Ovalbumin, unlike other secretory proteins, is synthesized and secreted without cleavage of a hydrophobic signal peptide. Kinetic experiments were performed in a cell-free translation system to measure the minimum size of ovalbumin nascent chains required for binding of both the nascent chain and the corresponding mRNA to microsomal membranes derived from dog pancreas. Results of these experiments revealed that 50 to 60 amino acid residues are sufficient to bind ovalbumin-synthesizing polysomes to membranes in vitro. When microsomes with associated polysomes were isolated from chick oviduct, nascent ovalbumin chains longer than 50 residues were protected from proteolysis as long as the membranes remained intact, suggesting that the polypeptides were sequestered by the endoplasmic reticulum. We conclude that the functional signal for membrane translocation of ovalbumin becomes accessible when the nascent chain is 50 to 60 residues long. We speculate that the hydrophobic sequence which lies between residues 25 and 45 folds back on the preceding residues to form an amphipathic hairpin structure which is the signal element recognized by the membrane.

In recent years, the mechanisms by which proteins cross or become inserted into cellular membranes have become more clearly understood (for reviews, see Refs. 1-5). Secretory proteins, as well as membrane proteins, are synthesized as precursors containing NH2-terminal, signal peptides of 15 to 30 amino acid residues. In both eukaryotes and prokaryotes, there is compelling evidence that the hydrophobic signal peptide is essential for the transport of secretory proteins (2-7). In eukaryotes, the signal sequence is removed from the growing nascent chain by a "signal peptidase" activity in membranes of the endoplasmic reticulum. The resulting polypeptides become segregated from the cytoplasm into the intracisternal space of the endoplasmic reticulum, where they proceed to the Golgi apparatus for subsequent modification, packaging, and exocytosis.

In contrast to these findings, ovalbumin, the major secretory protein from hen oviduct, lacks the transient NH2-terminal signal sequence found on all other secretory proteins studied so far (8). Yet, studies in cell-free protein synthesis systems show that ovalbumin is sequestered within microsomal vesicles like other secretory proteins (9, 10). In addition, proteins with NH2-terminal signal sequences compete with ovalbumin for sequestration within microsomes (9, 10). These data suggest that ovalbumin and other signal-containing proteins use a common component in the RER transport system. However, the lack of an obvious signal peptide in ovalbumin remained unexplained. Lingappa et al. (11) proposed that ovalbumin contains the functional equivalent of a signal sequence located between amino acid residues 234 and 253. Their conclusions were based largely on the isolation of Ovα, a peptide which inhibits the translocation of precursor proteins across dog pancreas microsomal membranes in a cell-free translation system.

We have conducted experiments to determine the location of a signal sequence in ovalbumin by measuring the shortest nascent chains that interact with microsomal membranes both in vivo and in vitro. The results of our studies are at variance with the proposal of Lingappa et al. (11) and indicate that the signal sequence for ovalbumin secretion resides within the NH2-terminal 50 to 60 amino acid residues.

**MATERIALS AND METHODS**

**Preparation of mRNA and cDNA—mRNAα and [3H]cDNAα were prepared as described by Senear and Palmiter (12). Hen oviduct poly A-containing RNA was enriched for mRNAα as described by Thibodeau and Walsh (13), and [3H]cDNAα was synthesized and purified as described by Compere et al. (14). Enriched mRNAα was prepared essentially as described by Haines et al. (15) and is >90% pure as judged by sodium dodecyl sulfate-acrylamide gel analysis of the total translation products. [3H]cDNAα was synthesized as described by Lee et al. (16). The cDNA probes were all greater than 90% pure and hybridized to greater than 80% with S1 backgrounds of less than 5%.

