Protein Synthesis in the Cultured Fetal Mouse Heart: Effects of Deprivation of Oxygen and Oxidizable Substrate†

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ABSTRACT: To define regulation of cardiac protein synthesis, we studied protein synthesis in cultured fetal mouse hearts injured by deprivation of oxygen and oxidizable substrates. Time-dependent changes in the rate of total cardiac protein synthesis, in tissue concentrations of amino acids, ATP, and GTP, and in the distribution and translatability of mRNA were measured. Injury reversibly inhibits protein synthesis. [^{3}H]Phe incorporation ceases within 30 min after the onset of deprivation, but, even after 5 h of deprivation, the rate of [^{3}H]Phe incorporation eventually returns to control rates after reoxygenation. After 30 min of deprivation, neither amino acid nor purine nucleotide pools are significantly depleted. After 5 h of deprivation, the cellular mRNA content and the rate, extent, and products of mRNA-directed cell-free translation are indistinguishable for mRNA isolated from deprived and control hearts. However, the percentage of cytoplasmic mRNA in polysomes decreases about 3-fold from ~50% in control hearts to ~18% in hearts deprived for 30 min or for 5 h, suggesting that initiation of translation is inhibited. In addition, after 5 h of deprivation (but not after 30 min), the ratio of rRNA:mRNA in polysomes from deprived hearts is 30% greater than that for polysomes from control hearts, a property consistent with a slightly reduced rate of elongation. Thus, inhibition of protein synthesis in the deprived heart results primarily from reduced entry of mRNA into polysomes.

The study of translational regulation in the cultured fetal mouse heart combines the advantages of controlled culture conditions with a system that simulates some important properties of ischemic injury, namely, deprivation of oxygen and oxidizable substrates (Ingwall et al., 1975, 1980). Regulation of translation has been investigated in several other mammalian cell systems: methods employed to modulate or perturb protein synthesis include growth of cultured cells at elevated temperatures (Kelley & Schlesinger, 1978; Schochetman & Perry, 1972), regulation of translation by hemin in reticulocyte lysates (deHaro & Ochoa, 1978; Hunt et al., 1972), anchorage dependence of protein synthesis in cultured cells (Benecke et al., 1978), starvation (Sonenshein & Brawerman, 1977), viral infection (Christman et al., 1973), serum-induced growth (Lee & Engelhardt, 1978; Rudland et al., 1975), and synchronization of cells by mitotic selection (Tobey et al., 1967). In these examples, inhibition of protein synthesis often results from conditions that cells usually do not encounter in situ. We report here that protein synthesis is inhibited rapidly and reversibly in hearts subjected to conditions simulating some of the important components of ischemia. Regulation of translation in the deprived heart occurs primarily at initiation and is not limited by depletion of amino acids, purine nucleotides, or mRNA.

Experimental Procedures

Preparation of Cultures of Fetal Mouse Heart and Experimental Conditions. Hearts weighing ~2.5 mg and measuring ~2 mm in diameter from 16- to 17-day fetal mice [Crl:CD-1 (ICR)BR, Charles River, Inc., Wilmington, MA] were removed aseptically, freed of pericardia and vessels, and placed on stainless-steel grids (two hearts per grid) in organ culture dishes as previously described (Ingwall et al., 1980; Wildenthal, 1971). Cultures were incubated overnight in 0.55 mL of minimum essential medium (MEM) (Grand Island Biological Co., Grand Island, NY) at 37 °C in sealed chambers in an atmosphere of 95% O_2 + 5% CO_2. After 18 h, hearts either were maintained as above (control) or were deprived of oxygen and oxidizable substrates (glucose or glucose and branched-chain amino acids) for up to 5 h by using special media prepared by Grant Island Biological Co. Chambers containing cultures of deprived hearts were gassed continuously with 95% N_2 and 5% CO_2 (medium pO_2 12–15 torr). In recovery experiments, hearts deprived for 5 h were resupplied with oxygen and complete culture medium for periods up to 6 h.

Incorporation of [^{3}H]Phe into Total Cardiac Protein. Hearts were cultured in MEM, MEM minus glucose, or MEM minus glucose and branched chain amino acids containing either 20 μCi of L-[*ring*-2,3,4,5,6-^{3}H]Phe/mL (104 Ci/mmol, New England Nuclear Corp., Boston, MA), 0.1 mCi of L-[U-^{14}C]Phe/mL (536 mCi/mmol, New England Nuclear Corp.), or 20 μCi of [^{3}H]Leu (59 Ci/mmol, New England Nuclear Corp.). For some experiments, the Phe concentration of the culture medium was increased from 0.2 to 0.7 nmol/μL. Hearts were labeled from 10 min to 5 h, weighed, and homogenized in 1.0 mL of 10% trichloroacetic acid by Dounce glass homogenization (two hearts from one dish in 1.0 mL). Acid-insoluble material collected on glass fiber filters (Whatman GF/A) was washed with ethanol–ether (3:1). Radioactivity in aliquots of heart homogenate and in acid-

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insoluble material was measured by counting in 0.5 mL of Protosol (New England Nuclear Corp., Boston, MA) in toluene-based scintillation fluid. The efficiency of counting was 39% for ^{3}H-labeled amino acids and >85% for ^{14}C-labeled amino acids for all samples. Approximately 85–95% of acid-insoluble radioactivity was resistant to extraction in 5% perchloric acid at 70 °C for 10 min, defining the material as protein (Schneider, 1945). Total protein content was measured by using the method of Lowry et al. (1951).

