Trimer Formation Determines the Rate of Influenza Virus Haemagglutinin Transport in the Early Stages of Secretion in *Xenopus* Oocytes

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Abstract. We have previously shown that influenza haemagglutinin (HA) acquires Endo H resistance en route to the cell surface after microinjection of its mRNA into *Xenopus* oocytes (Ceriotti, A. and A. Colman. 1989. *J. Cell Biol.* 109:1439–1444.) In this paper we use the injection of varying amounts of mRNA (0.05–5 ng/oocyte) to effect a 30-fold change in HA protein synthesis within the oocyte. Using the Endo H assay as an indicator of protein movement from the ER to the medial Golgi we find that this movement is reduced, sometimes dramatically, when intracellular HA levels fall. This reduction in movement is closely correlated with a decreased rate of trimer formation as assessed both by trypsin resistance and sedimentation analysis, leading us to conclude that trimer formation is not only, as has been shown before essential for ER-Golgi complex movement, but is the major rate limiting step in this movement. Interestingly at least 50% of unassembled HA monomers that accumulate after low HA synthesis can be rescued into trimers over 24 h later, after a second injection of concentrated HA mRNA.

In contrast when we repeated this experiment with another membrane protein, the human low density lipoprotein, or with murine secretory immunoglobulin we found that the rate of movement was insensitive to the protein concentration. This latter result seemed surprising since earlier work had shown that unassembled IgG heavy chains (like monomeric HA) remain in the oocyte ER; however in these present experiments we have been unable to detect any unassembled heavy chains even at the lowest expression levels, indicating that tetramerization of Ig is much faster than trimerization of HA.

IT is generally believed that movement of secretory and membrane proteins from the ER to the cell surface is a passive process and that specific signals are required for their retention at various points along the pathway (Pfeffer and Rothman, 1987). It has also been shown that different proteins can move at distinctive and varying rates, and it is thought that the upper limit for the rate of protein transport is determined by the rate of bulk flow of membrane to the cell surface (Wieland et al., 1987). All proteins for which the appropriate data are available travel much slower than this, and there is considerable evidence that most of the observed variation in the overall rate of movement is caused by differences in the ER-Golgi complex rate of transport (Lodish et al., 1983; Fitting and Kabat, 1982; Fries et al., 1984; Williams et al., 1985). What determines the speed at which the various proteins leave the ER is still ill-defined. Recent work in this area has focused on the contribution made by oligomerization to the movement of multimeric proteins from the ER to the Golgi complex. In several cases the single subunits are selectively retained in the ER so that only fully assembled and correctly folded oligomers are competent for transport (Mains and Sibley, 1983; Kvist et al., 1982; Chen

et al., 1988). The influenza virus hemagglutinin HA (HA)[1] has been extensively used as a model system to try to elucidate the relationships between protein conformation and intracellular transport. The mature protein is a trimer composed of identical subunits (Wilson et al., 1981), which are synthesized on the ER membranes. Trimerization is a posttranslational event (Boulay et al., 1988), which in mammalian cells, can occur with a half time of <10 min (Gething et al., 1986; Copeland et al., 1988; Yewdell et al., 1988). Trimerization occurs in the ER, and mutants that cannot trimerize or correctly fold are not transported to the medial Golgi complex both in mammalian cells (Copeland et al., 1986; Gething et al., 1986) and in *Xenopus* oocytes (Ceriotti and Colman, 1989). These data strongly suggest that correct folding and trimerization are necessary for transport out of the ER but do not pinpoint trimerization as the rate limiting step in this process. To test the possibility that the rate of oligomerization dictates the rate of delivery of this protein to the Golgi apparatus we have injected graded amounts of HA mRNA into *Xenopus* oocytes. By so regulating the amount

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1. *Abbreviation used in this paper*: HA, haemagglutinin.

of HA monomers made in the oocyte we hoped to influence the rate of oligomerization and to establish a link between the rate of trimerization and the rate of ER to Golgi apparatus movement. We and others have previously demonstrated the ability of the oocyte to transport a variety of monomeric and oligomeric foreign proteins to their correct intra and extracellular destinations (Colman, 1984) and have shown that for several proteins secretion is linearly related to synthesis (Colman and Morser, 1979; Cutler et al., 1981). Of particular relevance to the work described in this paper are studies on the synthesis of murine immunoglobulins where it was shown that oligomerization of heavy and light chains can be controlled in oocytes by the coincident or sequential injection of purified heavy or light chains mRNAs (Colman et al., 1982).

In this paper we show that the rate of ER to Golgi apparatus movement of HA is coupled to the degree of HA trimerization which itself is controlled through the amount of mRNA injected. This indicates that in the oocyte, trimerization is the rate limiting step in HA movement from the ER to the medial Golgi compartment. Surprisingly, when we performed similar experiments with tetrameric immunoglobulin (Ig) we found that tetramerization was essentially completed during the labeling period irrespective of the level of Ig synthesis, and the secretion was linearly related to synthesis. This shows that lowering the expression level can have markedly different effects on the rate of assembly and transport of two different oligomeric proteins. Finally we are able to demonstrate that at least 50% of HA monomers that remain unassembled in oocytes for 24 h can be rescued into trimers by the subsequent introduction of high concentrations of newly synthesized HA molecules.

Materials and Methods

Oocyte Microinjection, Labeling, and Homogenization

Oocytes were obtained from large females of *Xenopus laevis*, maintained and microinjected as described by Colman (1984). Cytosolic RNA from (γ2b) cells (Sitia et al., 1990), prepared as described by Neuberger (1983) was a kind gift from R. Sitia. For microinjection, natural and synthetic (see below) RNAs were resuspended in distilled water. Injected oocytes were cultured overnight at 20°C in modified Barths' saline (MBS) before labeling in MBS containing 1 mCi/ml [^{35}S]methionine (800 Ci/mmol; Amersham, UK) at 5 μl/oocyte. In pulse-chase experiments the medium was removed, the oocytes were washed thoroughly with MBS, and then transferred to a small petri dish containing MBS supplemented with 1 mM methionine. Oocytes were homogenized in buffer containing Triton X-100 (Colman, 1984) and 1 mM PMSF using 40 μl/oocyte, and the homogenate was frozen in liquid nitrogen and stored at −80°C, or directly analyzed.

Immunoprecipitation and Gel Electrophoresis

For immunoprecipitation from total oocyte homogenate, from oocyte incubation medium and from fractions recovered from sucrose gradients, the samples were diluted to 1 ml with NET-gel buffer (Gething et al., 1986) and one of the following antibody additions made: 0.4 μl of α HA (Gething et al., 1986), or 0.4 μl of R260 anti–influenza virus antiserum (a kind gift from Dr. J. Wood, National Institute for Biological Standards and Control, London, UK), both antibodies recognizing native and unfolded forms of HA; 3 μl of an antibody preparation which exclusively recognizes unfolded HA (αUHA, Gething et al., 1986); 5 μl of anti–human low density lipoprotein receptor antibodies (a kind gift from J. L. Goldstein, University of Texas, Dallas, TX); 0.5 μl of rabbit anti–mouse immunoglobulin antibodies (a kind gift from R. Sitia, University of Genoa, Genoa, Italy). Samples were incubated for 2 h on ice and then 150 μl of a 10% suspension of protein A-Sepharose CL 4B (Pharmacia, Uppsala, Sweden) were added. The incubation was continued for 2 h on a rotary shaker at 4°C, the Sepharose beads

were washed three times with NET-gel buffer, and the antigen was released from the beads by boiling the sample in SDS-PAGE loading buffer, with or without beta-mercaptoethanol. Reduced samples were analyzed by SDS-PAGE as described (Colman, 1984). Nonreduced samples were run on 7.5–15% gradient gels, with an acrylamide/bisacrylamide ratio of 36.4:1. Relative molecular mass markers were always run under reducing conditions. Gels were fixed and fluorographed as described (Colman, 1984). Densitometric scans were obtained using a densitometer (TLC Scanner II; CAMAG, Muttenz, Switzerland) and integration was performed by a D-2000 Chromato-Integrator (Hitachi, Tokyo, Japan). Film exposures were chosen so that the signal was within the linear range of the film.

