suggests that equilibration in vivo may be speeded up by the presence of the effectors.

In summary, we have shown that RNA polymerase can be functionally heterogeneous, different populations of polymerase molecules exhibiting distinguishable template preferences. These populations are functionally and structurally interconvertible. We suggest that regulators of polymerase specificity, such as ppGpp, alter the promoter preference of the enzyme by altering the position of an equilibrium between different forms of the enzyme.

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Preferential Stimulation of Ribosomal Protein Synthesis by Insulin and in the Absence of Ribosomal and Messenger Ribonucleic Acid Formation†

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ABSTRACT: Insulin stimulates the rate of production of cytoplasmic ribosomes in resting cultures of chick embryo fibroblasts. The effects of the hormone were studied on the synthesis of individual basic ribosomal and nonribosomal proteins and on total cell protein to gain insight into the mechanism. Insulin elevates by fourfold or more the formation of all the ribosomal proteins that were examined whereas the synthesis of individual nonribosomal proteins and of total cell protein is raised only ~1.5 times. In both control and insulin-treated cells, all or almost all newly made ribosomal proteins are used to make cytoplasmic ribosomes. The action of the hormone on ribosomal protein formation is rapid and is already more than one-half of maximum between 10 and 20 min after treatment of the resting cells. Insulin stimulates ribosomal protein synthesis in the presence of a level of actinomycin D that blocks completely the formation of rRNA and inhibits the synthesis of poly(A)-containing mRNA by 95%. Under these conditions, newly made ribosomal proteins are unstable and the majority of those that were measured decay with a half-life of 30 min or less. Rates of decay are not reduced by insulin. Inhibition of protein synthesis by cycloheximide has no effect on the rate of formation of pre-rRNA in control cells. With insulin-treated cultures, however, the antibiotic blocks completely the enhanced production of pre-rRNA that would otherwise occur. Similar results were obtained with puromycin. The observations on the synthesis of ribosomal proteins and pre-rRNA suggest that insulin acts directly to raise the production of ribosomal proteins and only secondarily to increase the formation of pre-rRNA. The effect of the hormone on ribosomal protein formation would seem to be at the level of translation.

Insulin stimulates the incorporation of radioprecursors into the cytoplasmic rRNAs[1] of cells in vivo (Wool, 1963; Steiner & King, 1966) and in organ fragment (Turkington & Riddle, 1970) and monolayer culture (Baseman et al., 1974; Baseman & Hayes, 1975). The effect of insulin on ribosome synthesis

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[1] Abbreviations used: rRNA, ribosomal ribonucleic acid; r protein, ribosomal protein; EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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in chick embryo fibroblasts was measured with [^{3}H]leucine by the ratios of the specific activities of ribosomal core protein to total cell protein (DePhilip et al., 1979) to explore the possibility that the hormone achieves this merely by increasing the specific activity of the labeled nucleotide in the nucleolus. Interpretation of the results depends only on the reasonable assumption that r proteins and non-r proteins are made from identical pools of leucyl-tRNAs. Insulin increases the labeling of cytoplasmic ribosomal core proteins in the resting chick cells by about fourfold whereas that of total cell protein is raised by only 1.5 times.

The study with the core proteins did not shed light on the mechanism by which insulin enhances the manufacture of ribosomes in the cultured fibroblasts. Three questions were of particular interest. (1) Is the immediate target of the hormone the synthesis of pre-rRNA, r protein, or both? (2) If the initial effect is on pre-rRNA, is there a secondary increase in r protein formation or are molecules salvaged that were already being made in excess by the resting cells? (3) If the direct action of insulin should prove to be on r protein synthesis, is a transcriptional or a posttranscriptional process involved?

It has been known that the production of pre-rRNA in eucaryotic cells is not contingent upon r protein formation (Ennis, 1966; Warner et al., 1966; Soiero et al., 1968). Furthermore, r proteins continue to be made after rRNA synthesis is turned off (Craig & Perry, 1971; Maisel & McConkey, 1971; Warner, 1977).

The lack of a coordinate regulation of the two processes made it possible to explore the three questions. The effects of insulin on r protein and pre-rRNA formation have been examined independently of one another by treating chick embryo fibroblasts with actinomycin D or inhibitors of protein synthesis. Broken cells were extracted with 67% acetic acid (Hardy et al., 1969; Warner, 1977) and basic proteins were separated from the bulk of the proteins and from each other by two-dimensional gel electrophoresis (Kaltschmidt & Wittman, 1970; Lastick & McConkey, 1976) to look at individual r proteins. Pre-rRNA formation was measured by the incorporation of [^{3}H]adenine.

Materials and Methods

Materials. Crystalline bovine insulin (~25 IU/mg) was from Sigma, and radioactive compounds and counting solutions were from New England Nuclear. Radioactive leucine and unlabeled leucine were in the L form. Specific activities of [^{3}H]leucine ranged from 40 to 60 Ci/mmol.

Tissue Culture. Primary cultures of chick embryo fibroblasts were made from trypsinized minces of 12-day-old embryos. The cells were grown at 38 °C in a CO_2–air atmosphere in 50-mm glass dishes in 4 mL of basal medium (Minimal Essential, Eagle) containing 4% calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Confluency was reached by 3 days of incubation, and the cultures were used 2 or 3 days later. At this time, each dish contained ~5×10^6 cells, 1 mg of protein, and 70 μg of RNA.

Radioactive Labeling and Extraction of Protein for Electrophoresis. At the time of implantation of the primary cells, 3 μCi of [^{14}C]leucine (270 mCi/mmol) was added to each culture. [^{3}H]Leucine (400 μCi/culture) was dried with a stream of air and dissolved in leucine-free basal medium immediately before use. Protein was extracted from the doubly labeled cells with 67% acetic acid as described by Warner (1977) except that the step with DNase was omitted. To each extract was added 400 μg of unlabeled r protein and the mixture was precipitated with acetone and dried in vacuo (Roberts & Ashby, 1978). Finally, the dry powder was dissolved in 150 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 extracts uniformly contained 90% or more of the radioactivity and the protein of the cells.

Unlabeled r proteins were from confluent chick fibroblast cultures. Polyribosomes that had been prepared by the method of Palmiter (1974) were washed once with ice-cold 0.5 M KCl, sedimented through a 1-mL cushion of 20% sucrose, and extracted with 67% acetic acid. The extracted proteins were precipitated with acetone and dissolved as for the radioactive cells.

