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# REGULATION OF CATIONIC AMINO ACID TRANSPORT: The Story of the CAT-1 Transporter

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■ **Abstract** The discovery of the function of the receptor for the ecotropic retrovirus as a membrane transporter for the essential amino acids lysine and arginine was a landmark finding in the field of molecular nutrition. This finding indicated that cationic amino acid transporters (CATs) act pathologically as viral receptors. The importance of this transporter was further supported by knockout mice that were not viable after birth. CAT-1 was the first amino acid transporter to be cloned; several other CATs were later characterized biochemically and molecularly. These transporters mediate the bidirectional transport of cationic amino acids, thus supporting important metabolic functions, such as synthesis of proteins, nitric oxide (NO) synthesis, polyamine biosynthesis, and interorgan amino acid flow. This review briefly describes the advances in the regulation of cationic amino acid transport, focusing on the molecular mechanisms that regulate the CAT-1 transporter. Of particular interest to this review is the regulation of CAT-1 by nutritional stresses, such as amino acid availability. The studies that are reviewed conclude that the CAT-1 gene is essential for cell survival during stress because it allows cells to resume growth as soon as amino acids become available.

## CONTENTS

## DISCOVERY OF THE CAT FAMILY

Albritton and coworkers identified the first member of the CAT family (mCAT-1, for mouse cationic amino acid transporter) as the receptor for murine ecotropic leukemia viruses (MuLV) (1). Similarities observed between the secondary structure of the MuLV receptor and L-histidine and L-arginine permeases from *Saccaromyces cerevisiae* led to the discovery of the physiological function of the virus receptor: the $Na^+$-independent transport of cationic amino acids (55, 97). Three additional related proteins, CAT-2A (also known as CAT-2a), CAT-2B (also known as CAT-2), and CAT-3, have since been identified in different mammalian species including humans; CAT-2A and CAT-2B are splice variants that differ only in a stretch of 42 amino acids (17, 18, 42, 45, 48, 52, 94). A more distantly related protein has been named CAT-4 (91). However, its expression in the plasma membrane of *Xenopus laevis* oocytes or mammalian cells is not sufficient to mediate amino acid transport, and its function remains therefore obscure (100). The CATs have been classified in the human gene family solute carrier family 7 (SLC7, members A1–4), together with the light chains (members A5–11) of heteromeric amino acid transporters (HATs) (95). While CATs are glycosylated and display 14 putative transmembrane segments, the light chains of the HATs (SLC7A5–11) have two fewer transmembrane segments, are not glycosylated, and need to associate with a glycoprotein of the SLC3 family (heavy chains 4F2*hc* or rBAT) for surface expression.

## FUNCTIONAL CHARACTERISTICS OF MEMBERS OF THE CAT FAMILY

All CAT proteins mediate $Na^+$-independent transport of cationic L-amino acids. The biochemical characteristics and the transport properties of CAT-1, CAT-2B, and CAT-3 are consistent with those originally attributed to the classical amino acid transport system $y^+$. A characteristic of the $y^+$ system is the concentration-dependent stimulation of transport by substrate at the opposite site of the

membrane. CAT-1 has the most pronounced *trans* stimulation among the CATs. In contrast, CAT-2A is a low-affinity carrier for cationic amino acids and relatively insensitive to *trans* stimulation. Apparent $K_m$ values for influx of L-arginine reported for CAT proteins expressed in *X. laevis* oocytes vary between 70–250 $\mu$M for CAT-1, 2–5 mM for CAT-2A, 38–380 $\mu$M for CAT-2B, and 40–450 $\mu$M for CAT-3 (16–18, 45, 48, 52, 55, 94, 97). $K_m$ values for L-lysine and L-ornithine are similar to those reported for L-arginine, suggesting that these three substrates are recognized with similar affinity. L-histidine is a poor substrate for CAT-1 at pH 7.4, but a good substrate at pH 5.5, when it is largely protonated (55). In contrast, CAT-3 does not recognize L-histidine even in its protonated form (94). Some other neutral amino acids have also been reported to be recognized by the CAT proteins, although with rather low affinity (48, 97). For CAT-1 and CAT-2A a voltage dependence of cationic amino acid transport has been described, with membrane hyperpolarization increasing the $V_{max}$ for influx (51, 71).



## EXPRESSION PATTERNS OF THE CAT GENES

The expression of the different CAT isoforms has been the subject of extensive reviews (15, 21, 65, 66). CAT-1 is expressed almost ubiquitously, with the exception of the adult liver. Its expression level varies considerably in different tissues and cell types and can be modulated by a variety of stimuli, including cell proliferation, growth factors, cytokines, hormones, and nutrients. This article mainly reviews reports on CAT-1 gene regulation. The two CAT-2 splice variants exhibit a quite distinct expression pattern. The low-affinity CAT-2A is most abundant in liver, and is also expressed in skeletal muscle and pancreas (65). A basal CAT-2A expression that can be further increased by cytokines has been found in cardiomyocytes, cardiac microvascular endothelial cells, and vascular smooth muscle cells (7, 87). Although a basal expression of CAT-2B has been observed in the same cell types, as well as keratinocytes and dermal endothelial cells (86), a significant expression of CAT-2B is found only after cytokine or lipopolysaccharide treatment. CAT-2B has therefore been referred to as the inducible CAT isoform. It is often induced together with the inducible isoform of nitric oxide synthase (iNOS) and is generally coexpressed with CAT-1. CAT-3 is found in mesoderm and in many developing tissues of mid streak mouse embryos (45), but seems to be confined to central neurons in adult mice and rats (9, 41, 42, 45). In humans, a strong CAT-3 expression was observed in thymus, a moderate expression in uterus, testis, mammary gland and brain, and a weak expression in ovary and stomach (94). No direct correlation between the expression of CAT-3 and the neuronal NOS could be detected. SLC7A4 (CAT-4) is expressed in brain, testis, and placenta (91).

