Glutamine Synthetase Induction in Embryonic Neural Retina: Immunochemical Identification of Polysomes Involved in Enzyme Synthesis

(differentiation/enzyme induction/immunoprecipitation/nascent enzyme/messenger RNA)

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ABSTRACT Glutamine synthetase (EC 6.3.1.2) in embryonic neural retina in culture is rapidly induced by hydrocortisone. Retina polysomes involved in translation of this enzyme were precipitated with a high degree of specificity by the gammaglobulin isolated from antiserum against the enzyme (anti-enzyme gammaglobulin). Using immunoprecipitation procedures, we determined that the amount of polysome-bound nascent enzyme was maximal in polysomes comprising 9–14 ribosomes and was about 3-fold higher in the induced than in the noninduced retina. Within this size group of polysomes, those comprising 11–13 ribosomes showed consistently greater binding of anti-enzyme [^{125}I]gammaglobulin than of normal [^{125}I]-gammaglobulin. This size of polysomes corresponds to that calculated for a monocistronic messenger RNA for the subunit of this enzyme, which has a molecular weight of 42,000. The application of immunochemical techniques to identification of templates for synthesis of an enzyme in embryonic cells that constitutes less than 1% of the total cellular proteins indicates the usefulness of this method for detailed studies on regulation of other quantitatively minor products significant in cell differentiation.

Identification of polysomes and isolation of mRNA involved in synthesis of specific proteins is of obvious importance in studies on cell differentiation. So far, this has been accomplished only for proteins like hemoglobin, which represent major and unique products of highly specialized cells. However, embryonic differentiation on the whole is marked by the appearance of quantitatively subtler disparities in levels of relatively minor cell constituents, especially enzymes. In connection with our studies on hormonal induction of glutamine synthetase (EC 6.3.1.2) (1), it became necessary to identify the polysomes involved in synthesis of this enzyme as a step towards further analysis of this developmental system. We report here that although the enzyme constitutes less than 1% of the total cellular protein in the retina, the polysomes involved in its synthesis can be identified by an immunochemical method based on the reaction of the polysome-bound nascent chains with the gammaglobulin against the enzyme.

The properties of induction of retinal glutamine synthetase as a model system for the study of regulation of gene expression in embryonic cells have been described (2). The molecular responses characterizing this induction include transcription of stable, active RNA templates for synthesis of the enzyme and of regulatory products (3, 4) and a rapid and specific increase of the enzyme due to acceleration of enzyme synthesis, as shown by radioimmunochemical methods (2, 5). Earlier attempts to identify the polysomes involved in synthesis of the enzyme were based on its relatively high aspartic

acid content. It was suggested that polysomes comprising 12–14 ribosomes were involved in synthesis of the enzyme, since pulse-labeling with [^{14}C]aspartic acid showed increased radioactivity in the region of the polysomal profile corresponding to 12–14 ribosomes (4). The present results strongly support the above suggestion by demonstrating preferential precipitation of polysomes in this size group by gammaglobulin against glutamine synthetase; they also indicate the potential usefulness of immunochemical procedures in studying synthesis of other quantitatively minor products in differentiating cells.

MATERIALS AND METHODS

Tissue Culture, Enzyme Induction, and Labeling. Neural retinas were isolated aseptically from 12-day chick embryos and cultured in flasks on a shaker (3, 4). Each culture consisted of four retinas in 8 ml of medium (Tyrode's solution with 10% fetal-bovine serum and with antibiotics) in 125-ml Erlenmeyer flasks. The flasks were swirled on a gyratory shaker (80 rpm) at 38°. After 16 hr of incubation, hydrocortisone was added to elicit a rapid induction of glutamine synthetase (2, 3); controls were without the inducer. 5 hr later, the retinas were labeled with [^{14}C]aminoacid mixture or with [^{3}H]aminoacid mixture for 10 min and were then harvested for cell fractionation and analysis.

Cell Fractionation and Purification of Polysomes. All buffers, sucrose solutions, and glassware were sterilized by autoclaving. The labeled retinas were washed with ice-cold Tyrode's solution (40 ml total) and with TKMH buffer [50 mM Tris·HCl, 100 mM KCl, 10 mM MgCl$_2$; 40 μg/ml of sodium heparin (pH 7.4)], and were then taken up in 0.5% Triton X-100 in TKMH (1 ml per eight retinas) containing 0.25 M sucrose. The cells were lysed by five gentle strokes with a loose pestle in a glass homogenizer and allowed to stand for 5–10 min. The lysates were spun at 25,000 × *g* for 10 min, and the postmitochondrial supernatants were used for purification of polysomes.

