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Genetics

Mutation creates an open reading frame within the 5′ untranslated region of macaque erythrocyte carbonic anhydrase (CA) I mRNA that suppresses CA I expression and supports the scanning model for translation

(*Macaca nemestrina*/reinitiation/carbonic anhydrase I deficiency/upstream open reading frame/translational regulation)

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ABSTRACT **A variant allele at the CA I locus that produces a deficiency of erythrocyte-specific CA I occurs as a widespread polymorphism in pigtail macaques from southeast Asia. Sequence analyses revealed a C → G substitution 12 nucleotides downstream of the cap site in the variant erythrocyte CA I mRNA. This mutation forms a new AUG start site and an open reading frame coding for 26 amino acids that terminates 6 nucleotides before the normal AUG initiation codon for CA I. It appears that the presence of this upstream open reading frame greatly diminishes reinitiation of translation from the normal start site, resulting in trace levels of CA I in erythrocytes. Preferential use of the first AUG codon supports the scanning model for translation initiation in eukaryotes.**

Open reading frames (ORFs) upstream of the normal AUG start codon occur only rarely in eukaryotic transcripts (1, 2) and usually have the effect of inhibiting the translation of the normal downstream ORFs (3). As yet no inherited deficiency of a mammalian enzyme has been attributed to a mutation that produces an upstream ORF. We describe here a naturally occurring base-change mutation that produces an upstream ORF in the erythrocyte CA I mRNA of the pigtail macaque *Macaca nemestrina*. This mutation results in the reduction (≈5000-fold) to trace levels of erythrocyte CA I (4, 5), suggesting that reinitiation at the normal start site occurs only infrequently, producing the CA I-deficiency phenotype. These findings are in agreement with the scanning model for initiation of translation in eukaryotes. This model has been thoroughly tested by *in vitro* mutagenesis experiments and predicts that translation will begin at the first AUG codon downstream of the cap site that is in a good sequence context; a purine at position −3 from the AUG is usually adequate for initiation (6–8).

Eight CA or CA-related genes are known in mammals (cf. refs. 9 and 10). The CA I gene is unique, however, in that it is under the control of two promoter regions (10–12). It appears that one promoter is essentially erythrocyte-specific, whereas the other controls CA I expression in the colonic mucosa and probably other tissues that normally express CA I (10, 11, 13). The erythrocyte CA I deficiency allele we have characterized is common in populations of pigtail macaques from southeast Asia where its frequency ranges from 0.55 in Thailand and 0.57 in Malaysia to 0.70 on the island of Sumatra (4, 14, 15). The finding of trace levels of CA I in erythrocytes and seemingly normal levels in the colonic mucosa of individuals homozygous for the erythrocyte CA I-deficiency allele suggested that the mutation was located either in an erythroid-specific promoter element or in the untranslated exon 1a that is unique to the seemingly specific CA I mRNA expressed in erythrocytes (10–12).

MATERIALS AND METHODS

PCR Amplification and Sequencing of the Amplified DNA. The PCRs were performed according to the protocol supplied with the Perkin–Elmer/Cetus PCR kit, with the only exception being a primer concentration of 0.1 μg/100 μl. Thermal cycling was performed in a Bellco DNA PaCeR for 30 cycles with the following parameters: 94°C for 15 s, 54°C for 15 s, and 71°C for 1.5 min. Sequencing was carried out by using the PCR to amplify all eight exons of the CA I gene. The gene fragments were electrophoresed on a polyacrylamide gel with 1× TBE (90 mM Tris, pH 8.3/90 mM borate/2 mM EDTA). The ethidium bromide-stained bands were cut from the gel, eluted into 0.5 M ammonium acetate by passive diffusion, followed by ethanol precipitation. The fragments were directly sequenced as double-stranded material. It is necessary to use a 360-nm light source to visualize the bands, as the use of a 300-nm light source results in unreadable sequencing ladders. The primers that were used are listed in Table 1. The primers were annealed to the template by boiling for 3 min in the presence of 0.5% Nonidet P-40 (16). The DNA was immediately snap-cooled in a dry-ice/ethanol slurry and then allowed to thaw on wet ice for 10 min. The DNA was then sequenced according to the protocol supplied with the Sequenase kit (United States Biochemical). The sequence is numbered from the translational start site.

