Maternal messenger RNA distribution in silkmoth eggs

I. Clone Ec4B is associated with the cortical cytoskeleton

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Summary

We have constructed a cDNA library from mature egg RNA of the silkmoth, Hyalophora cecropia. Differential screening of the library using cDNA made against mRNAs from the yolky cytoplasm (soluble fraction) and the cortical cytoplasm (cytoskeletal-associated or cortical fraction) resulted in several clones that hybridized to a higher degree to the cDNA from the cytoskeletal-associated fraction. We selected and analyzed the clone giving the strongest signal (designated Ec4b) for its distribution in situ and found that it bound to mRNAs in the nurse cell cytoplasm, in the cortex and in the follicle cells of oocytes. Hybridization of the insert from Ec4b to both detergent-soluble and -insoluble (cortical) RNA on dot blots further supported the observation that the mRNA corresponding to Ec4b was enriched in this cytoskeletal fraction. The mRNA for Ec4b was approximately 500 bases long and the gene seems to be a member of a large multigene family in the H. cecropia genome. Analyses of the nucleotide and amino acid sequences reveal similarity to lepidopteran chorion genes and a lesser but convincing similarity to vertebrate cytokeratins. The filter and in situ hybridization data point to the association of specific messenger RNAs with the cortical cytoskeleton of silkmoth oocytes. Aspects of the structure of the protein encoded by this mRNA suggest that it is a structural component necessary for formation of the cellular blastoderm of the embryo. The association of this maternal mRNA with the cortical cytoskeleton presents the interesting possibility that mRNA bound to the cytoskeleton may be capable of participating in the synthesis of new cytoskeleton or related structures during blastoderm formation. Such an association between maternal mRNA and the cytoskeleton may indicate a general mechanism for the spatial distribution of determinative elements in eggs.

Key words: maternal messenger RNA, cortical cytoskeleton, insect oogenesis, Hyalophora cecropia.

Introduction

One of the interesting features of insect development is the late compartmentalization of the egg that may occur after several thousand zygotic nuclei have formed. Cellularization of the zygote occurs in the thin rim of yolk-free cytoplasm on the periphery, the cortex. The cortex of several species of giant moths has been shown to contain a dense cytoskeleton composed primarily of actin fibers (Jarnot et al. 1988). It is this cortex that is subdivided when cellularization occurs after the nuclei migrate to this region. Since the cortex is included in these first cells, it might be anticipated that information in the form of mRNAs would be bound to the cortex, particularly mRNAs for proteins required for the cellularization process. In the case of most embryos examined to date, protein synthesis begins soon after fertilization, but transcription from the zygote genome is delayed until a stage analogous to the midblastula transition in amphibians (Newport and Kirschner, 1982). A major consequence is that early translation is dependent entirely upon maternal mRNA transcripts in the immediate post-fertilization period. We have demonstrated that this pattern of early activation of translation, but delayed initiation of transcription, is followed in the eggs of giant silkmoths (Kastern et al. 1982).

The ovarioles of giant moths are an excellent place to study the sequential biochemical events of oogenesis, because each of the eight ovarioles contains a continuous series of developing oocytes from the primordial germ cells near the terminal filament to completed eggs with chorion at the oviduct end. Thus the developmental history of the oocyte can be reconstructed by examining successive oocytes along the length of the ovariole. Maternal mRNAs are manufactured in the nurse cell cluster at one end of the follicle and transported to the ooplasm via cytoplasmic connectives (Paglia et al. 1976). In situ hybridization with [3H]-polyuridyline indicates that polyadenylated mRNAs accumulate in the cortex (Jarnot et al. 1988).

Association of mRNA with the cortex has been reported in Chaetopterus eggs (Jeffery and Wilson, 1983). These mRNA molecules appear to be tightly
associated with the cytoskeletal domain of the cortex since they are resistant to detergent extraction. The significance of this association of messenger RNA with cortical cytoskeletal elements is not clear at the moment, but it indicates that the cortex may play a role in holding messages in place until cellularization occurs.

The experiments described below represent the first in a series designated to determine what sorts of proteins are coded for by the 'cortical' maternal mRNA of the silkworm, how this information is distributed spatially, and the temporal sequence of synthesis of particular messages.

