Nucleic Acids Research, Vol. 19, No. 23 6405–6412

A highly efficient, cell-free translation/translocation system prepared from *Xenopus* eggs

Glenn Matthews and Alan Colman
Biochemistry Department, University of Birmingham, PO Box 363, Birmingham B15 2TT, UK

Received September 18, 1991; Revised and Accepted November 11, 1991

ABSTRACT

We describe the use of a *Xenopus laevis* egg extract for the in vitro translation and post translational modification of membrane and secretory proteins. This extract is capable of the translation and segregation into membranes of microgramme per millilitre levels of protein from added mRNAs. Signal sequences of segregated proteins are efficiently cleaved and appropriate N-linked glycosylation patterns are produced. The extract also supports the quantitative assembly of murine immunoglobulin heavy and light chains into tetramers, and two events which take place beyond the endoplasmic reticulum, mannose 6 phosphorylation of murine cathepsin D and O-linked glycosylation of coronavirus E1protein, also occur, but at reduced efficiency. The stability of the membranes allows protease protection studies and quantitative centrifugal fractionation of segregated and unsegregated proteins to be performed. Conditions for the use of stored extract have also been determined.

INTRODUCTION

Our present understanding of the molecular events involved in the segregation and processing of secretory proteins in eucaryotes owes a great deal to the use of cell-free translation and translocation systems. The first such assay was a combination of a wheat germ lysate and dog pancreas microsomes (1). Subsequently a number of alternative systems prepared from a variety of sources, plant and animal, have been described (see reference 2 for review). In most of the assays reported, a heterologous collection of components is utilised and, in studies on the same protein, different results can be obtained according to the exact source of the assay components (3,4,5). This has complicated intepretation of the results from these systems and, consequently, raised doubts about the validity of models based on the use of only one type of in vitro assay system. Clearly the development of systems where all the translation and translocational machinery derive from a single source would be helpful. Systems of this type already exist from fungal (6) and plant sources (5) but are generally inefficient and, in the case of fungal sources, may not be suitable models for events occuring in higher eucaryotes. In this paper we describe the preparation and characterisation of a homologous translation/translocation system from the unfertilised eggs of *Xenopus laevis*.

The *Xenopus* oocyte has proved a versatile cell for studies on the segregation, assembly, and movement of a variety of secretory or membrane proteins encoded on microinjected mRNAs (7). Cell-free translation systems have been reported from oocytes and show a similar activity to that shown by the intact oocyte (8). Preparation of the extract, however, is troublesome due to the resistance of the oocytes to centrifugally-induced lysis, an essential step in the formation of an active extract. The meiotic descendant of the oocyte, the unfertilised egg, is more amenable to disruption and has been used to make cell-free extracts active in chromatin assembly (9), nuclear envelope assembly (10), in vitro mutagenesis (11), DNA ligation (12), RNA duplex unwinding (13), DNA repair (14), DNA replication (15,16) and cell cycling(17). For several of these activities, on-going protein synthesis in the extracts is essential. In this paper we present the first detailed information on the translational characteristics of an egg extract. We find it is at least five times more active than that reported for oocyte extracts. This extract, which can be nuclease-treated to render it mRNA-dependent (17), is capable of the segregation, signal sequence cleavage, O- and N-glycosylation, mannose 6 phosphorylation, and assembly of microgramme per millilitre quantities of a variety of secretory and membrane proteins encoded on added synthetic and natural mRNAs.

MATERIALS AND METHODS

Preparation of *Xenopus* egg extract

This procedure, summarised in figure 1, was performed essentially as described by Murray and Kirschner (17), modified to the scale of the Beckman TL-100 ultracentrifuge. All operations, after dejellying, were performed at 4°C.

One or two females were induced to lay eggs into modified Barth's saline (7) supplemented with NaCl to 110mM. After rinsing in the same buffer, eggs were dejellied in 2% cysteine hydrochloride (pH 7.7), then transferred immediately to extraction buffer (50mM sucrose, 100mM KCl, 0.1mM $CaCl_2$, 1mM $MgCl_2$, 10mM Hepes-KOH pH 7.7), with multiple washes. Eggs were then pipetted gently into 2ml polyallomer tubes and allowed to settle for a few moments. Surplus buffer was removed and the tubes were topped up with Versilube F-50 (General Electric, USA) and centrifuged for 1 minute at 1000×g at 2°C. The supernatant oil and buffer were removed and the tubes centrifuged at 15,000 rpm (20,000×g) for 15 minutes at

2°C in a Beckman TLS 55 rotor. This results in a multi-layered lysate of which the translucent yellow cytoplasm forms approximately 40%. This was recovered with a pasteur pipette and fractions from two tubes pooled into thick walled, 1.5ml, polycarbonate tubes. Cytochalasin B (Sigma) was added to 50μg/ml, the lysate was recentrifuged under the same conditions as before and the cytoplasm recovered. Routinely between 1.5 and 2ml of product was recovered from 8ml of packed eggs.

Depletion of endogenous mRNAs

100μl aliquots of lysate were incubated with 1μg/ml heat treated RNAse A (Boehringer) at 10°C. After 20 minutes, 1μl of 100mM DTT and 50 units of placental RNAse inhibitor (Boehringer) were added and the incubation continued for 10 minutes. The lysate was then supplemented with 1μg/ml tRNA (calf liver, Boehringer) before use or freezing as 100μl aliquots in liquid nitrogen followed by storage at −70°C or in liquid nitrogen.

Preparation of reticulocyte lysate S-100 fraction

100μl aliquots of rabbit reticulocyte lysate (Bathesda Research Laboratories) were centrifuged at 50,000rpm for 2 hours at 4°C in a Beckman TLA 100 rotor. 80μl of supernatant was recovered, taking care not to disturb the ribosomal pellet. This was frozen, as 10μl aliquots, in liquid nitrogen and stored at −70°C

The translation reaction

Each 100μl aliquot of lysate was usually supplemented with 10μl reticulocyte lysate S-100, 2.5μl 350mM creatine phosphate, 1μl of 120mM spermidine (Sigma) and 100μCi ^{35}S-methionine (Amersham). After mixing, reaction volumes of 10−30μl were added to tubes containing appropriate mRNAs and incubated at 21°C for 1hour.

