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Signal sequences, secondary modification and the turnover of miscompartmentalized secretory proteins in *Xenopus* oocytes

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The cytoplasm of the *Xenopus* oocyte can be altered by the microinjection of proteins and the regulatory responses to such perturbations can then be studied. We have investigated proteolytic systems within the oocyte which may be involved in the maintenance of the integrity of the different subcellular compartments. Thus primary translation products, made in the wheat germ system under the direction of frog liver, chicken oviduct, rat liver rapidly sedimenting endoplasmic reticulum, rat seminal vesicle, guinea pig mammary gland or honey been venom gland RNA, were injected into oocytes. Their stability in the frog cell cytosol was in general low compared to that of their processed counterparts. The latter were usually obtained by collecting the heterologous proteins exported by RNA-injected oocytes. Electrophoretic analysis of oocytes injected with particular primary and processed polypeptides permitted measurement of the stabilities of proteins differing only by the presence or absence of a detachable signal sequence, or by the presence of a specific secondary modification. The effect of the latter on protein stability appears slight. However, the presence of a detachable signal sequence destabilizes those miscompartmentalized secretory proteins which are otherwise stable. Indeed all other results are consistent with this concept for they show that primary translation products are in general much less and are never more stable than their processed counterparts. Thus we provide evidence that errors of compartmentation can be corrected in living cells and that this process is often facilitated by the properties conferred on a protein by a detachable signal sequence.

Subcellular architecture and organisation can be studied to special advantage in *Xenopus* eggs or oocytes, giant cells capable of withstanding microinjection. Informational macromolecules [1–3] and organelles [4–6] can be introduced, directly or indirectly [7, 8] into different intracellular compartments. Oocytes possess an elaborate structure that reflects primarily the specialised storage functions carried out during oogenesis [9]. However, the injection of messenger RNA coding for exported proteins has revealed the presence of a functional secretory pathway [10, 11]. Thus the oocyte contains rough endoplasmic reticulum, golgi apparatus and secretory vesicles [12].

Whole cell systems are also useful [13] for investigating protein degradation because suitable cell-free systems are not generally available [14]. It seems possible that cells have evolved mechanisms that correct errors of compartmentation, and, in particular that miscompartmentalized secretory proteins are susceptible to proteolytic attack. In the present paper we have made use of the existence within oocytes of both degradative and secretory pathways to evaluate such homeostatic mechanisms. We provide further evidence [7] for the presence within frog cells of error-correcting machinery, and discuss the important but not exclusive role played by detachable signal sequences [15–18] in destabilizing miscompartmentalized secretory proteins.

MATERIALS AND METHODS

Radioactive proteins were injected into oocytes and their stabilities measured [7]: thus wheat germ extracts and oocyte incubation media were made 10 mM in methionine, were frozen, thawed and, without further treatment (thereby avoiding further possible denaturation artefacts), the protein solutions were introduced into the oocyte cytosol. In most experiments, a given protein solution was only thawed once: and, in certain experiments designed to control against denaturation artefacts, samples were injected without prior freezing. The recipient cells were both preincubated (4 h) and incubated in medium containing 15 mM methionine, thus preventing reincorporation of radioactive amino acid. The same micropipette, calibrated to contain about 350 nl of solution, was used in a given series of stability measurements. Two pipettefuls of solution, yielding about 14 injected oocytes, were used for each time point. The batches of injected oocytes were incubated for up to 24 h: at various times oocytes and their surrounding media were measured for their content of total and acid-insoluble radioactivity and then analyzed on SDS/polyacrylamide gels. Batches of frog cells showing leakage [11] were discarded. Radioactive polypeptides resolved on SDS/polyacrylamide gels were revealed by autoradiography or fluorography [19] and measured using a computer-aided integral densitometer.

Wheat germ cell-free systems were prepared and programmed with guinea pig mammary gland poly(A)-rich RNA [20]. Different magnesium ion concentrations were required by other species of messenger RNA for optimal translation *in vitro*, and the cell-free system was altered accordingly.

