ornithine and carbamyl phosphate to citrulline (Brusilow and Horwich 1989). The structural gene for the enzyme is localized on the short arm of the X, at Xp21.1 (Lindgren et al. 1984). Severe deficiency of OTC causes hyperammonemia with onset in the newborn period in affected males who present with vomiting, lethargy, and coma after a brief interval of wellness. Milder symptoms in males with partial defects and in heterozygous females have been reported (Rowe et al. 1986; Girgis et al. 1987). Biochemically the disorder is characterized by low plasma citrulline and orotic aciduria in addition to the hyperammonemia. Because of the severe symptoms and the limited usefulness of pharmacologic and dietary treatment, there is considerable demand for prenatal diagnosis and carrier detection in families with OTC deficiency. The human OTC cDNA was cloned in 1984 by Horwich et al. (1984) and since then has been used for the detection of intragenic RFLPs and linkage-based diagnosis (Rozen et al. 1985; Fox et al. 1986*a*, 1986*b*). Four useful polymorphisms have been described, and approximately 70%–80% of females are informative for one of the RFLPs (Schwartz et al. 1986; Fox and Rosenberg 1988; Svirklys et al. 1988; Spence et al. 1989). *Bam*HI detects two allelic bands, one each of 18 and 5.2 kb, and *Msp*I detects an "upper" polymorphism of 6.6 and 6.2 kb and a "lower" polymorphism of 5.1 and 4.4 kb. In addition, *Taq*I digests reveal a fairly uncommon RFLP consisting of 3.7- and 3.6-kb bands. Linkage-based diagnosis, however, is limited in OTC defi-

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ciency, as in other X-linked lethal disorders, because of the high frequency of new mutations (Haldane 1935). The principal question in families with an affected individual but with no other history of OTC deficiency is whether the patient's mother is a carrier. Recently, considerable progress has been made in biochemical testing of carriers in OTC deficiency, but even the allopurinol loading test is not 100% accurate (Hauser et al. 1990). Direct detection of the mutation responsible for the disease would solve the diagnostic difficulties in families in which uncertain biochemical carrier tests or lack of informative polymorphisms exist.

In the present paper we report the PCR amplification of OTC exons 1, 3, 5, 9, and 10 from genomic DNA, for the direct detection of deletions, point mutations altering *Taq*I restriction sites, and point mutations identified by chemical mismatch cleavage. The exon-specific amplification products were also used as probes in Southern blot analysis to clarify the restriction map of the gene and to map the polymorphic sites commonly used in RFLP analysis. Our experience in using these techniques and conventional linkage analysis in 18 families is reported.

Subjects and Methods

Families

The families utilized in the present study were referred to the Kleberg DNA Diagnostic Laboratory, Baylor College of Medicine, for diagnostic evaluation of OTC deficiency between January 1988 and March 1990. In all 18 families there was an index case with biochemically proved OTC deficiency, and in all cases the disease was of the severe form with neonatal onset. The biochemical tests (enzyme assays, plasma amino acid analysis, and orotic acid determinations) were performed in part here and in part in laboratories at referring centers.

Southern Analysis

Genomic DNA was isolated from peripheral blood leukocytes by using the proteinase K/phenol extraction procedure on a model 340 A nucleic acid extractor (Applied Biosystems). Five-microgram samples of DNA were digested with *Bam*HI, *Msp*I, or *Taq*I restriction endonuclease, electrophoresed through 1% agarose gels, and transferred to a nylon membrane by standard procedures (Southern 1975). The blots were then hybridized with a radiolabeled (Feinberg and Vogelstein 1983) full-length cDNA probe for human OTC, pHOC3 (Maddalena et al. 1988), containing approximately 100 bp of 5′ and 300 bp of 3′ untranslated sequence. Hybridizations were conducted in 0.5 M sodium phosphate pH 7.2, 1 mM EDTA, 7% SDS containing 2×10^6 cpm probe/ml and 100 μg of herring sperm DNA/ml at 65°C for 36 h or more. Washing was performed with 40 mM sodium phosphate pH 7.2, 1% SDS, once at room temperature for 15 min and twice at 60°C for 40 min. After the initial autoradiography the blots were stripped and then reprobed in a sequential fashion with exon-specific PCR products or a cDNA probe for exons 1-5 (the sequence of pHOC3 5′ to the *Xho*I site).

