

## RAPID COMMUNICATIONS

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### NASCENT CHICKEN OVALBUMIN CONTAINS THE FUNCTIONAL EQUIVALENT OF A SIGNAL SEQUENCE

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#### ABSTRACT

Highly purified mRNA for chicken ovalbumin has been translated in a cell-free protein synthesizing system from rabbit reticulocytes in the presence or absence of EDTA-stripped microsomal membranes from dog pancreas. Nascent—but not completed—ovalbumin was transferred across the microsomal membrane, as demonstrated by cotranslational core glycosylation of ovalbumin nascent chains, by resistance to posttranslational proteolysis of only the glycosylated ovalbumin chains, and by cosedimentation with the membrane of exclusively the glycosylated form. Furthermore, nascent chains of bovine prolactin were observed to compete with nascent ovalbumin for transfer across the microsomal membrane. However, no competition for membrane sites was observed between nascent chains of rabbit globin and either nascent ovalbumin or prolactin.

We interpret these results to suggest that nascent ovalbumin contains the functional equivalent of a signal sequence for transfer across membranes, and that membrane components involved in the segregation of secretory proteins with cleaved signal sequences also function in the segregation of ovalbumin.

**KEY WORDS** signal hypothesis · protein biosynthesis · cell-free translation · uncleaved signal peptide

In contrast to studies on the biosynthesis of a variety of other secretory proteins, it has been reported recently (7) that chicken ovalbumin is not synthesized with a cleavable amino terminal signal sequence. However, other hen-oviduct secretory proteins such as lysozyme, conalbumin, and ovomucoid that are produced by the same cells as ovalbumin, were shown to be synthesized with cleavable signal sequences (8). The suggestion was therefore made that in chick oviduct there are at least two distinct mechanisms of protein secretion (7).

In recent years, this laboratory has employed cell-free systems that were supplemented with microsomal membranes to analyze the events that

lead to segregation of secretory proteins within the lumen of the rough endoplasmic reticulum (RER) (1, 2, 5, 6, 10). It was shown that these *in vitro* systems have the capacity to effect cotranslational transfer of a large variety of secretory proteins across the microsomal membrane. However, all of the secretory proteins thus far analyzed have been shown to be synthesized with cleavable signal sequences. It was, therefore, of interest to investigate whether ovalbumin which lacks a cleavable signal sequence could be segregated in the membrane-supplemented cell-free system.

Our results demonstrate that ovalbumin is indeed segregated in this system, and furthermore, that nascent ovalbumin competes for segregation with another nascent secretory protein (bovine prolactin) which contains a cleavable signal sequence. These results will be discussed in terms of the signal hypothesis (1).

## MATERIALS AND METHODS

Most of the procedures used in the present study have been detailed previously. Among these are: the preparation of mRNA from chicken oviduct and the purification of mRNA for ovalbumin (12), the preparation of EDTA-stripped "microsomal membranes" from isolated dog pancreas rough microsomes (2), the translation of mRNA in a staphylococcal nuclease-treated rabbit reticulocyte lysate (9, 11), and various posttranslational assays such as polyacrylamide slab gel electrophoresis in sodium dodecyl sulfate and subsequent autoradiography (1).

Other posttranslational procedures such as incubation with proteinase K or concanavalin A (Con A) Sepharose affinity chromatography are detailed in the figure legends.

Total pituitary RNA was prepared from bovine anterior pituitary glands of steers by sodium dodecyl sulfate/phenol/chloroform/isoamyl alcohol extraction; a mRNA fraction was isolated from total RNA by oligo (dT) affinity chromatography (5). In the case of mRNA extracted from steer pituitary glands, prolactin mRNA was the single major species since ~90% of the total translation products were represented by preprolactin (data not shown).

[<sup>35</sup>S]methionine was purchased from Amersham Corporation, Arlington Heights, Ill., phenylmethylsulfonyl fluoride (PMSF) from Calbiochem, San Diego, Calif., proteinase K from Boehringer, Mannheim, Germany, and Con A Sepharose from Pharmacia Fine Chemicals, Uppsala, Sweden.

## RESULTS

### *Translation-Coupled Core-Glycosylation and Segregation of Ovalbumin*

Translation of highly purified ovalbumin mRNA in a staphylococcal nuclease-treated reticulocyte lysate system yielded, as expected, a major polypeptide (Fig. 1, lane 2) of ~40,000 daltons that represents an unglycosylated form (see below) of ovalbumin, designated here as Ov<sub>0</sub>. When microsomal membranes from dog pancreas were present from the start of translation, an additional and slower moving polypeptide of ~45,000 daltons was synthesized (Fig. 1, lane 4, downward pointing arrow). By analogy to our previous data on the cell-free synthesis of glycoproteins (3, 6), it was likely that this slower moving band (referred to as Ov<sub>1</sub>) represented a core-glycosylated form of ovalbumin that was segregated within the dog pancreas microsomal vesicles.

