Adaptive Regulation of the Cationic Amino Acid Transporter-1 (Cat-1) in Fao Cells*

Susannah L. Hyatt‡, Kulwant S. Aulak‡, Marc Malandro§, Michael S. Kilberg§, and Maria Hatzoglou¶

From the ‡Department of Nutrition, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106 and the §Department of Biochemistry and Molecular Biology, The University of Florida College of Medicine, Gainesville, Florida 32610-0245

The regulation of the high affinity cationic amino acid transporter Cat-1 in Fao rat hepatoma cells by amino acid availability has been studied. Cat-1 mRNA level increased (3-fold) in 4 h in response to amino acid starvation and remained high for at least 24 h. This induction was independent of the presence of serum in the media and transcription and protein synthesis were required for induction to occur. When Fao cells were shifted from amino acid-depleted media to amino acid-fed media, the levels of the induced cat-1 mRNA returned to the basal level. In amino acid-fed cells, accumulation of cat-1 mRNA was dependent on protein synthesis, indicating that a labile protein is required to sustain cat-1 mRNA level. No change in the transcription rate of the cat-1 gene during amino acid starvation was observed, indicating that cat-1 is regulated at a post-transcriptional step. System y⁺-mediated transport of arginine was reduced by 50% in 1 h and by 70% in 24 h after amino acid starvation. However, when 24-h amino acid-starved Fao cells were preloaded with 2 mM lysine or arginine for 1 h prior to the transport assays, arginine uptake was trans-stimulated by 5-fold. This stimulation was specific for cationic amino acids, since alanine, proline, or leucine had no effect. These data support the hypothesis: (i) the use of inhibitors of protein synthesis in starved cells inhibits the trans-zero transport of arginine; (ii) cells starved for 1–24 h exhibited an increase of trans-stimulated arginine transport activity for the first 6 h and had no loss of activity at 24 h, suggesting that constant replenishment of the transporter protein occurs; (iii) immunofluorescent staining of 24-h fed and starved cells for cat-1 showed similar cell surface distribution; (iv) new protein synthesis is not required for trans-stimulation of arginine transport upon refeeding of 24-h starved cells. We conclude that the increased level of cat-1 mRNA in response to amino acid starvation support the synthesis of Cat-1 protein during starvation and increased amino acid transport upon substrate presentation. Therefore, the cat-1 mRNA content is regulated by a derepression/repression mechanism in response to amino acid availability. We propose that the amino acid-signal transduction pathway consists of a series of steps which include the post-transcriptional regulation of amino acid transporter genes.

The signal(s) that initiate the molecular response to amino acid starvation and the mechanism of selectively increasing gene expression in mammalian cells are not known. Unlike mammalian cells, bacterial and yeast cells have the ability to compensate for the effects of amino acid starvation by inducing enzymes that promote de novo amino acid biosynthesis (1–3). In yeast, a specific protein (GCN4) compensates for amino acid starvation by activating the transcription of a group of genes encoding enzymes involved in amino acid biosynthesis (2). When yeast cells are grown in nutritionally complete medium the rate of translation of GCN4 is extremely low, but its translation is specifically enhanced in response to amino acid starvation. It has been suggested (4) that mammalian cells have a similar general control mechanism in response to amino acid starvation for genes which are involved in different aspects of amino acid metabolism (4–8).

It is clear that mammalian cells have mechanisms to respond to changes in amino acid availability (4, 5). These mechanisms may involve the regulation of transcription, translation, and (or) mRNA stability (9). However, there are very few studies of genes in which their synthesis is regulated by amino acid availability (9). Positive and negative effects of amino acid limitation on mRNA levels of specific genes have been reported (9). Specific examples of mRNAs or proteins for which synthesis is enhanced in response to amino acid deprivation include: serine dehydratase (10); asparagine synthase (7); ornithine decarboxylase (11, 12); System A (13) and System L (14) amino acid transport; IGFBP-1 (15); c-jun and c-myc (12); gadd153, b-actin, ubiquitin c, phosphoglycerate kinase, c/EBPα and c/EBPβ (9); ribosomal protein L17 and S25 (16, 17).

