

# Signal Peptides and Signal Peptidase in *Drosophila melanogaster*

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**ABSTRACT** Translation of poly(A)-containing RNA from the female fat body of *Drosophila melanogaster* in a rabbit reticulocyte cell-free system results in the synthesis of previtellogenin polypeptides (PVs) having higher apparent molecular weights (46,000 and 45,000) than the forms seen after an in vivo pulse labeling. However, when this RNA is translated in the presence of EDTA-stripped microsomal membranes from the dog pancreas, vitellogenin precursors are produced that, upon SDS-polyacrylamide gel electrophoresis, comigrate with the in vivo forms (apparent molecular weights, 45,000 and 44,000). These processed forms are sequestered within the microsomal lumen, as evidenced by their insensitivity to trypsin digestion. Neither processing nor sequestration occur posttranslationally. In addition, a microsomal membrane fraction derived from *Drosophila* embryos is able to cotranslationally process the PVs as well as a murine pre-light chain IgG. These observations support a signal-mediated mode of secretion in *Drosophila*, and suggest that signal sequence recognition and signal peptidase activities are conserved even between mammalian and insect systems.

The process of vitellogenesis in *Drosophila melanogaster* is one well suited for the study of the production of secretory proteins. The fat body in insects is known to be the site of vitellogenin synthesis (1), playing a role analogous to the vertebrate liver (2, 3). In *Drosophila*, vitellogenin production may account for as much as 30% of the protein synthesized by the mature female fat body (4). The vitellogenin polypeptides are secreted into the hemolymph and transported to the ovary, where they are accumulated and stored in the form of yolk, or vitellin.

We have shown previously that there are three distinct vitellogenin polypeptides produced by the fat body of *Drosophila melanogaster*, and that each of these undergoes at least two modifications before secretion (4). The vitellogenin polypeptides resulting from in vitro translation of female fat body poly(A)-containing RNA are ~1,000 daltons larger than the corresponding species seen after a 30-s in vivo pulse labeling. Such a difference in apparent molecular weight suggested an amino-terminal leader sequence (5) that is removed from the nascent polypeptide.

We present here further evidence that the vitellogenin polypeptides do, in fact, contain presecretory leader sequences. When translated in vitro in the presence of EDTA-stripped microsomal membranes from the dog pancreas, female fat body poly(A)-containing RNA directs the synthesis of polypeptides of the same size as seen in vivo. Consistently, the fat body is rich in rough endoplasmic reticulum,<sup>1</sup> as expected for the production of secretory proteins. Moreover, we demonstrate a membrane-associated signal peptidase activity in *Drosophila*

*melanogaster* itself. Taken together, these findings suggest that the signal hypothesis is a valid mechanism for protein secretion in invertebrate systems. A preliminary account of this study has been presented elsewhere (6).

## MATERIALS AND METHODS

*Drosophila melanogaster* (Oregon R, P2 strain) grown in mass culture (7) and aged 4–5 d after eclosion, were used for in vivo labeling and isolation of fat body poly(A)-containing RNA (both previously described in reference 4). All experiments concerning murine light-chain IgG were carried out with a cultured strain of mouse myeloma cell (MOPC-21; reference 8). Cells were grown at 37°C in Dulbecco's Modified Eagle's Medium (9) (Grand Island Biological Co. (GIBCO), Grand Island, N. Y.), containing 10% fetal calf serum (GIBCO), 25 mM glucose, 4 mM glutamine, 1 mM pyruvate, and 43 mM NaHCO<sub>3</sub>, pH 6.8. All protein products were labeled with L-[<sup>35</sup>S]methionine (Amersham Corp., Eastern Heights, Ill.; 600–1,300 Ci/mmol).

