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Control of Cationic Amino Acid Transport and Retroviral Receptor Functions in a Membrane Protein Family*

(Received for publication, November 19, 1993, and in revised form, February 28, 1994)

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A partial cDNA sequence indicated that the T lymphocyte early-activation gene (*Tea*) encodes a protein related to the dual-function ecotropic retrovirus receptor/cationic amino acid transporter (ecoR/CAT1), and RNA blots suggested highest *Tea* expression in T lymphocytes and liver (MacLeod, C. L., Finley, K., Kakuda, D. Kozad, C. A., and Wilkinson, M. F. (1990) *Mol. Cell. Biol.* 7, 3663-3674). The sequence of full-length *Tea* cDNA from liver (3683 bases) predicts a 657-amino-acid protein (CAT2 α) with 12-14 transmembrane domains. A long (515 base) region with six initiation codons and termination codons precedes the translation start codon. The liver *Tea* cDNA is identical to *Tea* cDNA from T lymphocytes (encoding CAT2 β) with the exception of an apparent alternatively spliced sequence encoding a hydrophilic loop of 43 amino acids. The liver-specific sequence contains unique consensus sites for phosphorylation by cyclic AMP-dependent protein kinase and by protein kinase C. Injection of *Xenopus* oocytes with CAT2 α or CAT2 β messenger RNA resulted in expression of Na⁺-independent cationic amino acid transport that was detected by current measurements under voltage-clamp. Although the amino acid sequences of the isoforms differ in only 21 of 43 residues with the majority of substitutions being conservative, the apparent affinity of CAT2 β for arginine uptake was 70-fold higher than the CAT2 α isoform (K_m 38 μ M versus 2.7 mM). Neither isoform functioned as a receptor for ecotropic or amphotropic murine retroviruses. However, CAT1-CAT2 chimeric proteins that contain the first three putative extracellular loops of ecoR/CAT1 functioned as ecotropic receptors despite a diminished capacity to bind the viral envelope glycoprotein. The chimeric proteins also functioned as basic amino acid transporters with substrate affinities corresponding to the CAT2 isoform constituting the carboxyl-terminal portion. These results demonstrate that domains of these transporters can function in chimeric combinations to control viral receptor and transport functions.

The cell surface receptor (ecoR) for ecotropic host-range murine retroviruses has been identified as a transporter for cationic amino acids (1, 2) and in this role is referred to as CAT1.

This protein has the functional characteristics of the cellular transport system classically known as y⁺ (for review, see Ref. 3) and is distantly related to yeast transporters for the cations arginine, choline, and histidine (1, 2). Thus, the dual-function ecoR/CAT1¹ protein occurs in an ancient diverse family of transporters. Moreover, because CAT1 is an electrogenic Na⁺-independent cation transporter, it can be analyzed by electrophysiological methods in *Xenopus* oocytes, thus providing an approach for thorough kinetic and molecular genetic studies of transporter functions. Studies of this protein family are also important because they may reveal properties necessary for retroviral reception and for retroviral-host coevolution.

The cloning of a partial cDNA for a T lymphocyte early activation gene (*Tea*) revealed significant sequence homology to ecoR/CAT1 (4). Synthesis of *Tea* and ecoR/CAT1 mRNAs is increased in T cells stimulated to proliferate (4-6) concomitant with increased high affinity cationic amino acid uptake activity (7). In addition, the *Tea* gene is expressed in nonproliferating liver cells (4) which lack high affinity cationic amino acid transport activity (8). Based on this information, we cloned and expressed the full-length mouse liver *Tea* cDNA and an apparent alternate splice form found in lymphocytes. During the course of our work, and after its initial submission, a series of independent reports described a partially overlapping investigation (9-12). The present report describes analysis of the viral receptor functions of *Tea* using chimera constructions and molecular genetic studies, and characterization of the transport properties of both splice forms by two-electrode voltage-clamp and radiolabel uptake methods.

EXPERIMENTAL PROCEDURES

Molecular Cloning—A partial mouse *Tea* cDNA clone from a T cell library (4), generously provided by C. MacLeod (University of California at San Diego, La Jolla, CA), was used as probe to isolate *Tea* cDNAs from a random-primed mouse liver λ gt11 cDNA library (Clontech, Palo Alto, CA). Sequences were determined in both directions by the method of Sanger *et al.* (13) and were confirmed by analyses of independent cDNA clones. Clones with overlaps were used to reconstruct the full-length mouse liver cDNA in the plasmid vector pGEM3, and the *Eco*R1 site in the cDNA sequence was confirmed by polymerase chain reaction of reverse-transcribed liver RNA (14). The T cell and liver *Tea* sequences appeared to be identical except for one substitution that was present in multiple independent cDNA clones (see below). Full-length T cell cDNA was thereby also reconstructed in the same vector. *Tea* cDNA clones with shorter 5'-untranslated sequences were then constructed by deleting a 468-base pair *Stu*I-*Sma*I fragment and relegating the re-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L29006.

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¹ The abbreviations used are: CAT1/ecoR, the dual-function cationic amino acid transporter/cell surface receptor for ecotropic murine retroviruses; system y⁺, the major Na⁺-independent transporter for basic amino acids arginine, lysine and ornithine; *Tea*, the murine T lymphocyte early activation gene; CAT2 α , the liver isoform of *Tea* encoding a low affinity cationic amino acid transporter; CAT2 β , the lymphocyte isoform of *Tea* encoding a high affinity cationic amino acid transporter.

