STIMULATION BY INSULIN OF RNA SYNTHESIS IN CHICK FIBROBLASTS

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ABSTRACT

After the addition of insulin to monolayers of chick fibroblasts previously incubated in
serum-free medium, the rates of protein and RNA synthesis increase continuously during
the first 8–10 h. Little stimulation of DNA synthesis or mitosis results with the addition
of insulin alone in contrast to the addition of fresh serum which stimulates both markedly.
The stimulation in RNA synthesis does not result from expansion of the nucleotide pool
but is correlated with increases in RNA polymerase activity. All major classes of RNA
are stimulated; processing of preribosomal RNA to 28S and 18S and the association of
this mature RNA with ribosomes appear to occur normally. The kinetics of stimulation
of 5S RNA differ from those of the synthesis of 4S and of ribosomal RNA. Insulin and
serum appear to affect the synthesis or stability of certain transcripts differentially.

INTRODUCTION

The role of hormones in the regulation of transcription and translation in mammalian cells has been widely investigated (1, 2). There is little doubt that hormones are required to maintain general and specialized metabolic activities of normal cells. Nevertheless, little progress has been made in assigning specific mechanisms to hormones at a molecular level, in part because much of the work has been accomplished in animals.

More recently the action of hormones on cells and organs in culture has attracted investigators (3–6). Here, the problems of mixtures of cell types and the complexity of the medium must be considered carefully when results are interpreted. Consequently, it has been difficult to establish the biological steps which implement the expression of hormonal function in target cells.

The work to be reported here describes the kinetics of the transient stimulation of RNA and protein synthesis when chick fibroblasts in culture are exposed to insulin after serum deprivation. The addition of insulin alone under such conditions has little effect upon DNA synthesis and does not lead to a round of cell division as does serum addition. Of particular interest are the patterns of accumulation of certain RNA fractions whose rates of synthesis or degradation are influenced by insulin in the incubation medium.

MATERIALS AND METHODS

Primary Cultures of Chick Embryo Fibroblasts

The preparation of monolayer cultures of cells from the 12-day chick embryo has been described (7). Monolayers (initial density of 1.5×10^6 cells/ml of medium, 10^5 cells/cm^2) were cultivated in Eagle's basal medium (BME) (8) supplemented with 3% calf serum. Cultures were incubated in glass bottles at 37°C for 72 h. The medium was then removed by washing twice with Hank's balanced salts (BSS) (9), and replaced with BME lacking serum. Cultures

were incubated in the nonserum medium for 24 h prior to the experiment.

Incorporation of Radioactive Precursor into Whole Cells

After exposure of cells to insulin, serum, or BSS, monolayers were labeled with mixtures of amino acids or uridine for 1 h. Cells were then washed in cold saline, harvested by scraping, and the acid-insoluble components were precipitated with cold 5% TCA. Protein concentrations were determined by the Lowry procedure (10) and samples for estimation of radioactivity were collected on Millipore filters (Millipore Corp., Bedford, Mass.).

Nucleotide Pool Determinations

The procedure employed was essentially that described by Horisberger and Amos (11). Cells were prelabeled with $^{32}PO_4^{-3}$ for 12 h before addition of insulin, serum, or BSS followed 4 h later by a 1-h pulse with [^{14}C]uridine. At the end of the exposure period, the monolayer was washed with BSS and the cells were treated with 5% TCA supplemented with 300 µg each of unlabeled uridine and UMP. The TCA-soluble fraction was treated with hot HCl to yield mixtures of monophosphates only. After charcoal adsorption and elution, eluates were evaporated, dissolved in weak acid, and applied to Whatman no. 3 paper for two-dimensional chromatography. Nucleotide pool equilibration was expressed as the ^{14}C to $^{32}PO_4$ ratio of the UMP. Recovery of UMP from charcoal was generally around 30%.

RNA Polymerase Assay

Nuclei from chick fibroblasts were obtained by the technique of hypotonic swelling and homogenization, as described by Penman (12). The reaction mixture (final volume = 0.5 ml) contained 30 µmol Tris-HCl, pH 7.5, 2.0 µmol mercaptoethanol, 4.0 µmol MgCl$_2$, 2.0 µmol MnCl$_2$, 1.0 µmol KCl, 0.1 µmol of ATP, GTP, and CTP, 0.005 µmol of UTP, 1.5 µCi of [^3H]UTP, and nuclei equivalent to 20 µg of nuclear protein. In high ionic strength assays, (NH$_4$)$_2$SO$_4$ was added to a final concentration of 7.5×10^2 µmol. Enzyme incubations were performed at 30°C for 10 min. Reactions were terminated by addition of 5% TCA-0.05 M sodium pyrophosphate. Samples were collected on Millipore filters, washed with TCA-pyrophosphate, and counted.

