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Stress differentially induces cationic amino acid transporter gene expression

Donald K. Kakuda [1], Kim D. Finley [2], Michio Maruyama [3], Carol L. MacLeod *

UCSD Cancer Center, Cancer Genetics Program and Department of Medicine, University of California, San Diego, CA, USA

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Abstract

The amino acid L-arginine plays a central role in several adaptive metabolic pathways and we postulate that regulated L-arginine transport contributes to important physiological responses. The majority of L-arginine flux is mediated by transport system y^+ that is encoded by at least three genes, *Cat1*, *Cat2* and *Cat3*. *Cat2* encodes two distinct protein isoforms (CAT2/CAT2a) that differ by 10-fold in their apparent substrate affinity. *Cat2* transcription is controlled by four widely spaced promoters. The expression of CAT2/2a transcripts was tested in skeletal muscle and macrophages following specific stresses or activators. Unexpectedly, CAT2a transcripts accumulated in skeletal muscle in response to surgical trauma (hepatectomy and splenectomy) as well as food deprivation, although neither high affinity CAT2 nor CAT1 were detectably altered. Activated macrophages decreased CAT1 levels, but accumulated CAT2 and iNOS mRNA and protein with parallel kinetics suggesting that CAT2 mediated L-arginine transport might regulate the L-arginine:nitric oxide pathway. In macrophages, liver and skeletal muscle, the most distal CAT2 promoter was predominant. No change in promoter usage was apparent under any stress conditions tested nor was alternate splicing of the CAT2 transcript dictated by promoter usage. The differential regulation of the *Cat* genes indicates their encoded transporter proteins meet different requirements for cationic amino acids in the intact animal.

Keywords: CAT; Arginine; Transport; System y^+; Macrophage; Inducible nitric oxide synthase; T-cell; Hepatectomy; Fasting

1. Introduction

Multicellular organisms regulate amino acid transport in specific organs and tissues in response to physiological demand [1]. The cationic amino acids L-lysine and L-arginine are essential and semi-essential, respectively; hence their transport is required to meet cellular needs and maintain homeostasis [1,2]. Apart from protein synthesis, L-arginine and L-lysine have numerous cellular functions [3,4]. L-Arginine and its transport are essential for the regulated production of nitric oxide (NO) via the Ca^{2+}-independent inducible nitric oxide synthase (iNOS). Not only is L-arginine the sole amino group donor for NO

Abbreviations: iNOS, inducible nitric oxide synthase or NOS_2; NO, nitric oxide; LPS, lipopolysaccharide; IFN-γ, Interferon-γ; ConA, Concanavalin A

* Corresponding author. Present address: UCSD Cancer Center, MC-0684, 9500 Gilman Drive, La Jolla, CA 92093-0684, USA. Fax: +1 (619) 534-7340;
E-mail: cmacleod@ucsd.edu

[1] Present address: ForBio Research Pty Ltd., St. Lucia, Australia.

[2] Present address: Salk Institute for Biological Studies, La Jolla, CA, USA.

[3] Present address: First Department of Surgery, Tokyo Medical and Dental University, Tokyo 113, Japan.

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synthesis, extracellular L-arginine is essential for NO synthesis via iNOS [3–5].

The identification of cationic amino acid transporter cDNAs was an important first step in determining the role of regulated transport in cellular responses (reviewed in [6–9]). The three known *Cat* genes encode similar, perhaps redundant proteins, CAT1, CAT2 and CAT3 [10–16] and they are thought to comprise the functionally defined high affinity transport system y^+ [6–9,14]. The *Cat2* gene, however, encodes two protein isoforms CAT2 [12] and CAT2a [15] that result from mutually exclusive alternate splicing [13]. The CAT2a protein exhibits a significantly lower apparent affinity for its substrates and appears to comprise the low affinity y^+ transport system found in liver [1,9,14]. The recently identified *Cat3* gene is expressed exclusively in adult brain [16].

