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Identification of chorion protein precursors and the mRNAs that encode them in *Drosophila melanogaster*

(wheat germ system/immunoprecipitation/membrane-dependent processing/follicular RNA)

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ABSTRACT **Specific antisera were generated for major size classes of *Drosophila* chorion proteins. These antisera were used in conjunction with cell-free translation of follicular RNA to identify precursors, containing signal peptides, for chorion components A_1, A_2, B_1, and B_2. An additional precursor cross-reacts with anti-B antisera, although its product appears to correspond to a protein distinct from authentic B proteins. The size classes of mRNA encoding A and B protein precursors have been identified by cell-free translations.**

Because of its genetics, *Drosophila* is a favorable organism for the study of cell-specific gene expression. A particularly suitable system is the production during the final stages of oogenesis of the complex extracellular eggshell, or chorion, in a few hours by the follicular epithelium surrounding the oocyte. We have previously identified six major size classes of chorion proteins that are synthesized in succession during choriogenesis (1). These have been further resolved, and additional components have been identified, by using two-dimensional gel fractionation (2). Classes of follicular poly(A)-containing RNAs have been identified as putative mRNAs for chorion proteins, and the corresponding genes have been studied by *in situ* hybridization and analysis of mutants (3, 4).

We are studying the chorion system by using recombinant DNA procedures. For recognizing cDNA or genomic DNA clones encoding chorion proteins, an important approach is cell-free translation of specifically hybridized mRNAs. Similarly, definite characterization of specific mRNAs requires their cell-free translation and product identification. However, as we show in this report, a complication arises because chorion proteins are translated in the form of precursors. By using immunochemical techniques, we have identified the precursors of specific classes of chorion proteins. As a consequence, we have been able to establish unequivocally the size classes of mRNAs encoding several of the chorion proteins.

MATERIALS AND METHODS

Materials. Follicles were dissected from *Drosophila melanogaster* (Oregon R) maintained at 25°C. [^{3}H]Leucine (145 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels), [^{3}H]proline (111 Ci/mmol), [^{3}H]uridine (40 Ci/mmol), and [^{32}P]H_3PO_4 (≥500 Ci/mmol) were obtained from New England Nuclear. Collagenase (830 units/mg) was obtained from Sigma.

Preparation of Immunogen. Chorions were purified as described (ref. 1, method II), washed extensively in Ringer's solution (5), and incubated overnight in 2% sodium dodecyl sulfate ($NaDodSO_4$)/62.5 mM Tris·HCl, pH 6.8. They were washed thoroughly with Ringer's solution and extracted overnight with 7 M guanidine hydrochloride/62.5 mM Tris·HCl (pH 6.8), and then dialyzed sequentially against the same buffer containing 3 M urea and then 2% $NaDodSO_4$. Dialyzed material was electrophoresed on preparative 10–21% exponential gradient, slab polyacrylamide gels containing $NaDodSO_4$, and the buffer systems developed by Laemmli (6). Appropriate bands were excised, minced, mixed with a mixture (1:8) of complete and incomplete Freund's adjuvant and injected directly into rabbits intradermally at multiple sites (7) and in the footpads (≈0.2 mg of protein per injection).

Organ Cultures. Female flies were cold-anaesthetized and dissected in Ringer's solution. To generate radioactively labeled proteins, ovaries were cultured for 30 min at room temperature in Robb's medium (8) supplemented with [^{3}H]proline at 1 mCi/ml. Ovaries were washed in Ringer's solution and dissolved in urea/$NaDodSO_4$ buffer [7 M urea/2% $NaDodSO_4$/5% 2-mercaptoethanol/62.5 mM Tris·HCl, pH 6.8]. To generate radioactively labeled RNA, ovaries were incubated as above, but for 3 hr with [^{32}P]H_3PO_4 at 3 mCi/ml or [^{3}H]uridine at 3 mCi/ml.

RNA Preparation. Hand-dissected ovaries were either used whole or dispersed into individual follicles that were size-selected for choriogenic stages by filtration (Nytex, 132 mesh). The tissue was homogenized briefly in 100 mM NaCl/10 mM Tris·HCl, pH 7.5/2% Triton X-100. The resulting homogenate was clarified for 5 min at 27,000 × *g*, made 0.5% in $NaDodSO_4$ and 10 mM in Na_2 EDTA (pH 8.0), and deproteinized by repeated extractions with phenol and Sevag (chloroform/isoamyl alcohol, 24:1). The RNA was ethanol-precipitated and used for translation either directly or after selection of poly(A)$^+$ RNA by using oligo(dT) cellulose chromatography. RNA specific for follicular epithelial cells was prepared as described (3).

