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Cell-Free Protein Synthesis in Lysates of *Drosophila melanogaster* Cells†

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ABSTRACT: A procedure is described for preparing cell-free protein synthesizing lysates from *Drosophila melanogaster* tissue culture cells and embryos. Preparation of translationally active lysates from tissue culture cells is dependent on the presence of rat liver supernatant during cell lysis to inhibit ribonuclease activity. After micrococcal nuclease treatment of the lysate, protein synthesis is dependent on the addition of exogenous messenger RNA. The fidelity of translation is very high. The conditions for optimal translation have been determined. In addition, the effects on translation of a variety of supplements, including rat liver supernatant, have been analyzed. The products of translation by the *Drosophila* lysate have been compared with those of wheat germ extracts and of micrococcal nuclease treated rabbit reticulocyte lysates. Translation in vitro of bovine parathyroid hormone messenger RNA yielded two products tentatively identified as preproparathyroid hormone and proparathyroid hormone, as well as an unidentified third product. This result suggests that insect enzymes can accurately process mammalian precursor proteins.

The use of cell-free protein synthesizing systems has facilitated two types of analysis. First, the protein synthesizing system can be dissected and reconstituted, using defined messenger RNAs and well-characterized products, in order to understand how ribosomes and messenger RNA interact with the various controlling signals and factors to synthesize proteins. Second, in vitro translation systems are important tools for determining the coding capacity of purified messenger RNAs. Primary translation products as well as processing and posttranslational modifications can be analyzed.

In general, the more closely the in vitro products made by a translation system resemble the in vivo protein(s) made from the same messenger RNA, the better the in vitro system is judged to be. Two cell-free protein synthesizing systems that have been particularly useful in this regard are translation extracts made from wheat germ embryo (Roberts & Paterson, 1973) and micrococcal nuclease treated rabbit reticulocyte lysates (Pelham & Jackson, 1976; Villa-Komaroff et al., 1974). It is often the differences, however, between in vivo and in vitro protein products that are most interesting, particularly differences that may be due to the ability of the translation system components to recognize signals encoded in the sequence or modifications of the messenger RNA. The capability to interpret (or misinterpret) such signals could have species or even tissue specificity.

Drosophila melanogaster is especially attractive for studies of gene control because of the extensive genetic studies conducted over the past half century, the small size of the genome, and the growing body of knowledge about the developmental biology of this species. In studying certain aspects of gene expression, we realized the potential usefulness of an in vitro translation system prepared from *Drosophila*. We report here the development of such a system from *Drosophila* tissue culture cells and embryos. In both cases the method is simple and the in vitro products closely resemble proteins synthesized in vivo. In addition, the system is responsive to exogenous mRNA[1] and becomes dependent upon such RNA after treatment with micrococcal nuclease.

Materials and Methods

RNA and Protein Extraction. Vesicular stomatitis virus (VSV) messenger RNA was prepared by phenol–chloroform extraction of infected Chinese hamster ovary (CHO) cells (Lodish & Froshauer, 1977). [^{35}S]Methionine-labeled VSV protein from infected CHO cells was a gift from Susan Froshauer. *Drosophila* RNA was extracted and purified from Schneider L-2 suspension culture cells by guanidine hydrochloride extraction (Strohman et al., 1977) as modified by Bruce Paterson (personal communication).

Five hundred milliliters of cultured cells was pelleted, resuspended in 5 mL of 8 M Gdn·HCl, 0.025 M NaAc, and sheared in a Dounce homogenizer. RNA was precipitated at −20 °C by the addition of 0.5 volume of ethanol. The precipitate was collected by a 10-min, 3000*g* centrifugation. The pellet was dissolved in 5 mL of 8 M Gdn·HCl, then brought to 25 mM EDTA, pH 7, and to 50 mM NaAc, pH 5. The ethanol precipitation and resuspension procedure were repeated. The resulting pellet was resuspended in 0.025 M EDTA, pH 7, and extracted with chloroform/butanol (4:1) twice. The RNA was then precipitated with 2 volumes of 4.5 M NaAc, pH 6, at 4 °C overnight.

Yeast transfer RNA was purchased from Boehringer-Mannheim. Yeast and wheat germ transfer RNA synthetases purified by DEAE-cellulose chromatography were gifts from Arnaud Ducruix. Purified wheat germ transfer RNA was

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[1] Abbreviations used: mRNA, messenger RNA; RLS, rat liver supernatant; $NaDodSO_4$, sodium dodecyl sulfate; VSV, vesicular stomatitis virus; RNase, ribonuclease; EDTA, ethylenediaminetetraacetate; Gdn·HCl, guanidine hydrochloride.

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from Thomas Fraser. *Drosophila* tRNA was prepared from 12–18 h old embryos by phenol–chloroform extraction and purified by Sephadex G-100 chromatography.

Wheat Germ Cell-Free System. Wheat germ cell-free extracts were prepared by the procedure of Roberts & Paterson (1973). The conditions used for translation have been described previously (Storti & Rich, 1976).

mRNA-Dependent Rabbit Reticulocyte System. mRNA-dependent rabbit reticulocyte lysates treated with micrococcal nuclease were prepared according to Pelham & Jackson (1976). The procedure was modified in that reticulocytes were lysed directly into water containing 40 μg/mL hemin. The assay system in a final volume of 25 μL was as described (Storti & Rich, 1976) using salt concentrations of 0.64 mM $MgAc_2$ and 80 mM KAc.

