effects should not be ignored.

Acknowledgments

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Regulation of Protein Synthesis in *Dictyostelium discoideum*: Effects of Starvation and Anoxia on Initiation†

James A. Cardelli* and Randall L. Dimond

ABSTRACT: A rapid reduction in the size and amount of total polysomes is one of the earliest observed changes when the cellular slime mold *Dictyostelium discoideum* is starved for a nutrient source. The measured elongation rate of protein synthesis is similar in both starved and vegetative cells and measures approximately 100 and 120 amino acids per min, respectively. Therefore, the reduction in the size and amount of polysomes following starvation is the result of a rapid decrease in the initiation rate of protein synthesis. Accompanying this reduction in the initiation rate is a decrease in the percentage of cellular messenger ribonucleic acid (mRNA) associated with polysomes from 85–90% in fed cells to 65% in starved cells. When the elongation rate in starved cells is reduced by the addition of cycloheximide to cultures, polysomes increase to vegetative levels in size and amount, demonstrating the lack of inactivation of either ribosomes or mRNA. Polysomes also rapidly increase in amount when starved cells are resuspended in growth medium. When axenic cultures containing exponentially growing cells are shifted from rapid shaking to gentle mixing conditions, the concentration of dissolved oxygen decreases by greater than 95% within 10–15 min. After a short period of time of continued gentle mixing, total polysomes disaggregate, and the rate of protein synthesis drops to 20% of the control rate. When cultures are reoxygenated by being returned to rapid shaking conditions or by bubbling air through the medium, polysomes increase in size and amount, and the rate of protein synthesis rapidly returns to control levels. We conclude from these data that the regulation of protein synthesis in *Dictyostelium* is exquisitely sensitive to the cellular environment. In response to starvation and anoxia, these cells rapidly reduce the initiation rate of protein synthesis. Furthermore, when these cells are returned to normal growth conditions, the initiation rate rapidly returns to vegetative levels.

Eukaryotic cells regulate the rate of protein synthesis in response to a wide variety of environmental conditions. As an example, polysomes disaggregate and the initiation rate of protein synthesis drops in many mammalian cells starved for amino acids or glucose (Christman, 1973; Hogan & Korner, 1968; Lee et al., 1971; Van Venrooij et al., 1972). This block in polypeptide chain initiation also leads to the accumulation in the cytoplasm of mRNA[1] in the form of nonpolysomal messenger ribonucleoprotein. When the missing nutrients are added back to cultures, the amount of polysomes and the initiation rate of protein synthesis in these cells rapidly return to vegetative levels. Furthermore, this increase in the rate of protein synthesis does not require new mRNA synthesis and instead appears to be dependent on more efficient translation of mRNA made prior to the starvation event. Mitosis, hy-

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[1] Abbreviations used: mRNA, messenger ribonucleic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Cl_3CCOOH, trichloroacetic acid; RNase, ribonuclease; EDTA, ethylenediaminetetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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pertonic growth, high temperature, and the transition from a growing to a resting state are some of the other conditions which trigger similar cellular responses (Christman, 1973; Fan & Penman, 1970; Rudland, 1974; Rudland et al., 1975; Schochetman & Perry, 1972). Little is known about the molecular details of these cellular regulatory events or other factors which may modify them.

In the absence of a nutrient source, the cellular slime mold *Dictyostelium discoideum* undergoes an orderly series of developmental changes leading to the formation of two differentiated cell types making up a well-defined morphological structure, the fruiting body. A reduction in the size and amount of free and membrane-bound polysomes is one of the earliest starvation responses known to occur in these cells (Alton & Lodish, 1977b; Cardelli et al., 1981; Margolskee et al., 1980). In addition, the relative rate of synthesis of a small number of proteins drops dramatically even though functional mRNAs coding for them remain intact in the cytoplasm (Alton & Lodish, 1977a,b).

The regulation of protein synthesis in response to starvation may play an important role in the differentiation of *Dictyostelium*. We have therefore determined the step in protein synthesis which is regulated in response to starvation. In addition, we describe the regulation of protein synthesis in exponentially growing cells in axenic cultures under anoxic conditions.

Materials and Methods

Conditions of Growth and Starvation. Strain Ax3 of *Dictyostelium discoideum* was used for all experiments. Cells were grown in axenic culture in TM medium (1% glucose, 1% trypticase, 0.5% yeast extract, 0.35 g of Na_2HPO_4, and 1.2 g of KH_2PO_4 per L) as described (Free & Loomis, 1974) and had a doubling time of 8 h at 21 °C. For initiation of starvation in suspension, cultures at densities between 3×10^6 and 5×10^6 cells/mL were harvested by centrifugation at 1000*g* for 2 min, washed once in Mes–PDF [1.5 g of 2-(*N*-morpholino)ethanesulfonic acid, 1.5 g of KCl, 0.11 g of $CaCl_2 \cdot 2H_2O$, and 1.07 g of $MgCl_2 \cdot 6H_2O$ per L], resuspended to 3×10^6 cells/mL in Mes–PDF, and placed back on gyratory shakers rotating at 240 rpm. Alternatively, for starvation of cells on solid supports under normal developmental conditions, cells were washed and resuspended in TM at a titer of 1.4×10^8 cells/mL. A 0.5-mL sample was then layered on top of black Millipore filters (HABP 047 00; Millipore Co., Bedford, MA) which in turn rested on top of absorbent pads soaked in TM medium. Starvation was initiated by transferring filters to fresh pads soaked with Mes–PDF buffer. The tops of the filters were kept moist with Mes–PDF.