For calculation of rates of protein synthesis (nanomoles of Phe incorporated per milligram of cardiac protein per hour), the specific activity of [^{3}H]Phe was determined for culture medium and intracellular water. Total water content was determined by drying 10–20 mg of tissue to constant weight. The volume of extracellular space was determined as sorbitol space (Ingwall et al., 1980). The amino acid compositions of culture media and acid-soluble fractions of homogenates (prepared from 7 to 30 mg of tissue) were made with Beckman Model 119 and Durrum 500 amino acid analyzers by using the ninhydrin reaction.

Purine Nucleotide Content. ATP and GTP concentrations were determined by using Partisil SAX ion-exchange (Whatman, Inc., Clifton, NJ) high-pressure liquid chromatography (DeBoer et al., 1980). Six to ten hearts were pooled for each analysis.

Isolation of Total RNA and Poly(adenylate)-Containing mRNA. RNA in heart homogenates, cytoplasm, or polysomal and postpolysomal ribonucleoproteins (RNPs)[1] was deproteinized by extraction with guanidine hydrochloride (Strohman et al., 1977). RNA precipitated from the third guanidine extraction was rinsed with 5 mL of 95% ethanol, drained briefly, dissolved in 20 mM EDTA, and extracted with chloroform–isoamyl alcohol (24:1) until the aqueous–organic interphase was clear of denatured protein. Deproteinized RNA from cytoplasmic samples was precipitated with 2 volumes of ethanol at −20 °C; RNA from total fetal heart was precipitated with 2 volumes of 4.5 of M sodium acetate (pH 6.0).

Poly(A)-containing mRNA separated from rRNA by two chromatographic passages on oligo(dT)–cellulose (Aviv & Leder, 1972; type T-2, Collaborative Research, Inc., Waltham, MA) was quantitated by the absorption at 260 nm.

Separation of Polysomal and Postpolysomal Ribonucleoproteins. Cytoplasmic RNPs were prepared from cultured fetal mouse hearts by sedimentation in sucrose density gradients (Ouellette et al., 1976, 1982). Homogenates of 50–100 hearts prepared by glass Dounce homogenization in 1 mL of 10 mM Tris-HCl, 0.1 M NaCl, 2 mM $MgCl_2$, and 0.25 M sucrose were centrifuged 10 min in an Eppendorf microfuge. The supernatants were sedimented in 7–47% (w/w) linear sucrose density gradients in 10 mM Tris-HCl (pH 7.4), 0.5 M NaCl, and 50 mM $MgCl_2$ for 3 h at 4 °C in the Beckman SW28 rotor at 27 000 rpm. Polysomes (>80 S) were distinguished from postpolysome RNPs (20–80 S) by continuous monitoring of the absorbance at 260 nm. RNPs were precipitated from appropriate fractions with 2 volumes of ethanol.

mRNA-Directed Cell-Free Protein Synthesis. Poly(A+) mRNA from control and deprived fetal mouse hearts was translated in the reticulocyte lysate system. Lysate was purchased from New England Nuclear Corp. as the [^{35}S]Met and [^{3}H]Leu translation systems. Each 12.5-μL reaction contained 0.5 μg of mRNA, 5 μL of micrococcal nuclease treated reticulocyte lysate, 80 mM potassium acetate, 0.65 mM magnesium acetate, 19 μCi of L-[^{35}S]Met (1119 Ci/mmol), and 1 μL of cocktail supplied with the kit containing ATP, GTP, polyamines, creatine phosphate, and creatine kinase. Incorporation of labeled amino acid into polypeptides was assayed as alkali-stable, acid-insoluble radioactivity in 1 μL of the reaction (Pelham & Jackson, 1976).

Analysis of mRNA-Directed Translation Products. Translation reactions containing 62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, and 0.0001% (w/v) bromophenol blue were boiled 3 min and applied to 1.5-mm 6–15% linear gradient polyacrylamide slab gels (Laemmli, 1970). Gels were run at 37 °C for 2 h at 120 V. Gels stained with 0.5% Coomassie Blue to locate molecular weight markers were prepared for fluorography (Laskey & Mills, 1975).

[^{3}H]Poly(uridylic acid) Hybridization. Heart RNA samples (1–100 ng) were hybridized with excess [^{3}H]poly(uridylate) [[^{3}H]poly(U)] in 200-μL reactions containing 0.10 μCi of [^{3}H]poly(U) (New England Nuclear Corp., 5.8 Ci/mmol UMP), 0.3 M NaCl, and 30 mM sodium citrate at 37 °C for 15 min (Wilt, 1973). After dilution of samples with 10 volumes of 0.30 M NaCl and 30 mM sodium citrate, they were incubated an additional 15 min at 37 °C with 10 μg of pancreatic RNase A. [^{3}H]Poly(U) hybridized with poly(A) was precipitated with 1 volume of ice-cold 20% (w/w) TCA after addition of 50 ng of yeast tRNA. Precipitates collected on nitrocellulose filters were counted in 5 mL of toluene-based scintillation fluid.

Results

Incorporation of [^{3}H]Phe into Total Cardiac Protein. Protein synthesis in the fetal mouse heart is inhibited after 30 min of deprivation, but even after 5 h of deprivation, the inhibition is reversed by resupplying O_2 and oxidizable substrate. Rates of Phe incorporation were measured by supplying hearts with [^{3}H]Phe in labeling intervals of 15, 30, or 60 min introduced during the deprivation or recovery periods (Figure 1). In deprived hearts, [^{3}H]Phe incorporation was reduced to 50% of the control rate by 30 min and to 15% of the control rate by 2 h (Figure 1A). The rate of incorporation returned to control values 3 h after resupply of oxygen and oxidizable substrates (Figure 1B). A similar pattern of inhibition and recovery was obtained by using [^{3}H]Leu in place of [^{3}H]Phe (not shown). In deprived hearts (but not recovery hearts), actual rates of [^{3}H]Phe incorporation could be overestimated by using this approach since exposure to room air at the beginning of each labeling interval may briefly relieve the extent of oxygen deprivation. Nevertheless, the kinetics of protein synthesis shown in Figure 1 are qualitatively correct.

