c-my c mRNA is Stabilized by Deprivation of Amino Acids in Primary Cultured Rat Hepatocytes

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Summary We investigated whether the expression of growth-related genes could be changed in primary cultured hepatocytes in response to changes in the nutritional environment. Hepatocytes were isolated from the liver of growing rats after collagenase perfusion and cultured in Williams' E medium (WE medium) containing 5% calf serum, 10⁻⁷ M insulin and 10⁻⁶ M dexamethasone for 24 h. When amino acids were removed from the culture, the level of c-my c mRNA increased more than 18-fold within 2–3 h, whereas replenishment of the amino acids to the medium caused rapid decrease in the mRNA level. We found that the half-life of the c-my c mRNA was prolonged more than 6-fold in the absence of amino acids. The mRNA levels of other proteins, such as ornithine decarboxylase, c-Ha-ras and actin, and their half-lives were not affected by amino acids. It is known that a short-lived protein is involved in the degradation of c-my c mRNA. In fact, the addition of cycloheximide to cultured hepatocytes increased the level of c-my c mRNA either in the presence or absence of amino acids, though the levels of other mRNAs were not changed significantly. These results suggest that the synthesis of the short-lived protein is suppressed and the c-my c mRNA is thereby stabilized in the absence of amino acids.

Key Words amino acids, c-my c, primary cultured hepatocytes, half-life

Cell growth and proliferation are controlled by extracellular signals through their transduction pathways. Not only various growth factors and hormones but also cell-cell and cell-extracellular matrix contact have long been recognized as important modulators of normal cell growth both in vivo and in culture (I–5). Although protein or amino acid nutrition is absolutely required for cell growth, the interaction between nutrition and growth signal transduction has not been studied much yet. We previously examined whether expression of growth-related genes in growing rat tissues could be modulated by manipulation of dietary nutrition and
demonstrated that the level of c-myc mRNA and the rate of DNA synthesis in the liver were changed depending on the nutritive quality of dietary protein (6, 7). In contrast to many reports in which a transient increase in c-myc expression was observed when cell growth was stimulated (8-11), the level of c-myc mRNA in the liver paradoxically increased by feeding of protein-free diet on which their growth was arrested. However, when a casein diet was fed to rats which had been maintained on protein-free diet, the level of liver c-myc mRNA decreased and DNA synthesis was induced. Whereas zein diet, which lacks tryptophan and lysine, neither suppressed the c-myc mRNA level nor induced DNA synthesis in the liver unless deficient amino acids were supplemented. From these results, we hypothesized that the liver cell cycle was arrested at G1 phase during feeding of protein-free diet, because c-myc mRNA was known to increase transiently at G1 phase when quiescent cells were stimulated by growth factors (11), and that good quality proteins were required for the cell cycle to progress to S phase. In this context, we suggested the possibility that protein nutrition not only supplies material for body components but also plays a role as a signal for the progression of the liver cell cycle in young growing rats (7).

To examine this possibility, we determined the levels of c-myc mRNA in primary cultured hepatocytes in the presence and absence of amino acids. Here we demonstrated that the cellular level of c-myc mRNA rose more than 18-fold by amino acid starvation and that the half-life of the c-myc mRNA was prolonged 6-fold under these conditions.

EXPERIMENTAL

Materials. Deoxycytidine 5'[^32P]triphosphate (3,000 Ci/mmol) and Multiprime DNA-labeling system were purchased from ICN Biomedicals Inc. (USA) and Amersham Corp. (England), respectively. Male Sprague-Dawley rats, 5 weeks of age, were obtained from CLEA Japan, Inc. (Tokyo). Tissue culture dishes (100×15-mm) were products of Nunc Inc. (USA). Collagenase was obtained from Nitta Gelatin (Tokyo). Human c-myc (0.467 kb) and Harvey murine sarcoma virus v-Ha-ras (0.56 kb) were purchased from Takara Shuzo (Kyoto). An 0.7 kb PstI fragment of rat liver ornithine decarboxylase cDNA (12) and a 2.0 kb HindIII fragment of β-actin cDNA (13) were prepared in our laboratory. Earle medium (salt/glucose medium) was purchased from Nissui Pharmaceutical (Tokyo). Williams' E medium, amino acids and other chemicals were purchased from Nacalai Tesque (Kyoto).

Cells. Parenchymal hepatocytes were isolated from the livers of male Sprague-Dawley rats after in situ perfusion of the liver with collagenase and the cells (5×10^4 cells/cm²) were cultured as monolayers in 100-mm dishes in 10 ml WE medium supplemented with 5% calf serum, 10⁻⁷ M insulin and 10⁻⁶ M dexamethasone (complete medium) at 37°C under 5% CO₂ in air. Three hours later, the medium was replaced with fresh complete medium and incubated under the same conditions.

conditions for 21 h.

