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Identification of proteins and mRNAs in isolated yellow crescents of ascidian eggs

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SUMMARY

The yellow crescent or myoplasm is a localized cytoplasmic region in eggs of the ascidian *Styela* that is partitioned to the larval tail muscle and mesenchyme cells during embryonic development. To determine whether the myoplasm contains a specific subset of maternal macromolecules, its abundant proteins and mRNAs were identified and compared to those present in the remainder of the egg. This was accomplished by exploiting a newly developed method for the mass fractionation of yellow crescents which is based on the presence of a unique cytoskeletal domain in the yellow crescent region. The fractionation yields a yellow crescent fraction (YCF) representing the myoplasm and a supernatant fraction representing the non-myoplasmic regions. The YCF comprises structures which contain the characteristic myoplasmic organelles and about 10% of the protein, 8% of the RNA, and 1–3% of the poly(A) of whole eggs. Two-dimensional gel electrophoresis indicated that the YCF contains 15 polypeptides that are undetectable in the supernatant fraction and 43 polypeptides that are significantly depleted in the latter fraction. The proteins restricted to the YCF are both of cytoskeletal and non-cytoskeletal origin. *In vitro* translation of RNA in a message-dependent lysate and analysis of [^{35}S]methionine-labelled polypeptide products by two-dimensional gel electrophoresis did not reveal qualitative differences between the YCF and the supernatant fraction. Furthermore, the mRNAs coding for two polypeptides that were localized in the myoplasm were not restricted to the YCF. The results suggest that qualitative differences in proteins but not in prevalent mRNAs exist between the yellow crescent and the other cytoplasmic regions of *Styela* eggs.

INTRODUCTION

The eggs and embryos of the ascidian *Styela* contain pigmented cytoplasmic regions which exhibit specific developmental fates (Conklin, 1905). One of these regions is the myoplasm, a yellow-pigmented cytoplasm which enters the tail muscle and mesenchyme cell lineages during embryogenesis. The myoplasm is a vegetal localization of mitochondria, yellow pigment granules, membrane systems, and cytoskeletal elements (Berg & Humphreys, 1960; Jeffery & Meier, 1983) that is restricted to the periphery of the unfertilized egg. During ooplasmic segregation this cytoplasmic region is condensed into a vegetal cap in the fertilized egg and is eventually extended into a yellow crescent in the vegetal-posterior region of the precleavage zygote (Jeffery, 1984*a*). The yellow crescent is divided equally between the blastomeres at first cleavage but it is subsequently partitioned

Key words: ascidian egg, yellow crescent, protein, mRNA, myoplasm, *Styela*.

to only two cells of the 4- and 8-cell embryo, four cells of the 16-cell embryo, six cells of the 32-cell embryo, and eight cells of the 64-cell embryo (Conklin, 1905). These eight cells are the progenitors of most of the larval tail muscle and mesenchyme cells.

There is evidence that cells which receive part of the yellow crescent contain factors capable of influencing the course of embryonic development. Whittaker (1980) has diverted some of the yellow crescent cytoplasm into other cells by compressing embryos at the third cleavage. Although the nuclear lineage of these cells was unaltered by this operation, some of the cells which received yellow crescent cytoplasm subsequently synthesized acetylcholinesterase, an enzyme which is normally expressed only in the larval tail muscle and mesenchyme cells and their progenitors. The simplest explanation for these results is that the yellow crescent contains factors that are responsible for acetylcholinesterase development. Similar explanations exist for localization phenomena in many other embryos (Wilson, 1925; Davidson, 1976) but in no case have the cytoplasmic factors been isolated or characterized.

Localized macromolecules of maternal origin are often cited as candidates for cytoplasmic factors which specify the fate of embryonic cells (Davidson, 1976), however, their existence has been difficult to demonstrate because methods do not exist for separating different egg cytoplasmic regions in quantities suitable for biochemical analysis. Consequently, most of the available information on the spatial distribution of maternal macromolecules has been obtained for individual molecular species by *in situ* hybridization with cloned nucleic acid probes (Jeffery, Tomlinson & Brodeur, 1983) or by immunocytology with antibodies (McClay *et al.* 1983). In the present investigation the distribution of abundant proteins and mRNAs in the yellow crescent cytoplasm of *Styela* eggs has been evaluated and localized species have been identified by exploiting a newly developed method for the mass isolation of yellow crescents.