Isolation of RNA

1 vol of cold phenol was added to 0.5% sodium dodecyl sulfate (SDS)-treated whole cells or poly-

ribosomes, and the mixture was shaken at room temperature for 5 min. After centrifugation the aqueous phase was reextracted twice with phenol. After two ether extractions of the aqueous phase to remove traces of phenol, 2 vol of 95% ethanol were added and the mixture was allowed to stand overnight at −20°C. The precipitate was centrifuged at 20,000 g and resuspended in SDS buffer. Optical densities (260/280) were determined and the RNA was applied to acrylamide gels for separation by electrophoresis.

Polyribosome Extraction

Polyribosomes were separated from crude cell extracts on 5–20% sucrose gradients. Gradients were centrifuged at 98,000 g for 90 min at 22°C. SDS was added to a final concentration of 0.5% prior to phenol extraction of RNA.

Gel Electrophoresis

RNA species were analyzed on acrylamide-bis-acrylamide gels as described by Loening (13). After electrophoresis of 10 µg of RNA at 5–7 mA/gel for specific time intervals, gels were treated in one of two ways: (a) Gels were sliced into sections 1 mm in thickness; RNA in each slice was hydrolyzed in the scintillation vial overnight by addition of 0.5 ml of concentrated ammonium hydroxide, and 5 ml of Bray's scintillation fluid was added prior to counting. (b) Other gels were sliced longitudinally in quarters or halves, dried under vacuum, and autoradiograms were prepared by exposing Kodak No-Screen Medical X-ray film to strips as described by Sirbasku and Buchanan (14).

Chemicals

[5-^3H]uridine, [5-^3H]uridine-(N)5′-triphosphate, H$_3$ $^{32}PO_4$, carrier-free, and [^{14}C]- and [^3H]amino acid mixtures were purchased from New England Nuclear, Boston, Mass. [2-^{14}C]uridine, [8-^3H] guanosine, [5-^3H]cytidine, and [8-^3H]adenosine were obtained from Schwarz Bio Research Inc., Orangeburg, N. Y. Bovine insulin was obtained from Sigma Chemical Company, St. Louis, Mo., and calf serum was purchased from Microbiological Associates, Inc., Bethesda, Md.

Mitotic Index

Cover slips with attached monolayers were fixed in absolute methanol and stained with May-Grunwald Giemsa stain according to the procedure of Jacobson and Webb (15). The stained coverslips were mounted on standard microscope slides in Permount (Fisher Scientific Co., Pittsburgh, Pa.). Cells in mitosis were

counted under ×540 magnification. Approximately 2,000 cells were monitored on each slide and the ratio of those in mitosis to the total recorded.

Estimates of DNA

DNA in populations of cells was estimated by the indole method of Ceriotti as modified by Keck (16).

RESULTS

Insulin Versus Serum Effect on Confluent Monolayers: DNA Synthesis and Mitosis

In experiments previously reported from this laboratory (17) protein accumulation and the rate of amino acid incorporation into protein were stimulated when insulin or serum was added to cultures maintained in serum-free medium for at least 24 h. Care was taken in the earlier experiments to confine the observations to fibroblast cultures not approaching confluency.

The current experiments deal exclusively with confluent cultures. Although the primary aim was the examination of the effect of insulin on RNA synthesis, it was essential first to follow for some
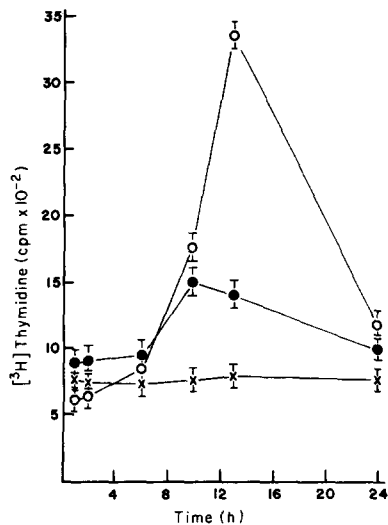

Figure 1   Thymidine incorporation after addition of serum or insulin. Monolayer cultures maintained in serum-free medium for 24 h were provided with insulin (10^{-2} U/ml), calf serum (3%), or BSS. Cells were harvested at 1, 2, 6, 10, 14, and 24 h. Each point (1-h pulse with 0.3 μCi/ml [H]thymidine) is the average of determinations from two monolayer populations. TCA-insoluble counts are reported as cpm/mg cell protein. ✕ = control, ● = insulin, ○ = serum.

