Studies on Template-Active Ribonucleic Acid from Nuclei of Insect Pupae*

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ABSTRACT: Messenger ribonucleic acid which can serve as a template for amino acid incorporation has been isolated from insect pupal nuclei by phenol extraction at 65°. Using an *Escherichia coli* S-30 protein-synthesizing system, this message can stimulate [^{14}C]leucine incorporation into polypeptide by about ninefold. The rate of incorporation is constant for 10 min then decreases. Maximal stimulation is reached after 30 min. Incorporation is linearly proportional to the amount of messenger ribonucleic acid up to 200 μg/ml. Omissions of adenosine triphosphate, guanosine triphosphate, and phosphoenolpyruvate or Mg^{2+} prevent [^{14}C]leucine incorporation. Additions of ribonuclease or puromycin abolish incorporation. The incorporation of all nine amino acids tested is stimulated, and the product differs in its relative amounts of amino acids from that of the endogenous *Escherichia coli* messenger ribonucleic acid in the control. It is rich especially in tyrosine, leucine, and valine and is susceptible to proteolysis. Sucrose density gradient analysis of the ribonucleic acid shows four distinct optical density peaks at 6–8, 12, 20, and 40 S. After 1-hr labeling by radioactive uridine the label is dispersed throughout the gradient. Base composition analysis of the messenger ribonucleic acid showed a significant difference from that of cytoplasmic ribonucleic acid. It is similar to that of deoxyribonucleic acid from higher organisms. Neomycin is able to increase the template activity of this messenger ribonucleic acid for the incorporation of many amino acids tested. The effective range of the antibiotic is in the range of 0.1–1 μg/0.25 ml of incubation mixture.

MRNA is usually characterized as a rapidly labeled RNA fraction, by its ability to anneal to DNA or by its template activity, that is, its ability to stimulate synthesis of specific proteins on heterologous ribosomes.

The latter has been made possible by the development of a cell-free extract from *Escherichia coli* (Nirenberg and Matthaei, 1961), which responds to both homologous and heterologous RNA. In some cases the product of the stimulation has been analyzed and shown to be a protein uniquely determined by the RNA. Thus, it has been shown that the synthesis of coat protein can be mediated by f2 RNA, when added to *E. coli* extract (Nathans *et al.*, 1962), or to extract of *Euglena gracilis* (Schwartz *et al.*, 1965). Also, a plant viral RNA when added to *E. coli* extract can direct the synthesis of its viral coat protein (Clark *et al.*, 1965).

Template-active RNA has been isolated from many tissues and organisms. An RNA fraction from the posterior silk glands of the silkworm was isolated, which could stimulate amino acid incorporation into *E. coli* extract in proportions similar to their content in the silk protein sericin (Szafranski *et al.*, 1963). Similar results were reported by Tanaka and Shimura (1965) who showed that two species of RNA fractions from silk glands stimulated the incorporation of amino acids in proportion to their relative contents in silk fibroins. Recently, Howells and Wyatt (1969) described the isolation of an RNA fraction from wing epidermis of the *Cecropia* silk moth. This RNA stimulates amino acid incorporation in a ribosomal system prepared from *E. coli*. However, the results suggest that at least part of the stimulation obtained with the *Cecropia* RNA may be due to mechanisms other than true template activity.

The common way of detection of mRNA, pulse labeling of the cells with a precursor of RNA, is based on the assumption that this material possesses a high rate of turnover (Jacob and Monod, 1961). In multicellular organisms, however, this may not be the case. Mammalian reticulocytes, while synthesizing hemoglobin, lack DNA and are incapable of RNA synthesis (Marks *et al.*, 1962). In sea urchin eggs, fertilization in the presence of actinomycin D initiates protein synthesis in the absence of RNA synthesis. Development is normal up to the blastula stage (Gross and Cousineau, 1963). In the cellular slime mold *Dictyostelium discoideum* mRNA for the enzyme UDP-galactose polysaccharide transferase is very stable (Sussman, 1966). Induction of glutamine synthetase by hydrocortisone in chick embryo retina cell cultures leads to the accumulation of stable RNA that is essential for the increase of the enzyme (Moscona *et al.*, 1968). Thus template-active RNA in such systems must be metabolically stable, and may not be detectable by the pulse-labeling technique.

In insects a stable mRNA was reported for a blood protein of a silk moth (Berry *et al.*, 1964), for adult cuticular protein in pupae of *Tenebrio molitor* (Ilan *et al.*, 1966), and for the cocoonase protein in the differentiated zymogen cell of a silk moth (Kafatos and Reich, 1968).