Secondary translation products were prepared using a whole cell system. Thus oocytes were injected with RNA [1] and after a 24-h wound-healing period they were cultured for two or three days in medium containing [^{35}S]methionine (300 μCi in 50 nl per 20 cells). The medium surrounding those batches of oocytes showing no signs of leakage was collected and used for injection into fresh oocytes. Heterologous secretory and endogenous proteins sequestered within oocytes were recovered

Abbreviation. SDS, sodium dodecyl sulphate.

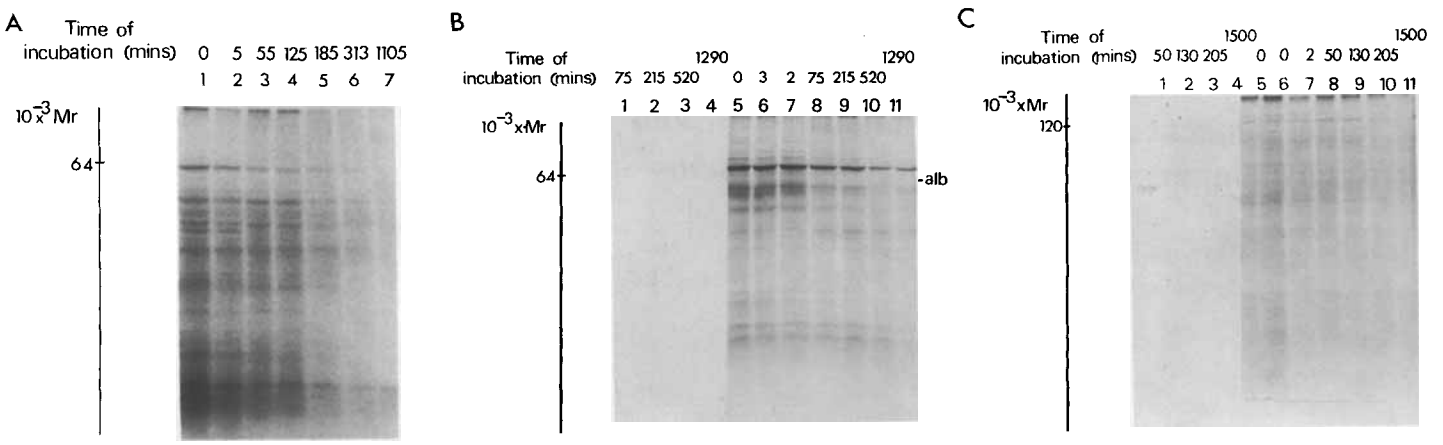

Fig. 1. *The stability within oocyte cytosol of rat liver proteins exported from oocytes or made in wheat germ extracts under the direction of rapidly sedimenting endoplasmic reticulum RNA.* Batches of oocytes were injected with protein solutions, homogenized after various times of incubation (indicated above each track) and analyzed on SDS gels. (A) The stability of rat primary translation products; (B) stability of reinjected rat and endogenous frog processed products; (C) the stability of frog oocyte protein alone. The medium surrounding the oocytes was sampled at various times, as shown in tracks 1–4 of B and C. Rat albumin (alb, M_r 64000) is known from immunoprecipitation data (not shown) to be the most abundant protein coded for by rapidly sedimenting endoplasmic reticulum RNA

by a single-step procedure [7] and were also used for reinjection studies.

Poly(A)-rich RNAs were made by established procedures. Thus guinea pig mammary gland RNA [20], rat liver rapidly sedimenting and rough microsomal RNA [21], chicken oviduct RNA [22], *Xenopus* liver RNA [23], honey been venom gland (total) RNA [24] and rat seminal vesicle RNA [25] were prepared and were stored as concentrated (> 1 mg/ml) solutions.