**Materials and methods**

**Isolation of RNA, DNA and construction of cDNA library**

Total mature egg RNA was prepared from *Hyalophora cecropia* charionated, but unfertilized, eggs solubilized in guanidine isothiocyanate and subjected to centrifugation through CsCl according to published procedures (Chirgwin et al. 1979). Egg cytoplasm RNA was obtained from mature eggs excised from the ovary and cut open manually. Ooplasm was gently washed out of the egg shell with WG/Triton X-100 buffer pH 7.0 (200 mM-Hepes, 100 mM-KCl, 5.0 mM-magnesium acetate, 1.0% Triton X-100). RNA was prepared from the eggshells remaining after several rounds of washing opened eggs with WG/Triton X buffer. This detergent-insoluble fraction will be referred to as cortical cytoskeleton.

Both cytoplasmic and cortical cytoskeleton RNAs were purified by solubilization in guanidine isothiocyanate and centrifugation through CsCl as described above.

Single-stranded cDNA was prepared using purified total egg RNA primed with oligo(dT) as template and AMV reverse transcriptase (Life Sciences; St. Petersburg, Fla). Second strand synthesis, nuclease S1 digestion, EcoRI methylation, and insertion into lambda gt11 (Promega Biotec, Madison, Wisconsin) with EcoRI linkers (New England Biolabs) were essentially as described previously (Kastern et al. 1986) using size-selected cDNA greater than 200 base pairs in length. A cDNA library of approximately 4×10^6 individual clones with inserts prior to amplification was obtained. 90% of the clones contained inserts. Length of inserts range from 200 to 900 base pairs.

Duplicate copies of the library were screened with 32P-labeled cDNAs synthesized using either egg cytoplasmic or cortical cytoskeleton-associated RNA as template. In the initial screening, approximately 2000 plaques per plate were screened.

DNA fragments subcloned into M13 mp18 were sequenced according to previously published procedures using dideoxynucleotide chain termination with 35S-dATP (New England Nuclear) and Klenow fragment of DNA polymerase I (Boehringer-Mannheim). Sequences were read, analyzed and compared to the GenBank database using an IBM-XT computer running Microgenie (Beckman Instruments). All enzymes were from New England Biolabs except as otherwise noted, and used according to the suppliers' suggestion.

**Results**

A cDNA library with a complexity of approximately 4×10^6 individual clones with inserts was constructed from mature *H. cecropia* egg RNA. The library was subjected to differential screening with cDNA made from either egg cytoplasmic RNA or cortical cytoskeleton-associated RNA. In the preparation of cytoskeleton-associated RNA, care was taken to ensure that all residual ooplasm was washed from the eggshells and were carried out using 35S-labeled cDNAs and H-labeled anti-sense RNA as probes. Sense H-RNA was used as a negative control. The hybridizations were carried out at 45°C for 18h as described by Lawrence and Singer (1985), followed by a 30 min treatment with RNase A in 0.5 m-NaCl, 10 m-Tris–HCl pH 8.0, 1 m-EDTA at 37°C. After rinsing several times at 37°C in the same buffer, the slides were dehydrated, dried and coated with Kodak NTB-2 radiographic emulsion. Slides were exposed for 8–16 days.

**Northern, Southern and dot blot hybridizations**

For Northern blot analysis, purified RNA was denatured with glyoxal (McMaster and Carmichael, 1977), subjected to electrophoresis on 1.4% agarose gels and blotted to activated DBM and nitrocellulose paper (Schleicher and Schuell, Keene, NH; Alwine et al. 1977). RNA dot blots were made by coupling total oocyte RNA and RNA from either egg cortical cytoskeleton or yolky cytoplasm to nitrocellulose filters using a manifold apparatus (Schleicher and Schuell). DNA was labeled by nick translation using 32P-dCTP and a nick translation kit (Amersham). Labeled, single-stranded RNA was made after subcloning the Eco4b insert into the EcoRI site of pIBI-30 (IBI Inc; New Haven, CT). Anti-sense RNA was made with T3 RNA polymerase and sense strand RNA with T7 RNA polymerase in a kit supplied by IBI using 32P-CTP as label. Hybridization was overnight at 42°C in a buffer composed of 50% formamide, 5×SSC (1×SSC=0.15 M-NaCl, 0.015 M-sodium citrate), 5×Denhardt's (1×Denhardt's=0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.05 M-NaPO4, pH 6.5, and 0.02 mg/ml—1 denatured salmon sperm DNA. Following hybridization, northern blot filters were washed in 0.1×SSC and 0.1% SDS at 52°C (or as otherwise noted), and exposed to Kodak XAR film. Filters from dot blots were washed at 52°C and exposed as mentioned above.

