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Regulation of CAT: Cationic Amino Acid Transporter gene expression

Review Article

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Summary. The majority of mammalian cationic amino acid transport is mediated by the transport system y^+ which facilitates Na^+ independent cationic amino acid (arginine, lysine, & ornithine) transport and Na^+ dependent zwitterionic amino acid (glutamine & homoserine) transport. Other transport systems y^+L, $b^{0,+}$ and $B^{0,+}$ also mediate cationic amino acid transport. Their broad substrate specificities and overlapping expression patterns confound biochemical analysis. The isolation of cDNA clones has permitted an analysis of their regulation and opens the opportunity to define the role of each protein in specific cell types. Two genes, *Cat1* and *Cat2* encode transporters with properties similar to the y^+ transport system. The *cat2* gene from the mouse encodes two distinct proteins. mCAT2, and mCAT2A via alternate splicing; each protein has distinctly different transport properties. The regulation of mCAT1, mCAT2 and mCAT2A proteins are reviewed here. The implications of this gene specific regulation on cationic amino acid transport is discussed.

Keywords: Cationic amino acid transport – Arginine transport – Regulation of mCAT2

Abbreviations: VSMC, vascular smooth muscle cells; NO nitric oxide; NOS, nitric oxide synthase; LPS, lipopolysacharide; INF-γ, interferon gamma; RT/PCR, reverse transcriptase, polymerase chain reaction

General overview

Mammalian amino acid transport systems were identified and characterized over the past several decades using their kinetic properties, substrate recogni-

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tion, and their requirement for sodium ions in substrate co-transport (reviewed, Christensen, 1989; Kilberg et al., 1993). The majority of mammalian cationic amino acid transport is mediated by the nearly ubiquitous transport system y^+ which facilitates Na^+-independent arginine, lysine, and ornithine transport and Na^+-dependent glutamine and homoserine transport (Christensen and Antonioli, 1969; Christensen et al., 1969; White and Christensen, 1982; White et al., 1982). Transport systems y^+L, $b^{0,+}$ and $B^{0,+}$ (Devès et al., 1992; Van Winkle et al., 1988; Van Winkle, 1992; Van Winkle, 1993) also mediate cationic amino acid transport in some tissues and organs while transport systems b^{1+}, and b^{2+} are expressed in the developing embryo (Van Winkle, 1992). Two related genes, *mCat1* and *mCat2* encode transporter proteins that exhibit several characteristics of the y^+ transport system (Kim et al., 1991; Wang et al., 1991; Kakuda et al., 1993; Closs et al., 1993c; Kavanaugh et al., 1994).

There are two structural classes of mammalian cationic amino acid transporters, those encoding large, hydrophobic, multiple (12–14) transmembrane-spanning domains (Kim et al., 1991; Wang et al., 1991; Kakuda et al., 1993; reviewed, MacLeod et al., 1994; Closs, 1996) and a second group predicted to span the membrane one to four times (Palacìn 1994; Mosckovitz et al., 1994). There are two known genes in the first category (*Cat* genes) that encode proteins that belong to the class of large, highly hydrophobic transporters; their proteins exhibit many properties of the well characterized transport system y^+ when expressed in *Xenopus* oocytes. The second category of proteins (rBAT/NBAT and 4F2hc) share transport properties with systems $b^{0,+}$ and y^+L respectively (Bertran et al., 1992; Tate et al., 1992; Markovich et al., 1993; Mosckovitz et al., 1994). rBAT and 4F2hc may function as independent transporter proteins or may form a complex with larger pore forming proteins (Palacín, 1994). Interestingly, a detailed analysis of the transport properties of rBAT reveal a potassium exchange function in the transport of zwitterionic amino acids (reviewed, Palacìn et al., 1996). The precise mechanism by which these proteins elicit amino acid transport remains to be elucidated although there is important evidence that mutations in the rBAT gene are related to the disease cystinuria (Colonge et al., 1994).

Since distinct biochemically defined transport systems each recognize multiple, sometimes overlapping substrates that are frequently co-expressed in the same tissue or cell type, the isolation of cDNAs that encode transporter proteins represent a significant advance (reviewed Kakuda and MacLeod, 1994; Molandro and Kilberg, 1996). However, with few exceptions, it has not been possible to directly assign a cloned gene to a specific, previously defined transport system. There are several reasons for this gap between knowledge of transporter genes and their cognate transport systems: (1) The transport characteristics of proteins encoded by cloned mammalian cDNAs are usually determined by expressing their cRNAs in *Xenopus leavis* oocytes because they have low endogenous amino acid transport activity and efficiently express these transporters (Van Winkle, 1993). However, their transport properties may not entirely mimic that in mammalian cells. (2) The fact and/or possibility of genetic redundancy. When co-expressed in the same cells,

transport properties of the individual transporters proteins might be difficult to distinguish. (3) Most cells co-express distinct transport systems that have broad and overlapping substrate specificity. (4) Transport systems identified in tissues and cells may derive some of their characteristics from the presence or absence of transport modifiers which may not be present in *Xenopus* oocytes. If so, it may explain that oocytes expressing mCAT proteins do not mediate efficient Na^+ dependent glutamine or homoarginine transport, whereas these substrates are classically recognized by transport system y^+. Similarly, *Xenopus* oocytes expressing rBAT protein transport cysteine, even though it is not a classic substrate for system $b^{0,+}$ (Van Winkle et al., 1988; Bertran et al., 1992).