**Cell-free Translation and Assay for Membrane-bound mRNA—**

Dog pancreas microsomes were prepared as described by Thibodeau and Walsh (13). Cell-free translations were performed using an mRNA-dependent rabbit reticulocyte lysate system treated either with micrococcal nuclease (Worthington) alone or with nuclease followed by Sephadex G-50 as described by Palmiter et al. (17). Prior to the addition of mRNA or dog pancreas microsomes, 150-μl aliquots were incubated at 27°C for 10 min. Translations were initiated by the simultaneous addition of microsomes (2.0 A280 units/ml) and mRNAα, mRNAα, or mRNAαβ (2.0 μg/ml). Control translations lacked microsomes or contained pactamycin (5 μM) during the 10-min incubation prior to the addition of mRNA. At various times, 10-μl aliquots were pipetted into 100 μl of ice-cold buffer P (25 mM Tris-Cl, pH 7.5, 25 mM NaCl, 5 mM MgCl2, 0.5 mM ethylene glycol bis[β-aminoethyl ether]-N,N,N',N'-tetraacetic acid. Microsomes were then pelleted through 0.5 to 1.0 M sucrose in buffer P for 30 min at 30,000 rpm (120,000 × g) in an SW 60 rotor (Beckman) or sedimented through 500 μl of 0.5 M sucrose in buffer P in culture tubes (6 × 50 mm) (Kimble) for 30 min at 10,000 rpm (14,000 × g) in adaptors fitted to a Sorvall HB-4 rotor. Pellets were dissolved in 100 μl of 0.5% SDS and, in some experiments, the solubilized pellets were extracted with phenol:chloroform (1:1).

1 The abbreviations used are: RER, rough endoplasmic reticulum; SDS, sodium dodecyl sulfate; ov, mu, and gb, subscripts to identify nucleic acids specifying ovalbumin, ovomucoid, and globin, respectively; Ovα, a tryptic peptide from ovalbumin representing residues 229 to 276.

**The Signal Sequence of Ovalbumin Is Located Near the NH2 Terminus**

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The endoplasmic reticulum. We conclude that the func-

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Aliquots were then assayed for specific mRNA by hybridization with the appropriate \[^{3}H\]cDNA as described by McKnight (18).

**SDS-Polyacrylamide Gel Electrophoresis**—Samples were dissolved in 65 mM Tris-Cl, pH 8.5, 2% SDS, 5% mercaptoethanol, 10% glycerol, deacylated by alkaline hydrolysis at 37 °C, and heated at 100 °C for 10 min. Samples were then subjected to SDS-polyacrylamide gels (10-20%) as described by Laemmli (19). Gels were processed for fluorography using Enhance (New England Nuclear) and exposed to x-ray film (Kodak X-Omat XR-5 or XAR-5). The relative positions of radioactive bands were compared with those of \(^{125}\)I-labeled protein molecular weight markers (Bethea Research Laboratories).

**Sedimentation of Polyosomes to Determine Ovumblin Nascent Chain Sizes and Immunoprecipitation of Ovumblin**—[\(^{35}\)S]Methionine (Amersham, >1000 Ci/\(\mu\)mol) was added to the translation system at 800 \(\mu\)Ci/ml and preincubated as described above. At various times after mRNA, med and mRNA, cytoplasmic were added, 10-\(\mu\)l aliquots of the translation mixture were removed and diluted into buffer P containing 0.1 mM cycloheximide and 0.5% Triton X-100. Polyribosomes were sedimented at 125,000 \(\times\) 10 min, and the supernatant was immunoprecipitated directly with 3 \(\mu\)g of ovumblin and an excess of specific goat anti serum (17). Both immunoprecipitates and polysomes were analyzed on SDS-polyacrylamide gels. In identical experiments performed in the presence of microsomes (2.0 \(A_{260}\) units/ml), no significant difference occurred in nascent chain distribution or time of ovumblin release was observed; however, the abundance of peptidyltransferase was reduced.

**Analysis of Nascent Ovumblin Polypeptide Segregation within Microsomes**—The sequestration of ovumblin nascent chains within microsomal membranes was assessed by their resistance to exogenous proteolysis (6, 7). After treatment of samples with 1 \(\mu\)m tetraacaine (20), proteinase K (EM Biochemicals) was added to yield a final concentration of 50 \(\mu\)g/ml. After 30 min at 4 °C, proteolysis was stopped by adding 3 volumes of buffer P containing 2 mM phenylmethylsulfonyl fluoride (Sigma). Microsomes were then pelleted by centrifugation through 500 \(g\) tubes (6 \(\times\) 50 mm) as described above. Some pellets were analyzed directly for ovumblin in polyribosomes (17) prior to deacylation and electrophoresis. In all experiments, the addition of Triton X-100 (0.5%) abolished sedimentation of membrane-associated polyribosomes.