30 h the sequence of events consequent to insulin addition to such cultures.

Fibroblasts were plated in BME supplemented with 3% calf serum at an inoculum density of 1.5 × 10^6 cells/ml (to provide 10^5 cells/cm² of the plating surface). After 72 h of incubation at 37°C the medium was removed; the monolayers were rinsed twice with BSS and fresh nonsupplemented BME was supplied.

After 24 h in serum-free medium, the monolayers were provided with serum (3%) or insulin (10^{-2} U/ml) and were permitted to incorporate radioactive [³H]thymidine, for 1 h at intervals up to 24 h after addition of the supplements. The addition of serum resulted in a stimulation of thymidine incorporation first detected at about 6 h and reaching a maximum of at least sevenfold between the 16th and 20th h (Fig. 1). Insulin resulted in a more modest stimulation (twofold) which reached its peak between the 6th and 10th h.

Cover slips inserted into the culture vessels were stained (Materials and Methods) and the mitotic index was obtained for monolayers at 12, 18, 20, 24, and 36 h after a single addition of serum or insulin (Table I). One series of cultures received fresh insulin at 12-h intervals. Mitotic figures were readily identified in serum-treated cultures. By 20–24 h a mitotic index of 4–6% was observed.

TABLE II

DNA Content of Insulin-Supplemented Cultures

Exp. no.	DNA per culture			
	0 time	Se⁻	Ins	Se⁺
	μg			
1	130 ± 7	106 ± 12	145 ± 3	204 ± 21
2	210 ± 9	150 ± 6	255 ± 8	372 ± 14
3	175 ± 18	227 ± 6	218 ± 12	312 ± 5

Monolayer cultures in 32-ounce bottles (120 cm² surface area) were grown for 96 h at 37°C, at which time the monolayers were confluent. The serum-containing medium was removed and replaced, after appropriate washing, with serum-free BME. All cells were incubated for 24 h in serum-free medium before addition of insulin (Ins) 10^{-2} U/ml or 3% calf serum (Se⁺) to some. The 0 time DNA content represents values obtained for cells at the time of addition of insulin or serum. After 24 h further incubation at 37°C, the cells were harvested by scraping and the DNA content was determined for the control (Se⁻) without serum as well as the Ins and Se⁺ populations. Each value is the average of DNA determinations on three monolayers with average deviations from the mean of the three values.

Very few mitotic figures were observed in cultures supplemented only with insulin.

Determinations of the DNA content of the cultures 24 h after supplementation revealed that the insulin-treated cells did not increase their DNA appreciably while the serum-treated monolayers nearly doubled their DNA (Table II). This last was accepted as evidence that the results from thymidine incorporation do indeed represent DNA synthesis and not an artefact of the thymidylate pool equilibration.

Protein Synthesis: Amino Acid Incorporation

Cultures exposed to radioactive amino acids ([³H]- or [¹⁴C]amino acid mixtures) were used to determine the effect of serum or insulin supplementation on the rate of protein synthesis at intervals up to 8 h after the additions. The results of 1-h pulses (Fig. 2) showed that the rate of protein synthesis increased progressively during 8 h of continuous exposure to insulin or to serum. At 8 h the rate observed was three to four times that of the control rate which remained unchanged.
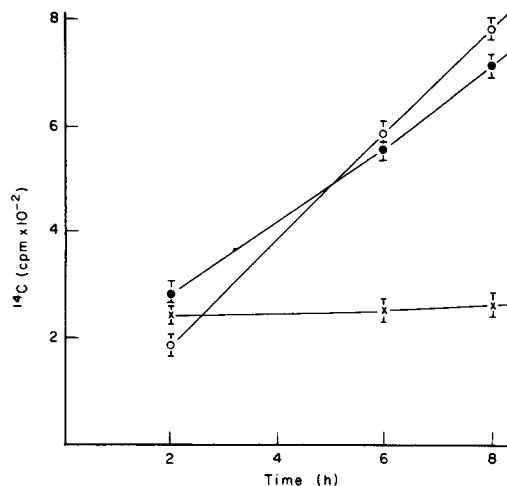

FIGURE 2   Stimulation of protein synthesis (amino acid incorporation) by serum and insulin. Confluent monolayers of chick fibroblasts incubated at 37°C in serum-free medium for 24 h were treated with insulin (10^{-2} U/ml), serum (3%), or BSS. Cells were harvested after 2-, 6-, and 8-h exposures to insulin or serum. Each point includes a 1-h pulse with 0.08 μCi/ml of [¹⁴C]amino acid mixture added 1 h prior to harvesting. TCA-insoluble counts are reported as cpm/mg cell protein. Each value is the average of determinations on two monolayer populations. O = serum, ● = insulin, X = BSS.