Little information is available concerning the protein expression and subcellular localization of the CATs. CAT-1 has been reported to colocalize with caveolin in endothelial cells (68) and baby hamster kidney (BHK) cells (63), but not in human embryonic kidney cells (57). In polarized epithelial cells, expression of fusion

proteins between CAT-1 and the green fluorescent protein (GFP) is restricted to the basolateral membrane (12, 57). In contrast, when expressed in glioblastoma cells, the majority of the CAT-1-GFP fusion protein is found in intracellular vesicles (100). The importance of the CAT-1 $NH_2$ terminus in subcellular localization of the protein in different cell types was recently demonstrated. Deletion of amino acids 4 through 33 led to retention of the majority of CAT-1 in the endoplasmic reticulum in BHK cells, but not in 293 cells (75). It is therefore possible that the correct subcellular localization of CATs is essential for interorgan amino acid flow. This is particularly important for cationic amino acid transport through the placenta, where system $y^+$ is the main transporter in the microvillus membrane and $y^+L$ in the basal membrane (5, 13, 46). The use of CAT-1-GFP fusion proteins will facilitate studies on cationic amino acid transport as it relates to intrauterine fetal growth, especially in cases such as intrauterine growth retardation (IUGR) (13).



## BIOCHEMICAL AND STRUCTURAL CHARACTERISTICS OF THE CATS

So far, little structural information is available for the CAT proteins and specific amino acid residues essential for substrate recognition and translocation. All CAT proteins are glycosylated and exhibit quite similar hydrophobicity plots predicting 14 putative transmembrane domains and intracellular $NH_2$- and COOH-termini. $Glu^{107}$ has been shown to be essential for the transport activity of mouse CAT-1 (mCAT-1) (96). Located in the third transmembrane domain and conserved in all other known CAT isoforms, this Glu residue is likely to be part of the substrate translocation pathway. A region of 80 amino acids spanning the fourth intracellular and fifth extracellular loops (transmembrane domains IX and X) has been shown to determine the substrate affinity of the CAT proteins and the sensitivity to *trans* stimulation (18). This region contains the stretch of 42 amino acids that differs in the two splice variants, CAT-2A and CAT-2B. The three isoforms exhibiting similar transport properties (CAT-1, CAT-2B, and CAT-3) also show the highest percentage of amino acid sequence identity in that region. Two amino acid residues within this sequence of human CAT-2A are responsible for the low substrate affinity of human CAT-2A:$Arg^{369}$ and a missing His at position 381 (31).

## REGULATION OF EXPRESSION OF THE CAT-1 TRANSPORTER

CAT-1 is the most extensively studied CAT at the molecular level. The molecular sites of regulation of its expression involve transcriptional, posttranscriptional and posttranslational mechanisms. This review will describe the recent advances in the understanding of the mechanisms of regulation of CAT-1 gene expression.

## Gene Complexity and Regulation

cDNAs for CAT-1 have been isolated for the human (106), rat (3, 82, 101), and mouse (1). The encoded CAT-1 proteins are highly homologous, consisting of 622 amino acids in the mouse, 624 in the rat, and 629 in the human. In all three species the CAT-1 mRNAs are approximately 8 kb, which is much larger than their coding regions (1866 bp for the mouse, 1872 for the rat, and 1887 for the human). Cloning of the full-length CAT-1 mRNA from the rat (3) revealed that the 3′-UTR is 4.5 kb and the 5′-UTR is 270 nucleotides (23). One interesting feature of the 5′-UTR was an open reading frame (uORF) encoding a predicted peptide of 48 aa. The role of this uORF in translation of the CAT-1 mRNA is discussed below. The full-length UTRs from the mouse and human have not been described. However, the uORF is conserved in the mouse 5′-UTR (102). Because of the importance of long 3′-UTRs (20) and uORF-containing 5′-UTRs (69) in regulation of mRNA turnover and translational control, CAT-1 seemed a good target gene for regulation of its expression. This was further emphasized by the cloning and characterization of the promoter region for the rat CAT-1 gene (21a). The promoter is TATA-less and does not contain any obvious initiator or CAT box sequences. In contrast to other TATA-less promoters, which have multiple transcription initiation sites, a single transcription start site was mapped, which suggests that transcription from the CAT-1 promoter may require a different combination of transcription factors.

Is expression of the CAT-1 gene regulated? The presence of CAT-1 mRNA in all rat, human, and mouse tissues was suggestive of ubiquitous expression of the gene. However, the mRNA levels in the different tissues varied significantly, which suggests regulation of its expression. The factors that determine tissue-specific expression of the CAT-1 gene are not known.

What regulates expression of the CAT-1 gene? Several regulators of CAT-1 mRNA levels have been described (21). As previously stated, cytokines, growth factors, and hormones regulate the CAT-1 mRNA levels in a manner specific to cell type. The regulation of CAT-1 mRNA levels in cells that use arginine for NO synthesis has been extensively described in previous reviews (e.g., 15). However, the molecular mechanism via which expression of the CAT-1 gene is regulated by these different agents has been studied in a limited number of investigations. Furthermore, a limitation in all these studies was the lack of information on the regulation of CAT-1 protein levels by different stimulants. This limitation made difficult a direct correlation of $y^+$ transport with expression of the CAT-1 gene, especially when $y^+$ transport involves a family of transporters (15). Therefore, the physiological significance of the different $y^+$-transporters in processes such as NO synthesis, cell differentiation, and cell growth needs further refinement. We would expect in future studies to use the technology of small interfering RNA as a gene-specific inactivation mechanism to determine the participation of the different CATs in regulated cellular processes. In fact, an antisense RNA against CAT-1 was previously used to demonstrate the importance of the CAT-1 transporter in NO synthesis in the renal medulla (47).

## Adaptive Regulation of CAT-1-Mediated $y^+$ Transport

Adaptive regulation links substrate availability to amino acid transport (28). The increased transport activity in cells exposed to limited substrate is called adaptive derepression, and the decreased transport activity in the presence of abundant substrate is called adaptive repression. This adaptive regulation implies that when there is amino acid sufficiency the activity of the amino acid transporter is low (adaptive repression). However, when there is amino acid limitation the activity of the transporter is high (adaptive derepression). Physiologically, adaptive regulation of amino acid transport may serve as part of the defense mechanism of cells to limited amino acid supply. During derepression, expression of the transporters may provide the cells with the proteins required for quick influx of amino acids once they become available, and therefore may promote faster recovery. Because amino acid limitation causes growth arrest, amino acid influx is required for the cells to resume growth.

This adaptive regulation was first described for the transport of neutral amino acids by system A in 1971 (28). The transporters known as system A are responsible for $Na^+$-dependent uptake of aliphatic neutral amino acids in most cell types. Evidence on the mechanism of this adaptive regulation came in 1981 (27) and 1985 (54), when it was shown that transcription and protein synthesis were required for the adaptive response of system A amino acid transport. Gazzola and coworkers (27) proposed a model in which "the concentration of Site A–reactive amino acids affects transport activity of System A by modulating transcription of mRNA species coding for transport proteins and their putative inactivators and by regulating the efficiency of transport protein inactivation at the cell membrane" (Figure 1*A*). This model was supported further a few years later by Kilberg, who showed that inhibition of protein glycosylation by tunicamycin abolished derepression of system A (54). No transporters had been cloned at that time to allow testing of this model. In fact, the cDNAs for system A transporters were cloned recently (84, 104), and molecular studies on adaptive regulation of system A transport are expected to emerge soon.