RESULTS

The stability of primary and secondary translation products in Xenopus *oocytes*

The stability of miscompartmentalized secretory proteins can be studied by injection into the cytosol of living cells. Primary translation products were prepared in wheat germ extracts, whilst processed proteins were recovered from the media surrounding oocytes programmed with heterologous messenger RNAs. Thus oocytes were injected with rat liver proteins, either those formed *in vitro* or those exported *in vivo* under the direction of rapidly sedimenting endoplasmic reticulum RNA. This particular RNA was chosen because, as indicated in Fig. 1 B (tracks 5–11) it codes for a wide range of proteins, many of which are topologically segregated [26]. Table 1 and Fig. 1 A show that the primary translation products are degraded rather rapidly by enzymes present in the cytosol of the oocyte, whilst the reinjected processed and secreted proteins (Fig. 1 B) are in general much more, and are never less, stable. Since endogenous proteins are exported from both the oocyte and its surrounding follicle cells, the reinjected proteins seen in Fig. 1 B are a mixture of frog and rat species. Gel analysis of the medium surrounding [^{35}S]methionine-labelled control oocytes (see Fig. 1 C) shows that nearly all the major bands seen, the obvious exception being the upper doublet, correspond to rat proteins. The study of Mohun et al. [41] and further defolliculation experiments (data not shown) reveal that the high-molecular-weight doublet represents protein exported by the follicle or theca cells which surround the oocyte. Thus Fig. 1 C shows that the major processed *Xenopus* proteins are, with the exception of the uppermost band, relatively stable when introduced into the cytosolic compartment. Neither reinjected heterologous proteins (Fig. 1 B, tracks 1–4) nor reinjected oocyte proteins are re-exported (Fig. 1 C, tracks 1–4).

The stabilities of products made *in vivo* and *in vitro* under the direction of chicken oviduct RNA have also been compared. The results reveal (Fig. 2 B) that the ovalbumin primary product, which lacks a detachable signal sequence [27], is stable whilst all the other primary species are unstable. Table 1 shows that primary translation products made by RNA from rat liver, frog liver, chicken oviduct and honey been venom gland are generally much less stable than the corresponding processed proteins. Both primary and processed proteins made under the direction of either guinea pig mammary gland or rat seminal vesicle mRNA are unstable.

The influence of detachable signal sequences and secondary modification on the stability of miscompartmentalized secretory proteins

The specific influence of detachable signal sequences on the fate of miscompartmentalized proteins can be evaluated by comparing the stabilities of pairs of proteins that differ in their N-terminal sequences but not in any other respect. An informative comparison can only be made if the processed form is relatively stable, although such stability is not a necessary condition of the proposed error-correction hypothesis. It has proven difficult to prepare suitable pairs of proteins, because most secretory proteins are secondarily modified. Fig. 2 (A and B) and Table 1 show the stabilities of chicken lysozyme and prelysozyme within frog cytosol: the presence of a signal sequence causes a fivefold reduction in stability. Lysozyme was identified by its gel mobility, abundancy and, for the processed form, by immunoprecipitation [28]. Similarly Fig. 2 C and Table 1 show the rapid destruction of honey bee prepromelittin, identified by its mobility and sequence [29], and the relative stability of promelittin. A half-life of 0.3 h was obtained with prepromelittin labelled either exclusively in the signal sequence or predominantly in the melittin region suggesting that proteolysis, once started, is both rapid and extensive.