The pupa of the yellow mealworm *Tenebrio molitor* represents a system which undergoes rapid differentiation. Experiments with actinomycin D *in vivo* suggested that at least part of the mRNA for adult cuticular protein synthesis is present on the first day of pupation and translated 5–7 (last) days later (Ilan and Quastel, 1966). The proportion of amino acids incorporated on microsomes isolated from the last day of pupation is similar to that of adult cuticular protein (Ilan and Lipmann, 1966), but differs with microsomes of the first-

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day pupa. However, recent studies in this laboratory (Ilan, 1968) with ribosomal preparations from the first day of pupation have shown that they contain the message for cuticular protein which can be translated when supplemented with tRNA and enzyme from the seventh-day pupae. The message is synthesized during pupation and accumulates on the ribosomes (Ilan, 1969). It was, therefore, interesting to isolate and characterize the mRNA fraction from nuclei of *Tenebrio* pupae.

The present paper describes a study on mRNA from nuclei of first-day *Tenebrio* pupae and its characteristics as template for amino acid incorporation into protein using *E. coli* extracts. Analysis of the data suggests that the nuclear RNA directs the synthesis of a novel product.

Material and Methods

A. Source of Insects and Chemicals. T. molitor larvae were obtained from a local biological supply house and maintained as previously described (Ilan *et al.*, 1966). Only first-day pupae were used. Uniformly labeled [^{14}C]amino acids with specific activities of about 200 mCi/mmole and [2-^{14}C]-uridine (30 mCi/mmole) were obtained from New England Nuclear Corp., Boston, Mass. RNase, thrice crystallized, and DNase, electrophoretically purified, were products of Worthington Biochemical Corp., N. J. Puromycin and chloramphenicol were obtained from Nutritional Biochemical Corp., Cleveland, Ohio. Neomycin was purchased from Mann Research Laboratories, New York, N. Y. Log-phase *E. coli* strain B paste was purchased from Grain Processing Co., Muscatine, Iowa. ATP, GTP, phosphoenolpyruvate, and pyruvate kinase (EC 2.7.1.40) were obtained from Sigma Chemical Co., St. Louis, Mo.

B. Isolation of Nuclear Ribonucleic Acid. For a typical preparation 100 g of first-day pupae was suspended in 250 ml of solution containing 10 mM $MgCl_2$, 25 mM KCl, 35 mM Tris-HCl (pH 7.6), 250 mM sucrose, 4 mM mercaptoethanol, 1 mM phenythiourea to inhibit phenol oxidase, and 4 μg/ml of polyvinyl sulfate. Homogenization of suspension was carried out for 40 sec at 4° in a Sorval Omni-Mixer at speed control 5. The homogenate was filtered through four layers of cheesecloth and centrifuged at 800*g* for 30 min at 4°. The pellet was resuspended in 0.140 M NaCl containing 4 mM $MgCl_2$, 2.2 M sucrose, and 4 μg/ml of polyvinyl sulfate and centrifuged for for 1 hr at 30,000*g*. The nuclear pellet consisted of a microscopically clean preparation of nuclei. It was resuspended in 10 ml of solution containing 0.14 M NaCl, 20 mM potassium acetate (pH 5), 1% sodium dodecyl sulfate, and 4 μg/ml of polyvinyl sulfate. An equal volume of freshly distilled water-saturated phenol was added, and the mixture was shaken vigorously for 1 hr at 45°, cooled, and centrifuged at 10,000*g* for 10 min. The water phase was aspirated and used to prepare nuclear ("ribosomal like") 45° RNA (Hadjiolov, 1966). The material accumulated in the interphase between the phenol and the aqueous layer was extracted once more with phenol for 5 min at 45° and after centrifugation the aqueous layer was discarded and the material in the interphase was suspended in 10 ml of NaCl–sodium dodecyl sulfate solution, and an equal volume of phenol was added. After shaking for 1 hr at 65°, the mixture was cooled and centrifuged at 10,000*g* for 10 min. The aqueous layer was used to prepare nuclear RNA. To the aqueous phase 0.1 volume of 20% (w/v) potassium acetate (pH 5) and 2 volumes of absolute ethanol were added. The mixture was allowed to stand overnight at −20° after which the RNA was collected by centrifugation, washed several times with ethanol, once with ether, dried in a stream of air, and dissolved in deionized water. The average yield of RNA was 5 mg/100g of pupae. The quantity of RNA was estimated by its absorbance at 260 mμ (24 absorbance units = 1 mg of RNA).