Endo H Treatment

Samples were immunoprecipitated as described above. Immunoprecipitates were subjected to Endo H digestion and analyses by SDS-PAGE were performed as described (Colman et al., 1985).

Sedimentation Velocity Centrifugation

Oocytes were injected, labeled and homogenized as described above. The homogenate was loaded on a linear 5–25% sucrose gradient (12 ml) in 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% Triton X-100. Gradients were centrifuged for 16 h at 20°C in a rotor (SW40; Beckman Instruments, Inc., Palo Alto, CA) at 40,000 rpm and fractionated from the top using an Auto-Densi-Flow IIC (Buchler Instruments, Fort Lee, NJ) into 20 × 0.6 ml aliquots. Immunoprecipitation of HA, SDS-PAGE on 10% gels, fluorography, and densitometry were performed as described above.

Protease Sensitivity

Oocytes were injected, labeled, and homogenized as described above, but PMSF was not included in the homogenization buffer. Oocyte homogenate (40 μl) was diluted 1:4.5 with PBS and incubated in the presence or in the absence of trypsin (10 μg/ml) for 10 min at 37°C. Digestion was stopped by adding soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) to a final concentration of 40 μg/ml. Trypsin-resistant fragments were immunoprecipitated and analyzed by 12.5% SDS-PAGE and fluorography as described above. To quantitate the amount of radioactivity contained in the protein bands, these were cut out of the dried gels and the polyacrylamide was depolymerized in 30% hydrogen peroxide (1 ml/gel slice, 50°C, 36 h). The sample was then mixed with 20 ml of scintillation fluid (Insta-gel; Packard Instrument Co., Inc., United Technologies, Downers Grove, IL) and counted in a scintillation counter (TRI-CARB 300; Packard Instrument Co., Inc.).

Production of Synthetic mRNAs

To produce synthetic mRNAs coding for the wild-type (HA$_{wt}$) or mutant (HA$_{env}$) hemagglutinins of the A/Japan/305/57 strain of influenza virus, pSP6HA$_{wt}$ or pSP6HA$_{env}$ DNAs (Ceriotti and Colman, 1988) were linearized with Sac I and then transcribed by SP6 polymerase in the presence of the capping dinucleotide m7G(5')ppp(5')G (Boehringer Mannheim Diagnostics, Inc., Houston, TX) as described by Krieg and Melton (1984). Trace amounts of [^{32}P]UTP were included in the reaction mixture for quantitation of reaction yields. The transcription mix was phenol extracted and the mRNAs were precipitated with ethanol and resuspended at concentrations of 100 μg/ml. Synthetic mRNA coding for human LDL receptor was a kind gift from J. L. Goldstein (Peacock et al., 1988).

Results

Expression Level Influences the Rate of HA Movement to the Golgi Complex

HA from the HA/Japan/305/57 strain contains several asparagine-linked (N-linked) oligosaccharide chains (Gething et al., 1980), all of which are modified in the Golgi complex to a form resistant to digestion by the enzyme Endo H. This enzyme removes N-linked chains that have not been processed by the Golgi enzymes *N*-acetylglucosamine transferase I and mannosidase II (Farquhar, 1985). When expressed
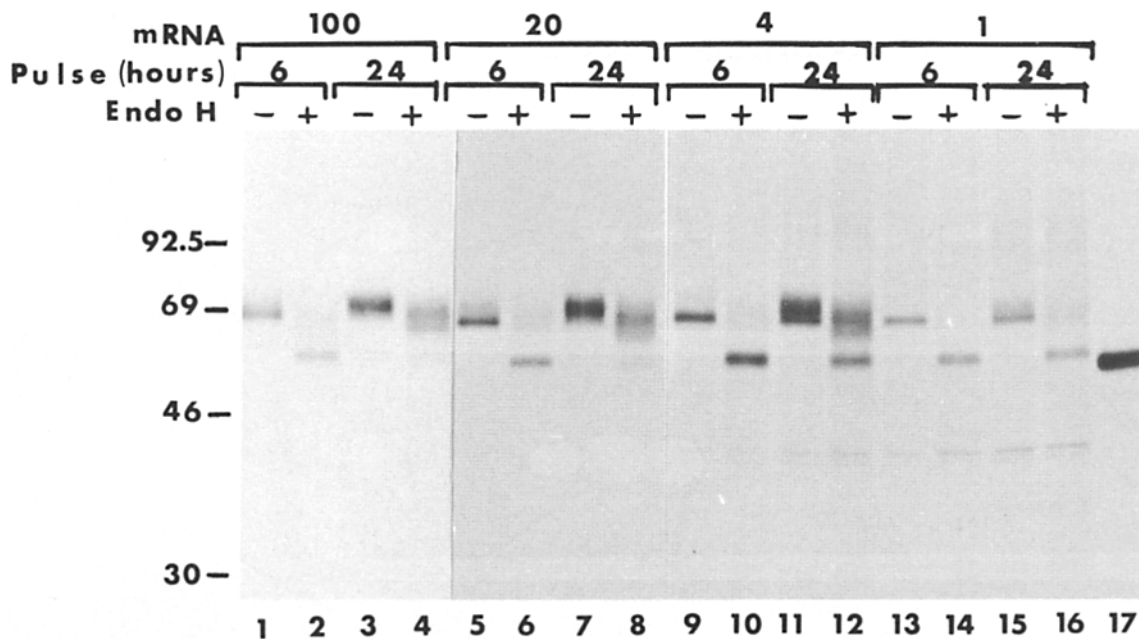
Figure 1. Effect of expression level on HA movement to the Golgi complex. Oocytes were injected with 50 nl of synthetic HA mRNA at concentrations of 100 (lanes *1-4*), 20 (lanes *5-8*), 4 (lanes *9-12*), or 1 μg/ml (lanes *13-16*). After culturing overnight, the oocytes were labeled continuously for the indicated time and then homogenized. HA was immunoprecipitated using a polyclonal antiserum (αHA). Half of the immunoprecipitate was incubated with Endo H(+). The other half was incubated without the enzyme (−). Proteins were analyzed by SDS-PAGE and fluorography. Lane *17* contains unglycosylated HA synthesized in the presence of tunicamycin. Band excision followed by scintillation counting indicated a 30-fold difference in the amounts of HA present in lanes *1* and *13*, and that the percent of Endo H-resistant HA in lanes *4* and *16* were >95 and 50%, respectively. Relative molecular mass marker positions (in kilodaltons) are indicated on left margin. Film exposure was 1 h for lanes *1-4*, 10 h for lanes *5-8*, and 8 d for lanes *9-17.*

in *Xenopus* oocytes, HA is synthesized as a glycosylated precursor containing Endo H-sensitive oligosaccharide chains, which are then modified and become resistant to the action of this enzyme (Ceriotti and Colman, 1989). The subcellular localization of the relevant processing enzymes has not been investigated in *Xenopus* oocytes but the fact that a mutant HA that does not leave the ER in mammalian cells is fully susceptible to digestion with Endo H when expressed in *Xenopus* oocytes (Ceriotti and Colman, 1989) strongly suggests that also in this system acquisition of Endo H resistance occurs after exit from the ER.

