THE MOLECULAR BASIS

OF CARBONIC ANHYDRASE II DEFICIENCY

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MOLECULAIR-GENETISCHE BASIS
VAN KOOLZUURANHYDRASE II DEFICIENTIE

PROEFSCHRIFT

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One fence needs three piles.
One hero needs three helpers.
(Chinese proverb)

To Xiao Mi and Tom Tian Mi
who sacrificed many many
things for the realization of
this thesis
To my parents
who encouraged and
supported me to pursue the
truth of nature
To all my teachers and friends
who taught me science
who helped me when needed
without whom this thesis would
have been impossible

CONTENTS

SCOPE

The association of renal tubular acidosis with osteopetrosis was first reported in three different families in 1972. Later, in 1980 cerebral calcification was added to the syndrome. The pattern of inheritance is autosomal recessive. In 1983, Sly et al. reported that the original patients lacked carbonic anhydrase II (CA II) in their erythrocytes and proposed CA II deficiency as the primary defect in this syndrome.

Carbonic anhydrase (CA) is the enzyme catalyzing the reversible hydration of CO_2 in the reaction $CO_2+H_2O<->HCO_3+H^+$ which contributes to acid-base balance in human body by acid base homeostasis, H^+ and HCO_3^- secretion and CO_2 exchange. Among seven CA isozymes, CA II deficiency is the only one known to attribute to a human disease. More than 50 patients have been reported to date from different geographical and ethnic origins. All patients described so far were totally deficient in CA II. However, the clinical manifestations in different pedigrees studied vary in severity, suggesting a genetic heterogeneity among different groups of patients.

The genes coding for CA I, II and III were found to be clustered in a stretch of about 180 kb on chromosome 8q22. In 1991 the CA II gene was cloned and the first mutation identified.

The aim of the work presented in this thesis was to characterize mutations in the structural gene of CA II in different groups of patients with a wide range of clinical manifestations, so as to delineate the genetic basis of the clinical heterogeneity.

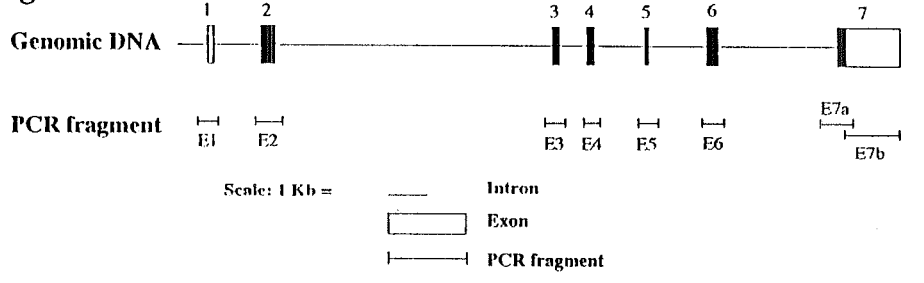
Chapter 1

Introduction to carbonic anhydrase II deficiency syndrome

1.1 Carbonic anhydrase: Isozymes, gene families, chromosome localization and physiological functions

The carbonic anhydrases (CAs, EC 4.2.1.1, carbonate dehydrolase), are a family of zinc metalloenzymes that catalyze the reversible hydration of CO_2 in the reaction $CO_2 + H_2O <-> HCO_3^- + H^+$. They vary in physicochemical properties, in sensitivity to various inhibitors, and in their subcellular localization (Sly and Hu, 1995). Seven isozymes, CA I - CA VII, have been identified in mammals (Tashian, 1989). CA I, CA II, CA III, and CA VII are cytosolic (Tashian, 1989; Deutsch, 1987). CA IV is anchored to the extracellular surface of the plasma membranes (Whitney and Briggle, 1982; Wistrand and Knuuttila, 1989; Zhu and Sly, 1990; Sly and Hu, 1995), of certain differentiated cells (Brown et al., 1990; Fleming et al.,1993; Ghandour et al., 1992; Hageman et at., 1991). CA V is mitochondrial (Storey et al., 1984, Nagao et al., 1993), and CA VI is secreted in saliva (Feldstein and Silverman, 1984; Murakami and Sly, 1987; Aldred et al., 1991). CA VII is a "virtual enzyme" which we know conceptually from its gene, its cDNA, and its mRNA detected by in situ hybridization in the cytosol of the salivary glands (Montgomery et al., 1991; Tashian, 1992).

Fig. 1



Genomic organization of human CA II gene and PCR fragments for mutational analysis. Seven exons are indicated by box and numbered. The filled box represents the coding region. PCR fragments are shown below the genomic organization, indicated by E1 to E7b.

Like most human CA genes, the human CA II (hCA II) gene contains 7 exons separated by 6 introns as shown in Fig. 1. The hCA II gene (CA2),

together with those of CA I and CA III (CA1 and CA3) are clustered in a stretch of about 180 kb on chromosome 8q22 in the order of CA1, CA3, CA2. The CA II and CA III genes are transcribed in the same direction and opposite to that of CA I (Lowe et al., 1991). CA IV was assigned to 17q23 (Okuyama et al., 1993), and CA VI was assigned to chromosome 1p36.22-33 (Sutherland et al., 1989). CA V was recently assigned to chromosome 16 (Nagao et al, 1993), where CA VII was previously mapped on 16q22 (Montgomery et al., 1991).

CA II is a high-activity isozyme with a maximum turnover rate for CO_2 hydration of 1×10^6 sec $^{-1}$ (Khalifah, 1971), and has the widest distribution, being expressed in the cytosol of cell types in virtually every tissue or organ (Tashian, 1992). Cell types expressing CA II include osteoclasts in bone, oligodendrocytes in brain, epithelium of the choroid plexus (brain) and the ciliary body (eye), lens, Müller cells in retina, liver (mainly perivenous hepatocytes), kidney (proximal tubule, distal tubule, and intercalated cells of the cortical collecting ducts), acinar cells in salivary glands, pancreatic duct cells, gastric parietal cells, endometrium of the uterus, endothelial cells, epithelial cells of seminal vesicle and ductus deferens (Kaunisto et al., 1990), spermatozoa (Parkkila et al., 1991), endothelial and epithelial cells of duodenum, intestine, and colon (Lonnerholm et al., 1985), erythrocytes, and platelets. It has also been reported recently in zona glomerulosa cells of the adrenal (Parkkila et al., 1993), neutrophils (Campbell et al., 1994) and type II epithelial cells of lung (Fleming et al., 1994).

The physiological roles of CA II in these cell types are diverse (Dodgson et al., 1991; Tashian, 1992). In some cells, CA II plays a major role in contributing to acid base homeostasis. It contributes to H^+ secretion by gastric parietal cells, by renal tubular cells that secrete H^+ to produce urinary acidification, and by osteoclasts which secrete H^+ to acidify the bone-resorbing compartment. CA II promotes HCO_3^- secretion by pancreatic duct cells which contribute HCO_3^- to pancreatic juice, by ciliary body epithelium (which produces aqueous humor), by choroid plexus (which produce cerebral spinal fluid), by salivary gland acinar cells (which produce saliva), and by distal colonic epithelium, where H^+ and HCO_3^- secretion are coupled to Cl^- and Na^+ reabsorption and contribute to electrolyte and water balance (Dagher et al., 1993). CA II also promotes CO_2 exchange in proximal tubules in the kidney, in the erythrocytes, and in lung. It has been suggested that it contributes to fatty acid and amino acid synthesis (Tashian, 1992).

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1.2 CA II deficiency syndrome: Heterogeneity in clinical manifestations in patients from different geographical origins.

To date, the only known disease attributable to a deficiency of CA is the CA II deficiency syndrome (Sly and Hu, 1994). The association of renal tubular acidosis with osteopetrosis was first reported in three different families in 1972 (Guibaud et al., 1972; Vainsel et al., 1972; Sly et al., 1972). The pedigree from these families suggested that the pattern of inheritance is autosomal recessive. In 1980, Ohlsson et al. (1980) described a syndrome that consisted of osteopetrosis, renal tubular acidosis and cerebral calcification in a Saudi Arabian family. The same year Whyte et al. (1980) reported intracranial calcifications in the American siblings with osteopetrosis and renal tubular acidosis that originally had been reported in 1972 by Sly et al. (Sly et al., 1972).
Metabolic acidosis can be produced by sulfonamide inhibitors of CA (Maren, 1967). CA inhibitors also inhibit the hormone-induced release of calcium from bone, suggesting a role for CA in bone resorption (Waite et al., 1970; Waite, 1972; Minkin and Jennings, 1972). The presence of CA II isozyme in renal and brain tissues (Kumpulainen and Nystrom, 1981; Wistrand, 1980; Dobyan and Bulger, 1982) together with pharmacological studies on the role of CA inhibitors in cerebrospinal fluid production (Vogh, 1980) led Sly et al. (1983) to predict a defect in CA in osteopetrosis, renal tubular acidosis and cerebral calcification. CA II deficiency seemed likely because complete absence of CA I in erythrocytes has no clinical symptoms (Kendall and Tashian, 1977). Since both CA I and II are present in erythrocytes, it was possible to check the activities of CA I and II enzymes in blood lysates of the patients. Sly et al. (1983) reported that the original American kindred with this syndrome were deficient in CA II and proposed CA II deficiency as the primary defect in this syndrome. Since then, more than 50 patients with this syndrome have been reported from different geographical and ethnic origins, all of whom were shown to have CA II deficiency (Sly and Hu, 1994).
Besides osteopetrosis, renal tubular acidosis, and cerebral calcification, other clinical features of CA II deficiency syndrome include mental retardation, seen in over 90% of reported cases, growth failure, and dental malocclusion. Complications of osteopetrosis include increased susceptibility to fractures which heal normally, and cranial nerve compression symptoms. Anemia and other hematological manifestations are mild or absent. The renal tubular acidosis is usually of a mixed type. A distal component is evident from inability to acidify the urine, and a

proximal component is evident from a decreased transport maximum for bicarbonate (Sly and Hu, 1994).

Interestingly, patients from different ethnic background tend to present different clinical manifestations and severity. One of the first cases reported with the syndrome of osteopetrosis, renal tubular acidosis and cerebral calcification was a Belgian patient who had normal intelligence and suffered from frequent skeletal fracture (Vainsel et al., 1972). Three sisters of an American family, had similar clinical manifestations (Sly et al., 1972). However, the third case reported in 1972, was an Arabic patient (Guibaud et al., 1972), who, like subsequently reported patients of Arabic descent from Kuwait, Saudi Arabia, Algeria, and Tunisia, was more severely affected. Mental retardation and metabolic acidosis were prominent in these patients, while bone fractures were less frequent (Strisciuglio et al., 1990).

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1.3 Mutational analysis on patients with CA II deficiency syndromes: PCR of genomic DNA, subcloning, nucleotide sequencing and RFLP.

The clinical heterogeneity in CA II deficient patients from different ethnic background makes it interesting to know whether there is any genetic heterogeneity, and whether possible differences in gene mutations can be related to the clinical variation. Although mRNA from CA II-deficient patients could not easily be obtained, knowledge of the genomic organization and of the intron sequences surrounding each exon has made mutational analysis on patient genomic DNA straightforward. Using intronic oligonucleotide primers (Table 1), polymerase chain reaction (PCR) was performed to amplify each exon including exon-intron boundaries from genomic DNA (Fig. 1). PCR fragments were either subcloned into sequencing vector and sequenced, or directly sequenced. If a mutation created or removed an unique restriction site, the mutation was confirmed with restriction fragment length polymorphism (RFLP) analysis. If the mutation did not change any restriction site, dot-blot hybridization with allele-specific oligonucleotide probes was used to distinguish mutant allele from the normal.

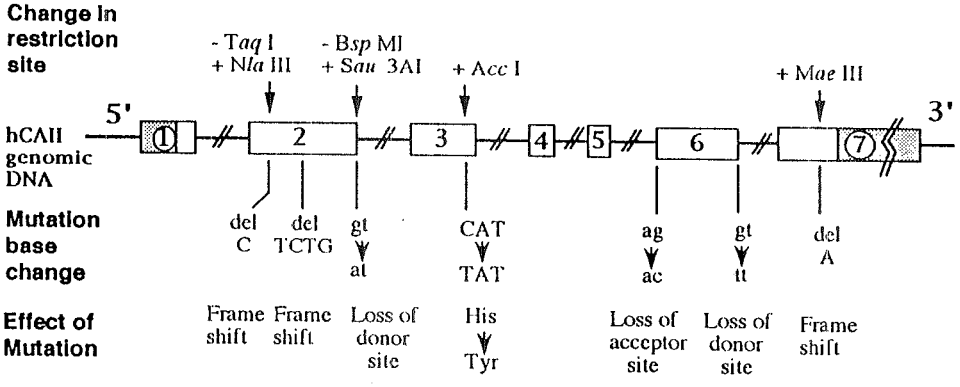
Table 1. PCR primers for analysis of CA II mutations.

Exon	Forward primers	Reverse primers	Size (bp)
Exon 1	5'CGCCCGTCACCTCCTCCCCT	3'TGCCGGTCGCGCCCCCGCGG	255
Exon 2	5'GATTGCTCTTCTCTAGGGGT	3'CACTGTTCCTAGCTCCTTCC	328
Exon 3	5'CTCTGGATTGAATTTTCAGAG	3'TCGTAATTAAAGTTTTCGAATTAA	230
Exon 4	5'TCACTCACTGTGGCTTTGTC	3'CTTACTGGTCTGTCTC	186
Exon 5	5'GGGGTCATGTATGAAGTGGAG	3'GTTATGAGGTAACCAAAATC	256
Exon 6	5'GTGATAGTTTGAAGCTGCG	3'TCAGTTCCGTCTTCTGGAAC	254
Exon 7a	5'ATTACAGCATGAGCCACTGC	3'TCTGATCTGGTTAACAGTAC	409
Exon 7b	5'AGCCACTGAAGAACAGGCAA	3'GGTAAGTCTGTTATATAGTA	688

Seven different mutations in the CA II structural gene have been identified (Fig. 2). The first mutation was identified in the mildly affected Belgian patient homozygous for a C to T transition in exon 3, which

results in replacement of the conserved histidine at position 107 with tyrosine (His107Tyr) (Venta et al., 1991). The three affected sisters in the American family were also found to have this mutation. However, they were compound heterozygotes, having inherited the His107Tyr mutation from their mother and a splice acceptor mutation in the 3' end of intron 5 from their father (Roth et al., 1992).

Fig. 2



Seven structural mutations found to date in the human CA II gene. + or - sign indicate the restriction sites introduced or removed by the mutation. Capital letters and the lower cases represent base pairs in exons and introns respectively. A *gt→at* change in the 5' end of intron 2 destroyed a splice junction donor site. This mutation is common in Arabic patients (Hu et al, 1992). A *C→G* transition in exon 3 results in a replacement of the conserved histidine at position 107 with tyrosine (His107Tyr). This mutation was identified in a homozygous Belgian patient (Venta et al, 1991), a homozygous Italian patient (Hu, Strisciuglio, Ciccolila and Sly, unpublished data), and also as one of two mutations in the three American sisters who were compound heterozygotes. Their second mutation is an *A→C* transversion at the 3' end of intron 5, which destroys a splice junction acceptor site (Roth et al, 1992). A single-base deletion in the coding region of exon 7 results in a frameshift at codon 227, which changes the next 12 amino acids and introduces a UGA stop codon 22 amino acids earlier than in the normal enzyme. This mutation was found to be common in Hispanic patients from Caribbean islands (Hu et al, 1992; Hu et al, 1994). Two different deletions in exon 2 and a *gt→tt* change in the 5' end of intron 6 were discovered recently in two Italian families and one American family (Hu, Strisciuglio, Ciccolila and Sly, unpublished data).

As mentioned earlier, neither the Belgian patient nor the American patients were mentally retarded. Frequent skeletal fractures were the most

disabling manifestation of their disease (Strisciuglio et al., 1990). When the CA II cDNA containing the His107Tyr mutation was expressed in E. coli, some CA activity was detected. These experiments led to the suggestion that a small amount of residual CA II activity in patients with the His107Tyr mutation may allow them to escape mental retardation (Roth et al., 1992

The third structural gene mutation identified is a splice junction mutation at the 5' end of intron 2, which was found in patients from Kuwait, Saudi Arabia, Algeria, and Tunisia and all of the patients of Arabic descent so far studied (Hu et al., 1992a). More than 75% of the patients so far recognized have been Arabic and have been severely affected. The new Sau 3AI restriction site created by this mutation made it easier for diagnosis and prenatal diagnosis in CA II deficient patients with Arabic background. An RFLP screening in Tunisia has discovered 11 patients homozygous with this mutation (Fathallah et al., 1993). The consequence of the early splice junction mutation may well explain the clinical severity for this group of patients.

A single base deletion in exon 7 resulting in a frameshift and truncated mutant protein was found in seven unrelated patients of Caribbean Hispanic background (Hu et al., 1994). Unlike Belgian and American patients with mild clinical course, or Arabic patients who were severely affected, the Hispanic patients presented with a wide range of clinical manifestations. Characterization of the Hispanic mutant protein in COS cells and bacteria revealed that the predicted 27-kDa truncated protein was produced, but it was insoluble, inactive and rapidly degraded. A 29-kDa mutant protein was also found in bacteria and COS cells expressing the Hispanic mutant cDNA. This full length mutant protein was identical with the wild type enzyme in both N- and C-termini, but it contains 11 missense amino acids between the codon containing the single base deletion and the point where a ribosomal frameshift restores the reading frame. These 11 missense amino acid may explain the reduction to 10% of wild type activity in this full length mutant protein. Whether individual variability in efficiency of ribosomal frameshift contributes to the clinical variability in patients with this mutation deserves further study (Hu et al., 1992b; Hu et al., in press).

Recently, three new mutations were discovered in the CA II structural gene using single strand conformational polymorphism (SSCP) and direct sequencing of the PCR products (Hu, Strisciuglio, Ciccolella, and Sly, unpublished data). Two deletion mutations were found in Exon 2, including a four base pair deletion identified in an American family, and a single base deletion identified in an Italian family. Two other Italian

families were studied recently, in one of which the CA II deficient patient was homozygous for the His107Tyr mutation. In the other Italian family, the CA II deficient patient was homozygous for a splice junction mutation at the 5' end of intron 6. A summary of the different mutations found in the CA II structural gene is presented in Table 2 and in Fig. 2.

Table 2 Different mutations in CA II structural gene

Ethnic background	Mutations	Reference
Belgian	His107Tyr (missense mutation in exon 3)	Venta et al.,1991
German/ Italian	His107Tyr from Italian originated mother Splice junction mutation at the 3'end of intron 5 from German originated father	Roth et al., 1992
Arabic	Splice junction mutation at the 5'end of intron 2	Hu et al., 1992
Caribbean Hispanic	Frameshift mutation due to single base deletion in exon 7 coding region	Hu et al., 1994
American	Frameshift mutation due to 4 base pair deletion in exon 2	Hu et al., unpublished
Italian	Frameshift mutation due to single base deletion exon 2	in Hu et al., unpublished
Italian	Splice junction mutation at the 5'end of intron 6	Hu et al., unpublished

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Chapter 2

Results and discussion of the experimental work on the CA II deficiency syndrome

The clinical heterogeneity of CA II deficient patients with different ethnic background raised the challenging question whether different phenotypes could be related to different gene mutations? Three publications in this chapter deal with the delineation of Arabic and Hispanic mutations in the human CA II gene, and with the characterization of Hispanic mutant protein expressed in mammalian cells and bacteria.

