J. exp. Biol. ***196****, 109–121 (1994)*
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y^+-TYPE CATIONIC AMINO ACID TRANSPORT: EXPRESSION AND REGULATION OF THE *mCAT* GENES

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Summary

The transport of cationic amino acids across animal cell membranes is largely mediated by a small group of well-described transport systems (y^+, $b^{o,+}$, $B^{o,+}$). Only recently have genes encoding transport proteins in some of these systems been isolated. Two genes, *mCAT-1* and *mCAT-2*, encode related multiple membrane-spanning proteins that share substantial amino acid sequence identity and virtually superimposable hydrophilicity profiles. mCAT-1 and mCAT-2 proteins expressed in *Xenopus* oocytes are functionally indistinguishable and similar to transport system y^+, but have distinct tissue distribution patterns. *mCAT-1* expression is nearly ubiquitious and produces a single protein, while *mCAT-2* is highly tissue-specific, has two distinct protein isoforms encoded by a single gene and is expressed in different tissues using at least two widely separated promotors. All three proteins facilitate the ion-independent transport of arginine, lysine and ornithine. Both mCAT-1 and mCAT-2 proteins have low amino acid sequence similarity but strikingly similar hydrophilicity profiles with amino acid antiporters, uniporters and symporters of yeast, fungi and eubacteria. Current work will elucidate whether any of the mCAT proteins interact with members of a newly identified family of single membrane-spanning proteins, such as rBAT, 4F2 and NAA-Tr, which are thought to modulate or activate y^+L and/or $b^{o,+}$ transport systems.

Introduction

Two major types of proteins have been identified that mediate the transport of cationic amino acids. In this paper, we review the *mCAT* (*m*urine *C*ation *A*mino Acid *T*ransporter) genes and their protein products (MacLeod *et al.* 1990*a*; Kim *et al.* 1991; Wang *et al.* 1991; Kakuda *et al.* 1993; Closs *et al.* 1993*a*). The mCAT proteins have striking structural similarity with the well-characterized multiple membrane-spanning transporters of eubacteria and lower eukaryotes (Reizer *et al.* 1993). These mammalian transporters show properties highly similar, but not identical, to transport system y^+. They carry cationic amino acids in a Na^+-independent manner, but require Na^+ as a cosubstrate for the transport of zwitterionic amino acids. In this review, we have adopted Van Winkle's proposed terminology that confines the term *transport system* to designate a functionally distinct membrane transport process and employs the term *transporter* to denote a protein, usually expressed from a cloned gene, that catalyzes amino acid transport across a biomembrane (Van Winkle, 1993).

Key words: arginine, amino acid transport, CAT, lysine.

A distinct group of proteins modulating amino acid transport are predicted to have a different structure, only a single membrane-spanning domain (reviewed Palacín, 1994; Bertran *et al.* 1992*a*,*b*; Wells and Hediger, 1992; Tate *et al.* 1992; Pickel *et al.* 1993; Hediger *et al.* 1993; Markovich *et al*. 1993). One of them was recently shown to play a crucial role in the heritable transport disease cystinuria (Calonge *et al.* 1994). It is possible that transporter proteins such as the mCATs can associate with other proteins and/or ions or other substrates for effective transport.

Serendipitous discovery of cDNAs encoding mCAT transporters

Subtraction-differential screening (MacLeod *et al.* 1990*b*) was employed to identify cDNA clones from two closely related lymphoma cell lines (MacLeod *et al.* 1984, 1985) and resulted in the isolation of several novel cDNAs. One of the novel genes was named *Tea*, because it is induced early in the response of normal T cells to mitogens (MacLeod *et al.* 1990*a*). The cDNA sequence revealed significant homology with only one protein, also derived from mouse, that was isolated on the basis of its capacity to function as an ecotropic retroviral receptor (ERR, Albritton *et al.* 1989). The natural function of neither gene was known, although both shared low amino acid sequence similarity and considerable predicted structural similarities with bacterial and yeast amino acid transporter proteins (Reizer *et al.* 1993). Fig. 1 shows an unrooted phylogenetic tree of the mCAT proteins and selected well-characterized yeast and eubacterial transporters. The similarity prompted three independent research groups to test the possible transport function of the proteins (Kim *et al.* 1991; Wang *et al.* 1991; Kakuda *et al.* 1993).