The rates achieved with insulin appear to be indistinguishable from those observed with serum.

Inhibition of RNA synthesis by 0.1 μg/ml of actinomycin D had no significant adverse effect on the enhanced rates of protein synthesis achieved by insulin or serum addition to starved cultures (Table III). RNA synthesis in the cultures proved very sensitive to actinomycin, 0.1 μg/ml effecting inhibition of 95%. Higher levels of actinomycin not only proved more effective inhibitors of uridine incorporation, but blocked the stimulation of protein synthesis by insulin or serum.

Effect of Insulin or Serum Addition upon Uridine Incorporation into RNA

The rate of RNA synthesis measured by 1-h pulses of [³H]uridine (0.3iμCi/ml) also increased with time after insulin or serum addition (Fig. 3). The kinetics of the change in rate are very similar to those noted for protein synthesis except that serum stimulation was always greater than that observed with insulin. Such stimulation of RNA synthesis was effected by as little as 10^{-5} U

Table III

Effect of Actinomycin D on Protein and RNA Synthesis

Actinomycin D (μg/ml)	Uridine				Amino acids			
	0	0.1	1.0	5.0	0	0.1	1.0	5.0
	cpm/mg cell protein							
Control	3,300	178	48	35	6,240	5,510	5,147	4,772
Insulin	11,760	374	50	32	14,472	12,800	6,405	6,285
Serum	13,236	420	72	38	15,624	13,840	4,206	4,357

Confluent monolayers of chick fibroblasts previously incubated in serum-free medium for 24 h were exposed to insulin (10^{-2} U/ml), serum (3%), or BSS, along with varying concentrations of actinomycin D for a total of 5 h. During the last hour, cells were doubly labeled with 0.3 μCi/ml of [^{3}H]amino acids and 0.08 μCi/ml of [^{14}C]uridine. Cells were harvested, and TCA-insoluble counts reported as cpm/mg cell protein. Each value is the average of determinations on two monolayer populations.
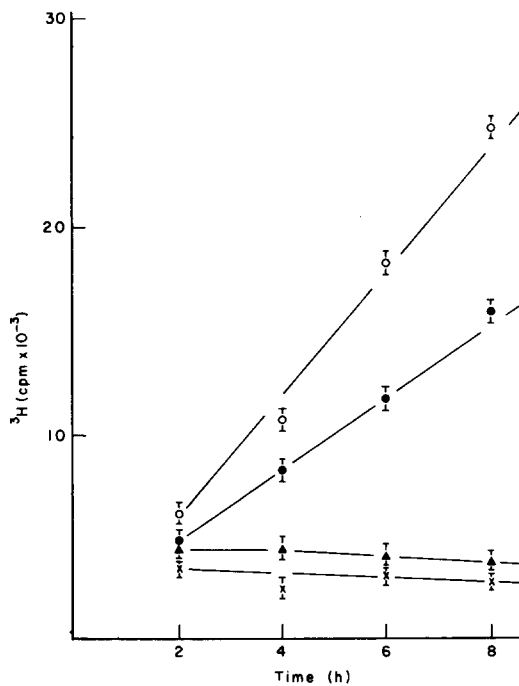

FIGURE 3 Stimulation of RNA synthesis (uridine incorporation) by serum and insulin. Confluent monolayers of chick fibroblasts deprived of serum for 24 h were treated with 10^{-2} U/ml insulin, 3% serum, or BSS for 2, 4, 6, and 8 h. [^{3}H]uridine (0.3 μCi/ml) was added to cells for 1 h prior to harvesting. In specific cultures, cycloheximide (5 μg/ml) and insulin were added at the same time, and representative data are presented. O = serum, ● = insulin, ▲ = insulin and cycloheximide, X = control.

(4.0 ng)/ml of beef insulin (unpublished observations). Newly stimulated RNA synthesis was as sensitive to actinomycin D as was the control

synthesis (unpublished results). Little increase in RNA synthesis was observed if protein synthesis was inhibited by cycloheximide (5–100 μg/ml) 1 h prior to addition of insulin or serum.

