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NUTRITIONAL CONTROL OF GENE EXPRESSION: How Mammalian Cells Respond to Amino Acid Limitation*

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■ **Abstract** The amino acid response (AAR) pathway in mammalian cells is designed to detect and respond to amino acid deficiency. Limiting any essential amino acid initiates this signaling cascade, which leads to increased translation of a "master regulator," activating transcription factor (ATF) 4, and ultimately, to regulation of many steps along the pathway of DNA to RNA to protein. These regulated events include chromatin remodeling, RNA splicing, nuclear RNA export, mRNA stabilization, and translational control. Proteins that are increased in their expression as targets of the AAR pathway include membrane transporters, transcription factors from the basic region/leucine zipper (bZIP) superfamily, growth factors, and metabolic enzymes. Significant progress has been achieved in understanding the molecular mechanisms by which amino acids control the synthesis and turnover of mRNA and protein. Beyond gaining additional knowledge of these important regulatory pathways, further characterization of how these processes contribute to the pathology of various disease states represents an interesting aspect of future research in molecular nutrition.

*__ABBREVIATIONS__: AAR, amino acid response (pathway); AARE, amino acid response element; ARE, AU-rich elements; ASNS, asparagine synthetase; Cdk, cyclin-dependent kinase; CdkI, cyclin-dependent kinase inhibitor; C/EBP, CCAAT-enhancer binding protein; ChIP, chromatin immunoprecipitation; CHOP, C/EBP homology protein/growth arrest and DNA damage 153; EMSA, electrophoresis mobility shift analysis; mTOR, mammalian target of rapamycin; NSRE, nutrient-sensing response element; UPR, unfolded protein response.

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DETECTION OF AMINO ACID AVAILABILITY

Mammalian cells have two recognized pathways for monitoring and responding to amino acid availability. These two pathways change the rate of protein synthesis in opposite directions, but whether they are linked in a more direct manner is not known. The mammalian target of rapamycin (mTOR) pathway functions to confirm a sufficient level of amino acids to support protein synthesis and cell growth. Through an unknown amino acid detection mechanism, amino acid sufficiency activates the mTOR kinase cascade that ultimately results in phosphorylation of the ribosome-associated S6 kinase. Activation of the S6 kinase permits a high level of translation of mRNA species that encode ribosomal proteins. In this way, protein synthesis and cell growth rates are maintained at a rate consistent with nutrient availability. How the extracellular or cellular amino acid level is detected remains unknown, as do the exact events that exist between that detection mechanism and the activation of the mTOR kinase. A number of excellent reviews on the mTOR pathway and control of translation have been published recently (35, 50, 65, 98). The second known amino acid responsive pathway is designed to detect amino acid deficiency. Limiting the availability for any single amino acid initiates this signaling cascade, which is referred to here as the amino acid response (AAR) pathway. The substrate specificity for this pathway is not well understood, but in general, if an amino acid is "essential" to the cell, an insufficient amount of that amino acid will trigger pathway activation. In this instance, the definition of an essential amino acid must be considered in the context of a particular cell type rather than the whole organism. For example, acute lymphoblastic leukemia cells express particularly low levels of asparagine synthetase, and therefore, asparagine is an essential amino acid for this specific cell type. In fact, this cell-specific need for extracellular asparagine is the basis for the use of asparaginase as a chemotherapeutic drug for childhood acute lymphoblastic leukemia (7), and as expected, the drug-induced asparagine deprivation triggers the AAR pathway in MOLT4 leukemia cells (8).

Limiting the extracellular supply of an essential amino acid or blocking the synthesis of an otherwise nonessential one results in an increase in uncharged tRNA that binds to and activates the general control nonderepressible protein 2 (GCN2) kinase (13, 110, 130). In this manner, the GCN2 protein serves as a sensor of amino acid insufficiency. It is interesting that suppressed activity for any one of the tRNA synthetases can also trigger the AAR pathway, despite a normal cytoplasmic level of that amino acid (6). Once activated, GCN2 kinase phosphorylates the translation initiation factor eIF-2α, which leads to a decrease in global protein synthesis (64). However, selected mRNAs exhibit increased translation under these circumstances, as first demonstrated for the yeast transcription factor GCN4 (52), and more recently shown for the mammalian GCN4 counterpart, activating transcription factor 4 (ATF4) (46, 103). This translational control is modulated by short upstream open reading frames in the GCN4 (52) and ATF4 (77, 115) mRNA, which permit translation of the transcription factor primarily when translation initiation has been slowed in response to eIF-2α phosphorylation. GCN4 has been called a "master regulator" of nutrient sensing in yeast, as its elevated production following amino acid deprivation results in a change in the transcriptional rate for literally hundreds of genes (85). Although the entire mammalian genome has yet to be screened for responsiveness to ATF4, it also appears to serve as an important regulator of cell stress based on an initial array analysis (47). In support of this interpretation, genomic analysis of specific genes for which the amino acid response elements have been identified has demonstrated that ATF4 plays a key role in the transcriptional control of these genes (see below).

AMINO ACID REGULATION OF RNA SYNTHESIS AND METABOLISM

Amino acid deprivation has been demonstrated to regulate gene expression at a number of steps along the pathway of DNA to RNA to protein (Figure 1). As mentioned above, amino acid–dependent control of protein synthesis has been reviewed elsewhere. Therefore, the following is a summary of the influence that amino acid availability has at steps from DNA to RNA, as well as mRNA turnover. The published observations are described briefly to illustrate the breadth of mechanisms that cells use to regulate gene expression in response to amino acid limitation. Examples from yeast are given for those steps not yet investigated in mammalian cells.

Chromatin Structure and Transcription Factor Recruitment

Eukaryotic DNA is packaged with core histone proteins to form chromatin, with the nucleosome serving as the basic structural repeating unit. In addition, the chromatin fiber contains a large number of other proteins, such as linker histones and nonhistone proteins, to stabilize the folded nucleosomal arrays and to perform specialized functions within tightly bundled chromatin domains. Transcriptional activation of a particular gene is thought to require a transition from a compacted

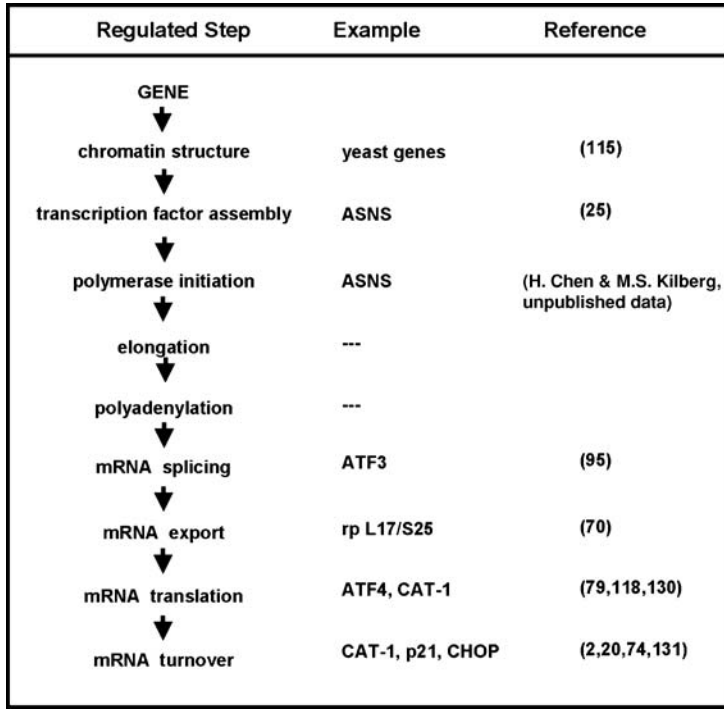

Figure 1 Amino acid limitation regulates multiple steps of gene expression.