Processing and analysis

Samples for electrophoresis were diluted in 4 volumes of 1% Triton X-100, 1mM phenylmethanesulphonyl fluoride (PMSF), on ice, before addition of 2-fold concentrated SDS gel sample buffer. Routinely, no more than 1μl of extract was analysed per 5mm wide×0.75mm thick gel track as the concentration of protein present causes distortions in the gel at higher levels.

Polyacrylamide gel electrophoresis was performed in the BioRad mini Protean II apparatus under the conditions specified by the manufacturer and gels were treated with Enhance (Du Pont) before drying and exposure to Kodak X-Omat AR film at −70°C.

Protease protection assay

After translation, the extract was divided into 10μl aliquots and 1μl proteinase K at either 1mg/ml or 2mg/ml added on ice. Parallel aliquots also received 1μl of 10% Triton X-100 in order to disrupt the membranes present, as a control for protease activity. After incubation for 1hour, on ice, PMSF was added to 2.5mM and samples incubated for a further 15 minutes before addition of 10 volumes 1×SDS gel sample buffer, including 0.5% Triton X-100.

Sucrose gradient fractionation

Translation products were diluted in ten volumes of 10% sucrose, 50mM KCl, 10mM Mg Ac, 100mM NaCl, 20mM Tris HCl pH 7.6, layered onto a 1ml step of 20% sucrose (in the same buffer), in a 2ml polyallomer tube, and centrifuged for 30 minutes at 30,000rpm (40,000×g) in a Beckman TLS-55 rotor. The top (10% sucrose) layer was recovered, the 20% step carefully removed and the membrane pellet was either resuspended in 10% sucrose buffer or dissolved in 1%Triton X-100, 1mM PMSF.

TCA precipitation assay

A portion of each reaction was diluted in ten volumes of 1%Triton X-100, 1mM PMSF and 2μl of this spotted to each of four 5mm squares of Whatman 3MM paper. Two of these were retained unprocessed to determine total radioactivity, while the other pair were washed in 10% TCA at 4°C for 30 minutes, brought to the boil in 5% TCA and washed twice in ice cold 5% TCA before rinsing in water, ethanol and ether and air drying. Radioactivity was determined by scintillation counting in Optiphase Hi-safe (LKB).

Preparation of synthetic mRNA

Synthetic mRNAs used in this study were prepared from the following plasmid templates: pSP64TVg1, encoding *Xenopus* Vg1 protein (18); cDpSP64 encoding human cathepsin D (19); pSPBP4, encoding bovine preprolactin (20); pSP64THAwt, encoding influenza virus haemagglutinin (21); pSP64TE1 encoding coronavirus E1 protein (22) and pSP64TMO15, encoding p40^{MO15} (23).

10μg of linearised caesium chloride-purified DNA template was transcribed in a 100μl reaction containing all four ribonucleotide triphosphates and ^{me}GpppGTP at 0.5mM in 40mM Tris-HCl pH 7.5, 6mM $MgCl_2$, 2mM spermidine, 50mM DTT, 100μg/ml BSA, 2units/μl ribonuclease inhibitor and 1unit/μl SP6 RNA polymerase. 1μCi of ^{32}P-UTP (3000 Ci/mmol, Amersham) was added before incubation at 37°C for 1 hour. DNA was removed by digestion with RNAse-free DNAse 1 (Pharmacia) for 15 minutes and the percentage of radioactive nucleotide incorporated was determined by the DE 81-binding assay (24). The reaction was extracted twice with phenol-choroform and RNA was recovered by ethanol precipitation in the presence of 0.7M

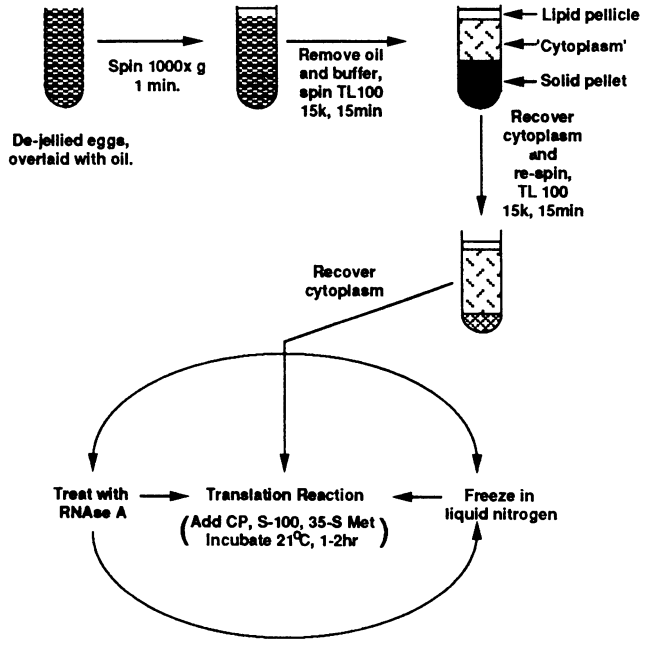

Figure 1. Preparation of the extract.

ammonium acetate, redissolved in water at 1μg/ml and stored at −20°C. The integrity of the product was checked by agarose gel electrophoresis and autoradiography.

Preparation of natural mRNAs

Xenopus embryo total RNA was prepared by homogenisation in 5mM EDTA, 300mM NaCl, 50mM Tris-HCl pH7.5, 1% SDS, and 1μg/ml proteinase K, followed by phenol-chloroform extraction and ethanol precipitation.

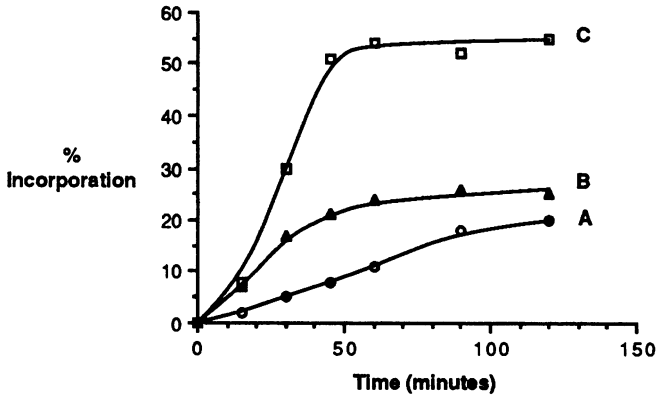

Figure 2. Kinetics of translation. *Xenopus* egg extracts extracts were prepared by the methods described by Lohka and Maller (A) (25) and Murray and Kirschner (B&C) (17) and incubated at 21°C in the presence of ^{35}S methionine. Creatine phosphate (7mM) and reticulocyte S-100 (10% by volume) were also added to reaction C. Samples were taken at the time points indicated and quenched by freezing on dry ice. Percentage incorporation of ^{35}S was determined by TCA precipitation as described in Materials and Methods.

