Molecular Sites of Regulation of Expression of the Rat Cationic Amino Acid Transporter Gene*

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Cat-1 is a protein with a dual function, a high affinity, low capacity cationic amino acid transporter of the y⁺ system and the receptor for the ecotropic retrovirus. We have suggested that Cat-1 is required in the regenerating liver for the transport of cationic amino acids and polyamines in the late G₁ phase, a process that is essential for liver cells to enter mitosis. In our earlier studies we had shown that the cat-1 gene is silent in the quiescent liver but is induced in response to hormones, insulin, and glucocorticoids, and partial hepatectomy. Here we demonstrate that cat-1 is a classic delayed early growth response gene in the regenerating liver, since induction of its expression is sensitive to cycloheximide, indicating that protein synthesis is required. The peak of accumulation of the cat-1 mRNA (9-fold) by 3 h was not associated with increased transcriptional activity of the cat-1 gene in the regenerating liver, indicating post-transcriptional regulation of expression of this gene. Induction of the cat-1 gene results in the accumulation of two mRNA species (7.9 and 3.4 kilobase pairs (kb)). Both mRNAs hybridize with the previously described rat cat-1/2.9-kb cDNA clone. However, the 3’ end of a longer rat cat-1 cDNA (rat cat-1/6.5-kb) hybridizes only to the 7.9-kb mRNA transcript. Sequence analysis of this cDNA clone indicated that the two mRNA species result from the use of alternative polyadenylation signals. The 6.5-kb clone contains a number of AT-rich mRNA destabilizing sequences which is reflected in the half-life of the cat-1 mRNAs (90 min for 7.9-kb mRNA and 250 min for 3.4-kb mRNA). Treatment of rats with cycloheximide superinduces the level of the 7.9-kb cat-1 mRNA in the kidney, spleen, and brain, but not in the liver, suggesting that cell type-specific labile factors are involved in its regulation. We conclude that the need for protein synthesis for induction of the cat-1 mRNA, the short lived nature of the mRNAs, and the multiple sites for regulation of gene expression indicate a tight control of expression of the cat-1 gene within the regenerating liver and suggest that y⁺ cationic amino acid transport in liver cells is regulated at the molecular level.

Cationic amino acids lysine, ornithine, and arginine are transported into cells through several transport systems that differ in their requirement for Na⁺ (1–2). Na⁺-independent cationic amino acid transport is mediated by the y⁺ and b₅⁻ systems (2). Transporters of the Na⁺⁻independent y⁺ system span the cellular membrane multiple times, whereas members of the b₅⁻ system may span the membrane once and therefore are considered as accessory proteins (3). A number of extensive reviews on amino acid transport systems have been published (1–4). In the liver, system y⁺ is of particular importance, since the activity of the transporter may represent the rate-limiting step in the conversion of arginine to urea by the hepatic arginase (1). The importance of dietary arginine in the urea cycle has been demonstrated in the cat, where restriction of arginine from the diet leads to hyperammonemic coma (5). System y⁺ activity is absent in the quiescent liver, therefore protecting the plasma pool of arginine from the hepatic arginase (1), and is increased in transformed (2, 6) or primary hepatocytes (2) in the liver of diabetic rats (7) and in liver diseases (8, 9).

Although the biochemical properties such as kinetics, substrate specificity, and cell type-specific regulation of y⁺ transport activities have been studied thoroughly (10), very little is known about the molecular mechanisms of regulation of expression of the transporter genes (11–16). The cloning of the first cDNA for an amino acid transporter was coincidental with the cloning of the receptor for the ecotropic retrovirus (17). This viral receptor was shown to be a cationic amino acid transporter of the y⁺ system (18, 19) and so named mCat¹ (mouse Cationic Amino acid Transporter) or EcOr (Ecotropic retrovirus receptor). Subsequently mcat-2 (also known as Tß; T-cell early activator) was cloned which encodes a protein with 61% homology to mCat-1 (20). Both proteins function as y⁺ system transporters for the cationic amino acids arginine, lysine, and ornithine (21). A second isoform of mcat-2 (mcat-2a) has been isolated that is an alternatively spliced mRNA encoded by the same gene as mcat-2 (21). The three transporters differ in their affinity for arginine with mCat-1 and mCat-2 having 10 times higher affinity than mCat-2a (21–23). Isolation of cDNAs for the Cat-1 transporter from mouse (18, 19), rat (24–26), and human cells (27) indicated that they are highly homologous and that the transporter activity of the protein is highly conserved through species (28–31). Interestingly, mammalian cationic amino acid transporters (3) are related to the yeast transporters for arginine, histidine, and choline (31), and this is supported by the recent findings of Kabat and associates (32) who demonstrated that the amino acids responsible for the function of the protein as a transporter are conserved through species.

