Organization of the Mouse and Human Carbonic Anhydrase II Genes[a]

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INTRODUCTION

The carbonic anhydrase (CA) multigene family is beginning to appear more complex than was considered possible during the earlier days of work on the different isozymes. The two classic erythrocyte isozymes found in amniotes, CA I and CA II, have been studied in considerable detail in terms of their kinetics, structures, and evolution.[1-7] Within the last few years, at least one other isozyme (CA III), which is coded by a separate locus, has been shown to exist.[7] In addition, membrane-bound carbonic anhydrases in brain,[8] lung,[9,10] and kidney,[11,12] and a soluble CA in mitochondria[13,14] have been characterized and may be the products of loci distinct from the genes coding for the CA I, CA II, and CA III isozymes. However, assignment of these CA isozymes to their respective loci has been a difficult task, complicated by the possibility that two or more genes can code for an identical protein product, as has been shown for human α-globin[15] and human placental lactogen,[16] or that one gene can code for different protein products, as in the case of the human calcitonin gene.[17,18] Clearly, determination of the number of expressed (as well as unexpressed) genes will require work at the DNA and RNA level.

The linkage relationships among the members of the CA multigene family are only partially known. In mice, the loci for CA I and CA II are tightly linked near the centromere of chromosome 3;[19] in humans, the CA II gene is present on chromosome 8.[20] The location of the human CA I gene is not yet known. However, additional linkage data from work on pigtail macaques[21] and guinea pigs[22] support the notion that CA I and CA II are linked in all mammals. No data are presently available concerning the location of the CA III gene in any species. If all the CA isozyme genes are indeed linked, they may constitute a complex regulatory system, the study of which could lead to insights into various levels of eukaryotic gene regulation.

Deficiencies of erythrocyte CA I and CA II have been found in humans. An inherited deficiency of CA I has been described in three members of a Greek family who, despite a virtually complete absence of the enzyme in erythrocytes,

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show no clinical manifestations.[23] On the other hand, humans with deficiency of red cell CA II suffer from a syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification.[24,25] While there are several genetically distinct forms of osteopetrosis in humans and other mammals,[26] the form caused by CA II deficiency has been found only in humans.[25] The molecular basis of the CA I and CA II deficiencies is unknown. As with the well-characterized globin system,[27] a fuller understanding of the genetic lesions that cause these deficiencies will require study of the genes at the DNA level.

As a first step to answering the questions raised above, we have set out to isolate the genes for the carbonic anhydrase isozymes. Here, we describe the isolation of clones containing mouse and human CA II sequences and the initial characterization of these clones.

MATERIALS AND METHODS

Mouse CA II cDNA Probe

The mouse CA II clone, pMCAII, (kindly provided by Dr. P. J. Curtis) was isolated from a cDNA library derived from splenic mRNA of an anemic Balb/c mouse.[28,46] The nearly full-length 1.5-kb insert was purified from the vector as previously described by centrifugation through a sucrose gradient and this insert was then nick-translated to $1–2 \times 10^8$ cpm/μg.[20] Probes representing the 5′, middle, and 3′ regions of the cDNA were derived from the *Pst* I fragments of pMCAII (see FIG. 1).

Cosmid and Lambda Libraries

The mouse lambda and cosmid libraries were constructed as described.[55] Briefly, high molecular weight DNA was prepared from the livers of YBR mice, partially digested with *Mbo* I, size-selected on a sucrose gradient,[56] and ligated into the *Bam* HI site of either the lambda vector Charon 30[30] or pJB8[31,57] (a cosmid vector partially derived from pBR322 and kindly provided by R. W. Padgett). The pJB8 vector was prepared for ligation as described.[31] Recombinant molecules were packaged *in vitro* into lambda phage particles[58] and used to infect *E. coli* K803[61] for the lambda libraries and *E. coli* 490A[62] for the cosmid libraries. The phages were plated at 50,000 plaques per 150-mm plate and the cosmids at 10,000 colonies per 150-mm plate. Each library was large enough to ensure that at least

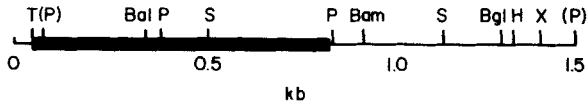

FIGURE 1. Restriction map of mouse CA II cDNA. This map was generated from two overlapping cDNA clones, one of which was pMCAII, which includes the entire sequence except for the 5′ untranslated region and the first eight amino acids of the coding region. Heavy line = translated region; thin line = untranslated regions. S = *Stu* I, P = *Pst* I, H = *Hind* III, X = *Xba* I, Bal = *Bal* I, Bam = *Bam* HI, T = *Tth* 111 I, and Bgl = *Bgl* II. (P) = the artificial *Pst* I sites generated during the cloning process in pMCAII.

one genomic equivalent was plated.[59] The DNA from colonies or plaques were fixed onto nitrocellulose filters and hybridized as described below.

