ABSTRACT

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Lysine and arginine transport is primarily mediated by cationic amino acid transporters (CATs) in cells. The chicken CAT-2 (cCAT-2) transcript is alternatively spliced to three isoforms. Transcriptional and cellular localization experiments were utilized to study their regulation. The mRNA abundance of cCAT-2 isoforms was estimated in body tissues, and although differentially expressed, all tissues expressed each cCAT-2 isoform gene, indicating that alternative splicing was not tissue-specific. Both cCAT-2A and cCAT-2B proteins localized to the plasma membrane and cCAT-2C protein was retained in the cytosol. Chicken CAT-2A functions as a low affinity transporter with specificity for lysine and arginine. Chicken CAT-2B and cCAT-2C transporter functions were not detectable. Our data indicates that CAT-2 transporters are conserved in non-mammalian vertebrates, but cCAT-2 isoforms differ in their tissue distribution and transporter function from previously characterized CAT-2 transporters. These results also indicate a

mechanism by which additional dietary lysine and arginine contribute to increased protein accretion in muscle tissue.

CHARACTERIZATION OF CHICKEN CAT-2 ISOFORMS

By

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Dedication

To my loving and patient boyfriend who helped me make it through.

Acknowledgements

First, I would like to thank Dr. Brooke Humphrey for all of his patience and instruction throughout the last two years. I am proud to have had you as my mentor, and I hope many other students benefit from your tutelage in the years to come.

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List of Abbreviations

ActD	Actinomycin D
ATF	activating transcription factor
ARE	adenosine-uracil rich elements
Arg1	arginase 1
CAA	cationic amino acid
CAT	cationic amino acid transporter
C/EBP	CCAAT/enhancer binding protein
CHO-K1	Chinese hamster ovary cell
CMV	Cytomegalovirus
FPP	fluoroscene protease protection
HAT	heterodimeric amino acid transporter
HEK	human embryonic kidney cell
IFN	Interferon
IL	Interleukin
iNOS	nitric oxide synthase
(K)bp	(kilo)base pair
LMH	chicken liver hepatocyte cell
NAA	neutral amino acid
NMD	nonsense mediated decay
NRC	National Research Council
Prefix c, h, m	chicken, human, mouse respectively
PTC	premature termination codon
RUST	regulated unproductive splicing and translation
SLC7	solute carrier family 7
TH1/2	T helper ½
TGF	transforming growth factor
TNF	tumor necrosis factor
TM	transmembrane domain
UTR	untranslated region

Chapter 1: Introduction

1.1 Lysine and arginine are essential for the growth and development of chickens

1.1.1 Lysine and arginine metabolism

The cationic amino acids lysine and arginine are limiting amino acids required for growth. They are polar, basic to strongly basic, and can act as antagonists (1) with indications for increased catabolism of arginine in the presence of excess lysine (2). While lysine is an essential amino acid for all animals, avians lack a functional urea cycle and, therefore, require arginine in the diet (3).

Metabolism of lysine and arginine begins with hydrolysis of intact dietary protein in the gastrointestinal tract. Exopeptidases and endopeptidases, such as amino peptidases, dipeptidyl peptidases, and carboxypeptidases secreted from epithelial cells in the gastrointestinal tract and the pancreas, cleave peptide bonds to allow the absorption of free amino acids from the intestinal lumen (4). Within chickens, absorption of amino acids has been observed in the crop, gizzard, proventriculus, small intestine, and colon (5). A balance of absorbable essential amino acids is important for maximum utilization of the dietary protein source. Three gene systems: $b^{0,+}$, $y^{+}L$ and y^{+} are responsible for cationic amino acid transport in enterocytes (6-9) and preferentially transport the L- form amino acid rather than the D- form amino acid (4).

Following absorption, amino acids may be used for synthesis of enzymes, hormones, muscle tissue or metabolized to other metabolites. They can also undergo deamination or transamination and may be used to generate endogenous non-essential amino acids, or their carbon skeletons can enter the Kreb's cycle for energy production or for gluconeogenesis (4). Excess dietary amino acids are not stored, but rather catabolized

for energy (10,11). In mammals, the ammonia resulting from deamination enters the urea cycle for urea synthesis and secretion. Birds, however, lack the enzymes of the urea cycle and excrete nitrogen as uric acid (3,11,12). Birds also express high levels of glutamine synthase in the mitochondria of hepatic and renal cells that functions to fix free ammonia to glutamate to form glutamine (3). In the final step of metabolism, bacteria in the colon and ceca recycle nitrogen by breaking it down to uric acid to ammonia, carbon dioxide and short chain fatty acids (13,14) and uric acid is secreted.

1.1.2 Lysine and arginine utilization for growth

Lysine and arginine utilization have been studied primarily at the whole-animal level as it pertains to animal health and growth. A landmark study by Dean and Scott in 1965 (15) established the "ideal protein concept" in which the ratio of essential amino acids to lysine could be used to formulate and evaluate diets. Since that time, many studies have been performed to identify the proper lysine:arginine ratio for increased weight gain, food efficiency, breast meat yield, and decreased fat percentage (16). Carcass deposition of protein has been directly correlated to the availability of limiting amino acids, most commonly lysine, arginine, and methionine (17). The pectoralis major muscle is more sensitive to dietary lysine levels than to dietary levels of neutral amino acids, including threonine and valine (18). Feeding lysine or arginine above NRC requirements (19) increases breast meat yield, total carcass weight, and reduces abdominal fat percentage (18,20). In addition, feeding arginine above NRC requirements (19) improves growth and feed efficiency during heat stress (21).

The majority of dietary amino acids in growing chicks is partitioned towards protein accretion (22,23) and chicks from hatch to 14 days of age have significantly higher lysine requirements than in later stages of life (24). Therefore, feed digestability and the dietary lysine:arginine ratio has been studied thoroughly to provide growing chicks with adequate supplies of these essential amino acids during times when amino acid deposition efficiency is at its peak (17,25).

1.1.3 Lysine and arginine utilization for immunity

In addition to protein synthesis, arginine is utilized by the immune system. In animals, arginine is required by T lymphocytes for proliferation, expression of the T cell receptor complex, cell surface peptides, and the generation of memory (26,27). In times of stress due to injury or infectious challenge, arginine may be utilized by inducible nitric oxide synthase (iNOS) in macrophages to produce nitric oxide (NO) for pathogen killing, or may be used for polyamine synthesis involved in wound healing by arginase 1 (Arg1) (28,29). These enzymes compete for arginine, therefore NO production is dependent on arginine availability in addition to regulation by Arg1 (30,31). In response to pro-inflammatory cytokines produced by T helper 1 (T_H1) cells, such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), iNOS converts arginine to NO which plays an important role in ridding the body of parasites, bacteria, viruses, and cancer cells (32). Anti-inflammatory cytokines produced by T_H2 cells, such as IL-4, IL-6, IL-10, IL-13, and transforming growth factor- β (TGF-β), up-regulate Arg1 which metabolizes arginine to polyamines that are excreted by macrophages at the site of

infection and are used by cells to promote wound healing, thereby depleting the available arginine pool (26) and helping to down-regulate the local inflammatory response (33,34).

Lee et al. (35) showed that increased dietary arginine in broiler diets increases the heterophil to lymphocyte ratio, resulting in a stronger innate immune response. Chicks fed arginine 25% above NRC recommendations (19) had increased resistance to microbial infections and maintained connective tissue integrity during processing (20). When comparing dietary arginine requirements, it is apparent that the immune system requirement is equal to or exceeds the requirement for growth, and that bird health would benefit from increased arginine supplementation (20,21,23,36).

1.2 Cationic Amino Acid Transporters

In the 1960's, Halvor Christensen demonstrated that the cationic amino acids, lysine, arginine, and ornithine share the same transport systems. At that time, amino acid transport studies were conducted *in vitro* to test substrate specificity by either limiting amino acid levels in cell culture media or by treating cells with chemicals known to inhibit specific transport systems (37). A single transport system termed y^+ was considered to be the major entry route for cationic amino acids in most cells and the y^+ genes were the first amino acid transporters to be cloned (38-40). More recently, several other transport systems (y^+L, $b^{0,+}$, and $B^{0,+}$) that also transport cationic amino acids have been identified (Figure 1) and their substrate specificity, transport mechanism, and sodium dependence have been determined (41,42) (Table 1).

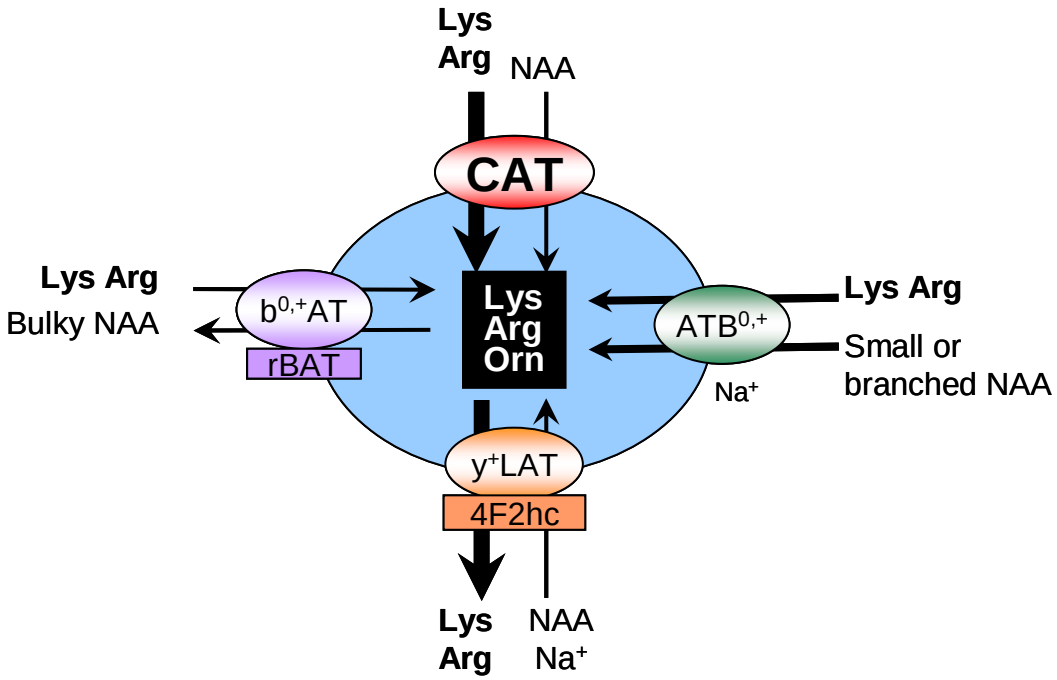
Table 1. Cationic amino acid transporter systems[1]

System	Substrate specificity	Transport mechanism	Sodium dependence	Gene product(s)	Tissue distribution
y^+	K, O, R	uniport; high affinity for CAA, low affinity for NAA	independent for CAA, dependent for NAA	CAT 1-4	Widespread, especially in liver, skeletal muscle, and macrophages
y^+L	K, L, M, Q, R	antiport;,efflux of CAA, influx of NAA and Na+	independent for CAA, dependent for NAA	LC: y^+LAT1/2 HC: 4Fhc	Basolateral membrane of enterocytes and renal tubule epithelial cells
$b^{0,+}$	K, L, R	antiport; influx of CAA and efflux of bulky NAA	No	LC: $B^{0,+}$AT HC: rBAT	Apical membrane of enterocytes and renal tubule epithelial cells
$B^{0,+}$	A, K, R, V	uniport; influx of CAA and small or branched NAA	Yes	$ATB^{0,+}$	Trachea, stomach, and glandular tissues

[1] Abbreviations used: CAA, cationic amino acids; NAA, neutral amino acids; LC, light chain; HC, heavy chain.

Figure 1. Cationic Amino Acid Transporters.

The cationic amino acids lysine, arginine, and ornithine are carried by four transporter gene families in non-polarized cells. System y^+ transport is mediated by the Cationic Amino Acid Transporter genes. System y^+L transport is mediated by the heterodimeric protein complex of y^+LAT light chain and 4F2hc heavy chain. System $b^{0,+}$ transport is mediated by the heterodimeric protein complex of $b^{0,+}$AT light chain and rBAT heavy chain. Na^+-dependent system $B^{0,+}$ is mediated by the $ATB^{0,+}$ gene. Neutral amino acids (NAA) are also transported by these systems.



1.2.1 Four systems transport cationic amino acids

Systems y^+L and $b^{0,+}$ are heterodimeric amino acid transporters (HATs). The system y^+L proteins, y^+LAT-1/2, and system $b^{0,+}$ proteins, $b^{0,+}AT$, dimerize with unique heavy chain glycoproteins that target their sub-cellular localization to the plasma membrane (43). System y^+L proteins are localized to the basolateral membrane by heavy chain 4F2hc while system $b^{0,+}$ proteins are localized to the apical membrane by heavy chain rBAT (9,42). Together, these HATs mediate the vectorial transport of cationic amino acids in polarized cells. System $b^{0,+}$ functions in the uptake of cationic amino acids into the cell while system y^+L functions in the efflux of cationic amino acids from the cell (44). These transport systems are particularly important in cationic amino acid absorption in the intestine and reabsorption in the kidney. System y^+L activity has been identified in chicken erythrocytes and brush-border membrane vesicles and exhibits both high and low transport properties with specificity for neutral, bulky amino acids (45). The gene products responsible for mediating system y^+L activity in chickens have not been cloned, however a candidate y^+L gene sequence has been identified in the chicken genome (Morris, Kirsch and Humphrey, unpublished). Torras-Llort et. al. (46) determined system $b^{0,+}$ activity and transport kinetics in chicken erythrocytes. The presence of the rBAT heavy chain has been verified by western blot, however it is not known if high and low affinity transporters are expressed (46).

Sodium-dependent cationic amino acid transport is conducted by system $B^{0,+}$, a glycoprotein with 12 transmembrane (TM) domains (42,46). Within mammals, this system exhibits high affinity for cationic, bulky neutral, and small neutral amino acids (44,47). The system $B^{0,+}$ protein product $ATB^{0,+}$ is expressed in glandular tissue in

mammals and may also be linked to gastric acid secretions (48). Research has not been conducted on system $B^{0,+}$ in chickens.

System y^+ is a subfamily of the solute carrier family 7 (SLC7) shown to transport via facilitated diffusion (49,50). System y^+ is considered to be the primary entry pathway for cationic amino acids in most non-polarized cells (42) and is required for the cellular uptake of lysine and arginine (29,51). In addition, system y^+ transport helps to maintain the intracellular lysine:arginine balance which is essential for both protein accretion and the immune response (39). System y^+ transporters have been characterized in the mouse, rat, human, pig, and chicken (52).

1.2.2 Cationic amino acid transporters (CATs)

System y^+ activity is mediated by cationic amino acid transporters (CAT) (Table 2). Mouse and human CAT (mCAT and hCAT respectively) proteins are encoded by 3 genes (CAT 1-3) that produce four isoforms involved in CAA transport (CAT-1, CAT-2A, CAT-2B, CAT-3). The human genome organization has assigned CAT-1, CAT-2A +-2B, and CAT-3 the gene names SLC7A1, SLC7A2 (A+B), and SLC7A3, respectively (43). These mCAT proteins are 70 kDa glycosylated proteins with 14 TM domains (41). Mammalian CAT proteins localize to the plasma membrane (43) and are closely related to each other with 60% identity at the nucleotide level. Human CAT-4 has been identified based on nucleotide homology with the hCATs. Human CAT-4 protein localizes in the plasma membrane, however, it does not exhibit transport activity for cationic, anionic, or neutral amino acids at varying pH levels (43,54).

Table 2. Description of y^+ system transporters

Gene name	Gene	Tissue Distribution in Mammals	Transport properties	Approximate K_m	pH sensitivity	Trans-stimulation
SLC7A1	CAT-1	Ubiquitous excluding liver	High affinity, low capacity	100-150 μM	Independent between pH 5.5-8	Highly sensitive
SLC7A2-A	CAT-2A	Throughout body, highest in liver	Low affinity, high capacity	2-5mM	Moderately dependent	Insensitive
SLC7A2-B	CAT-2B	Immune cells	High affinity, low capacity	70-400 μM	Highly dependent below pH 5.5	Highly sensitive
SLC7A3	CAT-3	Brain and placenta	High affinity, low capacity	40-165 μM	Independent between pH 5.5-8	Sensitive
SLC7A4	CAT-4	Placenta	Not known	Not known	Not known	Not known

Expression of mouse and human CAT proteins in *Xenopus laevis* oocytes and mammalian cells has shown that transport of CAAs by CAT-1, CAT-2A, CAT-2B, and CAT-3 are similar in their substrate specificity and sodium independence, yet differ in their substrate affinities, pH sensitivities, and response to trans-membrane stimulation (39). Mouse and human CAT-1 mRNA is ubiquitously expressed in tissues, excluding the liver (28), is pH-independent within a range of 5.5 to 8, is strongly stimulated by substrate on the trans- side of the plasma membrane, and exhibits high affinity transport (K_m of 100-150μM) (44).

While transport kinetics have not yet been established for chicken cationic amino acid transporter-1, Humphrey et. al. (55) quantified CAT-1 isoform mRNA levels in chicken tissues that were fed either a lysine adequate or deficient diet. Chicken CAT-1 mRNA is highest in the bursa of Fabricius, thymus, gastrocnemius, pectoralis major and liver, and absent in the heart and spleen. Chicken CAT-1 mRNA levels decrease when chicks are fed a lysine deficient diet and increase when feed intake is restricted.

Mouse and human CAT-3 has a slightly lower affinity for CAAs (K_m= 40-165μM) and is less sensitive to trans-membrane stimulation than mCAT-1 (44). Mouse and human CAT-3 is pH independent within a range of 5.5 to 8 and has been identified in the brain and placental tissue in humans and rodents (56).

Chicken CAT-3 is expressed in the bursa, thymus, heart and pectoralis major, and is undetectable in the gastrocnemius (52). Similar to CAT-1, CAT-3 mRNA levels are regulated by dietary lysine levels and feed restriction (55). Transport kinetics have not been determined for chicken CAT-3.

The mouse CAT-2 primary transcript, located on chromosome 8 (57), is alternatively spliced within the same region of the mRNA, at a codon that yields a Ser^{352} triplet (Appendix Figure 2) (58) to produce either mCAT-2A or mCAT-2B. Mouse and human CAT-2A is a low affinity transporter (K_m=2-5mM) of CAAs and mCAT-2B is a high affinity transporter (K_m= 70-400μM) of CAAs (41). Mouse and human CAT-2A has moderate pH dependence and is relatively insensitive to trans-membrane stimulation. Mouse and human CAT-2B is highly sensitive to trans-membrane stimulation and is pH dependent resulting in 50% lower activity at pH 5.5 compared to pH 7.5 (44). Both mCAT-2A and mCAT-2B are integral membrane proteins with 14 TM domains and contain intracellular N- and C- termini (59).

