**Molecular Cloning of an Amino Acid-regulated mRNA (Amino Acid Starvation-induced) in Rat Hepatoma Cells***

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Using the combination of a subtracted library and differential hybridization, a 409-base pair cDNA was identified that corresponds to a mRNA that is induced 2–3-fold when rat Fao hepatoma cells are subjected to amino acid starvation for 12 h. While this mRNA species was induced during starvation, others such as β-actin, Cu-Zn superoxide dismutase, glyceraldehyde-3-P, and histone H4 were decreased in abundance to 25–50% of their original levels. The induction of the amino acid starvation-induced (ASI) mRNA was repressed when starved cells were returned to a medium supplemented with amino acids. Tissue distribution analysis showed the ASI mRNA, approximately 650 base pairs in length, to be present in every rat tissue tested. The cDNA clone has been sequenced and appears to correspond to the 3’-most end of the mRNA. The cDNA sequence includes the poly(A) tail, two potential polyadenylation signal sequences, and an open reading frame that we presume to be a portion of the coding sequence. The ASI cDNA will be used to investigate the molecular mechanisms for amino acid-dependent regulation of protein expression by mammalian cells.

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Metabolite control of gene expression has been well documented in both bacteria and yeast, particularly control exerted by carbohydrates and amino acids. In bacteria for example, the nutrient-dependent regulation of the lactose, histidine, and tryptophan operons by the respective substrates has been well characterized (1). Starvation of bacteria for certain amino acids will activate genes coding for enzymes involved in the biosynthesis of those same amino acids (1). In yeast, a more general control of amino acid biosynthesis also exists which involves both transcriptional and posttranscriptional elements (2).

In mammalian cells, changes in amino acid availability initiate a response that is characterized by altered plasma membrane amino acid transport mediated by the System A carrier (3–5). Substrate starvation results in increased transport that can be blocked by inhibitors of transcription, translation, and glycoprotein biosynthesis (3–6). Furthermore, plasma membrane vesicles isolated from amino acid-starved cells retain elevated transport rates when compared to similarly prepared vesicles from control cells (7). These results suggest that elevated gene transcription and subsequent translation generate a membrane-bound glycoprotein, perhaps the transporter itself, that is responsible for the increased transport rates following amino acid starvation. Conversely, there is a repression of transport activity when cells are maintained in the presence of amino acid-containing medium (3–5, 8, 9). Although there are exceptions (10, 11), amino acids which are not System A substrates, such as the branched chain and aromatic amino acids, have no repressive effect on carrier protein expression. The amino acid-dependent repression of System A also occurs by a RNA- and protein synthesis-dependent mechanism (3, 8, 9). For liver tissue, alanine, asparagine, glycine, proline, serine, and threonine are the most effective repressor amino acids (9). It has been proposed that a molecular regulatory system functions to induce System A transporter gene expression when concentrations of intracellular amino acids become too low (12, 13). Conversely, when adequate substrate levels are restored, the excess functional plasma membrane transporters are inactivated or degraded and the elevated transcription rate is slowed. The laboratory of Englesberg (14–17) has employed genetic mutational analysis to obtain data showing that control of System A gene expression in Chinese hamster ovary cells is the result of at least two different regulatory genes. Translation of Chinese hamster ovary mRNA by microinjected Xenopus oocytes has confirmed the increased levels of System A mRNA in the mutant cell line (18). Interestingly, the Chinese hamster ovary mutant cell lines which exhibit constitutively high levels of System A activity also contain increased amounts of NaK-ATPase mRNA and protein (19).

To understand the molecular mechanisms responsible for amino acid-dependent regulation of gene expression in mammalian cells, it will be necessary to obtain both cDNA and genomic clones for an amino acid-regulated gene. To identify specific transcripts, other than that of System A, that are increased in abundance during amino acid starvation, we have used a combination of “subtracted” cDNA library construction and differential hybridization (20). We report here the cloning of a cDNA (amino acid starvation-induced, ASI), for which the corresponding mRNA concentration is elevated by amino acid starvation. This cDNA will be a useful tool for future studies on nutrient-dependent control of protein expression in mammalian cells.

**MATERIALS AND METHODS**

**Cell Culture and RNA Preparation**—Rat Fao hepatoma cells were maintained in modified Eagle’s medium, pHi 7.4, supplemented with 24 mM NaHCO₃, 2.5 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 28.5 μg/ml gentamicin, and 6% fetal bovine serum. Cells were transferred to Krebs-Ringer bicarbonate buffer as an

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1. The abbreviations used are: ASI, amino acid starvation-induced; MOPS, 4-morpholinepropanesulfonic acid; bp, base pair.
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Amino acid-free condition. To maintain cells in the "amino acid-fed" state, either modified Eagle's medium without serum or Krebs-Ringer bicarbonate containing 3 mM each of proline, asparagine, serine, threonine, alanine, and glycine were used. The latter amino acid-free medium was developed from data showing that these amino acids were the most efficient repressors of System A carrier activity (9).