Exon-specific PCRs

Amplification of 200 ng genomic DNA (Mullis and Faloona 1987) was carried out with the following primers: exon 1—A = 5′-TCACTGCAACTGAACACATTTCTTAG-3′ and B = 5′-CCTAAATCAAACCCAAGTCTCTGACC-3′; exon 3—C = 5′-GGGAAGTCCTTAGGCATGATTTTTG-3′ and D = 5′-GTGTGAATTTGGCAGTGGACTTACC-3′; exon 5—E = 5′-GGTTTACCACAGTGTATTGTCTAG-3′ and F = 5′-CAGCCAGGATCTGGATAGGATGGTAC-3′; exon 9—G = 5′-GGTCTTATCCCCATCTCTTT-3′ and H = 5′-CCATTCCTTGTTTCTTGCTT-3′; and exon 10—I = 5′-GTGTCATCAGGCTGTCATGGTGTC = 3′ and J = 5′-GATTCTCTTCTCTTTTCCCCATAAAC-3′. In the chemical mismatch cleavage analysis of the SZ family, primer E was replaced with primer K = 5′-TGGCAGATGCAGTATTGGCTCGAGTGT-3′. These primer sequences were based on the published sequence of the human OTC gene (Hata et al. 1988). Not all of the primer sites are in introns; there is some overlap with exon sequences. The expected sizes of the amplification products are as follows: exon 1, 215 bp; exon 3, 90 bp; exon 5, 148 bp; exon 9, 180 bp; and exon 10, 145 bp. The buffer, deoxynucleotide, and primer concentrations were the same as described by Kogan et al. (1987). Amplification conditions were as follows: denaturing at 94°C for 7 min, followed by 30 cycles of 90°C for 30 s, 50°C for 30 s, and 70°C for 90 s, and a final extension step of 70°C for 5 min. *Taq*I restriction digests were performed by adding 10 U of the enzyme to 15 μl of the completed PCR reaction and then incubating the mixture at 65°C for 1 h. The amplification products were analyzed by electrophoresis in a 2% Nusieve® gel and by ethidium bromide staining.

Automated Dideoxy Sequencing

Four units of Klenow enzyme (Pharmacia) were added to the PCR reaction after completion of amplification and were incubated for 30 min at 37°C. The PCR product was then gel purified and subjected to a kinase reaction (Maniatis et al. 1982, pp. 122-124) prior to ligation into vector pTZ18u (Mead et al. 1986). Two individual clones were picked, and single-stranded DNA was produced by superinfection with M13K07 helper phage (Vieira and Messing 1987). The single-stranded DNA was then sequenced by the dideoxy chain-termination method (Sanger et al. 1977) using fluorescently labeled sequencing primers and was analyzed on an Applied Biosystems automated sequencer as described in detail elsewhere (Gibbs et al. 1989).

Chemical Mismatch Cleavage Analysis

The method (Cotton et al. 1988) and family SZ (Grompe et al. 1989) have been described in detail elsewhere. In brief, PCR amplification of genomic DNA from individuals in the SZ family was performed with primers K and F. A wild-type antisense and mutant sense probe were produced by reamplification of the initial PCR product with end-labeled primers. In the wild-type PCR product, primer F was labeled; in the mutant, primer K was radioactive. These probes were then used to form heteroduplexes with the amplification products from various family members and were subjected to chemical modification. Heteroduplexes containing the labeled wild-type antisense probe were treated with hydroxylamine to detect guanosine mismatches, and those containing the mutant sense probe were treated with osmium tetroxide to detect thymine mismatches. This was followed by piperidine cleavage, electrophoresis in an 8% denaturing polyacrylamide gel, and autoradiography.

Results

RFLP Analysis

A complete RFLP analysis was performed in 62 individuals in 15 families. RFLP analysis was not performed fully in all families, since the mutation was detectable directly in three families. The haplotype was determined for 46 chromosomes, and, in addition, the data from the only other study with complete haplotype information (Svirklys et al. 1988) were included in the calculations (see table 1). The *Taq*I RFLP is rarely informative (it was so in only one of the families we studied) and thus has been excluded from the haplotype analysis. No correlation of the haplotype to ethnic subgroups was observed. The linkage disequilibrium Δ value (Ott 1985, pp. 150–152) was −.35 with 99% confidence levels of −.60 to −.03 for the upper *Msp*I and the *Bam*HI polymorphisms, indicating linkage disequilibrium as expected from the close physical distance of these two polymorphisms (see be-

Table 1

Haplotype Analysis

A. Size of Haplotypes

	Haplotype Size (kb)							
Enzyme Type	A	B	C	D	E	F	G	H
*Msp*I:								
Upper	6.6	6.6	6.6	6.6	6.2	6.2	6.2	6.2
Lower	5.1	5.1	4.4	4.4	5.1	5.1	4.4	4.4
*Bam*HI	18	5.2	18	5.2	18	5.2	18	5.2

