On the Mechanism by Which Insulin Stimulates Protein Synthesis in Chick Embryo Fibroblasts†

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ABSTRACT: It has been known that insulin raises the rate of incorporation of [^{3}H]leucine into the total protein of hormone-deficient chick embryo fibroblasts by ~1.5-fold. The elevation is not dependent upon the production of new messenger ribonucleic acids (mRNAs). Evidence is now presented in support of the following points: the greater labeling is due to more rapid polypeptide synthesis, not to an increase in the specific activity of leucyl-tRNA; the enhanced synthesis derives largely or entirely from a speeding up of the process of initiation, rather than that of elongation or termination; and the 1.5-fold stimulation is due to the elevated rates of formation of at least many of the fibroblast proteins. The hormone was shown before to stimulate posttranscriptionally and highly preferentially the formation of ribosomal proteins in the resting chick embryo cells. The question has been asked here whether insulin increases the production of total cell ribosomal protein by chemically altering preformed mRNAs. Results obtained by translating messages from deprived and hormone-treated cells in wheat germ and reticulocyte preparations do not support a mechanism involving covalent modification of preformed mRNAs. These observations, coupled with those previously made with inhibitors of translation, lead us to suggest that insulin stimulates protein synthesis in the resting chick embryo cells by activating limiting components of the initiation system. The effects of the hormone are greatest with messages, such as those for the ribosomal proteins, that have low affinities for the limiting initiation components.

Insulin stimulates the incorporation of [^{3}H]leucine into the total cell protein of hormone-deprived cultures of chick embryo fibroblasts by ~1.5-fold and into r proteins[1] by ~5-fold (DePhilip et al., 1979, 1980). The increased labeling of both total and r proteins begins within ~10 min after the resting cells are treated with the hormone. Neither change depends upon the synthesis of new messenger or ribosomal RNA.

Insulin may enhance the formation of non-r and r proteins by two different mechanisms. Simpler, and perhaps more attractive, however, is a hypothesis that invokes a single mechanism. Under this constraint, there would seem to be two ways in which the hormone could increase rapidly and posttranscriptionally the production of at least many cell proteins but highly preferentially that of the r proteins. First, insulin could transform untranslatable or poorly translatable cytoplasmic mRNAs into fully functional molecules, and a disproportionately large fraction of the chemically altered RNAs are those that code for r proteins. Second, the hormone could speed up a translational step that had been slowing marginally the rate of synthesis of many proteins but had been drastically limiting the formation of the r proteins.

Evidence has been presented (Ignotz et al., 1981) to support the conclusion that the selective depression of r protein production in resting chick embryo fibroblasts is due to the limited availability of two or more components of the initiation machinery for which the r protein mRNAs have especially low affinities. Conditions that are considered to increase the levels or activities of the limiting components strongly favor the synthesis of the r proteins.

The present work had two main goals. The first was to learn whether insulin stimulates total protein synthesis in the resting chick embryo cells by increasing rates of initiation, as has been suggested for r proteins (Ignotz et al., 1981), and the second, if this should prove to be the case, was to determine whether covalent alterations in total and r protein messages are responsible for the increased frequencies of initiation.

Materials and Methods

Materials. Crystalline bovine insulin was from Sigma Chemical Co., and radioactive compounds and counting solutions were from New England Nuclear. The specific activity of [^{35}S]methionine was ~1000 Ci/mmol. Wheat germ was a gift from the Dixie-Portland Flour Mills, Inc., Arkansas City, KS, and rabbit reticulocyte lysates were from Clinical Convenience Products, Madison, WI.

Tissue Culture. Primary cultures of chick embryo fibroblasts were grown in 50-mm glass dishes in 4 mL of basal medium (minimal essential, Eagle) that contained 4% calf serum, as previously described (DePhilip et al., 1979). Confluency was reached by 3 days of incubation, and the cultures were used 2 or 3 days later.

Specific Activity of [^{3}H]Leucyl-tRNA. Labeling was with [^{3}H]leucine, whereupon, culture by culture, the media were removed and 2 mL of ice-cold 95% ethanol was immediately added. The cells were detached by scraping, collected by centrifugation, and washed three times with ethanol. The washed cells were then lysed in 12 mL of a solution of 100 mM Tris (pH 5.4), 100 mM EDTA, and 0.5% $NaDodSO_4$, and the lysate was homogenized in 12 mL of water-saturated phenol. After extraction of the aqueous phase with 9 mL of chloroform/phenol/isoamyl alcohol (10:10:0.1), the solution was made 0.1 M in NaCl, and RNA was precipitated with ethanol. Purification of the low molecular weight RNAs, deacylation of the aminoacyl-tRNAs under alkaline conditions, and derivatization of the released amino acids with [^{14}C]dansyl chloride were exactly as described by McKee et al. (1978).

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[1] Abbreviations used: r protein, ribosomal protein; EDTA, ethylenediaminetetraacetate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; Mops, 3-(*N*-morpholino)propanesulfonate; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; RNA, ribonucleic acid; mRNA, messenger RNA; tRNA, transfer RNA; poly(A), poly(adenylic acid); ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; RNase, ribonuclease; DNase, deoxyribonuclease; dansyl, 8-dimethylamino-1-naphthalenesulfonate.