***In Vitro* Translation.** Translation assays are described (9) and contained 20 μg of poly(A)$^+$ RNA per ml or 200 μg of unfractionated RNA per ml. Translation in the presence of dog pancreatic microsomes (a gift of J. Majzoub) and subsequent proteolysis were carried out as described (10).

Immunoprecipitation Assays. Immunoprecipitations were performed according to a modification of the procedure that uses *Staphylococcus aureus* (gift of H. Ploegh) as immunoadsorbent (11). All reactions were performed in Tris-buffered saline/0.1% $NaDodSO_4$. Bacterial pellets were washed once with 0.1% $NaDodSO_4$/Tris-buffered saline and once with 0.01% $NaDodSO_4$/Tris-buffered saline before elution by boiling for 3 min in sample buffer (6). Whole ovaries labeled in organ culture provided authentic chorion proteins. *In vitro* translation products were precipitated with 2 vol of 95% ethanol. Samples were dissolved in urea/$NaDodSO_4$ buffer and diluted 1:15 into immunoprecipitation buffer. In order to make the antigen concentration comparable among samples, a standard amount

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Abbreviations: Chorion protein A_1, A_2, B_1, B_2, C_1, and C_2 classes correspond to c15, c16, c18, c19, c36, and c38 classes as defined in ref. 2; $NaDodSO_4$, sodium dodecyl sulfate.

of unlabeled, dissolved whole ovaries was added to the translation immunoprecipitation reactions. All samples were treated with preimmune serum prior to specific immunoprecipitation.

Polyacrylamide Gel Electrophoresis. Protein samples were electrophoresed in 10–15% linear gradient, slab $NaDodSO_4$/polyacrylamide gels, 17 cm long, using the buffer systems described by Laemmli (6). In experiments involving immunoprecipitations, it was necessary to add equivalent amounts of *S. aureus*-precipitated serum to all samples in order to obtain comparable migration of identical proteins. RNA samples were electrophoresed as described (12). Protein or RNA bands were detected by fluorography (13). RNA bands were recovered from gels as described (9) and translated after purification through oligo(dT) cellulose and subsequent ethanol precipitation.

RESULTS

Generation of Antisera Specific for Molecular Weight Classes of Chorion Proteins. The mass isolation procedure of Petri *et al.* (1, 14) yields highly purified eggshell fragments that can be dissolved and fractionated by electrophoresis into six major protein size classes (1). Such preparations were electrophoresed on preparative gels, and individual bands (A_1, A_2, B_1, and B_2) or pairs of bands (C_1 plus C_2) were excised as indicated (Fig. 1*a*) and injected directly into rabbits.

The specificity of the antisera was assayed by immunoprecipitation. The starting material was a complex mixture of labeled proteins from total ovaries cultured in [^{3}H]proline for 30 min. (Fig. 1*b*, lanes 1, 4, 6, and 9). Each serum reacted specifically with the corresponding molecular weight class of proteins used as immunogen (Fig. 1*b*, lanes 2, 3, 5, 7, and 8). The anti-A_2 serum precipitated additional antigens of slightly higher molecular weight (Fig. 1*b*, lane 3, dots), which we believe are chorion components (see *Discussion*). C_1 protein was precipitated specifically by the anti-C serum (Fig. 1*b*, lane 8), but the antigenicity of C_2 cannot be investigated adequately because of its apparent insolubility: C_2 is largely precipitated by preliminary treatment of our samples with preimmune serum (data not shown) and is also found as a minor contaminant in all specific immunoprecipitates.

Putative Precursors of Chorion Proteins Among the Cell-Free Translation Products of Ovarian RNA. We have used a mixture of the class-specific chorion antisera to detect chorion components among the cell-free translation products of follicular RNA. This has permitted us to identify precursors of chorion proteins.

As expected, a mixture of the antisera precipitated specifically the authentic As, Bs, and C_1 (Fig. 1*b*, lane 10; Fig. 2*a*, lane 1). When mixed with [^{3}H]proline-labeled cell-free translation products of total ovarian RNA in a wheat germ system (Fig. 2*a*, lane 2), the antiserum mixture precipitated a limited number of proteins (Fig. 2*a*, lane 3). However, these proteins did not comigrate exactly with any of the authentic chorion proteins, presumably because they are precursors.

