Ovalbumin Utilizes an NH₂-terminal Signal Sequence*

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In synchronized translation experiments in the wheat germ and reticulocyte lysate systems, ovalbumin (385 amino acids) was glycosylated by and segregated in dog pancreatic microsomes only if microsomes were added before the nascent ovalbumin polypeptide contained less than 150 amino acids. This would place the "signal" sequence of ovalbumin prior to residue 150, in contrast to a previous report.

In the study of the biosynthesis of secretory and membrane proteins, it is desirable to ascertain whether insertion of the newly synthesized protein occurs in a co-translational manner. This determination can be made by means of a synchronized translation experiment. In such a study, initiation of protein synthesis is allowed for a brief period, after which initiation is specifically inhibited, and the ribosomes undergoing translation of the messenger are allowed to traverse the message synchronously. The addition of dog pancreatic microsomes at various stages of growth of the nascent chain, followed by completion of the protein in the presence of the membranes, will show when such membranes must be present if insertion is to occur at all. The sequence mediating insertion into membranes, the "signal sequence," must have been synthesized prior to the time when addition of microsomes no longer brings about insertion.

This type of experiment has been performed with several proteins, including membrane proteins like vesicular stomatitis virus G and Sindbis PE2, pancreatic secretory proteins, and the peculiar case of ovalbumin (1–4). In all save the last case, insertion is prevented if more than about 100 amino acid residues are synthesized before the addition of membranes. This constitutes good evidence for the existence of an NH₂-terminal signal sequence which directs co-translational insertion; this sequence must act rapidly after its emergence from the ribosome or else become ineffective. The exception, ovalbumin utilizes an internal signal sequence made confiuation of the presence of such a sequence in ovalbumin a vital matter.

**EXPERIMENTAL PROCEDURES**

*Gel Electrophoresis and Autoradiography—SDS* 1-gel electrophoresis analysis was performed by the method of Laemmli (7), except that samples were prepared with a modified sample buffer (2% w/v) SDS, 1% (v/v) glycerol, 20 mM dithiothreitol, 0.7 M 2-mercaptoethanol, 0.015% bromphenol blue dye, and 80 mM Tris-HCl buffer, pH 6.8. Fluorography was performed by the procedure of Laskey and Mills (8). Autoradiography was performed with Kodak SB-5 film. Exposures were scanned on a microdensitometer (Joyce-Loebl and Co.) using a wedge with a full scale deflection of 1.16 optical density units. Such exposures are within the linear response region of the film and the microdensitometer.

Preparation of VSV Messenger RNA—BHK cells (9) were harvested 5 h after infection by 10 plaque-forming units/cell of the Glasgow isolate of VSV. The extraction of mRNA was as described in Ref. 9, except that 50 μg/ml of Proteinase K was added to the lysed cell suspension after the DNase I treatment, and the solution was incubated an additional 10 min at 37 °C. Typically, the RNA was not purified by oligo(dT) columns and was used in cell-free translations at concentrations of 300–1000 μg/ml.

**Cell-free Translation of Messenger RNA**—Reagents were prepared from sterile water and stored frozen at −20 °C for up to 1 month before discarding. ATP stock solution was neutralized with ammonium hydroxide before use. Hemin was prepared as a 4 mg/ml stock solution in ethylene glycol. Amino acids (5 mM each) were made up as a stock without cysteine and methionine; 5 mM cysteine was added separately. To some reactions, dog pancreatic microsomes, prepared by the method of Shields and Blobel (10), were added at 3–6 A₂₆₀ units/ml. Wheat germ extract was prepared as described by Roberts and Paterson (11). Reticulocyte lysate, prepared from rabbits and the micrococcal nuclease to destroy endogenous messenger RNA, as described by Pelham and Jackson (13), VSV messenger RNA was used as described above. Ovalbumin messenger RNA (oligo(dT)-cellulose-purified), the gift of Drs. R. Meer and R. D. Palmieri of the University of Washington, was used at 5 μg/ml. At all times, autoclaved or disposable utensils were used.