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Separation of the dansyl derivatives by two-dimensional chromatography on sheets of micropolyamide with a mixture of benzene and acetic was also according to McKee et al. (1978) but with one additional step. Dansylleucine and dansylisoleucine were incompletely resolved. For complete separation of the two compounds, the fused spot was cut out, eluted with ethyl acetate, and dissolved in 50% pyridine. The pyridine solution was then applied to a strip of micropolyamide (1.6 × 10 cm), and chromatography with the benzene/acetic acid solvent was repeated. The doubly labeled dansylleucine was counted according to McKee et al. (1978).

Protein Synthesis in Cells. Confluent cultures of the chick embryo cells were washed twice, preincubated for 45 min in 2 mL of basal medium, and labeled with 2 μCi of [^{3}H]leucine in 2 mL of fresh basal medium. After labeling, the medium was replaced with 2 mL of a solution of 1% trichloroacetic acid/50 mM leucine, and insoluble material was collected on a filter and washed with the acid amino acid solution, ethanol, and ether. The dried pad, suspended in 2 mL of 5% trichloroacetic acid, was boiled for 30 min, and insoluble material was again recovered by filtration. After washing and drying as before, the protein was dissolved by boiling for 30 min in 2 mL of 0.05 M NaOH, and 0.2 mL portions of the supernatant fluid were used to measure radioactivity and protein.

Absolute Transit Times. The time that elapses between the attachment of a ribosome to a mRNA and the release of a completed polypeptide was estimated by the procedure of Fan & Penman (1970). Resting cultures of chick embryo cells were labeled for 2 h with 3 μCi of [^{14}C]leucine. At the end of this time, the attached cells were washed twice and preincubated in 2 mL of basal medium for 45 min. Some of the cultures then received insulin (5 μg/mL), and labeling with [^{3}H]leucine (200 μCi/culture) was begun 30 min later. After labeling with [^{3}H]leucine, the media were removed, each culture received 2 mL of ice-cold phosphate-buffered saline solution, and the cells were immediately detached by scraping and homogenized (15-mL Dounce vessel, tight pestle, 20 strokes) in 5 mL of a solution (0 °C) that contained 25 mM Tris, 25 mM NaCl, 5 mM $MgCl_2$, 5 mg of heparin, 0.1 mL of Triton X-100, and 90 μg of phenylmethanesulfonyl fluoride. To 1 mL of the homogenate was now added 0.05 mL of 10 M NaOH, the remainder of the homogenate was centrifuged at 105000*g* for 2 h, and the soluble fraction then received 0.2 mL of 10 M NaOH. All the alkalinized samples were kept at 37 °C for 15 min, and the radioactivities of the acid-insoluble material were then determined as for Protein Synthesis in Cells. The ^{14}C provided a measure of recovery, and the ^{3}H/^{14}C ratios provided a measure of the production of completed plus incompleted (homogenate) or only completed (soluble fraction) polypeptide chains.

Relative Transit Times. The relationship between the times that elapsed between the initiation of protein synthesis and the release of completed polypeptides in control and insulin-treated cells was determined according to Palmiter (1972). Confluent cultures (three per group) of chick embryo fibroblasts were washed and preincubated for 45 min in basal medium. The medium was then replaced with 2 mL of fresh basal medium or basal medium supplemented with 10 μg of insulin. At the end of 30 min, each control culture received 100 μCi of [^{3}H]leucine, and each insulin-treated culture received 12.5 μCi of [^{14}C]leucine. After 8 min, the media were replaced with 2 mL of ice-cold phosphate-buffered saline solution containing 100 μg/mL cycloheximide, and the cells were detached by scraping. The control and insulin-treated cell suspensions were then pooled, sedimented, and homogenized (7-mL Dounce vessel, tight pestle, 20 strokes) in 0.5 mL of a solution that contained 25 mM Tris (pH 7.5), 25 mM NaCl, 5 mM $MgCl_2$, 0.5 mg of heparin, and 10 μL of Triton X-100. Large particles were discarded by centrifugation (800*g*, 10 min), and the postmitochondrial fraction was made 1% in sodium deoxycholate and put on 12 mL of a 15–45% linear sucrose gradient in 10 mM Tris (pH 7.5), 0.1 M NaCl, 5 mM $MgCl_2$, and 0.004% heparin. Sedimentation was in an SW 41 rotor (40000 rpm, 1 h, 4 °C), the gradients were then displaced upward through an absorbance monitor (254 nm), and fractions of ~0.5 mL were collected. The acid-insoluble radioactivities of the gradient fractions were measured as for the determination of absolute transit times. The relationship between the transit time of the hormone-deprived and the insulin-treated cultures was the quotient of the ^{3}H/^{14}C ratios in the prepolyribosomal area/the polyribosomal area of the gradient.

Isolation of Poly(A)-Containing mRNAs. Confluent cultures (24 per group) of the chick embryo cells were washed twice and then preincubated in basal medium for 45 min. At the end of this time, the medium was replaced with 2 mL of fresh basal medium or basal medium supplemented with 5 μg/mL of insulin. After 1 h, culture by culture, the media were removed, and 2 mL of 1% $NaDodSO_4$ (60 °C) was vigorously spewed into the dish. Total cell RNA was extracted at pH 5.4 with phenol according to Wolf & Schlessinger (1977), and the poly(A)-containing fraction, obtained by three passes on a column of oligo(dT)–cellulose (Groner et al., 1974), was dissolved three times in water and precipitated with ethanol to free it of $NaDodSO_4$. This procedure yielded mRNA preparations that were completely free of RNase as judged by a comparison of sedimentation patterns in sucrose gradients before and after incubation at 37 °C for 1 h. The sedimentation patterns of the mRNAs from the resting and insulin-treated cells were identical, the peak of absorbance (254 nm) was at ~19 S, and there was no detectable contamination with ribosomal RNAs.

