Post-transcriptional Regulation of the Arginine Transporter Cat-1 by Amino Acid Availability*

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The regulation of the high affinity cationic amino acid transporter (Cat-1) by amino acid availability has been studied. In C6 glioma and NRK kidney cells, cat-1 mRNA levels increased 3.8-18-fold following 2 h of amino acid starvation. The transcription rate of the cat-1 gene remained unchanged during amino acid starvation, suggesting a post-transcriptional mechanism of regulation. This mechanism was investigated by expressing a cat-1 mRNA from a tetracycline-regulated promoter. The cat-1 mRNA contained 1.9 kilobase pairs (kb) of coding sequence, 4.5 kb of 3'-untranslated region, and 80 base pairs of 5'-untranslated region. The full-length (7.9 kb) mRNA increased 5-fold in amino acid-depleted cells. However, a 3.4-kb species that results from the usage of an alternative polyadenylation site was not induced, suggesting that the cat-1 mRNA was stabilized by cis-acting RNA sequences within the 3'-UTR. Transcription and protein synthesis were required for the increase in full-length cat-1 mRNA level. Because omission of amino acids from the cell culture medium leads to a substantial decrease in protein synthesis, the translation of the increased cat-1 mRNA was assessed in amino acid-depleted cells. Western blot analysis demonstrated that cat-1 mRNA and protein levels changed in parallel. The increase in protein level was significantly lower than the increase in mRNA level, supporting the conclusion that cat-1 mRNA is inefficiently translated when the supply of amino acids is limited, relative to amino acid-fed cells. Finally, y⁺-mediated transport of arginine in amino acid-fed and -starved cells paralleled Cat-1 protein levels. We conclude that the cat-1 gene is subject to adaptive regulation by amino acid availability. Amino acid depletion initiates molecular events that lead to increased cat-1 mRNA stability. This causes an increase in Cat-1 protein, and y⁺ transport once amino acids become available.

The study of the mechanisms involved in the response of mammalian cells to amino acid availability lags behind the studies in bacteria and yeast cells. In mammalian cells, decreased amino acid availability causes substantially decreased protein synthesis (1), as well as changes in mRNA stability and gene transcription (2). Inhibition of protein synthesis involves phosphorylation of the translation factor eIF-2 (3). This results in the sequestration of eIF-2B, with a decrease in the availability of eIF2-GTP-ternary complexes for binding to the 40S ribosomal subunits (4). Saccharomyces cerevisiae yeast cells have the ability to compensate for the effects of total or individual amino acid starvation by activating the transcription of the genes involved in amino acid biosynthesis, through a general control mechanism (5). The mechanism involves the enhancement of translation of the transcription factor GCN4 (6, 7), which in turn induces transcription of the amino acid biosynthetic genes (8). It has been suggested that mammalian cells, like yeast, have a general control mechanism to respond to amino acid limitations for genes involved in different aspects of amino acid metabolism (5). Specific examples of mRNAs or proteins for which synthesis is enhanced in response to amino acid deprivation include serine dehydratase (9), asparagine synthase (10), ornithine decarboxylase (11, 12), and the glutamate transporter EAAC1 (13). However, the molecular mechanism of regulation of gene expression by amino acid availability has only been studied for the asparagine synthase (AS)1 gene (9, 14, 15). The steady state level of AS mRNA has been shown to be increased by amino acid starvation (15). This increase is caused both by an increase in gene transcription (15) and an increase in mRNA stability (10).

Changes in mRNA stability influence gene expression in mammalian cells (16). The steady state level of an mRNA is a reflection both of its rate of synthesis and its rate of degradation. Amino acid depletion has been shown to increase the stability of the AS mRNA (10). We have previously shown that expression of the cationic amino acid transporter gene, cat-1, is subject to adaptive regulation by amino acid availability in cultured hepatoma cells by a post-transcriptional mechanism of regulation (17). The cat-1 mRNA level increased in cells depleted of amino acids and returned to fed levels when starved cells were shifted to amino acid-containing medium (17). Although protein synthesis is decreased during amino acid depletion, some mRNAs are preferentially translated (1). Therefore, increased cat-1 mRNA levels could result in sustained or increased protein levels in depleted cells. Moreover, the increased cat-1 mRNA will be available for protein synthesis once amino acid levels return to normal.

In this paper we investigate the mechanism by which amino acid starvation causes an accumulation of cat-1 mRNA. First,

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1 The abbreviations used are: AS, asparagine synthase; ActD, actinomycin D; ARE, A/U-rich element; bp, base pair(s); kb, kilobase pair(s); Cat-1, cationic amino acid transporter 1; Cx, cycloheximide; Dox, doxycycline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KRB, Krebs-Ringer bicarbonate buffer; tet, tetracycline; UTR, untranslated region; FBS, fetal bovine serum; dFBS, dialyzed fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; TBS-T, Tris-buffered saline with Tween 20; MOPS, 4-morpholinopropanesulfonic acid.
we show that amino acid depletion causes accumulation of cat-1 mRNA in cultured C6 glioma and NRK kidney cells in addition to the hepatoma cells we studied previously (17). Second, we show that the mechanism of mRNA accumulation does not involve regulation of transcription. Rather, increased mRNA half-life in amino acid-starved cells is responsible for the accumulation. This stabilization appears to be caused by the interaction of trans-acting factors with sequences in the 3'-UTR of the cat-1 mRNA in amino acid-depleted cells. Finally, we show that increased cat-1 mRNA stability results in increased Cat-1 protein and increased cationic amino acid transport.

