Hybridization-selected translation of Bombyx mori high-cysteine chorion proteins in Xenopus laevis oocytes

(recombinant DNA/multigene families/diazobenzyloxyethylcellulose/specific mRNA purification)

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ABSTRACT Xenopus laevis oocytes were injected with poly(A)+-mRNA isolated from chorionating follicular epithelium of the domesticated silk moth (Bombyx mori). On two-dimensional gel electrophoresis, the resultant translation products comigrated with authentic, secreted, chorion standards, demonstrating that the frog oocyte system synthesizes and correctly processes virtually all major chorion components. A cDNA clone has been shown to contain sequences complementary to those of mRNAs encoding B. mori high-cysteine (He) chorion proteins He6-He11. mRNAs were selected by hybridization to plasmid m5000 DNA bound to diazo-benzyloxyethyl-cellulose and subsequently translated in X. laevis oocytes into forms that comigrated with authentic chorion standards. The selection of a distinct subset of He mRNAs under stringent hybridization conditions (70% formamide/0.2 M NaCl, 60°C) suggests that they are encoded by related genes. This is consistent with the pattern obtained by hybridizing radioactive m5000 DNA to Southern blots prepared from EcoRI-cleaved B. mori chromosomes.

The Bombyx mori chorion genes constitute an excellent model system for studying the control of gene expression during development. Late in oogenesis, the follicular epithelium surrounding each silk moth oocyte synthesizes about 60 well-defined structural proteins according to a complex temporal program (ref. 1; unpublished data). The chorion proteins have been divided into four major classes, designated A, B, C, and Hc (high cysteine), on the basis of their size and amino acid composition (2). Members of each class appear to be the products of related multigene families (3-7) and have characteristic periods of synthesis during choriogenesis (8, 9).

Although it has been shown that the accumulation of chorion mRNAs approximately parallels their translation (4, 10, 11), elucidation of the molecular mechanisms that regulate chorion gene expression will require a more detailed examination of the transcription, processing, and utilization of individual chorion messages. In vitro translation, immunoprecipitation, and peptide mapping techniques have been utilized frequently for the identification of mRNA and structural protein products encoded by cloned DNA sequences. Unfortunately, such methods are of limited utility in the chorion system because these proteins have evolved from several common ancestral genes and thus exhibit a high degree of structural similarity and amino acid sequence homology (6, 12, 13). The characterization of individual chorion mRNAs has been further complicated by the presence of NH2-terminal signal peptides on chorion precursors synthesized by conventional cell-free translation systems (11).

Xenopus laevis oocytes are capable of performing a wide variety of modifications on proteins synthesized from heterologous, microinjected mRNAs. These modifications include signal peptide (14) and post-translational cleavage (15), acetylation (16), and glycosylation (17). Moreover, the use of frog oocytes greatly enhances the sensitivity of experiments using hybridization-selected messages because these cells translate nanogram quantities of mRNA efficiently (18). We have circumvented the problems of chorion protein multiplicity and processing by utilizing microinjected X. laevis oocytes to translate chorion mRNAs and by developing a high-resolution two-dimensional gel electrophoresis system that resolves individual chorion components (2).

Our initial experiment with total mRNA has demonstrated that frog oocytes can translate microinjected follicular RNA encoding chorion proteins and correctly process the primary translation products to forms that comigrate with authentic chorion standards separated in the same two-dimensional gel. Subsequent microinjections with purified chorion mRNAs have established the desirability of the frog oocyte system for identifying protein products of specific chorion messages and for characterizing cloned DNA fragments thought to encode chorion proteins. Specifically, mRNA isolated by hybridization-selection to plasmid m5000 DNA immobilized on diazo-benzyloxyethyl-cellulose (DBM-cellulose) was shown to encode a subset of the Hc proteins He6-Hc11.

METHODOLOGY

Isolation of Follicular poly(A)+ mRNA. Strain C108 female pupae were dissected when air appeared in their galeae (stage 13). The chorionic section of each ovariole was removed in Grace’s tissue culture medium (GIBCO) and frozen in liquid nitrogen.

Follicular RNA was prepared according to the Glisin procedure (19), and poly(A)+ RNA was subsequently isolated on oligo(dT)-cellulose (Collaborative Research, Waltham, MA). Follicles pooled from 25–35 animals typically yielded 500–700 μg of poly(A)+ RNA, or 7–15% of the A450 applied to oligo(dT)-cellulose. The A260/A280 ratio for these poly(A)+ RNA preparations ranged from 1.6 to 2.0.