To eliminate uncertainties resulting from introduction of oxygen while labeling deprived hearts and to test the observation of Figure 1 under different labeling conditions, we labeled hearts continuously, beginning at the onset of deprivation for periods of 15 min to 5 h. Rates of [^{3}H]Phe incorporation were calculated from linear regression analyses of the relationship of nanomoles of Phe incorporated per milligram of cardiac protein per hour. Measurements of incorporation of [^{3}H]Phe per milligram of protein (Figure 2A), acid-soluble [^{3}H]Phe activity (Figure 2B), Phe concentration, and specific activity of phenylalanine in the intracellular fluid at frequent time intervals permit rates of incorporation of [^{3}H]Phe into protein to be calculated for control and deprived hearts. The rate of [^{3}H]Phe incorporation into total cardiac protein of control heart was linear at ~1 nmol of Phe (mg

[1] Abbreviations: poly(A+) mRNA, mRNA containing poly(adenylate); RNP, ribonucleoprotein; eIF, eukaryotic initiation factor; sRF, postribosomal supernatant factor; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TCA, trichloroacetic acid.

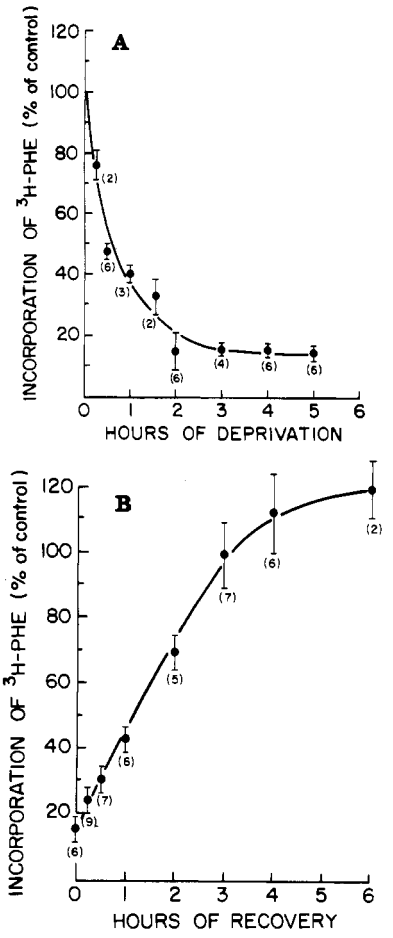

FIGURE 1: Effect of deprivation (A) and resupply (B) of oxygen and glucose on incorporation of [^{3}H]Phe into total cardiac protein. The amount of radiolabeled amino acid incorporated into trichloroacetic acid precipitable protein was determined by using 15-, 30-, or 60-min pulse labeling techniques (Experimental Procedures). The data are expressed as the mean percentage of control ± SEM. Incorporation into control hearts was 69 800 ± 3100 dpm (mg of cardiac protein)$^{-1}$ h^{-1} (n = 39). The numbers in parentheses are the number of cultures assayed.