Additional challenges also confound progress on these transporter proteins such as a severe paucity of immunological reagents of high specificity; especially those directed to specific functional domains. Even more problematic is a complete lack of information on the structure of these proteins and little information on their topological arrangement within membranes. In this volume, two reviews regarding different aspects of the rBAT, 4F2hc transporters are presented (Palacìn et al., 1996, Tate, 1996). Ellen Closs reviews aspects of cationic amino acid transporters (CATs) that include the function of rodent CAT1 as a retroviral receptor, the identification of a liver isoform of mouse mCAT2, important functional domains of CAT proteins, conservation these transporters in mammalian species, and the relation of arginine transport to the production of nitric oxide. In this report we focus on the expression and regulation of the two genes, the arrangement of the multiple mCAT2 promoters, their utilization in different physiological states, and some of our unpublished experience with the production of immunological reagents.

Characterization of the mCAT transporters

Isolation of the mCAT transporters

The mCATs were the first mammalian amino acid transporter cDNAs isolated and both were cloned serendipitously (Albritton et al., 1989; MacLeod et al., 1990a, 1990b). The original isolation of mCAT1 (previously called Rec1 or ERR), was identified by its capacity to function as an ecotropic retroviral receptor (Albritton et al., 1989). Although the mCAT cDNAs share very weak sequence similarity with yeast and bacterial permease genes, it was on the basis of their predicted structural similarity that their natural function as amino acid transporters was discovered (Kim et al., 1991; Wang et al., 1991; Reizer et al., 1993; Kakuda et al., 1993).

The original mCAT2 cDNA was isolated by subtractive hybridization (MacLeod et al., 1990a) from a mouse T-lymphoma cell line and the gene locus initially named *Tea* when it was mapped to chromosome 8 (MacLeod et al., 1990b). When we obtained several full length cDNAs and determined the function of the expressed protein it was renamed mCAT2 to indicate it's functional and genetic similarity to mCAT1 (Reiser et al., 1993; Kakuda

et al., 1993; reviewed, MacLeod et al., 1994). Using the Tea probe, Cunningham's laboratory and the Kabat group independently isolated a distinct isoform of mCAT2 from liver which was also initially designated mCAT2 (Closs et al., 1993a) and later changed to mCAT2A or 2α (Closs et al., 1993c; Kavanaugh et al., 1994). Subsequent to our reports, two papers described the isolation and characterization of cDNAs identical to mCAT2/Tea cDNA which were designated mCAT2B or 2β (Closs et al., 1993c; Kavanaugh et al., 1994). Table 1 provides several alternate names that have been used for these proteins. Some confusion remains regarding the nomenclature of the mCAT2 gene products. We propose the designation mCAT2 for the lymphocyte isoform on the basis of functional and genetic similarity to mCAT1 and the term mCAT2A for the liver isoform as suggested by Closs et al. (1993c) to indicate the distinct transport properties of this carrier. Our proposed terminology (MacLeod et al., 1994) has been adopted by several members of the transport community (Palacín, 1994; McGivan and Pastor-Anglada, 1994; VanWinkle et al., 1995; Finley et al., 1995; Molandro and Kilberg, 1996; Stevens et al., submitted). A review by Closs (1996) employs the term mCAT2 (B) to indicate that mCAT2 and mCAT2B name the same protein.

The properties of mCAT mediated amino acid transport

The genes encoding mCAT1 and mCAT2 proteins are located on mouse chromosomes 5 and 8 respectively (Kozak et al., 1990; MacLeod et al., 1990b) and when expressed in *Xenopus* oocytes exhibit high affinity, low capacity trans-stimulated cationic amino acid transport activity (Kim et al., 1991; Wang et al., 1991; Kakuda et al., 1993; Closs et al., 1993c). The *mCat2* gene encodes two distinct protein isoforms, mCAT2 and mCAT2A that differ in a 41–42 amino acid segment (Closs et al., 1993a, Kavanaugh et al., 1994). Since a single genomic fragment contains both exons, the isoforms clearly result from mutually exclusive alternate splicing of the primary transcript (DK and CM, unpublished). The mCAT2A protein mediates cationic amino acid transport that has a substantially lower apparent affinity (higher K_m), but a higher capacity (higher V_{max}), and it is not strongly trans-stimulated, features that distinguish it from mCAT2. All three proteins mediate the Na^+-independent transport of the L-isomer forms of arginine, lysine, ornithine, and protonated histidine. Both mCAT1 and mCAT2 but not mCAT2A mediate detectable Na^+-dependent transport of cysteine, leucine, and homoserine (Kakuda et al., 1993; Kavanaugh et al., 1994). An elegant exposition of biochemical and kinetic parameters of mCAT1 transport in single *Xenopus* oocytes provides concepts for the mechanisms by which transport and trans-stimulation are accomplished for these facilitated transporters (Kavanaugh, 1993).

The mCAT2 isoforms differ in only 20 amino acids within a domain of 41 amino acids, yet their transport properties (apparent K_m, V_{max}, and sensitivity to trans-stimulation) are quite distinct. These data suggest that their transport properties are determined by this small domain (Closs et al., 1993a; Kavanaugh et al., 1994). This posulate was confirmed by preparing chimeric

proteins among mCAT1 mCAT2 and mCAT2A in which this domain was exchanged (reviewed, Closs, 1996). The data indicate that substrate recognition and perhaps the mechanism of substrate translocation are established by this protein domain. A reassessment mCAT kinetic data suggests both a high and a low affinity component for each protein (Van Winkle et al., 1995). This analysis indicates that each protein can assume two kinetically distinct conformations, although this remains to be confirmed by more extensive kinetic analysis. However, when the arginine transport data was assessed using an area-based, rather than volume-based Hofstee plot, both mCAT1 and mCAT2 display linear kinetics while mCAT2A transport data indicate two-component transport kinetics. If the validity of a calculation of transport kinetics in two-dimensions (area) rather than in three-dimensions (volume) is confirmed by a more complete mathematical verification, it may be useful in assessing future transport kinetic studies.