C. Isolation of rRNA. Ribosomes were prepared as already described (Ilan, 1968). They were suspended in NaCl–sodium dodecyl sulfate solution and extracted with an equal volume of phenol at 4° or at 65°. rRNA was prepared from the aqueous phase as described above for nuclear RNA. Ribosomal subunits were prepared from ribosomes according to Tashiro and Siekewitz (1965), and RNA was extracted from each subunit as described above. The values of ribosomal subunits and that of RNA particles were obtained from linear sucrose gradients by application of the formula of Martin and Ames (1961), using monomeric 80S rat liver ribosomes as reference.

D. Zone Centrifugation. The RNA solution (0.5 ml) was layered over 4.5 ml of 10–40% (w/v) linear gradient, supplemented with 10 mM sodium acetate (pH 5.1), 0.1 M NaCl, and 1 mM EDTA (Scherrer and Darnell, 1962). The tubes were then centrifuged at 40,000 rpm in a Spinco SW65 swinging-bucket rotor for 4 hr at 4°. After puncturing the bottom of the tubes, four drop fractions were collected and diluted with water to 1 ml for absorbency measurement at 260 mμ. The RNA was precipitated with 5% cold trichloroacetic acid after addition of 50 μg of bovine serum albumin to each sample, washed three times with 5 ml of cold trichloroacetic acid on Millipore filters, and counted as described before (Ilan, 1968).

E. Assay for Template Activity. The RNA samples were incubated with the S-30 fraction of *E. coli* prepared according to the procedure of Nirenberg (1963). The reaction mixture, however, was modified by substituting NH_4Cl for KCl (Conway, 1964) and increasing the level of guanosine triphosphate. The reaction mixture (0.25 ml) contained per milliliter: 160 μmoles of NH_4Cl, 12 μmoles of $Mg(CH_3COO)_2$, 6 μmoles of mercaptoethanol, 35 μmoles of Tris-HCl (pH 7.6), 0.1 μmole of GTP, 1 μmole of ATP, 5 μmoles of sodium salt of phosphoenolpyruvate, 20 μg of pyruvate kinase, 4 mg of S-30 protein, 10 μmoles of each of 19 amino acids, 0.8 μCi of [^{14}C]amino acid, and RNA fraction as indicated for each table and figure. Incubations were carried out for 30 min at 30°. The reaction was terminated by the addition of 5 ml of trichloroacetic acid. Determination of incorporation of amino acids into protein was carried out as previously described (Ilan, 1968).

F. Base Composition. The RNA was hydrolyzed in 0.3 N KOH for 45 min at 80° or at 37° overnight. The solution was neutralized with 6 N perchloric acid at 4° and the $KClO_4$ precipitated was removed by centrifugation. Base composition was determined by optical density of the 2′,3′-nucleotides after separation of the nucleotides by paper electrophoresis at pH 3.5 (Markham and Smith, 1952).

Results

A. Template Activity of Insect Pupal Nuclear RNA. The capacity of different RNA fractions to stimulate [^{14}C]leucine incorporation into protein is given in Table I. Thus, 200 μg

TABLE I: Stimulation of Incorporation of [^{14}C]Leucine into Protein in *E. coli* S-30 by RNA Fractions from Insect Pupae.[a]

RNA Fraction Added (200 μg/ml)	[^{14}C]Leucine Incorp (μμmoles/ml of S-30)	
	Expt A	Expt B
None	7.15	7.15
Cytoplasmic		
18S RNA	8.57	8.62
28S RNA	7.85	7.91
rRNA	8.01	8.07
Nuclear		
45° extraction RNA	10.68	10.70
65° extraction RNA	64.34	

[a] The constitution of the assay system is presented in Material and Methods. 28S RNA and 18S RNA were extracted from 60S and 40S ribosomal subunits, respectively. rRNA was extracted from whole ribosomes. In expt A cytoplasmic RNA's were extracted at 4°. In expt B cytoplasmic RNAs were extracted at 65° and nuclear 45°-extracted RNA was resuspended in 10 ml of 0.14 M NaCl containing 1% sodium dodecyl sulfate and 4 μg/ml of polyvinyl sulfate and reextracted with phenol at 65°.