Analysis of mRNA transcripts for the cat genes in the mouse

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1 The abbreviations used are: mcat, mouse cationic amino acid transporter; cat-1, cationic amino acid transporter-1; CAT-2, cationic amino acid transporter-2; EcOr, ecotropic retrovirus receptor; Tß, T-cell early activator; PEPCK, phosphoenolpyruvate carboxykinase; TAT, tyrosine aminotransferase gene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UTR, untranslated region; bp, base pairs.

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(11) and the rat (13, 24, 25) indicate that the gene for the high affinity transporter Cat-1 is expressed in most tissues except the liver (13, 24, 25), while the gene for the low affinity transporter Cat-2a is expressed in the liver and other tissues but not in kidney or intestine (11). The pattern of expression of the two genes in the liver is in agreement with previous studies by Christensen and co-workers (1) who reported that normal hepatocytes lack \( y^+ \) cationic amino acid transport except under conditions where the substrate concentration exceeds the level found in systemic plasma. Therefore, lack of Cat-1 gene expression in the liver protects plasma arginine from degradation characterizing this transporter as a growth response gene.

In this paper that the cellular components necessary for growth. We demonstrate growth-related processes before liver cells enter mitosis. During this phase of the regenerating liver, early and delayed early response genes are induced in order to provide hepatocytes and non-parenchymal cells with the cellular components necessary for growth. We demonstrate in this paper that Cat-1 is a delayed early response gene implicating this transporter as a growth response gene.

Transport of cationic amino acids through the system \( y^+ \) has been demonstrated in different mammalian cells (2), and regulation of expression of the Cat-1 gene has been found in a wide variety of cells and tissues (6, 14–16, 24). The molecular sites of regulation of expression of the Cat-1 gene are not known. In the rat, two mRNA species of 7.9 and 3.4 kb have been found (13, 24, 25), with the 7.9-kb mRNA being five times more abundant than the 3.4 kb. The relative concentration of the two mRNAs is regulated by cell density (24). The 3.4-kb transcript has been shown to correspond to a 2.9-kb cDNA clone that was isolated from a rat hepatoma cDNA library (24). We describe here the characterization of a 6.5-kb cDNA clone (25) that contains the earlier cloned 2.9-kb rat Cat-1 cDNA (24), and we use this cDNA to characterize the two rat Cat-1 mRNAs. In addition, we report the molecular sites of regulation of expression of the Cat-1 gene in rat tissues and hepatoma cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—All DNA modifying enzymes and nucleotides were purchased from Boehringer Mannheim. \( \alpha^32P \mid \text{dCTP} \) (3000 Ci/mmol) and \( \text{[35S]} \mid \text{ATP} \) (1000 Ci/mmol) were purchased from DuPont NEN. Restriction enzymes were used as specified by the manufacturer.

**Cloning and Sequencing of the Rat cat-1 Gene**—A size-fractionated adult rat intestinal cDNA library was screened using the mouse EcoR cDNA (17) as a hybridization probe. A positive clone (MP10) containing an insert of 6.5 kb (25) was fragmented by restriction enzyme digestion and subcloned into pBluescript (KS–). Plasmids were purified by CsCl and then sequenced by the dideoxy chain termination method using Sequenase (U. S. Biochemical Corp.) and either internal oligonucleotide primers or the T7 and T3 primers within the vector.