The human genomic lambda library[32] was kindly provided by Dr. T. Maniatis.

Hybridizations and Southern Blots

After digestion with restriction enzymes and electrophoresis of 0.8% agarose gels, genomic DNA was transferred to nitrocellulose[33] and hybridized with the appropriate probe in 5XSSC (1XSSC = 15 m*M* sodium citrate, 0.15 *M* sodium chloride), 5X Denhardt's solution (1X = 0.02% each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin),[34] 10% dextran sulfate, 200 μg/ml denatured salmon testes DNA, and 35–50% formamide depending on the experiment. All hybridizations were performed at 42°C for 12–48 hr. Multiple washes (4–6) with 2XSSC, 0.1% SDS were done at 50°C sometimes followed by more stringent washes with 0.1XSSC, 0.1% SDS at 50°C. Hybridizations for screening of the mouse cosmid and lambda libraries were modified from previously existing protocols.[35,36,60] Hybridizations for the human lambda library were performed at reduced stringency to obtain better signals with the mouse probe.[20]

Isolation of Cloned DNAs

Cosmids and plasmids were isolated after amplification in L-broth[29] using the boiling method[37] followed by banding in CsCl-ethidium bromide gradients. Residual bacterial RNA was removed by chromatography on Sephacryl S-300.[38] Minipreparations were also performed using the boiling method. Lambda phase DNAs were isolated by standard techniques[29] after plaque purification. *Eco* RI fragments were subcloned using the plasmid vector pBR325[39,40] and *E. coli* DH1[41] as the host.

Restriction Mapping

Maps were constructed by performing single and double digestions for enzymes that cut only a few times and by partial digestion mapping for enzymes that cut many times.[42] Partial digestion mapping was carried out by first linearizing the cosmids by cutting with *Mlu* I, which has only one recognition site in pJB8 and none in any of the genomic DNA inserts. Following partial digestion with an appropriate second restriction enzyme (e.g., *Eco* RI), the DNA was run on a 0.8% agarose gel and blotted onto nitrocellulose. After hybridization with a probe that binds with only vector sequences to the left or right side of the cloning site (i.e., the *Eco* RI–*Bam* HI or *Eco* RI–*Pst* I fragment of pBR322, respectively), the blot was washed and autoradiographed. The banding pattern thus displayed the direct order and spacing of the restriction sites for the second enzyme from either end of the insert depending on which probe was used.

DNA Sequencing

DNA fragments were labeled at the 5′ ends using polynucleotide kinase, digested with a second enzyme, and separated by polyacrylamide gel electrophore-

sis. Bands were located by alignment with Polaroid Type 52 or 57 film that had been exposed to the gel for several minutes, cut from the gel, and eluted.[43] Fragments were subjected to the chemical degradation methods of Maxam and Gilbert,[43] electrophoresed in thin 8 *M* urea-containing polyacrylamide gels, and exposed to Kodak AR-5 film with Dupont Cronex Lightning Plus intensifying screens.[44]

RESULTS

Southern Blot Analysis of the Mouse CA II Genomic Sequences

A summary of the hybridizing bands seen on genomic blots of C57BL/6 DNA using pMCAII as the labeled probe is given in TABLE 1. A representative autoradiogram is shown in FIGURE 2. A comparison of C57BL/6 DNA with YBR DNA showed no discernible differences when using *Bam* HI, *Bgl* I, *Sph* I, or *Hind* III (data not shown). An apparent difference occurs with *Bgl*II as shown by a 4.4 kb fragment in C57BL/6 DNA (TABLE 1) instead of the 5.1 kb fragment found in cosmid clone A6 (TABLE 2).

Using the three *Pst* I fragments from pMCAII as probes, it was found that the 3.5-kb *Eco* RI and the 6-kb *Bam* HI bands contain the 3′ end of the gene, and the 2.7-kb *Eco* RI and the 15-kb *Bam* HI bands contain the 5′ portion of the gene.