Nucleotide Pool Equilibration

The possibility that the rise in RNA synthesis might be explained by increased transport of uridine and expansion of the uridylate pool was investigated. The UMP pool was permitted to equilibrate with $^{32}PO_4^{-3}$ for 12 h prior to insulin or serum addition. [^{14}C]uridine was supplied 4 h after the latter additions. At a time when the rate of specific incorporation of [^{14}C]uridine into RNA had increased threefold over control levels, as determined by TCA-insoluble counts, the cellular acid-soluble UMP was isolated as described in Materials and Methods. The equilibration of added [^{14}C]uridine with the existent uridine phosphate pools was evident from the ratio of ^{14}C/^{32}P (Table IVA). The data indicate that equilibration of [^{14}C]uridine with the nucleotide pool was relatively unchanged at a time of threefold difference in apparent rate of RNA synthesis in stimulated and control cells. Indeed, no significant difference was observed in insulin-treated cultures, while the ratio of ^{14}C/^{32}P is approximately 20% higher in serum-treated cells. Apparently nucleotide pool expansion could not account for the increased RNA synthesis observed. To another series of cultures, actinomycin D (5 μg/ml) was added 1 h prior to stimulation so that the effect of insulin and serum on nucleotide pools could be further examined when utilization of formed uridylate was inhibited. Inhibition by actinomycin D (Table IVB) prevented the formation of uri-

dylate. Such an effect was reported earlier by
Skehel et al. (18) in chick embryo fibroblasts.

RNA Polymerase Activity

Since the increased RNA synthesis in insulin and
serum-treated cultures could not be explained by
nucleotide pool expansion, the level of RNA
polymerase activity was examined. Nuclei pre-
pared from insulin- or serum-treated cultures were
assayed for polymerase activity as detailed in
Materials and Methods. No significant difference
in polymerase activity was observed after 2 h of
exposure to insulin or serum (Table V). However,
by the 6th h, when incorporation of labeled
uridine into RNA had increased four- to fivefold
in stimulated cultures, a similar rise in polymerase
activity could be demonstrated. The presence of
actinomycin D or DNase during the assay abol-
ished RNA polymerase activity. Conditions of high
and low ionic strength only slightly altered
enzymatic activity although previous reports
indicated direct influence of salt concentration on
the activity of RNA polymerase from other sources
(19, 20).

TABLE IV

Nucleotide Pool Equilibration

	Control	Insulin	Serum
	cpm/mg cell protein		
A.			
UMP			
^{14}C	1,045	1,110	1,450
^{32}P	1,519	1,610	1,629
Ratio			
^{14}C/^{32}P	0.69	0.69	0.85
B. Actinomycin D			
UMP			
^{14}C	171	70	379
^{32}P	966	1,002	1,440
Ratio			
^{14}C/^{32}P	0.18	0.07	0.26

The endogenous UMP pool was equilibrated with
^{32}PO$_4{}^{-3}$ (20 μCi/ml) for 12 h. Insulin (10^{-2} U/ml),
serum (3%), or BSS were added to cultures for 4 h
followed by a 1-h pulse with [^{14}C]uridine (2.0
μCi/ml). Selected cultures received actinomycin D
(5 μg/ml) 1 h prior to addition of insulin or serum.
TCA-soluble pools were analyzed as described in
Methods and Materials.

TABLE V

*Effect of Serum and Insulin on RNA Polymerase
Activity*

	[^{3}H] UTP Incorporated			
	$-$(NH$_4$)$_2$SO$_4$		$+$(NH$_4$)$_2$SO$_4$	
	2 h	6 h	2 h	6 h
	pmol/100 μg nuclear protein			
Control	32.2	45.2	37.1	45.2
Insulin	35.2	170.1	37.9	159.5
Serum	38.0	179.5	32.8	136.1
Control (zero time)				
Insulin		8.8		6.2
Serum		7.3		5.5
DNase-treated nuclei				
Insulin		6.5		6.8
Serum		11.7		4.4
Actinomycin D-treated nuclei				
Insulin		3.1		ND*
Serum		7.0		ND

Nuclei were isolated from chick fibroblasts previ-
ously exposed to insulin, serum, or BSS for 2 and
6 h (see Methods and Materials). Enzyme assay
was permitted to proceed for 10 min at 30°C. In
specific cases, nuclei were incubated with actino-
mycin D (5 μg), or DNase at 30°C for 10 min prior
to addition of reaction mixture. The zero time
control represents [^{3}H]UTP incorporation in reac-
tion mixture to whih 5% TCA was added at 0°C.
*ND, not determined.