## Labeling of Authentic Light Chain and Electrophoresis

Myeloma cells were grown to a high density, then collected and concentrated by centrifugation at 1,000 g in a swinging-bucket rotor at 4°C. Cells were suspended to a density of  $2 \times 10^6$  viable cells/ml in the above medium containing only 2.5% fetal calf serum and supplemented with 25  $\mu$ Ci/ml L-[<sup>35</sup>S]methionine. After incubation at 37°C for 4 h, cells were removed from the culture fluid by centrifugation at 1,000 g. Culture fluid was then centrifuged at 20,000 g for 15 min. Protein was collected from the supernate by precipitation with 10 vol of cold acetone, followed by centrifugation at 10,000 g for 10 min. The resulting pellet was air-dried and prepared for electrophoresis by heating at 100°C for 5 min in sample buffer (10). Insoluble material was removed by centrifugation at 10,000 g for 10 min. These products, as well as all others described here, were resolved by SDS (BDH Chemicals Ltd., Poole, England) polyacrylamide gel electrophoresis on 10% resolving gels (10). Gel patterns were visualized by fluorography (11) with preexposed Kodak RP Royal film (12).

<sup>1</sup> J. H. Caulton, and A. P. Mahowald, Indiana University, personal communication.

## Preparation of Myeloma RNA

Cells were grown to a density of  $2.5 \times 10^6$  cells/ml and collected by centrifugation at 1,000 g for 10 min at 4°C. They were then rinsed once in growth medium lacking fetal calf serum and repelleted. Cells were lysed in 10 vol of SDS buffer (0.5% SDS, 1 mM EDTA, 100 mM NaCl, 10 mM Tris-HCl, pH 7.4) containing 1 mg/ml heparin and 500 µg/ml proteinase K, and homogenized in a Dounce-type homogenizer with a B pestle. Purification of total poly(A)-containing RNA was as described for fat body RNA (4).

## Cell-free Translation

Preparation of the mRNA-dependent rabbit reticulocyte lysate and the reaction conditions were as previously described (4). Initial incubations were at 37°C for 60 min, after which time no additional increase in acid-precipitable radioactivity was observed (data not shown). Standard 25-µl reactions contained either 0.18 µg of female fat body or 1.0 µg of myeloma poly(A)-containing RNA. In addition, to control for components added along with microsomal membranes (see below), all reactions contained 85 mM sucrose and 330 µM dithiothreitol.

## Preparation of Microsomal Membranes

All manipulations were carried out at 0–4°C. Dog pancreas rough microsomal membranes were prepared and stripped of ribosomes by EDTA treatment as described (13). Before use, membranes were suspended in 0.25 M sucrose, 1 mM dithiothreitol, and treated with micrococcal nuclease (13). *Drosophila* membranes were prepared from embryos aged 3–9 h at 25°C. Embryos were dechorionated (7), rinsed three times with 10 vol of deionized water, and then twice with 10 vol of homogenization buffer (50 mM KCl, 5 mM MgCl<sub>2</sub>, 0.25 M sucrose, 2 mM dithiothreitol, 50 mM triethanolamine, pH 7.5). The embryos were then homogenized in 10 vol of the above buffer, using a Dounce-type homogenizer with a loose-fitting, motor-driven Teflon pestle. The homogenate was centrifuged twice at 10,000 g, with the supernate being retained at each step. Rough microsomal membranes and EDTA-stripped membranes were prepared from the second supernate as described for the dog pancreas (13).

## Posttranslational Treatments

After the initial 60-min incubation, the *in vitro* reactions were treated in one of the following ways: (a) The protein was immediately precipitated with 10 vol of acetone, air-dried, and either prepared for electrophoresis as described above, or prepared for nonequilibrium pH gradient electrophoresis as previously described (14). (b) For reactions containing *Drosophila* membranes, immunoprecipitation was used to visualize the products of interest. Reactions were diluted with 10 vol of phosphate-buffered saline containing 0.5% Nonidet P-40 and 0.1 mM phenylmethylsulfonyl fluoride, followed by incubation for 60 min with one-tenth the starting volume of the appropriate antiserum. Yolk polypeptide antiserum (15) was used for fat-body products, and goat antimouse IgG serum (Miles-Yeda LTD, Revot, Israel) was used for myeloma products. Immune complexes were precipitated as described (16). The final pellets were prepared for electrophoresis in a manner identical to that used for acetone precipitates. The gel patterns of the resulting supernate (not shown) and pellets confirmed nearly quantitative recovery of precipitated products. (c) Products were tested for resistance to proteolytic digestion by incubating 90 min at 4°C in the presence of 0.25 mg/ml trypsin (180–220 U/mg; Worthington Biochemical Corp., Freehold, N. J.). Digestion was terminated by adding a twofold weight excess of soybean trypsin inhibitor (Worthington Biochemical Corp.), followed by acetone precipitation and preparation for electrophoresis. Trypsin digestion was carried out either immediately or after a posttranslational incubation at 37° for 90 min in the presence of membranes, as indicated in the figure legends. In all cases, gel lanes compared within a given figure represent equal volumes of initial *in vitro* reactions.