Growth of Cells. The cells used in making *Drosophila* lysates were Schneider L-2 *Drosophila melanogaster* cultured cells (Schneider, 1972) adapted to spinner culture (Lengyel et al., 1975). Cells were grown at 25 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 0.5% lactalbumen hydrolysate, MEM nonessential amino acids, 50 units/mL penicillin, and 50 μg/mL streptomycin (all from Gibco). The cells doubled approximately every 25 h.

mRNA-Dependent Drosophila Schneider L-2 Cell Lysate System. The *Drosophila* cells were harvested by centrifugation in a GSA rotor (Sorvall) at 2500 rpm for 2 min and washed three times in a small volume of ice-cold phosphate buffered saline by centrifugation for 4 min at top speed in a clinical centrifuge. The cell pellets were suspended in 1 volume of 10 mM Hepes, pH 7.6, 6 mM β-mercaptoethanol, and 20% rat liver supernatant (RLS).

RLS was prepared (Roth, 1956; Blobel & Potter, 1966) by homogenizing the liver of a freshly killed rat in 3 volumes (w/v) of 10 mM Hepes, pH 7.6, 6 mM β-mercaptoethanol. A motor-driven loose-fitting Dounce-type homogenizer was used. The homogenate was centrifuged at 200000*g* for 4 h, and the supernatant removed and stored in small aliquots at −20 °C.

The *Drosophila* cell suspension was homogenized by 10–20 strokes of a loose-fitting Dounce homogenizer. Usually more than 80% of the cells were broken, as determined by light microscopy. The homogenate was centrifuged at 18 000 rpm (40000*g*) in a Sorvall SS-34 rotor for 20 min. After centrifugation the top clear supernatant was removed, taking care to avoid the opaque fluffy layer immediately above the packed pellet. All operations were at 4 °C.

The clear lysate was digested immediately with micrococcal nuclease (P-L Biochemical). To each 100 μL of lysate were added: 2 μL of a 50 mM $CaCl_2$ stock and 15 units of micrococcal nuclease. Digestion was at 20 °C for 10 min. Immediately following digestion, 2 μL of 100 mM EGTA and 0.5 μL of 40 mg/mL creatine phosphokinase (in 50% glycerol) were added.

The standard assay system in a final volume of 25 μL consisted of: 10 μL of lysate, 3 μL of a mixture of 0.25 mM amino acids minus methionine, 66.6 mM creatine phosphate, 10 mM dithiothreitol, 5 mM spermidine, and 200 mM Hepes, pH 7.6. The assay mix was made 0.33 mM $MgAc_2$ and 80 mM KAc. [^{35}S]Methionine (20–40 μCi, Amersham), RNA, and water were added to give the final volume of 25 μL. Incubation was at 28 °C for 45 min.

Protein synthesis was monitored by the incorporation of [^{35}S]methionine into trichloroacetic acid precipitable material. One-microliter aliquots were spotted onto 3MM Whatman filter paper discs, washed 5 min in 10% Cl_3CCOOH, 5 min in 5% Cl_3CCOOH, and 10 min in boiling 5% Cl_3CCOOH. They were then rinsed with methanol and ether and dried. Samples were counted in Liquiflor–toluene scintillant.

Drosophila Embryo Lysates. Twelve to eighteen hour *Drosophila* embryos were a gift from Sarah Elgin. The embryos were dechorionated and sterilized in 50% ethanol/50% Chlorox for 1 min followed by five washes in phosphate-buffered saline using a clinical centrifuge at top speed to collect the embryos.

Homogenization, lysate preparation, and protein synthesis were carried out using the same procedure described above for the Schneider cell lysate, except that RLS was omitted from the lysis buffer.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Samples of cell-free protein synthesis assays were analyzed by $NaDodSO_4$ slab gel electrophoresis as described by Maizel (1971) and Laemmli (1970). Slab gels were 12% (or 16%) polyacrylamide. After electrophoresis the gels were stained with Coomassie blue and the radioactive proteins detected by fluorography as described by Laskey & Mills (1975) using XR-5 X-ray film. Exposure was usually for 1–2 days.

Storage of the Lysate. We have not yet found a satisfactory method for preserving the activity of the lysate during storage. All the experiments described in this report were done using freshly prepared lysates. If the lysate derived from cultured cells is frozen before or after micrococcal nuclease digestion, about 50% of the activity is lost. This is true for lysates frozen at −70 °C or in liquid nitrogen. We usually add 10% glycerol to minimize freezing damage. The lysate derived from embryos behaves somewhat better than the tissue culture lysate, yielding up to 80% of its original activity after 1 freeze-thaw cycle.