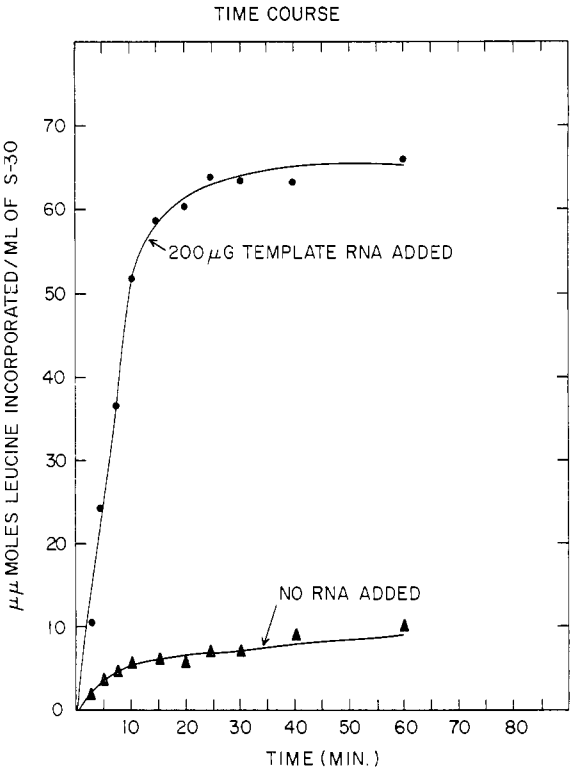

FIGURE 1: Kinetics of [^{14}C]leucine incorporation in the presence or absence of template-active RNA from insect pupae. Reaction conditions as described in Material and Methods except for the incubation time as indicated. (▲—▲) Endogenous activity in the absence of any messenger; (●–●–●) template-active RNA (200 μg/ml) as messenger.

of 18S RNA extracted from ribosomal subunits stimulates incorporation of leucine into protein in *E. coli* extract by about 20%. The 28S RNA stimulates the cell-free system by only 10%. This observation is in agreement with reported stimulation of 18S and 28S RNAs from rat liver (DiGirolamo *et al.*, 1964; Brawerman *et al.*, 1965; Hadjiolov, 1966). The nuclear RNA is much more active in stimulating leucine incorporation. RNA extracted at 45° has 50% stimulatory activity whereas RNA extracted at 65° stimulates the *E. coli* system by about ninefold. It was reported (Holland, *et al.*, 1966) that an *in vitro* protein-synthesizing system from *E. coli* is capable of using ribosomal RNA as a template for amino acid incorporation after heating the ribosomal RNA for 20 min at 100° in the presence of EDTA. We, therefore, investigated the effect of heating. When pupal cytoplasmic RNAs were extracted with phenol–sodium dodecyl sulfate at 65° or when 45° nuclear RNA was reextracted at 65°, almost no additional stimulation was observed (Table I, expt B).

Since RNA extracted from nuclei at 65° had the higher template activity, we decided to investigate this fraction and termed it template-active RNA.

The kinetics of the incorporation of labeled leucine into polypeptide in the presence of pupal template-active RNA are recorded in Figure 1. The rate of incorporation is constant for 10 min and then decreases. Maximal stimulation is observed after 30-min incubation. Figure 2 shows that [^{14}C]leucine incorporation is linearly proportional to the amount of added pupal template-active RNA up to 200 μg/ml. Above this concentration there is a decrease in template activity possibly due to an increased requirement for magnesium.

B. Energy Requirements and Susceptibility to Inhibitors. Omission of ATP, GTP, and phosphoenolpyruvate prevents [^{14}C]leucine incorporation almost completely (Table II). RNase and puromycin abolish incorporation, as previously shown for cases of stimulation by virus RNA and synthetic polyribonucleotides (Nirenberg and Matthaei, 1961) and for endogenous messenger activity on insect ribosomes (Ilan,

TABLE II: Effect of Inhibitors Or Omission of Energy Source on Amino Acid Incorporation Stimulated by Insect Pupal Template-Active RNA.[a]

Additions or Omissions	μμmoles of [^{14}C]Leucine Incorpd/ml of S-30
None	62.56
ATP, GTP, phosphoenolpyruvate omitted	0.51
25 μg/ml of ribonuclease added	0.05
0.5 mM puromycin added	0.12
Mg^{2+} omitted	0.55
Template-active RNA omitted	6.93
None (0° control)	0.16

[a] Reaction conditions are as described in Material and Methods. Reaction mixtures also contain 200 μg/ml of insect nuclear template-active RNA.