Separation of Basic Protein and Radioactivity Counting. Two-dimensional gel electrophoresis was by the method of Kaltschmidt & Wittman (1970) as modified by Lastick & McConkey (1976). The high pH of the first-dimension gel, 8.6, excluded all but very basic proteins. The second-dimension slab gels were stained and destained according to Cleveland et al. (1977).

Ribosomal proteins were named on the basis of the results of Ramjoué & Gordon (1977) with chick liver. The proteins designated as nonribosomal were not detectable in stained electrophorograms of acetic acid extracts of washed polyribosomes. Non-r proteins were assigned letters. Those that migrated in the slab gels exactly as chick histones were d, e, and f and one that coincided with r protein L26.

A third electrophoretic step was introduced to purify further the proteins that were resolved by the two-dimensional procedure. Plugs (4.8-mm diameter) from the stained slab gel were washed 3 times with a total of 15 mL of 30% methanol, and the washed plugs were cemented with 1% agarose to the tops of $NaDodSO_4$ cylinder gels (1 cm of 3% acrylamide stacking gel and 10 cm of 12% acrylamide separating gel) (Laemmli, 1970). After electrophoresis for 3 h at a constant current of 2.5 mA/cylinder, the gels were stained for 30 min in 0.1% Coomassie brilliant blue R in 50% methanol and 10% acetic acid and destained in 30% methanol. The stained bands, usually no more than 1 mm wide, were cut out, quartered, and incubated at 38 °C for 2 days in 10 mL of 5% Protosol in Econofluor. Before counting, the decolorized and swelled acrylamide gel was discarded by filtration through glass wool. Recoveries of radioactivity in the third electrophoretic step ranged from about 50 to 100%, and the ^{3}H/^{14}C ratios of the individual r proteins were reduced from 0 to 35%.

Under the counting conditions used, there was no carry-over of ^{3}H into the ^{14}C channel and only 0.40 of the ^{14}C was carried over into the ^{3}H channel. Almost all samples of individual proteins were counted to a probable error of less than 4% (Cooper, 1977).

Rates of Synthesis of Total Cell Protein. Relative rates of formation of total protein were measured in three ways: by the conversion of [^{3}H]leucine to a hot acid insoluble form in cells that had not been prelabeled with [^{14}C]leucine but were otherwise treated identically with those of the accompanying prelabeled cultures; from the ^{3}H/^{14}C ratios of the hot acid insoluble fraction of the acetic acid extracts that were used for gel electrophoresis; from the ^{3}H/^{14}C ratios of individual basic non-r proteins.

Estimation of total protein synthesis with previously unlabeled cells was with 0.2 μCi/culture of [^{3}H]leucine. Protein synthesis was stopped with 1 mL of a solution of 1% trichloroacetic acid–50 mM leucine, and insoluble material was collected on a filter and washed with trichloroacetic acid, ethanol, and ether. The dry residue was suspended in a so-

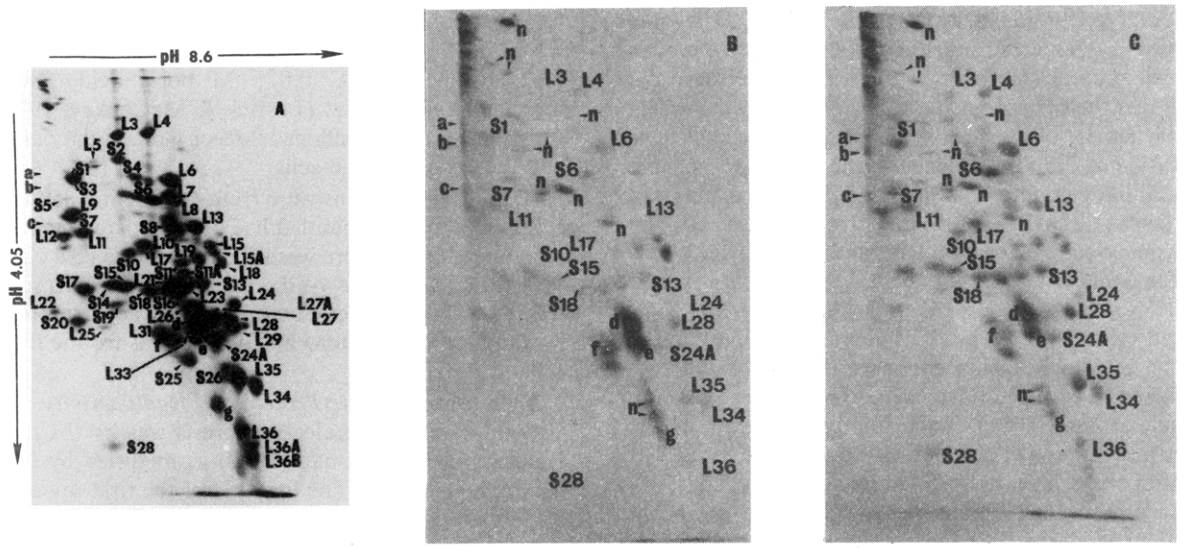

FIGURE 1: Map of basic proteins and preferential effect of insulin on the synthesis of r proteins as visualized by fluorography. Two pairs of unlabeled cultures of chick embryo fibroblasts were washed twice and preincubated for 30 min in modified basal medium (0.1 mM instead of 0.4 mM leucine). At zero time, the medium was replaced with 2 mL of fresh modified basal medium and one pair of cultures received, in addition, 10 μg of insulin. At the end of 30 min, each culture was labeled with [^{3}H]leucine for 0.5 h, the pooled cells from each pair of cultures were extracted with acetic acid, and carrier r proteins were added to the extracts. Approximately 5×10^6 cpm from each extract was used for two-dimensional gel electrophoresis, and the slab gels were stained, impregnated with 2,5-diphenyloxazole according to Bonner & Laskey (1974), dried, and exposed to X-ray film for 30 h at −70 °C. Panel A: stained slab gel. Panels B and C: fluorograms of slab gels from control and insulin-treated cells, respectively. The letters designated non-r proteins, and n designated those that were not assigned specific names.

lution of 10% trichloroacetic acid–50 mM leucine and boiled for 30 min, and the insoluble fraction was again collected on a filter and washed with acid, ethanol, and ether. Finally, the protein was dissolved by boiling for 60 min in 2 mL of 50 mM NaOH and a portion of the NaOH solution was counted and another was used for the colorimetric estimation of protein. Specific activity was the average of the results with four cultures.