The transport of cationic amino acids into most mammalian cells is mediated mainly by system y⁺ (18). Four related proteins have been identified that differ in their affinity for substrate (19–21). The genes of these proteins are expressed in a tissue-specific manner, thus regulating amino acid flux for tissue-specific nutritional needs (19). The Cat-1 transporter is the only member of the y⁺ transporter family that has been reported to be regulated by amino acid availability (17).

**Experimental Procedures**

*Materials—* All DNA modifying enzymes and nucleotides were purchased from Roche Molecular Biochemicals. [α-32P]UTP (3000 Ci/mmol) was purchased from NEN Life Science Products. DiaZyme bovine serum (dfBS) was purchased from Life Technologies, Inc. t-12,3,4,5-3H]Arginine monohydrochloride (63 Ci/mmol) was purchased from Amersham Pharmacia Biotech. All other chemicals and media were from Sigma.

**Cell Culture Cells—** C6 and NRK cells were maintained in 10% FBS-supplemented DMEM/F-12 medium. Amino acid-fed cells were cultured in dfBS-supplemented DMEM/F-12 (fed), whereas cells were amino acid-depleted by culture in dfBS-supplemented Krebs-Ringer bicarbonate (KRB) buffer (starved).

**Generation of the WCAT-1 Antibody—** Polyclonal antisera was raised against oligopeptide (LAAGQAKTPDSNLDQ), corresponding to amino acid residues 24–34 of the human cat-1 transporter (22). An antibody was generated by coupling to keyhole limpet hemocyanin. The complex (1.7 mg of peptide/mg of keyhole limpet hemocyanin) was injected intradermally into rabbits. Rabbits were boosted five times, and were bled 120 days after the first immunization. The antisera was purified on a protein A column (Sigma).

**Plasmids and Transfection of Cells—** The following plasmids were used to generate the pCat-1 and pCatR5 expression vectors: (i) pHUD10–3 containing the hCMV minimal promoter (23); (ii) pUDH12–1neoo, containing the RTA gene and the neo marker gene (23); and (iii) pMP10 (24) containing a 6.5-kb cat-1 cDNA (Fig. 1). The full-length cat-1 cDNA is 6.573 bp (24), suggesting an approximate 7.0-kb-cat-1 mRNA. However, in the present study, the size of the cat-1 mRNA is described as 7.9 kb, to be consistent with earlier reports that estimated the size based on the electrophoretic mobility of the cat-1 mRNA on agarose gels (25). To generate the pCat-1 expression vector, the XhoI/SmaI fragment containing the entire cat-1 cDNA (25) was isolated from pMP10 and ligated into the BamHI site of the pHUD10–3 plasmid by blunt-end ligation (Fig. 1). The pCatR5 expression vector was generated by cloning a 1.1-kb EcoRI fragment of the 5'-end of the cat-1 cDNA (25) into the EcoRI site of pHUD10–3 (Fig. 1). pUDH12–1neoo DNA was cotransfected with either pCat-1 or pCatR5 into C6 cells using the calcium phosphate precipitation method (26). Transfected cells were selected in G418 (0.1%), and individual clones were screened for responsiveness to Dox (5 μg/ml for at least 36 h). The clones C67-3, C67-5, and C6R5 were used in this study.

**DNA Hybridization Probes—** The following DNA fragments were used to generate the probes employed in this study: (i) cat-1, a 6.5-kb fragment of the rat cat-1 cDNA (25); (ii) 5'-cat-1, a 0.1-kb fragment within the 5'-UTR of the cat-1 cDNA; (iii) GAPDH, a 1.4-kb genomic fragment of the mouse gapdh cDNA (27); (iv) AS, a 0.9-kb fragment of the rat AS cDNA (14); (v) c-fos, a 1.0-kb fragment of the c-fos cDNA (25); (vi) c-jun, a human c-jun cDNA (25); (vii) tet, a 0.157-kb KpnI fragment from the tet promoter (23); (viii) c-myec, a 4.8-kb genomic fragment of the mouse c-myec oncogene (27); (ix) 18S, a 5.8-kb fragment containing the 18S mouse ribosomal DNA (28). Probes for Northern blot analysis were generated by random primed labeling with [α-32P]UTP in the reaction mix using a kit from Roche Molecular Biochemicals. The specific activity of the probes was 10⁸ to 10⁹ cpm/μg DNA.

**RNA Extraction and Analysis—** Methods described previously were used for RNA analysis (25). Tissue culture cells were placed into 4 ml guanidine thiocyanate, 0.5% sarcosyl, 25 mM sodium citrate, pH 7.0. The samples were immediately homogenized. The homogenate was then loaded onto a cushion of 5.7 M cesium chloride, 0.1 M sodium citrate, pH 7.0. The precipitate was then dissolved in 0.1 M guanidine thiocyanate, 0.5% sarcosyl, 25 mM sodium citrate, pH 7.0. The samples were immediately homogenized. The homogenate was then loaded onto a cushion of 5.7 M cesium chloride, 0.1 M sodium citrate, pH 7.0. 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 analyzed on a 1% agarose gel containing 0.6% formaldehyde, 0.02 μM MOPS, pH 7.2, and 0.002% sodium citrate. RNA was transferred onto GeneScreen Plus and hybridized with the appropriate DNA hybridization probes in 1.5 mM EDTA, 7% SDS, 0.5 M NaH2PO4, 0.5 M Na2HPO4, pH 7.0, at 65 °C for 24 h. Blots were washed in 0.1% SDS and 0.1× SSC (0.15 M NaCl and 0.015 M sodium citrate).