The function of the mCAT-2 gene product was investigated by examining *Xenopus* oocytes expressing mCAT-2 protein (Kakuda *et al.* 1993). The oocytes were assayed *via* a two-microelectrode voltage-clamp method at -60 mV, monitoring for changes in current resulting from specific amino acid transport. Significant saturable inward currents were detected with lysine, arginine, ornithine and histidine, while minor currents were observed with cysteine, leucine and homoserine. The small signal obtained with the latter amino acids requires further investigation. None of the other common amino acids used in protein synthesis nor MeAIB elicited significant transport (Fig. 2). mCAT-2-mediated transport is stereospecific for the L-isomers of arginine, lysine and ornithine and is Na^+-independent. In contrast, the dipolar substrate (tested with homoserine) transport requires Na^+. In all these properties, the mCAT-2 transport characteristics are highly consistent with those of the y^+ transport system originally described by Christensen and Antonioli (1969) and White and Christensen (1982*a*,*b*). A direct comparison of the transport properties of mCAT-2 and mCAT-1 reveals no significant differences, as shown in Table 1 (Kakuda *et al.* 1993; Closs *et al.* 1993*a*).

The function of mCAT-1 was established before that of mCAT-2 and was assessed both by voltage-clamp methods (Wang *et al.* 1991) and by measuring radiolabeled influx of amino acids (Kim *et al.* 1991) with results highly similar to those obtained for mCAT-2. Discrepancies in K_m values were noted (Kakuda *et al.* 1993) and recently resolved (Closs *et al.* 1993*a*; Table 1).

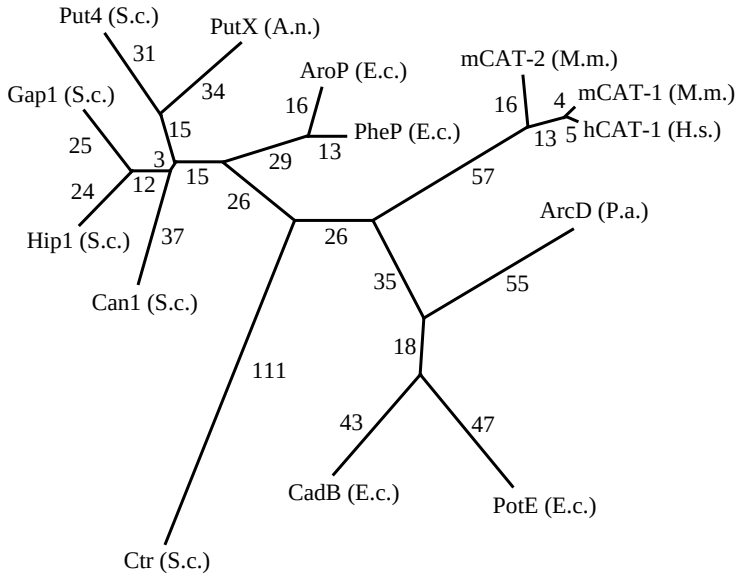

Fig. 1. Phylogenetic tree of selected proteins from unicellular and multicellular organisms belonging to the APC family. An unrooted phylogenetic tree of prokaryotic and eukaryotic transport proteins comprising the amino acid, polyamine, choline (APC) family. The tree was generated by the progressive alignment method of Feng and Doolittle (1990), as described by Reizer and Reizer (1994). The evolutionary distances are indicated. This family consists of sequence-related transport proteins predicted to contain 12 transmembrane domains that have been postulated to arise from a six transmembrane domain duplication (Saier, 1994). The proteins form four clusters of similarity as indicated. Cluster 1 contains Put4 (S.c.) proline permease, PutX (A.n.) proline permease, Gap1 (S.c.) general amino acid permease, Hip1 (S.c.) histidine permease, Can1 (S.c.) arginine permease, AroP (E.c.) general aromatic amino acid permease and PheP (E.c.) phenylalanine permease. Cluster 2 contains mCAT-1 (M.m.), mCAT-2/2a (M.m.) and hCAT (H.s.) cationic and selected dipolar amino acid transporters. Cluster 3 contains ArcD (P.a.) arginine/ornithine antiporter, PotE (E.c.) putrescine/ornithine permease and CadB (E.c.) putative cadaverine/lysine antiporter. Cluster 4 contains Ctr (S.c.) choline permease. S.c., *Saccharomyces cerevisiae*, A.n., *Aspergillus nidulans*, M.m., *Mus musculis*, H.s., *Homo sapiens*, E.c., *Escherichia coli*, P.a., *Pseudomonas aeruginosa*. Adapted from Reizer *et al.* 1993.

