Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 10330–10334, October 1994
Biochemistry

Mitochondrial carbonic anhydrase (isozyme V) in mouse and rat: cDNA cloning, expression, subcellular localization, processing, and tissue distribution

YOSHIRO NAGAO, MYTHILI SRINIVASAN, J. SUSO PLATERO, MARION SVENDROWSKI, ABDUL WAHEED, AND WILLIAM S. SLY*

Edward A. Doisy Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, 1402 South Grand Boulevard, St. Louis, MO 63104

Contributed by William S. Sly, July 11, 1994

ABSTRACT **When the human cDNA, isolated on the basis of homology to the murine carbonic anhydrase (CA) "Y" was expressed in COS cells, the human CA was targeted to and processed in mitochondria, as expected for CA-V. However, tissue distribution reported for the corresponding mouse CA Y mRNA was much more limited than that reported for the distribution of CA-V immunostaining in rat tissues. To determine whether the murine cDNA actually encodes a mitochondrial CA activity and to compare the tissue distribution of the homologous murine and rat gene products, we used reverse transcription-PCR to reisolate the murine CA-V candidate cDNA and used the murine cDNA probe to isolate the homologous rat cDNA. We compared the two cDNA sequences, the activities they expressed after transfection of COS cells, and the sites of N-terminal processing of expressed products. In addition, we used antibodies to the C-terminal peptides predicted from each cDNA to compare distribution of CA-V in mouse and rat tissues and to identify CA-Vs in mitochondria isolated from mouse and rat liver. From these studies, we conclude that both mouse and rat CA-V candidate cDNAs encode active CAs that are targeted to and processed in mitochondria and that there are real differences in tissue distribution of CA-V between mouse and rat. However, the findings that the M_r of CA-V in rat tissues is smaller than that previously reported and that the tissue distribution also differs lead us to conclude that the antibody used in prior reports most likely misidentified another antigen in rat tissues as CA-V.**

The carbonic anhydrase (CA) gene family in mammals encodes seven isozymes (CA-I–CA-VII), which differ in physicochemical and enzymatic properties. Some isozymes also differ in subcellular location, being cytosolic (CA-I, -II, -III, and -VII), membrane-associated (CA-IV), mitochondrial (CA-V), and secretory (CA-VI) (1–12). The presence of CA in mammalian mitochondria was recognized very early (13–15). From observed effects of CA inhibitors, roles for a CA in mitochondria have been suggested in gluconeogenesis (where HCO_3^- is required by pyruvate carboxylase) and in ureagenesis (where HCO_3^- is required by carbamyl phosphate synthetase) (16–21).

CAs have been purified from guinea pig and rat liver mitochondria using inhibitor affinity chromatography (22, 23) and N-terminal sequence information determined on purified guinea pig (22) and rat enzymes (24). A murine cDNA, which appeared to be a candidate to encode the mouse mitochondrial CA, was isolated by chance when a mouse liver cDNA library was screened with a repetitive sequence probe, and a positive clone was found to have sequence homology to mammalian CAs and also to predict an N-terminal sequence resembling a mitochondrial leader peptide (25). When this murine CA-V candidate cDNA was used to probe a human liver cDNA library, a full-length human homologue was isolated which, when expressed in COS cells, produced an active CA that was targeted to and processed in mitochondria. Further, an antibody to the C-terminal peptide predicted from the human cDNA sequence reacted with a protein of the expected molecular mass on immunoblots of mitochondria from human liver (26).

Although the evidence was compelling that the human homologue of this murine cDNA encoded human CA-V, tissue distribution of the corresponding murine mRNA was far more restricted than that reported for rat CA-V (27, 28). The CA "Y" mRNA was detected only in liver (25), whereas an antibody believed to be raised against rat CA-V reacted with an antigen present in many rat tissues in addition to liver, including myocardium and skeletal muscle (23, 27, 28). To determine whether the murine CA Y cDNA indeed encodes a mitochondrial CA activity and to compare the tissue distribution of the homologous mouse and rat gene products, we cloned the CA Y cDNA and the homologous rat cDNA.† In comparing the two cDNA sequences, we discovered an important correction for the murine cDNA sequence. We expressed both cDNAs in COS cells and compared the sites of N-terminal processing in the products they expressed. In addition, we developed antibodies to the C-terminal peptides predicted from both cDNA sequences and used these antibodies to identify the products expressed from the cDNAs to identify mouse and rat CA-Vs in mitochondria isolated from mouse and rat liver and to compare the distribution of CA-V in mouse and rat tissues.

MATERIALS AND METHODS

Isolation and Characterization of cDNA Clones. A mouse CA-V cDNA was prepared from C57BL total mouse liver RNA and sequenced as described (29). A rat liver cDNA library in λ-ZAP II vector (Stratagene) was screened by using the mouse CA-V cDNA as probe (30). The DNA insert in pBluescript was excised from the λ-ZAP II clone. The cDNAs of mouse and rat were inserted in M13 phage DNA, and the single-strand DNAs were sequenced by the dideoxynucleotide chain-termination method of Sanger *et al.* with deoxyadenosine [γ-[^{35}S]thio]triphosphate (29).