Wheat germ reactions contained the following: 300 μCi/ml of [35S] methionine (1000 Ci/mmol), 52 mM potassium acetate, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2, 16.8 mM creatine phosphate, 2.0 mM ATP, 0.3 mM GTP, 6 mM dithiothreitol, 1.5 mM Mg acetate, 800 μM spermidine, 19 amino acids (excluding methionine) at 120 μM each, 60 μg/ml of creatine phosphokinase, and 0.3 volume of wheat germ extract. Ribonuclease inhibitor, prepared by the modified procedure of Blackburn (14), was added to reactions at 10 μg/ml as described by Scheele and Blackburn (15). Reactions were run at 22 °C, for periods of time ranging from 90 min to 3 h. For synchronized translation experiments, elongation was slowed by diluting the wheat germ extract to 0.18 volume of the reaction. Synchrony was conferred by the addition of both 1.5 mM 7-methylguanosine 5'-phosphate (P-L Biochemicals) and sufficient KCl to give a

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*The abbreviations used are: SDS, sodium dodecyl sulfate; VSV, vesicular stomatitis virus.
Reticulocyte lysate reactions contained the following: 300 μCi/ml of [35S]methionine, 72 mM potassium acetate (or potassium chloride for the synchrony experiments), 20 mM ATP, 0.2 mM GTP, 4 mM dithiothreitol, 0.052 mM Mg acetate, 800 μM spermidine, 20 μg/ml of hemin, 19 amino acids (excluding methionine) at 60 μM each, 20 pg/ml of hemin, 19 amino acids (excepting methionine) at 60 μM each, 60 pg/ml of creatine phosphokinase, 128 pg/ml of tRNA, and 0.4 volume of nuclease-treated reticulocyte lysate. Ribonuclease inhibitor was added as described above for wheat germ translations. Reactions were performed at 30 °C, for times ranging from 90 to 120 min. Synchrony was conferred by the addition of 120 mM 7-methylguanosine 5’-phosphate and 1.5 μM edeine (Calbiochem-Behring) to the translations 2 min after RNA addition.

Endoglycosidase H Digestion of Reactions—Translation reactions were boiled 5 min in 4 volumes of 2% SDS and 2% 2-mercaptoethanol. These were made 0.25 M in sodium citrate buffer, pH 6.0, 0.01% NaN₃, and 1 mM in phenylmethylsulfonyl fluoride, diluting the boiled sample 2-fold. Each sample was split and incubated for 18 h at 37 °C with 0.1 volume of the same buffer or with 0.1 volume of endoglycosidase H (30 μg/ml), a gift from Dr. P. W. Robbins of MIT, essentially as described by Zilberstein et al. (16). Reactions were precipitated with acetone, and the pellets were lyophilized and dissolved in 60 μl of sample buffer, with heating at 100 °C for 5 min, in preparation for SDS-gel electrophoresis and fluorography.

Protease Treatment of Dog Pancreatic Microsomes—Cell-free translations which were to be analyzed for the degree of protection of the translated products from protease degradation were treated with 100 μg/ml of emetine at 25 °C for 5 min to inhibit further protein synthesis. In some cases, 3 mM tetracaine-HCl, pH 7, was also added during this period. This anesthetic apparently improves the efficiency of protease protection of dog pancreatic microsomes (3).

Protease digestion was at 0 °C for 60 min. For VSV G protein, trypsin, trypsin, treated with tosylphenylalanyl chloromethyl ketone, at 1 mg/ml was used, while for ovalbumin, Protease K (Merck) at 200 μg/ml was used. Trypsin was inhibited by the addition of excess soybean trypsin inhibitor, while Protease K was inhibited with 2 mM phenylmethylsulfonyl fluoride. Digested and inhibited samples were made up in sample buffer and heated 5 min at 100 °C prior to storage at −70 °C.