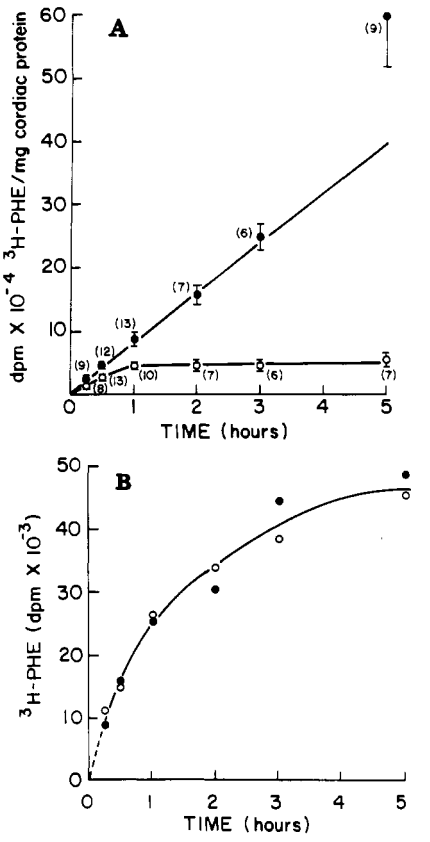

FIGURE 2: (A) Relationship between incorporation of dpm of [^{3}H]Phe per milligram of cardiac protein and labeling time for control hearts (solid circles) and hearts deprived of oxygen and oxidizable substrates (open circles). Hearts were supplied with 20 μCi of [^{3}H]Phe/mL of culture medium. Experimental conditions are as described (Experimental Procedures). Results are expressed as means ± SEM; numbers in parentheses are the numbers of cultures assayed. Protein contents of control and deprived hearts were 0.105 ± 0.002 (n = 15) and 0.100 ± 0.004 (n = 12) mg of protein/mg wet weight, respectively. (B) Acid-soluble [^{3}H]Phe was calculated as the difference between total dpm in heart homogenates and the sum of dpm in acid-insoluble material + dpm in extracellular space and assumes that the extracellular space and culture medium equilibrate rapidly. The relationship between the specific activity of the intracellular Phe pool (dpm per nanomole) and time was the same for control and deprived hearts determined by using the data shown and by the following analysis. Total tissue water was the same for control (0.825 ± 0.004 μL of H_2O/mg wet weight, n = 9) and deprived hearts [0.839 ± 0.003 (n = 3) and 0.833 ± 0.005 (n = 5) μL of H_2O/mg wet weight after 3- and 5-h deprivation, respectively]. Extracellular space was measured as sorbitol space and was the same for control (0.326 ± 0.007 μL of H_2O/mg wet weight, n = 18) and deprived hearts [0.338 ● 0.014 (n = 10) and 0.354 ± 0.025 (n = 3) μL of H_2O/mg wet weight after 3- and 5-h deprivation, respectively]. Thus, the intracellular space was ~0.50 μL for all conditions. The culture medium contained 0.21 nmol of Phe/μL, and Phe activity was ~40 000 dpm/μL. The intracellular Phe content was calculated as the difference between the nanomoles of Phe in the acid-soluble fraction and the nanomoles in the extracellular space and did not change during deprivation: linear regression analyses showed no correlation between the Phe pool and duration of deprivation (r^2 = 0.14). All values were normalized to the volume of the heart (milligrams wet weight corrected by the density of the heart, 1.054 mg/μL). Thus, the relationship for the specific activity of the Phe pool and time closely follows the pattern shown here.

of protein)$^{-1}$ h^{-1}. In contrast, after 30 min of deprivation, there was no further incorporation of Phe into protein (Figure 2A). In the deprived heart, the intracellular Phe pool remained constant and equal to that of the control heart (0.33 ± 0.03 nmol/0.5 μL) throughout the 5-h period of deprivation (averaging 0.38 ± 0.03 nmol/0.5 μL) and during recovery (0.36 ± 0.01 nmol of Phe/0.5 μL). In control and in deprived hearts, the specific activity of intracellular Phe increased with time at the same rate (not shown). These experiments independently confirm the findings shown in Figure 1A under different labeling conditions and show that the precursor pool equilibrates normally while protein synthesis is inhibited completely. Since no further radioactivity was incorporated into protein (Figure 2A), the conclusion that protein synthesis is severely inhibited by 30 min of deprivation can be made independent of any possible compartmentation of Phe or noncoordinate equilibration of aminoacyl-Phe-tRNA and intracellular Phe pools.

So that the inhibition of protein synthesis during the early phase of deprivation could be described more accurately, the extent of incorporation of [^{3}H]Phe into protein was measured at frequent intervals between 15 and 120 min of deprivation (Figure 3). The rates of Phe incorporation into total cardiac protein for control and deprived hearts are described respectively by the equations $y = 3.3 + 1.18x$ and $y = 0.48 + 0.08x$, where y is the nanomoles of Phe incorporated and x is time (hours). These results show that protein synthesis in the deprived heart is inhibited by 20–25 min after the onset of deprivation. In another experiment (not shown), hearts were preincubated with [^{14}C]Phe for 3 h before the continuous labeling experiment was begun. Again, Phe incorporation was completely inhibited by 20–30 min of deprivation of oxygen

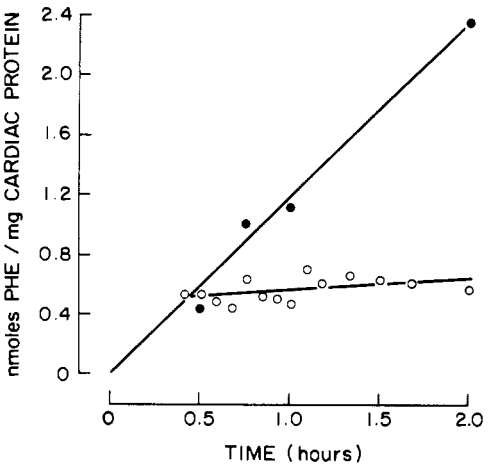

FIGURE 3: Representative experiment showing the relationship between nanomoles of Phe incorporated per milligram of cardiac protein and time for control (solid circles) and deprived (open circles) hearts. Data were obtained at 5–10-min intervals between 25 and 120 min for deprived hearts and at 15–60-min intervals for control hearts as described (Experimental Procedures). Each entry represents one culture dish.

Table I: ATP and GTP Concentrations of Control and Deprived Cultured Mouse Heart[a]

time (min)	ATP nmol/mg of protein	ATP nmol/μL of ICW	GTP nmol/mg of protein	GTP nmol/μL of ICW	n
0	35.5 ± 1.8	7.1	3.02 ± 0.26	0.60	25
15	35.0 ± 3.5	7.0	3.19 ± 0.32	0.64	23
30	31.0 ± 2.4	6.2	2.91 ± 0.17	0.58	9
60	23.0 ± 0.8	4.6	1.58 ± 0.12	0.32	13
120	16.9 ± 3.4	3.4	1.12 ± 0.11	0.22	15
300	4.18 ± 0.67	0.84	0.79 ± 0.16	0.16	10

[a] ICW = intracellular water (see legend to Figure 2).

and oxidizable substrates: the rates of Phe incorporation for control and deprived hearts were $y = 3.21 + 1.10x$ and $y = 3.45 - 0.03x$, respectively, where y is the nanomoles of Phe incorporated and x is time (hours). These results complement those in Figures 2 and 3 and eliminate the possibility that changing the specific activity of the [^{3}H]Phe pool introduced errors into these rate calculations. Thus, under all labeling conditions tested, the rate of Phe incorporation was ~1.1 nmol of Phe (mg of protein)$^{-1}$ h^{-1} for control heart and, after 20–25 min of deprivation, ~0 for deprived heart. Similar results were also obtained by using Leu (0.38 mM) and higher concentrations of Phe (0.7 mM) (not shown).

