Cloning of rat asparagine synthetase and specificity of the amino acid-dependent control of its mRNA content

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A full-length cDNA clone for rat asparagine synthetase (AS) was isolated from a cDNA library enriched for amino acid-regulated sequences. The AS cDNA was used to investigate the amino acid-dependent repression of AS mRNA content in rat Fao hepatoma cells. In response to complete amino acid starvation, there was an ~ 10-fold increase in the level of AS mRNA. Three species of mRNA, of approx. sizes 2.0, 2.5 and 4.0 kb, were detected and each was simultaneously regulated to the same degree. The expression of AS mRNA increased by 6 h after removal of amino acids, reached a plateau after 9 h, and was blocked by either actinomycin D or cycloheximide. Partial repression of the AS mRNA content was maintained by the presence of a single amino acid in the culture medium, but the degree of effectiveness for each one varied widely. Glutamine showed the greatest ability to repress the AS mRNA content, even at an extracellular concentration 10 times below its plasma level. Other effective repressors included the amino acids asparagine, histidine and leucine, as well as ammonia. Depletion of selected single amino acids from an otherwise complete culture medium also caused up-regulation. In particular, removal of histidine, threonine or tryptophan from the medium, or the addition of histidinol to inhibit histidinyl-tRNA synthetase, resulted in a significant increase in AS mRNA content. The data indicate that nutrient regulation of AS mRNA occurs by a general control mechanism that is responsive to a spectrum of amino acids.

INTRODUCTION

For mammalian cells, some of the general characteristics for nutrient control by carbohydrates, fatty acids, sterols and minerals have been described [1–7]. However, there is considerably less information available concerning the control of mammalian gene expression by amino acids. It is well documented that numerous bacterial operons are feedback-regulated by the specific amino acid end-products of the corresponding enzymes [8]. In addition to this type of specific control, in the yeast *Saccharomyces cerevisiae* a number of genes encoding enzymes in multiple amino acid biosynthetic pathways are under a common control process [9]. This 'general control' of nitrogen metabolism (GCN) is mediated by translational regulation of the protein transcription factor, GCN4, which in turn regulates the expression of over 30 different genes involved in nine independent amino acid biosynthetic pathways [9,10]. Starvation of yeast cells for a single amino acid causes an increase in translation of the GCN4 mRNA [11]. In the presence of an elevated level of an end-product amino acid, induction by the general control response can be overridden by pathway-specific repression [9].

A protein-rich diet or individual amino acids can modulate mammalian protein expression by either transcriptional or post-transcriptional processes. Specific examples include serine dehydratase [12], asparagine synthetase [13], ornithine decarboxylase [14], amino acid transport mediated by System A [15] or System L [16], the ribosomal proteins L17 and S25 [17–19], and the protein and mRNA content for a mitochondrial protein of unknown function [20,21]. The mRNA content for mammalian asparagine synthetase (AS), which catalyses the ATP-dependent conversion of aspartic acid to asparagine, is regulated by the availability of amino acids [13]. Both the human and hamster AS cDNAs have been cloned and are highly homologous [22–25]. Gong et al. [13] first reported that depletion of a complete culture medium for a single amino acid induced the AS mRNA content in both baby hamster kidney (BHK) cells and Hela cells. Their data suggested that the elevated AS mRNA content resulted from an increase in transcription as well as mRNA stabilization. Evidence for an amino acid response element within the human AS genomic promoter sequence has been reported recently [26].

We report here that the level of rat AS mRNA is increased in Fao hepatoma cells in response to amino acid starvation, and that the increase requires *de novo* RNA and protein synthesis. Supplementation of complete culture medium with histidinol, an inhibitor of histidinyl-tRNA synthetase, also caused an increase in the level of AS mRNA. Supplementing the amino acid-free medium with even a single amino acid was sufficient to maintain partial repression of the AS mRNA content, and the broad substrate specificity of this down-regulation suggests a general control response.

MATERIALS AND METHODS

Cell culture

Rat Fao hepatoma cells were maintained in minimal essential medium (MEM), pH 7.4, and 4% fetal bovine serum (FBS) as previously described [17]. The cells were diluted in MEM/4% FBS and 150 mm diameter dishes inoculated with 10.5×10^6 cells, such that after 48 h the dishes were 70% confluent as determined by cell number. In between all medium changes the cells were rinsed with PBS. Cells were then transferred to Krebs–Ringer bicarbonate (KRB) buffer, pH 7.4, as an 'amino acid-starved' condition [27]. To maintain cells in the 'amino

Abbreviations used: AIB, 2-aminoisobutyric acid; AS, asparagine synthetase; FBS, fetal bovine serum; GDH, glutamate dehydrogenase; KRB, Krebs–Ringer bicarbonate buffer; MEM, minimal essential medium; RT, reverse transcriptase.