Quantification of CA I mRNA. mRNA was quantified according to a modified "PATTY" protocol (17). Since no blood samples were available, 100 mg of spleen from normal and erythrocyte CA I-deficient animals were used to prepare RNA by the use of RNAzol (Cinna/Biotecx Laboratories, Friendswood, TX). First-strand cDNA synthesis, primed with the $stop_U$ primer (Table 1), was performed in a volume of 50 μl. The cDNA was used as a template with the $EX4\text{-}HI_D$ (the *Bam*HI mutagenesis primer) and $stop_U$ primers to introduce a *Bam*HI site (Fig. 1). A cloned CA I cDNA described below was used with the $H\text{-}ATC_D$ and $EX6_U$ primers to amplify the 5′ part of CA I cDNA. These two fragments were cut with *Eco*RI [at position 372, 12 base pairs (bp) upstream

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Abbreviations: ORF, open reading frame; CA, carbonic anhydrase.

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Table 1. Primers used for PCR amplification

Primer	Sequence	Complementary to positions	Mutation
-96_D	5′-GCTTGTGGTATCCAGTCCTCAGGTG	−96 to −71	None
ATG_D	5′-ATGGCAAGTCCAGACTGGGGA	1 to 21	None
$stop_U$	5′-GGGCTGTGTTCTTGAGGAAGG	828 to 808	None
$stop2_U$	5′-AGTTTCTTCTCCGACTCATC	804 to 785	None
$EX4\text{-}HI_D$	5′-CACTGGAATTCTGCAAAAGGATCCAG	367 to 392	*Bam*HI at position 372
$EX6_U$	5′-GTCAGAGAGCCAGGGTAGGTCCA	599 to 577	None
$anchorC_D$	5′-GGTCCAAATGCGTACGTGCCCCCCCCCCCC	—	None
$anchor_D$	5′-GGTCCAAATGCGTACGTG	—	None
ery_U	5′-ACTTCCGAATGAGAGAAGGC	−44 to −25	None
-25_U	5′-ACTTCCGAATGAGAGAAGGC	−25 to 44	None
$H\text{-}ATC_D$	5′-AAAAAGCTTGTGGTATCCAGTCCTCAGGTG	−101 to −72	*
$H\text{-}ATG_D$	5′-AAAAAGCTTGTGGTATGCAGTCCTCAGGTG	−101 to −72	**
8_U	5′-CTTGCCATTGTCTTGTACTGA	8 to −13	G → C at position −7
-22_D	5′-AAAGAAAACTCAGTACAAGACAATGGCAAG	−22 to 8	G → C at position −7
M13 rev	5′-AACAGCTATGACCATG	M13 polylinker	
M13-20	5′-GTAAAACGACGGCCAGT	M13 polylinker	

Subscript D indicates a downstream-directed primer, and a subscript U indicates an upstream-directed primer. *, Cytidine at position −85 as in the normal transcript; **, guanosine at position −85 as in the erythrocyte CA I-deficient transcript.

of the newly created *Bam*HI site] and gel-purified, and the 5′ and 3′ fragments were then ligated and amplified with the $H\text{-}ATC_D$ and $-stop_U$ primers. A portion was reamplified with the $H\text{-}ATC_D$ and $stop2_U$ primers. This results in a CA I cDNA with a *Bam*HI site between nucleotide positions 304 and 309. Different amounts of this mutated cDNA were added to a constant amount of cDNA from normal and erythrocyte CA I-deficient spleen mRNA. To analyze the erythroid-specific mRNA (containing exon 1a), the $H\text{-}ATG_D$ and $stop2_U$ primers were used, and the ATG_D and $stop2_U$ primers were used for the total level of CA I. In both cases the first PCR was followed by a second hemi-nested amplification with $EX6_U$ as the upstream-directed primer. Five units of *Bam*HI were added directly to the PCR mixtures. After 1 h at 37°C, 5 μl of the reaction mixture was electrophoresed on a 1% agarose gel. Several amplifications were made from different animals to ensure the consistency of the results.