A

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1  CTACGATGTTAGAGCCCAAGTCTCCTAAGGCCAAGGACAGGGGGGAGACTTCGACGGCAG
61  CAATCTCCTGAGGTTGTGTACTGTGTGATGGGTATGAGGAAGTTGAAAGGAGCAACCCC
121  CACACCAGCAGGTCATGTACAGGTGTGACAGCAAGTGAAGTACAAGACTGACACACAACA
181  CACTCCACCCCTCCCTCCCTGACTGCTTCAAGTCTGCTGAACTGTGTCCCTGTAGCTAA
241  CACCAATAGACCAATTAATTTACAGATTTCTTTTGAACCTTAGGTTGACATATTTCTT
301  TGGAAATTAATGTGGAGATAGATAGTAATAAATATGTGTGATGAAATACATAGAAATA
361  AGAAATTAATCACCATCAAAATCTTAAGCCTCCTGGGTGCTGTGAACCAAGTATCTATT
421  AAAGAGAGGCCCACTGCCCTTCTGTCTCAACGGAGGGGAGAGGCCCTCAACCCCTCC
481  CCTCCCCCTCTCCTACAGGTTGCTCTCTCCACCATGATTCCTCGAGAGCAGTGTCTGA
      M I P C R A V L      8
541  CTTTCGGCGATGTCTGATCCGGAGAAAATTGTACACACTGGACAGCCTTGAAGATTCCA
      T F A R C L I R R K I V T L D S L E D S      28
601  AACTCTGCCGCTGCTTAACACCAGTGGACCTCATCGCTTTGGGGGTGGAAAGCCTCTGG
      K L C R C L T T V D L I A L G V G S T L      48
661  GCGCTGGGCTACGCTCCTGGCTGGGGAAGTCCGCAAAAGCCGATTCGGGCCAGTATCG
      G A G V Y V L A G E V A A K A D S G F P S Y      68
721  TGGTGTCTTTCCTACGCTGCCCTGGCTCGGTTATGGCCGGCCTTTGCTATGTGTAAT
      V V S F L I A A L A S V M A G L C Y A E      88
781  TTGGGCGCGAGTACCAAGACTGATCTGCTATCTATACACTACGTCACGCTCGGAG
      F G A R V P K T G S A Y L Y T Y V T V G      108
841  AGCTGTGGCCCTTACACTGCTGGAATCTACCTGTCTATGTCTATAGTACGTCACCA
      E L N A F I T G W N L L I L S Y V I G T S      128
901  GTGTCCGAGAGCATGAGTGGACCTTTGACGACTCTTAATAAAGAGATTGGCAGT
      S V A R A W S G T F D E L L N K Q I G      148
961  TTTTCAAAACGTAATCAAAATGAATACACTGGTCTGGCAGAGTATCAGACTCTTTG
      F P K T Y F K M N Y T G L A E Y P D F F      168
1021  CCGTGTGCCCTGTATTACTCCTGGCAGGCTTTTATCTTTTGGAGTAAAAGAGCTGTCTT
      A V C L V L L L L A G L L S F G Y K E S A      188
1081  GGGTGAATAAATTTTACAGCTATTAAATACCTGGTCTCTCTTTGTCTAGTGGCTG
      W V N K F F T A I N I L V L L L F V M V A      208
1141  GGTTTGTGAAGGAAATGTGGCTAACGGAAGATCAGTGAAGATTCTCAAAATATAT
      G F Y K G N V A N W K I S E E F L K N I      228
1201  CAGCAAGTCTAGAGAACCCCTTCTGAGAACGGAACAGCATCTACGGGGCTGGCGGCT
      S A S A R E P P S E N G T S I Y G A G G      248
1261  TTATGCCCTATGGCTTACAGGACGCTGGCTGGTCTGCAAGCTGCTTTTATGCCCTTG
      F M P Y G P T G T L A G A A T C F Y A F      268
1321  TGGGCTTTGACTGATGCAACACCGGTGAAGAGTTCGGAAATCCACAAAAGCGGATCC
      Y G F D C I A T T G E E V R N P Q K A I      288
1381  CCATCGGAATAGTACGCTTACTTGTCTGCTTTATGGCTTACTTTGGGGTTCCTCGAG
      F I G I V T S L L V C M A Y F G V S A      308
1441  CTTTAAACGCTTATGATGCCCTTACTACCTCCTGGATGAGAAAAGTCCACTCCAGTCCGCT
      A L T L M M P Y Y L L D E K S P L P V A      328
1501  TTGAGTATGTCAGATGGGGCCCGCAAAATACGTTGTCCGACAGGCTCCCTCTGCGCCT
      F E Y V R W G P A K Y V V A A G S L C A      348
1561  TATCAACAGTCTTCTGGGTTCTATGTTCCTTACCCGAATCTGTTTGCATGGGCC
      L S T S L L G S M K F P L P R I L F A M A      368
1621  GGGATGGCTTACTGTTAGATTTCTTCCAAAGATGAGTAAAGGACGTCACCCCTGCTG