Transfection of COS-7 Cells. The cDNA of mouse or rat was subcloned into the pCAGGS vector originally described by Miyazaki *et al.* (31) and used by Yoshida *et al.* (32). COS-7 cells in 60-mm dishes were transfected with 15 μg of DNA per dish by the DEAE-dextran procedure (33) and chloroquine

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: CA, carbonic anhydrase.

*To whom reprint requests should be addressed.

†The sequence reported in this paper has been deposited in the GenBank data base (accession no. U12268).

treatment 12 hr after transfection (34). The transfected cells were harvested by scraping into phosphate-buffered saline 84 hr after transfection and disrupted and lysed by sonication (three times for 10 sec each) in 25 mM Tris sulfate, pH 7.2/1 mM benzamidine/0.05% Triton X-100.

CA Assay. CA activity was assayed in cell homogenates according to Maren (35) as described (36). The protein concentration was determined by the Lowry procedure (37) with bovine serum albumin as standard.

Purification of Mouse and Rat CA-V from COS Cells Transfected with cDNA. The COS cells transfected with CA-V cDNAs of rat or mouse were disrupted and lysed by sonication in 10 mM Tris sulfate, pH 9/1 mM benzamidine/2 mM phenylmethylsulfonyl fluoride/5 mM iodoacetamide/0.1% Triton X-100. The clear supernatant after centrifugation at 40,000 × *g* for 1 hr was applied to the benzene-sulfonamide affinity column. Unbound proteins were removed by washing with 0.2 M Tris sulfate, pH 9, and the bound enzyme was eluted in 100 mM sodium acetate, pH 5.5/0.5 M sodium perchlorate, as described (5).

Isolation of Mitochondria from Liver. Mitochondria were isolated from Dounce homogenates of mouse and rat liver in the presence of 1 mM phenylmethylsulfonyl fluoride/1 mM benzamide/5 mM iodoacetamide using discontinuous sucrose density gradient as described (38). Mitochondrial enrichment of the fraction was measured by determining activity of cytochrome oxidase (39) as a marker for mitochondria.

Immunochemical Methods. Antiserum against the carboxy-terminal 17-amino acid peptides were produced by injecting the synthetic C-terminal peptide of rat CA-V or that of mouse CA-V conjugated to porcine thyroglobulin into rabbits in complete Freund's adjuvant. The animals were boosted 4 weeks later with the same antigens in incomplete Freund's adjuvant (5). The titer and specificity of the antisera from rabbits were determined by immunoblots.

Immunoblotting and N-Terminal Amino Acid Sequencing. SDS/PAGE was done under reducing conditions according to Laemmli (40). The polypeptides were electrophoretically transferred to poly(vinylidene difluoride) membrane as described (5). The poly(vinylidene difluoride) membranes were incubated with rabbit antiserum to the C-terminal peptide of rat or mouse CA-V at 1:5000 dilution followed with goat anti-rabbit IgG peroxidase conjugate at 1:500 dilution. The immunoblots were developed with peroxidase activity as described (41). For N-terminal sequencing, the poly(vinylidene difluoride) membranes were stained with Coomassie blue to locate the polypeptides. After destaining, pieces of membrane containing the appropriate polypeptide were analyzed in an Applied Biosystems automatic A77 protein sequencer (5).

RESULTS

Reisolation and Characterization of Mouse CA Y cDNA. We used reverse transcription-PCR to reisolate the murine CA-V candidate cDNA using primers designed from the published sequence for CA Y (25) and total RNA from livers of C57BL mice. The nucleotide sequence we determined on the cDNA clone differed from the published sequence (25) in that we found an extra 3 bp in the coding region. The additional two guanines and one cytosine in the open reading frame result in replacement of two amino acids in the published sequence (phenylalanine and methionine) with three new amino acids (valine, histidine, and tryptophan) (Fig. 1). The Val-His-Trp specified by codons 122–124 of the new sequence are found in nearly all human CAs (26). Evidence that the CA Y cDNA sequenced is the authentic cDNA for murine CA-V is provided by sequence comparisons and by expression and subcellular localization studies described below.

```
               Glu Leu His Leu  Phe  Met   Asn Ser Thr Lys
Mouse CA'Y'
               GAG CTC CAT CTG |-TT CA- TG-|AAT TCC ACG AAA
              550         560  |           | 570
                               |           |
               GAG CTC CAT CTG |GTT CAC TGG|AAT TCC ACG AAA
Mouse CA V
               Glu Leu His Leu Val His Trp Asn Ser Thr Lys
                       120                 125
```

FIG. 1. Nucleotide sequence difference between the originally reported and reisolated CA Y cDNA. The differences between nt 560 and 570 of CA Y (25) and the reisolated CA-V candidate cDNA reported here are boxed. Deduced amino acids are indicated.