Sucrose Gradient Analysis of Polysomes. Approximately 5×10^7 cells were collected by centrifugation (1000*g* for 2 min) and resuspended by vortex mixing in 1 mL of lysis buffer consisting of 4% NP40, 0.25 M sucrose, 50 mM Tris-HCl, 25 mM KCl, 5 mM $MgCl_2$, and 0.25% diethyl pyrocarbonate, pH 7.5. Cell lysates were centrifuged at 3000*g* for 2 min to pellet nuclei. Postnuclear supernatants were then layered on linear 10–50% sucrose gradients made up in TKM buffer (50 mM Tris-HCl, 25 mM KCl, and 5 mM $MgCl_2$, pH 7.5) and centrifuged at 37 000 rpm in an SW41 rotor at 4 °C for 100 min. Following centrifugation, gradients were fractionated, and A_{260} scans were recorded by a Gilford spectrophotometer. The percentage of ribosomes associated with mRNA was determined planimetrically as previously described (Cardelli et al., 1981).

Pretreatment of postnuclear supernatants with 10 μg/mL pancreatic RNase prior to centrifugation eliminated the A_{260} absorbing material sedimenting in the polysome region of the sucrose gradient (Figure 1C). This result suggests that the majority of the rapidly sedimenting A_{260} adsorbing material observed in sucrose gradients is polysomal in nature and that our planimetric method of quantitating this material accurately assesses the percentage of total cellular ribosomes associated with mRNA.

Isolation of Polysomal and Nonpolysomal Poly(A+) RNA. Cellular RNA was labeled to steady state by allowing cells to grow for three generations in TM medium with 10 μCi/mL of [^{3}H]uridine (38.6 Ci/mmol). When the titer reached 4×10^6 cells/mL, the cells were harvested by centrifugation at 1000*g* for 2 min, washed, and resuspended at the same concentration in TM or Mes–PDF. After shaking at 180 rpm for 20 min, cells were recovered by centrifugation (1000*g* × 2 min), resuspended in 1 mL of lysis buffer, and centrifuged at 3000*g* × 2 min to pellet nuclei. The postnuclear supernatants were layered on 10–40% STKM gradients which were formed on top of a 1-mL cushion of 2.0 M STKM. Gradients were centrifuged for 105 min in a SW40 rotor at 39 000 rpm and fractionated as described above. The regions of the gradient corresponding to 0–90 S (nonpolysomal) and >90 S (polysomal) were pooled, and the RNA was extracted with phenol and chloroform as described (Cardelli et al., 1981). Poly(A+) RNA was isolated by two cycles of oligo(dT)–cellulose chromatography. RNA samples were adjusted to 500 mM NaCl, 10 mM Tris, pH 7.6, 1 mM EDTA, and 0.2% NaDodSO$_4$ (binding buffer) and applied to columns containing 0.2 g of oligo(dT)–cellulose (Collaborative type 3) equilibrated with binding buffer. Columns were washed with binding buffer, and the bound RNA was eluted with H_2O. The eluted RNA was heated to 65 °C for 5 min and refractionated on the columns as described above.

Measurement of Protein Synthesis. The in vivo rate of protein synthesis was monitored by measuring the incorporation of [^{3}H]leucine (130 Ci/mmol) into Cl_3CCOOH-precipitable material. At various times following the addition of [^{3}H]leucine (10 μCi/mL) to cultures, aliquots (~5×10^6 cells) were removed and pipetted into ice-cold TKM containing 5 mM leucine and 200 μg/mL cycloheximide. Cells were washed once by centrifugation (1000*g*, 3 min) in the same buffer, solubilized in 0.2 N NaOH, and heated to 35 °C for 20 min. Ice-cold 100% Cl_3CCOOH was then added to a final concentration of 10%. Precipitates were collected on GF/C glass-fiber filters and washed with 10 mL of 5% Cl_3CCOOH containing 2.5 mM leucine.

We measured the elongation rate of protein synthesis following the basic procedure of Fan & Penman (1970). Cells were allowed to incorporate [^{3}H]leucine for various periods of time and were then harvested and washed as described above. Cells were lysed in ice-cold TKM buffer containing 1.0% Triton X-100 and centrifuged at 12000*g* for 5 min. One-half of the postmitochondrial supernatant volume was centrifuged at 23 000 rpm for 2 h at 4 °C in a type 25 rotor to pellet ribosomes. The postribosomal and postmitochondrial supernatants were precipitated with Cl_3CCOOH and processed as described above. The incorporation of radioactivity into Cl_3CCOOH-precipitable material in the postmitochondrial supernatant reflects total protein synthesis while that in the postribosomal supernatant measures only completed polypeptide chains. Examination of the incorporation of radioactivity into the polysomal pellet showed a constant amount of radioactivity after the first 2 min. This indicates that the amino acid pool reaches a constant radioactivity very early in these experiments.

Agglomerate Formation in Axenic Cultures. Cells were grown in axenic culture in flasks containing TM medium that were shaken at 240 rpm on a gyratory shaker. The formation of cell agglomerates was initiated as follows. Cultures were removed from the shakers and gently mixed with a magnetic stir bar which rotated at the slowest speed sufficient to maintain a uniform distribution of cells throughout the flask. At various times, aliquots of the culture were examined under a phase contrast microscope, and the total number of single cells and cell agglomerates were counted. The concentration of oxygen dissolved in the medium was determined by using a YSI Model 53 oxygen monitor connected to a Sargent chart recorder.

Chemicals. Sucrose was purchased from Schwarz/Mann (Orangeburg, NY). [^{3}H]Leucine and [^{3}H]uridine were purchased from New England Nuclear (Boston, MA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Results

