The Regulation of Protein Synthesis in Animal Cells by Serum Factors†

John A. Hassell* and Dean L. Engelhardt

ABSTRACT: We have investigated the regulation of protein synthesis in animal cells by serum factors. Withdrawal of serum from the medium of actively dividing Vero cells resulted in an immediate decline in the rate of peptide chain elongation (Hassell and Engelhardt, 1973). Assay of elongation factor I (EFI) activity in the post-ribosomal supernatant as well as that associated with the ribosomes revealed that serum deprivation resulted also in reduction in the activity of this factor. The decline in the activity of EFI after serum deprivation occurred to the same extent and at the same time as the decline in the in vivo rate of protein synthesis and the in vitro peptide synthetic capacity of cell-free extracts. A temporal correlation therefore exists among the in vivo rate of protein synthesis, the peptide synthetic activity of cell-free extracts, and the activity of EFI. The activity of peptidyl transferase was not altered by serum deprivation. The loss of extract peptide synthetic activity resulting from serum deprivation was reversible since serum addition to previously serum-starved cultures resulted in full restoration of activity for polyphenylalanine (polyPhe) synthesis within 3 h. Moreover, RNA synthesis was not required for this turn-on of polyPhe synthesis. Based on these data we conclude that a translational control mechanism is operative in Vero cells deprived of serum.

The multiplication of many types of untransformed fibroblasts in culture is dependent on serum (Dulbecco and Elkington, 1973). Removal of serum from the medium of growing fibroblasts in culture causes a coordinate stepdown of cellular activity. Those reactions affected include RNA synthesis, protein synthesis, and nutrient uptake (Hershko et al., 1971). Many types of transformed cells, however, do not cease dividing when serum is removed from the culture medium. Moreover, cellular activity is not depressed by withdrawal of serum from the medium of these cells (Hershko et al., 1971; Hassell et al., 1975).

We have begun to study the regulation of the protein-synthetic machinery of cultured animal cells by serum factors (Hassell and Engelhardt, 1973). Our objective is to determine the mechanism whereby control of protein synthesis is effected in untransformed cells, and to learn how transformed cells escape this control.

There are many examples of variations in the rate of protein synthesis during growth rate transitions (Todaro et al., 1965; Levine et al., 1965; Soeiro and Amos, 1966; Ward and Plagemann, 1969; Engelhardt, 1971; Stanners and Becker, 1971; Hershko et al., 1971; Hassell and Engelhardt, 1973). Several translational mechanisms to regulate protein synthesis in nondividing cells have been proposed. They include a reduction in the number of ribosomes per cell (Stanners and Becker, 1971), a failure of mRNA to attach to ribosomes (Stanners and Becker, 1971; Rudland, 1974; Bandman and Gurney, 1975), and that an inhibitor or governor of ribosome function controls the rate of mRNA translation in nongrowing cells (Soeiro and Amos, 1966; Christopher et al., 1971; Engelhardt, 1971). We have reported previously that serum deprivation of Vero cells results in an immediate decline in the intracellular rate of protein synthesis which is accompanied by a reduced efficiency of peptide chain elongation by cell-free extracts prepared from these cells (Hassell and Engelhardt, 1973). The work reported here extends these observations by demonstrating that serum deprivation of growing cells depresses the activity of the translation factor, elongation factor I (EFI[1]), to the same extent and at the same time as it does the in vivo rate of protein synthesis and the in vitro peptide elongation capacity of cell-free extracts.

Materials and Methods

1. Cell Culture. Vero cells, a line derived from an African Green Monkey kidney (Yasumura and Kawakita, 1963), were maintained by transfer of 10^7 cells to a roller bottle (670 cm^2) every 3 days. The cells were grown in Dulbecco's Modified Eagle's medium (DME) supplemented with calf serum to 10% (v/v). Large quantities of Vero cells to be used in preparing cell-free extracts were grown on roller bottles. The cells were seeded at $1-1.5 \times 10^7$ cells per roller bottle and deprived of serum at $2-3 \times 10^7$ cells per bottle. To deprive cells of serum, the growth medium was poured off and the cell layer rinsed twice with 50-ml volumes of pre-warmed (37 °C) DME. DME (250 ml) was then added to half the cultures (serum-deprived cells), while the other half received an equal volume of DME plus serum. Unless otherwise stated, Vero cells were deprived of serum for 20 h before cell-free extracts were prepared.

2. Preparation of Cell Fractions. Cell-free extracts were prepared from Vero or HeLa cells as described (Hassell and Engelhardt, 1973). Crude ribosomes and post-ribosomal supernatants (S-100's) were prepared by centrifugation of

† From the Microbiology Department, College of Physicians and Surgeons, Columbia University, New York, New York 10032. *Received October 3, 1975.* This study was supported by Grants GB-40650 and BMS74-02201 A01 from the National Science Foundation.

* Predoctoral fellow of the National Institutes of Health (GM-00317). Present address: Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, New York 11724.