Mouse and human CAT-2A is the predominant isoform expressed in liver and is thought to mediate the important role of clearing excess lysine and arginine from the portal circulation after a meal (28). High affinity mCAT-2B was first identified as a T cell early activation protein (Tea) in B and T cells (60). Mouse and human CAT-2B was grouped into the y^+ transporter family due to high nucleotide homology with mCAT-1, sensitivity to trans-stimulation, and substrate specificity for cationic amino acids (42). Mouse and human CAT-2B is expressed in immune cells and has been intensively studied due to its role in transporting arginine for use as a substrate for nitric oxide synthesis (47). Genetic ablation of mCAT-2 affects both splice variants, since these isoforms arise from alternative splicing of the same gene (51,61). Knockout mCAT-2 mice lack expression of mCAT-2A in the liver, and do not result in the upregulation of other CAT mRNAs (52). It remains to be determined how these mice will respond to a diet high in CAAs. CAT-$2^{-/-}$ mice also lack mCAT-2B expression in dendritic cells,

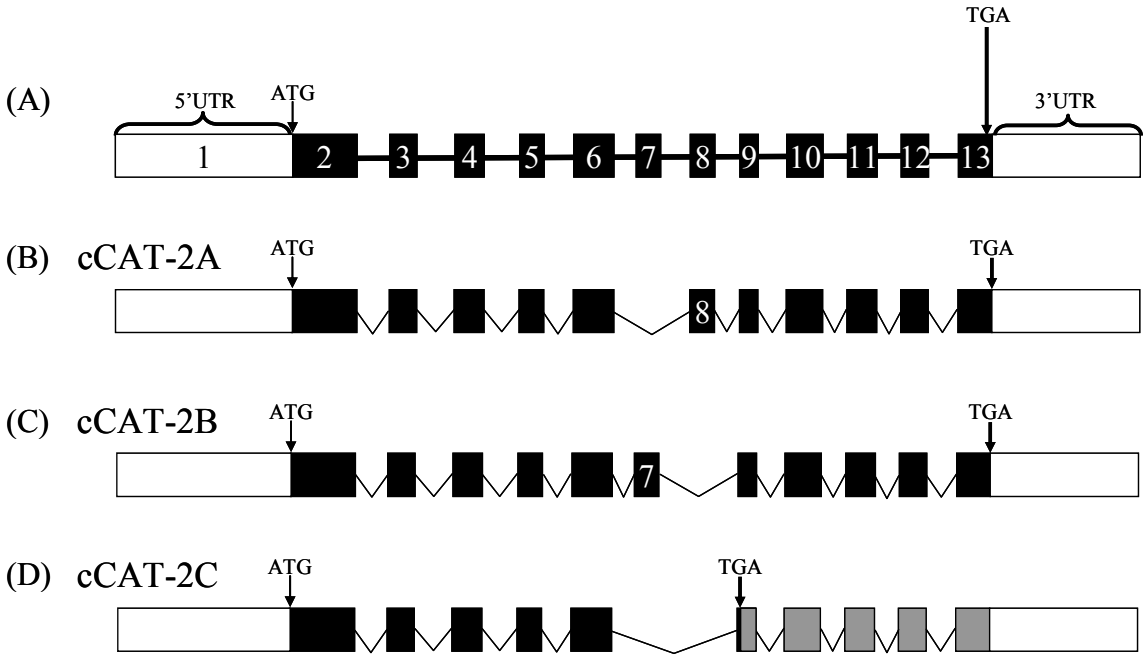
resulting in increased activation due to the absence of NO that suppresses dendritic cells (47,62).

1.2.3 Chicken CAT-2

Chicken total CAT-2 mRNA is expressed by many tissues with highest levels in the pectoralis major and liver, two tissues involved in amino acid homeostasis and protein accretion (28). Chicken CAT-2 (cCAT-2) sequences were identified by RT-PCR and RACE molecular cloning strategies (Morris, Kirsch, and Humphrey, unpublished) using primers in Appendix Table 1. The cCAT-2 gene is located on chromosome 4 (57) and is alternatively spliced to form cCAT-2A, cCAT-2B, and a novel transcript cCAT-2C. Chicken CAT-2A, -2B, and -2C have 100% nucleotide homology in the coding regions of exons 2-6 and 9-12, but differ in the use or deletion of exon 7 or 8 (Figure 2; Appendix Figure 1). The cCAT-2A isoform contains exon 8 while the transcript encoded by cCAT-2B contains exon 7. The cCAT-2C isoform contains neither exon 7 or 8, resulting in a shift in the reading frame and formation of a premature termination codon.

Figure 2. The genomic structure of chicken CAT-2 and alternatively spliced isoforms.

Diagram of (A) the genomic structure of the chicken CAT-2 (cCAT-2) gene, and alternatively spliced transcripts of (B) cCAT-2A, (C) cCAT-2B, and (D) cCAT-2C isoforms. Exon 1 and the region upstream of the ATG translational start site in exon 2 (white boxes) are part of the 5' UTR. Coding exons are numbered 2 through 13 (black boxes).

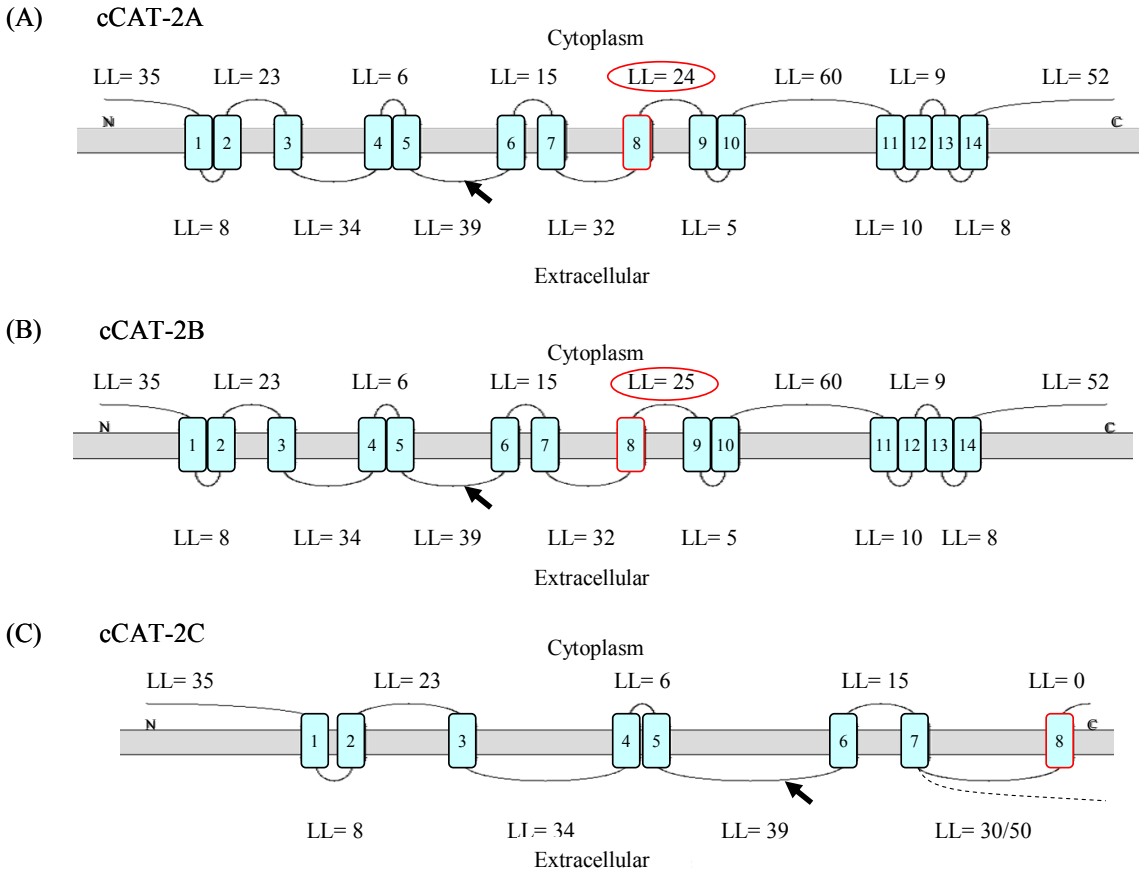


Chicken CAT-2A has an open reading frame consisting of 1965 base pairs and is predicted to encode a 654 amino acid protein with a molecular weight of 76 kDa. Chicken CAT-2A shares 78%, 81% and 78% nucleotide homology with human (GenBank accession # AAB62810), mouse (# AAA37350) and rat (# NP_072141) CAT-2A, respectively (Appendix Figure 3). Chicken CAT-2B has an open reading frame consisting of 1968 base pairs and is predicted to encode a 655 amino acid protein with a molecular weight of 76 kDa. Chicken CAT-2B shares 85%, 83% and 84% nucleotide homology with human (# NM_001008539), mouse (# M62838) and rat (# RNU53927) CAT-2B, respectively (Appendix Figure 4). The cCAT-2C isoform has an open reading frame of 1828 base pairs and is predicted to encode a 358 amino acid protein with a molecular weight of 40 kDa. Hydropathy plot analysis of predicted cCAT-2 isoform amino acid sequence was performed with TopPred software (63) (Figure 3). The cCAT-2A and cCAT-2B proteins are predicted to contain 14 TM domains, while the cCAT-2C protein is predicted to contain 8 TM domains. The N-terminus and C-terminus are predicted to be located intracellularly for all cCAT-2 proteins.

While human, mouse, and rat have 4, 5, and 6 predicted N-glycosylation sites, respectively, only two sites are located on an extracellular loop. Mammalian CAT-2A and mCAT-B contain $Asn^{227, 239}$ on the third extracellular loop (64,65), both of which have been shown to be glycosylated (66). This confirms the predicted 14 TM model of topology for mCAT-2 proteins. The cCAT-2 proteins also contain a predicted N-linked glycosylation site on the third extracellular loop, however it is located at Asn^{233} (65), which has not been examined for glycosylation (Figure 3; Appendix Figure 2).

Figure 3. Predicted transmembrane orientation of cCAT-2 isoforms.

TopPred prediction of transmembrane domains and orientation for (A) cCAT-2A, (B) cCAT-2B, and (C) cCAT-2C proteins. Membrane spanning regions are predicted to contain 20 amino acid residues. Transmembrane region 8 and the intracellular loop 4 contain the divergent region between cCAT-2 isoforms. Arrows indicate glycosylation site at Asn^{233}. Dashed line in (C) indicates an alternate orientation for the C-terminal residues of cCAT-2C. Abbreviation: LL, loop length.



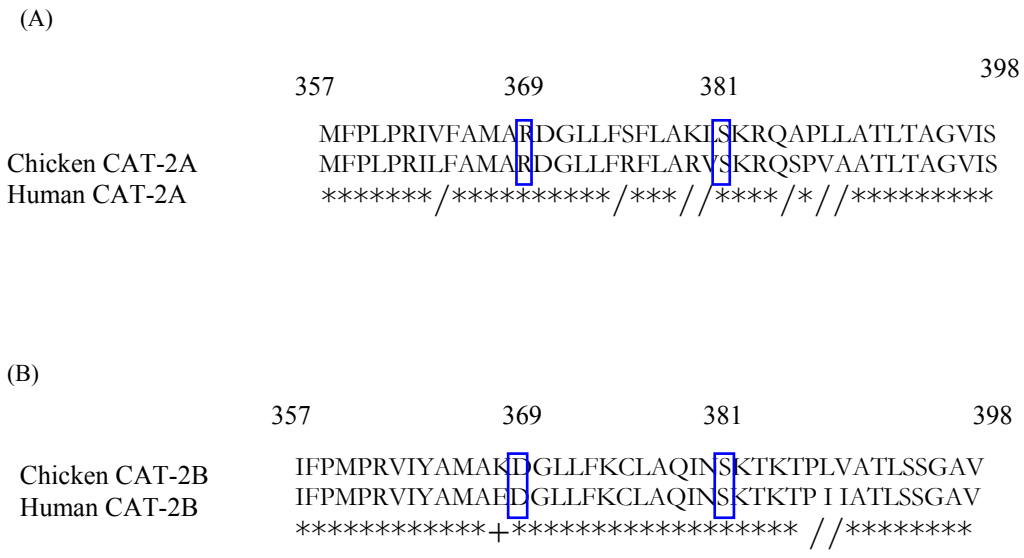
Studies by Closs et al. (49) identified that the 42 amino acid region that is divergent between mCAT-2A and mCAT-2B confers the transporter affinity. CAT-1 chimeras containing either the mCAT-2A or mCAT-2B divergent region resulted in transport characteristics for mCAT-2A and mCAT-2B, respectively (49). Habermeirer et al. (59) showed that low affinity transport by mCAT-2A is due to Arg^{369} and Ser^{381} while high affinity transport by mCAT-1, -2B, and -3 is due to Glu^{369} and His^{381} or Asn^{381}. Mutation of mCAT-2A Arg^{369} to Glu^{369} results in an intermediate K_m between high and low affinity transport, while mutation of both residues, Arg^{369} to Glu^{369} and Ser^{381} to His^{381} or Asn^{381}, results in a low affinity K_m value.

Chicken CAT-2A and cCAT-2B differ by 42 amino acid residues due to alternative splicing of exons 7 or 8. These residues are located within transmembrane region 8 and intracellular loop 4 (Figure 3A & B). Within this alternatively spliced region, hCAT-2A and cCAT-2A have 82% nucleotide identity, and hCAT-2B and cCAT-2B have 92% nucleotide identity. The residues conferring low affinity amino acid transport of hCAT-2A, Arg^{369} and Ser^{381}, are conserved in cCAT-2A (Figure 6A). This indicates that cCAT-2A may also function as a low affinity amino acid transporter. The residues conferring transport activity in hCAT-2B, Glu^{369} and Asn^{381}, are not entirely conserved in cCAT-2B (Figure 6B). The Asn^{381} is conserved, but Glu^{369} is replaced by Lys^{369}. This substitution results in a change in amino acid charge and may result in transport properties of cCAT-2B that differ from hCAT-2B. When comparing cCAT-2B peptide sequence to the peptide sequence of hCAT-2B, cCAT-1 and cCAT-3, all presumed to be high affinity transporters, the Glu^{369} residue is conserved in all but cCAT-2B (Appendix Figure 5), also indicating that cCAT-2B may not function as a high

affinity transporter of the CAA. Chicken CAT-2C, which is predicted to produce a truncated protein, does not appear to contain an amino acid binding domain due to exclusion of exon 7 and 8.

Figure 4. Amino acid alignment of divergent regions between cCAT-2A and cCAT-2B.

Comparison of predicted amino acid sequence between human and chicken CAT-2A and CAT-2B within the domain that confers the unique transport properties of these proteins. Amino acid sequences are aligned using the tBLASTx program. Numbers above alignments correspond to amino acid residues in the region of variation between cCAT-2A and cCAT-2B. Blue boxes indicate the residues that confer transport properties. * indicates identical residues. / indicates change in residue that does not result in change in charge. + indicate change in residue that results in a positively charged residue.



Mammalian CAT-2A and mCAT-2B mRNA expression is induced by stress. In times of surgery or fasting, skeletal muscle protein is catabolized and provides the major proportion of plasma amino acids (21). During these times, mCAT-2A mRNA expression is induced in skeletal muscle, presumably to release CAAs from skeletal muscle into the plasma (28,67). High affinity transporter mCAT-2B is an inducible isoform involved in arginine uptake for NO production, and is expressed in activated lymphocytes, macrophages, and other cell types (68). Mammalian CAT-2B expression has been induced in macrophages in the presence of lipopolysaccharide and IFN-γ (28).

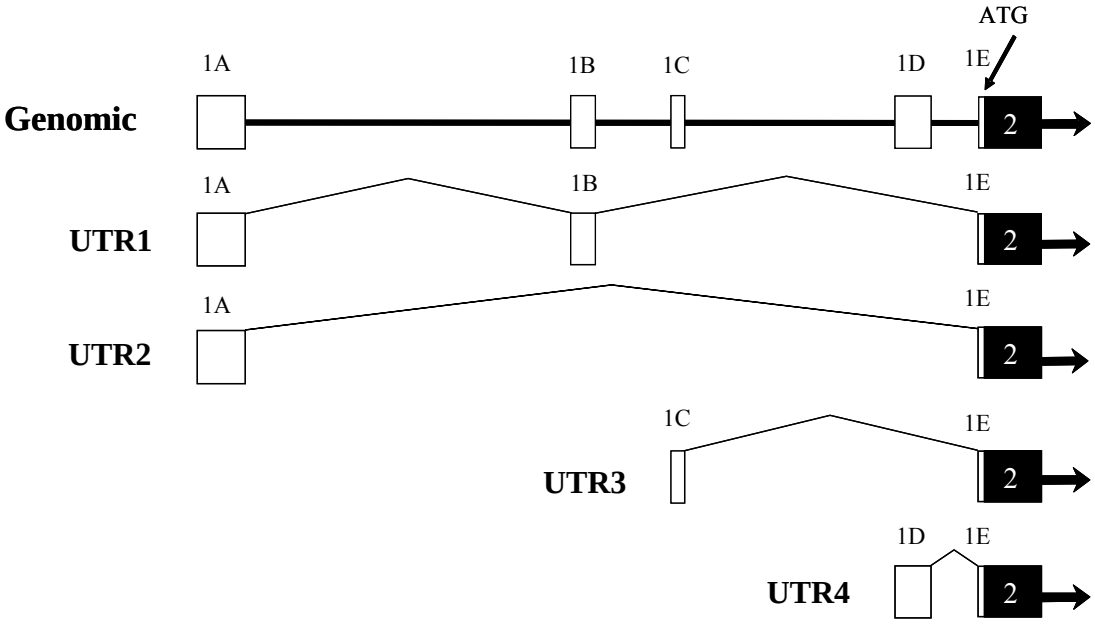
1.3 Regulation of cationic amino acid transporter (CAT) genes

It was previously shown the mCAT-2 transcription is initiated by multiple promoters. The 5' untranslated region (UTR) contains 4 exons and 5 possible promoter regions extending over 18kb from the AUG translational start site (69). These exon regions contain several classical promoter and regulatory elements, including TATA-boxes, (G+C)-rich sites, and CAAT boxes. Promoter usage was not correlated to stress response, nor was alternative splicing due to promoter usage (28).

The cCAT-2 5' UTR (Figure 4) contains 5 exons extending over 61 kb from the start codon. The 5' UTR exons are alternatively spliced, resulting in three promoter regions. Within the exons, TATA-boxes and TATA-less putative promoters were identified as well as activating transcription factor-4 (ATF-4) and CCAAT/enhancer binding protein (C/EBP) that are sensitive to availability of amino acids. Promoter usage differs by tissue, however promoter usage is not responsible for alternative splicing of cCAT-2 isoforms (Morris, Kirsch and Humphrey, unpublished).

Figure 5. The chicken CAT-2 5' untranslated region.

Five non-coding exons (1A, 1B, 1C, 1D, and 1E) and four exon combinations (UTR1, UTR2, UTR3, and UTR4) have been identified 5' of the AUG start site of cCAT-2. Lines between boxes indicate introns. 5'UTR sequences beginning with either exon 1A, 1C, or 1D suggest the presence of 3 distinct transcriptional start sites for cCAT-2.



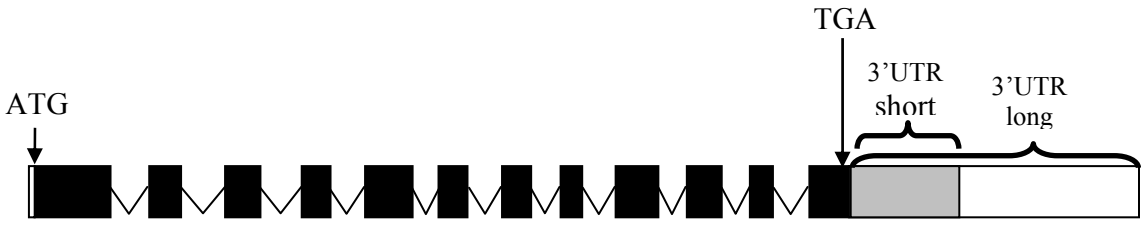
The mCAT-2 transcript contains a short (4.5 kbp) and long (8 kbp) 3' UTR. Presence of the long 3' UTR increases mCAT-2 transcript nuclear retention. Through unknown mechanisms, this transcriptional inhibition results in cleavage to the short message for nuclear export and allows translation to occur (47,72). The presence of this 3' UTR transcript modification indicates that mouse CAT-2 is post-transcriptionally regulated (28).