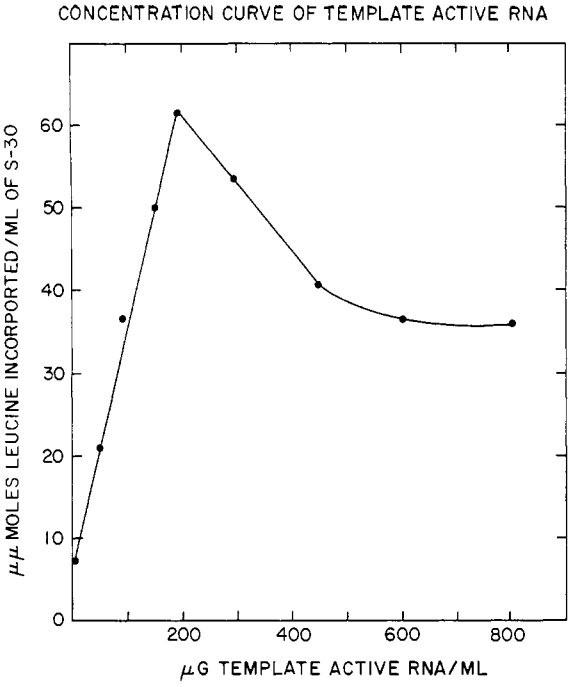

FIGURE 2: Effect of varying amounts of pupal template-active RNA on [^{14}C]leucine incorporation. Reaction conditions as described in Material and Methods.

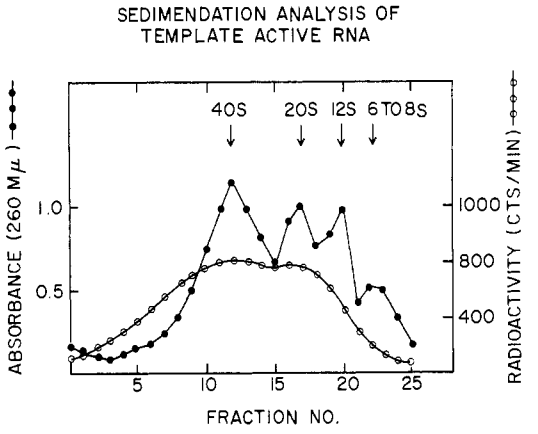

FIGURE 3: Sedimentation analysis of template-active RNA from insect nuclei. Fifty-first-day pupae (average wt 100 mg/pupa) were injected each with 1 μCi of [^{14}C]uridine. After 1 hr at 28° they were homogenized together with 100 g of first-day pupae. Template-active nuclear RNA was prepared as described under Methods. Twenty A_{260} units of RNA was applied on 10–40% (w/v) linear sucrose gradient as described under Methods.

1968). There is also an absolute requirement for Mg^{2+}. The reaction is temperature dependent.

C. Specificity of Amino Acid Incorporation by Insect Pupal Template-Active RNA. In order to obtain some information on the nature of the product synthesized as a result of the stimulation by the template-active RNA the extent of incorporation of several amino acids was examined in comparison to the endogenous incorporation. The results are depicted in Table III. Thus, the incorporation of all amino acids tested is stimulated in the presence of insect template-active RNA from pupal nuclei. The product of the stimulation has a different relative amount of amino acids from the endogenous product and is especially rich in isoleucine, leucine, tyrosine, and valine.

The amino acid labeled product, the formation of which is directed by insect pupal template-active RNA, is susceptible

TABLE III: Stimulation of Incorporation of Different [^{14}C]-Amino Acids by Template-Active RNA from Insect Pupal Nuclei.[a]

[^{14}C]Amino Acid	Incorporation (μμmoles/ml of S-30) Endogenous	+Template RNA	% of Endogenous
Alanine	5.10	11.40	223
Arginine	1.95	7.32	376
Isoleucine	0.56	10.20	1820
Leucine	6.95	64.34	925
Lysine	1.50	4.60	303
Proline	3.30	8.70	263
Threonine	4.12	13.40	326
Tyrosine	1.84	41.26	2240
Valine	1.25	10.36	830

[a] Preincubated *E. coli* S-30 is incubated with [^{14}C]amino acid (0.8 μCi/ml) together with 10 mμmoles of each of 19 [^{12}C]amino acids, in separate tubes, with or without 200 μg/ml of template-active RNA. Reaction conditions are as described in Material and Methods.