Furthermore, since cardiac protein content does not change even after 5 h of deprivation (0.21 ± 0.01 mg/control heart, $n = 12$, vs. 0.19 ± 0.01 mg/deprived heart, $n = 6$), it is unlikely that large changes in the rate of protein degradation can account for the inhibition of protein synthesis. Inhibition is not due to depletion of amino acids, since all amino acids present in control heart were also found in deprived heart (not shown). Protein synthesis is not limited by the availability of the purine nucleotides involved in initiation and elongation (Table I). Cardiac contents of ATP and GTP remained within 15% of control values for at least 30 min of deprivation; even after 5 h of deprivation, tissue ATP and GTP contents were in the millimolar range.

Cardiac mRNA Content during Deprivation. To describe the role of mRNA in the control of protein synthesis during deprivation-induced inhibition, we defined the content, synthesis, biological activity, and distribution of mRNA in control

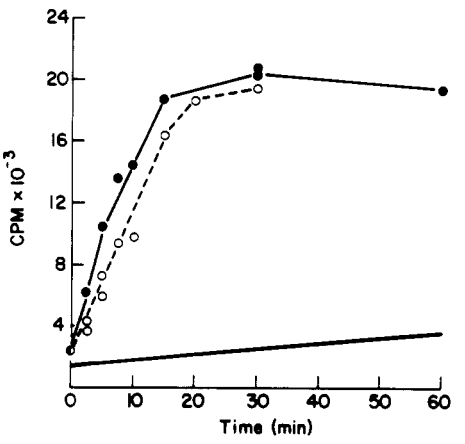

FIGURE 4: Cell-free protein synthesis directed by poly(A+) mRNA from control (solid circles) and deprived (open circles) hearts. The solid line without data points represents basal or background radioactivity. Reaction mixtures containing [^{3}H]leu were prepared and incubated as described under Experimental Procedures, except the quantity of each reagent was increased 4-fold to obtain a reaction volume of 50 μL. Amino acid incorporation was quantitated as alkali-stable, acid-insoluble radioactivity (Pelham & Jackson, 1976) in 5-μL aliquot samples removed at the times shown.

Table II: Cell-Free Translation of Control and Deprived Heart mRNA[a]

expt	sample	radioactivity (cpm) control	deprived	basal
1	poly(A+) mRNA	21 000	23 700	2 100
2	total RNA	7 300	7 000	950
	poly(A+) mRNA	12 000	12 200	950
3	poly(A+) mRNA	22 800	27 300	1 600

[a] Translation mixtures were prepared and assayed after 60 min at 37 °C as described under Experimental Procedures. Radioactivity values denote acid-insoluble, alkali-stable material in 1-μL samples. Basal activity refers to incorporation directed by lysates to which no exogenous mRNA was added.

hearts and in hearts deprived for 5 h. Since control and 5-h-deprived hearts have similar mRNA contents, averaging 8.8 ng of poly(A)/μg of total RNA for both, it is unlikely that the rapid and reversible inhibition of protein synthesis that occurs during deprivation results from intracellular hydrolysis of poly(A+) mRNA. In addition, poly(A) constitutes about 5% of poly(A+) mRNA from control and deprived hearts [50 and 56 pg of poly(A)/ng of poly(A+) mRNA, respectively], showing that mRNAs were of equivalent average length (about 1500 nucleotides) in hearts maintained under the two conditions. Because only mRNA regions covalently bound to poly(A) are retained on oligo(dT)–cellulose, selective degradation of poly(A+) mRNA in deprived hearts would have increased the ratio of poly(A) to poly(A+) mRNA artifactually, and regions of mRNA on the 5′ side of the hydrolytic site would have been lost as poly(A+) RNA fragments during chromatography. The similarity between the poly(A)/poly(A+) mRNA ratios for control and deprived hearts suggests that selective degradation did not occur in the deprived heart. Thus, protein synthesis in deprived hearts is not limited by loss of poly(A+) mRNA. Since deprivation did not alter mRNA content or size significantly, we compared the ability of control and deprived heart mRNAs to direct protein synthesis in vitro.

Translational Activity of mRNA from Deprived Fetal Mouse Hearts. Poly(A+) mRNA from control and 5-h-deprived fetal mouse hearts showed equivalent activity in the reticulocyte lysate system (Table II and Figure 4). Comparisons of unfractionated cytoplasmic RNA from control and

Table III: Cytoplasmic mRNA Distribution in Control and Deprived Hearts[a]

RNA	ng of poly(A)/ μg of RNA	total RNA μg	total RNA % in polysomes	poly(A) ng	poly(A) % in polysomes
control heart					
>80S polysomal RNP	8.38 ± 0.49	66	33	554	51
20–80S postpolysomal RNP	4.05 ± 0.39	132		540	
deprived heart					
>80S polysomal RNP	6.00 ± 0.21	14	13	82	18
20–80S postpolysomal RNP	4.05 ± 0.25	94		379	
adult mouse kidney					
>80S polysomal RNP	8.60 ± 0.23	222	75	1910	77
20–80S postpolysomal RNP	7.90 ± 0.20	74		588	