chromatin structure to a more extended DNA fiber. Recently, research has shown that the initial steps of transcriptional activation involve chromatin remodeling and histone modification, to provide a more accessible DNA for the recruitment of the general transcription machinery (84, 109). Unfortunately, there are not yet published detailed studies of chromatin remodeling associated with amino acid–responsive genes in mammalian cells.

Transcription Initiation

The transcription initiation site of a gene is established, in part, by core promoter elements that direct the assembly of the general transcription factors and RNA polymerase II to form the preinitiation complex (70, 84, 109). There are many reports showing the existence of multiple transcription initiation sites for a particular gene, but few examples of nutrient-regulated initiation. One example exists in the methylotropic yeast *Hansenula polymorpha*. In this organism, the methanol oxidase gene MOX is transcribed from two different transcription start sites under repressed and derepressed conditions. A shift from glucose-containing medium to glycerol or methanol medium causes repression of the longer transcript and

a strong activation from the initiation site that produces the shorter mRNA (39). The shorter mRNA is then efficiently translated into the functional MOX protein, leading to speculation that this shift of the transcription start site represents a regulatory mechanism to synthesize enough peroxisomal catalase for the removal of hydrogen peroxide.

During the process of determining the transcription start site for the human asparagine synthetase (ASNS) gene, it was discovered that for cells in amino acid–complete medium, transcription initiates at multiple sites within the promoter region (nucleotides –68 to –18), but only about 25% of these transcripts initiate at the nucleotide historically designated as the +1 site (H. Chen & M.S. Kilberg, unpublished results). Interestingly, induction of the gene by amino acid deprivation triggers the transcriptional machinery to strongly favor a single initiation site such that greater than 70% of the transcripts start at the +1 nucleotide. Consistent with these results, chromatin immunoprecipitation (ChIP) analysis of the ASNS promoter for the general transcription factors TBP and TFIIB showed that binding for both was enhanced following amino acid deprivation (24). TFIIB is thought to interact with TBP (66), and, relevant to the observations showing transcription start site alignment of ASNS after amino acid limitation, TFIIB has been proposed to play a significant role in determining the transcription start site (29). This preferential alignment of the ASNS initiation site coincides with a substantial increase in the overall transcription rate. Perhaps this higher rate of transcription is the result of the change in initiation sites, but given that the corresponding mRNA molecules will differ in the length of their 5′-UTR, it is also possible that this alignment of the ASNS transcription start site yields a translational advantage or a change in mRNA stability.

RNA Splicing

In higher eukaryotes, mRNA splicing provides a unique mechanism for expansion of the genome by assembling different mRNA units from a single gene, and thereby yielding a protein repertoire that exceeds the number of genes. Up to 60% of human genes are subject to alternative splicing (82), and many genes have multiple splicing patterns (41).

ATF3 is an ATF/cyclic-AMP-response element-binding protein (CREB) family member of the basic region/leucine zipper (bZip) transcription factor superfamily. ATF3 controls a wide variety of cellular activities and its expression can be induced in response to both intracellular and extracellular stress signals, including nutrient availability (44), as described in more detail in the "Transcription Factors" section below. The ATF3 gene is also known to produce an extensive set of proteins through alternative splicing. Whereas homodimers of the full-length ATF3 function to repress transcription, several truncated ATF3 isoforms, ATF3ΔZip (23), ATF3ΔZip2a and ΔZip2b (48), and ATF-3b (119) stimulate transcription of a reporter gene. ATF3ΔZip was identified in HeLa cells stimulated by serum (23), and ATF3ΔZip2a and 2b were induced in primary human umbilical vein endothelial cells after treatment by several stress-associated stimuli (48). They lack a functional

leucine zipper domain and therefore cannot form the mandatory dimers to bind to DNA, but they are believed to retain corepressor binding activity. Consequently, it has been proposed that the ATF3ΔZip isoforms cause transcriptional activation indirectly by sequestering corepressor proteins, a proposal supported by the observation that ATF3ΔZip can even activate promoters that lack an ATF3 binding site (23). The expression of two novel ATF3 isoforms (Figure 2), ATF3ΔZip2c and ATF3ΔZip3, was detected in HepG2 human hepatoma cells following histidine limitation (91). Polymerase chain reaction (PCR) analysis was used to confirm that these two new isoforms are also expressed in amino acid–deprived normal human hepatocytes. Whereas ATF3ΔZip2c had no regulatory effect on amino acid–responsive ASNS expression, ATF3ΔZip3 stimulated transcription from the ASNS promoter in amino acid–fed cells and further enhanced ATF4-dependent and amino acid starvation-dependent induction of ASNS-driven transcription (91). How amino acids control the mRNA splicing machinery is unknown, but future investigation of splicing regulation by amino acids and other nutrients may yield valuable information to show how nutrition can expand the proteome.

Nuclear Export of mRNA

Following amino acid deprivation, enhanced transcription increases the mRNA content for the mammalian ribosomal proteins L17 and S25 up to fivefold, but there is no concurrent increase in protein content (68). Cell fractionation demonstrated a nuclear retention of the increased mRNA, which suggests that amino acid deprivation blocked mRNA processing and/or transport to the cytoplasm. However, a block in L17 or S25 mRNA splicing or polyadenylation did not appear to be involved because a single L17 or S25 mRNA species of the mature size was detected in cells cultured in the presence or absence of amino acids. The nuclear retention of the induced L17 and S25 mRNAs was reversible, in that amino acid refeeding of the starved cells resulted in a rapid translocation of the previously synthesized L17 or S25 mRNA molecules to the cytoplasm and subsequent association with the polysomes (68). This transfer after refeeding occurred in the presence of actinomycin D, demonstrating that the polysome-associated mRNA was truly a release of the nuclear store of mRNA and not the result of de novo RNA synthesis. Adilakshmi & Laine (3) demonstrated that p53 binds to the S25 mRNA in the nucleus and may be associated with the nuclear retention process. Further investigation into how this nuclear retention is controlled should provide mechanistic insight into an interesting and novel cellular process regulated by amino acids.