Among more than 50 reported cases of CA II deficiency syndrome, 75% were from North African and Middle Eastern countries. Patients from these areas were severely affected with mental retardation besides their osteopetrosis and renal tubular acidosis. Mutation analysis on a severely affected proband from Algeria revealed a splice junction mutation in the 5' end of intron 2. Subsequently the same mutation was found in five other patients from other Arabic countries, such as Kuwait and Saudi Arabia. In six unrelated patients of Arabic descent, five were found to be homozygous for this splice junction mutation and one heterozygous, suggesting that this early splice junction mutation which seems to result in a clinically severe phenotype may account for most of the cases with the CA II deficiency syndrome in Arab communities.

The new Sau 3AI restriction site introduced by the G to A transition in the exon 2/intron 2 boundary allows screening for this mutation on the basis of the unique electrophoretic pattern of the PCR products of exon 2 following digestion with Sau 3AI. This finding provides a simple and accurate procedure for detecting this mutation in the Arabic communities and could be useful for the molecular diagnosis of index patients, carrier detection, and prenatal analysis (see **Publication II**).

A single base deletion in the coding region of exon 7 of the human CA II gene was found to be the predominant mutation in Caribbean Hispanic patients. A $2^1/_2$-year-old Hispanic girl of Puerto Rican ancestry was unique clinically in that she had no evidence of renal tubular acidosis which is seen in nearly all other patients with this disorder, even though she did have osteopetrosis, developmental delay, and cerebral calcification. For this reason, we suspected she would have a missense mutation that produced an enzyme with sufficient residual activity to spare the kidney. However, she proved to be homozygous for a single-base deletion in exon 7 that produces a frameshift which changes the next 12 amino acids before leading to chain termination. The 27-kDa truncated enzyme produced when the mutant cDNA was expressed in COS cells

was enzymatically inactive, present mainly in insoluble aggregates, and detectable immunologically at only 5% the level of the 29-kDa normal CA II expressed from the wild-type cDNA. Metabolic labeling revealed that the 27-kDa mutant protein has an accelerated rate of degradation.
Six subsequent Hispanic patients of Caribbean ancestry, all of whom had osteopetrosis and renal tubular acidosis but who varied widely in clinical severity, were found to be homozygous for the same mutation, Using the new Mae III restriction site introduced by the single base deletion. The results of this observation are discussed in **Publication III**.
In **Publication IV**, the gene products of the Hispanic mutation in human CA II deficiency are characterized in bacteria. When the Hispanic mutant cDNA was expressed in bacteria, a small amount of CA activity was detected in the crude cell lysate. The enzyme activity was not found in the 27-kDa truncated form of the protein, but rather in a form which resembled the 29-kDa normal length CA II in size and in binding to a sulfonamide inhibitor column. However, the affinity purified 29-kDa mutant enzyme had only 10% of the activity of the wild type enzyme. Biochemical and immunochemical analysis demonstrated that this protein is identical to wild type hCA II except for the 11 missense amino acids between the +1 frameshift at codon 227 (due to the single base pair deletion), and the -1 ribosomal frameshift at codon 237. After the reading frame is restored at codon 237, the protein is completed by translation of the last 23 amino acids in the normal reading frame. Immunochemical evidence is presented that the 29-kDa mutant protein is also produced in small amounts in COS cells from the Hispanic mutant cDNA and that the mutant protein is retained on the sulfonamide inhibitor column and reacts on Western blots with the frameshift peptide-specific antibodies. Although the mechanism underlying this particular frameshift is not clear, the evidence for ribosomal frameshift rescuing the original frameshift mutation suggests a mechanism whereby ribosomal frameshift could ameliorate the consequences of certain deleterious frameshift mutations. In this respect, the clinical variability of CA II-deficient patients homozygous for the same frameshift mutation is of considerable interest. Whether individual variability in efficiency of ribosomal frameshift contributes to the clinical variability in patients with this mutation deserves further study.

Publication I

THE CARBONIC ANHYDRASE II DEFICIENCY SYNDROME: OSTEOPETROSIS WITH RENAL TUBULAR ACIDOSIS AND CEREBRAL CALCIFICATION

William S. Sly and Peiyi Y. Hu

The Metabolic and Molecular Basis of Inherited Disease

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The Carbonic Anhydrase II Deficiency Syndrome: Osteopetrosis with Renal Tubular Acidosis and Cerebral Calcification

William S. Sly, M.D., Ph.D. and Peiyi Y. Hu, M.D.

1) The carbonic anhydrase II deficiency syndrome is an autosomal recessive disorder that produces osteopetrosis, renal tubular acidosis, and cerebral calcification. Other features include mental retardation (seen in over 90 percent of reported cases), growth failure, and dental malocclusion.
2) Complications of osteopetrosis include increased susceptibility to fractures (which do, however, heal normally) and cranial nerve compression symptoms. Anemia and other hematological manifestations of osteopetrosis are absent.
3) The renal tubular acidosis is usually a mixed type. A distal component is evident from inability to acidify the urine, and a proximal component is evident from a lowered transport maximum for bicarbonate.
4) About fifty patients have been reported, all of whom have a quantitative deficiency of carbonic anhydrase II activity and immunoreactivity in erythrocytes. Heterozygous carriers can be identified by simple tests.
5) The carbonic anhydrase II gene is 20 kb, contains seven exons, and maps to chromosome 8q22. Four different mutations in the structural gene have been identified by PCR amplification of genomic DNA from patients with this disorder. Two families with the His 107 → Tyr missense mutation have been notable for the relatively high frequency of skeletal fractures and absence of mental retardation. A splice junction mutation at the 5′ end of intron 2--the "Arabic mutation"--is found in most patients of Arabic descent, who account for over 75 percent of cases so far recognized. A frameshift mutation in exon 7 is the most common mutation in Hispanic patients from the Caribbean islands.
6) PCR-based diagnosis and prenatal diagnosis are available for these four mutations.
7) Symptoms of metabolic acidosis improve with treatment, but no specific treatment is available.

HISTORY

Osteopetrosis (marble bone disease) was first described in 1904 by Albers-Schonberg [1]. Subsequently, over 300 cases have been reported [2]. Among these, two principal types were distinguished. An autosomal dominant form was called the "adult, benign" form because of the relatively few symptoms and the benign course, which is compatible with a normal life span. This diagnosis is often made incidentally in adults evaluated for other complaints. At the other extreme is the clinically severe, autosomal recessive form which has its onset in infancy and produces anemia, leukopenia, hepatomegaly, failure to thrive, cranial nerve symptoms, and early death. This form is often referred to as the "infantile," " malignant," or "lethal" form. Beighton and colleagues have pointed out the existence of clinically intermediate forms of osteopetrosis [3]. Although this genetic heterogeneity indicates that multiple genetic causes produce osteopetrosis, the common mechanism underlying all forms is thought to be failure of bone resorption [4].

The association of renal tubular acidosis with osteopetrosis was reported independently from three different countries--France [5], Belgium [6], and the United States [7]--in 1972. These initial pedigrees suggested that the pattern of inheritance is autosomal recessive. The clinical course began with onset in infancy or early childhood. Though not entirely benign, it was much milder than the course of the recessive lethal form and was compatible with long survival. The hematologic abnormalities associated with the recessive lethal form of osteopetrosis were mild or absent. In 1980, Ohlsson et al. [8] reported the additional finding of cerebral calcification, documented by CT scans, in four children with osteopetrosis and renal tubular acidosis from Saudi Arabia. Calcification of the basal ganglia in the original American kindred was reported independently by Whyte et al. the same year [9].

In an effort to explain the pleiotropic effects of the mutation underlying this disorder by a single enzyme defect, we postulated a defect in one of the three isozymes of carbonic anhydrase (CA I, CA II, CA III) which are known to be under separate genetic control in humans [10-14]. This hypothesis seemed attractive for two reasons: (i) metabolic acidosis can be produced by sulfonamide inhibitors of CA [12], and (ii) several reports had shown that CA inhibitors can block the parathyroid hormone-induced release of calcium from bone, suggesting a role for CA in bone resorption [15-17].

The relationship of CA deficiency to cerebral calcification was less apparent, although it was known that CA II is present in brain [18] and

that CA inhibitors inhibit cerebrospinal fluid production [19] and affect electrical activity of the brain [20]. A defect in the CA II isozyme seemed most likely because this is the most widely distributed of the three known soluble isozymes of CA in human tissues [10,11] and CA II is the only soluble isozyme so far identified in renal and brain tissue [18,21,22]. In addition, a genetically determined, virtually complete absence of CA I in mature erythrocytes has been found to have no clinical consequences [23]. Because both CA I and CA II are expressed in human erythrocytes, it was possible to test this hypothesis by examining these isozymes in hemolysates of peripheral blood from the family we reported previously [8].

In 1983, Sly et al. [24] tested this hypothesis and found that the three sisters from the original American kindred with this syndrome (Fig. 1) CA II in their erythrocytes. Their normal-appearing parents and many first-degree relatives had half-normal levels of CA II in erythrocyte lysates. These observations, coupled with the fact that CA II was the only known soluble isozyme of CA in kidney and brain, led them to propose that CA II deficiency is the primary defect in the newly recognized metabolic disorder of bone, kidney, and brain [24].

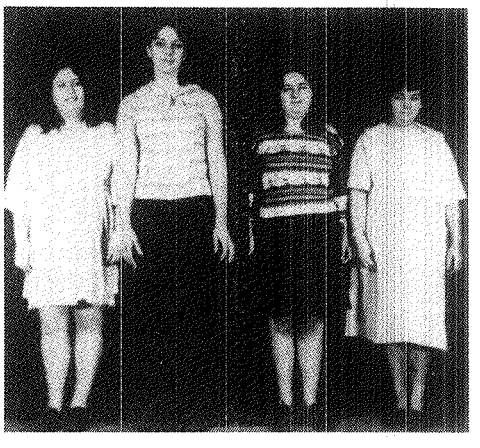

Fig. 1. American family with the CA II deficiency syndrome reported by Whyte et al.[9] From left to right are patient 3, an unaffected sister, patient 2, and patient 1 (proposita). This picture was taken in 1978 when the proposita was 29. Osteopetrosis had been diagnosed at age 2 following a pathological fracture[9]. Note the short stature, unusual facial features, and squint in the three affected sisters. Patients 2 and 3 had limited vision and were considered legally blind. Vision was nearly normal in patient 1. (*From Whyte et al.*[9] Used by permission of *The American Journal of Medicine.*)

Sly et al. [25] extended these studies to 18 additional patients in 11(proposita). This picture was taken in 1978 when the proposita was 29. Osteopetrosis unrelated families of different geographical and ethnic origins. Subsequently, Ohlsson et al. [26] reported four additional Saudihad been diagnosed at age two following a pathological fracture [9]. Note the short Arabian patients, including the first affected neonate, and

summarized the clinical features of 21 reported patients. Cochat et al. [27]stature, unusual facial features, and squint in the three affected sisters. Patients 2 and added an additional case and reviewed the clinical findings on the 30 patients reported by 1987, including a few who had not been3 had limited vision and were considered legally blind. Vision was nearly normal in completely described clinically. A few individual cases have been reported since, and many more have been recognized [28-32]. Whytepateint 1. (From Whyte et al. [9] Used by permission of the American Journal of reviewed nearly 50 cases reported up to 1992 [32a]. A deficiency of CA II has been found in erythrocyte lysates of everyMedicine.) patient so far identified with this syndrome.

NOMENCLATURE

The syndrome of osteopetrosis with renal tubular acidosis (McKusick catalog #259730 [33]) was recognized as a distinct entity in 1972 [5-7]. In 1980, when Ohlsson et al. [8] pointed out that cerebral calcification was part of the syndrome, they suggested that it be referred to as "marble-brain disease", by analogy with marble-bone disease, the name given earlier to inherited forms of osteopetrosis that did not involve the brain [2]. However, since the enzymatic basis for the disorder was established [24,25], it has been referred to as the "carbonic anhydrase II deficiency syndrome." [26,33] It has also been called the Guibaud-Vainsel syndrome after the authors of the first two full reports on the disorder [33].

CLINICAL MANIFESTATIONS

There is considerable variability in the age of onset and the severity of clinical manifestations among the reported cases [26,27]. All have renal tubular acidosis and eventually develop osteopetrosis and cerebral calcification. Additional features include growth failure, mental retardation, and dental malocclusion. In some patients, bone fractures and other complications of osteopetrosis have dominated the clinical picture [6,9]. In others, symptoms of metabolic acidosis including failure to thrive, developmental retardation, and growth retardation have been more prominent [5,8,26,27].

Osteopetrosis

The osteopetrosis results from a generalized accumulation of bone mass that is secondary to a defect in bone resorption [4]. This defect

prevents the normal development of marrow cavities, the normal tubulation of long bones, and the enlargement of osseous foramina. The clinical manifestations of osteopetrosis in the CA II deficiency syndrome tend to be milder than in the recessive, lethal form of osteopetrosis. They appear later [26,27], and they also tend to improve over time [9,32a].

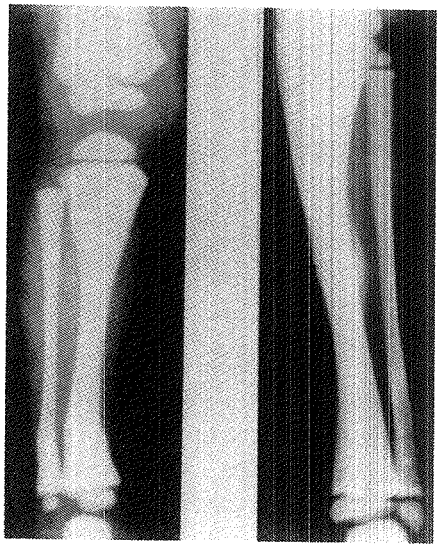

Fig. 2 Anteroposterior radiographs of the right tibia and fibula of patient 2 at 2 years of age and of the left tibia and fibula of patient 3 at age 6. Features of osteopetrosis include diffuse osteosclerosis with absence of medullary cavities and flared metaphyses containing transverse lines. Despite the increased bone density, healing fractures are evident in both radiographs.

Anemia is rarely profound in patients with CA II deficiency, though two patients had sufficient anemia to be referred for bone marrow transplantation. In fact, the first reported bone marrow transplantation for osteopetrosis was done on a patient who very likely had the CA II deficiency syndrome [34], and not the recessive lethal form of osteopetrosis, for which bone marrow transplantation has become an accepted form of therapy [35]. This patient was reported to have a favorable hematologic response, but to have been unimproved in terms of the metabolic acidosis following bone marrow transplantation [27].

The radiologic findings in patients with CA II deficiency syndrome are not distinguishable from those in patients with other forms of osteopetrosis [9,26,30-32]. Increased bone density (Fig. 2), abnormal modeling, delay or failure of normal tubulation of long bones, transverse banding of metaphyses, fractures, and "bone in bone" appearance are all seen, as in other forms of osteopetrosis. However, the changes can vary with age. In the only neonate studied to date, the radiologic features were too subtle to justify the diagnosis at 23 days of age [26], even though the hyperchloremic metabolic acidosis and alkaline urine were already prominent findings. This observation suggests that the osteopetrosis is a postpartum developmental abnormality that appears over the first year of life. The first patients reported by Guibaud [5, 27] also had no osteopetrosis at age four months, but typical findings evolved and

progressed over the first three years of life before stabilizing. In at least some patients followed into adulthood, the radiologic features of osteopetrosis, which were fully developed in childhood, improved substantially after puberty (Fig. 3). The radiographs may become nearly normal as the patients move into adulthood [9,32a].

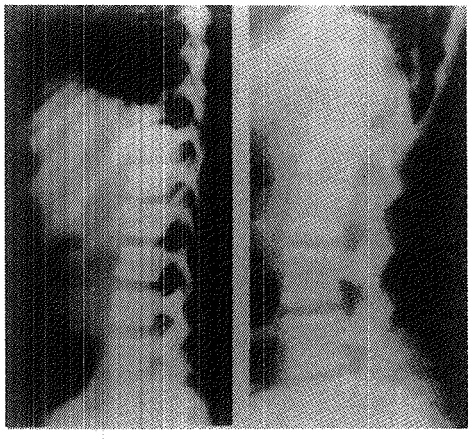

Fig. 3 Patient 1, lumbar spine (lateral radiographs). *A.* Age 8 years. *B.* Age 25 years. Osteosclerosis diminished greatly over this interval. Persistent osteosclerosis at the vertebral end plates characterizes the "sandwich vertebrae" of osteopetrosis. (*From Whyte et al.*[9] *Used by permission of The American Journal of Medicine.*)

Bone fractures are common in childhood in many patients, with some reporting 15-30 fractures by midadolescence [6,9,32a,36]. After puberty, the frequency of bone fractures decreases. Fractures were the most prominent symptoms in the American patients [7] and the Belgian patient [6] in whom mental retardation was not present (Fig. 2). Fractures were not seen in Guibaud's patients who were of Arabic descent [5].

The symptoms of cranial nerve compression secondary to osteopetrosis are milder than in the recessive, lethal form of osteopetrosis. However, the cranial nerve symptoms appear in 60 percent of reported patients [27]. Optic nerve pallor is common, but frank optic nerve atrophy is less frequent. Strabismus is also common, as is hearing impairment. Facial weakness has been noted in two reports.

Renal Tubular Acidosis

Patients typically have metabolic acidosis which varies considerably in type and severity in different pedigrees [27]. Metabolic acidosis was already present at 23 days of age in the first affected neonate [26]. Although one of the first patients reported had only proximal renal tubular acidosis, evidenced by low bicarbonate threshold, and had normal distal acidification [5,27], most of the patients have a combination of proximal and distal renal tubular acidosis [6,9,27,37,38]. Of 21 patients in whom the renal lesion was characterized, in four the renal tubular acidosis was felt to be proximal, in six distal, and in eleven both proximal and distal [26]. Most patients had hyperchloremia, a normal anion gap,

and inappropriately alkaline urine pH (>6.0). These findings are consistent with distal renal tubular acidosis. Symptomatic hypokalemia has been observed in four patients [9,27]. However, unlike other patients with distal renal tubular acidosis, these patients have neither hypercalciuria nor nephrocalcinosis. Glomerular filtration rate is not reduced, and serum creatinine and blood urea nitrogen are not elevated.

Most patients also have a reduced tubular maximum for bicarbonate. Although they usually have no bicarbonaturia when acidotic, they lose bicarbonate when plasma bicarbonate levels are raised to normal levels by loading. They do not have amino aciduria, glycosuria, or any other manifestations of the Fanconi syndrome.

Mental Retardation

The frequency and severity of mental retardation were not fully appreciated initially, because affected patients in two of the first four families recognized with this syndrome were not retarded [6,7,9]. However, over 90 percent of the patients reported to date have had significant mental retardation [26,27]. Even in the two families where intelligence was not below the normal range, some learning disabilities were observed. In most families, the mental retardation in affected patients has been severe enough to preclude education in regular schools [26,27].

