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Regulation of Protein Synthesis Initiation in HeLa Cells Deprived of Single Essential Amino Acids

(valine/histidine/methionine/high-temperature inhibition)

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ABSTRACT **In HeLa cells deprived of valine, histidine, or methionine initiation of protein synthesis decreases rapidly and disaggregation of polyribosomes also occurs. The mechanism of inhibition does not seem to involve the supply of RNA in the cell, and thus it differs from the initiation of inhibition at elevated temperatures. Polyribosomes rapidly form again if the missing amino acid is restored, even in the presence of actinomycin D.**

Studies of HeLa cells deprived of single essential amino acids have shown that protein synthesis continues in this situation at a reduced rate determined by the rate of endogenous protein turnover (1). Such cells are in a condition of suspended growth and offer a unique opportunity for the study of metabolic regulation. It has previously been established that the rate of synthesis of ribosomal precursor RNA is reduced in these cells (2, 3), and maturation into complete ribosomal subunits is restricted (3), though synthesis of ribosomal proteins is uninterrupted (4).

In several recent studies (5, 6), it was found that ascites tumor cells deprived of a complete mixture of essential amino acids had extensive disaggregation of polyribosomes into inactive monoribosomes. The disaggregation was reversed if the set of essential amino acids was restored. However, since all of the essential amino acids were withheld or supplied at the same time, it is uncertain whether the apparent regulation of protein synthesis that was observed involved the availability of amino acids *per se*, or amino acids in the role of donors of amide or methyl groups.

We have found that HeLa cells, deprived of any one of several essential amino acids, promptly exhibit a marked inhibition of translational initiation. This inhibition and its reversal have been studied and compared to previous demonstrations of control of translational initiation in cells undergoing mitosis (7) and in cells exposed to elevated temperatures (8).

MATERIALS AND METHODS

Cell culture

Suspension cultures of HeLa cells were maintained in Eagle's medium with 5% calf serum, as described (9, 10), at a concentration of 2–6 × 10^5 cells/ml. Most cultures were grown with [^{3}H]uridine (0.025 μCi/ml, 20 Ci/mmol) or [^{14}C]uridine (0.005 μCi/ml, 50 Ci/mol) for 20 hr before use, in order to label their ribosomes uniformly.

Amino-acid Deprivation, Cell Labeling, and Fractionation. Cells were collected by centrifugation and rinsed in Eagle's medium without valine, histidine, or methionine, but with leucine at a concentration of 0.02 mM (one-twentieth of the normal concentration), and 5% dialyzed calf serum. They were then resuspended in a medium of the same composition at a concentration of 3 × 10^6 cells/ml. Cells acting as growing controls were resuspended in medium to which the missing amino acid had been added to its normal concentration. All cultures were incubated at 37°C, except where noted

Suspension cultures deprived of an amino acid for specified times, or growing control cultures, were incubated for 45 min, and then labeled for various times with [^{14}C]leucine (0.25 μCi/ml, 12.5 Ci/mol final specific activity). 8-ml samples were then rapidly cooled and fractionated by methods previously described (11). This procedure yields an initial cytoplasmic fraction and first nuclear rinse, pooled and designated *cytoplasm 1;* a detergent rinse of the nuclei containing 10–20% of the cytoplasmic ribosomes, designated *cytoplasm 2;* and *clean nuclei.* Radioactivity in polysomes and smaller particles was analyzed (12) by making cytoplasmic fractions 0.5% in both Brij-58 nonionic detergent and sodium deoxycholate.

TABLE 1. *Distribution of [^{3}H]uridine-labeled ribosomes in the cytoplasm of growing and amino-acid-deprived cells**

Type of culture	Cpm in cytoplasm 1	Cpm in cytoplasm 2	% of Ribosomes in:† Polyribosomes	% of Ribosomes in:† Monoribosomes + subunits
Growing control	35,600	7,370	68	32
− methionine	30,100	4,140	26	74
− histidine	37,800	5,040	28	72
− valine	36,100	4,130	30	70

* The data are from the experiment of Fig. 1. Cells that had been previously labeled with [^{3}H]uridine for one generation were used.