If trimer availability is the rate limiting step in the export of the protein from the ER we would expect the rate of exit from the ER to be tightly coupled to the rate of trimerization. Since trimerization of HA is an intermolecular reaction, a reduction in the concentration of monomeric HA molecules should lead to a reduced rate of trimer formation and consequently, a reduced rate of exit from the ER. In the experiment shown in Fig. 1, we have generated a 30-fold range of HA concentrations in oocytes by the simple expedient of injecting solutions of HA mRNA which spanned a 100-fold concentration range. Export from ER to Golgi complex was assessed by the degree of Endo H resistance in HA at different times after incubation in radioactive media. After overnight incubation the oocytes were transferred to labeling medium for 6 or 24 h and then analyzed for the presence of Endo H-resistant HA. At both time points the percentage of HA containing Endo H-resistant chains was found to be related to the expression level, greater synthesis leading to

faster processing. Significantly, 24 h after the beginning of the labeling period, >95% (see legend, Fig. 1) of the HA synthesized in oocytes injected with mRNA at the highest concentration was processed to an Endo H-resistant form (Fig. 1, lane *4*). In contrast, ∼50% was still fully Endo H sensitive in oocytes injected with HA transcript at the lowest concentration (Fig. 1, lane *16*). The reduced movement seen after synthesis of lower amounts of HA protein was not affected by coexpression of compensatory amounts of another secretory protein, chick ovalbumin (data not shown). This excludes the unlikely possibility that the observed effects were a consequence of different local concentrations of newly synthesized secretory protein. Altogether these findings indicate that expression level (and hence, most probably, the concentration of HA monomers in the ER) influences a rate limiting step in HA delivery to the Golgi complex.

Similar results have been obtained in further experiments where either continuous labeling or pulse-chase protocols were used (data not shown). However differences were found in the proportion of Endo H-sensitive material found at a given time point in response to the injection of a particular concentration of mRNA. We attribute this to variation in the abilities of different batches of oocytes to use injected mRNA and note that a higher proportion of Endo H-resistant material was always found, at least after injections of 1 mg/ml HA mRNA, in the more biosynthetically active oocytes (e.g., Fig. 2).

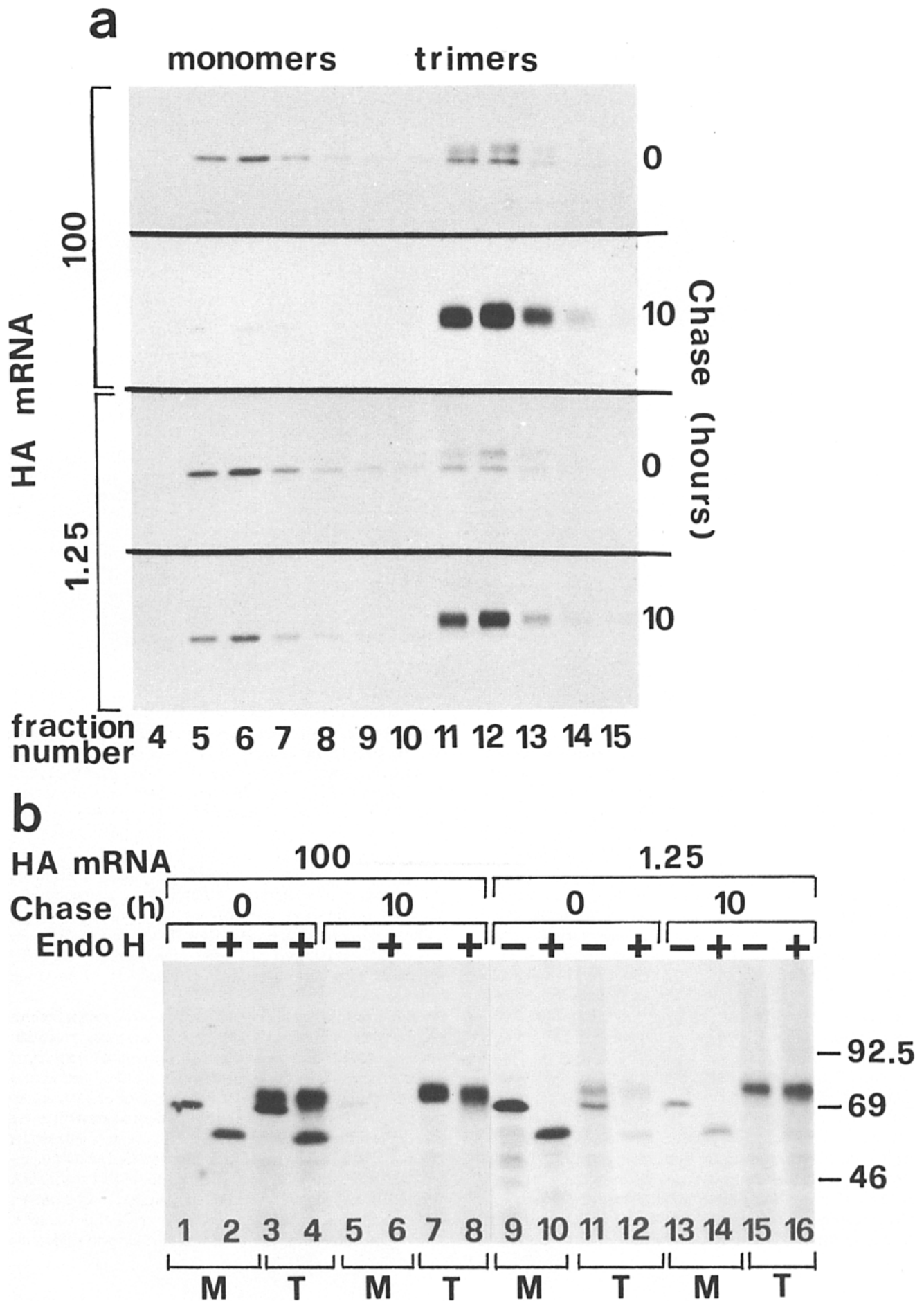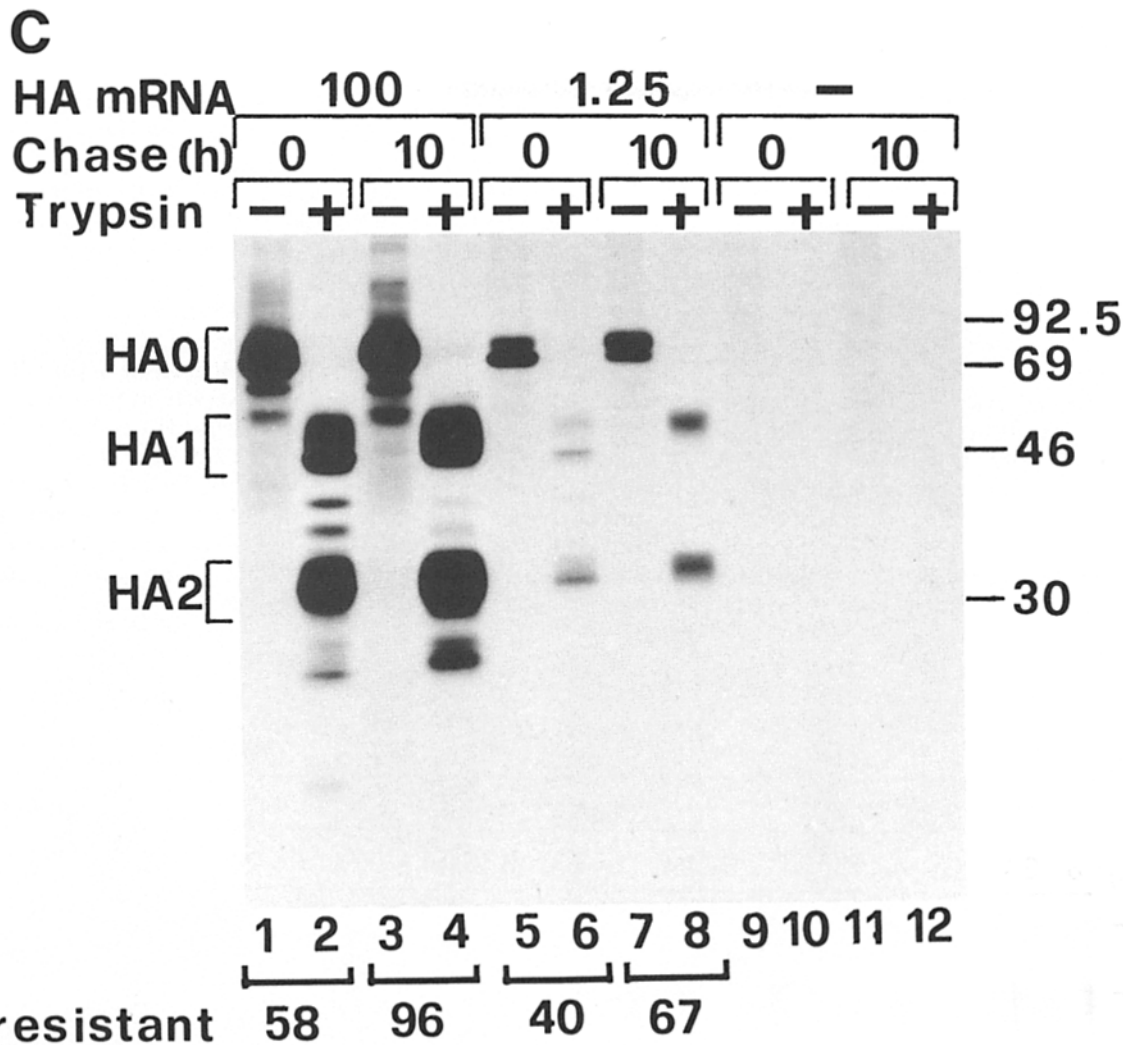We conclude that in the case of HA, the local rate of its