Stabilization of mRNA

The regulated degradation of many mRNA molecules is dependent on adenosine-uridine (AU)-rich elements (AREs) present within their 3′ untranslated regions. AREs, originally identified in cytokine genes (22), function as mRNA stability elements. A number of proteins, including HuR, TTP, BRF1, KSRP, AUF1/hnRNP

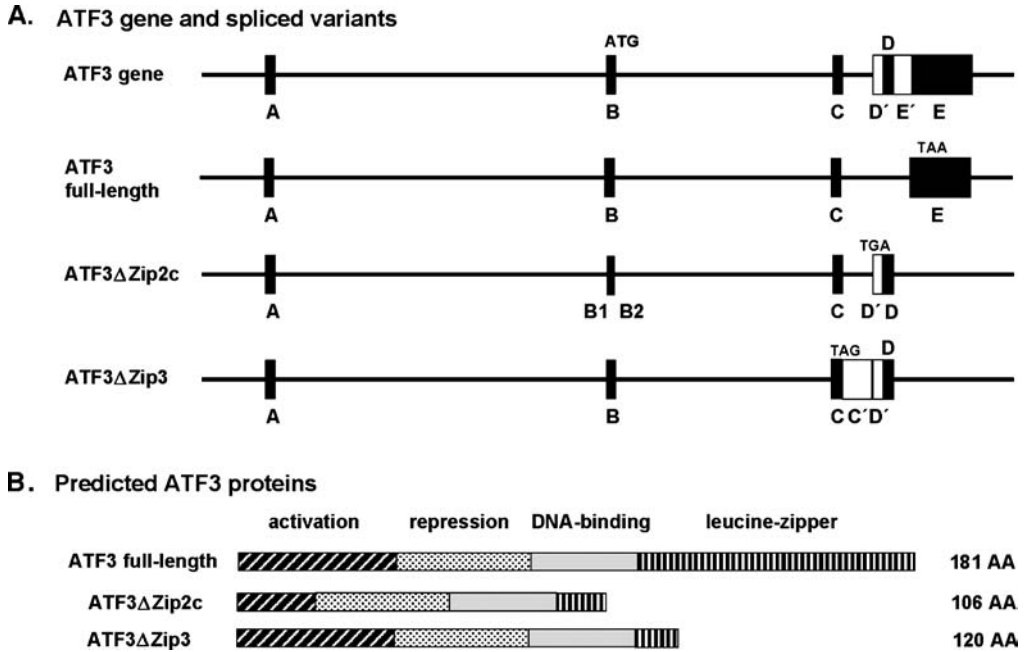

Figure 2 Amino acid–dependent alternative splicing of the ATF3 gene. Following amino acid deprivation of HepG2 hepatoma cells, synthesis of alternative mRNA species is observed (23). The ATF3 gene structure and the use of exons A–E in each of the ATF3 mRNA variants is shown. The translation start site (ATG) is the same for all three proteins, but the translation stop codon varies (TAA, TGA, or TAG) in its location and results in truncation of the leucine zipper for ATF3ΔZip2c and ATF3ΔZip3. ATF3ΔZip2c also lacks some of the activation domain as the result of a splice site within exon B.

D, and other members of the hnRNP family, interact with AREs and affect the stability of ARE-containing mRNA. All of the above-mentioned proteins except HuR function as destabilizers of the ARE-containing mRNA to which they bind (76). It has been shown that HuR and AUF1 can bind to common target mRNA molecules in a competitive fashion, but AUF1-bound mRNA undergoes exosome-mediated decay, whereas HuR-bound mRNA is stabilized (69). HuR is a member of the human embryonic lethal abnormal vision family of RNA-binding proteins, ubiquitously expressed in all cell types. In addition to stabilization of ARE-containing mRNA molecules, HuR also plays a role in the nuclear export of mRNA and in the control of translation (16).

Yaman et al. (128) demonstrated by in vitro RNA gel shift experiments that HuR could bind to an ARE within the 3′ UTR sequence of the CAT-1 cationic amino acid transporter mRNA. They were also the first to report that amino acid limitation causes increased HuR accumulation in the cytoplasm. Consistent with that observation, cytoplasmic HuR binding to the CAT-1 ARE was increased during amino acid deprivation, resulting in stabilization of the mRNA. Like CAT-1, the 3′ region of the ATF3 mRNA contains several AUUUA sequences. Immunoprecipitation of HuR-RNA complexes followed by real time PCR analysis showed that HuR also interacts with ATF3 mRNA in vivo and that the interaction increases following amino acid limitation of HepG2 hepatoma cells (Y.-X. Pan, H. Chen, & M.S. Kilberg, manuscript submitted). As originally shown by Yaman et al. (128) for C6 glioma cells, after amino acid limitation the total amount of HuR was increased in the HepG2 cells and it was translocated from the nucleus to the cytoplasm. Importantly, these changes in cytoplasmic accumulation of HuR paralleled the kinetics of ATF3 mRNA accumulation in vivo. Furthermore, suppression of HuR protein content using RNAi partially blocked the increase in ATF3 mRNA in histidine-deprived HepG2 cells.

Leung-Pineda et al. (72) have shown that the cyclin-dependent kinase inhibitors (CdkIs) p21 and p27 also represent an example of mRNA stabilization in response to amino acid deprivation. The progression of the cell cycle from one phase to the next is tightly controlled by three classes of proteins: cyclins, cyclin-dependent kinases (Cdks), and CdkIs. Cyclins and Cdks act as positive regulators by forming complexes with each other to cause phosphorylation of specific cell cycle–promoting substrates and thus advancement into the next phase. Individual CdkIs function as negative regulators of the cell cycle by inhibiting the action of specific cyclin-Cdk complexes.

A long-standing model of how amino acid limitation may block cell cycle progression is based on the observation that amino acid deprivation has the same effect as protein synthesis inhibition and serum starvation (83). All three treatments can lead to cell cycle arrest in G0, a resting state that the cell can enter from the G1 phase. This transition is thought to occur because of a general decrease in global protein synthesis, which leads to a decreased rate of cyclin D synthesis (105). However, more recent studies demonstrate that amino acids can act as signaling molecules, and this research has led to the hypothesis that amino acid limitation

controls cell cycle progression by specifically modulating the gene expression of individual regulators of the cell cycle, independent of global effects on protein synthesis. For example, Leung-Pineda et al. (72) observed that limitation for the essential amino acid histidine leads to increased mRNA and protein expression for the CdkIs p21 and p27 in HepG2 human hepatoma cells (72). This increase in p21 and p27 expression occurs in the presence of actinomycin D, and extensive promoter analysis provided no evidence for transcriptional control of the p21 gene (72). Additional experiments showed that the increase in p21 and p27 mRNA was dependent on mRNA stabilization rather than transcription. Furthermore, as described above for ATF3, siRNA-induced blockade of HuR protein expression following amino acid deprivation prevented the increase in p21 mRNA (Y.-X. Pan & M.S. Kilberg, unpublished observations). These observations not only provide an example of amino acid–regulated mRNA stabilization, but also are consistent with the proposal by Leung-Pineda et al. (72) that through the increased expression of the CdkIs p21 and p27, cells have more specific mechanisms for amino acid–dependent control of the cell cycle than relying on a global decline in protein synthesis with its subsequent pleiotropic effects. Collectively, the CAT-1, ATF3, and p21/p27 data illustrate the critical role that mRNA destabilization and stabilization plays in the amino acid response, and it is likely that many other examples will be discovered.