Cerebral Calcification

Cerebral calcifications, evident by CT scans, were first reported by Ohlsson [8]. They were not present at birth, but appeared some time during the first decade (in one case, by 18
months) [5,25,27]. Calcifications involved the caudate nucleus, putamen, and globus pallidus, and also appeared peripherally in the periventricular and subcortical white matter (Fig. 4). The variability in the rate of progression of cerebral calcification in different patients has not been determined.

Growth Retardation

Growth retardation is nearly a constant finding. Almost all reported patients had short stature and many were underweight. Bone age was retarded, and corresponded to height age. Genu valgum is a common finding in older patients. At least part of the growth retardation is due to the chronic metabolic acidosis. Guibaud reported acceleration of growth following correction of the acidosis [5], but later noted that growth retardation persisted, even after treatment [27]. The final height achieved

by the patient who responded initially to correction of the acidosis was still nearly four standard deviations below normal [5,27].

Dental Malocclusion

Dentition was typically delayed and dental malocclusion was a prominent finding in affected patients from several families. Dental malalignment and malocclusion complicate dental hygiene, and dental caries may be severe [9,26]. Enamel hypoplasia has also been noted [8,26].

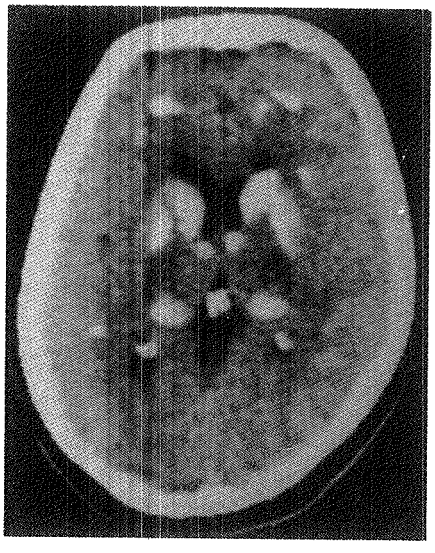

Figure 4 CT scan of the head of patient 3 at 33 years of age. Scattered dense cerebral calcifications are especially prominent in the basal ganglia. (*From Sly et al.*[25] Reprinted with permission from *The New England Journal of Medicine*, vol. 313, p.139, 1985.)

Other Features

Ohlsson [8,26] reported a characteristic facies in the patients from Saudi Arabia, and it is present in many patients from other ethnic groups as well. These features include craniofacial disproportion with a prominent forehead and a cranial vault large relative to the size of the face. The mouth is small, and there is micrognathia. The nose is narrow, but prominent. The philtrum is short, the upper lip thin, and the lower lip thick. Squint is common and contributes to the unusual facies (Fig. 1).

Ohlsson et al. [26] recently reported findings of restrictive lung disease in two patients. Chest films showed no signs of parenchymal lung disease, but the rib cages were very dense.

Optic atrophy has been found in patients in whom the optic foramina were of normal size [27]. The mechanism of optic atrophy in these patients is unclear.

Hematological disorders, including anemia, leukopenia, and thrombocytopenia, which are typically prominent in the recessive malignant lethal form of osteopetrosis, are usually not seen in osteopetrotic patients with the CA II deficiency syndrome. However, anemia and hepatosplenomegaly were seen in three unrelated patients, who were even considered candidates for bone marrow transplantation

until the anemia and hepatosplenomegaly improved without treatment and the more benign course became apparent.

PATHOLOGY

No autopsies have been reported on patients with the CA II deficiency syndrome. However, bone biopsy samples from iliac crest have been analyzed and showed histologic features typical of osteopetrosis [5,9,26]. The cortical bone showed small Haversian systems widely separated from dense bone. The separation of cortical and cancellous bone was generally indistinct. Trabeculae were broad and irregular. Osteoid and normal-appearing osteoblasts were seen lining trabecular bone in several areas. On routine microscopy, osteoclast morphology was unremarkable. A minute sample of femoral cortex was obtained during open reduction of a femoral fracture. Osteoclasts were normal in appearance on light microscopy. Four osteoclasts were identified on electron microscopy, and showed a normal rim of cytoplasm adjacent to the bone surface. This "clear zone" was free of organelles. The osteoclasts appeared normal, although no "ruffled borders" were seen. In summary, the histologic findings of osteopetrosis were present, but no features appeared to distinguish the osteopetrosis of the CA II deficiency syndrome from other forms of osteopetrosis.

PATHOGENESIS

The Carbonic Anhydrase Gene Family

All three soluble isozymes of CA in humans (CA I, II, and III) are monomeric, 29-kDa zinc metalloenzymes which catalyze the reversible hydration of CO_2 (reaction I) [39].

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

Reaction II involves an ionic dissociation, occurs virtually instantaneously nonenzymatically, and is not subject to enzymatic acceleration. The direction of the reaction in a given tissue or body fluid depends on the relative concentrations of CO_2, HCO_3^-, and on the H^+ ion concentration, i.e., the pH. There is also a distinctive membrane-bound CA in lung called CA IV [40], which was shown to be identical to the membrane-bound CA in the brush border lining the lumen of the proximal tubules of the kidney [41]. The CA IV cDNA [42] and CA IV genomic organization [43] were recently reported. A distinct, secretory form of CA

(CA VI) has been described in saliva of the rat [44], the human [45], and sheep [46]. The amino acid sequence of the ovine salivary CA was recently reported and shows 33 percent sequence identity with ovine CA II, though residues involved in the active site were more highly conserved. A distinct CA has also been reported in mitochondria in the liver and has been designated CA V [47].

Chromosome Localization of CA Genes

Genetic and structural evidence suggests that the CA isozymes constitute a multilocus enzyme family derived from a common ancestral gene by gene duplications [48]. CA I, II, and III are clustered at chromosome 8q22 [49], CA IV was assigned to 17q23 [43], CA VI was assigned to chromosome 1p [50]. CA V was recently assigned to chromosome 16 [47], to which CA VII was previously mapped [51].

Tissue Distribution and Properties of CA Isozymes

The kinetic parameters of the different isozymes, and their sensitivities to different inhibitors, and their tissue distributions, can differ markedly, indicating that they play different physiological roles [52,53]. The human CA II isozyme, whose turnover number for CO_2 hydration ($1.3\text{-}1.9 \times 10^6$/sec) is the highest known for any enzyme [54, 55], is widely distributed. It has been identified in erythrocytes, brain, eye, kidney, cartilage, liver, lung, skeletal muscle, pancreas, gastric mucosa, and anterior pituitary body [39,56]. The other isozymes, whose activities are lower than those of CA II, in the order CA II>CA IV>CA I>CA III>CA V, appear to have a more limited distribution [47,57]. CA I is found primarily in erythrocytes. CA III is found mainly in red skeletal muscle [52,53]. CA IV is expressed on the apical and basolateral surfaces of cells of the proximal tubule and thick ascending limb of the nephron [41], and on the plasma face of certain endothelial surfaces, including the pulmonary microvasculature [58], the choriocapillaris [59], and microcapillaries of brain [60], heart, and skeletal muscle [57, 61]. CA V is expressed in mitochondria of liver, kidney, intestine, heart, and spleen [62].

The Biochemical Defect

In 1983, the three affected sisters reported initially by Sly et al. [7] and described in detail by Whyte et al. [9] were shown to have no detectable CA II activity in their erythrocytes [24]. CA I was present in near normal levels. No immunoreactivity was detectable with specific antibody to CA II. The obligate heterozygote parents and several

additional family members were found to have half-normal levels of CA II activity. These findings were subsequently extended to 18 similarly affected patients from 11 unrelated families of different geographic and ethnic origins [25]. Every patient with osteopetrosis and renal tubular acidosis since tested has had no detectable CA II activity [27]. Thus, there has been no exception to the finding of a quantitative defect in CA II in erythrocytes of patients with this syndrome.

Mutations in the structural gene for CA II (summarized below) have been found in most patients with CA II deficiency [63-65,80,91]. Although the complete absence of CA II activity and immunoreactivity in erythrocytes have been consistent findings in affected patients, it should be stressed that the residual activity in cells that continue to synthesize protein (such as osteoclasts in bone and cells in the proximal and distal tubules of the kidney) might be significantly higher than in erythrocytes. In fact, we suspect that some of the clinical heterogeneity in this syndrome may be explained by differences in residual CA II activity in bone and kidney in patients with different mutations in the structural gene for CA II.

Pathophysiology

The finding of a quantitative defect in CA II in these patients provided an unusual opportunity to assess the function of this enzyme and to understand its importance for bone, brain, and kidney metabolism.

Bone Metabolism. All known forms of osteopetrosis involve the failure to resorb bone [4]. Studies showing inhibition of parathyroid hormone (PTH)-induced release of CA^{2+} from bone by CA inhibitors had suggested a role of CA in bone resorption [15-17]. Also, CA had been demonstrated histochemically in chick and hen osteoclasts [66] and CA II demonstrated immunohistochemically in rat [67] and human [15] osteoclasts. The osteopetrosis seen in patients with CA II deficiency provided genetic evidence for a role for CA in bone resorption, and implicated the CA II isozyme specifically [24].

It had been suggested that CA aids the resorptive process by mediating the secretion of H^+ [16,67]. We proposed that the role of CA II in acidifying the bone resorbing component is an indirect one, analogous to its role in supporting the acidification of the lumen in the distal tubule of the kidney. It has been suggested recently that the acidification of the bone-resorbing compartment is mediated by a proton-translocating ATPase [68], that secretes protons into the lumen. This reaction would simultaneously generate an OH^- ion in the cytoplasm for each H^+

translocated to the lumen. Titration of the OH^- ions produced in the cytosol by CA II might be required to allow the proton-translocating ATPase to maintain the pH gradient (7.0-4.5) between the cytosol of the osteoclast and the bone resorbing compartment. This model could explain the pharmacologic evidence that CA is required in bone resorption [15-17]. Since CA II is the only CA isozyme known to be expressed in osteoclasts [67,68], it could also explain the osseous manifestations of CA II deficiency.

Renal Tubular Acidosis. Three things need explanation in regard to renal metabolism in these patients. First, most CA II deficient patients have both a proximal and a distal component to the renal tubular acidosis [26,27]. Second, some patients have predominantly proximal renal tubular acidosis, while in others, the distal renal tubular acidosis predominates [26,27]. Third, CA II deficient patients have a nearly normal bicarbonaturia after ingestion or infusion of carbonic anhydrase inhibitors [69]. Some of these observations can be explained by a model in which the functions of CA II in the proximal and distal tubules are physiologically and biochemically distinct, and the major role of CA in bicarbonate reclamation is assigned not to CA II, but to CA IV, the luminal CA in the brush border of the proximal tubule [70,71]. CA IV is biochemically and immunologically distinct from CA II, and appears to be normal in CA II deficient patients [69], based on the evidence of the normal bicarbonaturia in response to infused acetazolamide. We recently showed that the affected patients in the original American family with CA II deficiency have normal CA IV levels in their urinary membranes [71a].

We deal first with the explanation for the proximal renal tubular acidosis. There is general agreement that renal reabsorption of bicarbonate is a major factor in acid-base homeostasis. Most of the bicarbonate reclamation takes place in the proximal tubule and is blocked by inhibitors of CA. However, two distinct CA isozymes participate in bicarbonate reclamation by the proximal tubule, and they play separate roles in bicarbonate reclamation.

Bicarbonate reclamation depends on H^+ secretion, which is mediated primarily by Na^+/H^+ exchange in the proximal tubule but also by the Mg^{2+}/H^+-ATPase on the apical membrane [72]. The H^+ secreted into the lumen of the proximal tubule is titrated by the HCO_3^- in the glomerular filtrate to produce H_2CO_3, which is in contact with the membrane-bound CA IV. The luminal CA IV catalyzes the dehydration of H_2CO_3 to H_2O and CO_2 [73,74]. The bicarbonaturia seen in response to infused

acetazolamide in already acidotic CA II deficient patients is attributed to inhibition of this luminal CA IV [69].

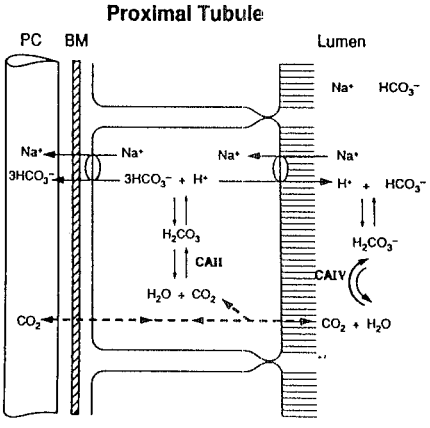

Figure 5 A. Proposed roles of carbonic anhydrases in bicarbonate reclamation in the proximal tubule. Na^+ and HCO_3^- enter the lumen of the proximal tubule. H^+ is secreted in exchange for Na^+, and H^+ and HCO_3^- are converted to CO_2 and H_2O in a reaction catalyzed by the luminal CA (CA IV). We propose that this enzyme functions normally in CA II-deficient patients, and its inhibition explains the positive response to acetazolamide (normal bicarbonate diuresis). CO_2 diffuses freely into the proximal tubular cell [and across the basement membrane (BM) and into the peritubular capillary (PC)], and is exposed to cytosolic CA II which catalyzes its rehydration to form HCO_3^- and H^+. Three molecules of HCO_3^- and one of the Na^+ are cotransported by the basolateral cotransporter from the contraluminal surface of the proximal tubular cell to the peritubular capillary (PC).

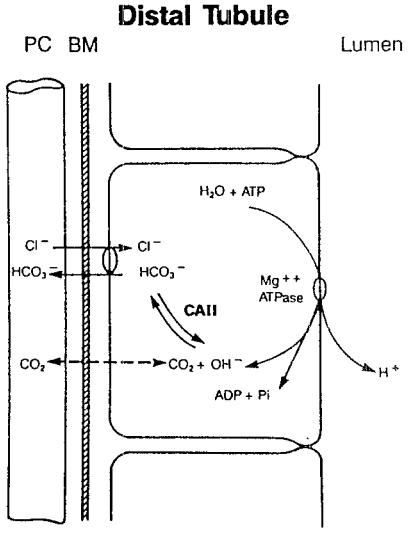

Fig. 5B. The H^+ generated by CA II is secreted in exchange for Na^+ to initiate another cycle of HCO_3^- reabsorption. Loss of CA II-mediated regeneration of H^+ is suggested as the cause of HCO_3^- wasting in CA II-deficient patients. B. Proposed role of CA II in distal urinary acidification. The H^+ is secreted into the lumen by a proton-translocating Mg^{++} ATPase, as in amphibians, which produces OH^- in the cytosol. CO_2 can condense with OH^- to form HCO_3^- in a CA II-catalyzed reaction, and HCO_3^- can be transported across the basement membrane and into the peritubular capillary. We suggested that failure to titrate the OH^- limits the ability to secrete H^+ and acidify the urinary appropriately in CA II-deficient patients. (From Sly et al. [69] Used by permission of Pediatric Research.)

The CO_2 produced by the CA IV-catalyzed reaction in the lumen diffuses freely into the cytosol of the proximal tubule. Here in the cytoplasm CO_2 encounters CA II, which acts to hydrate the CO_2 to produce H_2CO_3, which dissociates spontaneously to HCO_3^- and H^+. The HCO_3^- generated from CO_2 in the cytosol is transported from the cytosol to the interstitial fluid or peritubular capillary by the Na-$3HCO_3$

cotransporter, completing the reclamation of the filtered bicarbonate. The H^+ regenerated in the cytosol by the CA II-catalyzed reaction can be secreted in exchange for Na^+ to initiate another round of HCO_3^- reclamation [72,74].

Thus, both the luminal CA IV and the cytosolic CA II participate in the reclamation of HCO_3^- in the proximal tubule. The fact that CA II-deficient patients do not spill HCO_3^- when acidotic suggests that CA II is not required for HCO_3^- reclamation when patients have low bicarbonate loads, i.e., when acidotic. However, they have a lowered tubular maximum for bicarbonate and lose bicarbonate when the filtered load is increased by bicarbonate infusion or ingestion, indicating that CA II is required to regenerate H^+ for bicarbonate reclamation under normal bicarbonate loads. This requirement explains the proximal component of the renal tubular acidosis in CA II deficient patients (Fig. 5A).

The prominent distal component of the renal tubular acidosis in most CA I-deficient patients, evidenced by inappropriately high urine pH values when patients are acidotic, suggests that CA II is needed for distal acidification as well. This idea is consistent with the immunohistochemical evidence showing a much more intense reaction for CA II in the distal tubule and the intercalated cells of the collecting ducts than in the proximal tubules [71]. Why is there normally such an abundance of CA II in the distal tubules, when most of the HCO_3^- reclamation takes place in the proximal tubule? We suggested [69] that the explanation may be inferred from the analogous situation in the distal nephron and
collecting system in the amphibian. In the turtle bladder, for example, the "CA rich cells" are specialized cells which secrete H^+ and are capable of generating a steep pH gradient [75, 76]. However, the acidification of the lumen is sensitive to inhibition by acetazolamide. It has been proposed that CA is needed in the amphibian nephron to titrate the OH^- produced in the cytosol by the proton-translocating Mg^{2+} ATPase. We have suggested a similar role for CA II in the distal tubule of the human kidney, i.e., catalyzing the conversion of OH^- and CO_2 to HCO_3^- [69]. Unless the OH^- is titrated by CO_2, the proton-translocating ATPase cannot generate a pH gradient and acidify the lumen. The absence of CA II for this reaction in CA II deficient patients can explain their defect in distal tubular acidification (Fig. 5B).

The third point, the basis for heterogeneity in the renal lesion in CA II deficiency, still requires explanation. Why is there variation in prominence of the proximal and distal lesions in different pedigrees? The explanation for this heterogeneity is still speculative. The different

structural gene mutations producing CA II deficiency in different pedigrees may contribute to this heterogeneity in at least two ways. First, different mutations may affect the rate of enzyme turnover differently in proximal and distal tubular cells, resulting in different levels of residual enzyme activity in the two locations. Secondly, different structural gene mutations could affect the two enzymatic activities differently in the two locations. Thus, hydration of CO_2 to produce H^+ and HCO_3^- in the proximal tubule and the condensation of OH^- and CO_2 to produce HCO_3^- in the distal tubule might be differentially affected by different mutations in the CA II gene. Continued delineation of the mutations in different CA II deficient patients and studies of the enzyme produced after expression of the cloned mutant genes in prokaryotic and eukaryotic cells may allow one to test this hypothesis.

Brain Calcification and Cerebral Function. The mechanism of the cerebral calcification is unclear. CA II is primarily a glial enzyme that occurs predominantly in oligodendrocytes [77]. It is the only soluble carbonic anhydrase in brain homogenates. As much as 50 percent of the total CA II activity occurs in a membrane-bound or myelin-associated form [78]. The function of CA II in brain is not known. It is not clear whether the cerebral calcification in carbonic anhydrase II deficiency represents a direct effect of the deficiency of CA II in the brain or an indirect effect--for example, of carbonic anhydrase deficiency in erythrocytes or of chronic systemic acidosis.