      R D G L L F R F L A R V S K R Q S P V A      388
1681  CCACGATGACTGCAAGGGTCAATTTCTGCTGTGATGGCCCTTTCTTTTGGACCTGAAAGGCC
      A T M T A G V I S A V M A F L F D L K A      408
1741  TCGTGGACATGATGCTTATGGCACCTCATGGCCCTACTCTGTGGTGGCAGCTGTGTGC
      L V D M M S I G T L M A Y S L V A A C T      428
1801  TTATTCTCAGGTACCAACCTGCTGTGTGTACGAGCAGCCAAATACACCCTGAGAAAG
      L I L R Y Q P G L C Y E Q P K Y T P E K      448
1861  AAATCTGGAATCATGACCAATCGACTTTGAAGCAGGAGTCCAGGTCACCATGCTGC
      E T L E S C T N A T L K S E S S Q V T M L      468
1921  AAGACAGGGTTTCAGCCTACGACCCCTCTGACCCCTCTGCCCTGCCACACAGT
      Q G Q G F S L R T L F S P S A L P T R Q      488
1981  CCGCTTCCCTTGTGAGCTTTCTGGTGGGATCTGGCTTCTCATCTGGGCTGTAGTA
      S A S L Y S F L V Y G F L A F L I L G L S      508
2041  TTCTAACCACTATGCGCTCCAGGCCATGCCAGACTGGAAGCCTGGAGCCTGCGCTTC
      I L T T Y O V Q A I A R L E A W S L A L      528
2101  TCGCCCTGCTCCTTCTCTCTGCTGCCCTCATCTTACCATTTGGAGGCAGCCACAGA
      L A L F L V L C A A V I L T I W R Q P Q      548
2161  ATCAGCAAAAAGTAGCCTTATGCTCCCTTCTTACCCTTTCTGCCCGGCTTCAGCATCC
      N Q P K V A F M V L P L P L P A F S I      568
2221  TGGTCAACATTTACTGTGATGTCAGTAAAGTCCGACACTGGATCAGATTCAGCATCT
      L V N I Y L M V Q L S A D T W I R F S I      588
2281  GAGTGGGCTTGGCTTCTGATCTATTTCGCTATGCGCTATGACACAGCTTGGAGGATA
      M M A L G F L I Y F A Y Q I R H S L E G      608
2341  ACCCCAGGACGAAAGAGCATGAGGATGCCCTTTCAGCAAAACATCAATGTAGCAACAG
      N P R D E E D D E D A F S E N I N V A Y      628
2401  AAGAAAAGTCCGTCATGCAAGCAAAATGACCATCACCAGAAACCTCAGCTTACCTTTCA
      E E K S V M Q A N D H E Q R N L S L P F      648
2461  TACTTCATGAAAAGCAAGTGAATGTGATGCTGGCCCTCGGTCTTACCACGCATACCTT
      I L H E K T S E C *
2521  AACAAAGTACACTGTGGCCGATGCCACCATGCTGCTGGCTGTCTGGGCTGCTGT
2581  GGACATGGCTTGCCTAATCTGACTTCCCTCCAGACAGCTTCTCTCAGATGGTGGAT
2641  TCTGTCTGAGGAGACTGCTGAGAGCACTCCTCAGCATATATGTATCCCAAAACAGTA
2701  TGTCCGTGGCTACATGATGTCTGCGATGTGAGTGTCAATGTCTCCCTTATAGTC
2761  TTGACATAATCCAGCATGGTAAATGGTGGCATACTGCACACACTAGTAAACAGAT
2821  ATTGCTGAATAGAGATGATCTGTATATGTCTAGGTGGCTGGGAAATAGTGGT
2881  TCTTTATTAGGTATGACCATGATTTGGACATCTGAAATGCCATCCCTGTCCAGGAT
2941  GTTATACAGTGGTATGGGTTGGGAGGGGATAAGGAATGGGCATTTGTATAAATGTAA
3001  TGCAATATCTCTCTCTCTGCTGCTAAGACAGCTTCTTAAACGGCCAGGAGAGTGT
3061  CTTCTCTGTATGACAGATGAGAGGATGCTGTGGCTGGAGATGGCAATCCCTGGT
3121  TTGAGGCCCTGGCTTAAATATACACTCTTACCTGCTGTAATGATATACCATCAA
3181  CFCTTGAAAAGTCTCAGAAAAGTACCCTGCTTCTGGGACCTTTAAGCAGAGAT
3241  GAACGTATGCCAGTGGCTTCTAAGATCAGATGACTTACAAAGATGCTGGCAGAGAT
3301  GAAGGCAGAAATAGGCTCCGAAATCACACATTTGGTAGACTGGTTCAGGCTGTTCCTCAT
3361  ACTTCAGACCTAATCACAGCTCAGCTGCTGCTTCTCATCACAGAACCCAGTCTTTGT
3421  TCTCATCTTCTTGTATGCTGGATGAAAACGAGTGTGGTAGCAAAATCTTTTTTACTGT
3481  GGACAAAAGACACAGATGGAGGGGTATAGGAAAATGCCCTTAACTCAGCTCACAGGG
3541  CGCTTGAAGCAAGTGAAGGATTTAAACCTAATTTCCCTGATATTTCTTTTAGAAAT
3601  GTTTAGAGATCTATCTATCTCCTACCCATCTCATCATTTAGCACTTATAAATTTATT
3661  TCTCCCTGCTGCTGTCTCC
    
