Subcellular Compartmentation of Albumin and Globin Made in Oocytes under the Direction of Injected Messenger RNA

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Summary

The Xenopus oocyte can be used to study the nature and specificity of the translational and post-translational systems of a normal living cell. We describe experiments combining messenger RNA microinjection and subcellular fractionation. Total Xenopus liver RNA directs the synthesis of albumin and vitellogenin contained within membrane vesicles; similarly, guinea pig mammary gland mRNA codes for membrane-bound protease-resistant milk proteins. In contrast, iodinated albumin protein injected into oocytes remains in the supernatant fraction, as does globin made on mRNA. The information encoded in the albumin messenger is therefore sufficient to specify synthesis of a membrane-bound product; moreover, this highly efficient coupled translation-processing system may be of use in the study of the transfer of newly made proteins across membranes.

A significant proportion (up to 20%) of newly made oocyte proteins enter a light membrane fraction, and many remain there. We speculate that these vesicles represent part of an important storage system.

Introduction

Some of the proteins made in eucaryotes are transferred across cellular membranes, a process which involves synthesis on ribosomes attached to the rough endoplasmic reticulum (Palade, 1975). Such proteins are destined for secretion, for transport to other cellular organelles or for insertion into the noncytoplasmic face of cell membranes. The signal hypothesis (Blobel and Sabatini, 1971; Milstein, et al. 1972; Blobel and Dobberstein, 1975a, 1975b) states that affinity of the N terminal region of the nascent chain for the endoplasmic reticulum specifies membrane transfer of the newly made polypeptide. Alternative theories invoke specific mRNA-membrane (Cardelli, Long and Pitot, 1976) or mRNA-ribosome-membrane (Baglioni, Bleiburg and Zauderer, 1971) interactions.

We show that when injected into oocytes, the messenger RNA coding for a secreted protein directs the synthesis of a vesicularized product. The

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physical history (Faiferman et al., 1973; Branes and Pogo, 1975) of the messenger is therefore unimportant: the sequence alone contains all the information needed for membrane transfer. We find that the signals involved in this process are neither cell type- nor species-specific. Thus the oocyte is one of the few systems (Lane, Marbaix and Gurdon, 1971) that will both translate membrane-associated messengers efficiently, and package and process the products correctly. This frog cell can therefore be used to assay membrane-bound messengers, to prepare highly labeled processed products and to test theories of membrane transfer of proteins.

A surprisingly large proportion (up to 20%) of newly made oocyte proteins are found within vesicles after a pulse label. We speculate that synthesis and compartmentation of this distinct subclass of proteins are important functions of the oocyte, which is a cell specialized for the accumulation of products for use later in development (Davidson, 1968; Chase and Dawid, 1972; Adamson and Woodland, 1974; Merriam and Hill, 1976). Pulse-chase experiments suggest that the vesicle fraction purified from oocytes represents part of an intracellular storage system.

Results

Subcellular Fractionation of Oocytes Synthesizing Secretory and Nonsecretory Proteins under the Direction of Injected Messenger

The nature and specificity of the signals coding for membrane transfer of newly made proteins can be studied by introducing messengers for secretory proteins into oocytes. Microinjection of albumin mRNA, albumin protein, globin mRNA and milk protein mRNAs provides a test of the hypothesis that information present in the messenger, but only made available transiently following translation, is alone sufficient to define intracellular destination. Moreover, injected mRNAs also serve as probes, revealing the functions of membrane fractions isolated from oocytes.

Oocytes were injected with total male Xenopus liver RNA and incubated overnight before labeling for 3 hr with ^{35}S-methionine. Sucrose gradient centrifugation of a homogenate revealed two major optical density bands, designated heavy (H) and light (L), well separated from the supernatant fraction which constitutes the top of the gradient. When applied to a discontinuous gradient, the light fraction (L) splits into a light (L/L) and a heavy (L/H) band; similarly, the heavy fraction (H) yields a light (H/L) and a heavy (H/H) band. Figure 1 shows gel electrophoresis of albumin immunoprecipitates of cell fractions from RNA-injected and control oocytes. Relatively little albumin is found in the super-
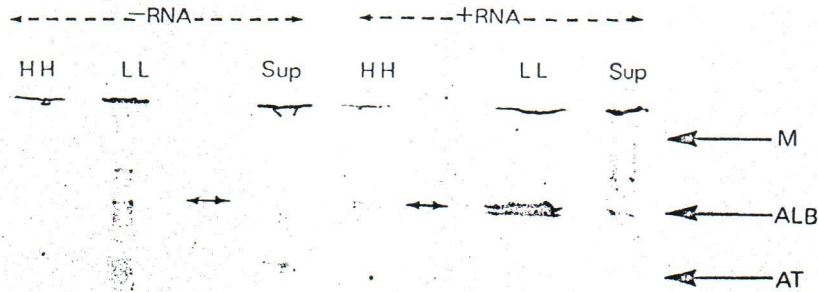
Figure 1. Albumin Made under the Direction of Injected Liver RNA Purifies with the Light Membrane Fraction

Total Xenopus liver RNA (10 mg/ml) was injected into oocytes, which were then left to incubate for 16 hr. The cells were labeled with 300 μCi of ^{35}S-methionine (1.5 mCi/ml) for 3 hr, while a group of 160 uninjected oocytes was treated similarly. Both batches were homogenized and fractionated; aliquots from the supernatant ($^1/_{10}$ by volume), light (L/L) and heavy (H/H) bands ($^1/_4$ by volume) were mixed with anti-albumin antibody and purified Xenopus albumin. The immunoprecipitates formed were run on 10% gels; arrows denote the positions of the internal albumin marker, which electrophoreses with the characteristic doublet present primarily in the L/L fraction of RNA-injected oocytes. The double arrows ($\leftrightarrow$) denote external frog albumin markers (ALB; 74,000 daltons); other markers include actin (AT; 42,000 daltons) and myosin (M; 200,000 daltons). The albumin doublet is also visible in other cell fractions; after allowing for aliquot size, densitometry reveals that 90% of the newly made albumin is present in the L/L, 4% in the H/H and 6% in the supernatant fraction. Other experiments confirm that the majority (>50%) of the albumin is present in the L/L fraction. Cell fractions from uninjected controls are labeled RNA.

natant or H/H fractions; the L/L fraction contains over 90% of the newly made albumin (as shown by densitometry), even though it is the supernatant that contains most (85%) of the newly synthesized protein. [The L/H and H/L fractions contained little radioactivity and were not analyzed; if the initial separation of light (L) and heavy (H) bands is poor, however, some of the newly made albumin may be found in the L/H band.]