Table 1. *The stability of primary and secondary translation products injected into the cytosol of* Xenopus *oocytes*
Oocytes were injected with heterologous secretory proteins exported by oocytes, or with primary translation products formed in the wheat germ cell-free system. At various times after injection batches of about 14 oocytes were homogenized, measured for their content of acid-insoluble radioactivity and analysed on SDS/polyacrylamide gels (see Fig. 1 – 3). A computer-aided integral densitometer was used to measure the relative amount of radioactivity in a given gel band or track. Measurements obtained for both populations and individual polypeptides were plotted, as a function of time, on semilogarithmic paper. The values shown represent the half-lives of species identified on gels or, for populations, the major (> 75%) component. Protein populations decaying with biphasic or multiphasic semilogarithmic plots were the exception and are indicated with an asterisk. The results of a previous study [7] are also indicated in square brackets. The half-lives of some proteins were measured several times, and results are expressed as average value, together with the standard deviation and, in parentheses, the number of observations. The oocyte system was, unless otherwise stated, used to prepare exported proteins: 'sequestered proteins' were prepared from the oocyte membrane vesicle fraction, whilst 'extracted proteins' were prepared by extracting whole oocytes with 0.0075 M EDTA, 0.078 M Tris pH 6.8

Origin of messenger RNA added to or present in translation system	Whole cell or cell-free protein synthesizing system	Half-life of proteins injected into oocytes		
		population	species	
		h		
Chicken oviduct	frog oocyte	52 ± 11 (4)	ovalbumin	35
			conalbumin[a]	6
			lysozyme[a]	23
	frog oocyte (+ tunicamycin)	43	ovalbumin	34
	wheat germ	28	ovalbumin	50 [22 ± 9 (4)]
			prelysozyme	4
Xenopus liver	frog oocyte	24 ± 4 (2)	albumin (doublet)[b]	22 ± 1 (2)
	frog oocyte (+ tunicamycin)		albumin[b,c]	20 ± 4 (2)
Rat liver rapidly sedimenting endoplasmic reticulum	wheat germ	1	preproalbumin[a]	1
	frog oocyte	22	proalbumin[a,d,e]	24 ± 11 (2)
Honey bee venom gland	wheat germ	2	preproalbumin	3
	honey bee venom gland cell[f]	> 10, > 10	–	
	wheat germ[g]	0.7 ± 0.1 (2)	prepromelittin[h]	0.3 ± 0 (2)
Rat seminal vesicle (S.V.)	frog oocyte	2.5*	fast and slow S.V. proteins	0.5
	wheat germ	2	fast and slow S.V. proteins	0.7
Xenopus oocytes (folliculated) endogenous	frog oocyte	39 ± 15 (4)	–	
	frog oocyte (internally sequestered species)	50	–	
Guinea pig mammary gland	wheat germ	1.0 ± 0.5 (2)	precasein A, B and C[b]	[0.6 ± 0.1 (3)]
			pre α-lactalbumin[b]	[0.6 ± 0.1 (3)]
	frog oocyte (exported proteins)	6 ± 2 (2)	Casein A, B and C	0.8
			α-lactalbumin	0.8
	frog oocyte (extracted proteins)[i]	[55 ± 18 (3)]	Casein A, B and C	[4 – 8]
			α-lactalbumin	[20 ± 8 (3)]
	mammary gland explant	2 ± 2 (2)	Casein A, B and C	0.5

[a] Identified from abundancy and molecular weight.
[b] Identified by abundancy, molecular weight and immunoprecipitation.
[c] Unglycosylated form identified by increase in gel mobility after tunicamycin treatment.
[d] The protein exported from oocytes might be albumin itself, although the frog cell fails to remove the pro region from at least two other secretory proteins [32, 40].
[e] Includes value obtained with proalbumin made in oocytes under the direction of rat liver rough microsomal RNA.
[f] Promelittin from venom glands was labelled with valine or leucine. Prepromelittin was labelled with leucine or methionine: the former is incorporated into both pre and promelittin regions, whilst the latter is confined to the pre sequence, yet the half-lives measured were about the same.
[g] Dialyzed against Barth X.
[h] Identified by abundancy, molecular weight and peptide mapping.
[i] Medium not frozen before injection.