Mouse hybridoma total RNA was prepared by the guanidinium isothiocyanate/caesium chloride method described by Sambrook et al (24).

Poly A+ RNA was prepared by fractionation of total RNA using oligo dT cellulose (Type 2 from Collaborative Research) by the method described by Sambrook et al (24).

Oocyte microinjection

Xenopus oocytes were obtained, cultured and microinjected as described by Colman (7). mRNA injections were 50nl of 1mg/ml mRNA per oocyte.

Labelled proteins were immunoprecipitated as described by Dale et al(18).

Mannose-6-phosphate receptor affinity chromatography

Samples of 10μl of extract were diluted 30-fold in Column Buffer (150mM NaCl, 5mM sodium β-glycerophosphate, 50mM imidazole-HCl pH 6.5 and 0.05% Triton X-100) and applied to a 1.75ml Affigel 15 column containing the bovine liver 215kD mannose-6-phosphate receptor, prepared as described by Faust et al (19). The column was washed with 20ml of column buffer and non-specifically bound proteins removed by washing with 2mM glucose-6-phosphate in the same buffer. Specifically bound proteins were eluted with 5mM mannose-6-phosphate in column buffer.

RESULTS

Translational activity of fresh extracts

The incorporation of ^{35}S methionine into protein by egg lysates prepared in different ways is shown in figure 2. Using isotope dilution methods (data not shown) we found the methionine pool

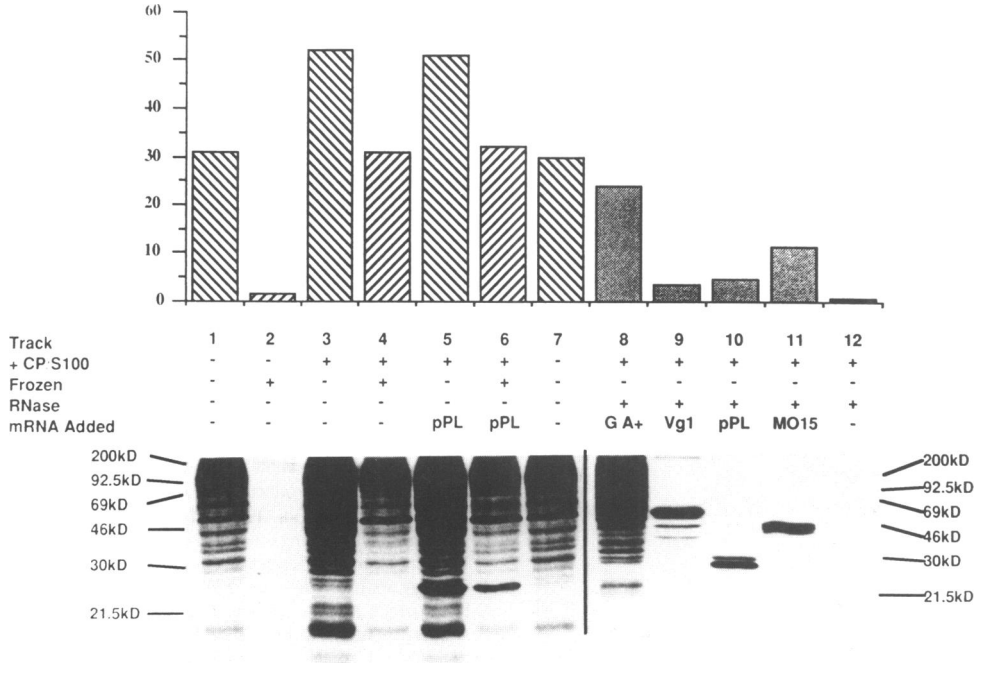

Figure 3. Translational activity before and after freezing and messenger depletion. A *Xenopus* egg extract was prepared as described and translation reactions performed under the conditions indicated. mRNAs were added at final concentrations of 100μg/ml. Percentage incorporation of ^{35}S methionine was measured by TCA precipitation and 1μl of each reaction was analysed by electrophoresis in 15% SDS gels and fluorography. Track 7 shows the result of preparing an extract from the same batch of eggs using an Eppendorf centrifuge at 4°C instead of the TL100. This shows equivalent activity but the recovery of extract is very low due to the fixed angle rotor. The mRNAs added are: pPL, preprolactin; G A+, *Xenopus* gastrula poly A+; Vg1, *Xenopus* Vg1; MO15, the *Xenopus* cytoplasmic protein $p40^{MO15}$.

of these extracts to be approximately 35μM. Using the assumption that the 'average' protein contains 2% by weight of methionine, each 10% incorporation represents 26μg/ml of newly synthesised protein. Early experiments, where the extract was prepared by the method described by Lohka and Maller (25), yielded 15−20% incorporation or 40−50μg/ml protein synthesised in a 2-hour reaction. Most experiments, however, used the method shown in figure 1, which included a low speed spin. The incorporation kinetics of this lysate differed from that of the earlier extract (figure 2) but produced a similar yield. Addition of creatine phosphate to 7mM and 10% (by volume) reticulocyte lysate S-100 to the extract increased total translation significantly, yielding up to 55% incorporation or approximately 140μg protein produced per ml of extract. Doubling the amount of creatine phosphate added had no effect on translational yield (data not shown). Each of these three approaches generates a similar spectrum of radioactive proteins as judged by electrophoresis (see figure 3, tracks 1 and 3).

Addition of synthetic mRNA to these extracts resulted in no increase in total translation, although the added RNA was translated as efficiently as in a message depleted extract, indicating that exogenous mRNAs can compete effectively with endogenous mRNAs for the translational apparatus (Figure 3, cf tracks 3 and 5).

Translational activity of mRNA-depleted fresh extracts

After ribonuclease treatment, addition of 100μg/ml *Xenopus* poly A+ RNA restores approximately half of the translational activity of the untreated extract (Figure 3, cf tracks 3 and 8). The extent of translation obtained on addition of synthetic mRNAs is variable, depending largely on the sequence being used (Figure 3, tracks 9−11) . Generally, the relative translational activities seen with various mRNAs upon injection into *Xenopus* oocytes are reflected in the extract (data not shown). Unsupplemented nuclease-treated extract shows incorporations of 0.1−1% (0.15−1.5μg/ml) for a single synthetic mRNA. Addition of creatine phosphate increases this by a factor of 6, and addition of reticulocyte lysate S-100 produces an independent 3-fold increase, as shown in figure 4. In the experiment shown in figure 4 over 13μg/ml of prolactin was made and over 95% of this was segregated into membranes.