***In Vitro* Translation.** The $H\text{-}ATC_D$ primers were used with the stop primer to amplify cDNA in two separate reaction mixtures (Fig. 2). After reamplification with the $H\text{-}ATC_D$ or $H\text{-}ATG_D$ and $EX6_U$ primers, the reaction products were cut with *Hin*dIII and *Eco*RI and cloned into pBSM13−.

To remove the UAG translational stop codon and to introduce the T3 RNA polymerase promoter upstream of the cap site, the pBSM13− clones were amplified with the M13 rev and 8_U primers. In parallel, cDNA was amplified with -22_D and stop primers. Portions of the amplified DNA were mixed and amplified with the M13 rev and $stop2_U$ primers. The mutated 5′ part of the cDNAs were in all instances sequenced twice. The PCR constructs were precipitated, dissolved in water, and added to 20 μl of T3 RNA polymerase reaction mixture, with [^{3}H]CTP and 7-methylguanosine(5′)triphospho(5′)guanosine to produce capped mRNA. The DNA was removed with DNase I and the amount of RNA was quantified by scintillation counting. The RNA (150 ng) was added to a reticulocyte lysate *in vitro* translation system (BRL, 8125SA, minus methionine) containing 5 μCi of [^{35}S]methionine (1 Ci = 37 GBq), 2.2 mM Mg^{2+}, 45 mM KCl, and 90 mM KOAc in a total volume of 30 μl. After 60 min at 30°C, 10 μg of RNase was added and the RNA was digested at 30°C for 15 min. Lysate (5 μl) was then mixed with 5 μl of SDS buffer and electrophoresed on a SDS/12.5% polyacrylamide gel (18). The gel was prepared for fluorography by soaking in 1 M sodium salicylate for 30 min (19). The gel was then dried and exposed to a Kodak XAR film for 3 days.

Determination of the Cap Site. The general method used has been described by Loh *et al.* (20). RNA was prepared as described under mRNA quantification. Total RNA (10 μg) was reversed-transcribed into single-stranded cDNA, which was dG-tailed with terminal deoxynucleotidyltransferase in 1 mM dGTP and 0.75 mM $CoCl_2$ (21). The dG-tailed cDNA was used as template in a PCR with the $anchorC_D$ and stop primers. The reaction was reamplified with the anchor and $EX6_U$ primers. To specifically amplify the erythrocyte CA I mRNA, a second reamplification with the $anchor_D$ and ery_U primers was performed. The PCR product of the latter

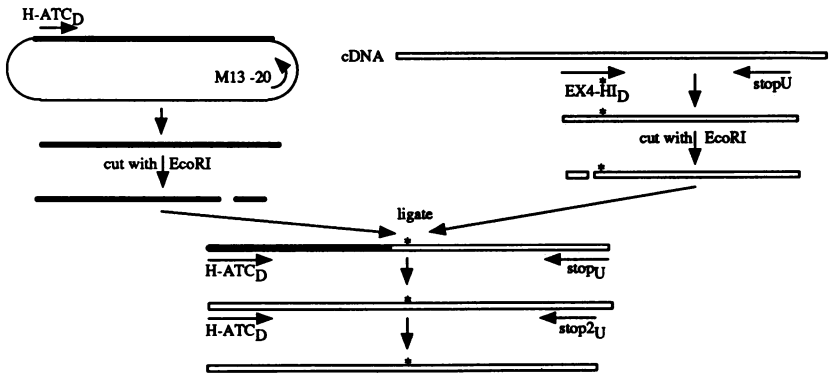

FIG. 1. Preparation of construct for mRNA quantitation. The M13 clone in the upper left is described in text. The asterisk indicates the position of the introduced *Bam*HI restriction site, which lies next to the *Eco*RI site. The final PCR products span the region from the cap site to 17 bp downstream of the UAG stop codon.