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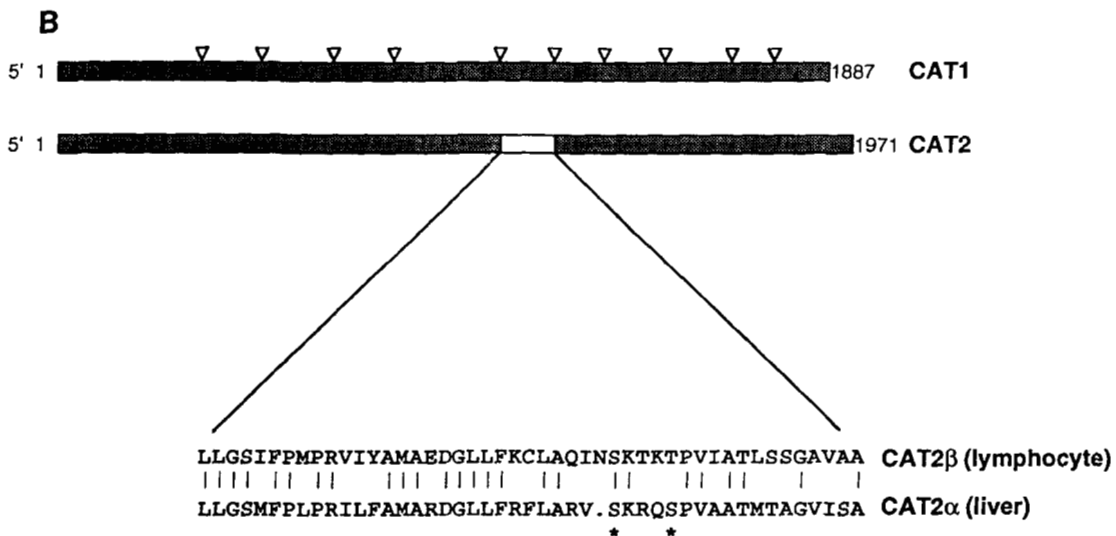


Fig. 1. A, nucleotide and deduced amino acid sequence of *Tea* cDNA isolated from mouse liver. The inverted triangles indicate a site in which G is substituted by T at nucleotide 721, and a site in which a T is inserted at nucleotide 1093 when the sequence is compared with the partial mouse T lymphocyte *Tea* sequence isolated by MacLeod *et al.* (3). The liver cDNA is extended by 721 nucleotides at the 5' end and 566 nucleotides at the 3' end compared with the previous partial cDNA (4). In addition, the corrected coding region of liver *Tea* was described in another report (12). The divergent region of the liver and lymphocyte cDNAs is indicated by a dotted line above the nucleotide sequence. Five potential sites for N-linked

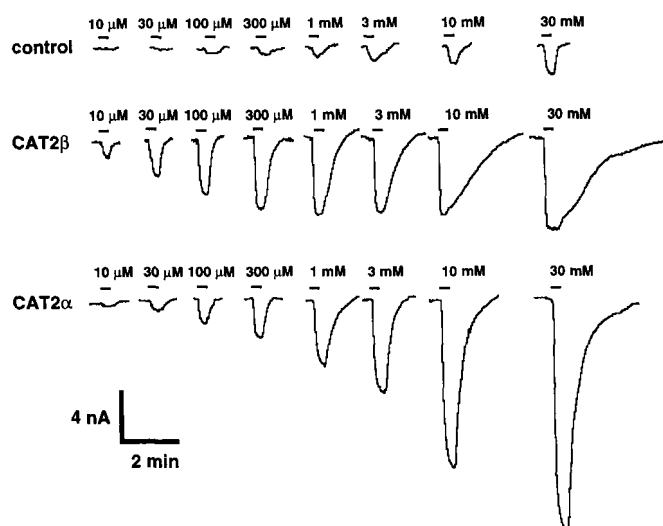


Fig. 2. Two-microelectrode voltage-clamp analysis of arginine-induced transport current (2). Representative records showing concentration dependence of the inward current induced by superfusion with L-arginine in a control (water-injected) oocyte and in oocytes injected with RNA transcribed from the low affinity liver (CAT2 β) or high affinity lymphocyte (CAT2 α) cDNA isoforms. Oocyte membrane was clamped at -60 mV, and varying concentrations of arginine were applied for duration indicated by the bar.

maining plasmid. To construct CAT1-CAT2 chimeras, site-directed mutagenesis was used to introduce a *Pst*I site at nucleotide 1091 of CAT1. The 5' portion of CAT1 cDNA was then ligated at this *Pst*I site to the 3' region of lymphocyte or liver CAT2 cDNA. The site of recombination in the chimeric protein occurs in the sequence PQKAIP at amino acid 275.

