Partial rescue of human carbonic anhydrase II frameshift mutation by ribosomal frameshift
(carbonic anhydrase II deficiency/osteopetrosis/renal tubular acidosis/genetic heterogeneity)

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ABSTRACT A single-base-pair deletion in exon 7 of the human carbonic anhydrase II gene was found to be the molecular defect in a group of independently ascertained, clinically heterogeneous, Hispanic carbonic anhydrase 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 position 239 produces a 27-kDa truncated carbonic anhydrase II. Expression of 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 that had 10% the specific activity of the wild-type enzyme after 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 that 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 indicate that ribosomal frameshifting can partially rescue the human carbonic anhydrase 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 frameshifting contribute to clinical heterogeneity in patients with such mutations deserves further study.

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 that 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 that 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 that 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 HindIII site in the bacterial expression vector pET11d (Novagen) was destroyed, the Nco I–Bgl II fragment of wild-type CA-II cDNA was subcloned into pET11d at Nco I–BamHI sites, and the plasmid was 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 BamHI (218 bp downstream of the AUG start codon) and HindIII (16 bp 5′ to the UAA stop codon) sites of Hispanic mutant and artificial double-frameshift cDNAs 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 cDNAs were excised as EcoRI fragments containing the whole coding sequences and 66- and 531-bp noncoding sequences at the 5′ and 3′ end, respectively. The EcoRI fragments were subcloned into the mammalian expression vector pCAGGS (a gift of A. Oshima), originally described by Miyazaki et al. (5) and used by Yoshida et al. (6).

Abbreviations: CA, carbonic anhydrase; hCA-II, human CA-II; EU, enzyme unit(s); BSA, bovine serum albumin; PVDF, poly(vinylidene difluoride).

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Expression of hCA-II in *Escherichia coli*. pET11d vectors containing wild-type, Hispanic mutant, and artificially made double-frameshift Hispanic mutant cDNAs were each transformed into *E. coli* BL21(DE3)pLysS cells (Novagen). Expression was induced after the plasmid-containing *E. coli* strains had been grown to OD₆₀₀ of 0.5 at 37°C, by adding isopropyl β-D-thiogalactopyranoside (to 0.6 mM) and ZnSO₄ (to 0.6 mM). *E. coli* cells were sedimented 3–5 hr after induction, resuspended, and sonicated with a Brinkman Polytron in lysis buffer containing 50 mM Tris-H₂SO₄ (pH 8.0), 0.1% Triton X-100, and 1 mM benzamidase. 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 by using the DEAE-dextran procedure (7) followed by a 3-h chloroquine treatment (8). The transfected cells were harvested 84 h after transfection by scraping cells into lysis buffer (20 mM Tris-H₂SO₄, pH 8.0/0.1% Triton X-100/1 mM benzamidase/1 mM phenylmethylsulfonyl fluoride/1 mM 3-phenanthroline/5 mM iodoacetamide). The cell homogenate was sonicated for two 15-sec periods 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) with bovine serum albumin (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-H₂SO₄ (pH 8.0) to remove the inhibitors and then concentrated in an ultrafiltration cell using YM10 membrane or Centricon-10 (Amicon).

**Production of Antiserum.** Anti-hCA-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 end as a spacer)] was synthesized in the protein sequencing facility of this department. The synthetic peptide was cross-linked to porcine thyroglobulin (Sigma) by using disuccinimidy suberate (Pierce). Antiserum against the synthetic Hispanic frameshift 11-amino acid peptide (anti-Hispanic FS peptide) was produced by injecting the Hispanic frameshafth thyroglobulin conjugates in rabbits with complete Freund’s adjuvant followed by a booster injection 4 weeks later with the same antigen in incomplete Freund’s adjuvant (12). The titer and the specificity of the antisera were determined by Western blot and dot blot analyses. Anti-hCA-II C-terminal 13-amino acid peptide antiserum was also raised in rabbits.