Unfortunately, it is not possible to kinetically distinguish the three high affinity forms of the transporters and no antisera is available that reliably distinguishes these four known CAT proteins. Hence, assessment of their mRNAs is the only practical way to determine *Cat* gene expression at this time. The CAT transcripts are expressed in distinct patterns [12]. CAT1 transcripts are constitutively and nearly ubiquitously expressed in normal tissues and cell lines, although the amounts vary widely among tissues [12]. The adult liver is *unique* in that it does not express CAT1 [7,10,12]; but rather, it constitutively expresses the low affinity CAT2a isoform exclusively. The constitutive expression of CAT2/2a is more limited; transcripts are abundant in liver, skeletal muscle and stomach as well as activated macrophages and skin [12,13]. The CAT2 gene is transcribed from four distinct, widely spaced promoters in a T-lymphoma cell line [17]. Both *Cat1* and *Cat2* genes are inducible in a variety of circumstances [18–23].

Dietary changes, trauma, immune challenge and some disease states greatly influence cationic amino acid uptake and metabolism [24–28]. Hence, changes in CAT1, CAT2 and CAT2a transcript expression were investigated in the liver and muscle, two organs involved in amino acid homeostasis. Their expression was also assessed in macrophages, a cell type that has special L-arginine requirements [3,4]. Resting splenocytes (largely comprised of quiescent T- and B-cells) express CAT1 mRNA predominately, yet lymphocytes exhibit extremely limited L-arginine and L-lysine transport [29,30]. However, following mitogen or antigen mediated T-cell activation, extracellular L-arginine is required [30] and both CAT1 and CAT2 transcripts rapidly accumulate [12]. Macrophages also require extracellular L-arginine transport for NO synthesis via the inducible form of nitric oxide synthase (iNOS; [3,5,31]). Furthermore, macrophages increase system y^+ transport following activation with lipopolysaccharide (LPS) and interferon-γ (IFN-γ; [32,33]) to provide these cells with adequate L-arginine for NO synthesis. In this study, the expression of CAT1, CAT2 and CAT2a transcripts were assessed in skeletal muscle, liver, and smooth muscle, and from macrophages before and following specific activation or pathophysiological stresses. CAT2 promoter utilization was assessed during each of these stress or activation responses.

2. Materials and methods

2.1. Surgical and dietary manipulation of mice

Female AKR/J mice, 6 weeks of age, were obtained from Jackson Laboratories, Bar Harbor, ME. They were treated in accordance with the University of California, San Diego and NIH guidelines for the humane treatment of laboratory animals. Surgery was performed on mice anesthetized with 70 mg of sodium pentobarbital per kg body weight; either the entire spleen or 60–65% of the liver was removed and RNA prepared. Sham surgery was identical to the partial hepatectomy except the organs remained intact. After 0, 1, 2 or 7 days mice were killed and RNA was prepared from liver, skeletal muscle (soleus and gastrocnemius), and from the uterus of partially hepatectomized, splenectomized or anesthetized control mice. Fasting studies were carried out with mice deprived of food for 24 or 48 h, after which they were killed and RNA prepared from specific organs.

2.2. Preparation of macrophage cell line

J774 mouse macrophages were cultured in RPMI

1640 medium in the presence or absence of lipopolysaccharide (LPS; 100 ng/ml) and IFN-γ (10 U/ml).

2.3. Preparation of radiolabeled probes

Probes for Northern blot analysis were a 2.4 kb *Eco*RI fragment of CAT1 cDNA [10,15], a 2.1 kb *Msc*I fragment of CAT2 cDNA [18], full-length iNOS cDNA (gift from Dr. Hume, [35]), a 0.7 *Pst*I/*Bam*HI fragment from cyclophilin cDNA and a 0.2 kb *Ava*I fragment from 18S ribosomal cDNA (ATCC). Isolated cDNA fragments were labeled with [^{32}P]dCTP by random priming (Amersham) and labeled DNA purified on a NACS-52 column (Gibco BRL). Oligonucleotides (22 mer) encoding CAT1, CAT2, and CAT2a [17], or iNOS (5′-GCAGCGGCTCCATGACTCC-3′) were 5′-end labeled with [γ-^{32}P]ATP.

2.4. RNA preparation and analysis

Preparation of total RNA from cultured macrophages, splenocytes or tissues and Northern blot analysis (10 μg/lane) used standard methods as previously described [18]. The membranes were exposed to XOMAT film (Kodak) within the linear range of detection. Stripping blots and sequentially probing with either 18S ribosomal cDNA assessed the amount of RNA loaded and transferred. Autoradiographs of Northern blots were quantitated by laser densitometry.