**RNA isolation and analysis.** Total cellular RNA was isolated by the acid phenol guanidinium thiocyanate method (14), and samples (10 μg each) were electrophoretically separated on a 1% agarose/formaldehyde gels and blotted onto nitrocellulose membranes. Hybridization analysis was conducted as described previously (6). As hybridization probes, the cDNAs were labeled with 32P using the multiprime DNA labeling system. Each band which hybridized with the probes was detected and quantitated using Fujix Imaging Analyzer Bas 2000 (Fuji Photo Film Co., Tokyo).

**RESULTS AND DISCUSSION**

We recently reported that hepatic levels of several growth-related gene transcripts, such as c-myc, ornithine decarboxylase (ODC) and c-Ha-ras mRNAs were elevated when growing rats were fed protein-free diets (6, 7). To determine whether the expression of these growth-related genes were affected directly by changes in the nutritional environment, we examined the levels of these mRNAs in cultured hepatocytes in the presence and absence of amino acids. Figure 1 shows that the levels of c-myc, ODC and c-Ha-ras mRNAs in isolated hepatocytes are essentially undetectable, but the mRNAs increased to a substantial level during culture in complete medium (Fig. 1). We also observed that the level of actin mRNA increased 10-fold gradually during the culture for 45 h (Fig. 1). Although the physiological significance of increased expression of these genes is not known, the above results may indicate that the hepatocytes dedifferentiate gradually during the culture, as suggested from the inverse expression of cytoskeletal- and liver-specific genes (15) and the reciprocal relation of growth and differentiated liver functions (1, 16, 17) of hepatocytes in culture.

When amino acids were removed from the medium, however, only the expression of c-myc mRNA was changed. Thus, the level of c-myc mRNA rapidly increased 18-fold in 5 h after the removal of amino acids by replacement of the complete medium with Earle medium, whereas the levels of other mRNAs were not affected by the medium change (Fig. 1). The level of c-myc mRNA also increased but to a lesser extent by replacement of the complete medium with WE medium (Fig. 1). Neither serum, insulin nor dexamethasone affected the change in the expression of c-myc mRNA by the medium (data not shown). Therefore, it seems likely that the level of c-myc mRNA in cultured hepatocytes is mainly regulated by amino acids. On the other hand, the level of ODC and c-Ha-ras mRNAs were not influenced by the medium change although they were increased in the growing rat liver by feeding of protein-free diet (7), suggesting that factors other than amino acids were involved in the regulation of the expression of these two mRNAs. In fact, we have reported that glucagon increases the level of ODC mRNA in cultured hepatocytes incubated either in the presence or absence of amino acids (12).

The elevated level of c-myc mRNA in the hepatocytes incubated in the Earle
medium decreased to 40% of the maximum level within 1 h upon replacement of
the Earle medium with WE medium (data not shown). Expression of c-myc
mRNA is known to be regulated at both transcriptional and posttranscriptional
levels (18–20). The c-myc mRNA is extremely unstable, with a half-life of less than
1 h (21, 22), which can be lengthened in response to growth factors and or
translational inhibitors (19, 23). We and others have reported independently that
protein deprivation induces c-myc expression in liver of rats and re-feeding of
protein diet to the rats causes a rapid decrease in the level of c-myc mRNA and
induces DNA synthesis (6, 7, 24–26). Mead et al. demonstrated that the increase in
hepatic c-myc mRNA level during protein deprivation is regulated by a post-
transcriptional mechanism (26). This indicated that the nutritional environment
can affect the stability of c-myc mRNA. We therefore determined the half-life of
c-myc mRNA in the presence and absence of amino acids.

Hepatocytes were pre-cultured in Earle medium for 3 h, then the medium was

c-myc mRNA IS STABILIZED BY AMINO ACIDS DEPRIVATION

Fig. 1. Time course of changes in the amounts of c-myc, ODC, c-Ha-ras and actin mRNAs during primary culture of rat hepatocytes. Cells were treated as described in the EXPERIMENTAL section and incubated in complete medium for 24 h and the medium was replaced with fresh WE medium (●) or Earle medium (○). As a control, a part of cells were incubated in complete medium without medium change (■). (a) Total cellular RNA was extracted from cells of each dish at indicated times and Northern blot analysis was performed with 32P-labeled c-myc, ODC, C-Ha-ras and actin cDNAs. (b) The intensities of the corresponding mRNA bands were quantitated with an imaging analyzer and the results are expressed as relative values compared with that of 24 h cultured cells.

changed to WE medium or fresh Earle medium and the decay of c-myc mRNA was examined in the presence of actinomycin D (Fig. 2). The result clearly indicated that the half-life of c-myc mRNA was prolonged about 6-fold in hepatocytes incubated in Earle medium. The change seems to be specific to c-myc mRNA, since the half-lives of actin, c-Ha-ras and ODC mRNAs were not influenced by the medium (Fig. 2).