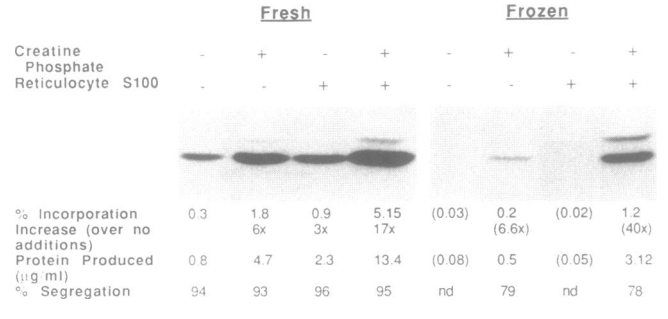

Figure 4. Effect of additions to fresh and frozen messenger-depleted extract. A messenger-depleted *Xenopus* egg extract was prepared and translation reactions, programmed with preprolactin mRNA at 50μg/ml, performed before and after freezing and in the absence and presence of creatine phosphate and reticulocyte S-100. The percentage of ^{35}S methionine incorporated was measured by TCA precipitation and the percentage segregation was determined by excision of gel bands and scintillation counting.

Translational activity of stored frozen extracts

Although the extract is relatively simple to prepare, it is time-consuming and it would be more convenient for many purposes to use stored material. Simple freezing in liquid nitrogen almost completely destroys activity in the unsupplemented extract (Figures 3 &4). It was determined, however, that some activity could be restored by the addition of creatine phosphate . It is not possible to produce a similar effect by adding ATP, GTP or creatine kinase. Addition of creatine kinase and creatine phosphate gave no stimulation beyond that seen in the presence of creatine phosphate alone, indicating the presence of an endogenous creatine kinase activity. Further stimulation was observed on the addition of 10% reticulocyte lysate S-100 but this had no effect when added alone. The effect of adding spermidine to 1mM was variable, increasing methionine incorporation in some extracts up to two-fold, while it had no effect on others. It was never seen to reduce activity and so became a standard component in later reactions.

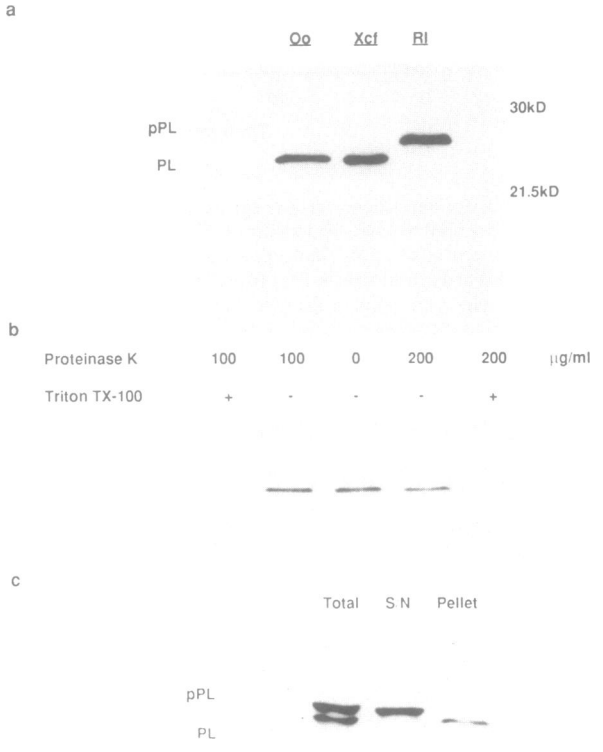

Figure 5. Segregation and signal sequence cleavage in messenger-depleted extracts. **a)** Synthetic preprolactin mRNA was translated in *Xenopus* oocytes (Oo) (30nl mRNA at 1μg/ml injected per oocyte), *Xenopus* cell-free extract (Xcf) (50μg/ml mRNA added) and rabbit reticulocyte lysate (Rl) (50μg/ml mRNA added). ^{35}S labelled translation products (immunoprecipitated in the case of the oocyte sample) were separated on a 12.5% SDS polyacrylamide gel and visualised by fluorography. Loadings were adjusted to give approximately equivalent signals. Efficient cleavage of preprolactin (pPL) to prolactin (PL) can be seen in the oocyte and egg extract while the reticulocyte lysate product remains uncleaved. **b)** Preprolactin mRNA was translated in the *Xenopus* egg extract at 50μg/ml and aliquots treated with proteinase K in the presence and absence of Triton X-100 as described in Materials and Methods before electrophoresis and fluorography. Essentially complete protection from the protease can be seen to have occured in the absence of detergent. **c)** Inefficient segregation of preprolactin was induced in the extract by adding mRNA to give a high concentration (0.5μg/ml). The resulting products were then fractionated by centrifugation on a sucrose step gradient as described in Materials and Methods. The quantitative fractionation of cleaved and uncleaved products demonstrates that no uncleaved protein is found within the membranes.

Routinely, this combination of creatine phosphate, reticulocyte lysate S-100 and spermidine restored activity to 25−60% of that observed with the unfrozen extract. Upon freezing, some reduction is seen in the efficiency of segregation, but the activity and fidelity routinely obtained are sufficient for most qualitative studies.

Addition of sucrose to 200mM before freezing has been used as a cryopreservative for similar egg extracts used in cell cycle studies (27). Since this might allow the thawed extract to function efficiently in the absence of any heterologous protein component, the effect of sucrose addition on translational yield was investigated. The activity of thawed extracts is enhanced two-fold after freezing in the presence of sucrose. This increase is significantly lower than that seen on supplementation with creatine phosphate and reticulocyte lysate S-100, but might prove useful if the addition of heterologous factors is proscribed (data not shown).

