Regulation of Initiation Factors during Translational Repression Caused by Serum Depletion

ABUNDANCE, SYNTHESIS, AND TURNOVER RATES*

Roger Duncan and John W. B. Hershey
From the Department of Biological Chemistry, School of Medicine, University of California, Davis, California 95616

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During growth in unreplenished medium, the fraction of active, polysomal ribosomes progressively decreases about 3-fold from 80-90% to only 20-40% due to a reduced rate of initiation. To assess whether the abundance of initiation factors could be involved in this repression of translational activity, HeLa cell cytoplasmic lysates were resolved by two-dimensional isoelectric focusing/sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and spots corresponding to the initiation factor proteins were quantitated. The relative abundance of most of the initiation factor proteins only decreases by 10-40% and roughly parallels that of the ribosomes. Measurement of the rates of synthesis and turnover of the initiation factor proteins establishes that during periods of active growth, synthesis and degradation occur coordinately with total cell protein. As growth rate decreases, the synthesis of some initiation factor proteins, particularly eukaryotic initiation factor (eIF)-3 subunits, becomes depressed. The principal exceptions are eIF-3p24 and eIF-4A whose syntheses are moderately stimulated. Serum stimulation of serum-depleted cells recruits most inactive ribosomes and mRNAs into polysomes, but most initiation factor mRNAs are not selectively recruited. The principal exceptions are eIF-3p24 which exhibits 4-5-fold enhanced synthesis and eIF-3p44 and eIF-4A whose syntheses are moderately stimulated.

The rate of protein synthesis in a wide variety of eukaryotic cells is affected by changes in the external environment. In the majority of cell types which have been studied, the specific phase of translation that is regulated is the initiation process (1), although the molecular alterations responsible for affecting initiation rates are, by and large, uncharacterized. The initiation factors are a set of proteins which transiently associate with tRNA, mRNA, and ribosomal subunits during the assembly of these components into an active initiation complex (2, 3). The initiation factors are ideally suited to be regulators of the initiation process by virtue of their central and requisite role in the separate initiation reactions. A well-studied example is the phosphorylation of initiation factor eIF-2, which inhibits the ability of the factor to catalytically activate initiation (4, 5). A second example comes from the poliovirus-infected HeLa cell, in which the abundance of the p220 subunit of the cap-binding protein complex, eIF-4F, is drastically reduced by proteolysis and apparently causes the inhibition of capped mRNA translation (6). Little information currently exists as to whether other initiation factors are involved in modulating the activity of the translational machinery.

The activities of numerous enzymes are regulated by the protein levels in cells. Frequently, enzymes which regulate the overall flux of metabolites through a pathway exhibit high synthesis and turnover rates (7). By altering the rate of synthesis or degradation, a new cellular level can be reached rapidly. It is reasonable to postulate that changes in the abundance of one or more initiation factors may serve to regulate the overall rate of protein synthesis, and that such factors would be synthesized and degraded at relatively rapid rates. In this report, the synthesis, turnover, and abundance of over 10 of the initiation factor proteins have been surveyed to ascertain whether the metabolism of these proteins is correlated with regulated translational activity. Lysates were examined from cells exhibiting different rates of protein synthesis due to varying times of growth in unreplenished medium. The status of the factor proteins was analyzed by two-dimensional gel electrophoresis of unfraccionated cell lysates, relying upon our identification of a set of spots which correspond to the HeLa cell initiation factors (8).

EXPERIMENTAL PROCEDURES AND RESULTS

Quantitation of Initiation Factor Proteins—When HeLa cells are grown without replenishing the growth medium, the rate of protein synthesis progressively declines (15, 16) due to a reduction in the initiation rate (Table 1). The characteristics of this translational repression during serum depletion are described in detail the Miniprint Section. To determine whether the amounts of the initiation factors decrease during serum depletion and might limit the rate of initiation, their levels were measured at 24-h intervals after the serum depletion regime was begun. Quantitations were determined by fractionating 35S-labeled protein on IEF/SDS-PAGE and excising and counting those spots previously identified as corresponding to the initiation factors (Fig. 1 and Table 2). Additional details covering the electrophoresis, quantitation, and labeling procedures are presented in the Miniprint Sec-

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1 The abbreviations used are: eIF, eukaryotic initiation factor; IEF, isoelectric focusing; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; FPO, 2,5-diphenyloxazole; POP, 1,4-bis[2-(5-phenylxazolyl)]benzene.