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acid-fed' state, MEM supplemented with 3 mM each of alanine, asparagine, glycine, proline, serine and threonine was used as the culture medium [17]. Amino acid-specificity experiments were carried out in MEM lacking individual amino acids (Selectamine, Gibco-BRL, Gaithersburg, MD, U.S.A.) or KRB buffer supplemented with a single amino acid.

Cloning of a rat AS cDNA

A human AS monoclonal antibody [28], kindly supplied to us by Dr. Sheldon Schuster (University of Florida), was used to determine that pancreas and testis showed the highest level of AS protein, in agreement with the data of Hongo et al. [29]. A partial cDNA sequence for rat AS, used as a probe for the Northern analysis reported here, was cloned using the reverse transcriptase (RT)–PCR as described by Kawasaki and Wang [30]. RNA was isolated from rat testis for RT–PCR and primers were chosen based upon sequence identity between the human and hamster AS cDNAs: 5′-CTTTTATCAGGGGGCTTGGACTCC-3′ and 5′-TAGTGGGTCAGCGTGCGGGCAGAAG-3′ respectively. Oligonucleotides were synthesized by the DNA Synthesis Core Facility of the Interdisciplinary Center for Biotechnology Research at the University of Florida.

Northern analysis

Total cellular RNA was prepared from tissue or cells in culture using the method of Chomcyznski and Sacchi [31]. RNA was size-fractionated by electrophoresis in a 1 % (w/v) agarose/6.6 % (v/v) formaldehyde gel in 20 mM Mops, pH 7.0, 5 mM sodium acetate, 1 mM EDTA [32]. After electrophoresis, equivalent loading of RNA samples was verified by quantification of the intensity of ethidium-bromide-stained rRNAs using a photographic negative and by probing the corresponding blot with a cDNA for rat glutamate dehydrogenase (GDH) (obtained from Dr. W. H. Lamers, University of Amsterdam) which does not respond to amino acid starvation. The RNA was capillary blotted to Hybond nylon membrane (Amersham, Arlington Heights, IL, U.S.A.) in 10 × SSPE (1 × SSPE contains 0.15 M sodium chloride, 0.01 M sodium phosphate, pH 7.4, 1 mM EDTA), u.v.-crosslinked to the nylon membrane, and then hybridized at 65 °C with a cDNA probe for either GDH or AS [32]. The cDNA probe was radioactively labelled before hybridization with 50 μCi [α^{32}P]dCTP (3000 μCi/mmol) using a random primer DNA labelling system (BRL, Gaithersburg, MD, U.S.A.). Blots were subjected to autoradiography, and the data as well as a photographic negative of ethidium bromide staining of rRNA were quantified by densitometry (Bio-Image Densitometer, Millipore, Ann Arbor, MI, U.S.A.). Northern blots with > 20 % variation in ethidium bromide staining were not used for quantification of AS mRNA content. All experiments were repeated at least once with independent cell populations to establish that the data were qualitatively reproducible. Where given, a mean ± S.D. represents the summary of three or more experiments.

RESULTS

Isolation and characterization of a rat AS cDNA

As predicted from the human AS cDNA sequence, a 900 bp rat cDNA fragment was isolated by RT–PCR of rat testis mRNA, which was 90 % identical to the human sequence at both the amino acid and nucleic acid level. The rat AS cDNA hybridized with three different mRNAs of approx. lengths 2.0, 2.5 and 4.0 kb in both hepatoma cells (Figure 1) and normal liver tissue (results not shown). The abundance of the 4.0 kb species was low compared with the 2.0 and 2.5 kb mRNAs, necessitating overexposure of the 2.0 and 2.5 signals to detect the 4.0 kb mRNA (Figure 1).