Expression in Oocytes—Capped *Tea* mRNAs were synthesized using SP6 RNA polymerase and injected into *Xenopus laevis* oocytes as previously described (2). 3–10 days after injection of 50 ng of RNA, two-electrode voltage-clamp recordings were made on oocytes continuously superfused with 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH 7.5 (ND-96). Substrate-induced currents were measured by rapidly changing the superfusing solution to one containing the indicated concentration of substrate. Current measurements were made at -60 mV. In some experiments sodium was replaced by Tris. Kinetic parameters were determined by least-squares fitting the concentration-response data to the following equation (Eq. 1):

$$I = I_{\max} \{ [S] / (K_m + [S]) \} + I_{\max \text{endo}} \{ [S] / (K_{\text{endo}} + [S]) \} \quad (\text{Eq. 1})$$

where I_{\max} and K_m are the kinetic parameters for the induced transport currents and I_{endo} and K_{endo} are the Michaelis parameters for the endogenous substrate-dependent currents as determined in control oocytes (uninjected or water-injected) from the same batch. Uptake was also studied by incubating oocytes in the same recording buffer containing 100 μ M L-[³H]arginine (50 Ci/mmol, Dupont-New England Nuclear) in the presence or absence of various competing substrates for 12 min at 22 °C followed by three rapid washes in cold buffer. Oocytes were then lysed in 100 μ l of 1% sodium dodecyl sulfate followed by liquid scintillation spectrometry. In these conditions, L-[³H]arginine uptake was determined to be linear with time for at least 18 min.

Expression in Mammalian Cells—Lymphocyte and liver CAT2 and chimeric CAT1-CAT2 cDNAs were expressed in the pSFF (15) retroviral vector after removal of transcriptional termination (poly(A)-addition) sequences as previously described (16). The expression vectors were transfected into 1:1 cocultures of Ψ 2 (17), and PA12 (18) cells and the mixture of helper-free amphotropic and ecotropic host-range viruses were recovered in the culture media (15). SFF-*Tea* virus was used to infect the BM3C3 derivative of E36 hamster cells; BM3C3 contains receptors for ecotropic murine retroviruses but not for amphotropic

retroviruses (19). To test whether the BM3C3 cells expressing *Tea* had amphotropic receptors, they were superinfected with a helper-free amphotropic pseudotype of MSV-*neo* virus followed by selection of G418-resistant colonies (20). This same virus preparation gave a titer of approximately 5×10^2 /ml on control mink CCL64 cells. To test whether *Tea* or *ecoR-Tea* chimeras encode functional ecotropic receptors, the viruses from Ψ 2/PA12 cocultures were used to infect mink CCL64 cells. Mink cells have amphotropic but not ecotropic receptors. The mink cells expressing *Tea* or *ecoR-Tea* chimeras were then superinfected with a helper-free ecotropic pseudotype of MSV-*neo* virus, as described previously (16). Measurement of L-[³H]arginine uptake into mammalian cells was performed as described previously (21, 22).

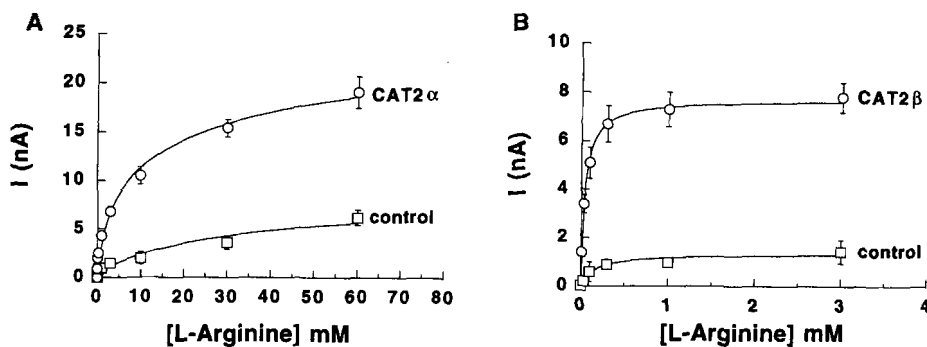
RESULTS AND DISCUSSION

Sequence Comparison of Liver And lymphocyte *Tea*—The full-length mouse liver *Tea* cDNA encodes a predicted protein of 657 amino acids (see Fig. 1) and contains long 5'- and 3'-untranslated sequences that were not previously reported. Although the translational start site contains a good Kozak consensus sequence (23), the preceding region contains six ATG initiation codons that are followed by in-frame termination codons. As expected (24), removal of these by deletion of 468 bases from the 5' end improves protein expression (see below). Immediately upstream from the translational start site is an oligopyrimidine tract, similar to those involved in coupling ribosomal protein synthesis to cellular growth (25). The 3'-untranslated sequence lacks an AATAAA poly(A) addition signal but contains a TATAAA at position minus 29–34 that could possibly serve this function (26). The encoded protein is homologous to *ecoR/CAT1* (58% identical and 76% similar) but is extended at the carboxyl terminus by 35 amino acids. Hydrophobicity plots of the predicted polypeptides encoded by *ecoR/CAT1* and *Tea* are nearly identical (results not shown), suggesting that their transmembrane topologies are common. Although Southern blot analyses have indicated that *Tea* is a single copy gene (4), liver and lymphocyte *Tea* cDNA diverge in one region that encodes 43 amino acids (357–396; see Fig. 1B). In this region, the two *Tea* protein isoforms contain 21 identical and 9 conservatively substituted residues. Major differences in the liver protein include 4 Arg substitutions (replacing Glu, Lys, Asn, and Thr in the lymphocyte form) and 1 missing residue. Interestingly, the liver isoform contains two unique consensus sequences (27) compatible with phosphorylation by cyclic AMP-dependent protein kinase and protein kinase C (see Fig. 1B). This divergent region, which comprises a hydrophilic loop that has been proposed to be oriented into the cytosol (28), corresponds precisely to the sixth exon of the human homolog of the murine *ecoR/CAT1* gene (see legend to Fig. 1B) (5). This strongly suggests that exonic structure of these two genes is at least partly conserved and that the *Tea* RNA transcript is alternatively spliced in T lymphocytes and liver.