Isolation and Characterization of the Rat CA-V Candidate cDNA. To do parallel studies on the homologous murine and rat CA-V candidate cDNAs and their gene products, we used the murine cDNA to probe a rat liver cDNA library and isolated the full-length rat cDNA. The sequence of the 1201-bp full-length rat cDNA is given in Fig. 2. It contains a 15-bp 5′ untranslated sequence, a 912-bp open reading frame beginning at the initiation ATG, and a 274-bp 3′ untranslated sequence that contains a polyadenylylation cleavage signal 11 bp upstream of the poly(A) tail.

Comparison of Amino Acid Sequences of Rat, Mouse, and Human CA-V. Fig. 3 presents the 304-amino acid sequence predicted from the rat CA-V candidate cDNA and its alignment with the recently reported human CA-V and the corrected sequence for mouse CA Y reported here. The rat sequence shows 85% and 65% similarity, respectively, to the murine and human sequences. The predicted molecular weights of the mitochondrial precursors would be 34,070 and 34,479 for mouse and rat CA-V, respectively. The solid triangle above the rat sequence indicates the proteolytic cleavage site where both the mouse and rat CAs are processed, based on the N-terminal sequences of the respective mature enzymes expressed in COS cells, determined as described below. The open triangle below the human sequence indicates the previously determined cleavage site in the human CA-V precursor. Assuming these cleavage sites, the molecular weights predicted for the mature mouse and rat CA-Vs would be 30,630 and 30,511, respectively.

Several other features can be noted from the sequence comparisons. (*i*) The mouse and rat mature enzymes each contain 270 amino acids, three more than the mature human CA-V. (*ii*) All three enzyme precursors have an arginine residue two amino acids upstream from the cleavage site (i.e., they fit the R-2 rule for cleavage of mitochondrial enzyme precursors) (42). (*iii*) Of 16 highly conserved amino acids in mammalian CAs, 14 (boxed in Fig. 3) are present in all three. However, all three lack a highly conserved tyrosine (Tyr-7 in human CA-I), which is replaced by histidine (His-8 in the rat and mouse sequences) and threonine in human CA-V. All three sequences also lack an important and highly conserved histidine (His-64 in human CA-I), which is replaced by tyrosine in all three CA-Vs. (*iv*) All three sequences contain three potential zinc-binding histidines. (*v*) All three enzymes have the Val-His-Trp sequence at positions 122–124 (of the mouse sequence), which, although not present in the sequence originally reported for mouse CA Y (Fig. 1), appears in nearly all human CAs.