B. Number of Haplotypes

	No. of Haplotypes Studied								
Source of Data	A	B	C	D	E	F	G	H	Total
Present study	8	6	11	2	14	1	4	0	46
Svirklys et al. (1988)	5	3	2	2	5	0	1	0	18
Total	13	9	13	4	19	1	5	0	64

Table 2

Characteristics of 18 Families with OTC Deficiency

	Frequency (%)
RFLP in patient's mother:	
Not done	3/18 (17)
Heterozygous	11/15 (73)
Homozygous	3/15 (27)
RFLP, all females:	
Heterozygous	29/38 (76)
Homozygous	9/38 (24)
Mutation known:	
Gene deletions	3/18 (17)
*Taq*I site mutations[a]	4/18 (22)
Other point mutations[b]	3/18 (17)
Total	10/18 (55)
Positive family history	4/18 (22)
Negative family history	14/18 (78)
Mothers carrier status known:	14/18 (78)
By family history	3/18 (17)
By family history alone	2/18 (11)
By biochemical testing	2/18 (11)
By direct mutation detection	10/18 (55)
Mother carrier status not known	4/18 (22)
Best diagnostic method:	
Not clear[c]	4/18 (22)
RFLP	6/18 (33)
Exon PCR and/or *Taq*I digest	7/18 (39)
Chemical cleavage or DGGE	1/18 (6)

[a] These are patients 573, JC, SM, and ZH in table 3.
[b] These are patients M, B, and SZ in table 3.
[c] These are the families in which the mothers carrier status is uncertain.

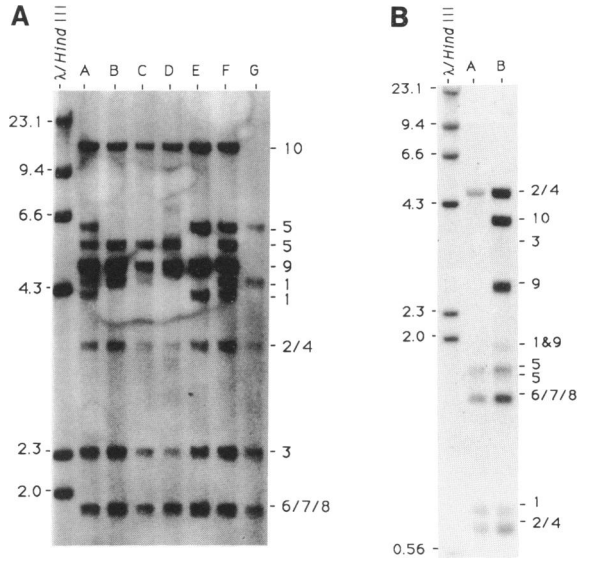

Figure 1 Assignment of exons to bands in OTC *Msp*I and *Taq*I Southern blots. The left lane in both blots contains *Hin*dIII-digested lambda DNA as a size marker. The numbers on the right side of the gel indicate specific exons. The bands representing exons 2, 4, and 6–8 could only be assigned to either the 3′ or 5′ half of the gene. The band marked "2/4" could represent either exon 2 or exon 4, and the band marked "6/7/8" could represent exon 6, exon 7, or exon 8. Panel *A*, *Msp*I digest probed with OTC cDNA. The two sets of polymorphic bands can be seen in lanes A–F. The upper (6.6/6.2-kb) alleles are detected by exon 5, the lower (5.1/4.4-kb) ones by exon 1. The strong constant band of 5.4 kb is detected by exon 9. Lane G, DNA from patient with deletion of 3′ end of OTC gene. Panel *B*, *Taq*I digest. Lane A, DNA from deletion patient. Lane B, DNA from normal individual. The bands detected by exons 1, 3, and 9 are very faint in a normal blot.

low). The Δ value of the lower *Msp*I RFLP was not significant with either of the other polymorphisms. Twenty-nine (76%) of 38 females tested were heterozygous for at least one of the alleles. In four (27%) of 15 families the mother of an index case was homozygous for all RFLP loci, but in two of those families this was due to a deletion of the entire OTC gene, and in one of the two other families there was an altered *Taq*I restriction site, allowing direct mutation detection. Thus a truly uninformative situation was encountered in only one (7%) of 15 families. This information is summarized in table 2.

The use of the exon-specific probes allowed the assignment of particular bands in a Southern blot to individual exons. In the exons not directly detected by probes (exons 2; 4, and 6–8), some assignment was possible by the use of a 5′ probe (exons 1–5). This information is summarized in figure 1. The exon 5 probe detected both the *Bam*HI and upper *Msp*I (6.6/6.2-kb) polymorphisms. When the gene restriction map (Hata et al. 1988) is taken into account, this means that the polymorphic *Bam*HI site maps 4 kb upstream of the 3′ end of intron 4. The polymorphic *Msp*I site could be located in either intron 4 or intron 5. The lower (5.1/4.4-kb) *Msp*I polymorphism is detected by the exon 1 probe and thus maps to the 5′ end of the gene. Exon 10 detects the 3.7/3.6-kb *Taq*I polymorphism.