A restriction map of the CA II gene region could not be constructed using these results plus data produced by digesting with two restriction enzymes, a method that has proven successful with some small genes such as rabbit β-globin.[45] It therefore seemed likely that the CA II gene was comparatively large, and that internal (i.e., intronic) nonhybridizing fragments were not detected, making map construction by this method unfeasible.

Mouse CA II Genomic Clones

Genomic sequences hybridizing to the labeled pMCAII probe were isolated from both cosmid and lambda genomic libraries. Four cosmid clones, A5, A6, 54, and 103, and several lambda clones were found. All of the cosmid and four of the lambda clones, 8.1, 8.2, 9.1, and 15.1, were studied in some detail.

Cosmid Clone A6

A complete mouse CA II gene is contained within this cosmid clone as shown by the correspondence of the cosmid A6 hybridizing bands with those seen on

TABLE 1. Restriction Fragment Sizes of Mouse CA II Hybridizing Bands from Genomic Blots[a]

Eco RI	*Bam* HI	*Hind* III	*Bgl* II
7.2	15	12	4.4
3.5	6.2	4.2	3.1
2.7		2.2	2.9
		0.6	2.8
			1.4
			1.2

[a] Fragment sizes are given in kilobase pairs.

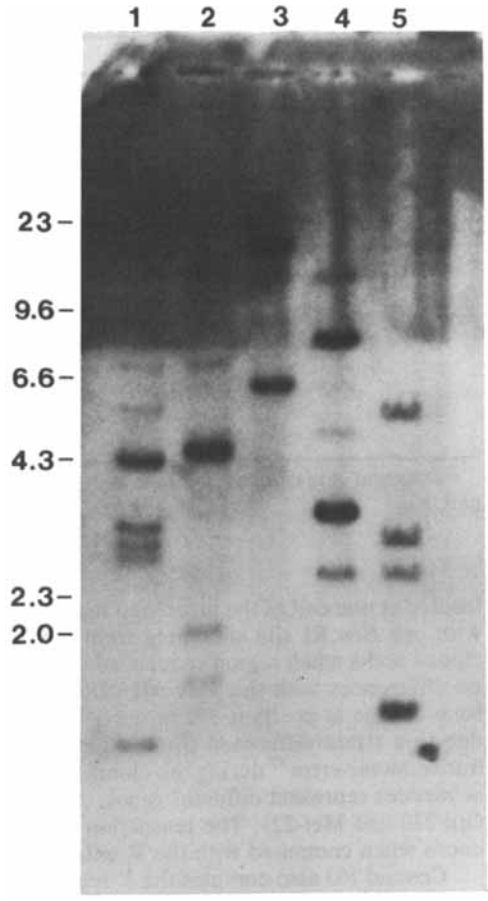

FIGURE 2. A representative autoradiogram used in genomic blot analysis of mouse CA II gene sequences. The lanes are: (1) *Bgl* II; (2) *Hind* III; (3) *Bam* HI; (4) *Eco* RI; and (5) *Xba* I. Bands smaller than 1.4 kb were lost off the end of this particular gel. The DNA in this gel is from mouse stain C57B1/6. This gel was hybridized under reduced stringency (40% formamide) and shows bands that are not present under more stringent conditions in addition to those listed in TABLE 1. Molecular weights on the left side of the figure are in kilobase pairs.

genomic blots (TABLES 1 and 2). A restriction map of this clone is shown in FIGURE 3. This map shows no inconsistencies with the data derived from the genomic blots. The 38-kb-long genomic fragment contains, in addition to the 16-kb-long gene, 12-kb of 5′ flanking region and 10-kb of 3′ flanking region. One intervening sequence is over 5-kb in length. From the presence of 6 *Bgl* II bands hybridizing to pMCAII, and the fact that pMCAII has only one *Bgl* II site, it can be deduced that there are at least 5 exons.

Cosmids Clones A5, 54, and 103

Cosmid A5 contains the 3′ end of a CA II gene as shown by the 3.5-kb *Eco* RI band that hybridizes with pMCAII. No other hybridizing regions are present. The *Eco* RI restriction pattern differs from that of the other cosmids (TABLE 2), although a few of the bands are of a similar size to those of the other cosmids.