a

monomers   trimers

HA mRNA

100

1.25

0

10

0

10

Chase (hours)

fraction
number 4   5   6   7   8   9   10 11 12 13 14 15

b

HA mRNA   100   1.25
Chase (h)   0   10   0   10
Endo H   − + − +   − + − +   − + − +   − + − +

—92.5

—69

—46

1 2 3 4   5 6 7 8   9 10 11 12   13 14 15 16

M   T   M   T   M   T   M   T

Figure 2.

Figure 2. Trimer production in oocytes is dependent on HA concentration. Oocytes were injected with HA mRNA at 100 or 1.25 µg/ml, cultured overnight, and then labeled for 3 h. Some oocytes were immediately homogenized while those remaining were chased for 10 h before homogenization. (*a*) Fractionation of monomeric and trimeric HA by sedimentation centrifugation. A fraction of the homogenate was subjected to sucrose gradient fractionation. HA was quantitatively immunoprecipitated from each fraction and analyzed on SDS-PAGE. The amount of labeled HA present in each fraction was quantitated by densitometry. (*b*) Endo H analysis of monomeric and trimeric HA. HA was immunoprecipitated from the pooled sucrose gradient fractions containing monomeric or trimeric HA. The immunoprecipitated HA was divided in two aliquots, one of which was digested with Endo H. *M*, monomeric HA; *T*, trimeric HA. (*c*) Trypsin sensitivity test. Aliquots of the oocyte homogenates were incubated with (+) or without (−) trypsin as described in Materials and Methods. After addition of the protease inhibitor, HA was recovered by immunoprecipitation and analyzed by SDS-PAGE. The positions of intact HA (*HA0*) and of the tryptic fragments (*HA1* and *HA2*) are indicated. Bands were excised and the amounts of radioactivity present in the HA0, HA1, and HA2 bands were determined by scintillation counting. The percentage of HA0 radioactivity recovered after trypsin treatment in the HA1 and HA2 fragments is indicated (*% resistant*). The positions of relative molecular mass markers (in kilodaltons) are shown on right margin.

synthesis determines its rate of movement from the ER to the medial Golgi compartment.

The Expression Level Influences the Rate of HA Trimer Formation

Biochemical and immunocytochemical data pinpoint the ER as the site of HA trimerization in mammalian cells and show that monomers are not transported out of this compartment (Copeland et al., 1988). Clearly HA movement to the medial Golgi complex is sensitive to the amount of HA made in the oocyte. To establish that the reduced exit rate reflects slower

trimer formation rather than, for example, a reduced absolute concentration of assembled timers, we performed additional chase experiments but this time also monitored trimer formation by two different methods. In the first method oocytes injected with HA mRNA at 100 or 1.25 µg/ml were labeled, chased, and then homogenized and subjected to sucrose gradient sedimentation analysis in the presence of Triton X-100; on these gradients monomeric HA sediments at 4.5S whereas trimers sediment at 9.0S (Doms and Helenius, 1986). HA was precipitated from each fraction and the immunoprecipitates were analyzed by SDS-PAGE (Fig. 2 *a*). Quantitative densitometry was then used to determine

Table 1. Quantitation of HA Assembly and Movement

HA mRNA	Chase	Trimeric	Endo H resistant	Trypsin resistant
µg/ml	h	%	%	%
100	0	60	27	58
	10	95	95	96
1.25	0	29	13	40
	10	80	60	67

The figures above were derived from Fig. 2, a and b by quantitative densitometry and from Fig. 2 c by band excision and scintillation counting.

the relative amount of HA present in the monomer and trimer peak (see Table I). At the end of the pulse, HA was found to be distributed between monomers and trimers, but the percentage of HA in trimers was dependent on the expression level, with 60 and 29% as trimers in oocytes expressing HA at high and low levels, respectively. In both cases trimerization continued during the chase period, at the end of which

~100% (high expression) and 80% (low expression) of the synthesized HA was recovered in the trimeric form. This shows that the expression level influences the rate of HA oligomerization, greater synthesis leading to faster assembly. Fig. 2 a also shows that monomeric HA always migrates as a single band, while trimeric HA at the beginning of the chase period migrates as a doublet. To see whether this was due to oligosaccharide processing, monomeric and trimeric HA recovered from the sucrose gradients were subjected to Endo H treatment (Fig. 2 b). Monomeric HA was always found to be fully Endo H sensitive, while trimeric HA was partially Endo H resistant at the end of the pulse, the Endo H-resistant material corresponding to the upper band of the doublet. We interpret these observations to indicate that the slower migrating, Endo H-resistant HA is derived from trimers that are in, or have passed through, the medial Golgi compartment, whereas the faster, Endo H-sensitive form is present as monomer in the ER or as trimers present in ER or cis-Golgi compartments. Using this information and reexamining Fig. 2 a, we note that the ratio of resistant to sensi-