Figure 2 shows that a similar experiment performed with reticulocyte polysomal RNA gives the opposite result. Newly made globin cannot be detected in any of the membrane fractions. [The strongly labeled band present in the ribosome fraction (Experimental Procedures) does not co-electrophorese with globin; nonetheless, it is a striking feature and perhaps worthy of study given the possible existence of inactive ribosomes within the

oocyte (Woodland, 1974).] The more specific technique of carboxymethyl cellulose chromatography (Table 1) also fails to reveal globin in the L/L or H/H fractions. Thus the results with globin messenger suggest that only the products of membrane-associated messengers can enter the light membrane fraction. In support of this idea, we find that as shown in Figure 3, total female Xenopus liver RNA directs the synthesis of a 210,000 dalton product (identified as vitellogenin by immunoprecipitation; data not shown) that after a 3 hr labeling period is found in the L/L fraction. The identity of the product was confirmed by two-dimensional (O'Farrell, 1975) gel electrophoresis (C. D. Lane, L. Wangh and W. M. Hunter, unpublished observations).

One can argue, however, that for example, the albumin found in the L/L fraction came from some specific yet artifactual adsorption effect. Microin-
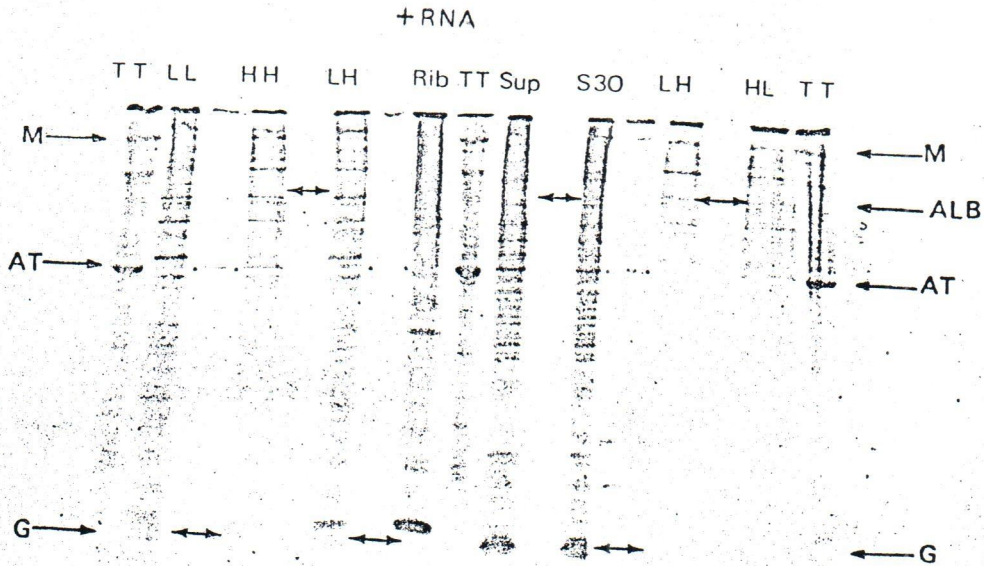
+RNA

Figure 2. Globin Made under the Direction of Injected Reticulocyte RNA Is Found in the Supernatant

The experiment described in Figure 1 was repeated using rabbit reticulocyte as opposed to Xenopus liver RNA. Aliquots of the various cell fractions were applied directly to a 12.5% gel. Arrows (→ and ↔) mark the position of globin (G; 16,000 daltons), actin (AT; 42,000 daltons), albumin (ALB; 74,000 daltons) and myosin (M; 200,000 daltons). The actin marker used was faintly labeled (dots marking the extremeties of the band), thereby enabling one to discriminate between the dark bands in the L/L fraction that run either side of the marker and the dark supernatant band that electrophoresis with actin. Globin is only detectable in the supernatant fraction of RNA-injected oocytes; control oocytes (not shown) are identical, except that the globin band is absent. Slots labeled S30 and Rib refer to post-mitochondrial supernatant and ribosome fractions, respectively, prepared as described in Experimental Procedures.

Table 1. Carboxymethyl Cellulose Chromtography of Cell Fractions from Oocytes Injected with Reticulocyte RNA

Cell Fraction	cpm Globin Region of Column Eluate[a]	
	+ RNA[b]	− RNA[b]
Supernatant	51,000	10,000
Light/Light (L/L) Fraction	4,400	3,000
Heavy/Heavy (H/H) Fraction	594	(Not analyzed)

[a] After subtraction of endogenous background, assumed to be linear.

[b] Results normalized to equal total TCA counts applied per column sample.

The table shows that globin made under the direction of injected messenger is confined to the supernatant. Cell fractions (isolated in the experiment described in the legend to Figure 2) were mixed with marker rabbit hemoglobin (40 mg), and globin was prepared. Carboxymethyl cellulose columns eluted with a gradient of sodium phosphate made up in 8 M urea were used to separate the rabbit globin [which elutes as two overlapping peaks: β-globin in the front peak, α- and β_2-globin in the second (C. D. Lane, unpublished results)] from the majority of the oocyte proteins. Radioactivity in the globin region of the supernatant fraction is significantly above the background of endogenous synthesis. Chromatography of cell fractions from controls confirmed the approximately linear nature of the endogenous background.

jection of iodinated Xenopus albumin helps to rule out this possibility; 2 or 16 hr after injection, <0.2% of the radioactivity can be recovered from the light fraction, yet as shown in Figure 4, most of the label is still in intact albumin molecules. Specific activity calculations show that the I^{125} albumin is within less than an order of magnitude of the specific activity of the albumin made following messenger injection (Eppig and Dumont, 1972; Lane and Knowland, 1975), and the experiment therefore constitutes a reasonable control. Thus entry into the light fraction appears to require translation or some event that takes place soon afterwards.