Cosmid 54 also contains the 3′ end of a CA II gene. The *Eco* RI restriction fragment sizes of the clone are given in TABLE 2. The 2.5-kb *Eco* RI fragment is

TABLE 2. Restriction Fragment Sizes of Mouse Cosmid Clones[a]

A6				54	103	A5
Eco RI	*Bam* HI	*Hind* III	*Bgl* II	*Eco* RI	*Eco* RI	*Eco* RI
7.2*	25	12.0*	18	8.9	8.7	8.3
7.0	15.0*	9.1	5.1*	7.2	5.4	6.9
5.4	6.2*	8.3	3.1*	5.4	5.1	5.4
5.0	0.7	6.0	2.9*	4.2	4.4	4.4
4.2		4.2*	2.8*	3.8	4.0	4.2
3.5*		2.2*	2.0	2.5*	3.3	3.5*
3.4		0.9	1.8	1.8	2.0*	2.8
2.8		0.9	1.6	1.8	1.8*	2.7
2.7*		0.9*	1.5	1.5	1.4	2.1
2.0		0.6	1.4*	1.2	1.0	1.8
1.0			1.3	1.0	0.9	1.5
			1.2*	0.9	0.4	1.4
				0.8	0.2	0.4
				0.4	0.1	0.3

[a] Fragment sizes are in kilobase pairs and an asterisk indicates hybridization with pMCAII.

located at one end of the insert and represents a truncated 3.5-kb *Eco* RI fragment with one *Eco* RI site stemming from the vector. The 2.5-kb fragment was subcloned and a small region sequenced (FIG. 4). This preliminary sequence showed no differences with the pMCAII cDNA sequence[46] (FIG. 4) except for a single base change at position 870 in the 3′ untranslated region. This change might be due to a strain difference (Balb/c for pMCAII, YBR for cosmid 54), a reverse transcriptase error[47] during the cloning of the cDNA, or the possibility that these sequences represent different genes. An intron-exon junction was found between Gln-220 and Met-221. The restriction map of the exonic region shows no differences when compared with the 3′ untranslated region found in pMCAII (FIG. 5).

Cosmid 103 also contains the 3′ region of a CA II gene. The *Eco* RI restriction fragment sizes are given in TABLE 2. The fragments were ordered by partial digestion mapping (data not shown). The restriction pattern of 103 is quite different from cosmids 54 and A5 that also contain 3′ hybridizing regions. FIGURE 5 shows the relative restriction maps of the subclones of hybridizing fragments from

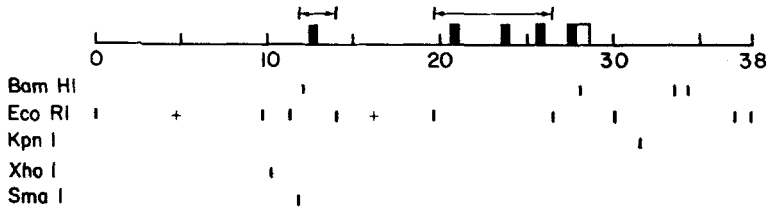

FIGURE 3. Restriction map of the cosmid clone A6. Plus marks indicate additional fragments that have not been mapped. The bars and arrows indicate the approximate location of the exons. The open box is the 3′ untranslated region. The minimum number of exons is shown.