[1] Abbreviations used: DME, Dulbecco's Modified Eagle's Medium; polyPhe, poly(phenylalanine); poly(U), sodium polyuridylate; Phe-tRNA, phenylalanyl-tRNA; EFI, elongation factor I; S-100, post-ribosomal cell supernatant; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Na_2EDTA, disodium ethylenediaminetetraacetate.

cell-free extracts at 100 000*g* for 90 minutes at 4 °C. The supernatant was carefully removed and the ribosomal pellet gently rinsed with 2 ml of ribosome buffer (250 mM sucrose, 6 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM Na_2EDTA). The centrifuge tubes were drained of buffer, and the ribosomal pellet was then resuspended at approximately 50–100 A_{260} units/ml in ribosome buffer and assayed for activity or stored in 0.05-ml aliquots at −70 °C. The S-100 fraction was assayed directly for EFI activity or could be stored frozen at −70 °C without loss of activity. Crude elongation factors were prepared from the S-100 by differential ammonium sulfate precipitation [between 25 and 70% (w/v)] according to the procedure of Engelhardt and Sarnoski (1975).

Washed ribosomes were obtained from actively dividing HeLa S_3 suspension cells. Crude ribosomes were prepared from HeLa S_3 cells as described above and washed according to the procedure of McKeehan and Hardesty (1969) including the second wash for an additional 24 h at 4 °C in 0.5 M KCl. This second wash resulted in a fivefold increase in the relative specific activity of these ribosomes in the EFI assay.

3. Isolation and Aminoacylation of Rat Liver tRNA. tRNA was isolated from whole organs as follows. Frozen livers (quick frozen at −90 °C after removal from the animal) were homogenized in buffer containing 25 mM NaCl, 25 mM Tris HCl, pH 7.6, 5 mM $MgCl_2$, in a Waring Blendor at 0–4 °C (2 ml of buffer per gram of tissue). The homogenate was centrifuged at 15 000*g* for 10 min at 4 °C. To the supernatant was added enough 2 M NaCl to bring the final sodium ion molarity to 0.1 M, and the supernatant was extracted with distilled phenol previously saturated with 100 mM NaCl, 50 mM Tris-HCl, pH 7.6 at room temperature. The aqueous phase was extracted twice more with phenol. One-tenth volume of 1.0 M potassium acetate, pH 5.5, and 2 volumes of absolute ethanol were added to the aqueous phase, and the RNA was allowed to precipitate for at least 2 h at −20 °C. This ethanol precipitation step was repeated twice more. The precipitate was then extracted twice with 1.0 M NaCl at 0–4 °C for 1 h at a time. Each extraction was followed by centrifugation at 27 000*g* for 10 min at 0 °C. The material soluble in 1.0 M NaCl was diluted with 5 volumes of glass-distilled water and precipitated with 2 volumes of absolute ethanol at −20 °C. The precipitate was then either lyophilized to dryness or dried in a stream of N_2 gas. The dry powder was resuspended in glass-distilled water and stored at −20 °C.

Several different sources of tRNA have been used in this study with identical results. These include bovine liver, rat liver, HeLa cell, rabbit reticulocyte, Krebs II ascites tumor cell, and commercial yeast.

Rat liver phenylalanyl-tRNA ligase was prepared exactly as described by Tscherne et al. (1973), up to and including the first wash step, from the ribosomal fraction. The ribosomal pellet was then resuspended in buffer (250 mM sucrose, 10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 60 mM KCl, 1 mM dithiothreitol) at 300 A_{260} units/ml and dialyzed against this same buffer for 12 h. This material was then divided into aliquots and stored at −70 °C.

To prepare [^{3}H]phenylalanyl-tRNA ([^{3}H]Phe-tRNA), reaction mixtures of 5–10 ml were used. The reaction mixture contained 60 mM Tes, pH 7.6, 6.0 mM $MgCl_2$, 3.0 mM Na_2ATP, 0.06 mCi/ml of [^{3}H]-L-phenylalanine (5.32 Ci/mmol). When tRNA was aminoacylated, it was at a final concentration of 0.56 mg/ml, and the phenylalanyl-tRNA ligase preparation was at 0.6 A_{260} unit/ml. The specific activity of the [^{3}H]Phe-tRNA after recovery was 7000 cpm per μg of tRNA. The reaction mixture was incubated at 32 °C for 20 min; at the end of this period, 0.1 volume of 1.0 M potassium acetate, pH 5.5, was added, and the mixture was extracted with distilled phenol saturated at room temperature with 50 mM potassium acetate, pH 5.5. The aminoacyl-tRNA was recovered from the aqueous phase by precipitation with absolute ethanol (−20 °C for 2 h). It was washed twice with ethanol and dried in a stream of N_2 gas. It was then dissolved in a small volume of glass-distilled water and stored in aliquots at −70 °C. Approximately 70–80% of the original trichloroacetic acid insoluble product was recovered by this procedure.

4. In Vitro Assay for Protein Synthesis. The assay conditions for in vitro polypeptide synthesis have been described (Hassell and Engelhardt, 1973). For reactions in which [^{3}H]Phe-tRNA was used, phenylalanine was added to the reaction mixture at a final concentration of 0.01 mM, and 50 mM NH_4Cl was substituted for 60 mM KCl.