Functional Expression of Liver and Lymphocyte Isoforms—Similar to previous studies of CAT1 (2, 29), L-arginine-specific inward currents were detected in voltage-clamped oocytes that had been injected with RNA transcribed from the liver (CAT2 α) or lymphocyte (CAT2 β) isoforms of *Tea* (Fig. 2). However, substantial expression was obtained only when 468 bases were removed from the 5' region of the mRNA (see "Experimental Procedures"). The inward currents induced by L-arginine were concentration dependent and obeyed Michaelis-Menten kinetics (see Fig. 3). Expression of CAT2 α results in a transport

glycosylation are indicated by asterisks at amino acids 157, 227, 239, 456, and 643. The putative membrane-spanning domains are underlined. B, a divergent sequence within the liver and T lymphocyte isoforms of TEA. The top line shows the exon boundaries (inverted triangles) of the human *ecoR/CAT1* gene (5) as they occur in the protein-encoding region of the mRNA. The second line shows the homologous murine *Tea* mRNA structure, with the divergent exon VI corresponding region represented by a light box. The amino acid sequences encoded by this exon VI region are compared in the lower lines for the lymphocyte and liver TEA isoforms. At the nucleotide sequence level, the divergence occurs entirely inside this exonic region and begins on each end within several nucleotides of the splice site. The liver TEA isoform contains two consensus sites for phosphorylation (27) indicated by asterisks.

FIG. 3. Comparison of concentration-response data for CAT2 α (A) and CAT2 β (B). Data from currents induced by arginine in control oocytes were fit by least squares to the equation: $I_{\text{endo}} = I_{\text{endo}} ([\text{Arg}]/(K_{\text{endo}} + [\text{Arg}]))$. Currents in cells expressing CAT2 α or CAT2 β were fit by to $I = I_{\text{max}} (K_m/K_m + [\text{Arg}]) + I_{\text{endo}} ([\text{Arg}]/(K_{\text{endo}} + [\text{Arg}]))$ (see "Experimental Procedures"). Data points shown are mean \pm S.E., normalized to the I_{max} for each cell ($n = 4-8$). The K_m for CAT2 β was 38 μM versus 2.7 mM for CAT2 α .



current characterized by a low millimolar affinity for arginine, consistent with a recent report (10) and characteristic of arginine uptake kinetics in cultured hepatocytes (30). In contrast, injection of RNA transcribed from the lymphocyte isoform CAT2 β resulted in induction of an arginine-dependent inward current with a 70-fold increase in apparent affinity (CAT2 α $K_m = 2.7$ mM; CAT2 β $K_m = 38$ μM) (Fig. 3). In order to analyze the substrate specificity of CAT2 β , several amino acids and analogs were tested in voltage-clamp and radiolabel flux assays. In voltage-clamped oocytes expressing CAT2 β , application of the acidic amino acid L-glutamate or neutral amino acid analog L-2-aminoisobutyric acid (1 mM) did not induce significant inward currents and did not antagonize the arginine transport current (Fig. 4). Currents were also not induced by application of 1 mM L-leucine, glycine, D-arginine, γ -aminobutyric acid, or choline (data not shown), while application of the cationic amino acids lysine and ornithine induced inward currents with K_m values of 51 ± 16 and 174 ± 32 μM , respectively ($n = 5$) (Fig. 4). Removal of Na^+ by substitution with equimolar Tris^+ did not significantly reduce the current induced by L-arginine for either CAT2 β or CAT2 α (Na^+ -free currents were $83.8 \pm 7.6\%$ of control ($n = 3$) and $82.1 \pm 9.2\%$ ($n = 3$, respectively). Cationic amino acid transport mediated by CAT2 β was also tested by measuring specific uptake of 100 μM L-[^3H]arginine. The uptake was blocked by coincubation with unlabeled L-arginine, L-lysine, or L-ornithine (each at 1 mM) but not by D-arginine (1 mM) or L-glutamate (10 mM) (data not shown). Following a 4-h preincubation with a saturating concentration of arginine, the initial rate of uptake mediated by CAT2 α was not changed in two separate experiments while that mediated by CAT2 β was increased by 32–49%. These results confirm the recent finding (11) that CAT1 and CAT2 β are subject to *trans*-stimulation, while CAT2 α is not.

Studies of CAT2 as a Retroviral Receptor—Hamster cells lack receptors for amphotropic murine retroviruses (31). Consistent with a recent independent study of CAT2 α (9), we found by other methods that hamster cells expressing either CAT2 α or CAT2 β remain completely resistant to infection by amphotropic host-range MSV-*neo* virus (see "Experimental Procedures"), confirming that CAT2 does not function as an amphotropic receptor.