Results

Lysates Prepared from Schneider L-2 Cells. Initially lysates were prepared by homogenizing cells directly in buffer containing rat liver supernatant (RLS) and centrifuging the homogenate at only 30000*g*. These centrifugation conditions are similar to those commonly used in preparing extracts from wheat germ or lysates from rabbit reticulocytes. The *Drosophila* lysates were highly active in endogenous protein synthesis as determined by the incorporation of [^{35}S]-methionine. Exogenous RNA directed the synthesis of the expected new protein products, as detected by gel electrophoresis, demonstrating that the lysates were capable of the initiation of protein synthesis as well as of the elongation of preexisting peptide chains. It was observed, however, that greater than 50% of the endogenous protein synthesis was completely resistant to even high micrococcal nuclease digestion. This suggested to us that some endogenous mRNA and polysomes might be shielded by microsomal vesicles formed during the initial homogenization. We therefore set out to find conditions which would reduce this resistant protein synthesis. Unfortunately, protein synthesis was completely inhibited by as little as 0.05% Triton detergent. However, we discovered that by centrifuging the initial homogenate at 40000*g*, the endogenous level of protein synthesis (incorporation of [^{35}S]methionine) was reduced to 40–60% of that in lysates which had been prepared by centrifuging at 30000*g*. The endogenous synthesis which remained after the 40000*g* centrifugation was completely sensitive to micrococcal nuclease digestion.

More importantly, addition of exogenous mRNA to these lysates after micrococcal nuclease digestion resulted in stimulation of incorporation of [^{35}S]methionine into protein.

Table I: Stimulation of Protein Synthesis in Micrococcal Nuclease-Digested *Drosphila* Schneider Cell Lysates

preparation of lysate	RNA (VSV)[a]	[^{35}S]Met incorpn $\times 10^{-3}$ (cpm/μL of reaction)	enhancement of stimulation[h]	stimulation: % of max[b]
+RLS	–	1.2	1.0	3.2
	+	37.0	30.8	100
–RLS	–	1.2	1.0	3.2
	+	1.3	1.1	3.5
	+(RLS)[c]	3.7	3.1	10.0
	+(RLS)[c]	2.2	1.8	5.9
+reticulocyte[d] supernatant	–	4.2	1.0	11.4
	+	6.3	1.5	17.0
+RLS[e] (heat-treated)	–	0.16	1.0	0.4
	+	0.60	3.8	1.6
+RLS[f] (trypsinized)	–	0.58	1.0	1.6
	+	8.9	15.3	24.1
+hemin[g]	–	0.61	1.0	1.6
	+	0.38	0.6	1.0

[a] VSV mRNA, when added, was at a concentration of 192 μg/mL. [b] Maximum = the amount of synthesis by lysates prepared in RLS. [c] RLS added to reaction mix rather than to lysis buffer. [d] Rabbit reticulocyte supernatant was prepared by centrifuging micrococcal nuclease-digested reticulocyte lysate at 100000g for 2 h at 5 °C. [e] RLS was heated to 65 °C for 5 min. [f] RLS was treated with 10 μg/mL trypsin at 34 °C for 15 min. After digestion, 100 μg/mL soybean trypsin inhibitor was added. [g] Hemin was added to lysis buffer at 200 μg/mL. [h] Expressed in multiples of the amount of incorporation obtained in reactions when no RNA was added.

The results of measurements of the amount of protein synthesis in the lysate are presented in Table I.

Drosophila melanogaster Schneider L-2 cultured cells contain high endogenous ribonuclease activity. Initial attempts to prepare an in vitro protein synthesizing system from these cells resulted in lysates which were inactive in protein synthesis. The more commonly used ribonuclease inhibitors (e.g., diethyl pyrocarbonate) unfortunately affect many proteins in addition to ribonuclease, and consequently interfere with protein synthesis. High-speed supernatants from rat liver, however, have been known for some time to contain a potent ribonuclease inhibitor which has been effectively used in tissue containing a high endogenous ribonuclease activity (Roth, 1956; Blobel & Potter, 1966). We have found this inhibitory activity useful for preparing lysates from Schneider L-2 cells.

Table I demonstrates the dependency on RLS. Neither rabbit reticulocyte lysate supernatant nor wheat germ extract supernatant could substitute for RLS. In addition, it can be seen that RLS must be present at the time of lysis; it did not stimulate protein synthesis if added after preparation of the lysate. When lysates were prepared without RLS, protein synthesis was not stimulated by subsequent addition of tRNA purified from *Drosophila* or wheat germ, or by yeast or wheat germ tRNA synthetases (Table II). Both the yeast and the wheat germ tRNA preparations did stimulate protein synthesis in rabbit reticulocyte lysates. These results indicate that RLS acts at the time of lysis and is not merely supplying tRNA or synthetases to the lysate.

Rabbit reticulocyte lysates contain a potent inhibitor of protein synthesis. Inhibition is mediated by activation of protein kinase activity which is believed to act upon initiation factor eIF-2 (Datta et al., 1978; DeHaro & Ochoa, 1978).

Table II: Effects on Protein Synthesis in Embryo and Schneider Cell Lysates of Addition of Translation Supplements[a]

supplement	embryo lysate	Schneider cell lysate[b]
RLS	+	–
reticulocyte supernatant	+	–
wheat germ extr. supernatant	–	NT[c]
tRNA		
wheat germ	+	–
yeast	+	NT
Drosophila	NT	–
tRNA synthetases		
wheat germ	–	NT
yeast	–	–
hemin	–	–

[a] The results from different experiments have been pooled and therefore are presented as stimulation (+) or no stimulation (–) when compared with those containing only VSV mRNA. Stimulation was considered "+" only when greater than 1.5 times the control. [b] The results shown apply both to lysates prepared with and to lysates prepared without RLS in the lysis buffer. [c] NT, not tested.