The presence of a short, 526bp, and long, 1.4 kbp, 3' UTR transcript for cCAT-2 (Figure 5) indicates that it may function similar to the mouse CAT-2 3' UTR. Within the long region of cCAT-2 there are several adenosine-uracil rich elements (ARE) that are characteristic of unstable mRNA, while no AREs are present in the short 3' UTR (Morris, Kirsch and Humphrey, unpublished).

Many studies have shown that mCAT-1 transcription, mRNA stability, and translation are increased in mice and rats fed an amino acid deficient diet (47). In addition, microRNA-122 binds to the 3' UTR of mCAT-1 and prevents mCAT-1 protein accumulation by increased mRNA degradation. Amino acid deprivation relieves this inhibition, resulting in increased mCAT-1 protein expression (71). Studies remain to be conducted on the effects of diet on gene regulation in chickens.

Figure 6. The chicken CAT-2 3' untranslated region.

The 3' untranslated region of chicken CAT-2 contains a short 526 bp fragment (gray box) and a long 1416 bp fragment (both gray and empty boxs). The coding exons of the primary transcript are shown as black boxes. Lines between exons denote introns.



In the current study we have cloned the cCAT-2 splice variants cCAT-2A, cCAT-2B, and cCAT-2C and quantitated their relative mRNA abundance in chicken tissues. We have induced cCAT-2 isoform protein expression by transient and stable transfection of mammalian cells to determine their sub-cellular localization, transporter kinetics, and transporter specificity. By characterizing the cCAT-2 isoforms, we have taken the first steps toward understanding the genes involved in the regulation of lysine and arginine utilization in chicken tissues.

Chapter 2: Materials and Methods

2.1 Animals and tissue sampling

Male Ross broiler hatchlings (*Gallus gallus domesticus*) were provided *ad libitum* access to water and a corn-soybean meal diet prepared according to the National Research Council recommendations for a young growing broiler chick (19). Chicks were raised in a Petersime Brooder battery (Petersime Incubator Co., Gettysburg, OH) located in an environmentally controlled room (25°C; 18h light:6 h darkness). At two weeks posthatch, chicks were euthanized by CO_2 overdose, and the bursa of Fabricius, gastrocnemius, heart, liver, pectoralis major, and thymus were collected and frozen between aluminum plates in liquid nitrogen (n=4/tissue). All tissue samples were stored at -80°C prior to analysis. All animal procedures were approved by the University of Maryland Institutional Animal Care and Use Committee.

2.2 RNA Isolation and Reverse Transcription

Total RNA was isolated from tissue samples using NucleoSpin RNA II Total RNA Isolation Kit (Macherey-Nagel, Easton, PA; #740933.10). Optical density absorbance at 260 nm was used to quantify total RNA concentrations. Total RNA was reverse transcribed to cDNA using the iScript Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA; #1708896) and oligo $(dT)_{15}$ primers according to the manufacturer's protocol. For cloning, total RNA (500 ng) was reverse transcribed from heart and pectoralis major collected from chicks 14 days old. For quantitative real-time PCR, total

RNA (200 ng) was reverse transcribed from day 14 bursa of fabricius, gastrocnemius, heart, liver, pectoralis major, thymus as described above.

2.3 Polymerase Chain Reaction (PCR) and Cloning

Pectoralis major and heart RT reactions were used to amplify cCAT-2 isoform open reading frames (ORF) by PCR. PCR reactions (50µl) utilized 20 mM Tris-HCl, 50 mM KCl, 0.2 mM dNTP's, 300 nM of cCAT-2 ORF primer pair 1 (Appendix Table 1), 1 µl of the RT product and 0.1 U Platinum *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA; #10966-018). Thermal cycling parameters were 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension step of 72°C for 8 min. PCR products containing CAT-2 isoforms were cloned into the pCR 2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA; #K4500-01) and sequenced (Genewiz, Inc. South Plainfield, NJ) using M13 and gene specific primers. Sequencing verified that cCAT-2A and cCAT-2C had been cloned from pectoralis major while cCAT-2B had been cloned from heart. Plasmids containing cCAT-2A, cCAT-2B or cCAT-2C were renamed to pCR-cCAT-2A, pCR-cCAT-2B and pCR-cCAT-2C, respectively. The ORF for cCAT-2A, cCAT-2B and cCAT-2C were amplified from pCR-cCAT-2A, pCR-cCAT-2B and pCR-cCAT-2C plasmid, respectively, using Platinum *Taq* DNA Polymerase and cCAT-2 ORF primer pair 2 (Table 1) that contained an added Kozak sequence on the sense primer and deleted stop codon on the anti-sense primer to allow for expression of a C-terminal V5 epitope for cellular localization studies. The cCAT-2A, cCAT-2B and cCAT-2C PCR products were cloned in-frame into pcDNA5/FRT/V5-His-TOPO mammalian expression vector (Invitrogen, Carlsbad, CA;

#K6020-01) to generate pcD-CAT-2A, pcD-CAT-2B and pcD-CAT-2C, respectively. Expression vectors were validated by sequencing (Genewiz, Inc., city, state) with T7, BGH and cCAT-2 specific primers.

2.4 Quantitative Real-Time PCR

Quantitative real-time PCR analysis of cCAT-2A, cCAT-2B, cCAT-2C and β-actin mRNA abundance in bursa of Fabricius, gastrocnemius, heart, liver, pectoralis major, and thymus was performed with an iCyclerIQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Reactions utilized the IQ SYBR Green Supermix (Bio-Rad, Hercules, CA; #170-8884), 1 µl of 1:2 diluted RT product and 300 nM of each cCAT-2 isoform specific primer (Table 1). Thermal cycling parameters were 1 cycle at 95°C for 3 min, 45 cycles of 95°C for 15 s, annealing temperatures as described in Table 1 for 30 s and 72°C for 1 min. Melting curve analysis was performed after each PCR run to confirm product specificity. Melting curve parameters were 1 cycle at 95°C for 1 min, 1 cycle at 55°C and then the temperature was increased 0.5°C/10 s to 95°C while continuously monitoring fluorescence. The $2^{-\Delta Ct}$ equation was utilized to determine the relative fold-change in mRNA abundance (73,74). The relative fold-change in tissue cCAT-2A, cCAT-2B and cCAT-2C mRNA abundance was normalized to each tissue's β-actin mRNA abundance. For tissue cCAT-2 isoform mRNA analysis, normalized values were expressed relative to each tissue's cCAT-2B mRNA abundance or the abundance of each isoform in the bursa.

2.5 Cell culture

The chicken liver hepatocellular carcinoma cell line (LMH, #CRL-2117), human embryonic kidney cell line (HEK 293 T cell, #CRL-11268), and Chinese hamster ovary-K1 cell line (CHO-K1, #CCL-61) were obtained from American Type Culture Collection (Rockville, MD). The human embryonic kidney Flp-In cell line (293 FLP-IN, #R75007) was obtained from Invitrogen. LMH cells were grown in 0.1% gelatin-coated cell culture dishes with complete medium (LMH-CM) consisting of Waymouth's medium with 10% fetal bovine serum (FBS) and 1% pen/strep (Invitrogen, Carlsbad, CA; #15140-163). HEK 293 T cells were grown in complete medium (HEK-CM) containing DMEM medium with 10% FBS, 1% pen/strep and L-glutamine (200mM). CHO-K1 cells were grown with complete medium (CHO-CM) containing Ham's F-12 medium with 10% FBS, 1% pen/strep and L-glutamine (200mM). Non-transfected 293 FLP-IN cells were maintained in media (293 non-trans CM) containing DMEM with 10% FBS, 1% pen/strep, L-glutamine (200mM) and Zeocin (100µg/ml; Invitrogen, Carlsbad, CA; #R250-01). Stably transfected 293 FLP-IN cells were maintained in complete media (293-CM) containing DMEM with 10% FBS, 1% pen/strep, L-glutamine (200mM) and hygromycin B (100µg/ml; Invitrogen, Carlsbad, CA; #10687-010). All cell cultures were maintained on 10 cm cell culture dishes at 37°C and 5% CO_2.

2.6. Transient Transfection

Prior to transfection, LMH, HEK 293 T, or CHO-K1 cells were plated at a density of 1 x 10^5 cells in a 6-well plate (Corning, Corning, NY; #3576) and grown to 90% confluency. Cells were chemically transfected in Opti-MEM (Invitrogen, Carlsbad, CA;

#31985-062) using 4 µl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA; #11668-027) and 1.5 µg of cCAT-2 isoform expression vector DNA, empty vector (pcDNA5/FRT/V5-His/CAT; Invitrogen, Carlsbad, CA; #K6020-01), or pECFP-Golgi (Clonetech, Mountain View, CA; #632464) marker according to the manufacturer's protocol. Transfected cells were incubated at 37°C and 5% CO_2 for 5-8 h. Following transfection, media was decanted and cells were grown for 48 h in their respective CM.

2.7. Stable Transfection

293 FLP-IN cells were grown in 293 non-trans CM to 75% confluency prior to transfection. FLP-IN cells were chemically co-transfected with Opti-Mem (Invitrogen, Carlsbad, CA; #31985-062) using 10 µl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA; #11668-027) and 12 µg of DNA from FLP-IN expression vector pcDNA5/FRT/V5-His-TOPO (Invitrogen, Carlsbad, CA; #K6020-01) containing the cCAT-2 cDNA or pcDNA5/FRT/V5-His/CAT control vector and Flp recombinase expression plasmid pOG44 (Invitrogen, Carlsbad, CA; #V6005-20) for 5-8 h according to the manufacturer's protocol. Following transfection, medium was decanted and cells were grown in DMEM with 10% FBS, 1% pen/strep and L-glutamine (200mM) for 48 h and then replated in 293-CM at 30% confluency. Two weeks following transfection, single cell colonies were harvested using clonal rings and 0.25% Trypsin-EDTA (Invitrogen, Carlsbad, CA; #25200-056) and were cultured in 293-CM. Stable expression of each gene of interest was verified by western blot using an anti-V5 antibody (Immunology Consultants Laboratory, Newberg, OR; #RV5-45A-Z), β-gal staining (Invitrogen, Carlsbad, CA;

#K1465-01) and zeocin sensitivity (Invitrogen, Carlsbad, CA; # R250-01) according to manufacturer's instructions. Stable cells were maintained at 37°C and 5% CO_2.

2.8. Immunoblotting

Transfected cells were collected in Delbucco's Phosphate Buffered Saline (DPBS) using cell scrapers (Fisher Scientific, Pittsburgh, PA; #08-773-2) and centrifuged at 14,000 x g for 15 min at 4°C. Cell pellets were lysed in membrane-stabilizing buffer (MS; 210mM mannitol, 70mM sucrose, 5mM Tris-HCl, pH 7.5, and 1mM EDTA) containing protease inhibitor cocktail III (Calbiochem, San Diego, CA; #539134) for 10 min at 4ºC according to the method of Krisnamurthy et al. (75). Total protein was centrifuged 100 x g at 4°C for 5 min to collect nuclei. The supernatant was collected and treated with a solution of DNase buffer and 1U/μL RNase-free DNase (Fisher Scientific, Pittsburgh, PA; #BP3223-1) for 20 min at 25ºC. Protein concentration was determined using the Bradford Assay Dye Concentrate (Bio-Rad, Hercules, CA; #500-0006). Protein samples (25 μg) were treated with 4 M urea in H_2O for 10 min and 100 mM DTT for 3 min and then electrophoresed under reducing conditions on 4% stacking and 10% resolving gels containing 8 M urea. Protein was transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA; #162-0112) at 0.07 amps using a semi-dry transfer cassette. Blots were blocked in 5% skim milk in 0.05% DPBS-Tween for 1 h at 25°C and probed with a 1:500 dilution of monoclonal rabbit anti-V5 (Immunology Consultants Laboratory, Newberg, OR; # RV5-45A-Z) followed by a 1:10,000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (Pierce Biotechnology, Rockford, IL; #31460) in a 5% skim milk in 0.05% DPBS-Tween solution for 1 h at 25°C or 16 h at 4°C. As a

control, blots were probed with a 1:5000 dilution of monoclonal mouse anti-tubulin (Sigma, St. Louis, MO; #T6199) followed by a 1:10,000 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (Pierce Biotechnology, Rockford, IL; #31430). Secondary antibodies were detected using SuperSignal West Pico Luminal Enhance for Horseradish Peroxidase (Pierce Biotechnology, Rockford, IL; #34078). All images were captured using Quality One Software Version 4.5.2 on a Chemidoc XRS (Bio-Rad, Hercules, CA; #170-8070).

2.9 Immunofluorescence confocal microscopy

At indicated times post-transfection, transfected cells were washed with DPBS (pH 7.4) and incubated with serum-free DMEM containing wheat germ agglutinin conjugate Alexa Fluor 633 (8 μg/mL; Invitrogen, Carlsbad, CA; #W21404) for 5 min at 25ºC. Cells were washed with DPBS, fixed with 4% paraformaldehyde in DPBS (pH 7.4) for 40 min, quenched with 0.1 M ethanolamine (pH 7.4) for 10 min and permeabilized with 0.2% Triton X-100 in DBPS for 10 min. Transiently transfected cells were blocked for 1 h with SuperBlock Blocking Buffer (Pierce Biotechnology, Rockford, IL; #37515) and incubated with 1:750 diluted anti-V5-FITC Antibody (Invitrogen, Carlsbad, CA; #R963-25) in Superblock Blocking Buffer for 1 h at 25ºC. Stably transfected cells were blocked for 1 h with SuperBlock Blocking Buffer at 25ºC and incubated overnight with 1:750 diluted anti-V5-FITC antibody in SuperBlock Blocking Buffer at 4ºC. Following incubation with primary antibody, cells were washed with 0.5% Tween 20 in DPBS and stained with 100 nM 4′,6-diamidino-2-phenylindole (DAPI) dilactate for 3 min (Invitrogen, Carlsbad, CA; #D3571) according to the manufacturer's

instructions. Coverslips containing transfected cells were mounted on glass slides using Prolong Antifade (Invitrogen, Carlsbad, CA; #P7481) and were viewed using a Zeiss LSM 510 confocal microscope with an Argon laser at 488 nm, a HeNe laser at 543 nm, and a HeNe laser at 633 nm using the Laser Scanning Microscope LSM 510 software version 3.2 SP2 (Zeiss, Thornwood, NY).

2.10 Amino acid transport kinetics

CHO cells were plated at a density of 1 x 10^5 cells/well and were transiently transfected as described previously and incubated for 48 h in CHO-CM at 37°C and 5% CO_2. Stably transfected 293 FLP-IN cells were plated at a density of 4 x 10^4 cells/well and maintained in 37°C and 5% CO_2 with 293-CM for 16 h. Cells containing pcD-CAT-2A, pcD-CAT-2B, pcD-CAT-2C or empty vector were washed and incubated at 25°C for 3 min with transport buffer (TB) pH 7.5 containing 140 mM choline chloride, 10 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, and 15 mM L-leucine. Cells received fresh TB containing 10 mM, 6 mM, 3 mM, 1 mM, 500 μM, or 50 μM, L-lysine or L-arginine with either 5μCi/ml L-[4,5-^{3}H(N)]-lysine monohydrochloride (92 Ci/mmol; Perkin Elmer, Waltham, MA; #NET376001MC) or 5μCi/ml of L-[2,3,4-^{3}H(N)]-arginine monohydrochloride (58 Ci/mmol; Perkin Elmer, Waltham, MA; #NET1123250UC) and were incubated at 37°C and 5% CO_2 for 0, 7, 21, or 35 min. Transport was stopped by washing cells with ice-cold TB and cells were lysed with 1% SDS in DPBS (pH 7.4). Lysed cell extracts in Ultima Gold Liquid Scintillation Cocktail (Perkin Elmer, Waltham, MA; #6013329) were used to determine radioactivity incorporated into cells. Total cell

protein was determined by the BCA protein assay (Pierce, Rockford, IL; #23225), and all samples were normalized to the protein concentration of each cell lysate. Velocity of lysine or arginine transport was expressed as pmol/mg protein/min based upon transport at 21 min. Velocity of transport for cells expressing the empty vector was subtracted from velocity of cells expressing pcD-CAT-2 isoforms. The K_m for each isoform was determined by setting transporter velocity as a function of substrate concentration using GraphPad Prism version 5.00 software (Graph Pad, San Diego, CA).

2.11 Transporter specificity assay

Cells stably transfected with pcD-CAT-2A or empty vector were plated at a density of 2 x 10^4 cells/well and maintained in 293-CM for 16 hours at 37°C and 5% CO_2. Cells were washed and incubated in TB for 5 min at 25°C and then received fresh TB with 5μCi/ml L-[4,5-^{3}H(N)]-lysine monohydrochloride, 7 mM L-lysine, and either 5- or 35-fold molar excess concentrations of L-lysine, L-arginine, L-glutamate, L-glutamine L-glycine, L-histidine, L-methionine, L-phenylalanine, L-serine or L-valine for 20 min at 37°C and 5% CO_2. Transport was stopped by aspirating transport medium and washing cells with ice-cold TB, and cells were then lysed with 1% SDS. Total protein content and radioactivity measurements were conducted as described for the transport kinetic studies. Velocity of L-[4,5-^{3}H(N)]-lysine monohydrochloride transport in 5- and 35-fold molar excess treatments were expressed relative to transport velocity in 7 mM L-lysine controls. Specificity of each transport protein was determined by comparing the differences between transporter velocity with and without additional amino acid, as measured by pmol/mg protein/minute.

2.12 Data Analysis

Statistical analysis for real-time PCR and transport kinetic studies were performed using one-way ANOVA with Student-Newman-Keuls post-test using GraphPad InStat version 3.06 for Windows (GraphPad Software, San Diego, CA). Sequence analysis was conducted using ClustalW multiple-alignment software (76) and BoxShade multiple-alignment shading software.

Chapter 3: Results

3.1 Tissue cCAT-2 isoform mRNA abundance

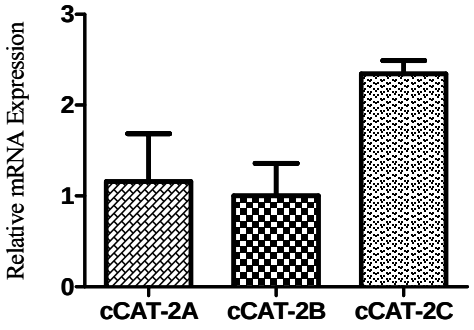
Quantitative real-time PCR analysis was performed to determine the relative abundance of cCAT-2 isoform mRNA in bursa of Fabricius, gastrocnemius, heart, liver, pectoralis major, and thymus. The mRNA abundance of cCAT-2 isoforms did not differ ($p>0.05$) in the bursa (Figure 7A), heart (Figure 7C), and thymus (Figure 7F). The mRNA abundance of cCAT-2 isoforms was differentially expressed in the gastrocnemius (Figure 7B), liver (Figure 7D), and pectoralis major (Figure 7E). In all of these tissues, the cCAT-2A isoform had the greatest mRNA abundance ($p<0.05$) while cCAT-2B and cCAT-2C mRNA abundance were similar ($p>0.05$). In the heart, cCAT-2B mRNA abundance was 2-fold greater than cCAT-2C ($p<0.05$; Figure 7C).

To compare mRNA abundance of cCAT-2 isoforms between tissues, the mRNA abundance of each isoform in each tissue was normalized to β-actin mRNA levels and was normalized relative to respective cCAT-2 isoform mRNA levels in the bursa, which had the lowest expression (Figure 8A-C). Chicken CAT-2A mRNA abundance was greatest in the gastrocnemius, liver, and pectoralis major ($p<0.05$; Figure 8A). Chicken CAT-2B mRNA abundance was greatest in the liver ($p<0.05$; Figure 8B). Chicken CAT-2C mRNA abundance was greatest in the gastrocnemius, pectoralis major, and liver ($p<0.05$; Figure 8C).