We also tested the ability of the two CAT2 isoforms and of the two CAT1-CAT2 chimeric isoforms to function as receptors for ecotropic retroviruses. Although the ecotropic receptor (CAT1) expressed by fibroblasts is encoded on mouse chromosome 5 (19, 28), whereas *Tea* (CAT2) is on chromosome 8 (4), retroviruses may utilize different receptors in different tissues (32, 33). The sequence of CAT2 in putative extracellular loop 3 is also compatible with possible function as a tissue-specific ecotropic receptor (12, 34). Mink CCL64 fibroblasts expressing CAT1, CAT2 α , CAT2 β , or CAT1-CAT2 chimeras were infected with an ecotropic host-range pseudotype of MSV-*neo* virus, followed by selection with G418 (see "Experimental Procedures").

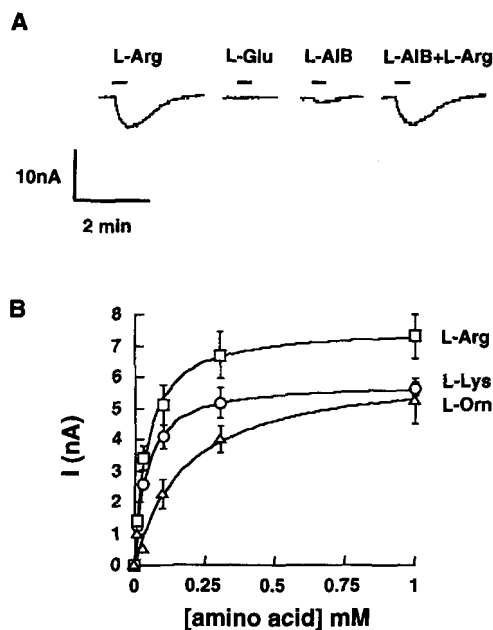


FIG. 4. A, substrate specificity of CAT2 β mediated current. L-Glutamate (1 mM) or the neutral amino acid transport analog 2-aminoisobutyrate (1 mM) did not induce a significant current or antagonize the response to 100 μM arginine. B, Michaelis-Menten fits of concentration-response data for currents induced by L-arginine, L-lysine, and ornithine. Data points are mean \pm S.E., $n = 5$ oocytes.

CAT1-CAT2 chimeras were constructed by ligating the 5' end of CAT1 to the 3' end of CAT2 α or CAT2 β at nucleotide 1091 of CAT1 (amino acid 275; NH_2 -terminal to the divergent sequences of CAT2 α and CAT2 β ; see "Experimental Procedures"). Large numbers of colonies formed in the cultures that expressed CAT1 or CAT1-CAT2 chimeras, whereas none formed in the cultures expressing CAT2 α or CAT2 β . This enabled us to readily isolate mink fibroblasts that expressed CAT1 as well as the CAT1-CAT2 chimeras. We then compared these cells for susceptibility to infection by a preparation of ecotropic virus that encodes human growth hormone (16). In one experiment, cells with CAT1 were infected to a multiplicity of 1.21 ± 0.08 , whereas a parallel infection of the cells with CAT1-CAT2 β chimera gave a multiplicity of infection of 0.53 ± 0.05 . In a second experiment, it was found that both chimeras functioned equally as ecotropic virus receptors. Although these studies are generally compatible with previous analyses of chimeras made with the more closely homologous murine and human CAT1 proteins, which suggested that the putative third extracellular loop of CAT1 is responsible for virus binding (12, 34), the CAT1-CAT2 chimeras bound ecotropic virus envelope glycoprotein (gp70) only relatively weakly (see Table I). Thus, although these proteins were expressed at similar levels as suggested by transport measurements (see below), the levels of gp70 binding

TABLE I

Binding of ecotropic envelope glycoprotein (gp70) to mink CCL64 fibroblasts that contain CAT1 or chimeric CAT1-CAT2 receptors

Experiment	Cells ^a	gp70 binding ^b
		counts/min/ μ g protein
1	CCL64 control	0.8
	CAT1	57.4
	CAT1-CAT2 β	3.1
2	CCL64 control	0.5
	CAT1	93.7
	CAT1-CAT2 α	1.7
	CAT1-CAT2 β	1.6

^a Cells used were the same as employed for the transport analysis in Fig. 5.

^b gp70 binding was determined by adsorption of excess unlabeled gp70 at 37 °C followed by addition of gp70 antiserum and 125 I-protein A as previously described (2,16,21).

were very different (Table I). One interpretation is that region(s) of CAT1 absent from the chimeras contribute to formation of a high affinity virus binding site, but that high affinity binding is not necessary for infection. Alternatively, the chimeric molecules might have much higher maximum velocities for transport than CAT1.

Transport Mediated by CAT1-CAT2 Chimeras—Fig. 5 shows analysis of L-[³H]arginine uptake induced by expression of CAT1 and the CAT1-CAT2 chimeras in mink CCL64 cells. The background transport that occurs in control CCL64 cells was subtracted as described elsewhere (3, 21, 30, 35). The CAT1-CAT2 α chimera, which contains the liver-specific spliced sequence, has a relatively high Michaelis constant compared with the CAT1-CAT2 β chimera that contains the lymphocyte sequence ($K_m = 4.4$ mM versus 200 μ M). Nearly identical results were obtained in multiple independent experiments, suggesting that the divergent sequence of the CAT2 isoforms strongly influences the kinetic parameters of transport in mammalian cells as well as in *Xenopus* oocytes.

