Regulation of Asparagine Synthetase Gene Expression by Amino Acid Starvation

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We have studied the regulation of expression of the asparagine synthetase (AS) gene in ts11 cells, a mutant of BHK hamster cells which encodes a temperature-sensitive AS and therefore does not produce endogenous asparagine at 39.5°C. Incubation of ts11 cells at the nonpermissive temperature drastically increases the level of AS mRNA, and the stimulation of AS mRNA expression is effectively suppressed by the addition of asparagine to the medium. We show here that regulation of AS gene expression involves *cis*-acting elements which are contained in the mRNA as well as in the 5' genomic region. When a plasmid containing the human AS cDNA under the control of the human AS promoter region was stably transfected into ts11 cells, the expression of human AS RNAs was regulated as that of the endogenous hamster transcripts, indicating that this construct contained all *cis* elements necessary for regulation. Expression of the AS cDNA in ts11 cells under the control of a constitutive foreign promoter was also regulated by the concentration of asparagine, and this regulation required translation. When we introduced by mutagenesis a number of stop codons in the AS cDNA, the mutant mRNAs with short open reading frames were expressed at low levels that were not increased by asparagine deprivation. Inhibition of protein and RNA synthesis also prevented down-regulation of AS mRNA levels by high concentrations of asparagine. In a parallel series of experiments, we showed that an AS DNA fragment including the promoter and first exon can also regulate RNA expression in response to asparagine concentration. Furthermore, similar increases in the levels of AS RNAs are produced not only by asparagine deprivation in ts11 cells but also by deprivation of human and wild-type BHK cells of leucine, isoleucine, or glutamine. Thus, regulation of AS gene expression is a response to amino acid starvation through mechanisms which appear to involve both changes in RNA stability and changes in the rate of transcription initiation or elongation.

Asparagine synthetase (AS) is expressed in most mammalian cells as a housekeeping enzyme responsible for the biosynthesis of asparagine from aspartate and glutamine. We and others have previously reported the cloning of human and hamster AS cDNAs (1, 7, 11) and genomic sequences (10). The human AS gene promoter has features such as high G+C content, multiple RNA start sites, absence of a TATA box, and presence of GC boxes that are common to those of housekeeping genes (10).

ts11 is a temperature-sensitive growth mutant of the BHK-21 Syrian hamster cell line that owes its phenotype to the production of a mutated, temperature-sensitive AS (7, 10). While asparagine is not an essential amino acid for mammalian cells, as a result of their mutation, ts11 cells are auxotrophs for asparagine at 39.5°C. We previously reported that a shift of ts11 cells to the nonpermissive temperature caused a decline in AS activity and a rapid increase in the levels of AS mRNA. The increase in AS mRNA could be prevented by addition of asparagine to the medium, and the high levels of AS mRNA expressed at 39.5°C drastically decreased after the addition of asparagine, indicating that the expression of AS transcripts was regulated by asparagine concentration (10). This study provided a possible explanation for the earlier observations (2) that mammalian cells regulate the level of AS activity in response to the concentration of asparagine in the medium and to the extent of tRNA^{Asn} aminoacylation, and the results suggested that the AS mRNA levels play a pivotal role in this regulation.

A salient feature of ts11 cells is that at the nonpermissive

temperature, they are blocked in progression through the G_1 phase of the cell cycle (10). In culture, G_1 arrest may be achieved by various means such as limiting the supply of essential nutrients or depriving the cells of essential growth factors. Much information has accumulated recently about changes in gene activities following stimulation of quiescent cells with growth factors (23). However, little is known about the genomic responses to nutrient deprivation in mammalian cells. Uncovering the molecular events that occur in ts11 cells following incubation at the nonpermissive temperature should provide some information about the mechanisms by which nutrient limitation leads to G_1 arrest in mammalian cells. We report here that the levels of AS gene transcripts are regulated both transcriptionally and posttranscriptionally and that AS gene expression is induced not only by asparagine starvation but also by deprivation of leucine, isoleucine, and glutamine.

MATERIALS AND METHODS

Cell culture. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. When indicated, DMEM was supplemented with asparagine (50 µg/ml). When indicated, DMEM lacking isoleucine, leucine, or glutamine was used. The concentrations of those amino acids in control medium were 104.8 µg/ml (leucine), 104.8 µg/ml (isoleucine), and 60 µg/ml (glutamine). ts11 cells were maintained at 33°C, and HeLa cells or wild-type BHK-21 cells were maintained at 37°C. All experiments involving amino acid starvation (including exposure of ts11 cells to 39.5°C) were performed with DMEM plus 10% dialyzed calf serum.

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DNA transfection. ts 11 cells were transfected by the calcium phosphate coprecipitation procedure as previously described (11), using 4 μg of the supercoiled plasmid DNA mixed with 500 ng of plasmid pCB7 (13) containing the neomycin resistance gene. The transfected cells were selected by growth in medium containing G418 (500 μg/ml) for 3 weeks. Thirty to fifty colonies were combined and grown in mass culture. During the experiments, the cells were grown in normal media.