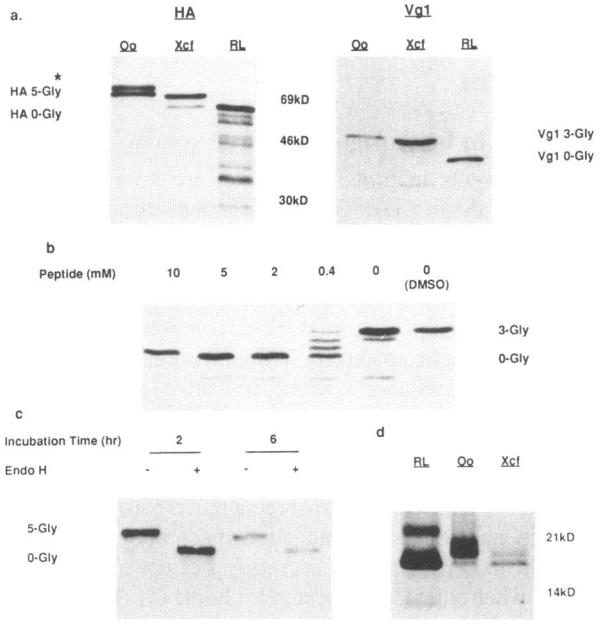

Figure 6. Glycosylation in messenger-depleted extracts. **a)** Shows a similar experiment to that in figure 5a, translating synthetic mRNAs encoding the glycoproteins HA and Vg1at 50μg/ml in the *Xenopus* egg extract (Xcf) and rabbit reticulocyte lysate (RL) or by injection of 30nl of 1μg/ml mRNA into *Xenopus* oocytes (Oo). The positions of the unglycosylated (HA 0-Gly, Vg1 0-Gly) and fully glycosylated (HA 5-Gly, Vg1 3-Gly) proteins are indicated. The band marked * results from terminal glycosylation of HA which occurs in the trans-Golgi in vivo. **b)** Aliquots of a *Xenopus* extract translation reaction, programmed with 50μg/ml Vg1 mRNA, were treated with the indicated range of final concentrations of the tripeptide acetyl-Asn-Tyr-Thr-amide before incubation. Complete and partial competitive inhibition of glycosylation can be seen as a shift in apparent molecular weight.The track marked DMSO received ten times the amount of this solvent (in which the stock of peptide was dissolved) as was introduced with the highest concentration of peptide, demonstrating that this had no effect on glycosylation.The positions of unglycosylated (0-Gly) and fully glycosylated (3-Gly) Vg1 are indicated. **c)** HA mRNA was translated at 50μg/ml in the *Xenopus* egg extract and aliquots removed after 2 and 6 hours. Half of each of these was treated with endoglycosidase H (Endo H) overnight before electrophoresis and fluorography. Sensitivity to the enzyme can be seen as a reduction in apparent molecular weight. **d)** E1 mRNA was translated at 50μg/ml in *Xenopus* cell free extract (Xcf) and rabbit reticulocyte lysate (RL) or by injection of 30nl of 1μg/ml mRNA into oocytes (Oo), and immunoprecipitated products analysed by SDS PAGE and fluorography. O-Glycosylation in the oocyte and cell-free extract can be seen to have caused a reduction in mobility.

Signal sequence cleavage and protein segregation

Co-translational cleavage of the signal sequence of pre-prolactin has been widely used as an assay for segregation into microsomes in cell-free extracts, and the ability of this extract to perform this reaction is shown in figure 5a. Signal cleavage alone, however, does not prove unequivocally that segregation is occuring since cleavage could occur with ruptured microsomes. The ability of the extract to protect the processed product from digestion by added protease provides a more reliable indication of the integrity of the ER membranes present. Figure 5b shows that practically all of the prolactin synthesised was stable under protease conditions which, after disruption of the membranes present with Triton X-100, caused complete digestion.

A parallel demonstration of segregation into membranes is provided by the quantitative separation of processed and unprocessed translation products on centrifugation through a 20% sucrose cushion shown in figure 5c. Protease protection studies (not shown) confirmed that the pellet fraction recovered was not simply an aggregate.

N-Glycosylation

A variety of mRNAs coding for glycoproteins were translated in the *Xenopus* egg extract, in intact oocytes and in rabbit reticulocyte lysate. Two examples of this, the *Xenopus* Vg1protein and influenza hemagluttinin (HA) are shown in figure 6a. It is evident that both proteins, when made in either intact oocytes or egg lysates, have a reduced mobility compared with the reticulocyte lysate product and we have previously shown that this is due to N-glycosylation (18, 26) a modification known to occur in the endoplasmic reticulum. Further evidence that the increase in apparent molecular weight observed is due to N-linked glycosylation was obtained from translations performed in the presence of the tripeptide (acetyl)-Asn-Tyr-Thr-(amide) (Alta Bioscience), which has been to shown to compete with proteins

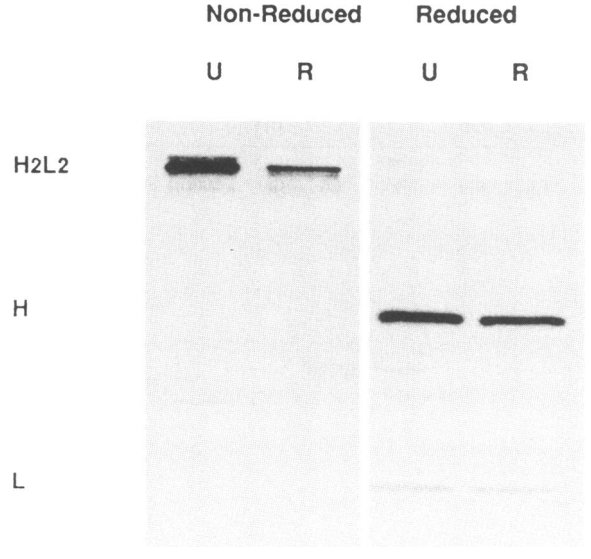

Figure 7. Immunoglobulin assembly in the extract. Mouse hybridoma poly A+ RNA was translated at 100μg/ml in whole, untreated (U) and in RNAse treated (R) *Xenopus* extract, immunoglogulin products were recovered by immunoprecipitation with anti-mouse immunobeads and analysed by SDS gel electrophoresis after denaturation in the absence (non-reduced) and presence (reduced) of b-mercaptoethanol. The positions of tetrameric immunoglobulin (H2L2), heavy (H) and light (L) chains are indicated.

for the transfer of sugar chains from their dolichol lipid carrier (28). As can be seen in figure 6b, not only do concentrations of peptide above 2mM completely abolish glycosylation, but partial conditions can be achieved, at around 0.4mM peptide, which allow the number of glycosylation sites in use to be determined. The DMSO-only control demonstrates that the effect was not caused by the presence of solvent added with the peptide. Protease protection and sucrose gradient fractionation confirmed that this effect was not the result of membrane disruption (data not shown).