TABLE IV: Sensitivity of the Product of Template-Active RNA to Proteolytic Enzymes.[a]

Addition	Acid-Insoluble [^{14}C]-Leucine (μμmoles/ml of S-30)
None	63.26
Trypsin (1 mg/ml)	4.14
Pronase (1 mg/ml)	3.28

[a] Preincubated *E. coli* S-30 incubated with [^{14}C]leucine and 200 μg/ml of template-active RNA. The reaction conditions were as described in Methods. After incubation the reaction mixture was cooled to 4° and passed through a column of Sephadex G-25 in buffer containing 10 mM magnesium acetate, 0.16 M NH_4Cl, 35 mM Tris-HCl (pH 7.6), and 4 mM mercaptoethanol. The front peak in the void volume containing the labeled product was divided into three aliquots of 1 ml, each of which was incubated for 45 min at 37° alone, or with 1 mg of either enzyme. Acid-insoluble material was precipitated with 5 ml of trichloroacetic acid, the mixture was heated for 20 min at 90°, washed five times with 5 ml of 5% trichloroacetic acid on Millipore filters, dried, and counted.

TABLE V: Base Composition of Nuclear Template-Active RNA and Cytoplasmic RNA.

	Base Composition (moles/100 moles)				
	C	A	G	U	(G+C)/(A+U)
Cytoplasmic RNA	31.7	17.0	31.8	19.5	1.74
Nuclear template-active RNA	21.2	27.3	24.7	26.8	0.89

to digestion by proteolytic enzymes. Both crystalline trypsin and pronase considerably reduced the labeled [^{14}C]leucine precipitated by hot trichloroacetic acid (Table IV).

D. Sedimentation Analysis of Template-Active RNA from Insect Nuclei. Sedimentation analysis on sucrose density gradient of template-active RNA is presented in Figure 3. There are four peaks of ultraviolet absorption at 6–8, 12, 20, and 40 S. Following injections of [2-^{14}C]uridine the distribution of radioactivity was recorded. After an *in vivo* pulse of 1 hr (Figure 3), the label is dispersed throughout the gradient with sedimentation coefficients mainly in the 10–50S range. The lack of concentration of isotope in the 6–12S region is evidence for the absence of degradation in the preparation and the absence of distinct labeled peaks at 45S, 28S, and 18S regions indicates that little of the labeled RNA is newly synthesized RNA.

RNA under each peak was tested for template activity. There was no difference in the stimulation of [^{14}C]leucine incorporation per A_{260} units between the various peaks. However, leucine is an evenly distributed amino acid in proteins. In order to determine whether each peak represents a particular mRNA, a wide range of amino acids should be tested for each peak. This was not possible as the amount of RNA was limited.

E. Base Compositions. The base composition of nuclear template-active RNA and that of cytoplasmic RNA is recorded in Table V. In higher animals typical base composition of rRNA is (G + C)/(A + U) = 1.6 (Georgiev, 1967). The cytoplasmic RNA approaches this value (1.74). This is due to the fact that in *Tenebrio* pupae most of the RNA in the cytoplasm is ribosomal and only a very small amount is tRNA (Ilan, 1968). The ratio (G + C):(A + T) in DNA of higher animals is 0.7–0.8 (Georgiev, 1967). In template-active RNA this ratio is 0.89, *i.e.*, it is "DNA like" in its base composition.

F. Effect of Neomycin on Template-Active RNA. It was shown (Holland *et al.*, 1966) that addition of neomycin increased the template activity of rRNA and tRNA. Cleavage of a number of phosphodiester bonds of these RNAs by heating at 100° in the presence of EDTA was required prior to the addition of neomycin. Also, neomycin enhanced the template activity of rat liver fetal nuclear RNA (Bresnick *et al.*, 1967).

The effect of varying concentrations of neomycin on increasing template activity of nuclear template RNA is recorded in Figure 4. In the presence of neomycin a greater stimulation of template-active RNA was observed in the

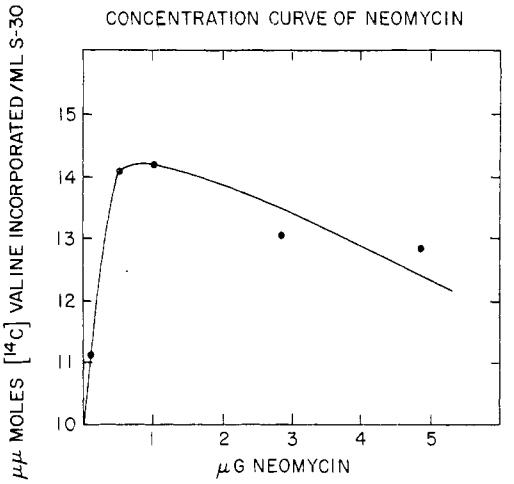

FIGURE 4: Effect of varying concentrations of neomycin on template activity of pupal nuclear template-active RNA. Reaction conditions as described under Methods except that varying amounts of neomycin were added to the 0.25-ml reaction mixture as indicated, and 200 μg/ml of template-active RNA was employed in each reaction mixture. [^{14}C]Valine (0.8 μCi/ml) is the labeled amino acid. The values shown express micromicromoles of [^{14}C]valine incorporated.

range of 0.1–1.0 μg/0.25 ml. The values shown express micromicromoles of valine incorporated.