**DNA Hybridization Probes**—The following probes were used in this study: (i) Cat-1/2, a 2.9-kb insert of the rat Cat-1 cDNA (24). (ii) PEPCK, a 1.1-kb PstI fragment from the 3′ end of the PEPCK cDNA (35). (iii) albumin, a 1.0-kb DNA insert from the pALB cDNA (32). (iv) TAT, the cDNA for the tyrosine aminotransferase gene (37). (v) c-jun, a human c-jun cDNA (35). (vi) c-fos, a 1.0-kb fragment of the c-fos cDNA, which contains the first three exons of the c-fos gene (37). (vii) ribo, a cDNA fragment for the 18 S ribosomal RNA (39). (viii) c-myc, mouse cDNA, purchased from American Type Culture Collection (ATCC No. 41029). The probes were labeled using the random priming kit from Boehringer Mannheim, and the specific activities were 10–1000 cpn/μg DNA.

**Partial Hepatectomy of Rats**—Hepatectomy (70%) was performed at 10 a.m., on Sprague-Dawley male rats (50 g) by removal of the median and left lateral lobes. Cycloheximide-treated rats were injected intraperitoneally with 40 mg/kg body weight cycloheximide in saline, 1/2 h before hepatectomy. Animals were sacrificed at the times given, and the livers, kidneys, spleens, and brains were frozen in liquid nitrogen and then stored at –80°C until required.

**RNA Extraction and Northern Blots**—RNA was purified and analyzed by Northern blotting as described previously (40). Briefly, frozen liver tissue pieces or tissue culture cells were taken and placed into 4 M guanidine thiocyanate buffer (4 M GTC, 0.5% sarcosyl, 25 mM sodium citrate, pH 7.0). The samples were immediately homogenized and the homogenate was then loaded onto a cushion of CsCl (0.5 M CsCl, 0.1 mM EDTA, pH 7.0) and spun at 175,000 × g for 16 h. After centrifugation, the pellet was dissolved in 10x Hepes buffer (1 mM Hepes, 1 μM EDTA, 0.1% SDS, pH 7.5) and precipitated with 2.5 volumes of ethanol and 0.3 M sodium acetate. The precipitate was then dissolved in diethyl pyrocarbonate-treated water, and samples were immediately frozen at –70°C until required. Poly(A)+ RNA was isolated using oligo(dT), as described previously (40).

For Northern blots, samples containing 25 μg of total RNA were denatured using methyl mercury (15 mM methyl mercury, 1% SDS, borate buffer, 15% glycerol) and analyzed on a 1% agarose gel. RNA was transferred onto GeneScreen Plus and probed in hybridization buffer (0.5 M NaCl, 0.25 mM sodium phosphate, 1 mM EDTA, 25 mg/ml salmon sperm DNA, 1 mg/ml milk, 50% deionized formamide) at 42°C. Blots were washed in 0.1% SDS and 0.1 × SSC (15 mM NaCl and 1.5 mM sodium citrate).
Evaluation of Transcriptional Activity and Densitometric Analysis—

The rate of transcription of the cat-1, PEPCK, and c-fos genes was quantified by densitometric analysis of the autoradiograms. The efficiency of transcription of the nuclei at different time points of liver regeneration was normalized against transcription of total rat genomic DNA. Since transcription of many genes is regulated during liver regeneration, the choice of genomic DNA was more reliable and gave us reproducible data. The fold induction of transcription over control (zero time point) was estimated as the ratio of the individual DNA autoradiographic signals over the signal of total rat genomic DNA. Scanning of autoradiographs was performed using the CS SCAN 5000 densitometer. Different timed autoradiographs were used for quantitation, per experiment, to ensure that the exposures were within the linear range of the x-ray film. The PhosphorImager (Molecular Dynamics) was also used for quantitation of the hybridized material on the blots.