Expression of CA Activity from the CA-V Candidate cDNAs and N-Terminal Processing of Expressed CA-Vs. Homogenates of COS cells transfected with either mouse or rat cDNA had four to five times the CA activity in COS cells transfected with vector only (Table 1). The levels of activity expressed are similar to the level reported in COS cells transfected with the human CA-V cDNA (26) but only 0.6% the level seen in cells transfected with the human CA-II cDNA in the same vector.

```
                Met Leu Arg Ala Lys Met Leu Gly Arg Gly Pro Tyr Lys Pro Leu Ala  -19
CGCCACCACCCCGCC ATG CTC AGA GCC AAG ATG CTC GGG AGA GGC CCC TAC AAG CCC TTA GCC

Ile Leu Arg His Met Gly Pro Leu Cys Ala Thr Arg Pro Gln His Trp Arg Phe▼Gln His  +2
ATC CTC AGG CAC ATG GGA CCT CTC TGT GCC ACA AGG CCA CAG CAC TGG CGC TTC CAG CAT

Ser Tyr Ala Glu Lys His Ser Asn Cys Ala Arg His Pro Leu Trp Thr Gly Pro Val Ser  22
TCC TAC GCA GAG AAA CAC AGC AAC TGT GCC CGG CAC CCT CTC TGG ACT GGC CCA GTG TCC

Ser Pro Gly Gly Thr Gln Gln Ser Pro Ile Asn Ile Gln Trp Thr Asp Ser Val Tyr Asp  42
TCA CCG GGA GGC ACC CAG CAG TCT CCC ATT AAT ATC CAG TGG ACG GAT AGT GTC TAT GAC

Pro Lys Leu Ala Pro Leu Arg Val Ser Tyr Asp Ala Ala Ser Cys Arg Tyr Leu Trp Asn  62
CCG AAG CTG GCA CCG CTC AGG GTC TCC TAT GAT GCT GCG TCC TGC AGA TAC CTC TGG AAC

Thr Gly Thr Phe Phe Gln Val Glu Phe Asp Asp Ser Cys Glu Glu Ser Gly Ile Ser Gly  82
ACT GGT TAC TTC TTC CAG GTG GAG TTT GAC GAT TCC TGT GAG GAG TCA GGG ATC AGT GGT

Gly Pro Leu Gly Asn His Tyr Arg Leu Lys Gln Phe His Phe His Trp Gly Ala Thr Asp  102
GGG CCT CTG GGA AAC CAC TAC AGG CTG AAG CAG TTT CAC TTC CAC TGG GGA GCA ACA GAT

Glu Trp Gly Ser Glu His Met Val Asp Gly His Ala Tyr Pro Ala Glu Leu His Leu Val  122
GAA TGG GGC TCT GAG CAC ATG GTG GAC GGC CAT GCC TAC CCG GCT GAG CTC CAT TTG GTT

His Trp Asn Ser Met Lys Tyr Glu Asn Tyr Lys Lys Ala Thr Thr Gly Glu Asn Gly Leu  142
CAC TGG AAT TCC ATG AAA TAT GAA AAT TAC AAG AAA GCC ACC ACG GGG GAG AAT GGA CTG

Ala Val Ile Gly Val Phe Leu Lys Leu Gly Ala His His Glu Ala Leu Gln Arg Leu Val  162
GCG GTG ATT GGA GTG TTT CTG AAG CTC GGG GCC CAT CAC GAG GCC CTG CAG AGG CTG GTG

Asp Ile Leu Pro Glu Val Arg His Lys Asp Thr Gln Val Thr Met Gly Pro Phe Asp Pro  182
GAC ATC TTG CCG GAA GTA AGA CAC AAG GAC ACA CAG GTG ACC ATG GGG CCC TTT GAC CCT

Ser Cys Leu Leu Pro Ala Cys Arg Asp Tyr Trp Thr Tyr Pro Gly Ser Leu Thr Thr Pro  202
TCT TGC CTG CTG CCT GCC TGC CGG GAT TAC TGG ACC TAC CCT GGC TCC CTC ACC ACC CCA

Pro Leu Ala Glu Ser Val Thr Trp Ile Val His Lys Met Pro Ile Glu Val Ser Pro Ser  222
CCA CTG GCT GAG TCA GTC ACC TGG ATT GTG CAC AAG ATG CCC ATT GAG GTG TCC CCG AGC

Gln Leu Ser Thr Phe Arg Thr Leu Leu Phe Ser Gly Arg Gly Glu Asp Glu Glu Val Met  242
CAG CTG TCC ACA TTC CGT ACA CTC TTG TTC TCC GGG CGA GGT GAG GAC GAG GAG GTG ATG

Val Asn Asn Phe Arg Pro Leu Gln Pro Leu Arg Gly Arg Asn Val Arg Ser Ser Phe Gln  262
GTG AAC AAC TTC CGC CCG CTC CAA CCA CTC AGG GGC CGC AAC GTT CGC TCC TCC TTC CAG

Val Pro Arg Val Gly Thr Lys Ser ***
GTC CCC AGG GTG GGA ACA AAG TCT TGA TCTCAGGATGAGGTCTGTAAGGATAGGCAGAGCGGATGGAAAA

GGGGGTGCGCATTTCCAGGGTGCGACGCCTGGATTAAAAAAAAAATGGCTGCAGAGATGGCTCAGGGGTTAAGAGCACT
GACTGCTCTTCCAGAGGTCCTGAGTTCAGTTCCCAGTAACCACTGGTGGCTCACAACCATCTGTAATGGGATCCGATGC
CCTCTTCTGGTGTGTCTGAAGAGAGCGACACTGCACTCATATGACATTAAATTAATAAATCTTTAAAAAA
```

FIG. 2. Nucleotide (lower) and deduced amino acid (upper) sequences of rat CA-V candidate cDNA. The putative leader sequence is amino acid −34 to −1. The solid triangle shows the cleavage site preceding the N-terminal amino acid determined for mature CA-V; the stop codon is indicated by asterisks. The putative polyadenylylation signal in the 3′ noncoding region is underlined.