## RESULTS

### *In Vitro* Processing of the Vitellogenin Polypeptides

After a 1-h *in vivo* labeling of the *Drosophila* ovary, the three major vitellin polypeptides are seen as a pattern of three bands when resolved by SDS-polyacrylamide gel electrophoresis (Fig. 1 a). The apparent molecular weights of these species are 46,000, 45,000, and 44,000 (15). In contrast, after a 1-min *in vivo* pulse labeling of the female fat body, the immature

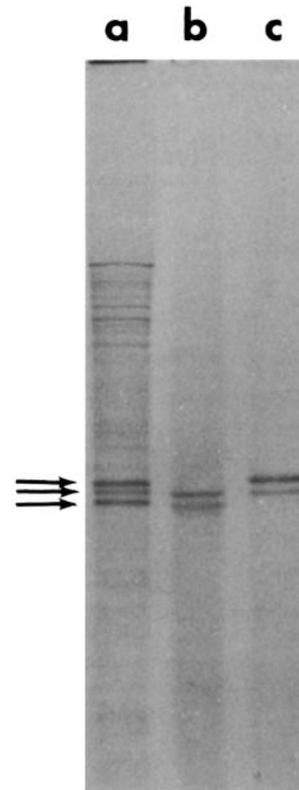


FIGURE 1 Comparison of *in vivo* labeled products and *in vitro* translation products. (a) Total ovary protein labeled for 1 h *in vivo*. Arrows indicate mature vitellin polypeptides (apparent molecular weight, 46,000, 45,000, and 44,000). (b) *In vivo* products of the female fat body after a 1-min pulse labeling. (c) *In vitro* translation products of female fat body poly(A)-containing RNA.

vitellogenins (4) produce a doublet, rather than a triplet, pattern (Fig. 1 b). It has been demonstrated that the top band, in this case, (apparent molecular weight, 45,000) contains two polypeptides and that these are precursors to the two larger vitellin polypeptides (4). Likewise, the lower band (apparent molecular weight, 44,000) contains the precursor to the smallest vitellin polypeptide (4). If, instead, female fat body poly(A)-containing RNA is translated *in vitro*, a doublet of vitellogenin polypeptide is increased in size by ~1,000 in apparent molecular weight (Fig. 1 c). Because with either the *in vivo* pulse-labeled products or the *in vitro* products the spatial separation of the doublet is unchanged, and because the lower *in vitro* product comigrates with the upper *in vivo* product (4), it proved unnecessary to include *in vivo* products as molecular weight markers for the remainder of the study. Also, all additional figures will show only the gel region containing the products of interest.

To determine whether the size difference in the *in vitro* and *in vivo* products is attributable to the presence of uncleaved signal peptides on the *in vitro* products (specified PV1 and PV2 at 46,000 daltons and PV3 at 45,000 daltons), translation of female fat body poly(A)-containing RNA was carried out in the presence of increasing concentrations of dog pancreas microsomal membranes. As can be seen in Fig. 2, as the level of membranes is raised, the *in vitro* products progressively begin to migrate as do the pulse-labeled *in vivo* ones. This is most easily followed by noting the disappearance of material migrating at 46,000 daltons and the appearance of a new

species at 44,000 daltons, as one proceeds from Fig. 2*b* to 2*f*. The greater intensity of the upper band even after complete processing (Fig. 2*f*) indicates that all three species are present after *in vitro* processing. At membrane levels in excess of 1.0 OD<sub>280</sub>/ml, no further change in the gel pattern has been observed.