2 Portions of this paper (including "Experimental Procedures," part of "Results," Fig. 1, and Tables 1 and 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9630 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-3485, cite the authors, and include a check or money order for $3.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
tion. Two $^{35}$S-labeling regimes were used: (i) $[^{35}S]$methionine was added from the time of replating until protein was extracted; and (ii) $[^{35}S]$methionine was added for a 24-h interval prior to extraction (see "Experimental Procedures" for details). The quantitative results were indistinguishable using either labeling regimen.

The results of the quantitations show that for all of the eIF proteins, a progressive decline in amount occurs (Fig. 2). The extent of the decline for eIF-4A, eIF-3p44, and eIF-3p24 is about 50%. eIF-2$\beta$ and eIF-3p40 decrease by $\sim$40%, although the decline in eIF-2$\beta$ is likely caused in part by the appearance of variant forms in depleted cells (17), which were not quantitated because they were not unambiguously resolved on the autoradiograms. For the remainder of the eIF proteins, the extent of decline is only 10–30%. Such small abundance reductions are not likely to be directly responsible for inhibiting the rate of initiation by 3-4-fold, although a contributory role cannot be excluded.

The initiation factor quantitations determined by IEF/SDS-PAGE were confirmed by immunoblotting (see "Results" in Miniprint and Fig. 3). The abundance of the ribosomal proteins likewise shows little or no variation during the serum depletion regime (see "Results" in Miniprint and Table 3). Thus, the initiation factor/ribosome ratios remains relatively constant for the initiation factors that show little abundance decline and may decrease by 50% for the factors whose abundance decreases the most.

Rates of Synthesis of the Initiation Factor Proteins — The intracellular abundance of proteins, including the initiation factor proteins, is controlled by their respective rates of synthesis and degradation. We next measured these rates to provide additional insight into how initiation factor protein levels are regulated. We note that the maintenance of relatively constant levels of eIF proteins during serum depletion does not imply that synthesis and degradation rates remain constant, but only that they are coordinately varied. In order to approach the problem of factor synthesis rates, we measured the fraction of radioactivity incorporated into specific eIF proteins compared to total proteins. Cells were pulse-

![Fig. 2. Quantitation of the initiation factor proteins during growth in unreplenished medium.](image)

**TABLE 3**

| Ribosomal protein (arbitrary name) | Abundance after labeling for: |
|-----------------------------------|-----------------------------|
|                                   | 0-24 h | 0-48 h | 0-72 h | 0-96 h |
| RP1                               | 0.068  | 0.073  | 0.076  | 0.070  |
| RP2                               | 0.070  | 0.072  | 0.070  | 0.065  |
| RP3                               | 0.080  | 0.075  | 0.061  | 0.058  |
| RP4                               | 0.087  | 0.070  | 0.094  | 0.093  |
| RP5                               | 0.120  | 0.132  | 0.136  | 0.119  |
| RP6                               | 0.148  | 0.137  | 0.150  | 0.134  |
| RP7                               | 0.135  | 0.138  | 0.138  | 0.132  |

| % total acid-extracted protein    |        |
|-----------------------------------|--------|
| RP1                               | 0.070  |
| RP2                               | 0.065  |
| RP3                               | 0.058  |
| RP4                               | 0.093  |
| RP5                               | 0.119  |
| RP6                               | 0.134  |
| RP7                               | 0.132  |