The $y^+$ system CAT-1 was the first transporter cDNA cloned. This discovery allowed studies on the regulation of the CAT-1/$y^+$ system by substrate availability. It was shown in different cell types (4) that depletion of cells of all amino acids results in coordinate increases of CAT-1 mRNA levels and $y^+$-mediated transport (22). In contrast, amino acid supplementation of depleted cells caused a decrease in CAT-1 mRNA levels (44). In agreement with the previous observations for system A transport, induction of $y^+$ transport required transcription and protein synthesis. This first observation supported that there is adaptive regulation of cationic amino acid transport and showed that amino acid limitation causes changes in cells that result in increased synthesis of CAT-1 protein. This additional CAT-1 protein can support $y^+$ transport upon substrate availability.

An unexpected observation was that adaptive regulation of CAT-1 occurred by limitation of any single essential amino acid (21a). This was different from the adaptive response of system A transport that was linked to the substrate amino

acids (60). It is therefore expected that the mechanism of adaptive regulation for the two transport systems will be similar, but not identical.

The observation that CAT-1 mRNA levels and $y^+$ transport (unpublished data) are increased by limitation of any single essential amino acid and are not altered by limitation of a nonessential amino acid (21a) suggested that a global cellular mechanism should be activated in response to amino acid limitation, leading to CAT-1 protein synthesis. This review focuses on the discovery of this mechanism. Because this mechanism involves the coordinate regulation of the CAT-1 gene at three levels—transcription, mRNA turnover, and mRNA translation—we review them in this order.



## Transcriptional Control of the CAT-1 Gene by Amino Acid Availability

The increased CAT-1 mRNA levels during amino acid starvation could derive from increased transcription and/or increased mRNA stability. Nuclear runoff studies demonstrated that the transcription rate of the CAT-1 gene did not increase in amino acid depleted cells, leading us to study first the regulation of mRNA turnover (4). However, because transcriptional control of the CAT-1 gene by hormones was previously reported (61), and negative results using nuclear runoff studies are not conclusive, we studied the transcriptional control of the CAT-1 gene by amino acid availability.

Our studies on transcriptional control by amino acid availability were triggered by the observation that the first exon of the CAT-1 gene contained the sequence CTGATGAAAC, which has been characterized as an amino acid response element (AARE) in the promoters for asparagine synthase (AS) and the CCAAT/enhancer-binding protein C/EBP homologus protein gene (GTGATGCAAT) (11). Transcription of these two genes is induced by amino acid depletion, and the potential transcription factors and their target *cis*-DNA elements have been identified (88, 89).

Cloning of the CAT-1 gene promoter revealed that the promoter has a single transcription start site although it does not have a TATA box (21a). Several SP1 and AP2 sites that probably modulate basal promoter activity were present within the promoter region. The TATA-less nature of the CAT-1 promoter can explain the low levels of expression of the CAT-1 gene in different tissues. It can also explain tissue-specific regulation of the CAT-1 gene, given that AP2 is a transcription factor implicated in modulation of basal promoter activity (109). A similar observation was made for the CAT $y^+$LAT-1 gene promoter, which is TATA-less and has a single transcription start site and AP-2 elements (70). The organization of the promoter for the alternatively spliced CAT-2 genes is more complex. TATA-containing and GC-rich TATA-less promoters transcribe the gene in different tissues (24). Although there is preferred usage of a transcription start site in different tissues, there are no reports on regulation of the activity of the different promoters by stress (49) or the amino acid supply.

The activity of the CAT-1 gene promoter was induced by amino acid limitation in a manner dependent on the AARE element that was located within the first exon of the gene (21a). The importance of this regulation is further supported by the fact that the AARE is conserved in the mouse and human genes (unpublished information). The transcription factors that interact with the CAT-1 gene promoter and the AARE during amino acid starvation are not known. However, it is known that transcriptional regulation during amino acid starvation requires protein synthesis. The lag of two hours in induction of the CAT-1 mRNA levels and the CAT-1 promoter activity during amino acid depletion (Figure 2) suggests that an AARE-binding transcription factor(s) may be synthesized or modified by amino acid starvation. These findings are in agreement with the regulation of the AS gene by the amino acid supply (88, 89).

The common DNA elements that regulate expression of the CAT-1, AS, and C/EBP homologus protein genes by amino acid depletion suggested that amino acid starvation activates a signaling pathway that results in synthesis of new transcription factors that modulate expression of stress-response genes (32). Such signaling pathways have recently been reviewed and are discussed below (56). The kinetics of accumulation of these factors during amino acid starvation will probably determine the need of different combinations of transcription factors for regulation of specific genes. It will therefore be required in future studies on adaptive transcriptional control to take into account the timing of cellular responses to amino acid depletion.

As previously stated, the mechanism of adaptive regulation of system A amino acid transport is not known. However, it has been shown that the adaptive regulation of system A amino acid transport is associated with changes in ATA2 mRNA levels (6, 29, 60). ATA2 is the ubiquitously expressed member of the system A transporter family. Although this regulation is abolished by actinomycin D, which suggests that it requires transcription, the mechanism remains to be found.



## Regulation of CAT-1 mRNA Turnover by Amino Acid Availability via a Nucleocytoplasmic Protein

The regulation of CAT-1 mRNA turnover by amino acid availability was examined after it was observed that the two CAT-1 mRNA transcripts of 7.4 and 3.4 kb accumulated with different kinetics during amino acid starvation (4). Although both mRNAs were induced after two hours of amino acid starvation, the levels of the 3.4-kb mRNA declined before the 7.4-kb. Both mRNAs contained the first exon of the gene, which suggests that transcriptional regulation should be similar. Therefore the hypothesis was put forward that the 3′-UTR of the 7.4-kb mRNA should contribute to its increased stability. In fact, it was shown that the 7.4-kb mRNA is more stable in amino acid–depleted than in amino acid–sufficient cells (4). The challenge was to identify the cis mRNA sequences and trans-acting factors that mediate regulation of mRNA turnover. Although the 3′-UTR contained several AU-rich elements (AREs) (3), a short 11-nucleotide AUUUUAUUUUA sequence

within the distal 217 nucleotides 3′-end of the mRNA was sufficient to mediate changes in CAT-1 mRNA stability during amino acid starvation (103). This 217 nt RNA was named nutrient sensor ARE (NS-ARE).