RESULTS
cat-1 Is a Delayed Early Response Gene in the Regenerating Liver—The orchestrated expression of genes in the regenerating liver following partial hepatectomy is required for the transition of liver cells from quiescence to growth (34). The genes induced in this transition are called immediate early response genes, and their induction occurs in the absence of protein synthesis (32). A second group of genes, called delayed early response genes, are induced between 3 and 24 h post-hepatectomy, and their expression depends on protein synthesis (33). At the cellular level, proteins encoded by the immediate early response genes may help control progression through the cell cycle, whereas proteins encoded by the delayed early response genes may be involved in the synthesis of cellular components required for the doubling of the cellular mass (33). Expression of the cat-1 gene in the rat peaks between 2 and 6 h following 70% partial hepatectomy, whereas expression of c-jun, an immediate early response gene, appears at 5 min (Fig. 1A). Expression of the genes involved in gluconeogenesis, PEPCK, and TAT also increases following partial hepatectomy (Fig. 1A) in order for the liver to maintain glucose homeostasis and compensate for the loss of mass (41–43). The level of the PEPCK mRNA, as expected, increased in 30 min and remained at the induced level until 6 h following hepatectomy (41–43). The induction of the PEPCK mRNA is probably due to transcriptional regulation of the PEPCK gene (through glucocorticoids and glucagon) and post-transcriptional stabilization of the mRNA by glucocorticoids (44). Comparison of the time course of induction of the mRNA levels for cat-1 and c-jun indicates that cat-1 is a delayed early response gene, whereas c-jun is an immediate early response gene (41–43). The induction of expression of the cat-1 gene in the regenerating liver involves two mRNA transcripts (7.9 and 3.4 kb), as is shown in poly(A)+ RNA from the regenerating liver (Fig. 1B). The 7.9-kb transcript is five times more abundant than the 3.4-kb mRNA.

The time course of expression of the cat-1 gene displays the characteristics of a delayed early response gene in the regenerating liver. Since expression of the delayed early response genes depends on protein synthesis (34), we determined the effect of inhibition of nascent protein synthesis on the expression of the cat-1 gene. Treatment of rats with cycloheximide for 30 min before partial hepatectomy completely abolished induction of expression of the cat-1 gene (Fig. 1C). As expected, mRNAs for c-fos and PEPCK were stabilized in the same rats (Fig. 1C) demonstrating the lack of requirement of protein synthesis on these immediate early growth response genes. Treatment of control rats with cycloheximide had no effect on the expression of the cat-1 gene in the liver but, as expected, stabilized the mRNAs for PEPCK and fos (Fig. 1C, lanes 1 and 2).

Effect of Cycloheximide on the Expression of the cat-1 Gene in Kidney, Spleen, and Brain—In contrast to the liver, treatment of rats with cycloheximide induced accumulation of the 7.9-kb cat-1 mRNA in kidney (4.16-fold) and spleen (4.1-fold) and to a lower extent in the brain (1.52-fold). The cat-1 3.4-kb mRNA was marginally induced (Fig. 1D). Densitometric analysis of the 7.9- and 3.4-kb mRNA bands in control and cycloheximide-treated brain, spleen, and kidney indicated that the intensity of the 7.9-kb mRNA was 8–10 times higher than the intensity of the 3.4-kb mRNA species in the cycloheximide-treated rats, which indicates that the larger transcript accumulates faster than the smaller transcript (Fig. 1D, bottom). These data suggest that labile factors are involved in the regulation of the concentration of the mRNA for the cat-1 gene in these tissues.

Correlation of Transcriptional Activity and mRNA Accumulation of the cat-1 Gene in the Regenerating Liver—In order to
determine the mechanism for increased mRNA accumulation of the cat-1 gene in the regenerating liver, we performed nuclear run-off experiments using nuclei isolated from 0, 30, 60, and 240 min after partial heptectomy. Six independent experiments demonstrated that the transcription rate of the cat-1 gene is not significantly altered in the regenerating liver (Fig. 2A). The transcriptional silence of the cat-1 gene was also observed in cycloheximide-treated, hepatectomized rats (Fig. 2A, last two lanes). As expected (45–46), transcription of the early response gene PEPCK (41–43) was induced 5.48-fold at 30 min after surgery. Transcription of the c-fos gene was only induced by 2.0-fold at 30 min (Fig. 2B, compare first two lanes) which is in agreement with the fact that regulation of expression of the c-fos gene in the regenerating liver is post-transcriptional (43). A darker exposure of the autoradiogram presented in Fig. 2B was used to evaluate the induction of transcription of the c-fos gene. Furthermore, following cycloheximide treatment and heptectomy, we have observed an expected induction of the transcription rate (12–17-fold) of the c-fos gene (Fig. 4, last two lanes). These data indicated that the change in the steady state levels of the cat-1 mRNA (Fig. 2C) from the regenerating liver is protein synthesis-dependent and it is controlled at the post-transcriptional level. Post-transcriptional regulation of expression of the cat-1 gene in the regenerating liver may involve stabilization of the cat-1 mRNAs. Regulation of mRNA stability has been shown to be the key mechanism in the regulation of expression of delayed early response genes in the regenerating liver (34).