Conversion of [^{3}H]Adenine to ATP. Cultures (seven/group) were labeled with 0.04 μCi/mL [^{3}H]adenine (19 Ci/mmol), and the labeled cells were quickly killed with ice-cold formic acid (1 M) and homogenized. To the supernatant fraction, obtained by centrifugation, was added 5 mg of activated charcoal (Sigma), the charcoal was washed twice with water, and adsorbed material was then eluted with 5% NH_4OH in 50% ethanol. The eluate was evaporated to dryness at 45 °C with a stream of air, and the dry residue, in 10 μL of water, was chromatographed on Whatman 1 paper with the isobutyric acid–NH_4OH solvent system of Hurlbert (1957). Elution of ATP from the paper was with 0.01 M HCl, and absorbance was measured at 257 nm.

Results

Fluorography of Stained Slab Gels. As a preliminary step in studying the effects of insulin on the synthesis of basic proteins, unlabeled control and hormone-treated chick cells were allowed to incorporate [^{3}H]leucine, acetic acid extracts of the labeled cells were fortified with carrier r proteins, the mixtures were subjected to two-dimensional gel electrophoresis, and the slab gels were stained and fluorographed. The distribution of the stained spots in one of the dried slab gels is depicted in Figure 1A, and parts B and C of Figure 1 represent the fluorograms of the basic proteins from the control and insulin-treated cells, respectively. It can be seen from Figure 1A that 56 r proteins could be identified and from parts B and C of Figure 1 that insulin induced a marked preferential incorporation of [^{3}H]leucine into the r proteins over that into the non-r proteins. In this experiment, nonribosomal spots a, b, and c appeared to have barely entered the first-dimension gels. These spots were usually much more heavily stained and radioactive.

Radioactivity Not Associated with Stained Spots. Figure 1 shows that at least most of the counts in the slab gels were associated with stained spots. Cells that had been prelabeled with [^{14}C]leucine were briefly incubated with [^{3}H]leucine, the doubly labeled cultures were extracted with acetic acid, and a mixture of the extract and carrier r proteins was used for two-dimensional gel electrophoresis to learn whether any radioactivity failed to migrate with stained spots. Table I presents the levels of radioactivity and the ^{3}H/^{14}C ratios of unstained plugs and adjacent r proteins from the four quadrants of the stained slab gel. It can be seen from the table that the unstained plugs from the upper left quadrant of the gel were highly labeled and that the ^{3}H/^{14}C ratios of both the unstained plugs and the r proteins from this region were greater than those in the other portions of the electrophorogram. The presence of weakly basic contaminant proteins in gels of r proteins has been recently reported by Boersma et al. (1979).

The remaining quadrants of the electrophorogram were not free of counts in between the stained spots. In these regions, however, the ^{3}H/^{14}C ratios in the unstained plugs were similar to those of the neighboring stained proteins, suggesting that the contaminating radioactivity was in r protein that had failed to migrate along with the majority of the molecules. One effort to find an alternative electrophoretic procedure (Gorenstein & Warner, 1976) that would focus the resolved proteins more sharply was not successful.

Table I also shows the effects of subjecting r proteins from the slab gel to a third electrophoretic step in cylinders of $NaDodSO_4$–acrylamide gel. With some r proteins the ^{3}H/^{14}C ratios were lowered appreciably whereas with others little or no change occurred. In the case of L26, as indicated in the table, most of the radioactivity proved to be in a non-r protein that migrated as a histone and that was separated from the r protein by the $NaDodSO_4$ gel. In all subsequent experiments with resolved proteins, the third electrophoretic step was used

Table I: Radioactivity Not Associated with Stained Spots and Effectiveness of Third Electrophoretic Step[a]

gel quadrant	unstained plug or protein no.	slab gel ^3H (cpm)	slab gel ^{14}C (cpm)	$NaDodSO_4$ gel $^3H/^{14}C$	$NaDodSO_4$ gel $^3H/^{14}C$
upper left	1	202	13	15.5	
	S3	782	127	6.16	4.62
	2	193	12	16.1	
	S5	357	54	6.61	4.79
	3	173	12	14.4	
	L5	545	81	6.73	6.31
lower left	4	47	8	5.88	
	S20	218	40	5.45	4.44
upper right	5	83	15	5.53	
	L8	460	98	4.69	4.83
	6	83	19	4.37	
	L13	371	75	4.95	4.57
lower right	7	13	3		
	L35	235	46	5.11	5.09
	L26[b]	946	239	3.96	4.31

[a] Four confluent cultures of chick embryo fibroblasts that had been grown up with [^{14}C] leucine were washed and then preincubated for 30 min in 2 mL of modified basal medium (0.1 mM leucine). At zero time the preincubation medium was replaced with 2 mL of fresh modified basal medium, and at 30 min [^3H]leucine was added to each culture. At 60 min, the cells were pooled and extracted with acetic acid and unlabeled r proteins were added to the extract. The mixture was then divided into two equal portions and each was used to separate basic proteins by two-dimensional electrophoresis. After staining, plugs (4.8-mm diameter) of r protein spots and adjacent unstained gel were cut out from each of the four quadrants of one of the slab gels. From the other slab gel, r protein plugs were processed further in $NaDodSO_4$ gels as described under Materials and Methods. [b] Most of the radioactivity resided in a nonribosomal contaminant that was discarded in the third electrophoretic step. The stained band from the $NaDodSO_4$ gel contained 125 cpm of ^3H and 29 cpm of ^{14}C.

in an effort to free the individual proteins of contaminating radioactivity.

Quantitation of Effects of Insulin. As described under Materials and Methods, the synthesis of basic proteins was measured with cells that had been grown up with [^{14}C]leucine and were then briefly labeled with [^3H]leucine. The ^{14}C provided a measure of the recovery of the individual protein. A minor modification of the equation of Dennis (1974) was used to relate the synthesis of protein i to that of total cell protein. In the modified equation

$$A_i = \frac{^3H/^{14}C \text{ in i protein}}{f}$$

f adjusts for the relative rates of total protein synthesis in the control and experimental cells as measured by the incorporation of [^3H]leucine into previously unlabeled cells. The specific activity of the total protein of control cells was set at 1.0, and the values of f for experimental cells were the quotients of the specific activities of (experimental cells)/(control cells).