† Data from the analyses of cytoplasmic fraction 1 shown in Fig. 1.

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Fractions were centrifuged for 75 min at 4°C, in a Spinco SW 40 rotor at 39,500 rpm, on 15–30% sucrose gradients in 0.01 M NaCl–0.0015 M $MgCl_2$–0.01 M Tris (pH 7.2). The pH of gradient fractions, from analyses of cytoplasm containing radioactive leucine, was adjusted to 10 with NaOH that contained excess unlabeled leucine and the fractions were incubated for 30 min at 37°C to discharge aminoacyl-tRNA.

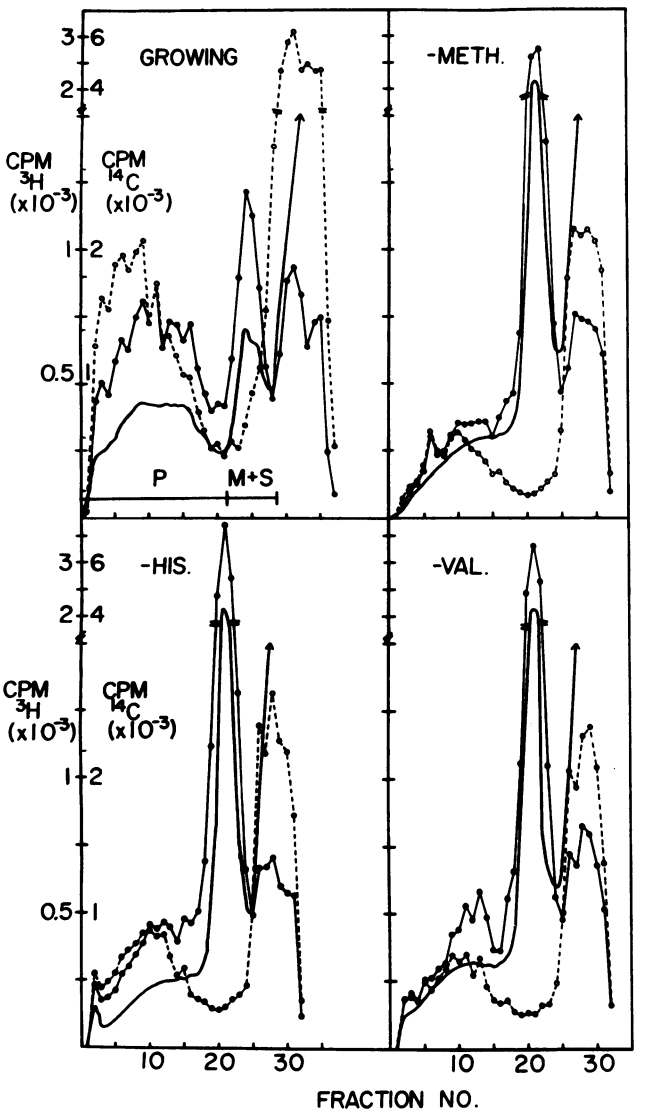

FIG. 1. Sedimentation analysis of polyribosomes from the cytoplasms of growing and amino-acid-deprived HeLa cells. Cytoplasm fraction 1 was prepared from cells pulse-labeled for 2 min with [^{14}C]leucine after incubation in normal growth medium, or in medium lacking methionine, valine, or histidine, for 2 hr. All cells were from a culture previously labeled with [^{3}H]uridine for one generation. The UV-absorption profiles of the gradients are shown as a continuous line, on an arbitrary linear absorbance scale. The trichloroacetic acid-precipitable radioactivity in each fraction is shown for ^{14}C, O—O, and ^{3}H, ●—●. The gradient regions containing polyribosomes, *P*, and monoribosomes and ribosomal subunits, *M* + *S*, are indicated from the analysis of growing control culture. [^{14}C]leucine in the polyribosome region was considered to be in nascent peptides, while that sedimenting more slowly was considered incorporated into finished protein.