For the ribonuclease protection assay, the DNA template was generated by cloning the pUDH10–3-derived XhoI/EcoRI 450-bp fragment containing the tet promoter, into the XhoI/EcoRI sites of plBluescript KS−. The template plasmid was digested with XhoI and antisense RNA was synthesized using the Maxiscript kit (Ambion), following the protocol provided by the manufacturer (Ambion). RNase protection was performed using the RPA II kit (Ambion).

**Nuclear Run-off Assays—** Nuclei were prepared from rat hepatoma cells as described previously (17). Briefly, plates were washed three times in phosphate-buffered saline and scraped into phosphate-buffered saline. Pelleted cells (600 × g, 4 °C for 5 min) were lysed in 10 ml 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 isolated by centrifugation at 800 g, 4 °C for 5 min. The pellet was resuspended in the same buffer and centrifuged again. Nuclei were suspended in 50 mM Tris/HCl, pH 8.3, 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. Frozen nuclei (2 × 10⁶ nuclei, 200 μl) were added to 200 μl of 25% glycerol, 10 mM MgCl2, 0.2 mM KCl, 1.2 mM ATP, 0.6 mM GTP, 0.6 mM CTP. After the addition of 40 units/ml RNase inhibitor and 100 μCi

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2 M. Hatzoglou, unpublished data.
of $[^{32}]$P|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 1/10 volume of 10 mM CaCl$_2$. This mixture 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 100 mM EDTA at 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 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 filters. Blots were hybridized in 1× SSC (0.15 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. Slots containing genomic DNA were used to normalize the efficiency of the nuclear run-off reactions.

**Evaluation of Transcriptional Activity and Quantitation of cat-1 mRNA Levels by Densitometric Analysis**—Signals on Northern blots and nuclear run off experiments were quantified by using either a PhosphorImager (Molecular Dynamics) or the densitometer CS SCAN 5000 (U. S. Biochemical Corp.). The efficiency of transcription of the nuclei in amino acid-fed and amino acid-depleted cells was normalized against the signal 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 cellular gene and gave us reproducible data. The relative transcription rate was expressed as the ratio of individual cDNA autoradiographic signals over the signal of total rat genomic DNA. Different autoradiographic exposure times were used for quantitation, to ensure that the exposures were within the linear range of the x-ray film and the detection instrument.

**Amino Acid Transport Assays**—C6 and NRK cells were plated in Costar 24-well plates at a density of 0.2–0.25 × 10$^5$ cells/well and cultured for 48 h (29). trans-Stimulation experiments were performed by incubating the cells for 1 h before the assay in 10% dFBS-supplemented DMEM/F-12 or KRB, containing 2 mM lysine. For the transport study, cells were lysed after washing twice with phosphate-buffered saline (PBS) and 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton P-40, 0.25% sodium deoxycholate, 2 mM EDTA, 10 mM NaF, 1 mM sodium phosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Cell lysates were stored in aliquots for 24 h at −70 °C, after removing cellular debris by centrifugation at 1000 g for 10 min. A membrane fraction was obtained by homogenizing cells in 20 mM Tris/HC1, pH 7.4, 1 mM EDTA, 255 mM sucrose, 10 μg/ml leupeptin, and 10 μg/ml aprotinin in an all-glass homogenizer for 40 strokes. The homogenate was centrifuged at 16,000 × g for 15 min. The resulting supernatant was centrifuged at 200,000 × g for 1 h. The membrane pellet was suspended in the same buffer, aliquoted, and stored at −70 °C. The protein concentration of the samples was assayed by the Bio-Rad DC protein reagent kit. Equal amounts of protein (20 μg) were prepared in sample buffer (2% SDS, 0.02% bromphenol blue, 20% glycerol, 0.001% Tris/HC1, pH 6.8, 1% mercaptoethanol) and analyzed on a 10% SDS-polyacrylamide gel. The gel was transferred onto Immobilon-P membrane at 65 mA for 2 h at 20 °C using the Bio-Rad Trans-blot apparatus. After transfer, the membranes were blocked overnight in TBS-T (0.1% Tween 20, 20 mM Tris-buffered saline, pH 7.6) containing 5% nonfat dried milk at 4 °C. Membranes were incubated with the primary antibodies for 2 h at 25 °C and then washed three times in TBS-T. Membranes were then incubated for 1 h with the appropriate secondary antibodies conjugated with horseradish peroxidase in TBS-T containing 5% non fat dried milk. Membranes were washed three times for 5 min each in TBS-T. Cat-1 and AS were detected by the Enhanced Chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech). The membranes were exposed to Kodak XAR film. The primary antibodies used were WCAT-1 anti-cat-1 antisemur and 3G6, a monoclonal antibody against human AS (30).