Plasmid construction. Plasmid p3.4-AS was constructed first by ligating the 3.4-kb HindIII fragment containing the 5'-flanking sequences, the first two exons and introns, and the first 6 bp of the third exon of the human AS gene to the human AS cDNA coding sequences (10), followed by deletion of an EcoRI fragment of about 120 bp toward the 3' end of the coding sequence. Plasmid p3.4-CAT was constructed by ligating the 3.4 kb of human AS gene 5' region to the chloramphenicol acetyltransferase (CAT) coding sequence and has previously been described (10). Plasmid pCD-15 was made by inserting a full-length hamster ts11 AS cDNA clone (approximately 2 kb) containing the coding sequence as well as the 5' and 3' untranslated regions into the BamHI site downstream from the simian virus 40 (SV40) early promoter in the expression vector pCD (22). The hamster AS cDNA clone 15 was isolated from ts11 cells and contained a single nucleotide substitution located within the coding region compared with the wild-type BHK AS cDNA (7). Plasmids pTK-CAT and pSV2-CAT have been previously described (8, 19). Plasmid pSV-β globin was made by inserting the 340-bp HindIII-PvuII fragment of the SV40 origin region upstream from the human β-globin coding sequences in place of the β-globin gene promoter region in plasmid pHBG 4.4 (17). The stop codon mutants of hamster AS cDNA were generated by the site-directed mutagenesis method of Kunkel (16).

The sequences of the oligonucleotides for generating the stop codon mutants of the ts11 AS cDNA were as follows: codon 23, 5'-GAGTGCTATGTAGATTGCACA-3'; codon 60, 5'-TTTGGAATGTAGCCAATAAG-3'; codon 204, 5'-ATG GTGAAGTAGCATCATTGTCG-3'; codon 398, 5'-AAGGA ACTCTAGCTGTTTGATGT-3'; and codon 464, 5'-AGAGA TCCTCTAGAGACCAAAAG-3'. The nucleotides that change the sequence of the wild-type cDNA are underlined.

RNA preparation and analysis. Total cellular RNA was extracted by the guanidine isothiocyanate-cesium chloride method and analyzed by Northern (RNA) blotting and primer extension as described previously (10).

RESULTS

Down-regulation of AS mRNA expression by asparagine requires RNA and protein synthesis. We previously showed that exposure of ts11 cells to 39.5°C, the temperature at which their AS is nonfunctional, results in a drastic increase of the levels of AS mRNAs. Under these conditions, addition of asparagine to the medium caused a rapid reduction of AS RNA levels, that by 6 h returned to the low levels of expression detected at 33°C (10; data not shown).

The stability of both hamster and human AS RNAs is very high ($t_{1/2} > 12$ h) when measured by using actinomycin D in cells grown without exogenous asparagine (data not shown). Thus, these results suggested that addition of asparagine to the media decreased AS mRNA stability, possibly through the induction of a short-lived nuclease. We therefore over-expressed AS mRNA in ts11 cells by first incubating them at 39.5°C for 12 h, and we then added asparagine to the medium
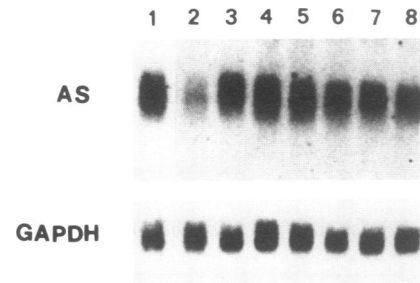

FIG. 1. Evidence that down-regulation of AS mRNA expression requires both transcription and translation. ts11 cells were shifted to 39.5°C in DMEM containing 10% dialyzed serum to induce the expression of AS mRNA. At 12 h (lane 1) after the shift, asparagine (50 μg/ml) was added to the medium either alone (lane 2) or with actinomycin D (10 μg/ml; lane 3), cycloheximide (10 μg/ml; lane 5), or puromycin (10 μg/ml; lane 7), and the cells were incubated for another 8 h. Cells treated for the same period of time (8 h) with actinomycin D (lane 4), cycloheximide (lane 6), or puromycin (lane 8) alone were also examined. At the end of incubation, total cellular RNA was extracted and analyzed (20 μg per lane) by Northern blotting, using a ^{32}P-labeled ts11 AS cDNA as a probe. The same filter was also hybridized to a rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe. As already described (10), hamster cells produce two major species of AS mRNA.

in the presence of inhibitors of transcription or translation. As shown in Fig. 1, in the presence of actinomycin D, which prevents RNA synthesis, no down-regulation of AS mRNA expression by asparagine was observed (compare lanes 2 and 3). In the presence of cycloheximide, which blocks translation elongation and traps mRNAs onto ribosomes, addition of asparagine to the culture did not result in decrease of the level of AS mRNA (compare lanes 2 and 7). In the presence of puromycin, which causes premature release of nascent polypeptides from the mRNA template, down-regulation of AS mRNA was also prevented (compare lanes 2 and 6). Thus, RNA and protein synthesis are both required for the down-regulation of AS mRNA levels in response to high concentrations of asparagine. These results suggested that addition of asparagine to the medium induces the expression of a short-lived nuclease and/or that AS mRNA degradation requires translation.