**DNA sequence analysis**

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**Microscopy**

Samples for light and electron microscopy were prepared using conventional techniques. Fully charionated eggs were extracted in Triton X-100, fixed with 4% glutaraldehyde/15% tannic acid, dehydrated and embedded in epon. 200 nm thin sections were viewed under the Phillips 300 electron microscope.

**Results**

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that all eggs were fully chorionated with no follicle cell layer remaining. Fig. 1 shows transmission electron micrographs of sections of the cortex before and after Triton X extraction. In the extracted egg, it can be seen that a substantial cortical cytoskeleton remains. The purified RNA from each preparation served as template to synthesize radioactive cDNA which was then used to screen duplicate copies of a portion of the amplified mature egg cDNA library. Identical amounts of cDNA labeled to similar specific activities were used as hybridization probe. Following autoradiography, the degree of hybridization of each plaque to the ooplasmic cDNA was compared to that of the cortical cDNA. The majority of plaques demonstrated similar levels of hybridization with both probes. However, a substantial number of clones showed increased hybridization with one or the other of the probes, suggesting that the mRNA for these clones was more abundant in one of the two RNA preparations.

Twenty clones showing markedly increased hybridization to cortical cytoskeleton cDNA were selected and subjected to three rounds of plaque purification and differential re-screening with both cytoplasmic and cytoskeletal cDNA's. Only one clone, lambda Ec4b, remained enriched in the cortical cytoskeleton fraction throughout the three rounds of re-screening and this clone was examined further.

The visual localization of mRNA corresponding to lambda Ec4b in the egg was studied by in situ hybridization. For this purpose, the cDNA insert of Ec4b was excised with EcoRI and subcloned into the vector pIBI-30. This vector contains the T3 and T7 RNA polymerase promoters flanking a polylinker cloning site, allowing the synthesis of radioactive single-stranded RNA from either the sense or anti-sense strand depending upon which polymerase is used. The resulting subclone, pEc4b, was used as a template to synthesize both the radioactive sense and anti-sense strands. Each of these served as probes of sections of fixed eggs from various stages of oogenesis. Very strong hybridization was seen in nurse cells (Fig. 2A-D) as well as follicle cells. The density of label in the follicle cells varied with the stage of development while it was reasonably constant in the nurse cell cytoplasm. Very little hybridization is seen in follicle cells during early stages, but the signal began to increase during this stage just before chorion synthesis was initiated. During chorion synthesis, there was a dramatic increase in signal (Fig. 2B,C,D). Hybridization in the egg was localized to the cortex and absent from the yolk platelets after chorion synthesis (Fig. 3A,A'). No specific hybridization was detected when the sense strand was used as a probe (Fig. 3B,B'). We conclude that mRNA corresponding to Ec4b gene, or related genes, was present in the nurse and follicle cells, as well as in the egg from which the cDNA clone itself was isolated. Fully chorionated eggs lack both nurse cells and follicle cells. The patterns of hybridization were similar for the Ec4b cDNA probe and the pEc4b antisense strand of the pEc4b riboprobe prepared as described above. To
ascertain that the cortical localization was not an artifactual binding, we used for in situ analysis, a cDNA clone that hybridized to both cortical and insoluble cDNA probes during the 1°, 2° and 3° differential screening of the Agt11 egg cDNA library. Unlike Ec4b, the clone bound equally to both cortex and deeper ooplasm of the oocytes (Fig. 3C).

A further test for the cytoskeletal localization of Ec4b mRNA within the egg was performed by coupling purified preparations of ooplasmic RNA and cortical RNA to DBM filters. These dot blots were hybridized with radioactively labeled anti-sense RNA prepared from pEc4b as described above. The results demonstrated that the cytoskeletal RNA preparations were indeed enriched for Ec4b mRNA (Fig. 4A). When the same filters were washed and re-hybridized with the sense strand RNA, no hybridization was detected, indicating that the hybridization was specific to Ec4b mRNA (data not shown). A control experiment using a clone with uniform distribution in the egg is shown in Fig. 4B.

The size of the mRNA corresponding to Ec4b was determined by Northern blot analysis. Total egg RNA was denatured with glyoxal, subjected to electrophoresis on agarose gels, and blotted to DBM filters. After hybridization of these filters with Ec4b anti-sense strand RNA, the size of the mRNA was found to be approximately 500 bases (Fig. 4C). Once again the radioactive sense strand did not hybridize.