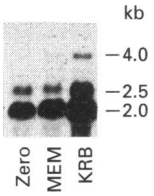

Figure 1 Three rat AS mRNA species are induced by amino acid starvation

Fao hepatoma cells cultured to 70% confluence in MEM containing 4% FBS (Zero) were incubated for 12 h in amino acid-fed (MEM) or amino acid-starved (KRB) media. Total RNA was isolated and subjected to Northern analysis with a ^{32}P-labelled rat AS cDNA. The AS mRNAs, estimated by RNA markers, are approx. 2.0, 2.5 and 4.0 kb. Autoradiographic quantification demonstrated that all three species were induced 10-fold.

The rat AS PCR product was used as a probe to screen an amino acid-starvation-induced Fao hepatoma cDNA library [17], from which a full-length rat AS cDNA was identified (Figure 2). The 1974 bp cDNA was sequenced in both directions and contained an open reading frame coding for 561 amino acids, a 123 nt 5′-untranslated region, and a 168 nt 3′-untranslated region that includes a single polyadenylation signal and a short poly-A sequence (Figure 2). In addition to the poly-A-containing clone, an additional clone was identified that began at the same 5′ site and extended an additional 266 nt in the 3′ direction. Northern analysis with this 3′ extended sequence as a probe revealed hybridization to the 2.5 and 4.0 kb sequences, but not to the 2.0 kb mRNA (results not shown).

At 'time zero', Fao hepatoma cells were transferred to amino acid-free (KRB) or amino acid-supplemented (MEM) medium, and total RNA was isolated from these cells after 12 h and then subjected to Northern analysis with a ^{32}P-labelled rat AS cDNA probe (Figure 1). After removal of amino acids from the medium for 12 h, there was a 10.7 ± 1.4-fold increase in AS mRNA content, compared with the level of AS mRNA in the presence of amino acids (Figure 1). KRB medium contains all of the components of MEM except amino acids and vitamins; the addition of vitamins to KRB had no effect on the expression of AS mRNA (Figure 8). Each of the three rat AS mRNA species were simultaneously regulated by amino acid starvation to the same degree (ratios of starved/fed mRNA expression for a typical experiment were: 2.0 kb, 9.5; 2.5 kb, 10.0; 4.0 kb, 10.5); therefore in the remaining experiments only the 2.0 kb transcript was quantified.

Time-course of AS mRNA up-regulation following amino acid deprivation

To establish the time-course of AS mRNA induction, cells were incubated in amino acid-free KRB for specific time intervals and then total RNA was isolated for Northern analysis. An increase in the level of AS mRNA could be detected as early as 6 h and reached a plateau by 9 h after the removal of amino acids (Figure 3). As an internal control, these blots were probed with a rat liver GDH cDNA. In contrast to AS, the level of GDH mRNA remained relatively constant in Fao cells incubated in the presence or absence of amino acids (Figure 3). The ratio of starved/fed GDH mRNA at 12 h was 0.7 ± 0.3. To determine whether the up-regulation of AS mRNA was dependent on either transcription or translation, cells were transferred for 0–12 h to