Translation of Follicular poly(A)+ RNA in X. laevis Oocytes. X. laevis adult females were obtained from the South African Snake Farm (Fish Hoek, Cape Province, South Africa) or from Nasco Biological Science (Fort Atkinson, WI). Some were in-
ected with pregnant horse serum 1 week before their oocytes were used.

Stage V and VI oocytes (20), from which the surface epithelium and theca layers had been removed by manual dissection, were injected (21) with 50–60 nl of poly(A)+RNA at 1 µg/µl or less in water. Control oocytes were injected with water. Six to 10 oocytes were injected with each RNA sample and cultured together in a half-dram vial containing modified Ringer’s solution (100 mM NaCl/2 mM Na phosphate, pH 7.7–1.8 mM KCl/2 mM CaCl2/1 mM MgCl2) supplemented with 50 µg of gentamycin and 500 µg of bovine gamma globulin per ml, 1 mM Na pyruvate, and 120–150 µCi (1 Ci = 3.7 × 1010 becquerels) each of [3H]glycine (20 Ci/mmol), [3H]leucine (136 Ci/mmol), and [3H]valine (39 Ci/mmol) per ml. The oocytes were incubated in this medium for 24 hr at room temperature and then removed from the liquid and quick-frozen in a dry ice/ethanol mixture.

Electrophoresis. Thirty microliters of solubilization buffer (8 M urea/50 mM Tris-HCl, pH 9.0/1.5 mM lystine/1.5 mM EDTA/70 mM dithiothreitol) was added to each sample of four X. laevis oocytes. The oocytes were homogenized in a 1.5-ml microcentrifuge tube by using a fire-polished glass rod and then carboxamidomethylated (22). The resultant sample was not homogeneous in appearance and contained some particulate matter and lipid droplets. However, upon subsequent electrophoretic analysis, chorion components appeared to be fully solubilized and carboxamidomethylated.

Two-dimensional electrophoresis was performed as described (23). Each gel received a sample corresponding to four X. laevis oocytes.

Radioactively labeled strain C108 chorion proteins were prepared by reduction with [14C]iodoacetamide (53 mCi/mmole) as described (1). To detect radioactivity, gels were treated with ENHANCE (New England Nuclear) and preflashed (24) x-ray film (XR, Kodak) was used.

Hybridization-Selection of Specific Chorion mRNAs with Cloned cDNAs. The two cDNA clones used in this work were generously provided by Kostas Iatrou. They were synthesized from poly(A)-containing chorion mRNA from strain 703 wild-type follicles as described (25). The plasmids were propagated under conditions set forth in the National Institutes of Health guidelines for recombinant DNA research. Supercoiled DNA's were purified from "cleared lysates" (26) of chloramphenicol-amplified transformants by acid/phenol extraction (27). Plasmid DNA was bound to DBM-cellulose according to the procedure of Noyes and Stark (28). HindIII- or BamHI-linearized plasmid DNA (0.5–10.0 µg) was added to a binding reaction mixture containing approximately 25 mg (dry weight) of DBM-cellulose. After 30–48 hr, the cellulose was washed twice for 5 min each in 80% dimethyl sulfoxide/20 mM sodium phosphate buffer (PB) at 50°C, four times for 2 min each in 15 mM NaCl/15 mM Na citrate at room temperature, twice for 1 min each in water at 100°C, three times for 10 min each in 99% deionized formamide/10 mM PB at 60°C, and three times for 5 min each in hybridization buffer at 60°C.

Hybridizations were performed in a minimal volume (<100 µl) of 70% deionized formamide/100 mM Na Pipes, pH 6.4/1 mM EDTA/0.1% NaDodSO4 containing 0.2, 0.4, or 0.6 M NaCl. The DNA-DBM-cellulose was incubated for 2–8 hr at 60°C in hybridization buffer containing 100 µg poly(rA) per ml. Hybridization followed for no more than 2 hr at 60°C in a minimal volume (50–100 µl) of hybridization buffer containing 100 µg of poly(rA) and 200 µg of follicular poly(A)+RNA per ml. After hybridization, the supernate was removed and the DNA-DBM-cellulose was washed twice with 0.3 M NaCl/0.3 M Na citrate/0.1% NaDodSO4 at room temperature, twice for 5 min with hybridization buffer [without poly(rA) and mRNA] at 60°C, and twice with water at room temperature. Complementary RNA was eluted from the DNA-DBM-cellulose during two 1-min incubations in water at 100°C. Before precipitation with ethanol, contaminating particulate cellulose was removed from the washes and eluted RNA samples by centrifugation. Each sample was dissolved in a minimal volume of water (5 µl or less) and heated to 60°C for 1 min before microinjection into X. laevis oocytes.