That segregation had indeed occurred was indicated by the following results: (a) Ov<sub>1</sub> but not Ov<sub>0</sub> could be quantitatively sedimented with the mi-

croosomal vesicles (Fig. 1, lane 7). (b) Posttranslational incubation with proteolytic enzymes yielded protection of Ov<sub>1</sub> (Fig. 1, lane 5) but not of Ov<sub>0</sub> (Fig. 1, lanes 1 and 5). (c) Protection was abolished when posttranslational incubation with proteolytic enzymes was performed in the presence of detergents that solubilized the microsomal vesicles (Fig. 1, lane 6), indicating that resistance of Ov<sub>1</sub> to proteolysis was not an intrinsic property of Ov<sub>1</sub> but was because of the impermeability of the membrane vesicle to the proteolytic probe.

That Ov<sub>0</sub> represented an unglycosylated form, and Ov<sub>1</sub> a glycosylated form of ovalbumin was suggested by the fact that Ov<sub>0</sub> did not bind to Con A Sepharose whereas Ov<sub>1</sub> did (Fig. 1, lane 8) and that Ov<sub>1</sub> could be specifically eluted by  $\alpha$ -methyl mannoside (Fig. 1, lane 9).

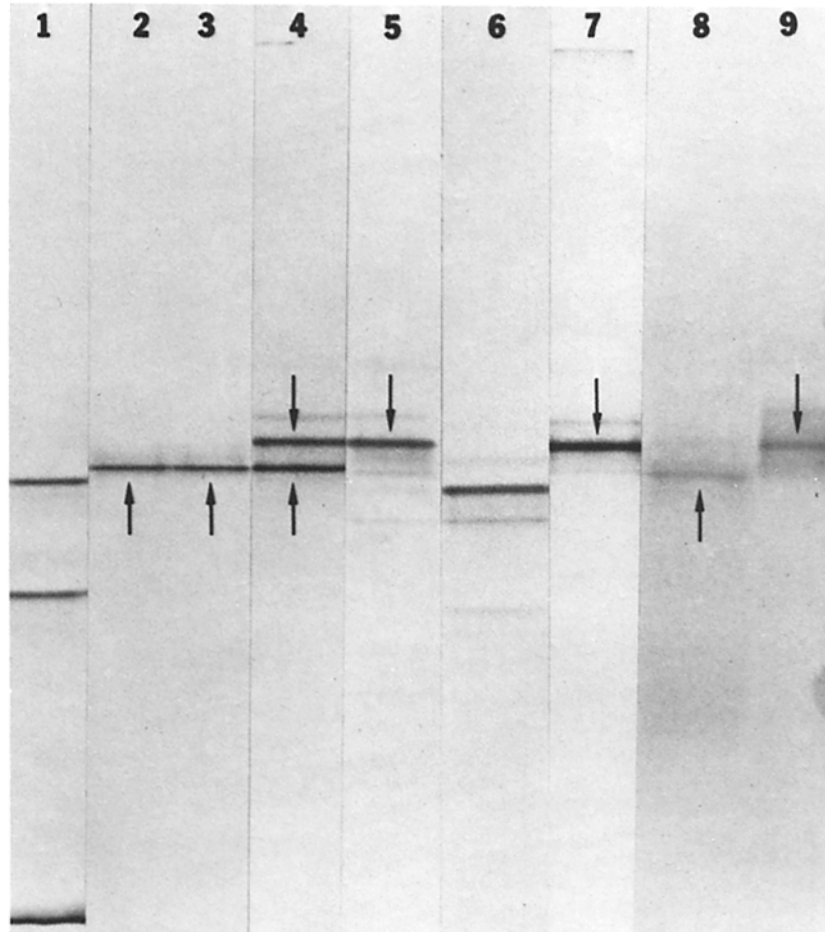
As previously observed for other glycoproteins (3, 6), segregation and glycosylation of ovalbumin is strictly coupled to translation. When membranes were added to the cell-free system only after completion of translation, followed by an additional incubation period, there was no synthesis of Ov<sub>1</sub> (Fig. 1, lane 3). Furthermore, the Ov<sub>0</sub> form that was synthesized in the absence of membranes was not segregated by a posttranslational incubation with membranes and, therefore, was found to be sensitive to added proteolytic enzymes (Fig. 1, lane 1).