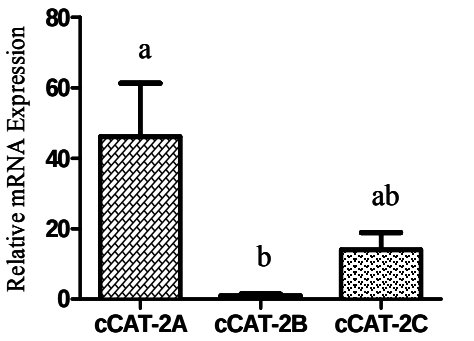
Figure 7. **Quantitative real-time PCR analysis of cCAT-2 isoform mRNA abundance by tissue.**

Chicken CAT-2 isoform mRNA abundance in the (A) bursa of Fabricius, (B) gastrocnemius, (C) heart, (D) liver, (E) pectoralis major, and (F) thymus was quantified by real-time PCR. Values were normalized to tissue β-actin mRNA abundance and expressed relative to the tissue cCAT-2B mRNA level. Graph bars not sharing common superscripts are significantly different, $p<0.05$. Values are means ± SEM, n=4.

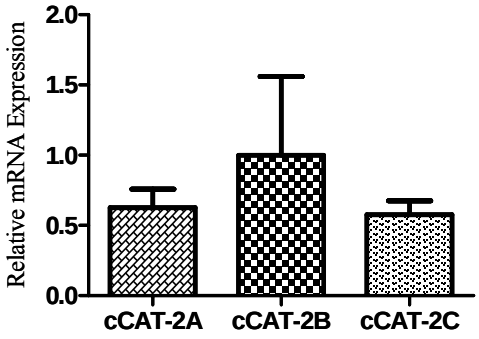
(A)


cCAT-2 Expression in the Bursa
Relative mRNA Expression
3
2
1
0
cCAT-2A
cCAT-2B
cCAT-2C

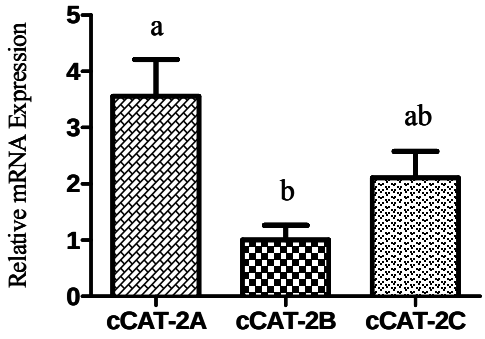
(B)


cCAT-2 Expression in the Gastrocnemius
Relative mRNA Expression
80
60
40
20
0
a
b
ab
cCAT-2A
cCAT-2B
cCAT-2C

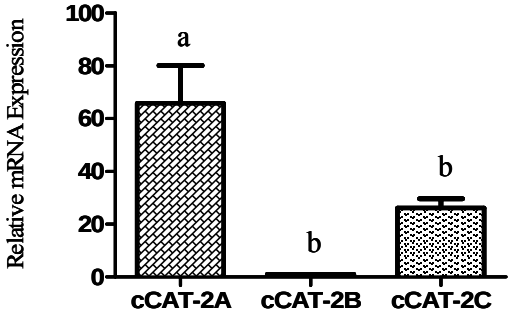
(C)


cCAT-2 Expression in the Heart
Relative mRNA Expression
2.0
1.5
1.0
0.5
0.0
cCAT-2A
cCAT-2B
cCAT-2C

(D)


cCAT-2 Expression in the Liver
Relative mRNA Expression
5
4
3
2
1
0
a
b
ab
cCAT-2A
cCAT-2B
cCAT-2C

(E)


cCAT-2 Expression in the Pectoralis
Relative mRNA Expression
100
80
60
40
20
0
a
b
b
cCAT-2A
cCAT-2B
cCAT-2C

(F)

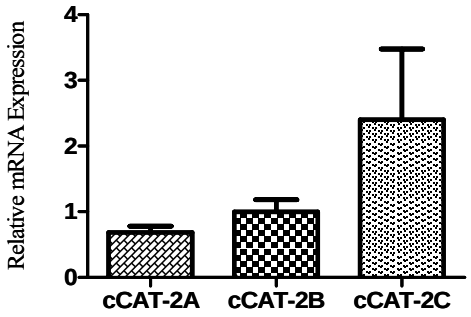
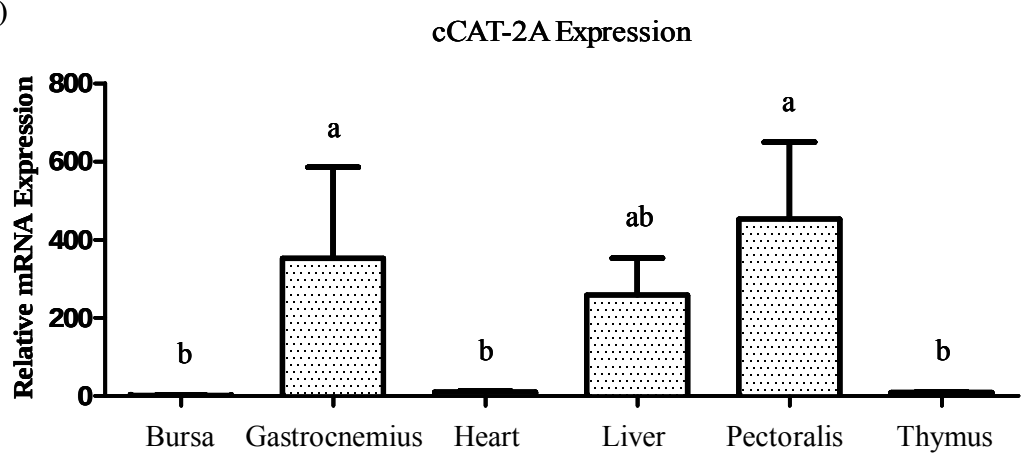

cCAT-2 Expression in the Thymus
Relative mRNA Expression
4
3
2
1
0
cCAT-2A
cCAT-2B
cCAT-2C

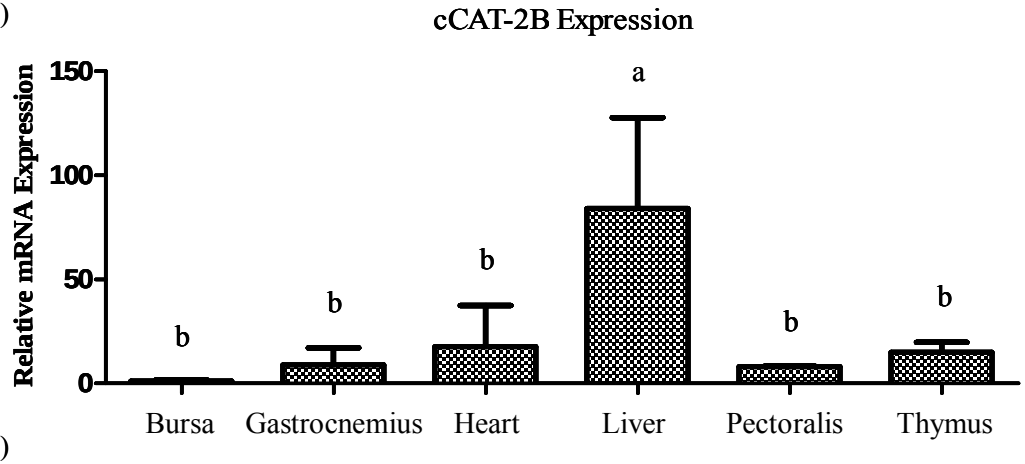
Figure 8. **Quantitative real-time PCR analysis of cCAT-2 isoform mRNA abundance by isoform.**

Quantitative real-time PCR analysis of (A) CAT-2A, (B) CAT-2B, and (C) CAT-2C in two-week old broiler chick tissues. Data are normalized to β-actin and expressed relative to the respective cCAT-2 isoform mRNA abundance in the bursa of Fabricius. Graph bars not sharing common superscripts are significantly different, $p<0.05$. Values are means ± SEM, n=4, of mRNA expression.

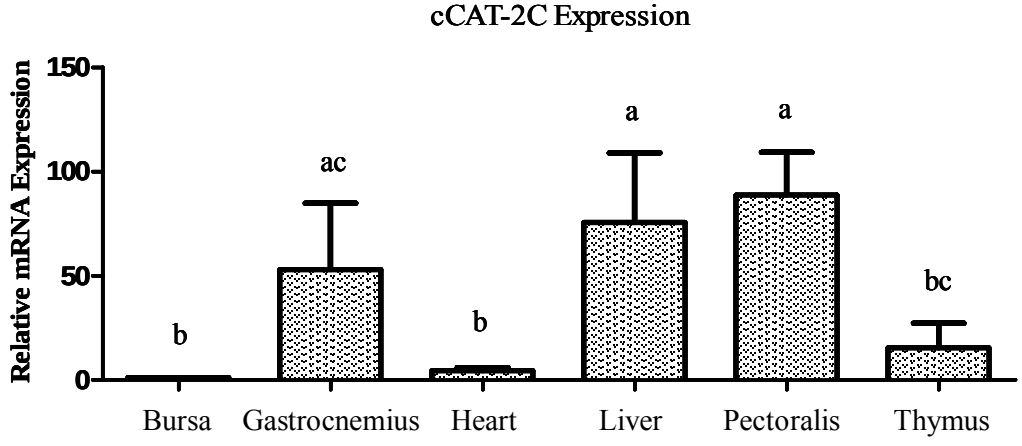
(A)


cCAT-2A Expression
Relative mRNA Expression
800
600
400
200
0
a
b
b
ab
a
b
Bursa
Gastrocnemius
Heart
Liver
Pectoralis
Thymus

(B)


cCAT-2B Expression
Relative mRNA Expression
150
100
50
0
b
b
b
a
b
b
Bursa
Gastrocnemius
Heart
Liver
Pectoralis
Thymus

(C)


cCAT-2C Expression
Relative mRNA Expression
150
100
50
0
b
ac
b
a
a
bc
Bursa
Gastrocnemius
Heart
Liver
Pectoralis
Thymus

3.2 Transient expression of cCAT-2 isoform protein

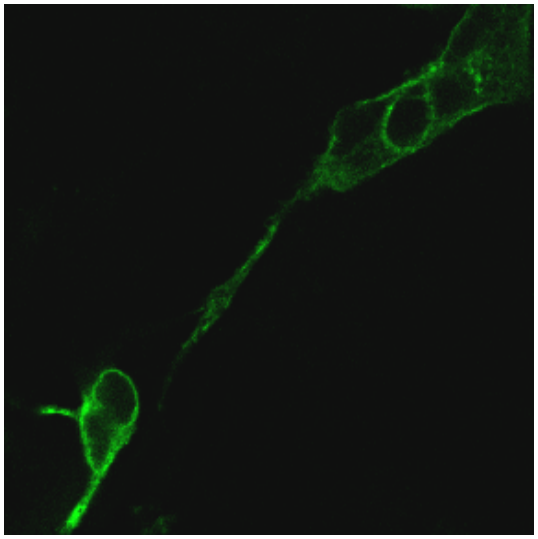
Preliminary localization studies with cCAT-2A and cCAT-2C utilized the chicken LMH and mammalian HEK 293 cell lines. In both cell types, cCAT-2A protein was localized to the plasma membrane (Figures 9A &10A), and cCAT-2C protein localized to the cytoplasm (Figures 9B & 10B). These cell types, however, proved difficult for image analysis by confocal microscopy due to the columnar growth of LMH cells and the low transfection efficiency of HEK 293 cells (<5%). As a result, CHO-K1 cells were used due to their morphology and higher transfection efficiency (10-15%).

Cellular localization of cCAT-2 isoforms was determined by immunocytochemistry in transiently transfected CHO-K1 cells. Chicken CAT-2A protein localized to the plasma membrane, as confirmed by colocalization with a plasma membrane marker (Figure 11A). A small amount of cCAT-2A protein also localized to the perinuclear space, indicative of the golgi. Chicken CAT-2B localized to the cytoplasm juxtaposed to the plasma membrane (Figure 11B). Chicken CAT-2C protein localized throughout the cytoplasm (Figure 11C) and control protein, chloramphenicol acetyltransferase, localized throughout the cell (Figure 11D).

Figure 9. Localization of chicken CAT-2A and CAT-2C in LMH cells.

LMH cells were transiently transfected with (A) pcD-CAT-2A and (B) pcD-CAT-2C and labeled by immunocytochemistry using an anti-V5-FITC conjugated antibody. This cell type has poor morphology and difficult image capturing due to the columnar growth pattern. Localization of cCAT-2A and cCAT-2C proteins differ.

(A) cCAT-2A



(B) cCAT-2C

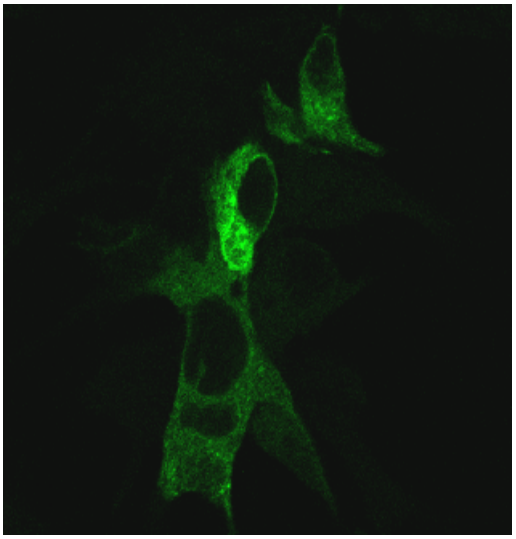
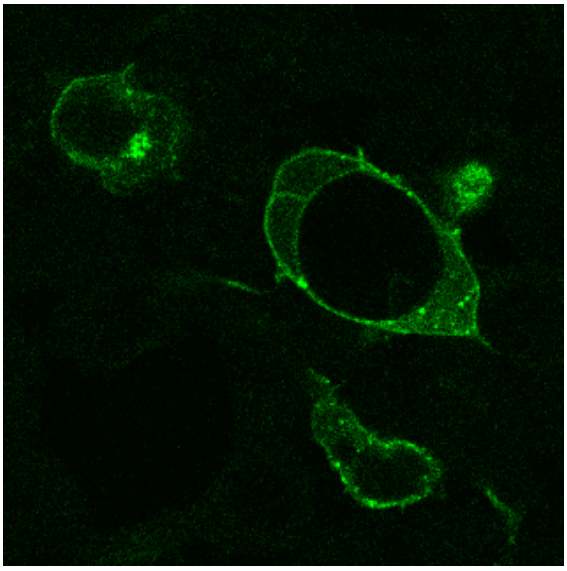


Figure 10. Localization of chicken CAT-2A and CAT-2C in HEK 293 cells.

HEK cells were transiently transfected with (A) pcD-CAT-2A and (B) pcD-CAT-2C and labeled by immunocytochemistry using an anti-V5-FITC conjugated antibody. This cell type had poor adherence to cell culture dish surface during staining procedure and low transfection efficiency. Localization of cCAT-2A and cCAT-2C proteins differ.

(A) cCAT-2A



(B) cCAT-2C

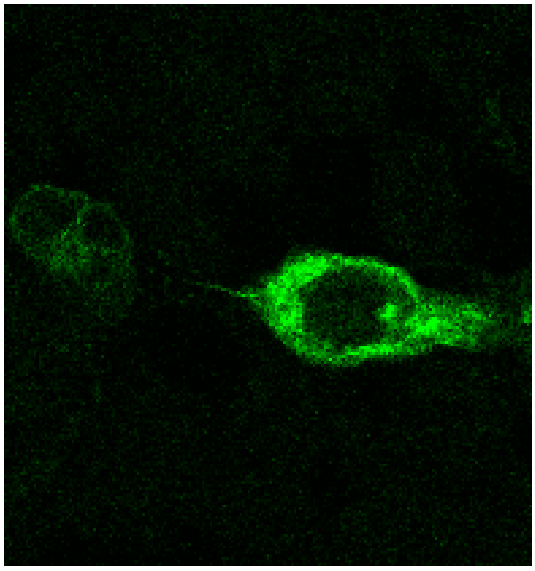
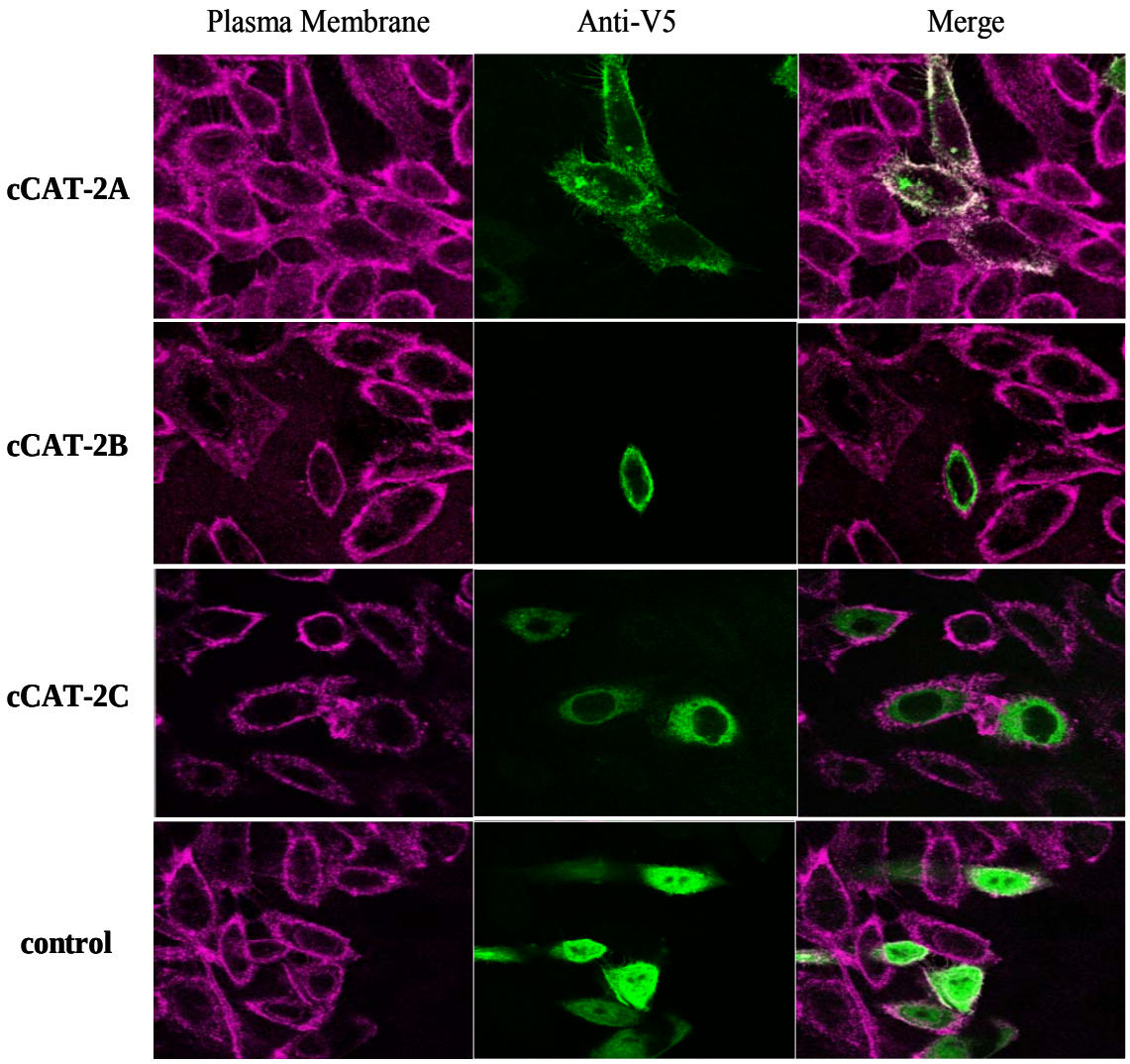


Figure 11. Immunocytochemistry of cCAT-2 isoform proteins in transiently transfected CHO-K1 cells.