Cell-Free Protein Synthesis. The concentrations of K^+ and Mg^{2+} were optimal. For wheat germ, extracts were made according to Roberts & Paterson (1973) with the two small modifications of Marcu & Dudock (1974). The S30 fraction was not incubated at 30 °C before gel filtration, and the size of the Sephadex G-25 column was reduced (24 × 1.1 cm). A test mixture (50 μL) contained 10 mM Hepes (pH 7.6), 2 mM dithiothreitol, 1 mM ATP, 20 μM GTP, 72 mM potassium acetate, 2 mM magnesium acetate, 8 mM creatine phosphate, 2.5 μg of creatine kinase, 19 amino acids (each 30 μM), [^{35}S]methionine, chick embryo fibroblast mRNA, and 10 μL of wheat germ extract (~250 μg of protein). Incubation was at 23 °C. For reticulocyte, a reaction mixture (25 μL) contained 20 mM Hepes (pH 7.6), 1 mM dithiothreitol, 1 mM ATP, 0.4 mM GTP, 95 mM potassium acetate, 1.5 mM magnesium acetate, 5 mM creatine phosphate, 1.5 μg of creatine kinase, 19 amino acids (each 50 μM), 5 μM hemin, [^{35}S]methionine, chick embryo fibroblast mRNA, and 10 μL of reticulocyte lysate (~1 mg of protein) that had been treated with micrococcal nuclease (Pelham & Jackson, 1976). Incubation was at 30 °C.

Unlabeled and ^{3}H-Labeled r Proteins. Unlabeled r proteins were from 12-day-old chick embryos. Each embryo was homogenized in 7 mL (0 °C) of a solution of 0.01 M Tris (pH 7.8), 1 mM $MgCl_2$, 0.3 M sucrose, and 0.05% Triton X-100, and to the homogenate was added 1 mL of 0.2 M Mops (pH 7.8). Particulates were discarded by centrifugation (12000*g*, 10 min, 3 °C), 0.9 mL of 10% sodium deoxycholate was added

to the postmitochondrial fraction, and the fraction was layered over 1 mL of 20% sucrose. Polyribosomes, collected by centrifugation (105000*g*, 2 h, 3 °C), were washed with 0.5 M KCl and then with acetone, and r proteins were extracted and stored in a solution of 67% acetic acid, 0.1 M $MgCl_2$, and 10 mM dithiothreitol. The preparations contained no non-r proteins that could be detected by staining two-dimensional acrylamide gels. Names were assigned to the r proteins on the basis of the results of Ramjoué & Gordon (1977) with chick liver.

Labeled r proteins were from cultures of chick embryo fibroblasts that had been grown with [^{3}H]leucine (20 μCi/culture). The preparation of polyribosomes and the extraction of the r proteins with 67% acetic acid were as described for the unlabeled r proteins. The yield of labeled protein was ~25 000 cpm/culture.

Separation of Basic Proteins Made by Wheat Germ and Reticulocyte Preparations, Fluorography, and Counting. The incorporation of [^{35}S]methionine was stopped by the addition to the test mixtures of 50 μL of unlabeled methionine (50 mM), 100 μL of water, 10 μL of 0.25 M $MgCl_2$, and 20 μg each of pancreatic RNase and DNase. After 60 min at 0 °C, the mixture received 300 μg of unlabeled (fluorography) or ^{3}H-labeled (counting) r proteins and 2 mL of acetone. Protein was precipitated for 15 h at −20 °C, and the precipitate was washed three times with acetone, dried, and dissolved in 200 μL of a solution of 8 M urea, 0.02 M Tris (pH 8.6), 1 mM EDTA, 0.026 M boric acid, and 5% 2-mercaptoethanol (v/v) (Lastick & McConkey, 1976). The entire solution was used for two-dimensional gel electrophoresis of basic proteins by the method of Kaltschmidt & Wittman (1970) as modified by Lastick & McConkey (1976). Fluorography involved impregnation of the slab gel with 2,5-diphenyloxazole (Bonner & Laskey, 1974), drying, and exposure to X-ray film.

For quantitation of the radioactive products, the second-dimensional slab gels were stained and destained according to Cleveland et al. (1977). Plugs from the slab gel were subjected to a third electrophoretic step in $NaDodSO_4$ cylinder gels (Laemmli, 1970), exactly as before (DePhilip et al., 1980), in order to purify further the proteins that had been resolved by the two-dimensional procedure. The stained bands from the $NaDodSO_4$ gels, usually no more than 1 mm wide, were cut out and kept in 10% methanol for 2–4 h, whereupon they were blotted, quartered, and incubated at 38 °C for 3 or 4 days in 10 mL of 7.5% Protosol in Econofluor. The decolorized and swollen acrylamide gels were discarded before counting. Under the conditions used, 80% or more of the radioactivity was routinely extracted from the gels. All samples were counted for 100 min. The probable error for samples with 50 cpm of ^{35}S was 4% (Cooper, 1977).