Carrier bovine serum albumin was added and the fractions were made 10% in trichloroacetic acid and collected on filters for scintillation or low-background Geiger counting.

The rate of [^{3}H]leucine incorporation into cell protein was measured essentially as previously described (13), including treatment of samples with dilute NaOH before acid precipitation in the presence of excess unlabeled leucine. Cultures were given [^{3}H]leucine (0.9 μCi/ml), and 0.5-ml samples were taken at 5, 10, 15, and 20 min for determination of the linear rate of incorporation.

RESULTS

Effects of essential amino-acid deprivation on ribosome distribution, and on translational initiation and elongation

Previous studies (1) have shown that the absolute rate of protein synthesis declines 3- to 4-fold when actively growing HeLa cells are placed in a medium lacking a growth-essential amino acid. Sedimentation analyses of cytoplasmic fraction 1 from growing cells or from cells deprived of methionine, histidine, or valine for 2 hr are shown in Fig. 1, which indicates that deprivation causes a substantial decrease in the number of ribosomes in polyribosomes, a substantial increase in free monoribosomes, and a decrease in the average size of the remaining polyribosomes. Cells have been previously labeled with [^{3}H]uridine for a period (20 hr) such that this radioactivity accurately indicates the distribution of ribosomal RNA; cells have been also pulse labeled (2 min) with [^{14}C] leucine, to demonstrate the distribution of nascent chains on active ribosomes. The monoribosomes of cells deprived of amino acid, like those of growing cells, are seen to be inactive in protein synthesis. If the size of polyribosomes is taken into consideration, the amount of nascent peptide chain per ribo-

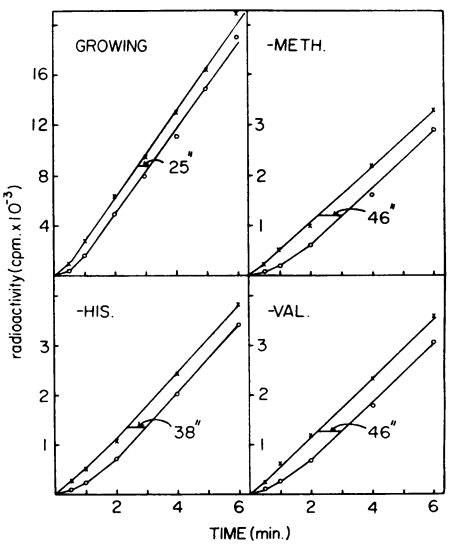

FIG. 2. Time required for the synthesis of a peptide chain in growing and in amino-acid-deprived cells. Pulse-labeling experiments with [^{14}C]leucine were performed and analyzed as in the experiment of Fig. 1, with various labeling times. The total amounts of ^{14}C incorporated in all peptides, ×—×, and in finished peptide chains, O—O, were determined and are shown versus the labeling time. Each point is the average of two or more determinations. The time lag between the lines fitted to the linear portion of the curves is equal to one-half the average peptide synthesis time (compare ref. 15).

TABLE 2. *Distribution of [^{14}C]leucine incorporated in a two-minute labeling of growing and amino-acid-deprived cells**

Type of culture	Total cpm incorporated	Cpm in fraction:			% cpm in:†	
		Nuclear	"Cytoplasm 1"	"Cytoplasm 2"	Nascent protein	Released protein
Growing control	57,400	4,260	44,000	9,130	30	70
− methionine	13,100	764	10,900	1,420	30	70
− histidine	17,000	988	13,700	2,270	36	64
− valine	13,900	691	11,800	1,340	32	68

* The data are from the experiment shown in Fig. 1, and described in its legend.

† Acid-precipitable, alkali-resistant ^{14}C radioactivity in the polyribosome and post-polyribosome sedimentation regions is considered to be localized in nascent and complete released peptide chains.

some is similar in the polyribosomes of growing and deprived cells. Thus, in comparison to growing control cells, about half of the ribosomes in the main cytoplasmic fraction of amino-acid-deprived cells are active in protein synthesis. This suggests the amino-acid deprivation affects not only the rate of peptide elongation, but also other steps in translation.