Based on their sequences and their effect on mRNA stability, three classes of AREs have been defined (10). Class I AREs contain scattered copies of the AUUUA sequence within a U-rich region. Class II AREs contain overlapping AUUUA motifs within a U-rich region. Class III AREs are U-rich sequences, but do not contain the AUUUA motif. The NS-ARE in the CAT-1 mRNA does not fit any of the three classes. Furthermore, it is the shortest ARE described that contributes to mRNA turnover. Other genes known to be regulated by amino acid availability also contain AU-rich elements in their 3′-UTRs. However, it is not known if they contribute to regulation of mRNA stability. It will be interesting to determine if other classes of AREs can regulate mRNA stability by amino acid availability.



What trans-acting factors bind AREs? Several binding proteins have been identified (10). However, there is no sequence-specific recognition by the ARE-binding proteins. It is likely that the ARE and the flanking ARE RNA sequences form a structure that is recognized by cellular proteins, leading to mRNA degradation or stabilization. Among the cellular proteins that have been shown to bind to AREs and to increase mRNA stability is the nucleocytoplasmic protein HuR (10, 53). We have shown that the cytoplasmic concentration of HuR increases during amino acid starvation (103). This increase was associated with increased binding to the NS-ARE from the CAT-1 mRNA. We therefore suggested that the nutritional status of cells influences the subcellular localization and binding ability of RNA-binding proteins such as HuR. This influence has also been observed for other stress conditions, such as UV radiation, heat shock, and inhibition of protein synthesis, that result in increased HuR accumulation in the cytoplasm (26). The amino acid starvation-induced transient translocation of HuR may assist the transient stabilization or translation of mRNAs at the adaptive phase of amino acid depletion. It is expected that amino acid starvation will promote a cellular response, which in its first phase protects the cells until amino acids become available. However, prolonged amino acid depletion will lead the cells to apoptosis. We therefore propose that transient changes in transcription factors and RNA-binding proteins at the early phase of amino acid starvation are part of the adaptive response to stress. HuR-mediated mRNA decay has been reported for growth factor, cell cycle, and glucose transporter mRNAs (62, 99), supporting an important role in the regulation of genes involved in growth and nutrient transport. CAT-1 mRNA is a new member of the downstream HuR targets. Regulation of other transporters by HuR has not been reported.

The mechanism of HuR cytoplasmic localization during amino acid starvation is not known. The amino acid specificity of this response is also not known. It has previously been shown that the cytoplasmic accumulation was dependent on the inhibition of the AMP-activated kinase (AMPK) (98). AMPK has been shown to be activated in low fuel conditions and to phosphorylate nuclear proteins (43, 59). It is not known if the activity of AMPK is regulated by amino acid depletion. However,

it is expected that target proteins of AMPK are involved in regulating nuclear-cytoplasmic shuttling of HuR. Interesting findings are expected to emerge by linking the nutritional status of the cell with transient changes of AMPK activity, leading to regulation of expression of genes that are important for adaptation to stress.

In summary, it has been concluded that amino acid depletion increases both transcription and mRNA stability of the CAT-1 mRNA, which leads to the next question: Is the CAT-1 mRNA translated in amino acid depleted cells?

## Regulation of Translation Initiation of the CAT-1 mRNA by Amino Acid Availability via Structural Remodeling of the mRNA Leader



Amino acid starvation results in the global decrease of protein synthesis (56). We therefore asked whether CAT-1 protein levels increase in amino acid depleted cells. After a good antibody against CAT-1 was obtained, we were able to show that CAT-1 protein levels increased 50-fold as compared to 10- to 15-fold mRNA levels (22). This was the first indication that the CAT-1 mRNA is being translated during amino acid starvation. Because cap-dependent translation of mRNAs decreases during amino acid starvation, we wondered how the CAT-1 mRNA is translated in amino acid depleted cells. Let us give you the rationale that we followed in order to discover an unusual mechanism of translational control (102).

Translation of the vast majority of the mRNAs is initiated via mRNA scanning of the 5′-end (36). This involves the recognition of the 5′-end of the mRNA and its $m^7$G-cap structure, followed by binding of the ternary complex/40S ribosomal subunit complex and scanning downstream to the initiation codon. The $m^7$G-cap structure is recognized by the translation initiator factor eIF4F, which is composed of eIF4A, eIF4G, and eIF4E. The ternary complex delivers the initiator Met-$tRNA_i$ to the P-site of the ribosome located at the correct AUG codon. An alternative mechanism of translation initiation has also been described (35). *Cis* mRNA elements, known as internal ribosome entry sequences (IRESs), can direct ribosome binding to an internal initiation codon, without the need of the eIF4F complex. This mechanism was first described for translation of viral mRNAs in infected cells, when cellular cap-dependent translation is inhibited. However, some cellular mRNAs continue to be translated under conditions of reduced eIF4F activity. In fact, 200 out of 7000 cellular mRNAs remained associated with polysomes in poliovirus-infected cells. Among the gene products of these mRNAs were transcription factors, kinases, phosphatases, and protooncogenes (35).

We therefore hypothesized that translation of the CAT-1 mRNA occurs via an IRES. This IRES should be located within the 5′-UTR. To test this hypothesis, we used a bicistronic mRNA expression vector (64). In this vector the chloramphenicol acetyltransferase is translated from the first cistron by a cap-dependent scanning mechanism. The second cistron, encoding the firefly luciferase (LUC), is translated by the CAT-1/5′-UTR in the intercistronic spacer region. If the CAT-1/5′-UTR functions as an IRES, the LUC cistron will be translated and LUC activity

will be measured. Using these vectors it has been demonstrated that the CAT-1/5′-UTR has IRES activity (22). An interesting observation was that the activity of the IRES was induced during amino acid starvation with a lag of 7 hours. Furthermore, the increase was transient. It peaked at 9 hours followed by a decrease to uninduced levels at 18 hours. These findings suggested that timing is important in the expression of genes during cellular adaptation to stress.

How does the mRNA leader function as an inducible IRES? We knew that IRESs are RNA structures that bind the ribosome (35). We hypothesized that induction of IRES activity during amino acid starvation may occur via formation of an mRNA structure with efficient ribosome binding. If this was the case how did the change of structure occur? A simple explanation was that a new protein interacted with the IRES, assisting its transformation.