There was no carry-over of ^{3}H into the ^{35}S channel, and only half of the ^{35}S spilled over into the ^{3}H channel. Carry-over of ^{35}S into the ^{3}H channel was measured by adding 1 μL of [^{35}S]methionine (20 000 cpm) to vials that had already been counted.

Results

Lack of Effect of Insulin on the Specific Activity of [^{3}H]Leucyl-tRNA. Specific activities of [^{3}H]leucyl-tRNA were measured to learn whether insulin enhances the labeling of total cell protein merely by increasing the specific activity of the activated amino acid. Control and insulin-treated cells were labeled with [^{3}H]leucine, the amino acids of the aminoacyl-tRNAs were derivatized with [^{14}C]dansyl chloride (McKee et al., 1978), and the doubly labeled dansylleucine was isolated. The ratios of ^{3}H/^{14}C gave a measure of the specific activity of the leucine that had been used by the cells to make protein. As Table I shows, insulin had no effect on the specific activity of the precursor leucine. In the four experiments at 45 min, the hormone did, however, increase the incorporation of [^{3}H]leucine into total cell protein by an average of 1.54 times (data not shown). The results with leucyl-tRNA are consistent with the observation of Vaheri et al. (1973) that insulin does not stimulate the transport of leucine into chick embryo fibroblasts.

Table I: Specific Activities of [^{3}H]Leucyl-tRNA in Control and Insulin-Treated Cells[a]

expt no.	^{3}H/^{14}C 0–15-min labeling period control	0–15-min labeling period insulin	0–45-min labeling period control	0–45-min labeling period insulin
1			5.71	5.28
2	3.84	4.35		
3	4.78	3.92	4.88	5.32
4	5.99	5.29	5.47	6.08
5			6.00	6.12

[a] Resting cultures of chick embryo cells (24 cultures per group) were washed and then preincubated in 2 mL of basal medium for 45 min. At zero time, the medium was replaced with 2 mL of fresh basal medium containing 10 μg of insulin, as indicated, and 2 μCi of [^{3}H]leucine. After 15 or 45 min, the cells were killed with 95% ethanol, and the specific activities of leucyl-tRNA were measured as detailed under Materials and Methods.

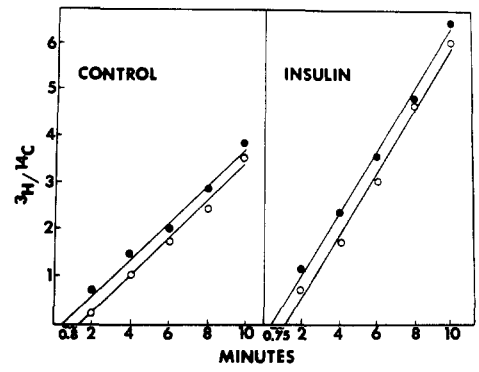

FIGURE 1: Estimation of absolute transit times in deprived and insulin-treated chick embryo cells. Half-transit times, shown in the figure, were measured as described under Materials and Methods. The upper curve in each panel shows the results with the homogenates (completed plus incompleted polypeptides), and the lower curves show the results with the soluble fractions (completed polypeptides). Each point represents the result with a single confluent culture labeled for 2–10 min, as shown.

Absolute Transit Times. Transit times were measured by the procedure of Fan & Penman (1970) to gain some insight into the means by which insulin increases the rate of protein formation in the resting chick embryo fibroblasts. Figure 1 shows that the control and hormone-treated cells had almost identical transit times, 1.6 and 1.5 min, respectively, even though the insulin raised protein synthesis by 1.65 times as judged from the slopes of the curves.

The possibility was tested that insulin did, in fact, enhance the rate of elongation or termination but that the increase was obscured by the preferential effect of the hormone on the synthesis of high molecular weight polypeptides. High molecular weight products would be expected to have longer than average transit times. Control cultures were given [^{3}H]leucine and insulin-treated cultures the ^{14}C-labeled amino acid, to test this point. After 2 h of labeling, the cells were pooled, and the doubly labeled soluble cell fraction was prepared and subjected to electrophoresis on a 10-cm cylinder of $NaDodSO_4$–acrylamide gel. The ratios of ^{3}H/^{14}C of the 20 gel slices

Table II: Comparison of the Effects of Insulin on the Conversion of Subribosomes to Polyribosomes and on the Rate of Synthesis of Total Cell Protein[a]

fraction	control	insulin	insulin/control
subribosome (% of total A_{254})	52, 61, 57	37, 37, 39	
polyribosome (% of total A_{254})	48, 39, 43	63, 63, 61	1.31, 1.62, 1.42
total protein (cpm/μg ± SD)	15.6 ± 1.16, 14.7 ± 1.09, 16.0 ± 0.84	20.0, 24.1, 22.6 0.63, 1.62, 1.28	1.28, 1.64, 1.41

[a] The results of three experiments are shown. Confluent cultures of chick embryo fibroblasts were washed and then preincubated in basal medium for 45 min. At zero time, the preincubation medium was replaced with 2 mL of fresh basal medium that was supplemented with 10 μg of insulin, as shown. Some of the cultures (five per group) were harvested after 45 min for the visualization of polyribosome profiles as for Figure 3. The proportions of subribosomes and polyribosomes were estimated by weighing paper cutouts. The remainder of the cultures (four per group) was labeled with 2 μCi of [^{3}H]leucine for from 30 to 60 min, and rates of synthesis of total cell protein were measured as described under Materials and Methods. The total ultraviolet-absorbing material in the gradient fractions of the first experiment was 3.4 units for the control cells and 3.5 units for the insulin-treated cells, and the comparable values for the next two experiments were 6.3 and 6.2 and 5.0 and 5.1, respectively.