a

b
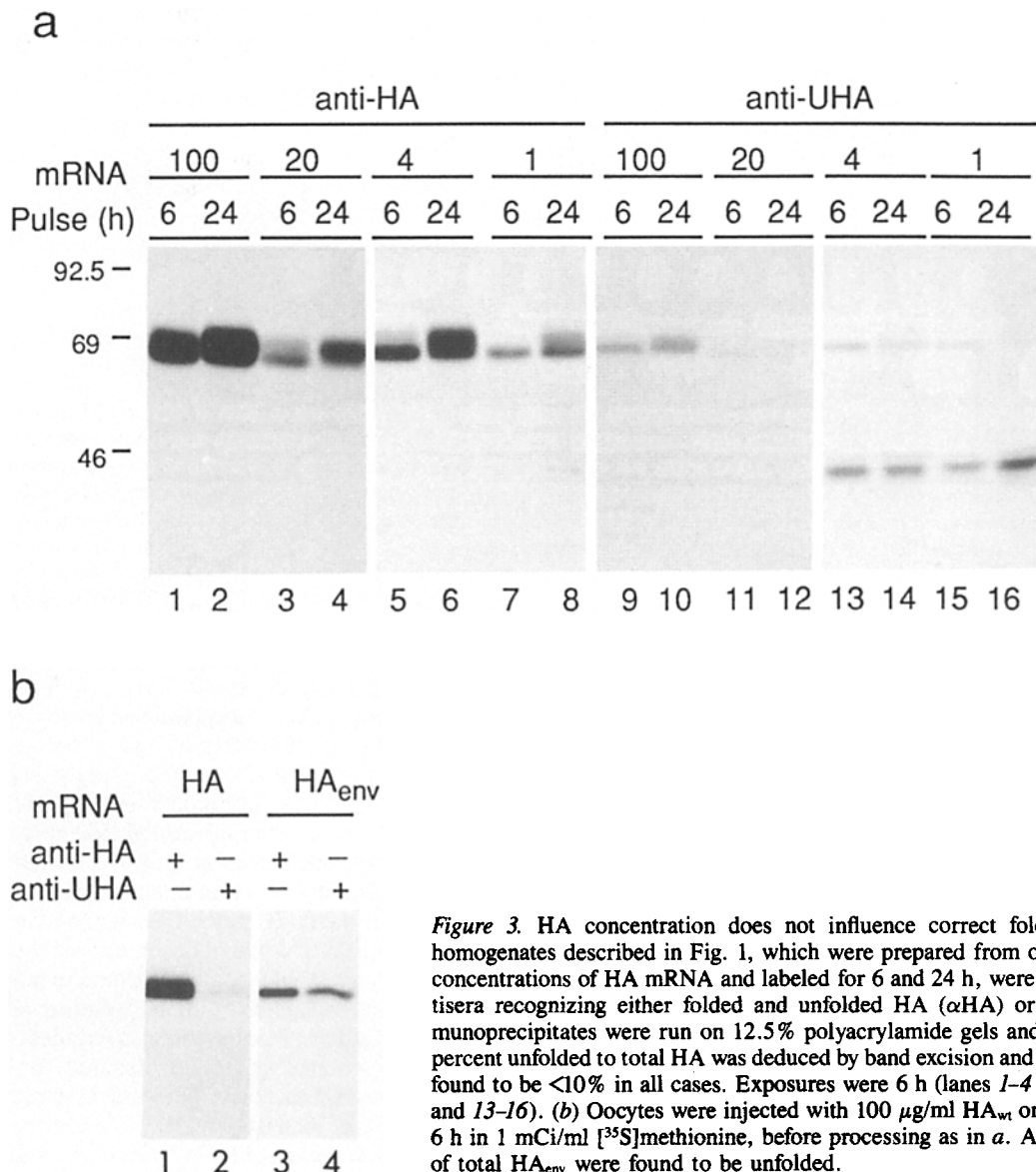
Figure 3. HA concentration does not influence correct folding of monomers. (a) The homogenates described in Fig. 1, which were prepared from oocytes injected with different concentrations of HA mRNA and labeled for 6 and 24 h, were immunoprecipitated with antisera recognizing either folded and unfolded HA (αHA) or unfolded HA (αUHA). Immunoprecipitates were run on 12.5% polyacrylamide gels and the gels fluorographed. The percent unfolded to total HA was deduced by band excision and scintillation counting and was found to be <10% in all cases. Exposures were 6 h (lanes 1-4 and 9-12) and 7 d (lanes 5-8 and 13-16). (b) Oocytes were injected with 100 µg/ml HA$_{wt}$ or HA$_{env}$ RNA and cultured for 6 h in 1 mCi/ml [35S]methionine, before processing as in a. About 15% total HA and 75% of total HA$_{env}$ were found to be unfolded.

414

tive trimers is similar at 0 h with both HA concentrations. This strongly suggests that the intrinsic rate of trimer movement and processing is unaffected by HA concentration.

The second method used to measure trimer formation in samples from the same experiment was a trypsin sensitivity assay. Trimeric HA is resistant to trypsin digestion, and is split into two fragments (HA1 and HA2) while monomers are very sensitive (Gething et al., 1986) and are rapidly degraded to many small fragments. The percentage of HA recovered in the two characteristic fragments is therefore a measure of the extent of trimerization. The results of this experiment are shown in Fig. 2 c and Table I, and confirm that the acquisition of a trypsin-resistant conformation is more rapid in oocytes injected with high HA mRNA concentrations.

When all these data are considered together (Table I) it is clear that HA trimer formation, whether assessed by sedimentation analysis or trypsin sensitivity, is more rapid in oocytes injected with higher concentrations of mRNA. Since it appears that only trimers can travel from the ER to Golgi compartment, Endo H resistance is only seen in the trimer form, and if we use this resistance as an indicator of HA transport, it is also clear that a reduced rate of trimer formation is associated with a similar reduction in the rate of acquisition of Endo H resistance. Since movement to the Golgi complex is so sensitive to the rate of trimer formation we conclude that trimer formation is not only obligatory for ER exit, but that it or some prior event is the rate limiting step in this process.

Monomer Folding Is Not Affected by HA Concentrations

The sequence of events leading to formation of HA trimers is translation of HA monomers, monomer folding, and finally the assembly of folded monomers into trimers. We show above that trimer formation is sensitive to the concentration of HA monomer synthesized. Although unlikely, it was possible that the correct folding of HA monomers was the concentration-sensitive step, and that the reduced rate of trimer formation was a secondary consequence. This possibility was excluded by the experiment shown in Fig. 3. The HA made in oocytes after the injection of different concentrations of HA mRNA (see Fig. 1), was immunoprecipitated either with an antibody which recognizes folded and unfolded forms of HA (αHA) or with an antibody recognizing only unfolded forms of HA (αUHA; Gething et al., 1986). Clearly very little of the HA (10%, see legend to Fig. 3) was unfolded after high or low doses of injected mRNA (Fig. 3 a, cf. lanes 1-8 with 9-16) unless denatured before immunoprecipitation (not shown); in contrast, the misfolded mutant HA$_{env}$ protein (Gething et al., 1986) was almost quantitatively precipitated (Fig. 3 b, cf lanes 3 and 4). We therefore conclude that the correct folding of monomers is not concentration dependent and that it is the process of monomer assembly into trimers that is concentration dependent.

HA Monomers Can Trimerize after a Long Stay In The ER

It has been shown (Colman et al., 1982) that light and heavy Ig chains synthesized separately in Xenopus oocytes remain intracellular but retain the ability to oligomerize with the complementary chains if these are synthesized later. These experiments demonstrated that marooned heavy and light chains had not been diverted out of the secretory pathway or irreversibly complexed with factors that could prevent proper assembly. In mammalian cells a small proportion of the monomeric HA never trimerizes or moves to the Golgi complex and is found associated with the heavy chain binding protein, Bip (Hass and Wolb, 1983). We were interested to see whether in oocytes, the events of monomer folding and productive trimerization could be separated by long periods of time. We therefore injected oocytes with diluted mRNA solution (1 μg/ml) so that trimerization was very slow. The oocytes were labeled for 3 h and then chased in cold medium, and rescue of the HA monomers was attempted after different times of chase by injecting a concentrated (100 μg/ml) HA mRNA solution. Trimer formation was directly assessed using sedimentation analysis; Golgi complex processing could be monitored from the same gradients by the upper shift in HA mobility. The results are shown in Fig. 4. In the absence of a second injection of HA mRNA, labeled HA monomers were found to be quite stable ($t_{1/2}$ of ~30 h). However trimer formation was low even after a 50-h chase. In contrast, a second injection of HA mRNA promoted trimerization of the previously synthesized monomers so that the majority of the HA molecules were in the trimer form. In all cases most of the trimeric HA showed the upward shift in mobility indicative of Golgi complex processing. We conclude that most of HA monomers can persist in the oocyte ER for a period as long as 24 h and still be in a conformational state and topological situation that permits trimerization and transport to the Golgi complex.