However, a second factor became important in this regulation. Translation of the uORF that is contained within the IRES was also found to be important for induction of the IRES activity (23). More interesting was the observation that the peptide encoded by the uORF was not important in this induction (102). Therefore, the data suggested that the active translation of the uORF was required for induction of IRES activity.

Does the unwinding of the RNA structure by the translating of the uORF ribosome cause the structural change of the mRNA leader? At this point, a simple observation provided a clue. We studied the structure of the CAT-1 mRNA leader and noticed that the RNA of the 5′-end of the uORF is involved in a strong secondary structure (102). RNA structures in solution are identified by determining the ability of the RNA to be digested by specific ribonucleases (102). A hypothesis therefore was put forward that translation of the uORF unwinds the strong RNA structure at the 5′-end of the mRNA, allowing new RNA-RNA interactions that result in formation of the new IRES.

What are the structures of the active and inactive forms of the CAT-1 mRNA leader? Furthermore, how is conversion of one to another demonstrated? To determine the conversion of the inactive RNA structure to the active structure it is necessary to identify regions of the RNA that participate in different interactions in the two structures. Using this strategy, we identified an RNA hairpin of six nucleotides to be present as a pseudoknot interaction in the inactive form and in a stem interaction in the active form (102). Translation of the uORF mediates unwinding of the pseudoknot, leading to formation of the stem. Studies are under way to determine all the structures of the induced and uninduced states of the CAT-1 mRNA leader. These studies suggested that dynamic interactions within the 5′-end of the mRNAs can modulate the efficiency of translation.

CAT-1 is the first example of nutritional control via structural remodeling of the mRNA leader. As studies on translational control by nutrients emerge we will likely discover many more genes to be regulated via this mechanism. However, we should not think that this mechanism of structural remodeling can be applied only in translational control. Rather, we should consider that any process that involves regulation via RNA structure may use this mechanism. Examples might be RNA

splicing and RNA stability, where small ORFs translated via IRESs may change a local structure within RNA molecules.

## Amino Acids as Signaling Molecules in Regulation of CAT-1 Gene Expression

Is regulation of CAT-1 gene expression specific for amino acids? The fact that limitation by any single essential amino acid induces CAT-1 gene expression supports the idea that amino acids may act as signaling molecules and the CAT-1 gene is a downstream target of the signaling pathway. In this section, we review the studies that determined the signaling pathway that is involved in induction of the CAT-1 gene expression during amino acid starvation.



Essential amino acids are nutrients that must be provided in the diet to sustain protein synthesis. However, recent studies indicate that amino acids act as signaling molecules, initiating a cascade of phosphorylation events that alter synthesis and function of translation factors, transcription factors, etc. Such signaling pathways have been reviewed recently (56, 81). Translation initiation is regulated by amino acid limitation in a positive and negative manner. Let us briefly summarize what is known about regulation of translation initiation during limited essential amino acid supply.

As described above, translation of the vast majority of eukaryotic mRNAs is initiated by scanning of the 5′-end. This mechanism involves recognition of the $m^7$G-cap structure by the translation initiator factor eIF4F, and delivery of the initiator Met-tRNA$_i$ by the eIF2·GTP·Met-tRNA$^{Met}$ ternary complex. The guanine nucleotide exchange factor, eIF2B, recycles the ternary complex for another round of initiation. Amino acid limitation inhibits both eIF4F activity and the ability of the ternary complex to recycle the Met-tRNA$^{Met}$ (56). The inactivation of recycling of the ternary complex occurs via phosphorylation of the $\alpha$ subunit of eIF2 (eIF2$\alpha$), which causes a tight association of the ternary complex with eIF2B, not allowing recycling for subsequent rounds of initiation. Inactivation of the eIF4F complex results in decreased cap-dependent protein synthesis. Phosphorylation of eIF2$\alpha$ causes global decrease of protein synthesis. However, two known mammalian mRNAs to be translated under conditions of eIF2$\alpha$ phosphorylation are the transcription factor ATF4 (32, 33) and the CAT-1 mRNAs (23).

The mechanism of the adaptive response to amino acid starvation for any single amino acid has been studied in yeast. Translation of the transcription factor *GCN4* increases during total or single amino acid starvation, causing a transcriptional induction of the amino acid biosynthetic genes (38, 39). Yeast has developed a sophisticated mechanism to increase translation of the *GCN4* mRNA when eIF2$\alpha$ is phosphorylated and levels of eIF2·GTP·Met-tRNA$^{Met}$ ternary complexes decrease (37); initiation at the *GCN4* ORF is inversely related to the concentration of ternary complexes in the cell (38). The regulatory system that mediates the response of yeast to amino acid starvation is known as a general amino acid control mechanism. This mechanism involves activation of the Gcn2p kinase by the uncharged tRNAs

that accumulate in the cell in response to limitation of essential amino acids (40). ATF4 is the only mammalian mRNA that is known to be regulated via a similar mechanism to the yeast GCN4 mRNA (32).

As described in this review, translation of the CAT-1 mRNA increased by amino acid limitation via a mechanism different from the GCN4 mRNA. However, CAT-1 mRNA translation also required phosphorylation of eIF2$\alpha$ (23). Our current hypothesis is that eIF2$\alpha$ phosphorylation modulates the synthesis of a protein that interacts with the remodeled IRES, stabilizing its structure and therefore increasing its activity. A model of this regulation has been proposed (102). Similar to the yeast system, activation of the mammalian GCN2 kinase that phosphorylates eIF2$\alpha$ (90) is required for induction of CAT-1 IRES-mediated translation during amino acid starvation (23). Taking into account the data on the transcriptional and translational control of the CAT-1 mRNA by amino acid limitation, it can therefore be proposed that the mammalian amino acid response shares features with the general "amino acid control mechanism" as described in yeast. A model of this regulation is shown in Figure 1.

Is regulation of translation of the CAT-1 mRNA limited to amino acid availability? Given the fact that amino acid limitation causes its effects via eIF2$\alpha$ phosphorylation, it would be expected that agents that cause eIF2$\alpha$ phosphorylation should also induce CAT-1 IRES activity. In agreement with this expectation, CAT-1 IRES activity was increased by endoplasmic reticulum stress, which activates the eIF2$\alpha$ protein kinase-like endoplasmic reticulum kinase and the double-stranded RNA-dependent protein kinase (23). The latter activation occurs during viral infection of cells and causes eIF2$\alpha$ phosphorylation (50). It is therefore now clear that a limitation in nutrients, such as amino acids or glucose (glucose starvation causes an endoplasmic reticulum stress response), activates signaling pathways that have as downstream targets amino acid biosynthetic genes, amino acid transporter genes, transcription factors, etc. (50, 74). The kinetics of regulation of expression of the CAT-1 gene during amino acid starvation is shown in Figure 2.