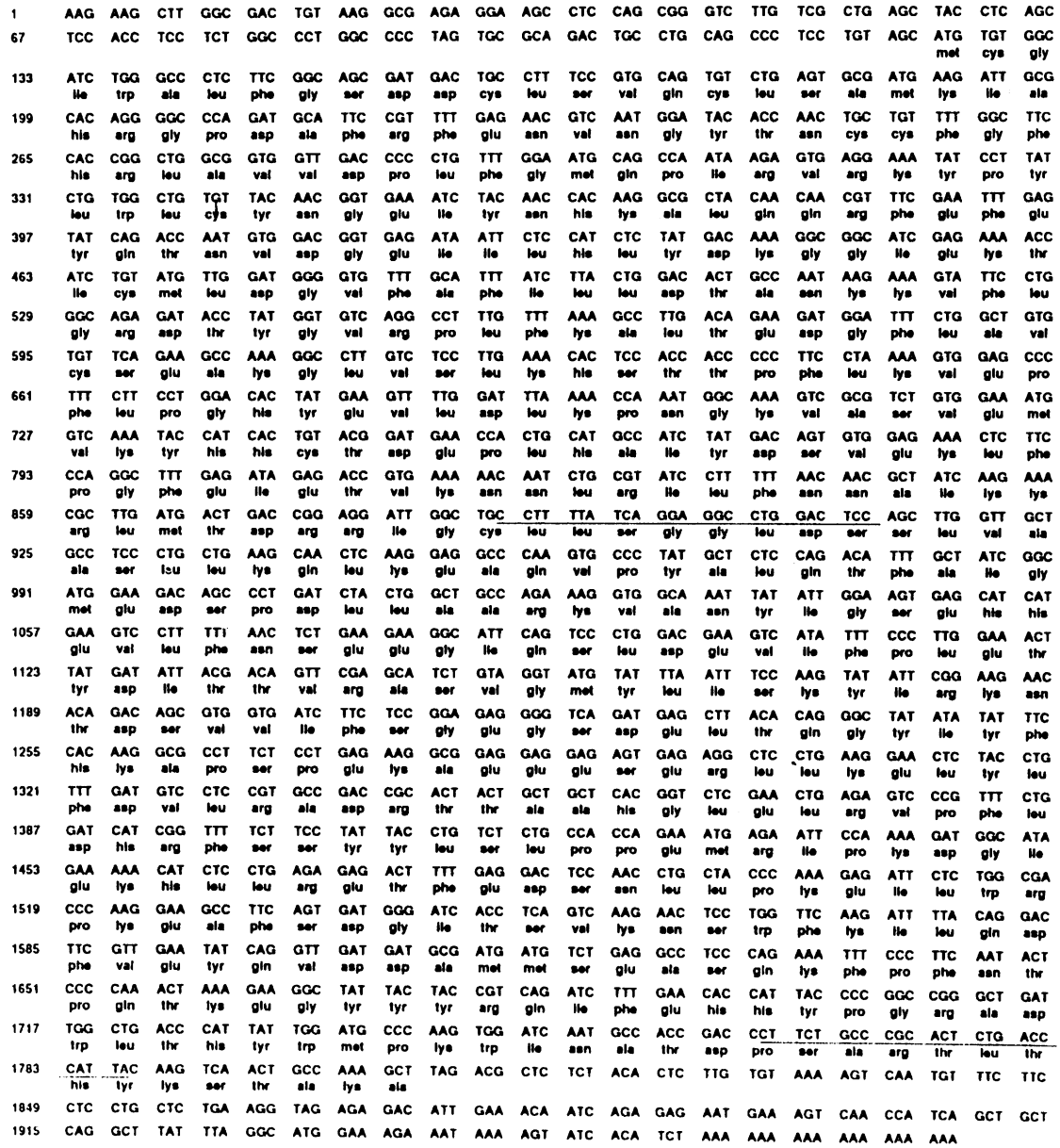

Figure 2 Nucleotide and predicted amino acid sequence of a rat AS cDNA clone

The 1974 bp rat AS cDNA and the predicted amino acid sequence is shown. Underlined are the sequences corresponding to the oligonucleotide primers used for RT–PCR to obtain an original 900 bp rat AS cDNA. This was used to screen a rat Fao hepatoma cDNA library enriched for starvation-induced mRNA sequences [17]. The clone contains a putative start codon (position 124), stop codon (position 1807) and polyadenylation signal (positions 1939–1945).

amino acid-free or amino acid-containing media in the presence of actinomycin D or cycloheximide (Figure 4). The presence of either of these inhibitors in the culture medium blocked the amino acid-dependent induction of AS mRNA. These results are consistent with data obtained by expression of the AS cDNA in hamster cells [13], which indicated a transcriptional and translational component to the starvation-dependent induction of AS mRNA.

Specificity of the amino acid-dependent control of AS mRNA

To better characterize the amino-acid-dependent repression of AS mRNA, Fao cells were transferred to a complete MEM medium or MEM deficient in a single amino acid. The media were replenished at 6 h and RNA was isolated at 12 h to assay the AS and GDH mRNA contents. Depletion of the medium of even a single amino acid resulted in increased AS mRNA content (Figure 5). Although the enhancement was not as great as that observed following complete amino acid starvation (10.1 ± 0.8-fold increase), depletion of histidine (5.6 ± 2.0-fold increase), threonine (5.4 ± 1.9-fold increase) or tryptophan (5.3 ± 1.9-fold increase) resulted in the greatest increase in AS mRNA.