Since the membrane-bound ribosomes found in the detergent rinse of the nuclei (cytoplasm 2) have been reported (14) to differ in some respects from the bulk of the ribosomes, found in cytoplasm 1, the relative amounts and activities of ribosomes in both fractions were compared. Amino-acid deprivation induces significant changes in the proportions of ribosomes in the two cytoplasmic fractions (Table 1). However, other experiments have shown that the disaggregation of polyribosomes induced by amino-acid deprivation was similar in both fractions.

The distribution between cytoplasmic fractions 1 and 2 of [^{14}C]leucine incorporated in a pulse label is shown in Table 2 to be similar in amino-acid-deprived and control cells. Thus, the relative protein synthetic activities of the ribosomes in the two cytoplasmic fractions seem to be similarly altered by amino-acid deprivation.

After 2 min of labeling with [^{14}C]leucine, the proportion of incorporated amino acid in nascent versus complete, released peptide chains is similar (Table 2) in cytoplasm fraction 1 of growing control and amino-acid-deprived cells. Similar ratios were also found for nascent versus released, complete chains in cytoplasm fraction 2 in the growing and deprived cell. These results suggested that amino-acid deprivation has relatively little effect on the time required to synthesize peptide chains. We investigated this point further by combining the results of several experiments of the type shown in Fig. 1, using different labeling times and sedimentation analyses of cytoplasm fraction 1. For such brief labeling periods the radioactivity in the nuclear fraction is negligible (Table 2), and there is no kinetic evidence that protein synthesis in cytoplasm fraction 2 differs significantly from that in fraction 1. The results of the analyses of labeling kinetics (Fig. 2) show that the average time for the synthesis of a peptide chain during amino-acid deprivation is not more than double that in growing cells.

Since the rate of peptide elongation and the number of active ribosomes are approximately halved in cells deprived of amino acid, the average size of the polyribosomes is reduced, and the absolute rate of protein synthesis is reduced 3- to 4-fold (Table 2), it follows that the *initiation* of translation is also reduced, 2- to 4-fold. This conclusion assumes that the peptide chains made by growing control cells and cells deprived of amino acid are of the same average size. The assumption is supported by studies to be published elsewhere (Pawlowski and Vaughan) on protein synthesis during valine deprivation.

Similar results are obtained if any one of the three different essential amino acids is omitted from the medium.

Ribosome distribution as a function of the duration of deprivation of an essential amino acid

The proportion of ribosomes in polyribosomes of cytoplasm fraction 1, and the relative rate of protein synthesis, are shown in Fig. 3 as a function of the time of histidine deprivation. Histidine deprivation was selected for further study since histidine is an infrequent amino acid in HeLa cell protein (16) and is not thought to function in these cells except as an amino-acid constituent of protein. The lowest ratio of active ribosomes to polysomes occurs after 1 hr of deprivation, at the cell concentrations used, but after 2 hr the proportion of ribosomes in polyribosomes consistently increases again. The absolute rate of protein synthesis falls rapidly and after

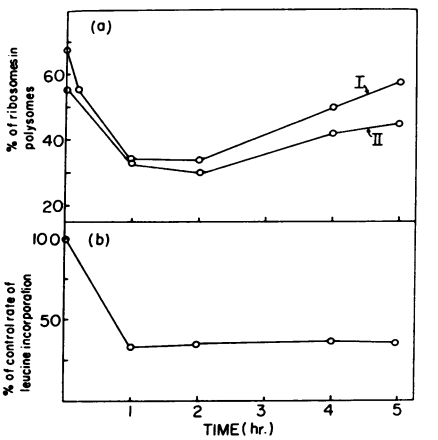

FIG. 3. Proportion of ribosomes in polyribosomes and the relative rate of protein synthesis as a function of the length of deprivation for histidine. The proportion of ribosomes in polyribosomes, (*a*) in histidine-deprived cells previously labeled with [^{14}C]uridine, was determined in experiments performed and analyzed as in Fig. 1. The curves *I* and *II* are from separate experiments. The relative rate of protein synthesis (*b*) was determined from [^{3}H]leucine incorporation into total cell protein as described in *Methods*. The culture was that of curve *II* in part (*a*).

