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Sequestration and Turnover of Guinea-Pig Milk Proteins and Chicken Ovalbumin in *Xenopus* Oocytes

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The stability and distribution of proteins within the living cell can be studied using *Xenopus laevis* oocytes. Microinjection of messenger RNAs and secretory proteins, followed by cell fractionation, shows that transfer of ovalbumin and milk proteins across intracellular membranes of the oocyte only occurs during their synthesis. Thus milk protein primary translation products, made in the wheat germ cell-free system, when injected into oocytes remain in the cytosol and are not recovered within membrane vesicles. Such miscompartmentalized primary milk proteins are rapidly degraded ($t_{1/2}$ 0.6 ± 0.1 h). In contrast, processed milk proteins, extracted from oocytes injected with mammary gland RNA, are relatively stable when introduced into the cytosolic compartment ($t_{1/2}$ α-lactalbumin 20 ± 8 h, casein A 6 h, casein B 4 h, casein C 8.3 h). The primary ovalbumin product is also stable ($t_{1/2}$ 22 ± 9 h).

Indirect evidence that rapid degradation of miscompartmentalized milk protein primary translation products may occur *in vivo* was obtained by the injection of massive amounts of ovalbumin and milk protein mRNA. Under these conditions there is no accumulation of primary milk protein translation products, but a polypeptide resembling the unglycosylated ovalbumin wheat germ primary product can be detected in the cytosol. Only the glycosylated forms of ovalbumin are found in the oocyte membrane vesicle fraction.

We discuss the roles played by the presence of detachable signal sequences and the absence of secondary modifications in determining the rate of degradation of primary translation products within the cytosol.

The molecular mechanisms involved in the export of proteins from cells are beginning to be understood [1] and the first event in the segregation of secretory proteins appears to be the initiation of protein synthesis on free polyribosomes [39]. The nascent peptide formed is believed to interact with a membrane receptor, resulting in the vectorial discharge of the growing polypeptide across the membrane and into the lumen of the endoplasmic reticulum. The specificity for this interaction is presumed to stem from the amino-terminal sequence of the nascent chain [2–4] and, with the one known exception of ovalbumin [5], secreted proteins are synthesized *in vitro*, in the absence of endoplasmic reticulum, as precursor proteins containing 15–30 additional amino-terminal residues [6]. These 'signal' sequences are rich in hydrophobic residues; generally (3,7,39] but not invariably [8–10] they are removed *in vivo* before completion of the nascent chain.

Abbreviation. Phosphate-buffered saline (0.12 M NaCl, 3 mM KCl, 3 mM KH_2PO_4, 8 mM $NaHPO_4$), P_i/NaCl.

Although there are cell-free systems which show tight coupling between polypeptide synthesis, segregation and core glycosylation of membrane and secretory proteins [11,12], such systems are not ideal [13] for studying the control of these processes, nor for examining the stability of the secretory polypeptides formed. Little is known of the mechanisms involved in protein turnover *in vivo*, largely because cell-free systems do not appear to reflect intact cells [14]. Thus it is of interest to study the synthesis and stability of primary and processed translation products in *Xenopus* oocytes, in particular to compare the stabilities of milk proteins with and without either their signal sequences or secondary modifications [12,15–17,31,38]. Ovalbumin, a secreted glycoprotein, lacks a detachable signal sequence and makes another interesting comparison.

Oocytes sequester secretory proteins made under the direction of injected messenger [18], and in this paper we show that vesicularization only takes place during synthesis of the nascent polypeptide. Further-

more, we demonstrate that milk protein primary translation products are rapidly degraded in oocyte cytoplasm whilst processed milk proteins are relatively stable, as is the ovalbumin primary translation product. The influence on protein stability of the presence of a detachable signal sequence and the absence of secondary modification is discussed.

MATERIALS AND METHODS

Injection and Incubation of Oocytes

Oocytes were injected and incubated in Barth x solution [19] usually for 16 h, to permit recruitment of the exogenous messenger [20] before being labelled with [^{35}S]methionine (150–450 Ci/mmol). Chase experiments were carried out by first transferring labelled oocytes to Barth x (for about 5 h) and then incubating in medium containing 10 mM methionine. Pipettes calibrated to deliver approximately 350 nl were used for experiments on protein stability: two full pipettes of radioactive protein solution (about 14 oocytes at 50 nl/cell) were used for each time point, and between 5 and 9 time points (including duplicates at zero time) were taken for each stability curve (24 h). Each batch of 14 oocytes was incubated in 200 µl of Barth x containing 10 mM methionine. Oocytes preincubated for about 4 h in 10 mM methionine were used to reduce reincorporation.

Subcellular Fractionation

Two different methods of fractionating oocytes have been used. The first, similar to that described by Zehavi-Willner and Lane [18], is based on sucrose density gradient centrifugation, whilst the second includes the use of appropriate detergent concentrations to solubilize membranous vesicles but only limited amounts of yolk. Thus oocytes were washed twice with Barth x and then twice with T buffer (0.05 M KCl, 0.01 M magnesium acetate, 0.02 M Tris pH 7.6) before being homogenized in 0.5 ml (per 40 oocytes) of T buffer containing phenylmethylsulfonyl fluoride (1.5 µg/ml). In the first method [18] the homogenate is frationated on a sucrose gradient into light (L) and heavy (H) bands: further fractionation of the light band yields an upper light-light (L/L) and a lower heavy-light (H/L) band, whilst the heavy band can be resolved into an upper light-heavy (L/H) and a lower heavy-heavy (H/H) band. This nomenclature applies to our previous work and corrects errors therein.

In the second method the oocyte homogenate (40 oocytes/0.3 ml T buffer) was layered above a 1-ml cushion of 20% (0.59 M) sucrose (in T buffer). After centrifugation at $10000 \times g_{av}$ for 15 min at 4 °C, the top 300 µl (sup.) was collected. This was sometimes combined with the sucrose cushion to ensure quantitative recovery of radioactivity in the supernatant fraction. The pellet was extracted with 200 µl phosphate-buffered saline (0.12 M NaCl, 3 mM KCl, 3 mM KH_2PO_4 and 8 mM $NaHPO_4$) containing 1% (v/v) NP40. Centrifugation ($10000 \times g_{av}$ for 10 min at 4 °C) yielded a detergent supernatant (DS1) and a pellet. Re-extraction of the pellet with a further 200 µl P_i/NaCl/1% NP40 yielded a supernatant (DS2) and a final pellet (P).

Biochemical Analysis

Sample Preparation and Immunoprecipitation. Light and heavy band fractions prepared by method 1 were immunoprecipitated as described by Zehavi-Willner and Lane [18] except that all solutions containing phenylmethylsulfonyl fluoride (1.5 µg/ml). Fractions obtained by the second method were treated similarly. Casein and α-lactalbumin antisera were prepared as described previously [15]. Antiserum raised against ovalbumin was the kind gift of Dr M. Doel. Immunoprecipitates were analysed on gels [20] which were fluorographed by the method of Bonner and Laskey [21].