For isolation of total polysomes, the procedure of Palacios *et al.* (6) was modified as follows: 2 ml of supernatant was layered over 1 ml of 2.5 M sucrose and 2 ml of 1 M sucrose (both made up in TKMH) and was spun at 46,000 rpm for 2 hr in an SW50.1 rotor of a Spinco model L2-65B centrifuge. The polysomes banded 1–2 mm below the interphase between 1 and 2.5 M sucrose and were collected by withdrawal of 0.6 ml with a sterile syringe (20 gauge needle) through a side puncture in this region of the tube. This procedure allowed

TABLE 1. *Precipitation of polysome-bound nascent labeled glutamine synthetase (GS) with anti-GS gammaglobulin*

Label	Source of polysomes	Total cold Cl₃CCOOH-insoluble dpm/mg of poly-somes*	Net GS dpm precipi-tated/ mg of poly-somes	Ratio of GS dpm in-duced/ control
Exp. I: [¹⁴C]amino-acid mixture	Noninduced retina	168,991	3294	2.9
	Induced retina	171,260	9579	
Exp. II: [³H]amino-acid mixture	Noninduced retina	142,857	2396	3.6
	Induced retina	222,222†	8793	

Retinas were labeled for 10 min with [¹⁴C]aminoacid mixture (5 μCi/ml) in Exp. I and with [³H]aminoacid mixture (12.5 μCi/ml) in Exp. II. Equal amounts of polysomes from noninduced and induced retinas were immunoprecipitated. In Exp. I, normal gammaglobulin precipitated 4–6% and anti-enzyme gammaglobulin precipitated 8–10% of the total input counts (about 20,000 dpm) in the assay mixture. In Exp. II, the corresponding values were 6–12% with normal and 8–17% with anti-enzyme gammaglobulin. Net GS dpm precipitated was calculated by subtraction of the radioactivity precipitated by normal gammaglobulin from that precipitated by anti-enzyme gammaglobulin.

* Concentrations of polysomes and gammaglobulins were determined on the basis of absorbance as follows: Polysomes-A¹%₁cm at 260 nm = 160; gammaglobulin-A¹%₁cm at 280 nm = 14.

† Glutamine synthetase is relatively high in aspartic and glutamic acids (7); the specific radioactivity of these amino acids is higher in the [³H]aminoacid mixture (NET 250, New England Nuclear Corp.) than in the uniformly labeled [¹⁴C]aminoacid mixture (NEC 445); this difference accounts for the greater total radioactivity in the polysomes from induced retina than in the control. This result is consistent with previous findings (4). The NET 250 preparation was used in subsequent experiments because of its lower price.

for practically complete separation of the polysomes from the soluble proteins, which banded over the 1 M sucrose layer; it also reduced by 50–80% the proportion of monosomes in the purified polysome preparation.

For fractionation of polysomes, the postmitochondrial supernatant was layered over 10–30% sucrose gradients and spun for 105 min at 27,000 rpm in a Spinco SW27 rotor; 1-ml fractions were collected with an Isco model D fractionator equipped with a UV-analyzer. The entire polysomal profile was divided into five groups, and the pooled fractions from each group were concentrated by sedimentation over 5 ml of 2.5 M sucrose in an SW 27 rotor at 27,000 rpm for 4 hr. The polysomes were collected from the interphase by puncturing the tubes as described above. They were dialyzed overnight against TKMH and then used in the immunoprecipitation tests.

Preparation and Purification of Gammaglobulins. Glutamine synthetase purified from chicken neural retina (7) was used as the immunogen for the enzyme antiserum. Rabbits were

injected with 0.75 mg of the enzyme in Freund's complete adjuvant and with three boosters of 0.5 mg each at 15-day intervals. Gammaglobulin was prepared from normal serum and from antiserum by two precipitations with ammonium sulfate (added to 40% saturation). The precipitates were dissolved in phosphate–saline [10 mM potassium phosphate buffer–0.9% NaCl (pH 7.1)], using half the original serum volume, and dialyzed overnight against the same buffer.

For removal of RNAse, the gammaglobulin solutions were passed through DEAE–carboxymethyl cellulose and Sephadex G-100 columns (6). From the eluant, gammaglobulin was concentrated by precipitation with ammonium sulfate; the resulting preparations did not measurably degrade polysomes after 30–60 min of exposure at 0–4°. We refer here to the gammaglobulin obtained from the antiserum against glutamine synthetase as *anti-enzyme gammaglobulin*, and to that from normal serum as *normal gammaglobulin*.