Characterization of the cat-1 mRNAs—Two mRNAs (7.9 and 3.4 kb) have been identified in FTO2B rat hepatoma cells using the rat cat-1/2.9-kb cDNA clone as a hybridization probe (24). We suggested earlier that the cat-1/2.9 cDNA (24) corresponded to the 3.4-kb cat-1 mRNA. In order to better understand the nature of the two mRNAs and in light of their differential regulation in response to cycloheximide (Fig. 1D), we have isolated (25) and sequenced a 6.5-kb cDNA (cat-1/6.5, Fig. 3) from a size-fractionated adult rat intestinal cDNA library.

Comparison of the sequence between the two cDNAs indicated that they share 2.9 kb of sequence at the 5’ end, with the cat-1/6.5-kb clone having additional sequence of 79 bp upstream of the translation start site and 3.6 kb of additional sequence at the 3’ end including a poly(A) tail (Fig. 3). The common 2.9-kb sequence is almost identical between the two cDNAs (>99% homology). Most base changes do not alter the amino acid sequence except for the region indicated in the figure legend. These changes result in an alteration of three amino acids, in a region where there is a low degree of homology between the mouse and human CAT-1 protein. Comparison of the sequence of the two cDNAs shows that they are derived from the usage of alternative polyadenylation sites within the same gene. Northern blot analysis of RNA isolated from FTO2B cells and hybridized with DNA probes corresponding to the 5’ and 3’ ends of the cat-1/6.5 cDNA indicated that the 3’ end probe does not hybridize to the 3.4-kb mRNA (Fig. 4A). Additionally, hybridization of the same RNA with a 3.5-kb cDNA probe (containing the entire uncommon 3’-untranslated region from nucleotide sequence 2962 to the end of the cat-1/6.5 cDNA, Fig. 3) indicated that only the 7.9-kb mRNA contained this sequence (data not shown). This confirms that the usage of alternative polyadenylation sites has generated the two mRNAs. The putative polyadenylation signals are indicated in Fig. 3.

Evaluation of the Half-life (t1/2) of the cat-1 mRNAs—The sequence analysis of the cat-1/6.5 cDNA clone shows the presence of a stretch of 11 AT repeats (double underlined, Fig. 3) and four copies of the ATTTA sequence motif (Fig. 3) within the 3’-untranslated region (3’-UTR). AU-rich mRNAs at the 3’-UTR are more unstable and susceptible to degradation (47). In order to accurately determine the half-life of the two mRNAs, we have isolated poly(A)+ mRNA from actinomycin D-treated cells at different time points and analyzed by a Northern blot. The t1/2 was evaluated by scanning different exposures of the autoradiograms from the analysis of total and poly(A)+ RNA. A representative experiment is presented in Fig. 4, B and C.
Evaluation of the 1/2 of the two mRNAs in FTO2B cells indicated that the 7.9-kb mRNA is short lived with 1/2 of 90 min and the 3.4-kb mRNA has a 1/2 of 250 min (Fig. 4D). As an internal control for our study, we estimated the 1/2 of the short lived c-myc mRNA (48) using the same Northern blots (Fig. 4, B and C). The latter was estimated to be 18 min. Comparison of the 1/2 of the two cat-1 mRNAs suggests that the destabilizing sequences in the 3'9-UTR of the 7.9-kb transcript may contribute to its shorter half-life than the 3.4-kb mRNA.