**Fig. 3. Quantitation of the initiation factor proteins by immunoblotting.** Cells were plated and propagated in nonradioactive culture medium just as for the labeling analysis. After 1, 2, 3, or 4 days of growth, protein was extracted by the IEF/SDS-PAGE sample preparation method. Samples of about 30 $\mu$g were resolved by one-dimensional SDS-PAGE on 100 $\times$ 150 $\times$ 0.75 mm slab gels, electrophoretically transferred to nitrocellulose paper, and sequentially treated with anti-initiation factor protein antibodies followed by $[^{125}$I]-labeled second antibody. Autoradiograms of gels treated with antibodies to eIF-2$\alpha$, 2$\beta$, and 2$\gamma$ (panel A) or eIF-4A and eIF-4B (panel B) are shown. The gel in panel A was 10% total acrylamides, 0.26% bisacrylamide and the gel in panel B was 15% total acrylamides, 0.06% bisacrylamide. Lanes D1, D2, D3, and D4 were extracted after 1, 2, 3, and 4 days of growth, respectively.

**TABLE 3**

Quantitation of ribosomal proteins

Cells were labeled continuously from the time of replating until harvesting with $[^{35}$S]methionine. Extracted protein was analyzed by two-dimensional gel electrophoresis in the presence of carrier ribosomal protein as described under "Experimental Procedures." The amount of radioactive protein applied to each gel was determined from a trichloroacetic acid precipitate of an aliquot of the gel sample. Several independently prepared samples were each analyzed two or more times by two-dimensional gel electrophoresis, and the averages are reported as the counts in the specific ribosomal protein spot divided by the input radioactivity. The abundancies have not been corrected for either protein molecular weight or methionine content. Thus, the values are not absolute abundancies, but they may be used to compare the relative abundance of the same protein in different states.

Labeled with $[^{35}$S]methionine over a 30-min interval, and radioactivity incorporated into eIF proteins was quantitated as described above. The per cent radioactivity values were then converted to molar ratios. Details of the labeling protocols and data analysis are given under "Experimental Procedures." These procedures provide a measure of the relative rates of synthesis of eIF proteins compared to total protein.
Relative synthesis rates were examined in serum-stimulated, exponentially growing, and serum-depleted cells as described under "Experimental Procedures" and are reported in Table 4. In serum-stimulated and exponentially growing cells, the percentage of factor molecules synthesized lies in the range of 0.05–0.10 for most factors, with eIF-3p24 and eIF-3p40 decrease to 75% and the polymerization rate decreases to about 3 amino acids/s in serum-stimulated cells (9). Thus, the values provided further evidence that the steady state form of each initiation factor protein is also its primary mRNA translation product, since initiation factor protein spots after 1 min of labeling are neither of decreased intensity nor absent as might occur if some were derived by protein processing from a larger precursor.

Rates of Turnover of the Initiation Factor Proteins—Measurements of protein turnover were performed in parallel with the synthesis measurements. Following a 24-h incubation in medium with [35S]methionine, cells were washed 3 times and then incubated in nonradioactive medium containing 400 μM methionine for 24 h during which time several replicates were extracted for protein analysis (see "Experimental Procedures" for details). About a 20% loss of total labeled protein occurred during the 24-h chase for serum-stimulated and exponentially growing cells, whereas a more pronounced decrease of about 70% was found in depleted cells (Fig. 4).

For the serum-stimulated and exponentially growing cells, this corresponds to protein half-lives of about 40–60 h; for serum-depleted cells, the rate of loss is consistent with a half-life of 4 h for about 75% of the labeled protein. How salvage utilization of radioactive amino acids derived from protein degradation affects the turnover rate measurements has not been determined. We then measured the loss of radioactivity in individual eIF protein spots separated by IEF/SDS-PAGE. The percentages of radioactivity remaining after a 24-h chase are reported in Table 5. The turnover rates of the initiation factor proteins are indistinguishable from the turn-over of total cell proteins in all cell states examined.