TARGET GENES FOR AMINO ACID LIMITATION

Array analysis of amino acid–deprived yeast has illustrated that the spectrum of GCN4-activated genes goes well beyond amino acid transport and biosynthesis (85). Although there is not yet an array covering an entire mammalian genome, partial screening has identified a large number of ATF4 target genes (47) and similarities to yeast counterparts are clear. In addition to those described in more detail in this section, other amino acid–responsive genes include transcription factors such as CCAAT-enhancer binding protein-α (C/EBPα) (79), as well as c-jun, junB, c-myc, and c-fos (96). As mentioned above, the mRNA content for the ribosomal proteins L17 and S25 is increased (67, 68, 104), as is expression of the ER chaperone protein calreticulin (51). Cytokines and growth factors that have been confirmed to be amino acid responsive include interleukin-8 (IL-8) (14) and glucagon (93). This list is increasing steadily and it is expected to get much longer. More important than the number of genes induced by amino acid limitation, the breadth of their function illustrates the organism's need to globally modulate its gene expression profile to adapt to a changing nutritional environment. A few examples of amino acid–regulated genes are described below in more detail to illustrate the molecular mechanisms by which gene expression is controlled.

Transporter Proteins

A number of plasma membrane amino acid transport activities have been demonstrated to be responsive to amino acid availability. The two most extensively studied

include neutral amino acid transport System A, mediated by the SNAT2 gene product, and cationic amino acid transport, mediated by the CAT-1 transporter. These two examples are discussed in greater detail below, but other examples include one or more members of the excitatory amino acid transporter (EAAT) family, which mediate the uptake of the anionic amino acids glutamate and aspartate. McGivan and colleagues (86, 94) have demonstrated that the transport of glutamate across the cell membrane of the kidney cell line NBL-1 increased following amino acid limitation. Glutamate transport in this cell line is thought to be mediated by the EAAT3 transporter (86, 94), but the EAAT3 protein content within the starved NBL-1 cells was not increased, a finding that suggests the increased glutamate transport activity was the result of a posttranslational mechanism on EAAT3 or that regulatory proteins or unidentified transporters contributed to this activity. Interestingly, the EAAT transporters have interacting proteins referred to as glutamate transporter-associated proteins (GTRAPs) that can modulate transport activity. For example, GTRAP3-18 binds to the C-terminal of EAAT3 and inhibits transport activity (21). Such regulatory proteins associated with specific transporters represent a relatively new area of investigation and should provide important new insight into transporter control.

Another example of amino acid–regulated transport has been demonstrated for System L transport activity mediated by the protein product LAT1, which exists as a heterodimer with the plasma membrane glycoprotein 4F2 heavy chain (116). Although not a substrate for the neutral amino acid transporter LAT1, removing arginine from the culture medium caused an activation of the transporter activity (87). It remains to be established whether this effect by arginine represents a regulatory mechanism associated with arginine itself or an indirect result of changing nitric oxide levels within the cell.

SNAT2 NEUTRAL AMINO ACID TRANSPORTER System A transport activity mediates the sodium-dependent, secondary active transport of neutral amino acids in all nucleated mammalian cells. Regulation of transport activity has been investigated extensively and is increased by a broad spectrum of hormones and growth factors as well as substrate availability and cellular growth rate (15, 62, 81, 89). Hepatic transport of alanine, in part mediated by System A activity, can be a rate-determining step for further alanine metabolism (27, 28), and thus it is hypothesized that glucagon-induced alanine delivery via System A contributes to the aberrant synthesis of glucose in diabetes (106). In addition to hormone signals, System A transport activity is increased by amino acid limitation. This activation occurs when mammalian cells are deprived of even a single amino acid that is essential for that particular cell; the amino acid may or may not be a substrate for the transporter (45). Recently, three distinct genes have been identified that encode System A transport activity, and a unified nomenclature has been proposed for these genes by Mackenzie & Erickson (78) using the term SNAT (systems N and A transporters). The genes representing System A transport activity are SNAT1 (formerly ATA1, SAT1, SA2, GlnT, NAT2), SNAT2 (formerly ATA2, SAT2, SA1), and SNAT4 (formerly ATA3, NAT3). Expression of these

genes is tissue specific and the functional properties are somewhat different between the three protein products. SNAT2 mRNA content is increased following amino acid deprivation (10, 38, 74), and Hyde et al. (55) have shown that there is an increase in plasma membrane SNAT2 protein following amino acid limitation of skeletal muscle cells or 3T3-L1 mouse adipocytes. The induction of System A activity (36), as well as the increase in SNAT2 mRNA (75), is largely prevented by the MAP kinase pathway inhibitor PD98059. Lopez-Fontanals et al. (75) used this sensitivity to show that the SNAT2 induction by amino acid limitation was mediated by the MAP kinase pathway, whereas transporter activation by hypertonicity was not. Two important questions about these observations remain unresolved. How is the MAP kinase pathway activated by amino acid limitation? What is the mechanism by which the kinase pathway increases SNAT2 mRNA expression?

Palii et al. (90) have established that the human, chimpanzee, mouse, and rat SNAT2 gene contains an amino acid response element (AARE) within the first intron. For the human gene, the AARE sequence is located at nucleotides +712 to +724 (Figure 3). The core sequence of the SNAT2 AARE (5′-TGATGCAAT-3′) is identical to that present in the promoter region of the human C/EBP homology protein (CHOP) gene (90). A unique feature of the SNAT2 AARE is the presence of a nearby CAAT box (Figure 3) that is not absolutely necessary for the amino acid response, but when it is mutated the response is blunted to about 60% of the maximum (90). The AARE functions as an enhancer element in that it is position and orientation independent and can convey amino acid responsiveness to a heterologous promoter. The SNAT2 intronic region near the AARE also contains a purine-rich sequence (PuR box) that contributes to maintenance of the gene's low rate of basal transcription but does not appear to play a direct role in the amino acid response (90). As shown in Figure 3, all three of these elements are completely conserved in sequence and relative intronic location across the human, chimpanzee, mouse, and rat species.