Cells were transfected with (A) pcD-CAT-2A, (B) pcD-CAT-2B, (C) pcD-CAT-2C, or (D) control vector. Wheat germ agglutinin plasma membrane marker (pink) and anti-V5-FITC antibody (green) identified subcellular localization of cCAT-2 isoforms and control proteins. Localization of cCAT-2A, cCAT-2B and cCAT-2C proteins differ.

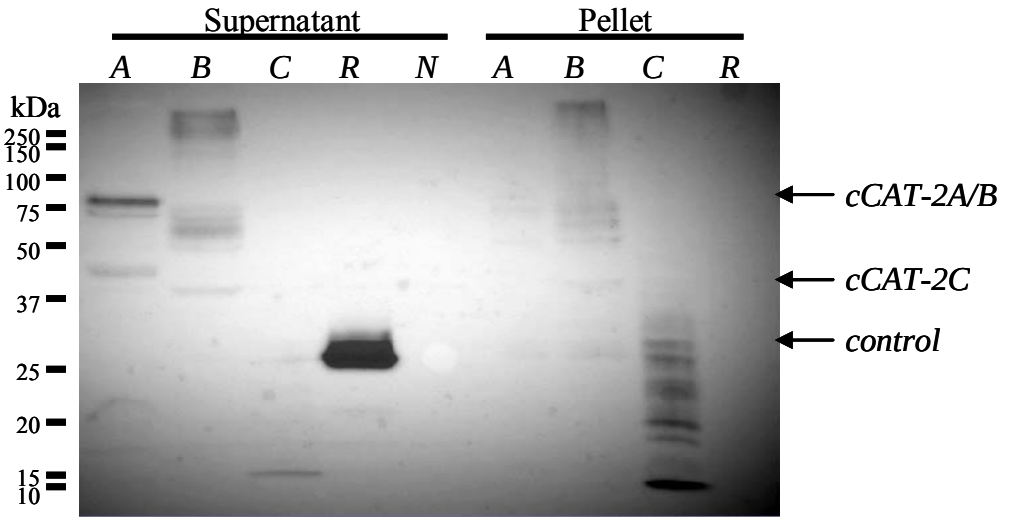


Chicken CAT-2 isoform protein expression was determined by western blot analysis of supernatant and nuclear fractions (Figure 12A). The predicted molecular weight of each V5 epitope-tagged protein is 76kDa for cCAT-2A and cCAT-2B, 40kDa for cCAT-2C, and 28kDa for chloramphenicol acetyltransferase control. Chicken CAT-2A protein was detected in the supernatant fraction at the predicted molecular weight. Bands for cCAT-2B protein were detected in both the supernatent and pellet fractions at 250, 75 and 50 kDa. Chicken CAT-2C protein was detected at 37 kDa in the supernatant fraction. Control bands were detected at the predicted molecular weight of 40 kDa.

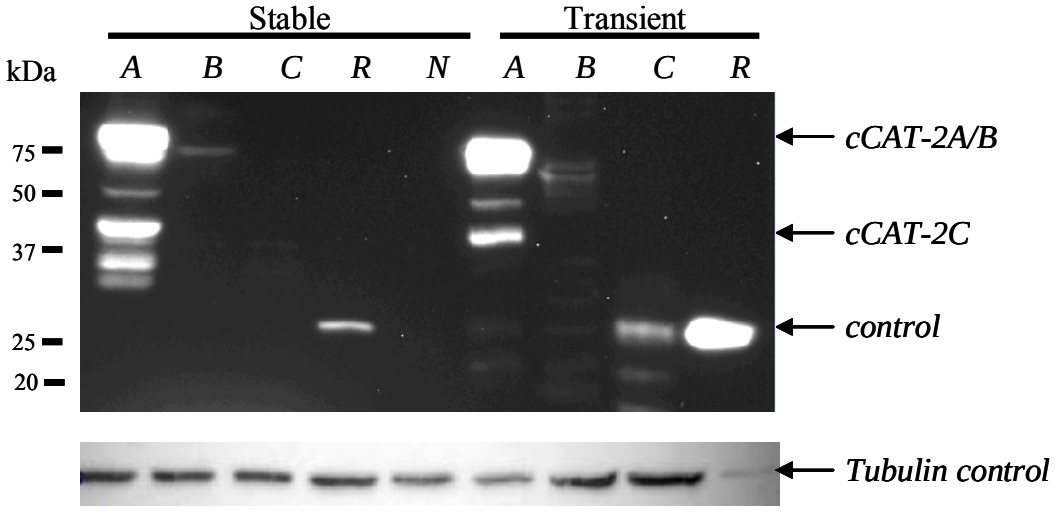
Figure 12. Immunoblot analysis of cCAT-2 isoform proteins from CHO-K1 cell fractions and stable cell lines.

Chicken CAT-2 proteins were extracted from transiently transfected CHO cells 48 h after transfection or stably transfected 293 Flp-In cells. Samples include cCAT-2A, *A*; cCAT-2B, *B*; cCAT-2C, *C*; control (chloramphenical acetyltransferase), *R*; and non-transfected cells, *N*. (A) Proteins were extracted from transiently transfected cells using hypotonic lysing solution and were separated into supernatent and pellet fractions by differential centrifugation. (B) Protein was extracted from transiently transfected and stably transfected cells using a hypotonic lysing solution. Total protein extract was centrifuged at 100 x g for 5 minutes to separate nuclei and supernatant fractions. All samples were treated with 4M urea, 100mM DTT, and Laemmli-SDS sample buffer and resolved using SDS-PAGE gel containing 8 M urea.

(A)


Supernatant
Pellet
A
B
C
R
N
A
B
C
R
kDa
250
150
100
75
50
37
25
20
15
10
cCAT-2A/B
cCAT-2C
control

(B)


Stable
Transient
kDa
A
B
C
R
N
A
B
C
R
75
50
37
25
20
cCAT-2A/B
cCAT-2C
control
Tubulin control

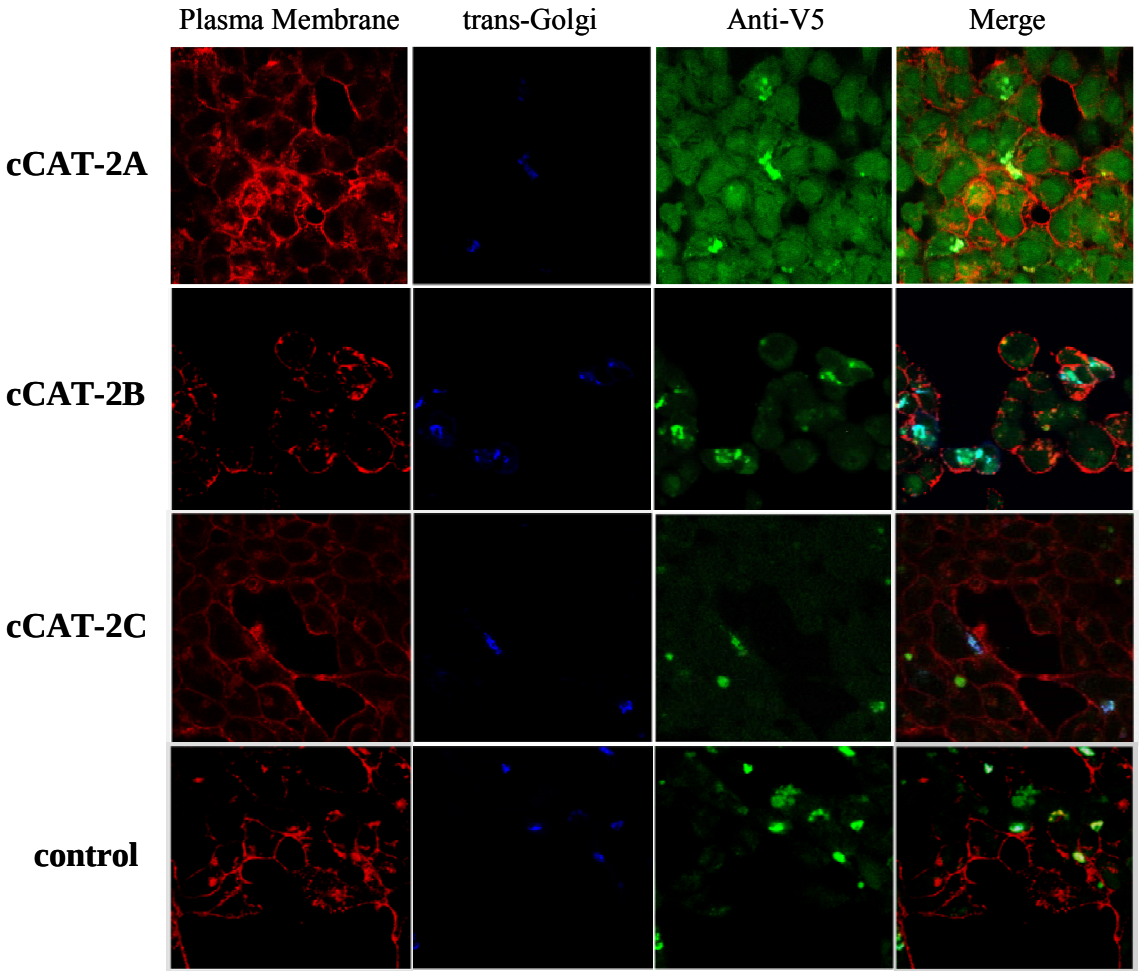
3.3 Stable expression of cCAT-2 isoform proteins

Due to the low transfection efficiency, variable protein expression level in transfected cells, and questions concerning degradation of the proteins that arose while using transiently transfected cells, it was decided to conduct stable transfection with pcD-CAT-2A, pcD-CAT-2B, pcD-CAT-2C, or empty vector. In Flp-In 293 cells, recombination of the cCAT-2 or control genes occurs singly in a transcriptionally active genomic locus and uniformly in all cells. As a result, the cCAT-2 and control proteins are expressed at low levels within all cells. Stably expressed cCAT-2A and cCAT-2B localize to the plasma membrane as well as in cytoplasmic vesicles and the golgi (Figure 13A & B). Chicken CAT-2C protein localizes to the golgi with very low levels in the cytoplasm (Figure 13C) and cells stably expressing the positive control protein, chloramphenicol acetyltransferase, show localization of protein throughout the cytoplasm and golgi (Figure 13D).

Western blot analysis of stable cCAT-2 isoform cells detected cCAT-2 isoform protein at the predicted molecular weight in supernatant fractions (Figure 12B). Protein expression of cCAT-2A was 25-fold and 40-fold higher than cCAT-2B and cCAT-2C respectively, as measured by relative brightness of each band. Relative intensity was calculated by measuring the brightness of anti-V5 antibody labeled bands compared to the brighness of anti-tubulin bands per mm^2 (Figure 12C) using Quantity One software (Bio-Rad, Hercules, CA).

Figure 13. Immunocytochemistry of cCAT-2 isoform proteins in stably transfected 293 Flp-In cells.

Cells were transfected with (A) pcD-CAT-2A, (B) pcD-CAT-2B, (C) pcD-CAT-2C, or (D) control vector. Plasma membrane marker (red), ECFP-trans-golgi marker (blue) and anti-V5-FITC antibody (green) identified subcellular localizion of cCAT-2 isoforms and control. Localization of cCAT-2A, cCAT-2B and cCAT-2C proteins differ.



3.4 Function of cCAT-2 isoforms

Uptake of [^{3}H]-L-lysine was time- (Figure 14) and concentration- (Figure 15A) dependent in CHO-K1 cells transiently transfected with cCAT-2A, indicating that cCAT-2A transport is saturable. The K_m and V_{max} for L-lysine by cCAT-2A transfected CHO-K1 cells were 2.644 ± 1.379 mM and 11.93 ± 2.152 pmol/mg protein/min, respectively (Figure 15B). In contrast, CHO-K1 cells transiently transfected with either cCAT-2B or cCAT-2C did not transport enough L-lysine to be detected during the time frame or amino acid concentration in which these experiments were performed (Figure 16A & B).

Figure 14. L-lysine uptake by CHO-K1 cells transiently expressing chicken CAT-2A is time dependent.

CHO-K1 cells were transiently transfected with chicken CAT-2A expression vector (filled circles) and 24 h later were cultured in transport buffer containing 6 mM L-[4,5-^{3}H(N)]-lysine monohydrochloride for 0-60 minutes. CHO-K1 cells transfected with empty vector were used as controls (open circles). Transport of L-lysine is time dependent and reaches saturation by 60 minutes. L-[4,5-^{3}H(N)]-lysine monohydrochloride in cell lysates was quantified using a β-scintillation counter and normalized to protein concentrations. Each data point represents the mean ± SEM, n=3. * indicates significant difference between cCAT-2A and empty vector ($p<0.05$).

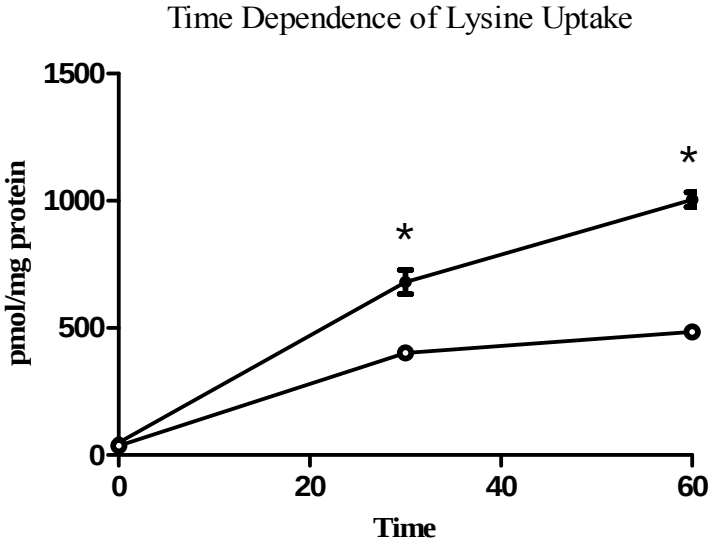
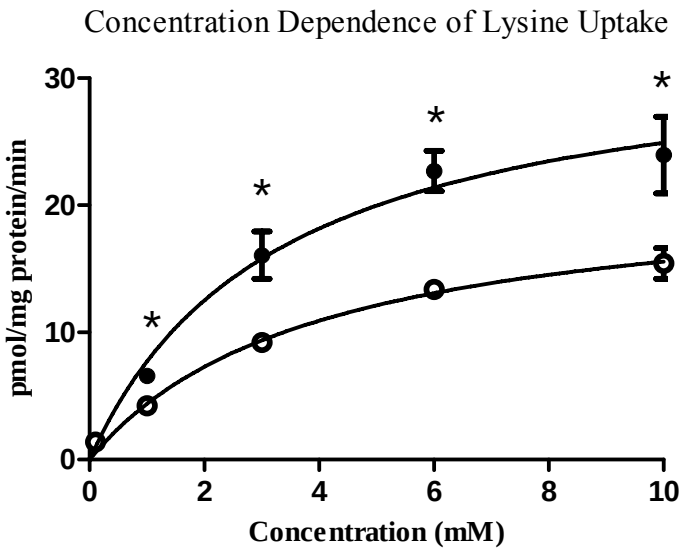


Figure 15. L-lysine uptake by CHO-K1 cells transiently expressing chicken CAT-2A is concentration dependent.

CHO-K1 cells were transiently transfected with chicken CAT-2A expression vector (filled circles) and 24 h later were cultured in transport buffer containing 100 μM , 1 mM, 3 mM, 6 mM, or 10 mM L-[4,5-3H(N)]-lysine monohydrochloride for 30 min. CHO-K1 cells transfected with empty vector were used as a control (open circles). (A) Transport of L-lysine is concentration dependent in cells expressing cCAT-2A and control. (B) Transport of L-lysine by CHO cells transiently transfected with cCAT-2A, the subtracted difference, has a K_m=2.644 ± 1.379 mM as determined by Michaelis-Menton Kinetics and non-linear regression analysis. L-[4,5-^{3}H(N)]-lysine monohydrochloride in cell lysates was quantified using a β-scintillation counter and normalized to protein concentrations. Each data point represents the mean ± SEM, n=3. * indicates significant difference between cCAT-2A and empty vector ($p<0.05$).

(A)


Concentration Dependence of Lysine Uptake
pmol/mg protein/min
30
20
10
0
0
2
4
6
8
10
Concentration (mM)
*
*
*
*

(B)

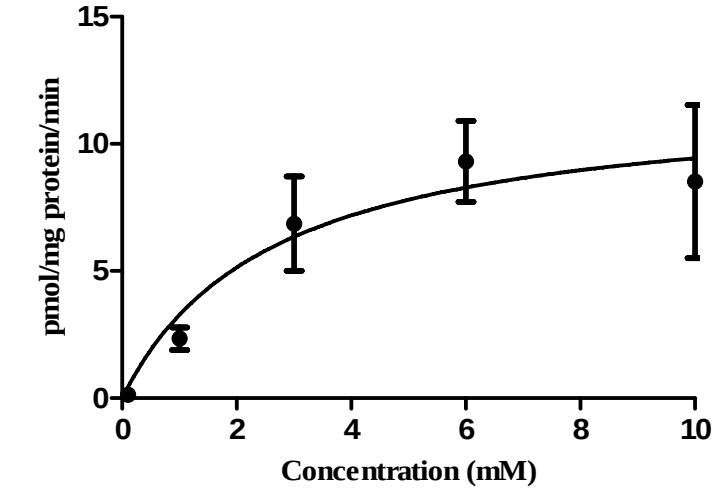
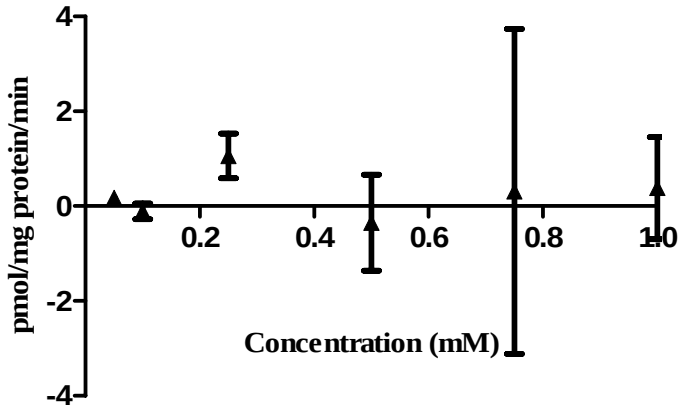

cCAT-2A Transport of Lysine in Transiently Transfected Cells
pmol/mg protein/min
15
10
5
0
0
2
4
6
8
10
Concentration (mM)

Figure 16. L-lysine is not transported by CHO-K1 cells transiently expressing chicken CAT-2B and CAT-2C.

CHO-K1 cells were transiently transfected with (A) chicken CAT-2B expression vector (filled triangles) and 24 h later were cultured in transport buffer containing 10 μM, 50 μM, 100 μM, 250 μM, 500 μM, 750 μM, or 1 mM L-[4,5-^{3}H(N)]-lysine monohydrochloride for 18 minutes and (B) chicken CAT-2C expression vector (filled diamonds) and 24 h later were cultured in transport buffer containing 10 μM, 50 μM, 100 μM, 500 μM, 1mM, or 5mM L-[4,5-^{3}H(N)]-lysine monohydrochloride for 18 min. Chicken CAT-2B and cCAT-2C did not transport L-lysine. L-[2,3,4-^{3}H(N)]-arginine monohydrochloride in cell lysates was quantified using a β-scintillation counter and normalized to protein concentrations. Each data point represents the mean ± SEM, n=3.