The *mCAT-2* gene encodes two distinct proteins

Using an mCAT-2 probe from our laboratory, Cunningham and his colleagues identified a variant isoform of mCAT-2 (mCAT-2a) from a murine liver cDNA library (Closs *et al.* 1993*a*). The truncated clones are identical to mCAT-2 over their length but contain a 123 base pair unique segment within the coding sequence. This fragment has been postulated to arise from alternate splicing of mCAT-2 mRNA during processing. Both alternately spliced segments encode highly similar amino acid sequences of either 40 or 41 amino acids (see below). Although no clones containing the entire coding sequence were obtained, usable clones were derived for cRNA production and expression studies in *Xenopus* oocytes by ligating mCAT-2 sequences from our clone or by reverse transcription/polymerase chain reaction and ligation (Closs *et al.* 1993*a*,*b*). The two

Table 1. *Apparent* K_m *values for the mCAT transporters in* Xenopus *oocytes*

Amino acid	mCAT-2 K_m (mmol l^{-1})	mCAT-2a K_m (mmol l^{-1})	mCAT-1 K_m (mmol l^{-1})
Arginine	0.187±0.028	2.2±0.15	0.206±0.020
Lysine	0.203±0.034		
Ornithine	0.419±0.053		
Histidine	3.887±0.099		

Values are means ± S.E.M.

The apparent K_m values were derived by non-linear regression analysis from dose–response data presented in Kakuda *et al.* (1993) fitted to a sigmoidal curve.

In vitro transcribed cRNA (30–185 ng) was injected into oocytes for these experiments (the mCAT-2a value is from Closs *et al.* (1993*a*)).