Inhibition of protein synthesis is prevented by the addition of hemin. A similar effect has been reported in extracts made from HeLa cells (Weber et al., 1975). We tested the possibility that *Drosophila* cells might contain a similar inhibitor by preparing lysates in the presence of hemin. Table I shows that lysates prepared in the presence of hemin were inactive. Although RLS may contain hemin, lack of hemin is not the primary cause of the requirement for RLS. Table I shows that the effect of RLS is completely abolished by prior heat treatment and is at least partially sensitive to trypsin pretreatment, suggesting that a protein component is responsible for RLS activity. Similar treatment of RLS has been previously reported to abolish the effects of the ribonuclease inhibitor (Blobel & Potter, 1966).

Optimum conditions for protein synthesis in the cultured cell lysate are presented in Figure 1. It can be seen that, as in rabbit reticulocyte lysates, protein synthesis is maximal at a KAc concentration of 80 mM (Figure 1A). The $MgAc_2$ optimum is 0.3 mM, approximately one-half that of rabbit reticulocyte lysates (Figure 1B). Protein synthesis is maximal at 28 °C and is linear for the first 30 min but continues for at least 1 h (Figures 1C and 1D). The rate of elongation (amino acids incorporated into protein per unit time) was estimated by electrophoresing aliquots of a reaction mixture at different times during synthesis. The minimum time required for the synthesis of VSV N (M_r 52 500) protein was between 5 and 10 min, and the rate of elongation was estimated from this to be approximately 50–100 amino acids per min. This rate is approximately half that of rabbit reticulocyte lysates and approximately twice that of wheat germ extracts (B. Roberts, personal communication).

The fidelity of translation was determined by $NaDodSO_4$–polyacrylamide slab gel electrophoresis of the products made in predigested *Drosophila* Schneider cell lysates. Figure 2 shows the products of the *Drosophila* system in comparison with proteins made from the same mRNA by wheat germ extracts and micrococcal nuclease-treated rabbit reticulocyte lysates. The mRNAs used were VSV mRNA and RNA from Schneider L-2 cells grown at 25 or 36 °C. It can be seen that *Drosophila* lysates synthesize high molecular weight proteins in greater abundance than wheat germ extracts and in amounts comparable to rabbit reticulocyte lysates. *Drosophila* lysates also synthesize fewer lower molecular weight polypeptides suggesting a reduced number of in-

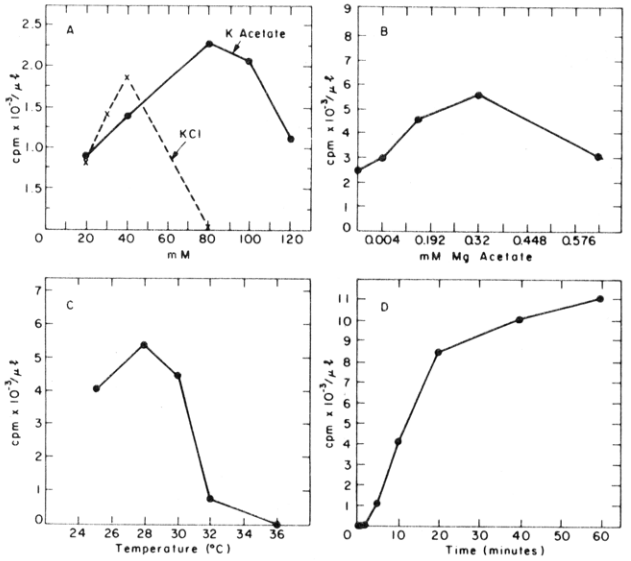

FIGURE 1: (A) Optimization of KAc and KCl concentrations. Reactions were at 28 °C for 45 min in 0.32 mM $MgAc_2$ using 96 μg/mL VSV mRNA. (B) Optimization of $MgAc_2$ concentration for protein synthesis. Conditions were the same as in A using 80 mM KAc. (C) Temperature optimization with 80 mM KAc, 0.32 mM $MgAc_2$, and 96 μg/mL VSV mRNA. (D) Time course of [^{35}S]-methionine incorporation. Conditions as in C except 192 μg/mL VSV mRNA was used.

complete polypeptides and a higher degree of translational fidelity. Note also the almost complete absence of endogenous protein synthesis in the absence of added mRNA (Figure 2, lane 1).

Bovine proparathyroid hormone (M_r 12 500) has been shown to be synthesized as a 14 000-dalton precursor protein in wheat germ extracts (Kemper et al., 1972). Synthesis in the presence of dog pancreas membranes results in the cleavage of preproparathyroid to proparathyroid. This phenomenon has now been described for a number of secretory proteins and forms the basis for the "signal sequence" hypothesis of membrane protein synthesis (Blobel & Dobberstein, 1975). When preproparathyroid mRNA was added to *Drosophila* lysates, it directed the synthesis of three major products (Figure 3). One of these products comigrated with a protein synthesized in wheat germ extracts previously identified as preproparathyroid hormone. In addition, a second major protein synthesized in *Drosophila* lysates comigrated with proparathyroid hormone suggesting that preproparathyroid was processed to proparathyroid during or shortly after synthesis. The identity of this putative proparathyroid hormone is currently being substantiated. In control experiments RLS was added along with preproparathyroid hormone mRNA to wheat germ extracts and rabbit reticulocyte lysates. In neither the wheat germ nor the rabbit reticulocyte lysates was a putative proparathyroid hormone product synthesized, indicating the processing activity is a function of the *Drosophila* lysate and not of RLS.