The nature of the immunoprecipitable cell-free products was confirmed by studies of tissue specificity. Choriogenic follicles were selected by size from dispersed ovaries, and follicular epithelial cell contents (as opposed to oocyte contents) were

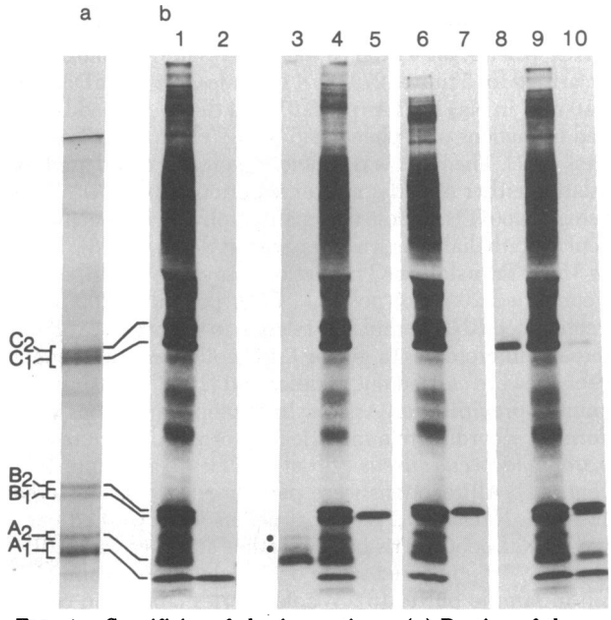

FIG. 1. Specificity of chorion antisera. (*a*) Portion of the preparative $NaDodSO_4$/polyacrylamide gel used for production of immunogens, showing the six major molecular weight classes of chorion proteins and the regions excised for immunogen preparation (brackets). (*b*) Lanes 1, 4, 6, and 9 show the pattern of labeled ovarian proteins used in immunoprecipitation reactions. Remaining lanes show immunoprecipitates of this preparation reacted with serum produced in response to specific gel bands A_1 (lane 2), A_2 (lane 3), B_1 (lane 5), B_2 (lane 7), and C_1 plus C_2 (lane 8). Lane 10 shows the immunoprecipitate obtained with a mixture of the above sera.

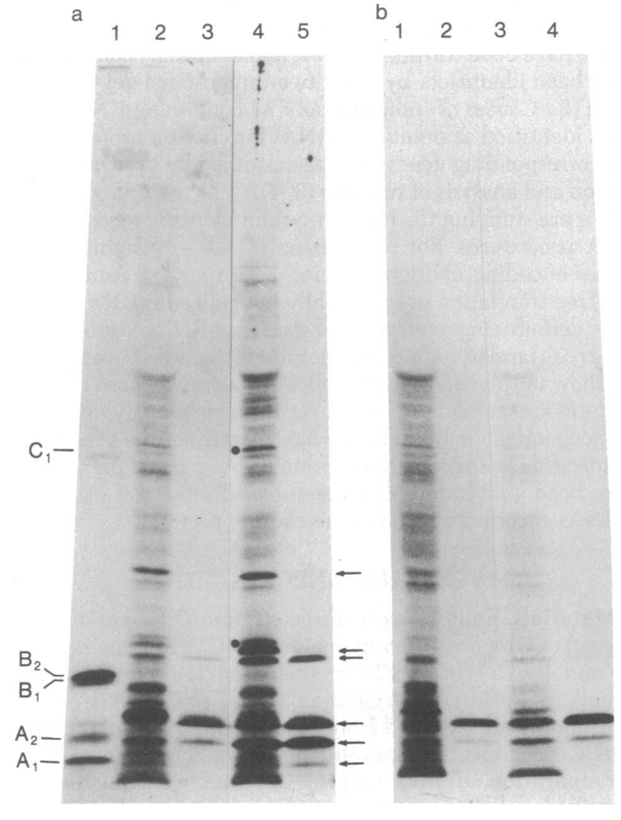

FIG. 2. Tissue-specific enrichment of chorion mRNAs. (*a*) Cell-free translation products, labeled with [^{3}H]proline, of RNA isolated from whole ovaries (lane 2) or from follicular epithelial cells derived from chloriogenic follicles (lane 4). The corresponding immunoprecipitates, produced by the antiserum mixture, are displayed in lanes 3 and 5; a reference immunoprecipitate from authentic ovarian proteins is displayed in lane 1. Arrows indicate immunoprecipitable cell-free products; dots indicate other tissue-enriched cell-free products. (*b*) Cell-free translation products labeled with [^{3}H]-leucine; lanes 1–4 correspond to lanes 2–5 in *a*.