Deletions

We detected a deletion of the OTC gene in three (17%) of the 18 families we studied. For one patient, missing bands were seen in a Southern blot (fig. 1), and a deletion of the 3′ end of the OTC gene was demonstrated by PCR (fig. 2). A PCR signal was obtained from exons 1, 3 and 5 but not from exons 9

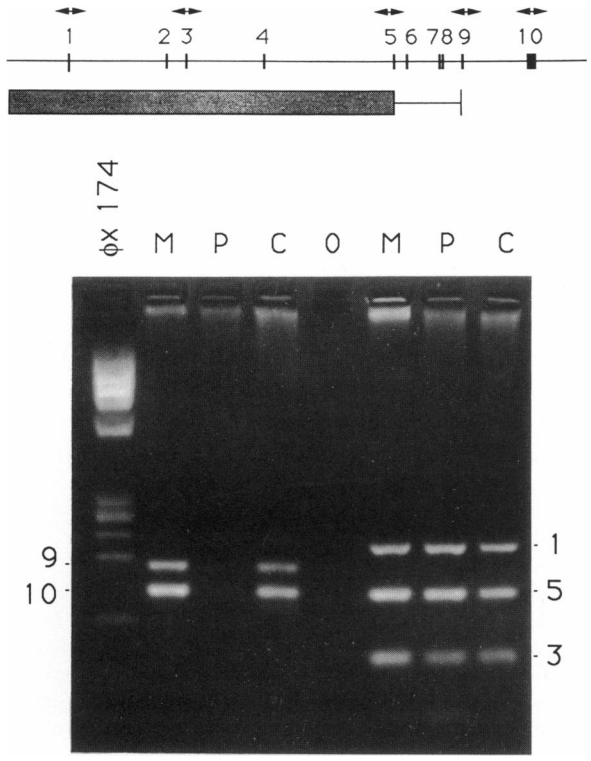

Figure 2 Multiexon amplification for deletion analysis. Ethidium-stained products of PCR amplification of OTC exons 9 and 10 (left half of gel) and of exons 1, 3, and 5 (right half). An *Hae*III digest of φx174 was used as a size marker in the left lane. M = patient's mother; P = patient; C = normal control; and 0 = negative control with no DNA. Exons 1, 3, and 5 were present in the patient, but exons 9 and 10 were not. The top half of the figure schematically shows the structure of the human OTC gene (adapted from Hata et al. 1988) with the localization of the amplification units. The grey bar below the gene diagram indicates the intact part of the gene, with the thin line showing the area in which the breakpoint must be located.

and 10. The deletion breakpoint therefore must lie between exons 5 and 9. In one other patient the deletion could not be demonstrated by Southern blot, since the DNA from the index case was obtained from a 5-year-old autopsy liver specimen and was severely degraded. However, a deletion of the entire OTC gene was revealed by PCR. As a control, the intactness of the X-linked dystrophin gene was demonstrated by multiplex amplification (Chamberlain et al. 1988) (data not shown). A third deletion was detected by failure to inherit maternal alleles in a large kindred with several females affected with OTC deficiency. In the latter two deletion cases, the patient's mother was initially scored as uninformative.

Taq *Site Alterations*

In three (17%) of our 18 families Southern blot analysis after *Taq*I digestion revealed abnormal bands of approximately 3.5 kb. These were initially thought to represent exon 5 *Taq*I-site alterations, of which four cases have been reported in the literature (Nussbaum et al. 1986; Maddalena et al. 1988; Hata et al. 1989). In two of the affected males however, the 1.8-kb *Taq*I band, which has been reported to disappear in exon 5 mutations, was preserved (fig. 3). After amplification of all *Taq*I restriction site–containing exons (exons 1, 3, 5, and 9), followed by *Taq*I digestion, the mutation in these two families was shown to affect the *Taq*I restriction site in exon 3. The third patient fit the "classic" pattern of an exon 5 alteration, and this was confirmed by PCR and *Taq*I digestion.