**DISCUSSION**

We show in this paper that cat-1 is a delayed early response gene that requires protein synthesis for an increase in the accumulation of the mRNA levels. Since we did not observe any
significant induction of transcription of the cat-1 gene in the regenerating liver, we conclude that post-transcriptional mechanisms are involved in the regulation of its expression. The mechanism of regulation of expression of the transporter gene is not known. We have shown earlier that glucocorticoids and insulin induce the level of the cat-1 mRNA in the quiescent liver and in hepatoma cells in culture. It is possible that glucocorticoids are involved in the increased stability of the cat-1 mRNAs in the regenerating liver, since the level of the serum glucocorticoids increases immediately following partial hepatectomy (49). Alternatively, the transcription of the cat-1 gene may slightly increase early after partial hepatectomy, followed by mRNA stabilization. However, we were unable to detect any significant change of transcription of the gene in the first 3 h of liver regeneration. Since protein synthesis is required for the transient increase in cat-1 mRNA levels, we suggest that a regulated labile factor controls the steady state mRNA levels in the regenerating liver. Since both mRNAs (7.9 and 3.4 kb) transiently accumulate in the regenerating liver, at least in part, the mRNA sequences that are associated with the rapid mRNA turnover should be contained within the 3.4-kb cat-1 mRNA. At this point, we do not know if the labile factor that controls turnover of the cat-1 mRNAs stabilizes or accelerates decay. Therefore, the expression of the labile factor in the regenerating liver is either induced (if it is a stabilizing factor) or inhibited (if it is a destabilizing factor) immediately following partial hepatectomy. Post-transcriptional mRNA stabilization of alternatively polyadenylated mRNA transcripts has also been observed for the mRNAs for the gene for asparagine synthase (50). Evaluation of the half-life of the cat-1 mRNAs in FTO2B cells indicated that there might be a regulatory protein involved in stabilization or turnover of the mRNA. Although

**FIG. 4. Characterization of the cat-1 mRNAs and their half-lives.** A. Northern blot analysis of RNA isolated from FTO2B cells grown in serum-supplemented media and hybridized with probes A–D. A diagram of the hybridization probes (A–D) is presented at the bottom of B and C; the t₁₀ of the cat-1 mRNAs was evaluated in FTO2B cells. Northern blot analysis of poly(A)⁺ RNA, isolated from FTO2B cells, following treatment with actinomycin D (ActD). Confluent cells were changed to serum-containing media (Con) or media containing actinomycin D and incubated for 0–3 h as indicated on the top of the figure. Northern blot analysis was performed using the cat-1/2.9 cDNA as a hybridization probe. The Northern blot of the 3-h time point is presented in C (the right panel in C is a darker exposure of the left panel). The same blot was hybridized with c-myc and GAPDH as hybridization probes. As expected the GAPDH mRNA is very stable and the c-myc mRNA has a half-life of 18 min. D, scanning of the autoradiogram of B and C was used to evaluate the half-life of the cat-1/7.9-kb mRNA (t₁₀ of cat-1/7.9; 90 min). The t₁₀ of the 3.4-kb mRNA was evaluated from a separate Northern blot of poly(A)⁺ RNA isolated from FTO2B cells treated with actinomycin D (t₁₀ of cat-1/3.4; 250 min). Each data point is expressed as a percent of the remaining of mRNA from time 0 and is normalized as a ratio of cat-1 mRNA to GAPDH mRNA. The slopes of the decay curves for the two cat-1 mRNAs were significantly different than one another. Scanning of the autoradiograms of five independent experiments gave identical results on the evaluation of the t₁₀.
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half-life measurements using actinomycin D may not reflect the true in vivo rate, due to the loss of labile stability or decay factors, the differences between the half-lives of the 7.9- and 3.4-kb cat-1 mRNA demonstrate differential regulation. If actinomycin D inhibits a stabilization factor for the cat-1 mRNA then it implies that expression of this factor is induced in hepatoma cells, is absent in the quiescent liver, and is induced in the regenerating liver. Future studies will determine the mechanism of post-transcriptional regulation of the cat-1 gene. The pattern of regulation of the cat-1 gene in the regenerating liver is similar to the regulation of expression of most genes during liver regeneration (34). Post-transcriptional regulation of gene expression has also been observed for the mcat-2 gene, another member of the cationic amino acid transporter family, in lymphoma cells and somatic cell hybrids (51).