5. Assay for Elongation Factor I Activity. The enzymatic binding of [^{3}H]Phe-tRNA to ribosomes mediated by EFI was performed in a 0.25-ml assay mixture containing 50 mM Tris-HCl, pH 7.4, 7.5 mM $MgCl_2$, 160 mM NH_4Cl, 1 mM dithiothreitol, 0.1 mM GTP, 20 μg of poly(U), and 1–2 A_{260} units of washed HeLa ribosomes or varying amounts of crude ribosomes as described in text. The assay mixture was incubated at 0 °C for 20 min, then diluted with 3 ml of buffer containing 50 mM Tris-HCl, pH 7.4, 7.5 mM $MgCl_2$, 60 mM NH_4Cl, and immediately filtered through a Millipore filter (0.4-μm pore size, 2.4-cm diameter). The filters were washed twice with the above buffer, dried, and counted in a scintillation counter. This procedure is a modification of that of Ravel and Shorey (1971). The validity of this assay as a measure of EFI activity has been discussed. In particular the radioactivity bound to filters has been identified as phenylalanine (Engelhardt and Sarnoski, 1975).

6. Assay for Peptidyltransferase Activity. The incorporation of [^{3}H]puromycin into nascent polypeptides has been used to assay for peptidyl group transfer on ribosomes from Vero cells, and the number of ribosomes active in this step was calculated according to the method of Wool and Kurihara (1967).

A modification of the assay procedure of Pestka (1974) was used as follows. Each 50 μl of assay mixture contained 800 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-acetate, pH 7.2, 5 μM [^{3}H]puromycin (3.8 Ci/mmol), and from 0.5 to 5.0 A_{260} units of crude ribosomes. The reaction mixture was incubated at 35 °C for 30 min. The reaction was stopped by adding 2 ml of ice-cold 10% (w/v) trichloroacetic acid to precipitate the peptidyl[^{3}H]puromycin, and the reaction mixture then filtered through a polyvinyl chloride Millipore filter (type BDWP, 0.6-μm pore size, 25-mm diameter). The tube and filter were washed three more times with 3-ml volumes of 5% trichloroacetic acid, and eight times with 3-ml volumes of absolute ethanol. This assay procedure was used in preference to those previously reported because less than 0.0017% of the input radioactivity was retained on the filter when ribosomes were omitted from the reaction mixture.

7. Protein and RNA Measurements. Protein determinations were performed at four different concentrations in duplicate by the method of Lowry et al. (1951), using bovine serum albumin as a standard. RNA concentrations were

Table I: The Translational Activity of Mixed Ribosomes from Control and Serum-Deprived Cells.[a]

Control Cell Ribosomes (A_{260} units)	Serum-Deprived Cell Ribosomes (A_{260} units)	Total (A_{260} units)	Amount of Phe Polymerized (nmol)	Expected Amount of Phe Polymerized (nmol)
0.2		0.2	5.7	
0.4		0.4	10.8	
0.5		0.5	12.7	
	0.2	0.2	2.2	
	0.4	0.4	4.0	
	0.5	0.5	4.7	
0.1	0.1	0.2	3.9	3.95
0.2	0.2	0.4	8.0	7.9
0.3	0.3	0.6	10.8	11.85

[a] Ribosomes from control, serum-deprived cells, and mixtures thereof were assayed for their capacity for polyPhe synthesis (see Materials and Methods). Each value is the average of duplicate determinations.

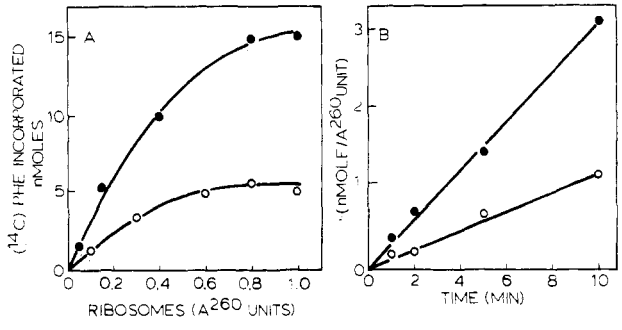

FIGURE 1: The effect of serum deprivation on the translational efficiency of ribosomes. To measure the time course of polyPhe synthesis, duplicate 0.1-ml aliquots were removed from a 1.5-ml reaction mixture containing 3.0 A_{260} units/ml of ribosomes and processed as described in Materials and Methods. Panel A: The capacity of ribosomes prepared from control and serum-deprived cells to synthesize polyPhe. Panel B: The time courses of polyPhe synthesis by control and serum-deprived cell ribosomes. (○) Ribosomes prepared from cells deprived of serum for 20 h; (●) ribosomes prepared from control cells.

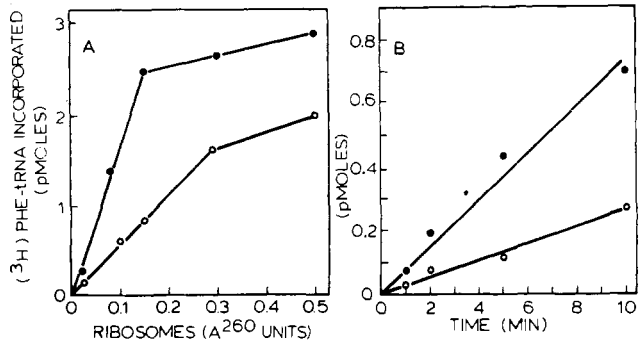

FIGURE 2: The effect of serum deprivation on the translational efficiency of ribosomes using [^{3}H]Phe-tRNA as a precursor. To measure the time course of polyPhe synthesis, a 1.5-ml reaction was used and 0.1 ml aliquots were removed at intervals as described previously in Materials and Methods. The ribosome concentrations were 3.0 A_{260} units/ml for both preparations. Panel A: The capacity of ribosomes to synthesize polyPhe. Panel B: The time course of polyPhe synthesis of ribosomes. (●) Ribosomes prepared from control cells; (○) ribosomes prepared from serum-deprived cells.

measured in duplicate at four different dilutions by the method of Munro and Fleck (1966).