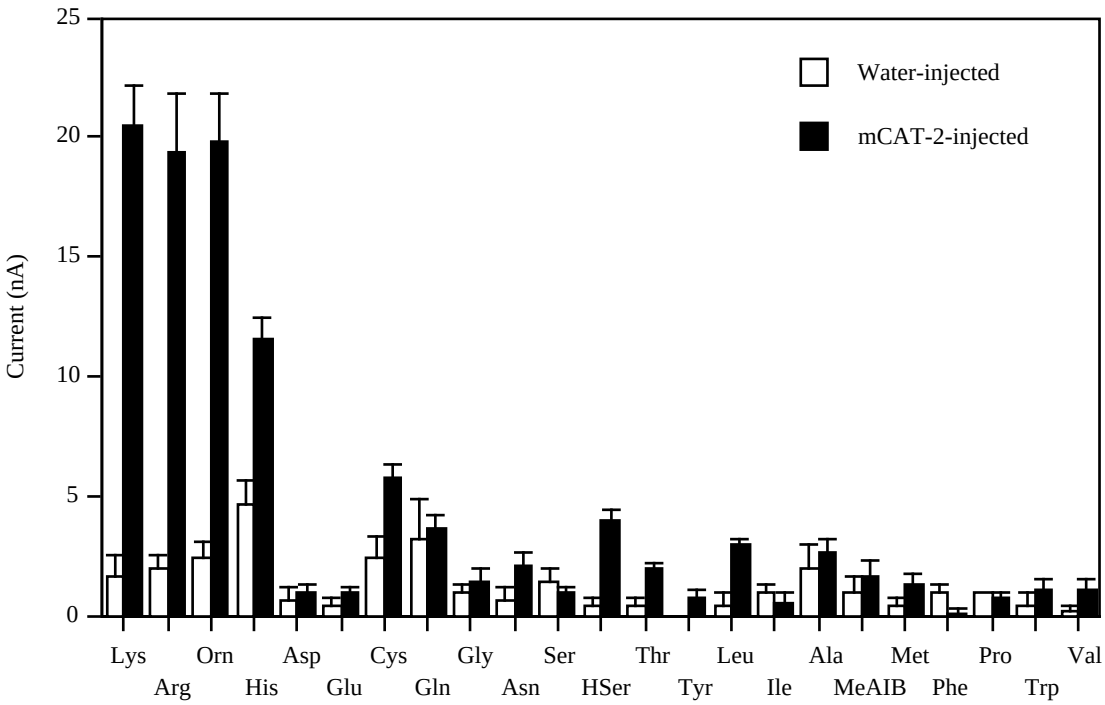

Fig. 2. Substrate specificity of mCAT-2-mediated amino acid transport in *Xenopus* oocytes. The transport of amino acids was assessed by measuring inward currents recorded in response to the application of 10 mmol l^{-1} amino acid solution (tyrosine at 5 mmol l^{-1}) for 30 s as described (figure adapted from Kakuda *et al.* 1993). These data were obtained from 4–14 oocytes injected with 30 or 75 ng of mCAT-2 cRNA or water. Values are the mean changes in current + S.E.M.; the holding potential was −60 mV.

mCAT-2 proteins are nearly identical (97 %) but differ significantly in their substrate affinity. Table 1 shows that the mCAT-2 and mCAT-1 proteins have a tenfold higher substrate affinity than mCAT-2a. In a nice series of experiments, Closs *et al.* (1993*b*)

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mCAT-1    M FPMPRVIYAM AEDGLLFKFL AKINNRTKTP VIATVTSGA IA
mCAT-2    I FPMPRVIYAM AEDGLLFKCL AQINSKTKTP VIATLSSGA VA
mCAT-2a   M FPLPRILFAM ARDGLLFRFL ARV-SKRQSP VAATMTAGV IS

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Fig. 3. A comparison of the protein isoforms from alternately spliced mCAT-2 RNA with the equivalent mCAT-1 sequence. Asterisks indicate sequences that are identical in all three proteins.

showed that chimeric proteins generated by swapping the relevant mCAT-2a domain with the corresponding mCAT-1 or mCAT-2 regions elicit reductions of their apparent affinities for arginine, providing evidence that this region of the protein is involved in substrate recognition. Although mCAT-2a-mediated transport of radiolabeled arginine is not detectable at concentrations below $100\,\mu mol\,l^{-1}$, arginine uptake is substantially greater than mCAT-1-mediated transport at $3.2\,mmol\,l^{-1}$ arginine. Hence, mCAT-2a has properties similar to the previously described low-affinity, high-capacity cationic transport system observed in liver tissue slices (Closs *et al.* 1993*a*; White and Christensen, 1982*a*). A comparison of the isoforms resulting from alternately spliced mCAT-2 RNA is shown in Fig. 3 with the equivalent mCAT-1 sequence.

The differences conferred on the protein by the substitution of these 41 amino acids has no predicted effect on the tertiary structure of the mCAT-2 proteins. In fact, all three proteins have highly similar predicted structures as modelled in Fig. 4. On the basis of the hydrophobicity profiles and computer analysis, the topology was depicted with 12 membrane-spanning domains. The three computer programs used (Fig. 4, legend) differ in the number of transmembrane domains that they predict: two of the three indicate 14 such domains for mCAT-1. Nevertheless, we chose to illustrate their obvious similarity and to take into account their membership in the APC family of proteins (Reizer *et al.* 1993), all of which are considered to have 12 membrane-spanning domains (Saier, 1994). None of these proteins contains a leader sequence, suggesting that the amino terminus will be intracellular and one or more of the hydrophobic regions may function as internal 'signal' sequences (Saier *et al.* 1989).

Expression of mCAT-1 and mCAT-2 RNAs in murine tissues

The striking similarity in the predicted structure of the two proteins and their highly similar amino acid transport properties prompted an analysis of their expression in the organs and tissues of normal mice. mCAT-1 mRNA is present in all murine tissues examined with the exception of liver (Kakuda *et al.* 1993). The absence of mCAT-1 expression in liver is supported by the failure of ecotropic retroviruses to infect this tissue (Closs *et al.* 1992). Among the 15 tissues tested, liver is unique in expressing only mCAT-2. Several organs, e.g. skeletal muscle, stomach, skin, brain, lung and uterus, express both mCAT-1 and mCAT-2 transcripts; conceivably, but not necessarily, within two distinct cell types (Kakuda *et al.* 1993). Indeed, activated lymphocytes express both