In mammals, dietary protein deficiency results in decreased levels of serum proteins including albumin (8). In the case of albumin, it has been suggested that the decreased protein and mRNA levels are mainly due to reduced mRNA stability (9). For asparagine synthase, amino acid starvation increases the mRNA level, and this increase depends on de novo protein synthesis (4–8).

In conditions of decreased amino acid availability, it is expected that there will be a coordinate increase in catabolism of cellular proteins, amino acid biosynthesis, and amino acid transport across the plasma membrane to provide cells with sufficient amino acids for cell survival. Regulation of amino acid transport by amino acid availability has been described for System A. System A-mediated transport of neutral amino acids is induced by amino acid starvation (18) and this induction is sensitive to actinomycin D and cycloheximide. These studies

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† To whom correspondence should be sent. Tel.: 216-368-3012; Fax: 216-368-6644; E-mail: mxh8@po.cwru.edu.

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support the concept of regulation of amino acid transport at a molecular level. Given that the gene for System y-mediated amino acid transporter has not been cloned, direct studies at a molecular level are not possible.

Transport of cationic amino acids into most mammalian cells is mainly mediated through System y+. Three related proteins encoding y+ like activity have been isolated (Cat-1, Cat-2, and Cat-2a) (19–20). All three proteins function as transporters for the cationic amino acids arginine, lysine, and ornithine, but they differ in their affinity for substrate. Cat-1 and Cat-2 have a 10-fold higher affinity than Cat-2a (19–20). The biological significance of having multiple transporters for cationic amino acids in mammalian cells is not known. However, it has been suggested that these genes may be expressed and regulated in a cell-specific manner, thus facilitating amino acid transport in and out of mammalian cells in response to specific nutritional needs within a particular tissue (21). In this regard, the family of amino acid transporters for cationic substrates resembles the family of Na+-independent glucose transporters (22).

No molecular studies regarding adaptive regulation of System y+ to amino acid starvation have been published. Studies of amino acid deprivation in entire animals are not feasible, mainly because of the dramatic changes that occur in the circulating hormones, which regulate the expression of a variety of genes. Therefore, the use of in vitro systems is initially required to identify the regulatory signals for amino acid-dependent gene expression. As shown in this report, Fao cells are ideal to study the adaptive regulation of Cat-1-mediated transport because they do not express the Cat-2 and Cat-2a transporters. Here, we show that the regulation of the cat-1 gene in rat hepatoma Fao cells is responsive to amino acid deprivation and re-exposure to individual amino acids.

EXPERIMENTAL PROCEDURES

Materials—All DNA modifying enzymes and nucleotides were purchased from Boehringer Mannheim. [γ-32P]dCTP (3000 Ci/mmol) was purchased from NEN Life Science Products. Actinomycin D, puromycin, cycloheximide, KB media, and MEM were purchased from Sigma-Dialyzed bovine serum was purchased from Life Technologies, Inc. The homogenate was then loaded onto a cushion of CsCl (5.7 M CsCl, 0.1 M EDTA, 0.1% SDS, pH 7.5) and precipitated with 2.5 volumes of ethanol.

The abbreviations used are: MEM, minimal essential medium; kb, kilobase pair(s); MOPS, 4-morpholinepropanesulfonic acid; PBS, phosphate-buffered saline; FBS, fetal bovine serum; KB, Krebs-Ringer bicarbonate; gapdh, glyceraldehyde-3-phosphate dehydrogenase; AS, asparagine synthase.

The homogenate was then loaded onto a cushion of CsCl (5.7 M CsCl, 0.1 M EDTA, pH 7.0) and spun at 125,000 x g for 16 h. After centrifugation, the pellet was dissolved in Hepes buffer (10 mM Hepes, 1 mM 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 −80 °C until required.