The effects of insulin on the radioactivities and A_i values of 30 r proteins and 7 non-r proteins are shown in Table II. With the exception of S6, insulin raised the $^3H/^{14}C$ ratios and the values of A_i of the r proteins by about 3.5- and 2.5-fold, respectively. The $^3H/^{14}C$ ratios of the basic non-r proteins, on the other hand, were increased by the hormone to about the same extent as total protein synthesis (1.3 times) and the values of A_i were, therefore, unaffected.

Exclusive of S6, 22 of the 29 A_i values of the control r proteins fell in the range of 5.0 ± 0.5 with 3 values falling below and 4 above this range. The proteins with low values

Table II: Radioactivities and A_i Values of r Proteins and Non-r Proteins from Control and Insulin-Treated Cells[a]

protein no.	no insulin $^3H/^{14}C$ (cpm)	no insulin A_i	insulin $^3H/^{14}C$ (cpm)	insulin A_i	A_i (insulin)/A_i (no insulin)
S1	464/87	5.33	1446/67	16.6	3.11
S2	161/38	4.24	724/48	11.6	2.74
S3	406/84	4.83	1355/70	14.9	3.08
S4	200/44	4.55	826/47	13.5	2.97
S5	408/80	5.10	1166/65	13.8	2.71
S6	487/81	6.01	2553/30	65.5	10.9
S7	435/82	5.30	2389/124	14.8	2.79
S8	236/48	4.92	1176/64	14.1	2.87
S9	331/61	5.43	1590/82	14.9	2.74
S10	344/68	5.09	1271/67	14.6	2.87
S11	402/83	4.84	909/53	13.2	2.73
S14	322/50	6.44	859/44	15.0	2.33
S15	314/63	4.98	1437/89	12.4	2.49
S17	199/33	6.03	763/38	15.4	2.55
S20	113/24	4.71	478/26	14.1	2.99
L3	184/46	4.00	741/47	12.1	3.03
L4	253/49	5.16	667/38	13.5	2.62
L6	458/91	5.03	2018/113	13.7	2.72
L7	456/93	4.90	1201/67	13.8	2.82
L8	370/74	5.00	1446/85	13.1	2.62
L9	311/61	5.10	1734/106	12.6	2.47
L11	341/57	5.98	1777/91	15.0	2.51
L12	212/44	4.82	763/38	15.4	3.20
L13	243/54	4.50	923/57	13.8	3.07
L15A	248/50	4.96	845/52	12.5	2.52
L17	264/44	6.00	1462/58	19.4	3.23
L22	216/42	5.14	550/36	11.8	2.30
L24	110/21	5.24	425/24	13.6	2.60
L26	162/38	4.26	564/32	13.6	3.19
L35	222/42	5.29	863/48	13.8	2.61
a	1476/211	7.00	1826/196	7.17	1.02
b	2256/218	10.3	1941/147	10.2	0.99
c	428/50	8.56	504/48	8.08	0.94
d	2351/389	6.04	4982/583	6.57	1.09
e	2190/496	4.42	1837/366	3.86	0.87
f	682/166	4.11	460/103	3.44	0.84
g	306/181	1.70	382/176	1.66	0.98

[a] Conditions were exactly as described for Table I except that one of the pairs of cultures received insulin (5 μg/mL) at zero time and the proteins resolved by the two-dimensional gels were all subjected to electrophoresis in cylinders of $NaDodSO_4$-acrylamide gel as described under Materials and Methods. The value of f for the insulin-treated cultures was 1.30. The $^3H/^{14}C$ ratio of the hot trichloroacetic acid insoluble fraction of the acetic acid extract of the insulin-treated cells was 1.29 times greater than that of the control extract.

were in the normal range in other experiments, but those with high values, S14, S17, L11, and L17, were consistently above the norm. The proximity of these four r proteins to each other in the slab gel (Figure 1A) suggested that the high values were due to contamination, but other explanations were not ruled out.

The A_i value of S6 appeared to be elevated by insulin to a much greater extent than that of any of the other r proteins. This difference proved to be an artifact and depended upon the portion of the elongate spot that was taken for counting. In other experiments with insulin-treated cells, it was found that the ^{14}C content of the left (more acidic) end of the spot was considerably higher than that of the right end whereas the levels of ^3H at the two ends were about the same. Insulin has been shown to stimulate the phosphorylation of S6 in 3T3-derived cells (Smith et al., 1979). One explanation for the changing $^3H/^{14}C$ ratios is that insulin induces much better the phosphorylation of the S6 in cytoplasmic ribosomes than of nascent molecules.

Speed of Action of Insulin. For a study of the kinetics of

Table III: Values of A_i of r Proteins and Non-r Proteins as a Function of Time after Treatment of Cells with Insulin[a]

	A_i (no insulin) at various labeling periods (min)				A_i (insulin) at various labeling periods (min)			
protein no.	0-10	10-20	20-30	50-60	0-10	10-20	20-30	50-60
S3	1.56	1.60	1.42	1.78	1.82	3.40	4.67	4.92
S7	1.73	1.70	1.53	1.80	2.34	4.23	5.90	6.05
S8	1.51	1.34	1.17	1.72	1.50	3.26	4.55	4.83
S11	1.33	1.49	1.22	1.62	1.52	2.88	3.85	4.19
S15	1.63	1.62	1.39	1.82	1.89	3.19	4.47	4.64
L4	1.27	1.38	1.01	1.45	1.39	2.80	3.82	4.12
L6	1.25	1.41	1.10	1.31	1.39	3.09	3.88	4.00
L8	1.27	1.02	1.07	1.40	1.40	2.75	4.00	4.19
L11	2.14	2.12	1.81	2.56	2.56	5.12	6.39	6.77
L12	1.29	1.87	1.78	1.66	1.63	3.33	4.82	5.15
a	6.50	6.75	5.22	6.30	6.18	6.52	5.79	5.40
b	6.58	6.20	6.09	5.50	6.50	6.89	6.17	6.85
c	5.60	4.97	4.75	5.85	5.67	4.75	5.23	5.45
d	2.69	3.42	2.53	2.36	2.96	2.48	2.54	2.32
e	1.85	1.95	1.68	1.43	1.89	1.50	1.27	1.13
f	1.61	1.79	1.52	1.24	1.61	1.44	1.20	1.20