Immunoprecipitation. For measurements by immunoprecipitation of total amount of newly-made enzyme in the cells, labeled retinas were sonicated in Tyrode's solution (1 ml per two retinas); 100,000 × g supernatants were prepared, filtered through Millipore filters, and used for immunoprecipitation (5). The reactions were done in 0.4-ml plastic tubes, in a total volume of 0.35 ml; the reaction mixture contained, in addition to aliquots of supernatants and gammaglobulin, 50 μg of bovine-serum albumin, 0.5% of Triton-X, and 0.5% of sodium deoxycholate (6). The antigen–antibody complexes were spun at 15,000 × g for 2 min in a Beckman model 152 microfuge and washed twice with phosphate–saline containing the above detergents. The final precipitates were transferred into counting vials with three changes (0.3 ml each) of NCS solubilizer (Nuclear Chicago); the bottom of the tube was cut off and was also placed in the vial. Radioactivity was determined in a toluene-based scintillation fluid with a Nuclear Chicago (Mark II) counter.

For the reaction of polysome-bound nascent enzyme chains with antibody, the procedure of Palmiter et al. (8) was used with modifications. In general, to 0.5–1.0 absorbance units of purified polysomes in 0.5–1.0 ml of TKMH, the following materials were added sequentially at 30-min intervals, at 4°: 0.01 ml (12 μg) of anti-enzyme gammaglobulin, 0.025 ml of 100,000 × g supernatant from sonicate of nonlabeled retina containing 1.0 unit of glutamine synthetase (equivalent to 12 μg of antibody), and 60 μg of anti-enzyme gammaglobulin. In controls, anti-enzyme gammaglobulin was substituted by normal gammaglobulin. After an additional 30 min, the reaction mixture was layered over 2 ml of 0.8 M sucrose in TKMH buffer in 15-ml Corex tubes and spun at 18,000 rpm in a Sorvall centrifuge for 30 min. The precipitates were washed twice in phosphate–saline with detergents and transferred into counting vials with NCS solubilizer.

Assays. Glutamine synthetase assay and protein determinations were as described (3).

RESULTS

To determine if polysomes carrying nascent chains of glutamine synthetase could be precipitated by anti-enzyme antibody, we labeled control and induced retinas for 10 min with a mixture of [¹⁴C]aminoacids or of [³H]aminoacids, and the total polysomes prepared from these tissues were subjected to immunoprecipitation. The data in Table 1 show that poly-

somes carrying nascent enzyme chains can be preferentially precipitated by antibodies directed against the antigenic determinants of the enzyme. The radioactivity precipitated specifically by the anti-enzyme gammaglobulin, i.e., net glutamine synthetase dpm (the difference between amounts of radioactivity precipitated by anti-enzyme and normal gammaglobulin) was about 3-times higher for induced polysomes than for the corresponding control. Taking the amount of nascent enzyme on the polysomes as indicative of the number of polysomes engaged in enzyme synthesis, the above difference implies that the rate of enzyme synthesis in the induced retina is, at this time point, at least 3-times greater than in the noninduced control. This result assumes that in both cases the rates of peptide-chain elongation and termination are equal.

The amount of polysome-bound nascent enzyme was then compared with that of released enzyme accumulated in the cells during the same period. These amounts should not differ significantly if the antibody reactions with polysome-bound nascent enzyme chains and with the complete enzyme molecules are equally effective, and if the rate of enzyme release from polysomes is similar in control and induced retina. Retinas were labeled with [³H]aminoacid mixture for 10 min; 100,000 × g supernatants were prepared from sonicates of these retinas, and the amount of labeled enzyme was determined immunochemically. The results (Table 2) showed that within the same interval, accumulation of newly-made enzyme in the induced retina is 4 to 5-times greater than in the noninduced tissues; thus, this value is somewhat higher than the 3-fold increase detected by immunoprecipitation of polysomes (Table 1). The most likely explanation is that the completed and released enzyme molecules react more effectively with the anti-enzyme gammaglobulin than the incomplete polysome-bound nascent enzyme, which undoubtedly contains fewer antigenic determinants. Other possibilities, e.g., differ-

TABLE 2. *Immunochemical determination of pulse-labeled glutamine synthetase (GS) in supernatants from noninduced and induced retina*