2.5. CAT2/2a reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription of total RNA (5 μg) from the mouse cell lines SL12.4 (T-lymphoma) and J774 (macrophage); mouse liver and skeletal and smooth muscle tissues was used to detect CAT2 promoter usage according to methods previously reported [17] and summarized here. The CAT2 and CAT2a isoforms were discriminated by priming the RT reaction with 5′-CGCGAATTCCGACTGTCGTGTGGGCAG-3′ (1453–1470 nt) located in a region 3′ of the alternately spliced exons. The cDNA product was amplified using nested primers located within the transcribed region and flanked the alternately spliced region: 5′-TATCCAGACTTCTTTGCCGTGTGC-3′ (489–513 nt) and 5′-GTAGGCTGAAACCCTGTCCTTGC-3′ (1406–1429 nt). The PCR products were authenticated using CAT2 and CAT2a specific 5′-end labeled oligonucleotides, CAT2: 5′-TCCCAATGCCTCGTGTAATCTA-3′ (1071–1093 nt); CAT2a: 5′-TGCAGTCATCGTGGCAGCAACG-3′ (1159–11810 nt). The details of this assay are contained in [17].

2.6. Western blotting

J774 total cell lysates were prepared by adding 1.0 ml RIPA buffer (1% deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.2, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, 0.5 μg/ml leupeptin) per 10 cm dish [34]. The 12 K supernatant was used for analysis following protein determination by Lowry assay (Bio-Rad, DC protein assay). Total cell lysates (200 μg/lane) were denatured with 6 molar urea and separated by 10% SDS-PAGE. The proteins were electrophoretically transferred to supported nitrocellulose (Trans-blot, Bio-Rad). The membranes were blocked for 2 h (5% milk/PBS/0.1% Tween-20/0.5 M NaCl), treated with anti-CAT2 antisera (1.0 μg/ml) for 1 h with or without treating with glutathione *S*-transferase (GST) or CAT2-carboxyl terminus-GST fusion (pGEX-3X, Pharmacia) proteins (2.5 μg/ml in 4 ml), and secondary goat-anti-rabbit IgG (1:5000, Bio-Rad) for 1 h. The proteins were detected by chemiluminescence (Renaissance, NEN Life Science Products), and the autoradiographs were quantified by scanning densitometry (Bio-Rad). Anti-CAT2 antisera was generated by injecting rabbits with CAT2-GST fusion proteins encoding the carboxyl-terminal 70 amino acids, and the IgG fraction was enriched using protein A column and ammonium sulfate fractionation. Membranes were treated at room temperature for all manipulations, and the anti-sera incubations contained blocking buffer.

2.7. Data analysis

Non-saturated autoradiograms were quantitated following RNA hybridization by densitometer scanning or Phosphoimager analysis. The absorbency ratios of hybridization signals from CAT and control

probes were calculated and the values were compared by the two-sample, two-tailed Student's *t*-test, using the INSTAT program and one way ANOVA followed by Duncan's test using the Number Cruncher Statistical System. Both tests detected the same level of significance.

3. Results

3.1. CAT2 and CAT2a tissue distribution

Prior to the identification of the CAT2a isoform [15], we reported CAT2 transcript expression in a variety of cell lines, tissues and organs [12,18,36]. To determine which CAT2 isoform is expressed in several organs under steady-state conditions, RNA samples were analyzed by RT-PCR (Fig. 1). The low affinity CAT2a isoform is expressed in skeletal muscle and liver, tissues with low amounts of the high affinity CAT1 [12] or CAT2 (Fig. 1A) isoforms. CAT2a mRNA is also predominant in skin. Reciprocal expression of the high and low affinity transporters is reasonable if they play different roles in the import or export of their substrates. CAT1 transcripts are present in all the tissues shown in Fig. 1 except liver [12].