Transport of amino acids is subjected to metabolic regulation (2, 4). It has been shown that System A, one of the most widely found amino acid transport systems, is sensitive to inhibition of transcription and inhibitors of protein synthesis, which indicates that there is a molecular site of control of amino acid transporters. The pattern of regulation of System A involves amino acid transporters, which is sensitive to actinomycin D inhibition (52). Furthermore, Kilberg and co-workers (7) have shown that induced y' activity is sensitive to actinomycin D and cycloheximide. Since Cat-1 is the ubiquitous high affinity cytosolic amino acid transporter, it plays an important role in supplying arginine and lysine to the peripheral tissues. We have shown that cycloheximide differentially alters the steady state mRNA levels of the cat-1 gene in the liver as compared with other tissues such as brain, kidney, and spleen, demonstrating that tissue-specific labile factors are involved in the regulation of expression of this gene.

We have isolated (24, 25) and sequenced a cDNA clone of 6.5 kb (cat-1/6.5) from a rat intestinal cDNA library, which probably represents the 7.9-kb transcript detected by Northern blot analysis in a variety of rat tissues (13, 21, 24, 25, 53). The difference in size between the cDNA and the 7.9-kb mRNA may be due to the difficulty in accurately measuring the molecular weight of RNA at this large size. However, the 5'-untranslated region may contribute to the larger than the cDNA size of the 7.9-kb mRNA. We have demonstrated that the 5' end of the 6.5-kb cDNA is almost identical to the 2.9-kb cDNA that we cloned earlier using a rat hepatoma cDNA library (24). This 2.9-kb cDNA clone recognized a 3.4-kb mRNA transcript and the 7.9-kb mRNA. Based on sequence homology we conclude that both cDNAs encode the same protein, since they have the same open reading frame. The two cDNAs had minor differences in the DNA sequence that resulted in three amino acid substitutions. The significance of these substitutions is not known. However, the amino acid substitutions are not in the region that has been shown to be involved in the function of the protein as a viral receptor (29, 30, 54) or amino acid transporter (32). Furthermore, sequence analysis of a partial cat-1 cDNA that was isolated from rat vascular smooth muscle cells contained the identical sequence variation that we have found for the 6.5-kb cDNA clone (14). Since the rat, human, and mouse proteins are highly homologous (90%) but are variable at these altered amino acids, we conclude that the observed amino acid differences between the translated proteins of the rat cat-1 cDNAs are not functionally important. The differences in the nucleotide sequence between the two cDNA clones may indicate either a normal polymorphic variation of the alleles of the cat-1 gene or variations due to the rat strains that were used to generate the cDNA libraries (24, 25).

The most significant difference between the two cat-1 cDNA clones is found in the 3'-untranslated region. The 3.4-kb mRNA derives from the usage of the non-canonical polyadenylation signal AATAAT (at 2940 bp), whereas the 7.9-kb mRNA derives from the usage of a consensus AATAAA polyadenylation signal (at 6433). Three consensus polyadenylation signals have been found (743, 5206, and 6433). The first at 743 is present within the coding region of the protein (80–155), and the other two are present within the 3'-UTR. A nucleotide sequence TTTTTTATTA that confers efficient polyadenylation in yeast primary transcripts (55) is also present at position 4336. Northern blot analysis of poly(A)+ mRNA isolated from FTO2B rat hepatoma cells demonstrated the presence of two additional minor transcripts that may correspond to inefficient use of the polyadenylation signals at 4336 and 5206 (Fig. 1B). Transcripts of different lengths due to the usage of alternative polyadenylation signals have been reported for many genes including genes encoding for membrane transport proteins. b+/-like amino acid transporter is a protein with one transmembrane domain involved in sodium-independent transport of L-basic amino acids, L-cysteine, and some neutral amino acids through a system related to cat-1 (56). This protein is encoded by two mRNAs with the major difference in the 3'-UTR (56). The mouse cationic amino acid transporters mcat-1 and mcat-2, also have two transcripts each. Although complete cDNA sequences are not available, a recent report indicates that the mcat-2 mRNA contains a long 3'-untranslated region (21). The functional significance of these differences is unknown.