Measurements were also made of the relative stabilities of pre-α-lactalbumin ($t_{1/2}$ 0.6 h) and α-lactalbumin ($t_{1/2}$ 0.8 h). The latter was ascertained by the injection into fresh oocytes of radioactive α-lactalbumin exported from oocytes, the identity of the milk protein having been established by immunoprecipitation. The half-lives recorded (Table 1) are much shorter than those obtained (20 ± 8 h) injecting back a crude cell extract containing α-lactalbumin and oocyte proteins. Mixing experiments (data not shown) suggest the presence of a stabilizing factor in such extracts.

The influence of secondary modification of protein stability can be studied using pairs of proteins differing only in, for example, their degree of *N*-glycosylation or phosphorylation. Tunicamycin-treated oocytes [8] can be used to prepare unglycosylated proteins, such as chicken ovalbumin. *Xenopus* albumin runs as a doublet unless tunicamycin is present, both

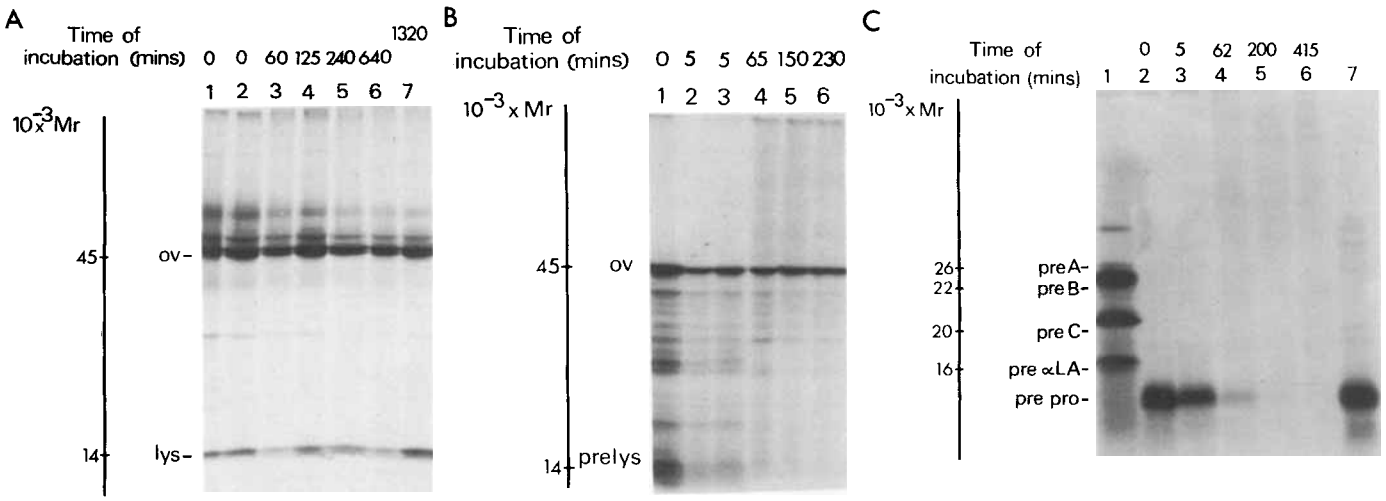

Fig. 2. *The stability of chicken lysozyme and prelysozyme injected into oocyte cytosol.* Chicken ociduct RNA was translated in the wheat germ system and the products formed were injected into batches of oocytes, which were incubated for various times. (B) Gel analyses of oocyte homogenates, the time of incubation being shown above each track; (A) similar experiment, except that oocytes were injected with proteins exported from oocytes programmed with oviduct RNA. (The reinjection of frog proteins exported by control oocytes is shown in Fig. 1C: in the experiment shown in B exported frog proteins are swamped by chicken proteins.) (C) The rate of degradation of honey bee prepromelittin made in the wheat germ system under the direction of venom gland RNA. The track marked shows the mobilities of guinea pig precaseins A, B and C (preA, preB and preC) and pre-α-lactalbumin (pre-α-LA) made in the wheat germ extract and used as molecular weight markers. Lysozyme (lys) was identified by its abundancy, molecular weight and, for the processed protein, by immunoprecipitation [28]. Conalbumin was not identified but, from its molecular weight and abundary, is likely to be the uppermost major band. Ovalbumin (ov) was identified by its molecular weight, by immunoprecipitation and by two-dimensional gel analysis (data not shown). Prepromelittin (pre pro) was identified by its apparent molecular weight and by amino acid sequencing [29]