To provide additional documentation of the substrate specificity, Fao cells were transferred for 12 h to amino acid-free medium (KRB) or KRB supplemented with individual amino acids. The level of GDH mRNA exhibited relatively small

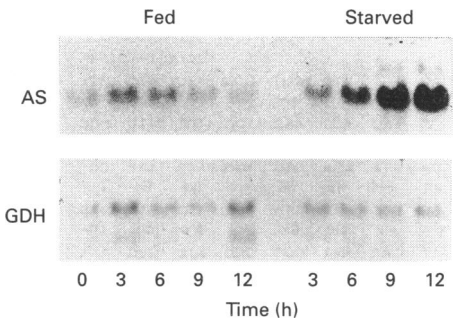

Figure 3 Induction of steady-state AS mRNA level during amino acid starvation

Fao hepatoma cells were cultured in MEM containing 4% FBS and grown to 70% confluence. The cells were transferred to either amino acid-fed (MEM) or amino acid-starved (KRB) medium and total RNA was isolated after 3, 6, 9 and 12 h. Northern analysis was performed with a ^{32}P-labelled cDNA probe for either rat AS or GDH.

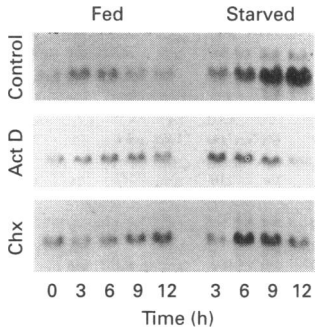

Figure 4 Actinomycin D and cycloheximide blockade of the starvation-dependent increase in AS mRNA

Fao hepatoma cells, cultured in MEM containing 4% FBS, were grown to 70% confluence and then transferred to amino acid-fed (MEM) or amino acid-starved (KRB) medium. Total cellular RNA was isolated after 3, 6, 9 and 12 h. Northern analysis was performed with a ^{32}P-labelled rat AS cDNA probe. Some incubations included either 25 μM actinomycin D (Act D) or 100 μM cycloheximide (Chx) in both the MEM and KRB media.

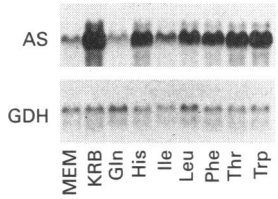

Figure 5 Depletion of individual amino acids from complete MEM medium

Fao hepatoma cells were cultured to 70% confluence and then transferred for 12 h to amino acid-fed (MEM), amino acid-starved (KRB) or MEM that was deficient in a single amino acid. Total RNA was isolated for Northern analysis with ^{32}P-labelled cDNA probes for either rat AS or GDH. The Northern blot shown is representative of multiple experiments for which the statistical analysis is given in the text.

changes as a result of complete amino acid starvation, and the presence of individual amino acids had only a minor influence on its content (Figure 6). The usual enhancement of AS mRNA attributable to amino acid deprivation was observed (10.7 $\pm$ 1.5-fold increase) and each of the amino acids tested was able to partially repress the level of AS mRNA relative to amino acid-free medium (Figure 6). Glutamine (2.2 $\pm$ 0.8-fold increase) suppressed the AS mRNA induction to the greatest degree, i.e. $\sim$ 85% as well as the complete MEM culture medium, whereas asparagine (4.9 $\pm$ 1.5-fold increase) suppressed the AS mRNA enhancement by 60% compared with complete MEM. Likewise, KRB medium supplemented with either histidine (4.4 $\pm$ 1.5-fold increase) or leucine (3.5 $\pm$ 0.4-fold increase) suppressed the AS mRNA induction 65 and 74% respectively. The amino acids proline, serine, and threonine were moderate repressors of the AS mRNA content, and aspartate, glycine and glutamate were the weakest repressors. Interestingly, supplementation of KRB media with 5 mM ammonia (2.6 $\pm$ 0.2-fold increase) was able to partially suppress the level of AS mRNA better than many of the individual amino acids, perhaps indirectly by promoting the synthesis of several amino acids.

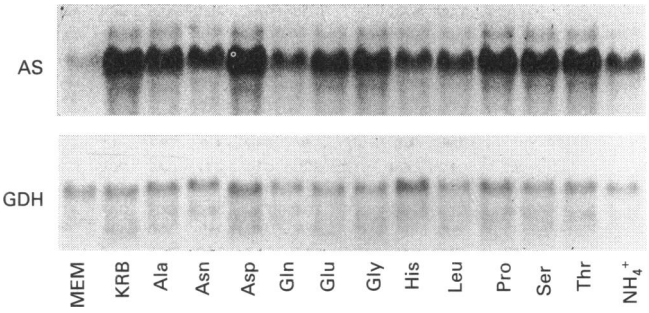

Figure 6 Specificity of the amino-acid-dependent repression of AS mRNA content

Fao hepatoma cells were cultured to 70% confluence and then transferred for 12 h to amino acid-fed medium (MEM), amino acid-starved (KRB), or KRB medium supplemented with 5 mM of the indicated amino acid or ammonia. Total cellular RNA was isolated for Northern analysis with ^{32}P-labelled cDNA probes for either rat AS GDH. The Northern blot shown is representative of multiple experiments for which the statistical analysis is given in the text.