CAT-1 CATIONIC AMINO ACID TRANSPORTER The CAT-1 gene product mediates the sodium-independent transport of arginine, lysine, and, to a lesser extent, histidine and ornithine. Prior to cloning of the cDNA and gene for CAT-1, CAT-2, and CAT-3, the collective transport activity for cationic amino acids was referred to as System Ly^+ (122). Characterization of Ly^+ activity and its regulation has been described over the last three decades, and two recent reviews summarize the current knowledge regarding the transport activity and the CAT family of transporter genes (49, 116). Interestingly, the CAT-1 transporter serves as the receptor for the murine ecotropic retrovirus, and it was on the basis of this receptor function that the original mouse cDNA was cloned (63, 118). The CAT-1 transporter protein in other species does not function as a retroviral receptor because of specific amino acid differences (5), but stable expression of the murine CAT-1 protein has been used to render human cells susceptible to the murine ecotropic retrovirus (7). While investigating the viral receptor properties of the rat CAT-1 protein, Hatzoglou and colleagues determined that expression was regulated by amino acid

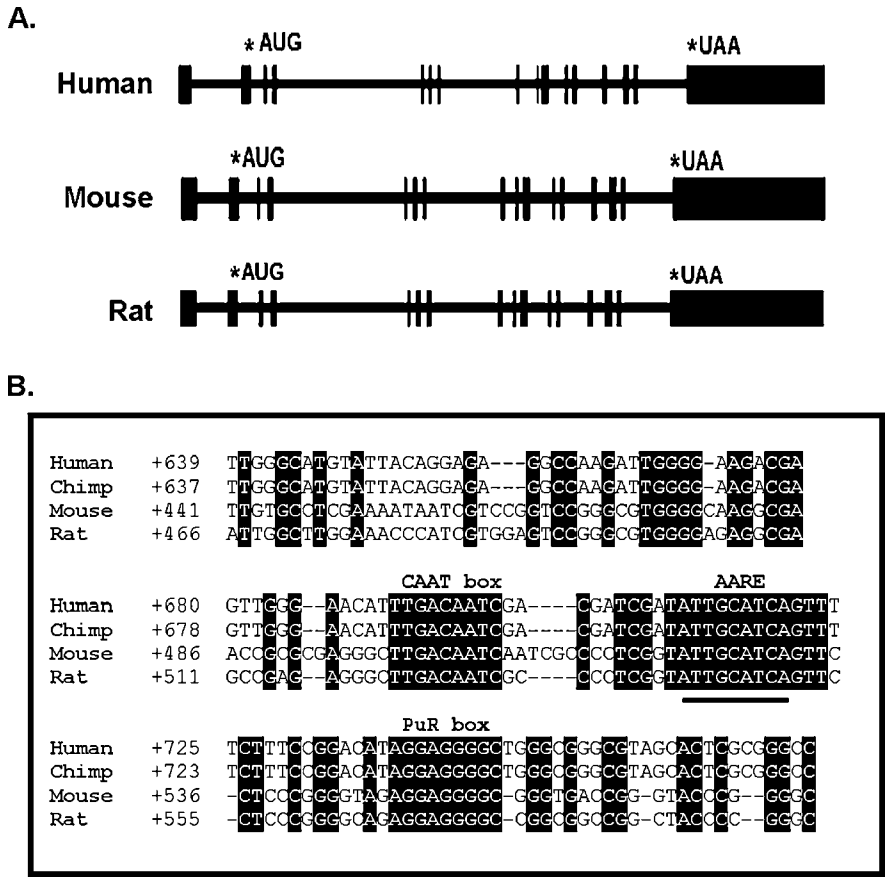

Figure 3 SNAT2 gene structure and the conserved sequences within intron 1. *Panel A* depicts the structure of the 16 exons and 15 introns within the human, chimpanzee, mouse, and rat SNAT2 genes (90). The translation start (AUG) and stop (UAA) sites are also shown. *Panel B* shows the conservation of sequence within intron 1 that contains the amino acid response element (AARE), the CAAT box, and the purine-rich element (PuR). The core sequence of the AARE that is identical to that in the C/EBP homology protein (CHOP) promoter is underlined. The numbers are the nucleotide for that particular species relative to the predicted transcription start site as +1.

availability (54). Their studies showed that removal of a single essential amino acid or all amino acids induced an increase in CAT-1 mRNA. More recently, it has been documented that expression of CAT-1 protein is increased by amino acid limitation through both transcriptional and posttranscriptional mechanisms (49). The transcriptional activation is mediated by an AARE identified within the first exon of the genomic sequence (nucleotides +45 to +53) that is identical in sequence to the

nutrient-sensing response element-1 (NSRE-1) nutrient control site of the ASNS gene (125). These two genes differ in that the combination of NSRE-1/NSRE-2 sites in the ASNS gene responds to both the AAR and the unfolded protein response (UPR) stress response pathways (discussed below), whereas the CAT-1 gene lacks an NSRE-2 sequence, and control through the AARE is limited to amino acids (31). Point mutation of the CAT-1 AARE sequence abolished the amino acid–induced transcription, although the corresponding transcription factors that mediate this induction have yet to be identified.

As described above, using CAT-1 as a model, Hatzoglou and coworkers were the first to demonstrate that regulatory proteins associated with mRNA stabilization are modulated by amino acid availability (128). Those authors documented that after amino acid limitation, nuclear HuR protein was translocated to the cytoplasm and the interaction between CAT-1 and HuR protein was increased. A third form of regulation impacting CAT-1 expression arises from the presence of an internal ribosome entry sequence (IRES) in the CAT-1 mRNA, such that m^7G-cap independent translation initiation occurs, and this internal initiation is increased following amino acid deprivation (33, 34). Translation of a short uORF in the 5′ end of the CAT-1 mRNA is necessary for this amino acid–dependent translational control (32). Translation of the uORF leads to a change in mRNA structure that allows ribosome entry at the IRES, and consequently, selective translation of the CAT-1 coding sequence (127). Thus, synthesis of the CAT-1 cationic amino acid transporter is a highly regulated process that displays amino acid–dependent control at the transcriptional, mRNA stability, and translational steps of synthesis.

Transcription Factors

ACTIVATING TRANSCRIPTION FACTOR 4 The ATF family of proteins represents a subclass of the bZIP superfamily (43). Selected ATF members are known to heterodimerize within the ATF family as well as with members of another bZIP subgroup, the C/EBP family. In particular, a C/EBPβ-ATF4 complex has been detected at cAMP response elements (114) and the crystal structure of the C/EBPβ–ATF4 heterodimer has been obtained (95). As described below, the human CHOP promoter contains a C/EBP-ATF composite site (123), at which Fawcett et al. (30) observed by electrophoresis mobility shift analysis (EMSA) a transient ATF4 binding 2 hours after initiating arsenite-induced stress. The nuclear extract ATF4 binding activity was replaced at 6 hours by ATF3 binding activity that was coincident with a suppression of the gene back toward the basal rate. Bruhat et al. (17) later showed that this same C/EBP-ATF composite site functions as an AARE. Other C/EBP-ATF composite sites that function as AAREs have been reported for ASNS (107), the SNAT2 neutral amino acid transporter (90), and the CAT-1 cationic amino acid transporter (31). ATF4 has been shown to bind to the ASNS (107) and CHOP (9) sites in vitro by EMSA, and to the ASNS promoter in vivo by ChIP (24). These observations, coupled with increased translation of ATF4 following amino acid deprivation (46), are consistent with the proposed role for ATF4 as the key regulator of the AAR pathway.