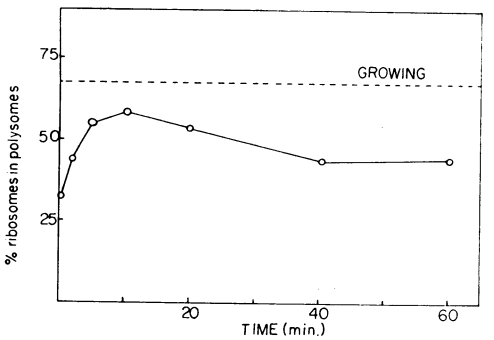

FIG. 4. The re-formation of polyribosomes after restoration of histidine to cells deprived of histidine for 2 hr. The proportion of the total ribosomes in polyribosomes was determined as a function of time after the restoration of histidine to its normal concentration (0.2 mM) in a culture previously labeled with [^{3}H]uridine for one generation and deprived of histidine for 2 hr. All details as for Fig. 1. The dotted line indicates the percentage of ribosomes in polyribosomes in a control culture.

the first hour remains steady, limited by endogenous protein turnover at about 1%/hr (1). The increase of ribosomes in polyribosomes after a 2-hr is thus probably due to a further increase in the average time of peptide synthesis. We conclude from these results that histidine deprivation rapidly triggers a mechanism for inhibiting translational initiation.

Recovery from amino-acid deprivation and the effects of inhibiting RNA synthesis or incubating at elevated temperatures

The kinetics of polyribosome re-formation when histidine is restored after a 2-hr deprivation are shown in Fig. 4. Consistently, there is a rapid increase in the number of ribosomes in polyribosomes, which reaches nearly the proportion found in growing cells within 10 min. If the average peptide synthesis time promptly returns to its normal value, these results suggest that the addition of histidine rapidly reverses the inhibition of translational initiation. The subsequent decrease of ribosomes in polyribosomes, which lasts an hour or more, was seen repeatedly and is not readily interpreted at present.

The rapid reversal of polyribosome disaggregation upon the restoration of histidine does not depend upon simultaneous RNA synthesis, since it is essentially insensitive to the addition of actinomycin D, as shown in Table 3. Actinomycin D had little effect on polyribosome recovery even when added 50 min before histidine was restored. Since the recovery process does not require the synthesis of new messenger or other RNA, the supply of RNA does not seem to be directly involved in the inhibition of translational initiation after amino-acid deprivation.

The effect of incubation at 42°C was investigated (8). Other workers found evidence for a factor, apparently an RNA, that limits translational initiation. Table 3 presents data confirming extensive disaggregation of polyribosomes in growing cells exposed to a temperature of 42°C, but it also shows that incubation at 42°C of cells previously deprived of histidine for 2 hr actually causes a small, reproducible increase of ribosomes in polyribosomes. The factor that normally limits translational initiation at 42°C is apparently more abundant in histidine-deprived cells and thus is probably not limiting for translational initiation in these cells. In the previous study (8), it was found that if protein synthesis was reduced for 2 hr with cycloheximide, cells responded by considerably increasing synthesis of the factor that limits for translational initiation at 42°C. Hence, one might expect a similar response during histidine deprivation. This has been confirmed by our finding that there is little disaggregation of polysomes in cells exposed to 42°C after 10 min of recovery from a 2-hr histidine deprivation.

DISCUSSION

These experiments have revealed what appears to be a significant metabolic control in HeLa cells, a prompt inhibition of translation initiation when cells are deprived of single essential amino acids. It is known that cultured human cells can adapt to this deprivation and retain their viability for many days. Protein synthesis in these cells declines to 25–30% of the rate in actively growing cells (1), as endogenous protein turnover makes the limiting amino acid available for reutilization.