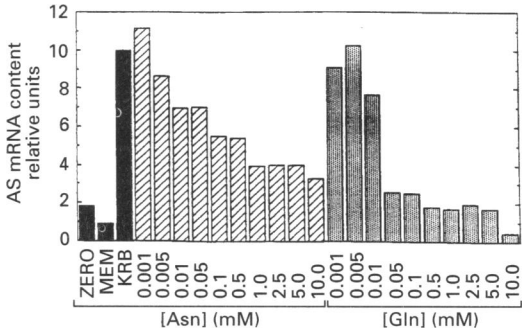

Figure 7 Concentration dependence for amino-acid-dependent regulation of AS mRNA content

Fao rat hepatoma cells were cultured to 70% confluence (Zero) and then transferred for 12 h to amino acid-fed (MEM), amino acid-starved (KRB) or KRB medium supplemented with specific concentrations of either glutamine or asparagine as shown. After 12 h, total cellular RNA was isolated and AS mRNA content assayed with a ^{32}P-labelled AS cDNA probe. Densitometry was performed on both the autoradiograms from Northern analyses and the photographic negatives of ethidium-bromide-stained RNA. The ethidium bromide staining of the rRNA served as a quantitative reference for comparison of the amount of total RNA loaded in each lane. The data presented are a summation of AS mRNA levels quantified from three independent experiments.

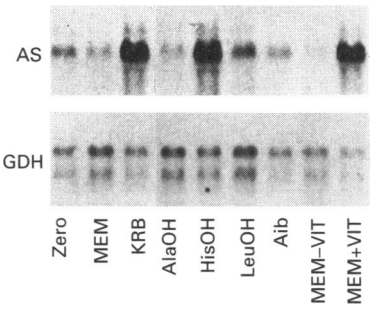

Figure 8 Supplementation of complete MEM medium with amino alcohol analogues

Fao hepatoma cells were cultured to 70% confluence (Zero) and then transferred for 12 h to amino acid-fed (MEM), amino acid-starved (KRB) or MEM supplemented with 5 mM of the amino acid analogues alaninol (AlaOH), histidinol (HisOH), leucinol (LeuOH) or AIB. Additionally, some cells were transferred to MEM deficient in vitamins (MEM — VIT) or KRB supplemented with vitamins (KRB + VIT). Total RNA was isolated for Northern analysis with ^{32}P-labelled cDNA probes for either rat AS or GDH. The Northern blot shown is representative of multiple experiments.

To determine whether the amino acid-dependent regulation of AS mRNA occurred at physiological substrate levels, Fao cells were transferred to amino acid-free medium (KRB) or KRB supplemented with specific concentrations of either asparagine or glutamine (Figure 7). At concentrations of $< 5\ \mu$M, neither amino acid affected the induction of AS mRNA. With increasing asparagine concentrations of $10\ \mu$M to 10 mM, repression gradually increased from 10 to 60% of that observed with complete MEM medium. The ability of glutamine to repress the induction of AS mRNA increased significantly between 10 and $50\ \mu$M, and then reached a plateau at $\sim 80\%$ repression.

The alcohol derivatives of specific amino acids inhibit their corresponding tRNA synthetases and thus prevent aminoacyl-tRNA formation [33,34]. To determine whether aminoacylated tRNA is important for amino acid-dependent repression of AS mRNA content, histidinol, alaninol or leucinol was added to complete MEM to block acylation of the corresponding amino acid. Addition of histidinol (5 mM) to MEM caused a degree of induction of AS mRNA equal to or greater than that seen in the complete absence of amino acids, whereas alaninol and leucinol did not significantly affect the level of AS mRNA (Figure 8). The addition of the non-metabolizable alanine analogue 2-amino-isobutyric acid (AIB) did not significantly affect the AS mRNA content. These results suggest that there exists a pathway for amino-acid-dependent repression of AS mRNA content involving recognition of certain aminoacyl-tRNA(s).