Regulated expression of mCAT genes

Virtually all cells (except liver) express mCAT1 and y^+ transport which is considered to be the major cationic amino acid transport system (White and Christensen, 1982). The *mCat1* and *mCat2* genes encode proteins with transport properties similar to the y^+ transport system. However both mCAT1 and mCAT2 proteins share highly similar transport properties, a fact that raises questions regarding the purpose of this apparent gene redundancy. Further questions surround the requirement for two highly dissimilar proteins encoded by the *mCat2* gene. What is the reason two proteins with nearly identical transport properties (mCAT1 and mCAT2) are required, and why does mCAT2A exhibit such distinct transport properties (Closs et al., 1993a; Kavanaugh et al., 1994)? It is possible that tissue specific or regulatory requirements could necessitate two genes performing similar functions and that similar signals in different tissues lead to changes in splicing that have profound effect on transport properties. These questions have led to the investigation of the regulation of the two genes and their expression patterns in normal and manipulated tissues and cells. In the following sections, the expression characteristics of mCAT mRNA and protein (when possible) is presented and discussed. It is not a comprehensive review of this subject and we apologize to those investigators whose research was not included.

Expression pattern of the mCAT genes in normal tissues

The expression of the *Cat* genes have been examined by Northern analysis and by reverse-transcriptase polymerase chain reaction (RT/PCR) and the data are summarized in Table 1. All tissues or cell types examined express at least one of the *mCat* genes, in some tissues both genes are expressed, while liver is the only tissue that exclusively expresses mCAT2A and no mCAT2 or mCAT1. In fact, the *mCat1* gene is expressed nearly ubiquitously except in

Table 1. Expression of mCAT transcripts in mouse or rat tissue

Alternate transporter names	**mCAT1** EcoR, REC1, ERR MLV-R, $y1^+$	**mCAT2** [mCAT2B] Tea, mCAT2-β	**mCAT2A** $y2^+$, mCAT-2α mCAT-2
Brain	++	+++	−
*Astroglial cells (steady state)	++	−	−
(LPS + INF-γ)	++	++++	−
Heart	+	+/−	+
Intestine (large)	+++	+/−	−
(small)	++	−	−
Kidney	++	−	−
Liver (steady state)	−	−	++++
(regenerating)	+ (Transient)	−	++++
Lung	++	++++	+
Macrophages (resident)	++	−	−
(activated)	++	++	−
Ovary	++	++	++
Skeletal muscle	+/−	++	++
(post trauma)	+	++	++++
Skin	+++	++	+++
Splenocytes (quiescent)	+++	−	−
(activated)	+++	++	−
Stomach	+	++	++
Testis	++	++++	+
Thymocytes	++	++	N/D
Uterus	++	+++	+/−
#Vascular Smooth Muscle Cells	++	+	ND
(activated with IL-1β + TNF-α)	++	+++	ND
SL 12.4 Thymoma cells	++	+++++	+/−
SL 12.3 Thymoma cells	+++	−	−

The nomenclature was recently reviewed in Malandro and Kilberg, (1996). The term mCAT2 for the high affinity form emphasizes it's similarity to mCAT1 and was the first of the two forms to be isolated. The mCAT2A has a lower affinity and is most predominent in liver (Closs et al., 1993a; Kavanaugh et al., 1994). The number of pluses indicate the relative amount of the two mCAT2 isoforms present in the RNA of tissues harvested from normal mice. The data for mCAT1 was from Nothern analysis (Kakuda et al., 1993), except for * CNS astroglial cells (Bruce Stevens, personal communication, submitted) and #Vascular smooth muscle cells (Gill et al., 1996). The mCAT1 values are not comparible to the mCAT2/2A data which were generated by semi-quantitative RT/PCR using a series of nested oligonucleotide primers, similar to the approach previously reported (Finley et al., 1995). *ND* not determined.

liver (Table 1). In contrast, *mCat2* gene expression is restricted to a more limited number of tissues and cell types, including the liver. The exclusive expression of mCAT2A isoform in the liver is consistent with the previously identified low affinity cationic amino acid transporter (White and Christensen, 1982). Furthermore, mCAT2A mRNA is abundantly expressed in skeletal muscle under some specific conditions (Finley et al., 1995; DK and CM, unpublished data).

Regulation of cationic amino acid transport in T-cells and thymocytes

Arginine and lysine play important roles in the mitogenic activation of T cells apart from protein synthesis (Segal, 1992). Both genes are expressed in the thymus where stem cells undergo differentiation to T-cells; however, only mCAT1 mRNA is constitutively expressed in mature resting and activated T-cells. A cell line model system of thymocyte differentiation is represented in SL12 thymoma cell clones and illustrates developmental regulation of mCAT2 during thymocyte maturation (Wilkinson et al., 1991). From this model system the first mCAT2 cDNA was identified from a clone of intermediate thymocyte maturation, but was not expressed in a less mature, related cell clone (MacLeod et al., 1990a). When fully mature, normal thymocytes leave the thymus and migrate to Peyer's patches, spleen, lymph nodes and peripheral blood. The *mCat2* gene is down-regulated in these mature cells until activated by mitogens or antigen (MacLeod et al., 1990b; Kakuda et al., 1993). Quiescent lymphocytes from spleen, Peyer's Patches and lymph nodes constitutively express mCAT1. In spite of this mCAT1 expression, peripheral blood lymphocytes and quiescent splenocytes exhibit little lysine transport via systems y^+ or y^+L (Boyd and Crawford, 1992; Devés et al., 1992). Upon activation however, T-cells rapidly accumulate mCAT2 mRNA with kinetics similar to a strong increase in transport system y^+ activity (Boyd and Crawford, 1992). Activation also results in the transient induction of mCAT1 mRNA that shows different kinetics (Finley, 1993). Since there is an absolute requirement for arginine during T-cell activation, the induction of mCAT2 gene expression may be important (Moriguchi et al., 1987; reviewed, Segal, 1992).