That all three polypeptides undergo processing has been confirmed by two-dimensional gel electrophoresis (Fig. 3). Here are seen the vitellogenin polypeptides produced by translation in the presence of 0.5 OD<sub>280</sub>/ml dog pancreas membranes. The resulting gel pattern, at this intermediate level of processing, is virtually identical to that obtained by electrophoresing a mixture of unprocessed *in vitro* products and *in vivo* pulse-labeled products (4). Thus, within the limits of our gel system, cotranslational processing produces polypeptides indistinguishable from those seen *in vivo*.

Two further tests confirm that the vitellogenin precursors are secreted according to a signal-type model. First, the processed forms are sequestered within the microsomal lumen and as such are rendered insensitive to proteolytic digestion (Fig. 4*d* and *e*), whereas the unprocessed forms are sensitive (Fig. 4*a* and *b*). Second, the processing and sequestration of the polypeptides occur only cotranslationally, requiring action on the nascent polypeptide. Fig. 4*f* and *g* show that no processing

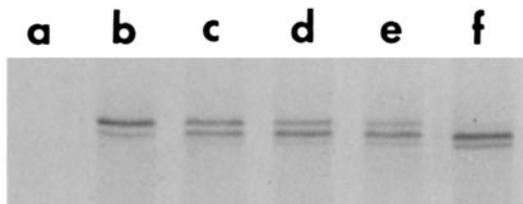


FIGURE 2 Cotranslational processing of previtellogenin polypeptides by dog pancreas microsomal membranes. (a) Endogenous synthesis in presence of 2.0 OD<sub>280</sub>/ml micrococcal nuclease-treated membranes. (b) Translation of female fat body poly(A)-containing RNA in the absence of membranes. (c-f) Translation of female fat body poly(A)-containing RNA in the presence of increasing concentrations of micrococcal nuclease-treated membranes. c, 0.2 OD<sub>280</sub>/ml; d, 0.3 OD<sub>280</sub>/ml; e, 0.5 OD<sub>280</sub>/ml; f, 1.0 OD<sub>280</sub>/ml.

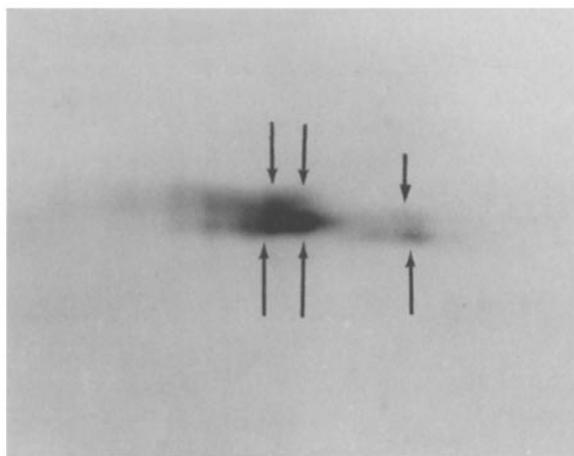


FIGURE 3 Two-dimensional electrophoresis of translation of fat body poly(A)-containing RNA in the presence of 0.5 OD<sub>280</sub>/ml dog pancreas membranes. The first dimension (migration left to right) was run by the modified nonequilibrium pH gradient method of Waring et al. (14). The second dimension was on a 10% SDS gel. Downward pointing arrows indicate unprocessed forms, and upward pointing arrows indicate processed forms.

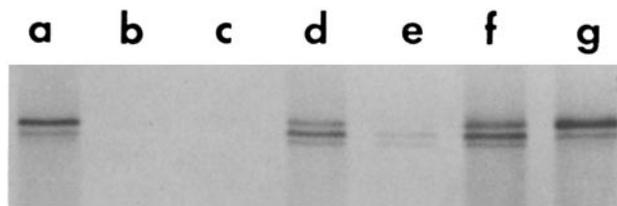


FIGURE 4 Trypsinization of *in vitro* products in absence or presence of dog pancreas membranes. (a) Translation of female fat body poly(A)-containing RNA in the absence of membranes. (b) Same as a, after trypsin digestion. c Membranes were added to a concentration of 1.0 OD<sub>280</sub>/ml posttranslationally. After a 90 min incubation at 37°C, the reaction was treated with trypsin. (d) Translation in the presence of 0.5 OD<sub>280</sub>/ml of membranes. (e) Same as d, after trypsin treatment. (f) Additional 90 min incubation at 37°C with 0.5 OD<sub>280</sub>/ml membranes present cotranslationally. (g) Same as c before trypsin treatment.

occurs posttranslationally, and Fig. 4*c* demonstrates no post-translational sequestration.