The data obtained from the use of the exon-specific probes on *Taq*I Southern blots allowed us to predict the band pattern, which would be seen with an exon 1 *Taq*I mutation. Bands of 0.7 and 1.9 kb would fuse to form a new 2.6-kb fragment. However, this new fragment is difficult to detect, because of the 2.6-kb constant band detected by exon 9. In addition, the absence of the 1.9-kb and 0.7-kb bands can easily be

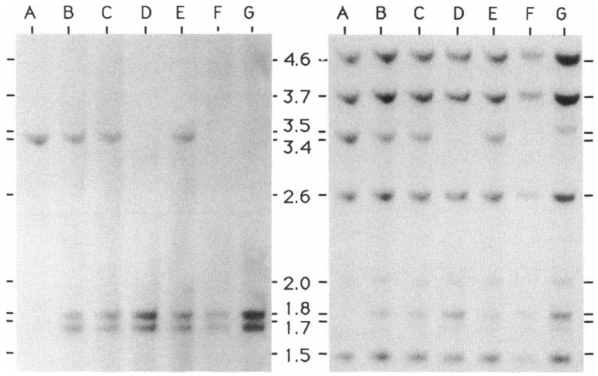

Figure 3 Southern analysis of *Taq*I-site mutations in exons 3 and 5. *Taq*I-digested DNA from different individuals probed with OTC exon 5 (*left panel*) and with full-length cDNA (*right panel*). The numbers in the middle give the band sizes in kilobase pairs. Lane A, DNA from an individual with an exon 5 *Taq*I-site mutation, lane B, DNA from individual's mother. Lanes C and E, DNA from carrier females in this pedigree. Lanes D and F, DNA from normal individuals. Lane G, DNA from mother of patient with an exon 3 *Taq*I-site mutation. The left panel illustrates our use of exon-specific probes for the assignment of bands. Exon 5 detects two bands of approximately 1.7 kb. In individuals with the *Taq*I-site mutation, these two bands disappear and become replaced by a new band of 3.4 kb; this is not the case with an exon 3 mutation. Note, however, that the pattern looks very similar between an exon 3 carrier (lane G) or patient and exon 5 carrier females (lanes B, C, and E).

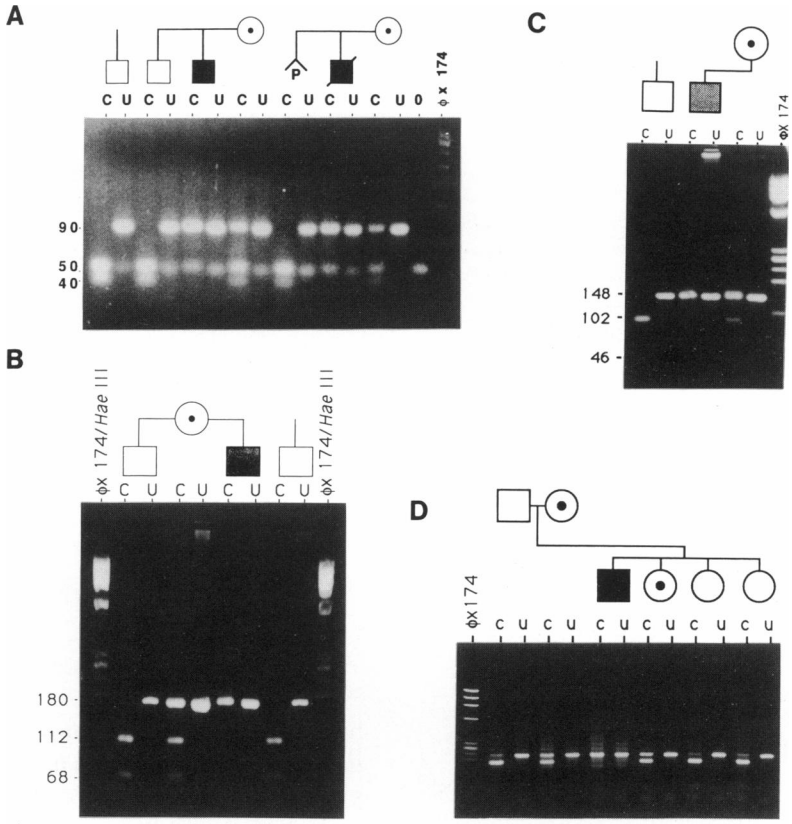

Figure 4 PCR detection of *Taq*I-site mutation in exons 1, 3, 5, and 9. Ethidium-stained gels containing amplified OTC exon 3 (panel *A*), exon 9 (panel *B*), exon 5 (panel *C*), and exon 1 (panel *D*) in five different families with OTC deficiency. Lanes C, Amplified DNA digested with *Taq*I. Lanes U, Uncut PCR product. Band sizes, in basepairs, are indicated by numbers at the side of the gels. The PCR products from all patients failed to be digested by *Taq*I, whereas complete digestion occurred in normal controls. In all five families, the patient's mother showed evidence of both cut and uncut PCR product, indicating carrier status. In the second family in panel *A*, this technique was used for prenatal diagnosis.

missed, since the lower fragment is often run off the gel and since the 1.9-kb band is extremely faint. We therefore rescreened by PCR all families in which the mutation was unknown, and, indeed, we found one family with an exon 1 *Taq*I-site mutation. No abnormal band pattern had been noticed on the Southern blot.