[a] RNA recovered from RNP fractions was analyzed for poly(A) by hybridization with excess [^{3}H]poly(U). Total RNA was measured by the absorbance at 260 nm. A total of 100 hearts (~250 mg of tissue) were pooled for control heart data; 50 hearts (~125 mg of tissue) were pooled for deprived heart data. Adult mouse kidney samples were prepared from one pair of kidneys (500 mg of tissue). Values shown denote means of three replicates ± the standard error from the mean.

deprived heart also showed similar levels of translation (Table II), but the extent of the reaction was lower relative to poly(A+) mRNA. Therefore, accumulation of "translational control" nucleic acids (Bester et al., 1975; Pluskal et al., 1979) that inhibit translation generally or selectively seems unlikely. Although levels of activity varied slightly with the RNA and lysate preparation (Table II), the mRNA-directed incorporation of [^{3}H]Leu or [^{35}S]Met was usually 10–15 times greater than the basal protein synthetic activity of the mRNA-depleted lysate. The rate of mRNA-directed [^{3}H]Leu incorporation into protein was ~1 pmol min^{-1} (μg of RNA)$^{-1}$ for mRNAs from control and deprived hearts. In every instance, mRNAs isolated from the same batch of control and deprived hearts were equally active, although slight variations were observed between batches prepared at different times (Table II). Thus, hearts deprived for 5 h retain mRNA in the active form but do not utilize it in translation.

Translation Products of Control and Deprived Fetal Mouse Heart mRNA. Poly(A+) mRNA isolated from control and deprived hearts directs the synthesis of the same 35–40 abundant polypeptides (Figure 5), suggesting that the effect of deprivation on the potential coding function of abundant mRNA is minimal. Accumulation of new biologically active, abundant mRNAs in deprived hearts was not observed. Possible adverse effects of adaptation to organ culture are ruled out by the similarity of the translation products directed by mRNA from freshly explanted hearts and hearts cultured for 18 h (not shown). Activity of a particular mRNA in a cell-free translation system does not prove that in situ translation of that mRNA occurs. However, selective mRNA inactivation by removal or modification of capped 5′ termini, by cryptic mRNA strand scissions, or by other mechanisms caused by deprivation is inconsistent with these results. Since electrophoresis of cell-free translation products permits visualization of only the 30–40 most abundant and efficient mRNA-coded polypeptides, altered coding patterns in low-abundance mRNAs, which represent >95% of total mRNA diversity, cannot be discounted (Paterson & Bishop, 1977). For example, translation of myosin heavy chain mRNA, a relatively inefficient template in the reticulocyte lysate system, was rarely observed in vitro (Lodish, 1971), although myosin accumulates in these hearts. Nevertheless, these results suggest that abundant translatable mRNAs of control and deprived hearts are similar.

Cytoplasmic Distribution of Poly(A+) mRNA in Deprived Fetal Mouse Heart. To distinguish between inhibition of protein synthesis at initiation and elongation, we quantitated the distribution of poly(A+) mRNA in polysomal and nonpolysomal 20–80S RNP of control and deprived hearts by poly(U) hybridization (Table III). Polysomes and nonpolysomal 20–80S RNP were separated by sedimentation of postmitochondrial supernatants in sucrose density gradients. Because the cytoplasmic distribution of mouse kidney poly(A+) mRNA is established (Ouellette et al., 1982), identical gradients containing adult mouse kidney postmitochondrial supernatants were also analyzed during each experiment and served as reference standards. In all gradients, the polysomal–nonpolysomal RNP boundary was the same. Fetal heart differs from adult mouse kidney in two major respects: fetal heart contains lower levels of recovered RNA, and a smaller fraction of both total RNA and poly(A+) mRNA is found in polysomes (Table III). In control fetal mouse heart, ~33% of total cytoplasmic RNA and ~51% of poly(A+) mRNA were recovered from polysomes; in contrast, for hearts deprived for either 30 min (data not shown) or 5 h, only ~13% of total cytoplasmic RNA and only ~18% of poly(A+) mRNA were found in polysomes (Table III). This 65% reduction in the amount of mRNA in the translational apparatus shows that a defect occurs in initiation as early as 30 min from the onset of deprivation.

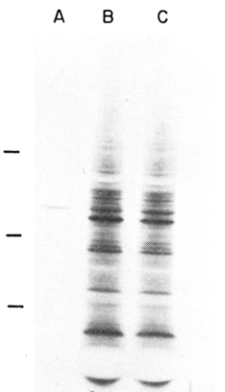

FIGURE 5: Polyacrylamide gel electrophoresis of translation products directed by poly(A+) mRNA from control and deprived hearts. [^{35}S]Met-labeled translation products (see Experimental Procedures and Table II) were electrophoresed in vertical, sodium dodecyl sulfate containing linear 6–15% polyacrylamide gradient slab gels (Experimental Procedures). Approximately 350 000 cpm were applied to lanes B and C. Gels were stained to locate molecular weight markers, prepared for fluorography (Laskey & Mills, 1975), and exposed for 18 h on Kodak X-Omat RP film. Bars at left denote the following molecular weight markers: top, phosphorylase *a* (M_r 92 500); center, ovalbumin (M_r 45 000); bottom, trypsinogen (M_r 24 500). Lane A, products synthesized without addition of mRNA; lane B, products coded by control heart mRNA; lane C, products coded by deprived heart mRNA.