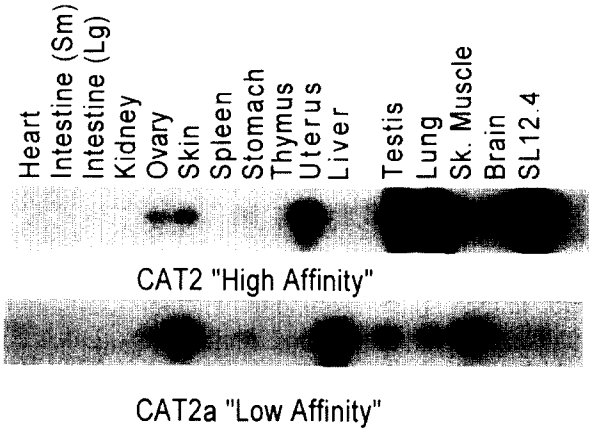

Fig. 1. CAT expression in normal mouse tissues. The two isoforms of CAT2 were distinguished in the 15 indicated mouse tissues by RT-PCR; CAT1 RNA expression in these tissues was previously reported [12]. The reverse transcribed cDNA was amplified using common CAT2/2a primers flanking the unique alternate spliced region and distinguished by probing Southern blots of the amplified products with CAT2 and CAT2a specific oligonucleotides as described in Section 2.

3.2. CAT mRNA expression in unactivated and activated macrophages

Macrophage activation by cytokines and LPS results in the induction of iNOS mRNA and protein together with a coordinate increase in L-arginine transport via system y^+ [14,32,33]. Activated macrophages produce large amounts of NO for a sustained period; a response that forms part of their immunological defense function [3–5]. iNOS mediated NO synthesis requires the transport of extracellular L-arginine (e.g. [31,37]). To determine which transporters contribute to transport system y^+ mediated L-arginine uptake, J774 macrophage cells were activated with LPS and IFN-γ. Northern analysis (Fig. 2A) shows that CAT1 is expressed in unactivated cells and message levels do not increase during activation, but instead, they actually decrease by 24 h following activation. In contrast, CAT2 and iNOS mRNAs increase in parallel from undetectable levels in unstimulated cells to substantial amounts by 3 and 6 h. Only the high affinity CAT2, but not the low affinity CAT2a transcript was detected (Fig. 2B) using RT-PCR. This finding is concordant with reported transport studies and our own work that demonstrates high affinity L-arginine transport is increased during activation ([32,33], Kakuda and MacLeod, unpublished). CAT2 protein is also induced from undetectable levels in unactivated cells to abundant expression in LPS/IFN-γ activated cells (Fig. 2C). From this data, it appears that CAT2 is responsible for the increase in system y^+ transport induced in activated macrophages. We postulate that CAT2 mediated transport is required to supply iNOS with its essential substrate, L-arginine. We tested the utilization of alternate CAT2 promoters during macrophage activation. Like splenocytes [17] only the most distal promoter is used (data not shown).

3.3. CAT mRNA expression in the liver

Normal adult liver expresses exclusively CAT2a transcripts although both established hepatoma cell lines and freshly explanted hepatocytes accumulate CAT1 mRNA [12,38]. It is not clear whether *Cat1* gene expression in cultured normal and neoplastic hepatocytes is triggered by active cell proliferation