Table VI shows that incorporation of different amino acids stimulated by template-active RNA is enhanced by neomycin (except leucine).

Discussion

Recent achievements in methodology have made it possible to study growth and differentiation of embryonic tissues in terms of RNA and protein synthesis, the aim being the elucidation of the mechanism controlling gene expression.

The synthesis of protein and different species of RNA have been followed during embryogenesis of sea urchins (Monroy and Tyler, 1963; Gross and Cousineau, 1963; Nemer, 1963; Comb, 1965), of *Xenopus laevis* (Brown and Littna, 1964; Decroly *et al.*, 1964), of *Rana pipiens* (Brown

TABLE VI: Effect of Neomycin on the Template RNA-Stimulated Incorporation of Different Amino Acids.[a]

	Incorporation (μμmoles/ml of S-30)	
[^{14}C]Amino Acid	Without Neomycin	With Neomycin
Leucine	63.22	63.34
Valine	10.25	15.02
Proline	8.68	43.16
Lysine	4.80	38.40
Arginine	7.32	51.52

[a] Reaction conditions are as described for Table III, except that each tube contains 200 μg/ml of template-active RNA. Neomycin when used is at a level of 0.5 μg/tube.

and Caston, 1962), and of embryonic liver (Bresnick *et al.*, 1967).

The goal of these studies is to reveal the mechanism of gene action, the end product of which is either a structural or a specific enzyme protein. Therefore, embryonic growth, differentiation, and morphogenesis were followed as a function of the synthesis of protein- and species-specific RNA at particular times during ontogeny.

RNA synthesis in the wing epidermis of the silk moth has been followed during the first 24 hr following termination of diapause (Wyatt and Linzen, 1965). Analysis of the ^{32}P-labeled RNA lead to speculation that it might be largely mRNA. Incorporation of labeled uridine into a 12S fraction of RNA from pupae of the oak silkworm *Antherae pernyi* lead to the assumption that this is a newly synthesized mRNA (Barth *et al.*, 1964). Experiments with *Tenebrio* pupae using actinomycin D or colchicine and labeled uridine indicated the existence of stable mRNA (Ilan *et al.*, 1966; Ilan and Quastel, 1966).

In this study template activity and base composition were the main criteria for defining mRNA. Thus, RNA isolated from pupal nuclei at 65° is able to stimulate significantly amino acid incorporation into the hot trichloroacetic acid precipitate in *E. coli* preincubated S-30. Slight stimulation was observed using the 18S rRNA subunit as template. This is in agreement with observations obtained with mammalian 18S RNA (DiGirolamo *et al.*, 1964; Brawerman *et al.*, 1965; Hadjiolov, 1966).

The nuclear RNA extracted at 65°, which exhibited greatest stimulatory activity, was termed template-active RNA and was further investigated. The stimulation was dependent on an energy source and Mg^{2+}. Puromycin and RNase inhibited this stimulation completely (Table II). Moreover, the reaction product was sensitive to proteolytic enzymes indicating its protein nature (Table IV). The template-active RNA stimulated the incorporation of nine amino acids in comparison with the endogenous *E. coli* product. A similar test with liver nuclear template-active RNA showed high incorporation of lysine and arginine which suggested a product related to nuclear histones (Hadjiolov, 1966).

It has been postulated by Roberts (1965), Osawa (1965), and Yoshikawa-Fukuda (1966) that rRNA when in an unmethylated, *i.e.*, nascent, state may possess template properties for the construction of ribosomal protein. That could be the case with the liver nuclear template RNA (Hadjiolov, 1966). However, the product of stimulation by template-active RNA from insect nuclei is relatively low in lysine and arginine. Moreover, it is rich especially in tyrosine, isoleucine, leucine, and valine (Table III).

Supporting evidence for the messenger character of the nuclear template-active RNA is its sedimentation profile (Figure 3). The peaks do not coincide with known *s* values for RNA or rRNA precursors. Moreover, base composition analysis shows (Table V) a marked difference from cytoplasmic RNA, the composition resembling that of DNA. Pulse labeling revealed incorporation of radioactive uridine throughout the gradient. The lack of accumulation of counts near the top of the gradient (Figure 3) indicates that there is no detectable degradation of the product.