The Dependence of the Rate of Transport on the Level of Expression Is Not a General Feature of Secretory and Plasma Membrane Proteins Expressed in Xenopus Oocytes

To extend our analysis we also studied the effect of the expression level on the rate of assembly and movement of murine IgG. Xenopus oocytes have been shown to be an appropriate surrogate system for the study of the synthesis, assembly, and secretion of IgG molecules (Valle et al., 1981, 1983; Colman et al., 1982). Both in mammalian cells (Kohler et al., 1976) and in Xenopus oocytes (Valle et al., 1981) the heavy (H) chain of IgG is secreted only when assembled with light (L) chain in a H$_2$L$_2$ tetrameric structure. Since assembly is a prerequisite for secretion, we wanted to see whether as in the case of HA, the rates of both processes were dependent on the rate of synthesis of the individual subunits. The experiment reported in Fig. 5 shows that this is not the case. Xenopus oocytes were injected with total cytoplasmic RNA from a cell line secreting IgG$_{2b}$, at concentrations of 2.0, 0.08, or 0.02 mg/ml. After overnight incubation the oocytes were labeled for 24 h with [³⁵S]methionine and then the IgG present in the oocyte homogenate and media was immunoprecipitated using a polyclonal antiserum. Immunoprecipitates were analyzed by SDS-PAGE under nonreducing conditions. Only two major bands, corresponding to H$_2$L$_2$ tetramers and excess free light chains, are evident both inside the oocyte and in the secreted medium. Quantitative densitometry of the H$_2$L$_2$ bands showed the ratio between intracellular and secreted IgG was similar for all
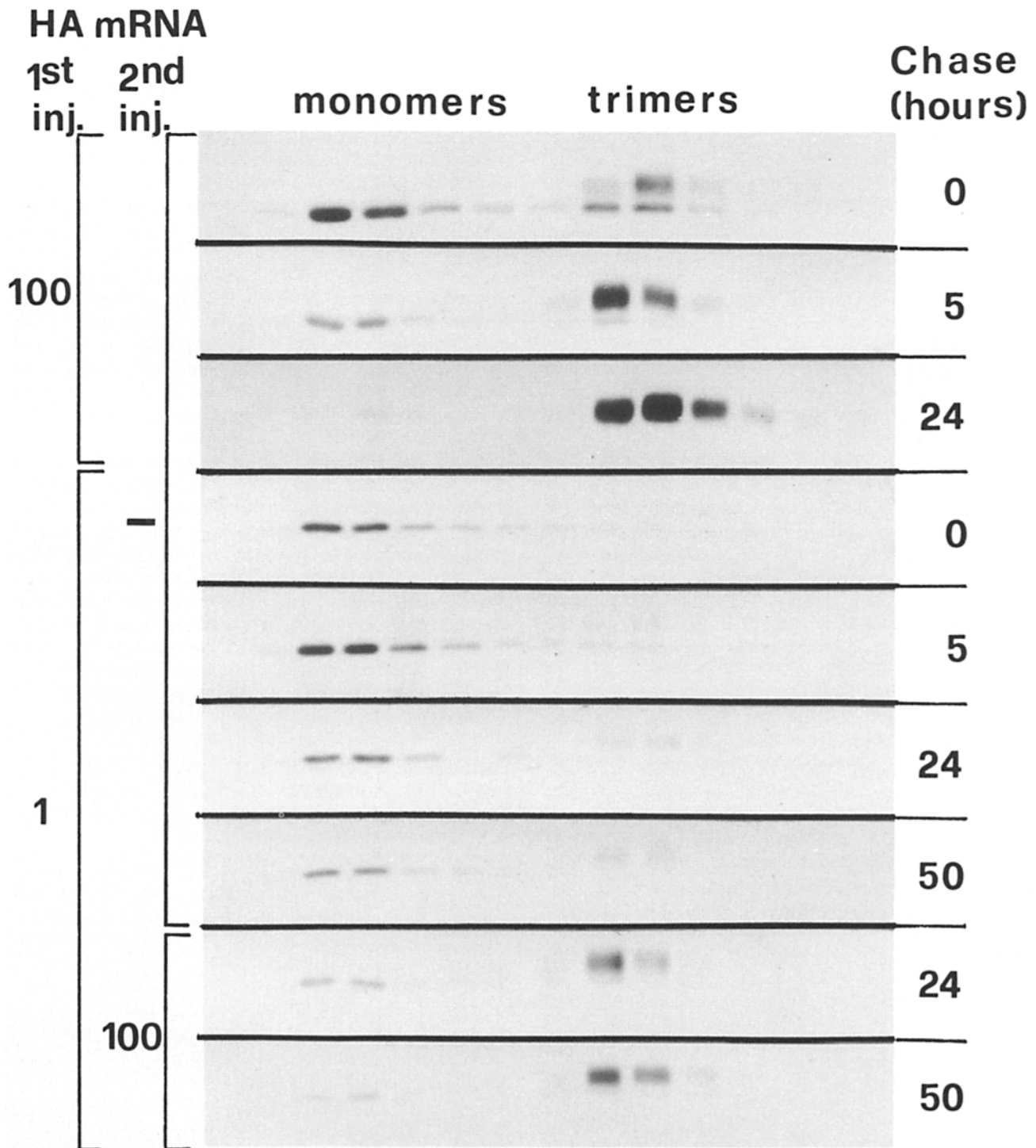
Figure 4. Rescue of monomeric HA. Oocytes were injected with HA mRNA at 100 or 1 μg/ml, cultured overnight, and then homogenized (0-h chase) or chased in cold medium for 5, 24 or 50 h (*Chase*). At 5 and 24 h of chase some of the labeled oocytes that had received HA mRNA at 1 μg/ml were injected with HA mRNA at 100 μg/ml. These oocytes were homogenized either 24 (second injection at 5 h) or 50 h (second injection at 24 h) into the chase. The chase conditions were validated by parallel sets of injections of HA mRNA (100 μmg/ml) into control oocytes that had been labeled and chased; very little labeled HA was detected in such oocytes. Aliquots of the oocyte homogenates were fractionated by sedimentation velocity centrifugation and analyzed as in Fig. 2 *a*.