![Image](https://example.com/image.png)

**FIG. 2. Effect of amino acid depletion on the concentration of the cat-1 mRNAs in C6 and NRK cells.** A and C, NRK and C6 cells, respectively, were maintained in DMEM/F-12 medium supplemented with 10% FBS until they were 70% confluent (first lane). Medium was changed to KRB (S) or DMEM/F-12 (F) supplemented with 1% dFBS. At the indicated times, RNA was isolated and Northern blot analysis was performed using probes for cat-1, AS, GAPDH, and 18S ribosomal RNA. B and D, relative amounts of cat-1/7.9 mRNA were determined in NRK and C6 cells from the autoradiograms in A and C. The level in amino acid-fed cells was arbitrarily set to 1.

**RESULTS**

Amino Acid Depletion Induces cat-1 mRNA Levels in C6 Glioma and NRK Kidney Cells—cat-1 gene expression results in accumulation of a major mRNA species of 7.9 kb and a minor one of 3.4 kb (25). The two mRNAs result from alternative polyadenylation at two sites within the 3’-UTR (25). We have previously shown that the levels of both mRNAs are increased when Fao hepatoma cells are depleted of amino acids (17). To determine whether this regulation is a general phenomenon, the effect of amino acid depletion on the accumulation of the cat-1 mRNAs in C6 glioma and NRK kidney cell lines was studied. The cat-1 gene is expressed in both cell lines grown in amino acid-containing medium, as evident from the presence of the 7.9- and 3.4-kb mRNAs (Fig. 2, A and C). In both cell lines, amino acid depletion caused an induction of both cat-1 mRNA species. In NRK cells, the level of the 7.9-kb mRNA increased 3.8-fold in 2 h, reached a peak (6.5-fold) in 6 h, and declined after 24 h (Fig. 2, A and B). In C6 cells, the 7.9-kb cat-1 mRNA levels peaked at 6 h (18-fold) and remained elevated throughout the 36-h duration of the experiment (Fig. 2, C and D). A smaller increase in the level of the 3.4-kb mRNA was observed (Fig. 2, A and C). The increase was 2-fold for C6 and 3-fold for NRK cells. As a positive control for regulation of gene expression by amino acid depletion, we measured the levels of AS mRNA (14), which is known to be induced by amino acid depletion (10, 14). As expected, the AS mRNA was increased similarly to the cat-1 mRNA (Fig. 2C). The levels of the mRNA
Amino Acid Starvation Induces cat-1 Gene Expression

The cat-1 mRNA Is Stabilized in Amino Acid-depleted Cells by Sequences in the 3' UTR—Our data suggest that amino acid depletion increases cat-1 gene expression by stabilizing the mRNA. To test this hypothesis, we studied the levels of a chimeric tetcat-1 mRNA expressed from a regulated promoter. The tetcat-1 gene was made by linking the cat-1 cDNA to a tetracycline-inducible promoter. The tetcat-1 cDNA contained 80 bp of vector sequence beginning at the transcription start site followed by 80 bp of the 5'-UTR (Fig. 1), the entire coding region, and entire 3'-UTR of the cat-1 cDNA (25). Expression of the tetcat-1 cDNA in cells will result in accumulation of two mRNAs, of approximately 7.9 and 3.4 kb. These mRNAs result from the use of alternative polyadenylation signals within the cat-1 cDNA. Both tetcat-1 mRNAs contain 80 bp of tet vector sequence at the 5'-UTR. C6 cells were transfected with the tetcat-1 plasmid, along with a plasmid that expresses the tetracycline-regulated transcriptional activator, rtTA (a fusion between the Tet repressor and the activating domain of the vp16 protein). Two stably transfected tet-responsive cell lines (C6/7-3 and C6/7-5) were chosen for further studies. The cell lines gave identical data; results from C6/7-3 are described below.

We first determined the effect of amino acid starvation on the level of the chimeric tetcat-1 mRNA in C6/7-3 cells. Cells were treated with the tetracycline analog, Dox, for 36 h or were kept in Dox-free medium. Following Dox treatment the medium was changed to either amino acid-containing or deficient medium in the presence of Dox. RNA was isolated at 1–8 h of incubation and analyzed on Northern blots, using the tet DNA probe, which is specific for the chimeric tetcat-1 mRNA (Fig. 4, top). This probe hybridizes with both the 7.9- and 3.4-kb tetcat-1 mRNAs. The concentration of both tetcat-1 mRNAs increased following treatment of amino acid-fed cells with Dox (Fig. 4, compare first and second lanes), indicating that expression of the chimeric construct showed the expected regulation by Dox. When Dox-treated cells were shifted to amino acid-depleted medium, there was a further time-dependent increase in the level of 7.9-kb tetcat-1 mRNA. Starting at 2 h of depletion, the level of this mRNA increased 5-fold over Dox-treated cells in amino acid-containing medium and remained elevated throughout the 8-h course of the experiment. The level of the 7.9-kb tetcat-1 mRNA showed a transient small increase when Dox-treated cells were fed with fresh amino acid-containing...
medium (Fig. 4, last three lanes). This transient increase was probably due to the change of medium, an effect that has been observed for other mRNAs (33). The 5-fold increase of the tetcat-1 mRNA in amino acid-depleted cells has been calculated over the transient 2-fold increase of this mRNA in amino acid-fed cells. In contrast to the induction of the 7.9-kb tetcat-1 mRNA in amino acid-depleted cells, the 3.4-kb tetcat-1 mRNA was not changed by amino acid depletion of Dox-treated cells (Fig. 4, compare lanes 2 and 9). The same blot was hybridized with a probe specific for the endogenous cat-1 gene. This probe was directed against a region of the cat-1 5′-UTR that was not contained in the tetcat-1 cDNA (Fig. 1). As shown in Fig. 4, the endogenous cat-1 mRNA was not affected by Dox treatment, but was induced 10-fold by amino acid depletion, following the same time course as the 7.9-kb tetcat-1 mRNA. Because the transcription rate of the tetcat-1 construct is not affected by amino acid depletion, we conclude that the increase in mRNA level is caused by stabilization of the message. Moreover, because the 3.4-kb tetcat-1 mRNA was not induced by amino acid depletion, we conclude that the 5′-UTR region that is present in the 7.9-kb but not in the 3.4-kb mRNA (2860–6433, Fig. 1) contains cis mRNA sequences involved in the stabilization of the cat-1 mRNA in amino acid-depleted cells.