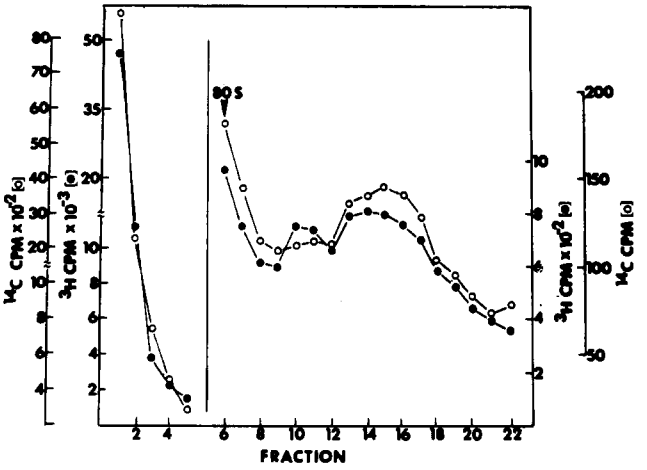

FIGURE 2: Relative transit times. The procedure detailed under Materials and Methods was used to estimate the ratio of the transit times in control and insulin-treated cells. Sedimentation was from left to right. Gradient fractions 1–5 were taken to contain polypeptides that had been completed and released, and the remainder of the fractions were taken to contain growing polypeptides. The increase in protein synthesis in the hormone-treated cells, measured with [^{3}H]leucine, was 1.6-fold. (●) No insulin; (○) insulin.

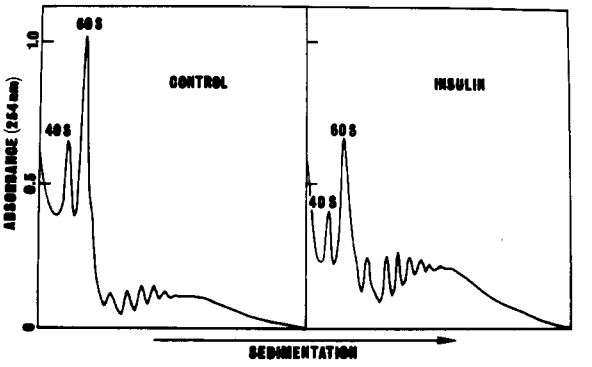

FIGURE 3: Effect of insulin on the polyribosome pattern of resting chick embryo cells. Polyribosomes were prepared (five confluent cultures per group) and displayed exactly as described under Materials and Methods for relative transit times, except that treatment with insulin was for 45 min and no radioactivity was used. Sedimentation was from left to right.

varied by no more than ±2%. This result was taken to mean that insulin stimulated the rate of production of at least many species of fibroblast polypeptides regardless of molecular weight.

Relative Transit Times. The procedure of Palmiter (1972) gives the relationship between the transit times of two populations of cells rather than the absolute times required to make a polypeptide. Relative transit times were determined by mixing resting cells that had been labeled with [^{3}H]leucine with insulin-treated cells that had been labeled with the ^{14}C-labeled amino acid. The mixture was homogenized, and the postmitochondrial fraction was sedimented in a sucrose gradient.

Figure 2 shows the results that were obtained. The ratio of ^{3}H/^{14}C (69 300 cpm/13 100 cpm) of the completed and released polypeptides (prepolyribosomal area of the gradient) was 5.29, the corresponding ratio for the growing chains (polyribosomal area) was 5.52 (11 200 cpm/2030 cpm), and the quotient of the two ratios, 0.96, was the relative transit time of the resting cells/insulin-treated cells. In agreement with the absolute measurements, the hormone had, at most, an insignificant effect on rates of elongation and termination.

Effects of Insulin on the Quantity of Polyribosomes. The measurements of absolute and relative transit times led to the conclusion that insulin stimulated total protein synthesis by increasing initiation rates. Polyribosomal profiles were examined to provide additional support. Figure 3 shows that less than half of the ribosomal particles of resting chick embryo cells were associated with mRNA. Treatment of the cells with insulin increased the amount of ribosomal material in the polyribosomes and decreased the sizes of the 40S and 60S subribosomal peaks, as would be expected from a stimulation of the process of initiation. The reduction in ribosomal material in the prepolyribosomal area of the gradient from the insulin-treated cells was due to a transfer of 40S and 60S subunits to the polyribosomes and not to a destruction of subribosomes by the stimulated cells. Thus, the averages (nine experiments) of the total absorbing material in the gradients from control and hormone-treated cells (five cultures per group) were 5.2 and 5.1 A_{254} units, respectively.

Insulin was previously shown to cause a shift of free subribosomes into polyribosomes in perfused rat heart (Morgan et al., 1971) and rat adipocytes (Lyons et al., 1980). The altered polyribosomal patterns led the authors to conclude that the hormone stimulates the initiation of polypeptide synthesis.