[a] Conditions were as for Table I except that half of the cultures received 10 μg of insulin and labeling with [^{3}H]leucine was for 10 min only beginning immediately after the preincubation period. The values of f for the control cultures labeled at 0-10, 10-20, 20-30, and 50-60 min after preincubation were 1.00, 0.98, 1.24, and 1.37, respectively. The comparable numbers for the insulin-treated cultures were 1.14, 1.41, 1.85, and 2.00. The differences between the values of f and the equivalent numbers as calculated from the $^3H/^{14}C$ ratios of the acid-insoluble fraction of the acetic acid extracts ranged from 1 to 14%.

insulin action, ^{14}C-prelabeled cultures were given [^{3}H]leucine for 10-min periods starting immediately after the addition of the hormone. Table III shows the results with the 10 r proteins that were examined. Insulin had little or no effect during the first 10-min labeling period, but the values of A_i were raised to more than half the maximum between 10 and 20 min. It may be worth pointing out that exactly the same kinetics were found for the stimulation by insulin of pre-rRNA synthesis in the resting chick fibroblasts (DePhilip et al., 1979).

Labeling of r Proteins in the Presence of Actinomycin. The effect of insulin on the incorporation of [^{3}H]leucine into r proteins could result from an enhanced rate of synthesis or a decreased rate of breakdown. The hormone might conserve newly made r proteins by a generalized dampening of protein turnover as has been shown with a variety of other cell types (Mortimore & Mondon, 1970; Goldberg & St. John, 1976) or, perhaps more likely, by increasing the availability of pre-rRNA to stabilize the newly made proteins.

The effects of insulin were studied in cells that were labeled with [^{3}H]leucine in the presence of actinomycin D to evaluate the contribution of pre-rRNA. The level of antibiotic used, 10 μg/mL, was 250 times greater than required to inhibit completely the synthesis of pre-rRNA in the cultured chick cells (DePhilip et al., 1979). Table IV shows the results that were obtained with 15 r proteins in two separate experiments. It can be seen from columns 2 and 3 that actinomycin depressed the values of A_i of about half of the r proteins, but the extent of the reductions was similar for the proteins from the control and hormone-treated cells. In consequence, as can be seen from columns 4 and 5, insulin raised the A_i values of the r proteins to about the same degree whether or not the cells were labeled in the presence of the antibiotic.

Table IV also shows the results with seven non-r proteins. The A_i values of four of the proteins, three of them histones (d, e, and f), were severely reduced by actinomycin D, but the effects of the antibiotic were not influenced by insulin.

Conditions of Chase. To compare the stabilities of nascent r proteins made by control and insulin-treated cells in the presence of actinomycin, it was necessary to establish conditions under which the incorporation of [^{3}H]leucine into cell protein could be abruptly stopped so that the fate of the newly formed proteins could be followed. Figure 2 shows that immediate termination of [^{3}H]leucine incorporation into total cell protein was achieved by simply replacing the radioactive culture medium with unlabeled medium containing 4.4 mM leucine.

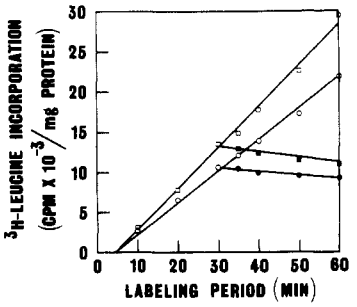

FIGURE 2: Immediate cessation of [^{3}H]leucine incorporation into total cell protein after chase. Confluent cultures of chick embryo fibroblasts were washed with modified basal medium (0.1 mM leucine), and each was then incubated in 2 mL of the modified medium containing 0.25 μCi of [^{3}H]leucine and, as indicated, 10 μg of insulin. After 30 min, the labeled medium on some of the cultures was replaced with 2 mL of unlabeled medium containing 4.4 mM leucine and, as appropriate, 10 μg of insulin. Specific activities of total protein were measured as described under Materials and Methods. (○) Control; (□) insulin; (●) control after chase; (■) insulin after chase.

Actinomycin and Instability of Newly Formed r Proteins. As part of the first experiment of Table IV, control and insulin-treated cultures that had been labeled with [^{3}H]leucine for 30 min in the presence of actinomycin D were incubated for an additional 30 min in unlabeled culture medium containing 4.4 mM leucine, 10 μg/mL actinomycin, and, as appropriate, 5 μg/mL insulin. In the second experiment of Table IV, chase periods of 30 and 60 min were included.

Table V shows the fractions of the ^{3}H-labeled r proteins and non-r proteins that survived the chase periods. It can be seen from the table, first, that the rates of decay of the individual r proteins varied considerably, as was shown to be the case with HeLa cells (Warner, 1977). The half-lives of the fibroblast r proteins ranged from less than 15 min to more than 1 h. Second, the kinetics of decay of the r proteins was exponential in both the control and insulin-treated cells. Third, insulin did not stabilize any of the r proteins and, indeed, the rates of degradation of most of them were increased slightly

Table IV: Effects of Actinomycin D on Values of A_i of r Proteins and Non-r Proteins from Control and Insulin-Treated Cells[a]