For Northern blots, samples of 25 µg of total RNA were dissolved in denaturing solution (5 mM Hepes, 0.05% SDS, 8% formaldehyde), heated at 65 °C for 5 min, and then analyzed on an 1% agarose/MOPS, 6.8% formaldehyde gel (MOPS consists of 0.02 M MOPS and 0.002 M sodium citrate). RNA was transferred onto GeneScreen Plus and hybridized with the appropriate DNA hybridization probes in hybridization buffer (1.5 × M EDTA, 7% SDS, 0.5 M NaH2PO4/Na2HPO4, pH 6.8 at 65 °C for 24 h. Blots were washed in 0.1% SDS and 1 × SSC (0.15 M NaCl and 0.015 M sodium citrate).

Nuclear Run-off Assays—Nuclei were prepared from rat hepatoma cells as described previously (25, 26). Briefly, plates were washed three times in PBS and scraped into PBS. Pelleted cells (600 × g, 4 °C for 5 min) were resuspended in lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 0.5% Nonidet P-40) and incubated for 7 min at 4 °C. Nuclei were pelleted (600 × g, 4 °C for 5 min), resuspended in lysis buffer and repelletted as before. Nuclei were suspended in storage buffer (50 mM Tris-HCl, pH 8.3, 5.5 mM MgCl2, 0.1 mM EDTA, 40% glycerol) and aliquots were stored at −80 °C. Nuclear run-off assays were performed by the following method. 200 µl of frozen nuclei (2 × 106 nuclei) were added to 200 µl of reaction mixture (25% glycerol, 10 mM MgCl2, 0.2 M KCl, 1.2 mM ATP, 0.6 mM GTP, 0.6 mM CTP). After the addition of 40 units of RNase inhibitor and 100 µl of [γ-32P]UTP, this mixture was incubated at room temperature for 45 min and the reaction was stopped by the addition of RNase-free DNase I and 10% (v/v) 10 mM CaCl2. This was incubated at 37 °C for 30 min after which 40 µl of 10 × SET buffer (5% SDS, 50 mM EDTA, 100 mM Tris-HCl, pH 7.0), 20 µl of 2 mg/ml proteinase K and 10 µl of 10 µg/ml yeast RNA was added. The reactions were then incubated at 37 °C for 30 min, extracted with 1 ml of RNAzol-B mixed with 10% (v/v) chloroform and then precipitated with isopropanol alcohol at −20 °C. Finally, the purified and washed RNA was dissolved in 100 µl of 0.5% SDS. The radiolabeled RNA from each sample was denatured and hybridized to dot blots containing 2 µg of purified cDNA fragments or total rat genomic DNA immobilized onto nitrocellulose paper. Blots were hybridized for 72 h at 45 °C in 1 ml of hybridization buffer containing 1% bovine serum albumin, 0.5 M sodium phosphate, and 7% SDS. The blots were washed with 1 × SSC, 0.1% SDS at 45 °C for 1 h and exposed to film. The genomic DNA was used to normalize the efficiency of the nuclear run off reactions in each sample.

Evaluation of Transcriptional Activity and Quantitation of cat-1 mRNA Levels by Densitometric Analysis—The rate of transcription of the cat-1 gene was determined using a PhosphorImager (Molecular Dynamics). Densitometric analysis of the autoradiograms was also performed using the CS SCAN 5000 densitometer (U. S. Biochemical Corp.). The efficiency of transcription of the nuclei in fed and starved cells was normalized against transcription of total rat genomic DNA. Given that transcription of many genes may be regulated in hepatoma cells, the choice of genomic DNA was more reliable than a particular cell line or templateable DNA. The percentage rate was estimated as the ratio of the individual DNA radiographic signals over the signal of total rat genomic DNA. Different timed autoradiographs of nuclear run-off and Northern blots were used for quantitation, per experiment, to ensure that the exposures were within the linear range of the x-ray film and the detection instrument.