Source of supernatant	Total cold Cl₃CCOOH-insoluble dpm/mg of protein	Net GS dpm precipitated/mg of protein	Ratio of GS dpm induced/control	GS dpm as % of total protein dpm
Noninduced retina	60,000	775		1.2
			4.4	
Induced retina	88,235*	3397		3.8

Noninduced and induced retinas were labeled for 10 min with 12.5 μCi/ml of [³H]aminoacid mixture. Each sample was sonicated in Tyrode's solution; 100,000 × g supernatants were prepared and immunoprecipitated. The radioactivity precipitated by normal and anti-enzyme gammaglobulin represented 2–3% and 4–6%, respectively, of the total input counts (about 25,000 dpm) in the assay mixture. GS specific activity (expressed as μmol of γ-glutamylhydroxamate formed per mg of total protein per hr) was 0.44 in noninduced and 1.45 in induced retinas.

* The reasons for the greater total radioactivity in the polysomes from induced retina relative to that in the noninduced control are explained in the footnote to Table 1.
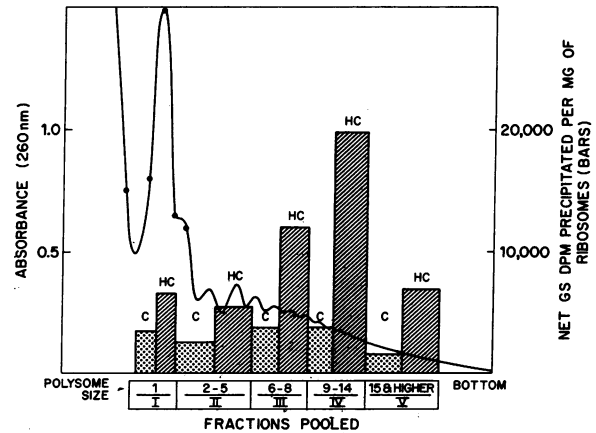

FIG. 1. Immunoprecipitation of fractionated polysomes. Non-induced and induced retinas (48 each) were labeled with 12.5 μCi/ml of [³H]aminoacid mixture, and total polysomes were fractionated in 10–30% sucrose gradients. Equal amounts of polysomes (0.5 absorbance units) from each fraction were immunoprecipitated with anti-enzyme gammaglobulin. (Controls were treated with normal gammaglobulin.) The absorbance profile is represented by the *solid line*. From it, polysome sizes were estimated by counting the peaks. Designation of polysome sizes higher than 12 is approximate. Net glutamine synthetase (GS) dpm (*bars*) refers to radioactivity precipitated by anti-enzyme gammaglobulin minus normal gammaglobulin. *C*-control (noninduced); *HC*-induced.

ences between control and induced tissue in the rates of peptide termination or release, are not excluded. Table 2 also shows that of the total proteins accumulated during the 10-min pulse period, glutamine synthetase represents about 1% in the control retina and 4% in the induced retina.

The reactivity of the polysome-bound nascent enzyme with the antibody made it possible to determine by immunoprecipitation the size-group of polysomes with the longest enzyme peptide, i.e., those involved in completion of its synthesis. Polysomes from control and induced retinas were fractionated in sucrose gradients, and the fractions were pooled into five groups (Fig. 1). These pooled fractions were concentrated, and each was reacted with normal and anti-enzyme gammaglobulin to measure the amount of immunoprecipitable radioactivity. The results showed that the maximum amount of immunoprecipitable counts were obtained from Group-IV polysomes comprising 9–14 ribosomes (Fig. 1). Accordingly, polysomes of this size group carry the longest chains of nascent enzyme and are involved in completion of its synthesis. The reason for immunoprecipitable counts in other regions of the polysomal profile may be due, in part, to some degradation of the polysomes during their handling, and in part to the presence of partially completed enzyme chains on the smaller polysomes. A similar distribution of radioactivity on both sides of the main peak was observed also in studies with F(ab')₂ antibody fragments for precipitation of polysomes synthesizing gammaglobulin (9).

Palacios *et al.* (6) reported that the polysomes that synthesize ovalbumin in chick oviduct cells can be identified by their preferential binding of anti-ovalbumin gammaglobulin labeled with ¹²⁵I relative to their binding of ¹²⁵I-labeled anti-bovine-serum albumin gammaglobulin. Ovalbumin constitutes as much as 60% of the total protein in the cells that make it (8), while glutamine synthetase repre-

sents less than 1% of the total protein content of the retina. Considering this difference, we attempted to determine if binding of iodinated gammaglobulin to polysomes could be used in our system to corroborate the immunochemical identification of the polysomes involved in the synthesis of glutamine synthetase.