**Isolation of Chicks Oviduct Microsomes**—Chicks were given secondary estrogen stimulation for 2 days as described (14). The magnum portion of the oviduct was excised, connective tissue removed, and the magnum minced finely with razor blades. The microsome isolation procedure was a modification of the method described by Hanover and Lennarz (22). The minced tissue (1 g) was homogenized in 50 ml of buffer (30 mM triethanolamine, pH 7.5, 25 mM KCl, 5 mM MgCl\(_2\), 2 mM dithiothreitol, 0.25 mM sucrose containing 1 mg/ml of heparin) (Sigma) and centrifuged for 10 min at 13,500 \(\times\) 10 min (13,300 \(\times\) \(g\)) in an SS-34 rotor (Sorvall). The resulting pellet was homogenized with four strokes of a motor-driven Thomas homogenizer in the same buffer with 0.5 mg/ml of heparin. Following centrifugation at 5,000 \(\times\) 10 min (3,000 \(\times\) \(g\)) for 8 min, the postmitochondrial supernatant was centrifuged at 27,000 \(\times\) 10 min (14,000 \(\times\) \(g\)) for 90 min in a Beckman SW 27 rotor. The resulting pellet of total microsomes was resuspended in 10 ml 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, pH 7.5, containing 1 mM MgCl\(_2\), 2 mM dithiothreitol, and 50 \(\mu\)g/ml of heparin to a final concentration of 20 \(A_{260}\) units/ml. Aliquots were frozen quickly and stored at \(-70^\circ\)C.

**RESULTS**

**Kinetics of mRNA Binding to Dog Pancreas Microsomes**—Current concepts of protein secretion suggest that polysomes form stable associations with membranes via their nascent chains (3, 5-7). Thus, in a synchronized cell-free translation system containing membranes, one would expect that the mRNA for a secreted protein would become associated with membranes only after the peptide sequence that is recognized by the RER membrane became exposed. We compared the time course of mRNA, med binding to membranes with that of mRNA, globin, which codes for a typical presecretory protein (21), and mRNA, globin, which codes for a cytoplasmic protein.

**Stability of mRNA to Membranes**—The binding of mRNA to membranes was monitored by sedimenting the membranes and quantitating mRNA in the pellet by solution hybridization with \[^{3}H\]cDNA. Binding of mRNA, med and mRNA, globin to membranes was first detected 1 to 2 min after the respective mRNAs were added to translation mixtures (Fig. 1). By 4 min, the amount of membrane-bound mRNA, med and mRNA, globin plateaued at 3 and 7 times background, respectively. Omission of microsomes or disruption of microsomes with 0.5% Triton X-100 reduced the amount of mRNA in the pellet to very low levels (~20 pg). Globin mRNA did not exhibit nascent chain-dependent binding to the membranes (Fig. 1). Inclusion of pactamycin, an inhibitor of initiation, prevented the time-dependent binding of mRNA (Fig. 1). When pactamycin was added 15 min after translation started, there was a lag of ~10 min prior to the release of mRNA, whereas puromycin released the mRNAs from the membranes immediately (data not shown). These results are consistent with the mode of action of these inhibitors and indicate that the mRNAs are bound to membranes via nascent chains rather than directly (23-25).

**Binding of mRNA, med and mRNA, globin to membranes** was also observed when translations were stopped with cycloheximide prior to the addition of membranes. The kinetics and quantity of bound mRNA were essentially identical with the experiment described in Fig. 1. Using this approach, it was also possible to show that 0.5 mM KCl did not inhibit binding of ovumblin and ovomucoid polysomes to membranes, suggesting that binding was not potentiated by ionic interactions between ribosomes and membranes.

**Nascent chain elongation rates on mRNA, med and mRNA, globin were estimated by determining the time required for the release of completed polypeptides from polysomes after mRNA was added to the translation reaction (Fig. 2).** Aliquots of cell-free translation mixtures (identical with those used in Fig. 1) were incubated with \[^{3}H\]valine. Polysomes were then sedimented, and the proteins remaining in the supernatant were precipitated with specific antisera. Ovumblin was first released from polysomes 9 min after mRNA addition, whereas ovomucoid was released after 5 min. Therefore, the average elongation rate of ovumblin was 43 amino acid residues/min, while the rate for ovomucoid was 37 residues/min. Taken together with the binding kinetics (Fig. 1), we calculate the binding of mRNA, med to membranes was half-maximal (2 min) when the longest nascent chains were ~90 amino acid residues.