The lack of increased stimulation of protein synthesis in Schneider cell lysates by the added supplements listed in Table II is consistent with the hypothesis that RLS is acting as a RNase inhibitor. We wished to test directly, however, the ribonuclease inhibition by RLS during the preparation of Schneider cell lysates. Accordingly, cells were pulse labeled with [^{3}H]uridine to label RNA and then homogenized in the presence or absence of RLS. The RNA was then purified from lysates at various times after homogenization by phenol–chloroform extraction and the RNA analyzed by acrylamide gel electrophoresis. Figure 4 shows a comparison of the

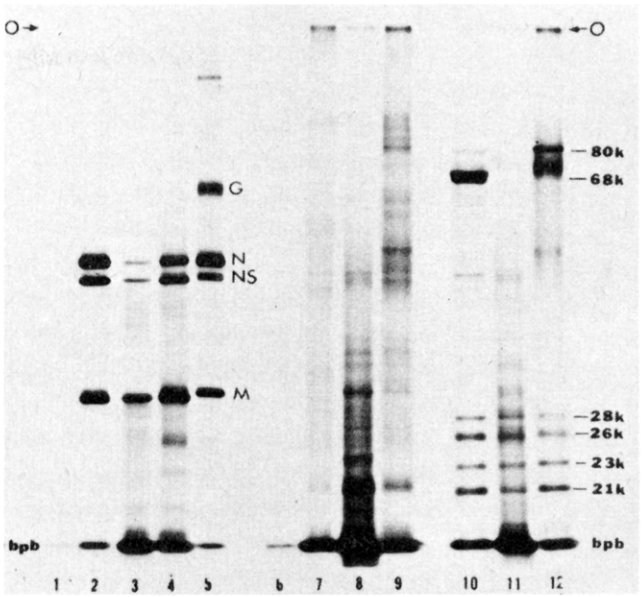

FIGURE 2: Products of cell-free translation in *Drosophila* lysates, wheat germ extracts, and rabbit reticulocyte lysates. Electrophoresis was in a 12% $NaDodSO_4$–polyacrylamide slab gel. (1) Two microliters (3200 cpm) of a 25-μL reaction mix of *Drosophila* lysate with no added RNA (endogenous synthesis); (2) Two microliters (32 000 cpm) of a 25-μL reaction mix of *Drosophila* lysate with 192 μg/mL VSV mRNA added; (3) 32 000 cpm of the products translated from VSV mRNA in wheat germ extract; (4) 33 000 cpm of the products translated from VSV mRNA in rabbit reticulocyte lysate; (5) proteins labeled in vivo in VSV-infected Chinese hamster ovary (CHO) cells; (6) 5μL (8000 cpm) of a 25-μL reaction mix of *Drosophila* lysate without added mRNA (endogenous synthesis). (7) 7 μL (33 000 cpm) of a 25-μL reaction mix of *Drosophila* lysate containing approximately 320 μg/mL total RNA from Schneider cells grown at 25 °C; (8) same RNA as in 7, translated in wheat germ extracts (30 000 cpm); (9) same RNA as in 7, translated in rabbit reticulocyte lysate (30 000 cpm); (10) 5 μL (33 000 cpm) of a 25-μL reaction mix of *Drosophila* lysate containing approximately 320 μg/mL total RNA from Schneider cells "heat shocked" to 36 °C for 2 h; (11) same RNA as in 10, translated in wheat germ extracts (33 000 cpm); (12) same RNA as in 10, translated in rabbit reticulocyte lysates (29 000 cpm). Exposure was for 2 days. (O) Origin; (bpb) bromphenol blue dye marker.

densitometer scans of RNA from cells lysed in buffer with and without RLS. It can be seen that the amount of high molecular weight RNA is significantly reduced in the sample prepared without RLS. There is also a corresponding increase in the amount of RNA in the low molecular weight region of the gel. These results demonstrate directly the RNase inhibition by RLS in lysates.

Lysates Prepared from Drosophila Embryos. We also tested the possibility of preparing lysates from *Drosophila* embryos. Embryos between the ages of 12 and 18 h were used in these studies since they were assumed to be more active in protein synthesis than earlier stages. Lysates prepared from dechorionated embryos were comparable to Schneider cell lysates in endogenous protein synthesizing activity (compare Tables I and III). Unlike Schneider cell lysates, however, embryo lysates did not show a requirement for RLS. Addition of VSV and mRNA stimulated incorporation of [^{35}S]-methionine into protein above endogenous levels. This increase in stimulation was the result of initiation of protein synthesis on added mRNA as determined by the observation of VSV N, NS, and M proteins on acrylamide gels (Figure 5).