Effect of Starvation on the Size and Amount of Total Cellular Polysomes. A reduction in the size and amount of total polysomes is the earliest known macromolecular change to occur in developing cells of *Dictyostelium discoideum* (Alton & Lodish, 1977b; Cardelli et al., 1981; Margolskee et al., 1980). It has been shown that total polysomes are reduced in size from 10–12 ribosomes per mRNA in vegetative cells to 6–8 ribosomes per mRNA in cells 15 min after development is initiated by the removal of the food source (Alton & Lodish, 1977b; Cardelli et al., 1981; Margolskee et al., 1980). In addition, only 40–45% of the total ribosomes are associated in polysomes in starved cells compared to 87% in vegetative cells. In order to measure how rapidly starvation initiates a change in the polysome level, we measured the size and amount of polysomes in developing cells at times earlier than 15 min. Cells growing in TM medium were washed with TM or Mes–PDF (starvation medium) at 22 °C, resuspended to the original titer in the medium in which they were washed, and placed back on gyratory shakers. At various times, cells were collected by centrifugation, lysates prepared, and total polysomes analyzed in sucrose gradients. The A_{260} profiles of the fractionated sucrose gradients shown in Figure 1B,F indicate that total polysomes prepared from growing cells washed once and resuspended in TM at 22 °C for 5 min appear similar in size and amount to polysomes prepared from unwashed growing cells harvested by centrifugation from TM medium. This initial experiment was done as a control to show that the physical handling of the cells which occurs during washing by centrifugation does not itself contribute to the disaggregation of polysomes observed in starved cells. Unexpectedly, however, total polysomes appear reduced in size and amount in lysates prepared from cell pellets from the last centrifuge step prior to resuspension in TM medium (Figure 1A). Identical results are found when polysomes are examined from cells washed in Mes–PDF medium and not resuspended (results not shown). These results and data shown in Figure 1B,F suggest that washing cells by centrifugation in TM medium at 22 °C can by itself affect a transient decrease in the size and amount of total polysomes. Figure 2A reveals how rapidly polysomes return to vegetative levels after washed cells are resuspended in TM medium. Contrary to this, and not unexpectedly, polysomes remain reduced in size and amount in cells washed and resuspended in Mes–PDF (Figures 1D,E and 2A). We also performed the cell washes at 4 °C instead of 22 °C to determine if a lower temperature would prevent the disaggregating effect the washing procedure appears to have on

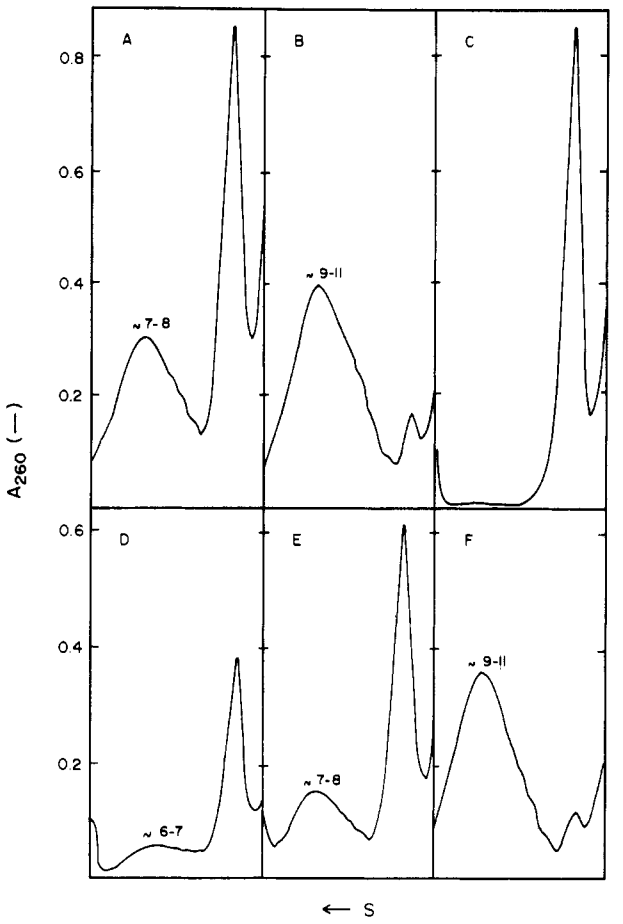

FIGURE 1: Sucrose gradient analysis of total polysomes prepared from starved and vegetative cells. Cells growing in axenic culture at a density of 3 × 10^6 cells/mL were washed once by centrifugation in TM (growth medium) or Mes–PDF (starvation medium) at 22 °C and resuspended in the same medium at 22 °C. At indicated times, cells were harvested, postnuclear supernatants were prepared, and aliquots were fractionated in linear sucrose gradients as described under Materials and Methods. In panel C, postnuclear supernatants prepared from axenically growing cells were treated with 10 μg/mL pancreatic ribonuclease for 30 min on ice prior to gradient fractionation. A_{260} scans of fractionated sucrose gradients containing polysomes prepared from (A) pelleted cells after washing in TM, (B) cells washed and resuspended in TM for 5 min, (D) cells washed and resuspended in Mes–PDF for 1 min, (E) cells washed and resuspended in Mes–PDF for 5 min, and (F) cells harvested by centrifugation from axenic cultures (no washes).

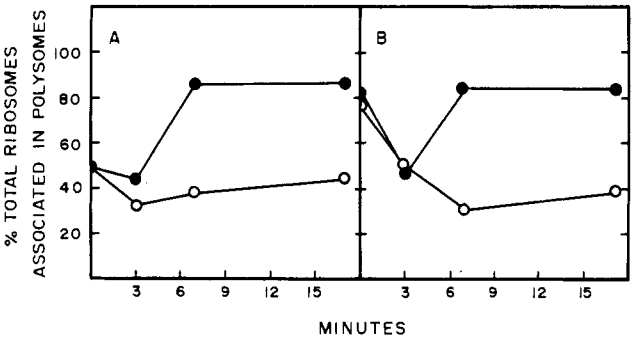

FIGURE 2: Percent of ribosomes associated in polysomes in starved and fed cells. Exponentially growing cells were harvested by centrifugation, washed in TM (●) or Mes–PDF (○) at (A) 22 °C or (B) 4 °C, and resuspended in the same medium at 22 °C. The 0-min time point represents results found when lysates of the final washed cell pellets were fractionated in sucrose gradients prior to resuspension.

polysomes. As indicated in Figure 2B, the size and amount of polysomes appear similar to those from vegetative cells when lysates of TM or Mes–PDF washed cell pellets are subjected