RESULTS

Most of the ovalbumin synthesized in the wheat germ cell-free system containing dog pancreatic microsomes was in two slowly migrating glycosylated forms, OV₁ and OV₂ (Fig. 1, lane a). Upon treatment with endoglycosidase H these convert to a form that co-migrates with the unglycosylated form made by cell-free systems without microsomes, OV₀ (Fig. 1, lanes e and f). OV₁ and OV₂ resist degradation by Protease K, unlike OV₀, unless detergent is present during digestion (Fig. 1, lanes b, c, and g). OV₀, OV₁, and OV₂ are specifically immunoprecipitated by anti-ovalbumin serum (data not shown). Thus, OV₁ and OV₂ are forms of ovalbumin that

Fig. 2. Synchronized completion of ovalbumin. Ovalbumin RNA was translated without added microsomes in the wheat germ system at 22 °C, with 1.5 mM 7-methylguanosine 5’-phosphate added after 2 min to inhibit initiation. Aliquots of the reaction were delivered into SDS gel sample buffer at various times after inhibitor was added, and heated 5 min at 95 °C. The times shown are elapsed time from the beginning of translation of RNA. Lanes a through h, respectively, show the completion of ovalbumin at 2, 8, 12, 14, 17, 20, 24, and 62 min of translation. Lanes i and j show product synthesized in the presence of dog pancreatic microsomes, without and with Protease K digestion, respectively, as described in the legend to Fig. 1. Lanes k and l show the products synthesized with no added membranes, without and with protease digestion, respectively. Quantitation of completion is shown in Fig. 6.
Ovalbumin Utilizes an NH_2-terminal Signal Sequence

FIG. 3. Synchronized segregation of ovalbumin. Translation reactions were synchronized as described in the legend to Fig. 2. At various times, pancreatic microsomes (6 A_260 units/ml) were added to aliquots of the reaction mix. Translation was continued at 22 °C for the remainder of a 65-min period from the time of inhibitor addition. The times shown are dates from the time of RNA addition. Lanes a and b, respectively, show the translation products made without and with added microsomes. Lanes c through j, respectively, show the products made when membranes were added at 2, 4, 7, 10, 14, 18, 22, and 62 min after RNA addition. Lanes j through r, respectively, show the results of Proteinase K digestion of the samples which had membranes added at 2, 4, 7, 10, 14, 18, 22, and 62 min after RNA addition. Quantitation of the segregation of ovalbumin is shown in Fig. 6.

FIG. 4. Synchronized completion and protection of VSV G.
In parallel with the experiment of Figs. 2 and 3, VSV messenger RNA was translated in the wheat germ system under identical conditions of synchronization. In lanes a through k, translation was in the absence of microsomes. To measure the degree of completion of G protein, samples were removed into SDS gel sample buffer at 2, 4.5, 7, 12, 17, 22, 32, and 62 min after RNA addition. In lanes i through p, microsomes were added at 2, 4.5, 7, 12, 17, 22, 32, and 62 min after RNA addition. The reactions were then incubated for the remainder of a 65-min period from the time of inhibitor addition. All of the reactions were then treated with trypsin. The G species in lanes i, g, and h is unglycosylated. The slower migrating G species in lanes i through k contain two Asn-linked high mannose oligosaccharides, has lost the 16 NH2-terminal amino acids (the "signal sequence") and has had removed, by the trypsin, the 30 COOH-terminal amino acids that are accessible to the cytoplasm (1). The labels on the left denote the principal VSV proteins.

contain at least one high mannose oligosaccharide, and are segregated in the microsomal vesicles. OV_o is not segregated within the microsomes. As judged by its resistance to endoglycosidase H (data not shown). OV_o is unglycosylated. Palmieter et al. (17) have obtained similar results on the OV_o, OV_1, and OV_2 forms of ovalbumin.

The synchronized membrane addition experiment was performed first in the wheat germ system using inhibitors that inhibit initiation to 99% or better (1.5 mM 7-methylguanosine 5'-phosphate) (1). The degree of completion of ovalbumin was monitored by delivery of aliquots of reaction at various times directly into SDS gel sample buffer and analyzing by SDS-gel electrophoresis and fluorography (Fig. 2). The timing of translation of ovalbumin was monitored by adding microsomes to aliquots of the synchronized reaction at various times, completing read-off of the polypeptide, and analyzing both the degree of formation of glycosylated ovalbumin, and its protection from Proteinase K (Fig. 3).