As a further test of the hypothesis that the tetcat-1 mRNA is stabilized by amino acid depletion, we looked at the effect of amino acid depletion on the level of tetcat-1 mRNA after removal of Dox (Fig. 5A–E). In this experiment, C6/7-3 cells were treated with Dox for 36 h in amino acid-containing media. The cells were then shifted to Dox-free medium for 4 h, either in the presence or absence of amino acids (Fig. 5A). It was expected that transcription of the chimeric tetcat-1 gene would cease when cells were shifted to Dox-free medium, leading to a decrease in the tetcat-1 mRNA level. Our hypothesis was that if amino acid depletion stabilizes the tetcat-1 mRNA, the decrease in the chimeric message would be faster in amino acid-depleted than in amino acid-starved cells. The data in Fig. 5B (lane 2, bottom) support our hypothesis. Whereas the elevated tetcat-1/7.9 mRNA level seen in Dox-treated cells (Fig. 5B, lane 2, bottom) returned to base line when cells were shifted to Dox-free medium in amino acid-containing medium (Fig. 5B, lane 3, bottom), the level remained elevated when cells were shifted to Dox-free medium without amino acids (Fig. 5B, lane 4, bottom). This conclusion was verified by performing an RNase protection assay specific for the tetcat-1 RNA on the samples shown in Fig. 5B (Fig. 5D). Surprisingly, the concentration of the tetcat-1 mRNA was higher in amino acid-depleted cells shifted to Dox-free medium than in Dox-treated fed cells (Fig. 5, B and C). This could be due to the fact that removal of Dox from the medium does not turn off transcription of the tetcat-1 gene immediately. As transcription continues in amino acid-free medium, the tetcat-1 mRNA is stabilized, resulting in an increase in the mRNA level (Fig. 5C). A lag for cessation of transcription following removal of Dox is better shown in Fig. 6 (A and B). C6/7-3 amino acid-fed cells maintained in Dox-containing media for 36 h were shifted to Dox-free media for 8 h. The tetcat-1 mRNA decayed with a half-life of ~3.5 h, with a 2-h lag before the message level began to decrease (Fig. 6B). However, an accurate evaluation of the mRNA half-life could not be made using this method because the lag time varied between experiments. Fig. 5B also shows that the concentration of the endog-
enous cat-1 mRNA is not affected by the treatment of cells with Dox and it is induced during the 4 h of culture in amino acid-deficient medium (Fig. 5B, cat-1/7.9). It is noticeable that the cat-1/7.9 mRNA was induced 4-fold in Fig. 5B, whereas the induction obtained in the experiments described earlier was 10-fold (Fig. 4A). We should mention that we have experienced a variation in the level of cat-1 mRNA induction between experiments, possibly due to the quality of the dialyzed FBS.

In order to further support our conclusion that degradation of the cat-1 mRNA is slowed in amino acid-depleted cells, the half-life of the cat-1 mRNA was evaluated in either amino acid-fed or amino acid-depleted C6 cells treated with ActD. Cat-1 mRNA levels were assessed in cells treated with ActD for less than 4 h because it is well known that accurate evaluation of mRNA half-life cannot be obtained from cells treated with ActD longer periods. As shown in Fig. 7 (A and C), cat-1 mRNA decayed with a half-life of approximately 120 min in amino acid-fed cells. In order to determine the cat-1 mRNA half-life in amino acid-depleted cells, cells were depleted of amino acids for 4 h and then treated with ActD for an additional 4 h in the same medium. As shown in Fig. 7 (B and C), the level of cat-1 mRNA did not change in depleted cells during the 4 h of ActD treatment, suggesting an mRNA half-life much longer than 4 h. In order to demonstrate that the cells remained healthy during the experiment, the cat-1 mRNA level was assessed in cells incubated in amino acid-deficient medium without ActD for 8 h. As expected, the level of cat-1 mRNA in these cells was similar (1.5-fold higher) to that found in cells depleted for 4 h (Fig. 7B, compare lanes 1 and 6). In order to demonstrate the efficacy of ActD, the half-life of c-myc mRNA was measured (Fig. 7, A and B). As expected, the half-life of this mRNA was approximately 30 min in both amino acid-depleted and -fed cells (Fig. 7D). The experiments in Figs. 6 and 7 demonstrate a striking difference between the half-life of cat-1 mRNA in amino acid-fed and -depleted cells using two different methods. In amino acid-fed cells the half-life is approximately 120 min, whereas the half-life in depleted cells is much longer. We conclude that degradation of cat-1 mRNA is slowed by amino acid depletion.