Acquisition of endoglycosidase H resistance is confered on many glycoproteins on their passage through the medial Golgi compartment (29). In order to see whether transfer to the appropriate compartment occurs in the extract, HA was translated in the extract and treated with endoglycosidase H. The protein produced was completely sensitive to the enzyme indicating that these modifications were not occuring in the extract, even when the translation mixture was incubated for extended periods (Figure 6c). This lack of processing accounts for the difference seen between HA made in the oocyte and that made in the *Xenopus* egg extract, since we have shown previously that the slowest migrating oocyte band (marked by * in figure 6a) results from terminal glycosylation in the Golgi complex (26).

O-Glycosylation

We have shown previously that the corona virus E1 glycoprotein is post-translationally modified in the oocyte and that this modification is almost certainly a result of O-linked glycosylation (22). In figure 6d it is clear that whilst the extent of E1 modification performed in the extract is less than that seen in the oocyte, some modification has taken place, indicating that O-glycosylation can be initiated in the extract.

Protein folding and assembly

We have previously shown that *Xenopus* oocytes and eggs are capable of assembling murine immunoglobulins into functional tetramers (30, 31). Poly A+ RNA, prepared from a mouse hybridoma cell line which secretes IgG tetramers, was translated in both whole and ribonuclease treated extract and the immunoglobulin products recovered by immunoprecipitation. Electrophoresis under reducing and non reducing conditions clearly demonstrates that the majority of protein was assembled into a tetrameric complex which dissociated in the presence of reducing agent to yield heavy and light chains (Figure 7). We conclude that the extract is capable of assembling tetrameric immunoglobulin while the similarity between the results obtained with the two extracts indicates that assembly does not depend on co-translation of *Xenopus* proteins.

Addition of mannose 6 phosphate to lysosomal enzymes requires the appropriate juxtaposition of two non-contiguous regions of the protein and is thought to occur in an intermediate compartment between the endoplasmic reticulum and the Golgi apparatus (32). Figure 8 shows that both fresh and frozen extracts are capable of mannose 6 phosphorylation of cathepsin D, although at reduced efficiency compared with injected oocytes. We conclude that the correct folding of at least some cathepsin D molecules can occur in the extract.

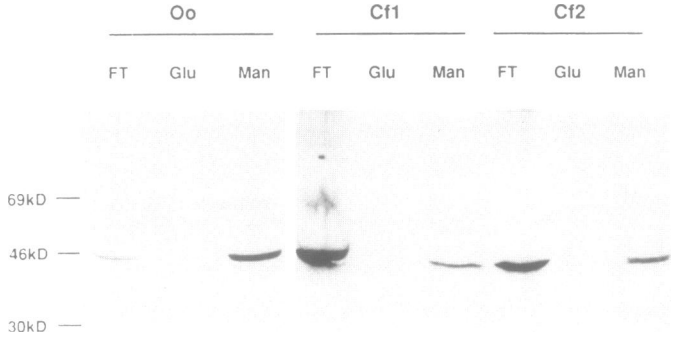

Figure 8. Mannose-6-phosphorylation of cathepsin D. Synthetic cathepsin D mRNA was translated by microinjection of 30nl of 1μg/ml mRNA into *Xenopus* oocytes (Oo), in whole fresh *Xenopus* egg extract (CF1) and in RNAse treated frozen *Xenopus* egg extract (CF2). The final concentration of mRNA in both extracts was 50μg/ml. ^{35}S labelled proteins were applied to a mannose-6-phosphate receptor column and the flow through (FT), 2mM glucose-6-phosphate wash (Glu) and mannose-6-phosphate elution (Man) were collected. Equal portions of each were analysed by immunoprecipitation and electrophoresis. Sizes of molecular weight markers are indicated.

DISCUSSION

Translation in heterologous cell-free systems

The early events in protein secretion are now understood in considerable detail (33). To a large extent this knowledge has been acquired by using a combination of in vitro translation and translocation systems to reconstitute the initial stages of segregation into the endoplasmic reticulum. Frequently the final assay systems comprise a mixture of components derived from a wide phylogenetic spectrum. Whilst it soon became clear that not all cell-free translation systems were alike [eg. the wheat germ lysate but not the reticulocyte lysate, is inhibited by dog pancreas microsomes (34)] , it was not readily appreciated that there could be qualitative differences.

For example, the canine signal recognition particle, SRP, which is a component of the translocation machinery, was shown to cause translational arrest of secretory but not cytosolic protein synthesis when added to a wheat germ lysate (3). This arrest could be relieved by the addition of translocation-competent membranes. These results were incorporated into a general model of protein translocation where one role of SRP was to prevent the premature elongation and consequent misfolding of nascent secretory proteins prior to their encounter with a translocation-competent membrane (33). This arrest did not occur, however, in the reticulocyte or HeLa cell lysates (35) nor did the addition of wheat germ SRP cause arrest in the wheat germ lysate (5). Furthermore the reticulocyte lysate contains endogenous SRP yet manages to translate secretory mRNAs in the absence of added membranes. Wolin and Walter (35) solved this paradox by showing that in the reticulocyte lysate the SRP causes translational 'pausing' but not arrest and speculate that this is the normal course of events in the living cell.

A further example of differences due to the use of cross-species combinations of translation and translocation systems comes from studies on the post-translational translocation of proteins into microsomes. It has been found that *S. cerevisiae* pre-proalpha factor can be post-tranlationally segregated into yeast microsomes in vitro, but only if it is first synthesised in a yeast lysate (36); the same protein made in the wheat germ lysate will not enter such membranes post-translationally although it will enter dog

pancreas membranes, but only co-translationally.Such examples serve to illustrate the difficulty in formulating general models of protein segregation on the basis of hybrid segregation systems.