While brain development and central nervous system function are not profoundly deranged in patients with this syndrome, psychomotor delay, learning disabilities, and even mental retardation are evident in most affected patients [26,27]. The mental retardation was not so obvious in the initial reports of patients with CA II deficiency syndrome, but it is now clear that over 90 percent of the reported patients have mental retardation severe enough to prevent school attendance. Whether this is a direct consequence of the CA II deficiency, or an indirect effect, is not yet clear.

Although CA II is the only soluble CA expressed in brain, CA IV is expressed on the plasma face of cerebral capillaries and is anchored to the capillary membrane by a glycosylphosphoinositol linkage [60].

Growth Failure. Growth failure appears to result from a combination of the effect of the osteopetrosis on bone elongation and of the chronic

metabolic acidosis on general health. Correction of the acidosis was followed by a growth spurt in one patient [27], but the fact that final height achieved by this patient was still dramatically low makes it clear that the growth retardation is not due to the acidosis alone.

GENETICS

Inheritance

The CA II deficiency syndrome is inherited as an autosomal recessive trait. Affected patients are offspring of normal-appearing heterozygote carrier parents who have half-normal levels of CA II in their erythrocyte lysates. Heterozygotes have no symptoms or signs of the disorder. Males and females are affected with equal frequency and severity. Consanguinity is very common (87 percent) in parents of affected offspring [27].

The geographical distribution of this syndrome is striking, with more than half the known cases observed in families from Kuwait, Saudi Arabia, and north Africa [25]. This probably results from both an high frequency of the carbonic
anhydrase II deficiency allele in these regions and an high frequency of consanguineous marriages, particularly in the Bedouin tribes from which many of these patients originated.

Molecular Genetics

In humans, the CA I, II, and III genes (CA1, CA2, CA3) are clustered within around 180 kb on chromosome 8q22 [48]. The entire 20-kb CA II gene has been cloned and the intron/exon organization determined [63,64] (Fig. 6). The human CA II gene contains seven exons, as does the mouse gene, and intron/exon junctions 2-7 are conserved in all human CAs so far examined. The 5′ flanking region of the human CA II gene contains a TATA box and a possible CAAT box. It also contains nine potential Sp1 binding sites. Deletion analysis of the human 5′ promoter region showed a gradual but differential loss in promoter activity with loss of Sp1 binding sites [79].

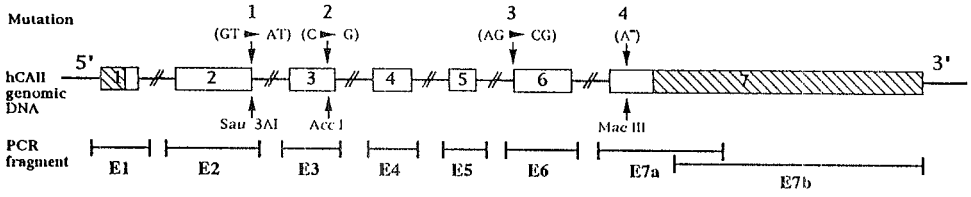

Figure 6 Genomic organization, PCR fragments, and structural mutations found to date in the human CA II gene. The human CA II gene contains seven exons and six introns. Except exon 7, each exon including exon/intron boundaries can be amplified individually by PCR using intronic PCR primers. Exon 7 has 928 bp which can be amplified in two overlapping fragments for sequencing. Similar PCR primers were described by Venta et al.[63] and Roth et al. [64]. Four structural mutations have been found in the CA II gene, which are numbered from the 5′ end. Mutation 1, which is common in Arabic patients, changes the first nucleotide of intron 2 from G to A and destroys a splice junction donor site. This change creates a new Sau 3AI restriction site which can be used for diagnosis and prenatal diagnosis [32,65]. Mutation 2 is a C → G transition in exon 3, which results in replacement of the conserved histidine at position 107 with tyrosine (His 107 → Tyr) and introduces a new Acc I restriction site. This mutation was identified in a homozygous Belgian patient [63] and also as one of two mutations in the three American sisters (shown in Fig. 1), who were compound heterozygotes and their mother [64]. Mutation 3 is an A-to-C transversion at the 3′ end of intron 5 which destroys a splice junction acceptor site. This mutation was the second mutation found in the American sisters, the one they inherited from their father [64]. Mutation 4 is a single base pair deletion in the coding region of exon 7a which creates a new Mae III restriction site. This single base deletion results in a frameshift at codon 227, which changes the next 12 amino acids and introduces a UGA stop codon 22 amino acids earlier than in the normal enzyme. This mutation was found to be common in Hispanic patients from the Caribbean islands [80,91].

The full-length human cDNA has been expressed in both prokaryotic and eukaryotic cells. Although mRNA from CA II deficient patients could not easily be obtained, knowledge of the genomic organization and of intron sequences surrounding each exon has made mutational analysis on patient genomic DNA straightforward. Four mutations have been identified so far in CA II deficient patients (Fig. 6). The first mutation (mutation 2 in Fig. 6) was identified in a Belgian patient and was a C-to-T transition in exon 3 which results in replacement of the conserved histidine at position 107 with tyrosine (His 107 → Tyr) [63]. The three affected sisters in the American family in which CA II deficiency was first reported were also found to have this mutation. However, they were compound heterozygotes, having inherited the His 107 → Tyr mutation from their mother and a splice acceptor mutation in the 3′ end of intron 5 from their father [64]. Neither the Belgian patient nor the American patients were mentally retarded. Frequent skeletal

fractures were the most disabling manifestation of their disease [28]. When the CA II cDNA containing the His 107 → Tyr mutation was expressed in E. coli, the activity of the mutant protein was drastically lower than that of the normal enzyme. However, residual activity could be easily demonstrated in cells induced at 30°C and 20°C where a larger fraction of the expressed enzyme remained soluble (80 percent at 20°C). These experiments suggested that a small amount of residual CA II activity in patients with the His 107 → Tyr mutation may allow them to escape mental retardation [64]. The third structural gene mutation identified is a splice junction mutation at the 5' end of intron 2 which was found in most of the patients of Arabic descent from Kuwait, Saudi Arabia, Algeria, and Tunisia [65]. More than 75 percent of the patients so far recognized have been Arabic [28] and have been severely affected. Mental retardation and metabolic acidosis were prominent in these patients, while bone fractures were less frequent [28,65].

A novel frameshift mutation resulting from a single base deletion in the coding region of exon 7a was found in a mildly affected Hispanic girl, who is the only patient reported so far with no renal tubular acidosis [80,91]. This single base deletion results in a frameshift at codon 227 that changes the next 12 amino acids and introduces a UGA stop codon at codon 239. The truncated enzyme resulting from this mutation is 22 amino acids shorter than the 260 amino acids in normal CA II. When expressed in bacteria, the mutant allele produced 0.07 percent of the activity expressed by the normal allele. Unexpectedly, this mutant enzyme activity was not due to the truncated form of the mutant enzyme (27-kDa), but to a small fraction of near normal size enzyme (29-kDa) which had about 10 percent of normal specific activity. Protein sequencing showed that the first 11 amino acids were abnormal in the 29 kDa mutant protein, as predicted by the frameshift, after which the reading frame was restored. The last 23 amino acids of the 29 kDa mutant protein were the same as in normal CA II. These results can be explained by a ribosomal -1 translational frameshift that restores the reading frame 11 codons after the original mutation and allows completion of full-length CA II. Subsequently, patients referred from seven independent Hispanic families, some having severe clinical manifestations including severe renal tubular acidosis, anemia, and hepatosplenomegaly, were found by sequencing or restriction site analysis to be homozygous for the same mutation. The basis for the wide clinical variability in these patients is unclear. However, these findings raise the possibility that individual variation in

efficiency of frameshift suppression may contribute to clinical heterogeneity among patients with identical frameshift mutations.

DIAGNOSIS

Clinically, CA II deficiency should be suspected in any newborn infant with metabolic acidosis and failure to thrive, especially if the urine pH is alkaline. Osteopetrosis may not be present initially, but it usually develops over the first year of life. If osteopetrosis and renal tubular acidosis coexist, the diagnosis is virtually certain. No patient with this combination has yet been found who does not have CA II deficiency. Cerebral calcification, evident by CT scan, is usually present by the end of the first decade.

Enzymatic confirmation can be made by measuring the CA II level in erythrocyte lysates [81,82]. A relatively easy assay has been described that allows one to quantitate both CA I and CA II levels in erythrocyte lysates. This method takes advantage of the large difference in sensitivity of CA I and CA II to
inhibition by sodium iodide. Normally CA I and CA II each contribute about 50 percent of the total activity, and the CA I activity is virtually completely abolished by inclusion of 8 mM sodium iodide in the assay. One simply measures the total activity (CA I + CA II), and also the activity seen in the presence of 8 mM sodium iodide (CA II). Patients with CA II deficiency have no iodide-resistant enzyme (i.e., no CA II). Obligate heterozygotes have about half-normal levels of iodide-resistant activity. Other assays have been described, including staining of individual isozymes following electrophoresis, quantitation of CA I/CA II ratios by high-pressure liquid chromatography, and immunologic identification of the isozymes on immunodiffusion with specific antisera [24].

The identification of the structural gene mutations underlying CA II deficiency has provided simple and accurate molecular techniques for detecting these mutations which should be useful for diagnosis, genetic counseling, and prenatal diagnosis, and also for carrier detection in certain populations. As shown in Fig. 6, three of the four mutations in the CA II gene have introduced new restriction sites in the mutant DNA alleles. The Arabic mutation introduces an extra Sau 3AI site in exon 2, the His 107 → Tyr mutation creates a new Acc I site in exon 3, and the Hispanic mutation creates an additional Mae III site in exon 7a. Digestion of PCR amplified genomic DNA fragments with the appropriate

restriction enzymes followed by agarose gel electrophoresis allows one to make accurate diagnoses [63,65,80,91]. The splice-junction mutation at the 3′ end of intron 5 does not create or destroy a restriction site. In this case, dot-blot hybridization using allele-specific oligonucleotide probes proved to be useful in distinguishing patients, carriers, and normal individuals [64].

GENETIC COUNSELING

The counseling appropriate for an autosomal recessive trait is indicated. First degree relatives can be tested for heterozygosity. Prenatal diagnosis using the techniques described above under DNA diagnosis is now available to families where the mutation has been established. Prenatal diagnosis is not available to families in which the mutation has not yet been established. In addition, the osteopetrosis does not appear prenatally and the diagnosis cannot be made in the fetus radiologically or by ultrasound. Carbonic anhydrase levels in erythrocytes are normally extremely low at birth in normal infants, and it is unlikely that CA II deficiency could be diagnosed by measuring CA II activity in samples of fetal blood.

TREATMENT

No specific treatment for CA II deficiency is available. Treatment for the metabolic acidosis is recommended, at least until after adolescence [27]. It appears that the renal tubular acidosis may stabilize at a milder level after puberty. Frequent fractures require conventional orthopedic management. Bone healing is usually normal. Most patients require special education because of mental retardation. There is no specific treatment for the cranial nerve abnormalities, which may lead to impaired vision, hearing deficits, and facial nerve weakness. Attention to dental hygiene is important because of the susceptibility to caries.

In the early course of the initial American family, treatment with bicarbonate was withheld for fear that the acidosis may be compensating for the osteopetrosis and that treatment of the acidosis might aggravate the osteopetrosis, resulting in further loss of vision and hearing. However, prolonged treatment of several patients by Dr. Guibaud and colleagues appeared to have a beneficial effect on general health without any marked progression of the osteopetrosis and with no aggravation of

cranial nerve symptoms [27]. It is not clear whether the development of cerebral calcification is influenced by correction of the acidosis.

Bone marrow transplantation is not indicated, since the hematologic manifestations that make it appropriate in the infantile, recessive lethal form of osteopetrosis [34] are usually not present in the CA II deficiency syndrome. Although the bone manifestations should theoretically improve following bone marrow transplantation, because stem cells from the donor marrow would provide CA II-containing osteoclasts, the renal insufficiency will not improve. This was actually the observation reported in the first patient treated with transplanted for osteopetrosis who, in retrospect, appears to have had CA II deficiency [33].

We had the opportunity to replace the CA II deficient red cells with CA II replete blood cells following severe uterine hemorrhage in one of the patients we followed [83]. Raising the circulating erythrocyte levels of CA II to the heterozygote range by transfusion with replete erythrocytes had no effect on plasma pH or urine pH. These observations was supported by the proposal that the metabolic acidosis is due to the renal CA II deficiency, and not a secondary consequence of CA II deficiency in erythrocytes.

FUTURE PROSPECTS

Delineation of the molecular defect has made prenatal diagnosis possible in many families. The PCR-based RFLP analysis may be practical for population-based screening in certain restricted populations. Perhaps the clearest example is that of the Arabic mutation which accounts for more than 75 percent of cases reported to date. Using the PCR based RFLP analysis, Fathallah et al. found that every affected member in 14 families in Tunisia had this mutation [32].

Another potentially important development is the description of a mouse with CA II deficiency [84]. The mutation was produced intentionally by exposing mice that were heterozygotes for electrophoretically distinguishable CA II gene products to a powerful mutagen and screening progeny electrophoretically for loss of one of the alleles. A null mutation was found, and a breeding colony established. The affected mouse has severe acidosis, but has not been found to have osteopetrosis or cerebral calcification. Although this animal model lacks some of the components of the human CA II deficiency syndrome, it is certain to be a profitable model for studying many facets of CO_2 and

HCO_3^- metabolism and for studying certain experimental therapies, such as bone marrow replacement and gene therapy.

Finally, the remarkable utility of this human disease in shedding light on the physiological roles of the various carbonic anhydrases should stimulate clinical research aimed at identifying disorders caused by deficiencies of other members of the CA gene family [56]. An inherited deficiency of CA I has already been found, and proved to have no clinical consequences [56]. Presumably, the late finding reflects the facts that CA I is expressed primarily in the erythrocytes and CA II, which is expressed at normal levels in CA I-deficient patients, can more than handle the requirements for CA activity in the erythrocytes [55]. It seems likely that deficiencies of CA III, CA IV, and CA V would produce significant clinical abnormalities. Such experiments of nature probably exist, and once they are identified, they will likely add greatly to our understanding of why we have evolved so many isozymes to catalyze a reaction as simple as the reversible hydration of CO_2.

IMPLICATIONS FOR OSTEOPOROSIS

By now, there is considerable histochemical, pharmacologic, and genetic evidence that CA II plays an important role in the generation of hydrogen ion gradients, and is required for the normal functioning of osteoclasts in bone resorption [85]. Although osteopetrosis results from a defect in bone resorption, there are other metabolic disorders in which the reverse is true, and accelerated bone loss is the problem. Can one take advantage of the CA dependence of bone resorption process to inhibit accelerated bone loss? A number of organ culture systems have been developed to study bone resorption [55,86,87]. In organ culture, Ca^{2+} release from bones was shown to be hormone responsive (parathormone and dibutyryl cyclic AMP) and sensitive to inhibition by acetazolamide and other inhibitors of CA [87-89]. Animal studies have suggested that bone loss associated with disuse can be partially prevented by CA inhibitors [90]. This observation raises hope that CA inhibitors might have a role in treating common causes of bone loss like osteoporosis. One problem, however, is that chronic administration of the currently available agents produces a systemic acidosis due to their actions on the kidney, and systemic acidosis itself can lead to calcium mobilization from bone. It has been suggested [87] that development of effective inhibitors that might be useful in metabolic bone disease may require development of agents that act selectively on CA II in bone or that can be selectively

targeted to bone-resorbing osteoclasts to avoid inhibition of CA II in kidney and other sites.

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Publication II

A SPLICE JUNCTION MUTATION IN INTRON 2 OF THE CARBONIC ANHYDRASE II GENE OF THE PATIENTS FROM ARABIC COUNTRIES.

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A Splice Junction Mutation in Intron 2 of the Carbonic Anhydrase II Gene of Osteopetrosis Patients From Arabic Countries

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Clinical manifestations in patients with carbonic anhydrase (CA) II deficiency include osteopetrosis, renal tubular acidosis, and cerebral calcification. Of the 39 reported cases of the carbonic anhydrase II deficiency syndrome, 72% were patients from North African and Middle Eastern countries, most, if not all, of whom were of Arabic descent. We have analyzed DNAs from members of six unrelated Arabic kindreds and found five to be homozygous and one heterozygous for a novel splice junction (donor site) mutation at the 5′ end of intron 2. These findings suggest that a common "Arabic" mutation may be the predominant cause of CA II deficiency in this region. The mutation introduces a new Sau3A1 restriction site which allows polymerase chain reaction (PCR)-based diagnosis of this mutation that should be useful in diagnosis, carrier detection, and prenatal diagnosis. The presence of mental retardation and relative infrequency of skeletal fractures distinguish the clinical course of the patients with the Arabic mutation from those of the American and Belgian patients with the His 107 → Tyr mutation.

KEY WORDS: Carbonic anhydrase II, Osteopetrosis, Renal tubular acidosis, Arabic mutation

INTRODUCTION

Osteopetrosis in association with renal tubular acidosis was recognized in three independent kindreds in 1972 (Sly et al., 1972; Guibaud et al., 1972; Vainsel et al., 1972). It later became clear that cerebral calification also developed in those patients (Whyte et al., 1980; Ohlsson et al., 1980). The pattern of inheritance is autosomal recessive. In 1983, Sly et al. presented evidence that the primary defect in this disorder is a deficiency of carbonic anhydrase II, one of the two isozymes of carbonic anhydrase (CA) present in erythrocytes (Sly et al., 1983). In 1985, this finding was extended to 18 additional patients from 11 additional kindreds (Sly et al., 1985). By 1987, 30 cases had been reported (Cochat et al., 1987). To date, no patient with the combination of osteopetrosis and renal tubular acidosis has been identified who does not have a deficiency of CA II.

Carbonic anhydrase II deficiency syndrome has been diagnosed in patients from different ethnic backgrounds including Italian, German, Belgian, French, Hispanic, and Arabian (Sly et al., 1985). Recently, the Belgian patient (Vainsel et al., 1972) has been shown to be homozygous for a missense mutation which results in replacement of the conserved histidine at position 107 with tyrosine (His 107 → Tyr) (Venta et al., 1991). The three affected sisters in the American family in which CA II deficiency was first reported were found recently to be compound heterozygotes, having inherited the His 107 → Tyr mutation from their mother and a splice acceptor mutation in the 3′ end of intron 5 from their father (Roth et al., 1992). Neither the Belgian patient nor the American patients was mentally retarded, although all

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subsequently reported patients with CA II deficiency had mental retardation. It has been suggested that a small amount of residual CA II activity in patients with the His 107 → Tyr Mutation allows them to escape mental retardation (Roth et al., 1992).

Among the 39 patients reported with this disease (Strisciuglio et al., 1990; Bejaoui et al., 1991; Schwartz et al., 1991), the clinical manifestations in different pedigrees vary in severity, suggesting genetic heterogeneity. The patients of Arabic descent from Kuwait, Saudi Arabia, and Algeria, who represented more than half of the patients so far recognized (Strisciuglio et al., 1990), were severely affected. Mental retardation and metabolic acidosis were prominent in these patients, while bone fractures were less frequent. By contrast, the patients from Belgium and America were not mentally retarded, and repeated bone fractures were the most common manifestation of their disease (Strisciuglio et al., 1990). The significant difference in the clinical symptoms between Belgian, American, and Arabic patients prompted us to extend the mutational analysis to Arabic patients.