General Implications—Our results support other evidence (9–12) that alternative splicing of an mRNA precursor leads to production of CAT2 α and CAT2 β protein isoforms that have markedly different kinetic properties. The liver isoform CAT2 α is a Na⁺-independent basic amino acid transporter with a relatively low affinity for arginine (10). This conclusion is compatible with the fact that unstimulated normal adult liver cells appear to lack a system for high affinity uptake of cationic amino acids (8). Because liver cells contain large amounts of arginase and other urea cycle enzymes, their low affinity CAT2 α transporter probably serves to conserve normal blood levels of arginine in fasting animals. Interestingly, however, cationic amino acid transport in liver can be stimulated by hormones, such as glucagon, that activate adenylate cyclase (36), implying that transport is subject to metabolic regulation. In contrast, the lymphocyte isoform CAT2 β has a relatively high affinity for arginine (see Figs. 2–5), and its activity seems to be primarily controlled by messenger RNA synthesis following activation of T lymphocytes (4–6). In this case, it has been suggested that increased uptake of arginine may be related to its role as the precursor of nitric oxide (7, 37). The apparent tissue-specific splicing of the CAT2 messenger RNA precursor, therefore, gives rise to functional differences likely to have important physiological consequences.

An important aspect of our analysis has involved transport assays in oocytes using voltage-clamp methods. The results demonstrate that transport mediated by both CAT2 α and CAT2 β is electrogenic and Na⁺-independent. Because expression of these transporters in oocytes can alter membrane po-

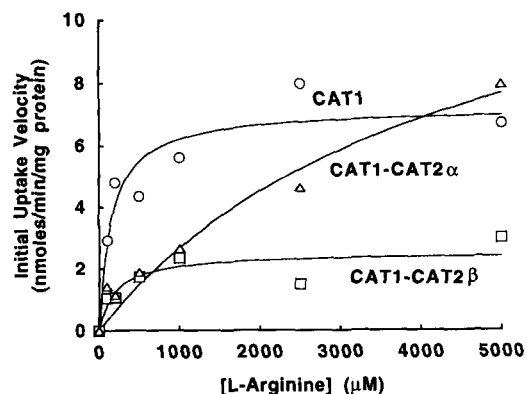


FIG. 5. Analysis of L-[³H]arginine uptake into mink CCL64 fibroblasts that express CAT1 (ecoR) and the chimeric constructs CAT1-CAT2 α and CAT1-CAT2 β , showing uptake above control CCL64 cells. Curves were drawn by computer as best fits to the Michaelis-Menten equation. Assay methods were previously described (21, 22). For this experiment, K_m values were 155 μ M (CAT1), 4366 μ M (CAT1-CAT2 α), and 203 μ M (CAT1-CAT2 β).

tential², and because transport is highly dependent on membrane potential (29), voltage-clamp conditions are necessary for accurate kinetic analyses of these transporters. In a recent report it was suggested that steady-state ratios of intracellular to extracellular arginine differ substantially in oocytes expressing CAT2 α and CAT2 β or CAT1, and these ratios were used to infer relative apparent affinity constants for arginine at the intracellular membrane face (11). However, this result is incompatible with a Na⁺-independent facilitated diffusion mechanism for arginine transport, which is suggested by the present studies. In this type of transport process, the steady-state distribution of arginine will depend only on the membrane potential according to the Nernst equation. This will in turn determine the ratio of the kinetic parameters for influx and efflux ($K_m^{in} I_{max}^{out \rightarrow in} / (K_m^{out} I_{max}^{in \rightarrow out})$), which should not differ for the transporters (29). Consequently, we infer that the oocytes examined in these previous studies may have had different membrane potentials or that measurements were not made under steady-state conditions.

An implication of this work and of the independent results of Closs and co-workers (9–11) is that the proteins in this family contain domains that can function independently in chimeric contexts to control specific aspects of transporter and retroviral receptor functions. For example, the 43-amino-acid region responsible for the CAT2 α and CAT2 β divergence appears to affect transport similarly whether in a CAT2 context or in the CAT1-CAT2 chimeras (see Fig. 5). In addition, the viral receptor properties of CAT1 are independent of the kinetic differences caused by the divergent CAT2 sequences. The change in the kinetic properties of the CAT2 isoforms is striking (70-fold difference in K_m) considering the relatively discrete structural change. The large difference in apparent affinity constant associated with the substituted domain could arise from differences in the intrinsic substrate dissociation constants or from changes in other rate constants in the transport cycle (see Ref. 38). In agreement with this, *trans*-stimulation of radiolabel uptake by intracellular arginine preloading is much lower for CAT2 α than for CAT2 β (11; also, see above). These results suggest the possibility that the alternate domain is involved in determining the rate of the conformational transition of the unloaded transporter relative to other rate constants in the cycle (38). In this case, the voltage dependence of transport mediated by the two isoforms would be predicted to differ ac-

² M. P. Kavanaugh, unpublished observations.

ording to the voltage dependence of the respective rate-limiting steps (29). Although the precise mechanism by which this hydrophilic domain influences transport kinetics is unknown, these issues should now be amenable to further investigations.

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