obtained by Triton X-100 lysis after collagenase treatment. Sequences encoding the immunoprecipitable products were highly enriched in the follicular epithelial cells of choriogenic follicles compared to total ovaries (Fig. 2*a*, lanes 4 and 5 vs. lanes 2 and 3, respectively).

A second confirmation of the nature of the immunoprecipitable cell-free products was obtained from their apparent amino acid content. The chorion proteins of *Drosophila* are enriched in proline but not in leucine (1). Translations were repeated by using the same RNA preparations but with [^{3}H]-leucine rather than [^{3}H]proline. In this case, most immunoprecipitable products were much less prominent (Fig. 2*b*, lanes 1–4; see Fig. 2*a*, lanes 2–5).

Although RNA isolated from follicular epithelial cells through collagenase treatment shows the most enrichment, total RNA isolated from choriogenic follicles is also somewhat enriched (data not shown) and is obtained in better yields. Identical patterns of immunoprecipitable products are obtained by translation of total and poly(A)-containing RNA fractions (data not shown). Therefore, for subsequent translations we used poly(A)$^+$ RNA from choriogenic follicles. Proline was used as the label to obtain maximum incorporation into chorion-specific products. Precursors were identified with the prefix p.

Identification of Putative Precursors for Individual Chorion Protein Classes. Fig. 3 shows that, by using individual chorion class-specific antisera, we have identified the precursor forms of four major chorion protein classes. The A_1, A_2, and B_2 precursors (lanes 3, 5, and 10, respectively) show comparable shifts to decreased mobility relative to the corresponding authentic proteins (lanes 2, 6, and 11, respectively). The B_1 precursor (lane 8) shows a more extensive mobility shift, migrating slower than the B_2 precursor (lane 10), even though the authentic B_1 protein (lane 7) migrated faster than authentic B_2 (lane 11). In addition, a single band of considerably slower mobility was precipitated by B_2-specific, and to a lesser extent by B_1-specific, antiserum (component pX in Fig. 3; lanes 8, 10, and 13). This band shows the tissue and amino acid specificities expected of chorion components (Fig. 2) and is thought to be the precursor of a chorion protein not readily identified in authentic chorion protein samples (see *Discussion*). The precursor forms of A_1 and A_2 proteins show structural similarity, as indicated by the precipitation of pA_1 by anti-A_2 antiserum (Fig. 3, lane 5).

We have been unable to identify convincingly precursors of class C proteins for various reasons. Although some tissue-specific, proline-rich components are evident among the cell-free products in the C region (e.g., upper dot in Fig. 2*a*, lane 4), these are neither as efficiently immunoprecipitated nor as prominent as might be expected from the abundance of C proteins in *in vivo*-labeled ovarian protein samples. It may be either that the class C mRNAs are not efficiently translated in the wheat germ system, or that the class C precursors are not very crossreactive with an antiserum directed against the corresponding authentic proteins, or both.

Membrane-Dependent Processing of Chorion Precursors. Precursors of nearly all secretory proteins are known to contain an amino-terminal "signal peptide" that is cleaved off as the nascent protein is transported across the membrane of the endoplasmic reticulum (15). By using a wheat germ system supplemented with microsomal membranes from dog pancreas, we have been able to process *in vitro* the chorion precursors. This suggests that the reduced mobility results from the presence of uncleaved signal peptides in the precursors.

Translation of follicular RNA in the presence of membranes (Fig. 4*a*, lane 2) resulted in the disappearance of the precursor bands identified previously in the absence of membranes (Fig.