MATERIALS AND METHODS

DNA samples from patients with the CA II deficiency syndrome and normal individuals were isolated from leukocytes. Individual exons and surrounding intronic sequences were amplified by PCR. The primers and procedures for PCR were the same as described (Roth et al., 1992). PCR products were subcloned into M13 sequencing vectors and DNA sequencing was carried out by the dideoxynucleotide chain termination method of Sanger et al. (Sanger et al., 1977) on single-stranded DNA. At least 2 separately amplified PCR products were cloned and a minimum of 10 clones were sequenced for each exon. The sequence of the mutant allele was also determined directly from the PCR product (Bergenhem et al., 1992).

Sau3A1 Restriction Site Analysis

About 0.2 μg of DNA from PCR was digested with the restriction enzyme Sau3A1 (New England Biolabs) at 37°C for 3 hr. Products were analyzed by electrophoresis through 12% acrylamide/8 M urea denaturing gels in 89 mM Tris, 40 mM boric acid, and 0.2 mM EDTA buffer. The electrophoresis was carried out at 50 mA per plate, and the gel was stained with ethidium bromide.

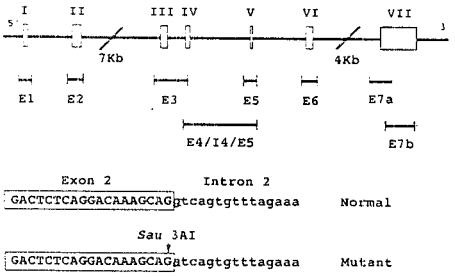

FIGURE 1. Genomic organization of the CA II gene and the Arabic splice junction mutation. Open boxes show exons. Bars E1 through E7b represent PCR fragments which were sequenced in mutational analysis. Sequences around the exon 2/intron 2 boundary are shown. At 5′ of intron 2, a G in the normal sequence was replaced with an A in the mutant. This change created a new Sau3A1 restriction site (GATC) which is absent in the normal allele.

RESULTS

Splice Junction Mutation

Genomic DNAs from normal individuals and CA II-deficient patients were amplified and sequenced exon by exon, including exon-intron boundaries as shown in Figure 1. A base transition from G to A at the 5′ end of intron 2 was found in all clones sequenced from two unrelated patients from Algeria (SB and MER), one from Kuwait (72), and one from Saudi Arabia (75). Sequencing results from one patient (SB) are shown in Figure 2. Sequencing of other exons showed no differences from the normal sequence for the human CA II gene (Venta et al., 1991).

Identification of the Arabic Mutation by Sau3A1 Digestion

As shown in Figure 1, the G to A base change introduces an extra Sau3A1 restriction site 88 bp from the 3′ end of the PCR product. The normal 371 base pair (bp) PCR product for exon 2 contains a single Sau3A1 site in the coding region which, upon digestion, produces 5′ and 3′ fragments of 211 bp and 160 bp (see lane of control in Fig. 3a). The digestion of PCR products of exon 2 from five patients produced extra 88 bp and 72 bp bands which were the result of further cleavage of the 160 bp fragment (Fig. 3a).

Figure 3b shows the analysis of an Egyptian family in which Sau3A1 restriction digestion of the PCR product from the patient BT showed all four bands, indicating a heterozygote pattern. The same restriction pattern was seen with DNA from

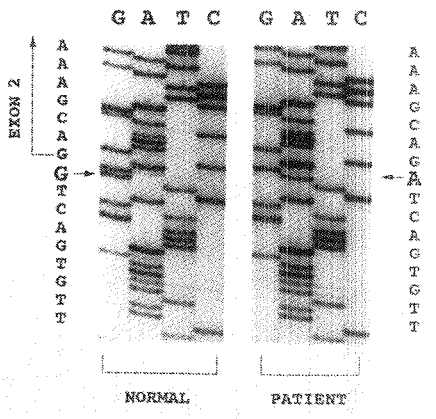

FIGURE 2. Sequencing gels showing the sequence of the exon 2/intron 2 boundary of a normal individual and patients. Arrows indicate the G to A transition at 5' of intron 2.

his asymptomatic brother (HT) and father (AT), but not with DNA from his mother (AET), who was indistinguishable from controls in this analysis. The maternal exon was sequenced completely and found to be normal. Thus, the maternal CA II mutation must be present in sequences outside this exon, although it has not yet been identified by sequencing all of the exons and intron-exon boundaries of the maternal CA II gene.

DISCUSSION

The CA II deficiency syndrome appears to occur more frequently in North African and Middle Eastern countries such as Algeria, Kuwait, and Saudi Arabia than in other parts of the world, based on the relatively large fraction of total cases that have been reported from this region. How much of this increased disease frequency results from increased frequency of the mutant gene in these populations, and how much can be attributed to the relatively high incidence of consanguineous marriages, is uncertain. What is clear is that most of the reported cases from North Africa and the Middle East are products of consanguineous marriages (Sly et al., 1985). In fact, all five of the patients studied here who were homozygous for the Arabic mutation are products of consanguineous marriages. The Egyptian patient who is a compound heterozygote is not the product of a consanguineous marriage, although both parents are Arabic.

The fact that five of the six patients from unrelated kindreds from different countries in this region were homozygous for the same mutation, and the sixth was heterozygous for this mutation, suggests that this mutation may account for most of the cases of CA II deficiency syndrome in Arab communities. Should larger studies confirm this hypothesis, the identification of the common Arabic mutation would have important practical implications. The new Sau3A1 restriction site introduced by the G to A transition in the exon 2/intron 2 boundary allows screening for this mutation on the basis of the unique electrophoretic pattern of the PCR products of exon 2 following digestion with Sau3A1. This finding provides a simple and accurate procedure for detecting this mutation in the Arabic communities that could be useful for diagnosis, carrier detection, and prenatal diagnosis. The PCR-based assay can also be used to determine the gene frequency of this splice-junction mutation in Arabic communities, in order to decide whether screening for this mutation is practical in Arab populations.

The number of pedigrees analyzed for the molecular basis of the CA II dificiency syndrome is not yet sufficient to allow us to predict with confidence the severity of clinical symptoms of patients with specific mutations in the CA II gene. However, affected patients in both families in which the His 107 → Tyr missense mutation was found, have had multiple fractures but escaped mental retardation. By contrast, those who were homozygous for the Arabic splice junction mutation have all had mental retardation, but relatively few bone fractures. The Egyptian patient who is a compound heterozygote with one Arabic mutation allele appears comparable in phenotype and severity to the patients who are homozygous for the Arabic mutation.

Since metabolic acidosis can lead to enhanced bone resportion, it has been suggested that the metabolic acidosis in the CA II deficiency syndrome may partially compensate for the CA II requirement for bone resorption by CA II deficient osteoclasts (Whyte et al., 1980; Cochat et al., 1987). If this were true, it might follow that patients with CA II mutations producing the most severe renal tubular acidosis, such as the patients with the Arabic mutation, might have milder osteopetrosis and fewer fractures than patients with less severe acidosis. It might also raise the concern that one could aggravate the skeletal complications of osteopetrosis in CA II deficient patients by correcting their metabolic acidosis. However, no deleterious effects have been reported in patients

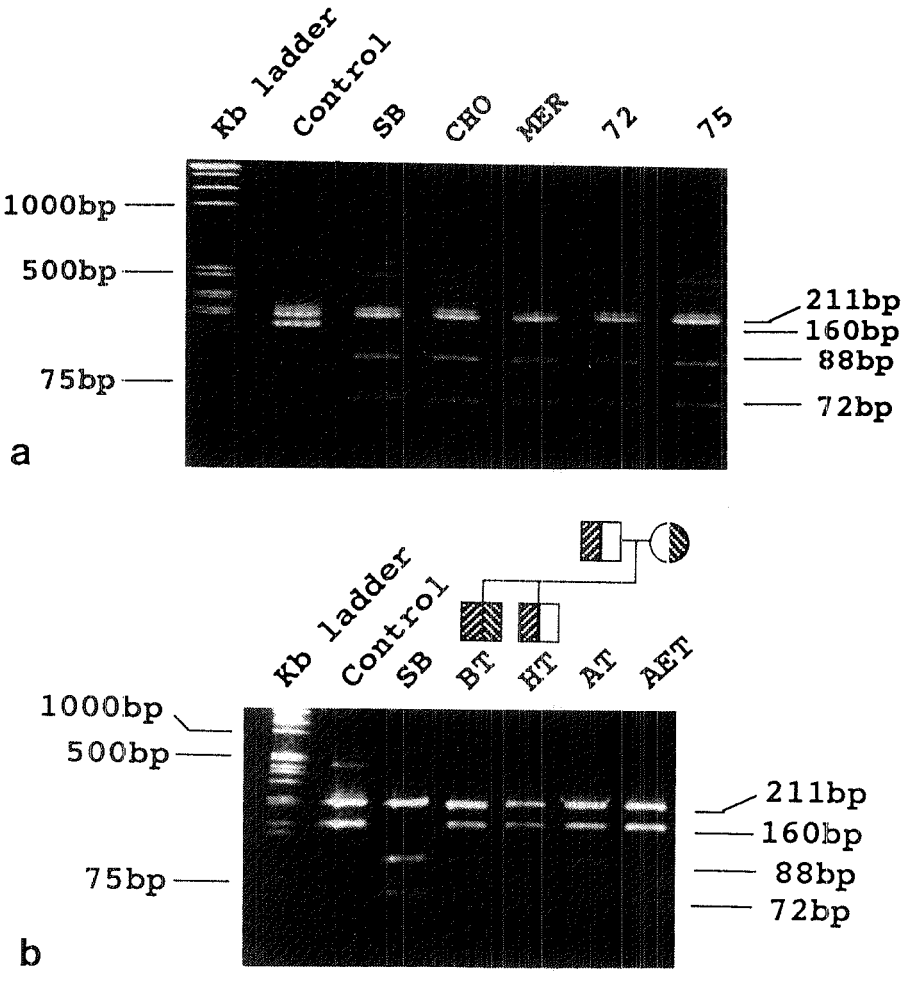

FIGURE 3. (a) Detection of the mutation by Sau3A1 digestion. PCR products of exon 2 from normal and CA II-deficient patients from Algeria (SB, CHO, and MER), Kuwait (72), and Saudi Arabia (75) were digested with restriction enzyme Sau3A1. The resulting fragments were electrophoresed through 12% acrylamide/8 M urea denaturing gel. The molecular weights of the fragments are indicated. (b) Sau3A1 digestion of exon 2 PCR products on an Egyptian patient (BT), his asymptomatic brother (HT), his father (AT), and mother (AET). DNA from Algerian patient (SB) were included as a positive control.

whose acidosis was corrected by treatment (Cochat et al., 1987).

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Publication III

CARBONIC ANHYDRASE II DEFICIENCY: SINGLE-BASE DELETION IN EXON 7 IS THE PREDOMINANT MUTATION IN CARIBBEAN HISPANIC PATIENTS.

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Carbonic Anhydrase II Deficiency: Single-Base Deletion in Exon 7 Is the Predominant Mutation in Caribbean Hispanic Patients

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Summary

To date, three different structural gene mutations have been identified in patients with carbonic anhydrase II deficiency (osteopetrosis with renal tubular acidosis and cerebral calcification). These include a missense mutation (H107Y) in two families, a splice junction mutation in intron 5 in one of these families, and a splice junction mutation in intron 2 for which many Arabic patients are homozygous. We report here a novel mutation for which carbonic anhydrase II–deficient patients from seven unrelated Hispanic families were found to be homozygous. The proband was a 2½-year-old Hispanic girl of Puerto Rican ancestry who was unique clinically, in that she had no evidence of renal tubular acidosis, even though she did have osteopetrosis, developmental delay, and cerebral calcification. She proved to be homozygous for a single-base deletion in the coding region of exon 7 that produces a frameshift that changes the next 12 amino acids before leading to chain termination and that also introduces a new *Mae*III restriction site. The 27-kD truncated enzyme produced when the mutant cDNA was expressed in COS cells was enzymatically inactive, present mainly in insoluble aggregates, and detectable immunologically at only 5% the level of the 29-kD normal carbonic anhydrase II expressed from the wild-type cDNA. Metabolic labeling revealed that this 27-kD mutant protein has an accelerated rate of degradation. Six subsequent Hispanic patients of Caribbean ancestry, all of whom had osteopetrosis and renal tubular acidosis but who varied widely in clinical severity, were found to be homozygous for the same mutation. These findings identify a novel mutation common to Hispanic patients from the Caribbean islands and provide a ready means for PCR-based diagnosis of the "Hispanic mutation." The basis for their phenotypic variability is not yet clear.

Introduction

Carbonic anhydrase II (CA II) is a monomeric cytosolic isozyme of carbonic anhydrase with no posttranslational modification. Deficiency of this enzyme was found to produce osteopetrosis, renal tubular acidosis, cerebral calcification, and several other abnormalities (Sly et al. 1985).

Three previous studies (Venta et al. 1991; Hu et al. 1992; Roth et al. 1992) suggested that clinical heterogeneity among CA II–deficient patients is due to different structural gene mutations. A mildly affected Belgian patient homozygous for the H107Y mutation had frequent skeletal fractures and no mental retardation (Venta et al. 1991). The original American kindred included three patients who were compound heterozy-

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gotes for the H107Y mutation and for a splice junction mutation in intron 5. They resembled the Belgian patient, in that they had many skeletal fractures, but were not mentally retarded (Roth et al. 1992). A large number of Arabic patients who have osteopetrosis, severe renal tubular acidosis, and moderate to severe mental retardation were found to be homozygous for a splice junction mutation in intron 2 (Hu et al. 1992). Skeletal fractures are infrequent in these Arabic patients.

In the present study, we analyzed the gene mutation in seven unrelated CA II–deficient patients of Hispanic origin who have a wide range of clinical manifestations. All seven patients were found to be homozygous for a single-base deletion producing a frameshift in the coding region of exon 7 of the CA II gene. The mutation is easily identified by PCR-based diagnosis, since it introduces a new *Mae*III restriction site. When expressed in COS-7 cells, the mutant cDNA produced the predicted 27-kD truncated polypeptide, which is functionally inactive, is present in reduced amounts compared with the 29-kD normal protein, is in large part due to accelerated degradation, and has a tendency to form insoluble aggregates.

Material and Methods

DNA samples from patients and normal individuals were isolated from leukocytes. *Taq* polymerase and buffer were purchased from Promega (Madison) for amplification of genomic DNA. Restriction enzymes were purchased from Promega and New England Biolabs (Beverly, MA). M13mp18, M13mp19, Sequenase, and dideoxynucleotide sequencing kit were from United States Biochemicals (Cleveland). A kit for site-directed mutagenesis and [^{35}S]-α-dATP were purchased from Amersham (Arlington Heights, IL). [^{35}S]-methionine/cystine translabel was from ICN Biomedicals (Irvine).

Amplification and Sequencing of Genomic DNA

Individual exons and surrounding intronic sequences were amplified by PCR. The primers and procedures for PCR were the same as described (Roth et al. 1992). PCR products were subcloned into M13mp18 and M13mp19 vectors, and DNA sequencing was carried out by the dideoxynucleotide chain-termination method of Sanger et al. (1977) on single-stranded DNA. At least two separately amplified PCR products were cloned, and a minimum of 10 clones were sequenced for each exon.

MaeIII Restriction-Site Analysis

About 0.2 μg of DNA from PCR was digested with 1 unit of restriction enzyme *Mae*III (New England Biolabs) at 55°C for 2 h. The products of the digestion were analyzed by electrophoresis through 1.2% agarose gel in 1 × TAE buffer and stained with ethidium bromide.

Construction of Expression Vectors

The full-length cDNA of CA II was modified by site-directed mutagenesis in M13 to create an *Nco*I site at the ATG start codon (pDRM12) as described elsewhere (Roth et al. 1992). The single-base deletion corresponding to the Hispanic mutation was introduced in pDRM12 by site-directed mutagenesis with the Amersham oligonucleotide mutagenesis kit and the mutant clone designated "pHispa." Single-stranded DNAs from several clones were sequenced to ensure that no other mutations were introduced. The normal and mutant cDNA from pDRM12 and pHispa were excised as *Eco*RI fragments containing the whole coding sequence and subcloned into the mammalian expression vector, pCAGGS, described by Miyazaki et al. (1989) and utilized by Yoshida et al. (1991), which was a gift of Dr. A. Oshima. The constructs containing the normal and mutant CA II cDNA were designated as "pCAGCA2" and "pCAGHisp," respectively.

Expression of Human Mutation in COS-7 Cells

COS-7 cells in 60-mm dishes were transfected with 10 μg of DNA per dish by using the DEAE dextran procedure (Lopata et al. 1984), followed by a 3-h chloroquine treatment 12 h after transfection (Luthman and Magnusson 1983). The transfected cells were harvested by scraping into PBS 84 h after transfection and were homogenized in 50 mM Tris-SO_4 (pH 8.0), 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM iodoacetamide (IAA), 1 mM benzamidine, and 0.1% Triton X-100, with sonication for 8 s twice in an ice bath. The supernatant was collected after centrifugation of the cell lysate at 44,000 *g* for 30 min, at 4°C.

Metabolic Labeling

Metabolic labeling of transfected COS cells was carried out in 60-mm dishes 60 h after transfection with 100 μCi (1 Ci = 37 GBq) of [^{35}S]-methionine/cysteine translabel for 1 h, followed by a 22-h chase with unlabeled methionine/cysteine. Cells were harvested in PBS and lysed in lysate buffer (10 mM Tris/HCl, 150 mM NaCl, 0.5% deoxycholate [DOC], 0.1% SDS, 1% Triton × 100, and containing 1 mM PMSF, 5 mM IAA, 1 mM

benzamidine). Cell homogenates were produced by sonication for 8 s twice in an ice bath. The supernatants were collected after centrifugation of the cell lysates at 44,000 *g* for 1 h. The insoluble cell membrane aggregates were washed with 1 ml of lysate buffer and centrifuged again as above and then directly solubilized in 1 × SDS-PAGE solubilization buffer.

CA Assay

CA activity was assayed by the end-point titration method as described (Sundaram et al. 1986). The protein concentration was determined by Lowry procedure (Lowry et al. 1951) using BSA as standard.

Immunochemical Methods

Antiserum against CA II was produced in rabbits immunized with recombinant human CA II (Roth et al. 1992). The titer and specificity of the antiserum from rabbits were determined by western blot analysis.

SDS-PAGE and Western Blotting

SDS-PAGE was performed under reducing conditions according to Laemmli (1970). The polypeptides were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Zhu and Sly 1990). The PVDF membranes were incubated with rabbit antiserum against the total CA II at a 1:1,000 dilution followed with goat anti-rabbit IgG peroxidase conjugate at a 1:500 dilution. The immunoblots were developed with peroxidase activity (Waheed et al. 1992). When cells were labeled with [^{35}S]-methionine/cysteine translabel, SDS-PAGE gel and PVDF membranes were developed by autoradiography using Kodak XQR-5 film.