We have been unsuccessful in making the embryo lysate completely dependent on exogenous mRNA. Table III shows that lysates treated with micrococcal nuclease sufficient to eliminate endogenous protein synthesis also rendered the lysate

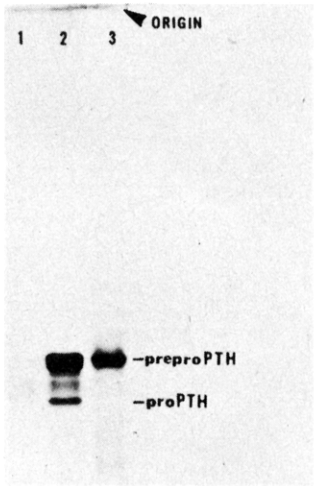

FIGURE 3: Synthesis of preproparathyroid hormone in *Drosophila* lysates. Electrophoresis was in a 16% polyacrylamide slab gel. (1) Ten microliters (16 750 cpm) of a 25-μL reaction mix of *Drosophila* lysate without added mRNA (endogenous synthesis); (2) 10 μL (30 100 cpm) of a 25-μL reaction mix of a *Drosophila* lysate programmed with parathyroid mRNA. (3) 20 000 cpm of the products of synthesis by wheat germ extract directed by parathyroid mRNA. Exposure: 1 day.

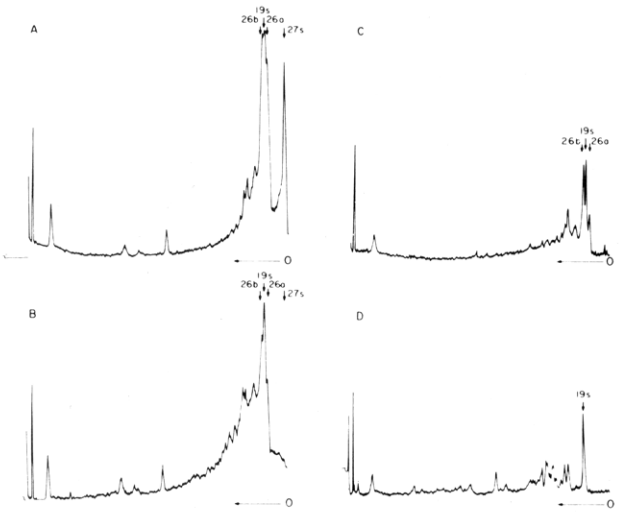

FIGURE 4: Effect of rat liver supernatant on endogenous ribonuclease. One thousand milliliters of Schneider cells at 3×10^6 cells/mL were concentrated fivefold and labeled for 1 h with 40 μCi/mL of [^{3}H]uridine. Lysates were then prepared as described in Materials and Methods. Half the cells were lysed in buffer containing 20% RLS; half in buffer without RLS. Immediately after homogenization 50 μL of each homogenate was removed and phenol/chloroform extracted. The remaining homogenates were centrifuged at 40000*g* for 20 min and the supernatants removed. These were left for 30 min on ice; then another 50 μL was removed from each lysate and the RNA was purified by phenol/chloroform extraction. The yield of Cl_3CCOOH-precipitable radioactivity was about 1.25×10^6 cpm from each homogenate, and 7.5×10^5 cpm from each supernatant; 125 000 cpm of each sample was loaded onto a 2.5–8% urea–acrylamide slab gel (Spradling et al., 1977) and electrophoresed for 600 V h. The fluorograph of this gel was traced on a Joyce-Loebl densitometer. 27S indicates the nuclear precursor to the 26S ribosomal RNA (Levis & Penman, 1978). 26a and 26b indicate the two parts of the nicked 26S ribosomal RNA. 19S indicates the RNA of the small ribosomal subunit. (A) RNA from the initial homogenate prepared with RLS; (B) same as A but prepared without RLS; (C) RNA from lysate incubated 30 min on ice, with RLS; (D) same as C, but without RLS. Exposure was for 6 h.

less sensitive to exogenous mRNA. In Figure 5 products of translation by the embryo lysate are shown. Several different combinations of centrifugation conditions, micrococcal nuclease concentrations, and EGTA concentrations yielded lysates that

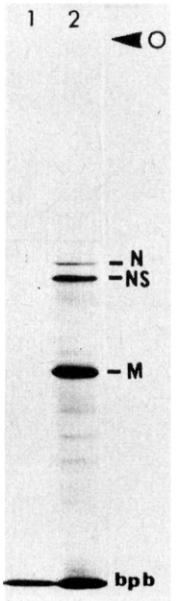

FIGURE 5: Cell-free protein synthesis in *Drosophila* embryo lysate. Electrophoresis was in a 12% polyacrylamide slab gel. (1) Endogenous synthesis: 5 μL (10 500 cpm) of a 25-μL reaction mix; (2) 5 μL (22 400 cpm) of a 25-μL reaction mix directed by 192 μg/mL VSV mRNA. Exposure: 2 days. (O) Origin; (bpb) bromphenol blue dye marker. N, NS, and M are the VSV proteins.

Table III: Stimulation of Protein Synthesis in *Drosophila* Embryo Lysates

micrococcal nuclease	RNA (VSV)	[^{35}S] Met incorpn $\times 10^{-3}$ (cpm/μL)
0	0	18.4
	+	29.9
+	0	1.1
	+	1.3

either maintained a high level of endogenous synthesis or were completely inactive.