Ovalbumin was completed after about 17 min of translation, yielding a rate of elongation of about 22 amino acid residues/min. The amount of ovalbumin completed in the system did not increase upon longer incubation (Fig. 2), demonstrating that the inhibition of initiation had been effective. In the same system, VSV G was completed after 27 min, yielding a rate of elongation of 21 residues/min (Fig. 4). Ovalbumin was protected from protease as long as membranes were added before
Ovalbumin Utilizes an NH₂-terminal Signal Sequence

Figure 5. Synchronized completion and protection of ovalbumin in the reticulocyte lysate system. Ovalbumin RNA was translated without added microsomes in the reticulocyte lysate system at 22 °C, with 1.5 μM eukine and 4.5 mM 7-methylguanosine 5'-phosphate added after 2 min to inhibit initiation. Aliquots of the reaction were delivered into SDS gel sample buffer at various times and heated at 95 °C for 5 min in order to monitor completion, while other aliquots were delivered into 6 A₅₅₀ units/ml of dog pancreatic microsomes at various times, and incubated at 22 °C for the remainder of an 80-min period, in order to monitor insertion of ovalbumin. The upper panel shows the degree of completion of ovalbumin at 2, 22, 27, 32, 42, 47, 52, 57, and 62 min of translation, respectively. The lower panel shows the degree of glycosylation (left) and protection from proteinase K digestion (right) of ovalbumin when microsomes were added at 2, 8, 12, 14, 16, 18, 20, 22, 32, and 62 min of translation, respectively. Quantitation is shown in Fig. 6.

Figure 6. Kinetics of protection and completion of ovalbumin and VSV glycoprotein. The fluorographs of the experiments of Figs. 2 through 5 were scanned with a microdensitometer, and the relative areas under the relevant peaks plotted against time. 0, values for ovalbumin; O, values for VSV glycoprotein. A and B show the results for completion (A) and protection from protease (B) in the wheat germ system. C and D show the results for completion (C) and protection from protease (D) in the reticulocyte lysate system. For all panels, the times of translation at which 50% completion or protection were observed were taken as the mean values for these parameters. Arrows indicate the times of addition of inhibitors of initiation of protein synthesis.

Translation had proceeded for about 7 min, corresponding to the synthesis of about 154 amino acid residues (Figs. 3 and 6B). Also, the glycosylated forms of ovalbumin, OV₁ and OV₂, were synthesized if membranes were added prior to the 7-min time, whereas OV₀, the unglycosylated form, was synthesized if membranes were added after this time (Fig. 3). VSV G was protected if membranes were present before 5 min had elapsed, corresponding to a synthesis of about 100 amino acid residues (Figs. 4 and 6B). These results demonstrate that the sequence which directs the translocation of ovalbumin occurs prior to about residue 150. This is much more in keeping with an NH₂-terminal signal sequence for ovalbumin.

As the experiment seeming to show that ovalbumin has an internal signal sequence (4) was performed in the reticulocyte lysate system, rather than the wheat germ system, it could be argued that the latter system gives anomalous results. We therefore repeated the synchrony experiment in the reticulocyte lysate system, using 1.5 μM eukine and 4.5 mM 7-methylguanosine 5'-phosphate to inhibit initiation of protein synthesis. This combination of inhibitors, first used by Scheele and co-workers (3), inhibited initiation greater than 99% (data not shown). Synthesis of ovalbumin was completed after about 46 min (Figs. 5 and 6C) giving a rate of elongation of 8.5 amino acid residues/min. Glycosylation and protection from protease
were prevented if microsomes were added after about 16 min of translation (Figs. 5 and 6D) placing the signal sequence prior to residue 140. This result agrees well with the result obtained in the wheat germ system. Again, synchronized translation of VSV glycoprotein in the synchronized reticulocyte lysate system placed the VSV G signal sequence prior to residue 100, although the rate of elongation, 13 amino acids/min, was significantly faster than for ovalbumin (Fig. 6, C and D).