Results

Serum Deprivation and Ribosome Function. Serum deprivation of Vero cells results in a decline in vivo in the rate of protein synthesis. However, after serum deprivation the number of ribosomes in polyribosome structures does not decrease. These data indicate a decrease in the rate of peptide elongation (Hassell and Engelhardt, 1973). Cell-free extracts prepared from these cells (or chicken embryo fibroblast cells) exhibit a diminished capacity for peptide chain elongation (Soeiro and Amos, 1966; Hassell and Engelhardt, 1973). Soeiro and Amos concluded that the peptide elongation defect resided in the crude ribosomal fraction. We therefore inquired if serum deprivation of Vero cells impaired ribosome function.

Crude ribosomes were prepared (Materials and Methods) from serum-deprived and control cultures and their capacity to synthesize polyphenylalanine (polyPhe) was determined. When [^{14}C]phenylalanine was used as a precursor, the extent of polyPhe synthesis by crude serum-deprived cell ribosomes was reduced to 40% of the control after 20 h of serum deprivation (Figure 1A). The time course of incorporation of [^{14}C]phenylalanine into trichloroacetic acid insoluble material by serum-deprived cell ribosomes was also reduced to 45% of the control rate (Figure 1B). Therefore both the extent and rate of polyPhe synthesis by ribosomes were reduced when Vero cells were deprived of serum. The extent of this reduction, as determined by ten separate repetitions of this measurement, was from 30 to 50% of the control value.

This decreased activity in protein synthesis displayed by serum-deprived cell ribosomes could have been caused by a decreased rate of aminoacylation of tRNA. If this were true, then the rate of protein synthesis using [^{3}H]Phe-tRNA as a precursor should be the same with control and serum-deprived cell ribosomes. We therefore assayed the extent and rate of polyPhe synthesis using [^{3}H]Phe-tRNA as a precursor.

The results show that the difference in the extent (Figure 2A) as well as the rate (Figure 2B) of polyPhe synthesis between control and serum-deprived cell ribosomes was maintained. Therefore reactions subsequent to aminoacylation of tRNA must be affected by serum deprivation of Vero cells.

To test the possibility that serum deprivation induces the appearance of an inhibitor of in vitro protein synthesis, we mixed ribosomes prepared from control and serum-deprived cells and measured their capacity for polyPhe synthesis. Table I shows that mixed ribosomes displayed additive synthetic capacities. Therefore the loss of activity of serum-deprived cell ribosomes is not due to the action of an inhibitor.

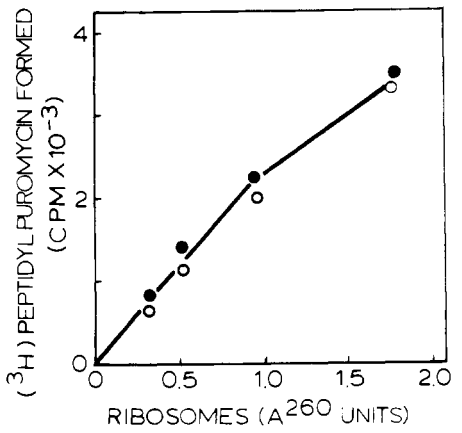

FIGURE 3: The effect of serum deprivation on the peptidyltransferase activity of ribosomes. Varying concentrations of crude ribosomes were tested for their capacity to catalyze bond formation between peptidyl-tRNA and [^{3}H]puromycin. (○) Crude ribosomes prepared 20 h after serum deprivation; (●) crude ribosomes prepared from control cells.

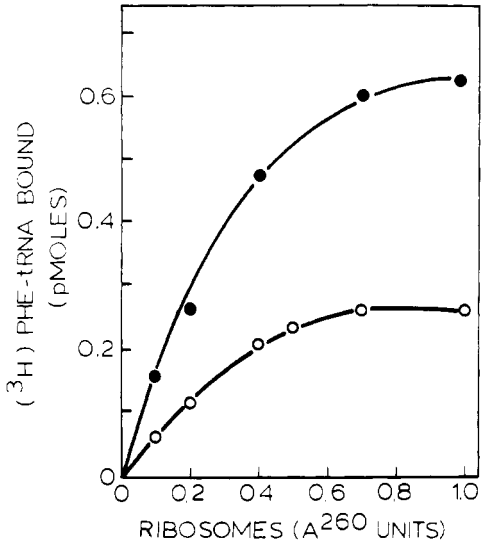

FIGURE 4: The effect of serum deprivation on the capacity of crude ribosomes to bind [^{3}H]Phe-tRNA in the presence of poly(U). Each value is the average of duplicate determinations. (●) Ribosomes prepared from control cells; (○) ribosomes prepared from serum-deprived cells.