Not only does amino acid limitation induce translation of pre-existing ATF4 mRNA, Siu et al. (107) demonstrated that there is also an increase in ATF4 mRNA expression. The initial increase in ATF4 protein synthesis occurs within 30–45 minutes of amino acid removal, whereas the mRNA increase does not begin until 2–4 hours of amino acid removal (24). Whether this increase in ATF4 mRNA results in a second phase of ATF4 protein production has not been established. If it occurs, it would be similar to the biphasic response of yeast GCN4 (4), in which amino acid limitation causes an initial burst of GCN4 protein within minutes via translational control, and a second, more sustained increase after 3–4 hours due to transcriptional activation of the GCN4 gene. How the long-term regulation of the ATF4 gene contributes to the later stages of the AAR pathway remains to be established, as does the mechanism. Given the activation of ATF4 transcription, another interesting question is whether ATF4 induces its own gene in an autoregulatory cycle.

CCAAT/ENHANCER BINDING PROTEINS The AARE (NSRE-1) in the ASNS promoter (described below) was used as bait for a yeast one-hybrid screen to identify potential transcription factors specific for this sequence (108). Those results and subsequent EMSA data indicated that several members of the C/EBP family could recognize the NSRE-1 sequence. This observation is consistent with the fact that the NSRE-1 sequence is a C/EBP-ATF composite site. The C/EBP proteins represent a subclass of the bZIP superfamily of transcription proteins (73, 113), and most have been shown to homodimerize and heterodimerize with C/EBP members or to heterodimerize with members of other bZIP subfamilies (117). The C/EBP family includes individual members designated α, β, Υ, δ, ε, and CHOP. Collectively, they have a wide array of effects on cellular metabolism, differentiation, growth, and cell stress responses. Consequently, the level of expression for each of these family members varies widely among tissues and cell types. In liver cells, C/EBPβ mRNA is subject to differential translational start site selection such that both an activating (liver-enriched transcriptional activator protein, LAP) and an inhibitory (liver-enriched transcriptional inhibitory protein, LIP) isoform can be generated from the same mRNA (25). LIP is a shorter protein (147 amino acids in human cells) that corresponds to the C-terminal portion of LAP (322 amino acids in human cells). LIP lacks the transactivation domain of LAP, but contains the bZIP dimerization region and therefore, through dimerization with other C/EBP members, acts as a dominant negative repressor of C/EBP function. For example, coexpression of LIP can override the LAP-dependent cell cycle arrest in hepatoma cells (20).

Supershift analysis using the ASNS NSRE-1 sequence as an EMSA probe and antibodies specific for individual C/EBP family members demonstrated that C/EBPβ binding was increased when nuclear extracts from cells subjected to amino acid limitation or activation of the ER stress response were tested (108). These results are consistent with the report that C/EBPβ mRNA content is increased in rat hepatoma cells following amino acid starvation (79), although the Northern analysis in the latter study did not allow those authors to establish whether LAP

or LIP was produced from the increased mRNA. Siu et al. (108) confirmed the increased expression of C/EBPβ mRNA following amino acid limitation, and subsequent experiments documented that this increase was the result of transcriptional induction rather than mRNA stabilization (C. Chen & M.S. Kilberg, unpublished results). Immunoblot analysis of both whole cell and nuclear extracts from HepG2 human hepatoma cells showed that C/EBPβ-LAP migrates as a group of four distinct bands, some of which are increased in abundance by histidine deprivation (24). In contrast, histidine removal from the medium produced a slight decrease in the C/EBPβ-LIP content. Consistent with these results, Marten et al. (80) showed that the hepatic LAP/LIP ratio was increased in rats fed a low-protein diet. Additional investigation is necessary to better characterize the amino acid–dependent regulation of C/EBPβ expression and the subsequent impact on cellular metabolism.

C/EBP HOMOLOGY PROTEIN Originally identified as a gene that was induced by DNA damage, it is now recognized that CHOP (also known as growth arrest and DNA damage protein 153) expression is activated by a number of stress stimuli. A link between CHOP expression and apoptosis induced by cellular stress has been established by deleting the CHOP gene (132). Originally, it was believed that CHOP formed heterodimers with other C/EBP family members only to inhibit their action, and thus it served as a negative regulator (100). However, additional evidence has documented that CHOP-C/EBP heterodimers are capable of activating a number of genes (120).

Although amino acid limitation increases CHOP expression by posttranscriptional mechanisms (2, 19), there is transcriptional control of the CHOP gene by both amino acid and glucose starvation (59, 97). Glucose starvation of eukaryotic cells results in aberrant accumulation of glycoproteins in the endoplasmic reticulum that activates the UPR, also known as the ER stress response (60, 88, 92). The UPR signal transduction pathway culminates in increased transcription of a number of genes, many of which are involved in protein processing and trafficking within the ER. The prototypical example is the ER resident chaperone GRP78. Amino acid deprivation (i.e., the AAR pathway) does not induce UPR-activated genes such as GRP78 (12); obviously, slowing of protein synthesis will not result in an accumulation of misfolded proteins within the ER, and, therefore, no UPR is triggered. In mammalian cells, many target genes for the UPR pathway contain one or more copies of a highly conserved *cis* element (ER stress element) for which the consensus sequence is 5′-CCAAT-N_9-CCACG-3′ (101, 129). Jousse et al. (59) used deletion analysis of the human CHOP gene to show that the *cis* element necessary for activation of transcription by the UPR pathway was different from that responsible for induction following amino acid deprivation. After establishing that the C/EBP-ATF core sequence (5′-TGATGCAAT-3′, nucleotides –302 to –310), originally identified as a stress-responsive element (30, 123), also functions as an AARE, Bruhat et al. (17) employed EMSA and ATF2 knockout cells to establish a role for the ATF2 transcription factor. More recently, the Fafournoux laboratory has extended its studies to show that induction of the CHOP gene

following amino acid limitation requires both ATF2 phosphorylation and increased ATF4 expression (9).

Interestingly, there may be graded differences in response to depletion of a particular amino acid within a given cell type (58). Whereas the relative activation of the ASNS promoter was greater than that for CHOP when HeLa cells were deprived of leucine, cysteine, asparagine, or histidine, the activation of the CHOP promoter was nearly twice as great as that for ASNS when the cells were starved for methionine (58). Furthermore, Bruhat et al. (17) demonstrated that the relative magnitude of transcriptional activation from the CHOP AARE following depletion of leucine, methionine, or lysine was different in HeLa, Caco-2, and HepG2 hepatoma cells. How amino acid specificity relates to possible in vivo tissue-specific effects, especially at the initial detection steps in the AAR pathway, remains to be established. With regard to other factors that may influence amino acid control, Entingh et al. (26) showed that the induction of CHOP by amino acid deprivation of mouse fibroblasts required serum; they went on to document that the serum-required component was insulin-like growth factor-1 (IGF-1). Interestingly, induction of the System A amino acid transporter activity following amino acid starvation in fibroblasts is also dependent on serum (37, 61), but that is not the case in rat hepatoma cells (61).