protein no.	A_i (actinomycin)/A_i (no actinomycin)		A_i (insulin)/A_i (no insulin)	
	no insulin	insulin	no actinomycin	actinomycin
S3	0.65, 0.75	0.67, 0.67	3.30, 3.14	3.38, 3.01
S4	0.57, 0.64	0.59, 0.60	3.21, 4.12	3.40, 3.89
S5	1.04, 1.08	0.86, 0.89	3.72, 3.62	3.22, 3.30
S7	0.87, 1.04	1.19, 1.14	2.99, 2.40	4.10, 2.64
S8	0.97, 1.04	0.91, 0.93	3.07, 3.27	2.88, 2.92
S11	0.71, 0.76	0.50, 0.45	3.10, 3.46	2.16, 2.02
S15	0.68, 0.62	0.41, 0.35	2.93, 2.64	1.77, 1.61
L3	0.96, 1.11	0.85, 0.81	4.40, 4.13	3.78, 3.02
L4	0.77, 0.91	0.77, 0.87	2.78, 2.21	2.76, 2.50
L6	1.16, 1.09	1.00, 0.96	3.29, 3.42	2.82, 3.02
L8	0.77, 0.93	0.74, 0.83	3.31, 3.82	3.64, 3.68
L11	1.06, 0.92	1.09, 0.92	2.90, 2.70	2.98, 2.68
L12	1.16, 1.09	1.19, 1.18	3.19, 2.28	3.26, 2.47
L17	0.55, 0.55	0.61, 0.56	3.44, 2.92	3.86, 4.05
L24	0.94, 0.90	0.78, 0.88	2.68, 2.76	2.22, 2.70
a[b]	1.22, 1.16	0.86, 1.13	0.92, 0.93	0.65, 0.90
b[b]	1.11, 1.07	1.01, 0.95	1.03, 0.97	0.94, 0.85
c	1.14, 1.07	1.25, 1.10	0.83, 0.91	0.89, 1.09
d	0.46, 0.47	0.61, 0.55	0.83, 0.76	1.10, 0.89
e	0.20, 0.21	0.22, 0.26	0.69, 0.69	0.73, 0.84
f	0.21, 0.29	0.20, 0.31	0.71, 0.82	0.66, 0.86
g	0.20, 0.46	0.21, 0.47	0.74, 1.07	0.80, 1.08

[a] The results of two experiments are shown. In each, four pairs of confluent cultures of chick fibroblasts that had been labeled with [^{14}C] leucine during growth were preincubated for 15 min in 2 mL of modified basal medium (0.1 mM leucine) with one-half of the cultures receiving, in addition, 20 μg of actinomycin D. At zero time 10 μg of insulin was added to one of the pairs of cultures that had not received antibiotic and to one of the pairs that had, and at 30 min [^{3}H] leucine was added to each of the cultures. Labeling was for 30 min. Extraction of the cells with acetic acid and separation of the basic proteins on two-dimensional gels followed by electrophoresis of individual stained spots in cylinders of NaDodSO$_4$-acrylamide gel were as described under Materials and Methods. Values of f for the two experiments were the following, respectively: actinomycin D, 0.79 and 0.87; insulin, 1.55 and 1.61; insulin and actinomycin D, 1.28 and 1.36. The comparable values calculated from the ^{3}H/^{14}C ratios of the hot acid insoluble fraction of the acetic acid extracts were 0.80, 1.64, and 1.28 for the first experiment and 0.99, 1.69, and 1.46 for the second. [b] The quantity of stained protein in the slab gels of the second experiment was judged to be insufficient for detection in NaDodSO$_4$ gels, and plugs from the slab gels were counted directly.

Table V: Rates of Degradation of Proteins Synthesized and Chased in the Presence of Actinomycin D[a]

	(^{3}H/^{14}C after 30-min chase)/(^{3}H/^{14}C before chase)		(^{3}H/^{14}C after 60-min chase)/(^{3}H/^{14}C before chase)	
	no insulin	insulin	no insulin	insulin
S3	0.29, 0.26	0.16, 0.17	0.14 (0.08)	0.06 (0.03)
S4	0.23, 0.23	0.16, 0.18	0.12 (0.05)	0.06 (0.03)
S5	0.62, 0.56	0.41, 0.38	0.34 (0.35)	0.17 (0.16)
S7	0.76, 0.79	0.55, 0.63	0.52 (0.60)	0.49 (0.35)
S8	0.87, 0.97	0.76, 0.73	0.84 (0.85)	0.65 (0.56)
S11	0.31, 0.28	0.10, 0.08	0.15 (0.09)	0.05 (0.01)
S15	0.37, 0.43	0.20, 0.38	0.29 (0.16)	0.31 (0.08)
L3	0.59, 0.52	0.60, 0.46	0.43 (0.31)	0.29 (0.28)
L4	0.45, 0.60	0.60, 0.41	0.34 (0.28)	0.23 (0.26)
L6	0.61, 0.59	0.47, 0.43	0.40 (0.36)	0.22 (0.20)
L8	0.48, 0.50	0.28, 0.29	0.26 (0.24)	0.18 (0.08)
L11	0.60, 0.52	0.40, 0.39	0.27 (0.31)	0.15 (0.16)
L12	1.13, 1.01	1.14, 1.00	0.72 (1.14)	0.86 (1.14)
L17	0.15, 0.19	0.11, 0.11	0.13 (0.03)	0.01 (0.01)
L24	0.87, 0.89	0.81, 0.64	0.65 (0.77)	0.40 (0.53)
a[b]	1.41, 1.04	1.49, 1.07	0.93	1.03
b[b]	1.09, 0.96	1.15, 0.95	0.89	1.02
c	1.07, 0.96	1.10, 0.95	0.99	1.04
d	0.88, 0.79	0.74, 0.64	0.56	0.56
e	0.96, 0.86	1.01, 0.86	0.86	0.85
f	0.83, 0.70	1.14, 0.86	0.74	0.84
g	1.22, 0.73	0.96, 0.96	0.68	0.62

[a] As part of the first experiment of Table IV, pairs of control and insulin-treated cultures that had been labeled with [^{3}H] leucine for 30 min in the presence of actinomycin D were incubated for an additional 30 min in fresh basal medium containing 4.4 mM leucine, 10 μg/mL actinomycin, and, as appropriate, 5 μg/mL insulin. The same conditions were used as in the second experiment of Table IV, except that pairs of labeled cultures were chased for 30 or 60 min. The results of both experiments are shown. The numbers in parentheses represent values for exponential decay as calculated from averages of the results after the 30-min chase. [b] Plugs from the slab gels of the second experiment were counted directly.

by the hormone. Finally, unlike the r proteins, the non-r proteins were largely or completely stable during the periods of chase.

A comparison of the results of Tables IV and V shows that during the labeling period, actinomycin reduced considerably more the A_i values of the relatively unstable r proteins than of the more stable r proteins. These correlations were taken to mean that the antibiotic depressed the accumulation of ^{3}H-labeled r proteins during the labeling period by provoking the destruction of nascent molecules rather than by interfering with synthesis.

Inhibition of Synthesis of Poly(A)-Containing mRNA by Actinomycin. Table VI shows that 10 μg/mL actinomycin D blocked almost completely the incorporation of [^{3}H]adenine into the 28S rRNA and poly(A) mRNA of control and insulin-treated cells. The table also shows that the reduced labeling of the RNAs could not be explained by an inhibition of the conversion of [^{3}H]adenine into ATP as measured in total cell extracts.