The decreased protein synthetic capacity of serum-deprived cell ribosomes could have been caused by a reduction of peptidyltransferase activity. Peptidyltransferase activity can be determined by measuring the transfer of the nascent, ribosome-bound peptidyl moiety to [^{3}H]puromycin. The amount of peptidyl[^{3}H]puromycin formed is measured by determining the [^{3}H]puromycin incorporated into trichloroacetic acid precipitable material (Pestka, 1974). To determine if serum regulated the activity of peptidyl transferase, ribosomes were prepared from control and serum-deprived cells (Materials and Methods) and assayed for their capacity to incorporate [^{3}H]puromycin. Using this reaction we found no difference between control and serum-deprived cell ribosomes (Figure 3). From the amount of peptidyl [^{3}H]puromycin formed, we calculate that 21–24% of the ribosomes from control and serum-deprived cells are actively engaged in protein synthesis (see Wool and Kurihara (1967) for calculation). We conclude that the functionality of the ribosome as measured by the puromycin reaction is not impaired by serum deprivation of Vero cells.

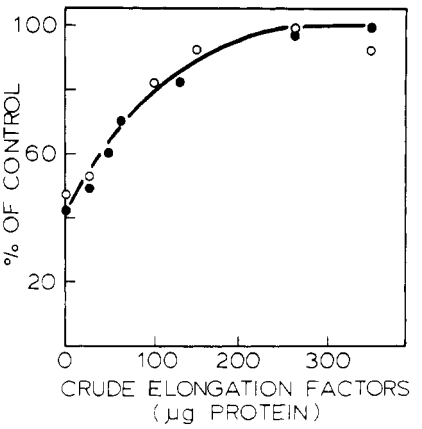

FIGURE 5: Restoration of ribosome activity by crude elongation factors. Crude elongation factors prepared from growing HeLa cells (Materials and Methods) were added to ribosomes prepared from serum-deprived and control Vero cells. These were then assayed for their capacity to synthesize polyPhe (Materials and Methods) using [^{3}H]phenylalanine as a precursor, and to bind [^{3}H]Phe-tRNA. The results were calculated per A_{260} unit and expressed as the percentage of control preparations. Each value is the average of triplicate determinations. (○) The capacity of ribosomes to synthesize polyPhe using [^{3}H]phenylalanine as a precursor; (●) the capacity of ribosomes to bind [^{3}H]Phe-tRNA.

The Effect of Serum Deprivation on Ribosome Associated Elongation Factor I Activity. Crude ribosomes prepared from Vero cells were capable of engaging in polyPhe synthesis in the absence of added elongation factors (Figure 1). These ribosomes therefore must have elongation factors associated with them. Since ribosomes from serum-deprived cells were inefficient in polyPhe synthesis relative to controls (Figure 1) but were as active as controls in the peptidyl transfer reaction (Figure 3), we investigated the possibility that serum deprivation of Vero cells elicited a reduction in the activity of these ribosome associated elongation factors.

Endogenous elongation factor I (EFI) activity was measured in crude ribosome preparations from control and serum-deprived cells. The results are shown in Figure 4. Serum-deprived cell ribosomes were 40–50% less active in this assay relative to control cell ribosomes. To ensure that free as well as bound EFI was being assayed, we added washed HeLa ribosomes to these crude ribosome preparations. The addition of washed ribosomes resulted in a 1.5–2.0-fold stimulation of binding of [^{3}H]Phe-tRNA for both control and serum-deprived cell ribosomes (data not shown), but the difference between control and serum-deprived cell ribosomes remained. We conclude that serum deprivation results in a decline in the capacity of ribosomes to bind [^{3}H]Phe-tRNA.

To determine if there was simply less EFI activity associated with serum-deprived cell ribosomes or if the decrease were due to an impairment of the capacity of the ribosome per se to bind [^{3}H]Phe-tRNA, we supplemented ribosomes from control and serum-deprived cells with a crude elongation factor preparation from HeLa cells (Materials and Methods). The results in Figure 5 show that at saturating concentrations of crude elongation factors the activity of serum-deprived cell ribosomes could be restored to 95–100% of the control cell ribosomes for binding of [^{3}H]Phe-tRNA. The capacity of these same ribosomes for polyPhe synthesis was also restored by crude elongation factors to 90–100% of the control level (Figure 5). Therefore we con-

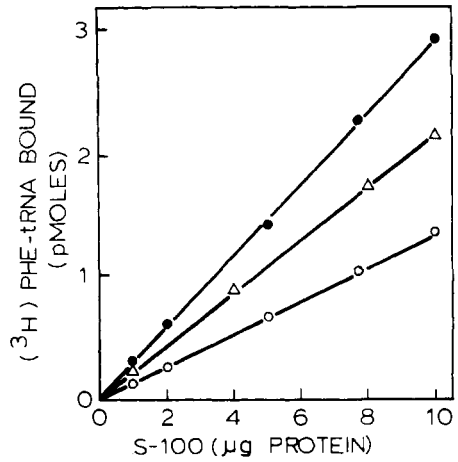

FIGURE 6: The effect of serum-deprivation on EFI activity in the S-100. S-100's were prepared from control and serum-deprived cells (Materials and Methods), and these were then assayed for EFI activity (Materials and Methods). Each value is the average of duplicate determinations. (○) EFI activity in the S-100 from serum-deprived cells; (△) the EFI activity of a 1:1 mixture (on a protein basis) of serum-deprived and control cell S-100's; (●) the EFI activity of a control cell S-100.