Results

Identification of Caribbean Hispanic Mutation

Table 1 summarizes the clinical information on the seven unrelated Hispanic patients who were found to be homozygous for the same mutation. Exons 1–7 of the human CA II gene were sequenced using PCR-amplified genomic DNAs from three of these unrelated patients (patients JP and ES in fig. 2 and DR in table 1). A single-base deletion of an adenosine in the coding region of exon 7 (CA II cDNA744) was observed in all three patients (fig. 1). The mutation appeared in all clones from each patient, suggesting that each was homozygous for this mutation. No change from the normal CA II sequence (Venta et al. 1991) was found in any other exon of CA II in DNA amplified from the patients.

The single-base-pair deletion created a new *Mae*III restriction site 150 bp from the 5′ end of the 440-bp PCR product for exon 7a. Figure 2 shows the *Mae*III restriction pattern of the PCR products for exon 7a from a normal individual and six unrelated patients of Hispanic origin. The 440-bp PCR product for exon 7a of the normal individual was not digested. The PCR product for exon 7a from Hispanic patients was digested into two fragments of 290 bp and 150 bp. However, DNAs from their heterozygous parents showed one normal allele of 440 bp and one mutant allele, which was digested by *Mae*III into two fragments of 290 bp and 150 bp.

Translation of the nucleotide sequence of the Hispanic cDNA predicts a frameshift at amino acid 227, resulting in incorporation of 12 abnormal amino acids before termination at a TGA codon (see fig. 3). This mutation would result in production of a truncated 27-kD protein containing 238 amino acids—22 amino acids shorter than the 29-kD normal enzyme. In order to test this prediction, we expressed the normal and Hispanic mutant cDNAs in COS-7 cells.

Expression of the Hispanic Mutation in COS-7 Cells

COS-7 cells were transiently transfected with a eukaryotic expression vector containing either the normal CA II cDNA (pCAGCA2) or the Hispanic mutant CA II cDNA (pCAGHisp). CA activities were determined in the cell homogenates, 44,000-*g* supernatants, and membrane pellet fractions. The expression of the normal CA II cDNA in COS-7 cells produced 195, 206, and 14 CA units/mg protein in the total cell homogenate, 44,000-*g* supernatant, and membrane pellet fractions, respectively. However, the expression of the Hispanic mutant cDNA in COS-7 cells had only 0.07, 0.03, and 0.02 CA units/mg protein in the corresponding fractions. These activities are close to the limits of detection and of questionable significance. When fractions from COS-7 cells expressing normal and mutant CA II cDNAs were characterized by SDS-PAGE followed by Coomassie blue stain, 29- and 27-kD proteins appeared in the normal and mutant cell extracts, respectively (fig. 4*A*). When equal amounts of cell protein were loaded on the SDS-PAGE, there was about 20-fold less 27-kD protein produced by the mutant cDNA transfection than 29-kD normal protein produced by the wild-type cDNA transfection. The majority of the 29-kD normal protein was easily solubilized and was present in the supernatant fraction. However, only 30% of the 27-kD mutant protein was in the supernatant. The remaining 70% sedimented with the cell membrane

Table 1

Clinical Summary of Hispanic Patients with CA II Deficiency

Patient	Age at Diagnosis	Radiologic Features of Osteopetrosis	Renal Tubular Acidosis	Cerebral Calcification	Anemia	Hepato-splenomegaly	Skeletal Fracture	Mental Retardation
JP	2.5 years	+	−	+	Mild	−	−	Mild
LL	33 years	+	+	+	−	−	−	Mild
DS	7 years	+	+	+	Mild	−	+	+
ES	10 mo	+	+	−	Mild	+	−	+
DR	9 mo	+	+	−	−	−	−	+
7546	5 years	+	+	+	−	−	−	Mild
DA	21 mo	+	+	−	−	−	−	+

NOTE.— + = presence of symptom; and − = absence of symptom.

fraction. Three possibilities for this observation were considered. The first possibility is that the transfection efficiency of the mutant construct was less than that of the wild type. To test this, the cDNA for human β-glucuronidase was cotransfected with both normal and mutant CA II cDNAs. As shown in fig. 4*B*, the coexpression of β-glucuronidase appeared to be similar in both normal and mutant CA II cDNA transfections. The 27-kD mutant CA II was still about 20-fold less abundant than the 29-kD normal CA II. This result does not support differing transfection efficiencies.

Two other possibilities for the reduced amount of mutant protein are reduced synthesis and accelerated degradation of the mutant protein. To test these possibilities, we did metabolic labeling experiments. The COS cells were transfected with normal and mutant cDNAs and were labeled with [^{35}S]-methionine/cysteine translabel for 1 h. As shown in figure 5 (homogenate panel at 1-h pulse and 0-h chase), the density of the autoradiogram for the 27-kD mutant protein is half that of the 29-kD normal protein. The supernatant and the cell membrane panels in figure 5 show that the

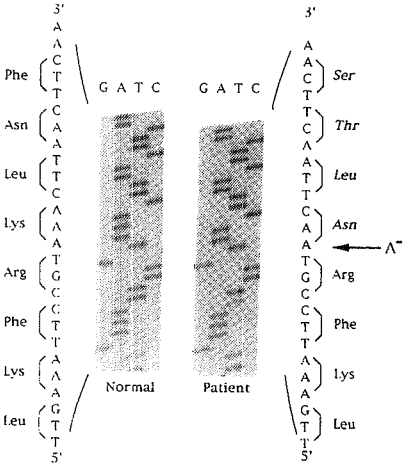

Figure 1 Partial sequence of PCR fragments of exon 7a from normal and patient DNA. The amino acids specified by each codon are indicated. The arrow points to the base deletion in the patient's sequence; the new amino acids specified by the frameshifted sequence are shown in boldface letters.

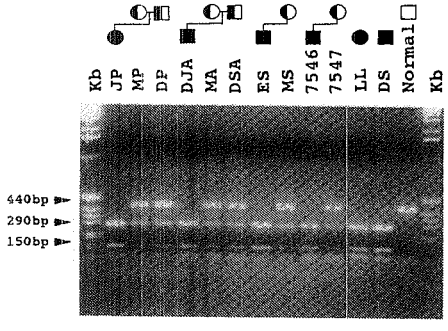

Figure 2 *Mae*III digestion of PCR fragments to detect Hispanic mutation. PCR products of exon 7a (440 bp) from normal and CA II–deficient Hispanic patients (JP, DJA, ES, 7546, LL, and DS) and their parents (MP, DP, MA, DSA, MS, and 7547) were digested with restriction enzyme *Mae*III. The resulting fragments were electrophoresed through 1.2% agarose gel and stained with ethidium bromide. The molecular weights of the fragments are indicated.

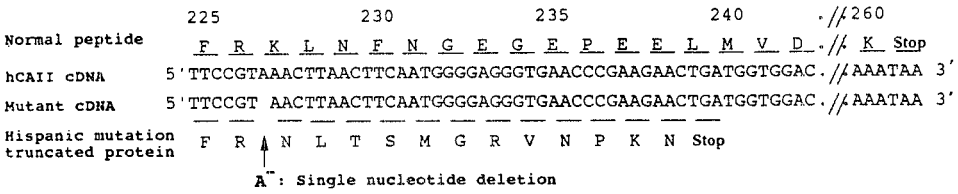

Figure 3 Comparison of the partial sequences of normal and Hispanic mutant cDNAs and their deduced amino acid sequences. Arrow indicates single-base deletion. This deletion results in a frameshift that changes the next 12 amino acids (from 227) before encountering a TGA stop codon at codon 239. The short bars above the normal sequence and below the mutant cDNA sequence show the reading frames.

27-kD mutant protein was mostly soluble at 0-h chase, although a small amount of it already started to appear as membrane-associated aggregates (cell membrane panel at 0-h chase). After a 22-h chase, the 27-kD mutant protein was reduced to one-third of the level seen at 0 h (see homogenate panel of fig. 5). More than 90% of the 29-kD normal protein remains at the same chase time. The panels showing results of fractionation show that the 27-kD mutant protein has almost disappeared from the supernatant at 22 h. The increase in the membrane pellet represents the insoluble material in 22 h. No comparable precipitation is seen with the 29-kD normal protein. Taken together, these results indicated that the 27-kD mutant protein is produced at a lower rate and also has a faster rate of degradation than wild-type enzyme. What residual 27-kD protein escapes degradation is mostly present in the form of insoluble aggregates.

These results (*a*) agree with the prediction that the single-base deletion in the cDNA will result in a frameshift producing a truncated protein on translation and (*b*) provide two explanations for the reduced amount of mutant protein. It is made more slowly and also is degraded more rapidly. The mutant enzyme that survives degradation forms insoluble aggregates that sediment with membranes.

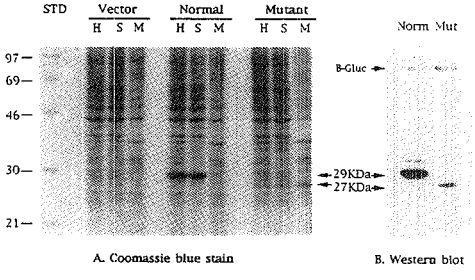

Figure 4 SDS-PAGE of transfected COS-7 cell lysates expressing normal and Hispanic mutant cDNAs. *A*, COS-7 cells transfected with plasmids of pCAGGS without cDNA (Vector) and with CA II cDNA inserts pCAGCA2 (Normal) and pCAGHisp (Mutant), which were electrophoresed through a 12% SDS-PAGE gel and stained with Coomassie blue. Protein (100 μg) of cell homogenate (H), 44,000-*g* supernatant (S), and cell membrane pellets (M) were loaded on each lane. Lane STD contains low-molecular-weight protein markers (Bio-Rad). The arrows indicate the 29-kD normal human CA II and the 27-kD Hispanic mutant CA II. *B*, Western blot of COS cells cotransfected with 1 μg β-glucuronidase cDNA and 10 μg normal or mutant CA II cDNAs. Cell homogenate (50 μg) was loaded on each lane. The immunoreaction was carried out with both anti-β-glucuronidase and anti-CA II antisera. Seventy-eight kilodaltons of β-glucuronidase is indicated by an arrow with "β-Gluc."

Discussion

This study is important in defining what appears to be the predominant mutation producing CA II deficiency in a particular group of Hispanic patients. All

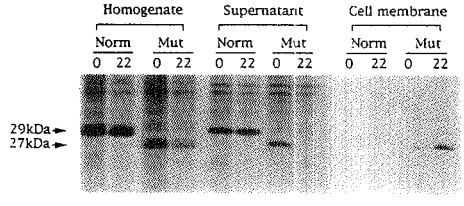

Figure 5 SDS-PAGE of the [^{35}S]-labeled COS-7 cell lysates transfected with normal and mutant CA II cDNA. At 60 h posttransfection, COS cells were labeled for 1 h with 100 μCi [^{35}S]-methionine/cysteine translabel and chased for 22 h (for details, see Material and Methods). Protein (100 μg; about one-eighth of the protein from a 60-mm dish) of each fraction of cell homogenate, supernatant, and one-eighth of the cell membrane from one 60-mm dish were loaded on each lane for SDS-PAGE followed by western blot on PVDF membrane. The PVDF membrane was autoradiographed on Kodak film.

seven patients were born to parents who came from, or are direct descendants of Hispanic parents from, the Caribbean islands (Puerto Rico and the American Virgin Islands).

The new *Mae*III restriction site introduced by this mutation provides a straightforward method to identify the mutation in affected patients, to determine heterozygosity in relatives of affected patients, and could be used for prenatal diagnosis. It might also be used to screen the Caribbean island Hispanics to determine the carrier frequency of the CA II mutation in this population.

The clinical variability of patients with this mutation is puzzling. The proband from the initial Hispanic family attracted our interest because, though she had typical osteopetrosis and cerebral calcification on computed-tomography scan, she had no evidence suggesting renal tubular acidosis, which is seen in nearly all other patients with this disorder. For this reason, we suspected she would have a missense mutation that produced an enzyme with sufficient residual activity to spare the kidney. The finding of the truncated, inactive, quickly degraded, and nearly insoluble enzyme expressed from this mutant cDNA in COS cells did not confirm this prediction. Furthermore, other Hispanic patients were soon identified who had a more severe phenotype, including severe renal tubular acidosis, who proved to be homozygous for the same mutation.

One possible explanation for the absence of renal tubular acidosis in the initial proband might be that the product of an alternate CA gene, such as CA I, which is normally not expressed in kidney, might be expressed in the kidney of the patient. However, we have no experimental evidence to support this speculation. The basis for the interesting differences in severity among patients homozygous for this mutation, including the absence of renal findings in the initial proband, deserves further study.

Although detailed pedigrees were not available for most of the families, consanguinity was denied in four of five families on which family information was available. The absence of known consanguinity in most of these families raises the possibility that the mutant gene may be relatively common in this Hispanic population from the Caribbean islands. How common this mutation is in this population can now be established using the PCR-based assay that is described here.

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Publication IV

PARTIAL RESCUE OF HUMAN CARBONIC ANHYDRASE II FRAMESHIFT MUTATION BY RIBOSOMAL FRAMESHIFT.

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Proc Natl Acad Sci USA (in press).

Partial Rescue of Human Carbonic Anhydrase II Frameshift Mutation by Ribosomal Frameshift

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ABSTRACT

A single base pair deletion in exon 7 of the human carbonic anhydrase II (CA II) gene was found to be the molecular defect in a group of independently ascertained, clinically heterogeneous, Hispanic CA II-deficient patients, all of whom had ancestors from the Caribbean islands. This mutation predicts a +1 frameshift at codon 227 and incorporation of 12 missense amino acids before an early stop codon at 239 produces a 27-kDa truncated CA II. Expression o9f the Hispanic mutant cDNA in bacteria produced predominantly the 27-kDa protein, which was inactive. However, a minor 29-kDa polypeptide species was also produced which had 10% the specific activity of the wild type enzyme following affinity purification.

Amino acid sequencing showed that the 29-kDa mutant protein was produced by two frameshift events: a +1 frameshift at codon 227 due to the single base deletion, and a -1 ribosomal frameshift at codon 237, which restored the original reading frame after 11 missense amino acids were incorporated. Antibody against the 11-amino acid frameshift peptide detected the 29-kDa mutant protein in lysates of transfected COS cells. These results indicated that ribosomal frameshift can partially rescue the human CA II frameshift mutation and suggest a mechanism whereby a compensatory ribosomal frameshift can ameliorate the consequences of certain frameshift mutations. Whether individual differences in efficiency of ribosomal frameshift contribute to clinical heterogeneity in patients with such mutations deserves further study.

INTRODUCTION

Carbonic anhydrase II (CA II) is a 29-kDa cytosolic zinc-metalloenzyme with wide tissue distribution (1). Deficiency of CA II in humans produces the syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification. Variability in clinical severity of this autosomal recessive

disorder in different kindreds has been attributed to different structural gene mutations in different families (2).

We recently reported that a frameshift mutation in exon 7 is the mutational basis for CA II deficiency in a group of independently ascertained, Hispanic patients, all of whom had ancestors from the Caribbean islands (3). The original proband was relatively mildly affected, in that she had no renal manifestations. Six subsequently identified Hispanic patients were found to be homozygous for the same mutation. However, all of them had renal tubular acidosis and showed considerable variation in the severity of their clinical manifestations (3). When the Hispanic mutant cDNA was expressed in COS cells, the predominant product was the 27-kDa truncated CA II predicted by the frameshift mutation in exon 7. The 27-kDa truncated CA II had no enzymatic activity and was not retained on a sulfonamide inhibitor column. It had an accelerated turnover and was largely present in insoluble aggregates in COS cell lysates (3).

When the mutant cDNA was expressed in bacteria, a small amount of CA activity was produced. The enzyme activity was not found in the 27-kDa truncated form of the enzyme, but rather in a form which resembled the 29-kDa normal length CA II in size, and in binding to a sulfonamide inhibitor column. In an effort to define the mechanism of expression of this activity from the mutant cDNA, and to explore its possible relationship to the clinical variability in CA II-deficient patients homozygous for this mutation, we characterized the 29-kDa mutant enzyme protein biochemically and immunochemically. In this report, we present evidence that this activity results from a ribosomal frameshift which restores the normal reading frame after incorporation of 11 missense amino acids and allows completion of translation in the normal reading frame. An antibody was produced to the 11 amino acid frameshift peptide which is unique to the Hispanic mutant protein, and evidence is presented that the 29-kDa "double frameshift" protein is also produced in COS cells expressing the Hispanic mutant cDNA.

MATERIALS AND METHODS

Construction of expression Vectors An *Nco* I site at the ATG start codon was created in full-length, human CA II (hCA II) cDNA by site-directed mutagenesis as described (4). A single-base deletion in exon 7 corresponding to the Hispanic mutation was introduced in the above cDNA as described (3). After the Hind III site in the bacterial expression vector pET11d (Novagen, Madison, WI) was destroyed, the *Nco* I/*Bgl* II fragment of wild type CA II cDNA was subcloned into pET11d at *Nco*

I/*Bam* HI sites, and the plasmid designated pETCA2. pETCA2 contains the entire CA II coding sequence and 62 bp of 3′ untranslated sequence. To introduce the mutant cDNAs into the expression vector, the fragments between the internal *Bam* HI (218 bp downstream of the AUG start codon) and *Hind* III (16 bp 5′ to the UAA stop codon) of Hispanic mutant and artificial double frameshift cDNA from the mutagenesis vectors were swapped with the fragment from pETCA2.

The pCAGGS mammalian cell expression vectors containing wild type and Hispanic mutant cDNAs have been described (3). In brief, the normal and mutant cDNA were excised as *Eco* RI fragments containing the whole coding sequences and 66 and 531 bp noncoding sequences at the 5′ and 3′ end, respectively. The Eco RI fragments were subcloned into the mammalian expression vector, pCAGGS, originally described by Miyazaki et al. (5) and utilized by Yoshida et al. (6), which was a gift of Dr. A. Oshima.

Expression of hCA II in E. coli pET11d vectors containing wild type, Hispanic mutant, and artificially made double frameshift Hispanic mutant cDNAs were each transformed into *E. coli* strain, BL21(DE3)pLysS cells (Novagen, Madison, WI). Expression was induced after the plasmid containing the *E. coli* strains had been grown to OD_{600} of 0.5 at 37°C, by adding isopropyl β-D-thiogalactopyranoside (to 0.6 mM) and $ZnSO_4$ (to 0.6mM). E. coli cells were sedimented 3 to 5 hr after induction, resuspended, and sonicated with a Brinkman polytron in lysate buffer containing 50 mM Tris/SO_4 pH 8.0, 0.1% of Triton x 100 and 1 mM benzamidine. A clear supernatant was obtained after centrifugation at 44,000 g for 45 min at 4°C.

Transfection of COS-7 Cells COS-7 cells in 150 mm dishes were transfected with 75 μg of DNA per dish using the DEAE-Dextran procedure (7) followed by a 3 hr chloroquine treatment (8). The transfected cells were harvested 84 hr after transfection by scraping cells into lysate buffer (20 mM Tris/SO_4, pH 8.0, 0.1% Triton x 100, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mM O-phenanthroline, 5 mM iodoacetamide). The cell homogenate was sonicated for 15 sec twice in an ice bath. The supernatant was recovered after centrifugation of the cell lysate at 44,000 g for 45 min at 4°C.

CA Assay CA activity was measured by the end-point titration method of Maren (9) as described (10). Protein concentration was determined by micro Lowry assay (11) using BSA as standard.