Gel Analysis of Classes of RNA Stimulated by Insulin

Cultures incubated with insulin for various
time intervals were labeled for 1 h with either
[^{3}H]uridine, [^{14}C]uridine, or ^{32}PO$_4{}^{-3}$. After phenol
extraction of RNA from whole cells, 10 μg of
RNA were layered on individual acrylamide-
bisacrylamide gels (see Materials and Methods)
and species of RNA were separated by electro-
phoresis. A typical profile of RNA labeled with
[^{3}H]uridine and electrophoresed on 2.6% gels
appears in Fig. 4. The rate of synthesis of pre-
ribosomal and ribosomal species (45S, 32S, 28S,
and 18S) continued to increase during the 9-h
exposure to insulin, and cleavage of 45S to smaller
ribosomal RNA species apparently proceeded
normally (12). Also, the synthesis of small molecu-
lar weight RNA including the 4–5S species was
stimulated. Throughout the period of insulin
treatment, control cultures synthesized RNA at a
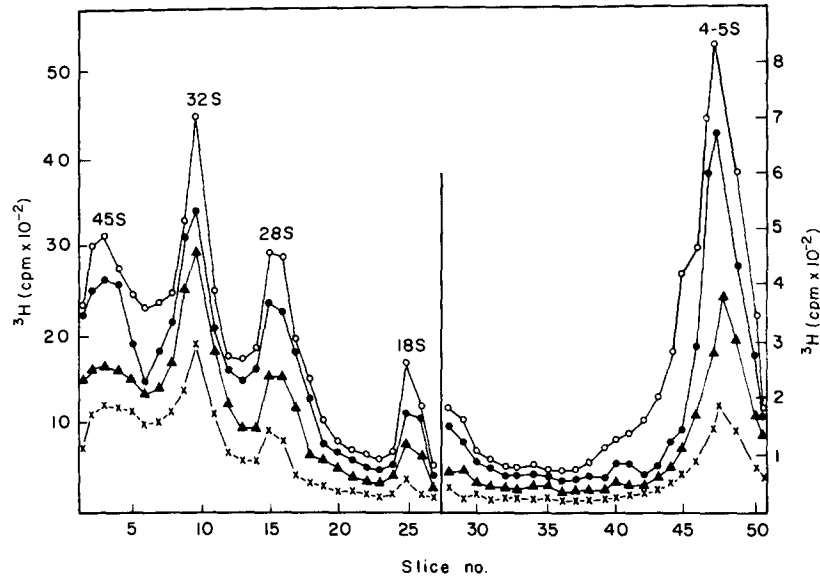constant but significantly lower rate.

FIGURE 4 Insulin stimulation of RNA synthesis. Monolayers of chick fibroblasts incubated in serum-free medium were exposed to insulin $(10^{-2}$ U/ml) for 3, 6, and 9 h. During the last hour of the incubation, [^{3}H]uridine (100 μCi) was added. RNA was extracted by the cold phenol method as described in Materials and Methods. 10 μg RNA were layered on 2.6% acrylamide gels and electrophoresis carried out for 2 h. $\bigcirc$ = 9 h, $\bullet$ = 6 h, $\blacktriangle$ = 3 h, $\times$ = control.

Since 4S and 5S RNA cannot be properly separated on 2.6% gels, electrophoresis was accomplished on 7.5% acrylamide gels (Fig. 5). From the latter gels it appears that the synthesis of both 4S and 5S RNA was clearly stimulated by insulin. Apparently 5S RNA synthesis reached its maximal rate after a 4–5 h exposure to insulin, while the rate of synthesis of 4S and of larger ribosomal species continued to increase for a somewhat longer period. Indeed, the final stimulation of 5S RNA was approximately threefold while that of 4S RNA was five- to sixfold. The considerable broadening of the 4S region might indicate the increased synthesis and accumulation of pre-tRNA (21). The kinetics of RNA synthesis in serum-treated cultures are similar to those resulting from insulin stimulation.