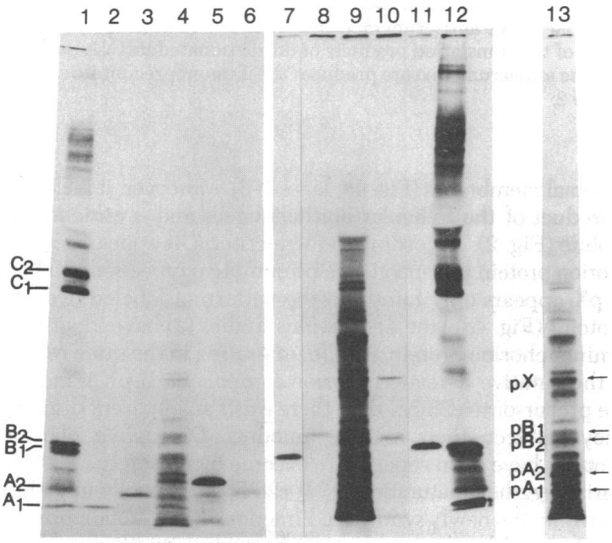

FIG. 3. Immunoprecipitation of cell-free translation products with specific sera. [^{3}H]Proline-labeled translation products of poly(A)-containing RNA from choriogenic follicles (lanes 4, 9, and 13) were immunoprecipitated with anti-A_1 (lane 3), anti-A_2 (lane 5), anti-B_1 (lane 8), and anti-B_2 (lane 10) antisera. Immunoprecipitation of labeled ovarian proteins (lanes 1 and 12) with the same antisera is shown in lanes 2, 6, 7, and 11, respectively. The positions of all identified chorion precursors among the translation products are indicated in lane 13.

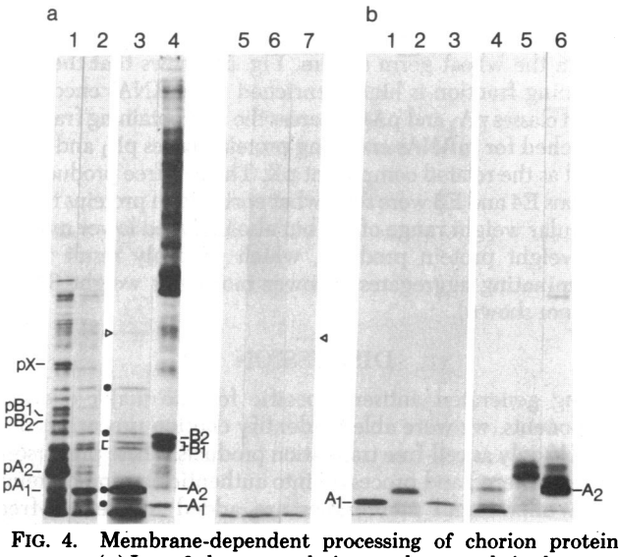

FIG. 4. Membrane-dependent processing of chorion protein precursors. (*a*) Lane 2 shows translation products made in the presence of membranes compared to the products made in their absence (lane 1). Lane 3 shows the resistant fraction of the membrane-supplemented translation after treatment with proteolytic enzymes. Controls show the effect of proteolysis on the translation reaction without membranes (lane 5) and on the membrane-supplemented translation in the presence of Triton X-100 (lane 6). Lane 4 shows the pattern of labeled ovarian proteins. The membrane preparation contains endogenous mRNA that produces a single translation product indicated in lane 7 and also present in lane 3. (*b*) Immunoprecipitates of products shown in *a*, lane 3, with anti-A_1 antiserum (lane 3) and anti-A_2 antiserum (lane 4) comigrate with immunoprecipitated authentic A_1 (lane 1) and authentic A_2 (lane 6), respectively. The immunoprecipitated precursors of A_1 (lane 2) and A_2 (lane 5) are also included.

4a, lane 1); thus, the precursors appear to be efficiently processed. Only a limited number of proteins synthesized in the presence of membranes were specifically protected from posttranslational proteolysis (Fig. 4a, lane 3), indicating that they are sequestered within the microsomal vesicles. Sequestration is confirmed by the observation that sensitivity to proteolysis (Fig. 4a, lane 5) is restored by Triton X-100, which is known to disrupt membranes (Fig. 4a, lane 6). The protected products comigrated with authentic chorion proteins, with the exception of a single band that migrated to a position between that of pX and the Bs, presumably the processed form of pX (see *Discussion*). In the absence of immunoglobulin proteins, the B_1 region resolved into two components (Fig. 4a, lane 4).* Likewise, the pattern of the membrane-protected products also shows two bands in this region (Fig. 4a, lane 3). Comigration of membrane-protected products with authentic As is confirmed by their immunoprecipitation with anti-A_1 antiserum (Fig. 4b, lanes 1 and 3) and anti-A_2 antiserum (Fig. 4b, lanes 4 and 6). As expected, the membrane-protected B proteins are also precipitated by individual class B-specific antisera (data not shown).