Embryo lysates also differed from Schneider lysates in their response to added supplements (Table II). Unlike Schneider cell lysates, embryo lysate protein synthesis was enhanced two- to threefold by the addition of RLS or rabbit reticulocyte lysate supernatant. Wheat germ supernatant did not stimulate activity. Addition of tRNA also increased protein synthesis; the stimulation was approximately equal to that produced in rabbit reticulocyte lysates by this tRNA. Addition of tRNA synthetase or hemin to the lysis buffer or later to the reaction mix failed to stimulate any increase in protein synthesis.

Discussion

Cell-free protein synthesizing systems made from *Drosophila* and other insects have been reported previously (Fox et al., 1965; Goldstein & Snyder, 1972; Ilan & Lipmann, 1966). Most notably these include fractionated and unfractionated systems derived from *Drosophila* embryos and adults. Each of the systems reported, however, was cumbersome to prepare and usually suffered from a low level of protein synthesis. More importantly, these systems were neither dependent upon the addition of mRNA nor were they demonstrated to be capable of polypeptide chain initiation—both important requirements for studying protein synthesis.

The cell-free protein synthesizing lysate system we have characterized and reported here is made from *Drosophila* Schneider L-2 tissue culture cells. These cells have been grown in culture for several years and can be obtained easily in large quantities. More importantly, the lysate can be rendered

mRNA dependent by micrococcal nuclease digestion and has been demonstrated to be highly efficient in protein synthesis. We have now successfully translated a number of invertebrate, vertebrate, and viral mRNAs in the micrococcal nuclease digested system.

It has been our experience that Schneider L-2 cells contain a high level of endogenous ribonuclease activity. We have had difficulty in obtaining undegraded RNA from these cells except in the presence of high concentrations of inhibitors of RNase activity or strong protein denaturants. Indeed, our initial lysates were completely inactive in protein synthesis. Rat liver supernatant proved to be an effective inhibitor of RNase activity in our system without also inhibiting protein synthetic activity. It has been shown that RLS contains a potent inhibitor of RNases which are optimally active at acidic pHs (Blobel & Potter, 1966). It has been widely used for isolation of high molecular weight RNA. The observation that RLS inhibits RNases which are optimally active at acidic pHs may explain why micrococcal nuclease digestion (pH optimum is 9.2) is not inhibited by RLS, although the concentration of micrococcal nuclease used here is somewhat higher than that needed for reticulocyte lysates.

The absolute requirement for RLS at the time of cell lysis strongly suggests that the principal effect of RLS is the inhibition of RNase activity. We nevertheless tested this and, as demonstrated in Figure 4, RNA degradation is substantially reduced when RLS is present. It is interesting to note that the large ribosomal RNA (26a and 26b) is considerably more sensitive to degradation than is the 19S RNA. We were also concerned that RLS might have additional effects such as supplementation of the lysate with additional factors required for protein synthesis. We tested this possibility by adding to the lysate system purified tRNA, tRNA synthetases, or supernatant fractions from reticulocyte lysates or wheat germ extracts, both of which contain the soluble factors required for protein synthesis. None of these stimulated protein synthesis in Schneider cell lysates, although each addition effectively stimulated protein synthesis in either *Drosophila* embryo lysates or reticulocyte lysates. It is likely, therefore, that the lysates prepared from Schneider L-2 cells are complete in their requirements for protein synthesis.

Translation of preproparathyroid hormone mRNA in the Schneider cell lysate resulted in synthesis of three sizes of protein products. While the nature of these products has not yet been rigorously determined by sequence analysis, two of the proteins comigrate on $NaDodSO_4$–polyacrylamide gels with preproparathyroid hormone and proparathyroid hormone. Neither band was found in the absence of added mRNA. The third product remains mysterious, as it has not been observed in wheat germ extracts with membranes added to allow processing of the hormone. Further characterization of the in vitro products is in progress, but if the *Drosophila* lysate does in fact cleave off the signal sequence accurately some interesting conclusions may be drawn. While insects have been reported to use hydrophobic N-terminal oligopeptides in protein precursors (Suchanek et al., 1978), this has not yet been demonstrated for any *Drosophila* protein. The existence of processing enzymes would suggest that *Drosophila* also uses "signal sequences" and furthermore that the instructions for proper cleavage of the signal sequence have apparently been conserved during the divergent evolution of the chordate and arthropod phyla.

We were initially interested in preparing a cell-free protein synthesizing system from cultured cells. We also recognized the utility of a similar system from embryos and, therefore, tested our methods of preparation and protein synthesis by preparing lysates from *Drosophila* embryos. Unlike Schneider cell lysates, embryo lysates do not require RLS, although RLS does stimulate protein synthesis if added later. This stimulation is probably due to tRNA present in RLS since purified tRNA had a similar stimulatory effect. Embryo lysates are also capable of polypeptide chain initiation as evidenced by the synthesis of VSV proteins in response to the addition of VSV mRNA. We were not able to make the embryo system completely dependent on the addition of exogenous mRNA, in contrast to our results with the cultured cell lysate. Conditions of micrococcal nuclease digestion which eliminated endogenous protein synthesis also completely inhibited synthesis dependent on exogenously added mRNA. Nonetheless, the embryo lysate should prove very useful in characterizing *Drosophila* mutants which are defective in some aspects of protein synthesis. An established cell line is not necessary.