Preparation of RNA and Protein for Microinjection. Guinea-pig mammary gland poly(A)-rich RNA was prepared, and translated in the wheat germ system, by the procedure of Craig et al. [15]. Poly(A)-rich chicken oviduct RNA, the kind gift of Drs M. Doel and N. Carey, was prepared by the method of Doel and Carey [22]. Cell-free extracts, containing labelled proteins for injection, were frozen, thawed and then dialysed overnight at 4 °C against 1 mM Tris/0.1 mM methionine, pH 7.5; after lyophilization the protein was resuspended, in water, in one-tenth of the original volume of the cell-free translation system. Centrifugation at $10000 \times g_{av}$ for 3 min at 4 °C yielded a supernatant that was subdivided and then stored at −20 °C ready for injection. Labelled oocyte proteins were prepared from cells injected with mammary gland RNA incubated overnight in [^{35}S]methionine. The oocytes were homogenized (10 oocytes/100 µl) in a freshly prepared mixture (1/1/3) of 0.05 M EDTA/0.625 M Tris/0.01 M EDTA pH 6.8, and water. After centrifugation at $3000 \times g_{av}$ for 10 min at 4 °C the supernatant was frozen ready for injection. Caseins were prepared [15], iodinated by the method of Hunter and Greenwood [23], and dialysed against 1 mM Tris/0.1 mM methionine pH 7.5. Lyophilized iodinated proteins were dissolved in one-tenth of their original volume of water.

Measurement of Protein Stability. Oocytes were injected with protein solutions, as described, and incubated in batches for a defined time period in a known volume (200 µl). Duplicate aliquots (25 µl) of incubation medium were taken for measurement of acid-precipitable and total radioactivity. Batches of

14 oocytes were homogenized in 200 µl of P_i/NaCl/1% NP40 containing phenylmethylsulfonyl fluoride (1.5 µg/ml). Duplicate aliquots (10 µl) were taken for counting. Immunoprecipitation was carried out on a supernatant obtained by centrifugation at $10000 \times g_{av}$ for 4 min at 4 °C. Oocytes showing signs of extensive leakage (> 20% of trichloroacetic-acid-precipitable radioactivity) were disregarded. Half-lives were calculated by plotting on semi-logarithmic graph paper the ratio of (acid precipitable radioactivity in the homogenate plus incubation medium) to (total radioactivity in the system), against time. In experiments where there is little variation in the amount of protein injected, plotting the decrease in trichloroacetic-acid-precipitable radioactivity per oocyte versus time yields similar half-lives. Values for putative fast and slow components can be calculated if the stability curve is biphasic: in the present paper we only report half-lives for majority (> 75%) components.

RESULTS

Subcellular Distribution and Stability of Secretory Proteins Synthesized in Xenopus *Oocytes*

The specificity and control of the transfer of newly made proteins across intracellular membranes can be studied by the injection of secretory protein messenger RNAs into oocytes. Milk proteins made in oocytes under the direction of poly(A)-containing mammary gland RNA are found sequestered within membrane vesicles. Thus RNA injected and control oocytes were labelled with [^{35}S]methionine for 4 h, homogenized and vesicle fractions (designated L/L, H/H and L/H) were isolated by sucrose gradient centrifugation. Dodecylsulphate/polyacrylamide gel electrophoresis of oocyte supernatant (cytoplasmic fraction) and vesicles reveals (Fig. 1 A) the preferential accumulation, in the vesicle fractions, of four distinct polypeptides; the synthesis of these four proteins is directed by the injected mammary gland RNA, for they cannot be detected in control oocytes. As shown in Fig. 1 C antibody precipitation of the oocyte subcellular fractions, using a mixture of antisera raised against purified guinea-pig caseins and α-lactalbumin, identifies these products as caseins A, B and C and α-lactalbumin. Densitometery reveals that at least 80% of the newly made milk proteins are associated with L/L membrane vesicles. α-Lactalbumin synthesized in the frog cell electrophoreses with α-lactalbumin from guinea-pigs: casein C from oocytes can only just be distinguished from its mammary gland counterpart, but oocyte caseins A and B can be well resolved from caseins A and B of guinea-pig milk. Such differences probably stem from the inability of oocytes correctly to phosphorylate or glycosylate guinea-pig caseins.

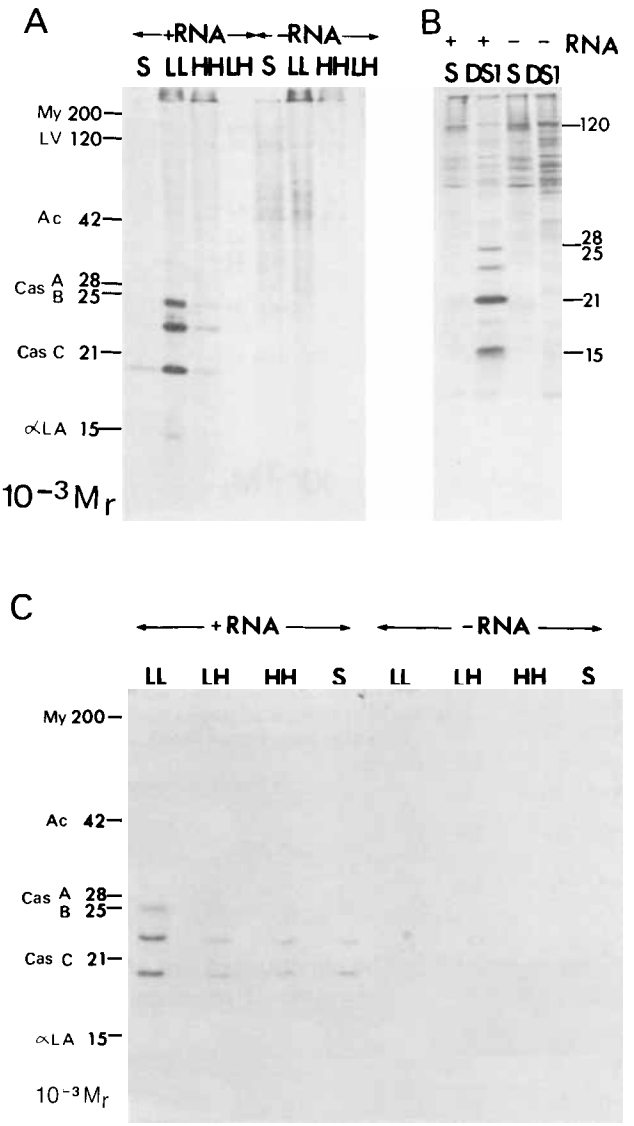

Fig. 1. *Identification and subcellular distribution of milk proteins made under the direction of injected mammary gland RNA.* RNA injected (25 ng/cell) and control oocytes were labelled for 4 h with [^{35}S]methionine and then fractionated by the double gradient (A) or the rapid procedure (B). Aliquots of the cell fractions were run on gels, before (A, B) or after (C) antibody precipitation. The cell fractions shown in (A) and (C) are labelled S (supernatant), LL (light/light band), LH (light/heavy band) and HH (heavy/heavy band), whilst those in (B) are labelled S (supernatant) and DS1 (first detergent supernatant). Molecular weight markers include casein A (CAS A, 28000), casein B (CAS B, 25000), casein C (CAS C, 21000) and α-lactalbumin (αLA, 15000), all prepared from guinea-pig milk: other markers include actin (Ac, 42000), lipovitellin (LV, 120000) and myosin (My, 200000). Cell fractions were prepared from RNA injected (+RNA) and control (−RNA) oocytes