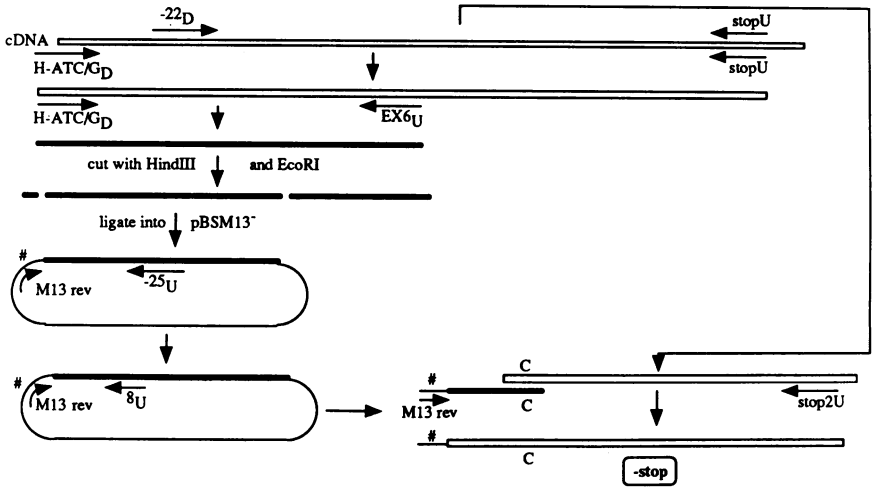

FIG. 2. Preparation of constructs for mRNA translation. #, T3 RNA polymerase promoter; −stop, construct with the UAG stop codon at positions −9 to −7 mutated to UAC. Two constructs are synthesized with a guanosine or cytidine at position −85. Due to the T3 promoter, the transcribed RNA will have the sequence m^7GpppGGGAACAAAA preceding the natural cap site, where m^7GpppG is 7-methylguanosine(5′)triphospho(5′)guanosine.

reaction was purified by PAGE on a 6% gel and sequenced with the ery_U primer. Primer extension was performed as described (22). ^{32}P-labeled ery_U primer (1×10^5 dpm) was annealed to 50 μg of total spleen RNA at 25°C overnight.

RESULTS

Characterization of the Mutant CA I. To determine the cause of this erythrocyte-specific deficiency, the PCR methodology was used to amplify and directly sequence the seven coding exons of CA I, the upstream noncoding exon 1a and the exon–intron boundaries (13, 23). In this way, we sequenced the CA I genes from a homozygous normal and erythrocyte CA I-deficient animal. The only difference between the normal and the erythrocyte CA I-deficient mRNAs was the presence in the mutant mRNA of a translational AUG start codon 87 bp upstream of the normal start site (Fig. 3). The cap site was shown to be 9 bp upstream of the mutant AUG start codon (Fig. 4). This start codon is in-phase with a UAG stop codon 9 bp upstream from the normal AUG start codon. Because this mutation is present only in the erythrocyte CA I mRNA (Fig. 3), it will not affect the expression of CA I in any tissue where the nonerythroid (e.g., colon)