When the mouse and rat CAs expressed in COS cells were purified by inhibitor affinity chromatography and subjected to SDS/PAGE, 31- and 30-kDa polypeptides were obtained (data not shown), which were transferred to membranes and directly sequenced. The N-terminal sequences for mouse and rat enzymes (in one-letter code) were QHSXAEEHSNXA and QHSYAEKHSNXA, respectively. When compared with Fig. 3, these sequences suggest that the active mouse and rat CAs produced in COS cells from the CA-V candidate cDNAs are, in fact, the mature mouse and rat CA-Vs

```
                                    -20                              ▼1
RCAV        M L R A K M L G R G P Y K P L A I L R H M G P L C A T R P Q H W R F Q H S Y A
MCAV                  M L R R D P R K P L A L L R H V G L L C A T G P Q R W R F Q H S C A
HCAV    M L G R N T W K T S A F S F L V E Q M W A P L W S R S M R P G R W C S Q R S C A
                                                                               △
                                 20                                      40
RCAV    E K H S N C A R H P L W T G P V S S P G G T Q Q S P I N I Q W T D S V Y D P K L
MCAV    E E H S N C A R H P L W T G P V S S A E G T R Q S P I N I Q W K D S V Y D P Q L
HCAV    W Q T S N N T L H P L W T V P V S V P G G T R Q S P I N I Q W R D S V Y D P Q L

                                 60                                      80
RCAV    A P L R V S Y D A A S C R Y L W N T G Y F F Q V E F D D S C E E S G I S G G P L
MCAV    A P L R V S Y D A A S C R Y L W N T G Y F F Q V E F D D S C E D S G I S G G P L
HCAV    K P L R V S Y E A A S C L Y I W N T G Y L F Q V E F D D A T E A S G I S G G P L

                          *   *      100                                 *120
RCAV    G N H Y R L K Q F H F H W G A T D E W G S E H M V D G H A Y P A E L H L V H W N
MCAV    G N H Y R L K Q F H F H W G A T D E W G S E H A V D G H T Y P A E L H L V H W N
HCAV    E N H Y R L K Q F H F H W G A V N E G G S E H T V D G H A Y P A E L H L V H W N

                                 140                                     160
RCAV    S M K Y E N Y K K A T T G E N G L A V I G V F L K L G A H H E A L Q R L V D I L
MCAV    S T K Y E N Y K K A S V G E N G L A V I G V F L K L G A H H Q A L Q K L V D V L
HCAV    S V K Y Q N Y K E A V V G E N G L A V I G V F L K L G A H H Q T L Q R L V D I L

                                 180                                     200
RCAV    P E V R H K D T Q V T M G P F D P S C L L P A C R D Y W T Y P G S L T T P P L A
MCAV    P E V R H K D T Q V A M G P F D P S C L L P A C R D Y W T Y P G S L T T P P L A
HCAV    P E I K H K D A R A A M R P F D P S T L L P T C W D Y W T Y A G S L T T P P L T

                                 220                                     240
RCAV    E S V T W I V H K M P I E V S P S Q L S T F R T L L F S G R G E D E E V M V N N
MCAV    E S V T W I V Q K T P V E V S P S Q L S T F R T L L F S G R G E E E D V M V N N
HCAV    E S V T W I I Q K E P V E V A P S Q L S A F R T L L F S A L G E E E K M M V N N

                                 260
RCAV    F R P L Q P L R G R N V R S S F Q V R R V G T K S
MCAV    Y R P L Q P L R D R N L R S S F R L D R T K M R S
HCAV    Y R P L Q P L M N R N V W A S F Q A T N E G T R S
```

FIG. 3. Comparison of amino acid sequences of candidates for CA-V from rat (RCAV), mouse (MCAV), and human (HCAV). Fourteen amino acid residues thought to be near the active site are common to nearly all CAs (boxed). The proteolytic cleavage site of rat and mouse CAs are indicated by solid triangles and that of human CA-V is indicated by an open triangle. Arginine residues at −2 positions from the cleavage sites are circled. Three potential zinc-binding histidines are indicated by asterisks. The C-terminal polypeptides (aa +254 to +270) used for producing antibodies are underlined.

Table 1. CA activities in homogenates of COS cells transfected with mouse and rat CA-V cDNAs

Enzyme	CA activity,* unit per mg of cell protein
Vector only	0.04 ± 0.01 (0.03–0.05)
CA-V (mouse)	0.19 ± 0.05 (0.12–0.24)
CA-V (rat)	0.18 ± 0.05 (0.11–0.23)

*CA activities were measured on homogenates from four different transfections. Numbers in parentheses are the range of CA activities.

produced by removal of 29- and 34-amino acid mitochondrial leader sequences, respectively.

Immunological Identification of Expressed CA-Vs. To identify the expressed CA-Vs immunologically, we prepared polyclonal antibodies against peptides corresponding to the 17-amino acid C-terminal peptides predicted from the mouse and rat cDNA sequences (underlined in Fig. 3). Each of these antibodies reacted on immunoblots with proteins expressed from the respective cDNA but not with proteins from COS cells transfected with vector only (Fig. 4). The predominant bands expressed in COS cells transfected with the mouse cDNA were 33 and 31 kDa, close to the molecular weights predicted for precursor and mature forms of mouse CA-V. The predominant polypeptides in COS cells expressing the rat cDNA were 34 and 30 kDa, again close to the sizes predicted for precursor and mature forms of rat CA-V.