RESULTS

Translation of Follicular poly(A)+RNA in X. laevis Oocytes. Translation of poly(A)+RNA isolated from pooled chorionating follicles in wheat germ lysates results in products whose molecular weights are significantly higher than those of authentic chorion proteins. Even though zones corresponding to A and B precursor proteins are recognizable in both NaDodSO4/urea (29) and two-dimensional gels (9), the patterns are sufficiently altered so that it is not possible to correlate individual bands or spots unequivocally with their native counterparts. Thus, additional processing of products is necessary before hybridization-selected translation can be used to identify the proteins encoded by purified chorion mRNAs.

The X. laevis oocyte system was chosen for further translation studies because it is capable of many kinds of protein processing and can utilize trace amounts of message with great efficiency.

Stage V and VI frog oocytes were mock-injected with water and cultured in the presence of labeled amino acids to determine the extent of endogenous protein synthesis. Only a small fraction of the proteins labeled under these conditions migrated in the low molecular weight, acidic region of our two-dimensional gel system where the majority of chorion proteins are found (Fig. 1A).

Upon injection with follicular poly(A)+RNA, X. laevis oocytes translated and correctly processed chorion proteins to forms that comigrated with unlabeled authentic chorion standards separated in the same gel (Fig. 1B). Of the 66 components detectable in [3H]carboxamidomethylated authentic chorion (Fig. 1C), 49 were positively identified among the proteins synthesized by frog oocytes injected with silk moth chorion RNA, establishing the utility of this preparation for elucidating the kinds of sequences present on cloned chorion DNAs. Of the remaining 17, 16 were minor constituents that are difficult to detect even in pure chorion. The X. laevis translation product apparently corresponding to the last protein, C3, exhibited an altered mobility (see Figs. 1 and 3).

Identification of C105 Chorion Proteins Encoded by cDNA Plasmid m5000. Plasmid m5000 is a cDNA clone from the B. mori strain 703 follicular poly(A)+ cDNA library constructed by Iatrou et al. (25). Our laboratory elected to work with a different wild-type strain, C108, because its simpler two-dimensional chorion protein pattern and excellent growth characteristics were expected to make it superior for developmental and genetic studies. However, strains 703 and C108 are closely enough related that cDNA clones from strain 703 hybridize specifically with the related mRNAs from strain C108 (9). Hybridization-selected translation experiments were undertaken to identify the chorion protein(s) whose mRNA(s) are homologous to sequences in plasmid m5000. We were particularly interested in m5000 because in Northern blots it hybridized to follicular RNA present during the very late period of choriogenesis that exactly corresponds to the interval during which a unique group of chorion components, the Hc proteins, are synthesized (9). These proteins constitute an extremely interesting family of chorion components due to their unusual amino acid composition and their distinctive pattern
of synthesis during development (2). As a class they are produced in the last stages of oogenesis and are utilized in the final maturation of the chorion, probably for crosslinking and for forming the outer layers of the eggshell (2, 30).

We also utilized a second cloned CDNA, plasmid m2774, in parallel experiments. This clone has been reported to encode a chorion protein of the A class (25) which differs markedly from the Hc proteins in relative amino acid composition and molecular weight (2). We have found that in reverse Southern blots it hybridizes to mRNA that accumulates in the middle period of choriogenesis. This corresponds to a period when A proteins are synthesized but, because of the homology of A and B proteins (4, 5, 13), we expected it to select mRNAs of both the A and B classes in our system. Nevertheless, we used it as a control for the m5000 hybrid selections.

Plasmid DNA was coupled to DBM-cellulose and hybridized to poly(A)+ RNA from choriogenic follicles under conditions of high stringency. The cellulose was washed extensively, and then specifically bound RNA was eluted in water at 100°C. This eluate as well as representative samples from earlier washes were injected into X. laevis oocytes for translation and protein modification in the presence of tritiated amino acids known to be abundant in B. mori chorion (2). Excess nonradioactive chorion standard was added to homogenates of the frog eggs prior to two-dimensional gel electrophoresis and fluorography of each sample.