Nonetheless, it is still possible to explain the results obtained in terms of adsorption artifacts, since newly made secretory proteins might have some special affinity for the light membrane fraction. Protease digestion experiments can rule out this possibility and, more importantly, show that secretory proteins made under the direction of injected messenger are contained within vesicles. Oocytes were injected with guinea pig mammary gland mRNA, incubated overnight and then labeled with ^{35}S-methionine for 4 hr. Uninjected control
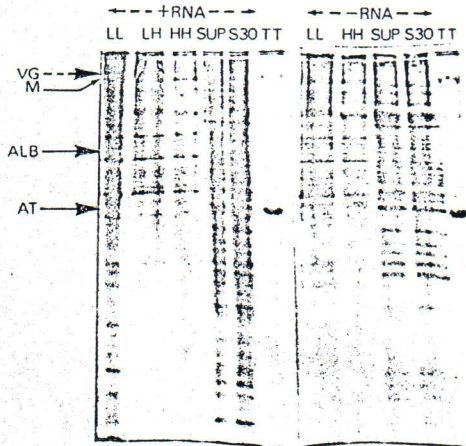
Figure 3. Total Female Xenopus Liver RNA Directs the Synthesis of a Vitellogenin-Like Species That Purifies with the Light Membrane Fraction

The experiment described in Figure 1 was repeated using female as opposed to male total liver RNA. Aliquots of the various cell fraction (light-light (LL), light-heavy (LH), heavy-heavy (HH), gradient supernatant (SUP), whole oocyte (S30) supernatant (30,000 g at 4°C for 10 min) and tadpole tail (TT) marker were applied directly to a 10% gel. The broken arrow marks the high molecular weight species (210,000 daltons approximately) that appears in the L/L fraction of RNA-injected but not of control oocytes. Gel markers include actin (AT; 42,000 daltons), frog albumin (ALB; 74,000 daltons), myosin (M; 200,000 daltons) and vitellogenin (VG; 210,000).

oocytes were treated similarly. The light-light (L/L) membrane fraction was prepared, and aliquots were treated with proteases (trypsin plus chymotrypsin) or detergents (deoxycholate plus Triton X-100) or a combination of the two. Figure 5 shows gel electrophoresis of L/L fractions, from control and RNA injected oocytes, after various enzyme and detergent treatments. The bands made under the direction of injected mammary gland mRNA are resistant to proteases except in the presence of detergents, while detergents alone have no effect (although α-lactalbumin is resistant even in the presence of detergents). Similarly, the proteins made on endogenous oocyte messengers are only susceptible in the presence of detergents. Newly made albumin found in the L/L fraction of RNA injected oocytes is also resistant to proteases (data not shown). Protease resistance argues strongly (Blobel and Dobberstein, 1975a) against adsorption artifacts and suggests that nearly all the labeled proteins of the L/L fraction are within detergent-soluble vesicles. Immunoprecipitation is not required to reveal the products of the highly active mammary gland mRNA; however, specific antibodies can be used to identify three caseins and α-

lactalbumin as the major product coded for by the guinea pig messenger (C. D. Lane and R. K. Craig, unpublished observations). Gel electrophoresis of whole membrane and whole supernatant fractions, followed by densitometry, shows that 60-80% of the newly made milk proteins recovered are found in the L/L fraction; in contrast, injected I^{125}-labeled casein and α-lactalbumin are confined to the supernatant fraction.