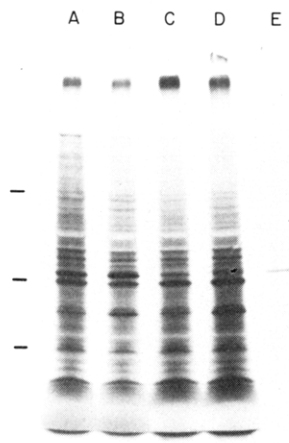

FIGURE 6: Translation products directed by polysomal and postpolysomal mRNA from control and deprived hearts. Translation products were prepared and electrophoresed as in Figure 5. Bars at left denote molecular weight markers: top, phosphorylase *a* (M_r 92 500); center, ovalbumin (M_r 45 000); bottom, trypsinogen (M_r 24 500). Translation products were directed by poly(A+) mRNA from control heart polysomes (A), control heart postpolysomal RNP (B), deprived heart polysomes (C), deprived heart postpolysomal RNP (D), and lysate without mRNA addition (E).

It seems likely that inhibition of initiation alone would result in all poly(A+) mRNA eventually accumulating in 20–80S nonpolysomal RNP. This was not observed, suggesting that the composition of polysomes might be altered as well. The ratio of poly(A+) mRNA to polysomal RNA (nanograms per microgram) was the same for hearts deprived for 30 min (not shown) and for control hearts, but was reduced ~30% in hearts deprived for 5 h (from 8.4 to 6.0 ng/μg) (Table III). The significance of the lower poly(A):polysomal RNA ratio for hearts deprived for 5 h is not totally clear, but these results suggest that polysome composition changes only after prolonged deprivation. Thus, protein synthesis is not limited by the availability of functional mRNA. Early during deprivation, protein synthesis is regulated primarily by a block in initiation (~65% inhibition). Since protein synthesis is completely inhibited by 30 min, however, inhibition of initiation may not be the sole mechanism regulating translation.

Translational Products Directed by Polysomal and Nonpolysomal Fetal Heart Poly(A+) mRNA. [^{35}S]Met-labeled translation products directed by polysomal and nonpolysomal mRNA from control and deprived hearts were examined in polyacrylamide gels (Figure 6) to test for selective expression of mRNAs in situ during deprivation. Qualitatively, their products were similar to each other and to those directed by total cardiac poly(A$^+$) mRNA (Figure 5). Small differences observed in the synthesis of high molecular weight cell-free synthesized polypeptides observed are unreliable since high molecular weight mRNAs initiate translation poorly. Minor quantitative differences possibly exist in the relative labeling of 40–80-kdalton translation products. In addition to the similar pattern of translation products, the translational activity of polysomal and nonpolysomal mRNA also was equivalent for control and deprived hearts (not shown). Thus, no major qualitative differences were observed in biologically active mRNAs recovered from the translational apparatus. We conclude that large changes in the selective expression of abundant fetal heart mRNA during deprivation do not occur.

Discussion

In the cultured fetal mouse heart, translational controls operate during the reversible inhibition of protein synthesis caused by deprivation of oxygen and oxidizable substrates. Analyses of the mRNA content, translatability, and the cytoplasmic distribution of poly(A+) mRNA of control and deprived hearts all support the conclusion that protein synthesis is not limited by the availability of biologically active mRNA. Our results suggest that regulation of protein synthesis in the deprived fetal heart occurs at both initiation and elongation of translation. Based on analyses of rates of protein synthesis, purine nucleotide contents, and ribosomal subunit distribution, Morgan and colleagues (Kao et al., 1976) concluded that regulation of protein synthesis in anoxic and ischemic isolated perfused rat hearts occurs primarily at elongation. On the basis of direct analyses of biologically active mRNA in polysomal and nonpolysomal RNA, our results show that the primary block of protein synthesis is at initiation. Differences in the nature and severity of injury, the species, and the degree of differentiation may be important in defining changes in initiation and elongation rates.

In both fetal and adult heart, deprivation of oxygen and oxidizable substrates leads to many biochemical and physiological derangements. The rate and extent of recovery from deprivation are reduced the longer the period of deprivation, and if deprivation is sufficiently prolonged, cell death occurs. Cultured fetal mouse hearts transiently deprived of oxygen and oxidizable substrates undergo mechanical, biochemical, and ultrastructural changes similar to those that occur in ischemic adult myocardium (DeBoer et al., 1980; Ingwall et al., 1975, 1980). Fetal mouse hearts cease beating within 8 min of deprivation, concomitant with a rapid depletion of creatine phosphate. Tissue contents of ATP and GTP remain close to those of control heart even 30 min after the onset of deprivation; even after 5 h of deprivation, purine nucleotide contents are still relatively high (in the millimolar range, Table I). Ultrastructural changes characteristic of ischemic injury occur early in deprivation: margination of nuclear chromatin, mitochondrial swelling, and glycogen depletion. In spite of these changes, cells remain intact even after 5 h of deprivation; release of cytoplasmic enzyme does not occur and protein and RNA contents are unchanged. Moreover, 5 h of deprivation does not prevent return to control rates of protein synthesis. Taken together, these results suggest that the inhibition of protein synthesis that occurs during the first hour of deprivation cannot be attributed to irreversible injury or cell death. Furthermore, the early inhibition of protein synthesis cannot be due to large increases in the rate of protein degradation nor to decreased availability of amino acids or purine nucleotides. Our results show that even after 5 h of deprivation, the availability of biologically active mRNA does not limit translation. Rather, protein synthesis is inhibited primarily at the level of translation. The cultured fetal heart should become a useful system in elucidating the mechanisms of translational regulation as well as providing other molecular details of myocardial injury.