Amino Acid Transport Assays—Fao cells which were maintained in MEM supplemented with 6% fetal bovine serum (FBS), were plated in Costar 24-well plates at a density of approximately 0.2–0.25 × 105 cells/well for 48 h (27). For the transport study, the cells were depleted for 5 min in choline KRP (119 mM choline chloride, 5.9 mM KCl, 1.2 mM MgSO4, 1.2 mM KHCO3, 5.6 mM glucose, 0.5 mM CaCl2, 25 mM choline HCO3) at 37 °C. Following depletion, transport was measured by incubating the cells in the labeled arginine solution (choline KRP including [1H]arginine (10 µCi/ml), 50 µM cold arginine and 2.5 mM cold leucine) or to show saturable transport the labeled arginine solution containing 2.5 mM unlabeled arginine, for 30 s at 37 °C. The cells were then washed 3 times with ice-cold choline KRP, labeled to air dry, and resuspended in 0.2% SDS, 0.2 M NaOH. Cell suspension aliquots were used for scintillation counting and subjected to protein analysis using the Lowry method.

Immunocytology—Fao cells were plated onto glass coverslips. Forty-eight hours after plating, the cells were washed 3 times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 30 min. Following fixation, the cells were washed 3 times with PBS, incubated in 50 % glycerol for 30 min, and washed again 3 times with PBS. The cells were blocked in 20% goat serum/PBS for 1.5 h, washed
3 times in PBS, incubated in the primary antibody cat-1 ABY4 (1:50 dilution) for 1 h, washed with PBS, and then mounted onto glass slides using Fluoromount mounting solution. A fluorescent microscope was used for observation and photography.

**RESULTS AND DISCUSSION**

**Time Course of cat-1 mRNA Accumulation following Amino Acid Starvation**—cat-1 gene expression results in accumulation of two mRNAs of 7.9 and 3.4 kb. We have previously shown that the two mRNAs derive from the usage of alternative polyadenylation signals within the 3'-untranslated region of the cat-1 gene (25). To determine the effect of amino acid starvation on the accumulation of cat-1 mRNA, we cultured rat hepatoma Fao cells in Krebs-Ringer bicarbonate buffer (KRB) as an amino acid free condition. KRB was either used without FBS or supplemented with 6% dialyzed FBS. For the amino acid fed state, we used Eagle's MEM with or without 6% dialyzed FBS (MEM).

3 times in PBS, incubated in the primary antibody cat-1 ABY4 (1:50 dilution) for 1 h, washed with PBS, incubated in the secondary antibody tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit IgG for 1 h, washed with PBS, and then mounted onto glass slides using Fluoromount mounting solution. A fluorescent microscope was used for observation and photography.

**Infection with Retroviruses**—Fao cells, which were maintained in KRB for 24 h, were incubated for 2 h with an ecotropic retrovirus containing the β-galactosidase gene, with a multiplicity of infection 1/1 (28). Fao cells do not efficiently become infected (28), therefore, a multiplicity of infection 1/20 is required for infection of large numbers of cell. We have maintained the multiplicity of infection low in this study to avoid infection of cells by using an excess of virus over cell number. Following infection, cells were washed three times with PBS and amino acid complete MEM was added for 48 h. The infectibility was determined by staining cells with X-galactosidase and counting the cells with blue nuclei.

**Amino Acid Regulation of the y+ Arg/Lys Transporter**

The cat-1 gene is expressed at high levels in Fao cells, as evident from the high levels of cat-1 mRNAs relative to its expression in rat tissues in vivo (23, 25). Expression of the other transporters Cat-2 and Cat-2a, is undetectable in Fao cells (Fig. 1A, right panel). Therefore, given that Cat-1 and Cat-2/2a are the only known proteins to mediate leucine-insensitive Na⁺-independent cationic amino acid transport (19–21), measurement of System y+ transport activity in Fao cells...
should reflect cat-1 expression.

The amount of cat-1 mRNAs increased (3-fold) following amino acid starvation (Fig. 1, A and B). The peak of induction occurred at 4 h for the 7.9-kb mRNA and at 8 h for the 3.4 kb (Fig. 1B). Accumulation of the 7.9-kb mRNA was maintained up to 24 h in a manner independent of the presence of serum in the media (Fig. 1C). Refeeding of cells which previously had been starved for 10 h, with MEM amino acid-containing media, decreased cat-1 mRNA levels to the fed state (Fig. 1, D and E).