Induction of mCAT2 mRNA in macrophage activation

Macrophages play a central role in both antigen independent and antigen specific immune responses. The release of nitric oxide (NO) is an important mediator of macrophage function (Takema et al., 1991; Nathan, 1992; Mosckovitz et al., 1994) and arginine is the sole amino group donor for the enzyme nitric oxide synthase (NOS, Mondada and Higgs, 1993; Hibbs et al., 1987). Macrophages activated with lipopolysacharide (LPS) and gamma interferon (INF-γ) rapidly accumulate mRNA encoding the inducible form of NOS (iNOS) and produce large amounts of nitric oxide (Ding et al., 1988; Lorsbach et al., 1993; Sato et al., 1991; Stuehr et al., 1989; Xie et al., 1992). In parallel with iNOS induction, macrophages exhibit an increase in cationic amino acid transport mediated by system y^+ (Baydoun et al., 1993). Exogenous arginine is required to sustain the high levels of NO synthesis in cytokine and endotoxin treated macrophages (Iyengar et al., 1987; Wu and Brosnan, 1992). Hence, iNOS and arginine transporter co-induction in activated macrophages may together facilitate NO synthesis. The transporters mCAT1, mCAT2, rBAT, and/or 4F2hc may mediate arginine uptake in activated macrophages although the mCAT proteins are likely candidates because they exhibit transport properties consistent with the induced macrophage system y^+ (Sato et al., 1991).

In the macrophage cell lines Raw 264.7 and J774, mCAT1 but not mCAT2 is expressed. Following activation with LPS and INF-γ the high affinity arginine transporter mCAT2 (but not mCAT2A) mRNA accumulates (DK and CM, unpublished data; Closs et al., 1993c). In a similar manner, cytokines induce resident peritoneal macrophages to accumulate mCAT2 mRNA. If mice are treated with thioglycolate (an irritant), inflammatory macrophages harvested subsequently show a similar accumulation of mCAT2, but to a lesser extent (DK and CM, unpublished data). Hence mCAT2 mRNA accumulation correlates well with increased system y^+ activity in these cells. This induction is similar to what is observed in mature resting T-cells that also constitutively express mCAT1, but no mCAT2 until the cells are activated (MacLeod et al., 1990b; Finley et al., 1995).

Knowledge of transporter gene expression in activated macrophages will improve our understanding of arginine and NOS inhibitor uptake and possibly lead to improved NOS inhibitors. Several NOS inhibitors structurally resemble arginine (Schmidt et al., 1995) and uptake of these inhibitors is mediated through previously identified transport systems. The transport of the cationic NOS inhibitor L-N-methyl-arginine (L-NMA) is mediated by system y^+; whereas the dipolar inhibitor L-N-nitro-arginine (L-NNA) enters the cell via transport systems L and T (Schmidt et al., 1995). Either or both of the mCAT proteins could mediate transport system y^+ arginine and NOS inhibitor uptake.

Regulation of mCATs in vascular smooth muscle cells

Vascular smooth muscle cells (VSMC) are known to secrete NO under various physiological stimuli. A recent report from Girgor's group (Low and Grigor, 1995) demonstrate a substantial increase in mCAT1 mRNA in response to angiotensin II (Ang II) in VSMC which correlated with stimulated arginine uptake and efflux rates that were dependent on protein synthesis and blocked by valsartan, a specific Ang II (subtype-1) antagonist. Subsequently, they found that Ang ll has no effect on iNOS or mCAT2 mRNA. In contrast, IL-1β/TNF-α treatment increases both mCAT2 and iNOS mRNA while mCAT1 mRNA levels remain constant (Gill et al., 1996). In this volume, Rivera-Correa et al. (1996) present data on the parallel inhibition of IL-1β induced arginine transport and nitric oxide synthesis by angiotensin II in vascular smooth muscle cells. In their study, Ang II had no effect on y^+ transport but it decreased arginine transport mediated by $B^{0,+}$.

Expression of mCATs in regenerating liver and stress induced skeletal muscle

Replicating hepatoma cells and cultured hepatocytes express both mCAT1 and mCAT2A mRNA although only mCAT2A is expressed in the intact rodent liver. However, human liver membranes transport arginine mediated by both high and low affinity systems that are sodium independent (Inoue

et al., 1993). Earlier studies of fresh liver slices from rodents failed to demonstrate high affinity y^+ transport activity but did observe a low affinity transport (White and Christensen, 1982). The fact that only mCAT2A is expressed in the adult liver is consistent with the detection of a low affinity system y^+ activity (Kakuda, 1995; Kakuda, in preparation). In regenerating mouse liver, we found no change in mCAT1 or mCAT2A expression 24 or 48 hours after partial hepatectomy (Finley, 1993; Kakuda et al., 1993). In independent studies no CAT1 protein was detected in regenerating rat liver 12 or 24 hours post-surgery (Closs, et al., 1993c). However, a more extensive analysis of rat liver regeneration was reported by Hartzoglou and her colleagues (Wu et al., 1994) which demonstrated clear induction of mCAT1 expression in the liver between 2–6 hours following partial hepatectomy. Further, mCAT1 mRNA was induced following treatment with insulin, dexamethasone or arginine (Wu, et al., 1994). It is not known whether mCAT2 mRNA changes very early after partial hepatectomy or in response to hormones.