ACTIVATING TRANSCRIPTION FACTOR 3 ATF3 is expressed at low levels in normal and quiescent cells, but can be rapidly induced in response to diverse stress signals and is likely to be involved in controlling a wide variety of cellular activities (48, 56, 91). Pan et al. (91) and Jiang et al. (56) demonstrated that the expression of ATF3 is induced in response to activation of the AAR and the UPR pathways, and the latter group showed these inductions required the eIF-2α kinases GCN2 and PERK, respectively (56). Ron and associates (47) have shown through a microarray survey that in ATF4-deficient fibroblasts, tunicamycin-induced ATF3 mRNA induction is reduced as compared with the wild type. Wek and colleagues (56) have shown that in both GCN2$^{-/-}$ and ATF4$^{-/-}$ fibroblasts, induction of ATF3 was impaired following amino acid limitation. These results illustrate the importance of ATF4 on ATF3 expression in response to a variety of stress stimuli.

The amino acid–dependent stabilization of ATF3 mRNA is briefly summarized above, but relatively little is known about the mechanisms controlling transcription of the ATF3 gene under nutritional stress conditions. The human ATF3 promoter has a consensus ATF/CRE site at nucleotides –93 to –85 (5′-TTACGTCAG-3′) and a C/EBP-ATF composite site at nucleotides –23 to –15 (5′-TGATGCAAC-3′), but there are no in vivo studies of transcription factor binding. The C/EBP-ATF element has been identified as an ATF3 binding site responsible for autoregulation of the ATF3 gene (124). The sequence of this element differs from the NSRE-1 in ASNS (5′-TGATGAAAC-3′) and the AARE in CHOP (3′-TGATGCAAT-5′) by only one nucleotide each, and ATF4 has been shown to bind this ATF3 sequence in vitro (30). However, whether this AARE-like element contributes to the increase in ATF3 mRNA following histidine deprivation is unknown.

METABOLISM AND GROWTH CONTROL

VASCULAR ENDOTHELIAL GROWTH FACTOR Vascular endothelial growth factor (VEGF) has potent angiogenic properties and is expressed as four alternative spliced variants that exert similar activity on endothelial cell proliferation and permeability. VEGF levels are higher in the serum and urine of patients with tumor or metastatic disease relative to healthy individuals (126). Abcouwer et al. (1) showed that deprivation of the amino acid glutamine caused increased VEGF mRNA expression in a retinal pigmented epithelial cell line. This induction was the result of both increased transcription and mRNA stabilization. Although glutamine deprivation induced the transcription factors NF-κB and activating protein 1 (AP-1) activities in human breast carcinoma cells, Bobrovnikova-Marjon et al. (14) showed that the induction of VEGF expression was independent of those two factors. Roybal et al. (102) reported that homocysteine or other thiol-containing compounds induced expression of VEGF and also caused transient phosphorylation of eIF2α, which, as expected, caused an increase in ATF4 protein content. Those authors showed that the VEGF induction in response to homocysteine was abolished by blockade of ATF4 function through the use of a dominant negative or in ATF4-deficient mouse embryonic fibroblasts. Collectively, these results suggest that control of expression for critical growth factors such as VEGF is sensitive to amino acid availability.

INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-1 The importance of amino acid–dependent regulation of insulin-like growth factor binding protein-1 (IGFBP-1) has been reviewed by Bruhat et al. (18), and is briefly summarized here. Protein malnutrition has been documented to decrease the circulating level of IGF-I, but to increase the level of IGFBP-1 (111). The effect on IGFBP-1 expression was demonstrated to be the result of increased mRNA content. Straus (112) showed that single amino acid limitation of hepatoma cells in culture also resulted in an increase in IGFBP-1 mRNA. Jousse et al. (57) investigated the expression of IGF-I, IGF-II, and IGFBP-1 in response to amino acid limitation in cultured cells and showed that IGFBP-1 mRNA and protein content was increased significantly, whereas the expression of the IGF-1 and IGF-II were either unaffected or slightly reduced. These observations appear to conflict with the observations of Entingh et al. (26), who reported that the activation of the CHOP gene by amino acid deprivation of fibroblasts requires the presence of IGF-1. However, those authors suggested that even a decreased level of IGF-I might still be sufficient for induction of the CHOP gene. The exact role of IGF-I in modulating the AAR pathway and the apparent cell specificity of this involvement requires further investigation. The regulation of IGFBP-1 is of particular interest because given the broad nature of its physiologic function, nutrient-dependent control of its expression is likely to impact a number of organs and cell types. An investigation of the influence of amino acid availability on the entire spectrum of hormones and cytokines has not been reported, but such a study would contribute to our understanding of the interorgan

effects that protein nutrition has on cell growth and metabolism. For example, it has been demonstrated in cell culture that histidine deprivation of pancreatic cells was unique among the amino acids in causing suppression of the synthesis of glucagon (93). Relative to this observation, it will be interesting to discover how dietary amino acid availability impacts metabolic homeostasis in diabetic patients and their nutrition.

ASPARAGINE SYNTHETASE ASNS catalyzes the ATP-dependent synthesis of asparagine and glutamate from aspartate and glutamine (99). Gong et al. (40) were the first to determine that ASNS mRNA content increased in cells deprived of amino acid; subsequently, Hutson & Kilberg (53) also demonstrated increased ASNS mRNA content following total amino acid deprivation or depletion of a single essential amino acid. Guerrini et al. (42) analyzed the ASNS promoter region and determined that an AARE was present at nucleotides –70 to –64. In vivo footprinting by Barbosa-Tessmann et al. (11) revealed five protein binding sites within the ASNS proximal promoter region that contribute to nutrient control of the human ASNS gene, three GC boxes (GC-I, GC-II, and GC-III), and two novel sequences, originally labeled sites V and VI, and later renamed nutrient-sensing response element (NSRE)-1 and -2. The three GC-rich sequences are necessary to maintain the basal transcription rate and to permit maximal activation of the ASNS gene by the AAR or UPR pathways (71). Expression of either Sp1 or Sp3 in *Drosophila* SL2 cells supported basal ASNS promoter activity, but interestingly only Sp3 expression permitted the starvation-induced ASNS-driven transcription. Barbosa-Tessmann et al. (12) demonstrated that both NSRE-1 and NSRE-2 were required for activation of the human ASNS gene following activation by either the AAR or the UPR pathways. EMSA experiments revealed increased nuclear protein binding to the NSRE-1 binding site (5′-TGATGAAAC-3′, nucleotides –68 to –60) when extracts from either amino acid or glucose-deprived cells were tested (11). This broader nutrient-detecting capability is the reason that the site is referred to as an NSRE rather than solely as an AARE. Mutagenesis of the ASNS promoter confirmed the in vivo footprinting, which had suggested the presence of a second element, NSRE-2 (nucleotides –48 to –43, 5′-GTTACA-3′), positioned eleven nucleotides downstream of NSRE-1. NSRE-2 is also absolutely required for induction of the ASNS gene by both the AAR and the UPR pathway (11). The NSRE-1 and NSRE-2 sequences, as well as the 11 bp between them, are completely conserved in rat, mouse, hamster, and man. Mutagenesis studies have documented that these two elements must be aligned on the same side of the DNA helix and must be only one turn away from each other (131). It is postulated that this spatial relationship is required for protein-protein interactions that occur between the transcription factors that bind to these two sites.