The milk proteins of the L/L band are sequestered within vesicles, for they remain intact, as judged by gel analysis of antibody precipitates, after protease treatment (chymotrypsin and trypsin 15 µg/ml for 3 h at 4 °C) unless detergents (Triton X-100 1.25%, deoxycholate 0.125%) are present; the small amounts

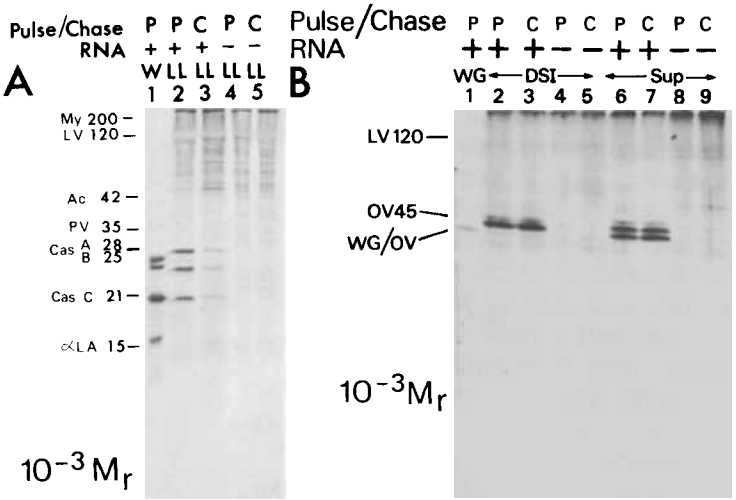

Fig. 2. *The stability and subcellular distribution of milk proteins, and ovalbumin made under the direction of injected messenger RNA.* (A) Oocytes injected with mammary gland RNA, labelled with [^{35}S]methionine for 4 h, transferred to unlabelled medium for 4 h, and then incubated in saline-containing 10 mM methionine for 24 h. Fractionation by the double-gradient procedure both at 4 h and after the full 24-h chase yielded light/light (LL) vesicles: aliquots were then run on a sodium dodecylsulphate gel (slots LL). Mammary gland wheat germ (W) products were run for comparison: the identity and molecular weights of these species are given in Fig. 5. The molecular weight markers shown in (A) include casein A (CAS A, 28000), casein B (CAS B, 25000), casein C (CAS C, 21000) and α-lactalbumin (αLA, 15000), all purified from guinea-pig milk, as well as phosvitin (PV, 35000), actin (Ac, 42000), lipovitellin (LV, 120000) and myosin (My, 200000). (B) An experiment of similar design (pulse 19 h, chase 24 h) performed using oocytes programmed with poly(A)-rich oviduct RNA (1 mg/ml). Cells were fractionated by the rapid procedure which yields supernatant (Sup), vesicle (DS1) and yolk extracts. These fractions were analyzed on gels (data not shown) and immunoprecipitates obtained using anti-ovalbumin antibodies were also analysed, as shown in (B). Immunoprecipitated ovalbumin wheat germ products are shown in the track labelled WG1, and this unglycosylated species is labelled WG/OV. The mobility of ovalbumin from chickens (OV, 45000) is also marked. (A) and (B) show fractions from RNA-injected (+) and control (−) oocytes, and from pulse-labelled (P) or chased (C) oocytes, or cell-free systems

of casein present in the supernatant are not protease resistant. Increasing amounts of processed secretory proteins appear in the supernatant if homogenates are left to stand, suggesting that their presence is caused by vesicle rupture after cell breakage. Recently we have found that adding 10% sucrose to the homogenization medium greatly reduces release of vesicle contents.

A simpler method for obtaining vesicle contents also revealed sequestration of newly made milk proteins. Thus homogenates from RNA-injected and control oocytes were layered over sucrose cushions, and in a single centrifugation step yolk and vesicles were separated from soluble oocyte proteins: vesicles were preferentially solubilized using detergent. Gel analysis of oocytes injected with milk protein mRNA and then fractionated in this manner shows (Fig. 1 B) that most of the newly made milk proteins (> 90%) were present in the vesicle (DS1) extract.

Studies of the stability and subcellular distribution of secretory proteins made in the oocyte (Fig. 2) show that after a pulse of [^{35}S]methionine followed by a cold chase of at least 20 h, milk proteins and ovalbumin are still present, still mainly within the vesicle (L/L or DS1) fractions. Fig. 2 A reveals a decrease in the milk protein content of vesicles, and other experiments have shown a similar loss of sequestered ovalbumin (see Discussion). There is no evidence either in the vesicle or supernatant fractions for primary milk protein translation products, the mobilities of which are known from gel analysis (Fig. 2 A) of wheat germ cell-free system translation products. Even after antibody precipitation casein and α-lactalbumin precursor proteins are not detectable in oocytes.

In contrast, one of the oviduct RNA-directed products, precipitable with anti-ovalbumin antibodies and found exclusively in the oocyte supernatant fractions, does electrophorese with the primary ovalbumin product made in the wheat germ system. Within the oocyte vesicle (DS1) fraction there are two additional forms of ovalbumin which also react with antibody: probably as a result of vesicle breakage, the sequestered forms are also found free in the supernatant. Further analysis, using Con-A-Sepharose, of the different oocyte ovalbumin species shows (Boulton, Craig and Lane, unpublished observations) that only the sequestered ovalbumin products are glycosylated. The formation of unglycosylated ovalbumin in the supernatant provides evidence that miscompartmentalization of secretory proteins may occur in living cells. This presumably reflects ovalbumin synthesis on free polyribosomes, due to the saturation by endogenous or injected mRNAs of putative polyribosome binding sites on the endoplasmic reticulum. As shown in Fig. 3 A miscompartmentalized ovalbumin