Size Identification of mRNAs Encoding A and B Classes of Chorion Proteins. Spradling and Mahowald (3) have identified as putative chorion mRNAs certain *in vivo*-labeled poly(A)-containing RNAs that show the expected developmental specificity and are enriched in the follicular epithelial cells. We have identified these same RNAs after their extraction from choriogenic follicles labeled in organ culture with [^{3}H]-uridine (Fig. 5a, lane 1, E3–E6). The pattern of the poly(A)$^-$ fraction of the RNA is also included to provide a reference for band identification (Fig. 5a, lane 2).

Poly(A)$^+$ RNA from choriogenic follicles labeled in organ culture with [^{32}P]H_3PO_4 was mixed with an excess of cold RNA of similar origin and electrophoresed on a polyacrylamide gel. Gel regions were excised, and the RNA was eluted and translated in the wheat germ system. Fig. 5b shows that the E6-containing fraction is highly enriched for mRNAs encoding protein classes pA_1 and pA_2, whereas the E5-containing fraction is enriched for mRNAs encoding protein classes pB_1 and pB_2, as well as the related component pX. The cell-free products of fractions E4 and E3 were somewhat enriched in proteins in the molecular weight range of Cs, but also included lower molecular weight protein products, which probably result from contaminating aggregates of lower molecular weight RNAs (data not shown).

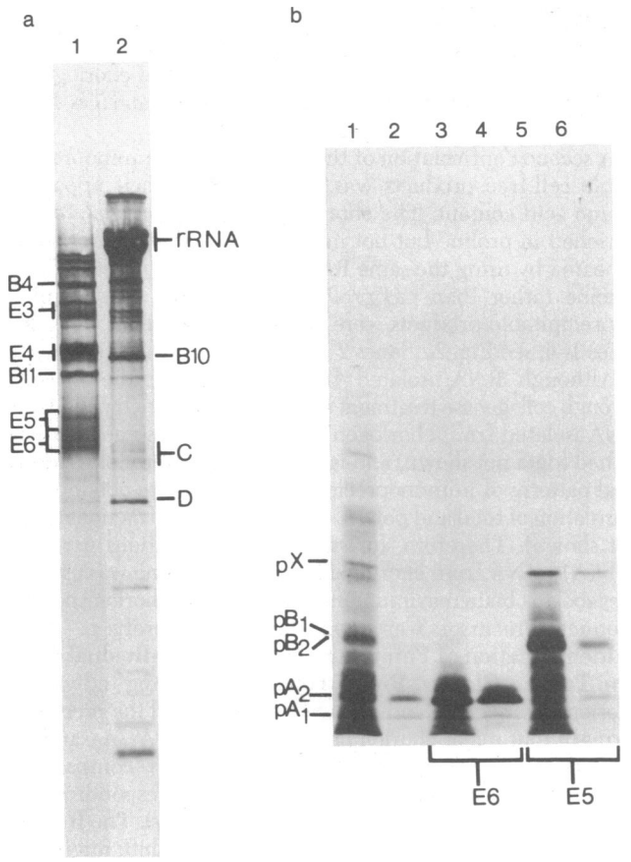

FIG. 5. Characterization of size classes of chorion mRNAs. (a) Lane 1 shows the pattern of the poly(A)$^+$ fraction, and lane 2 shows that of the poly(A)$^-$ fraction of [^{3}H]uridine-labeled RNA from choriogenic follicles. The B RNA classes correspond to mitochondrial species; the C region contains the presumptive histone mRNAs; D is an unknown intensively labeled species; and E classes have been identified as putative chorion mRNAs (3, 12). (b) The translation products of mRNA eluted from E6 and E5 regions are shown in lanes 3 and 5, respectively, and their corresponding immunoprecipitates, produced by the antiserum mixture, are shown in lanes 4 and 6. Reaction of the translation products of unfractionated mRNA (lane 1) with the antiserum mixture produces the immunoprecipitate shown in lane 2.

DISCUSSION

Having generated antisera specific for the major chorion components, we were able to identify chorion precursors detectable only as cell-free translation products. These precursors are sequestered and processed into authentic chorion proteins when microsomal membranes are added to the cell-free translation system—i.e., they show the behavior predicted for secretory proteins by the "signal" hypothesis (15).