The extent of segregation and glycosylation was, as previously observed, dependent on the amount of microsomal membranes that were added to the cell-free system. Increasing amounts of microsomal membranes resulted in increased synthesis of Ov<sub>1</sub> and in decreased synthesis of Ov<sub>0</sub> (Fig. 2).

### *Competition for Membranous Receptors*

We have demonstrated previously that nascent secretory proteins from a variety of sources can be segregated by dog pancreas microsomal membranes (1, 2, 5, 6, 10). Remarkable, however, in the present case is that a secretory protein was segregated, i.e., transferred across the microsomal membrane, that is not synthesized with a cleavable signal sequence. It is conceivable that similar secretory proteins are synthesized also in dog pancreas. Microsomal membranes from dog pancreas could therefore be endowed with a separate set of receptors which function only in the transfer of secretory proteins that are not synthesized with cleavable signal sequences.

To investigate this possibility, experiments were



**FIGURE 1** Coupled cell-free synthesis, core-glycosylation and segregation of ovalbumin. Ovalbumin mRNA was translated in the staphylococcal nuclease-treated rabbit reticulocyte lysate (9), in the absence or presence of nuclease-treated dog pancreas microsomal membranes (11). This was followed by various posttranslational assays, as specified for each lane, and by preparation of aliquots (11) for polyacrylamide slab gel electrophoresis in sodium dodecyl sulfate. Lanes 1-9 were derived from several slab gels; they were aligned according to protein standards on each slab gel. Incubation mixtures for translation (50  $\mu$ l) contained 12  $\mu$ Ci of [ $^{35}$ S]methionine, 0.05  $A_{260}$  U of purified ovalbumin mRNA and either no microsomal membranes (lanes 1-3) or 0.5  $A_{260}$  U of microsomal membranes (lanes 4-9). Incubation was for 90 min at 22°C. The following posttranslational assays were performed on aliquots. *Lane 1*: a 10  $\mu$ l aliquot was adjusted to 10 mM CaCl<sub>2</sub> and incubated at 0°C and for 4 h with a final concentration of 100  $\mu$ g/ml of proteinase K; digestion was terminated by the addition of 2 mM PMSF, sodium dodecyl sulfate to 2%, and incubation at 100°C for 5 min. *Lane 2*: a 10  $\mu$ l aliquot was incubated for 90 min at 22°C with 0.1  $A_{260}$  U of microsomal membranes. *Lane 3*: as Lane 2, except that microsomal membranes were omitted. *Lane 4*: a 10  $\mu$ l aliquot was processed for electrophoresis without posttranslational incubation. *Lane 5*: a 10  $\mu$ l aliquot, digested with proteinase K as detailed above (Lane 1). *Lane 6*: as Lane 5, except that digestion was in the presence of 0.5% Triton X-100. *Lane 7*: a 50  $\mu$ l aliquot, adjusted to 0.01 M CaCl<sub>2</sub> and layered over 500  $\mu$ l of a solution of 0.6 M sucrose, 0.5 M NaCl, 0.01 M Tris HCl, pH 7.5, 0.005 M CaCl<sub>2</sub>, and 0.002 M MgCl<sub>2</sub> was centrifuged for 1 h at 105,000  $g$  in a Spinco no. 40 rotor (Beckman Instruments, Inc. Spinco Div., Palo Alto, Calif.). The resulting pellet was rinsed with H<sub>2</sub>O, and prepared for electrophoresis. *Lane 8*: a 50  $\mu$ l aliquot was adjusted to 2% sodium dodecyl sulfate, incubated at 60°C for 2 min, diluted with 4 ml of Solution A (0.15 M NaCl, 0.02 M Tris HCl, pH 7.5, 0.001 M MgCl<sub>2</sub>, and 0.001 M dithiothreitol) and loaded onto a column of Con A Sepharose 4B (50  $\mu$ l bed volume) that was equilibrated with Solution B (Solution A containing 0.05% sodium dodecyl sulfate). The unbound fraction was collected; an 0.8 ml aliquot was incubated at 0°C for 1 h with 2 vol of ethanol/ether (50:50). The precipitate was collected and prepared for electrophoresis. *Lane 9*: after collection of the unbound fraction (see Lane 8), the column was washed with 5 ml of Solution B. The bound fraction was then eluted with 5 ml of Solution B containing 0.4 M  $\alpha$ -methyl mannoside, and a 1-ml aliquot of this eluate was precipitated as in Lane 8, but with 25  $\mu$ g of tRNA as a carrier. Upward pointing arrows indicate unglycosylated ovalbumin (Ov<sub>0</sub>) and downward pointing arrows indicate core-glycosylated ovalbumin (Ov<sub>1</sub>).