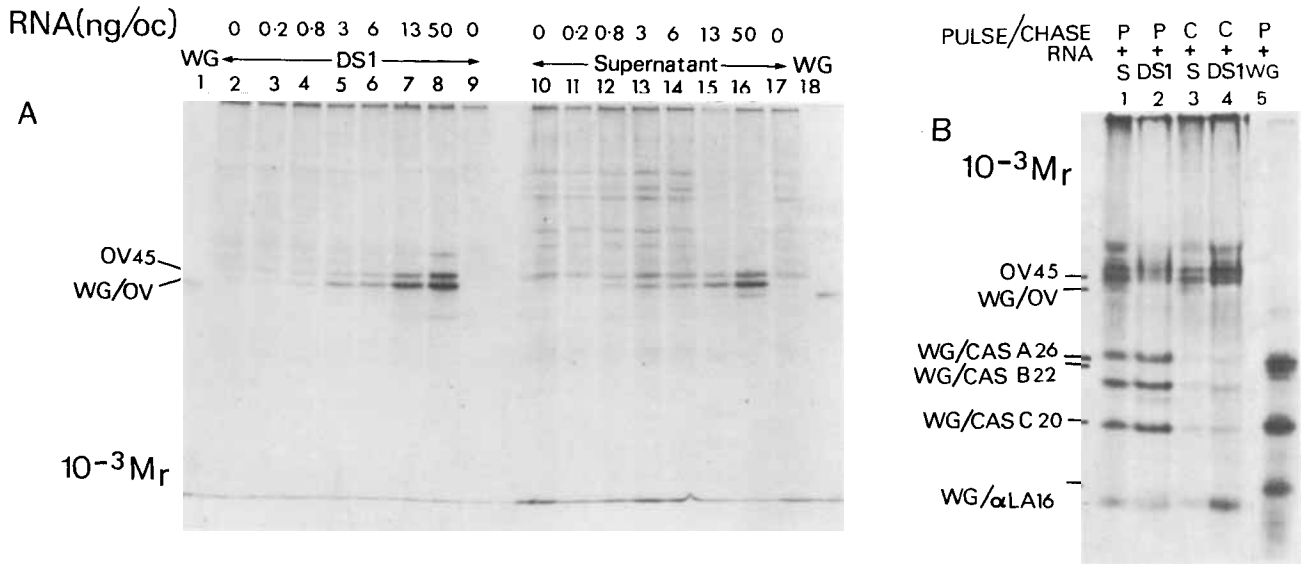

Fig. 3. *Secretory proteins made in oocytes injected with increasing amounts of oviduct mRNA, or with combined mammary and oviduct RNA.* Batches of oocytes were injected with varying amounts of oviduct poly(A)-rich RNA, left overnight and then incubated in [^{35}S]methionine for 4.5 h. After fractionation by the rapid procedure, anti-ovalbumin immunoprecipitates from supernatant and vesicle extract (DS1) were run on a $12^1/_2$% dodecylsulphate gel (A). An anti-ovalbumin immunoprecipitate from a wheat germ system programmed with oviduct RNA is shown in track 1 and also in track 18 (WG), and the unglycosylated ovalbumin species is labelled WG/OV. The mobility of chicken ovalbumin (OV, 45000) is also shown. In the experiment shown in (B) mammary and oviduct poly(A)-rich RNA were mixed and injected in such proportions that each oocyte received a large quantity (20 ng/cell and 25 ng/cell respectively) of both messenger preparations. RNA-injected and control oocytes were incubated with [^{35}S]methionine, pulsed (P) for 20 h and then chased (C) for 20 h with non-radioactive methionine: both pulsed and chased oocytes were fractionated by the rapid procedure and combined anti-casein, anti α-lactalbumin and anti-ovalbumin immunoprecipitates were run on $12^1/_2$% dodecylsulphate gels (B). Products made in the wheat germ system under the direction of mammary gland RNA were run for comparison (track 5), and the previously identified milk proteins are as follows: precasein A (WG/CAS A, 26000), precasein B (WG/CAS B, 22000), precasein C (WG/CAS C, 20000) and pre-α-lactalbumin (WG/α-lactalbumin, 16000). Other markers include chicken ovalbumin (OV, 45000) and wheat germ synthesized ovalbumin (WG/OV)

is readily seen at high mRNA levels; however, densitometry reveals that the ratio of cytosolic to sequestered ovalbumin does not fall markedly at lower levels of injected oviduct RNA, suggesting that endogenous oocyte messengers also play a role in the competition for membrane receptors. In contrast to the results observed with oviduct RNA, there is no accumulation of milk protein primary translation products and even immunoprecipitation of oocytes labelled for a short time (10 min) revealed only modified proteins. Such observations suggest that either the milk protein primary translation products are rapidly degraded when released into the cytosol, or alternatively that the membrane receptor sites are not saturated with polyribosomes synthesizing milk proteins. As a test for the latter (see Discussion), we have mixed and injected milk protein and ovalbumin at concentrations of each mRNA known to be greater than that required for maximal milk protein or ovalbumin synthesis (C. Lane, unpublished observation). The oocytes were then either pulsed with [^{35}S]methionine and then fractionated, or pulsed and then chased with non-radioactive methionine before analysis. Gel electrophoresis of the antibody-precipitable proteins synthesized by the mixed messengers (Fig. 3B) shows that unglycosylated ovalbumin is present in the cytoplasm even after a lengthy chase, whilst the milk protein primary products (typified by the mobility of wheat germ synthesized polypeptides) are absent, at least for the bands where precursor and processed product are well resolved. Nonetheless such negative experiments provide no direct evidence that miscompartmentalized milk proteins are unstable.

Subcellular Distribution and Turnover of Processed and Unprocessed Secretory Proteins Injected into Oocytes

The injection of primary and processed milk proteins provides a direct test of the idea that there is preferential degradation within the cytosol of pre-α-lactalbumin and precaseins. Moreover protein injection combined with cell fractionation permits analysis of the coupling between translation and membrane transfer.

Injection of ^{125}I-labelled α-lactalbumin and ^{125}I-labelled caseins (Fig. 4B) followed 3 h later by subcellular fractionation showed that nearly all the iodinated milk proteins were present in the supernatant fraction, as less than 5% of the trichloroacetic-acid-precipitable radioactivity was associated with the L/L vesicle band. Calculation of the turnover rate, based