**FIG. 1.** Time course of mRNA binding to microsomes during cell-free translation. Translation reaction mixtures were incubated for 10 min at 27 °C prior to addition of microsomal membranes (2.0 \(A_{260}\) units/ml) and mRNAs (2 \(\mu\)g/ml). At the indicated times after these additions, translations were stopped with cycloheximide, and membrane-bound mRNA was determined by hybridization with \[^{3}H\]cDNA (see Materials and Methods). •, mRNA, med; □, mRNA, globin; ▲, mRNA, med. Translation mixtures in which pactamycin (5 \(\mu\)M) was added 10 min prior to addition of mRNA are indicated by the corresponding open symbols.
while at 1 min, when mRNA<sub>ov</sub>, binding was first detected, the chains were ~45 residues long. Similar calculations indicate that mRNA<sub>ov</sub>, was first bound to membranes when nascent chains were ~75 residues long. Using this kinetic approach, we have consistently observed mRNA<sub>ov</sub>, binding to membranes prior to mRNA<sub>ov</sub>,.

Since the rate of ribosome movement along a mRNA molecule may not be constant, an independent determination of nascent chain length was also performed. Nascent ovalbumin chains were recovered from polysomes which had been pelleted from aliquots of a cell-free translation mixture at intervals during a 12-min translation period. These pellets and the corresponding supernatants were then analyzed on SDS-polyacrylamide gels (Fig. 3). Mature ovalbumin was released from polysomes between 8 and 10 min of translation (Fig. 3b), confirming the results described above. Fig. 3a shows that 45 s after translation was initiated, the largest ovalbumin nascent chains were approximately 4,000 daltons. By 2 min, the largest chains were approximately 8,000 daltons and, by 3 min, they were 13,000 daltons. In general, these measured maximum nascent chain lengths and the lengths predicted by interpolations of the transit time are similar. After 45 s, 2 min, and 3 min, chains of 37, 75, and 120 residues were observed, while calculations based on a uniform transit time predict 32, 86, and 129 nascent residues at these time intervals. Based upon these measurements and the time course of binding to membranes (Fig. 1), we estimate that when ovalbumin nascent chains are ~50 residues long (1 min), they promote binding of the polysome to membranes, and, by the time the nascent chains are 120 residues long (3 min), binding is complete.

Using another experimental approach, we determined the smallest nascent chains of ovalbumin that were capable of binding to microsomal membranes. Toward this end, mRNA<sub>ov</sub>, was translated in the presence of [<sup>35</sup>S]methionine and microsomes as in Fig. 1. At various times, aliquots were treated with cycloheximide and the nascent chains were examined by SDS-polyacrylamide electrophoresis. We have observed that while mRNA<sub>ov</sub>, binding occurs prior to mRNA<sub>ov</sub>, synthesis, mRNA<sub>ov</sub>, binding to membranes becomes detectable earlier, which is consistent with the observed kinetic approach.
gel electrophoresis. Each aliquot was separately analyzed for the total set of polysomal nascent chains (including those released from membranes with 0.5% Triton X-100; Fig. 4b) and for membrane-bound nascent chains (those remaining with a microsomal pellet after treatment with 10 mM EDTA; Fig. 4a). The only observed difference between nascent chains in the total polysomal pellet (Fig. 4b) and those in the microsomal pellet (Fig. 4a) was that chains with apparent molecular masses less than ~5500 daltons were not retained with the microsomes. Evidently, nascent chains shorter than ~50 residues are unable to bind to microsomes.