Polysomes from induced retina were incubated for 30 min with (a) normal [^{125}I]gammaglobulin or (b) anti-enzyme [^{125}I]gammaglobulin, or (c) nonlabeled anti-enzyme gammaglobulin followed by 30 min of incubation with anti-enzyme [^{125}I]gammaglobulin. The distribution of radioactivity in the polysomes was examined after their fractionation in sucrose gradients (Fig. 2, *bottom*). Analysis of the amounts of ^{125}I-radioactivity bound to the polysomes in the region of Fractions III–V of Fig. 1 showed a significant difference between those treated with normal and anti-enzyme [^{125}I]gammaglobulin; this difference [(b) minus (a)] was maximal in the polysomal region corresponding to 11–13 ribosomes (Fig. 2, *top*), i.e., in the size group that showed the greatest reactivity with anti-enzyme gammaglobulin (see Fig. 1). The specificity of this reaction was further supported by the control provided by (c) in which the sites available for binding with anti-enzyme gammaglobulin were first saturated with an excess of the unlabeled anti-enzyme gammaglobulin and then exposed to the ^{125}I-labeled anti-enzyme gammaglobulin; in this case, the amount of [^{125}I]radioactivity bound to polysomes comprising 11–13 ribosomes was again significantly lower than that bound by the direct reaction of polysomes with anti-enzyme [^{125}I]gammaglobulin [Fig. 2; (b) minus (c)]. It should be pointed out that unlike in the work with ovalbumin (6), in the present experiments there was considerable nonspecific binding of [^{125}I]gammaglobulins throughout the entire polysomal profile for reasons that could not be ascertained; however, since the greater binding of the isotope by the polysomes comprising 11–13 ribosomes was significant and reproducible, these results support the conclusion derived
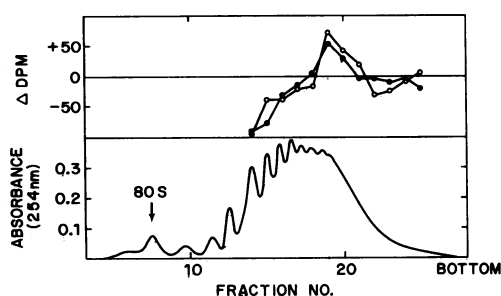

Fig. 2. Binding of [^{125}I]gammaglobulins* to polysomes purified over 2.5 M sucrose. Aliquots of polysomes (7 absorbance units) from induced retinas were incubated at 4° as follows: (a) with 12 μg of normal [^{125}I]gammaglobulin for 30 min; (b) with 12 μg of anti-enzyme [^{125}I]gammaglobulin for 30 min; (c) with 60 μg of nonradioactive anti-enzyme gammaglobulin for 30 min, followed by 12 μg of anti-enzyme [^{125}I]gammaglobulin for 30 min. The absorbance profiles in all three cases were similar to that shown at the *bottom*. dpm represents radioactivity: [(b) − (a)] O——O; [(b) − (c)] ●——●.

* Iodinated gammaglobulins were prepared (8) by incubation of normal or anti-enzyme gammaglobulin with 0.1 ml of Na ^{125}I, 0.25 ml of lactoperoxidase (0.8 mg/ml), and 0.25 ml of H$_2$O$_2$ (90 μM). RNase was removed as described in *Methods*.

from the immunoprecipitation experiments that polysomes of this size group are involved in completion of enzyme synthesis.

DISCUSSION

The specific reactivity of polysome-bound nascent glutamine synthetase chains with anti-enzyme gammaglobulin can be used for preferential precipitation and identification of polysomes that are active in synthesis of the enzyme molecule. Polysomes synthesizing specific proteins have been previously identified by different methods in several specialized and favorable systems. In reticulocytes, which make predominantly hemoglobin, the major polysome species is a ribosome-pentamer (10). Myosin-synthesizing polysomes are exceptionally large and sediment much faster than the rest (11). Polysomes for the synthesis of β-galactosidase (12) and catalase (13) have been identified by enzymatic activity of the nascent proteins attached to them.