It has been reported that constitutively expressed cytosolic factors are involved in the degradation of c-myc mRNA. Two proteins (37 and 40 kDa) were found to bind to AU-rich sequences located in the 3'-untranslated region of c-myc and accelerate c-myc decay in vitro (27). It is conceivable that synthesis of these factors

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Fig. 2. Effect of amino acids on the half-lives of c-myc, β-actin, c-Ha-ras, and ODC mRNAs in primary cultured hepatocytes. Cells were prepared and cultured in complete medium for 24 h as described in the EXPERIMENTAL section, then the medium was replaced with Earle medium to increase the level of c-myc mRNA. After 3 h, the medium was replaced with fresh WE medium (●) or fresh Earle medium (○) containing 2.5 μg/ml actinomycin D. Total cellular RNA was prepared from triplicate dishes at indicated times and Northern blot analysis was performed with 32P-labeled c-myc, ODC, and β-actin cDNAs, respectively. For determination of the level of c-Ha-ras mRNA, total cellular RNA was extracted from pooled cells of three dishes at each time point, and Northern blot analysis was performed using 32P-labeled c-Ha-ras cDNA. The intensities of the bands of each mRNA were determined with an imaging analyzer and the values are expressed in arbitrary units.

is suppressed in hepatocytes incubated in Earle medium, and consequently the c-myc mRNA is stabilized. To examine this possibility, we compared the effect of cycloheximide on the expression of c-myc mRNA, since the cytosolic factors may be labile and their destabilizing activity may be abolished by the inhibition of protein synthesis caused by cycloheximide (19). When cycloheximide was added to the medium, the level of c-myc mRNA increased dramatically in hepatocytes incubated in either Earle or WE medium, whereas cycloheximide did not affect the expression of actin, c-Ha-ras, and ODC mRNAs (Fig. 3). Although cycloheximide affected specifically the expression of c-myc mRNA as we expected, the level of c-myc mRNA in cycloheximide-treated hepatocytes was 3-fold higher than in the
Fig. 3. Effect of cycloheximide on the expression of c-myc, β-actin, c-Ha-ras, and ODC mRNAs in primary cultured hepatocytes. Cells were prepared and cultured in complete medium for 24 h as described in the EXPERIMENTAL section. Then, the medium was replaced with Earle medium alone (a), Earle medium containing 10 μg/ml cycloheximide (b) or WE medium containing 10 μg/ml cycloheximide (c). The cells were harvested 2 h after the medium change. Total cellular RNA was prepared and Northern blot analyses were performed with 32P-labeled c-myc, ODC, β-actin and c-Ha-ras cDNAs, respectively. The intensity of the band of each mRNA was determined with an imaging analyzer and the results are expressed as relative values compared with that of cells cultured in Earle medium alone. The values represent the means of duplicate dishes.

c-myc mRNA is stabilized by amino acids deprivation

It has been reported that cycloheximide does not affect the transcription of normal cells (28). For instance, cycloheximide-caused superinduction of c-myc mRNA in regenerating rat liver was solely due to stabilization of the mRNA but not to transcriptional stimulation (9). Therefore, it is likely that the transcription rate of c-myc gene was not affected by amino acids, since the level of c-myc mRNA was not different in cycloheximide-treated cells incubated either in Earle or in WE medium (Fig. 3). However, the c-myc mRNA increased when the medium was changed to fresh WE medium (Fig. 1), suggesting that components other than amino acids contained in the fresh medium may stimulate the transcription of c-myc gene. It can be concluded that the increase in the level of c-myc mRNA in hepatocytes cultured in Earle medium was caused mainly by stabilization due to suppression of the synthesis of cytosolic factor.
involved in degradation of c-myc mRNA.

Although it is not known yet whether the increased level of c-myc mRNA in the hepatocytes cultured in amino acid-deficient medium affects the cell cycle of the hepatocytes, our present results as well as previous results reported by us (6, 7) and by other workers (24–26) suggest the possibility that amino acid nutrition affects the expression of growth-related genes and modify the signal transduction for cell growth.

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