The human AS gene is regulated in ts11 cells like the endogenous hamster gene. We wished to identify the cis-acting elements involved in the regulation of AS gene expression by asparagine. Intracellular concentrations of asparagine cannot be easily manipulated in normal cells, and use of the ts11 mutant was essential to these experiments. We thus had to determine whether expression of the human AS gene was subjected to the same regulation as was expression of the endogenous hamster gene in ts11 cells. We therefore constructed a plasmid (p3.4-AS) in which a DNA fragment containing the promoter region (~300 nucleotides [nt]) as well as the first two exons (noncoding) and introns of the human AS gene was fused to the human AS cDNA at the common HindIII site as described in Materials and Methods (Fig. 2). A segment of about 120 bp near the 3' end of the coding region was deleted to render the DNA biologically inactive (data not shown). This genomic-cDNA hybrid construct was transfected into ts11 cells, about 30 independent clones of stable transfectants were pooled, and expression of the human AS mRNA was examined.

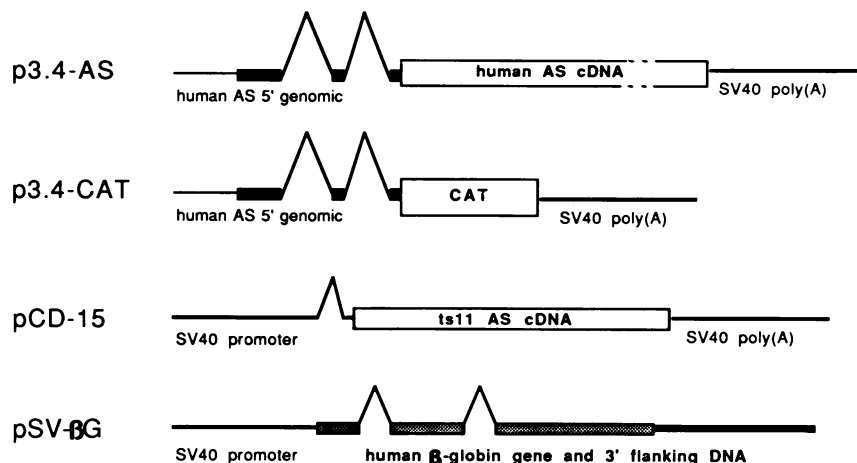Expression of the human AS mRNA was determined by primer extension analysis using a human-specific oligonucle-

FIG. 2. Schematic representation of the plasmid constructions used in this study. Plasmid p3.4-AS contains a 3.4-kb HindIII fragment of human genomic DNA including the promoter region and the first two exons (solid bar) and introns (bent lines) and the first 6 bp of the third exon of the AS gene. This genomic fragment was fused through a common HindIII site to a human AS cDNA containing the coding region and the 3' untranslated sequences including a poly(A) addition site. The human AS cDNA contains a deletion (dashed lines) of 120 bp toward the 3' end of the coding region in order to render the expression of the plasmid biologically inactive. The plasmid also contains the SV40 late gene polyadenylation signal sequences. Plasmid p3.4-CAT was constructed by inserting the same 3.4-kb human AS genomic DNA as in p3.4-AS at the HindIII site upstream of the CAT gene coding sequences. Plasmid pCD-15 contains a full-length hamster ts11 cDNA encoding a temperature-sensitive AS, including a 56-base 5' untranslated region placed downstream from the SV40 early promoter in the expression vector pCD (13). Plasmid pSV-βG contains the SV40 early promoter in place of the human β-globin gene promoter and its associated 5' untranslated region.

otide corresponding to a region in the second exon (10) to detect the AS RNAs initiated from the expected start sites. For determination of the expression of endogenous hamster AS mRNAs, an oligonucleotide corresponding to a sequence in the 5' untranslated region (7) and specific for hamster AS mRNAs was used. The level of mRNAs from the human AS constructs increased severalfold following incubation of the stable integrants of ts11 cells at 39.5°C (Fig. 3A and data not shown), and the increase was suppressed by the presence of asparagine. Thus, expression of the human AS gene is regulated in ts11 cells like that of the endogenous hamster gene, and our hybrid genomic-cDNA construct appears to contain all cis elements necessary for regulation.

Regulation of AS mRNAs levels by 5' elements. To determine whether the regulation of AS mRNA expression involved transcriptional components, we stably transfected ts11 cells with a plasmid in which the 3.4-kb human AS 5' fragment described above was fused to the bacterial CAT gene (p3.4-CAT; Fig. 2). Transfectants were shifted to 39.5°C in the presence or absence of asparagine. The level of CAT RNAs under the control of the AS 5' region was substantially increased by asparagine starvation (Fig. 3A, p3.4-CAT). As a control, we used a plasmid in which the CAT gene was under the control of the herpes simplex virus type 1 thymidine kinase (TK) promoter with its associated 5' untranslated region (pTK-CAT). In ts11 cells stably transfected with plasmid pTK-CAT, the level of CAT mRNAs was higher when cells were cultured in the presence of asparagine than when they were cultured in its absence. In all cases, the increases in the level of endogenous hamster AS mRNAs in each pool of transfectants in response to amino acid deprivation were similar (Fig. 3B). Thus, the human AS 5' region contains elements which contribute to the regulation of AS mRNA levels in response to asparagine. However, since the AS 5' DNA fragment used in these experiments contains, in addition to the AS promoter, also

the first two exons and introns, these results do not indicate whether these elements are operating at the transcriptional or posttranscriptional level (see Discussion).