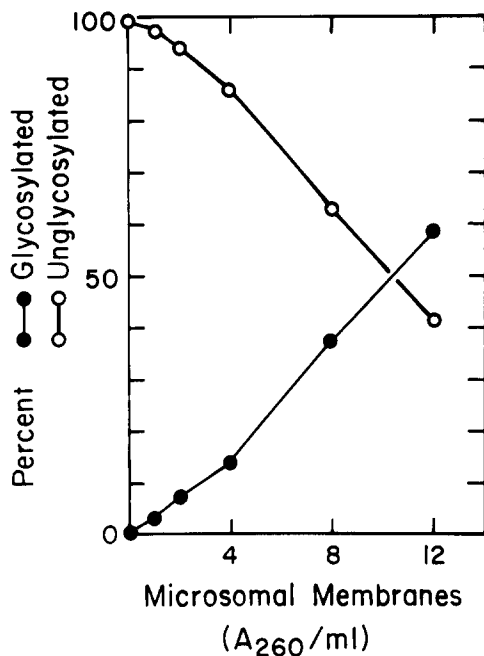


FIGURE 2 Effect of membrane concentration on the extent of cotranslational glycosylation and segregation of nascent ovalbumin. Ovalbumin mRNA was translated in the absence or in the presence of increasing amounts of microsomal membranes as indicated on the abscissa. Translation products were analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and autoradiography. Gel slices containing  $Ov_0$  (unglycosylated) and  $Ov_1$  (glycosylated) were excised, and radioactivity determined as previously described (11). Extent of ovalbumin glycosylated (closed circles) or unglycosylated (open circles) is expressed on the ordinate as a percentage of total ovalbumin synthesized. Percent glycosylated ovalbumin ( $Ov_1$ ) =  $Ov_1/Ov_0 + Ov_1$ ; percent unglycosylated ovalbumin ( $Ov_0$ ) =  $Ov_0/Ov_0 + Ov_1$ .

designed to determine whether nascent ovalbumin would compete for membranous receptors with a nascent secretory protein (bovine prolactin) that contains a cleavable signal sequence and with a cytosolic protein (rabbit globin) that contains no signal sequence at all. In all experiments the amount of membranes was kept constant and subsaturating with respect to segregation activity. However, since different batches of membranes were used, slightly different segregation activities were observed (e.g., see open and closed circles on Fig. 3, *a* and *b*). Competition was monitored by assaying for either glycosylation of ovalbumin or processing of prolactin. Both events, glycosylation and processing, have been shown previously to occur exclusively on the luminal aspect of mi-

croosomal membranes and to be tightly coupled to segregation (6). Thus, the extent of glycosylation and processing are accurate and readily quantifiable indicators of the extent of segregation.

In the first experiments, the concentration of ovalbumin mRNA was kept constant and the amount of a second species of mRNA, either for globin or prolactin, was varied. The radioactivity in the  $Ov_0$  and  $Ov_1$  bands was determined separately and segregation was expressed as a percentage of radioactivity in  $Ov_1$  ( $Ov_1/Ov_0 + Ov_1$ ). It can be seen from Fig. 3*a* that increasing amounts of prolactin mRNA reduced the percentage of  $Ov_1$  from ~60 to 30% (i.e., by one half), whereas increasing amounts of globin mRNA had no significant effect on percent ovalbumin segregated. In subsequent experiments the concentration of prolactin mRNA was kept constant and increasing amounts of ovalbumin mRNA or of globin mRNA were used as a second species of mRNA. The