forms being precipitable by anti-albumin antiserum. Westley et al. [30] have shown that these two albumin species are the products of different genes. (It is interesting therefore that only one form has an altered gel mobility if made in tunicamycin-treated oocytes.) As shown in Fig. 3 and Table 1, *N*-glycosylation appears to have no great effect on the rate of degradation of these two rather stable processed albumins, although the test is not a very sensitive one because after tunicamycin treatment the two albumin species cannot be resolved on our gel system. Fig. 3B shows that deglycosylated ovalbumin is also stable.

Guinea pig caseins exported from oocytes are, compared to those secreted by mammary gland explants, underphosphorylated [42]. Nonetheless, both forms are unstable in frog oocytes (Table 1), showing that phosphorylation has, for the caseins at least, no profound influence on protein stability.

DISCUSSION

The oocyte contains structurally and functionally distinct subcellular compartments whose integrity is preserved by systems of membranes. It is conceivable that mechanisms have evolved for correcting errors of compartmentation because maintenance of these subdivisions is of paramount importance for cell functions. Thus a compartmentalized system is required for the synthesis and export of proteins [31]. The topological segregation of secretory proteins is believed to be irreversible and therefore, as confirmed in the present study, proteins reinjected into frog cells are not re-exported [32]. The nascent polypeptide chain contains the information specifying segregation of secretory proteins and particular regions of the nascent chain are believed to direct vectorial transfer across the endoplasmic reticulum. Such signal sequences are hydrophobic N-terminal peptides which are usually removed by membrane-bound peptidase [15–17]. The signal sequence of chicken ovalbumin is internal and remains attached [27].

We have explored the possibility that oocytes contain cytosolic enzyme systems which correct errors of compartmentation and, in particular, that detachable signal sequences play a specific role in destabilising secretory proteins which fail to cross the endoplasmic reticulum. We have demonstrated the instability within frog cell cytosol of diverse populations of rat, frog, honey bee and chicken primary translation products, thereby generalizing results obtained with the four major guinea pig milk proteins [7]. Half-lives of greater than 20 h cannot be measured accurately if microinjection is followed by an incubation period amounting to only one half-live. Half-lives of less than 30 min are also difficult to measure. Within these limits, however, our results show that the primary products of secretory protein messengers are usually less, sometimes equally, but never more stable than their processed counterparts. Thus all our results are consistent with a destabilizing role being played by the presence of detachable signal sequences, but data for chicken ovalbumin and frog albumin show that proteins can be quite stable in the absence of some at least of their normal secondary modifications. Experiments with promelittin and lysozyme gene products (Table 1) establish that for some polypeptides at least it is the detachable signal sequence itself which can lead to rapid degradation of miscompartmentalized secretory proteins. If one assumes that oocytes export rat proalbumin, as seems likely from results obtained with other such precursor proteins [32, 40], then the instability of preproalbumin also demonstrates the major influence exerted by a detachable signal sequence. However, there is no evidence that such regions are specifically recognised by proteases, and enhanced susceptibility may result from structural perturbations of other regions of the protein molecule [14].