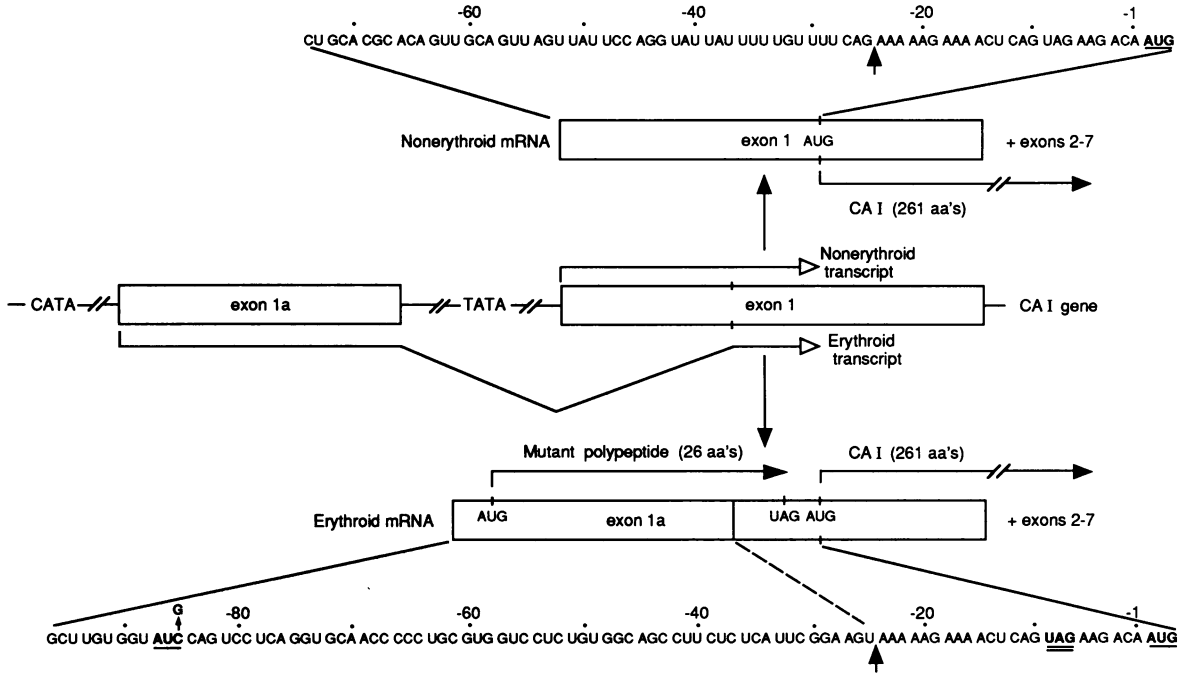

FIG. 3. Diagram of the 5′ end of the pigtail macaque CA I gene and the two mRNAs (erythroid and nonerythroid) produced by the two promoters. The proposed upstream ORF formed by the C → G mutation at position −85 is shown along with the normal erythroid CA I mRNA. Sequences of the 5′ ends (cap sites to AUG start) of the erythroid and nonerythroid mRNAs for CA I found in erythrocytes and colon, respectively, in pigtail macaques are shown. The TATA boxes purportedly used for the erythroid and nonerythroid CA I mRNA expression are shown preceding the respective cap sites. The C → G mutation at position −85 is shown in the erythroid CA I mRNA. Transcription of the 5′ part of the mRNA from the colon promoter begins at the 5′ end of exon 1, and transcription from the erythroid promoter includes exon 1a, which is spliced into exon 1 at the position indicated by the vertical arrows. The double underlined UAG stop codon was changed to UAC in two constructs (Fig. 2) used in the *in vitro* translations (see Fig. 6). The mutant and normal AUG start sites are underlined.

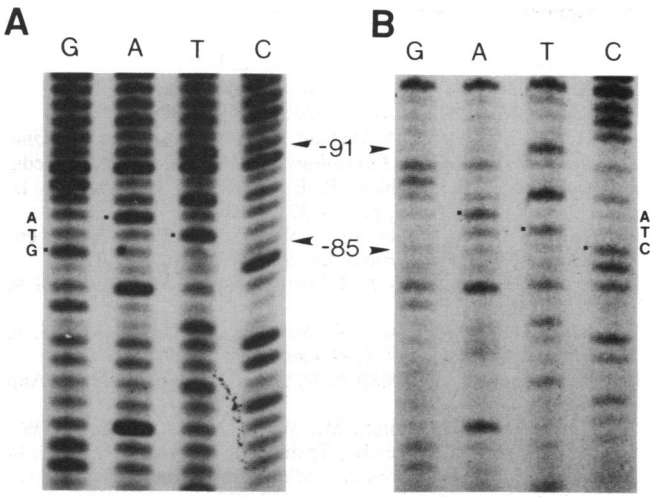

FIG. 4. Determination of the cap site. (*A*) Erythrocyte CA I-deficient. (*B*) Normal. At position −85 there is a cytidine in the normal mRNA and a guanosine in the erythrocyte CA I-deficient mRNA. The marked sequence reads upstream toward the cap site; it is readable to position −91, indicating that the 5′ end of the mRNA is at least this far upstream. Primer extension (data not shown) indicates the cap site is at position −96.

promoter is used. Exon 1a was sequenced from two homozygous-deficient, one homozygous-normal, and two heterozygous animals. In all of these cases, a guanosine at position −85 (AUG) was associated with the deficiency.