### Demonstration of Signal Peptidase in *Drosophila*

Strong evidence for a signal mechanism in the secretion of *Drosophila* proteins is the demonstration of a membrane-associated signal peptidase activity in *Drosophila* itself. Though the fat body is rich in rough endoplasmic reticulum, fat body preparations can be made only by time-consuming dissection, and not enough material can be generated to allow large-scale preparation of microsomal membranes. In addition, contaminating vitellogenin mRNA would complicate analysis of *in vitro* products. As an alternative, we used *Drosophila* embryos as a source of membranes. Embryos have the advantage that before extensive gut formation, contaminating protease and nuclease activities are expected to be minimal.

Electron microscopy of our *Drosophila* membrane preparation (not shown) has indicated that the membranes may be incompletely freed of ribosomes. This is possibly attributable to a high level of divalent cations in the embryonic lysate. It is consistent with this interpretation that it has proven impossible to treat the *Drosophila* membranes with micrococcal nuclease without severe inhibition of the *in vitro* system. This result was apparently the result of failure of EGTA to inactivate the nuclease, even at levels up to 40-fold over the added Ca<sup>++</sup>. The ions responsible are apparently not free Ca<sup>++</sup>, because no nuclease activity was observed in the absence of added Ca<sup>++</sup>. As an alternative to nuclease treatment, products translated in the presence of *Drosophila* membranes were analyzed after indirect immunoprecipitation.

As can be seen in Fig. 5, the *Drosophila* microsomal membranes isolated do indeed have an associated signal peptidase activity. However, on the basis of membrane concentration, about an eightfold higher amount of the *Drosophila* membranes is needed to give processing comparable to the dog pancreas membranes (cf. Fig. 2 and 5). At the membrane levels required to give total processing (Fig. 5*d*), the *in vitro* system is inhibited by ~50% with regard to immunoprecipitable material, but the processing is easily seen by adjusting exposure time (Fig. 5*e*). At least a part of the reduction in immunoprecipitable material may be accounted for by competition between membrane-associated mRNA and exogenously added mRNA.

A further demonstration of the *Drosophila* signal peptidase activity is seen in Fig. 6. Here, for purposes of comparison, is

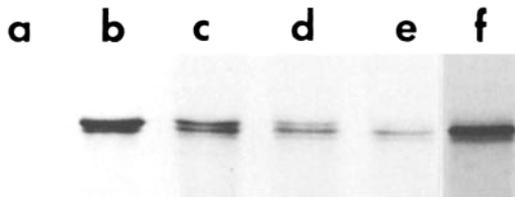


FIGURE 5 Cotranslational processing of previtellogenin polypeptides by *Drosophila* microsomal membranes. All lanes are immunoprecipitate pellets. (a) Endogenous synthesis in the presence of 8.5 OD<sub>280</sub>/ml membranes. (b) Translation of female fat body poly(A)-containing RNA in the absence of membranes. (c–e) Translation in the presence of increasing concentrations of membranes. c, 2.0 OD<sub>280</sub>/ml; d, 5.0 OD<sub>280</sub>/ml; e, 8.5 OD<sub>280</sub>/ml; f, Same gel lane as in e, exposed twice as long.