Sulfonamide Inhibitor Affinity Chromatography The clear supernatant after centrifugation at 44,000 g was applied to the inhibitor affinity column. The flowthrough was reapplied two times to ensure complete

binding of the enzyme, and unbound proteins were removed by washing the column. The bound enzyme was eluted in 100 mM sodium acetate pH 5.6, containing 0.5 M sodium perchlorate (12). Eluted protein was dialyzed against 50 mM Tris/SO_4, pH 8.0 to removed the inhibitors and then concentrated in Amicon using YM-10 membrane, or Centricon-10 (Amicon, Lexington, MA).

Production of Antisera Anti-human CA II polyclonal antiserum was as described (4). A 12-amino acid synthetic peptide including 11 amino acids in Hispanic mutant frameshift region, i.e., Asn-Leu-Thr-Ser-Met-Gly-Arg-Val-Asn-Pro-Lys (with an extra Gly at the N-terminal as a spacer), was synthesized in the protein sequencing facility of this department. The synthetic peptide was cross-linked to porcine thyroglobulin (Sigma) using disuccinimidyl suberate (DSS, Pierce). Antiserum against the synthetic Hispanic frameshift 11 amino acid peptide (Anti-Hispanic FS peptide) was produced by injecting the Hispanic frameshift peptide-thyroglobulin conjugates into rabbits with complete Freund's adjuvant followed by a booster injection four week later with the same antigen in incomplete Freund's adjuvant (12). The titer and the specificity of the antiserum were determined by Western blot and dot blot analyses. Anti-human CA II C-terminal 13 amino acid peptide antiserum was also raised in rabbits.

Purification of the Hispanic Frameshift 11 Amino Acids Peptide Specific IgG The synthetic Hispanic frameshift peptide was cross-linked with bovine serum albumin (BSA) using DSS at the ratio of 1 part peptide to 10 parts BSA. The peptide-BSA conjugate was then coupled to AH-Sepharose 4B (Pharmacia) with 1-ethyl-3-[3-(dimethylamino) propyl]carbodiimide (EDC, Sigma). The Hispanic frameshift peptide-Sepharose affinity column was equilibrated with phosphate buffered saline (PBS), after with the antiserum, diluted 1:1 in PBS, was passed through the affinity column. The column was washed with PBS until OD_{280}=0, and then eluted with 100 mM glycine/HCl, pH 2.5 and neutralized immediately with 1/10 volume of 0.5 M Na_2HPO_4. The eluted antibodies were concentrated with Amicon using YM-10 membrane (Amicon) and stored in 50% glycerol at -20°C.

SDS-PAGE and Western Blot Analysis SDS-PAGE was carried out under reducing conditions according to laemmli (13). The SDS-PAGE gels were stained with Coomassie blue. The polypeptides were electrophoretically transferred to poly(vinylidene difluoride) (PVDF) membranes as described (12). The PVDF membranes were incubated with anti-hCA II and anti-hCA II C-terminal 13 amino acid peptide antisera at 1:1000 and 1:500 dilutions respectively. Purified anti-Hispanic frameshift 11 amino acid peptide antibodies, 50 µg/ml were used. After incubation with the first

antibodies, the PVDF membranes were incubated with goat anti rabbit IgG peroxidase conjugate at 1:500 dilution. The immunoblots were visualized using 4-chloro-1-naphthol and H_2O_2 as described (14).

Cyanogen Bromide (CNBr) Treatment and N-Terminal Amino Acid Sequencing The HPLC purified 29-kDa wild type and Hispanic mutant proteins were lyophilized and dissolved in 100 µl of 70% HCOOH. The protein mixtures were sonicated 5 sec twice in an ice bath. To 20 µg protein, 70 µg CNBr (5 µg/ul in H_2O) was added and the mixture incubated in the dark at room temperature for 24 hr. The reactions were terminated with addition of 500 µl H_2O and then lyophilized. CNBr cleaved polypeptides were subjected to amino acid sequencing with Edman degradation method using an Applied Biosystems model A77 automatic protein sequencer (14).

RESULTS

Expression of the Hispanic mutation in E. coli Expression of the wild type cDNA in pET11d produced a large amount of CA II activity, most of which remains in the supernatant following sedimentation at 44,000 x g (Table 1).

Table 1. CA activities in E. coli expressing wild type and Hispanic mutant CA II cDNA

Fraction	Wild type CA 11 (EU/mg)	Hispanic mutant (EU/mg)	Vector only (EU/mg)
Total Homogenate	433	0.11	0.003
Supernatant (44,000 g)	505	0.26	0.003
Pellet (44,000 g)	68	0.02	0.001

Expression of the Hispanic mutant cDNA produced only 0.03% as much activity in the total homogenate, most of which was present in the supernatant. The low level of activity in the supernatant (specific activity 0.05% of the wild type supernatant) was still 50-100 times the activity seen in the corresponding fractions of the induced control (vector only). SDS-PAGE (Fig. 1A) revealed a 29-kDa protein in the wild type CA II homogenate and fractions, that was primarily in the supernatant, and was the predominant band with anti-CA II immunoreactivity on western blotting (Fig. 1B). SDS-PAGE and western blot analyses of the mutant

homogenate and frameshift mutation, most of which sedimented into the pellet. Note, however, that the mutant fractions also contain a smaller amount of a 29-kDa immunoreactive protein (Fig. 1B).

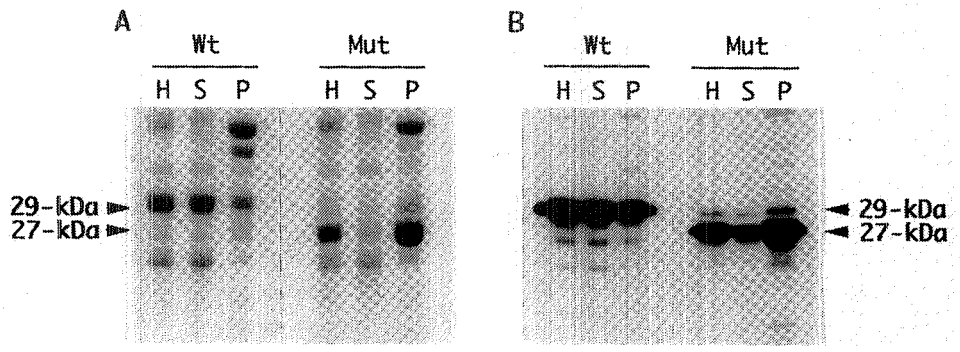

Figure 1 Expression of the wild type (Wt) and Hispanic mutant (Mut) CA II cDNA in *E. coli*. The *E. coli* cell homogenate (H), supernatant (S), and pellet (P) containing 50 μg protein were analyzed on SDS-PAGE and stained with Coomassie blue (a), and in parallel, by Western blot using anti-human CA II (b). The apparent molecular weights of the polypeptides are indicated.

Characterization of the Hispanic mutant CA II protein The wild type and Hispanic mutant CA IIs were purified from the E. coli lysates by sulfonamide inhibitor affinity chromatography and the affinity-purified enzymes analyzed by SDS-PAGE (Fig. 2A). The 29-kDa wild type CA II was purified to near homogeneity. The eluate from affinity chromatography of the extract of bacteria expressing the mutant cDNA contained a major polypeptide of 29-kDa and several high molecular weight polypeptides (Fig. 2A). None of the 27-kDa truncated form of the mutant CA II was retained by the inhibitor affinity column. Furthermore, the flowthrough fraction of the inhibitor affinity column containing the 27-kDa mutant protein (not shown) had no CA activity. The affinity-purified wild type and mutant proteins were both further purified by HPLC and subjected to N-terminal amino acid sequencing. The N-terminal amino acid sequences of both the 29-kDa wild type and the 29-kDa mutant proteins were identical with the sequence reported for CA II (15). The N-terminal sequences of three higher molecular weight proteins which copurified with the mutant CA II on affinity chromatography, but were separated by HPLC, indicated that they were unrelated proteins. The specific activity of the affinity purified mutant CA

II was 594 enzyme units/mg (EU/mg), only 10.2% of the activity of the wild type enzyme (5788 EU/mg).
The immunoreactivities of the affinity-purified with type and mutant CA IIs were compared following SDS-PAGE by western blotting (Fig. 2B). Two polyclonal antisera were used, one against human CA II, and one against the synthetic C-terminal. 13-amino acid of human CA II. The 29-kDa products of wild type and mutant cDNAs both reacted with both antisera with similar intensities (Fig. 2B). These results suggested that these two proteins share epitopes and have similar, if not identical, C-terminal sequences.

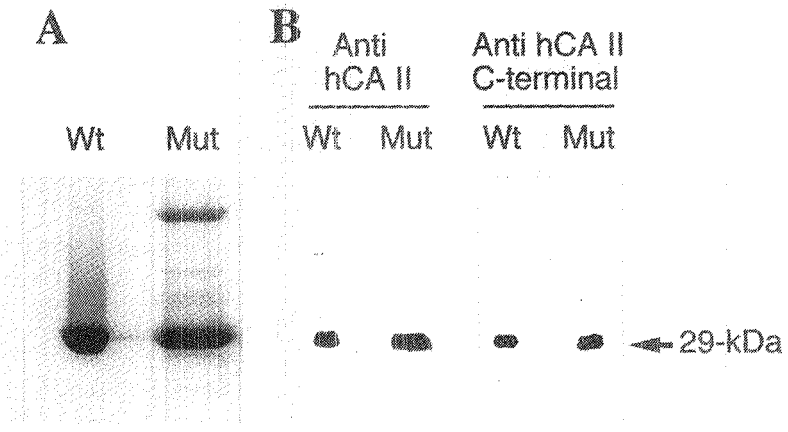

Figure 2 (a) Analysis of affinity-purified wild type (Wt) and Hispanic mutant (Mut) CA II from *E. coli* lysates by SDS-PAGE followed by Coomassie blue staining. (b) Immunochemical characterization of affinity-purified wild type (Wt) and Hispanic mutant (Mut) human CA IIs expressed in *E. coli*. After SDS-PAGE, polypeptides were electrophoretically transferred to PVDF membrane and probed with anti-native human CA II (left), and anti-CA II C-terminal 13 amino acid peptide antisera (right), respectively.

Elucidating the mechanism of producing the 29-kDa mutant protein The results so far indicated that the activity seen following expression of the Hispanic mutant cDNA in bacteria could not be attributed to residual activity in the truncated protein, but was instead present in the small amount of normal-size protein expressed from mutant cDNA. We considered three possible mechanisms whereby the Hispanic frameshift mutant cDNA could produce a 29-kDa CA II. The first was reversion of

the mutation to wild type in a small part of the bacterial population. The fact that the normal-sized mutant protein had greatly reduced specific activity argued against it being a wild type enzyme resulting from reversion of the original mutation to wild type in a small fraction of the bacteria. The second explanation considered was UGA suppression, ion which case insertion of an amino acid rather than termination at the UGA codon would lead to a longer translation product. However, the reactivity of the mutant protein with antiserum to the normal C-terminal 13-amino acid peptide argued against it being the product of suppression of the UGA stop codon, since UGA suppression would extend translation of the mutant protein in the wrong reading frame, incorporating only missense amino acids until it encountered the next stop codon. Furthermore, when we substituted Ser or Trp codons for the UGA at codon 239 by site-directed mutagenesis in order to mimic UGA suppression, the longer products produced were mostly insoluble and had no CA activity (data not shown).

The third mechanism considered to explain the normal length mutant enzyme was a ribosomal frameshift which restored the reading frame between the site of the original frameshift mutation in codon 227 and the UGA codon (38 nucleotides downstream). Such an event would allow completion of the mutant protein in the proper reading frame, and explain its reactivity with antiserum to the normal C-terminal peptide. The only way to test this hypothesis would be to determine the amino acid sequence from the mutant protein. Fortunately, the position of the only methionine in normal human CA II at codon 240, and that of the newly introduced methionine in the frameshift protein at codon 231, made it practical to determine the relevant amino acid sequences by microsequencing the CNBr fragments of normal and mutant CA IIs.

HPLC-purified wild type and mutant CA IIs were treated with CNBr as described in Methods, and sequenced directly without separating the CNBr fragments. Sequencing of the CNBr fragments from the normal human CA II revealed two amino acids per cycle for the first 19 of 20 cycles, and disclosed the residues predicted for the two fragments of the wild type enzyme. The Ser_2-Pro_{21} sequence from the N-terminus and the Val_{241}-Phe_{259} sequence from the C-terminal CNBr fragment (Fig. 3), could be identified in the 20 cycles of automated microsequencing. Microsequencing the CNBr fragments of the mutant protein revealed three amino acids per cycle. Two of them corresponded to the same two amino acids seen each cycle on sequencing the wild type enzyme, namely the Ser_2-Pro_{21} from the N-terminus, and the Val_{241}-Lys_{257} from the CNBr fragment created by cleavage at Met_{240} (Fig. 3).

Figure 3 C-terminal amino acid sequences of 29-kDa wild type and Hispanic mutant CA II determined by microsequencing the CNBr fragments. The unseparated CNBr fragments were subjected to N-terminal sequencing. The first 20 cycles from the wild type CNBr fragments included amino acid Ser_2-Pro_{21} from the N-terminus (not shown) and Val_{241}-Phe_{259} (solid underline). The amino acids in the 20 cycles from the mutant CNBr fragments included Ser_2-Pro_{21} from the N-terminus (not shown), $Val_{241}Lys_{257}$ (solid underline), and a new sequence Gly_{232}-Gln_{248} that was not present in the sequence from the wild type CA II. The arrow indicates the site of the single base deletion found in the Hispanic mutant cDNA. An open and filled bar at amino acid 237 of the mutant proteins shows the reading frames of lysine (K) and glutamic acid (E), respectively, which indicate the point of the ribosomal frameshift (indicated by the step-down in the dotted line under the sequence of the mutant peptide.

The Met_{240}-Lys_{257} sequence argued that the 29-kDa Hispanic mutant protein is completed in the normal reading frame. Further support for this conclusion was provided by a third sequence which was evident in the first 17 cycles of sequence from the CNBr fragments of the mutant protein. These amino acids correspond to Gly_{232}-Gln_{248}, as indicated in Fig. 3. The first part of this sequence was predicted by the single base pair deletion creating the original frameshift mutation at codon 227. Thus, the first six residues correspond to the Gly_{232}-Lys_{237} portion of the missense peptide predicted to follow Met_{231} in the mutant protein. However, the next 11 residues correspond to the Glu_{238}-Gln_{248} sequence of the normal,

wild type human CA II. These findings indicate that the reading frame was restored by a translational frameshift after codon 237. The fact that this sequence extends beyond Met_{240} indicates that the CNBr cleavage at Met_{240} had been incomplete.

These results from microsequencing the CNBr fragments of the wild type and mutant human CA IIs indicate that the 29-kDa Hispanic mutant protein is the product of two frameshift events, a +1 frameshift at codon 227 resulting from the 1 bp deletion, and a -1 ribosomal translational frameshift after codon 237 which restores the reading frame after incorporation of 11 missense amino acids. We refer to the 29-kDa protein as the "double frameshift" product.

Having established ribosomal frameshift as the mechanism whereby the Hispanic mutant cDNA produces a small amount of normal-size, partially active, mutant protein, we produced a "double frameshift" mutant cDNA containing the -A deletion in codon 227 (a +1 frameshift) and a +G insertion at codon 237 (to mimic the -1 ribosomal frameshift). We predicted that expression of this "double frameshift" cDNA would produce a 29-kDa protein with properties of the 29-kDa Hispanic mutant protein. The protein produced in bacteria by this "double frameshift" cDNA had the expected properties, namely a specific activity around 550 EU/mg, and reactivity with antibody to the 11 amino acid frameshift peptide (Figure 4) (described below) and also with the antibody to the normal C-terminal, 13 amino acid peptide (Fig. 4).

Characterization of frameshift peptide-specific antibodies In an attempt to produce an immunologic reagent to recognize the ribosomal frameshift protein in mammalian cell extracts, we raised an antibody in rabbits to a synthetic peptide containing the 11 missense amino acids (Asn_{227}-Lys_{237}, Fig. 3) present in the Hispanic mutant protein. The synthetic Asn_{227}-Lys_{237} frameshift peptide used to immunize the rabbits was conjugated to thyroglobulin.

The antibody was affinity-purified on immobilized Asn_{227}-Lys_{237} peptide conjugated to bovine serum albumin, and used to identify CAs containing the corresponding 11-amino acid peptide. Figure 4 compares the reactivities of this reagent with those of two other polyclonal antisera, one to the native, normal human CA II, and one to the synthetic C-terminal, 13-amino acid peptide of normal CA II. The three antibodies are compared for reactivity to three antigens, all expressed in bacteria: 1) the wild type 29-kDa CA II, 2) the 29-kDa protein produced by the "double frameshift" cDNA described above, and 3) the 27-kDa truncated Hispanic mutant protein (the predominant protein expressed in bacteria from the Hispanic mutant cDNA).

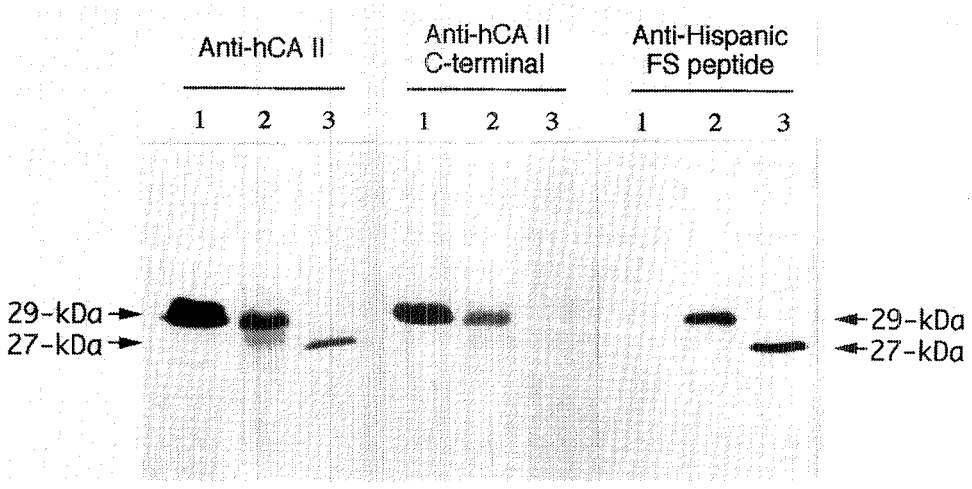

Figure 4 Comparison of the reactivities of three antibodies with three antigens expressed in *E. coli*. Lanes 1, affinity purified wild type CA II (0.5 µg); lanes 2, affinity purified double frameshift mutant protein (0.5 µg); lanes 3, affinity column flowthrough containing the 27-kDa Hispanic mutant protein (5 µg). After SDS-PAGE, polypeptides were electrophoretically transferred to PVDF membrane and probed with anti-hCA II (left), anti-hCA II C-terminal 13 amino acid peptide (center), and anti-Hispanic frameshift peptide (right) antisera.