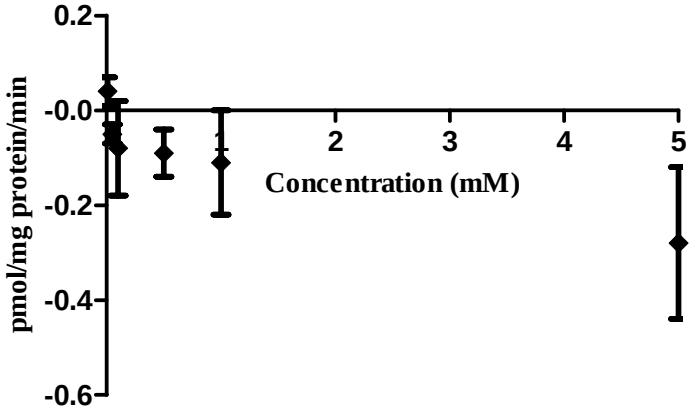
(A)

cCAT-2B Transport of Lysine in Transiently Transfected Cells



(B)

cCAT-2C Transport of Lysine in Transiently Transfected Cells

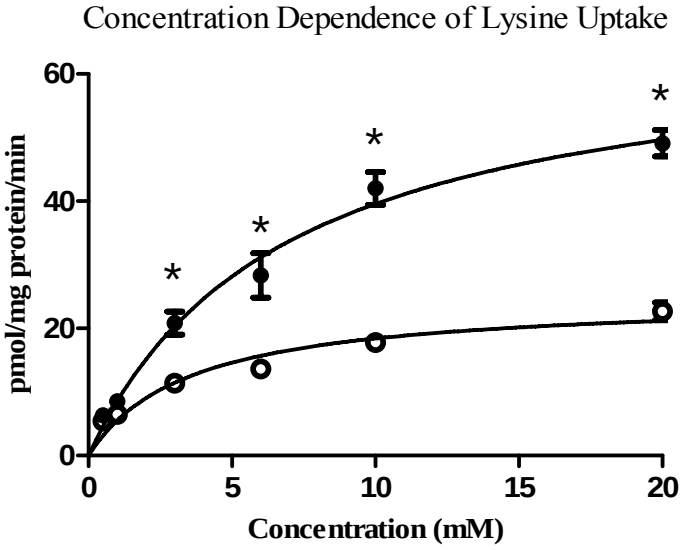


Transport of [^{3}H]-L-lysine in stable cCAT-2A cells was concentration dependent with a K_m of 7.982 ± 1.655 mM and a V_{max} of 12.79 ± 1.175 pmol/mg protein/min (Figure 17A & B). Uptake studies with [^{3}H]-L-arginine showed a K_m of 6.520 ± 1.876 mM and a V_{max} of 11.47 ± 1.358 pmol/mg protein/min (Figure 18A & B). Transport of [^{3}H]-L-lysine by stable cells expressing cCAT-2B and transport of [^{3}H]-L-lysine and [^{3}H]-L-arginine by stable cells expressing cCAT-2C was not detectable during the time frame and amino acid concentrations in which these experiments were performed (Figure 19A & B, Figure 20A & B).

Figure 17. L-lysine uptake by 293 Flp-In cells stably expressing chicken CAT-2A is concentration dependent.

293 Flp-In cells were stably transfected with chicken CAT-2A expression vector (filled circles) and were cultured in transport buffer containing 50 μM, 500 μM, 1 mM, 3 mM, 6 mM, 10 mM, or 20 mM L-[4,5-^{3}H(N)]-lysine monohydrochloride for 20 min. 293 Flp-In cells stably expressing empty vector were used as a control (open circles). (A) Transport of L-lysine was concentration dependent in cells expressing cCAT-2A and control. (B) Transport of L-lysine by cCAT-2A has K_m=7.982 ± 1.655 mM and V_{max}= 12.79 ± 1.175 pmol/mg protein/min as determined by Michaelis-Menton Kinetics and non-linear regression analysis. L-[4,5-^{3}H(N)]-lysine monohydrochloride in cell lysates was quantified using a β-scintillation counter and normalized to protein concentrations. Each data point represents the mean ± SEM, n=3. * indicates significant difference between cCAT-2A and empty vector ($p<0.05$).

(A)


Concentration Dependence of Lysine Uptake
pmol/mg protein/min
60
40
20
0
0
5
10
15
20
Concentration (mM)
*
*
*
*

(B)

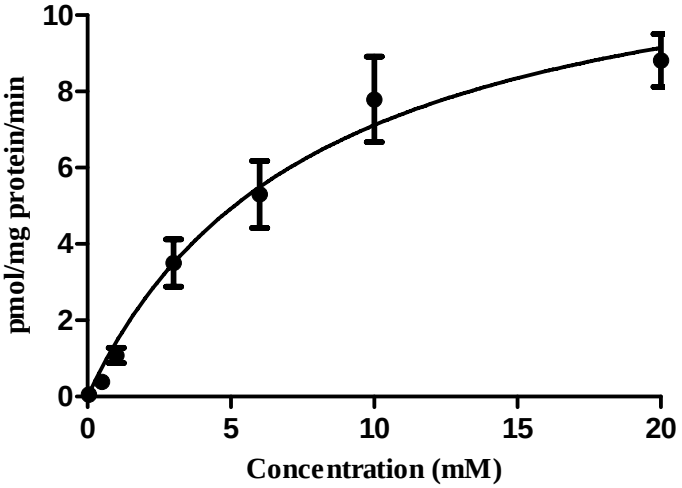
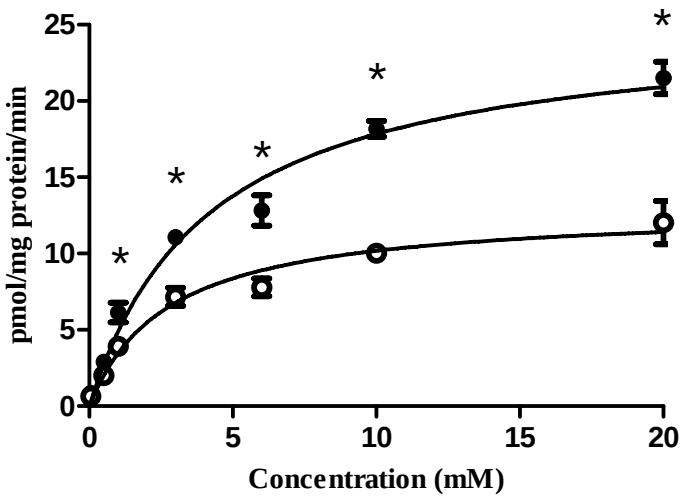

cCAT-2A Transport of Lysine in Stably Transfected Cells
pmol/mg protein/min
10
8
6
4
2
0
0
5
10
15
20
Concentration (mM)

Figure 18. L-Arginine uptake by 293 Flp-In cells stably expressing chicken CAT-2A is concentration dependent.

293 Flp-In cells were stably transfected with chicken CAT-2A expression vector (filled circles) and were cultured in transport buffer containing 50 μM, 500 μM, 1 mM, 3 mM, 6 mM, 10 mM, or 20 mM L-[2,3,4-^{3}H(N)]-arginine monohydrochloride for 20 min. 293 Flp-In cells stably expressing empty vector were used as a control (open circles) (A) Transport of L-arginine was concentration dependent in cells expressing cCAT-2A and control. (B) Transport of L-arginine by cCAT-2A has K_m=6.520 ± 1.876 mM and V_{max}=11.47 ± 1.358 pmol/mg protein/min as determined by Michaelis-Menton Kinetics and non-linear regression analysis. L-[2,3,4-^{3}H(N)]-arginine monohydrochloride in cell lysates was quantified using a β-scintillation counter and normalized to protein concentrations. Each data point represents the mean ± SEM, n=3. * indicates significant difference between cCAT-2A and empty vector ($p<0.05$).

(A)


Concentration Dependence of Arginine Uptake
pmol/mg protein/min
Concentration (mM)
0
5
10
15
20
25
*
*
*
*
*

(B)

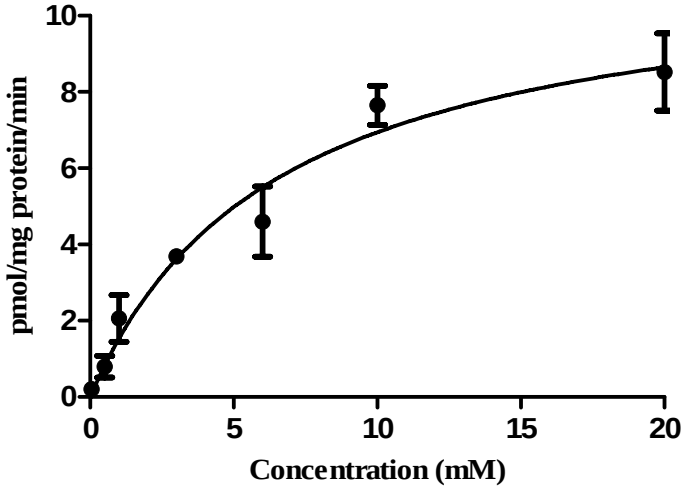
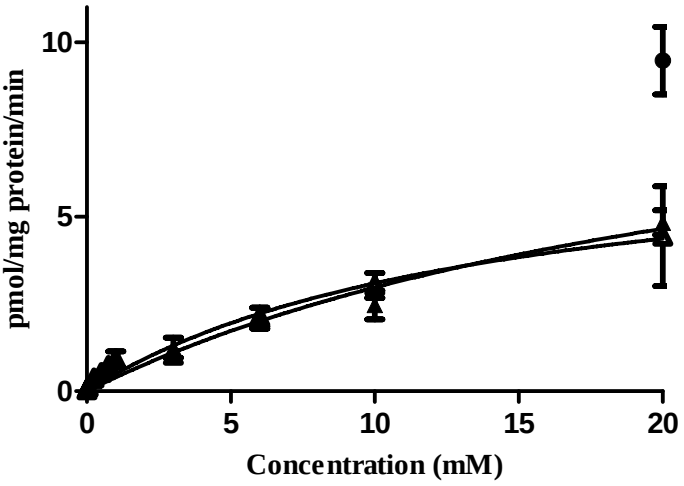

cCAT-2A Transport of Arginine in Stably Transfected Cells
pmol/mg protein/min
Concentration (mM)
0
2
4
6
8
10
0
5
10
15
20

Figure 19. L-Lysine is not transported by 293 Flp-In cells stably expressing chicken CAT-2B.

293 Flp-In cells were stably transfected with chicken CAT-2B expression vector (filled triangles) and were cultured in transport buffer containing 10 μM, 50 μM, 250 μM, 500 μM, 750 μM, 1 mM, 3 mM, 6 mM, 10 mM, or 20 mM L-[4,5-^{3}H(N)]-lysine monohydrochloride for 20 min. 293 Flp-In cells stably expressing empty vector were used as control (empty triangles). (A) Transport of L-lysine was not significantly different from controls (open triangles). Transport of L-[4,5-^{3}H(N)]-lysine monohydrochloride by cCAT-2B is shown compared to transport of L-[4,5-^{3}H(N)]-lysine monohydrochloride by cCAT-2A (filled circles). (B) Transport of L-lysine does not increase with increasing concentration. L-[4,5-^{3}H(N)]-lysine monohydrochloride in cell lysates was quantified using a β-scintillation counter and normalized to protein concentrations. Each data point represents the mean ± SEM,n=3.

(A


Concentration Dependence of Lysine Uptake
pmol/mg protein/min
10
5
0
0
5
10
15
20
Concentration (mM)

(B)

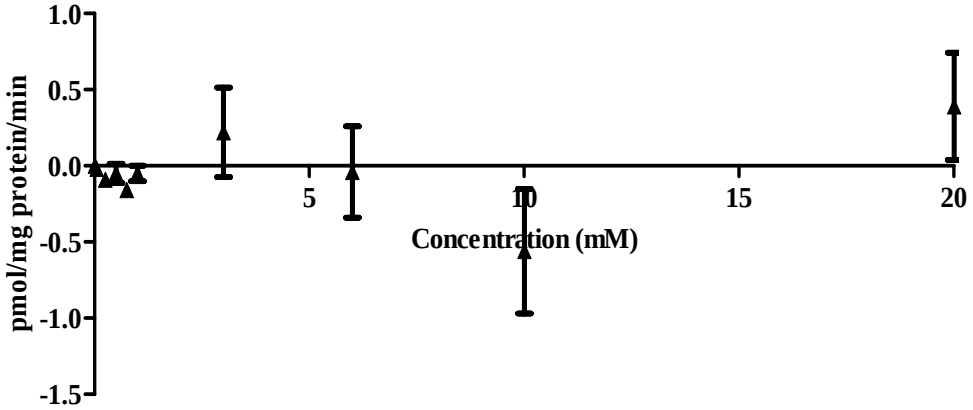
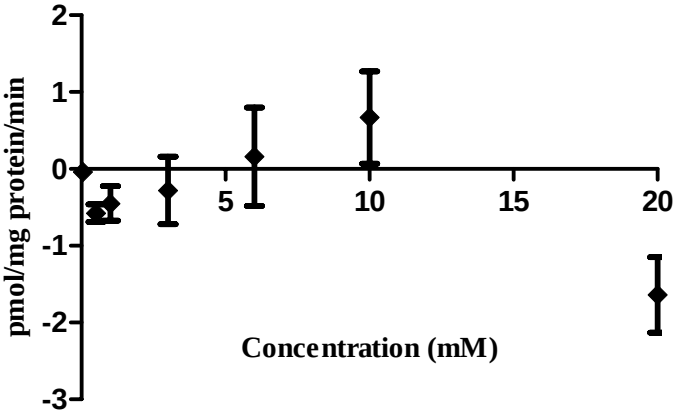

cCAT-2B Transport of Lysine in Stably Transfected Cells
pmol/mg protein/min
1.0
0.5
0.0
-0.5
-1.0
-1.5
5
10
15
20
Concentration (mM)

Figure 20. L-lysine and L-arginine are not transported by 293 Flp-In cells stably expressing chicken CAT-2C

293 Flp-In cells were stably transfected with chicken CAT-2C expression vector (filled diamonds) and were cultured in transport buffer containing 50 μM, 500 μM, 1 mM, 3 mM, 6 mM, 10 mM, or 20 mM (A) L-[4,5-^{3}H(N)]-lysine monohydrochloride or (B) L-[2,3,4-^{3}H(N)]-arginine monohydrochloride for 18 min. cCAT-2C transport of L-lysine or L-arginine does not significantly differ from transport by control cells. L-[2,3,4-^{3}H(N)]-arginine monohydrochloride in cell lysates was quantified using a β-scintillation counter and normalized to protein concentrations. Each data point represents the mean ± SEM, n=3.

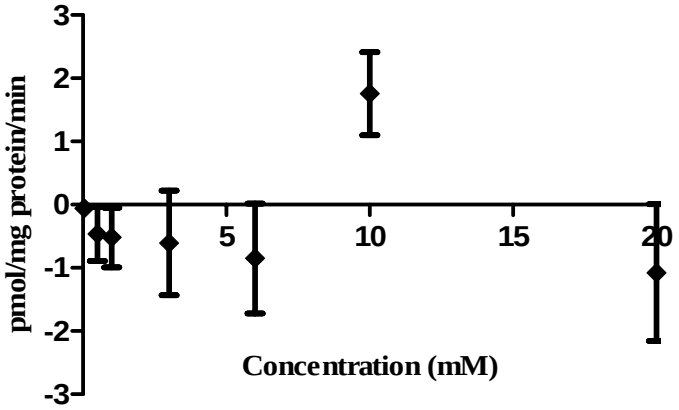
(A)

cCAT-2C Transport of Lysine in Stably Transfected Cells



(B)

cCAT-2C Transport of Arginine in Stably Transfected Cells

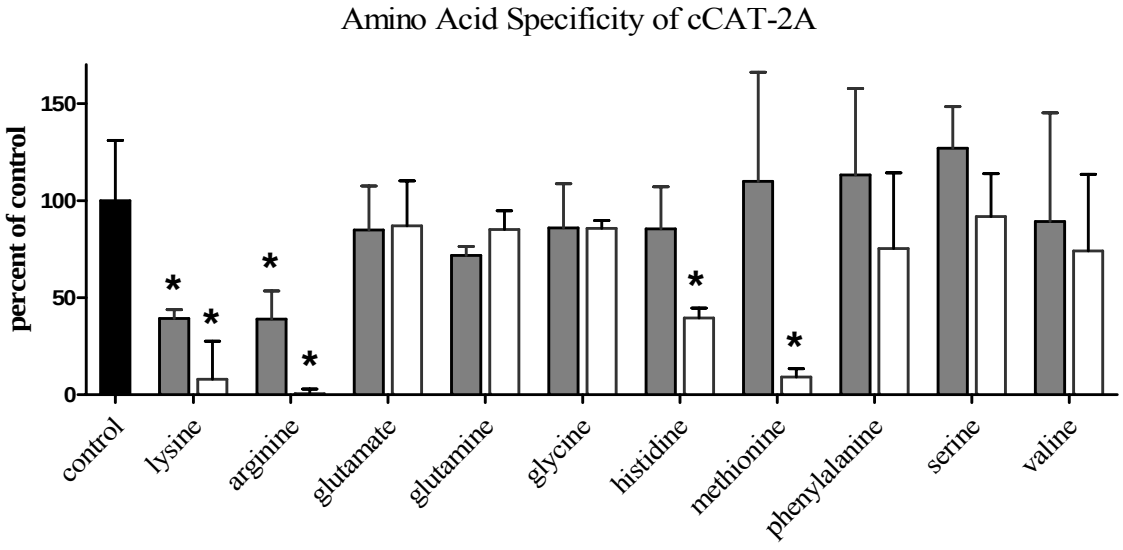


3.5 Chicken CAT-2 transporter specificity

Transport specificity was determined by measuring [^{3}H]-L-lysine uptake by stable cCAT-2A cells in transport buffer containing competitor amino acids differing in their R-group. Specificity assays for cCAT-2B and cCAT-2C were not performed since cells stably expressing these isoforms did not result in transport of L-lysine. As expected, 5-fold or 35-fold molar excess of L-lysine or L-arginine in the transport buffer decreased [^{3}H]-L-lysine uptake ($p<0.05$). A 35-fold molar excess of histidine and methionine also inhibited [^{3}H]-L-lysine uptake ($p<0.05$). No significant inhibition of [^{3}H]-L-lysine uptake was observed in treatments containing 5-fold excess of histidine or methionine and 5-fold and 35-fold molar excess of glutamate, glutamine, glycine, phenylalanine, serine, or valine ($p>0.05$) (Figure 21).

Figure 21. Chicken CAT-2A transport specificity

Stable chicken CAT-2A or empty vector control cells were cultured in transport buffer containing L-[4,5-^{3}H(N)]-lysine monohydrochloride and 5-fold (gray bars) or 35-fold (white bars) molar excess of L-lysine, L-arginine, L-glutamate, L-glutamine, L-glycine, L-histidine, L-methionine, L-phenylalanine, L-serine, or L-valine. Transport was expressed as a percentage relative to L-lysine uptake with transport buffer containing 7 mM L-lysine (control; black bars) and was normalized to cell lysate protein concentrations. Bars represent means ± SEM, n=3. * indicates significant difference between control treatment and treatment with other amino acids ($p<0.05$).