To summarize, in both the wheat germ and reticulocyte lysate synchronized translation systems, microsomes had to be added before 150 amino acid residues of ovalbumin had been synthesized if glycosylation and segregation were to occur. This would place the ovalbumin signal sequence within the first 150 amino acids, rather than around residue 250 as was suggested by Lingappa et al. (4). In agreement with these results, investigators in Palmiter’s laboratory (18) have found that nascent chains of ovalbumin as short as 50 amino acid residues are bound to and inserted into microsomes.

**DISCUSSION**

The experiments on ovalbumin, performed by Lingappa et al. (4), centered on the isolation of a tryptic peptide of ovalbumin which, when present at concentrations of several milligrams per milliliter, was a competitive inhibitor of the translocation of in vitro synthesized prolactin into dog pancreatic microsomes. This peptide was localized to residues 229 to 276 in ovalbumin, a protein of 385 total residues. On this basis, it was suggested that this peptide acted as an internal signal sequence for insertion of nascent ovalbumin into microsomal membranes. The evidence that this peptide was the actual signal sequence for ovalbumin was provided by a synchronized membrane addition experiment as outlined above, using the reticulocyte lysate system with 1 mM pactamycin to inhibit initiation. In this experiment, ovalbumin was no longer glycosylated if membranes were added after about 5.5 min, in a system where the protein was completed in 8.5 min. This placed the signal sequence prior to residue 250 of ovalbumin, and was interpreted as evidence for an insertion signal at that point. By contrast, our results, using both wheat germ and reticulocyte cell-free systems, position the “signal” sequence prior to residue 150, and exclude any signal function for an internal set of amino acids in the COOH-terminal two-thirds of the protein.

Exactly why Lingappa and co-workers observed for ovalbumin a critical point for insertion of 250 amino acids is not clear. One problem was the choice of inhibitor, pactamycin. The synchrony experiment depends on a virtually complete shut-off of initiation, without inhibition of elongation, in order to give accurate results. This is because the time allowed to complete the polypeptide is much longer than the time window allowed for polypeptide chain initiation. For example, if the initiation window is 5% of the completion period, 95% inhibition of initiation will allow as much initiation after the inhibitor is added as before. This nonspecific initiation generates an artifactualy longer time before membranes no longer can effect insertion. For accurate results, therefore, initiation must be inhibited 99% or better. 1 μg/ml of pactamycin inhibits initiation less than the 99% required, and at higher concentration also slows the elongation rate, making the time needed for completion even longer (19).

Another possibility is suggested by our own results in the reticulocyte lysate system. Ovalbumin was observed by us to take longer to complete in this system than VSV glycoprotein, although VSV glycoprotein (570 amino acids) is larger than ovalbumin (385 amino acids). If Lingappa et al. (4) misconstrued the completion time of ovalbumin to have been shorter than it actually was, an artifactualy high elongation rate would be obtained which would skew their results. As the completion data were not shown, however, this can only be a speculation. Nonetheless, this result indicates that elongation rates for one protein should not be applied to different proteins translated in the same system, at least for the reticulocyte lysate system.

The actual location of the signal sequence of ovalbumin is not determined by our results, except that it is NH2-terminal to residue 150. A combination of our results with those of Meek et al. (18) would seem to place the time of the sequence’s action as between the times when residues 50 and 150 are synthesized. Allowing for 40 residues residing within the ribosome (3), it is clear that the nascent chain of ovalbumin interacts with membranes almost as soon as it emerges from the ribosome. The sequence thus constitutes an actual NH2-terminal signal sequence, which need not be cleaved from the completed polypeptide in order to effect segregation.

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Ovalbumin utilizes an NH2-terminal signal sequence.

W A Braell and H F Lodish

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