The data described above support the hypothesis that transport factors in amino acid-depleted cells interact with sequences within the 3′-UTR and not within the coding region of the cat-1 mRNA, resulting in increased stability. This conclusion is drawn from the data showing that the 3.4-kb tetcat-1 mRNA is not stabilized in amino acid-depleted cells (Fig. 4A). The 3.4-kb tetcat-1 mRNA contains the entire coding region and 980 bp of 3′-UTR. To further support this finding, we analyzed the expression of a chimeric tetcat-1 gene (tetR5) that contained the tet promoter linked to 1.1 kb of the 5′-end of the coding region of the cat-1 cDNA (Fig. 1). When the experiment described in Fig. 5A was carried out on cells expressing tetR5, there was no difference in the level of the tetR5 mRNA between amino acid-fed and -depleted cells (Fig. 5E, compare last two lanes), confirming the importance of the distal end of the message in the stabilization of cat-1 mRNA in amino acid-depleted cells.

**Cat-1 Protein Synthesis Is Sustained in Amino Acid-depleted Cells**—To test whether the observed increases in cat-1 mRNA result in accumulation of the protein in amino acid-depleted cells, Cat-1 protein levels were examined. Studies on the Cat-1 protein have been hampered by the difficulties investigators met in generating anti-cat-1 antibodies. These studies use an antibody, Wcat-1, prepared against a C-terminal peptide. To test the specificity of this antibody, Western blot analysis was performed on cell extracts from the mouse fibroblast cell line KO47, which contains a homoygous knockout of the cat-1 gene (34), in parallel with extracts from C6 cells. As expected, a set of protein bands at 80 kDa were seen in C6 cells but not in the knockout cell line (Fig. 8A), probably representing different degrees of glycosylation of the Cat-1 protein as has been shown for other transporters (35). The specificity of the antibody was demonstrated by performing Western blots in the presence of the antigenic peptide, which blocked the appearance of the 80-kDa protein bands (Fig. 8D).

Western blot analysis was used to determine whether the increased cat-1 mRNA in amino acid-depleted cells leads to new Cat-1 protein synthesis. Because protein synthesis is significantly decreased in depleted cells, an increase in Cat-1 protein will indicate that the cat-1 mRNA is translated under conditions where the amino acid supply is derived from the breakdown of cellular proteins (36). In Fig. 8B it is demonstrated that membrane-associated Cat-1 protein increased by 2-fold after 3 h of amino acid deprevation, and remained elevated for the 36-h duration of the experiment. In a parallel experiment with the one presented in Fig. 8B, the level of the cat-1 mRNA was assessed in C6 cells depleted of amino acids (Fig. 2, C and D). Comparison of induction of the C6 cat-1 mRNA (18-fold, Fig 2D) and protein (2-fold, Fig. 8B) levels suggests that the cat-1 mRNA is not efficiently translated in amino acid-depleted cells when compared with amino acid-fed cells. However, the cat-1 mRNA may be translated more efficiently than other mRNAs in amino acid-depleted cells, because translation is significantly reduced under these conditions (1). The 2-fold increase in the Cat-1 protein level was observed in four independent experiments. The level of the cat-1 mRNA was induced 15–18-fold in 6 h in all four experiments (data not shown). To compare the behavior of the Cat-1 protein with a protein known to be regulated by amino acid starvation, a Western blot of total cell extracts from the same experiment described in Fig. 8B was immunoblotted with an antibody against AS (Fig. 8C). As expected (14), AS was induced in amino acid-depleted cells following the same time course as AS mRNA (Fig. 2C). This behavior is similar to that observed with Cat-1 protein, with the difference that the AS protein and mRNA are induced to similar extents. Therefore, we conclude that the induced cat-1 mRNA, like the AS mRNA, is translated in cells where the principal source of amino acids is protein catabolism.

**System y′ Arginine Transport Is Induced in Amino Acid-depleted Cells**—To determine whether the changes in cat-1 mRNA and protein induced by amino acid depletion are reflected in a change in transport activity, we evaluated the

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**Fig. 7.** A myc (Fig. 7A, compare lanes 1 and 6) is drawn from the data showing that the 3.4-kb tetcat-1 mRNA level was assessed in cells expressing AS (Fig. 8C). As expected (14), AS was induced in amino acid-depleted cells following the same time course as AS mRNA (Fig. 2C). This behavior is similar to that observed with Cat-1 protein, with the difference that the AS protein and mRNA are induced to similar extents. Therefore, we conclude that the induced cat-1 mRNA, like the AS mRNA, is translated in cells where the principal source of amino acids is protein catabolism.
Amino Acid Starvation Induces cat-1 Gene Expression

**Fig. 7.** Effect of amino acid depletion on the half-life of the cat-1/7.9-kb mRNA in C6 cells. A, Northern blot analysis of RNA isolated from C6 cells maintained in dFBS-supplemented DMEM/F-12 and incubated with ActD for 0–150 min. Northern blot analysis was performed using the cat-1/6.5, c-myc, and GAPDH hybridization probes. B, Northern blot analysis of RNA isolated from C6 cells, which were depleted of amino acids for 4 h (lane 1) and then treated with ActD for an additional 4 h in the same medium (lanes 2–5). The cat-1 mRNA level was also assessed in cells incubated in amino acid-deficient medium without ActD for 8 h (lane 6). Northern blot analysis was performed using the cat-1/6.5, c-myc, and 18 S hybridization probes. C and D, quantitation of the cat-1 (C) and c-myc (D) mRNA levels from amino acid-fed (closed symbols) and amino acid-depleted cells (open symbols) from the Northern blots in A and B.