Increase in Polyribosomal Ribosomes and Rate of Total Protein Synthesis. The stimulation by insulin of the rate of total protein production varied with the batch of resting fibroblasts over the range 1.3–1.7-fold. It was of interest to compare the effect of the hormone on the shift in the polyribosome pattern with that on the rate of total protein synthesis with different batches of cultures. Table II shows that there was an exact correlation between the extent to which the subribosomes became associated with polyribosomes and the size of the increase in [^{3}H]leucine incorporation into protein.

Cell-Free Protein Synthesis with mRNA from Control and Insulin-Treated Chick Embryo Cells. Wheat germ and reticulocyte preparations were not unwilling to accept fibroblast mRNAs for protein synthesis. The avian messages, however,

Table III: Cell-Free Translation of r Protein mRNAs from Control and Insulin-Treated Cells[a]

	$^{35}S/^{3}H$										
	wheat germ					reticulocyte					
spot	no mRNA	control mRNA		insulin mRNA		spot	no mRNA	control mRNA		insulin mRNA	
no.	cpm	cpm	ratio	cpm	ratio	no.	cpm	cpm	ratio	cpm	ratio
S2	2/1184	117/1513	0.077	96/1388	0.069	S1	0/628	47/742	0.063	39/676	0.058
S3	0/1157	544/1484	0.367	513/1545	0.332	S2	0/304	16/263	0.061	15/233	0.064
S4	1/187	49/219	0.224	50/264	0.189	S3	0/681	133/936	0.142	127/883	0.144
S8	1/586	157/384	0.409	236/621	0.380	S4	0/420	14/277	0.051	15/331	0.045
S10	2/768	480/715	0.671	635/845	0.751	S10	0/693	53/758	0.070	56/842	0.067
S11	1/249	163/298	0.547	199/387	0.514	S11	9/354	36/364	0.099	39/560	0.068
L3	1/623	34/732	0.046	31/775	0.040	S13	3/1035	15/1279	0.012	13/1245	0.010
L11	2/693	137/763	0.179	124/756	0.164	S16	0/1154	75/1184	0.063	81/1327	0.061
L15	1/307	94/328	0.287	86/355	0.242	S19	3/674	40/778	0.090	75/852	0.088
L17	0/834	359/868	0.414	370/959	0.386	L8	1/718	18/888	0.020	14/763	0.018
L24	3/318	381/714	0.534	456/918	0.497	L11	2/970	74/1077	0.069	70/1088	0.064
L26	2/1181	402/1377	0.292	314/1344	0.234	L13	4/610	21/648	0.032	13/399	0.033
L31	6/1422	796/1506	0.529	897/1688	0.531	L17	1/554	79/707	0.112	75/662	0.113
L34	0/344	90/398	0.226	108/466	0.232	L26	2/658	55/718	0.077	48/624	0.077
L36A	0/680	211/825	0.268	411/1175	0.350	L27	1/794	53/921	0.058	63/1232	0.051

[a] The test mixtures, the preparation of samples for electrophoresis, and the resolution of the basic proteins in two-dimensional gels were exactly as described for Figure 5 with two exceptions. First, duplicate 5-μL portions of each mixture were set aside for the estimation of acid-insoluble ^{35}S just before treatment with the nucleases, and, second, each mixture received 300 μg of ^{3}H-labeled chick r proteins (500 000 cpm) instead of the unlabeled preparation. All the r proteins from the slab gels were subjected to electrophoresis in cylinders of $NaDodSO_4$-acrylamide gels as described under Materials and Methods. The incorporation of [^{35}S]methionine into protein in the reaction mixtures with wheat germ extract (uncorrected for a zero time blank) was as follows: no added mRNA, 4.3×10^5 cpm; resting chick fibroblast mRNA, 3.3×10^6 cpm; and insulin-treated fibroblast mRNA, 3.5×10^6 cpm. The comparable values for the mixtures with reticulocyte lysate were 2.6×10^5, 6.7×10^5, and 6.5×10^5. In all cases, the radioactivity applied to the first-dimension gels represented more than 95% of the starting acid-insoluble counts.

were only 60% as effective as globin mRNA in the wheat germ system and <15% as active with the reticulocyte lysate.

In our hands, the incorporation of [^{35}S]methionine into protein with both cell-free preparations was essentially linear for only 30 min. A relatively brief incubation period of 20 min was, therefore, selected in order to compare the rates of protein synthesis that were supported by mRNAs from resting and insulin-treated chick embryo cells. Figure 4 shows that the efficacies of the two message preparations were indistinguishable at all the levels tested in both the wheat germ and reticulocyte systems. Exactly the same picture emerged with test mixtures that were incubated for 60 min.

Cell-Free Synthesis of r Proteins As Judged by Fluorography. Wheat germ extract and reticulocyte lysate were incubated with resting fibroblast mRNA and [^{35}S]methionine, unlabeled chick r proteins were then added, basic proteins were resolved by two-dimensional electrophoresis, and the slab gels were stained and fluorographed. In the fluorograms depicted in parts A (wheat germ) and B (reticulocyte) of Figure 5, names were assigned only to the radioactive spots that migrated exactly with stained r proteins. It was possible to identify 43 stained r proteins in the slab gels and, as can be seen from the figures, 33 and 28 labeled r proteins in the fluorograms of the products of the wheat germ and reticulocyte preparations, respectively.

Figure 5 does not show the results that were obtained with test mixtures to which no mRNA was added. Only negligible amounts of radioactivity entered the first-dimension gels, and none of the labeled spots in the slab gels corresponded to chick r proteins.