Polyribosomal RNA

In order to look more closely at the small RNA species associated with polyribosomes, the cytoplasmic fraction from chick fibroblasts previously incubated with insulin or serum for 6 h was layered on 5–20% sucrose gradients. The polyribosomes were collected and RNA isolated as described in

Materials and Methods. The electrophoretic profile of polyribosomal RNA on 12% gels appears in Fig. 6. It is evident that substantial amounts of polyribosome-associated RNA are synthesized in response to insulin or serum. Throughout the gel, the specific activity of RNA extracted from insulin- and serum-treated cultures is considerably higher than that of controls, and several distinct peaks are indicated. Association of newly synthesized 4S and 5SRNA with polyribosomes is clear. Electrophoresis of RNA on 2.6% gels demonstrated the presence of 28S and 18S species with the total radioactive counts of each being approximately 2.0 to 1, respectively (Fig. 7).

DISCUSSION

The sequence of events that occurs following the addition of fresh serum to confluent monolayers of "density sensitive" cells is well documented (5, 22–24). It is not surprising that insulin alone added to cells conditioned by 24 h in protein-free medium fails to effect the same sequence. Admittedly ultimate DNA synthesis and cell multiplication have been observed after addition of
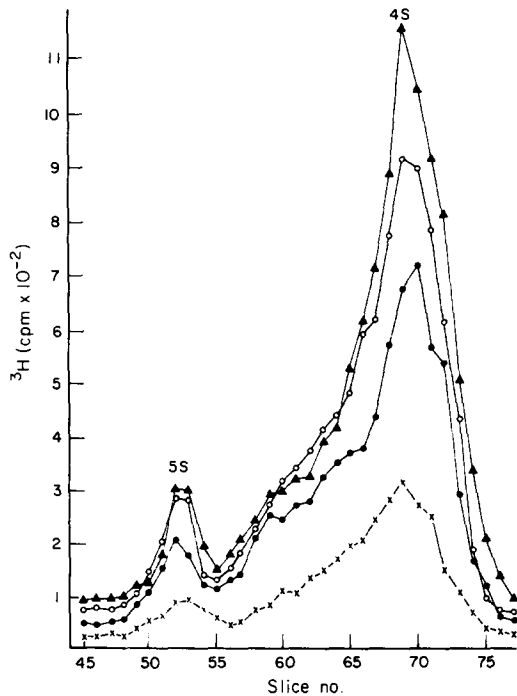
FIGURE 5 Insulin stimulation of 4S and 5S RNA. Cells were exposed to insulin for 3, 5, and 9 h and labeled as described for Fig. 3. Electrophoresis of RNA was performed on 7.5% acrylamide gels for 7 h. ▲ = 9 h, ○ = 5 h, ● = 3 h, × = control.

insulin as the sole new macromolecule. Most of the experiments have been conducted with organ cultures (25, 20, 5), or under conditions in which residual serum proteins in the cultures (24) may have contributed to the results obtained. More recent work by Turkington and Ward (20) assigns to insulin a less complete effect and the work of Holley and his collaborators (26) confirms the inability of insulin alone to initiate DNA synthesis. The serum growth factor or another serum macromolecule is required by 3T3 cells for the initiation of DNA synthesis.

Of interest in this series of experiments is the stimulation of RNA synthesis by insulin principally through increased RNA polymerase activity which is progressive for at least 8 h and requires protein synthesis. Indeed a more careful examination of RNA accumulation during 1-h pulses of [^{3}H]uridine or ^{32}PO$_4^{-3}$ (unpublished data) revealed that all classes of RNA are not equally stimulated or protected by insulin and serum. 5S RNA, for example, reached its maximal rate of synthesis several hours earlier than other ribosomal species and 4S RNA. Also 4S RNA synthesis is ultimately stimulated to a higher degree than 5S, a reflection perhaps of different polymerases for the two species or distinct nuclease activities. Knight and Darnell (27) have suggested separate
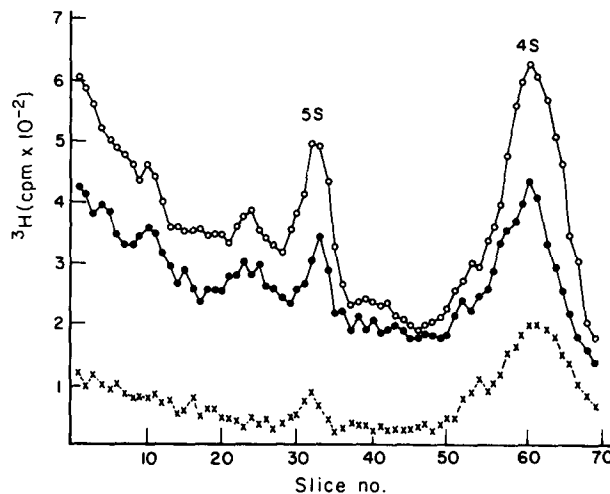