A graphical representation of these data is shown in Fig. 1, B and E. As expected (8), the level of gapdh mRNA decreased or remained unchanged in response to amino acid starvation, whereas the levels of AS mRNA were induced (7). The increase of the cat-1 mRNAs in response to amino acid starvation was also observed in human HepG2 (Fig. 2A), C6 rat glioma, NRK rat kidney, and mouse NIH3T3 fibroblasts (not shown), which indicates that the regulation of cat-1 gene expression by amino acid availability is not restricted to one cell type. Therefore, induction of cat-1 mRNA in amino acid-depleted cells is not specific for Fao cells.

The increase of cat-1 mRNA levels in amino acid-starved cells could either be due to an increased transcriptional rate of the gene or increased mRNA stability, or both. To determine if starvation alters the transcription rate of the cat-1 gene, we performed nuclear run-off assays using nuclei isolated from starved Fao cells for 15 min, 30 min, 1 h, 2 h, 4 h (Fig. 2B), and 24 h (data not shown) and fed cells for 4 (Fig. 2B) and 24 h (data not shown). Three experiments were used to determine the relative changes of the hybridization signal for cat-1 over genomic DNA, which would reflect changes in cat-1 transcriptional activity during amino acid starvation. The transcription rate was unchanged during starvation. In parallel, we measured the transcription rate of the c-jun and AS genes. The transcription rate of c-jun remained unaltered. The hybridization signal for the AS gene was low in all three experiments and an accurate evaluation of the transcription rate was not possible. However, given that the AS mRNA level increased 10-fold upon amino acid starvation, the transcription rate of the AS gene did not show this fold change (Fig. 2B). These data support that transcription of cat-1 was not induced during amino acid starvation and therefore, we conclude that amino acid starvation alters expression of the cat-1 gene at the posttranscriptional level. The mechanism of post-transcriptional regulation should involve mRNA stabilization by trans-acting factors through cis-mRNA sequences. Given that the amount of both cat-1 mRNAs increased in response to amino acid starvation, we conclude that the cis-mRNA regulatory sequences should at least be partly contained within the 3.4-kb mRNA. However, because accumulation of the 7.9- and 3.4-kb mRNAs occurred at different time points of amino acid starvation, mRNA sequences outside the 3.4-kb mRNA must also play a role in increased cat-1 mRNA stability. The half-lives for the cat-1 mRNAs in fed cells were evaluated in previous studies, following the mRNA decay in the presence of actinomycin D (25). These half-lives were 60 min for the 7.9-kb and 240 min for the 3.4-kb mRNAs. However, evaluation of the half-lives in amino acid-starved cells using actinomycin D cannot be conclusive because it has an effect on both, the transcription of the cat-1 gene and the labile factor which affects mRNA stability.

**Effect of Actinomycin D and Protein Synthesis Inhibitors on cat-1 mRNAs Level in Response to Amino Acid Starvation**—The induction of cat-1 mRNAs in response to amino acid starvation was inhibited by the inhibitor of transcription, actinomycin D (Fig. 2A), and the inhibitors of protein synthesis, puromycin (data not shown) and cycloheximide (Fig. 2A). Furthermore, the induced cat-1 mRNA level upon starvation decayed rapidly in the presence of inhibitors of protein synthesis (data not shown). These data indicate that a labile, inducible factor is involved in either cat-1 transcriptional induction and (or) mRNA stabilization during amino acid starvation. Our in vitro transcription data (Fig. 2B) support that the labile factor induces cat-1 mRNA stability. This factor may regulate the expression of a group of genes, because induction of AS mRNA was also blocked in amino acid-starved cells in the presence of the inhibitors of transcription or translation (Fig. 2A). A labile
factor is also required to sustain cat-1 mRNA levels in amino acid-fed cells. This is evident from the fact that accumulation of cat-1 mRNAs in cells maintained in MEM (F) or KRB medium (S) for 1–24 h. In a parallel study, Fao cells maintained in KRB for 24 h, were changed to KRB containing 2 mM lysine for 1 h before transport was measured (last lane). Transport was measured at the same time for all samples. b, arginine transport into Fao cells which were either starved (S) or fed (F) for 24 h and shifted to MEM or KRB containing 2 mM lysine, for 1 h before the transport assay. B, effect of protein synthesis inhibitors on the trans-stimulation of y+ transport in starved cells: Fao cells which were starved of amino acids for 24 h by maintaining them in KRB media (2d lane), were subjected to the following treatments: (i) the medium was changed to KRB + 2 mM lysine (first lane); (ii) the medium was changed to KRB + 2 mM lysine in the presence of 1 mM puromycin (third lane); (iii) the medium was changed to KRB in the presence of 1 mM puromycin (fourth lane). In a parallel study, Fao cells were maintained in KRB media in the presence of 2 mM lysine for 24 h (last lane). C, time course of trans-stimulated y+ transport in amino acid-starved cells: Fao cells were starved of amino acids by maintaining them in KRB media for 1–6 h. The media was changed to KRB containing 2 mM lysine 1 h before the transport assay. In a parallel study Fao cells were maintained in MEM for 24 h and 2 mM lysine was added in the medium, 1 h before the transport assay (first lane). D, effect of protein synthesis inhibitors on the trans-zero (basal) transport activity in amino acid-starved cells. Fao cells were maintained in KRB (first lane) or KRB in the presence of puromycin (second lane) for 6 h. Values are means of four independent determinations.