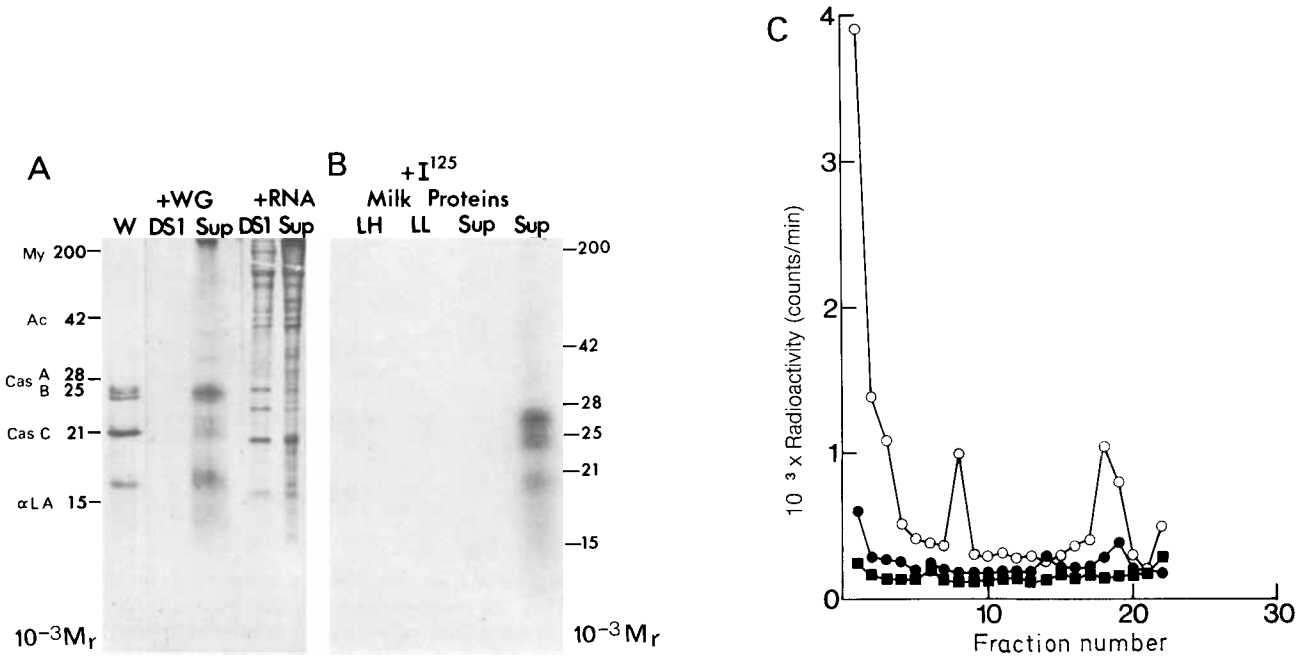

Fig. 4. *Subcellular distribution of iodinated milk proteins and milk protein primary translation products after injection into oocytes.* Oocytes were injected with mammary gland RNA wheat germ products, and after 3 h incubation the cells were separated into supernatant (Sup) and vesicle (DS1) fractions by the rapid procedure. (A) Shows the products before (track W) and after injection (tracks + WG Sup and DS1). For comparison the newly made proteins of [^{35}S]methionine-labelled RNA injected oocytes are shown in the tracks labelled + RNA Sup and DS1. (B) Shows a similar study except that (in separate experiments) ^{125}I-labelled caseins and α-lactalbumin were injected and the oocytes were fractionated by the double-gradient procedure. The light/heavy (LH) and light/light (LL) vesicle fractions of oocytes injected with ^{125}I-labelled α-lactalbumin contained no detectable radioactivity in these two fractions. Both ^{125}I-labelled α-lactalbumin (left-hand track labelled Sup) and ^{125}I-labelled caseins (right-hand track) are found in the supernatant. Molecular weight markers include casein A (CAS A, 28000), casein B (CAS B, 25000), casein C (CAS C, 21000) and α-lactalbumin (αLA, 15000), all prepared from guinea-pig milk, as well as actin (Ac, 42000) and myosin (My, 200000). (C) Sucrose gradient analysis of oocytes (preincubated in 10 mM methionine for several hours) injected with mammary gland wheat germ products and incubated for 18 h. Trichloroacetic-acid-precipitable radioactivity before (○) and after (●) protease treatment (trypsin 300 μg/ml and chymotrypsin 300 μg/ml for 3.5 h at 4 °C) of each gradient fraction. The distribution of trichloroacetic-acid-precipitable radioactivity following injection of control wheat germ products (produced by incubation without RNA) is also shown (■)

on a series of time points followed by densitometry of the iodinated supernatant proteins, showed that all three iodinated caseins (or the iodine linkages *themselves*) were unstable, all having half-lives of about 2–3 h (Table 1). A similar study of the fate of [^{35}S]methionine-labelled primary milk protein translation products showed that after a 3-h incubation some intact antibody-precipitable milk proteins could still be detected albeit at low levels in the supernatant fraction (Fig. 4A). The primary milk protein translation products were prepared using wheat germ cell-free systems [38] and products recovered from the oocyte supernatant fraction retained their characteristic electrophoretic mobilities. There was no evidence for the entry of primary products into vesicles (DS1 extract). Although low levels of trichloroacetic-acid-precipitable radioactivity were associated with the vesicle fraction, almost none was protease resistant (Fig. 4C) at an enzyme concentration (600 μg/ml) known to digest less than 50 % of mRNA-programmed vesicle caseins. Primary milk protein translation products injected into oocytes are rapidly degraded (Fig. 5A and Table 1), having on average half life of 0.6 ± 0.1 h. Half-lives calculated by trichloroacetic acid precipitation (Table 1) are 2–3 times longer than those based on gel densitometry.

Injection of [^{35}S]methionine-labelled ovalbumin synthesized in the wheat germ system showed that unlike the milk protein primary translation products, the ovalbumin primary product is quite stable (Fig. 5B), having a half-life of 22 ± 9 h. In order to exclude various artefactual explanations of the difference, oocytes were injected with an equal mixture (in terms of trichloroacetic-acid-precipitable radioactivity) of primary milk and ovalbumin translation products. Gel analysis of a total detergent extract (Fig. 5C) or of antibody precipitates (Fig. 5D), obtained with a mixture of antisera raised against all four major milk proteins and ovalbumin, showed that over the time course examined, much of the ovalbumin remained resistant to proteolytic digestion, whereas the milk proteins were rapidly degraded.

The results of both protein and mRNA injection studies are consistent with the idea that *in vivo* the transfer of secretory proteins across membranes is a function of the nascent peptide/polyribosome com-

Table 1. *The stability in oocytes of injected proteins as measured by trichloroacetic-acid precipitation and gel densitometry*
Radioactive proteins were prepared, injected into oocytes and their degradation was monitored over a 24-h (or longer) period by trichloroacetic acid precipitation and by gel analysis: by plotting data on semi-logarithmic paper half-lives were calculated (Materials and Methods). Both acid precipitation and gel data were corrected (Materials and Methods) for variation in the total radioactivity injected. The symbols mp^+ and ov^+ denote proteins derived from systems programmed with milk protein or ovalbumin mRNAs, whilst the letters WG and Oc denote the wheat germ or oocyte systems. Radioactive proteins were prepared with or without dialysis and lyophilization. Injected oocytes were homogenized and where indicated (Ab_{ppt}) proteins were immunoprecipitated, and then quantitated by gel densitometry. Other species were identified using gel markers. Numbers in brackets signify the number of experiments used to calculate standard deviations. Some trichloroacetic acid precipitation stability curves were biphasic, but all values recorded in Table 1 refer to majority (> 75% components). Thus no values are given in lines 4 and 5, for in these experiments fast and slow components were present in similar amounts