Under these conditions, six Hc proteins, Hc6–Hc11, were specifically translated, confirming that plasmid m5000 encodes a protein of the Hc class (Figs. 2C and 3B). Hc6–Hc11 clearly were not synthesized in the endogenous sample (Figs. 2A and 3A). Plasmid m2774 failed to select mRNA for these Hc proteins (Figs. 2D and 3C) and instead selected mRNA for two proteins, B6 and B18, which are not seen at all in m5000 gels. At least two others, A9 and B4/5, were differentially translated—that is, their expression was 10 times greater with m2774 selection. A fifth protein spot detectable with the m2774 selection (Fig. 2D, arrow) also was found as a faint product in the total poly(A)+ translation (Fig. 2B, arrow). However, it was not detectable in the authentic chorion protein gel. This spot may represent a protein that is translated but not properly processed in the oocyte or a protein whose RNA is in the follicle but is not significantly associated with the chorion.

Low levels of synthesis of several other proteins—notably Hc3, A1, A3, and A4—were observed in both m5000 (Fig. 2C) and m2774 (Fig. 2D) selected reactions. This observation and the low-level selection of B4/5 and A9 by m5000 may indicate that these proteins have partial sequence homology with the cloned probes. On the other hand, in control experiments performed under identical hybridization conditions the mRNAs for these proteins bound to roughly the same extent when a filter containing the pMB9 vector DNA alone was used. Thus, the background appearance of these proteins may simply reflect a higher level of nonspecific binding of their messages to DBM-cellulose or weak homology with pMB9 DNA.

**DISCUSSION**

The use of CDNA clones as probes in developmental studies requires the knowledge of which proteins are encoded by those clones. In the past, this kind of identification depended heavily upon in vitro translation experiments (31–34). Wheat germ and reticulocyte lysates have been used successfully to identify the template specificities of mRNAs arrested or selected by hybridization to cloned DNAs when the products of cell-free synthesis did not require cotranslational or post-translational modification.

However, as in many other secretory systems, study of silk moth chorion proteins is complicated by protein processing steps. Newy synthesized chorion proteins appear to have NH2-terminal signal peptides (11, 29) that must be cleaved prior to their transport through the endoplasmic reticulum, Golgi apparatus, and secretory vesicles (35). Moreover, after removal of the signal peptide, certain chorion proteins of the wild silk moth *Antheraea polyphemus* are known to undergo charge modification by cyclization of an NH2-terminal glutamine (36). Thireos and Kafatos (11) used a membrane fraction from dog pancreas to process chorion proteins synthesized by wheat germ extracts. To date, the processed products have been resolved only on NaDodSO4 gels which separate major classes of chorion proteins but not individual species. The comigration of proteins produced by *Xenopus* oocytes with mature chorion components extracted from laid eggshells presented here demonstrates that the oocyte translates and processes all classes of chorion proteins. This occurs with great sensitivity and in the absence of added protein fractions from either homologous or heterologous sources.

The poly(A)+ RNA preparation used in these studies was obtained from a whole-follicle preparation that included diverse cell types which could contribute various nonchorion mRNAs.
Probable nonfollicular epithelial contaminants include fat body and oocytes and, to a lesser extent, nurse cells, which largely have degenerated by this state of oogenesis (37). In particular, the existence of masked messengers in other early development systems suggests that similar preformed but inactive templates might be isolated from preparations of whole follicles (38, 39). However, careful evaluation of the proteins produced after injection of follicular poly(A) mRNA into *X. laevis* oocytes did not reveal any nonfrog, nonchorion proteins. The assay may be somewhat biased due to the design of the two-dimensional gel system which resolves acidic, low molecular weight chorion components optimally but has decreased sensitivity for other types of proteins. Nevertheless, the results indicate an extremely high enrichment for chorion mRNA and a notable lack of contamination by other messages.