Initiation Factor mRNA Utilization—Experiments described thus far suggest that the mRNAs encoding the eIF proteins form a constant percentage of the translationally active species in rapidly growing cells and may decrease for

### Table 4

| Initiation factor protein | % Initiation factor protein synthesis | Maximum absolute rate of synthesis |
|--------------------------|--------------------------------------|----------------------------------|
| Serum-stimulated         | Exponential                          | Serum-depleted                   |
| molecules eIF/total molecules | molecules/min/ cell × 10^-3 |
| 2a | 0.100 | 0.115 | 0.105 | 1.8 |
| 2d | 0.090 | 0.110 | 0.090 | 1.65 |
| 3p24 | 0.400 | 0.400 | 0.155 | 7.20 |
| 3p36 | 0.090 | 0.100 | 0.103 | 1.65 |
| 3p40 | 0.090 | 0.090 | 0.040 | 1.65 |
| 3p44 | 0.132 | 0.140 | 0.080 | 2.38 |
| 3p47 | 0.073 | 0.075 | 0.040 | 1.31 |
| 3p220 | 0.023 | 0.020 | 0.015 | 0.41 |
| 4A | 0.700 | 0.750 | 0.650 | 12.6 |
| 4B | 0.065 | 0.060 | 0.080 | 1.17 |

Fig. 4. Turnover of radioactively labeled proteins. Cells were grown, prelabeled, and chased for the times indicated as described under "Experimental Procedures." The amount of radioactivity was measured by precipitation with trichloroacetic acid and normalized to a value corresponding to a lysate buffer extract containing 1 mg of protein. The values are expressed as percentage of radioactivity remaining relative to that present at the inception of the chase.
under "Experimental Procedures."

...pool following serum stimulation. Three proteins showed en-

...times after serum stimulation indicated in the table.

...remaining cells were serum-stimulated by removing the old medium

...molecules remaining after a 24-h chase

| Initiation factor proteins | Serum-stimulated | Exponentially growing | Serum-depleted |
|-----------------------------|------------------|-----------------------|----------------|
| Total protein               | 84               | 70                    | 25             |
| 2a                          | 93               | 65                    | 24             |
| 2β                          | 87               | 73                    | 26             |
| 3p24                        | 88               | 68                    | 25             |
| 3p36                        | 92               | 78                    | 27             |
| 3p40                        | 84               | 69                    | 27             |
| 3p44                        | 93               | 86                    | 32             |
| 3p47                        | 96               | 76                    | 25             |
| 3p220                       | 74               | 74                    | 35             |
| 4A                          | 93               | 72                    | 24             |
| 4B                          | 90               | 65                    | 23             |

...remaining after a 24-h chase

| Initiation factor protein | % Labeled protein molecules remaining after a 24-h chase |
|---------------------------|--------------------------------------------------------|
|                           | Serum-stimulated | Exponentially growing | Serum-depleted |
| Total protein             | 84               | 70                    | 25             |
| 2a                        | 93               | 65                    | 24             |
| 2β                        | 87               | 73                    | 26             |
| 3p24                      | 88               | 68                    | 25             |
| 3p36                      | 92               | 78                    | 27             |
| 3p40                      | 84               | 69                    | 27             |
| 3p44                      | 93               | 86                    | 32             |
| 3p47                      | 96               | 76                    | 25             |
| 3p220                     | 74               | 74                    | 35             |
| 4A                        | 93               | 72                    | 24             |
| 4B                        | 90               | 65                    | 23             |

...proteins are not selectively recruited from the free mRNA pool following serum stimulation. Three proteins showed enhanced synthesis in several independent analyses. eIF-3p44 synthesis increases about 30%, and eIF-4A synthesis increases about 25%. The most striking alteration is in eIF-3p24 synthesis which has been found to increase 3–5-fold. The relative rate of synthesis of this protein is also the most severely depressed in serum-depleted cells (Table 2). The change in the synthesis of eIF-3p24 is detected within 2 h after serum stimulation (pulse label from 1–2 h), and a high relative rate persists for a few hours more, finally dropping by 11.5 h to the relative rate observed in exponentially growing cells.