Posttranscriptional regulation of AS mRNA expression by asparagine deprivation. While the results presented in the previous section suggested transcriptional regulation of AS RNA expression, the findings that addition of asparagine led to a rapid decrease in AS RNA levels in ts11 cells at 39.5°C and that this decrease was prevented by inhibitors of RNA and protein synthesis indicated that posttranscriptional mechanisms were also involved in the regulation of AS mRNA expression. To verify this hypothesis, we constructed a plasmid in which the hamster ts11 AS cDNA was linked to the SV40 early promoter (which also provides a 65-base 5' untranslated region) in a mammalian expression vector (Fig. 2). For comparisons, similar plasmids containing the human β-globin or the bacterial CAT gene were also constructed. The plasmids were independently transfected into ts11 cells, and pools of about 30 independent clones were expanded for analysis. The ts11 AS cDNAs, the human β-globin gene, and the CAT gene sequences expressed from the SV40 early promoter all contained an SV40 early gene 5' untranslated sequence of about 65 nt at their 5' termini. The exogenous ts11 AS RNAs and the CAT RNAs were detected by primer extension analysis using an oligonucleotide corresponding to a sequence in the SV40 early 5' untranslated region, while the human β-globin mRNA was detected by an oligonucleotide containing sequences derived from the β-globin coding region.

As shown in Fig. 4A, the level of the relatively stable human β-globin mRNAs (pSV-βG) in ts11 cells incubated at 39.5°C in the presence of asparagine was slightly higher than that in its absence. The level of the relatively labile CAT mRNA (pSV2-CAT) was similar in ts11 cells incubated with or without asparagine. In contrast, the level of the RNAs expressed from full-length ts11 AS cDNA (pCD-15) contain-
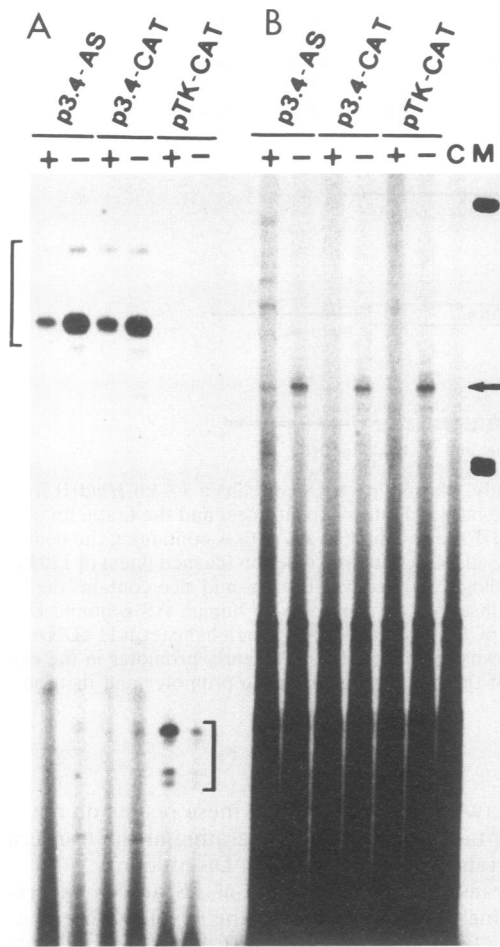
FIG. 3. Regulation of expression of the human AS gene in ts11 cells. Structures of plasmids p3.4-AS and p3.4-CAT are shown in Fig. 2. Plasmid pTK-CAT contains the herpes simplex virus type 1 TK promoter and its associated 5' untranslated region upstream from the CAT coding sequences and has been described previously (19). Stable transfectants of ts11 cells transfected with the plasmids were obtained as described in Materials and Methods. More than 30 independent clones transfected by each plasmid were pooled and grown in mass culture at 33°C. The cells were then shifted to 39.5°C in fresh DMEM containing 10% dialyzed serum for 12 h in the presence (+) or absence (−) of added asparagine (50 μg/ml). Then 30 μg of total RNA from each treatment of plasmid p3.4-AS and p3.4-CAT transfectants and 60 μg from pTK-CAT transfectants were analyzed by primer extension analysis (A); 15 μg of total RNA of each sample were also analyzed by primer extension for the levels of the endogenous hamster AS mRNA (B). The oligonucleotide used for detection of the human AS mRNAs expressed from the human AS promoter contains the sequence 5'-TC AAGTGATTTATTCAGATG-3', corresponding to a region in the second exon; the oligonucleotide for mRNA expressed from the viral TK promoter contains the sequence 5'-ACGCTGTTGACGCTGTTAAGC GGGT-3', corresponding to nt −50 to −25 of the TK gene. The extended cDNA products (indicated by brackets) obtained from end-labeled oligonucleotides by the reverse transcriptase were electrophoresed on a 7% polyacrylamide-urea gel along with a φX174 DNA HaeIII digest (lane M) as the size marker, of which the two bands shown are 118 and 72 nt in length. The sizes of the cDNA products were those expected of the RNAs initiated from the respective AS and TK gene RNA start sites determined previously (10, 20). The oligonucleotide used for detection of the endogenous hamster AS mRNA contains the sequence 5'-ATGCTGCAGGAGAGCTTCAGGCACT-3', and the major cDNA product is indicated by an arrow. This oligonucleotide did not detect the human AS mRNA, as hybridization to the HeLa mRNA did not produce any detectable cDNA products (lane C).
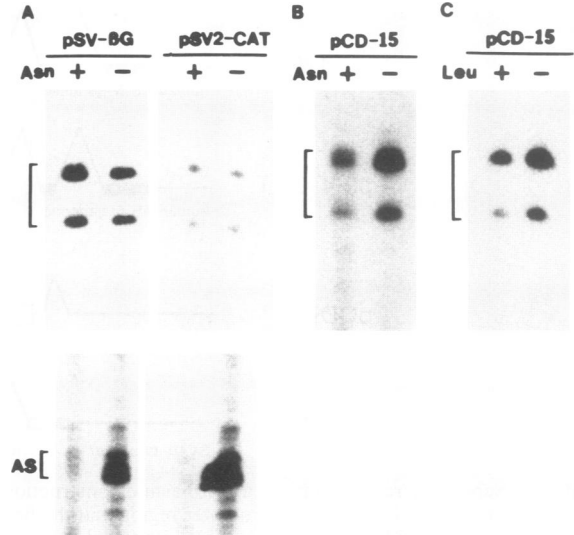