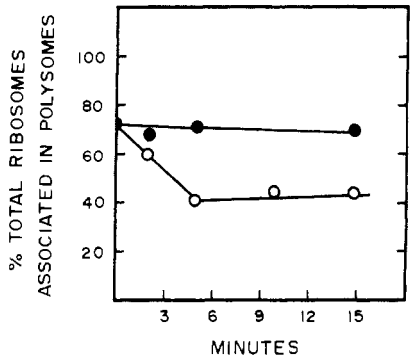

FIGURE 3: Percentage of ribosomes associated in polysomes in filter-attached starved and fed cells. Exponentially growing cells were harvested and resuspended in TM medium at a final concentration of 1.4×10^8 cells/mL; 0.5 mL was layered on Millipore filters which in turn rested on top of TM soaked pads. After 15 min, filters were transferred to pads soaked in TM (●) or Mes–PDF (○). At the indicated times, postnuclear supernatants were fractionated in sucrose gradients. Polysomes were quantitated as described under Materials and Methods.

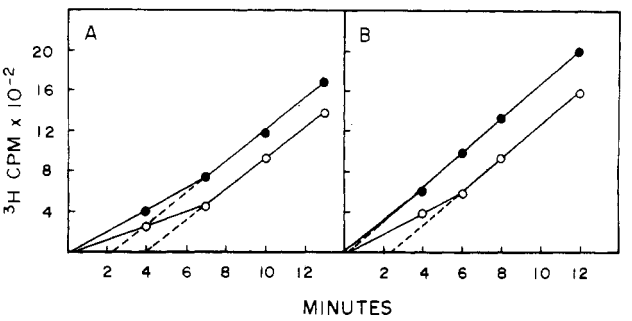

FIGURE 4: Elongation rate of protein synthesis in vegetative and starved cells. Exponentially growing cells (5×10^6 cells/mL) were harvested by centrifugation, washed, and resuspended in Mes–PDF. After 15 min, [^{3}H]leucine was added to these cultures and to cultures containing cells growing in TM medium. At the indicated times, 2-mL aliquots of cells were pelleted and fractionated into total lysates and postribosomal supernatants as described under Materials and Methods. Fractions were precipitated with Cl_3CCOOH, collected on filters, and counted in a liquid scintillation counter. Incorporation of [^{3}H]leucine into total protein (●) or completed polypeptide chains (○) in vegetative cells (A) or starved cells (B).

to sucrose gradient analysis. Polysomes probably remain intact in these cells during the wash steps because the low temperature prevents polysome runoff. However, polysomes rapidly run off after the cells are resuspended at 22 °C in TM or Mes–PDF (Figure 2B). This suggests that the low wash temperature does not entirely prevent the centrifugation-related disaggregation of polysomes. Consistent with the results shown in Figure 2A, polysomes rapidly increase in amount to vegetative levels within 5 min after pelleted cells are resuspended in 22 °C TM medium, but they remain reduced in amount in cells resuspended in 22 °C Mes–PDF medium (Figure 2B). These results suggest that in response to some unknown signal generated during washing by centrifugation, these cells reduce the ratio of the initiation rate to the elongation rate of protein synthesis.

On the basis of the above results, one could reasonably conclude that starvation does not trigger a decrease in the amount of cellular polysomes but instead actually inhibits cells from re-forming polysomes that were disaggregated during the washing steps. The following experiment was performed to determine if starvation itself effects a reduction in the size and amount of polysomes. The experiment was designed to avoid the washing of cells by centrifugation in Mes–PDF which is normally done in preparation for starvation. Vegetative cells were pelleted and resuspended in 1.4×10^8 cells/mL in TM medium, and 0.5 mL was gently layered on top of Millipore filters which in turn rested on TM soaked pads. After 15 min, filters were transferred to pads soaked with TM or Mes–PDF. Filters were kept moist by the addition of small volumes of TM or Mes–PDF to the top of the filter. At the indicated times, filters containing attached cells were removed, placed in lysis buffer, and vortexed. Postnuclear supernatants were fractionated in sucrose gradients as described under Materials and Methods. Figure 3 indicates that the amount of polysomes in cells that are attached to filters soaking in TM is only slightly less than the amount in vegetative cells shaking in TM (compare Figure 3 with Figure 2). Furthermore, no decrease in the amount of polysomes occurs when filters containing attached cells are transferred to fresh TM-soaked pads. However, when filters with attached cells are transferred to pads soaked with Mes–PDF, the percentage of total ribosomes associated with mRNA rapidly drops to 43% (Figure 3). This value for the amount of polysomes in starved cells that are attached to filters agrees closely with the value determined for the amount of polysomes in cells suspended in Mes–PDF medium. We conclude from this that starvation can directly and rapidly effect a decrease in the amount of cellular ribosomes associated in polysomes.

Starvation Effects a Decrease in the Cellular Initiation Rate of Protein Synthesis. The decrease in the size and amount of polysomes which occurs in starved cells does not appear to be due to the activation of latent cellular ribonucleases (Alton & Lodish, 1977b). This disaggregation event is also not caused by the premature termination of translation and subsequent release of mRNA-associated ribosomes because the size distribution of newly made proteins in starved cells is the same as the size distribution in unstarved cells (results not published). The decrease is most likely due to a change in the initiation and/or elongation rate or protein synthesis. One approach in determining the basis for the starvation-induced reduction in the size and amount of polysomes which occurs in *Dictyostelium* is to measure the elongation rate of protein synthesis in starved and fed cells. This is preferable to measuring rates of incorporation of radioactivity labeled amino acids because the elongation rate determinations are relatively unaffected by differences in rates of uptake or size of precursor pools in starved cells compared to fed cells. The procedure we used measures the length of time it takes a mRNA-associated ribosome to complete the synthesis of an average polypeptide chain. The measurement, termed the transit time, is inversely proportional to the elongation rate. The results from one such experiment are shown in Figure 4. The lines drawn in each panel of Figure 4 represent the rate of incorporation of [^{3}H]leucine into completed and total (completed plus nascent) cellular polypeptide chains. When the linear portions of the curves are extrapolated back to the abscissa, the distance in minutes between the two intercepts equals one-half the transit time (Fan & Penman, 1970). The measured transit times in starved and unstarved cells in this experiment are 4.0 and 3.2 min, respectively (Figure 4). The results of three separate experiments, summarized in Table I, indicate that the elongation rate in starved cells is slightly less than the rate in vegetative cells. We conclude from these results that the 50% decrease in the amount of total polysomes that occurs in cells upon starvation is due entirely to a decrease in the initiation rate of protein synthesis from approximately 3.0 polypeptide chains min^{-1} per mRNA to 1.3 polypeptide chains min^{-1} per mRNA (Table I).