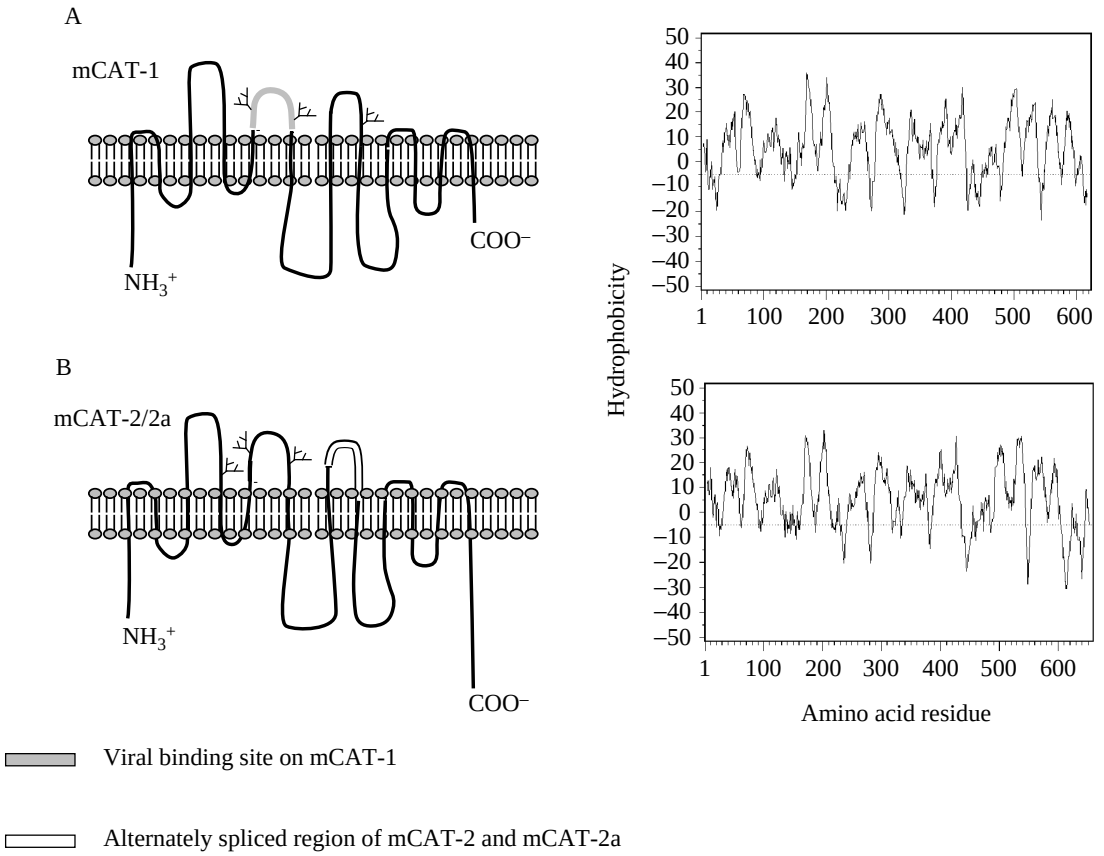

Fig. 4. Model of proposed structure of mCAT proteins in the plasma membrane. A proposed model for the membrane association of mCAT-1 (A) and mCAT-2/2a (B) proteins was derived using the PC Gene suite of programs, SOAP, Helixmem and RaoArgos, from IntelliGenetics after taking into consideration known evolutionary relationships within the APC family of transporters (see Fig. 1) as well as the conserved nature of structural features. For these reasons, they are modeled as proteins containing 12 transmembrane domains, although the hydrophilicity profiles (on the right) and putative hydrophobic transmembrane domains also predict 14 membrane-spanning regions for mCAT-1 (Albritton *et al.* 1989). The viral binding site (Albritton *et al.* 1993) was positioned extracellularly. The 'positive-inside rule' was applied by placing positively charged amino acids inside the cell (von Heijne, 1992).

genes, whereas quiescent lymphocytes express only mCAT-1 RNA (K. Finley, M. Maruyama, A. Barrieux and C. L. MacLeod, in preparation). The tissues with relatively high levels of mCAT-2/2a (skeletal muscle, stomach, skin, brain and lung) may express the high-affinity isoform of mCAT-2 (Kakuda *et al.* 1993), whereas liver expresses the low-affinity mCAT-2a isoform mRNA (Closs *et al.* 1993*a*). Some tissues, including large and small intestine and kidney, express no detectable mCAT-2 transcripts. Several human tissues were also tested and found to express the genes in the same pattern found in mouse (Finley, 1993).