FIG. 4. Regulation of expression of ts11 AS cDNA by amino acid starvation. Plasmids pSV-βG and pCD-15 are shown in Fig. 2. Plasmid pSV2-CAT contains the SV40 early promoter upstream from the CAT coding sequences. (A) About 30 independent clones of ts11 cells stably transfected with either plasmid pSV-βG or plasmid pSV2-CAT were pooled and grown at 33°C. The cultures were shifted to 39.5°C for 10 h in DMEM containing 10% dialyzed serum in the presence (+) and absence (−) of asparagine (50 μg/ml). Total cellular RNA was extracted, and 30 μg was analyzed for the levels of β-globin or CAT RNAs by primer extension analysis. The oligonucleotide used for detection of the human β-globin RNA contains the sequence 5'-GGGCAGTAACGGCAGACTTCT-3', complementary to a sequence located 43 to 23 nt downstream from the globin AUG start codon; the oligonucleotide for detection of CAT RNAs contains the sequence 5'-GGAGCTTTTTGCAAAAGCCT AGGCC-3', which corresponds to a region in the SV40 early gene 5' untranslated sequences. The cDNA products of the expected sizes that were extended from each end-labeled oligonucleotide are indicated by brackets in the upper panel. The SV40 early promoter contains two major RNA start sites spaced 5 bp apart, and therefore two major cDNAs whose sizes differ by 5 nt are produced from each oligonucleotide. Twenty micrograms of each RNA sample was also analyzed for the endogenous hamster AS RNA by primer extension analysis using an oligonucleotide as described in the legend to Fig. 2. The major AS cDNA products (AS) detected by this oligonucleotide are shown in the lower panel. (B) Stable transfectants of ts11 cells transfected with plasmid pCD-15 were generated as described in Materials and Methods. The cultures were then shifted to 39.5°C for 12 h in DMEM containing 10% dialyzed serum in the presence (+) or absence (−) of asparagine (50 μg/ml). Total cellular RNA was extracted, and 80 μg of each sample was analyzed for AS RNAs expressed from the transfected gene by primer extension analysis using an oligonucleotide as described for panel A for the detection of CAT RNAs. The extended cDNA products expected for RNA expressed from the SV40 promoter are indicated by a bracket. (C) Culture of the stable transfectants of ts11 cells transfected with plasmid pCD-15 was shifted to 39.5°C in DMEM with (+) or without (−) leucine and containing added asparagine (50 μg/ml) for 12 h. Total RNA (100 μg) extracted from the cells was analyzed for AS RNAs expressed from the transfected gene, using an oligonucleotide as described for panel B.

ing the coding region as well as 5' and 3' untranslated sequences was increased about fivefold in ts11 cells in the absence of asparagine (Fig. 4B). Thus, expression of the full-length AS cDNA linked to a foreign constitutive promoter was substantially increased by the deprivation of asparagine in ts11 cells, strongly suggesting that changes in
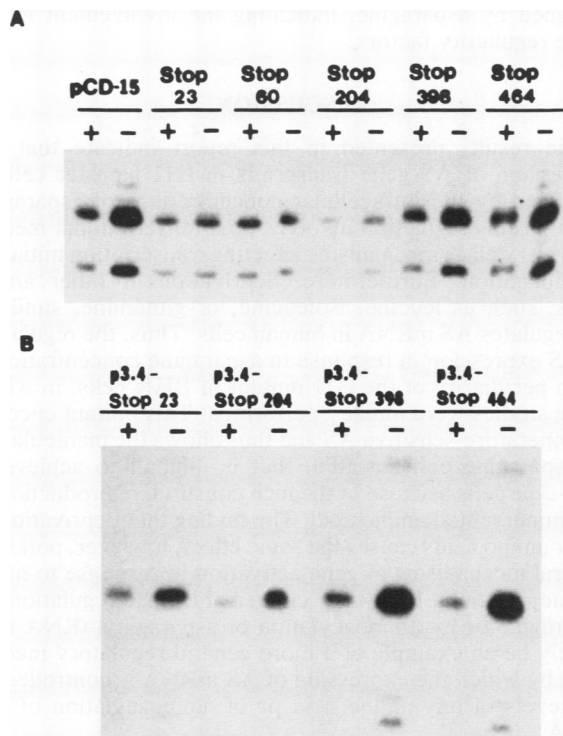
A