Starvation Causes a Decrease in the Percentage of Cellular Poly(A+) RNA Associated with Polysomes. We next de-

Table I: Initiation and Elongation Rates of Protein Synthesis in Starved and Growing Cells

condition of cells	transit time (min)[a]	elongation rate (aa min^{-1})[b]	initiation rate (polypeptide chains initiated[c] min^{-1} per mRNA)
starved	4.0 ± 0.2	103	1.3
unstarved	3.3 ± 0.2	121	3.0

[a] The transit time for ribosomes translating the average length mRNA was determined as described in Figure 4. The numbers represent the mean ± standard error for three separate experiments. [b] The elongation rate was determined by dividing the transit times into the average size polypeptide chain in *Dictyostelium* taken to be 400 amino acids (Bergmann & Lodish, 1979). [c] The initiation rate in growing cells was determined by dividing the average polysome size calculated to be 10 ribosomes per mRNA by the transit time. The initiation rate in starved cells was calculated by multiplying the initiation rate in growing cells by the ratio of the percentage of total cellular ribosomes associated in polysomes in starved cells vs. growing cells 43%/87% corrected for the difference in elongation rates, i.e., 103/121.

Table II: Percentage of Polysomal vs. Nonpolysomal Poly(A+) RNA in Starved and Fed Cells[a]

expt	condition of cells	total RNA (cpm × 10^{-2}) nonpolysomal	total RNA (cpm × 10^{-2}) polysomal	poly(A+) RNA (cpm × 10^{-2}) nonpolysomal	poly(A+) RNA (cpm × 10^{-2}) polysomal
1	fed	3848 (18.3)	17171 (81.7)	23 (9.0)	233 (91.0)
	starved	11116 (58.8)	7770 (41.2)	112 (38.1)	182 (61.9)
2	fed	4303 (24.9)	12984 (75.1)	66 (16.9)	323 (83.1)
	starved	12327 (58.9)	8613 (41.1)	161 (36.4)	282 (63.6)

[a] Postnuclear supernatants prepared from 10^8 cells labeled with [^{3}H]uridine were fractionated on sucrose gradients and the appropriate regions pooled and extracted with phenol and chloroform. The extracted RNA was subjected to two cycles of chromatography through oligo(dT)-cellulose as described under Materials and Methods. Numbers in parentheses represent the percentage of total or poly(A+) RNA in the nonpolysomal or polysomal fractions.

termined if the starvation-induced reduction in the initiation rate of protein synthesis was accompanied by a shift in the distribution of total cellular mRNA between functional (polysomal) and nonfunctional (nonpolysomal) states. For these experiments, we assume that the majority (>90%) of *Dictyostelium* mRNA has an associated poly(A) segment (Palatnik et al., 1979). Axenically growing cells labeled with [^{3}H]uridine for three generations were washed, resuspended in TM or Mes–PDF, and shaken at 180 rpm. After 20 min, the cells were harvested and lysed, and the postnuclear supernatants were fractionated on sucrose gradients. RNA from the nonpolysomal (0–90 S) and polysomal (>90 S) regions of the gradients was extracted with phenol and chloroform and subjected to two cycles of chromatography through oligo(dT)-cellulose. As shown in Table II, 85–90% of the total cellular poly(A+) RNA is associated with polysomes in growing cells. In contrast, in starved cells, only 60–65% of the cellular poly(A+) RNA is associated with polysomes. Relative to vegetative cells there was no loss of recoverable poly(A+) RNA in cells starved for 20 min (Table II). We

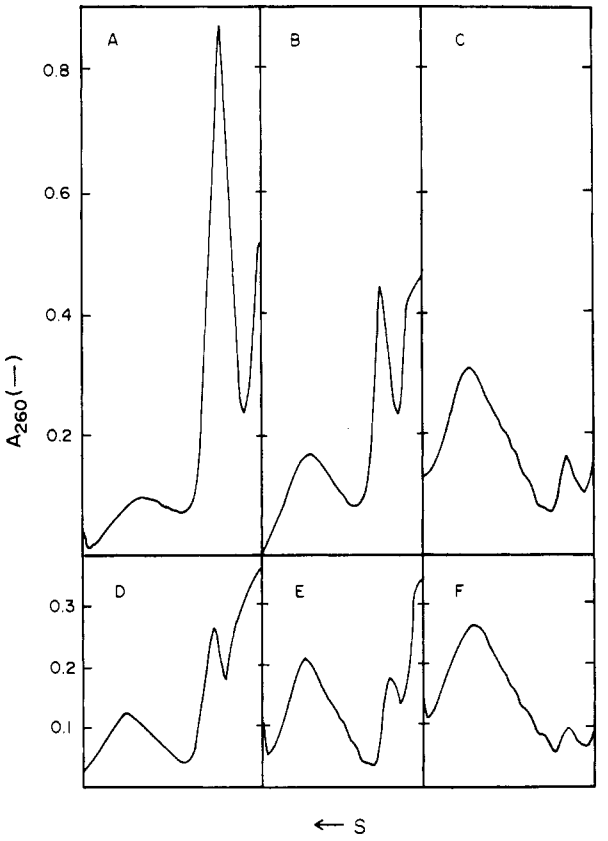

FIGURE 5: Sucrose gradient analysis of polysomes in starved cells that are refed. Cells shaking for 15 min in Mes–PDF at a concentration of 3 × 10^6 cells/mL were pelleted and resuspended in 22 °C TM. Alternatively, one-tenth the volume of 10× TM was added directly to cultures. Cycloheximide instead of TM was added to one culture to a final concentration of 50 μg/mL. At the indicated times, cells were harvested and lysed, and polysomes were fractionated in sucrose gradients. Size distribution of polysomes in starved cells pelleted and resuspended in TM for (D) 15 and (E) 26 min. Distribution of polysomes in cells (A) 15, (B) 32, and (C) 45 min after the addition of 10× TM to cultures or (F) 30 min after the addition of cycloheximide.