Fig. 4 shows that the antiserum to normal human CA II reacts with all three antigens. The antiserum to the C-terminal 13-amino acid peptide recognizes the first two antigens, both of which are expected to contain the C-terminal peptide, but fails to react with the 27-kDa Hispanic mutant species that is expected to lack the C-terminus. The antibody to the 11-amino acid frameshift peptide does not react with wild type CA II, but does react with both the 29-kDa "double frameshift" product and with the 27-kDa truncated enzyme, both of which should contain the unique 11-amino acid sequence. These results establish the specificity of the antibody to the frameshift peptide.

Detection of 29-kDa immunoreactive species in lysates of COS cells transfected with Hispanic mutant cDNA We previously reported that when the Hispanic mutant cDNA is expressed in COS cells, the predominant protein expressed is the 27-kDa truncated CA II predicted by the frameshift mutation. The "single frameshift" product is inactive and

is not retained on the inhibitor affinity column. To determine whether the "double frameshift" product could also be detected in transfected COS cells expressing the Hispanic mutant cDNA, we prepared lysates from transfected COS cells (200 mg cell protein), applied the lysate to a sulfonamide inhibitor affinity column, and analyzed the proteins eluted from the column by SDS-PAGE and western blots. Lysate from COS cells transfected with vector only was analyzed in parallel as a control. As a positive control for reactivity on western blots with the anti-Hispanic frameshift peptide antibody, we used the 27-kDa truncated protein expressed in bacteria from the Hispanic mutant cDNA.

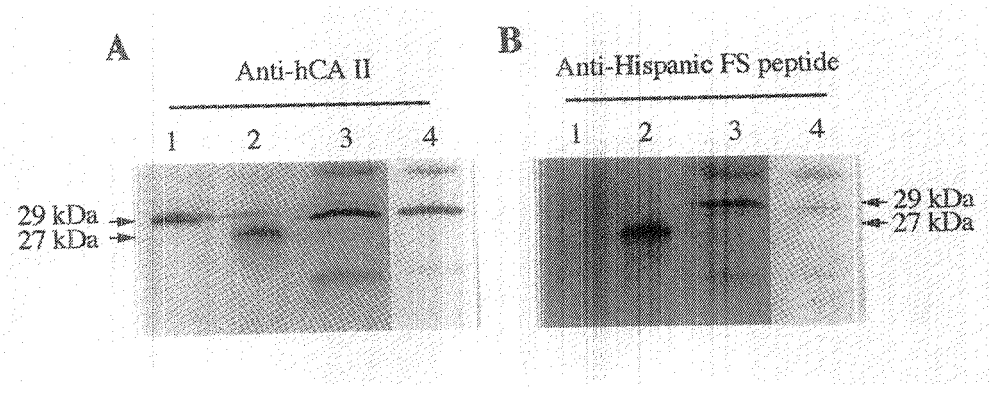

Figure 5 Immunochemical characterization using anti-human CA II antiserum and anti-Hispanic frameshift (FS) peptide antibodies. Lanes 1, affinity purified wild type CA II expressed in COS cells (30 ng); lanes 2, total homogenate of E. coli expressing the 27-kDa Hispanic mutant protein as 10% of the total protein (300 ng); lanes 3, proteins retained on and eluted from affinity column to which 200 mg of lysate protein from COS cells transfected with vector containing the Hispanic mutant CA II cDNA were applied; and lanes 4, proteins retained on and eluted from affinity column to which 200 mg of lysate protein from COS cells transfected with vector only were applied.

Figure 5A shows that the anti-human CA II antiserum recognizes all CA II species, including that expressed in COS cells from the wild type cDNA (lane 1), the 27-kDa truncated mutant CA II expressed in bacteria (lane 2), the 29-kDa endogenous monkey CA II which is expressed in COS cells transfected with vector only (lane 4), and a 29-kDa band (most, if not all, of which is endogenous COS cell CA II) expressed in COS cells

transfected with the Hispanic mutant cDNA (lane 3). (Note also some higher and lower molecular weight unidentified cross-reactive species that were contained in the column eluates from COS cells lysates.)
The affinity-purified antibody to the Hispanic 11 amino acid frameshift peptide (anti-Hispanic FS peptide) does not react with normal human CA II (lane 1), does react with the 27-kDa Hispanic mutant protein (lane 2) (as predicted), and reacts strongly with the 29-kDa protein expressed in COS cells transfected with the Hispanic mutant cDNA (lane 3). These data suggest that the 29-kDa "double frameshift" product, that was identified in bacteria expressing the Hispanic mutant cDNA, is also present in COS cells transfected with the Hispanic mutant cDNA.

DISCUSSION

Several lines of evidence presented here indicate that the CA II activity expressed in bacteria from the Hispanic mutant cDNA is the result of two frameshift events. Although the predominant protein expressed is the 27-kDa truncated protein predicted by the frameshift mutation at codon 227 and the premature UGA termination codon at 239, the 27-kDa "single frameshift" product does not bind to the sulfonamide inhibitor column, and has no activity. The CA II activity expressed in bacteria is explained by the small amount of 29-kDa, "double frameshift" product which is retained by the affinity column and, though active, has only 10% of the activity of the wild type enzyme. Biochemical and immunochemical analyses demonstrated that this protein has the same N-terminus as the normal human CA II and is most likely identical to normal human CA II except for the 11 missense amino acids between the +1 frameshift at codon 227 and the -1 ribosomal frameshift at codon 237. The data indicate that, after the reading frame is restored at codon 237, translation of the last 23 codons proceeds in the normal reading frame. The 11 missense amino acid peptide is present in both the 27-kDa truncated, inactive, "single frameshift" product, and in the 29-kDa full-length, active "double frameshift" product. Presumably, the 11 missense amino acids explain the reduced specific activity of the 29-kDa mutant protein. Immunochemical evidence indicates that both the 27-kDa and 29-kDa products are also expressed in COS cells from the Hispanic mutant cDNA. As was true in bacteria, the small amount of 29-kDa mutant protein reacted on western blots with the frameshift peptide-specific antibodies.
The phenomenon of ribosomal frameshift was discovered in the early 1970s (16), and has been studied in *E. coli* proteins, yeast retroposon Ty, and *Gag-Pol* fusion proteins of several retroviruses (17,18). Intensive mutagenesis studies revealed the mechanisms of several of these

ribosomal frameshifts. Ribosomal frameshift is facilitated by "shifty sequences" formed by a string of four or more single-base repeats in the primary structure, and a "stimulator," i.e., a secondary structure such as a stem-loop or pseudoknot in the mRNA downstream from the shifty site (17,18). In the case of the Hispanic mutant CA II mRNA, there is no obvious string of four or more single base repeats. However, the mRNA does have an AAG for lysine and AAC for asparagine near and at the shift site, and these "hungry" codons have been implicated in other ribosomal frameshifts (19,20). The persistence of expression of the 29-kDa Hispanic mutant protein from Hispanic mutant cDNAs with different replacements at the UGA stop codon at codon 239 indicated that the ribosomal frameshift at 237 did not depend on the UGA at 239 (data not shown).

Although the mechanism underlying this particular frameshift is not clear, the evidence for ribosomal frameshift rescuing the original frameshift mutation suggests a mechanism whereby a translational frameshift could ameliorate the consequences of certain deleterious frameshift mutations. In this respect, the clinical variability of CA II-deficient patients homozygous for the same frameshift mutation is of considerable interest. Whether individual variability in efficiency of ribosomal frameshift contributes to the clinical variability in patients with this mutation deserves further study. The antibody reagent with specificity for the Hispanic frameshift peptide described here should allow screening for the 29-kDa product of the ribosomal frameshift in erythrocyte lysates from Hispanic CA II-deficient patients, in the same way it allowed its detection in the COS cell lysates. If such a protein can be identified in erythrocyte lysates from patients with this mutation, it will be of great interest to determine whether the amount expressed in different patients correlates inversely with the clinical severity of their CA II deficiency syndrome.

ACKNOWLEDGMENTS

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Summary

This thesis deals with the molecular genetic basis of carbonic anhydrase II deficiency in man. The features of this disease are outlined in **Publication I.** Carbonic anhydrase II (CA II) is one of seven CA isozymes known to date in humans. Deficiency of CA II produces a genetic disorder with clinical manifestations including osteopetrosis with renal tubular acidosis and cerebral calcification. CA II deficiency syndrome has been diagnosed in more than 50 patients with different ethnic background, including Italian, German, Belgian, Hispanic, American and Arabic. Patients with the CA II deficiency syndrome show considerable clinical heterogeneity. These observations suggested that the clinical heterogeneity among CA II deficient patients might be due to different structural gene mutations.

In **Publication II**, we described a splice junction mutation at the 5′ end of intron 2 of the CA II gene in patients of Arabic descent. This mutation introduces a new Sau 3AI restriction site. Using this restriction enzyme we analyzed several other Arabic patients. Out of six unrelated Arabic kindreds, five patients were homozygous, and one was heterozygous for this splice junction mutation. This results suggest that a common "Arabic" mutation may be the predominant cause of CA II deficiency in this region. The presence of mental retardation and relatively infrequency of skeletal fractures distinguish the clinical course of the patients with "Arabic" mutation from those with Belgian and American patients with His107Tyr mutation.

The gene mutation in seven unrelated CA II deficient patients of Hispanic origin who had wide range of clinical manifestations was determined (**Publication III**). All patients were found to be homozygous for a single base deletion in the coding region of exon 7 that produces a frameshift which changes 12 amino acids before leading to chain termination. The single base deletion in exon 7 also introduces a new Mae III restriction site. The cDNA for Hispanic mutant was characterized by expressing it in COS-7 cells. A 27-kDa truncated mutant protein was observed but no enzyme activity was detected. The mutant protein was mainly present in insoluble aggregates and detectable immunologically at only 5% the level of the 29-kDa normal CA II expressed from wild type cDNA. Metabolic labeling revealed that this 27-kDa mutant protein has an accelerated rate of degradation. Therefore, these results did not provide any evidence to explain the basis of the phenotypic variability in Hispanic patients.

In **Publication IV**, we present results on expression of the Hispanic mutant cDNA in bacteria. The predominant protein was a 27-kDa protein which was inactive. However a minor 29-kDa polypeptide was also produced which had 10% the specific activity of the wild type enzyme. Amino acid sequencing showed that the 29-kDa mutant protein was produced by two frameshift events: a +1 frameshift at codon 227 due to the single base deletion, and a -1 ribosomal frameshift at codon 237, which restored the original reading frame after 11 missense amino acids were incorporated. Antibody against the 11-amino acid frameshift peptide was produced, and the antibody detected a 29-kDa protein in COS-7 cell lysates transfected with Hispanic mutant cDNA that is consistent with the 29-kDa double frameshift product see in bacterial expression. These results suggest that ribosomal frameshift can partially rescue the human CA II frameshift mutation. Whether individual differences in efficiency of the ribosomal frameshift contribute to clinical heterogeneity in patients with such mutation is not yet clear.

Samenvatting

Dit proefschrift gaat over de moleculair-genetische basis van koolzuuranhydrase II deficiëntie bij de mens. De klinische, pathologische en moleculaire karakteristieken van deze erfelijke ziekten met een autosomaal recessief overervingspatroon zijn beschreven in **Publicatie I**. koolzuuranhydrase II (CA II) is een van de zeven CA isoenzymen die tot nu toe bekend zijn bij de mens. Deficiëntie van CA II leidt tot een erfelijke ziekte met als belangrijkste klinische verschijnselen osteopetrosis, metabole acidosis en cerebrale calcificatie. Het CA II-deficiëntie syndroom is sinds de eerste beschrijving in 1972 gediagnostiseerd bij meer dan 50 patiënten met een verschillende etnische achtergrond, waaronder Italiaanse, Duitse, Belgische, Spaanse, Amerikaanse en Arabische patiënten. Patiënten met het CA II-deficiëntiesyndroom tonen een aanzienlijke klinische heterogeniteit. De veronderstelling, die het uitgangspunt vormde voor het experimentele werk beschreven in dit proefschrift, is dat de klinische heterogeniteit gerelateerd is aan verschillende structurele genmutaties.

In **Publicatie II** wordt een "splice junction" mutatie aan het 5′ einde van intron 2 van het CA II-gen in patiënten van Arabische afkomst beschreven. Deze mutatie veroorzaakt een nieuw Sau 3AI restrictieplaats in het gen. Met behulp van dit restrictie-enzym onderzochten wij verschillende andere Arabische patiënten. Van de zes niet verwante patiënten bleken er vijf homozygoot en één heterozygoot voor deze "splice junction" mutatie. Deze resultaten suggereren dat een veel voorkomende "Arabische" mutatie de voornaamste oorzaak is van CA II deficiëntie in dit geografische gebied. De aanwezigheid van mentale retardatie en het relatief weinig voorkomen van botfracturen onderscheiden het klinische beeld van de patiënten met deze "Arabische" mutatie van dat bij Belgische en Amerikaanse patiënten met een HisI07Tyr mutatie.

Mutatie-analyse in zeven niet verwante CA II deficiënte patiënten van Spaanse afkomst met een breed scala van klinische verschijnselen werd uitgevoerd via experimenten beschreven in **Publicatie III**. Alle patiënten bleken homozygoot voor een enkele basedeletie in het coderende deel van exon 7, waardoor er een "frameshift" ontstaat met als gevolg veranderingen in 12 aminozuren voordat de eiwitketen wordt afgebroken. De basedeletie in exon 7 introduceert ook een nieuwe Mae III restrictieplaats. Het cDNA voor de "Hispanic" mutant werd gekarakteriseerd door het tot expressie te brengen in COS-7 cellen. Een

27-kDa getrunceerd mutant eiwit werd hierbij aangetoond dat geen enzymactiviteit vertoont. Het mutant eiwit was voornamelijk aanwezig in de vorm van onoplosbare aggregaten en werd immunologisch slechts in een hoeveelheid van 5% van het normale 29-kDa CA II, dat tot expressie kwam na introductie van wild type cDNA, aangetoond. Metabole labeling studies toonden aan dat het 27-kDa mutant eiwit versneld wordt afgebroken. Deze resultaten geven geen verklaring voor de phenotypische heterogeniteit in deze groep patiënten.
In **Publicatie IV** worden de resultaten beschreven van de expressie van het "Hispanic" mutant cDNA in bacteriën. Het overheersende eiwitprodukt is een enzymatisch inactief 27-kDa eiwit. Daarnaast werd echter in veel geringere hoeveelheden een 29-kDa polypeptide geproduceerd met ongeveer 10% van de specifieke activiteit van het wild type enzym. Bepaling van de aminozuurvolgorde toonde aan dat dit 29-kDa mutant eiwit tot stand komt door twee "frameshift" gebeurtenissen: een +1 "frameshift" in codon 227 als gevolg van een enkele basedeletie, en een -1 ribosomale "frameshift" in codon 237, waardoor na 11 missense aminozuren het oorspronkelijke "reading frame" weer tot stand komt.
Antilichamen tegen het "11-aminozuren frameshift" peptide werden geproduceerd en met deze antilichamen werd een 29-kDa eiwit aangetoond in COS-7 cellysaten die getransfecteerd waren met het "Hispanic" mutant cDNA. Dit eiwit bleek overeen te komen met het 29-kDa dubbele "frameshift" produkt dat was aangetoond in bacteriële expressie-experimenten. Deze resultaten maken het waarschijnlijk dat een ribosomale "frameshift" de humane CA II "frameshift" mutatie gedeeltelijk kan compenseren. Of individuele verschillen in de effectiviteit van de ribosomale "frameshift" de klinische heterogeniteit in patiënten met de hierboven beschreven mutatie kunnen verklaren, is nog niet duidelijk.

Curriculum Vitae

Peiyi Hu was born in Wuhan, Hubei province, the People's Republic of China on Oct. 13, 1956.
Her high school education was given in the 11th high school in Wuhan from 1969 to 1974. Due to the "Cultural revolution", she had two options for her carrier after high school: to be a professional athlete or to be a farmer. It became the first.
In 1977, she passed the first national examination after the "Cultural revolution", became a medical student in Tongji Medical University in Wuhan, and graduated at the end of 1982 with a Bachelor degree of Medicine.
She was chosen to work as a teaching and research assistant and resident in Tongji medical university and associated hospital from 1982 to 1988. She participated in teaching a course on medical genetics, and worked in the clinic as a genetic counselor, especially for patients with inherited and metabolic diseases. She was registered as a Pediatrician in 1989.
A meeting with Professor Galjaard during one of his visits to Wuhan, led to a fellowship to stay in Rotterdam as a young researcher. She joined the department of Clinical Genetics at Erasmus University Rotterdam in May, 1988, where she stayed two and a half years. During this period she became acquainted with modern biochemical diagnostic methods and worked on research projects aimed at the elucidation of the molecular basis of some lysosomal storage diseases. At the end of 1990 she was fortunate that Professor Sly offered her a postdoctoral fellowship in the department of Biochemistry, Medical School of St. Louis University.
She joined Professor Sly's lab in December of 1990 and worked since then on mutational analysis of human carbonic anhydrase II deficiency syndrome using DNA technology and biochemical methods.

List of publications

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THE MOLECULAR BASIS OF CARBONIC ANHYDRASE II DEFICIENCY

THESIS

TO OBTAIN THE DEGREE OF DOCTOR
AT THE ERASMUS UNIVERSITY OF ROTTERDAM
BY AUTHORIZATION OF
THE RECTOR MAGNIFICUS PROF. DR. P.W.C. AKKERMANS
AND BY THE DECISION OF THE COUNCIL OF DEANS.

THE PUBLIC DEFENSE WILL TAKE PLACE
ON WEDNESDAY, MARCH 22nd 1995 AT 3.45 p.m.

by

PEIYI Y. HU

born in Wuhan, the People's Republic of China

STELLINGEN

behorende bij het proefschrift
The molecular basis of carbonic anhydrase deficiency

I

Crick's "Central dogma" that genetic information flows from DNA to RNA and then to protein (DNA → RNA → protein) does not hold since the discovery of reverse transcriptase.

II

The chemiosmotic theory of Dr. Peter Mitchell for which he got the Nobel Prize in 1978 is untenable for mitochondria after the discovery of carbonic anhydrase in these organelles.

III

Among seven carbonic anhydrase (CA) isozymes, the only one attributable to date to a CA deficiency syndrome is CAII. In some instances the heterogeneous clinical manifestations can be related to the specific nature of the gene mutation involved.
This thesis

IV

The emphasis of clinical genetics will gradually shift from the early diagnosis and prevention of congenital handicaps in children to risk prediction of a variety of diseases in adulthood.

V

The fact that ribosomes are capable of unexpected gymnastic feats will throw light on the great area of darkness remaining in this area of research.
A.E. Dahlberg, 1989

VI

The existence of nonstandard decoding events involving ribosomal hopping, frame-shifting, and reading through stop codons implies that the protein sequence cannot always be simply deduced from the mature mRNA sequence.
J.F. Atkins et al, 1990

VII

On persistent sanding, iron rod becomes needle.
Ancient Chinese proverb

VIII

Thousands miles travelling starts from the first step.
Ancient Chinese proverb

IX

Knowing but not doing is equal to not knowing.
(Kong Zi, Chinese teacher and philosopher in 2000 BC)

X

There is no easy road in science; Only the people who dare to climb the hardest trail can reach the brilliant top.
Karl Marx

Rotterdam, 22 maart 1995

Peiyi Hu