B

FIG. 5. Regulation of expression of the stop codon mutants of hamster AS cDNA by asparagine deprivation. (A) Hamster ts11 AS cDNA plasmid pCD-15 was as described in the legend to Fig. 4. Mutants of ts11 AS cDNA each containing a stop codon (codon positions indicated by the numbers) in the AS coding region were generated as described in Materials and Methods and were placed in the pCD vector similarly as in pCD-15. Cultures of pooled ts11 transfectants were shifted to 39.5°C for 12 h in DMEM containing 10% dialyzed serum with (+) or without (−) added asparagine (50 µg/ml). Total cellular RNAs were then extracted, and 80 µg of each sample was analyzed for levels of AS RNAs expressed from the transfected gene by primer extension analysis using an oligonucleotide as described for Fig. 4B. The two cDNA products extended from the end-labeled oligonucleotides are shown. (B) Plasmids p3.4-stop 23, 204, 398, and 464 contain the 3.4-kb human AS 5' genomic DNA as described for p3.4-CAT (Fig. 2) in place of the SV40 early promoter in plasmids pCD-stop 23, 204, 398, and 464, respectively, as described for panel A. Stable transfectants of ts11 cells of each plasmid were obtained as described in Materials and Methods. Culture dishes of pooled transfectants were shifted to 39.5°C for 12 h in DMEM containing 10% dialyzed serum, asparagine (50 µg/ml) was then added to half of the dishes, and the cells were incubated for another 10 h with (+) or without (−) added asparagine. Total cellular RNA (10 µg) from each sample was analyzed for levels of AS RNAs expressed from the transfected gene by primer extension analysis using an oligonucleotide corresponding to a region in the human AS second exon as described for Fig. 3.

stability of the AS mRNAs play a significant role in the regulation of AS RNA levels by asparagine.

Regulation of AS mRNA stability by asparagine requires translation. The experiments shown in Fig. 1 using cycloheximide and puromycin indicated that continued protein synthesis is required for the down-regulation of AS mRNA expression by asparagine. To address the hypothesis that translation of AS mRNA constitutes an important step in the regulation of its stability by asparagine, we constructed several translation mutants of AS cDNA. The full-length ts11 AS cDNA contains 561 codons for the translation of AS. By

site-directed mutagenesis, we constructed ts11 AS cDNAs that contained an in-frame stop codon at position 23, 60, 204, 398, or 464. To minimize structural perturbations of the RNA, all of the mutants contained a single nucleotide substitution. The mutant cDNAs were then inserted into the expression vector containing the SV40 early promoter described above (pCD-15; Fig. 2), and their expression in response to asparagine deprivation was examined in pools of stable transfectants of ts11 cells. As shown in Fig. 5A, the level of the wild-type (pCD-15) AS mRNA increased severalfold following asparagine deprivation, as shown previously. On the other hand, levels of the AS mRNAs containing a stop codon at position 23 or 60 were not changed. Levels of the mutant RNA containing a stop codon at position 204 increased by about twofold, and those of the translation mutants at codon 398 or 464 increased substantially.

The results of this experiment were somewhat surprising. Not only they do not substantiate the hypothesis that degradation of AS RNA requires translation, but they actually suggest that translation contributes to the stabilization of AS RNAs. In the absence of a long open reading frame (e.g., stop 23, 60, and 204), the levels of AS RNAs are uniformly low with or without asparagine, and asparagine deprivation elevates the levels of only those RNAs that can be extensively translated. These results thus indicate that translation is an obligatory requirement for the regulation of AS mRNA stability by asparagine (see Discussion).

To evaluate further the role of translation in the regulation of AS mRNA abundance by asparagine, we examined the expression in ts11 cells of the same translation mutants of ts11 AS cDNAs that were now placed under the control of the human AS promoter and first two exons and introns. We used the same 3.4-kb 5' AS DNA fragment that was shown (Fig. 3) to be capable of mediating some degree of asparagine-regulated gene expression. In line with this finding, comparison of the results of Fig. 5A and B shows that while the levels of mutant RNAs with short open reading frames (e.g. stop 23 and 204) expressed from the SV40 promoter are not increased by asparagine deprivation, the abundance of the same mutant RNAs under the control of the AS promoter region is increased to a significant extent. However, the levels of AS RNAs containing a stop codon at position 398 or 464 were severalfold more inducible by asparagine deprivation than those containing a stop codon at position 23 or 204. Thus, a substantial portion of AS mRNA needs to be translated for its levels to be maximally increased by asparagine deprivation.