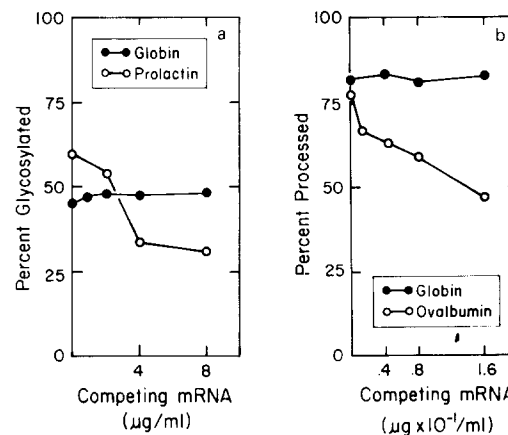


FIGURE 3 Competition for membranous receptors by nascent ovalbumin and nascent preprolactin. (*a*) A constant amount of ovalbumin mRNA ( $0.5 A_{260}$  U/ml) was translated in the presence of  $10 A_{260}$ /ml of microsomal membranes, in the absence or presence of increasing amounts of an additional mRNA (open circles, prolactin mRNA; closed circles, globin mRNA). Translation products were analyzed and the radioactivity in gel slices containing  $Ov_0$  and  $Ov_1$  was determined as described in Fig. 2. Percent of ovalbumin chains glycosylated (see Fig. 2) is plotted as a function of competing mRNA concentration. (*b*) As in *a* except that a constant amount of bovine pituitary prolactin mRNA ( $0.5 A_{260}$  U/ml) was translated in the presence of increasing amounts of either ovalbumin (open circles) or globin (closed circles) mRNA as the competing species of mRNA. Percent of prolactin chains processed (processed prolactin/preprolactin + processed prolactin) is plotted as a function of competing mRNA concentration.

radioactivity in the preprolactin and processed prolactin bands (5) was determined and segregation was expressed as percentage of processed prolactin. It can be seen from Fig. 3b that increasing amounts of ovalbumin mRNA reduced the percentage of processed prolactin from 76 to 45%, whereas increasing amounts of globin mRNA had no effect. It should be pointed out that the addition of increasing amounts of either second mRNA yielded approximately proportional translation of these mRNAs (data not shown). Moreover, competition was observed only under conditions of limiting membrane concentration and could be abolished by raising the membrane concentration to a saturating level (data not shown).

## DISCUSSION

Our results demonstrate that nascent chicken ovalbumin chains are recognized by receptors in dog pancreas microsomes involved in the transfer of proteins across the microsomal membrane. Furthermore, as in the case of other secretory proteins with cleavable signal sequences, transfer cannot be accomplished after completion of ovalbumin chains. This suggests that the information for segregation is expressed only in the nascent ovalbumin chain but not in the completed and presumably folded molecule.

The most plausible interpretation of these findings is that ovalbumin contains the functional equivalent of a signal peptide, most likely at the amino terminus, that is not removed, and therefore is retained in the mature ovalbumin molecule. It should be noted here that such a possibility had been clearly anticipated in the signal hypothesis (1), where it was proposed that point mutations could conceivably affect only the signal peptidase site so that the membrane recognition site of the signal peptide could remain functional for transfer across the microsomal membrane. Implicit in this prediction is that removal of the signal peptide is not a prerequisite for transfer of the nascent chain across the membrane. Thus, ovalbumin could be the first representative of a number of secretory proteins where—in the course of evolution—the amino-terminal signal sequence has remained functional for membrane transfer but has lost its site for signal peptidase. This conjecture is supported by a recent report (4) on an *Escherichia coli* mutant with structural alterations in the outer membrane lipoprotein. It was observed (4) that replacement of a glycy-

residue by an aspartyl residue at position 14 of the signal sequence that comprises 20 amino acid residues, resulted in a loss of cleavage but not of transfer across the inner membrane.

Although Palmiter and his colleagues have considered the possibility that ovalbumin contains an uncleaved signal sequence they have argued against it because of a lack of similarity of the amino terminal sequence of ovalbumin to the signal sequences of various presecretory proteins (7). However, in view of the considerable differences in the primary structure of signal sequences thus far elucidated, it is not yet possible to discern in them those unique structural features which confer on them their common biological function.

The competition for segregation observed in our *in vitro* assay indicates that at least one membranous component is common in the catalytic assembly engaged in segregation of prolactin and of ovalbumin. It is likely that this common component is a receptor involved in the initial recognition of the signal region of nascent chains. Since the two competing polypeptides undergo different and nonoverlapping cotranslational modifications (ovalbumin is glycosylated but not processed by signal peptidase, whereas prolactin is proteolytically processed but not glycosylated), the competition observed is unlikely to be for access to modifying enzymes on the luminal face of the membranes.

Our conjecture that the amino-terminal sequence of ovalbumin functions as a signal sequence can be tested experimentally by investigating whether an amino-terminal fragment of ovalbumin has the ability to serve in a cell-free system supplemented with microsomal membranes as a competitive inhibitor of *in vitro* segregation of nascent ovalbumin or of nascent presecretory proteins that contain cleavable signal peptides. Such experiments are now in progress.

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