Human quiescent T-cells have a limited capacity to transport lysine and do so, in part, *via* both the y^+ and the y^+L transport systems (Devés *et al*. 1992). The transport of amino acids by both systems increases in activated T-cells; the increase in y^+ transport activity corresponds to the kinetics of mCAT-2 mRNA induction (Boyd and Crawford, 1992; MacLeod *et al.* 1990*a*). Replicating hepatoma cells and cultured hepatocytes express both mCAT-1 and mCAT-2 mRNA, although only mCAT-2 is expressed in the intact liver. Souba and coworkers recently demonstrated both high- and low-affinity Na^+-independent arginine transport in fresh human liver membranes (Inoue *et al.* 1993), even though it has been difficult to demonstrate high-affinity y^+ transport system activity in fresh liver slices from rodents (White and Christensen, 1982*b*). In regenerating liver, mCAT-1 mRNA is not present when the cells are replicating, and there was no significant change in *mCAT-2* expression following hepatectomy (Kakuda *et al*. 1993). Unexpectedly, skeletal muscle mCAT-2 mRNA increased substantially (8.7-fold) 1 day following partial hepatectomy and, to a lesser but significant extent, after splenectomy and fasting. These physiological manipulations elicited no alteration in the amount of mCAT mRNA in a non-striated muscle, the uterus. Taken together, it appears that *mCAT-2* is under multifactorial regulation and in the conditions tested mCAT-1 mRNA is unaltered. This mutifactorial regulation of *mCAT-2* may reflect its requirement for transport of cationic amino acids, present in most cells at a low and steady level, but physiological stress may require regulated expression of transport (Finley, 1993; K. Finley, M. Maruyama, A. Barrieux and C. L. MacLeod, in preparation).

The *mCAT-2* gene appears to be expressed and/or induced in precisely those tissues that also express the inducible form of nitric oxide synthase (iNOS; Bandeletova *et al.* 1993; Liu *et al.* 1993), the enzyme which generates NO from arginine and oxygen (for a review, see Moncada and Higgs, 1993). The *mCAT-2* gene is different from *mCAT-1* in that *mCAT-1* encodes a single protein (L. M. Albritton, personal communication) with expression characteristics of a 'housekeeping' gene: it is constitutively expressed almost ubiquitously and is not (or is only minimally) induced under conditions that significantly increase mCAT-2 transcription (K. Finley, M. Maruyama, A. Barrieux and C. L. MacLeod, in preparation). The mCAT-2 proteins may play roles that are unique to cells involved in protecting the whole organism against pathogens and tumor cells, e.g. macrophages, lymphocytes and liver in which arginine metabolism plays a central role and mCAT-2/2a is expressed in a regulated manner (Nussler and Billiar, 1993; Billiar *et al.* 1991; Baydoun *et al*. 1993; Wilkinson *et al*. 1991).

mCAT-2 mRNAs have multiple 5′ termini

Investigation of the mCAT-2 transcription start site led to the isolation of four clones containing the entire coding sequence, but the DNA sequences differed in 5′ untranslated regions (UTRs). They diverged 16 base pairs 5′ of the initiation methionine codon (Fig. 5). Twenty-four independent clones sequenced in this region revealed four types of 5′ UTRs summarized in Fig. 5. The two forms of B sequences result from alternate splicing. DNA sequencing of genomic clones immediately 5′ of the putative splice

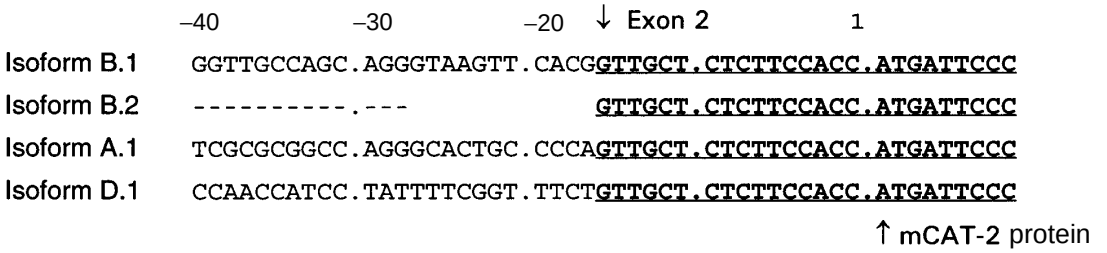

Fig. 5. The mCAT-2 splice start site. Four distinct untranslated regions are shown. There is a typical splice acceptor site upstream of exon 2 (downward arrow), and the single initiation codon common to all clones is marked with the upward pointing arrow. The dashes indicate identity with isoform B.1, the gap shows the alternate splice of isoform B.1 and B.2.