[^{35}S]Methionine proteins injected into oocyte	Dialysis and lyophilization of protein before injection	Additions to oocyte incubation medium	Measurement of half-life by trichloroacetic acid precipitation	Measurement by gel densitometry	
				species quantified	half-life
			h		h
1. WG/mp^+	+	–	3.1 ± 1.0 (5)	–	–
2. WG/mp^+	+	10 mM Met	2.6 ± 0.9 (3)	caseins A, B, C and α-lactalbumin	0.66
3. WG/mp^+	–	15 mM Met	1.2 ± 0.6 (2)	caseins A, B, C and α-lactalbumin	0.5
4. WG/ov^+	+	10 mM Met	–	ovalbumin	20
				Ab_{ppt}	33
5. (WG/mp^+) + (WG/ov^+) ratio 1:1 (counts/min)	+	10 mM Met	–	caseins A, B, C and α-lactalbumin	0.5
				ovalbumin	13
				caseins A, B, C and α-lactalbumin Ab_{ppt}	0.5
				ovalbumin Ab_{ppt}	20
6. WG/mp^+ (unlabelled)	+	[^{35}S]Met then 10 mM Met	100	–	–
7. Oc/mp^+	–	10 mM Met	–	casein A	6
			55 ± 18 (3)	casein B	4
				casein C	8.3
				α-lactalbumin } Ab_{ppt}	20 ± 8 (3)
8. ^{125}I-labelled caseins (denatured)	+	–	3	caseins A, B, C	2.3
Average values for half-lives					
WG/mp^+				caseins A, B, C α-lactalbumin	0.6 ± 0.1 (3)
WG/ov^+				ovalbumin	22 ± 9 (4)
Oc/mp^+				α-lactalbumin	20 ± 8 (3)

plex: but they also suggest that the primary translation product often contains the structural information not only for vectorial transfer but also for rapid degradation within the cytosol. A rigorous test of this idea requires measurement of the stability, within the cytosol, of the primary and secondary forms of a given protein. Thus oocytes were injected with mammary gland mRNA, and an extract of total [^{35}S]-methionine-labelled proteins was prepared. Labelled proteins having the mobilities of oocyte milk proteins are present in this total extract, and, like the endogenous protein (Fig. 6A) are relatively stable when reinjected into oocytes. However, antibody precipitation is necessary both to establish the presence of milk proteins, and to permit a quantitative estimate of stability (Table 1 and Fig. 6B). α-Lactalbumin with a half-life of 20 ± 8 h (as measured in three separate experiments) is probably more stable than the caseins ($t_{1/2}$ casein A 6 h, casein B 4 h, casein C 8.3 h, measurements based on one experiment); but all the processed products are much more stable than milk protein primary translation products ($t_{1/2}$ 0.6 ± 0.1 h).

DISCUSSION

It is now well established that secretory proteins [1,6] and also certain integral viral proteins [24,25] are synthesized on polyribosomes bound to the endoplasmic reticulum. The formulation [2,26] and subsequent elaboration of the signal hypothesis [3,4], has led to the view that the specificity of this interaction stems from the sequence of the amino-terminus