The 'incompatibilities' of the various participating components can, however, be advantageous: The purification of SRP exploited the absence of this particle from the wheat germ lysate (37) and wheat germ lysate provided the appropriate environment for the identification of yeast components involved in post-translational translocation (38). Nevertheless, one major reason for developing cell-free systems is to allow the events taking place in the living cell to be examined in closer detail. Clearly this will be somewhat compromised unless homologous systems are available where all the components have the same origin. Several such systems have been developed but all are relatively inefficient . In this paper we describe the preparation and characterisation of a homologous system from the eggs of the amphibian, *Xenopus laevis*. The egg is the meiotic descendant of the oocyte, a large (1.2mm diameter) cell which has been widely used to investigate the translation, assembly and transport of a variety of secretory, membrane, and lysosomal proteins (6). Cell-free translation systems have been described from both oocytes and eggs, although the oocyte lysates are technically more troublesome to make and are less active. In contrast, as we show in this paper, the egg lysates are extremely active translationally, are simple to make, can be stored at −70°C and are efficient at protein translocation, signal cleavage and glycosylation.

Quantitation of protein synthesis in *Xenopus* egg extracts

It is difficult to obtain quantitative estimates of protein synthesis from the literature on cell-free translation systems. Jackson and Hunt (39) estimate that a reticulocyte lysate could synthesise about 130μg protein/ml/h. Clearly the reticulocyte lysate is a very durable cell-free translation system since Ryabova et al (40) obtained 2mg of α and β globin from 0.5ml of extract in a 100h incubation. However mRNA-dependent reticulocyte lysates are much less efficient (39). Whilst it is clear that other cell free systems are much less active than the reticulocyte lysate, we have not been able to find useful comparative data. We have quantitated protein translation in egg extracts by monitoring ^{35}S-methionine incorporation in extracts in which we had determined the free methionine pool using isotope dilution. The most active extract we examined synthesised 140μg/ml/h, after supplementation with an energy generating system. This is greater than the 23μg/ml/h value reported for oocyte extracts (8) or the 10μg/ml/h cited for egg extracts by Blow and Laskey (41). On a per egg basis our value converts to about 50ng/egg/h which compares favourably with a value, in vivo, of 40−60ng/egg/h, a figure which is the quotient of the calculated rate in oocytes (42) and the 2−3 fold increase which occurs on maturation of the oocyte into the egg (43). This supplemented extract, however, is only active for about 1h. This incorporation rate could be further increased by 50% by doubling the methionine pool, providing that other amino acids were also added in excess(data not shown). Without supplementation the fresh extract can synthesise protein at 25μg/ml/h, but linear incorporation kinetics have been observed for 3h. Supplementation is essential for the activity of frozen extracts where 60% of original, supplemented activity can be restored after storage at minus 70°C.

mRNA depletion of egg extracts cannot be effected with micrococcal nuclease because Ca2+ ions needed for the activity of this nuclease are rapidly sequestered into Ca2+ stores within the extract. However treatment with pancreatic ribonuclease followed by the addition of placental ribonuclease inhibitor is effective (16). We show (Figure 3) that such extracts are capable of translating a variety of different mRNAs and that synthetic rates of 65μg/ml/h can be achieved.

Post-translational modifications in *Xenopus* egg extracts

The original coupled translation-translocation system combined the wheat germ lysate with dog pancreas membranes (1). It was found that the addition of membranes resulted in inhibition of protein synthesis. Whilst this inhibition is not observed if the reticulocyte lysate is used, the ability of all the coupled systems to translocate and process secretory proteins is easily saturated by raising the level of added mRNA. At saturation in assays containing dog pancreas membranes, it is the ability to glycosylate which is the first post-translational processing step to be affected (44). In contrast, with yeast membranes it is signal sequence cleavage rather than glycosylation which is first affected (45). The *Xenopus* egg extract is tolerant of a broad range of mRNA levels: no discernable saturation occurs as the concentration of a single added synthetic mRNA is increased in the range 2.5 to 50μg/ml. Beyond this the proportion of marginal translation products which are segregated progressively reduces. Generally, a progressive reduction in glycosylation efficiency is seen at approximately the same point, but segregation without signal cleavage has never been seen in our extracts (cf figure 5c). We calculate that for one of the proteins used in this study, bovine prolactin, more than 13μg/ml/h of translocated protein can be made, demonstrating the high capacity of these extracts to segregate proteins encoded on added mRNAs.

The *Xenopus* egg extracts are made by centrifugal lysis. This would be expected to cause minimum disruption to the integrity of organelles of the secretory system and the pathways between them. The post-translational events we describe above occur in the endoplasmic reticulum which is the initial site of protein translocation. We have also tested for post-translational modifications which are known to occur at later stages of the secretory pathway. We have not been able to demonstrate the acquisition of endoglycosidase H resistance in any of our glycosylated proteins. Since some of these proteins (eg HA) become resistant in oocytes and eggs *in vivo* (26), we conclude that a processing step present before disruption and known to be located in a medial Golgi compartment, at least in mammalian cells (29), does not occur in these extracts. In contrast, the addition of mannose residues to the lysosomal protein, cathepsin D, does occur quite efficiently. Mannose 6 phosphorylation of exogenous cathepsin D has been previously demonstrated in *Xenopus* oocytes. Two sequential enzymic reactions are responsible for the addition of mannose 6 phosphate to N-linked oligosaccharide side chains. Although the site of the modifying enzymes is still controversial, the ER does not seem to be involved whereas the cis-Golgi has been implicated (32). We cannot say whether both modifications have occured in our extract though the reported preference of the mannose 6 phosphate receptor for an unmasked mannose 6 phosphate residue (46) suggests that this is the case.

We also find that the corona virus E1 glycoprotein is post-translationally modified, although the extent of the modification is less than seen in oocyte. We have previously argued (22) that in mRNA-injected oocytes this protein is O-glycosylated (E1 lacks N-glycosylation acceptor sites). We therefore believe that the observed modification represents at least the first step in O-glycosylation. In mammalian cells this occurs in a compartment

distal to the ER (47). We conclude that in the *Xenopus* egg extract both ER and some post-ER events have been preserved.

Finally we present evidence that proteins can fold and assemble correctly in the *Xenopus* egg extract. Mannose 6 phosphorylation requires the juxtaposition of distant regions of the polypeptide chain in order to form a three dimensional 'signal patch'(48). It seems that the requisite folding events necessary to form this patch are occuring in the extract. More direct evidence for correct assembly is seen in the demonstration (Figure 7) that tetrameric immunoglobulin is formed quantitatively in this system. Since it is believed that the ER is the major site of protein folding and assembly (49), this extract could provide a useful alternative to in vivo systems for studying structure/function relationships where these depend on protein folding or on certain covalent modifications, like mannose 6 phosphorylation.