expression levels, at least within the tested range. Free heavy chains or heavy chain dimers (Valle et al., 1981) were not present under any conditions indicating the quantitative tetramerization of heavy chains even at low expression lev-

els. This result indicates that the low level of expression did not detectably inhibit oligomerization. This is in clear contrast with what was observed in the case of HA (see Figs. 2 *a* and 4) and quite surprising considering the tetrameric,
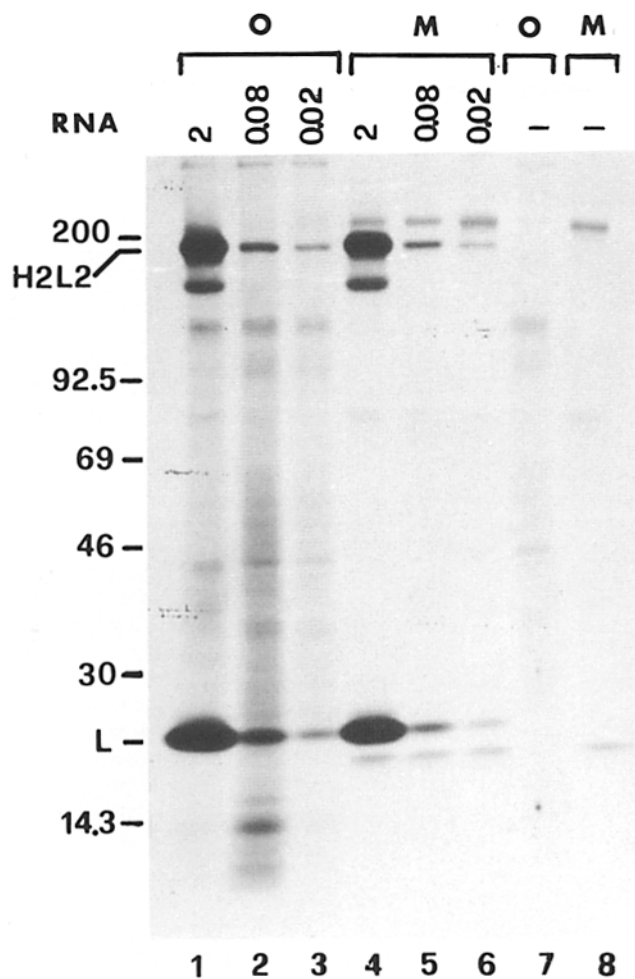
Figure 5. Effect of the expression level on the rate of secretion of tetrameric IgG. Oocytes were injected with total cytosolic mRNA isolated from a cell line producing murine IgG2b, at concentrations of 2, 0.08, and 0.02 mg/ml. Some oocytes were left uninjected. After overnight incubation the oocytes were labeled for 24 h and then homogenized in the presence of 75 mM iodoacetamide. Homogenate equivalent to 1 oocyte (O) and medium (M) equivalent to 2.5 oocytes were used to immunoprecipitate IgG using a polyclonal rabbit anti-mouse Ig serum. The immunoprecipitates were eluted from the protein A-Sepharose bead by heating in sample buffer without reducing agent, at 100°C for 2 min, and loaded on a 7.5-15% gradient gel. The positions of tetrameric IgG (H2L2), of free light (L) chain, and of relative molecular mass markers are indicated.

rather than trimeric, nature of IgG. Unfortunately this rapid oligomerization prevents identification of any rate limiting step with this approach.

Finally, in the case of monomeric proteins the expression level would be expected not to influence the rate of secretion. It has been previously shown (Cutler et al., 1981) that in Xenopus oocytes, the kinetics of secretion of the chick oviduct proteins, ovalbumin and lysozyme, are independent of their expression level. To see whether this was true also in the case of a monomeric membrane protein we studied the effect of the rate of synthesis on the intracellular transport of the receptor for the human LDL. It has been recently shown (Peacock et al., 1988) that when synthetic mRNA

coding for this LDL receptor is injected into Xenopus oocytes, the receptor is efficiently synthesized and glycosylated with O-linked and N-linked sugars. The latter are then modified to an Endo H-resistant form. We therefore used acquisition of Endo H-resistance to evaluate the effect of expression level on the rate of movement to the Golgi complex. Oocytes were injected with synthetic mRNA coding for the human LDL receptor (Peacock et al., 1988) at two different concentrations, labeled for 3 h in [^{35}S]methionine, and then chased for 7 h in unlabeled medium. Immunoprecipitates were subjected to Endo H digestion and analyzed by SDS-PAGE (Fig. 6). As previously reported, the precursor form the LDL receptor synthesized in the oocyte (Fig. 6, arrowhead) was always Endo H sensitive while the mature form (Fig. 6, asterisk) was found to be Endo H resistant. Both forms were present at the end of the labeling period and the ratio between them was not influenced by expression level (Fig. 6, cf. lanes 1 and 2 with 5 and 6). By the end of the chase period LDL receptor was in all cases completely converted to the Endo H-resistant form. These data indicate that as in the case of soluble monomeric secretory proteins, expression level does not influence the rate of movement of a monomeric membrane receptor. Interestingly, although the frog oocytes would appear to have no need for an LDL receptor since liver-derived vitellogenin is thought to perform a similar cholesterol transport role to LDL, an endogenous oocyte protein similar in molecular weight to mature LDL is immunoprecipitated by the anti-LDL antiserum (Fig. 6, lanes 9 and 10).

Discussion

Lowering HA Expression Reduces both Trimer Formation and Movement to the Medial Golgi Complex

It is well established that different proteins can move from their site of synthesis in the ER, to the cell surface at very different rates (Lodish et al., 1983; Fries et al., 1984; Williams et al., 1985; Fitting and Kabat, 1982; Cutler et al., 1981). It is also accepted that the rate of movement from the ER to the Golgi apparatus is in most cases the one that determines these differences, the rate of the subsequent delivery to the cell surface being comparable between the different proteins (Lodish et al., 1983; Fitting and Kabat, 1982; Fries et al., 1984). On the other hand it is still unclear what determines these differences in the rate of delivery to the Golgi complex. In many cases it has been shown that oligomeric cell surface or secreted proteins are transported to the cell surface only if assembled into the correct quaternary structure (Yu et al., 1983; Peters et al., 1985; Takei et al., 1987; Severinsson and Peterson, 1984; Kondor-Koch et al., 1982; Kishimoto et al., 1987; Ohashi et al., 1985; Mains and Sibley, 1983). Whereas some exceptions do exist (Potter et al., 1985; Kondor-Koch et al., 1982; Corless et al., 1987), it does seem for several proteins that some structural characteristic of the individual subunits is not compatible with transport, and that oligomerization either conceals or modifies these structures. In the case of HA it has been shown that monomers are not trimmed by Golgi mannosidase I (Copeland et al., 1988) and are not modified by the Golgi enzymes that confer Endo H resistance to oligosaccharide

mRNA 100 4 —

Chase (hours) 0 7 0 7

Endo H − + − + − + − + − +
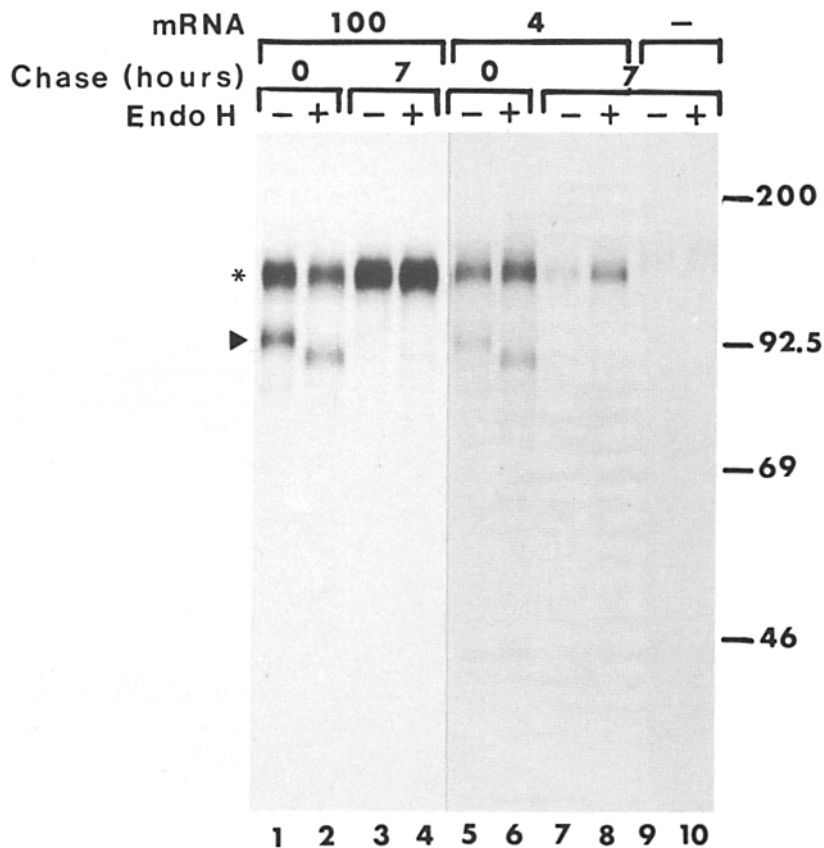
Figure 6. Effect of the expression level on the rate of transport of human LDL receptor. Oocytes were injected with synthetic mRNA coding for human LDL receptor at the concentration of 100 (lanes *1-4*), 4 μg/ml (lanes *5-8*), or left uninjected (lanes *9* and *10*). After overnight incubation the oocytes were labeled for 3 h, chased for 7 h, and homogenized. LDL receptor was immunoprecipitated using a polyclonal antiserum and the immunoprecipitates were incubated with (+) or without (−) Endo H and analyzed by SDS-PAGE (8% acrylamide). Precursor and mature forms of the receptor are indicated by the arrow and asterisk, respectively (see text). Relative molecular mass markers (in kilodaltons) positions are indicated on right margin. Film exposure was 16 h for lanes *1-4* and 5 d for lanes *5-10*.