Amino Acid Specificity of cCAT-2A
percent of control
150
100
50
0
*
*
*
*
*
*
control
lysine
arginine
glutamate
glutamine
glycine
histidine
methionine
phenylalanine
serine
valine

Chapter 4: Discussion

Lysine and arginine acquisition and transport from the plasma membrane to subcellular locations are critical processes for providing the cationic amino acids to all tissues for body processes and protein synthesis. Similarity between hCAT-2A and hCAT-2B protein structure and predicted amino acid sequence with chicken CAT-2 isoforms suggested that cCAT-2A and cCAT-2B would function as high- and low-affinity transporters, respectively. Direct functional analysis, however, was required to test the hypothesis that the cCAT-2 isoforms function as cationic amino acid transporters. Evidence from this work, including tissue distribution, cellular localization, transport kinetics and transport specificity supports the hypothesis that cCAT-2A functions as a low affinity transporter. Surprisingly, these studies showed that despite its subcellular localization pattern and sequence similarity to hCAT-2B, cCAT-2B does not transport lysine or arginine. Chicken CAT-2C neither localizes to the plasma membrane nor transports lysine or arginine, and therefore may be an example of non-productive alternative splicing to regulate functional cCAT-2 isoforms.

Alternative splicing of genes is often regulated in a temporal or tissue-specific fashion to give rise to different isoforms in different tissues or life stages (78). While mCAT-2A mRNA is primarily expressed in the liver, cCAT-2A mRNA is expressed in all tissues examined in these studies and was highest in muscle and liver. Following a meal, transporters in the liver remove excess amino acids from the portal circulation in order to maintain a plasma cationic amino acid concentration of 45-60 μM (79). Increased expression of cCAT-2A would enable the liver to remove excess amino acids from the portal circulation after a meal rich in protein (49). Based upon cCAT-2A

mRNA expression in chicken tissues, skeletal muscle (e.g. gastrocnemius and pectoralis major) and liver would be the primary tissues absorbing lysine and arginine post-prandially while immune tissue (e.g. bursa of Fabricius and thymus) would be the least. In addition, the high levels of cCAT-2A mRNA expressed in skeletal muscle may be required to supply rapidly developing muscles with sufficient quantities of lysine and arginine required for optimal protein synthesis (18,20). In the liver, high levels of cCAT-2A mRNA may permit this tissue to catabolize lysine when requirements for protein synthesis have been met (80). Unlike mCAT-2A, cCAT-2A mRNA was expressed in all tissues examined, and may be due to the bird's requirement for both lysine and arginine.

Our studies showed that cCAT-2A localizes to the plasma membrane and mediates low affinity, high velocity transport of lysine and arginine. The cellular localization and transport properties of cCAT-2A for lysine and arginine are similar to mCAT-2A (66) and indicates that the function of this transporter is conserved in chickens. Transport of lysine and arginine was also examined in cells stably expressing cCAT-2A due to the increased uniformity and intensity of cCAT-2A expression compared to transient expression. This is the first report of a CAT-2 stable cell line in any species, and kinetic studies with mouse and human CAT-2A primarily utilized *Xenopus* oocytes or cells transiently expressing mouse and human CAT-2A (42). The affinity constant for cCAT-2A differed between stable and transient expression of cCAT-2A, with cells stably expressing cCAT-2A having a higher K_m than in cells transiently expressing cCAT-2A. The K_m of cells transiently expressing cCAT-2A (K_m=2.644 ± 1.379mM) was similar to that of mCAT-2A while the K_m of cells stably expressing cCAT-2A (K_m=7.982 ± 1.655mM) was higher. The maximum velocity of cCAT-2A

transport for lysine and arginine did not differ between transient and stable expression. The difference in K_m between transient and stable cCAT-2A expression may be due to the uniform expression of cCAT-2A in stable cells. Stable expression of cCAT-2A resulted in a much lower coefficient of variation compared to transient expression of cCAT-2A (20.7% Vs. 52.15%) and indicates that stable expression increased the accuracy of transport measurements and represents a more accurate estimate of cCAT-2A affinity. Regardless of stable or transient expression, cCAT-2A exhibited transport properties of a low affinity, high capacity transporter similar to mouse and human CAT-2A (42).

Transporter specificity studies showed that cCAT-2A preferably transports lysine and arginine, and transport of these amino acids is inhibited by the presence of 35-fold molar excess of histidine and methionine, or 175mM of histindine and methionine, at physiological pH, as has previously been shown in mammals (40). Although transport of lysine was inhibited at 175mM concentration of histidine and methionine, plasma concentrations of these amino acids are within the micromolar range (81,82) and therefore it is unlikely that these levels would ever be present in order to inhibit lysine and arginine transport. Histidine is another basic amino acid at physiological pH that has been shown to be transported with low affinity by the CAT transporters (40). It is not clear whether inhibition by methionine is due to a direct competitive transport, which is unlikely due to the lack of inhibition in cells treated with 5-fold molar excess, or due to the transient and/or reversible decrease in function of cCAT-2A in the presence of high concentrations of this amino acid. The former scenario could be tested by incubating cells expressing cCAT-2A with [^{3}H] L-methionine to determine the amount of

radioactivity incorporated into the cells. The latter could be tested by pre-treating cells expressing cCAT-2A with 35-fold molar excess of L-methionine, followed by incubation with L-lysine and tritiated L-lysine to test for lysine uptake inhibition. Regardless, cCAT-2A is specific for cationic amino acids and competition for transport is physiological limits restricted to either lysine or arginine.

Unlike mammalian and chicken CAT-2A, the tissue distribution and functional properties of cCAT-2B differed markedly from mCAT-2B. While mCAT-2B is only expressed in activated macrophages, cCAT-2B mRNA was expressed in all chicken tissues and was highest in the liver. Though not examined in these studies, splenic macrophages and the avian HD11 macrophage cell line do not express cCAT-2B mRNA (Laing and Humphrey, unpublished). These cCAT-2B mRNA expression patterns indicate that cCAT-2B is not likely to play an important role, if any, in supplying activated macrophages with arginine for NO synthesis. Activated chicken macrophages produce NO and require extracellular arginine for NO synthesis (80). Therefore, other arginine transport systems, such as other system y^+ or y^+L genes, may be responsible for providing chicken macrophages with arginine for NO synthesis.

In addition to the divergence of the affinity-conferring residues Glu^{369} to Lys^{369} in the region specific to cCAT-2B and hCAT-2B, transport between these homologues also differed. Transient expression of cCAT-2B did not result in lysine transport and this was thought to be due to the localization of cCAT-2B inside of the plasma membrane. Stable expression of cCAT-2B resulted in cCAT-2B localizing to the plasma membrane, yet lysine transport still did not occur. Taken together, these data suggest that cCAT-2B is not a lysine transporter. However, mCAT-2B does not transport substrate unless

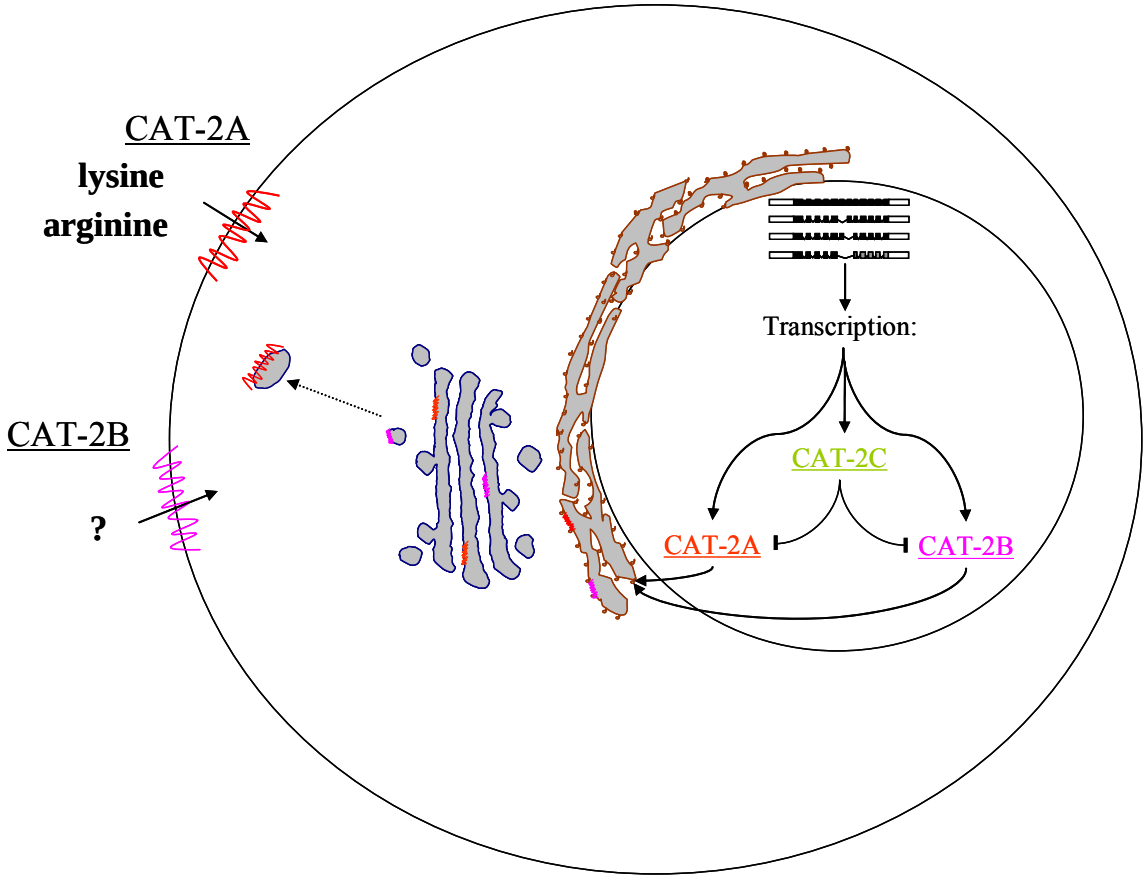
stimulated by bacterial LPS or proinflammatory cytokines, such as interleukin-1β and tumor necrosis factor-α (39,42), and transport by cCAT-2B may also have a similar requirement. Additionally, cCAT-2B protein expression was 25-fold lower than cCAT-2A protein expression, so perhaps cCAT-2B protein levels permitted amino acid uptake that were below the limit of detection. Conversely, cCAT-2B may transport other amino acids due to a modified amino acid binding site. It is surprising that cCAT-2B does not transport lysine, however in humans the system y^+ gene CAT-4 is also localized to the plasma membrane, yet does not transport lysine or arginine (54). However, no conclusions can be made concerning the ability of cCAT-2B to function as amino acid transporter without additional functional analysis of cCAT-2B transport with other amino acids.

Results presented by Auboeuf et al. (83) showed that 75% of all known human transcripts are subject to alternative splicing in a translated region of mRNA. One-third of alternative splicing events, however, result in the production of a transcript with a premature translation-termination codon (PTC) that are targeted for degradation by the nonsense-mediated mRNA decay pathway (NMD). Chicken CAT-2C has a PTC and is degraded by NMD as shown by increased mRNA stability in the presence of cycloheximide (Morris, Kirsch, and Humphrey, unpublished). These characteristics indicate the cCAT-2C transcript may be involved in regulated unproductive splicing and translation (RUST) (84) and consequently may delay or limit the transcription of cCAT-2A and cCAT-2B isoforms. A similar form of regulation has been observed for fibroblast growth factor 2 (FBGR-2) isoforms which include a spliced variant missing two exons. In the presence of this spliced variant, transcription of other functioning

isoforms are repressed in a cell-type dependent manner, and the variant is degraded by the NMD pathway (85). The data from our experiments suggest that rather than functioning to transport cationic amino acids, transcription of the cCAT-2C isoform may regulate the expression of cCAT-2A and cCAT-2B isoforms in tissues.

Our results establish distinct properties for all three chicken CAT-2 isoforms. The structural as well as functional similarities between chicken and mammalian CAT-2A proteins demonstrate that these carriers are highly conserved across species, and indicate a significant physiological function. While the structure of cCAT-2B is conserved, the function differs from mammalian CAT-2B, and additional experiments are required to elucidate the exact function of this isoform. Finally, the splice variant cCAT-2C is not conserved in mammals, and may play a role in post-transcriptional regulation of functional cCAT-2 isoforms.

Figure 22. Model of CAT function in the chicken



Chapter 5: Conclusion

5.1 Summary

Our studies have shown that a major transporter involved in cationic amino acid transport is conserved between mammals and non-mammalian vertebrates. Chicken and human CAT-2 show high nucleotide homology, possess 5' and 3' UTR regions that are similar in size and type of regulatory motifs, and maintain an alternative splicing mechanism. Tissue mRNA expression of cCAT-2A differed from mCAT-2A as it was expressed throughout the body, and functional analysis indicated that this may be due to lack of CAA transport by other cCAT-2 isoforms. Chicken CAT-2A was also highly expressed in both the liver and muscle tissues, which are sites of high amino acid requirement for production animals. Overall, cCAT-2A has similar functional properties to mammalian CAT-2A, including low affinity transport and high specificity for L-lysine and L-arginine. Chicken CAT-2B mRNA expression and transport properties varied greatly from mCAT-2B. Primarily, cCAT-2B did not transport CAAs, and this may be due to a modified amino acid residue in the amino acid binding site. In addition, cCAT-2B, which is expressed in all tissues and is not expressed in macrophages, may not play a role in nitric oxide synthesis, which is a major function of mCAT-2B. Finally, since cCAT-2C does not localize to the plasma membrane, does not transport CAAs, contains a premature termination codon, and is degraded by the NMD pathway, this isoform may participate in RUST and may function to post-transcriptionally regulate transcription of functioning cCAT-2A and cCAT-2B isoform proteins.

5.2 Future Studies

Many additional studies should be conducted to further elucidate the biochemical and physiological function of cCAT-2 isoforms. First, the N- and C- terminal transmembrane orientation of cCAT-2A and cCAT-2B could be determined. Glycosylation of the endogenous cCAT-2A or cCAT-2B peptide could be examined by treating the protein with N-glycosidase. Since cCAT-2A and cCAT-2B have one common glycosylation site that is predicted to be extracellular, a shift in protein molecular weight for both cCAT-2A and cCAT-2B following N- glycosidase treatment of intact cells would indicate that the glycoslyation site is extracellular and is common to both isoforms. An additional experiment which would test N- and C-terminal orientation of cCAT-2A or cCAT-2B around the membrane would require the expression of cCAT-2A/B fused to a fluorophore, such as GFP. Using the fluorescence protease protection assay (FPP) technique, incubation of the cells expressing cCAT-2-GFP with protease K, followed by cleavage of the GFP signal would indicate extracellular ends, while absence of cleavage would indicate intracellular ends (86).

Second, it would be interesting to compare the transport of cells transiently and stably expressing hCAT-2A to determine whether the type of transfection alters the K_m, as was seen for transient and stable expression of cCAT-2A. In addition, cCAT-2A and cCAT-2B should be tested for transport while transiently expressed in *Xenopus* oocytes, without the contribution of endogenous transporters, and with treatments of other tritiated amino acids. As long as cCAT-2 isoforms are being expressed in *Xenopus* oocytes it would be easy to determine whether cCAT-2A functions via facilitated diffusion, as has been shown in human, mouse, and rat CAT-2A(43). If no change in transporter function

is noted for uptake of [^{3}H] L-lysine in transport buffer containing choline chloride or sodium chloride, transport is Na^{+} independent and therefore the transporters functions via facilitated diffusion as has been shown for mouse and human CAT transporters.

Third, cCAT-2A response to an amino acid deficient diet induced stress state would be very interesting to study in regards to promoter regulation, mRNA expression of cCAT-2 isoforms in tissue, and change in response of chicken CAT-2A mRNA expression at different life stages. In addition, lysine levels have been directly correlated to protein accretion (87), so it would be interesting to determine if cCAT-2A expression in muscle could also be correlated to protein accretion. Muscle protein synthesis, as measured by incorporation of stable isotope tracers (^{13}C) of amino acids in biopsied muscles, could be linearly related to cCAT-2A expression, although a time delay in protein synthesis would be expected.

Finally, cCAT-2C should be further examined for its role in RUST and in regulation of transcription of cCAT-2A and cCAT-2B. In order to test this mechanism mRNA expression of cCAT-2C in chicks fed high levels of lysine and arginine with an amino acid sufficient diet and an amino acid deficient diet should be compared. In these studies I would expect cCAT-2C mRNA expression to be regulated by availability of CAAs and be several-fold greater in chickens fed excess amino acids compared to chickens fed a diet deficient in amino acids, as has been shown to be the case in spermidine N-acetyltransferase (88).

5.3 Implications

We have shown that, while the CAT-2 cationic amino acid transporters are conserved between mammalian and non-mammalian vertebrates, many differences remain. Results from these studies indicate that the differing requirement for CAAs, in mammals and chickens, may be due to a difference in use of these amino acids by body tissues. As a result, the cCAT-2 transporters have adapted to provide these amino acids as required. Using the knowledge gathered from these experiments, additional lysine and arginine in the diet would positively impact protein accretion, and would be transported by cCAT-2A. In regards to animal health, cCAT-2 isoforms are not directly involved in nitric oxide production in macrophages, and therefore the presence of other cationic amino acid transporters should be examined in macrophages. Finally, cCAT-2 plays an important role in transporting lysine and arginine from the extracellular environment into cells, and the substrate they provide can be used for protein synthesis or catabolized as an energy source.