The mechanisms that regulate cardiac protein synthesis during deprivation and recovery remain to be elucidated, but several possibilities are suggested by information derived from other systems. For example, phosphorylation of eukaryotic initiation factor eIF-2 by a cAMP-independent protein kinase inhibits further initiation of translation in the reticulocyte lysate (deHaro & Ochoa, 1978; Ranu et al., 1978; Tahara et al., 1978); a similar inhibitory factor has been isolated from rat liver (Delaunay et al., 1977). Furthermore, rabbit reticulocytes contain a postribosomal supernatant factor (sRF) that reverses the inhibition by phosphorylated eIF-2 and the inhibition of ternary complex eIF-2–GTP–Met-tRNA$_f$ formation (Ralston et al., 1978, 1979). Thus, deprivation of fetal heart may result

in phosphorylation of eIF-2 with a concomitant inhibition of protein synthesis. If a factor similar to sRF exists in mouse heart, it could be involved in the mechanism of recovery during reoxygenation. In addition to kinase-mediated inhibition, protein synthesis is subject to regulation by ADP-ribosylation of eIF-2 (Hayaishi & Kunihiro, 1977), by polyamines (Odom et al., 1978), by levels of aminoacyl-tRNAs (Zilberstein et al., 1976), and by translational control RNAs (Heywood et al., 1975). Furthermore, some protein synthetic reactions may be sensitive to small changes in the intracellular pools of nucleoside triphosphate catabolites, and translation may be limited at that level. Regulation by any of these mechanisms should be testable in this preparation, and results may provide insights into cellular events associated with ischemic injury.

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References

Aviv, H., & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1408–1412.

Benecke, B.-J., Benz-Ze'ev, A., & Penman, S. (1978) *Cell (Cambridge, Mass.) 14*, 931–939.

Bester, A. J., Kennedy, D. S., & Heywood, S. M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1523–1527.

Christman, J. K., Reiss, B., Kyner, D., Leven, D. H., Klett, H., & Acs, G. (1973) *Biochim. Biophys. Acta 294*, 153–164.

DeBoer, L. D. V., Ingwall, J. S., Kloner, R. A., & Braunwald, E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5471–5475.

deHaro, C., & Ochoa, S. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2713–2716.

Delaunay, J., Rajinder, S. R., Levin, D. H., Ernst, V., & London, I. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2264–2268.

Hayaishi, O., & Kunihiro, U. (1977) *Annu. Rev. Biochem. 46*, 95–116.

Heywood, S. M., Kennedy, D. S., & Bester, A. J. (1975) *Eur. J. Biochem. 58*, 587–593.

Hunt, T., Vanderhoff, G., & London, I. M. (1972) *J. Mol. Biol. 66*, 471–481.

Ingwall, J. S., Deluca, M., Sybers, H. D., & Wildenthal, K. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2809–2913.

Ingwall, J. S., Roeske, W. R., & Wildenthal, K. (1980) *Methods Cell Biol. 21A*, 167–186.

Kao, R., Rannels, D. E., & Morgan, H. E. (1976) *Circ. Res., Suppl. 38*, 124–130.

Kelley, P. M., & Schlesinger, M. J. (1978) *Cell (Cambridge, Mass.) 15*, 1277–1286.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335–341.

Lee, G. T.-Y., & Engelhardt, D. L. (1978) *J. Cell Biol. 79*, 85–96.

Lodish, H. F. (1971) *J. Biol. Chem. 246*, 7131–7138.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Odom, O., Kramer, G., Henderson, A. B., Pinphanichakarn, P., & Hardesty, B. (1978) *J. Biol. Chem. 253*, 1807–1816.

Ouellette, A. J., Kumar, A., & Malt, R. A. (1976) *Biochim. Biophys. Acta 425*, 384–395.

Ouellette, A. J., Ordahl, C. P., Van Ness, J., & Malt, R. A. (1982) *Biochemistry 21*, 1169–1177.

Paterson, B. M., & Bishop, J. O. (1977) *Cell (Cambridge, Mass.) 12*, 751–765.

Pelham, H. R. B., & Jackson, R. J. (1976) *Eur. J. Biochem. 67*, 247–256.

Pluskal, M. G., Roy, R. K., & Sarkar, S. (1979) *Biochem. Soc. Trans. 7*, 1091–1093.

Ralston, R. O., Das, A., Dasgupta, A., Roy, R., Palmieri, S., & Gupta, N. K. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4858–4862.

Ralston, R. O., Das, A., Grace, M., Das, H., & Gupta, N. K. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5490–5494.

Ranu, R. S., London, I. M., Das, A., Dasgupta, A., Majumdar, A., Ralston, R., Roy, R., & Gupta, N. K. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 745–749.

Rudland, P. S., Weil, S., & Hunter, A. R. (1975) *J. Mol. Biol. 96*, 745–766.

Schneider, W. C. (1945) *J. Biol. Chem. 161*, 293–303.

Schochetman, G., & Perry, R. P. (1972) *J. Mol. Biol. 63*, 577–590.

Sonenshein, G. E., & Brawerman, G. (1977) *Eur. J. Biochem. 73*, 307–312.

Strohman, R. C., Moss, R. S., Micou-Eastwood, J., Spector, D., Pryzbyla, A., & Paterson, B. (1977) *Cell (Cambridge, Mass.) 10*, 265–273.

Tahara, S. M., Traugh, J. A., Sharp, S. B., Lundak, T. S., Safer, B., & Merrick, W. C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 789–793.

Tobey, R. A., Anderson, E. C., & Petersen, D. F. (1967) *J. Cell. Physiol. 70*, 63–68.

Wildenthal, K. (1971) *J. Appl. Physiol. 30*, 153–157.

Wilt, F. H. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2345–2349.

Zilberstein, A., Dudock, B., Berissi, H., & Revel, M. (1976) *J. Mol. Biol. 108*, 43–54.