Quantitation of CA I mRNA. As shown in Fig. 3, the CA I mRNA in colon and erythroid cells differ in their 5′ untranslated ends due to the use of two different promoters (10–12). The levels of CA I mRNA expressed as the relative amounts of the erythroid mRNA/total CA I mRNA were the same in the spleen of normal and erythrocyte CA I-deficient macaques (Fig. 5).

***In Vitro* Translation.** To prove that the mutant upstream translational start site is used, cDNA sequences from normal and mutant mRNAs were first cloned into pBSM13−, PCR-amplified, and then transcribed and translated *in vitro* (Fig. 6). Because the amplified constructs included the T3 promoter and the *Hin*dIII site of pBSM13−, the transcripts have

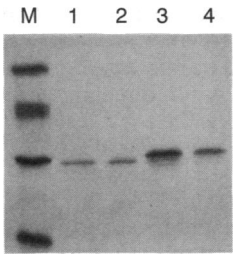

FIG. 6. *In vitro* translation of CA I mRNAs in reticulocyte lysates with upstream nucleotides at positions −7 to −9 changed from UAG (stop) to UAC (tyrosine). Lanes: 1 and 2, cytidine at position −85 and cytidine at position −7; 3 and 4, guanosine at position −85 and cytidine at position −7. The reaction mixture contained 150 ng of RNA and 2.2 mM Mg^{2+}. Note increased size of mutant protein in lanes 3 and 4 (289 residues) compared to normal CA I in lanes 1 and 2 (260 residues). Molecular mass markers (Sigma) in kDa (lane M), from the bottom, are as follows: 14.3, 18.4, 29.0 (bovine CA II), and 45.0.

the sequence 5′-GGGAACAAAA preceding the natural cap site. Also, since this added sequence does not include an AUG and since translation can be initiated as close as 3–5 bp from the cap site (24, 25), we feel that this extra sequence does not change the initiation properties of the mRNAs. Two constructs were transcribed, a normal mRNA and a deficient mRNA where the UAG stop codon between the two start site was removed (Fig. 3). These constructs were made because of the difficulty in detecting a 26-amino acid polypeptide. With the stop codon removed, the new start site, if used, will translate into a protein that is 29 amino acids longer than the normal CA I (i.e., 260 + 29 amino acids) and can easily be detected by SDS/PAGE. As can be seen in Fig. 6, translation is initiated at the mutant translation start site.

DISCUSSION

Because we have demonstrated that the erythrocyte promoter sequence, the location of the cap sites, the sequences of all eight exons and their intron boundaries, and the levels of erythrocyte CA I mRNA are the same in normal and erythrocyte CA I-deficient animals, we conclude that the mutation responsible for the erythrocyte CA I deficiency is the formation of an upstream AUG initiation codon. It is