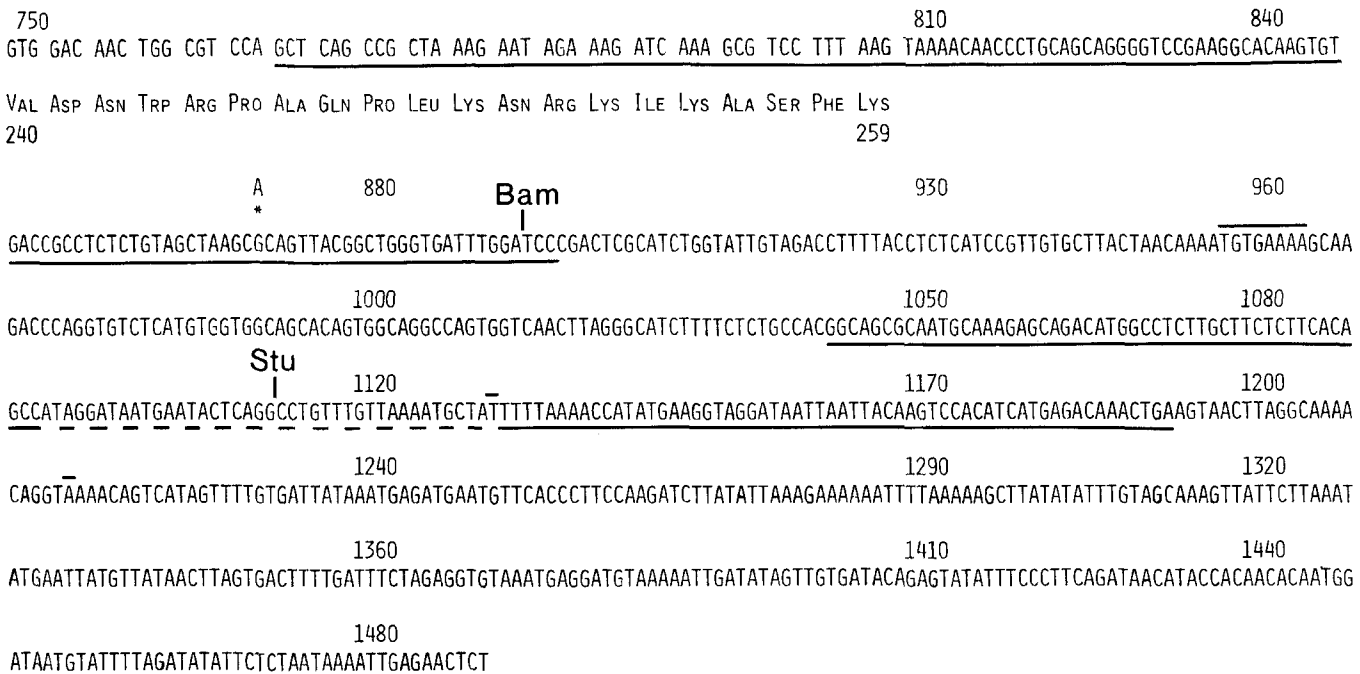

FIGURE 4. Nucleotide sequence of the 3′ half of pMCAII[46] and the corresponding regions of cosmid clones 54 and 103. The underlined region from the *Stu* I site is exactly the same sequence derived from cosmid clone 103. The underlined region from the *Bam* HI site is the same sequence derived from cosmid clone 54 with the exception of the single nucleotide difference shown at the asterisk (G is in the cDNA sequence). Overlined regions represent sequences present in cosmid 54 and the cDNA, but omitted from the original cDNA sequence.[46]

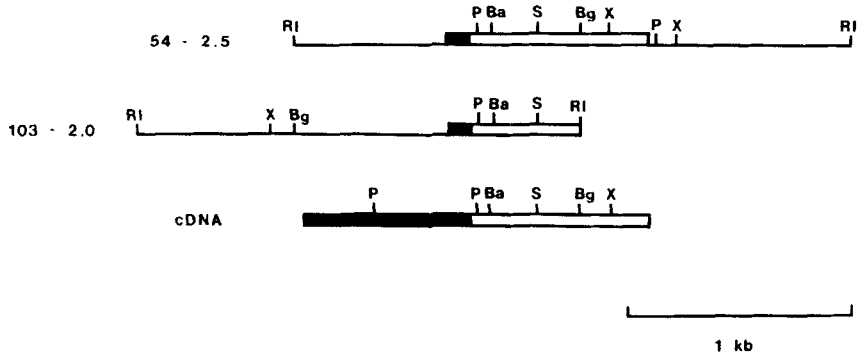

FIGURE 5. Comparison of the restriction maps of two cosmid subclones with the restriction map of the mouse CA II cDNA. The two subclones map at the end of the genomic inserts of the respective cosmid clones. Thus, the *Eco* RI site at the 5′ end of subclone 54-2.5 and the *Eco* RI site at the 3′ end of subclone 103-2.0 are artificial sites derived from pJB8.[31] The thick bars represent structural gene regions (i.e., those that are present in the cDNA) and the black bars represent coding sequence. Note that the restriction sites are the same in these regions between the cosmid subclones and the cDNA. Restriction site designations are as in FIGURE 1.

two of the three cosmids. Sequences surrounding the *Stu* I site in the subclone from cosmid 103 show that this site is homologous to the site in pMCAII (see FIG. 4).