The nucleotide sequence of the insert of lambda Ec4b consisted of 243 base pairs of which 18 nucleotides were contributed by the synthetic EcoRI linkers (data not shown). In the derived amino acid sequence, there was a single open reading frame extending the entire length of the insert. Comparison of the nucleotide sequence with the GenBank database revealed similarities both to vertebrate cytokeratins (58 % similarity) and some insect chorion proteins (75 % similarity with the B-type chorion proteins), and Drosophila vitelline protein (70 %) (Fig. 5).

The similarity of the Ec4b sequence with that of other known proteins led us to investigate the possibility that Ec4b was a member of a family of similar genes in the
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Fig. 3. *In situ* localization of Ec4b mRNA in mature eggs. (A) Darkfield image of mature dechorionated egg hybridized with 3H-labelled anti-sense RNA. Note strong hybridization in the remaining cortical area. A' is a magnified area of A. (B) Mature dechorionated egg hybridized with 3H-Ec4b sense-strand RNA as a control. No hybridization above background was detected. B' is enlarged area of B. (C) Dark-field image of a mature egg hybridized with 35S-labelled clone that hybridized equally to cortical and cytoskeletal cDNA probes in 1st, 2nd, and 3rd screens of Agt11 egg cDNA library. This is used as a positive control to show that some clones hybridize uniformly to both cortical and ooplasmic regions of the egg. Key to symbols: c, cortex; o, ooplasm.
Fig. 4. Characterization of the mRNA corresponding to Ec4b.
(A) Comparison of levels of Ec4b mRNA in total egg, cortical cytoskeleton RNA and the soluble RNA fraction. RNA was filtered onto nitrocellulose at 10 μg, 1.0 μg and 0.1 μg and probed with 32P-Ec4b cDNA.
(B) mRNA dot blot probed with a Agt11 clone that hybridized equally to both cortical and soluble cDNA probes during 1°, 2° and 3° differential Agt11 egg cDNA library screens. This control hybridization shows a uniform pattern of mRNA distribution for this clone in the RNA preparations.
(C) Northern blot of Ec4b mRNA. 40 μg each of soluble RNA (S), total RNA (T) and cytoskeleton-associated cortical RNA (Cy) was denatured with glyoxal and subjected to electrophoresis. Sizes of molecular weight markers are indicated.

Fig. 5. Comparison of the protein coded for by Ec20 with a portion of Antheraea polyphemus chorion protein B, clone pc 401 (Jones et al. 1979) representing a 75% amino acid homology; vertebrate 56K type II cytokeratin, 58.6% homology; Drosophila melanogaster vitelline membrane protein (Mindrinos et al. 1985), 69.8% homology for the regions compared. Positions of amino acids compared are shown on the right.
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Fig. 6. Genomic blots probed with Ec4b insert. DNA from a single cecropia pupa was digested with EcoRII, subjected to electrophoresis on agarose gels and blotted to nitrocellulose. Following hybridization at either 37°C (a) or 42°C (b,c) the strips were washed at either 37°C (a, 52°C (b) or 65°C (c), followed by autoradiography. The sizes of the HindIII markers are indicated at the right.

tained a single open reading frame identical to that of Ec4B. This open reading frame extended from the beginning of the insert until position no. 337 and encoded 108 amino acids. The size of this insert indicated that it contained nearly the entire coding sequence of the mRNA. The sequence of Ec20 was compared with that of Ec4b (Fig. 7), and it showed that 93% of the nucleotides were identical. A number of the mismatches were in fact silent since comparison at the amino acid level showed even higher similarity (95%).

Discussion

The results above are consistent with the binding of particular maternal messages by the cortex. The binding is tight enough to resist extraction by detergent, and is therefore not simply a loose or adventitious association. The significance of this observation is reinforced by the fact that particular messages are associated only with the cortical or with the ooplasmic fractions, and that this relationship is consistently maintained through multiple screenings. The in situ hybridizations confirm that Ec4b is located only in the cortex of the mature egg, but also can be detected in the cortex, the central ooplasm, and the nurse cell, and follicle cell cytoplasm of the developing follicle.