The long 3'-UTR of the rat cat-1 gene provides an insight into the post-transcriptional mechanism of regulation of expression of this gene. The 3'-UTR region of the 7.9-kb mRNA contains a number of AU-rich motifs, including four copies of the consensus AUUUA sequence, which is characteristic of unstable mRNAs (47). Consistent with this observation is that in rat hepatoma cells the t1/2 of the 7.9-kb mRNA species is shorter than the 3.4-kb (90 min compared with 250 min). Although the larger transcript has a shorter half-life, it is more abundant than the 3.4-kb, in all rat tissues and cell types examined, even after treatment with the protein synthesis inhibitor cycloheximide. The abundance of the two major mRNA species (3.4 and 7.9 kb) is also regulated by cell density (24). At a low density, the 3.4-kb mRNA is present at almost 1:1 ratio to the 7.9-kb mRNA. At higher confluency the ratio becomes 1:5 (24). The mechanism of regulation is not known, but since the two cat-1 mRNAs are generated from different polyadenylation signals resulting in different 3'-UTRs, we suggest that sequences within the 3'-UTR may be involved in the regulation of polyadenylation or stability of the two cat-1 mRNAs. Recently it has been suggested that the AU-rich 3'-UTR of the tumor necrosis factor mRNA negatively influences the translational efficiency of the tumor necrosis factor mRNA (57). Since the 3.4-kb mRNA is more abundant in rapidly growing cells, we can speculate that the 3.4-kb mRNA may be translated more efficiently than the 7.9-kb mRNA leading to a rapid production of the Cat-1 protein when cationic amino acid transport is required.

A number of interesting yet unknown functional sequences are also found within the 3'-UTR of the cat-1/6.5 cDNA clone. This includes 11 repeats of the dinucleotide AT (at 4049). AT repeats are found in numerous genes mainly in the promoter region (58), in the 3'-UTR (59), and in between coding regions of virucide (60). It has been suggested that these sequences possess protein binding properties such as nucleosome packaging (61). A number of proteins have been isolated from Drosophila and dictyostelium which specifically bind to these sequences. The role of the AT repeat within the 3'-UTR of the cat-1 gene needs to be investigated.

The 6.5-kb cDNA, in contrast to the previously cloned 2.9-kb, contains 79 bp upstream of the translation start site. A recent report by Finley et al. (62) has shown that differential splicing
or different promoters are being used to generate alternative 5'UTRs of the mcat-2 mRNAs. Furthermore, Kavanaugh et al. (28) have demonstrated that one of these mcat-2 mRNAs has 515 bp of 5'-UTR that is subjected to post-transcriptional regulation. Six initiation and termination codons within the 515-bp UTR precedes the translation start codon (28). Since the available 5'-untranslated region of the cat-1 is only 79 nucleotides, we do not know if the efficiency of translation of the cat-1 mRNAs is another site of regulation of expression of the cat-1 gene. Preliminary studies on the structure of the 5'-UTR of the cat-1 mRNAs by our group suggests that multiple promoters are being used to express the rat cat-1 gene. This may suggest that transcripts with different 5' ends may be generated from different promoters under the influence of stimulants.

Studies on the regulation of expression of genes encoding for amino acid transporters is just beginning (2, 4, 11, 14, 15, 16, 62), and as we mentioned above, the recent literature indicates that there are multiple levels of regulation of expression of the cat genes, including multiple promoters (62), alternative splicing (21) and efficiency of translation (28). Although substrate availability is considered the major regulator of transporter activity in mammalian cells, we present evidence in this paper that the expression of the cat-1 gene is regulated at the molecular level. The association of the cat-1 transporter with growth response genes and it being a receptor for the ecotropic retrovirus speaks about their co-evolution, since retroviruses infect only rapidly dividing cells. Future studies will determine the mechanisms of regulation of expression of the receptor/transporter gene and may bring a better insight in the retrovirus life cycle.

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