Immunological Identification of CA-Vs in Mouse and Rat Tissues and in Mitochondria from Mouse and Rat Liver. We next used the antibodies described above to identify CA-V in mouse and rat tissues and in mitochondria isolated from mouse and rat liver. Fig. 5 *A* and *B* present immunoblots of mouse and rat tissues in which the anti-mouse and anti-rat CA-V peptide antibodies were used as first antibody on the appropriate blots. In mouse tissues, CA-V immunoreactivity was detected as a 31-kDa band, which was detected only in liver of nine tissues examined. In rat tissues, two polypeptides of 30 kDa and 28 kDa were detected in six of the nine tissues. While the signal was most intense in rat liver, CA-V immunoreactivity was also detected in heart, lung, kidney, spleen, and intestine; no signal was detected in brain, testes, or muscle. The relative proportions of 30- and 28-kDa bands varied in different tissue preparations. The 30-kDa band predominated in fresh tissue samples, in some of which it was the only form seen. The 28-kDa band tended to become more prominent with storage and on freezing and thawing, as the 30-kDa band diminished.

To verify the mitochondrial localization of the immunoreactive CA-V in mouse and rat tissues, mouse and rat livers were subcellularly fractionated, and the postnuclear supernatants, cytosol, and mitochondrial fractions were analyzed by SDS/PAGE and then immunoblotted. Fig. 5*C* shows that most immunoreactivity seen in postnuclear supernatants from mouse and rat liver is present in the mitochondria

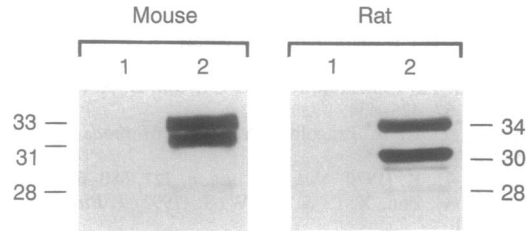

FIG. 4. Expression of mouse and rat CA-V in COS cells. Sonicated cell lysates of COS-7 cells transfected with vector only (lanes 1) and candidate CA-V cDNAs from mouse and rat (lanes 2) were analyzed by SDS/PAGE followed by immunoblotting. The apparent molecular masses of the polypeptides are marked in kDa.

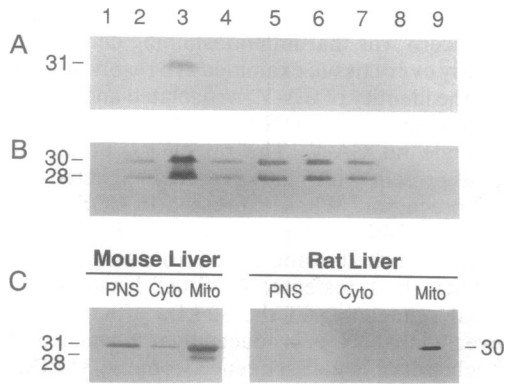

FIG. 5. Immunoblot analyses of CA-V in mouse and rat tissues and in subcellular fractions from mouse and rat liver. Homogenates of different tissues from mouse (*A*) and rat (*B*) equivalent to 100 μg of protein were analyzed on SDS/PAGE followed by immunoblotting, using anti-mouse and anti-rat C-terminal peptide antibodies and peroxidase-conjugated goat anti-IgG. Lanes: 1, brain; 2, heart; 3, liver; 4, lung; 5, kidney; 6, spleen; 7, intestine; 8, testis; and 9, muscle. In mouse tissues (*A*), liver shows a 31-kDa band. Polypeptides of 30 and 28 kDa are seen in rat liver and five other rat tissues (*B*). (*C*) Subcellular localization of CA-V in mouse and rat liver. After subcellular fractionation of mouse and rat liver, postnuclear supernatant (PNS), cytosol (Cyto), and mitochondria (Mito) fractions equivalent to 100 μg of total protein were subjected to SDS/PAGE and immunoblotting. The apparent molecular masses are indicated in kDa. No polypeptides larger than mature CA-V were detected in mouse or rat liver mitochondria.

derived from these postnuclear supernatant fractions. In both cases, the largest band in mitochondria had the molecular mass expected for the mature CA-V—i.e., 31 kDa for mouse and 30 kDa for rat.

DISCUSSION

Our first goal was to establish whether the reported cDNA called CA Y encodes the murine mitochondrial enzyme CA-V, as does its human homologue. The murine CA Y cDNA sequence we isolated encodes three highly conserved amino acids not predicted from the initially reported sequence (25). Whether the additional 3 nt that change the coding sequence in this way indicate strain variation or errors in the original sequence is unclear. What is clear is that expression of the cDNA encoding the three highly conserved amino acids in COS cells produces an active CA which, on immunostaining (data not shown), was localized to mitochondria. Transfected COS cells contained both the immunoreactive precursor (33 kDa) as well as the mature (31 kDa) enzyme, although only the 31-kDa enzyme was isolated when transfected COS cell homogenates were applied to an inhibitor affinity column. N-Terminal sequencing of the affinity-purified enzyme defined the site of proteolytic cleavage and indicated that a 29-amino acid mitochondrial leader sequence was removed to produce the 31-kDa mature enzyme. Only the 31-kDa mature enzyme form was detected in mouse tissues, and this form was only detected in mouse liver. The mouse liver enzyme was localized to mitochondria by subcellular fractionation. These studies clearly indicate that the CA Y cDNA described encodes the murine CA-V. Furthermore, its tissue distribution agrees with that reported for CA Y mRNA based on RNA blot analyses of mouse tissues (25).