AS gene expression is regulated by starvation for several different amino acids. In yeast cells, starvation for different amino acids activates several biosynthetic pathways through the induction of common transcriptional regulators (12). To determine whether increased abundance of AS gene transcripts could be obtained by different nutrient deprivation, we measured AS expression in cells which had been deprived of the essential amino acid leucine, isoleucine, or glutamine. We found that starvation for these amino acids induces AS gene expression as much as does asparagine deprivation in ts11 cells.

Figure 6A shows that AS mRNA was expressed at drastically elevated levels in ts11 cells incubated at 39.5°C in DMEM without added asparagine (compare lanes 1 and 2), while the level of glyceraldehyde 3-phosphate dehydrogenase was little changed (lower panel). In medium lacking the essential amino acid leucine, AS mRNA was expressed at high levels either with or without added asparagine (compare
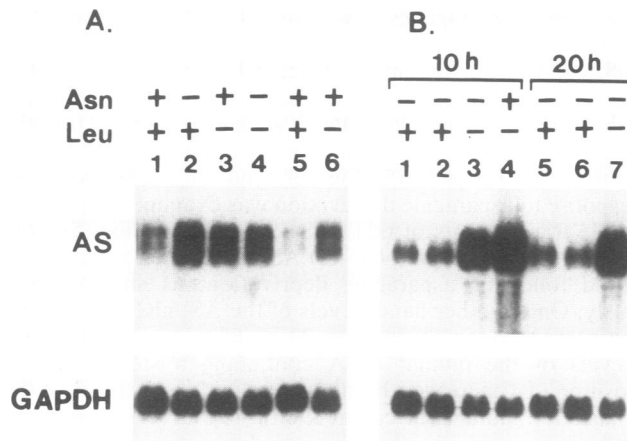
A.

B.

FIG. 6. Regulation of AS mRNA expression by starvation for many amino acids. (A) ts11 cells growing at 33°C were shifted to 39.5°C for 10 h (lanes 1 to 4) in DMEM containing added asparagine (lane 1) or in DMEM lacking (−) either leucine (lane 3) or added asparagine (lane 2) or both (lane 4). ts11 cells starved for both leucine and asparagine for 10 h at 39.5°C were supplemented with both leucine and asparagine (lane 5) or asparagine alone (lane 6) and were incubated at 39.5°C for another 8 h. At the end of the incubations, total cellular RNA was extracted and analyzed by Northern blotting (15 µg per lane) with a ts11 AS cDNA probe or a rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe. (B) HeLa cells were plated in DMEM (lane 1 and 5), in DMEM lacking leucine (lane 3 and 7), or in DMEM lacking leucine but supplemented with either leucine (lane 2 and 6) or asparagine (lane 4) and incubated at 37°C for 10 or 20 h. Total cellular RNA (15 µg per lane) was analyzed by Northern blotting with a human AS cDNA and a rat GAPDH cDNA probe.

lanes 3 and 4 with lane 1 or lane 5 with lane 6). Deprivation of these two amino acids did not act synergistically. Approximately the same level of AS RNA was achieved in response to double rather than single amino acid deprivation (compare lanes 2 and 4).

We further tested AS mRNA expression in response to amino acid deprivations in other cell lines. In HeLa cells, which do not require exogenous asparagine for normal growth, the level of AS mRNA also increased dramatically in medium lacking leucine (Fig. 6B; compare lane 3 with lanes 2 and 1). The activation of AS mRNA expression in HeLa cells was not due to deficient production of asparagine caused by leucine deprivation, since addition of asparagine to the medium did not inhibit the activation (Fig. 6B; compare lanes 3 and 4). Similar responses were obtained following isoleucine or glutamine deprivation, and the same was true also in wild-type BHK cells (data not shown). Thus, expression of AS mRNA in mammalian cells is also regulated in response to changes in the concentration of leucine, isoleucine, glutamine, and possibly most of the essential amino acids in the medium.

We then examined ts11 cells stably transfected with the various AS plasmid constructs described above, including those in which AS mRNA transcription was driven by the SV40 promoter (pCD-15) and those in which CAT expression is under the control of the AS 5′ region (p3.4-CAT), to determine whether expression of these constructs was also regulated by starvation of other amino acids. In both cases (Fig. 4C and data not shown), the transfected constructs were regulated by leucine starvation in the same manner as

obtained by asparagine, indicating the involvement of the same regulatory factors.

DISCUSSION

The results presented in this report indicate that the expression of AS gene transcripts in ts11 hamster cells is regulated by the intracellular concentrations of asparagine and that this regulation involves posttranscriptional mechanisms as well as mechanisms affecting transcription initiation or elongation. Furthermore, deprivation of other amino acids, such as leucine, isoleucine, or glutamine, similarly up-regulates AS mRNA in human cells. Thus, the regulation of AS expression in response to asparagine concentration is not a peculiarity of the ts11 mutant of BHK cells, in which these studies were initially performed. This mutant encodes a temperature-sensitive AS and thus allows the manipulation of asparagine concentration that is difficult to achieve in wild-type cells because of the high constitutive production of this nonessential amino acid. The finding that deprivation of other amino acids causes the same effect, however, points to general mechanisms of gene activation in response to nutrient deprivation. It is thus conceivable that regulation by asparagine or by aminoacylation of asparaginyl-tRNA may merely be an example of a more general regulatory mechanism by which the expression of AS mRNA is controlled by the levels of any amino acid or of aminoacylation of any tRNA.