It is unlikely that the effect observed on translational initiation can be a simple, direct consequence of limited loading of the tRNA carrying the amino acid in question. In general, reduced loading of one or more tRNA species will be rate-limiting for peptide elongation, and causes a build-up of ribosomes in polyribosomes, rather than a decline, as ribosomes queue before the relevant codons in mRNA. This is in fact observed (17) in rabbit reticulocytes deprived of single essential amino acids, which include two of the amino acids used in the present work, valine and histidine. Theoretically, a decrease in supply of an amino acid whose codon was at the point of translational initiation could cause decreased initiation and thus polysome disaggregation. However, in the present study essentially the same degree of inhibition of translational initiation, affecting the majority or all of the polyribosomes, was seen when HeLa cells were deprived of any one of the three amino acids. Decreased

TABLE 3. *Effects of actinomycin D and incubation at 42°C on ribosome distribution in growing and histidine-deprived cells*

Type of culture	Cpm in polyribosomes	Cpm in monoribosomes + subunits	Ribosomes in polyribosomes (%)
Growing control	15,700	7,570	68
Growing, 30 min with actinomycin D	11,500	13,800	45
Growing, 10 min at 42°C	5,550	30,100	16
− histidine, 2 hr	8,380	18,100	32
− histidine, 2 hr, plus 10 min at 42°C	9,760	15,700	38
− histidine, 2 hr, then* actinomycin D and 30-min recovery	12,300	15,100	45

The data are from sedimentation analyses of cytoplasm 1, from cells previously labeled for 20 hr with [^{14}C]uridine. Actinomycin D was used at a concentration of 5 μg/ml to effect total cessation of RNA synthesis (18). All cultures were incubated at 37°C, unless stated otherwise.

* After 2 min with actinomycin D, histidine was added to the normal concentration. Cells were harvested after a 30-min recovery.

loading of particular tRNA species may be a signal for the regulation of translational initiation in HeLa cells, but it is probably not directly limiting this process.

Control of translation at the level of initiation has previously been seen in cultured mammalian cells during mitosis (7), and during incubation at 42°C (8). The limiting factor for initiation at 42°C seems to involve the cellular content of an unidentified RNA, whose synthesis is increased by the cells in response to decreased translation. This factor seems to be enhanced in amino-acid-deprived cells. Furthermore, the substantial recovery of polyribosomes when missing amino acids are restored to deprived cells previously treated with actinomycin D suggests that decreased translational initiation during amino-acid deprivation is not primarily due to a lack of any required RNA. The factors limiting initiation at 42°C and during amino-acid deprivation are thus almost certainly different.

The inhibition of translational initiation during mitosis is also reported (7) to involve some limiting factor other than RNA. It is possible that the limiting factors for translational initiation in mitosis and amino-acid deprivation are related, but, if so, the factor involved during mitosis must remain potentially active throughout all or most of the cell cycle, since we have used asynchronous cultures.

It is interesting that in both mitosis and the early phase of amino-acid deprivation, the proportion of active ribosomes declines to the same level, approximately one-third of the total, which is also the proportion in HeLa cells, which have adapted to incubation at 42°C. This is probably the minimum number of active ribosomes needed to keep the total cell protein content from decreasing, if we assume essentially normal translation rates, in the face of constant protein turnover at about 1%/hr.

Our results raise the problem of the mechanism by which HeLa cells can regulate translational initiation according to the availability of single essential amino acids. This unknown mechanism may also have a role in the 2- to 3-fold decrease in synthesis of 45S ribosomal precursor RNA that occurs in amino-acid deprivation (2, 3). Work is in progress on the nature of the signal that triggers the inhibition of translational initiation. It may be significant that amino-acid deprivation does not cause polyribosome disaggregation in rabbit reticulocytes (17), with the exception of deprivation for tryptophan, which occurs only adjacent to the N-terminus of rabbit globin. Perhaps in the process of differentiation and enucleation, these cells have lost certain controls over the translational process.

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