## Posttranslational Regulation of the CAT-1 Transporter

There is accumulating evidence that the transport activity of the CAT proteins can be regulated posttranslationally. Activation of protein kinase C (PKC) leads to a pronounced downregulation of the transport activity of human CAT-1 (30, 58). L-Arginine uptake in porcine pulmonary artery endothelial cells (PAEC) is inhibited upon prolonged hypoxia, concomitant with an activation of calpain and subsequent degradation of fodrin, but with no effect on the total CAT-1 protein content (107). This hypoxia-induced inhibition of L-arginine transport can be prevented by calpain inhibitors. The authors concluded that the association of fodrin with CAT-1 is necessary for the full activity of CAT-1. The same group found 25% induction of L-arginine transport in PAEC by the actin-stabilizing toxin jasplakinolide and the same 25% reduction of L-arginine transport by the actin-disrupting toxin swinholide (108), again without effect on the total CAT-1 protein content. Taken

together, these data suggest that CAT-1 activity can be modulated by interactions with cytoskeletal proteins.

Nitration of the CAT-1 protein has also been suggested as a posttranslation modification in glial cells (93). Bolanos and coworkers (93) demonstrated that the NO-derivative peroxynitrite anion may nitrate the CAT-1 protein, thereby increasing arginine efflux from the glial cells. It was suggested that this process may protect glial cells from continuing synthesis of NO and at the same time provide arginine to the neighboring neurons.

## Regulation of CAT-1 Gene Expression by Protein Kinase C



PKC potentially regulates CAT-1 gene expression at all molecular levels discussed above. PKC isozymes are a family of serine/threonine protein kinases that have tissue-specific expression and are developmentally regulated (67). PKC is involved in diverse cellular processes, such as growth, differentiation, and apoptosis. These diverse functions are associated with regulation of the subcellular localization of the PKC isoforms. It is intriguing that activation of PKC has opposing effects on system $y^+$-mediated transport in different cell types. Changes in CAT-1 mRNA levels upon PKC activation have been reported. A massive induction of CAT-1 mRNA upon phorbol-12-myristate-13-acetate (PMA) treatment has been observed in B-lymphocytes (106) and EA.hy926 endothelial cells (30). While the increased CAT-1 mRNA in EA.hy926 cells is not translated into protein, this might be different in other cell types or under different experimental conditions, particularly because translation of the CAT-1 mRNA is extensively controlled by the nutrient supply (102).

A posttranslational mechanism of regulation of CAT-1-mediated amino acid transport has also been suggested. Our preliminary experiments indicate that PKC activation decreases the activity of the three $y^+$ carriers, CAT-1, CAT-2B, and CAT-3. Activation of PKC has a pronounced downregulation of the transport activity of human CAT-1, expressed either endogenously in mammalian cells or exogenously in oocytes of *X. laevis* (30, 58). PKC-mediated downregulation of human CAT-1 is due to an internalization of the transporter (A. Rotman, D. Strand, U. Martinez, & E.I. Closs, manuscript in preparation). The reduced cell surface expression does not seem to be the result of a direct phosphorylation of CAT-1 because PKC-induced internalization and reduction in transport are also observed in a mutant CAT-1 lacking all three intracellular potential PKC-binding sites (A. Rotman, D. Strand, U. Martinez, & E.I. Closs, manuscript in preparation). The mechanism of this posttranslational control is not known. PMA treatment of cells induces system $y^+$-mediated transport in human umbilical vein endothelial cells (78) and in human Caco intestinal epithelial cells (76, 77). This occurred only after several hours of treatment and required protein synthesis. Similar induction has been observed in pulmonary artery endothelial cells several hours after treatment (58). In contrast to the latter studies, a fast PMA-mediated induction of system $y^+$ activity independent of protein synthesis has been observed in rat, rabbit, and

mouse peritoneal macrophages, suggesting activation or cell-surface recruitment of preexisting transporter protein (83). The abundance of CAT-1 in intracellular membranes in nonepithelial cells makes the latter mechanism very likely. Can all of these contradictory reports be explained? The diverse effects of PKC on system $y^+$ activity in different cell types might be explained by differences in PKC isoforms or in the complement of intermediate proteins (protein kinases and/or regulatory proteins) conveying the respective PKC effect. However, it is also likely that PMA may have short-term effects via PKC that decrease $y^+$ transport, and long-term effects that increase CAT-1 protein. Again, this may occur by regulation of the subcellular localization of the different PKC isoforms.

## THE PHYSIOLOGICAL ROLE OF CAT PROTEINS

CAT-1 seems to be the major system $y^+$ transporter in most cells (including NO-producing cells) mediating the basic supply of cationic amino acids. Studies in mice demonstrate that deletion of the CAT-1 gene is lethal (79). The homozygous knockout mice die on day one after birth, exhibit a 25% reduction in size compared to the wild-type littermates, and suffer from severe anemia, whereas the heterozygous mice exhibit no phenotypical abnormalities. The relatively normal development of most tissues in the homozygous CAT-1 knockout mice up to birth is probably due to the expression of CAT-3 during embryogenesis and fetal development (73). However, the inhibition of erythropoiesis suggests that CAT-1 may be the only transporter expressed in proerythroid cells. If this is the case, inhibition of erythropoiesis may be the result of inhibition of gene expression due to a stress response caused by the limited supply of arginine and lysine.

Surprisingly, homozygous CAT-$2^{-/-}$ mice show no apparent phenotypical abnormalities, which indicates that the expression of both CAT-2 splice variants is primarily dispensable. However, sustained NO production in peritoneal macrophages from these mice is almost abolished, underlining the important role of CAT-2B for the substrate supply of iNOS. Neither the expression of iNOS nor the intracellular L-arginine concentrations are reduced in these cells (72). The latter strongly indicates the presence of different exchangeable L-arginine pools in macrophages. This situation seems to be cell type–specific, as fibroblasts from CAT-$2^{-/-}$ mice can sustain NO production (73). Gene knockout studies have not been performed for the other CATs. The importance of a $y^+$ CAT transporter was recently demonstrated in *Drosophila*. It was shown that downregulation of a CAT transporter in the fat body caused a global growth defect similar to that seen in *Drosophila* under low nutrient supply, such as amino acid restriction (19).