The PCR analysis also led to the identification of the mutation in a patient previously described elsewhere (Spence et al. 1989) who is not part of the current series of 18 families. The abnormal band pattern in this patient is due to the abolishment of the exon 9 *Taq*I site and consisted of a new band of 4.5 kb together with the disappearance of the 2.6-kb constant band. The detection of these mutations by PCR, both in the four families which are part of the present study and in the one family previously described by Spence et al. (1989), is shown in figure 4. All five mutations were sequenced and can be found in table 3. This table also lists all other point mutations identified by our laboratory, as well as their locations within the OTC gene.

Chemical Mismatch Cleavage

Chemical mismatch cleavage was used for prenatal diagnosis and carrier detection in the SZ family, in which the mutation had been previously determined to be a GAA→TAA change in exon 5, codon 154 (Grompe et al. 1989). As can be seen in figure 5, the full-length uncleaved heteroduplex is 122 bp long, and the cleavage products are 63 bp with the wild-type probe and 59 bp with the mutant probe. Presence of a cleavage band with a given probe indicates a sequence mismatch between that probe and the DNA which is

Table 3

Point Mutations and Their Distribution in the OTC Gene

Patient	Codon Change		Exon	Amino Acid Change	Restriction-Site Alteration
	Position	Change			
573[a]	23	CGA→TGA	1	Arg→stop	*Taq*I
M[b]	26	CGG→CAG	1	Arg→Gln	None
B[b]	45	CTA→CCA	2	Leu→Pro	None
JC[a]	92	CGA→CAA	3	Arg→Gln	*Taq*I
SM[a]	92	CGA→TGA	3	Arg→stop	*Taq*I
RC[b]	111	CTT→CCT	4	Leu→Pro	None
ZH[a]	141	CGA→TGA	5	Arg→stop	*Taq*I
SZ[b]	154	GAA→TAA	5	Glu→stop	None
S[b]	216	CAG→GAG	6	Gln→Glu	None
RZ[a,c]	320	CGA→CTA	9	Arg→Leu	*Taq*I

NOTE.—Listed are all the point mutations of the OTC gene that have been identified in our laboratory to date.

[a] Mutations were discovered and sequenced during present study.

[b] Mutations have been published elsewhere (Grompe et al. 1989).

[c] Patient was not part of the current series of 18 families but has been described elsewhere by Spence et al. (1989).

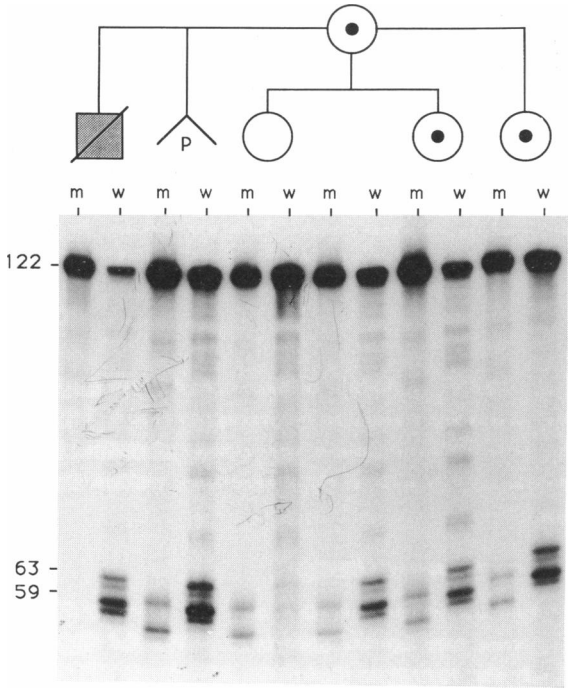

Figure 5 Prenatal diagnosis and carrier detection by chemical mismatch cleavage. Shown is an autoradiogram of a chemical mismatch cleavage analysis in a family with OTC deficiency due to a GAA→TAA change in codon 154. Lanes m, Heteroduplex formed with radiolabeled mutant sense strand and cleaved with osmium tetroxide. Lanes w, Radiolabeled wild-type antisense probe used for heteroduplex formation and cleaved with hydroxylamine. Individuals, who show cleavage of the wild-type probe have the mutant allele; conversely, those individuals who harbor a wild-type allele, show cleavage of the mutant probe. DNA from the index case (*far left*) only cleaved with the wild-type probe, since he is hemizygous for the mutation. The fetal DNA gave cleavage of both probes. The fetus therefore is a female carrier, as are the patient's mother and two of her daughters. DNA from a third daughter (*third from left*) did not cause cleavage of the wild-type probe, and therefore she is not a carrier.