In our liver regeneration experiments mCAT2 expression was also examined in tissues other than liver. To our surprise, a 9 fold increase in mCAT2 skeletal muscle mRNA was detected 24 hours following partial hepatectomy. To a lesser extent, mCAT2 was also induced in skeletal muscle following other surgical trauma and fasting (see Fig. 3 in Finley, 1993). In skeletal muscle, mCAT1 mRNA is barely detectable, while mCAT2 mRNA is somewhat more prevalent (Kakuda et al., 1993). In these stress conditions the mCAT2 and mCAT1 mRNA levels remain constant while only the mCAT2A isoform is induced (Finley, 1993). This isoform may facilitate the export of cationic amino acids released from skeletal muscle proteolysis which occurs during trauma and sepsis (Clowes et al., 1980). A low affinity, high capacity transporter such as mCAT2A could prevent the depletion of the cationic amino acids from skeletal muscle following stress. In contrast, the same manipulations that induced mCAT2A is skeletal muscle elicited no alteration in the amount of mCAT mRNA in a non-striated muscle, the uterus. From these examples it is clear that mCAT1 and mCAT2 transcripts are differentially regulated in some tissues following partial hepatectomy. In the case of skeletal muscle, the splicing of the mCAT2 mRNA to encode either the high or low affinity isoforms is also regulated (DK and CM, unpublished data).

Promoter analysis of mCAT2

Analysis of variability in the 5′ UTR of mCAT2 transcripts

mCAT2 and mCAT2A are products of the same gene yet they show tissue specific expression and they are specifically regulated in response to physiological stress. An analysis of mCAT2 promoter region was undertaken because we found that transcripts from a single thymoma cell line exhibit several distinct 5' UTRs (Finley, 1993; Finley et al., 1995). We sought to determine whether this 5' sequence variation resulted from the activity of several distinct promoters or whether the transcripts originated from a single promoter,

which was then alternatively spliced downstream. Several thymoma mCAT2 cDNA libraries were prepared using a region of mCAT2 common to both isoforms. Of several hundred mCAT2 clones obtained, 22 contained additional 5' untranslated sequence. Their DNA sequences identified 5' exon 1 isoforms 1a, 1b, 1b/c, 1c and 1d (Finley et al., 1995). These 5' isoforms splice into a common sequence 16 bp 5' of the first AUG start methionine codon. Isoform 1b/c and 1c exhibit variability at the splice junction (−27 bp to −17 bp; Finley et al., 1995). An additional 5' UTR sequence was reported from a liver cDNA clone (Kavanaugh et al., 1994) that was completely distinct from the sequences we obtained from lymphoma cells and is denoted 1e. It is not yet clear whether this cDNA was synthesized from incompletely processed RNA (DK, unpublished data) or represents an additional 5' UTR form of mature mRNA (see below).

mCAT2 promoter regions

A sketch of the organization of the 5' region of the *mCat2* gene is shown in Fig. 1 which illustrates that these regions are widely spaced from the first coding exon. At least 9 Kb separate exon 1a from exon 1b, 1c is adjacent to 1b, while an additional 9 Kb (at least) separate exon 1c from the first coding region. Genomic DNA sequences in the regions surrounding exons 1a, 1b, and 1c (Finley et al., 1995) identified typical promoter motifs adjacent to each of these 5' UTR exons. The region surrounding exon 1d was not identified due to the highly reiterated sequences in this region. The promoter upstream of exon 1a has a configuration consistent with the transcriptional start sites of a number of well characterized TATA-less genes that show staggered initiation, a feature of mCAT2 transcripts from this promoter. The region is GC-rich and contains several high affinity SP1 binding sites and CAC boxes. Our data indicate it is the most active promoter in all tissues examined (DK and CM unpublished data). The exon 1b promoter is a typical TATA promoter, it contains several CAAT boxes and a single TATA box 25 bp 5' of the transcription start site. It also contains putative enhancer motifs for different sets of regulatory proteins. Both 1a and 1b 5' regions can function as promoters in skeletal muscle and SL 12.4 T-lymphoma cells. Exon 1c is sometimes spliced to 1b and in some circumstances the 1c promoter initiates mCAT2

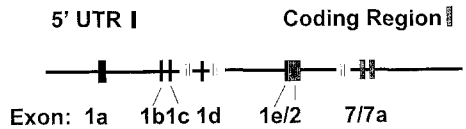


Fig. 1. Promoter structure of the *mCat2* gene. A schematic of the arrangement of the 5′ untranslated exons (UTRs) shown as filled dark bars and exon 2, which is the first coding exon, shown as a stippled bar. Exons 7/7a represent the exons that undergo mutually exclusive alternate splicing to encode transcripts for the two protein isoforms mCAT2 and mCAT2A. Transcripts isolated from the T-lymphoma cell line SL12.4 express exons 1a, 1b, 1c, or 1d that result from alternate promoter usage (Finley et al., 1995)