Quantitation of r Protein Synthesis with mRNAs from Insulin-Deprived and Insulin-Treated Chick Embryo Cells. Messages from control and hormone-treated fibroblasts were compared for their abilities to support the synthesis of r proteins in the cel-free systems. As part of the fluorographic experiment of Figure 5, wheat germ and reticulocyte preparations were incubated with the chick embryo mRNAs and [^{35}S]methionine, the mixtures were then supplemented with

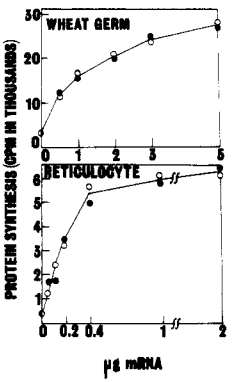

FIGURE 4: Cell-free protein synthesis supported by mRNAs from hormone-deprived and insulin-treated chick embryo cells. Each test mixture was prepared as described under Materials and Methods and contained 0.5 μCi of [^{35}S]methionine and the indicated quantity of mRNA from control (○) or insulin-treated (●) chick embryo cells. With the batch of cells from which the mRNAs were prepared, insulin stimulated the incorporation of [^{3}H]leucine into total protein by 1.5 times. After 20 min of incubation, unlabeled methionine (0.1 mL, 50 mM) and NaOH (0.5 mL, 0.2 M) were added, and the mixtures were kept at 37 °C for 15 min. At this time, protein was precipitated with 1 mL of 10% trichloroacetic acid, and the insoluble fraction was collected on a filter and washed with acid, ethanol, and ether. The filter pad was then dissolved by boiling in 2 mL of 50 mM NaOH, and 0.2 mL of the supernatant fraction was counted. The values shown represent the total acid-insoluble counts after correction for a zero time blank of 1160 (wheat germ) or 1960 (reticulocyte) cpm.

^{3}H-labeled r proteins, and the basic proteins were resolved by two-dimensional gel electrophoresis. Plugs from the slab gels were subjected to a third electrophoretic step with $NaDodSO_4$ cylinder gels to purify the r proteins further. The ^{3}H served as a measure of the recovery of the individual r protein. Included in the experiment were test mixtures to which no mRNA was added.

Table III shows the numbers of counts incorporated into total protein and the radioactivities and the $^{35}S/^{3}H$ ratios of the 15 r proteins that were examined. For the most part, the wheat germ products were selected from the portions of the

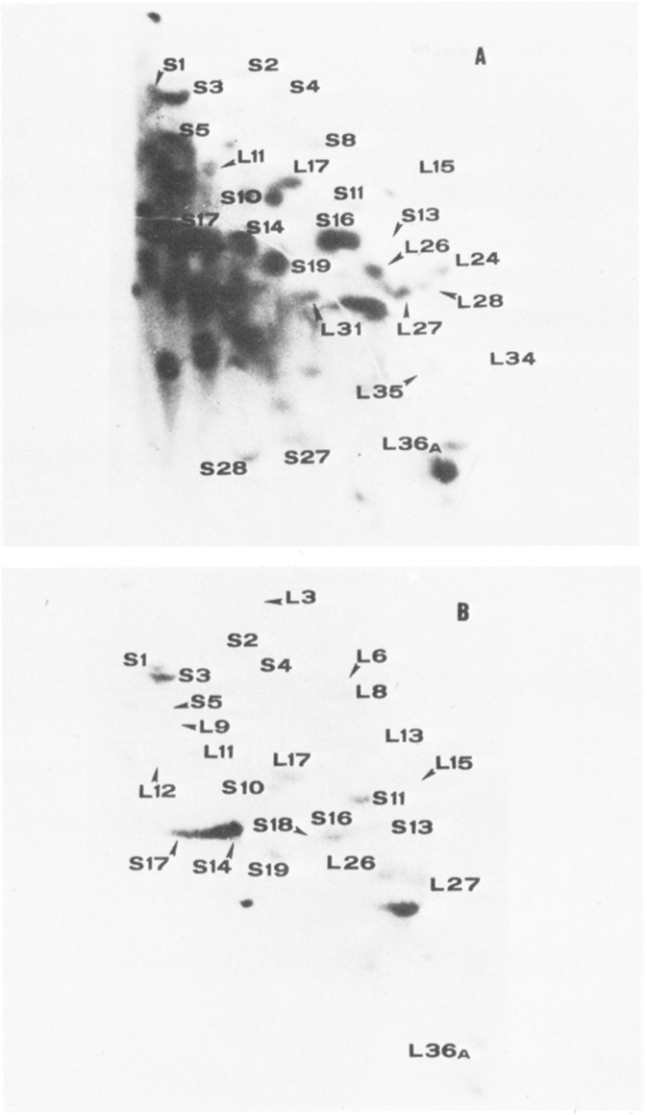

FIGURE 5: Cell-free synthesis of r proteins as judged by fluorography. The reaction mixtures, resolution of basic proteins, and fluorography were as described under Materials and Methods. The wheat germ mixture (100 μL) contained 6 μg of mRNA from resting fibroblasts, 40 μCi of [^{35}S]methionine, and 20 μL of extract. Incubation was for 120 min at 23 °C. Exposure to the X-ray film was for 5 days at −80 °C. The reticulocyte mixture (125 μL) contained 5 μg of mRNA from resting fibroblasts, 100 μCi of [^{35}S]methionine, and 50 μL of lysate. Incubation was at 30 °C for 60 min. Exposure to the X-ray film was for 23 days at −80 °C. (Panels A and B) Wheat germ and reticulocyte preparations, respectively.

slab gel with the least background radioactivity (Figure 5A). It can be seen from the table that the r protein messages from the control and insulin-treated cells behaved in an identical manner.