being studied. In the lanes with positive cleavage, bands additional to the expected ones are present, a phenomenon commonly seen with this technique and indicating destabilized bases in the neighborhood of the mutation (Cotton and Campbell 1989). The fetus was identified as a female carrier, since cleavage occurred with both the mutant and the wild-type probe. Two of the patient's half-sisters were identified as carriers, despite the unavailability of DNA from any of the fathers involved. These results were confirmed by allele-specific oligonucleotide hybridization (data not shown).

Discussion

Since the cloning of the OTC cDNA in 1984, Southern blot analysis for the detection of intragenic polymorphisms has been the mainstay of carrier and prenatal diagnosis. Most (70%–80%) females are informative for one of the RFLPs, thus allowing link-

age-based diagnosis if the phase can be set. In OTC deficiency this can be difficult for several reasons, the most important one of which is the high incidence of new mutations in this disorder. Many of the mothers in monoplex families might not be carriers. In a recent study, one-third of the mothers of isolated male patients and two-thirds of the mothers of isolated female patients were found to be noncarriers by biochemical carrier testing (Hauser et al. 1990). Isolated cases of OTC deficiency are very common, and in our own series of 18 families a positive family history was obtained in only four instances. Biochemical carrier testing therefore is very important and often is needed to interpret data obtained from DNA analysis. Recently Hauser et al. (1990) have shown that allopurinol loading and the measurement of orotidine rather than orotic acid is a reliable test. A positive allopurinol loading test is very strongly suggestive of carrier status. However, a negative test does not rule out carrier status for OTC deficiency, and the remaining risk is estimated to be 10%–15%. This is not unexpected in an X-linked disease, since bias of X inactivation can mask the carrier status.

Despite these difficulties, in several reports on OTC deficiency the overall success rate when RFLP analysis is used in conjunction with carrier testing is 60%–80% (McClead et al. 1986; Schwartz et al. 1986; Fox and Rosenberg 1988; Svirklys et al. 1988; Wendel et al. 1988; Spence et al. 1989). However, there are two groups of families in which the linkage-based approach is unsatisfactory. The first group are those in which the patient's mother is noninformative by RFLP. In those families, prenatal diagnosis is impossible, even if the carrier status of the mother is known. The second group are those families is which RFLPs cannot be used for positive fetal diagnosis because the mother has not been confirmed to be a carrier by allopurinol loading. In these families, only the direct detection of the disease-causing mutation can resolve the diagnostic issues. In OTC deficiency there are three classes of changes in regard to direct mutation detection: large deletions, point mutations affecting *Taq*I restriction sites in the cDNA, and other single-base or small mutations not detectable by Southern blot. Their incidence and the strategies for their detection will be discussed in the following.

Large deletions were found in three of the 18 families we studied and are readily detectable by Southern blot analysis, if high-quality DNA is available from the affected male. However, in two of these three deletion families this was not the case, and the deletions only became apparent when an extended family analysis was performed that showed failure to inherit maternal alleles in some cases. RFLP studies in the extended family of such "noninformative" females is therefore valuable and indicated. Simultaneous amplification of several OTC exons, as demonstrated in the present paper, facilitates and simplifies diagnosis in deletion cases and permits the use of degraded DNA sources.

Single-base changes which abolish one of the four *Taq*I restriction sites in the OTC cDNA constitute an important subgroup of mutations in OTC deficiency. Four of 24 patients in the study by Spence et al. (1989) and four of the 18 families in our study harbored such mutations. The last three bases of all four TCGA *Taq*I recognition sites in the OTC cDNA code for an arginine. The functional importance of these arginines and the increased susceptibility to transition mutations at CpG dinucleotides probably account for the high incidence of changes at these sites in patients with severe OTC deficiency. Previously several groups have reported mutations affecting the exon 5 *Taq*I restriction site (Maddalena et al. 1988; Hata et al. 1989; Lee and Nussbaum 1989). We now have also identified and characterized such mutations in exons 1, 3, and 9. Although each of these changes is associated with a typical band pattern in *Taq*I Southern blots probed with the full-length cDNA (see Results), the PCR-based method of detection described in the present paper is superior. The PCR-based test is quicker, can be performed with degraded DNA or small quantities of DNA, and is able to detect exon 1 mutations, which are otherwise easily missed.