Immunochemical procedures have so far been used successfully only for identification of polysomes in cells that make large amounts of a major protein, e.g., gammaglobulins in mouse myeloma cells (9) or ovalbumin in chick oviduct cells (8). Identification of polysomes for proteins constituting minor components of the total cell protein would be very useful for studies on mechanisms of differentiation and especially of tissue-specific enzyme inductions. The results presented here show that immunological precipitation of polysomes carrying specific nascent polypeptide chains offers a useful approach. Recent methodological advances in this area (8, 9) have enabled us to adapt this technique to identification of polysomes involved in synthesis of glutamine synthetase in embryonic neural retina. Using purified polysomes and RNase-free anti-enzyme gammaglobulin, we precipitated polysomes involved in the synthesis of this enzyme with a high degree of specificity, even though it constitutes less than 1% of the total proteins and less than 5% of the newly-made proteins in the induced retina.

That the above results are not due to nonspecific reactions is evident from the following: (a) There is practically no detectable labeling of ribosomal RNA or ribosomal proteins during a 10-min pulse with [^{3}H]aminoacid mixture (14, 8). (b) The radioactivity precipitated by the anti-enzyme gammaglobulin per mg of ribosomes peaked in fraction IV (polysomes with 9–14 ribosomes) of the polysomal profile (Fig. 1) and did not increase in the larger polysomes. (c) In the peak region, the radioactivity precipitated from the induced retina polysomes was 3-times higher than that precipitated from the control retina polysomes. (d) Within fraction IV, polysomes containing 11–13 ribosomes showed greater binding of anti-enzyme [^{125}I]gammaglobulin than of normal [^{125}I]gammaglobulin. (e) Retinal glutamine synthetase consists of eight identical subunits with a molecular weight of 42,000 each (7); assuming 365 amino acids for the subunit, the corresponding mRNA should contain about 1100 nucleotides; with an internucleotide distance of 3.4 Å, the estimated length of the mRNA would be about 3740 Å. With an interribosomal distance of 300–350 Å (10), polysomes comprising 10–13 ribosomes would be expected to accommodate a monocistronic mRNA for a subunit of glutamine synthetase; and, in fact, this size-group of polysomes showed the maximum reactivity with the anti-enzyme gammaglobulin. This conclusion agrees with previous identification of these polysomes by incorpora-

tion of [^{14}C]aspartate, an amino acid abundant in glutamine synthetase (4) and by evidence concerning the size of mRNA for this enzyme (15).

There is evidence (8, 9, 13) that immunoprecipitation of polysomes yields a complex that includes the mRNA. Thus, the above procedures may be useful in this and other systems in isolation, characterization, and analysis of mRNA for quantitatively minor, but functionally important, proteins.

1. Moscona, A. A. (1971) in *Biochemistry of Cell Differentiation*, eds. Monroy, A. & Tsaneo, R. (Academic Press, London and New York), Vol. 24, pp. 1–23.
2. Moscona, M., Frenkel, N. & Moscona, A. A. (1972) *Develop. Biol.* **28**, 229–241.
3. Moscona, A. A., Moscona, M. & Saenz, N. (1968) *Proc. Nat. Acad. Sci. USA* **61**, 160–167.
4. Sarkar, P. K. & Moscona, A. A. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2308–2311.
5. Alescio, T. & Moscona, A. A. (1968) *Biochem. Biophys. Res. Commun.* **34**, 176–182.
6. Palacios, R., Palmiter, R. D. & Schimke, R. T. (1972) *J. Biol. Chem.* **247**, 2316–2321.
7. Sarkar, P. K., Fischman, D., Goldwasser, A. & Moscona, A. A. (1972) *J. Biol. Chem.* **247**, 7743–7749.
8. Palmiter, R. D., Palacios, R. & Schimke, R. T. (1972) *J. Biol. Chem.* **247**, 3296–3304.
9. Delovitch, T. L., Davis, B. K., Holme, G. & Sehon, A. H. (1972) *J. Mol. Biol.* **69**, 373–386.
10. Rich, A., Warner, J. R. & Goodman, H. M. (1963) *Cold Spring Harbor Symp. Quant. Biol.* **28**, 269–285.
11. Heywood, S. M., Dowben, R. M. & Rich, A. (1967) *Proc. Nat. Acad. Sci. USA* **57**, 1002–1009.
12. Kiho, Y. & Rich A. (1964) *Proc. Nat. Acad. Sci. USA* **51**, 111–118.
13. Uenoyama, K. & Ono, T. (1972) *J. Mol. Biol.* **65**, 75–89.
14. Sarkar, P. K. & Moscona, A. A. (1971) *Develop. Biol.* **26**, 187–200.
15. Schwartz, R. (1972) *Nature New Biol.* **237**, 121–125.