The data presented in this report clearly establish that the regulation of AS mRNA levels has a posttranscriptional component affecting RNA stability. The stability of AS mRNA, when measured by blocking transcription with actinomycin D (Fig. 1 and data not shown), is very high both in the presence and in the absence of asparagine. Since addition of asparagine to ts11 cells expressing high levels of AS mRNA results in a rapid and drastic decrease of these levels, it is possible that asparagine induces the expression of a nuclease and that actinomycin D prevents this induction.

The mechanisms affecting AS RNA stability, however, appear to be complex. We found that the expression of mRNAs containing stop codons shortening the open reading frame was low and not regulated by asparagine. Thus, translation appears to be required for regulation of AS RNAs levels. Translation is linked in many cases to the regulation of mRNA metabolism. Premature translation termination was shown to reduce the half-life of stable mRNAs, such as that of β-globin (14, 25). It has also been shown that the autoregulatory feedback control of β-tubulin mRNA turnover requires it to be ribosome bound (31). Moreover, for cell cycle-dependent control of histone mRNA stability to occur, translation must proceed close to the 3′ end of the mRNA (9). While the exact mechanism by which translation might be coupled to the control of mRNA turnover in each case remains to be determined, translation of ribosomes could protect mRNA from nucleolytic attack or could influence mRNA structure and affect its metabolism (30). It has been proposed that ribosomes may contain an associated RNase whose activity can be regulated (4, 26, 31). Translation could also facilitate the processing or transport of mRNA molecules (3, 5, 6, 22, 28).

We have observed that the transcripts of mutant AS cDNAs containing a stop codon 5′ to codon 204 not only were not increased in levels by asparagine deprivation but were expressed at consistently lower levels than those of the wild type in ts11 cells supplemented with asparagine (Fig. 5 and data not shown). It is possible that the entire sequence of

the coding region of AS mRNA, and minimally that residing between codon 205 and the normal stop codon at position 562, is accessible to cytoplasmic nuclease degradation when free of ribosomes. Amino acid starvation could potentially result in inhibition of the synthesis of this putative endonuclease, which may be associated with ribosomes, and thus cause an increase in the levels of AS mRNA. While this hypothesis does not explain why mutant AS RNAs containing stop codons at position 23, 60, or 204 would not be stabilized by asparagine deprivation, it is possible that ribosomes transit also prevents the formation of a structure that destabilizes the AS mRNA. Since ribosome translation would operate only marginally on the three mutant AS mRNAs, they were thus rapidly degraded and not subjected to regulation by asparagine deprivation. In this regard, it was shown that adenovirus VAI-RNA could function to stabilize and increase the level of cytoplasmic mRNAs, but not if their translation was prevented by elimination of the initiator AUG codon or by introduction of stop codons early in the coding region (26), possibly because a major effect of VAI-RNA consists in stimulating translation.

Our experiments using cycloheximide and puromycin further indicated that down-regulation of AS mRNA expression requires protein synthesis. Thus, paradoxically, both the increase in AS RNA levels caused by asparagine deprivation and its down-regulation in response to asparagine require translation. mRNAs whose instability appears to be obligatorily linked to ongoing protein synthesis include those of c-*fos* and c-*myc* (15, 18, 21). While both mRNAs contain in their 3' untranslated region AU-rich elements that can mediate mRNA destabilization, each also contains distinct sequences in the coding region that can specify rapid mRNA turnover in a translation-dependent manner (25, 29). It remains to be determined whether the AS mRNA contains specific sequences that can mediate regulation of mRNA stability in a translation-dependent manner in response to amino acid starvation.

Finally, some of the results presented in this report indicate that the 5' region of the human AS gene also contains elements involved in the regulation of mRNA expression by asparagine. Since this region contained, in addition to the AS promoter, also the first two exons and introns, these results could be still due to posttranscriptional mechanisms. We attempted to measure the rate of transcription of the endogenous AS gene in hamster and HeLa cells by nuclear run-on experiments, but the results were inconclusive, probably because of the low rate of transcription of this gene (data not shown). Results to be reported at a later date (11a), however, indicate that the regulatory elements involved are located in the promoter and first exon and thus are likely to affect transcription initiation or elongation. In *Saccharomyces cerevisiae*, transcriptional induction of many amino acid biosynthetic genes in response to amino acid starvation is mediated by the *GCN4* gene product, which acts as a positive regulator (12). In *Escherichia coli*, expression of the tryptophan biosynthesis operon is regulated by many factors, including (i) repression of transcription in which tryptophan acts as a corepressor and (ii) attenuation of transcription (24). The role of transcription factors such as Sp1, which has three potential binding sites in the AS promoter region, as well as that of the sequences of the first exon in regulating AS gene expression is being investigated. The finding that starvation for a variety of amino acids has the same effect as asparagine deprivation on AS gene expression seems to suggest that a mechanism similar to that operating in yeast cells could be operative

here. Alternatively, transcription elongation in a region corresponding to the first exon could be under the control of a putative elongation blocker, whose synthesis could be affected by amino acid starvation. Further analysis of AS gene regulation should uncover the common motifs through which amino acid starvation regulates gene expression and affects cell proliferation.

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