Although little was known about mouse CA-V when this work began, there had been several reports on rat CA-V (23, 24, 27, 28), some of which raised questions whether the correct protein had been identified. One problem was that the protein identified as rat CA-V on immunoblots had the molecular mass expected for the mitochondrial precursor (34

kDa), rather than that of the mature enzyme (30 kDa). The second concern was that immunostaining detected this protein in nearly every tissue examined. To resolve the questions regarding the identity of CA-V, we isolated and characterized the rat homologue of the cDNA for CA Y, which we assumed would encode rat CA-V. The rat cDNA predicted a 304-amino acid protein with many features expected of a mitochondrial precursor. When expressed in COS cells, it produced 34- and 30-kDa polypeptides. Only the 30-kDa mature enzyme was adsorbed to and eluted from an inhibitor affinity column when the transfected COS cell lysate was applied. N-Terminal sequencing of the 30-kDa affinity-purified enzyme revealed that it was produced by removal of a 34-amino acid mitochondrial leader sequence from the 34-kDa precursor. In rat tissues, only 30-kDa and smaller-molecular-mass species were detected by the anti-CA-V C-terminal peptide antibody. Immunoreactivity was most abundant in liver, but it was also seen in five other tissues. The immunoreactivity in rat liver was localized to mitochondria by subcellular fractionation. From these studies, we conclude that the rat homologue of CA Y encodes rat CA-V.

Earlier reports (23, 27, 28) that CA-V is present in rat tissues as a 34-kDa peptide with a tissue distribution different from that reported here probably reflect their dependence on an antibody that misidentified another antigen in rat tissues as CA-V. Ohlinger *et al.* (24) recently reported that malate dehydrogenase (M_r 34,000) copurified with rat CA-V on the inhibitor affinity column used to purify rat liver CA-V. Misidentification of malate dehydrogenase as CA-V could easily account for the anomalously large size of the polypeptide (34 kDa instead of 30 kDa) and the different tissue distribution from that reported here.

We also observed copurification of malate dehydrogenase with CA-V to various degrees in our affinity-purified preparations of CA-V. Although we could not use these preparations to raise CA-V-specific antibodies, we did obtain CA-V-specific antibodies by immunizing rabbits against the C-terminal peptides predicted for the mouse and rat CA-V cDNAs. Whether the copurification of malate dehydrogenase with CA-V simply reflects a measure of nonspecificity of the affinity chromatography step or indicates that the two enzymes are associated *in vivo* and remain associated on purification is an interesting question that deserves further study.

The N-terminal sequence, which we determined on mature rat CA-V from transfected COS cells, begins 22 amino acids upstream of the N-terminal sequence reported (24) for the mitochondrial CA purified from rat liver. Probably the N-terminal sequence reported for rat liver CA-V was from enzyme that had undergone partial degradation after initial processing. We frequently observed conversion of the 30-kDa mature CA-V to a 28-kDa form in homogenates of rat tissues (Fig. 4).

Rat and mouse CA-V are both "low activity" CAs (compared with CA-II), as was true for the human and guinea pig (6) CA-Vs. This lower activity may result from differences in two amino acids, which are part of the hydrogen bonding network associated with the zinc ion and which are thought crucial to the active sites of CA-I and CA-II (10). Tyr-7 is replaced by histidine in rat and mouse CA-V and by threonine in human CA-V. His-64 is replaced by tyrosine in all three CA-Vs. It has been suggested that replacement of His-64 by lysine in CA-III at least partially explains its relatively low activity compared with CA-II (43).

We thank Drs. Jung Huang and H. P. Zassenhaus for help and discussion, Dr. Sanae Okuyama for the immunofluorescence staining of transfected COS cells (data not shown), and Elizabeth Torno for editorial assistance. This work was supported by National Institutes of Health Grants GM34182 and DK40163.