A cortical cytoskeletal network, consisting of actin and intermediate filaments, has been described for several species (Franke et al. 1976; Gall et al. 1983; Spudich and Spudich, 1979; Jeffery and Meier, 1983). An extensive cortical cytoskeleton has also been demonstrated in silkmoth oocytes (Jarnot et al. 1988). Of particular interest is the observation in this study and others that maternal messenger RNA is associated with this area (Jeffery, 1982, 1984, 1985; Moon et al. 1983; Weeks and Melton, 1987; Pondel and King, 1988). Such association is potentially significant, because the egg cortex is the region of later cellularization. Cytoskeletal elements could provide an anchor for maternal messages, holding them in place until cytoplasmic cleavage occurs at blastoderm formation. Messenger RNA is associated with cytoskeletal elements in cells other than oocytes (Lenk et al. 1977; Cervera et al. 1981; Bagchi et al. 1987), suggesting that association with cytoskeletal elements may subserve other translation requirements as well. Polysomes have been found to be associated with the cytoskeleton of Hela cells (Lenk et al. 1977).
Cervera et al. (1981) proposed that translational activity may require the presence of a cytoskeleton.

The presence of Ec4b transcripts in nurse cell cytoplasm as shown by in situ hybridization and dot blots indicates that the nurse cells are the source of this mRNA, and not the follicle cells. Thus, although oocyte Ec4b/Ec20 resembles, or could be identical to, chorion messages (Jones et al., 1979), it apparently does not arise in the follicle cells, but follows the conventional path of information flow from the nurse cells to oocyte via ring canals. The lack of appreciable hybridization in follicle cell cytoplasm until chorion synthesis commences also supports the conclusion that Ec4b/Ec20 mRNA in the oocyte is of nurse cell origin. The similarities with chorion proteins and with vertebrate cytokeratins may arise from the fact that all three are essentially fibrous proteins. Gly-X-Gly sequences typical of fibrous proteins are seen in the Ec4b sequence. The genomic Southern blot data indicate that Ec4b is a member of a family of genes, but closely related to only 4 or 5, and only one or possibly two of the genes are transcribed into the maternal mRNA pool. Some of the weakly hybridizing bands seen in the genomic blot at the lower temperatures (Fig. 6, lane a) are probably attributable to members of the chorion multigene family (Jones and Kafatos, 1981), since hybrids between them and Ec4b were melted at 65°C, indicative of the low levels of homology (60–75 %) between them. The high degree of homology (95 % at the amino acid level) between Ec4b and Ec20 may indicate that these genes are alleles, and it may also account for one of the bands still present at 65°C in the genomic blot (Fig. 6, lane c).

Ec4b was selected as the first clone to be examined because of the strong signal obtained when screened against cortical RNA and the weak signal with opolysaccharide RNA. The strength of the signal suggested that this message should be abundant, and the in situ hybridization data indicated that it is present at early stages of oogenesis, and is finally distributed ubiquitously in the cortex of the egg. This suggests that Ec4b is unlikely to be a 'determinative' molecule involved in regionalization of the embryo.

It is unclear what the role of the protein encoded by Ec4b/Ec20 may be. Its similarity to the insect chorion proteins and to vertebrate cytokeratins suggests that it plays a role in the structure of some of the components of early embryogenesis. Indeed, the abundance of the amino acids glycine and cysteine in a repeating fashion supports this conclusion. Significantly, there was a high degree of similarity (i.e. 70 % at the nucleotide level) for a protein that is a component of the Drosophila vitelline membrane (Mindrinos et al., 1985). Perhaps the Ec4b and Ec20 proteins are components of the fibrous cytoskeletal elements that are required for the cellularization of the cortical region during blastoderm formation. A high demand for synthesis at this time would necessitate that the components be encoded for by maternal mRNA, since embryonic transcription is negligible prior to this stage (Kastern et al., 1982). We are now examining clones with weak signals against cortical mRNA in hopes of finding mRNAs with less ubiquitous distributions. Evidence of asymmetric distribution of maternal messages in eggs have been reported (King and Berkls, 1985; Weeks and Melton, 1987). However, only very few of these genes have been identified. A major problem in identifying maternal genes of interest is the low copy number of many of the transcripts. These genes might play more regulatory roles in development and are the ones that we are interested in. We are developing new strategies to screen for these genes.

We are particularly grateful to Dr Howard Schneiderman and the Monsanto company for a grant to support this research. SJB was the recipient of a NATO grant for travel to Belgium and Denmark. Finally, Funds were made available by Wesleyan University.

References


(Accepted 13 November 1989)