conclude from this experiment that the reduction in the initiation rate of protein synthesis in starved cells is accompanied by a 3-fold increase in the percentage of cellular messenger RNA not associated with polysomes.

Re-formation of Polysomes in Starved Cells Resuspended in Growth Medium. For determination of how rapidly polysomes in starved cells increase in size and amount when returned to growth conditions, cells shaking in Mes–PDF were centrifuged and resuspended to the same titer in TM medium. Alternatively, one-tenth the volume of 10× TM medium was added directly to cells shaking in Mes–PDF medium. At various times, lysates were prepared and polysomes analyzed in sucrose gradients. Figures 5 and 6 indicate that after a 10-min lag polysomes begin to increase in size and amount in starved cells pelleted and resuspended in TM medium. A similar rate of increase in the amount of polysomes occurs in starved cells when TM medium is added directly to the starvation buffer (Figures 5 and 6). An unexpected result consistently observed in the latter experiment is that the percentage of ribosomes associated with polysomes actually decreases from 43% to 25% before polysomes begin to increase (Figure 6). This transient decrease in the amount of polysomes upon addition of TM to the starvation buffer also occurs when PDF containing 17 mM potassium phosphate instead of Mes is used as the starvation buffer (results not shown). This suggests that this decrease is not dependent on the buffer composition of the starvation medium.

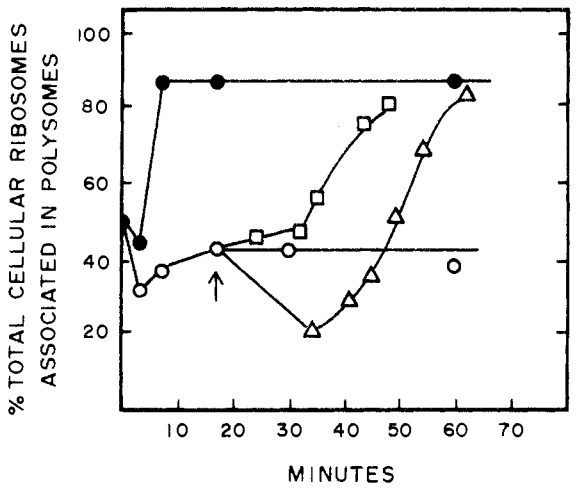

FIGURE 6: Percentage of ribosomes associated in polysomes in starved cells that are refed. The percent of ribosomes associated with mRNA was calculated from the recorded A_{260} scan resulting from the fractionation of sucrose gradients. The amount of polysomes in cells washed and resuspended in TM (●) or Mes–PDF (○). The amount of polysomes in starved cells pelleted and resuspended in TM (□) or exposed directly to TM as described in Figure 5 (△). The arrow marks the time when starved cells were reexposed to TM.

Polysomes increase to vegetative levels 25–30 min after starved cells are harvested by centrifugation and resuspended in TM medium (Figure 6). This increase in the amount of polysomes appears too rapid for it to be dependent on the synthesis of new ribosomes and mRNA. Instead it appears that a signficant percentage of single ribosomes (monosomes) and translated mRNA already existing in starved cells must be utilized in the re-formation polysomes. If this is true, then these components cannot have been irreversibly inactivated in starved cells. The following experiment suggests that this is the case. When cycloheximide is present in cultures at a concentration of 50 μg/mL, the elongation rate of protein synthesis in growing cells is reduced by greater than 50% (results not shown). When starved cells are washed and suspended in Mes–PDF for 30 min in the presence of this concentration of cycloheximide, total polysomes increase to vegetative levels in size and amount (Figure 5F). All the ribosomes and mRNA in starved cells appear capable of functioning in the translation process when the elongation rate is reduced, suggesting that none of these components are irreversibly inactivated when cells are starved.

Effect of Anoxia on Protein Synthesis. Results discussed at the beginning of this paper and summarized in Figure 2 show that polysomes undergo a transient decrease in size and amount in cells that are washed by centrifugation in TM. We also find that a similar decrease occurs in log phase cells growing in TM that are allowed to settle for 10–15 min prior to harvesting (results not shown). These data suggest that the decrease in size and amount of polysomes that occurs in these cells under these conditions may be triggered by an increase in cell density and subsequent decrease in the local concentration of dissolved oxygen. The following experiments provide additional evidence to support this idea and in addition describe an unusual cell–cell association event. In axenic cultures of *Dictyostelium* shaking in TM medium at 240 rpm, 70% of the cell population is found as single cells, and 30% consists of two to three cell agglomerates. When cultures are removed from shakers and gently mixed with a magnetic stir bar, greater than 95% of the cells associate in agglomerates containing an average of 70–80 cells (Figure 7). EDTA present at a final concentration of 5 mM inhibits the formation of these cell agglomerates. This concentration of EDTA is known to inhibit

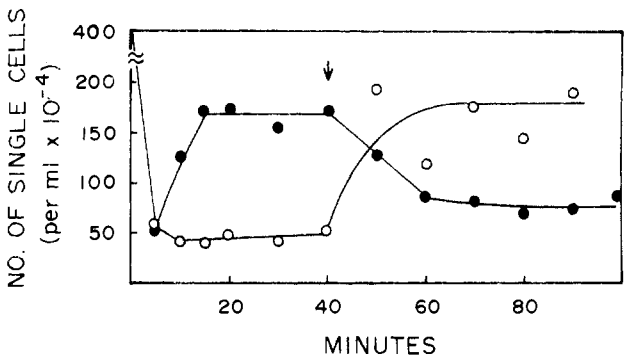

FIGURE 7: Formation of cell agglomerates in axenic cultures. Two cultures containing cells growing exponentially in TM were removed from gyratory shakers (240 rpm) and gently mixed with a stir bar as described under Materials and Methods. Air was gently bubbled through one of the gently mixed cultures. At various times, aliquots were removed and examined under a phase contrast microscope to determine the number of single cells. The arrow indicates the time at which we stopped aerating the one culture (○) and started to aerate the other culture (●).