junction revealed a typical splice acceptor site upstream of exon 2 (Fig. 5). A single initiation codon is located in the sequence common to all clones, indicated by the arrow in Fig. 5 (K. Finley, A. Barrieux, J. Kleeman, P. Huynh and C. L. MacLeod, in preparation).

mCAT-2 is transcribed from alternate promotors

Fig. 6 shows a sketch of the genomic organization of the *mCAT-2* gene, the arrangement of the 5′ UTR exons and the first coding exon (exon 2). The region illustrated on the right contains the entire coding sequence. The genomic organization of the *mCAT-2* gene is complex and contains two promotors with highly distinct properties (Finley, 1993). Sequences upstream of exon 1b contain a classic TAATA promoter. The region surrounding exon 1a contains no TAATA box but it is GC-rich, has two CAC boxes and two high-affinity SP1 binding sites; features commonly found in the promoter region of TAATA-less genes. Exons 1b and 1c are always expressed together and no promoter motifs are apparent around exon 1c. Hence, *mCAT-2* transcription is initiated at the promoters present upstream of exons 1a and 1b and at one as yet unidentified site within the *mCAT-2* gene (K. Finley, A. Barrieux, J. Kleeman, P. Huynh and C. L. MacLeod, in preparation). A genomic clone containing the exon for isoform D has not yet been isolated.

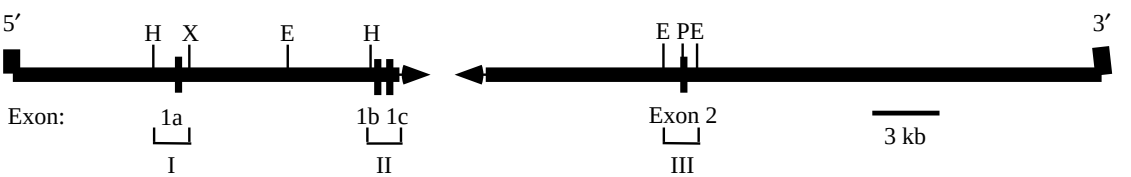

Fig. 6. Organization of the *mCAT-2* gene. A composite diagram of several clones, indicating the relative positions of exons encoding isoform A (exon 1a), isoform B (exon 1b and exon 1c) and the first coding region (exon 2). A limited endonuclease map is also illustrated. The clones cover 54 kb of genomic DNA. The numbered fragments were sequenced. H, HindIII; X, XhoI; E, EcoRI; P, PvuII.

How system y^+ may concentrate substrates intracellularly

Many transport systems take advantage of the Na^+ chemical gradient to cotransport substrates. This cotransport can lead to the accumulation of the substrate against its concentration gradient in cells. By contrast, system y^+ does not require Na^+ for the transport of cationic amino acids (White and Christensen, 1982*a*). These charged molecules can be concentrated within cells in the absence of evidence for symport or antiport mechanisms by using the negative resting membrane potential (Kavanaugh, 1993). The voltage gradient serves as a driving force for the influx of positively charged molecules, such as cationic amino acids, as discussed in Gerenscer and Stevens (1994). This proposed model would explain the observation that the concentration of cationic amino acids is generally higher inside, than outside, most cells (with the notable exception of liver and kidney, where arginase activity is high).

Kavanaugh (1993) has recently shown that changes in the membrane potential can contribute to differences in apparent K_m and I_{max} (maximum current) for mCAT-1 expressed in *Xenopus* oocytes. His results are consistent with the conclusion that hyperpolarization leads to an increase in the rate constants for the transition of the substrate-bound transporter from the extracellular to the intracellular side of the membrane and for the return of the unbound site to the extracellular side of the membrane. Hence, the membrane potential may participate in the regulation of cationic amino acid transport. The steady-state distribution of arginine in mammalian cells indicates that influx is favored. This may result from the asymmetric recognition of substrates on either side of the membrane, since higher substrate affinity and rate of transport from the extracellular side of the membrane occur. The use of the analogue 4-amino-1-guanylpiperidine-4-carboxylic acid (GPA) demonstrates differences in substrate recognition depending upon the interior or exterior location of the substrate with respect to the membrane (White and Christensen, 1982*b*). Their data show that GPA inhibits lysine influx, but not efflux, and that extracellular GPA *trans*-stimulates the efflux of intracellular homoarginine, but not of GPA. Conversely, intracellular loading with GPA fails to induce *trans*-stimulated influx of arginine, whereas homoarginine is a strong stimulant (White *et al.* 1982).