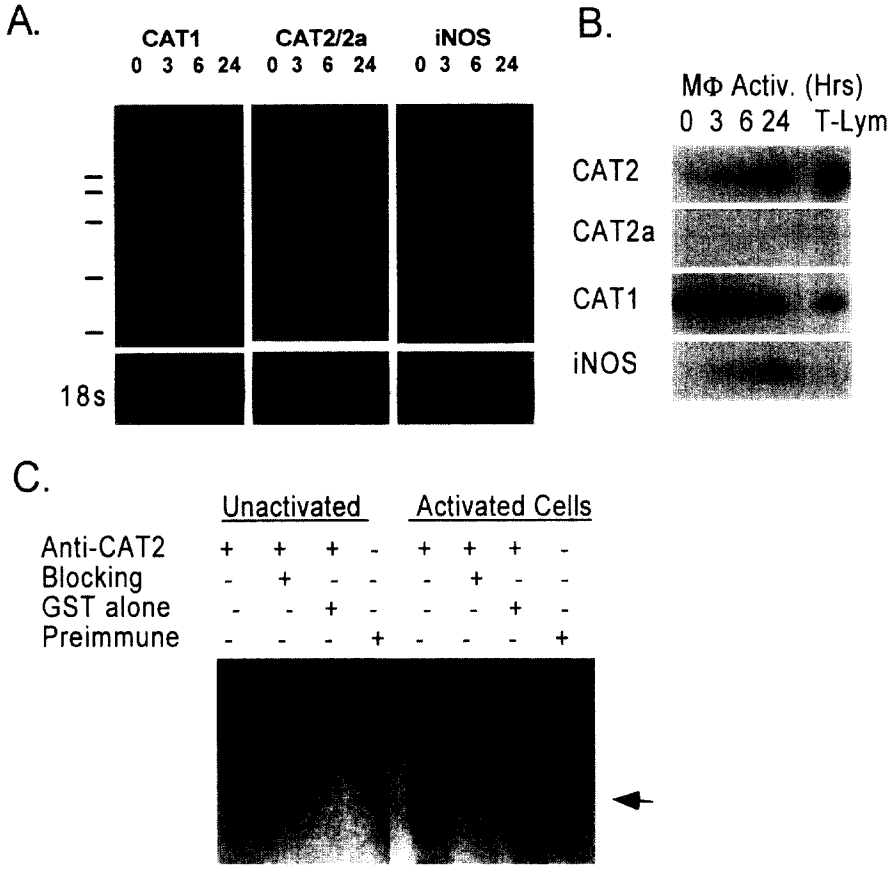

Fig. 2. Coordinate induction of CAT2 and iNOS in activated J774 macrophages. (A) Northern analysis of CAT and iNOS expression from untreated J774 macrophage cells (0 h), or following the addition of LPS and IFN-γ for 3, 6, and 24 h as indicated. Blots were probed with CAT1, CAT2/2a, iNOS and 18S cDNA probes. (B) RT-PCR analysis of CAT and iNOS expression in activated J774 macrophage RNA. (C) Western blot of cell lysates from J774 macrophage cells unactivated or activated for 24 h and probed with anti-CAT2 antisera as described in Section 2. Arrowhead indicates the CAT2 specific reactivity that migrated with an estimated mass of 92 kDa based on molecular mass markers (not shown).

or is a peculiar response of hepatocytes growing in monolayer culture [39]. To address this question, the expression of both genes was examined during hepatocyte replication in vivo following surgical ablation of 60–70% of the liver. The remaining hepatocytes undergo DNA synthesis and mitosis 24–48 h after surgery [40]. We assessed the expression of CAT2 and CAT2a mRNA in liver following partial hepatectomy and found no detectable change in expression of either mRNA 24 or 48 h or 7 days following surgery, sham surgery or fasting (Fig. 3A). In all conditions tested only the CAT2a transcript was expressed (Fig. 3B). Furthermore, there was no detectable change in CAT2 promoter usage in the liver during the most active period of cell proliferation (Fig. 3C.). From these data we conclude that CAT2a message accumulation is not changed in liver cells during active mitosis following partial hepatectomy; the promoter usage remains unchanged and there is no detectable expression of the transcript encoding the high affinity isoform CAT2.

3.4. CAT mRNA expression in skeletal and smooth muscles

Surgical trauma results in the loss of substantial protein mass from skeletal muscle [24,25] and fasting induces skeletal muscle catabolism to provide circulating amino acids [41]. Since both CAT1 and CAT2 are co-expressed in skeletal muscle [12], we tested whether surgical trauma or fasting altered their expression. Northern analysis of skeletal muscle expres-