In addition to the major precursor proteins that are shifted to slightly higher apparent molecular weights relative to the authentic proteins, A_1, A_2, B_1, and B_2, some antisera precipitate additional cell-free products. The anti-B_1 antiserum and, especially, the anti-B_2 antiserum precipitate a single component, pX, that has a mobility much lower than that of the B_1 and B_2 authentic proteins and their respective precursors. Component pX is the precursor of a secretory protein, because it is sequestered and processed to lower molecular weight by microsomal membranes (Fig. 4a, lanes 1–3). Moreover, it is clearly a product of the follicular epithelial cells and is enriched in proline (Fig. 2). According to these criteria, it appears to be a chorion protein precursor. The presumptive processed product of pX appears only faintly in the pattern of labeled ovarian proteins (Fig. 4a, lane 4). Previous studies (2) have identified a minor chorion protein, c28-58, migrating in the same region as the putative X-related chorion component. If pX is indeed the precursor to c28-58, then there exists an apparent discrepancy between their relative amounts. *Drosophila* chorion proteins have been reported to become increasingly insoluble during eggshell maturation (1). If c28-58 is unusually insoluble, then only the newly synthesized fraction of this protein present *in vivo* will be detectable. Alternatively, c28-58 may show typical solubility, but its mRNA may be more efficiently translated *in vitro* than *in vivo*. The c28-58 protein migrates well above the region of the B proteins, which argues against its having been included in the original immunogen preparation. The crossreactivity of pX with B_1 and B_2 classes suggests that distinct chorion proteins might be structurally related, perhaps being encoded by multigene families as are the chorion

* Also L. H. Margaritis, F. C. Kafatos, and W. H. Petri, unpublished data.

proteins of silkmoths (16). A similar indication may be the apparent crossreactivity of pA_1 with anti-A_2 antiserum. We have also observed substantial immunological crossreactivity within and even between protein size classes (A and B) under conditions of great antibody excess (data not shown).

The anti-A_2 antiserum precipitates a single major, and one or more minor, components from both authentic proteins and cell-free products (e.g., Fig. 4*b*, lanes 5 and 6). At least one of the minor components is secretory, because it is sequestered and processed by microsomal membranes (Fig. 4*b*, lane 4). A minor authentic A_2 component, c16-35, has been defined unambiguously by two-dimensional gel electrophoresis (2). The minor components may be immunoprecipitable because of the presence of the corresponding proteins in the A_2 immunogen, although the possibility of structural similarities between major and minor A_2 components resulting in their crossreactivity cannot be dismissed.

Under certain conditions, it has been reported that cultured follicles synthesize heat-shock mRNAs (4). However, it has been shown that Robb's tissue culture medium supports normal follicular development from stage 10 to stage 14 with kinetics very similar to those *in vivo* (17). Using this medium we have reproduced *in vitro* the *in vivo* labeling pattern of putative chorion mRNAs (3), without appreciable labeling of material above the rRNA region (19S) where major heat-shock mRNAs are known to migrate (12). Likewise, we have not detected major heat-shock protein synthesis in protein-labeling experiments. Thus, we believe that the *Drosophila* chorion system can be studied *in vitro*, as well as *in vivo* (3).

The immunological identification of chorion precursors made it possible for us to determine directly the size classes of mRNA that encode the A and B molecular weight classes of chorion proteins. These RNA size classes correspond to zones E5 and E6 as described by Spradling and Mahowald (3). Our findings indicate that E6 encodes both A_1 and A_2 chorion proteins, whereas E5 encodes both B_1 and B_2 proteins and the related component X. We have been unable to identify conclusively the proteins encoded by E3 and E4 RNAs, but our preliminary data are consistent with the interpretation that these RNAs encode class C proteins (3, 4).

The *ocelliless* mutation, which reduces production of C and B class proteins, also reduces the abundance of E3, E4, and E5 RNAs (4). Our data indicate that the reduced synthesis of B proteins results from the reduced level of E5 RNA. E3 and E4 RNAs hybridize *in situ* to chromosome region 7E11 in the vicinity of the *ocelliless* locus, and C protein electrophoretic variants map in the same region (4). *In situ* chromosomal mapping of E5 RNA should reveal whether class B as well as class C genes are clustered, and more specifically, whether all genes affected by *ocelliless* are clustered in its vicinity.

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