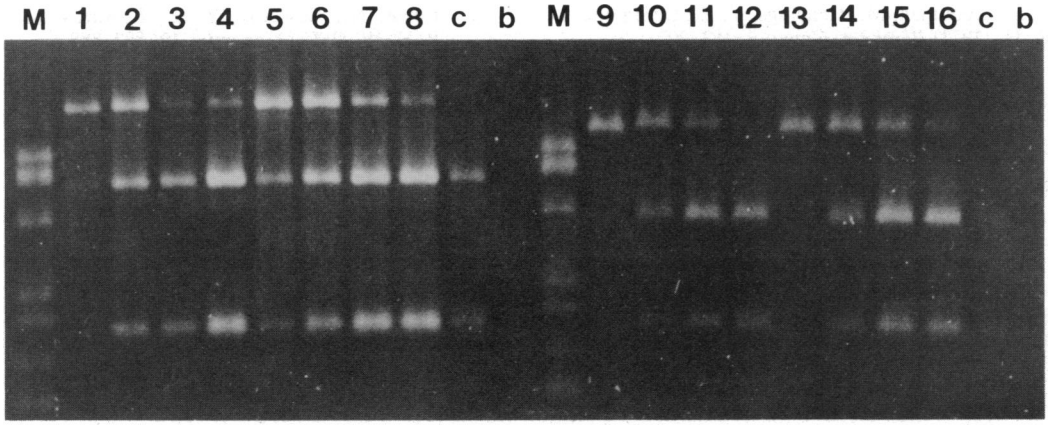

FIG. 5. Quantitation of CA I mRNA in spleen by a modification of the PATTY method (17). Lanes: 1–8, erythroid-specific CA I mRNA (using an exon 1a-specific -96_D primer); 9–16, total CA I mRNA (using the ATG_D and $stop_U$ primers common to both erythroid and nonerythroid mRNAs); 1–4 and 9–12, 1 μl of 1:50 diluted spleen cDNA from an erythrocyte CA I-deficient animal; 5–8 and 13–16, 1 μl of 1:125 diluted spleen cDNA from a normal animal. The amount of internal standard mRNA, in which a *Bam*HI site has been artificially created, is as follows. Lanes: 1, 5, 9, and 13, 5×10^{-18} g; 2, 6, 10, and 14, 2.5×10^{-17} g; 3, 7, 11, and 15, 1.3×10^{-16} g; 4, 8, 12, and 16, 6.3×10^{-16} g. Samples were digested with *Bam*HI after PCR amplification. Lanes: M, *Msp* I-digested pBSM13; b, no DNA template; c, 1.3×10^{-16} g of internal standard only. In samples 2, 6, 10, and 14, the internal standard is present in about the same amount as the endogeneous cDNA, showing that the relative levels of the erythroid-specific mRNA is the same in normal and erythrocyte CA I-deficient animals. Comparison of these lanes also shows that almost all of the CA I mRNA in spleen is transcribed from the erythroid-specific promoter.

possible that there may be a low level of expression from the nonerythrocyte (e.g., colon)-specific promoter in CA I-deficient erythrocytes. In this case reinitiation on the erythrocyte-specific mRNA (or direct internal ribosome binding) would be predicted to be even less frequent than 1 in 5000.

Although almost all cellular mRNAs use only one initiation and termination codon, there is now evidence that reinitiation can occur in some mRNAs (3, 26, 27). In general, the reinitiation is more efficient as the distance increases between the stop codon of the upstream ORF and the normal start site. Thus, the fact that only 6 nucleotides separate the termination of the upstream ORF and the normal AUG initiation further supports our conclusion that the mutation we have described is responsible for the erythrocyte CA I deficiency.

Even though CA I is the second most abundant nonglobin protein in most mammalian erythrocytes [e.g., 7–19 μg/mg of hemoglobulin in pigtail macaques (5)], its virtual absence in erythrocytes does not appear to be associated with any physiological abnormality. Inherited deficiencies of erythrocyte CA I in humans and pigtail macaques exhibit no detectable hematological or other clinical disorders (4, 5, 28, 29). Furthermore, CA I is characteristically absent in the erythrocytes of all ruminants (e.g., ox, goat, and sheep) and felids (e.g., cat, lion, and tiger) examined to date (cf. ref. 30).

An intriguing feature of the CA I deficiency mutation is a related cis-acting reduction of $\approx$60% in the levels of the other erythrocyte CA isozyme, CA II, in individuals homozygous for the CA I deficiency (5). If the order of the CA I, CA II, and CA III genes is the same in pigtail macaques as it is in humans (31), then the promoter regions of the human CA I and CA II genes are separated by $\approx$110 kilobases, with the CA III gene located between them (31). The basis for what seems to be a secondary effect on the expression of CA II by the CA I mutation would appear to be particularly worthwhile to investigate.

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