The vesicular nature of the L/L fraction, deduced from experiments with injected albumin and milk protein messengers, is confirmed by electron microscopy. Thus Figure 6 shows that the light (L) band obtained from the first gradient contains mitochondria, a few yolk platelets, a dense granular matrix and abundant smooth vesicles. The heavy band (H) is rich in yolk platelets and also contains much granular matrix. Discontinuous gradient centrifugation of the light (L) band separates smooth vesicles (L/L) from much of the contaminating material (H/L); less pure smooth vesicles (L/H) are separated from yolk and granular matrix when the heavy band (H) is recentrifuged. Electron microscopy reveals structures within the whole oocyte (Figure 6d) that could give rise to smooth vesicles; these include rough as well as smooth endoplasmic reticulum, since the former can be stripped of ribosomes during centrifugation or fixation. Moreover, biochemical evidence suggests that the L/L fraction contains some membrane-associated ribosomes. Detergent treatment (0.5% NP40) of this fraction released 80S particles that constituted, in terms of optical density at 260 nm, 5% of the free ribosomes (Experimental Procedures). If detergent was omitted, no 80S peak was seen in the 10-50% sucrose gradient. In oocytes labeled for 30 min, about 15% of the ^{35}S-methionine incorporated into the L/L fraction was released associated with the ribosome-like particles. Marker enzyme studies add little further weight to the evidence that the vesicles of the light fraction are derived from the endoplasmic reticulum, although the results obtained are consistent with this speculation. Antimycin A-resistant (Tolbert, 1974) membrane-associated NADH-ferricyanide reductase is found in the endoplasmic reticulum (Ohlsson and Jergil, 1977), although it is not a stringent marker enzyme. Such enzyme activity is found in the L/L (0.24 μmoles ferricyanide reduced per min per mg protein), L/H (0.27 units), H/L (0.03 units) and H/H (0.03 units) fractions, and in the former two fractions, there may be as much as a 4-fold enrichment over the crude homogenate (0.06 units). Glucose-6-phosphatase and NADP-ferricyanide (or cytochrome c) reductase, endoplasmic reticulum markers in liver cells, were not present in detectable amounts in oocytes (see Experimental Procedures). Cytochrome c oxidase activity confirmed that mito-
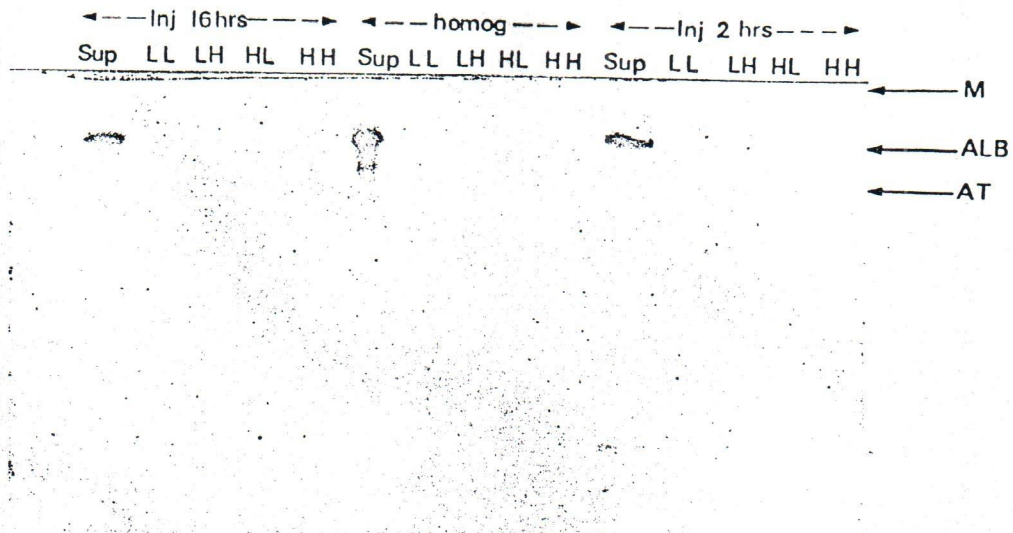
◄ — —Inj 16hrs— — —► ◄ — —homog— — ► ◄ — —Inj 2 hrs — — — ►

Sup LL LH HL HH Sup LL LH HL HH Sup LL LH HL HH

—— M

—— ALB

—— AT

Figure 4. ^{125}I-Albumin Injected into Oocytes Is Restricted to the Supernatant

Purified Xenopus albumin was iodinated to a specific activity of approximately 5×10^6 dpm/μg (each cell in a batch of 40 receiving about 2 $\times 10^4$ dpm) and injected into oocytes which were then fractionated after either 2 hr (gel slots 1–5) or 16 hr (slots 6–10) of incubation. Gel analysis was also performed on subcellular fractions from oocytes homogenized with ^{125}I-albumin (slots 11–15). Markers include albumin (ALB; 74,000 daltons), actin (AT; 42,000 daltons) and myosin (M; 200,000 daltons). Radioactivity is confined to the supernatant fraction [<0.2% is found in the light membrane (L/L) fraction], and the major band electrophoresis with marker frog albumin, although slight degradation is seen, especially in the oocytes incubated for 16 hr.

chondria contaminated all four membrane fractions, but each contained 10-fold less activity (per mg of protein) than the crude homogenate. 5'-nucleotidase activity was not detectable, as would be expected given the small amounts of plasma membrane present in oocytes.

Compartmentation of Newly Synthesized Oocyte Proteins

Newly made endogenous proteins that are contained within vesicles are of interest given the role of the oocyte in the storage of materials employed later in development and its use for the assay of membrane-bound messenger RNAs. Batches of oocytes were labeled with ^{35}S–methionine for 5 or 10 min or for 6 hr, homogenized separately and then fractionated on sucrose gradients. Figures 6a–6c show the distribution of incorporated radioactivity at different incubation times and also the effect of a 30 min chase of cold methionine.

At short times, a surprisingly large proportion (>50% in some experiments) of newly made proteins are associated with the light (L) and heavy (H) bands; label in the heavy band certainly equals and even exceeds label in the light band. With longer incubation times, or after chasing, radioactive proteins predominate in the light band and contribute about 20% of total incorporated label; SDS-gel electrophoresis shows a binding pattern quite different from that of the supernatant fraction. Electron microscopy and mRNA injection experiments have already suggested that this subpopulation represents protein secreted within vesicles. Further purification of the light membrane fraction by discontinuous gradient centrifugation yields an L/L fraction that (after our standard 3 hr labeling period) contains most of the newly made membrane-associated proteins and constitutes as much as 12 ± 4% of total incorporated radioactivity. Moreover, some breakage and loss of vesicles probably occur, resulting in an underestimate. Figures 2 and 3 [and the results of two-dimensional gel electrophoresis (O'Farrell, 1975)] show that the newly made proteins of the L/L fraction form a distinct subpopulation and, therefore, that cytoplasmic contamination must be rather small. Further gels of the L/L fraction showed that the proteins present were resistant to concentrated mixtures of trypsin and chymotrypsin (2–150 mg/ml per enzyme incubated at 4°C for 3.5 hr), unless detergents (1% Triton, 0.1% deoxycholate) were present; this argues against contamination by the cytosol and suggests that the newly made proteins are inside vesicles. The possibility that this subclass was of mitochondrial origin was ruled out by showing that mitochondria prepared (by the efficient method of Chase and Dawid,
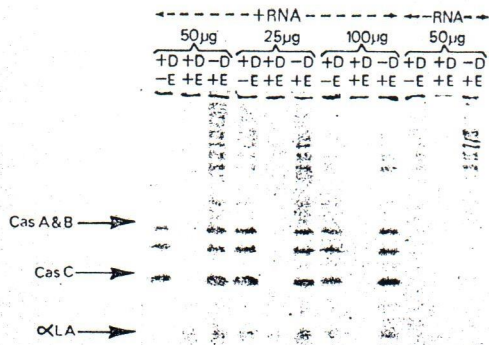
Figure 5. Milk Proteins of the Light Membrane Fraction Made under the Direction of Guinea Pig Mammary Gland mRNA Are Resistant to Proteases, Except in the Presence of Detergents