Efficiency of Use of r Proteins for Cytoplasmic Ribosome Synthesis. Ribosomal subunits are processed at the same rate in control and insulin-treated chick embryo fibroblasts. In both, nascent 40S (R. M. DePhilip and I. Lieberman, unpublished experiments) and 60S (DePhilip et al., 1979) subribosomes begin to emerge from the nucleus at 30 and 40 min, respectively. Based on this information and the instability of free r proteins, it seemed reasonable to expect that, within 2 h, most or all new molecules of a r protein would either have returned to the cytoplasm in combination with a rRNA or would have been destroyed.

Cultures that had been prelabeled with [^{14}C]leucine were given [^{3}H]leucine for 30 min and some of the cultures were then incubated under chase conditions for 2 h to estimate the efficiency with which nascent r proteins are converted to cytoplasmic particles by control and insulin-treated cells. Total protein was extracted with 67% acetic acid from the unchased cultures and from some of the chased cultures, and acetic acid extracts of polyribosomes were made from the remainder of the chased cultures.

Table VII shows the results that were obtained with 10 r proteins that had been resolved from the acetic acid extracts of the total cells and the polyribosomes by the three electrophoretic steps. It can be seen from the table that the ^{3}H/^{14}C ratios of the r proteins were reduced little or not at all during the 2-h chase period. It must be concluded, therefore, that both the control and insulin-treated cells utilized r proteins with great efficiency for the production of cytoplasmic ribosomes.

The data of Table VII provide information on two additional points. First, they show that r proteins that were synthesized

Table VI: Effects of Actinomycin D on the Incorporation of [^{3}H] Adenine into rRNA, mRNA, and ATP[a]

actinomycin	insulin	28S rRNA (cpm/μg)	mRNA (cpm/μg)	ATP (cpm/nmol)
−	−	47	771	369
+	−	0	43	355
−	+	165	1012	317
+	+	0.35	45	318

[a] 28S rRNA and mRNA were from 24 cultures/group. The cultures were washed twice with a total of 4 mL of basal medium, and each was then given 2 mL of basal medium and, as indicated, 20 μg of actinomycin D. At 15 min 10 μg of insulin was added, as shown, and after an additional 15 min of incubation each culture received 0.5 μCi of [^{3}H] adenine (19 Ci/mmol). Labeling was for 90 min, and the cells were broken in the detergent mixture of Perry & Kelley (1968) that had been supplemented with *N*-ethylmaleimide, polyvinyl sulfate, and spermine (Herman et al., 1976). RNA was extracted from the postmitochondrial supernatant fraction with phenol-0.2% $NaDodSO_4$, freed of protein by repeated extractions with $NaDodSO_4$-hot phenol-chloroform (Penman, 1969), and precipitated with ethanol. Poly(A)-containing mRNA was isolated by two passes on columns of oligo(dT)-cellulose according to Groner et al. (1974). A portion (2.5 A_{260} units) of the RNA that failed to hybridize with the oligo(dT)-cellulose was used as the source of 28S rRNA. 28S rRNA was purified by sedimentation in a sucrose gradient as previously described (DePhilip et al., 1979). Specific activities of ATP were estimated as detailed under Materials and Methods with cultures that were treated exactly as for the RNA measurements except that labeling was with 0.04 μCi of [^{3}H] adenine.

Table VII: Efficiency of Utilization of r Proteins for Cytoplasmic Ribosome Synthesis[a]

protein no.	^{3}H/^{14}C (no insulin) cell extract before chase	after chase	polysomes	^{3}H/^{14}C (insulin) cell extract before chase	after chase	polysomes
S7	4.86	4.49	4.06	22.4	24.0	20.6
S10	3.72	4.21	4.09	19.8	19.4	18.9
S11	4.22	4.26	4.16	15.6	16.2	16.5
S13	4.40	4.50	3.86	18.7	16.6	16.7
S20	4.12	4.28	4.22	17.7	18.2	18.4
L3	3.72	4.16	3.54	17.3	17.8	14.6
L6	3.86	4.25	3.46	18.5	17.1	14.4
L8	3.64	4.04	4.18	16.2	15.6	14.4
L24	4.24	4.20	4.04	16.8	14.4	13.1
L35	4.12	4.03	3.92	18.0	14.9	15.0

[a] Confluent cultures of chick embryo fibroblasts that had been grown up with [^{14}C] leucine were washed and then preincubated for 30 min in modified basal medium (0.1 mM leucine). At zero time, the medium was replaced with 2 mL of fresh modified basal medium and one-half of the cultures received, in addition, 10 μg of insulin. Labeling with [^{3}H] leucine was from 30 to 60 min. At the end of this time, pairs of control and hormone-treated cultures were extracted with 67% acetic acid and the radioactive medium from the rest of the cultures was replaced with 2 mL of unlabeled basal medium containing 4.4 mM leucine and, as appropriate, 10 μg of insulin. After 2 h of chase, pairs of control and hormone-treated cultures were extracted with acetic acid, the remaining two sets of cultures (five/group) were harvested, and the cells were mixed with those from 24 unlabeled cultures and sedimented. Polyribosomes were prepared by the method of Palmiter (1974) from the postmitochondrial fraction of the mixed cells, and they were extracted with 67% acetic acid. Finally, carrier r proteins were added to the acetic acid extracts of the whole cells and basic proteins were resolved with three electrophoretic steps as described under Materials and Methods.

and chased in the absence of actinomycin were stable, and, second, they show that 67% acetic acid extracted with equal effectiveness newly made r proteins that were considered to be in the nucleus (Warner, 1979) and those in cytoplasmic ribosomes.