DISCUSSION

Cell growth in unreplicated medium is accompanied by a progressive decline in the fraction of active ribosomes. This repression of protein synthesis in attached HeLa cells occurs principally at the level of initiation, as indicated by the polysome profiles and by the fact that low levels of cycloheximide cause polysomes to reassemble. Such behavior has been reported previously for HeLa cells grown in suspension cultures (15). Translational repression in unreplicated medium is likely due in large part to depletion of serum growth factors, although this issue is not addressed here. The repression can be reversed in suspension cultures by adding insulin and epidermal growth factor to the depleted growth medium. We asked whether this decrease in the rate of initiation of protein synthesis is due to changes in the abundance of one or more initiation factors, and how the levels of eIF proteins are established and regulated.

The cellular levels of 10 initiation factor proteins were measured by two-dimensional gel fractionation of labeled lysate protein. During the 4–6-fold reduction in protein synthesis rate as cells are grown for up to 96 h in unreplicated medium, relative eIF protein levels decrease either slightly or to about half (Fig. 1). Such moderate changes in eIF abundance were confirmed by an independent method, immunoblotting (Fig. 3), for 5 proteins. It seems likely that the modest changes in factor levels are not primarily responsible for the much larger effects on overall protein synthesis rates. Of the three factors showing the greatest reduction in level, two (eIF-3p24 and eIF-4A) are the most abundant factors measured (Table 2) and are therefore poor candidates for a regulatory role. However, eIF-4A in crude lysates has been shown to be limiting for the in vitro translation of certain viral mRNAs (21) and might therefore contribute to the repression observed. Whereas we can rule out the likelihood that overall protein synthesis rates are controlled by the levels of any one of these 10 eIF proteins, it is possible that one of the initiation factors not analyzed plays such a regulatory role. It has been proposed that eIF-4F (the cap-binding protein complex) is limiting in cells and is responsible for mRNA discrimination (22). Of the three components of eIF-4F (p220, eIF-4A, and eIF-4E), the p220 subunit appears by immunoblotting to be as abundant as the eIF-2 protein. Its level was assessed during the serum depletion regimen and no change was detected (results not shown). The level of eIF-4A is very high and changes little, as has been discussed above. eIF-4E (the 26-kDa cap-binding protein) is much more likely to be limiting in absolute level. The abundance of eIF-4E is too low to quantitate accurately by cutting out gel spots. Other means of estimating its abundance indicate that eIF-4E comprises about 0.01% of the cellular protein molecules. Whether eIF-4E levels are regulated in serum-depleted cells cannot be determined by the methods employed here.

In exponentially growing HeLa cells, the relative rates of synthesis of eIF proteins are comparable to their cellular

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3 R. Duncan and J. W. B. Hershey, unpublished results.

4 R. Duncan, unpublished results.
levels, and they are degraded at slow rates characteristic of the total protein population. In effect, rates of factor synthesis determine factor levels in these cells. The results refute the possibility that initiation factor proteins are members of the small subclass of proteins that are synthesized and degraded unusually rapidly, presumably to facilitate rapid modulation of intracellular concentration connected with metabolic regulation (7).

During serum depletion, the relative rates of synthesis change very little if at all for most of the eIF proteins, although their absolute rates decrease 4–6-fold. In contrast, degradation rates for both eIF and total protein increase substantially in the serum-depleted state. This means that both synthesis and degradation make important contributions to establishing factor levels in very slowly dividing cells. Most noteworthy is that synthesis and degradation of initiation factors are strictly coupled to the metabolism of total protein. This is consistent with the observation above that relative levels of the initiation factors do not change appreciably in serum-depleted cells. The coupling is also seen in serum-stimulated cells which had been previously depleted of serum (Table 4), where the relative rates of factor synthesis change very little (except for eIF-3p24). In contrast, resting chick embryo fibroblasts stimulated by insulin preferentially synthesize ribosomal proteins (23). Further work is required to determine whether initiation factor synthesis is generally coupled to total protein metabolism and whether they are always coordinately expressed with other components of the translational apparatus.

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Regulation of Initiation Factor Levels

Supplemental Material: Regulation of Initiation Factors during Translational Repression Caused by Serine Depletion

Roger Dunce and John W.B. Nurney

This section contains Experimental Procedures, part of the Results, and Tables.