Mouse CA II Lambda Clones

Four lambda clones were studied in some depth. The sizes of the fragments produced by digestion with *Eco* RI are given in TABLE 3. Clone 8.2 contains the 5′ end of a CA II gene and clones 8.1, 9.1, and 15.1 all contain 3′ ends as determined by hybridization with the 5′ and 3′ specific probes. The restriction map of subclone 8.2–2.7 (derived from clone 8.2 and containing the 2.7-kb *Eco* RI hybridizing fragment) is given in FIGURE 6. From the cDNA restriction map, it is known that a *Tth* 111 I site is located at the start codon of the mouse CA II cDNA (see

TABLE 3. *Eco* RI Fragment Sizes for Mouse Lambda Clones[a]

Clone			
8.1	8.2	9.1	15.1
22.0∠	22.0∠	22.0∠	22.0∠
10.0*∠	10.0∠	11.0*∠	10.0*∠
7.0	4.9	7.0	7.0
3.5*	2.7*	4.2	3.5*
	1.9		
	0.8		

[a] Fragment sizes are given in kilobase pairs. ∠ hybridizes to Charon 30; * hybridizes to pMCAII.

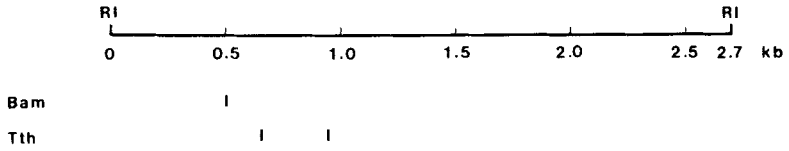

FIGURE 6. Restriction map of mouse lambda subclone 8.2-2.7. This subclone contains the 5′ region of the mouse CA II gene.

FIG. 1). Sequencing from the *Tth* 111 I site proximal to the *Bam* HI site revealed no homology with the sequence of the mouse CA II cDNA near the *Tth* 111 I site. This strongly suggests that the start codon is located at the *Tth* 111 I site distal to the *Bam* HI site.

Human CA II Lambda Clones

Several human lambda clones containing CA II sequences were isolated and identified by hybridization with pMCAII under low stringency conditions (i.e., 35% formamide). These were designated H10, H11, H17, H23, H24, and H25; clones H10, H11, and H25 were studied in some detail. The *Eco* RI restriction band sizes are given in TABLE 4 and the hybridizing bands are indicated. The most intense hybridization occurred with the 3.8-kb band in H25 that was subcloned for further study. The restriction maps of H25 and this subclone, H25-3.8, are shown in FIGURE 7. DNA sequencing from one of the *Bam* HI sites revealed part of the coding region for a CA II gene as shown in FIGURE 8 along with the homologous region from the mouse gene. The sequence codes for amino acids from approximately 58 to 77 and agrees exactly with the known amino acid sequence.[48,49] To the 3′ side of the first nucleotide of the codon for Val-77 is the beginning of an intervening sequence. The beginning of the next exon was not found within the 50 nucleotides sequenced downstream from position 77. There is good agreement between the donor splice site found in H25-3.8 and the consensus donor splice site sequence.[50] This subcloned fragment contains no repetitive elements as defined by genomic blot analysis using the subclone as a probe.

TABLE 4. *Eco* RI Restriction Fragments of Human CA II Clones[a]

Clone		
H10	H11	H25
7.6*	8.7*	5.2
4.3	3.0	3.8**
3.0	0.4	2.1
		1.8
		1.7
		0.6

[a] Fragment sizes are in kilobase pairs. The single asterisk indicates hybridization to the middle *Pst* I fragment of pMCAII. The double asterisk indicates hybridization to the 5′ *Pst* I fragment of pMCAII. Charon 4A arms are not included in the table.

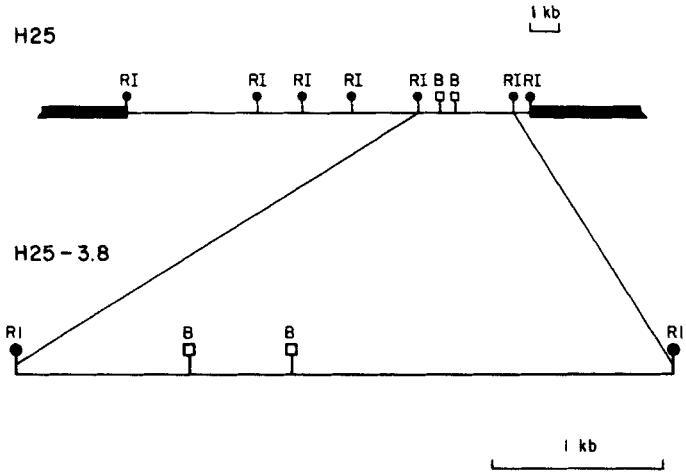

FIGURE 7. Restriction map of human CA II genomic clone H25 and subclone H25-3.8. The subclone hybridizes with the 5′ probe derived from pMCAII.