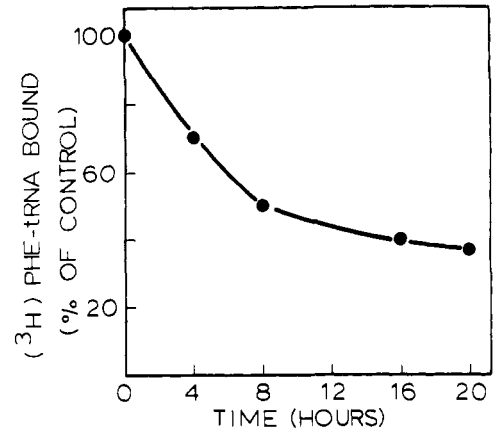

FIGURE 7: The temporal decline of EFI activity in the S-100 after serum deprivation. S-100's were prepared at intervals after serum deprivation and their EFI activity measured (Materials and Methods). The results are expressed as the percentage of a control S-100 prepared at the same time as the serum-deprived cell S-100. Each S-100 was assayed in duplicate between 1 and 10 μg of protein at five different protein concentrations and the percentage difference between the control and serum-deprived cell S-100's calculated. (●) The percentage difference in EFI activity between control and serum-deprived cell S-100's.

clude that the reduced capacity of ribosomes prepared from serum-deprived cells to bind [^{3}H]Phe-tRNA relative to control cell ribosomes is not due to impaired ribosome function per se, but due to a decline of EFI activity associated with these ribosomes. Moreover, the serum-deprivation-induced reduction of ribosome-associated EFI activity (30–50% determined five times) is sufficient to account for the decreased capacity of serum-deprived cell ribosomes to engage in polyPhe synthesis (30–50%, see above).

The Effect of Serum Deprivation on Elongation Factor I Activity. Our previous experiments indicated that serum deprivation of Vero cells led to a relative decrease in the activity of EFI associated with ribosomes (Figure 4). We therefore inquired whether serum deprivation of Vero cells resulted in a decrease in the activity of EFI in the post-ribosomal supernatant (S-100). When Vero cells were deprived of serum for 20 h, the EFI activity in the S-100 of these cells was reduced to 42% relative to the control (Figure 6). Moreover a 1:1 mixture of the S-100's showed strict additivity of EFI activity in the mixed S-100's, suggesting that stimulators or inhibitors were not present (Figure 6).

The rate of protein synthesis in vivo and cell-free peptide elongation capacity of extracts in vitro decline at the same rate after serum starvation of Vero cells (Hassell and Engelhardt, 1973). When S-100's were prepared from Vero cells at intervals following serum deprivation, a decline in EFI activity was observed which paralleled this reported decline in the in vivo and in vitro rate of protein synthesis and peptide chain elongation (Figure 7). A temporal correlation therefore exists among the in vivo rate of protein synthesis, the capacity of cell-free extracts for peptide chain elongation, and the activity of EFI. The decline in the specific activity of EFI is not a consequence of a general decline in the amount of protein per cell. The overall protein content per cell after serum starvation for 20 h was 90% ± 5 (three determinations) of the control. Moreover the rate of protein degradation is unaffected within this time period of serum starvation (Hassell, unpublished). Therefore the loss of EFI activity occurs during a period when the amount of protein per cell is relatively unaffected.

The Effect of Serum Stimulation on in Vitro Protein Synthesis. To determine if serum factors reversibly affect the protein synthetic capacity of cell-free extracts, serum was added to Vero cells previously deprived of serum for 20 h. Cell-free extracts were prepared from these cells and assayed for their capacity to synthesize polyPhe. Within 3 h after serum stimulation of serum-deprived cells, the peptide chain elongation capacity of their cell-free extracts was restored to a level equal to the control cell extract (Table II). Moreover, RNA synthesis was not required for this recovery of activity since pretreatment of cells for 1 h prior to and during the time of serum addition with 10 μg/ml of actinomycin D failed to prevent this turn-on of the translational machinery (Table II). This was not a consequence of the toxic effects of the drug since control experiments demonstrated that actinomycin D alone did not reduce the peptide chain elongation activity of either control or serum-deprived cell extracts. The addition of actinomycin D with serum enhances the capacity of cell-free extracts to synthesize polyPhe. This resembles the situation in rat hepatoma cells where the addition of glucocorticoids with actinomycin D results in superinduction of tyrosine aminotransferase activity (Tomkins et al., 1969). While the mechanism whereby superinduction occurs is unknown, it seems clear that in this system serum factors act reversibly to regulate the translational machinery by a post-transcriptional mechanism.

Discussion

The results reported here lead to a more precise understanding of Vero cells' response to serum deprivation. From previous data we know that serum deprivation of Vero cells rapidly lowered the in vivo rate of protein synthesis per cell as well as the peptide elongation capacity of cell-free extracts prepared from these cells. These two effects occurred at the same time and to the same extent, suggesting that they were different measures of the same event (Hassell and Engelhardt, 1973). The ribosome sedimentation profile of Vero cells was not changed after 20 h of serum deprivation except for a slight shift to heavier polysomes (Hassell and Engelhardt, 1973). This coupled with the observation that polyPhe synthesis in vitro was reduced after serum de-

Table II: The Effect of Serum Stimulation on the Capacity of Cell-Free Extracts to Synthesize Polyphenylalanine.[a]