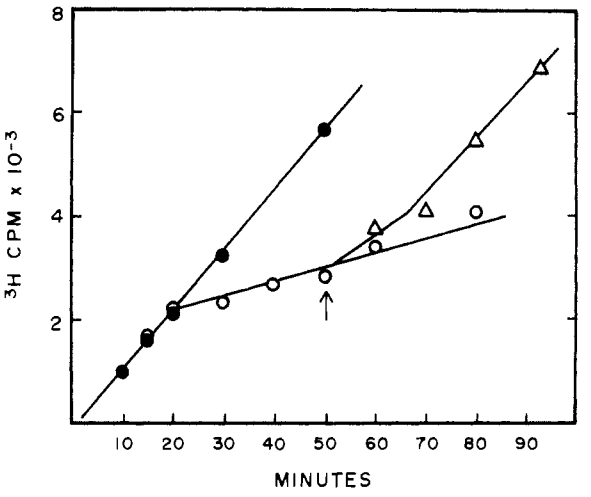

FIGURE 8: Rate of protein synthesis in cells during agglomerate formation and dispersion. [^{3}H]Leucine was added (10 μCi/mL) to 50-mL cultures of exponentially growing cells (4×10^6 cells/mL) which were shaking at 240 rpm (control flasks). After 10 min, 25 mL was transferred to a new flask, and cells were gently mixed with a stirring bar to induce agglomerate formation. After 40 min, 10 mL of this culture was pipetted into a new flask which was then placed on a shaker gyrating at 240 rpm (time of transfer marked by an arrow). Aliquots of cells were then taken from cultures at the indicated times and processed, and the ^{3}H-labeled Cl_3CCOOH precipitable material was counted as described under Materials and Methods. Incorporation of [^{3}H]leucine into cellular proteins in control cultures (●), gently mixed cultures (○), and gently mixed cultures returned to rapid shaking (△).

contact site B mediated cell cohesion in *Dictyostelium*, suggesting that the cell–cell associated event reported here may be mediated by these previously described cell surface ligands (Beug et al., 1973). These agglomerates can be rapidly dispersed by vortex mixing (results not shown), suggesting that the forces of cohesion between these cells are not very strong. In addition, we found that they disperse spontaneously after a short period of time upon continued gently mixing in TM (Figure 7). The length of time prior to agglomerate dispersal is inversely proportional to the initial concentration of cells. In some experiments where the total of cells was less than 2×10^6 cells/mL, agglomerates were stable for up to 1 h. Agglomerate formation does not reoccur in gently mixed cultures once the original agglomerates have dispersed (results not shown). We examined the rate of protein synthesis in these cells during the following agglomerate formation. Figure 8

indicates that the rate of protein synthesis does not change in cells associated in these agglomerates. However, when these cell agglomerates disperse, the rate of protein synthesis rapidly drops to 20% of the control level (Figure 8). Protein synthesis remains at the reduced level for at least 60 min. A similar reduction in the amount of polysomes also occurs (results not shown).

The following experiments implicate anoxia as the cause of both the decrease in protein synthesis and agglomerate dispersal in gently mixed cultures. First, the concentration of oxygen dissolved in the growth medium measured by using an oxygen probe decreases by greater than 95% in 10 min when rapidly shaking cultures at a titer of 6×10^6 cells/mL are shifted to gently mixing conditions. This suggests that logarithmically growing cells utilize oxygen at a rapid rate. Second, if air is bubbled through gently mixed cultures following agglomerate dispersal, cells rapidly reassociate (Figure 7). In addition, agglomerates formed during gentle mixing of cultures are stable only as long as air is bubbled through the medium (Figure 7). The reduction in the rate of protein synthesis which occurs in cells following agglomerate dispersal is reversed if gently mixed cultures are shaken rapidly at 240 rpm (Figure 8). Furthermore no reduction in the rate of protein synthesis occurs in gently mixed cultures if air is bubbled through the medium (results not shown). If rapidly shaking cultures of cells are extensively flushed with nitrogen for 30 min, polysomes disaggregate, and the rate of protein synthesis drops by 80% (results not shown). This suggests that the reduction in protein synthesis which occurs in gently mixed cultures is effective by anoxia and is not dependent on prior agglomerate formation and dispersal.

We conclude from these experiments that the initiation rate of protein synthesis rapidly decreases in vegetative cells under conditions of anoxia. In addition, the initiation rate rapidly returns to control levels when the cultures are reaerated by being returned to normal rapid shaking conditions.

Discussion

We have described in this report a protein synthesis regulatory mechanism in the cellular slime mold, *Dictyostelium discoideum*, which is responsive to at least two different environmental conditions. These cells reduce the initiation rate of protein synthesis in response to starvation for a nutrient source and during conditions of anoxia. Previous reports have already shown that starvation induces a rapid decrease in the size and amount of polysomes in *Dictyostelium* (Alton & Lodish, 1977b; Cardelli et al., 1981; Margolskee et al., 1980). We examined the size and amount of polysomes in starved cells at earlier times than those previously reported in an attempt to measure the rate of decrease. We found, to our surprise, that total polysomes transiently decrease in size and amount in cells simply washed by centrifugation and resuspended in growth medium. The transient nature of this response may explain why it was not previously observed (Alton & Lodish, 1977b; Cardelli et al., 1981; Margolskee et al., 1980). Not unexpectedly, polysomes remain reduced in size and amount in cells washed and resuspended in starvation medium. When cells are starved under conditions not requiring their washing by centrifugation, polysomes still rapidly decrease in amount. This suggests that both starvation and the washing of cells by centrifugation can effect a disaggregation of polysomes in *Dictyostelium*. Starvation alone is sufficient when the physical manipulation of cells which occurs during washing is avoided.