**Translocation of Nascent Chains**—Although short ovalbumin nascent chains were bound to microsomes, it could still be argued that this interaction did not lead to productive insertion of the ovalbumin nascent chains into the membrane. Instead, the initial binding could serve to anchor the nascent chain to the membrane until another portion of the molecule, e.g. the OV, region proposed by Lingappa et al. (11), was available for membrane interaction which might then trigger ovalbumin translocation. To determine whether the short

Fig. 5. **Microsome-associated ovalbumin (OV) nascent chains and their resistance to proteolysis.** a, immunoprecipitated membrane-bound ovalbumin nascent chains. A cell-free translation mixture containing [35S]methionine (1.0 mCi/ml) was incubated for 10 min at 27 °C prior to the addition of mRNAov (5 μg/ml) and dog pancreas microsomes (2.0 A260 units/ml). After a 10-min translation period, the reaction was terminated with 0.1 mM cycloheximide and polysomes were dissociated with EDTA (10 mM). This preparation was incubated with 1 mM tetracaine for 10 min at 27 °C to stabilize the membranes. Membranes were then sedimented and bound nascent chains analyzed on SDS-polyacrylamide gels (lane 1). To assay for protected nascent chains (see "Materials and Methods"), a duplicate aliquot was treated with proteinase K prior to sedimentation (lane 2). Lane 3 was prepared as in lane 1 but the sedimented microsomal pellet was disrupted in 0.5% Triton X-100 and then treated with proteinase K. Lane 4 shows the nascent chains that bind to membranes when membranes were added after translation was terminated with cycloheximide. After a 5-min incubation with membranes, EDTA was added and the membranes were pelleted. Lane 5 is the same as lane 4 except for treatment with proteinase K prior to membrane sedimentation. Lane 6 is the same as lane 4 except that EDTA was added prior to the membranes. Lane 7 is the same as lane 6 except for proteinase K treatment before sedimentation of membranes. Lane 8, no mRNAov added, b is the same as a but shows the total membrane-bound polyepitides. Refer to "Materials and Methods" for specific procedures.

**Fig. 6. Profiles of free and membrane-bound polysomes after density gradient centrifugation.** a, free polysomes in un-treated reticulocyte lysate; b, membrane-bound polysomes in oviduct microsomes. Free (c) and bound (d) polysomes after RNase A treatment, 10 μg/ml for 25 min at 27 °C. Free (e) and bound (f) polysomes after treatment with 10 mM EDTA for 15 min at 4 °C. In each case, 50 μl of lysate or 1.0 A260 units of microsomes were diluted with 3 volumes of buffer P containing 0.5% Triton X-100, layered over a linear sucrose gradient (0.5-1.0 M) in the same buffer, and centrifuged for 110 min at 40,000 rpm in a Beckman SW 41 rotor. The contents were then pumped from the bottom and monitored at 260 nm with an Isco type 4 flow cell.

microsome-bound ovalbumin nascent chains were being translocated across the microsomal membrane, we measured their protection from proteolysis, a characteristic of proteins sequestered within membrane vesicles (1, 3, 5-7). Ovalbumin mRNA was translated in the presence of microsomes for 10 min, translation was stopped with cycloheximide, and then EDTA was added to dissociate polysomes and release unbound nascent chains prior to sedimenting the microsomes. Fig. 5 (lane 1) shows that nascent ovalbumin polypeptides ranging from 6000 daltons to nearly complete molecules sedimented with the membranes. Addition of proteinase K had little effect (lane 2) unless the membranes were disrupted with Triton X-100 (lane 3), suggesting that the nascent chains are sequestered within the microsomal vesicles. No radioactive peptides precipitated in the absence of added mRNA (lane 8) and immunoprecipitation with anti-ovalbumin demonstrate that the radioactive peptides correspond to ovalbumin (all lanes of Fig. 5a). When ovomucoid and lysozyme mRNAs were substituted for mRNAov, the smallest nascent polypeptides that were sedimented and protected from proteolysis were also in the 5500- to 6000-dalton range (data not shown).

As a control for these experiments, membranes were added to reaction mixtures after translation was terminated with cycloheximide. Fig. 5 (lanes 4 and 6) shows that small nascent ovalbumin polypeptides bound to membranes whether ribosomes were dissociated with EDTA before or after the membranes were added. However, these nascent chains were completely degraded by proteinase K, suggesting that they are bound to the outside surface of the membrane. These experi-
ments indicate that nascent ovalbumin chains may bind to RER in the absence of protein synthesis, but they are not translocated into the lumen, either because they are not bound appropriately or because protein synthesis provides the motive force for insertion into the lumen.