In summary, cell-free protein synthesizing lysates from *Drosophila* tissue culture cells or embryos are easy to prepare and are fully active in protein synthesis and responsive to exogenous mRNA. *Drosophila* has long been used as an effective tool for genetic analysis of growth and development. We foresee the utility of this cell-free protein synthesizing system particularly for studying genetic lesions causing specific defects in the machinery required for protein synthesis.

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Transcriptional Regulation in Avian Erythroid Cells†

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ABSTRACT: Both the translational and transcriptional repertoires of nearly mature avian erythroid cells appear to be highly restricted: molecular hybridization experiments demonstrate the presence of about 4000 species of poly(A)$^+$ nRNA and fewer than 100 species of poly(A)$^+$ mRNA. This paper addresses the question of whether the nRNA of erythroid cells contains sequences which, although not expressed in the erythroid cells, are found on polysomes in another cell type. We have prepared cDNA from liver mRNA and have determined the representation of liver mRNA sequences in the erythroid cell nRNA. Liver mRNA consists of about 14000 species of poly(A)$^+$ RNA. Of these only about 100 species are detectable in erythroid cell nRNA. The vast majority of liver mRNA species is undetectable in erythroid cells; i.e., they are present at less than 0.03 copies per cell. The few species of liver mRNA that are detectable in erythroid cells are present in both the nuclear and polysomal RNA at concentrations less than 0.1 copies per cell. These data suggest that gene expression in avian erythroid cells is highly regulated at the transcriptional level.

Messenger RNA populations are developmentally regulated; that is, some RNA species are present on polysomes of one cell type but absent from those of another cell type (Galau et al., 1976; Ryffel & McCarthy, 1975; Axel et al., 1976). Such developmental regulation could result from selective transcription or from posttranscriptional selection among primary transcripts. Recent work with sea urchin embryos has suggested that the major control point in developmental regulation may be posttranscriptional. Although some polysomal mRNA species are stage specific in sea urchin embryos, the hnRNA[1] populations of different stages are experimentally indistinguishable (Kleene & Humphreys, 1977; Wold et al., 1978).

In this paper we examine transcriptional and posttranscriptional sequence selection in avian erythroid cells. These cells are well suited for such experiments, since they contain about 4000 species of poly(A)$^+$ hnRNA but only about 100 species of poly(A)$^+$ mRNA (Lasky et al., 1978). The specific question we address here concerns the erythroid cell hnRNA sequences that do not appear on erythroid cell polysomes: do these molecules contain sequences that appear on polysomes in another cell type?

We have approached this question by using cDNA complementary to liver mRNA. Our results show that, of the approximately 14000 mRNA sequences in liver mRNA, a maximum of 100 are found in the hnRNA of erythroid cells. This suggests that gene expression in avian erythroid cells is highly regulated at the transcriptional level.

Materials and Methods

Isolation of Liver Poly(A)$^+$ mRNA, Erythroid Cell hnRNA, and Erythroid Cell Poly(A)$^+$ mRNA. Polysomal poly(A)$^+$ mRNA was isolated from liver tissue as described by Axel et al. (1976), except that the liver was minced and homogenized in a loose-fitting Dounce homogenizer. Polysomal material was isolated by centrifugation at 26000 rpm for 2 h at 4 °C in a Beckman SW27 rotor through a linear 10–40% sucrose gradient in 25 mM NaCl, 5 mM $MgCl_2$, and 25 mM Tris-HCl, pH 7.5. The gradients were collected through a flow cell, and the absorbance was monitored at 260 nm. Material sedimenting more rapidly than 100 S was pooled and precipitated with ethanol. The precipitated polysomes were then treated with 25 mM EDTA for 20 min at 0 °C. Material sedimenting more slowly than 80 S after EDTA release was isolated by centrifugation at 26000 rpm for 3.5 h at 4 °C in a Beckman SW27 rotor. Poly(A)$^+$ mRNA was isolated by oligo(dT)-cellulose chromatography as described previously (Lasky et al., 1978). Erythroid cell hnRNA and poly(A)$^+$ mRNA were isolated from anemic chickens as previously described (Lasky et al., 1978).

Preparation of Liver cDNA and "Selected Liver cDNA". Single-stranded DNA complementary to liver poly(A)$^+$ mRNA (liver cDNA) was prepared as described by Efstradiatis et al. (1975). RNA-dependent DNA polymerase from avian myeloblastosis virus was provided by Life Sciences, Inc., through the Viral Oncology Branch of the National Institutes of Health. The specific activity of the liver cDNA was (4–6) $\times 10^6$ cpm/μg.

To isolate "selected liver cDNA", we hybridized liver cDNA to excess erythroid cell hnRNA to R_0t = 250 M s in 0.5 M

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[1] Abbreviations used: nRNA, nuclear RNA; hnRNA, heterogeneous nuclear RNA; poly(A)$^+$, poly(A)-containing; EDTA, ethylenediaminetetraacetate; NT, nucleotide; R_0t, the product of RNA concentration, in moles of nucleotides per liter, and time, in seconds; PB, an equimolar mixture of Na_2HPO_4 and NaH_2PO_4.

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