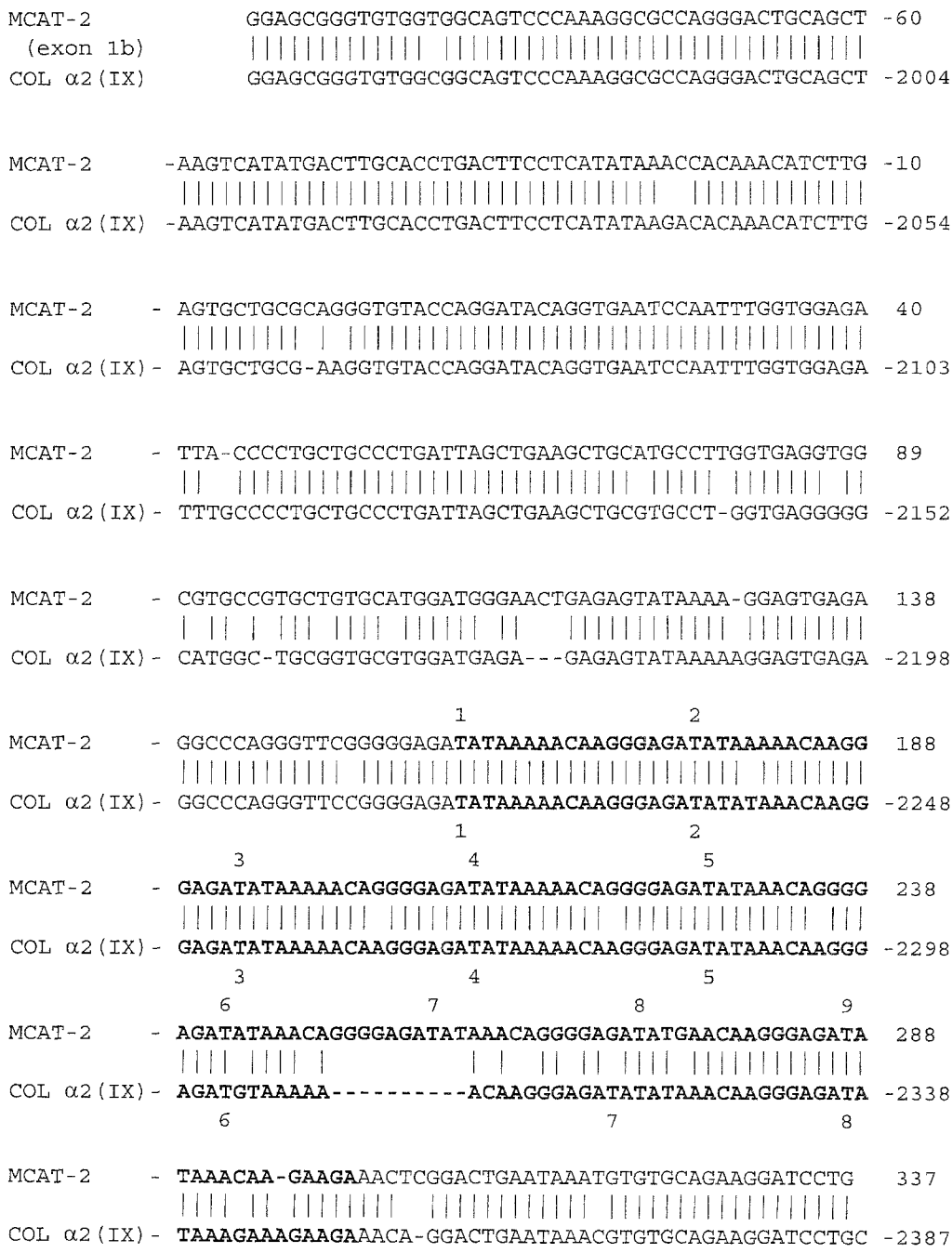


Fig. 2. Sequence comparison of the mCat2 promoter with an enhacer region of collagen. The DNA sequence of a region of mCAT2 which is located at least 13 kb 5′ of the first coding exon (exon 2) is shown aligned with an enhancer region of collagen *α2* (IX). The numbers above the sequence mark the ATG rich, reiterated sequences. There are 8 copies of the reiterated sequence in the mCAT2 sequence and 7 copies in the collagen enhancer sequence. The collagen sequence was obtained from genebank assession number z22923

transcription. It has similarity to the TATA-less promoter for thymidylate synthase.

A striking sequence similarity was found between the enhancer portion of the murine alpha 2 (IX) collagen gene (Swiderski and Solursh, 1992) and exon 1b with it's downstream sequence. The region of 90% sequence identity comprises over 443 bp (Fig. 2). This is a striking level of identity between non-coding regions of two unrelated genes. The region includes and A/T-rich oligomer (TATAAAAACAGGGGAGA) that is reiterated 8 times within one promoter region of the *mCat2* gene and 7 times in the collagen gene. Because A/T-rich DNA sequences are recognized by DNA binding proteins, (Duncan et al., 1994) they have been postulated to facilitate the concentration and localization of transcription factors in active regions of chromatin (Jenuwein et al., 1993). Hence, this region might function as a regulatory sequence modulating access of specific factors to their respective enhancers or silencers or could regulate the activity of distantly located promoters and thereby control one or more of the mCAT2 promoters.

The tissue specific expression of the two known *mCat* genes demonstrates their expression is under some regulation (Table 1). Because *mCat2* transcription is initiated at several widely spaced promoters, it is reasonable that their activation might respond to specific environmental signals (Finley et al., 1995). The promoter arrangement of mCAT1 has not been reported, but no variation has been found in the 5' UTR (J. Cunningham, personal communication).

Utilization of the mCAT2 promoters

The utilization of the mCAT2 promoters was determined using reverse transcription (RT)/PCR assays. PCR product specificity was confirmed with nested, isoform specific probes (Finley et al., 1995). These data document that all these promoters are used in the cell line from which the cDNAs were isolated and none are used in a sister cell line that does not accumulate detectable mCAT2 RNA. In every cell and tissue type examined, promoter 1a predominates. This promoter lies at least 19 kilobases 5' of the first coding exon. Liver cells use promoter 1a to the exclusion of all the others. Skeletal muscle uses the promoter adjacent to exon 1a and, when undergoing catabolism following stress, both 1a and 1b promoters are used. Table 1 lists a number of conditions in which mCAT2 transcripts accumulate. The promoter utilization in these conditions is currently under investigation. The SL 12.4 cell line, from which the original mCAT2 cDNA was cloned, uses at least four promoters, 1a, 1b, 1c, and 1d (Finley et al., 1995). In summary, the tissues previously shown to express mCAT2 transcripts by Northern analysis (Kakuda et al., 1993) were found to accumulate RNA transcribed from the 1a promoter most predominately. The preponderance of promoter 1a usage in both liver (where the mCAT2A isoform is exclusively expressed) and in macrophages (where only the mCAT2 isoform is expressed) demonstrate that the promoter used does not dictate downstream splicing events that give rise to the two protein isoforms.