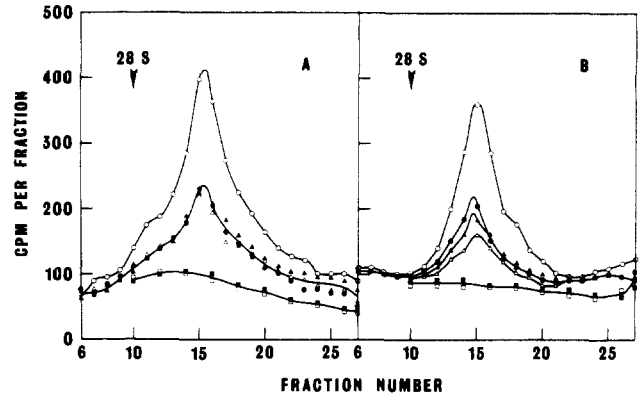

FIGURE 3: Effects of cycloheximide and puromycin on the synthesis of pre-rRNA in control and insulin-treated cells. Confluent cultures (five/group) were washed twice with a total of 4 mL of basal medium, and each was then given 2 mL of basal medium containing, as appropriate, 10 μg of insulin, 200 μg of cycloheximide, 80 μg of puromycin, and 0.08 μg of actinomycin D. At 30 min each culture received 20 μCi of [^{3}H]adenine (19 Ci/mmol), and after 10 min of labeling the medium was removed and the attached cells were lysed with 1 mL of 1% $NaDodSO_4$ (55 °C). The lysates were extracted with water-saturated phenol (55 °C, pH 5.4) according to Wolf & Schlessinger (1977), and the RNA was collected, dried, and dissolved in 0.25 mL of NETS buffer. The solution was heated at 60 °C for 10 min and quickly chilled in an ice slush. Sedimentation was in an SW 41 rotor for 4 h at 40000 rpm (25 °C) in a 10–30% linear sucrose gradient that contained NETS buffer. Sedimentation was from left to right. Fractions (0.45 mL) were collected by upward displacement. The peak of 18S RNA was in the sixth fraction. Panels A and B: cycloheximide and puromycin, respectively. (●) Control; (○) insulin; (△) cycloheximide or puromycin; (▲) insulin + cycloheximide or puromycin; (□) actinomycin; (■) insulin + actinomycin.

Effects of Cycloheximide and Puromycin on the Synthesis of Pre-rRNA. As already mentioned, insulin rapidly elevates the rate of synthesis of pre-rRNA in resting chick fibroblasts (DePhilip et al., 1979) as well as that of r proteins. The experiments with actinomycin showed clearly that the enhanced production of r proteins does not depend upon rRNA formation. Cells were treated with insulin in the presence of a level of cycloheximide or puromycin that reduced the uptake of [^{3}H]leucine into total cell protein by slightly more than 90% to determine whether the increase in pre-rRNA synthesis is dependent upon protein synthesis. Figure 3 shows that cycloheximide had no effect on the incorporation of [^{3}H]adenine into the pre-rRNA of the control cells but blocked completely the increased labeling that would otherwise have been induced by insulin. The same results were obtained with puromycin except that, in this case, there was a small reduction in the radioactivity of the pre-rRNA of the control cells.

The selective inhibition by the antibiotics of the incorporation of [^{3}H]adenine into the pre-rRNA of the insulin-treated cells could not be explained by a decreased labeling of ATP as measured with total cell extracts. Thus, the specific activities of ATP (cpm/nmol) in cells labeled with [^{3}H]adenine (0.04 μCi/mL) under exactly the same conditions as for the experiment of Figure 3 were as follows: control, 248; cycloheximide, 250; puromycin, 229; insulin, 216; insulin + cycloheximide, 221; insulin + puromycin, 226.

Discussion

Cultures that had been grown with [^{14}C]leucine and had come to rest were briefly labeled with the ^{3}H-labeled amino acid to compare the incorporation of [^{3}H]leucine into the r proteins of control and insulin-treated chick embryo fibroblasts. The ^{14}C content of an individual r protein served as a measure

of its recovery, and the $^3H/^{14}C$ ratio served as a measure of its synthesis or accumulation. Insulin raises the $^3H/^{14}C$ ratios of r proteins by almost 3 times more than those of isolated non-r proteins and total cell protein.

There does not seem to be a trivial explanation for the preferential effect of the hormone on the $^3H/^{14}C$ ratios of the r proteins. Insulin influences the extractability with 67% acetic acid of neither newly made molecules that are considered to be in the nucleus (Warner, 1979) nor r proteins that have reentered the cytoplasm. The hormone does not contribute to the higher $^3H/^{14}C$ ratios by causing the destruction of prelabeled r proteins. Resting cultures that are incubated with insulin for as long as 5 h suffer no loss of total RNA, and prelabeled r proteins from control and insulin-treated cultures have indistinguishable levels of ^{14}C.

There are two good reasons to believe that insulin works by increasing the rate of synthesis of r proteins rather than by decreasing the rates of degradation. For one thing, there is little or no breakdown of nascent r proteins in either control or hormone-treated cells. Almost all the molecules that are made end up in cytoplasmic ribosomes. For another, insulin does not protect r proteins even under conditions where the nascent molecules are caused to be unstable.

Ribosomal proteins are translated on poly(A)-containing messages in yeast (Warner & Gorenstein, 1977), Ehrlich ascites cells (Hackett et al., 1978), and rat liver (Nabeshima et al., 1979), and it is reasonable to assume that this is also the case in the chick cells. Yet, the hormonal induction of r protein synthesis in the resting fibroblasts is not at all depressed under conditions where poly(A) mRNA formation is reduced by 95%. This observation, coupled with the speed of the effect of the hormone, would seem to rule out any role for newly made messages in the action of insulin on r protein synthesis.

What cannot be excluded is the involvement of a small RNA whose production is unaffected by 10 μg/mL actinomycin D. Even this possibility is weakened, however, by the fact that such a high concentration of actinomycin does inhibit by almost 80% the synthesis by the chick cells of a species of low molecular weight RNA, tRNA (data not shown).

Two additional observations are pertinent to the mechanism of insulin action. The enhancement of r protein synthesis by the hormone is completely independent of pre-rRNA formation. In the reverse situation, however, where protein synthesis is blocked by cycloheximide or puromycin, insulin fails to stimulate the production of pre-rRNA, as it ordinarily does (DePhilip et al., 1979). Taken together with the absence of a requirement for mRNA synthesis, these observations suggest that insulin acts directly and posttranscriptionally to raise r protein in the resting chick cells and that the increase in pre-rRNA production is secondary to the elevated synthesis of one or more proteins, possibly r proteins.

Preliminary experiments involving treatment of chick cells with a variety of translational inhibitors of initiation and elongation, modeled after the studies of Lodish (1974) with globin and Jen et al. (1978) with viral proteins, lend support to the idea that, in the resting chick cells, r protein messages compete poorly with other messages for initiation. It may be that insulin stimulates r protein formation by selectively encouraging the initiation of r protein mRNAs.

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