The majority (11 of 18 in our study) of mutations in OTC deficiency are not detectable by Southern blot analysis but can be identified in the majority of families, should this become necessary diagnostically. Two principal approaches have been reported. In the first strategy, cDNA is generated from mRNA from an index patient and is amplified by PCR, and the mutation is identified by chemical mismatch cleavage (Grompe et al. 1989). In three of the families in our series the mutation was identified in this fashion. The second technique involves amplification of individual OTC exons by PCR, followed first by denaturing-gradient-gel electrophoresis to identify the exon with the mutation and then by subsequent sequencing. This approach has been successfully used in one reported case (Finkelstein et al. 1990). The knowledge of the mutation obtained from such studies can then be utilized for accurate diagnosis. In one of the families reported here we performed prenatal diagnosis and car-

rier detection by chemical mismatch cleavage, on the basis of previous knowledge of the mutation. This represents the first use of this technique in a prenatal test.

Although the direct detection of the disease-causing sequence alteration is technically feasible and would be the most accurate mode of diagnosis in a new mutation disease such as OTC deficiency, this approach is not practical or necessary in most cases (see table 2). Conventional Southern analysis in the core family was performed as the initial approach in all of the cases we studied. In four families this revealed the disease-causing mutation (one partial deletion, one exon 5 *Taq*I-site alteration, and two exon 3 *Taq*I-site alterations), and in three additional families the mutations were identified by RFLP family studies (two deletions) or PCR analysis (exon 1 *Taq*I-site mutation). In these seven families the PCR-based test is the diagnostic method of choice. In 10 of the remaining 11 families, the mother of the index case was heterozygous for one of the RFLPs, and thus the standard linkage-based approach in connection with biochemical carrier testing was the most efficient approach. Thus only one of the 18 families we studied was truly uninformative, and direct mutation detection was needed for accurate diagnosis. Chemical mismatch cleavage on amplified liver mRNA is currently being performed in this family. We also anticipate the need for direct mutation analysis in some of the 10 families in which the patient's mother is heterozygous for an RFLP. In six of these 10 women biochemical carrier testing has not been performed (in two cases, because of carrier status known by family history); in two of these 10 women the results were ambiguous; and in two of these 10 women the results were positive. Direct mutation detection has been performed in the two families which showed ambiguous test results (Grompe et al. 1989) and has been used in prenatal testing in one family. Whether it is worthwhile to determine the disease-causing mutation in those families (approximately 25% of all cases) in which a female's carrier status cannot be confirmed by biochemical carrier testing remains a matter for discussion, since the remaining risk is relatively low (5%–8% for an affected male). If these are included in addition to the families of known carrier status but uninformative by RFLP (approximately 10% of all cases), the need for direct mutation detection would arise in approximately 35% of all OTC families.

The proportion of families in which a given diagnostic procedure is indicated is also illustrated in figure 6, which contains a diagnostic algorithm for OTC deficiency. The successive steps of Southern analysis, biochemical carrier testing, and direct mutation detection, as well as the approximate percentage of patients which we expect to fall into each of the categories, are shown. Accurate diagnosis can be achieved in at least 90% of families by a combined use of the available methods. This algorithm represents our own current approach to OTC diagnosis but may change with improvement in diagnostic techniques.

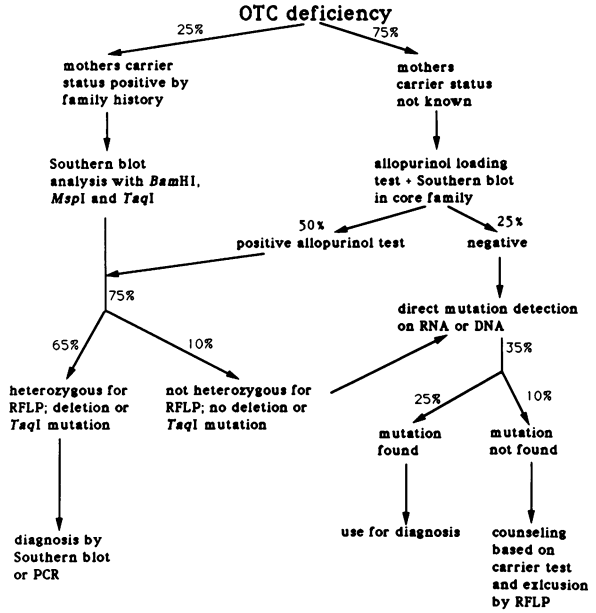

Figure 6 Diagnostic algorithm in OTC deficiency. The sequential diagnostic steps are outlined along with the percentage of patients which we estimate to fall into each category. These estimates are based on the known incidence of the RFLPs, the proportion of families thought to represent new mutations, and our own experience in how often we find positive family histories. The term "core family" is used to denote the patient's immediate family plus mother's siblings and parents.

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