Finally, it is important to assess the physiological relevance of regulation of CAT-1 by amino acid availability in vivo. There are limited molecular studies in animals. However, we would expect CAT-1-mediated amino acid transport to increase under conditions of eIF2$\alpha$ phosphorylation. In fact, it has recently been shown that eIF2$\alpha$ phosphorylation increases in the livers of young rats that were first deprived of food and then fed an imbalanced amino acid mixture (2). Our

studies in tissue culture suggest that y+-mediated transport of cationic amino acids should increase in these livers. Because such studies have not been published, we discuss previous studies on the effect of amino acid–imbalanced diets on amino acid metabolism and growth.

An amino acid–imbalanced diet (devoid of histidine or threonine) fed as a single meal to rats resulted in increased amino acid incorporation into liver protein (105). Harper and coworkers (105) stated that "it is suggested that an imbalance leads to more efficient incorporation of the growth limiting amino acid to tissues with the result that its concentration in blood plasma decreases within a few hours after ingestion of the imbalanced meal." In 1967, Harper & Rogers (34) measured the effect of an amino acid imbalance on the metabolism of the most limiting amino acid in rats. By determining the incorporation of the rate-limiting amino acid in total protein and protein-free portions of rat tissues (8), they found that incorporation of the limiting amino acid into protein synthesis increased in the livers of rats fed an imbalanced diet. They proposed that "[i]ncreased free amino acid concentrations in liver may contribute to increased incorporation of the limiting amino acid into liver proteins in animals fed the imbalanced diet." These studies support the theory that amino acid imbalance may result in essential amino acid deficiencies in specific tissues, which in turn upregulate synthesis of amino acid transporters, increase influx, and increase protein synthesis. We can therefore expect tissue-specific adaptive responses to amino acid imbalance, depending on the degree of reduction of the intracellular concentration of essential amino acids.

The effect of amino acid imbalance caused by a diet devoid of histidine has also been studied in rats maintained in a cold environment (34). Harper & Rogers in 1966 reported that "in rats kept in the cold the limiting amino acid is efficiently absorbed and utilized for protein synthesis even in the presence of a large surplus of all of the other indispensable amino acids." The utilization in diet-treated cold-stressed animals was more efficient than in the untreated cold-stressed controls. Cold stress results in decreases in protein synthesis and growth (25, 80). However, expression of a small number of proteins increases. Interestingly, it has also been shown that the 5′ leader of Rbm3, a cold stress–induced mRNA, mediates internal initiation of translation with increased efficiency under conditions of mild hypothermia (14). We are not aware of studies on phosphorylation of eIF2$\alpha$ during cold stress. However, given that CAT-1 uses protonated histidine as a substrate, one explanation for the studies of Harper & Rogers may be the adaptive response of the CAT-1 gene during cold stress. Induction of histidine influx by other transporters may also occur. Future studies will determine if cold stress induces CAT-1 gene expression.

Although it is not known if eIF2$\alpha$ phosphorylation occurs in other tissues, it is likely that imbalanced amino acid diets result in stress responses in vivo. It will be interesting to determine the stress response in different tissues and how this relates to the intracellular concentration of limiting amino acids. Part of the adaptive response of tissues to imbalanced amino acid diets is the regulation of amino acid flux at the molecular level. However, if the imbalanced diet has an excess of

an essential amino acid, this amino acid will compete for transport of other amino acids that share the same transport system. In the case of the CATs it will also increase efflux of the substrate amino acids, owing to trans stimulation. Therefore, an excess of an essential amino acid could be expected to cause competitive inhibition of transport and to decrease intracellular concentration of another amino acid that uses the same transporter. In fact, several studies in rats have shown this. Harper and coworkers reported that feeding rats a diet with increased arginine caused growth depression, decreased lysine levels in the brain, and increased arginine and ornithine levels in other tissues (92). Interestingly, growth depression was prevented by providing dietary lysine. The authors concluded, "The lysine preventable growth depressions (lysine imbalance) caused by dietary arginine or ornithine and the inverse correlations between brain lysine and plasma arginine and ornithine concentrations are consistent with the suggestion that transport mechanisms may be involved in induction of amino acid imbalances. . . . There may be an increase of the efficiency of utilization of the limiting amino acid (lysine) for protein synthesis, an observation which has been made with amino acid-limited diets." Studies at the molecular level today support these conclusions. Lysine or arginine deficiency increased synthesis of the CAT-1 transporter and increased $y^+$-mediated amino acid transport. Furthermore, protein synthesis of survival proteins occurs during amino acid limitation, suggesting that influx of the limiting amino acid may be linked to incorporation into protein. In tissue culture cells, CAT-1 adaptive regulation is observed when the lysine concentration in the media is lower than 50 $\mu$M. Therefore, we can extend Harper's conclusion that regulation of transport systems at the molecular level contributes to the depressed growth observed in diets with imbalanced amino acid.

## SIGNIFICANCE AND INTERVENTION

Transcriptional and translational control by nutrients is an emerging field. The identification and characterization of the adaptive mechanisms of cells to stress will not only uncover this important survival mechanism of the cells, but may also have a therapeutic value. It is worth considering whether patients undergoing severe medical treatments such as surgery, chemotherapy, etc., could benefit from the deliberate activation of the stress pathway. We could someday encounter "programmed adaptive stress" as a treatment for faster recovery of patients.

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report by SS Palii (*J. Biol. Chem.* 279:3463–71) showed that an intronic enhancer element regulates transcription of the neutral amino acid transporter System A gene by amino acid availability.