Source of Cell-Free Extract	Treatment[b]	Polyphenylalanine Synthesis[c]	Percentage of Control
Control cells	None	400	100
	Serum (10%)	422	106
	Act-D (10 μg/ml)	390	98
	Act-D (10 μg/ml); serum (10%)	383	96
Serum-deprived cells	None	181	45
	Serum (10%)	436	109
	Act-D (10 μg/ml)	175	44
	Act-D (10 μg/ml); serum (10%)	815	204

[a] Actinomycin D (final concentration 10 μg/ml) was added to the medium of some cultures 1 h prior to adding serum (containing 10 μg/ml of Actinomycin D) to a final concentration of 10% (v/v). Cell-free extracts were prepared 3 h after serum addition and assayed in the standard reaction mixture, except that the amount of [^{14}C]phenylalanine was increased to 0.5 μCi. Cell-free extracts were assayed over the range of 0.3–1.5 mg/ml of extract protein. Incorporation of [^{14}C]phenylalanine was linear over this range. Each value is the average of three determinations. [b] Act-D is acytinomycin D. [c] In cpm per μg of protein per 30 min.

privation suggested that the elongation step of protein synthesis was affected.

Soeiro and Amos (1966) first reported, and we have reproduced the observation, that the peptide elongation capacity of ribosomes is reduced upon serum deprivation of cells. Those ribosomes have a decreased capacity to add amino acids to the nascent peptide chain. This is not a consequence of the production of an inhibitor of peptide chain elongation during serum deprivation since mixtures of control and serum-deprived cell ribosomes displayed strict additivity when their activities for polyPhe synthesis were measured.

The effect of serum deprivation on the activity of peptidyltransferase, an integral part of the 60S ribosomal subunit (Vazquez et al., 1969; Falvey and Staehelin, 1970), was measured and found not to change. We calculate that 21–24% of the ribosomes from both control and serum-deprived cell preparations were actively engaged in protein synthesis (see Wool and Kurihara (1967) for calculations).

The capacity of ribosomes prepared from serum-deprived cells and control cells to bind [^{3}H]Phe-tRNA was tested. Serum-deprived cell ribosomes were 40–50% less efficient in this assay compared with control preparations. Under the conditions used (see Materials and Methods) only enzymatic binding was assayed. Hence we conclude that serum deprivation of Vero cells results in a reduction of EFI activity associated with ribosomes. Supplementation of serum-deprived cell ribosomes with a crude preparation of elongation factors from growing HeLa cells resulted in a restoration of the capacity of those ribosomes to bind [^{3}H]Phe-tRNA to a level of 95–100% of the control cell ribosomes. The impaired capacity of serum-deprived cell ribosomes to synthesize polyPhe was also restored to 100% of the control value by crude elongation factors. Identical results were obtained when crude preparations of elongation factors from exponentially dividing Vero cells were used (Hassell, unpublished). We conclude that serum deprivation of Vero cells does not lead to an impairment of ribosome functioning per se. We further conclude that the activity of ribosome associated EFI is reduced.

The activity of EFI in the S-100 was reduced by serum deprivation of Vero cells. Moreover the decline in the activity of EFI during serum deprivation occurred to the same extent and at the same time as the decline in the in vivo rate of protein synthesis and the decline in the translational activity of cell-free extracts. These experiments suggest that a common mechanism is operating to produce these three effects.

This mechanism is reversible. Readdition of serum to cells which had been serum deprived leads to a rapid restoration of polyPhe synthetic capacity. This is true even in the presence of 10 μg/ml of actinomycin D. Based on these data we conclude that a translational control mechanism is operating in Vero cells deprived of serum. Furthermore our data suggest that this translational control is expressed by regulating the activity of EFI.

References

Bandman, E., and Gurney, T. (1975), *Exp. Cell Res. 90,* 159.

Christopher, C. W., Ishikawa, K., and Amos, H. (1971), *Cell Tissue Kinet. 4,* 75.

Dulbecco, R., and Elkington, J. (1973), *Nature (London) 246,* 197.

Engelhardt, D. L. (1971), *J. Cell. Physiol. 78,* 333.

Engelhardt, D. L., and Sarnoski, J. (1975), *J. Cell. Physiol. 86,* 15.

Falvey, A. K., and Staehelin, T. (1970), *J. Mol. Biol. 53,* 1.

Hassell, J. A., Colby, C., and Romano, A. H. (1975), *J. Cell. Physiol. 86,* 37.

Hassell, J. A., and Engelhardt, D. L. (1973), *Biochim. Biophys. Acta 324,* 545.

Hershko, A., Mamont, P., Shields, R., and Tomkins, G. M. (1971), *Nature (London), New Biol. 232,* 206.

Levine, L., Becker, Y., Boone, C., and Eagle, H. (1965), *Proc. Natl. Acad. Sci. U.S.A. 53,* 350.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

McKeehan, W. L., and Hardesty, B. (1969), *J. Biol. Chem. 244,* 4330.

Munro, H. N., and Fleck, A. (1966), *Analyst (London) 91,* 78.

Pestka, S. (1974), *Methods Enzymol. 30,* 261.

Ravel, J. M., and Shorey, R. A. L. (1971), *Methods Enzymol. 20,* 306.

Rudland, P. S. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 750.

Soeiro, R., and Amos, H. (1966), *Science 154,* 662.