Total cellular RNA was prepared from cells in culture or tissue samples using the method of Chomczynski and Sacchi (21) modified by including an additional phenol/chloroform extraction. Two rounds of oligo-dT chromatography were performed to purify poly(A)" mRNA from the total RNA samples (22).

cDNA Library Construction and Screening—A subtracted library (20, 23) was prepared from Fao hepatoma cells that had been cultured in amino acid-free or amino acid-supplemented KRB for 3 h. First-strand cDNA was made from the mRNA of starved cells using avian myeloblastosis virus reverse transcriptase (Seikagaku). After hydrolyzing the RNA by boiling for 5 min in 0.1 M NaOH, the cDNA was hybridized with a 10-fold excess of poly(A)" mRNA prepared from the amino acid-fed cells. The hybridization was performed at 65 °C to a R_{t,v} value of 1500. After hybridization in 0.5 M sodium phosphate, pH 6.5, containing 0.1% sodium dodecyl sulfate, the mixture was passed over a hydroxyapatite column equilibrated with 0.1 M sodium phosphate. The single-stranded cDNA fraction was eluted off the column with 0.12 M sodium phosphate. After generating double-stranded cDNA using random hexadeoxynucleotide primers and DNA polymerase I (24), the cDNA was used to prepare λ gt11 library (23). The unamplified library contained 200,000 independent plaque-forming units.

The library was plated using Escherichia coli LEB92 and duplicate replica lifts prepared. The first filter was left on the agar plate until uniformly moist, while the second lift was left on for an additional 2 min to equalize the amount of DNA bound by the two filters. Filters were treated to fix the DNA (25), prehybridized for 2-6 h at 42 °C, and then hybridized for 48 h at 42 °C in 50% formamide, 5 X SSC (1 X SSC contains 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 20 mM sodium phosphate, pH 6.5, and 1 X Denhardt's solution containing 10^5 cpm/ml of a first-strand cDNA probe prepared using mRNA from either amino acid-fed or starved cells. After hybridization, the nitrocellulose filters were washed in 1 X SSC containing 0.1% sodium dodecyl sulfate at 65 °C for 1 h, and then subjected to autoradiography using standard protocols (26). Specific conditions for the agarose gel electrophoresis and hybridization are reported in the text. Specific conditions for the agarose gel electrophoresis and hybridization are reported in the text.

DNA Sequencing—After isolating a 409-bp insert from a purified λ gt11 clone, the insert was subcloned into the vector M13 and sequenced using standard techniques (25) with the TaqTrack sequencing kit following the manufacturer's protocol (Promega Biotec, Madison, WI).

RESULTS

Isolation of AS1 Clone—The λ phage gt11 cDNA subtracted library was screened with radioactive cDNA probes derived from Fao hepatoma cells cultured in either the presence or absence of amino acids for 3 h. This time period of incubation was chosen using the time course of induction for the hepatic System A-mediated transport as a guide (8). Approximately 50,000 plaque-forming units from the unamplified library were screened. After three successive rounds of screening, over 50 potential positives were eliminated because they were not consistently induced by starvation. Following Northern analysis with several clones, a 409-bp insert was chosen that reproducibly yielded increased hybridization to RNA from amino acid-starved cells. We have called this mRNA and the corresponding cDNA "amino acid starvation-induced," or AS1.

Northern analysis of RNA from several normal rat tissues revealed a single AS1 mRNA species of about 650 bp in length as estimated by migration of RNA molecular size markers on a denaturing agarose gel.

Induction of AS1 mRNA by Starvation—Experiments utilizing RNA prepared from hepatoma cells incubated for 0-12 h in amino acid-free or amino acid-supplemented media confirmed that the cDNA identified by differential hybridization corresponded to an mRNA which exhibited elevated levels of approximately 2-3-fold in the nutrient-starved cells (Fig. 1). Equal loading of RNA was demonstrated as described under "Materials and Methods." The increase in the absolute abundance of AS1 mRNA was first detected after a lag period of 2-4 h. On a relative basis, the induction of the AS1 mRNA was greater than 2-3-fold when compared to the abundance of any one of several "control" mRNAs. Each of these mRNAs, β-actin, histone H4, Cu-Zn superoxide dismutase, and glyceraldehyde-3-phosphate dehydrogenase, was reduced in absolute amount by amino acid starvation of the hepatoma cells (Fig. 2). With time, histone H4 also declined in the amino acid-fed cells, presumably due to its coordinated expression during the cell cycle (29).