Appendix

Appendix Figure 1. Nucleotide alignment of chicken CAT-2A, CAT-2B and CAT-2C

```
cCAT-2A     1 ATGTTGCCCTGTGGACCAGCTTTGACCTTTGTTCGGTGCCTGGTGCGTAAGAAGAATATC
cCAT-2B     1 ATGTTGCCCTGTGGACCAGCTTTGACCTTTGTTCGGTGCCTGGTGCGTAAGAAGAATATC
cCAT-2C     1 ATGTTGCCCTGTGGACCAGCTTTGACCTTTGTTCGGTGCCTGGTGCGTAAGAAGAATATC

cCAT-2A    61 AAGGGGGAAGGTCTTGAGGACTCGTTGTGCCGATGCTTATCTACGCTGGACCTTATAGCA
cCAT-2B    61 AAGGGGGAAGGTCTTGAGGACTCGTTGTGCCGATGCTTATCTACGCTGGACCTTATAGCA
cCAT-2C    61 AAGGGGGAAGGTCTTGAGGACTCGTTGTGCCGATGCTTATCTACGCTGGACCTTATAGCA

cCAT-2A   121 CTGGGAGTTGGAAGTACCCTTGGTGCTGGTGTCTATGTGCTTGCTGGAGAAGTTGCCAAA
cCAT-2B   121 CTGGGAGTTGGAAGTACCCTTGGTGCTGGTGTCTATGTGCTTGCTGGAGAAGTTGCCAAA
cCAT-2C   121 CTGGGAGTTGGAAGTACCCTTGGTGCTGGTGTCTATGTGCTTGCTGGAGAAGTTGCCAAA

cCAT-2A   181 TCTGATTCTGGACCTAGCATTGTTGTTTCCTTCCTCATTGCTGCCCTGGCATCTGTGATG
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cCAT-2C   181 TCTGATTCTGGACCTAGCATTGTTGTTTCCTTCCTCATTGCTGCCCTGGCATCTGTGATG

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cCAT-2C   301 TATACTTACGTAGCTGTTGGTGAACTGTGGGCCTTTATCACTGGTTGGAATCTCATTTTA

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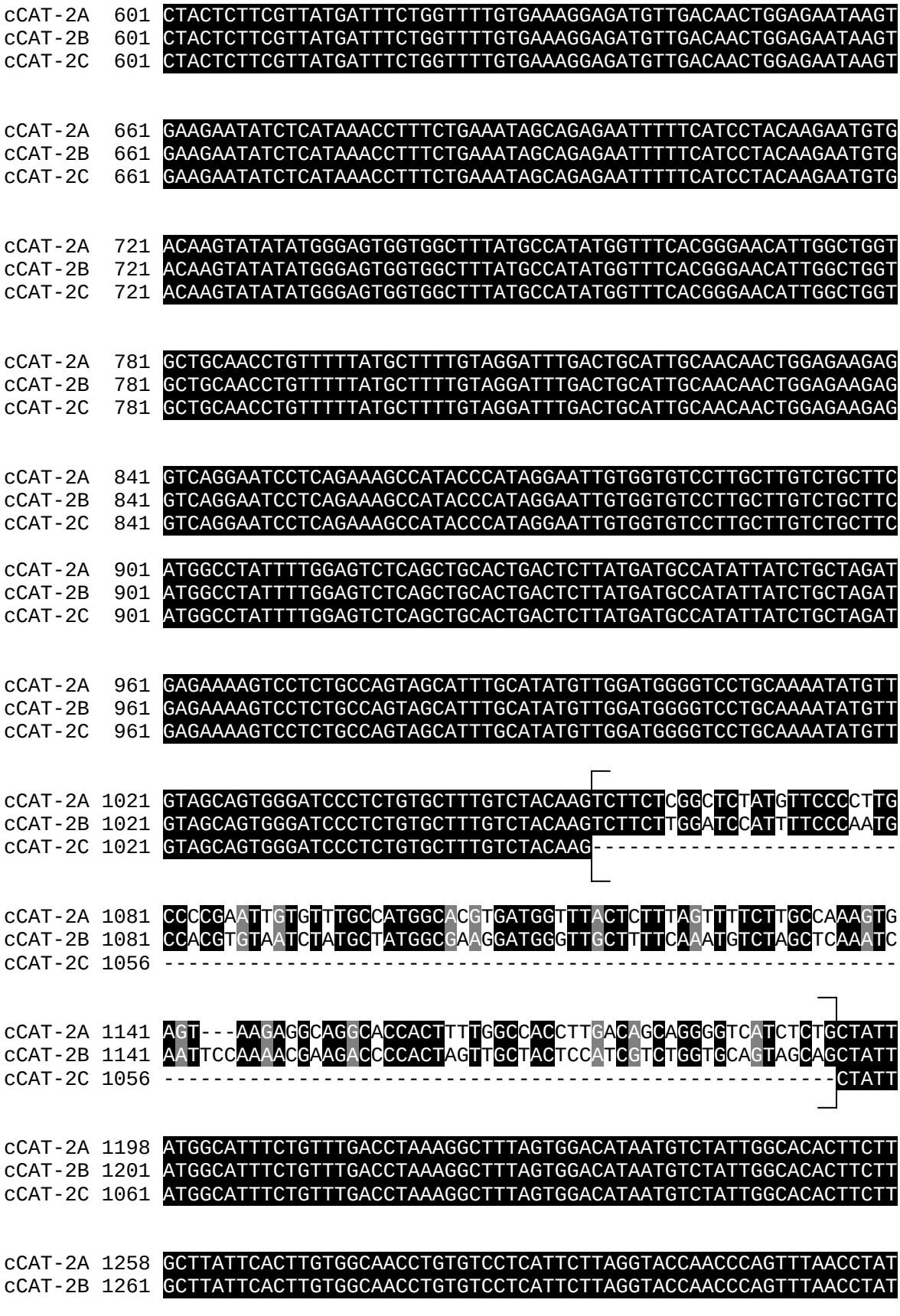
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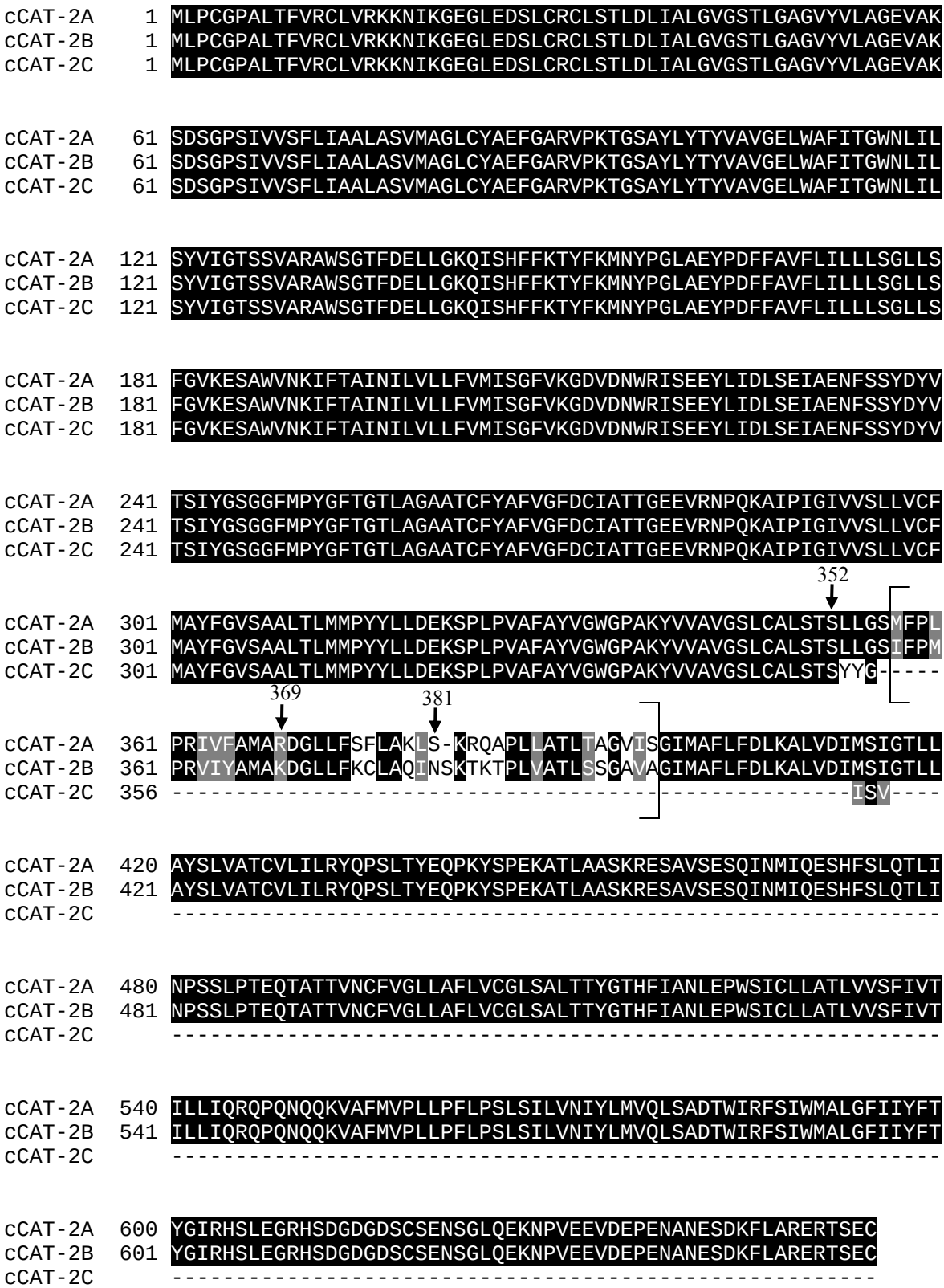
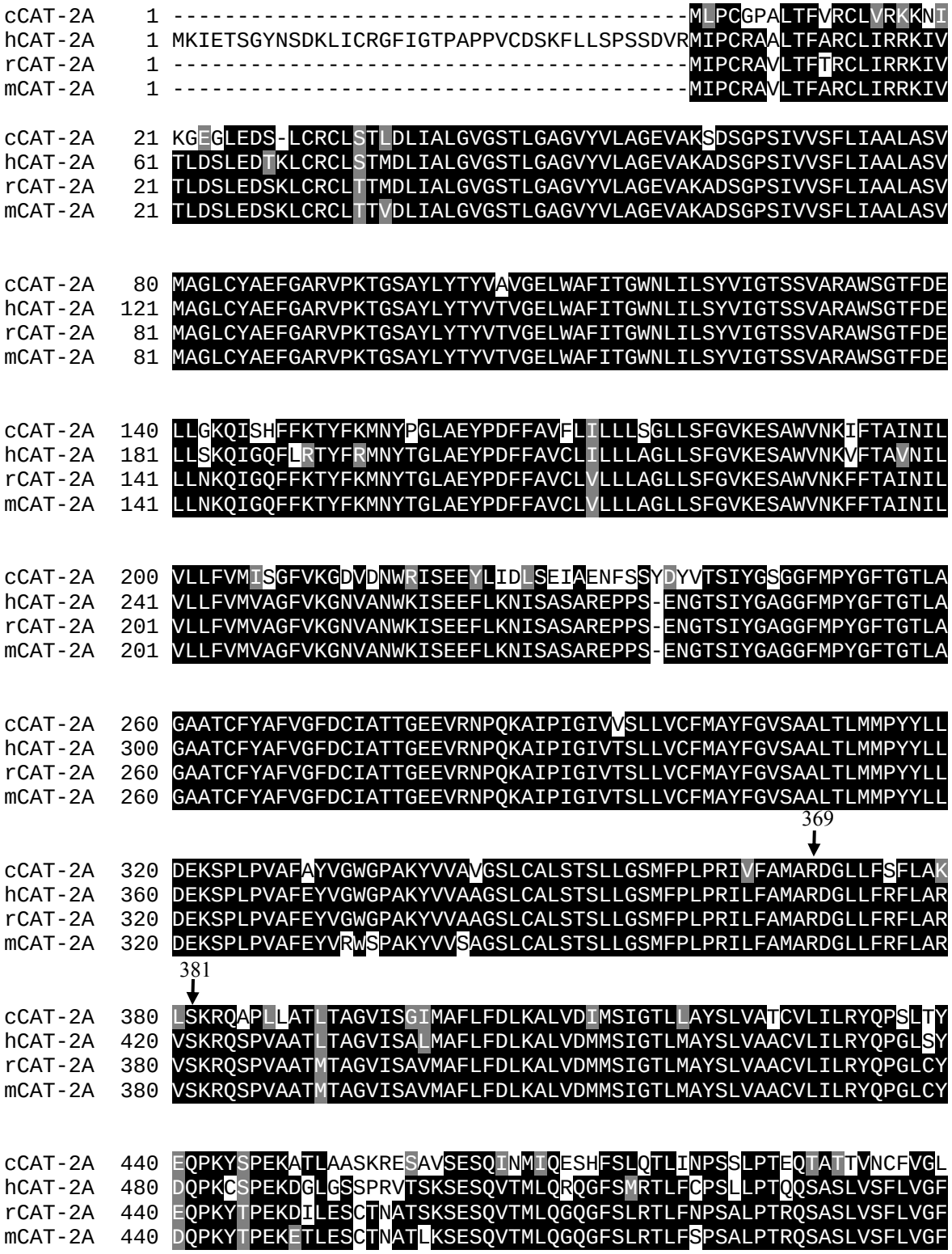
Appendix Figure 2. Peptide alignment of chicken CAT-2A, CAT-2B, and CAT-2C



Appendix Figure 3. Peptide alignment of CAT-2A from chicken, human, rat, and mouse

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cCAT-2A 560 LPFLPSLSILVNIYLMVQLSADTWIRFSIWMALGFIIYFTYGIRHSLEG--RHSDGDGDS
hCAT-2A 600 LPFLPAFSILVNIYLMVQLSADTWVRFSIWMAIGFLIYFSYGIRHSLEGHLRDENNEEDA
rCAT-2A 560 LPFLPAFSILVNIYLMVQLSADTWVRFSIWMVLGFLIYFAYGIRHSLEGNPRDEEEDEDV
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hCAT-2A 660 YPDNVHAAAEEKSAIQANDHHPRNLSSPFIFHEKTSEF
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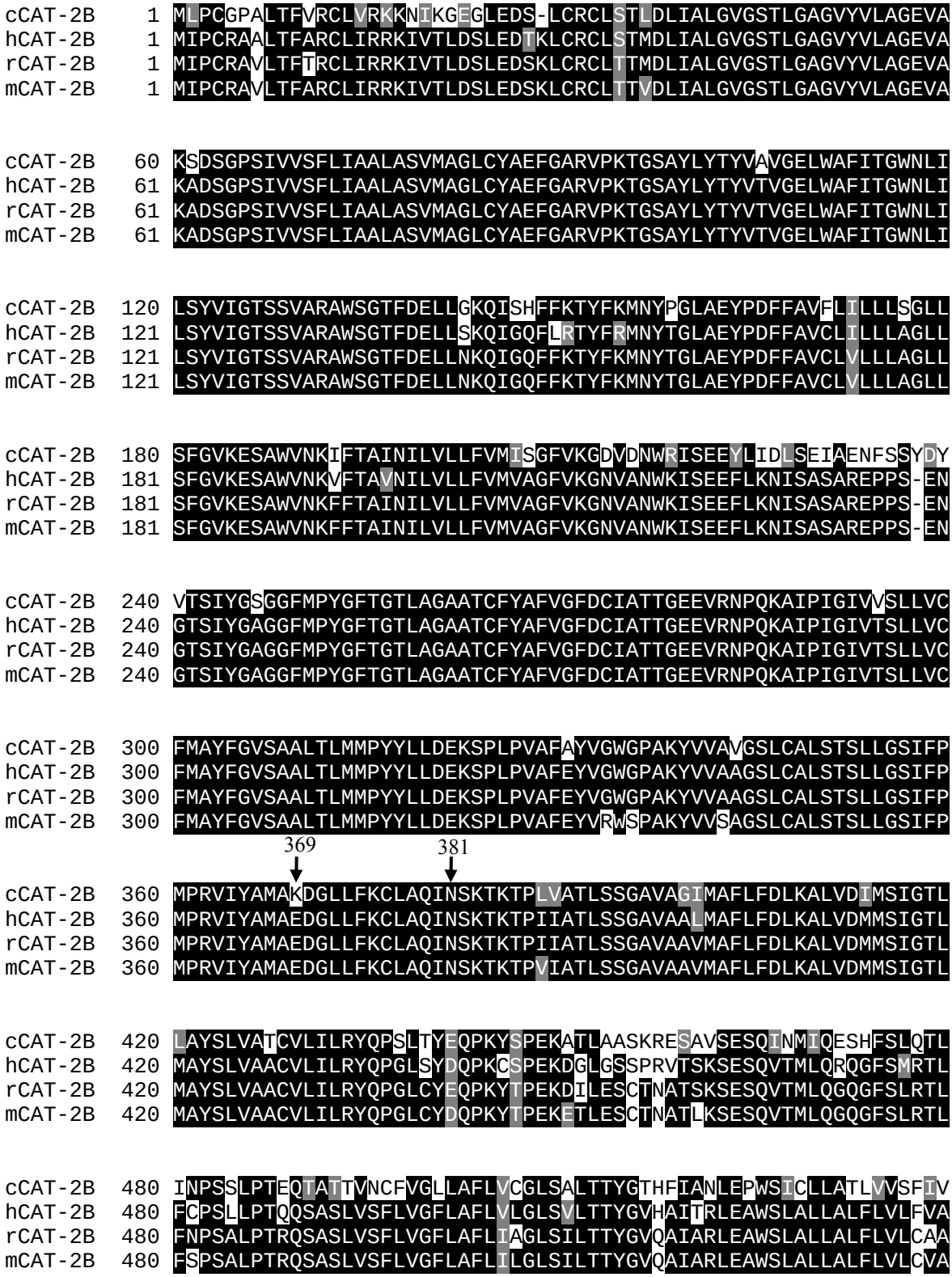
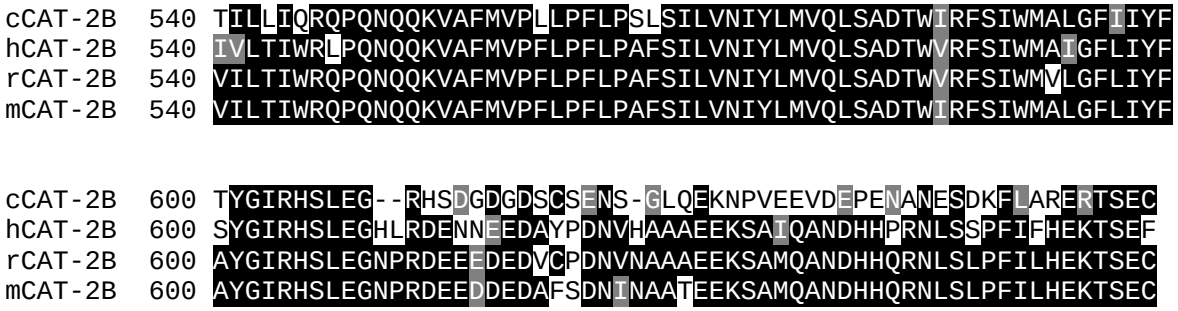
Appendix Figure 4. Peptide alignment of CAT-2B from chicken, human, rat, and mouse

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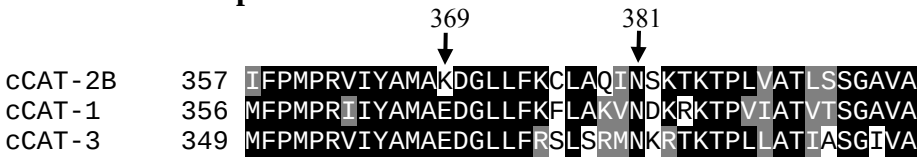
cCAT-2B 600 TYGIRHSLEG--RHSDGDGDSCSENS-GLQEKNPVEEVDEPENANESDKFLARERTSEC
hCAT-2B 600 SYGIRHSLEGHLRDENNEEDAYPDNVHAAAEEKSAIQANDHHPRNLSSPFIFHEKTSEF
rCAT-2B 600 AYGIRHSLEGNPRDEEEDEDVCPDNVNAAAEEKSAMQANDHHQRNLSLPFILHEKTSEC
mCAT-2B 600 AYGIRHSLEGNPRDEEDDEDAFSDNINAATEEKSAMQANDHHQRNLSLPFILHEKTSEC

Appendix Figure 5 Peptide alignment of the 42 amino acid region that differs between cCAT-2B as compared to cCAT-1 and cCAT-3



Appendix Table 1. **Primer sequences for RT-PCR and real-time PCR analysis of chicken CAT-2 isoforms and β-actin mRNA[1]**

Target	Accession number[2]	Orientation	Primer Sequence (5'→3')	Annealing Temp (°C)	Product size (bp)
ORF CAT-2		Forward	ATGTTGCCCTGTGGACCA	55	1965 (2A)
		Reverse	TTAACATTCACTTGTCCTTTCACG		1968 (2B)
cCAT-2C ORF		Forward	CCCAAGCTTGCCACCATGTTGCCCTGTGGACCA	56	1100
		Reverse	TCCCCGCGGTCAAACAGAAATGCCATAGCT		
cCAT-2d TOPO		Forward	CACCATGATGCCCTGTGGACCA	56	1965 (2A)
		Reverse	ACATTCACTCGTCCTTTCACG		1968 (2B)
cCAT-2C TOPO		Forward	CACCATGTTGCCCTGTGGACCA	58	1077
		Reverse	AACAGAAATGCCATAATAGCTTGTAG		
cCAT-2A[3]		Forward	TGCTTTCTGTACAAGTCTTCTCG	55	165
		Reverse	AATGCCATAATACCAGAGATGACC		
cCAT-2B[3]		Forward	CCTTGCTTGTCTGCTTCATGG	58	272
		Reverse	CTTCGTTTTGGAATTGATTTGAGC		
cCAT-2C[3]		Forward	ACTGCATTGCAACAACTGGA	55	251
		Reverse	GCCATAATAGCTTGTAGACAAAGCA		
β-actin	NM_205518	Forward	CCCCAGCCATGTATGTAGCC	55	199
		Reverse	TCTGTCAGGATCTTCATGAGGTAG		
β_2-M	Z48921	Forward	TGGAGCACGAGACCCTGAAG	59	161
		Reverse	TTTGCCGTCATACCCAGAAGTG		

[1] Abbreviations: β_2-M, β_2-microglobulin; bp, base pair; CAT, cationic amino acid transporter.
[2] GenBank accession numbers.
[3]Primers used for Real-Time PCR amplification

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