FIGURE 6 Insulin and serum stimulation of polyribosomal RNA. Chick fibroblasts were incubated with insulin (10^{-2} U/ml) or serum (3%) for 7 h. During the last 2 h, 100 μCi each of [^{3}H]guanosine, [^{3}H]-adenosine, [^{3}H]cytidine, and [^{3}H]uridine was added. Polyribosomes were isolated and RNA extracted as described in Materials and Methods. Electrophoresis was carried out for 12 h on 12.5% acrylamide gels. ○ = serum, ● = insulin, × = control.
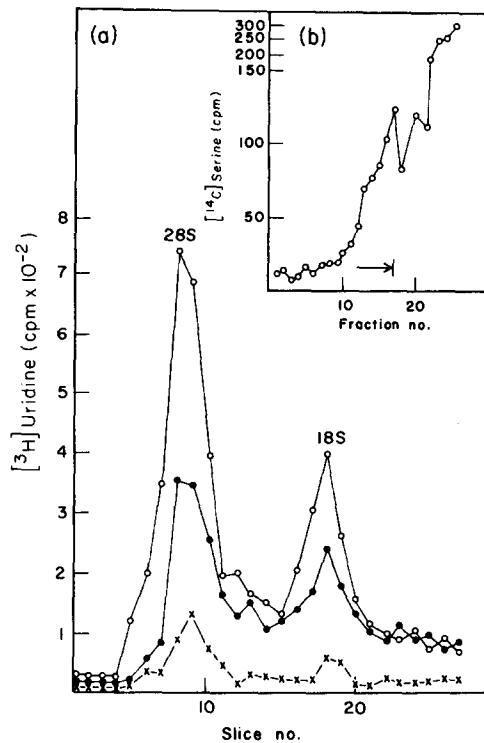
FIGURE 7 Insulin stimulation of 28S and 18S poly-ribosomal RNA. Chick fibroblasts were treated as described for Fig. 5, and RNA examined on 2.6% gels. A representative polyribosome profile also appears in the figure as an insert. Samples to the left of the indicator mark on the insert profile were considered polyribosomes and were pooled prior to extraction as described in Materials and Methods. O = serum, ● = insulin, X = control.

mechanisms for the synthesis of 5S and 4S RNA on the basis of comparative labeling patterns.

It is difficult to assign a primary or secondary role to insulin either for the increased polymerase activity or for the more subtle changes in RNA observed. Given the well-documented role of insulin in repairing a lesion in protein synthesis (28, 17), all other effects could be secondary, i.e., the result of the synthesis of particular proteins by the restored protein synthetic system. In addition, we must not overlook the possibility of a contribution by residual serum proteins associated with the cells despite attempts to remove them by washing. They may well remain sequestered between cells even after a 24-h incubation in serum-free medium.

It is generally agreed that proteins interact with nascent RNA molecules in the nucleus to form ribonucleoprotein particles which govern the processing of ribosomal precursor RNA to mature

ribosomes (29, 30) as well as the delivery of mRNA to the cytoplasm (31, 32, 3) in the transport of RNA from the site of synthesis to the cytoplasm of the cell (33, 34). Of potential serum macromolecules, the putative "RNA migration factor" reported in Cohn fraction III of bovine serum (35) is of particular interest. This factor promotes the transport of RNA from the nucleus of the primary cell maintained in serum-free medium. A similar factor could account for the accumulation of a particular species of RNA by protecting it against degradation by nucleases. The disappearance of other RNA species could be ascribed to serum macromolecules that interfere with the synthesis or accelerate the degradation of particular molecules at or near the site of synthesis.

The possibility remains that specific transcripts are affected by insulin in a manner that does not rest solely upon the observed increase in activity of one or more of the RNA polymerases of vertebrate cells (36). The mRNA fraction is being examined.

The finding that the stimulation of protein synthesis observed after insulin or serum addition is blocked by levels of actinomycin D of 1 μg/ml and above suggests that concomitant RNA synthesis is required for the effect. Concentrations of actinomycin D (0.1 μg/ml) that prevent ribosomal RNA synthesis with little influence on other species of RNA (37) have no adverse effect on the increased rate of protein synthesis. This result is contradictory to that reported by Eboué-Bonis et al. (38), Wool and Moyer (39), and to that observed in nonconfluent chick fibroblasts in earlier studies from this laboratory (17, 40). This difference is one additional instance of dissimilarity between confluent and nonconfluent cultures uncovered in the course of these studies and is undergoing further investigation.

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