Discussion

The incorporation of [^{3}H]leucine into the total protein of confluent cultures of chick embryo fibroblasts is invariably raised by insulin by ~1.5 times. It has now been shown that the enhanced labeling of total cell protein is due to an elevation in the rate of protein synthesis and not to an increase in the specific activity of leucyl-tRNA.

Polypeptide transit times have been compared in deprived and insulin-treated chick embryo cells by two independent methods. The results of both procedures indicate that the hormone does not increase rates of elongation and termination. With the procedure of Fan & Penman (1970), the times between initiation and release of a finished product were found to be 1.6 and 1.5 min for the control and insulin-treated cells, respectively. These results are similar to those of Meedel & Levine (1978), who showed that serum stimulates the incorporation of [^{3}H]leucine into the total protein of resting human diploid fibroblasts by almost 3-fold whereas the transit time, 1.7 min, was the same as in the resting cells, 1.6 min.

The results of the measurements of transit times point, by exclusion, to a mechanism of insulin action that involves rates of initiation. Support for this contention was provided by observations made with polyribosomes. As would be expected from an increase in the frequency of initiation, insulin causes free 40S and 60S subribosomes to become associated with mRNAs.

It was also found that the quantity of additional ribosomal particles that appears in the polyribosomes of insulin-treated chick embryo cells correlates exactly with the elevation in protein production. Such a result would seem to be in agreement with the kinetic model of Bergmann & Lodish (1979b). They showed that protein synthesis rises proportionately with initiation rate constants, even with large increases in the constants, so long as polypeptides grow at 400 amino acids/min. It is only when the rate of elongation falls to 100 amino acids/min that newly initiated ribosomes begin to hinder the binding to the message of waiting ribosomes as the initiation rate constant is raised. Even in this case, an almost proportional relationship is maintained between intiation rate constants and protein synthesis over the initial severalfold increase in the constants. The rate of polypeptide elongation in the chick embryo fibroblast would seem to be closer to 400 than to 100 amino acids/min, ~270, as calculated from the measured value for transit time, 1.5 min, and with the assumption of an average of 50 000 daltons for fibroblast polypeptides and 120 daltons for amino acids.

One tenable mechanism to account for the greater frequency of initiation in the insuin-treated cells supposes that the hormone induces covalent changes in preformed messages. According to this proposal, nonfunctioning messages, present in a nonpolyribosome cell fraction (Rudland, 1974; Bandman & Gurney, 1975; Meedel & Levine, 1977, 1978; Lee & Engelhardt, 1979), or poorly translatable molecules, already present in polyribosomes, would be transformed into fully effective mRNAs.

It seems unlikely that insulin acts by inducing covalent changes in preformed mRNAs. For one thing, wheat germ extracts and reticulocyte lysates fail to recognize any difference between the total poly(A)-containing messages of resting and insulin-treated fibroblasts. At least alterations involving the 5′ termini of the RNAs from the hormone-treated cells would be expected to influence markedly the competence of the messages in the two cell-free systems (Both et al., 1975; Muthukrishnan et al., 1975, 1976; Shih et al., 1976; Lodish & Rose, 1977; Weber et al., 1977; Zan-Kowalczewska et al., 1977; Bergmann & Lodish, 1979a). For another, insulin stimulates with strong preference the synthesis of r proteins by the deprived chick embryo cells (DePhilip et al., 1979, 1980). In spite of this, as with the total cell messages, the cell-free preparations do not use the r protein mRNAs from the hormone-treated fibroblasts any more effectively than those from control cells.

Finally, it is difficult to reconcile the hypothesis of chemically altered mRNAs with the highly selective action of the hormone. Fluorography of two-dimensional gels has shown that insulin preferentially stimulates the incorporation of [^{3}H]leucine into much less than 1% of the total species of

nonbasic polypeptides of the soluble cell fraction (W. A. Rudert and I. Lieberman, unpublished observations).

A more likely mechanism of action of insulin is that the hormone raises the activities of components of the initiating machinery that limit the synthesis of protein in the resting chick embryo cells. At the extremes, messages that were already being initiated with maximum frequencies under the stringent conditions of the resting cells would be unaffected by the change, and those that were functioning poorly, such as r protein messages (Ignotz et al., 1981), would be strongly affected. For the majority of the cellular mRNAs, the average increase in the frequency of initiation would be ~1.5-times.

A special benefit may accrue from the identification of the components of the initiation system whose activites are raised by insulin. If the same components prove to be involved in the stimulation of total and r protein synthesis, a mechanism would be provided, on the basis of inherent differences in mRNAs, for converting a small translational change with a broad impact into a much larger increase in the rates of synthesis of a specific set of polypeptides. An analogous mechanism can even be envisioned for the differential production of some cytoplasmic messages from primary transcripts. In this case, it is only necessary to postulate a modification of a processing enzyme or a protective or transport protein that preferentially increases the ability of the protein to bind to low-affinity RNA sequences.

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