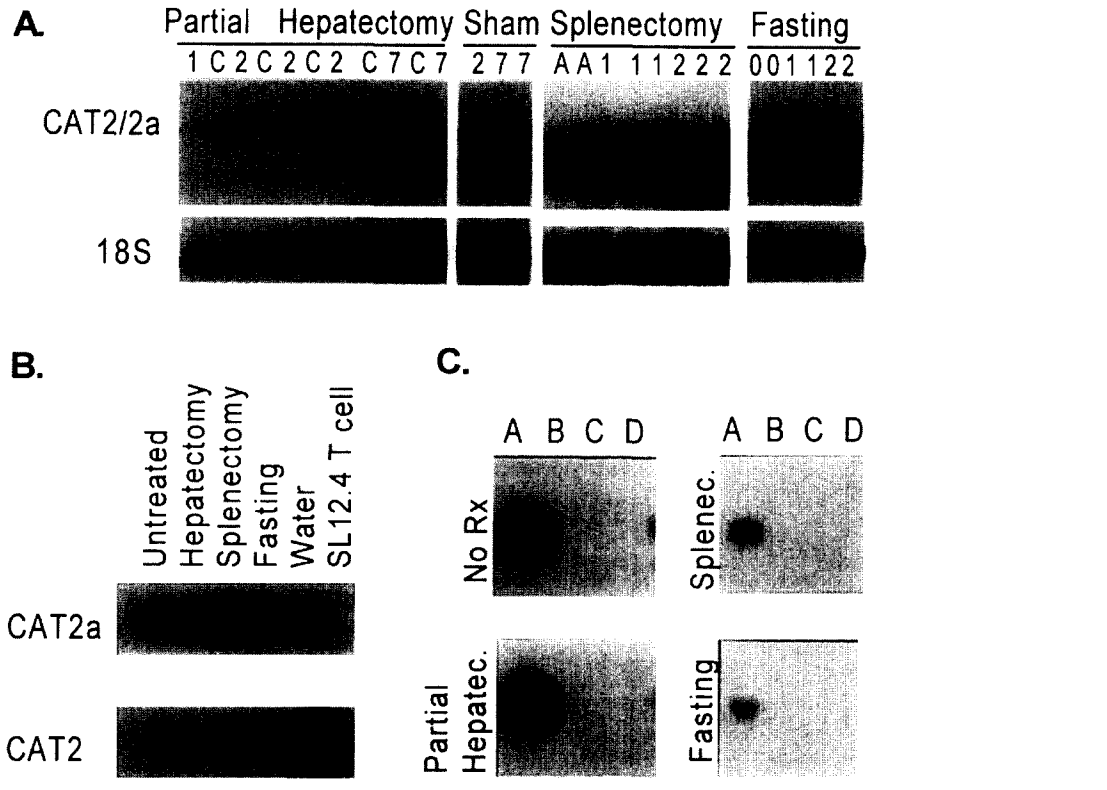

Fig. 3. CAT mRNA in liver following hepatectomy, sham surgery and fasting. Northern analysis (10 μg/lane) of CAT2/2a RNA expression from the liver of a representative group of animals. The left panel shows data from resected liver (C) or from liver 1, 2 or 7 days following partial hepatectomy or sham-operated animals as indicated. The middle and right panels shows CAT2/2a liver expression 0, 1 or 2 days following splenectomy, 1 day after anesthesia alone (A), or after 1 or 2 days of fasting. RNA loading controls are shown at the bottom of the panel. (B) RT-PCR analysis of CAT2 and CAT2a expression in liver following hepatectomy, splenectomy, and fasting (24 h). RNA from SL12.4 T-cells served as a positive control for CAT2. (C) CAT2 promoter usage in liver following the indicated treatments. RNA was prepared separately from six animals in each treatment or control group; representative data are shown. Details are provided in the legend to Fig. 2 and in Section 2.

sion illustrates CAT2/2a transcripts are substantially induced (Fig. 4A) following partial hepatectomy, whereas CAT1 mRNA levels remain low and unaltered (not shown). The most pronounced increase, 8.7-fold ($P=0.03$), occurred 1 day after hepatectomy (Fig. 4A). This increase was linked to liver regeneration, since it was not observed in sham-operated animals. Two days after hepatectomy, CAT2/2a mRNA had returned to control levels (not shown). Splenectomy also significantly induced CAT2/2a mRNA 3.5-fold ($P=0.001$) and it decreased to baseline 2 days later (not shown). Finally, Fig. 4A, right shows fasting produced a 2.4-fold, significant increase in CAT2a mRNA ($P=0.0005$). CAT1 mRNA levels were not significantly altered by these treatments (not shown). Interestingly, only isoform CAT2a is induced in response to hepatectomy, splenectomy and fasting (Fig. 4B). Perhaps the expression of this low affinity CAT2a isoform spares the muscle cell from excessive release of its L-arginine stores (see Section 4). Only a subtle change in promoter usage was detected in skeletal muscle following hepatectomy (Fig. 4C). As yet, this utilization of the 1D promoter is the only condition in which promoters other than 1A are utilized except in cell lines [17]. We can conclude, however, that promoter usage does not dictate the selection of splicing events that produce CAT2/2a isoforms since the same promoter is used exclusively in macrophages that exclusively express the high affinity CAT2 isoform and in liver and muscle, where only the low affinity isoform is expressed. In addition, *Cat* gene expression was assessed in the uterus, a smooth muscle. No change in expression of either CAT1 or CAT2 was detected