Detection of mCAT protein expression

The analysis of transport regulation mediated by the mCAT proteins requires the capacity to detect the proteins that comprise the transport systems. It is quite possible, even probable, that important steps in the regulation of mCAT expression occur via posttranscriptional and translational events. To begin such an analysis requires useful, highly specific antisera or monoclonal antibodies.

mCAT protein expression

The investigation of mCAT1 protein expression has exploited the fact that it serves as a retroviral receptor (Albritton et al., 1989). Expression can be detected on cycling cells by virtue of their infectivity or by employing a gp70 viral coat protein binding assay (Wang et al., 1991; Closs et al., 1993c; Wu et al., 1994). Virus infectability was used to document changes in mCAT1 protein expression in liver because normal liver does not express mCAT1, but it is induced following specific treatments (Wu et al, 1994). Ecotropic retrovirus containing the β-galactosidase gene (β-gal) was used by Closs et al. (1993b) in experiments that failed to detect induction of mCAT1 in regenerating liver but could identify mCAT1 protein expression in cultured mouse hepatocytes, hepatoma and fibroblast cell lines, and mCAT1 transfected CHO-K1 cells. However, a similar, more detailed analysis demonstrated transient mCAT1 induction in liver tissue of animals treated with insulin, dexamethasone, arginine and during liver regeneration (Wu et al., 1994). In most cases, virus infection was correlated with mCAT1 mRNA expression. In a similar vein, viral coat protein gp70 binding demonstrated mCAT1 protein expression either directly with ^{125}-Iodide labeled gp70 (Kim et al., 1991) or indirectly (Wang et al., 1991). Expression of mCAT1 protein in *Xenopus* oocytes was also documented using gp70 binding assays (Kim, et al., 1991). Unfortunately, mCAT2 and -2A do not appear to mediate viral entry (Closs et al., 1993a; Kavanaugh et al., 1994) although it permits discrimination of mCAT1 and mCAT2/2A protein expression.

Several groups have made rigorous attempts to generate mCAT1 antisera, but only a few have been successful. For example, either TrpE or GST fusion proteins (Closs et al., 1993a, 1993c) or synthetic oligopeptides linked to carriers (Woodward et al., 1994) were used as antigens. Western blots demonstrated that mCAT1 expressed in *Xenopus* oocytes is glycosylated (Closs et al., 1993a). Interestingly, immunocytochemical analysis using one of these antisera demonstrated mCAT1 (or a related) protein is localized in clusters on the plasma membrane of human fibroblasts and no protein was detected in human or rat liver (Woodard et al., 1994).

Antisera that can discriminate the mCAT2 and -2A isoforms will be required to accurately determine their localization within polarized cells since physiological studies have noted different cationic amino acid transport systems to basolateral or apical faces of intestinal epithelia and placenta cells (Moe, 1995). When appropriate reagents become available, it will be possible

to assess cell surface expression, determine whether the proteins are stored in intracellular compartments awaiting a cellular signal (like Glut 4), or are consitutively expressed in one cellular location. Further, such reagents are required to associate protein expression with transport properties to permit a more complete understanding of the relation between mRNA encoding putative transporters with a specific mammalian transport systems such as system y^+. In this regard, antibodies that block substrate recognition, like those we previously produced to the EGF receptor (MacLeod et al., 1986), would be highly desirable.

Although our attempts to produce specific antisera enjoyed limited success, several potentially useful mCAT2/2A vectors were constructed and are offered to encourage continued efforts. The reason for the limited success in producing antisera may result from the highly hydrophobic nature of the proteins. Initial efforts employed synthetic oligopeptides (carboxyl domain) of mCAT2. While the sera were reactive with the immunizing antigens, neither reacted with intact mCAT2 protein (Finley, 1993).

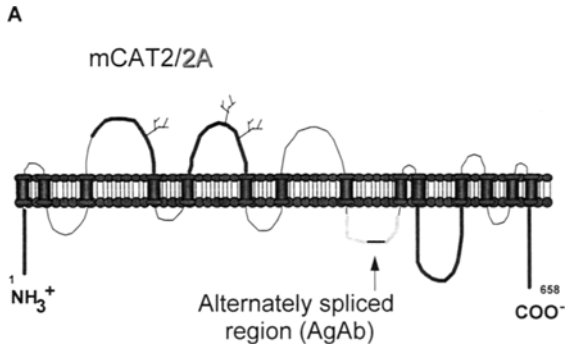
Fusion proteins produced in bacterial cells

Regions of mCAT1 and mCAT2 encoding hydrophilic carboxy-terminal domains were inserted in pGEX-3X and pATH vectors and expressed as fusion proteins with glutathione-S-transferase (GST) and TrpE (Closs et al., 1993a). Western blot analysis of protein lysates from *Xenopus* leavis oocytes injected with either mCAT1 or mCAT2 detected the predicted proteins with the appropriate mCAT antisera. Further, only mCAT2 (not mCAT1) was detected in liver lysates (Closs et al., 1993a) and it's apparent size was reduced by N-glycosidase treatment. The data are consistent with a pattern of glycolsylation similar to mCAT1. Similarly, mCAT1 glycoslyation was determined using mCAT1 antisera for immunoprecipitations (Kim and Cunningham, 1993).

mCAT2-GST-fusion protein antisera directed towards five hydrophilic domains (Fig. 3) were tested for reactivity to the immunizing antigen and its capacity to detect mCAT2 protein in cell lines and liver lysates (Kakuda, 1995). Each of the five antisera preparations recognized the appropriate fusion protein although only antisera produced to the carboxy-terminal fusion protein identified protein in lysates that could be competed with the immunizing antigen. A similar carboxy-terminal region produced a successful antisera (Closs et al., 1993a). If antisera to the other mCAT2 regions noted in Fig. 3 could be produced they would permit experiments to determine the orientation of each hydrophilic domain with respect to the cell membrane.