Association of Nascent Ovalbumin with the RER in Vivo—
We also examined RER derived from the magnum portion of chicken oviduct to determine the shortest length of ovalbumin nascent chains that are bound to and translocated across this membrane in vivo. Oviduct microsomal membranes were isolated as described under “Materials and Methods,” and their nascent chains were pulse-labeled with [35S]methionine in the reticulocyte lysate. Two methods were employed to release nascent chains not directly bound to membranes. In one, EDTA was used to dissociate the ribosomes into subunits and to release peptidyl-tRNA; in the other, the mRNA was digested with RNase A, yielding monoribosomes with associated nascent chains (26).

Control experiments were performed to ensure the efficiency of these treatments both on membrane-free reticulocyte polysomes (Fig. 6a) and on membrane-bound ovalbumin polysomes (Fig. 6b). Treatment with RNase A depleted the samples of polysomes in both cases and resulted in an accumulation of particles sedimenting at approximately 80 S (Fig. 6, c and d). These are presumably monomeric peptidyl-tRNA-ribosome complexes with fragments of associated mRNA (26).

There was also a small peak of faster sedimenting material from the oviduct-derived polysomes that appears to be a dimer of these complexes. The addition of EDTA dissociated both free and microsomal polysomes into ribosomal subunits (Fig. 6, e and f).

After treatment of the pulse-labeled oviduct membranes with EDTA or RNase A, the membranes were pelleted and subjected to protelysis to determine whether the adherent nascent chains were bound on the surface or sequestered within the microsomes. The ovalbumin nascent chains were then immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis (Fig. 7). The spectrum of membrane-associated ovalbumin polypeptides included chains as small as 5500 daltons and as large as completed ovalbumin (lane 1). After EDTA or RNase A treatment, the distribution of bound ovalbumin polypeptides remained the same (lanes 2 and 3) although the labeling abundance of the smaller polypeptides was reduced. This may be due to preferential immunoprecipitation of the larger peptides or it may result from the microsomal isolation procedure. Treatment with Triton X-100 prevented sedimentation of all ovalbumin polypeptides (lane 4). Significantly, lanes 5 and 6 show that the ovalbumin polypeptides which remained bound after EDTA or RNase A treatment were resistant to protease K unless the membranes were disrupted with Triton X-100 before or after sedimentation (lanes 7 and 8). These results indicate that ovalbumin nascent chains as small as ~50 amino acid residues are bound to microsomal membranes in vivo. More importantly, chains which remain bound to membranes after EDTA or RNase A treatment are resistant to protease attack, as were the corresponding chains synthesized in vitro (Fig. 5).

**DISCUSSION**

Ovalbumin represents an exception to the general observation that secreted proteins have transient, NH$_2$-terminal, signal peptides which are necessary for their vectorial transport through the membranes of the rough endoplasmic reticulum. It is difficult to reconcile its lack of a transient signal peptide with its ability to compete with other secretory proteins for segregation within microsomal vesicles during cell-free protein synthesis unless it has a functional equivalent of a signal peptide that is not proteolytically removed during membrane transport. Apparently, at least one component of the RER membrane transport system is common to the process of segregation of ovalbumin and of the other secretory proteins (9, 10).

A possible location of the signal peptide in ovalbumin was suggested in a report that described a tryptic fragment of ovalbumin (OV,) that inhibits the segregation of prolactin into microsomal vesicles (11). This putative signal sequence is located between amino acid residues 229 and 276. Thus, a physical model of ovalbumin secretion required that a nascent chain of ~250 residues would be the shortest unit recognized by the microsomal components and that this recognition would result in vectorial transport. This concept of an internal signal for a secreted protein poses several conceptual difficulties (1, 5) and would require a special translocation mechanism (27). Although ovalbumin secretion could be compatible with the spontaneous insertion models of Engleman and Steitz (28) and Wickner (29), these models do not provide simple explanations of the competition between ovalbumin with an internal signal and other secretory proteins with NH$_2$-terminal signal peptides (9, 10). Since membrane receptors apparently interact with hydrophobic signal peptides, one might expect that any hydrophobic peptide would also bind to these receptors and compete for the segregation of a secretory protein (11). Thus, a crucial question is whether hydrophobic peptides, like OV, or synthetic signal peptides (30), inhibit translocation in a specific or a nonspecific manner.