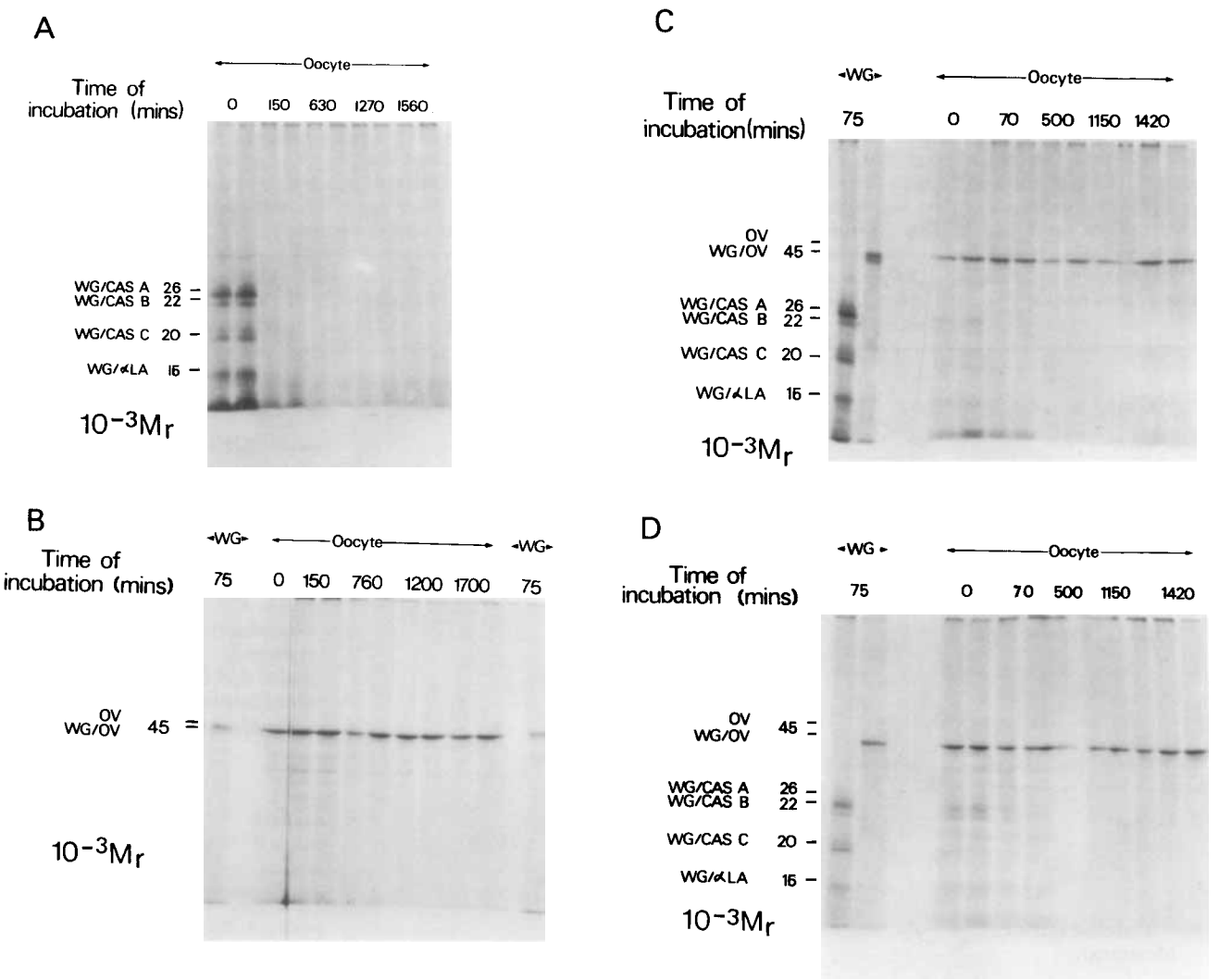

Fig. 5. *The turnover of ovalbumin and milk protein primary translation products after injection into oocytes.* Wheat germ cell-free systems were programmed with poly(A)-rich mammary gland RNA or with poly(A)-rich chicken oviduct RNA, and the [^{35}S]methionine-labelled products obtained were concentrated approximately tenfold by dialysis against 1 mM Tris-HCl, 0.1 mM methionine, pH 7.5, followed by lyophilization and resuspension in water. After clarification ($3000 \times g_{av}$ for 10 min) both preparations contained approximately 30000 *counts* min^{-1} μl^{-1} trichloroacetic-acid-precipitable radioactivity. The ovalbumin and milk protein products were injected separately (A and B), or after mixing (C) in roughly equal amounts in terms of trichloroacetic-acid-precipitable radioactivity. Precautions were taken (Materials and Methods) to inject constant volumes, and batches of oocytes were incubated for the times indicated: they were then homogenized in P_i/NaCl/1% NP40 and a constant amount (10 µl) of each (clarified) homogenate was applied per gel slot (A, B, C). (D) shows gel analysis of immunoprecipitates obtained using mixed anti-ovalbumin, anti-casein and anti-α-lactalbumin antibodies. The symbol WG denotes wheat germ cell-free system products before injection into oocytes, and the previously identified proteins are as follows: precasein A (WG/CAS A, 26000), precasein B (WG/CAS B, 22000), precasein C (WG/CAS C, 20000), pre-α-lactalbumin (WG/αLA, 16000), wheat germ ovalbumin (WG/OV). The mobility of chicken ovalbumin (OV, 45000) is also shown

of the nascent chain, usually from the presence of a detachable hydrophobic signal sequence. Clearly there is no absolute requirement for a detachable sequence, as ovalbumin, a secreted protein, is synthesized and exported without removal of a peptide; nor can there be an obligatory role for long stretches of hydrophobic residues at the N-terminus, for only the first 13 residues of the ovalbumin molecule can be classified as such [5]. Lingappa et al. [27] have shown that in a cell-free system ovalbumin is segregated, core glycosylated and competes for transfer across the endoplasmic reticulum with prolactin, a protein known to be made with a detachable signal peptide. Ohlsson, Lane and Craig (unpublished observations) have shown competition *in vitro* between ovalbumin and milk proteins for entry into *Xenopus* blastulae vesicles.

The selective transfer of proteins across the endoplasmic reticulum and the regulation of this process can be studied in the oocyte by microinjection of

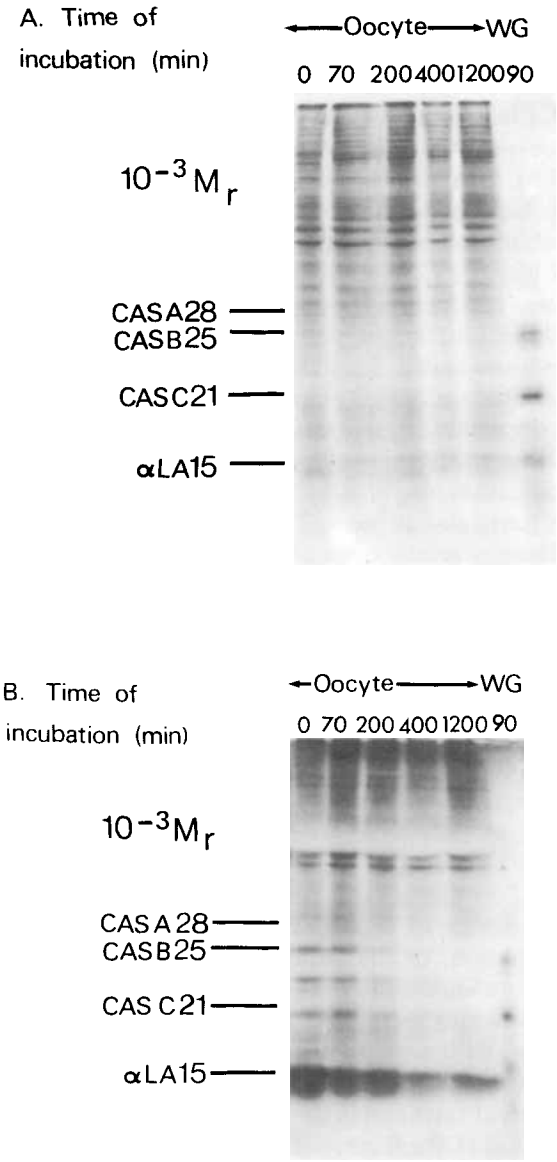

Fig. 6. *The stability of proteins extracted from oocytes injected with mammary gland RNA, reinjected into oocytes.* Oocytes programmed with approximately 50 nl mammary gland RNA (1 mg/ml) were incubated in [^{35}S]methionine for 20 h, homogenized in Tris-EDTA buffer (Materials and Methods), and the supernatant obtained (by centrifugation at $3000 \times g_{av}$ for 10 min) was made 10 mM in methionine before injection into oocytes (preincubated for 3 h in 10 mM methionine). The injected oocytes were incubated for various times and then extracted with detergent (P_i/NaCl/1% NP40); gel analysis of the total extract is shown in (A), whilst analysis of immunoprecipitates, obtained with mixed anti-casein and anti-α-lactalbumin antibodies, is shown in (B). Markers of guinea-pig milk proteins are as follows: casein A (CAS A, 28000), casein B (CAS B, 25000), casein C (CAS C, 21000) and α-lactalbumin (αLA, 15000)

proteins and messengers. We have shown in whole cells, as is true of cell-free systems [28], that the transfer mechanism lacks both species and cell type specificity, for there is sequestration of such diverse proteins as chicken ovalbumin, guinea-pig milk proteins, *Xenopus* albumin and *Xenopus* vitellogenin [18] honey-bee promelittin (Lane and Kreil, unpublished work) and rat albumin (Ohlsson and Lane, unpublished work). We now believe that the membrane systems that give rise to the vesicle fraction are part of a functional secretory pathway and that intracellular storage [18] is at least partly offset by secretion. Thus interferon made under the direction of injected messenger is secreted [29] as are guinea-pig milk proteins [40], chicken ovalbumin and certain endogenous oocyte proteins (Lane, Colman and Craig, unpublished observations). The time-dependent decrease in milk proteins within vesicles (shown in Fig. 2A) has now been correlated with the accumulation of milk proteins in the oocyte incubation medium.

Both core and peripheral [30] glycosylation of sequestered oocyte ovalbumin may occur as judged by elution from Con-A-Sepharose with α-methylmannoside and *N*-acetylglucosamine. However, caseins made in oocytes or cell-free systems do not electrophorese with the glycosylated and phosphorylated caseins isolated from guinea-pig milk [15, 31, 38]. Although oocytes (and the isolated vesicle fraction) contain functional endoplasmic reticulum, oocytes lack appreciable levels of casein kinase, and labelling studies with RNA-injected whole oocytes fail to reveal significant levels of phosphorylated casein (A. P., Boulton, J. Pascall, R. Craig and C. Lane, unpublished work). Thus we believe that lack of phosphorylation contributes to the differences in electrophoretic mobility.