DISCUSSION

We have isolated a rat AS cDNA using RT–PCR with oligonucleotide primers chosen from the human cDNA sequence [20]. The rat expresses three AS mRNA species of approx. 2.0, 2.5 and 4.0 kb. The predominant species is 2.0 kb, followed by the 2.5 and 4.0 kb species. Human and hamster each express an AS mRNA of 2.0 kb and the hamster expresses an additional AS mRNA that is somewhat larger than 2.0 kb [20,21]. We have cloned the 2 kb rat AS cDNA from an amino acid starvation-induced rat Fao hepatoma cDNA library [17]. In addition to this clone, an additional clone was isolated that extended 266 nt beyond the polyadenylation site contained within the shorter clone. Both the nucleotide coding sequence and the protein translation product of the rat 2 kb cDNA are 90% identical to the corresponding AS sequences of human and hamster [20,21].

Mammalian cells respond to complete amino acid starvation by altering the expression of specific mRNAs and proteins. Marten et al. [35] have categorized a number of genes based on the abundance of their mRNAs in cells grown in medium deficient in a single amino acid. The mRNA content for β-actin, histone H4, Cu-Zn superoxide dismutase and glyceraldehyde 3-phosphate dehydrogenase is decreased by complete amino acid starvation [17], whereas the mRNA content for ribosomal proteins L17 and S25 is increased 3–5-fold [17–19]. Our experiments demonstrating a blockade of the AS induction in hepatoma cells by actinomycin D are consistent with transfection experiments of others indicating a transcriptional component to the starvation-dependent increase in human AS mRNA [13,24]. Although inhibition of the induction by cycloheximide may reflect a requirement for translation of the complete AS mRNA [13], the data also are consistent with the hypothesis that the synthesis of a *trans*-acting regulatory factor may be needed. Such a regulator may be analogous to the yeast starvation signalling transcription factor GCN4 [9,10]. The general control response in the yeast *S. cerevisiae* is mediated through translational control of the positive transcription factor GCN4 which in turn modulates expression of numerous structural genes under general control [9,10]. The initial signal for derepression of this set of genes is increased translation of the GCN4 mRNA that results from assembly of the translational machinery at specific short upstream open reading frames found in the 5′ untranslated region of the GCN4 mRNA [9,11]. A similar signalling pathway for amino acid availability may occur in *Neurospora crassa* which contains cpc-1, a homologue of GCN4 [36].

Our results show that depletion of MEM for any one of several essential amino acids resulted in a greater AS mRNA induction than removal of the non-essential amino acid glutamine, although this induction was not as great as complete amino acid starvation. Interestingly, glutamine, a substrate of the AS reaction, when added to amino acid free media, was able to substantially down-regulate the level of AS mRNA, at even concentrations far below its plasma level of 0.6 mM. Conversely, asparagine, the end-product of the AS reaction, was not any more effective at repression of the AS mRNA content than several other amino acids. Given glutamine's unique role as a carrier of nitrogen, this substrate-dependent repression of AS may maintain asparagine synthesis at a low rate so that cellular glutamine levels are not depleted. Obviously, if a comparable degree of repression occurs *in vivo*, there may be other regulatory signals that override the glutamine repression when additional asparagine is required.

The signalling pathways that recognize amino acid availability in mammalian cells have not been investigated as extensively as those in bacteria or yeast. For AS, it has been recognized that a correlation exists between asparagine starvation, aminoacylation of $tRNA^{Asn}$, and AS activity [37]. These previous observations suggested that tRNA charging was involved in the sensing of amino acid levels. Presumably, supplementing complete medium with amino alcohol analogues does not reduce the cellular level of the corresponding amino acid, indeed it may increase in concentration. However, the addition of histidinol resulted in an induction of AS mRNA to a degree nearly as great as that observed for complete amino acid starvation. In contrast, alaninol and leucinol did not enhance the level of AS mRNA. These data suggest that the mechanism for amino-acid-dependent control in mammalian cells responds to the cellular content of specific aminoacyl tRNAs. Interestingly, it appears that histidine, as is the case in yeast [38], may be unique in the degree of its effectiveness.

Collectively, the data are consistent with the conclusion that the steady-state AS mRNA content is regulated by a general control mechanism in mammalian cells. Whether or not this signalling pathway is mechanistically analogous to the general control response of yeast is not yet known.

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