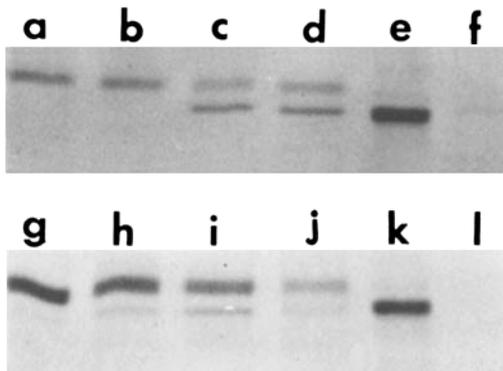


FIGURE 6 Comparison of cotranslational processing of murine light-chain IgG by dog pancreas and *Drosophila* microsomal membranes. All lanes except e and k are immunoprecipitate pellets. (a) Myeloma poly(A)-containing RNA translated in the absence of membranes. (b–d) Myeloma translation with increasing concentrations of dog pancreas membranes. b, 0.2 OD<sub>280</sub>/ml; c, 0.5 OD<sub>280</sub>/ml; d, 1.0 OD<sub>280</sub>/ml. (e) Authentic light chain marker. (f) Endogenous synthesis in the presence of 1.0 OD<sub>280</sub>/ml dog pancreas membranes. (g) Same as a. (h–j) Myeloma translation in presence of increasing concentrations of *Drosophila* membranes. h, 2.0 OD<sub>280</sub>/ml; i, 5.0 OD<sub>280</sub>/ml; j, 8.5 OD<sub>280</sub>/ml. (k) Same as e. (l) Endogenous synthesis in the presence of 8.5 OD<sub>280</sub>/ml *Drosophila* membranes.

shown in vitro processing of myeloma light-chain IgG by dog pancreas membranes and *Drosophila* embryo membranes. Again the inhibition at higher levels of *Drosophila* membranes is well demonstrated (Fig. 6j). It is, however, clear that the *Drosophila* signal peptidase recognizes and cleaves the signal peptide of this mammalian polypeptide. As with the vitellogenin polypeptides, comparable levels of processing are seen with about an eightfold higher level of the *Drosophila* membranes.

## DISCUSSION

As predicted by the signal hypothesis, the vitellogenin polypeptides are produced initially in presecretory forms that are proteolytically modified while the polypeptide chain is nascent. This is consistent with our earlier finding that even with in vivo pulse labeling as short as 30 s, the vitellogenin precursors produced are smaller than the initial translation products seen after in vitro translation. Larger in vitro precursors have recently been described for several of the major chorion proteins of *Drosophila melanogaster*, and, like the vitellogenin polypep-

ptides, they may be processed cotranslationally to give forms comigrating with those seen in vivo (17, 18). It is interesting to note that in the case of the chorion proteins, there appear to be two size classes of leader sequences (17). Two proteins have precursors 2,000–3,000 daltons larger than the processed forms, whereas a third has a precursor only about 1,000 daltons larger. Of the large number of vertebrate presecretory proteins studied (see reference 19 for review) amino-terminal leader sequences have been found to range from 15 to 29 amino acid residues. It is quite likely that *Drosophila* contains, within the limits of present analysis, presecretory proteins having leader sequences that fall within this range as well. For the case of honeybee prepromelittin it has been shown (20) that there is an amino-terminal leader sequence of about 21 amino acids, again indicating that insect presecretory proteins probably present no major exceptions to a signal-mediated secretion process.

The demonstration of a membrane-bound signal peptidase activity in *Drosophila* argues strongly for a signal-type mechanism in the secretion of invertebrate proteins. Though use of the *Drosophila* microsomal membranes with the rabbit reticulocyte in vitro system poses problems with regard to nuclease treatment and incorporation into immunoprecipitable material, the difficulties are not entirely unexpected. A similar problem with nuclease treatment has been found by treating embryonic lysates of *Drosophila melanogaster* used for in vitro translation (21). Moreover, the inhibition of protein synthesis seen here is reminiscent of that seen with wheat-germ extracts supplemented with dog pancreas microsomal membranes (13). Thus, the inhibition might in part be related to the divergent sources of translation machinery and microsomal membranes.

The striking finding of the present work is the reciprocity in processing found between an insect and mammalian system. The apparent generality of the signal hypothesis throughout animal systems is well supported. Perhaps most interesting is the fact that peptide insertion and cleavage signals as well as membrane transposition and signal peptidase activities are highly conserved. A critical test of this assumption will depend ultimately on sequence determinations. Any differences, as well as identities, in peptide removal should serve to elucidate the process of protein secretion in general.

We thank K. L. Page and J. Atkin for providing a dog pancreas, and M. E. Digan for help in preparing membranes. This work was supported by National Institutes of Health (NIH) grant no. HO-07983. M. D. Brennan is an NIH service award trainee (PHS-T32-GM07227).

Received for publication 9 July 1980, and in revised form 18 August 1980.

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