In vitro binding analysis revealed ATF4 binding to the NSRE-1 sequence and that this binding activity was increased when nuclear extracts from either histidine-deprived (AAR pathway) or glucose-deprived (UPR pathway) cells were tested (107). Consistent with the translational control of ATF4, inhibition of protein synthesis blocked the starvation-dependent enhancement in ATF4-NSRE-1

complex formation. ATF4 regulation of ASNS expression in vivo was illustrated by the observation that ASNS promoter-driven transcription was induced by ATF4 overexpression (107). Subsequently, ChIP analysis documented in vivo that ATF4 binds to the ASNS promoter following amino acid deprivation (24).

Both EMSA experiments and transient expression of ATF3-FL (107, 121) and C/EBPβ (108) suggested that these bZIP transcription factors also exhibit affinity for the ASNS NSRE-1 site and that both contribute to the regulation of transcription driven by the ASNS promoter. Transient expression studies using combinations of ATF4, ATF3-FL, and C/EBPβ suggest that ATF3-FL serves as the primary antagonist to ATF4 function (24, 91). Consistent with this hypothesis, as mentioned above, Fawcett et al. (30) used nuclear extracts from arsenite-treated cells and EMSA studies to show a transient increase in ATF4 binding activity at the C/EBP-ATF composite site in the human CHOP promoter. ATF4 binding activity peaked at 2 hours after arsenite exposure, but that activity was subsequently replaced by elevated ATF3 binding activity at about 6 hours, a time at which transcription from the gene declined back toward the basal expression rate. Fawcett et al. (30) also used overexpression studies to document that elevated ATF4 activates the CHOP gene through the C/EBP-ATF composite site and that increased ATF3 production antagonizes the ATF4 function. The data of Chen et al. (24) extend the observations of Fawcett et al. (30) by showing through ChIP analysis that a similar sequence of events can be observed in vivo at the ASNS C/EBP-ATF composite site (NSRE-1).

Chen et al. (24) have proposed a working model for ASNS transcription in response to amino acid limitation (Figure 4). Phase I encompasses the first 4 hours and phase II covers the time from 4–24 hours. During the first 30 minutes, translational control of ATF4 mRNA results in increased binding of ATF4 to the NSRE-1 site (24). A low level of C/EBPβ is constitutively bound to the ASNS promoter and this amount only increases after the transition to phase II has begun. In parallel with ATF4 binding, acetylation of histones H3 and H4 is increased, and subsequently, the general transcription factors TBP and TFIIB, as well as RNA Pol II, are recruited to the promoter. It is assumed that the ATF4 complex requires a coactivator or other bridging proteins to the general transcription machinery, which are shown as gray modules in the model (Figure 4). In phase II of amino acid deprivation, C/EBPβ binding increases at a time when the transcription rate has peaked. The synthesis and action of ATF3-FL and its alternatively spliced variant ATF3ΔZip3, also increases during this period (24), and it is believed that these three proteins act in concert to suppress, but not to completely reverse, the increased transcription. Transcription in phase II, even out to 24 hours, remains elevated relative to the status of the gene in amino acid–complete medium (24). Given that the rate of change in intracellular amino acid concentration surely varies from cell to cell and that the cells used by Chen et al. (24) were not cell cycle synchronized, it is proposed that individual cells make the transition from the "fed" state to phase I and from phase I to phase II at somewhat different rates. Therefore, it is hypothesized that what is measured as the "net" transcription rate and the "net" binding result by ChIP is actually the ratio of the number of cells in these three conditions at any single time point.

SUMMARY AND FUTURE DIRECTIONS

The understanding of how nutrients such as amino acids control gene expression in mammalian cells remains relatively limited, but significant progress has been achieved during the past few years, in particular with regard to amino acid control of transcription and mRNA stabilization. The full complement of target genes has not yet been established, but microarray technology should reveal many of the remaining candidates. Interestingly, even among the relatively small number of amino acid–regulated mammalian genes that have been identified thus far, there is already emerging evidence that multiple mechanisms exist. Clearly, there is much yet to learn about the nutrient-sensing signal transduction pathways upstream of transcription, the transcriptional steps themselves, and posttranscriptional mRNA metabolism. Beyond gaining a basic understanding of these important biological control mechanisms, the characterization of how these processes contribute to the pathology of various disease states represents an exciting aspect of future investigations in molecular nutrition.

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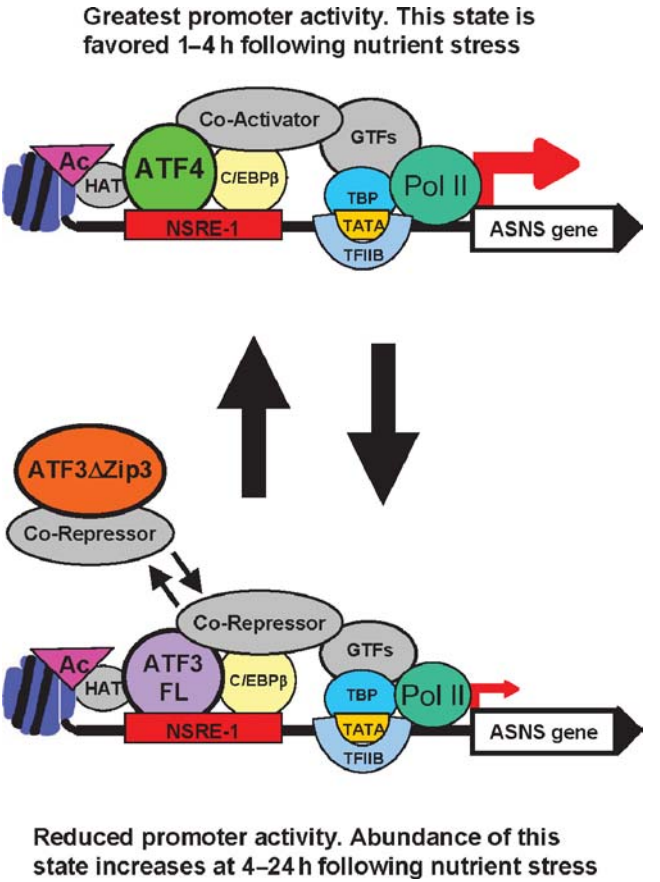

Figure 4 Working model for control of the asparagine synthetase (ASNS) gene (see text for details). Transcription factors shown in color have been localized to the ASNS promoter by chromatin immunoprecipitation analysis (24). Unidentified proteins are shown in gray.

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