Antigenized antibodies designed to discriminate mCAT2 and mCAT2A

The two isoforms of mCAT2 are 98% identical and differ only in one short region (41 amino acids) of the protein. Elegant experiments, performed and reviewed by Closs (1996), demonstrated that this short region significantly



B

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
	. **.**...** * *****. * *.. .....* *.** ..** ..

Fig. 3. Regions of mCAT2 protein used for production of antisera. **A** shows the mCAT2 protein as one that spans the membrane 14 times. In other models, the alternately spliced region is oriented extracellularly. The top of the figure is extracellular. The regions of the protein shown as heavy dark grey lines were expressed as GST fusion protein for antisera production. The light grey region represents the alternately spliced region, and the black bar within it is the most divergent region within this sequence that was used to produced antigenized antibodies used as immunogens (see text). **B** shows an alignment of the comparible region on mCAT1, mCAT2 and mCAT2A. The * indicates the amino acids identical in the three proteins, the (•) indicates conservative substitutions. The bold letters contain the region used for antigenized antibody production

alters the transport properties of the mCAT2 isoforms. Our attempts to produce antisera capable of distinguishing the two isoforms using small peptide antigens presented on an "antigenized antibody" (Billeta et al., 1991; Kakuda, 1995) were not successful. Antigenized antibodies (AgAb) are hybrid proteins containing a selected short peptide inserted within an immunoglobulin protein. Since the longest consecutive region of diversity between mCAT2 and mCAT2A proteins is contained within eight amino acid residues (Fig. 3B), the antigenized antibody approach was used in an attempt to generate isoform specific antibodies. Since the AgAb strategy presents short epitopes in a highly immunogenic region of the immunoglobulin protein it resulted in the production of highly specific antisera reactive towards one of two related proteins that had been previously refractive to immunological discrimination (c-src and n-src, Billetta and Zanetti, 1992; Rigaudy et al., 1994; Sollazzo et al., 1989 and 1990).

The most divergent mCAT2 and mCAT2A sequences (Fig. 3B) were introduced into the third hypervariable complementarity determining region (CDR3). The hybrid vector containing the coding region for the mCAT2/2A epitopes were separately introduced and expressed in a cloned B-cell line lacking a functional heavy chain gene. The expressed IgG secreted from these

cells contain the mCAT2 or -2A epitopes; they were separately purified and each of four different IgG/mCAT proteins were used as antigens in rabbits. Although the experiments were a technical success, the purified antisera did not detect the native or denatured mCAT2/2A protein. This considerable effort was made because the mCAT2/2A specific epitopes contain regions within a putative substrate recognition domain (Closs et al., 1993c; Kavanaugh et al., 1994), hence such antisera might competitively inhibit cationic amino acid transport. If so, they would be powerful reagents, since amino acid transport analysis is otherwise complicated by the existence of several different transport systems that mediate cationic amino acid flux (reviewed, Christensen, 1989; Kakuda and MacLeod, 1994). In particular, it has been difficult to discriminate between mCAT1 and -2 mediated transport using their known biochemical parameters. If these antisera can specifically inhibit transport mediated by any one of the mCAT proteins, the analysis of cationic amino acid transport via these proteins would be advanced considerably.

Production of antiserum in more divergent species

It is possible the conservation of the proteins between species may hamper the production of antisera in rabbits that are used by most laboratories. For example, the CAT1 amino acid sequence is 95% identical between rat and mouse and retains 86% sequence identity between mouse and human (Yoshimota et al., 1991; Wu et al., 1994; Puppi and Henning, 1995). A more divergent species such as the chicken may yield higher mCAT antisera titers. This approach is now widely used, but to our knowledge, it has not been attempted for the CAT proteins.

Summary

The two high affinity, low capacity mCAT transporters mCAT1 and mCAT2 are differentially expressed in specific tissues. Both genes are regulated in response to specific physiological stress in distinct tissues. *mCat1* gene expression changes in response to several physiological variables, but is constitutively expressed in all mouse tissues except liver. *mCat2* expression results in the synthesis of two distinct transporters, each of these (mCAT2 or mCAT2A) is found in a much smaller number of tissues. Both forms are inducible in specific cell types, many of which also express the inducible form of iNOS. *mCat2* gene expression occurs from widely spaced, multiple promoters that appear to be controlled by different transcription factors. The multiple promoters do not dictate downstream splicing events that change the functional properties of the mCAT2/2A transporter proteins.

The investigation of cationic amino acid transport expression and regulation requires the development of immune reagents capable of distinguishing each isoform and their localization within cells. Epitope specific antisera directed to functional domains may help determine the topological arrangement of the proteins within the membrane, they could block transport function and provide information on their physiological role in a variety of tissues.

This information, combined with genetic approaches such as targeted gene ablation, tissue specific gene knockout and transgenic overexpression will determine the physiological role and significance of these transport proteins.

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