**Fig. 8.** Regulation of the level of the Cat-1 protein in C6 cells by amino acid depletion. A, C6 and KO47 cells were maintained in DMEM/F-12 medium supplemented with 10% FBS until they were 70% confluent. Whole cell lysates (20 μg of protein each) were prepared and analyzed by immunoblotting using the WCAT-1 antibody. B and C, C6 cells were maintained in DMEM/F-12 medium supplemented with 10% FBS until they were 70% confluent (F). Medium was changed to dFBS-supplemented KRB (S), and samples were collected at the indicated times and analyzed by Western blotting as described under “Experimental Procedures.” A cell membrane fraction was used for Cat-1 protein analysis (B). Whole cell lysates were used to analyze AS protein (C). D, Western blot analysis of whole cell lysates from C6 cells incubated in dFBS-supplemented KRB and analyzed by immunoblotting using the WCAT-1 antibody (–) or the WCAT-1 antibody in the presence of 0.1 μg/ml antigenic peptide (+).

**DISCUSSION**

The sequence of molecular events leading to increased arginine transport in amino acid-deprived cells was studied here. Amino acid deprivation induced cat-1 mRNA levels by 4–18-fold in the cell lines studied (Fig. 1). The data clearly establish that this regulation occurs primarily at the post-transcriptional level, because the effect of amino acid depletion on the transcription rate is minimal. We therefore suggest that amino acid depletion increases cat-1 mRNA stability. The post-transcriptional regulation is not a general response to stress because heat shock had no effect on cat-1 mRNA levels (data not shown).

**cis-Acting** sequences of mRNAs that increase stability in response to biological and pharmacological stimuli, have mainly been found within the coding regions (37), or the 3' UTRs (38). Our studies of a chimeric tetcat-1 gene in C6 cells suggest that sequences within the 3'-UTR of the cat-1 mRNA are involved in this stabilization. Two tetcat-1 chimeric mRNAs, at 7.9 and 3.4 kb, were expressed from the tetcat-1 cDNA. These mRNAs result from the use of alternative polyadenylation signals (Fig. 1). Expression of the tetcat-1/3.4-kb mRNA was not affected by amino acid depletion, whereas depletion caused a 5-fold increase in the tetcat-1/7.9-kb. This demonstrates that only the larger mRNA contains sequences necessary for stabilization. Therefore, we suggest that the cis mRNA sequences involved in increased mRNA stability are found in the 3'-UTR sequence of the 7.9-kb mRNA, which is not present in the 3.4-kb mRNA (25). The precise cis mRNA sequences and trans-acting factors that are involved in stabilizing the cat-1 mRNA are not known. Our hypothesis is that, in amino acid-depleted cells, either the degradation of the cat-1
mRNA is inactivated or a protein factor is synthesized that stabilizes the mRNA.

Because the half-life of the cat-1 mRNA in C6 amino acid-fed cells is 120 min (25) and the 3'-UTR sequences contribute to this short half-life (25), it is possible that A/U-rich sequences (ARE) within the 3'-UTR (25) are involved in its rapid turnover. It is well known that AREs in the 3'-UTR of short-lived mRNAs facilitate their destabilization (39, 40). The 3'-UTR of the cat-1 mRNA contains three dispersed copies of the ARE sequence AUUUA and the sequence (AU)_{11} (25). It has been suggested that the decay of ARE-containing mRNAs is promoted by a family of ARE-binding proteins known as AUF1 (41). Recently, Schneider and co-workers (42) demonstrated that the ubiquitin-proteasome pathway is involved in regulating the degradation of these A/U-rich mRNAs. Rapid decay involves a complex of AUF1 with heat shock proteins, translation initiation factor eIF4G, and poly(A)-binding protein (42). Induction of hsp70 by heat shock and inactivation of the ubiquitin-proteasome pathway result in sequestration of AUF1 by hsp70 and inhibition of AU-rich mRNA decay (42). Sequestration of AUF1 could participate in the inhibition of cat-1 mRNA turnover in amino acid-depleted cells. However, inactivation of AUF1 cannot be the only regulator of cat-1 mRNA turnover in amino acid-depleted cells, because heat shock had no effect on cat-1 mRNA stability.

An alternative mechanism that could stabilize the cat-1 mRNA in amino acid-depleted cells is the synthesis of a protein factor(s) that binds to the mRNA preventing its degradation. This type of regulation has been described for regulation of mRNAs encoding transferrin receptor (43) and other proteins (44). Furthermore, it has been shown that a strong secondary structure 5' to an ARE within the 3'-UTR of short-lived mRNAs blocks rapid degradation of the corresponding mRNA (45). The 3'-UTR of the 7.9-kb but not the 3.4-kb cat-1 mRNA contains ARE elements and a stable secondary structure upstream of the (AU)_{11} sequence (data not shown). It is therefore possible that a protein synthesized in amino acid-depleted cells stabilizes the cat-1 mRNA by enforcing a stable secondary structure upstream of the (AU)_{11} sequence. This sequence is presently under investigation for its involvement in the regulation of cat-1 mRNA decay in amino acid-depleted cells.