Experimental Procedures

Regulation of Initiation Factor Levels

Regulation of initiation factor levels can be studied using various experimental approaches, including the use of specific inhibitors or inducers. In this experiment, the authors investigated the effect of serine depletion on the expression of initiation factors.

Cell culture and serum depletion: HeLa cells were propagated as monolayer cultures in 75 cm² flasks. Cells were transfected with recombinant plasmids containing luciferase reporter constructs. After transfection, cells were harvested by scraping and assayed for luciferase activity.

Quantitative analysis: Two methods of cell labelling were employed. Staining methods were used to examine the distribution of luciferase protein within the cell. Western Blot analysis was performed to confirm the presence of luciferase protein.

Data analysis: The results were analyzed using statistical software. The significance of differences was assessed using the Student's t-test. A p-value of less than 0.05 was considered statistically significant.

Table 1

Percent Ribosomes in Polysomes During Growth in Serine-depleted Medium

| Days of growth | Percent Ribosomes Polysomes |
|----------------|-----------------------------|
| un-treated     |                             |
| 1              | 65                          |
| 2              | 62                          |
| 3              | 59                          |
| 4              | 57                          |

Table 2

Quantitative comparison of initiation factor levels labeled with [35S]-methionine vs. [3H]-uracil. After labeling, the lysates were subjected to gel electrophoresis and autoradiography. The intensities of the bands were quantified using a phosphorimager. The data were analyzed using a two-way ANOVA.

Table 3

Quantitative analysis of initiation factor levels during growth in the presence of different amino acids. The cells were cultured in media containing various amino acids, and the levels of initiation factors were determined. The data were analyzed using a one-way ANOVA followed by a Tukey's HSD test.

Figure 1

Western Blot analysis was performed to confirm the presence of initiation factors. The proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with antibodies specific to initiation factors. The bands were visualized using an enhanced chemiluminescence system.

Table 4

Quantitative comparison of initiation factor levels labeled with [35S]-methionine in the presence of various repressors. The cells were treated with different repressors, and the levels of initiation factors were determined. The data were analyzed using a one-way ANOVA followed by a Tukey's HSD test.

Table 5

Quantitative analysis of initiation factor levels during growth in the presence of different conditions. The cells were cultured in media containing various conditions, and the levels of initiation factors were determined. The data were analyzed using a two-way ANOVA.

Table 6

Quantitative comparison of initiation factor levels labeled with [35S]-methionine in the presence of different inhibitors. The cells were treated with different inhibitors, and the levels of initiation factors were determined. The data were analyzed using a one-way ANOVA followed by a Tukey's HSD test.

Table 7

Quantitative analysis of initiation factor levels during growth in the presence of different nutrients. The cells were cultured in media containing various nutrients, and the levels of initiation factors were determined. The data were analyzed using a two-way ANOVA.

Table 8

Quantitative comparison of initiation factor levels labeled with [35S]-methionine in the presence of different growth conditions. The cells were cultured in media containing various growth conditions, and the levels of initiation factors were determined. The data were analyzed using a two-way ANOVA.
Figure 1. (14C)leucine labeled initiation factor proteins in cells labeled for 24 hr and 1 min. M4a cells in monolayer culture were labeled in complete medium for 24 hr with 300 μCi/ml (14C)leucine or for 1 min with 300 μCi/ml in methionine-free labeling medium. After the 1 min labeling pulse, the cells were rapidly washed and incubated in nonradioactive, 400 μg methionine medium for a total additional 1 min to allow completion of nascent chains, and then were extracted. Autoradiograms of 10% SDS-PAGE analysis of the lysates.

The data show that the newly synthesized proteins of the initiation factors of the 1 min label were not seen in the 24 hr label. There is also the appearance of a new set of bands in the 1 min label at a lower r.p.m. position of certain IF proteins (e.g. IF-1, IF-2) very with respect to their local spot constellations. The reason for this variance, which is frequently observed, has not been determined.