Injection experiments using saturating amounts of ovalbumin and milk protein mRNA either alone or as a mixture, reveal facets of the regulation of sequestration of secretory proteins. Saturation was defined as the level above which doubling the amount of mRNA injected caused a less than 15% increase in secretory protein synthesis (including exported proteins). Ovalbumin mRNA directs the synthesis of a stable polypeptide, identified as the unglycosylated primary product, that accumulates in the cytosol. Thus miscompartmentalized synthesis can occur in living cells. The proportion of the newly made ovalbumin that is found in the cytosol does not fall dramatically at low levels of injected messenger, suggesting that some of the oocytes own messengers are also competing for membrane receptors. Guinea-pig α-lactalbumin [15] and caseins [38] are synthesized in the wheat germ cell-free system as precursor polypeptides modified at the amino terminus, yet even high levels of milk protein mRNA (at least twice saturation, and over ten times the total polysomal poly[A]-rich mRNA of the oocyte, as calculated from Rosbash and Ford [32] and Woodland [33]), cause no accumulation of precaseins or pre-α-lactalbumin. Nonetheless, the results with ovalbumin mRNA show there are no regulatory mechanisms preventing the complete translation of secretory protein mRNAs

on free polyribosomes. Even if one assumes a very high affinity of nascent milk proteins (as compared to that of nascent oocyte or ovalbumin proteins) for the endoplasmic reticulum receptor, self-competition (see below) would be expected to lead to accumulation of primary products.

Low affinity or use of a different receptor could explain why levels of ovalbumin mRNA which lead to the appearance of unglycosylated ovalbumin fail, in mixed-messenger injection experiments, to reveal accumulation of primary milk protein translation products. However, studies *in vitro* using vesicles from *Xenopus* blastulae show competition for entry between caseins and ovalbumin; moreover the affinity of ovalbumin for the common receptor is only about threefold lower than that of casein C (Ohlsson, Lane and Craig, unpublished observation). Caseins A and B have higher affinities, suggesting that precasein C should accumulate at high levels of injected milk protein mRNA. Thus indirect arguments suggest that primary milk proteins are unstable in the cytosol. As we were unable to detect even the transient appearance of primary milk proteins, microinjection experiments were performed to provide direct evidence of their instability within the cytosol. We place much greater weight on the results of such direct experiments than we do on the essentially negative results described above.

Precaseins and pre-α-lactalbumin injected into oocytes were found to be degraded rapidly ($t_{1/2}$ 0.6 ± 0.1 h). In contrast, the primary ovalbumin protein was rather stable ($t_{1/2}$ 22 ± 9 h) even when coinjected with the primary milk proteins. Reinjected oocyte proteins are also stable, even when mixed with wheat germ extracts containing milk proteins. Such experiments, and others summarised in Table 1, rule out many trivial explanations of rapid degradation: for example, increased susceptibility caused by denaturation during sample preparation, or activation of proteolytic enzymes within oocytes caused by injection of wheat germ extracts. The rapid secretion of precursor proteins has also been ruled out, by gel analysis of the oocyte incubation medium. The stability of a protein is generally believed to be a consequence of its three-dimensional structure [14]: thus it is of interest to compare primary and secondary translation products, for differences in stability may be correlated with differences in structure. The relative stability of processed caseins A, B and C ($t_{1/2}$ casein A 6 h, casein B 4 h, casein C 8.3 h) as compared to that of the precaseins ($t_{1/2}$ 0.6 ± 0.1 h) is consistent with a destabilizing effect of the lack of secondary modification, for oocyte, unlike wheat germ products, are perhaps glycosylated, although probably not phosphorylated; however, the results obtained are also consistent with a destabilizing effect caused by the presence of a detachable signal sequence. A more rigorous test of the latter idea requires injection of a given protein with and without its signal sequence, and differing in this one respect. Pre-α-lactalbumin and α-lactalbumin meet these requirements [34] and processed α-lactalbumin is indeed very stable ($t_{1/2}$ 20 ± 8 h). Ovalbumin provides a direct test of the idea that lack of secondary modification causes great instability of the primary translation product: the half-life ($t_{1/2}$ 22 ± 9 h) obtained for the primary ovalbumin product argues against this idea, although modifications might well render the primary species even more stable. Similarly, the results obtained with α-lactalbumin, which emphasize the destabilizing role of a detachable signal sequence, cannot be generalized to other proteins; and even if they were, secondary modification might still contribute to the stability of a particular protein. The stability of the primary ovalbumin product, which lacks a detachable signal sequence but may have an internal one, is also consistent with the destabilizing effect of a detachable N-terminal peptide. Thus, based on the study of five different primary translation products and four corresponding processed products, we can state the following: secretory protein primary translation products unless they lack a detachable signal sequence are unstable when injected into the oocyte cytosol, whilst processed secretory proteins are relatively stable. ^{125}I-labelled caseins are relatively unstable ($t_{1/2}$ 2.3 h) despite the lack of a signal sequence and the presence of secondary modifications; however, deiodination, or the influence of the iodine residues and more importantly the denaturing conditions used to isolate and iodinate caseins invalidate direct comparisons with normal milk proteins.

Thus in the oocyte different proteins are degraded at different rates and in the absence of suitable cell-free systems [14], the factors affecting protein turnover are best studied using such a whole cell system. Nonetheless we do not know where miscompartmentalized proteins are degraded; the hydrophobic signal sequence could cause preferential adhesion to lysosomal membranes, and Dean [35] has reported selective association between hydrophobic proteins and lysosomal pellets. Since alterations in tertiary structure often affect protein stability it would not be surprising were the presence of a hydrophobic amino-terminal signal sequence to cause a dramatic loss of stability: a stabilizing role for glycosyl residues has already been proposed [36, 37]. Nonetheless, such effects may not be general ones, and we have measured stabilities of only four proteins with an without signal sequences or secondary modifications. There is also no direct evidence that miscompartmentalized synthesis occurs in normal living cells, although short-lived primary translation products have been detected in detergent extracts of whole cells; these products include preproparathyroid hormone [8], preproinsulin [9] and

preprolactin [10], each with an estimated half-life of less than 5 min. Inititation of mammary milk protein synthesis [39], and presumably that of other secretory proteins *in vivo*, takes place on free polyribosomes, and given the temporal requirements for successful interaction betweeen signal sequence and membrane receptor, the appearance of transient preproteins in the cytosol seems quite possible. Thus we believe that the short-lived precursors seen [8–10], represent miscompartmentalized proteins of the intact cell. We believe that structural aspects of the completed primary translation product usually leads to recognition by peptidases, and the correction of such errors of compartmentation.

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