A definitive assay for eucaryotic mRNA, one which would demonstrate the production of new distinct protein, is not presently available. However, considering its template characteristics, sedimentation profile in sucrose gradient, and base composition, we tentatively identified an RNA fraction from insect nuclei as mRNA.

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Preparation of an Exonuclease from *Lactobacillus* Which Releases 5′-Mononucleotides from Polynucleotides*

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ABSTRACT: A phosphodiesterase can be recovered from *Lactobacillus acidophilus* R26 cells which has the properties of an exonuclease with specificity for 5′-nucleotide esters. It hydrolyzes oligonucleotides of the ribo- and deoxyribonucleotide series, and *p*-nitrophenyl-5′-thymidine phosphate, to give exclusively 5′-mononucleotides. These substrates appear to compete for the same catalytic agent. Enzymatic activity is optimal at pH 8 with $MnCl_2$ at 5×10^{-4} M and in presence of a dithiol-stabilizing agent. Ca and Mg salts are also effective activators. High molecular deoxyribonucleic acid is degraded by the enzyme preparation with no evidence of intermediates other than mononucleotides. No evidence was obtained of activity on high molecular ribonucleic acid.

In a search for exonucleases capable of genetic variation, a study was undertaken in this laboratory of the nucleases of *Lactobacillus acidophilus* R26. The first enzyme found (R. D. Hotchkiss and H. G. Khorana, 1961, unpublished) was a diesterase hydrolyzing 3′-deoxyribonucleotide esters. This enzyme was then purified and shown to be an exonuclease hydrolyzing both ribo- and deoxyribooligonucleotides (Fiers and Khorana, 1963).

The present paper reports the discovery in *Lactobacillus* cells of an exonuclease of opposite polarity, *i.e.*, liberating 5′-ribo- and 5′-deoxyribomononucleotides. The substrate and cofactor requirements of partially purified preparations of the 5′-exonuclease are described.

Materials and Methods

Materials. Alkaline phosphatase, DNase I,[1] and RNA core were purchased from Worthington Biochemical Corp., Freehold, N. J. Cleland's reagent (dithiothreitol), *p*-nitrophenylthymidine 5′-phosphate (sodium salt), *p*-nitrophenol, and *p*-nitrophenyl phosphate (disodium salt, hexahydrate) were obtained from California Corp. for Biochemical Research, Los Angeles, Calif.

The following compounds (designated according to their 3′-phospho-5′ sequences) were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.: ApA,[1] CpA, CpU, ApG, ApC, GpC, and UpC. [*methyl*-^{3}H]Thymidine in H_2O (specific activity 3,47 Ci/mmole) was purchased from International Chemical and Nuclear Corp., City of Industry, Calif. Uridylate oligonucleotides and adenylate oligonucleotides were obtained from Miles Chemical Co., Elkhart, Ind.

DEAE-cellulose was Whatman microgranular DE32 from H. Reeve Angel, Clifton, N. J. Sephadex G-100, Sephadex G-15, and DEAE-Sephadex A-50 were from Pharmacia, Piscataway, N. J.

Preparation of Extracts. Bacterial strain *L. acidophilus* R26 was obtained from E. Travaglini of the Institute for Cancer Research, and also from the American Type Culture Collection (ATCC No. 314). The medium used for mass growth contained 40 g of Bacto Folic Acid Assay medium, 400 mg of ascorbic acid, 10 g of $NaAc \cdot 3H_2O$, 880 mg of $CaCl_2$, 6 g of casein hydrolysate (Nutritional Biochemicals, enzymatic), 400 mg of DL-alanine, 40 mg of L-tryptophan, 0.24 ml of glacial acetic acid, and 0.56 ml of Tween 80 per l. *L. acidophilus* was inoculated in the medium and grown at 37°; when cell density reached 5–10 $\times 10^8$ cells/ml, bacteria were

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[1] In this paper, nucleosides and nucleotides are represented by sequences in which A, C, G, U, represent the ribonucleoside residues adenosine, cytidine, etc., and p the phosphate residues starting from the left at the 5′ end. Deoxyriboside derivatives bear the d- prefix (except thymidylate). Other abbreviations are: PN-pT, *p*-nitrophenylthymidine 5′-phosphate; DNase I, pancreatic deoxyribonuclease; TDM 7.9, 0.05 M Tris-HCl buffer (pH 7.9) containing 0.075 M dithiothreitol and 10^{-3} M $MnCl_2$.