The experiment described in Figure 1 was repeated, except that mammary gland poly(A)-rich RNA (500 μg/ml) was used; the L/L fraction from both RNA injected and control oocytes was divided into aliquots, which were incubated with enzymes (chymotrypsin + trypsin, labeled + E) or detergents (0.2% deoxycholate + 1.5% Triton X-100, labeled + D) or both (+D+E). The experiment was performed at three different enzyme concentrations—25, 50 and 100 μg/ml. Results for control oocytes (−RNA) are shown at only one enzyme concentration (50 μg/ml).

1972) from oocytes labeled with ^{35}S–methionine contain <2% of the radioactivity associated with the L/L fraction. Moreover, gel analysis revealed a different banding pattern. The possibility that the vesicle fraction, or the newly made proteins present in it, arose from the follicle cells that surround the oocyte was ruled out by subcellular fractionation of 5 hr pulse-labeled defolliculated oocytes. The L/L fraction contained 9% of the incorporated radioactivity, similar to the proportion (8.6 ± 1.25%) found in the same fraction from controls. Moreover, removal of the follicle cells did not alter the gel banding pattern of the L/L fraction. The pattern yielded by the L/L fraction, although quite different from that of the supernatant, is quite similar if not identical to the L/H, H/L and H/H banding

patterns, suggesting that similar kinds of vesicles are present, albeit in very different amounts, in all these fractions.

The possibility that the smooth vesicle fraction represents a medium term repository for a particular subclass of oocyte proteins was investigated by pulse-chase experiments. Oocytes were injected with rabbit reticulocyte polysomal RNA and, after an overnight incubation, were labeled for 3 hr with ^{35}S–methionine (newly made globin serves as a supernatant protein marker). The oocytes were transferred to unlabeled Barth X for 4 hr and then placed in medium containing 10 mM methionine for 40 hr. Figure 7 shows gel analysis of cell fractions obtained from normal as well as RNA injected oocytes. The banding pattern is hardly altered by the chase (compare Figures 2 and 3, slots L/L; running samples on adjacent gel slots also fails to reveal any significant differences). As expected, globin is found exclusively in the supernatant. The chase conditions are probably effective, since incorporation ceases <2 hr after transfer to methionine-containing medium, and yet all known oocyte proteins are stable for several days (data not shown). In quantitative terms, the proteins of the vesicle fraction appear reasonably constant in their location, since at the end of the chase, they still constitute a significant (although somewhat variable) fraction (10 ± 7%) of total incorporated radioactivity. Such a figure is probably an underestimate, since recovery of vesicles may be low, especially with (certain batches of) oocytes incubated for several days in vitro.

The stability within the vesicle fraction of secretory proteins made on injected messenger has been investigated (C. D. Lane and R. K. Craig, unpublished observations) using mammary gland mRNA. The milk proteins formed are not chased out of the light (L/L) membrane fraction by an overnight (16 hr) incubation in cold methionine. Thus all our results are consistent with the view that the vesicles of the light membrane fraction are part of an important storage system.

Discussion

The Xenopus oocyte can be used to study the con-

Figure 6. Electron Microscopy and ^{35}S-Methionine Labeling of Oocyte Cell Fractions