DISCUSSION

One of the interesting results of the present study is that there may exist more than one CA II gene as indicated by the nonhomologous 3′ regions of cosmids A5, A6, 54, and 103. Since there is always a possibility of generating artifacts during the cloning process, it is necessary to remain cautious in the interpretation of these data until a more extensive analysis has been carried out. Several lines of reasoning argue against cloning artifacts. Rearrangements after cloning are one source of artifact. This would seem unlikely in the present case because very few of the restriction fragments are of equivalent size between the three cosmid clones that contain only the 3′ hybridizing regions. Massive rearrangement of each clone would have had to occur for this to happen. In addition, such rearrangements would in all likelihood also produce deletions. This does not appear to be the case here since all the clones are large and in the size range of normal cosmid clones.

The incorporation of two or more pieces of noncontiguous genomic DNA into a single cosmid clone is another possibility. This seems unlikely since the DNA was size fractionated into pieces of 35–45 kb, the maximum size for a cosmid insert that still allows packaging of the entire recombinant cosmid into a lambda phage particle.

One interesting point is the presence of a 1.8-kb hybridizing *Eco* RI fragment found in cosmid 103. This fragment has only been seen in a genomic blot of *Eco* RI digested mouse DNA hybridized with pMCAII at low stringency (i.e., 35% formamide). On a blot of clone 103, the 1.8-kb fragment is weaker than the 2.0-kb fragment (which is of artificial size, because it is one of the two end fragments of the genomic insert).

Duplicated genes that give rise to the same protein product with no (or very few) amino acid differences have been described for other gene systems. One of the best characterized is the case of human α-globin where two genes actively

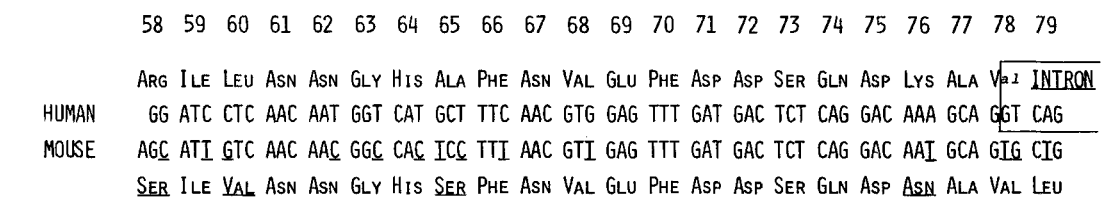

FIGURE 8. Comparison of human and mouse CA II nucleotide and amino acid sequences. Position numbers correspond to human CA I. The boxed region represents the beginning of an intron that starts after the first nucleotide of 78 Val in the human DNA sequence. Underlined residues are differences of the mouse sequences from the human sequences.

produce products distinguishable at the mRNA level by differences in the 3′ untranslated region but indistinguishable at the protein level.[18] The goat $^{I}\alpha$- and $^{II}\alpha$-globin genes, while differing by one amino acid in the coding region, have identical 3′ untranslated regions.[51] Several other examples also exist.[52–54] Gene conversion events or recent duplications have been postulated to produce these nearly identical gene copies. Assuming that our isolated mouse CA II genes do not represent cloning artifacts, a number of questions immediately arise. Do several CA II genes produce the same product? Or will the genes be found to differ slightly and have differential tissue expression? Clearly, further efforts toward obtaining overlapping clones containing entire genes, finer restriction maps, and additional sequence data are necessary to answer these questions.

The isolation of at least a portion of a human CA II gene provides a probe that will obviously facilitate our ongoing studies on the human CA II gene (or genes). Hybridizing fragments contained in the other human CA II clones have not yet been as extensively characterized as the one in H25. If multiple CA II or CA II-like genes do exist in humans, the differences in clinical severity of the CA II deficiencies might therefore be explained by lesions involving one or more of these genes. With the availability of a human CA II probe, such possibilities can now be investigated.

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