Completed preproteins are rarely seen in whole cells: those that have been detected have very short half-lives [33–35]. The

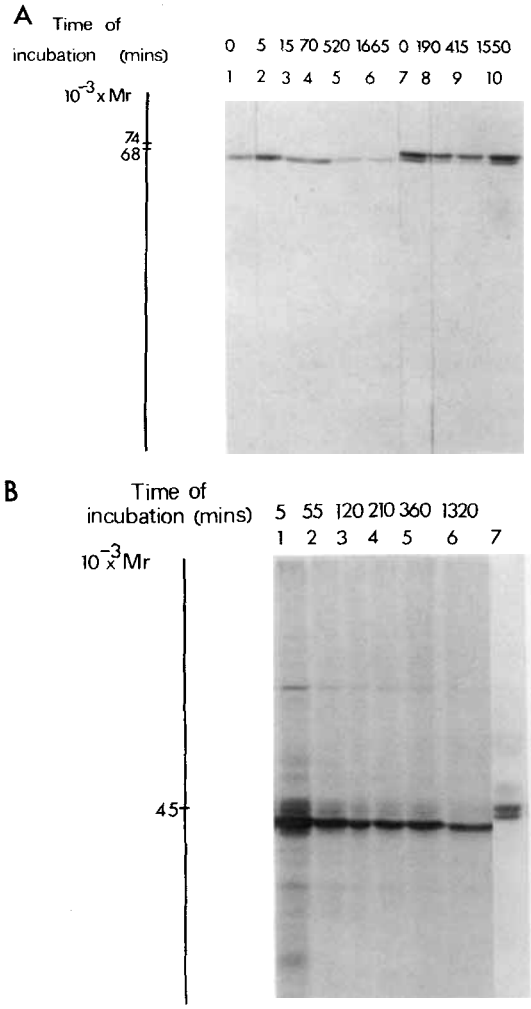

Fig. 3. *The stability of* Xenopus *albumins and chicken ovalbumin exported from tunicamycin-treated cells after reinjection into oocytes.* (A) *Xenopus* liver RNA was injected into tunicamycin-treated and normal oocytes which were then incubated with [^{35}S]methionine. The exported proteins were then injected into the cytoplasm of fresh oocytes. At various times, batches of oocytes were frozen and assayed, by immunoprecipitation followed by gel analysis, for their albumin content. Thus tracks 1–6 show the gradual decrease with time of albumin prepared from tunicamycin-treated cells, whilst tracks 7–10 show the slow decay of the two gene products which are characteristic of albumin synthesis in untreated oocytes [30, 32]. (B) A similar experiment in which ovalbumin exported by tunicamycin-treated oocytes was reintroduced. Tracks 1–6 show the gradual decrease with time of the deglycosylated ovalbumin, which has a higher mobility than the glycosylated forms (track 7), which are present in small amount even in the tunicamycin-treated oocytes

injection of large amounts of secretory protein mRNA is consistent with heterotopic synthesis followed by destruction of proteins bearing detachable as opposed to internal signal sequences [7]. However, these observations are also compatible with stringent coupling between synthesis and vectorial transfer [42, 44]. Nonetheless, preproteins of unknown localization or stability have been detected in oocytes [36, 37]. Furthermore, unglycosylated ovalbumin accumulates in the cytosol of injected oocytes [7, 8] and does so over a wide range (0.8–50 ng/cell) of messenger concentrations (unpublished observations). If mixtures of mammary gland and oviduct RNA are coinjected, both milk and oviduct proteins are made, but only the primary ovalbumin translation product can be detected (data not shown). Clearly, the coupling between synthesis and membrane transfer can be overridden but even in a cell where this is occurring, primary milk protein products do not accumulate in the cytosol.

We speculate that even the low levels of heterotopic synthesis predicted by thermodynamic considerations are of harm to the unperturbed cell and that error-correcting as well as error-preventing machinery [43, 44] may have evolved. Our results to date merely show that, at least in oocytes, there exists appropriate degradatory machinery. Wyllie et al. [38, 39] discovered that DNA introduced into the oocyte cytosol is rapidly degraded, whilst DNA injected into the nucleus is relatively stable. Furthermore, some transcripts normally processed within the nucleus are unstable when introduced into the cytosol. It seems possible that the integrity of the different subcellular compartments is of such importance that mechanisms have evolved for the removal of a variety of miscompartmentalized macromolecules.

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