**Fig. 7. Resistance to proteolysis of chick oviduct membrane-bound ovalbumin nascent chains.** A cell-free translation mixture containing [35S]methionine (1 mCi/ml) was incubated for 10 min at 27 °C prior to addition of chick oviduct microsomes (4.0 A$_{260}$ units/ml). After 1 min, cycloheximide was added and membranes were sedimented as outlined under “Materials and Methods.” The microsomal pellet was dissolved in phosphate-buffered saline, pH 7.5, containing 10 mM EDTA and 0.5% Triton X-100 and centrifuged for 10 min at 14,000 X g. Ovalbumin (OV) nascent chains were immunoprecipitated from the supernatant as described under “Materials and Methods” and analyzed on SDS-polyacrylamide gels (lane 1). Duplicate aliquots were treated with 10 mM EDTA (lane 2), RNase A (lane 3), or Triton X-100 (lane 4) prior to sedimentation. Lanes 5, 6, and 7 illustrate samples which correspond to lanes 2, 3, and 4 except that each was treated with protease K as described under “Materials and Methods” just prior to sedimenting the microsomes. A control aliquot (lane 8) was prepared as in lane 1 except that the sedimented microsomal pellet was disrupted in 0.5% Triton X-100 before treatment with protease K.
In this study, the experimental approaches were directed at identifying the shortest nascent chain of ovalbumin that would interact productively with RER membranes. We found that ovalbumin nascent chains bind to membranes when they are 50 to 60 residues long and, as judged by protease protection experiments, they are bound in a productive manner. As protein synthesis continues, the nascent chains remain protected, presumably due to sequestration within the microsomes. Analysis of the size of nascent ovalbumin chains associated with chicken oviduct RER in vitro fully corroborates the results obtained with the in vitro system. These observations form the basis of our proposal that the physiologically relevant signal is located toward the NH₂ terminus of ovalbumin. In accord with our data, Braell and Lodish (31) have shown by using a synchronized translation protocol that only those ovalbumin chains shorter than 150 residues are capable of segregating within dog pancreas microsomes; with longer chains, the signal is presumably inaccessible for functional interaction with the membranes. If the signal were between residues 228 and 276 as proposed (11), then ovalbumin nascent chains would be expected to bind to microsomes and become protected only after the synthesis of about 250 residues. Our data and those of Braell and Lodish (31) are inconsistent with this prediction and, thus, with the suggestion that the OV region of ovalbumin is the functional signal. It should be noted that 50% inhibition of prolactin translocation required about 1 mg/ml of OV, (11), an amount of peptide in excess of the total membrane protein in the experiments. This high concentration of hydrophobic peptide may have resulted in nonspecific membrane disruptions which inhibited prolactin secretion.

The fact that nascent ovalbumin chains compete with nascent chains of other secretory proteins for membrane transport suggests that ovalbumin may possess a structural feature in common with that of typical secretory proteins bearing transient, hydrophobic signal peptides. Because residues 1 to 25 of ovalbumin do not possess a hydrophobic stretch similar to that of signal peptides, whereas residues 26 to 45 are all either hydrophobic or uncharged, a unifying model is envisaged in which the hydrophobic sequence forms one limb of a hairpin loop structure and this structure is the signal element responsible for membrane interaction (Fig. 8). Thus, ovalbumin and other secretory proteins are envisaged as forming similar hairpin structures during their synthesis, but the amphiphatic character of the ovalbumin hairpin is reversed. This reversed orientation may also account for the lack of proteolysis in the case of ovalbumin. One concern about this model is the fact that part of the nascent chain is masked by the ribosome. However, estimates of chain lengths protected by the ribosome have been as high as 39 residues (22) and as low as 15 residues (33). Our studies indicate that the initiator methionine is cleaved from ovalbumin when the nascent chain is 19 residues long (8). If 15 to 19 residues are subtracted from our estimate that nascent chains 50 to 60 residues long bind to the RER, one is left with an exposed sequence of 31 to 45 residues, which is marginally compatible with the loop structure presented in Fig. 8 being the signal element. Because the signal for ovalbumin secretion is maintained within the mature protein, perhaps chemical manipulation can yield a peptide capable of interacting with membrane components in a physiologically relevant manner.

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