Batches of 40 cells were incubated in ^{35}S–methionine for 5 min (a), 10 min (b) and 6 hr (c); another batch was chased with 10 mM methionine after a 10 min labeling period (b; O——O). The oocytes were homogenized and then fractionated (90 min at 155,000 g_{Av} at 4°C) on an 11 ml 10–50% sucrose gradient layered above a 2 ml cushion of 2 M sucrose (Experimental Procedures). Two bands were visible and were recorded (optical density at 260 nm) (- - -). The distribution of incorporated radioactivity (●——●) was measured, and the heavy labeling of the light (L) and heavy (H) bands prompted further purification by equilibrium centrifugation in step gradients. The light band splits up into further bands—light/light (L/L) and light/heavy (L/H); the H band also splits up into two bands (H/L and H/H) of densities similar to L band subfractions. Electron microscopy of cell fractions from a batch of unlabeled oocytes [e(L), f(H), g(L/L), h(L/H) and j(H/H); magnification 15,000×] shows that some further purification has been achieved. (d) shows a small section of whole oocyte cytoplasm: mitochondria, rough endoplasmic reticulum, smooth vesicles, part of a dense yolk platelet, golgi cisternae and other structures are visible (magnification 10,000×).
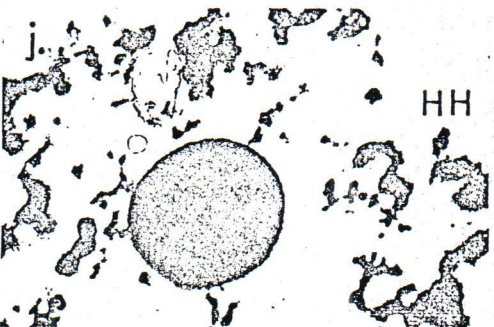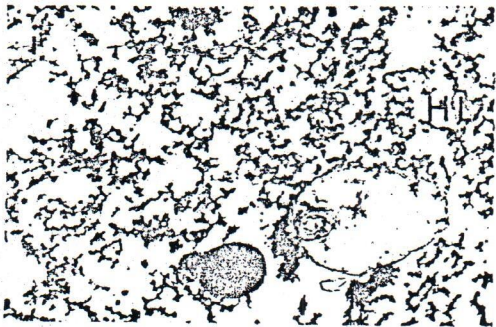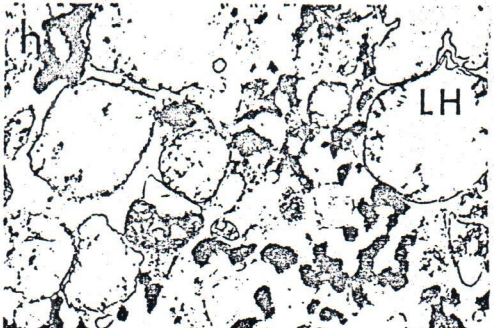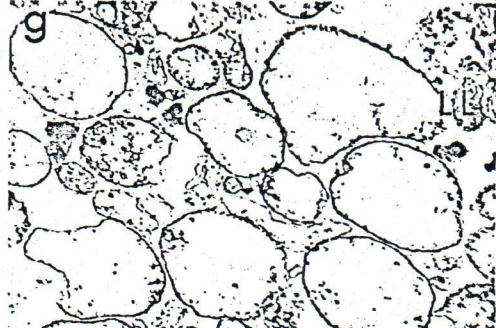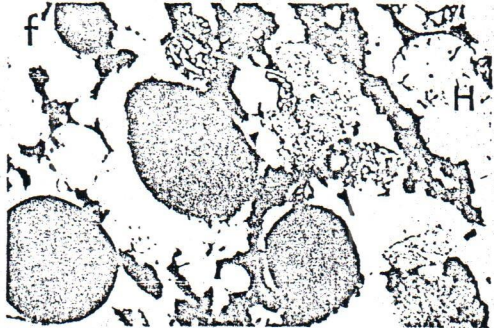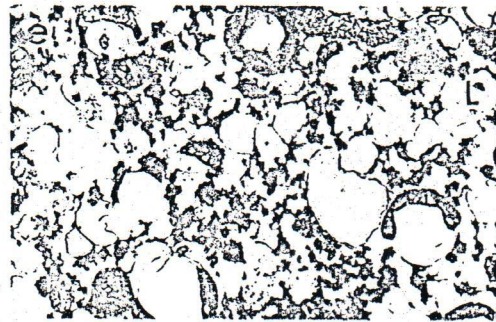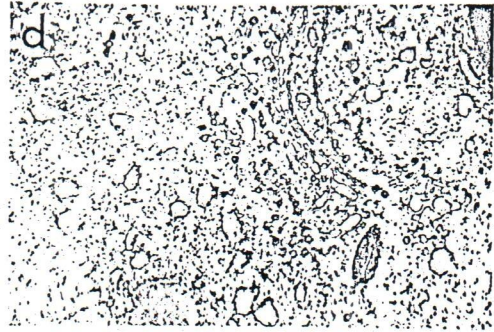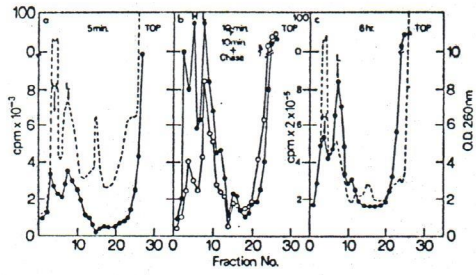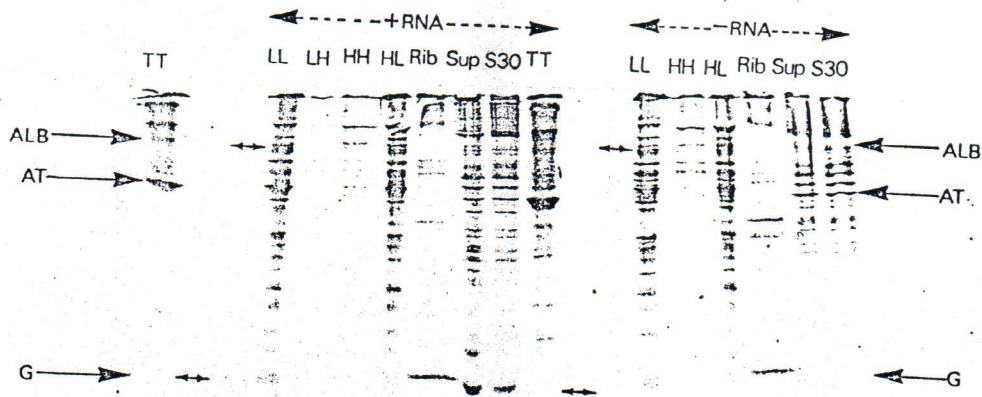
Figure 7. Gel Analysis of Subcellular Fractions from Oocytes Labeled with Methionine and Then Chased with Cold Methionine for 40 Hr

The experiment described in Figure 2 was repeated, except that after labeling with ³⁵S–methionine for 3 hr, the cells were washed extensively and incubated for a further 4 hr; they were then transferred to medium containing 10 mM methionine, and after 40 hr of incubation, the oocytes were fractionated. Slots labeled +RNA depict gel analysis of pulse-chased oocytes programmed with globin messenger, the globin made serving as a supernatant protein marker; slots labeled –RNA show analysis of fractions from uninjected oocytes. Markers include globin (G; 16,000 daltons), albumin (ALB; 74,000 daltons), actin (AT; 42,000 daltons) and myosin (M; 200,000 daltons).