**Purification of the Hispanic Frameshift 11-Amino Acid Peptide-Specific IgG.** The synthetic Hispanic frameshift peptide was cross-linked with BSA by using disuccinimidyl suberate 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 (Sigma). The Hispanic frameshift peptide-Sepharose affinity column was equilibrated with phosphate-buffered saline (PBS), after which the antiserum, diluted 1:1 in PBS, was passed through the affinity column. The column was washed with PBS until the *A₂₈₀* was zero, and then material was eluted with 100 mM glycine hydrochloride (pH 2.5) and neutralized immediately with 0.1 vol of 0.5 M Na₂HPO₄. The eluted antibodies were concentrated with an ultrafiltration cell using YM10 membrane (Amicon) and stored in 50% (vol/vol) glycerol at -20°C.

**SDS/PAGE and Western Blot Analysis.** SDS/PAGE was carried out under reducing conditions as described by 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 antiserum at 1:1000 and 1:500 dilutions, respectively. Purified anti-Hispanic frameshift 11-amino acid peptide antibodies at 50 μg/ml were used. After incubation with the first antibodies, the PVDF membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG at a 1:500 dilution. The immunoblots were visualized with 4-chloro-1-naphthol and H₂O₂ 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% (vol/vol) HCOOH. The protein mixtures were sonicated for two 5-sec periods in an ice bath. To 20 μg of protein, 70 μg of CNBr (5 μg/μl in H₂O) was added and the mixture was incubated in the dark at room temperature for 24 h. The reactions were terminated with addition of 500 μl of H₂O₂ and then lyophilized. CNBr-cleaved polypeptides were subjected to amino acid sequencing by Edman degradation on 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 remained in the supernatant after sedimentation at 44,000 × g (Table 1). 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 by Western blot analysis (Fig. 1B). SDS/PAGE and Western blot analyses of the mutant homogenate and fractions revealed the 27-kDa truncated hCA-II predicted by the frameshift mutation, most of which sedimented into the pellet. Note, however, that the mutant fractions also contained a smaller amount of a 29-kDa immunoreactive protein (Fig. 1B).

**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 were 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 (data 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 the

<table>
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<tr>
<th>Fraction</th>
<th>Wild-type</th>
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<td></td>
<td>CA-II</td>
<td>Vector</td>
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<td></td>
<td>EU/mg</td>
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<tr>
<td>Total homogenate</td>
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<tr>
<td>Pellet (44,000 × g)</td>
<td>68</td>
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EU/mg: EU/mg
29-kDa wild-type and the 29-kDa mutant proteins and the sequence reported for CA-II (15) were identical. The N-terminal sequences of three higher molecular weight proteins that 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 (EU)/mg, only 10.2% of the activity of the wild-type enzyme (5788 EU/mg).

The immunoreactivities of the affinity-purified wild-type and mutant CA-IIs were compared after SDS/PAGE by Western blot analysis (Fig. 2B). Two polyclonal antisera were used, one against hCA-II and the other against the synthetic C-terminal 13 amino acids of hCA-II. The 29-kDa products of both wild-type and mutant cDNAs 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.

Elucidating the Mechanism of Producing the 29-kDa Mutant Protein. The results so far indicated that the activity seen after 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, in 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 Thr codons for the UGA at codon 239 by site-directed mutagenesis 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 that restored the reading frame between the site of the original frameshift mutation in codon 227 and the UGA codon (38 nt 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 positions of the only methionine in normal hCA-II at codon 240 and 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 and sequenced directly without separating the CNBr fragments. Sequencing of the CNBr fragments from the normal hCA-II revealed 2 of 3 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-Pro21 sequence from the N terminus and the Val241-Phe259 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 3 amino acids per cycle. Two of them corresponded to the same 2 amino acids seen each cycle on sequencing the wild-type enzyme—namely, the Ser-Pro21 from the N terminus and the Val241-Lys251 (the CNBr fragment created by cleavage at Met240) (Fig. 3). The Met240-Lys259 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 that was evident in the first 17 cycles of sequence from the CNBr fragments of the mutant protein. These amino acids correspond to the C-terminal 42 residues as indicated by 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 6 residues correspond to the Gly232-Lys237 portion of the missense peptide predicted to follow Met231 in the mutant protein. However, the next 11 residues correspond to the Gln238-Glu248 sequence of the normal wild-type hCA-II. These findings indicate that the reading frame was restored by a translational frameshift after codon 237. The fact that this sequence extends beyond Met240 indicates that the CNBr cleavage at Met240 had been incomplete.