We propose that the cell washing related decrease in the amount of total polysomes is triggered by the increase in cell density which occurs during centrifugation and the subsequent decrease in the local concentration of dissolved oxygen (see below). Supporting this idea is our observation that polysomes disaggregate in vegetative cells allowed to settle out of culture for 10–15 min. Furthermore, polysome disaggregation is more extensive in cells that settle out of cultures grown to higher titers. Along these lines, we also find that when cultures over a range of cell densities are harvested quickly to avoid any cell settling, no significant decrease in the size and amount of polysomes occurs until titers of greater than 10^7 cells/mL are reached. These data suggest that rapid harvesting of high titer cultures is critical for reproducibility in investigating polysome size or the control of protein synthesis in these cells.

The 50% decrease in the initiation rate which occurs in cells upon starvation does not appear to be due to an irreversible inactivation of a certain percentage of ribosomes or mRNA because disaggregated polysomes re-form in starved cells when the elongation rate is reduced by the addition of cycloheximide. Consistent with this idea is our data showing that when starved cells are pelleted and resuspended in growth medium, polysomes rapidly re-form. Similar results were previously reported (Alton & Lodish, 1977b). Surprisingly, if growth medium is added directly to cells starving in suspension, polysomes actually decrease by 50% in amount during the first 15 min after which they re-form at a rate similar to that in cells resuspended in TM. This further decrease in the amount of total cellular polysomes which occurs when growth medium is added directly to starved cells in suspension is probably due to a further decrease in the ratio of the initiation rate to the elongation rate and may be effected by an increase in osmolarity (results not shown).

Accompanying the starvation-induced reduction in the initiation rate of protein synthesis is a 3-fold increase in the amount of untranslated mRNA. This increase can be partially accounted for by assuming that the binding of ribosomes to mRNA is a random (stochastic) event. Using Poisson's equation and assuming an average of two ribosomes per polysomal mRNA in starved cells, Alton & Lodish (1977b) calculated that 10% of the mRNA should be untranslated. Since we find that 35% of the total cellular mRNA is found as nonpolysomal mRNP in starved cells, this suggests that factors in addition to the reduction in the rate of binding of ribosomes to mRNA may effect an increase in the amount of untranslated mRNA. Such factors might include the sequestering of some mRNA through association with proteins or the reduction in the activity of an initiation factor(s) which preferentially recognizes a certain class of mRNA.

Mammalian cells respond to starvation in a manner similar to *Dictyostelium* by reducing the initiation rate of protein synthesis (Christman et al., 1973; Christman, 1973; Hogan & Korner, 1968; Van Venrooij et al., 1972). Upon refeeding, the initiation rate and amount of polysomes in these cells also rapidly return to vegetative levels. The increase in the rate of protein synthesis in starved mammalian cells which occurs upon refeeding appears to be due to a more efficient utilization of mRNA synthesized prior to starvation and is not dependent on the synthesis of mRNA. Although not conclusive, our data presented here and the data of Alton & Lodish (1977b) suggest that a similar mechanism may operate in *Dictyostelium*. Because starvation initiates development in this organism, it has been thought that this reduction in the initiation rate of protein synthesis may be related to development. However, we know of no evidence which would indicate whether this effect on protein synthesis is actually important for development or is a separate starvation response. A number of biochemical mechanisms have been proposed to explain how

mammalian cells regulate protein synthesis in response to starvation but none have been conclusively proven (Christman, 1973; Clemens et al., 1976; Grummt & Grummt, 1976; Sonenshein & Brawerman, 1977; Vaughan & Hansen, 1973).

We have described culture conditions which favor the transient formation of cell–cell association complexes referred to in this report as cell agglomerates. Agglomerates containing an average of 70 cells form within 10 min when rapidly shaking cultures of cells growing in TM medium are shifted to gentle mixing conditions. These multicellular agglomerates probably form because the shear force generated in the gently mixed culture is not great enough to counter the cohesive forces existing between cells. Our data suggest that this cell–cell association event may be mediated by previously characterized cell surface components termed contact sites B (CS B) present on both vegetative and developing cells (Beug et al., 1973). We have found that under our experimental conditions cell agglomerates spontaneously disperse after a variable period of time of continued gentle mixing. This change in the cohesive properties of these cells resulting in the dispersal of agglomerates appears to be triggered by a decrease in the concert ration of dissolved oxygen.

At the time cell agglomerates disperse, polysomes disaggregate, and the rate of protein synthesis drops to 20% of the control rate. Our results suggest that agglomerate dispersal and subsequent polysome disaggregation are independent events, both occurring at the same time as responses to anoxia. Recently it has been shown that *Dictyostelium discoideum* exists as an obligate aerobe (Srinivas & Katz, 1980). Our data are consistent with this and suggest additionally that the rate of protein synthesis may be regulated in part by levels of ATP. When gently mixed cultures are reaerated by shifting them to fast shaking conditions, polysomes re-form, and protein synthesis rapidly returns to control rates, demonstrating that the reduction in the initiation rate that occurs following anoxia is not permanent but can be reversed if cells are again reaerated. Experiments are now in progress to determine the molecular nature of the various regulatory signals, how they are sensed, and what biochemical mechanisms *Dictyostelium* uses to alter the initiation rate of protein synthesis in response to starvation and anoxia. In addition, it will be important to determine if these protein synthesis regulatory events play an important role in the development of this organism.

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