The population of chorion proteins synthesized by frog oocytes accurately reflects the natural distribution of components found in authentic chorion, with the single exception of protein C3. No spot was observed at the coordinates of authentic C3 protein (Figs. 1C and 3E) in the two-dimensional gel pattern from *X. laevis* oocytes injected with total follicular poly(A) mRNA (Figs. 1B and 3D). However, it is possible that a protein of slightly higher molecular weight and more basic pI than authentic C3 (Figs. 1B and 3D) may represent an unprocessed, incompletely processed, or incorrectly processed form of the *X. laevis*-synthesized C3 product. This protein migrated in the

**FIG. 2.** Autoradiographs of two-dimensional gels from oocytes injected with water (A), total follicular poly(A) mRNA (B), m5000-specific mRNA (C), and m2774-specific mRNA (D), showing translation of purified chorion mRNAs in *X. laevis* oocytes. Specific mRNAs were isolated by hybridization-selection to plasmid m5000 DNA immobilized on DBM-cellulose. This RNA was subsequently microinjected into *X. laevis* oocytes, which were cultured, homogenized, and electrophoresed. The small box encloses the region of the two-dimensional profile where chorion proteins Hc6–Hc11 migrate (see Fig. 1). Numbered proteins are referred to in the text. Arrows in B and D indicate proteins not detected in authentic chorion.

**FIG. 3.** Translation of purified chorion mRNAs in *X. laevis* oocytes. Autoradiographs of the Hc6–Hc11 region of two-dimensional gels prepared from *X. laevis* oocytes injected with water (A) or with purified chorion mRNAs isolated from follicular poly(A) mRNA by hybridization-selection with plasmid m5000 DNA (B) or plasmid m2774 DNA (C) or total follicular poly(A) mRNA (D). (E) Proteins Hc6–Hc11 from [14C]carboxamidomethylated, laid eggshells. The arrow in E indicates authentic C3 protein. The arrow in D marks putative *X. laevis*-synthesized C3 product. Note the absence of this spot in B which shows proteins synthesized from m5000 hybridization-selected mRNA.
vicinity of the Hc6–Hc11 proteins (Figs. 3 A and D), but its mRNA was not recovered in hybridization-selected experiments with m5000 plasmid (Fig. 3B). If the assignment is correct, translation of mRNA preparations from young follicles in X. laevis oocytes should result in enrichment of the putative C3 spot because C proteins are synthesized during the earliest period of chorionogenesis (unpublished data).

The hybridization of mRNA(s) for several Hc proteins to cDNA clone m5000 could indicate that members of this group of proteins result from modification of a single precursor which is encoded in a single structural gene or that the proteins are encoded by separate genes which are homologous to each other. We have found that a number of genomic DNA fragments digested with EcoRI from strain C108 hybridize to 32P-labeled nick-translated m5000 DNA in Southern blots (data not shown), suggesting that Hc6–Hc11 are encoded by different but partially homologous structural genes. Eickbush et al. (40) recently reported the isolation of several related chorion genes from B. mori strain 703 by using a cloned cDNA probe that is homologous to m5000 (T. Eickbush, personal communication).

Hc6–Hc11 comprise less than half of the 15 proteins in the Hc family of strain C108. They appear to represent a distinct chorion subfamily based on their electrophoretic mobility and nucleic acid homology. They are found in a localized region of the two-dimensional gel pattern, emphasizing their physical similarity to each other and a dissimilarity with the remaining Hc proteins which are smaller and more basic in pl. Structural features underlying these electrophoretic mobility differences may reflect unidentified differences in the functional roles of Hc proteins. The hybridization-selection experiments reported here indicate that, in addition to differences in physical properties, subgroups of Hc proteins also differ in nucleotide sequence and may thus represent evolutionary subfamilies such as those found among the class A and B chorion proteins (3–7, 12, 13).

Analysis of synthetic profiles for individual chorion proteins has demonstrated that, whereas all members of the Hc protein group are synthesized during the very late period of chorionogenesis, individuals in the Hc6–Hc11 subset are coordinately expressed at the 2.5-hr temporal resolution separating adjacent follicles of a developing ovarioly (ref. 9; unpublished data). The identification of a cloned probe for Hc6–Hc11 sequences makes it possible to study the regulatory events underlying the tightly coordinated expression of these genes and to investigate their organization and fine structure. To these ends, we have recently used m5000 as a hybridization probe to show that accumulation of RNAs encoding these coordinately expressed proteins is tightly coupled to their translation (9). We have also isolated a genomic clone complementary to m5000 from a B. mori strain C108 genomic DNA library.

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