1. Tashian, R. E. (1989) *Bioessays* **10**, 186–192.
2. Deutsch, H. F. (1987) *Int. J. Biochem.* **19**, 101–113.
3. Whitney, P. L. & Briggle, T. V. (1982) *J. Biol. Chem.* **257**, 12056–12059.
4. Wistrand, P. J. & Knuuttila, K. G. (1989) *Kidney Int.* **35**, 851–859.
5. Zhu, X. L. & Sly, W. S. (1990) *J. Biol. Chem.* **265**, 8795–8801.
6. Storey, B. T., Dodgson, S. J. & Forster, R. E., II (1984) *Ann. N.Y. Acad. Sci.* **429**, 210–211.
7. Feldstein, J. B. & Silverman, D. N. (1984) *J. Biol. Chem.* **259**, 5447–5453.
8. Murakami, H. & Sly, W. S. (1987) *J. Biol. Chem.* **262**, 1382–1388.
9. Aldred, P., Fu, P., Barrett, G., Penschow, J. D., Wright, R. D., Coghlan, J. P. & Fernley, R. T. (1991) *Biochemistry* **30**, 569–575.
10. Tashian, R. E. (1992) *Adv. Genet.* **30**, 321–356.
11. Sly, W. S., Hewett-Emmett, D., Whyte, M. P., Yu, Y.-S. L. & Tashian, R. E. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 2752–2756.
12. Sly, W. S. (1989) in *The Metabolic Basis of Inherited Disease*, eds. Scriver, C. R., Beaudet, A. L., Sly, W. S. & Valle, D. (McGraw-Hill, New York), 6th Ed., pp. 2857–2866.
13. Datta, P. K. & Shepherd, T. H. (1959) *Arch. Biochem. Biophys.* **81**, 124–129.
14. Lehninger, A. L., Carafoli, E. & Rossi, C. S. (1967) *Adv. Enzymol.* **29**, 259–320.
15. Dodgson, S. J., Forster, R. E., II, Storey, B. T. & Mela, L. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5562–5566.
16. Coulson, R. A. & Herbert, I. D. (1984) *Ann. N.Y. Acad. Sci.* **429**, 505–515.
17. Rognstad, R. (1983) *Arch. Biochem. Biophys.* **222**, 442–448.
18. Marsolais, C., Huot, S., David, F., Garneau, M. & Brunengraber, H. (1987) *J. Biol. Chem.* **262**, 2604–2607.
19. Dodgson, S. J. (1987) *J. Appl. Physiol.* **63**, 2134–2141.
20. Wanders, R. J. A., Van Roermund, C. W. T. & Meijer, A. J. (1984) *Eur. J. Biochem.* **142**, 247–254.
21. Dodgson, S. J. & Cherian, K. (1989) *Am. J. Physiol.* **257**, E791–E796.
22. Dodgson, S. J. (1991) in *The Carbonic Anhydrases*, eds. Dodgson, S. J., Tashian, R. E., Gros, G. & Carter, N. D. (Plenum, New York), pp. 297–306.
23. Väänänen, K., Carter, N. D. & Dodgson, S. J. (1991) *J. Histochem. Cytochem.* **39**, 451–459.
24. Ohlinger, D. E., Lynch, C. J., Forster, R. E., II, & Dodgson, S. J. (1993) *FASEB J.* **7**, A676 (abstr.).
25. Amor-Gueret, M. & Levi-Strauss, M. (1990) *Nucleic Acids Res.* **18**, 1646.
26. Nagao, Y., Platero, J. S., Waheed, A. & Sly, W. S. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 7623–7627.
27. Carter, N. D., Dodgson, S. J. & Quant, P. A. (1990) *Biochim. Biophys. Acta* **1036**, 237–241.
28. Carter, N. D. (1991) in *Carbonic Anhydrase*, eds. Botre, F., Gros, G. & Storey, B. T. (VCH, Weinheim, Germany), pp. 168–177.
29. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
30. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
31. Miyazaki, J., Takaki, S., Araki, K., Tashiro, F., Tominaga, A., Takatsu, K. & Yamamura, K. (1989) *Gene* **79**, 269–277.
32. Yoshida, K., Oshima, A., Shimmoto, M., Fukuhara, Y., Sakuraba, H., Yanagisawa, N. & Suzuki, Y. (1991) *Am. J. Hum. Genet.* **49**, 435–442.
33. Lopata, M. A., Cleveland, D. W. & Sollner-Webb, B. (1984) *Nucleic Acids Res.* **12**, 5707–5717.
34. Luthman, H. & Magnusson, G. (1983) *Nucleic Acids Res.* **11**, 1295–1308.
35. Maren, T. H. (1960) *J. Pharmacol. Exp. Ther.* **130**, 26–29.
36. Sundaram, V., Rumbolo, P., Grubb, J., Strisciuglio, P. & Sly, W. S. (1986) *Am. J. Hum. Genet.* **38**, 125–136.
37. Lowry, O. H., Rosebrough, M. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
38. Tapper, D. P., Van Etten, R. A. & Clayton, D. A. (1983) *Methods Enzymol.* **97**, 426–434.
39. Wharton, D. C. & Tzagoloff, A. (1967) *Methods Enzymol.* **10**, 245–250.
40. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
41. Waheed, A., Zhu, X. L. & Sly, W. S. (1992) *J. Biol. Chem.* **267**, 3308–3311.
42. Hendrick, J. P., Hodges, P. E. & Rosenberg, L. E. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 4056–4060.
43. Tu, C. K., Paranawithana, S. R., Jewell, D. A., Tanhauser, S. M., LoGrasso, P. V., Wynns, G. C., Laipis, P. J. & Silverman, D. N. (1990) *Biochemistry* **29**, 6400–6405.