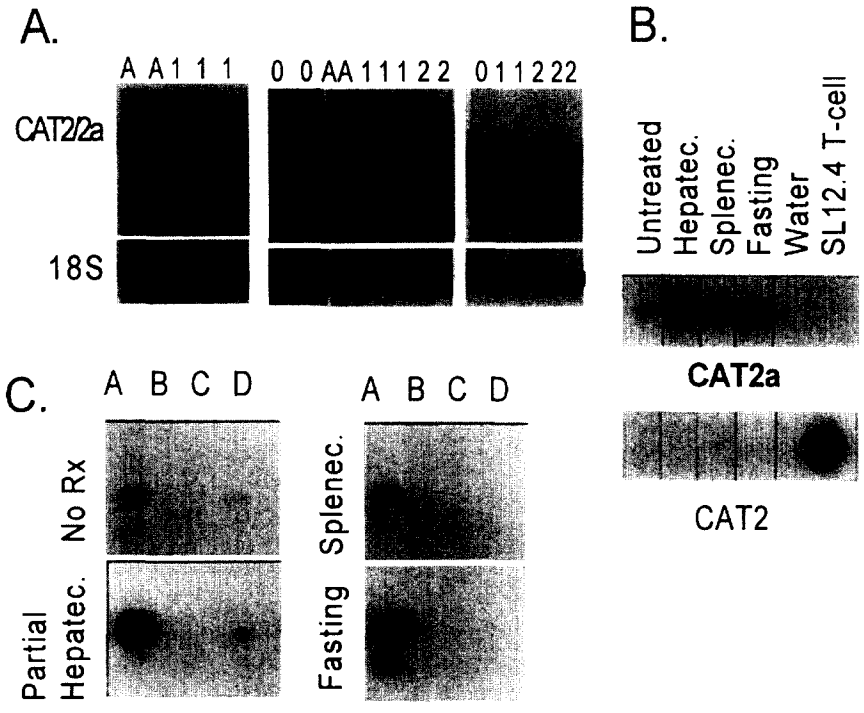

Fig. 4. CAT mRNA in skeletal muscle following hepatectomy, splenectomy and fasting. (A) Left side: Northern analysis (10 μg/lane) of skeletal muscle CAT2/2a mRNA expression 1 day following partial hepatectomy (lanes labeled 1) or the anesthesia control (A) or from untreated mice (0). Middle: Northern analysis of skeletal muscle CAT2/2a mRNA expression following splenectomy. Right: Northern analysis of skeletal muscle following 1 or 2 days of fasting all compared to 18S ribosomal RNA as a control for loading. Procedures are described in Section 2. (B) RT-PCR analysis of CAT2 and CAT2a isoform expression in skeletal muscle following the indicated conditions. (C) Promoter usage in skeletal muscle following the indicated treatments as previously described [17]. The letters designate the distinct CAT2 promoters [17].

following hepatectomy, splenectomy or fasting (data not shown) in response to the same conditions that stimulated CAT2a mRNA expression in striated muscle. Unlike skeletal muscle, the uterus expresses only the CAT2 and not the CAT2a isoform. Hence, CAT2a induction is specific for skeletal muscle, and is not induced in this smooth muscle.

4. Discussion

Three related genes encode four proteins that resemble the Na^+-independent low and high affinity y^+ transport system [10–12,16] when expressed in *Xenopus* oocytes and thereby appear to have overlapping functions [13,16]. However, their expression patterns are quite distinct; CAT1 expression is nearly ubiquitous, CAT2 has more limited expression and CAT3 is expressed exclusively in the adult brain, (where both CAT1 and CAT2 are also expressed [16]). *Cat1* and *Cat2* genes have distinct expression patterns from one another [12]. *Cat2* is expressed from multiple promoters [17] and encodes two mutually exclusive alternate splicing isoforms [8,13] that have substantially different transport kinetic properties [8,42,43]. We postulated that regulatory requirements might explain the apparently redundant function of these genes.