These results from microsequencing the CNBr fragments of the wild-type and mutant hCA-IIIs 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 that 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 adenosine deletion in codon 227 (a +1 frameshift) and a guanosine 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—

![FIG. 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 of protein were examined by SDS/PAGE and Coomassie blue staining (A) and, in parallel, by Western blot analysis with anti-hCA-II (B). The apparent molecular masses of the polypeptides are indicated.](image-url)
namely, a specific activity of ~550 EU/mg and reactivity with antibody to the 11-amino acid frameshift peptide (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 (Asn227-Lys237, Fig. 3) present in the Hispanic mutant protein. The synthetic Asn227-Lys237 frameshift peptide used to immunize the rabbits was conjugated to thyroglobulin. The antibody was affinity-purified on immobilized Asn227-Lys237 peptide conjugated to BSA and used to identify CAs containing the corresponding 11-amino acid peptide. Fig. 4 compares the reactivities of this reagent with those of two other polyclonal antisera, one to the native normal hCA-II and the other to the synthetic C-terminal 13-amino acid peptide of normal CA-II. The three antibodies are compared for reactivity to the following three antigens, all expressed in bacteria: (i) the wild-type 29-kDa CA-II, (ii) the 29-kDa protein produced by the double-frameshift cDNA described above, and (iii) the 27-kDa truncated Hispanic mutant protein (the predominant protein expressed in bacteria from the Hispanic mutant cDNA). Fig. 4 shows that the antiserum to normal hCA-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 27-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 have reported (3) 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 of cell protein), applied the lysate to a sulfonamide inhibitor affinity column, and examined the proteins eluted from the column by SDS/PAGE and Western blot analyses. 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. Fig. 5A

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**Fig. 3.** C-terminal amino acid sequences of 29-kDa wild-type and Hispanic mutant CA-IIIs determined by microsequencing the CNBr fragments. The unseparated CNBr fragments were subjected to N-terminal sequencing. The first 20 sequencing cycles from the wild-type CNBr fragments included amino acids Ser2-Pro21 from the N terminus (not shown) and Val241-Phc259 (solid underline). The amino acids in the 20 sequencing cycles from the mutant CNBr fragments included Ser2-Pro21 from the N terminus (not shown), Val241-Lys257 (solid underline), and a new sequence, Gln232-Gln248 (dotted underline), 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. The open and solid bars at amino acid 237 of the mutant proteins show the reading frames (K) and glutamic acid (E), respectively, which indicate the point of the ribosomal frameshift (indicated by the stepdown in the dotted line under the sequence of the mutant peptide).

**Fig. 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); 2, affinity-purified double-frameshift mutant protein (0.5 μg); 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, anti-hCA-II C-terminal 13-amino acid peptide, and anti-Hispanic frameshift peptide antiserum, as indicated.

**Fig. 5.** Immunochromatic characterization using anti-hCA-II antiserum and anti-Hispanic frameshift (FS) peptide antibodies. Lanes: 1, affinity-purified wild-type CA-II expressed in COS cells (30 ng); 2, total homogenate of *E. coli* expressing the 27-kDa Hispanic mutant protein as 10% of the total protein (300 ng); 3, proteins retained on and eluted from an affinity column to which 200 mg of lysate protein from COS cells transfected with vector containing the Hispanic mutant CA-II cDNA was applied; 4, proteins retained on and eluted from an affinity column to which 200 mg of lysate protein from COS cells transfected with vector only was applied.
shows that the anti-hCA-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 that 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 crossreactive species that were contained in the column eluates from COS cell lysates.)

The affinity-purified antibody to the Hispanic 11-amino acid frameshift peptide (anti-Hispanic FS peptide) does not react with normal hCA-II (lane 1), reacts 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 mechanism 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 that 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 hCA-II and is most likely identical to normal hCA-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-aminoc acid missense peptide is present in 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 with the frameshift peptide-specific antibodies on Western blots.

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" [e.g., a secondary structure such as a stem–loop or pseudoknot in the mRNA downstream from the shifty site (17, 18)]. However, the Hispanic mutant CA-II mRNA has no obvious string of four or more single-base repeats but 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 codon 237 did not depend on the UGA at codon 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.

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