side chains (Gething et al., 1986). This observation, together with the fact that a mutant HA that cannot form trimers is retained in the ER (Gething et al., 1986), strongly suggests that trimerization is a prerequisite for the exit of HA molecules from the ER. However trimerization per se isn't sufficient for movement since trimer "quality" can also be a contributory factor; mutant HA$_{env}$ trimers, which are assembled from incorrectly folded mutant monomer chains, are also retained in the ER of mammalian cells (Gething et al., 1986) and, probably, the ER of oocytes (Cerrioti and Colman, 1989). Similar data have been obtained for vesicular stomatitis virus G (VSV G) protein although in the case of several retarded mutant proteins, faulty trimerization was not detected (Doms et al., 1988). In the following discussion only transport competent trimers are considered. In mammalian cells at 37°C, HA trimerization is a very fast process and trimers can be detected as little as 3 min from the beginning of a labeling period (Gething et al., 1986). Trimerization has been shown to be a posttranslational process independent of cytosolic ATP but dependent on temperature (Copeland et al., 1988). In *Xenopus* oocytes trimerization and transport are much slower than in mammalian cells. One factor contributing to this difference is probably the different temperatures at which mammalian cells (37°C) and *Xenopus* oocytes (19°C) are normally maintained. Our data indicate that transport of HA to the Golgi complex can occur with a $t_{1/2}$ of <4 h, when HA mRNA is injected at a relatively high concentration (100 μg/ml or more) (Ceriotti and Colman, 1989); lowering the concentration of the injected mRNA resulted in a longer $t_{1/2}$

of movement to the Golgi complex. Trimer formation was similarly affected by the change in expression level thus indicating the presence of a link between the rates of the two processes.

If a step distal to trimer formation were or became rate limiting to the rate of ER-Golgi complex movement in our experiments, the modulation in trimer formation observed would not have affected net movement. We conclude that trimer formation alone determines the rate of this movement in oocytes. This conclusion is reinforced by the finding that the proportion of trimers that are Endo H resistant is similar at low and high HA concentrations (Fig. 2 *a*)—an observation that indicates that once formed, the movement of a trimer is uninfluenced by local HA trimer or monomer concentrations. If trimer clustering played a significant rate-limiting role in protein transport, as has been suggested by Doms et al. (1988) from their studies on wild-type and mutant VSV G proteins, then an effect of a trimer dilution might have been expected. A different result may be anticipated for hepatitis B surface antigen translocation. In the case of this protein, a self-assembly process occurring at the level of the ER is presumably responsible for the creation, by budding, of 22-nM particles that are subsequently transported through the hepatocyte secretory pathway and exocytosed (Standring and Rutter, 1986). This process also occurs in oocytes after RNA injection (Standring et al., 1986) and we would expect in this case that lowering the concentration of surface antigen monomers through mRNA dilution would result in significant attenuation in the rate of secretion.

Reduced Expression Has No Effect on Ig Tetramerization or Secretion

We attempted to use the above experimental strategy to investigate tetrameric IgG secretion since only the tetramers are secreted by mammalian cells (Kohler et al., 1976) or *Xenopus* oocytes (Valle et al., 1981). In view of the results with HA trimerization we anticipated, if anything, an even more marked effect of protein concentration on Ig tetramerization, simply due to the laws of mass action. However we were unable to detectably perturb Ig tetramerization, which was effectively completed during the labeling period, irrespective of the level of Ig synthesis. Previous studies attest to the rapidity of the Ig assembly process (Shapiro et al., 1966; Askonas and Williamson, 1986; Bergman and Kuehl, 1979; Sitia et al., 1987). Intrinsic factors, for example differences in the affinity between subunits, the nature of the intersubunit linkages (covalent, Ig; noncovalent, HA), and in the stability of assembly intermediates, could explain the differential effect of the level of expression on the assembly of Ig and HA. And while some extrinsic factors, for example, the heavy chain binding protein BiP (Haas and Walb, 1983; Bole et al., 1986), have been implicated in the assembly of both HA (Gething et al., 1986) and Ig (Bole et al., 1986), others, for example protein disulphide isomerase, assist only Ig assembly (Roth and Koshland, 1981; Lambert and Freedman, 1985; Roth and Pierce, 1987). However even without these differences our expectations were probably based on too simplistic a view of the pathways of protein assembly and did not accommodate the additional complication of excess light chains in the oocyte, which might result in the assembly reaction being kinetically dependent on the concentration of heavy chains only.

Monomers Are Stable for Long Periods in Oocytes

Another issue investigated in this work relates to the destiny of subunits of oligomeric proteins when they are retained in the ER. Recent work in this area has indicated the existence of a pre-Golgi pathway for the fast degradation of newly synthesized proteins (Lippincott-Schwartz et al., 1988). It is not clear whether the proteolytic compartment is part of the ER or constitutes a separate compartment, but there is evidence supporting the idea of a mechanism by which proteins whose destiny is to be rapidly degraded, are sorted from proteins whose fate is to be simply retained in the ER (Chen et al., 1988). Entering such a compartment would most likely correspond to exiting the secretory pathway. Previous work on Ig assembly in *Xenopus* oocytes has shown that Ig heavy chains are not secreted when synthesized in the absence of L chains but are stably maintained within the oocyte and can be rescued as a consequence of a subsequent injection of the mRNA coding for the complementary chain (Colman et al., 1982). In contrast, data obtained in mammalian cells have provided examples of a "time elapsed after synthesis" dependent loss of ability to participate in an assembly process (Peters et al., 1984; Bergman and Kuehl, 1979). Here we have shown that HA monomers are maintained stably in the oocyte ER and that >50% of them are still available for trimerization and transport even 24 h after synthesis. We believe that the correct folding of these monomers is a rapid process since even at the beginning of our assay periods they

cannot be efficiently precipitated by an antibody specific for unfolded molecules unless they are denatured before immunoprecipitation. These results show that long-term retention of HA monomers in the oocyte ER is not associated with a rapid loss of the conformational features necessary for trimer formation and that a considerable delay can exist between folding and trimerization.

Both these data and those previously obtained with the Ig heavy chains are consistent with the idea of subunit conformation being stabilized for long periods if necessary, by interaction with chaperonin-type proteins (Ellis, 1989). In the context of the development biology of the oocyte and its descendant, the fertilized egg, we believe that the ability to store isolated protein subunits for subsequent assembly and exocytosis may provide one of a repertoire of strategies involved in the temporal and spatial control of specific protein targeting during early development.

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