Control of mRNA turnover has been linked to its ability to be translated. Although the mechanism of mRNA turnover is not well understood, there is convincing evidence that activation of decay requires translation of the message (16). However, increased stability of mRNAs caused by biological stimuli can be independent of translation of the corresponding message (45). In our studies we have shown that protein synthesis is required for cat-1 mRNA induction in amino acid-depleted cells. This suggests either that cat-1 mRNA stability in these cells is associated with its ability to be translated or that translation is required for the synthesis of a protein factor that stabilizes the mRNA. Our data support the possibility that stabilization of the cat-1 mRNA in amino acid-depleted cells does not depend on its ability to be translated. Because the increase in the level of the Cat-1 protein (2-fold) was lower than the increase in the level of mRNA (18-fold), we conclude that the cat-1 mRNA is inefficiently translated in amino acid-depleted cells, relative to amino acid-fed cells. Furthermore, induction of the Cat-1 protein was similar in amino acid-depleted C6 cells and C6/7-3, cells which express the tetracycline chimeric gene (data not shown). The latter suggests that the tetracycline mRNA may not be translated in amino acid-depleted cells. An inability of the tetracycline mRNA to be translated may be due to the absence of the entire 5'-UTR (Fig. 1). Therefore, it is likely that translation during amino acid depletion is required for the synthesis of a regulatory protein that increases cat-1 mRNA stability. This conclusion is supported by the finding that treatment of amino acid-depleted cells with a protein synthesis inhibitor leads to a decrease of cat-1 mRNA to fed levels (data not shown). Further support is given by the finding that levels of both tetracycline and endogenous cat-1 mRNAs did not increase during the first 2 h of amino acid depletion. The lag time of 2 h may be required for the synthesis of the regulatory protein that increases cat-1 mRNA stability. An alternative explanation is that the 2 h lag is required to generate the signal that triggers changes in gene expression in amino acid-depleted cells.

The question raised from the studies described in this paper is why cat-1 mRNA level increases in amino acid-depleted cells if the mRNA is not going to be translated into more Cat-1 protein. As is well known (1), 5'-cap-dependent protein synthesis decreases significantly in amino acid-depleted cells. Similar to the majority of cellular mRNAs, cat-1 mRNA is inefficiently translated in amino acid-depleted cells, relative to amino acid-fed cells. This suggests that the increased cat-1 mRNA level compensates for the inefficient translation and sustains the level of Cat-1 protein during the time of depletion. The fact that Cat-1 protein accumulates for as long as 36 h in amino acid-depleted cells indicates that it is either a stable protein or that continuous synthesis occurs. An alternative explanation for the induction of the Cat-1 protein in amino acid-depleted cells is that it results from translation of the cat-1/3.4-kb mRNA, whereas the cat-1/7.9-kb is not translated. If this is true, the 2-fold induction of the protein would agree with the 2–3-fold induction of the cat-1/3.4-kb mRNA. Future studies will determine the efficiency of translation of the two cat-1 mRNAs in amino acid-depleted cells.

The level of y' arginine transport in amino acid-depleted cells is induced to the same extent as Cat-1 protein. We have previously shown that system y' arginine transport is induced in trans-stimulated amino acid-depleted Fao hepatoma cells (17). We have shown in this report that system y' transport is also induced in amino acid-depleted NRK and C6 cells. We
conclude that induction of the cat-1 mRNA in amino acid-depleted cells occurs to sustain Cat-1 protein level and cationic amino acid transport both during depletion and once amino acids become available.

Levels of the mRNAs for cat-1 and AS show similar regulation (10, 15). Regulation of the AS gene by amino acid depletion has been shown to occur at the transcriptional and post-transcriptional levels (10, 15). Although the transcription rate of the AS gene in amino acid-fed and -starved cells could not be detected by nuclear run-off (Fig. 2A), studies of chimeric genes indicated that the AS promoter is subject to transcriptional regulation in amino acid-depleted cells (15). It is therefore possible that cat-1 gene transcription is regulated by amino acid depletion, even though this was not detected by our methods. In support of this possibility is the finding that a 2–3-fold increase in the level of the cat-1/3.4-kb mRNA was observed in amino acid-depleted cells (17).

The pathways by which amino acid depletion triggers changes in gene expression in mammalian cells are not known. It has been suggested that increased levels of uncharged tRNAs trigger the regulation of gene expression by either increasing transcription or mRNA stability (5). These events may involve the synthesis of new proteins or the modification of existing proteins that may act as transcription factors or mRNA stabilizing/binding proteins. In support of a signal transduction pathway linked to modulation of gene expression is the report that p70 s6 kinase is dephosphorylated in amino acid-depleted cells leading to its deactivation, probably through a negative regulation of its activity by a pathway involving suppression of tRNA aminoaacylation (46).

The molecular events that regulate gene expression in response to changes in the nutrient supply have been extensively studied in prokaryotes and lower eukaryotes (5). Mammalian cells also have mechanisms to respond to nutrient availability (5). We have shown here that part of this response involves the induction of cat-1 transporter gene expression by a mechanism that involves post-transcriptional stabilization of the mRNA. Future studies will show if other transporter proteins are also induced in response to amino acid starvation. Studies on the regulation of amino acid transporter genes by amino acid availability will increase our understanding of regulation of amino acid transport in animal and human cells during periods of limited dietary protein supply.

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