Effect of Refeeding—To demonstrate the amino acid dependence of the AS1 regulation, Fao cells were incubated for 12 h in amino acid-free media and then returned to amino acid-supplemented media (Fig. 3). After a 3-h delay, the AS1 mRNA level increased during the starvation period reaching a maximum between 9 and 12 h. Amino acid refeeding resulted in a decrease in the AS1 mRNA level. After 24 h in the

Fig. 1. Induction of AS1 mRNA following amino acid starvation of rat hepatoma cells. Total RNA was isolated from amino acid-starved Fao cells and 10 μg of RNA was electrophoretically size-fractionated, blotted, and then hybridized with either "P-labeled AS1 or β-actin (ACT) cDNA probes. Quantitation of the autoradiograph using laser scanning densitometry allowed an estimation of the AS1 mRNA induction. Equality of gel loading was established by quantitating the ethidium-stained ribosomal RNA bands as described under "Materials and Methods." Specific conditions for the agarose gel electrophoresis and hybridization are reported in the text.
presence of amino acids, the mRNA content was reduced to near basal levels. Concurrently, β-actin mRNA abundance was significantly reduced by starvation and upon refeeding the cells with amino acid rebounded to a level even greater than that present initially (Fig. 3). Cells maintained continuously for 12 h in amino acid-containing medium did not exhibit significant changes in either AS1 or β-actin mRNA levels.

**Tissue Distribution**—Equal amounts (10 μg) of total RNA prepared from various rat tissues were tested for the presence of the AS1 mRNA (Fig. 4). The highest abundance of AS1 mRNA was detected in adipose, lung, spleen, and testes, whereas the lowest content was in liver and whole blood. The size of the single mRNA species detected was similar in each tissue. By comparing the abundance of the AS1 mRNA in various amounts (0.5-10 μg) of RNA from Fao hepatoma cells, the abundance of AS1 mRNA in the hepatoma was shown to be approximately 7-fold higher than in normal liver tissue (Fig. 5).

**Partial cDNA Sequence**—The nucleotide sequence of the 409-bp cDNA insert isolated from the differential screening is shown in Fig. 6. The sequence contains a potential open reading frame from bases 2 to 361. At nucleotides 360 and 362, the sequence includes a potential open reading frame from nucleotides 2 to 360, two potential polyadenylation signal sequences (underlined), and remnants of the poly(A) tail of the AS1 mRNA.
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DISCUSSION

The processes responsible for nutrient-dependent gene regulation have been described extensively for bacteria, in particular, the well defined systems of E. coli. The foremost example is the binding of lactose to a repressor protein that releases genetic repression and allows increased transcription of the lactose permease and β-galactosidase (1). These protein products allow the cell to respond to changes in lactose availability. Also in bacteria, depletion of a single amino acid leads to increased transcriptional activity of the genes responsible for enzymes in the cognate pathway. For example, bacteria adapt to starvation of L-tryptophan or L-histidine by elevating expression of several of the enzymes responsible for the biosynthesis of the corresponding amino acid (1).

In yeast, a more general genetic control also exists. Like bacteria, amino acid starvation of yeast causes an induction of mRNAs responsible for amino acid biosynthesis pathways. However, starvation of a single amino acid in yeast can up-regulate numerous amino acid biosynthetic pathways, not just the one associated directly with the limiting amino acid (2). Up to nine different pathways, involving 30 or more genes, can be induced by this starvation procedure. An example of a starvation-induced gene in yeast is GCN4, a DNA-binding protein that serves to transduce the starvation signal by functioning as a trans-acting positive regulator of several genes coding for amino acid biosynthetic enzymes (30). Coordinate regulation of the GCN system depends on a short nucleotide sequence (5'-TGACTC-3') found in the upstream region of each GCN-regulated structural gene (31). Despite the elaboration of the GCN control system, the exact mechanism by which the cell initially detects the amino acid starvation remains unclear. Some evidence (32, 33) suggests that either a reduced tRNA charge or a decreased rate of protein synthesis is the initial signal.