2 M. Malandro and M. S. Kilberg, unpublished data.
Amino Acid Regulation of the y+ Arg/Lys Transporter

In summary, our data indicate that amino acid starvation regulates the levels of cat-1 mRNAs at a post-transcriptional level, in a manner dependent on both transcription and protein synthesis. Our data are consistent with earlier findings on amino acid-regulated genes (4, 8, 9), which indicate that de novo protein synthesis is required for starvation-dependent increases in several mRNA species (9). Most of the data accumulated thus far suggest that the amino acid signaling pathway consists of a sequence of steps before gene regulation occurs. It has been suggested that there is a regulated labile protein which is involved in the positive regulation of genes by amino acids (4). Our data support the following as one model for regulation of genes by amino acid starvation: amino acid starvation initiates the signal for the synthesis of a labile protein which in turn stabilizes the mRNAs of a group of genes which are up-regulated in response to amino acid starvation (Fig. 5).

Evaluation of Transport of Arginine in Amino Acid-deprived Cells—There are many ways by which amino acid transport activity can be modulated (34). These include alterations in the synthesis and breakdown of transporter proteins and (or) modulation of their activity. In addition to changes in amino acid transport activity due to transcriptional or translational regulation, the activity of existing transporter proteins can be changed by ion concentration, membrane potential, recruitment from an intracellular compartment, and the intracellular or extracellular concentration of amino acids (34). Arginine transport mediated by System y+ activity (Cat-1) is stimulated by the intracellular cationic amino acids, a property called trans-stimulation (35). A model of trans-stimulation predicts that a conformational change of a transporter protein occurs much more rapidly in the presence of substrate. According to this model (20, 36), the return of the carrier to the cis conformation will be slow when the substrate concentration is low at the trans side, resulting in low level of amino acid flux from the cis side. Trans-zero influx concerns an ideal situation in which the initial velocity of substrate uptake is measured in the absence of transport reactive compounds inside of the cell (35). An allosteric modification of the System y+ carrier in kidney and intestine has also been suggested in response to leucine (37).

We have evaluated the transport of L-[3H]arginine (50 μM) in Fao cells that were maintained in MEM amino acid containing media or KRB amino acid-free media, for 1–24 h (Fig. 3A, a). Transport of arginine was reduced by 46% in 1 h after amino acid starvation, and by 75% in 24 h (Fig. 3A, a). At the same time points, the cat-1 mRNA levels increased by 3-fold (Fig. 1). The initial 46% reduction in the transport activity following amino acid starvation is probably due to the loss of trans-stimulation which is present in the amino acid-fed cells (Fig. 3A, a; F24). The loss of activity (29%) between 4 and 24 h of amino acid starvation may indicate that amino acids are sequestered in an intracellular compartment and depletion is slow. Alternatively, the loss of activity may reflect a reduced Cat-1 protein synthesis. However, when 24-h amino acid-starved Fao cells were preloaded with 2 mM lysine for 1 h prior to assaying transport, arginine uptake was stimulated by 5-fold (Fig. 3A, a, last lane). This induction of arginine transport was 25% higher than the transport of arginine in either Fao-fed cells (Fig. 3A, a, compare first and last lanes), or Fao cells which were maintained in KRB media containing 2 mM lysine, for 24 h (Fig. 3B, compare first and last lanes). Additionally, trans-stimulated arginine transport in 24-h starved Fao cells was similar to trans-stimulated transport in fed Fao cells (Fig. 3A, b). For the trans-stimulated transport assay of fed cells, Fao