Stanners, C. P., and Becker, H. (1971), *J. Cell. Physiol. 77,* 31.

Todaro, G. J., Lazar, G. K., and Green, H. (1965), *J. Cell. Comp. Physiol. 66,* 325.

Tomkins, G. M., Gelehrter, T. D., Granner, D., Martin, D., Samuels, H. H., and Thompson, E. B. (1969), *Science 166,* 1474.

Tscherne, J. S., Weinstein, I. B., Lanks, K. W., Gersten, N.

B., and Cantor, C. R. (1973), *Biochemistry 12*, 3859.
Vazquez, D., Battaner, E., Neth, R., Heller, G., and Monro, R. E. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34*, 369.
Ward, G. A., and Plagemann, P. G. W. (1969), *J. Cell. Physiol. 73*, 213.
Wool, I. G., and Kurihara, K. (1967), *Proc. Natl. Acad. Sci. U.S.A. 58*, 2401.
Yasumura, Y., and Kawakita, Y. (1963), *Nippon Rinsho 21*, 1209.

Amino Acid Sequence of Rabbit Light Chains: Variable Region of a Light Chain from a Homogeneous Immunoglobulin Raised by Streptococcal Immunization†

A. L. Thunberg‡ and T. J. Kindt*

ABSTRACT: The variable region sequence has been determined for the light chain (L) from a rabbit homogeneous immunoglobulin (3547) produced by immunization with group A streptococcal vaccine. Unlike most immunoglobulins produced by these vaccines, this immunoglobulin had no binding activity for the group A polysaccharide nor for any of a wide range of streptococcal cell components tested, nor did it have binding activity for rabbit IgG. Tryptic digestion of the citraconylated L chain and acid hydrolysis of the aspartyl-proline bond at positions 109–110 were used to obtain two variable region peptides comprising residues 1–61 and 62–109, respectively. Automated sequence analysis of these peptides and the peptides obtained from them by complete tryptic digestion gave sequence data for the entire L-chain variable (V) region. Comparison of the 3547 L chain V region sequence with other data supports the observations that only two hypervariable regions are present in rabbit κ chains and that the hypervariable region beginning at residue 90 may vary in length by as much as six residues.

Rabbit homogeneous antibodies induced by immunization with various bacteria are currently employed in chemical studies to explore the structure-function relationships of antibodies. Allotypic and idiotypic markers of homogeneous antibodies induced in genetically defined animals are also used to study the genetic basis of antibody diversity (Kindt et al., 1974). Extensive sequence data on homogeneous antibodies are needed, however, to unravel the complexity of the antigen binding site and to reveal the precise structures for the various genetic markers. Sequence data are accumulating for rabbit antibody light (L^1) chains directed against *p*-azobenzoate (Appella et al., 1973) and polysaccharides from the group C streptococcus (Chen et al., 1974), the type 3 pneumococcus (Jaton, 1974a,b, 1975), and the type 8 pneumococcus (Margolies et al., 1975).

An unexpected feature of rabbit antibody V_L region structure is the presence of only two hypervariable regions. Although three hypervariable regions have been described for mouse and human κ chains (Wu and Kabat, 1970), only the first and third are present in the rabbit L chains. Another interesting aspect of rabbit antibody light chains concerns the high degree of variability observed among the N-terminal residues. Variation in this region has been used to divide V regions of κ chains into subgroups (Hood et al., 1970). Recent studies on N-terminal hypervariability suggest there are a larger number of subgroups than were previously estimated (Thunberg and Kindt, 1975). More sequence information will be required to define the variable region subgroups and to resolve questions concerning the relationship between variable region sequences and the group b allotypes of the constant region of the rabbit κ chain.

The present report describes sequence analysis of the V region of an allotype b4 L chain from a homogeneous immunoglobulin produced by a rabbit hyperimmunized with group A streptococci. While most homogeneous immunoglobulins produced in this fashion have antibody activity for the group A carbohydrate, homogeneous components for which no antibody activity can be detected have been observed in concentrations as high as 40 mg/ml. The immunoglobulin studied here is such an example. The strategy for the sequence determination relied heavily on automated sequence analysis of peptides obtained by tryptic digestions carried out after various chemical modifications of the L chain.

Materials and Methods

Isolation of 3547 L Chains. An immunoglobulin was isolated from the serum of rabbit 3547 by chromatography on DEAE-cellulose (Kindt et al., 1972). This isolated IgG was homogeneous by cellulose acetate electrophoresis and by alkaline urea disc electrophoresis of the L chains (Reisfeld

† From The Rockefeller University, New York, New York 10021. *Received October 17, 1975.* This work was supported by PHS Grant A108249 from the NIAID and a Grant-in-Aid from the American Heart Association. This work was performed during the tenure of an Established Investigatorship of the American Heart Association awarded to Dr. T. J. Kindt.

‡ Current address: The Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218.

[1] Abbreviations used are: Pth, phenylthiohydantoin derivative of an amino acid; SPITC, 4-sulphophenyl isothiocyanate; DMAA, dimethylallylaminetrifluoroacetic acid buffer, pH 9.5; AECys, *S*-2-aminoethyl-L-cysteine; H chain, heavy chain; L chain, light chain; V region, variable region; TC and TA, peptides from tryptic digests for the carboxamidomethylated and aminoethylated L chains, respectively; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.