Hepatic low affinity transport system y^+ is likely to be encoded by the constitutively expressed CAT2a isoform [8,13,15]. Although hepatic system y^+ transport activity is stimulated following glucagon administration and requires new RNA synthesis [25], our data show that food deprivation, which elevates plasma glucagon, does not alter CAT2a RNA levels in liver. CAT1 is transiently induced 2–6 h following partial hepatectomy and in response to insulin, dexamethasone, or L-arginine treatment [19]. Our partial hepatectomy studies were conducted to examine the effects of mitosis per se on CAT expression, hence

regenerating liver was examined during the time characterized by hepatic cellular proliferation (24–48 h). No change in CAT1 or CAT2/2a mRNA was observed during this time period even though the regenerating liver is synthesizing L-lysine- and L-arginine-rich histones and shows no decrease in free intracellular L-lysine and L-arginine concentrations [44]. These data suggest there is increased cationic amino acid transport, perhaps mediated by the transient induction of CAT1 [19], although the expression levels of CAT2, CAT2a and CAT1 under these conditions have yet to be ascertained.

Skeletal muscle plays a unique role in amino acid homeostasis, since its protein mass serves as the major source of plasma amino acids in several physiological conditions. CAT2a expression predominates in skeletal muscle and is induced in response to surgical trauma and fasting. However, pathophysiological conditions that alter both liver and skeletal muscle metabolism induce CAT2a mRNA levels in skeletal muscle, but not in the liver. The apparent K_m of skeletal muscle y^+ transport is around 2 mM [28], which is similar to the low affinity CAT2a isoform [8,15]. CAT2a induction could increase cationic amino acid release into the systemic circulation during fasting and surgical trauma when there is a net amino acid export of lysine from skeletal muscle [45]. Because the low affinity isoform would require a high intracellular concentration of free amino acid to stimulate export of lysine, it might spare the muscle from excess loss of their cationic amino acid stores. Changes in unknown circulating factors after fasting, splenectomy and partial hepatectomy might account for the quantitative difference in skeletal muscle CAT2 induction in response to such stress. However, the fact that promoter 1A utilization remains predominant seems puzzling since each CAT2 promoter contains distinct potential transcription factor binding motifs that indicate a range of regulatory factors could influence *Cat2* gene expression. Nevertheless, our data show that the CAT2 promoter 1A is predominant in all tissues and cell lines examined regardless of: (1) the stresses applied; (2) the tissue examined; or (3) which alternate splicing event occurs during processing of the transcript. Furthermore, our data show that promoter usage *does not dictate* the alternate splicing events that give rise to the two protein isoforms, CAT2/2a since both liver (that expresses CAT2a exclusively) and macrophages (that express CAT2 exclusively) both utilize only promoter 1A.

The regulated expression of Cat genes has been investigated in several distinct cell types [18–23]. These investigations identified regulated expression of CAT2 and cationic amino acid transport in response to a variety of physiological stimuli, such as cytokines, bacterial products (LPS) and hormones [19,21–23]. Similar to our results with activated macrophages, CAT2 (but not CAT2a or CAT1) and iNOS were co-induced in astrocytes and vascular smooth muscle cells [21,20] when the demand for cellular arginine sharply rises.

With regard to possible redundancy of the Cat gene family of transporters, Perkins et al. found that ablation of *Cat1* is lethal following birth, although embryonic development is almost completely normal in $Cat1^{-/-}$ knockout embryos [46]. Therefore, *Cat2*, *Cat3* or other genes have the capacity to substitute for the absence of *Cat1* gene function during embryonic development, but cannot sustain viability for more than 6 h following birth [46,47]. When the *Cat2* and *Cat3* genes are ablated, it will be possible to determine whether these genes are required for normal development or the maintenance of cationic amino acid homeostasis. Furthermore, if they are viable, such knockout animals will make it possible to determine whether *Cat* regulatory responses are altered by mutational loss of one or more *Cat* genes.

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