ACKNOWLEDGEMENTS

We wish to thank Chris Ford, for assistance and advice on preparing extracts, Stuart Kornfeld, for the gift of the mannose 6-phosphate receptor column; Les Dale and Arthur Chakrabarti, for the providing *Xenopus* eggs; Monica Zoppe, for assistance with the mannose 6 phosphate experiment; John Davey, for comments on the manuscript, and the Medical Research Council and the Wellcome Trust for financial support.

REFERENCES

1. Blobel, G. and Dobberstein, B. (1975) J. Cell Biol.., **67**, 852–862.
2. Walter, P. and Blobel, G. (1983) In Fleischer, S. and Fleischer, B. (eds.), Methods in Enzymology, **96**, pp.84–93.
3. Walter, P., Ibrahimi, I. and Blobel, G. (1981) J. Cell Biol.,**91**, 545–550.
4. Meyer, D.I. (1985) EMBO J., **4**, 2031–2033.
5. Prehn, S., Weidman, M., Rappaport, T.A. and Zwieb, C. (1987) EMBO J., **6**, 2093–2097.
6. Devchand, M., Gwynne, D., Buxton, F.P. and Davies, R.W. (1988) Current Genetics, **14**, 561–566.
7. Colman, A. (1984) In Hames, D. and Higgins, S. (eds), Transcription and Translation–A Practical Approach. IRL Press, Oxford. pp271–302.
8. Patrick, T.D., Lewer, C.E. and Pain, V.M. (1989) Development, **106**, 1–9.
9. Laskey, R.A., Mills, A.O. and Morris, N.R. (1977) Cell, **10**, 230–243.
10. Lohka, M.J. and Masui, Y. (1983) Science, **220**, 719–721.
11. Almouzni, G., Mousseron-Grall, S. and Mechali, M. (1988) Nucleic Acids Res., **16** (17), 8525–8539.
12. Hardy, S., Aoufouchi, S., Thiebaud, P. and Prigent, C. (1991) Nucleic Acids Res., **19**, 701–705.
13. Bass, B.L. and Weintraub, H. (1987) Cell, **48**, 607–613.
14. Legerski, R., Penkala, J., Peterson, C. and Wright, D. (1987) Mol. Cell Biol., **7**, 4317–4323.
15. Blow, J.J. and Laskey, R.A. (1986) Cell, **47**, 577–587.
16. Hutchison, C.J., Cox, R. and Ford, C.C. (1988) Development, **103**, 553–566.
17. Murray, A.W. and Kirschner, M.W. (1989) Nature, **339**, 275–280.
18. Dale, L., Matthews, G.M., Tabe, L. and Colman, A. (1989) EMBO J., **8**, 1057–1066.
19. Faust, P., Wall, D., Perara, E., Lingappa,V. and Kornfeld, S. (1987) J. Cell Biol., **105**, 1938–1945.
20. Leaf, D.S., Roberts, S.J., Gerhart, J.C. and Moore, H-P. (1990) Developmental Biology, **141**, 1–12.
21. Ceriotti, A. and Colman, A. (1988) EMBO J., **7**, 633–638.
22. Armstrong, J., McCrea, M. and Colman, A. (1987) J. Cell. Biochem., **35**, 129–136.
23. Shuttleworth, J., Godfrey, R. and Colman, A. (1990) EMBO J., **9**, 3233–3240.
24. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual (Second Edition).Cold Spring Harbour Laboratory Press, Cold Spring Harbour.
25. Lohka, M. L. and Maller, J. (1985) J. Cell Biol., **101**, 518–523.
26. Ceriotti, A. and Colman, A. (1989) J. Cell Biol., **109**, 1439–1444.
27. Murray, A.W. Cell Cycle Extracts, Methods in Cell Biology, in press.
28. Lau, J. T., Welply, J. K., Shenbagamartin, P., Naider, F. and Lennarz, W. J. (1983) J. Biol. Chem., **258**, 15255–15260.
29. Kornfeld, R. and Kornfeld, S. (1985) Ann. Rev. Biochem., **54**, 631–664.
30. Valle, G., Besley, J. and Colman, A. (1981) Nature, **291**, 338–340.
31. Valle, G., Jones, J. and Colman, A. (1982) Nature, **300**, 71–74.
32. Kornfeld, S. and Mellman, I. (1989) Ann. Rev. Cell Biol., **5**, 483–525.
33. Walter, P. and Lingappa, V.R. (1986) Ann. Rev. Cell Biol., **2**, 499–516.
34. Shields, D. and Blobel, G. (1978) J.Biol. Chem., **253**, 3753–3756.
35. Wolin, S.L. and Walter, P. (1989) J.Cell Biol., **109**, 2617–2622.
36. Hansen, W., Garcia, D.P. and Walter, P. (1986) Cell, **45**, 397–406.
37. Walter, P. and Blobel, G. (1980) PNAS, **77**, 7112–7116.
38. Deshaies, R.J., Koch, B.D., Werner-Washburne, M., Craig, E.A. and Schekman, R. (1988) Nature, **332**, 800–805.
39. Jackson, R.J. and Hunt,T. (1983) In Fleischer, S. and Fleischer, B. (eds.) Methods in Enzymology, **96**, 54–74.
40. Ryabova, L. A., Ortlepp, S. A. and Baranov, V. I. (1989) Nucleic Acids Res., **17** (11), 4412.
41. Blow, J.J. and Laskey R.A. (1988) Nature, **332**, 546–548.
42. Taylor, M.A. and Smith, L.D. (1982), Dev. Biol., **110**, 230–237.
43. Wasserman, W.J., Richter, J.D. and Smith, L.D. (1982) Dev. Biol.,**89**, 152–158.
44. Dobberstein,B., Garoff, H., Warren, G. and Robinson, P.J. (1979) Cell, **17**, 759–769.
45. Rothblatt, J. and Meyer, D.I. (1986) Cell, **44**, 619–628.
46. Varki, A. and Kornfeld, S. (1983) J. Biol. Chem., **258**, 2808–2818.
47. Tooze, S.A., Tooze, J. and Warren, G. (1988) J. Cell Biol., **106**, 1475–1487.
48. Baranski, T.J., Faust, P.L. and Kornfeld, S. (1990) Cell, **63**, 281–291.
49. Rose, J.K. and Doms, R.W. (1988) Ann. Rev. Cell Biol., **4**, 257–288.