Trans-stimulation of y^+ transport systems

Trans-stimulation, in which increased intracellular concentrations of substrate stimulate amino acid influx, has been demonstrated for the system y^+ (White and Christensen, 1982*b*). Efflux studies of radiolabeled arginine in *Xenopus* oocytes show that mCAT-1-injected oocytes are most sensitive to arginine *trans*-stimulation, mCAT-2a-injected oocytes are least sensitive, and mCAT-2-injected oocytes are intermediately sensitive. These apparent differences in *trans*-stimulation may be explained by the lower K_m value of the mCAT-2a protein for arginine. If the intracellular concentration of arginine were made equal to the K_m values for transport by mCAT-2a, mCAT-2 and mCAT-1, it is possible that the differences in *trans*-stimulation would be minimized. Chimeric proteins of mCAT-1 substituted with the divergent regions of either mCAT-2 or 2a, or mCAT-2 replaced with the corresponding mCAT-1 region, elicit *trans*-stimulation

properties associated with the substituted domain (Closs *et al.* 1993*b*). These findings indicate that the divergent region of mCAT-2/2a and the corresponding region on mCAT-1 contribute to the *trans*-stimulation observed in the *Xenopus* oocyte.

Cationic amino acid transport systems, transporters and accessory proteins

In summary, arginine is one of the three cationic amino acids transported by the functionally defined y^+ transport system (for a review, see White, 1985; Christensen, 1989). It now appears that the mCAT-1 and mCAT-2 gene products are likely to mediate y^+-like transport. The two genes are highly similar in sequence and in structure. They differ in expression; *mCAT-1* appears to represent the 'housekeeping' constitutively expressed form, whereas *mCAT-2* is more restricted in tissue expression and inducible in tissues that also induce nitric oxide synthase (Bandelotova *et al.* 1993; Liu *et al.* 1993; K. Finley, M. Maruyama, A. Barrieux and C. L. MacLeod, in preparation).

Although mCAT-1 and mCAT-2 are thought to be the transporters for system y^+, other proteins, such as rBAT and 4F2hc, mediate or modulate cationic and zwitterionic amino acid transport in *Xenopus* oocytes. These cDNAs have been isolated and their transcribed mRNAs expressed in oocytes. The transport properties of these expressed proteins are similar to the transport systems $b^{o,+}$ and y^+L (Van Winkle *et al.* 1988; Van Winkle, 1992; Devés *et al.* 1992). The encoded proteins contain only one consensus membrane-spanning domain and, hence, are quite distinct from previously cloned transporters that generally possess multiple membrane-spanning regions. It has been postulated that they function as regulatory subunits, although their precise role in controlling transport and/or the intracellular fate of the amino acids in mammalian cells remains to be established (Bertran *et al.* 1992*a*,*b*; Wells and Hediger, 1992; Pickel *et al.* 1993; Hediger *et al.* 1993; Calonge *et al.* 1994). It is possible that individual transporters could associate with other membrane proteins, such as 4F2hc and/or rBAT, in different tissues to modulate or regulate the influx or efflux of amino acids under different physiological conditions (Van Winkle, 1993). The rBAT protein has recently been shown to contain mutations that are associated with the inheritable disease cystinuria (Calonge *et al.* 1994). It is possible that the mCAT genes are implicated in lysinuric protein intolerance, since it has been postulated that this disease results from defects in y^+-like transport (for a review, see Simell, 1989). It is now possible to determine whether these newly described gene products play a role in amino acid transport defects.

We are indebted to Dr Halvor Christensen for stimulating discussion and thoughtful teaching, Dr Lon Van Winkle and Dr Alice Barrieux for critical reading of the manuscript and members of the laboratory for help and advice. Research support was provided by the American Cancer Society, Grant IM653. C.L.M. is a Clayton Foundation Investigator; D.K. is a predoctoral fellow partially supported by USPHS GM07752 Training Grant ACS-IM653.

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