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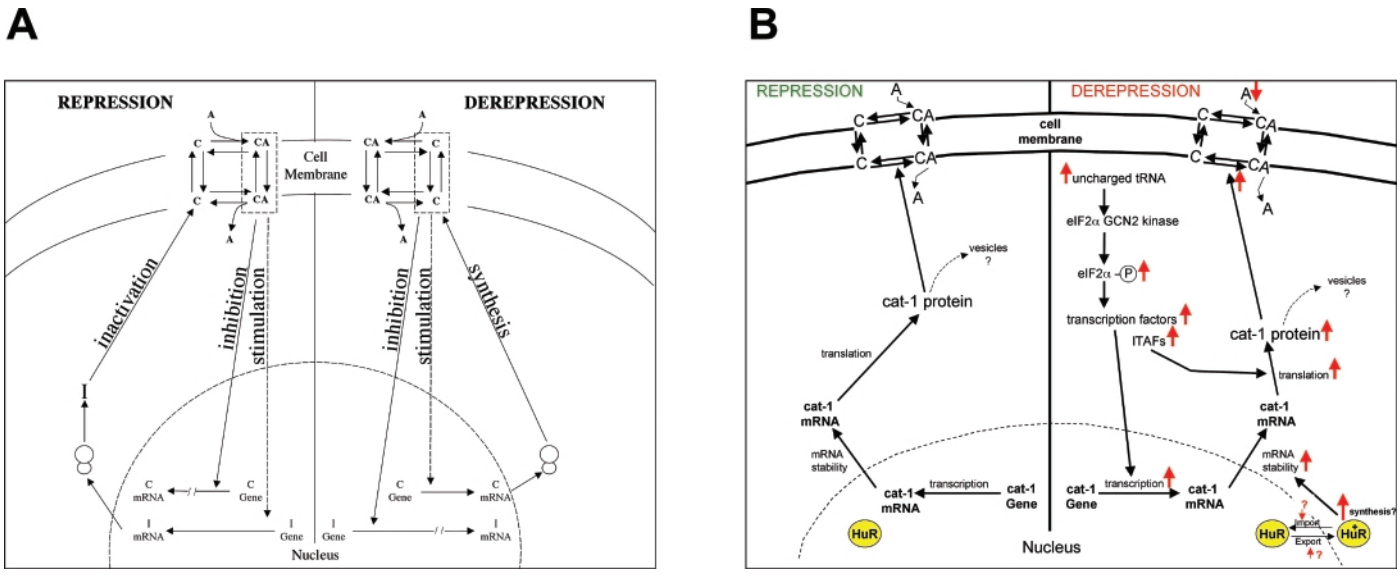


**Figure 1** Models of adaptive regulation of amino acid transport of systems A and $y^+$. The color highlights the new discoveries in the pathway of regulation of amino acid transport from the gene to amino acid flux. (*A*) Scheme for discussion of the molecular mechanism of adaptive regulation of system A amino acid transport. C is the transporter gene and I is a regulatory gene. This model was presented in the first report on the molecular mechanism of adaptive regulation (redrawn from Reference 27). (*B*) Mechanisms of CAT-1 gene regulation during amino acid starvation. Amino acid starvation causes concomitant increases in transcription (involving eIF2α signaling), mRNA stability (involving the nucleocytoplasmic protein HuR), and translation (involving an IRES). IRES, internal ribosome entry sequence; ITAF, IRES trans acting factor.

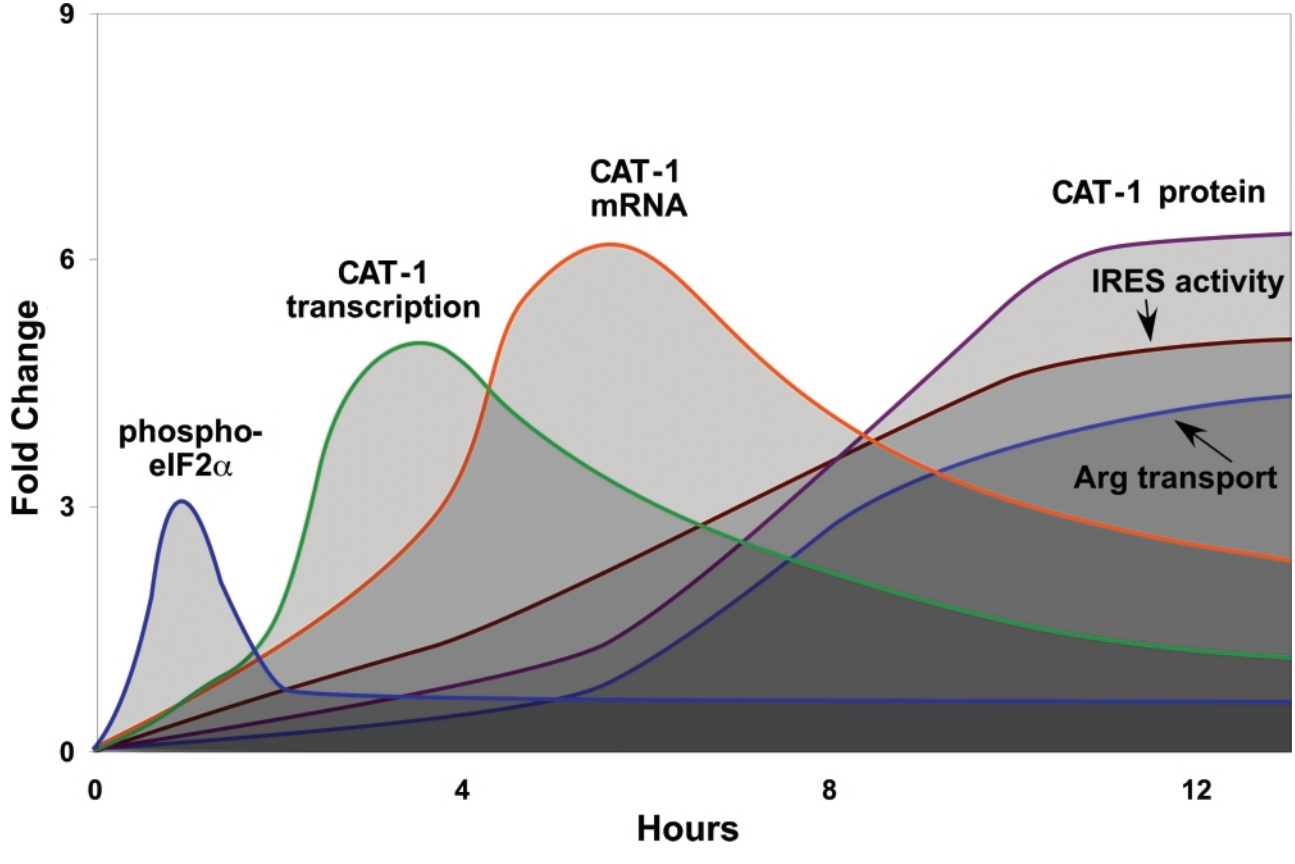


**Figure 2** Temporal expression of the CAT-1 gene during amino acid starvation. eIF2α phosphorylation, which is required for transcriptional and translational control of the CAT-1 gene, occurs after the first hour of amino acid starvation.

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# CONTENTS