for expression of the regulatory protein that is induced in response to amino acid starvation (Fig. 1).
cells were maintained in MEM in which 2 mM lysine was added 1 h before the transport assay. Furthermore, the stimulation was specific for cationic amino acids. Proline, alanine, and leucine did not show any stimulation of arginine uptake (data not shown). The latter indicates that the Cat-1 protein is fully functional and still responds to trans-stimulation after 24 h of amino acid deprivation. The increase of transport upon lysine refeeding was independent of new protein synthesis, because lysine was able to trans-stimulate arginine transport (3.3-fold) even in the presence of inhibitors of protein synthesis (Fig. 3B, compare second and third lanes).

To determine the time course of arginine transport during amino acid starvation, arginine transport was trans-stimulated in Fao cells starved for 1–6 h (Fig. 3C). Transport of arginine gradually increased (1.8-fold at 2.5 h) in starved cells when compared with trans-stimulated transport of amino acid fed cells (Fig. 3C, first lane). The increase of arginine transport in starved cells paralleled the increase of cat-1 mRNA level (Fig. 1A). The trans-stimulated arginine transport was found to be higher for the first 6 h of amino acid starvation, when compared with 24 h (compare Fig. 3, A and C). The latter may indicate that either the cat-1 mRNA is not as efficiently translated during prolonged starvation, or that there is another limiting factor that influences cationic amino acid transport in amino acid-starved cells. A possible factor might be a transport facilitator, such as the neutral and basic amino acid transporter. It has recently been suggested (38) that neutral and basic amino acid transporter functions as an activator of γ-like activity in Xenopus oocytes. These data demonstrate that Cat-1 protein was synthesized during starvation and trans-stimulated transport of arginine occurred when cationic substrate became available.

To determine if protein synthesis is important for the trans-zero transport of arginine in starved cells, we measured uptake of arginine in Fao cells starved for 6 h in the presence or absence of puromycin. Arginine uptake was reduced by 47% (Fig. 3D) in the puromycin-treated cells. The cat-1 mRNA levels were reduced by 90% in the 6-h starved cells treated with puromycin, a similar result to the decrease observed after 4 h in cycloheximide (Fig. 2A). The data suggest that synthesis of the Cat-1 protein takes place in the starved cells and is required for maintenance of the basal (trans-zero) transport activity. As a control, we have also measured transport by System A in the same cells and we found the expected (27) increase following amino acid starvation (data not shown).

Finally, our hypothesis of Cat-1 protein synthesis during starvation is further supported by indirect immunofluorescent staining of 24-h fed and starved cells using a Cat-1 antibody (Fig. 4, A and B). Cat-1 protein was present in both amino acid-starved and -fed cells, without any obvious qualitative difference in the distribution or intensity of fluorescent staining (Fig. 4, A and B). The cells which were starved of amino acids for 24 h, resume growth upon stimulation with amino acids since they can be infected with ecotropic retroviruses (Fig. 4C). It is well known that ecotropic retroviruses bind the cell-surface receptors of either dividing or quiescent cells, but can only infect dividing cells (39).

We conclude that cells respond to amino acid starvation by up-regulating specific amino acid transporter genes; among these is the cationic amino acid transporter Cat-1. We propose that the molecular mechanism of this up-regulation is increased mRNA stability which is dependent on new synthesis of a trans-acting labile regulatory protein. A model of this regulation is presented in Fig. 5. Synthesis and maintenance of the amino acid transporter protein content despite amino starvation prepares the cells to transport amino acids once they become available.

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