In mammalian cells, few systems have been examined for regulation by nutrients or metabolites at the transcriptional level. One of the inherent difficulties in studying substrate-dependent control in higher organisms is the fact that a change in metabolite concentration in vivo can cause changes in enzyme activity via complex hormonal or neural processes rather than by direct transcriptional or translational control by the substrate itself (34). Perhaps the best studied example of substrate-dependent gene regulation in mammalian cells is control of HMG CoA reductase mRNA synthesis in liver by cholesterol availability (35). Assays for mRNA abundance and mRNA transcription have shown that the presence of sterols act to reduce the transcription rate of the HMG CoA reductase gene (36). Sequences upstreaming to either positive or negative regulatory elements have been identified (37-38). Another example is the effect of glucose availability on the expression of a family of genes termed the glucose-regulated proteins (grpS). Some of these proteins are associated with the endoplasmic reticulum and may serve some function related to the processing of proteins within this intracellular compartment (39). Deprivation of glucose causes a 10-20-fold increase in the steady-state levels of mRNA for these proteins (40). With respect to amino acid-dependent control of cellular processes, regulation of general protein catabolism (41) and protein synthesis (42) in hepatic tissue has been documented. Increased enzymatic activity of the methionyl-tRNA synthetase in response to methionine deprivation results in an increase in the amount of the enzyme, although the mechanism has not yet been determined (43). Increased biosynthesis of four specific proteins was detected in amino acid-deprived chick embryo cells, but no delineation between translational or transcriptional control was reported (44).

Our laboratory has spent a considerable effort in attempts to detect amino acid starvation-induced protein synthesis by combining pulse-labeling techniques with two-dimensional polyacrylamide gel electrophoresis (45). A limited number of liver membrane proteins have been detected for which synthetic rates appear to be increased by 2-fold following amino acid deprivation of rat hepatocytes. We have prepared a monospecific polyclonal antibody against one of these proteins, a 73-kDa polypeptide that resides in the inner mitochondrial membrane (45). The protein-labeling results appear to be consistent with the present observations indicating the presence of only a few detectable amino acid-regulated mRNAs in a subtracted library, presumably enriched for such sequences. Together these two approaches, both designed to identify amino acid-regulated genes, argue that gene control with respect to starvation of mammalian cells is considerably different than that in bacterial or yeast in which expression of a large number of proteins is enhanced by several-fold or more.

After screening 50,000 plaques from a subtracted library that was enriched 7-fold for amino acid starvation-induced clones, we obtained a cDNA clone for which the corresponding mRNA concentration was elevated reproducibly by starvation. There may be many mRNAs that are induced between 1- and 2-fold, but the method of differential hybridization makes identification of these clones difficult. Furthermore, some mRNAs may be induced to a degree greater than 3-fold, but if their relative abundance is below 0.1%, the method of differential hybridization also will be unable to detect them (20). The clone, AS1, was obtained as a 409-bp cDNA insert and hybridizes to a 650-bp mRNA. We have found no significant homologies to sequences included in the Gen Bank sequence data base. The AS1 message is substantially more abundant in the Fao rat hepatoma cell line than in normal rat liver, but the significance of this difference is unclear. A number of tissues contain AS1 mRNA levels that exceed those in normal liver, but their diversity of function does not aid in narrowing the list of possible functions for the putative AS1 protein. Northern analysis of “total” versus “membrane-bound” RNA indicates that the AS1 mRNA is translated on free ribosomes.

Although the 2-3-fold induction of the AS1 mRNA level might be considered modest, with regard to actual protein production this increase may be more significant considering the drop in absolute amounts of a number of other mRNAs during the starvation period. Thus, on a relative basis, the AS1 mRNA abundance is increased by 4- to 5-fold when compared to actin, for example. Furthermore, the absolute amount of the AS1 mRNA is high even in the basal state; the content in the hepatoma cells maintained in amino acid-containing medium is nearly equal to that of β-actin mRNA. Whether the changes in AS1 mRNA in response to substrate deprivation are due to mRNA stabilization or transcriptional control of the AS1 gene is being determined. Our failure to find any mRNA species induced greater than 3-fold leads us to speculate that after 3 h of amino acid starvation, there are few highly abundant mRNA species that are induced to a high polyadenylation signal sequences. The cDNA contains a poly(A) sequence of 17 nucleotides that presumably represents at least part of the poly(A) tail. Given that the apparent size of the mRNA is approximately 250 bases longer than the cDNA, and that the open reading frame begins at the second nucleotide of the insert and does not begin with a start codon, we assume that there is additional coding sequence and experiments are in progress to isolate a full-length clone.
degree. This observation may indicate that in mammalian cells regulation of mRNA species during catabolic states may be driven primarily by turnover of RNA species having little value to the cell, rather than high rates of transcription for mRNA species that are important for the cellular response.

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