trol and specificity of the translational and post-translational systems within a living cell. Results based on the microinjection of 24 different kinds of messenger RNA (Knowland, 1974; Bergeron, Berridge and Evans, 1975; Eggitt and Scragg, 1975; Lane and Knowland, 1975; Van der Donk, 1975; Vassart et al., 1975; Yip, Hew and Hsu, 1975; Reynolds, Premkumar and Pitha, 1975; Chan, Kohler and O'Malley, 1976; Hew and Yip, 1976; Lane, 1976; Nickol et al., 1976; Salden, Asselbergs and Bloemendal, 1976) suggest that once a free all translational and nearly all post-translational events take place automatically; thus many of the oocyte systems are neither cell type- nor species-specific. The oocyte, however, does not seem to carry out proteolytic changes associated with secretion (Kindas-Mugge, Lane and Kreil, 1974) and may not be capable of processing preproinsulin (Yip et al., 1975). We show that the oocyte can recognize (directly or indirectly) that information in Xenopus liver cell mRNA or guinea pig mammary gland mRNA which directs the synthesis of a vesicularized product. The encoded information is alone sufficient, and the physical history (Faiferman et al., 1973; Branes and Pogo, 1975) of the membrane-associated messenger does not seem to have an obligatory role in the manufacture of a membrane-bound product. Results obtained with partially purified vitellogenin mRNA and the ob-

served processing of immunoglobin light chains made in oocytes under the direction of purified messenger (Faust, Vassalli and Rungger, 1973) suggest that encoded information within just one kind of mRNA can specify vectorial discharge, since here the information cannot be provided by translation of other injected mRNAs.

Total female Xenopus liver RNA directs the synthesis of a large protein (molecular weight approximately 210,000 daltons), identified as vitellogenin using specific antibodies, which is found in the light vesicle fraction; the initial translation product probably moves from here to the yolk platelet fraction, the postulated site of processing and the known respiratory of the cleavage products (Berridge and Lane, 1976). Cell fractionation (Jared, Dumont and Wallace, 1973) suggests that absorbed vitellogenin follows such a route. The identity of the 210,000 dalton product and its transient presence in the L/L fraction have been confirmed by immunoprecipitation and by two-dimensional gel analysis of cell fractions from pulsed and chased oocytes. (Such results do not, however, establish the site of cleavage of vitellogenin.) In contrast, milk proteins made under the direction of injected mRNA remain within the light vesicle fraction (C. D. Lane and R. K. Craig, unpublished observations), but since it is not known that vitellogenin and milk proteins are even secreted within the same type of

vesicle, one cannot speculate as the discriminating mechanisms involved.

Newly made oocyte proteins are also segregatated, and as shown in Figure 2, the actin markers present indicate that most (>90% after allowing for aliquot sizes) of the oocyte actin is confined to the supernatant fraction, as one might expect (Pollard and Weihing, 1974). Two-dimensional gel electrophoresis (O'Farrell, 1975) of cell fractions confirms this finding. One problem in the use of the oocyte as a coupled transcription-translation system (Gurdon, de Robertis and Partington, 1976) is the high background of newly made supernatant proteins; genes coding for vesicularized products can now be studied with much less interference from endogenous synthesis.

The signal hypothesis would predict that it is the nascent polypeptide chains that are recognized by sites on the endoplasmic reticulum of the frog cell. The oocyte is one of the few (Blobel and Dobberstein, 1975b) coupled translation-processing systems that reinitiate with added messenger and is perhaps the only efficient one. Thus it is an ideal system for testing the signal hypothesis and other theories (Baglioni et al., 1971; Cardelli et al., 1976), as well as for studying post-translational events as they occur in the living cell. Isolation of the rough endoplasmic reticulum is required before factors affecting the localization of messenger as well as product can be studied in the oocyte system. Detergent treatment releases a small population of ribosomes from the light (L/L) membrane fraction, suggesting the presence of at least some rough endoplasmic reticulum. Smooth vesicles predominate in electron micrographs of the L/L band (but stripping of rough membranes could occur during centrifugation or fixation). Marker enzyme studies are consistent with our speculation that the vesicles of the light (L/L) membrane fraction are derived from the endoplasmic reticulum. Messenger localization studies should rule out the remote possibility that secretory protein mRNAs are translated on free as opposed to bound ribosomes, and that the completed, as opposed to the nascent, polypeptide is transferred across the membrane. Moreover, fractionation of oocytes injected with wheat germ system mammary gland messenger products argues against this possibility, since intact precursor polypeptides (such as pre-α-lactalbumin) are formed, yet little of the labeled material introduced (<2%) enters the L/L fraction, even after an overnight incubation (C. D. Lane and R. K. Craig, unpublished observations).

The oocyte is specialized for storage of components for use later in development. It seemed probable that some of these—for example, mitochondrial proteins (Chase and Dawid, 1972)—would re-

quire synthesis on membrane-bound ribosomes, and indeed, that vesicularization might prove to be a convenient storage mechanism for as yet unidentified components. Moreover, cell fractionation studies could test these ideas. Labeling oocytes for a few hours with ^{35}S-methionine shows that a significant amount of protein synthesis is directed in the short term toward manufacture of membrane-bound products; chasing for 1 day produces no great qualitative changes in the labeled proteins within vesicles, and quantitative changes are on average small (although there is considerable variation). The constant location within the vesicles is confirmed by two-dimensional gel electrophoresis of pulsed and chased oocyte L/L fractions, which reveals a remarkable similarity in labeled protein patterns between the two samples. Milk proteins made under the direction of injected messenger also remain within the light vesicles for at least 24 hr (C. D. Lane and R. K. Craig, unpublished observations). Thus we speculate that the light membrane fraction purified from oocytes is derived from the endoplasmic reticulum and represents part of an important storage system.

Experimental Procedures

Injection and Subcellular Fractionation of Oocytes

Oocytes were injected and incubated in Barth X solution (Lane et al., 1971), usually for 16 hr, before being labeled for 3 hr with 300 μCi of ^{35}S-methionine (150–450 Ci/mmole at a concentration of 1.5 mCi/ml). Chase experiments involved transfer of the oocyte to Barth X for 5 hr, followed by incubation in medium containing 10 mM methionine for up to 40 hr. Defolliculated oocytes were prepared manually.

Cell fractionation was usually carried out on batches of at least 40 oocytes, thus enabling the yolk and light membrane bands to be seen by eye. The cells were washed 3 times with Barth X and 3 times with T buffer [0.05 M KCl, 0.01 M Mg Acetate, 0.02 M Tris (pH 7.6)], then mixed with 0.5 ml of T buffer (made 2 × 10⁻⁴ M in cycloheximide) and given 10 strokes with a loose-fitting glass homogenizer. 11 ml 10–50% w/v sucrose gradients, having 2 ml cushions of 68% sucrose (all sucrose solutions were made up in T buffer), were layered with 0.5 ml of homogenate. All the above washings and fractionations were carried out at 4°C and as quickly as possible. After centrifugation in the Beckman SV40 rotor (155,000 g$_{Av}$ for 90 min at 4°C), two bands could be seen. (In recent experiments, better separations have been achieved using shorter centrifugation times—130,000 g$_{Av}$ for 60 min.) Following removal of the supernatant (2 ml), the bands were removed in turn and diluted (at least 1:1 by volume) with T buffer; the diluted bands, designated heavy (H) and light (L), were then layered separately on discontinuous gradients (comprising 2.5 ml of 2.0 M sucrose, 1.0 ml of 1.8 M sucrose and 2.5 ml of 1.5 M sucrose; all solutions made up in T buffer).

After centrifugation in the SW40 rotor at 155,000 g$_{Av}$ for 16 hr at 4°C, two bands were again visible, except when a high degree of purification was obtained with the first gradient. The bands, now designated light-light (L/L) and light-heavy (L/H) when they arose from the L fraction, or H/L and H/H if derived from the H fraction, were removed with pasteur pipettes. After dilution with 4 vol of T buffer, the cell fractions were pelleted by centrifugation at 4°C for 60 min at 12,000 g; they were stored at −20°C. Oocyte mitochondria were prepared by the method of Chase and Dawid (1972). The method was checked both by electron microscopy and by cyto-

chrome oxidase assay (Schnaitman and Greenawalt, 1968). Oocyte ribosomes were prepared by spinning homogenate (as described) at 30,000 g for 10 min at 4°C to obtain a supernatant which was fractionated on a sucrose gradient (10–50% gradient with cushion, as described previously). The ribosome region of the gradient was pooled, diluted 1:3 by volume with T buffer and spun at 215,000 $g_{A \lambda}$ for 90 min.

Biochemical Analysis
Immunoprecipitation
Pelleted light and heavy band fractions were resuspended in 100 μl of phosphate-buffered saline [PBS; 0.15 M NaCl, 3 mM KCl, 2 mM KH_2PO_4 and 8 mM Na_2HPO_4] containing 1% w/v NP40, and after clarification (300 g at 4°C for 10 min), one aliquot (5 μl) was removed for spotting onto filter discs (Mans and Novelli, 1961), while another aliquot (30 μl) was mixed with double-strength SDS sample buffer (ready for gel analysis of total incorporated radioactivity). Then PBS containing NP40 was added to the remaining liquid, yielding a final volume of 400 μl and a final concentration of 1% w/v NP40. After clarification (3000 g at 4°C for 10 min), 2.5 μl of phneylmethylsulfonylfouride (15 mg/ml in ethanol), 4 μl of Xenopus albumin solution (2 mg/ml) and 40 μl of rabbit anti-albumin serum were added to the supernatant. Immunoprecipitates were allowed to form overnight (at 4°C). The oocyte supernatant, which constitutes the top (1–2 ml) of the 10–50% gradient, was treated similarly: aliquots (100–200 μl) were mixed with PBS-NP40 (to yield a final volume of 400 μl and a final concentration of 1% NP40) and were then immunoprecipitated. The precipitates from supernatant and membrane fractions were washed and then electrophoresed on SDS gels (Berridge and Lane, 1976).
Carboxymethyl Cellulose Chromatography of Globin
Carrier rabbit hemoglobin was mixed with cell fractions, and globin was prepared (Lane, Gregory and Morel, 1973) and chromatographed by the method of Clegg, Naughton and Weatherall (1965), modified in that carboxymethyl cellulose columns were eluted with a gradient of 2 × 250 ml of 5 mM Na_2PHO_4 (pH 6.7) – 40 mM Na_2HPO_4 (pH 6.9) made up in 8 M urea. Column fractions were collected and counted.
Enzyme Assays
Glucose-6-phosphatase (de Duve, et al., 1949), 5′-nucleotidase (the routine method of Widnell and Unkeless, 1968), cytochrome c oxidase (Schnaitman and Greenawalt, 1968), NADH-ferricyanide reductase, NADPH-ferricyanide (or cytochrome c) reductase [Hrycay and O'Brien (1974), but in the presence of antimycin A, as described by Tolbert (1974)] assays were performed on fresh as well as frozen cell fractions.

Preparation of RNA

Xenopus liver RNA and rabbit reticulocyte polysomal RNA were prepared by the method of Berridge and Lane (1976). Aliquots were assayed for biological activity in the nuclease-treated reticulocyte lysate (Pelham and Jackson, 1976). Guinea pig mammary gland poly(A)-rich RNA (50 $\mu g/ml$), prepared by the method of Craig et al. (1967), was a gift from Dr. R. K. Craig.

Preparation of I^{125}-Albumin
Xenopus albumin was prepared (Berridge et al., 1976) and iodinated (Hunter and Greenwood, 1962) to a specific activity of 5 × 10^6 dpm/μg.

Electron Microscopy
Whole oocytes and pelleted cell fractions were fixed overnight at 4°C in 1% gutaraldehyde-1% paraldehyde made up in 0.1 M phosphate buffer (pH 7.2); samples were dehydrated, embedded, sectioned (LKBI Ultrotome) and then viewed under a Philips EM 201 microscope after staining with uranyl acetate and Reynolds lead citrate.

Source of Materials
Adult Xenopus laevis, obtained from the South African Snake Farm (Fish Hoek, Cape Province), were kept at 20°C and fed chopped heart twice weekly. ^{35}S-methionine (150–450 Ci/mM) was obtained from the Radiochemical Centre (Amersham, England). Nonidet P40 came from BDH Chemicals (England).

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