MATERIALS AND METHODS

Biological materials

Styela plicata was obtained from Pacific Biomarine Inc. (Venice, CA). The procedures used to maintain, ripen, spawn, and fertilize these animals have been described previously (Jeffery & Meier, 1983). The fertilized eggs were cultured in Millipore-filtered sea water at 18°C until the yellow crescent stage, about 30–40 min after insemination.

Isolation of yellow crescents

The conditions for yellow crescent isolation were developed empirically; the following procedure produced the highest yield of yellow crescents. Eggs at the yellow crescent stage were concentrated by centrifugation for 30 s at 200 *g* and washed twice in 10 volumes of ice-cold isolation medium. The isolation medium contained 50 mM-Tris-HCl (pH 7·2), 500 mM-NaCl, 10 mM-$MgCl_2$, 5 mM-$CaCl_2$, 10 $\mu g\,ml^{-1}$ leupeptin, and 10 mM-vanadyl ribonucleoside complex (VRC; Berger & Birkenmeier, 1979); the VRC was omitted in situations in which intact RNA

was not required. The washed eggs were permeabilized by treatment with 0·1 % Triton X-100 in 20 volumes of isolation medium for 5 min on ice. The permeabilized eggs were centrifuged at 500 *g* for 1 min to remove the follicle cells, which are released from the chorion in the presence of detergent, and resuspended in 20 volumes of isolation medium. The suspension was homogenized by 10–20 up and down strokes of a teflon pestle fitted into a Potter-Elvehjem glass homogenizer (clearance 0·10–0·15 mm) and centrifuged at 500 *g* for 3 min. The supernatant fraction was decanted. The pellet, which at this point contained chorion pieces as well as yellow crescents, was resuspended in 10 volumes of isolation medium, homogenized by five up and down strokes of the pestle, centrifuged, and examined by phase microscopy. Cycles of homogenization, centrifugation, and microscopic examination (usually three or four) were continued as described above until the pellet contained relatively clean yellow crescents. Homogenization cycles beyond about five were avoided because they tended to fragment the isolated crescents. The combined supernatant fractions, which primarily represent the non-yellow crescent cytoplasm of eggs (the ectoplasm and endoplasm; Conklin, 1905), were pooled. The major contaminants of the crude yellow crescent fraction were yolk particles, free yellow pigment granules, and pieces of the chorion. A large proportion of these contaminants were removed by centrifuging the crude yellow crescent fraction, after resuspension in 2 ml of isolation medium, through 10 ml of 0·2 M-sucrose suspended over an 8 ml pad of 1·9 M-sucrose. Centrifugation at 15 000 *g* for 45 min (4 °C) produced a band of yellow crescents at the interface between the sucrose solutions, while most of the chorionic fragments were driven into the pellet. The yield of yellow crescents was estimated by comparing the numbers of intact crescents in aliquots from the final yellow crescent fraction (YCF) to the numbers of whole eggs in equivalent aliquots of the original egg suspension. The isolated yellow crescents were solubilized for biochemical analysis by resuspension in 10 mM-Tris-HCl, 50 mM-KCl, 1 mM-$MgCl_2$, 10 $\mu g\,ml^{-1}$ leupeptin, and 10 mM-VRC (pH 7·2) and homogenization.

Microscopy

Eggs or isolated yellow crescents were fixed for 30 min at 20 °C with 2 % glutaraldehyde in 0·1 M-phosphate buffer (pH 7·2), washed three times in 0·1 M-phosphate buffer, and postfixed in 1 % OsO_4 for 5 min (whole mounts) or 1 h (other specimens) at 20 °C. For transmission electron microscopy, specimens were embedded in Spurrs, thin sectioned, stained with lead citrate and uranyl acetate, and examined with a Hitachi H-300 transmission electron microscope. The procedures for preparing whole mounts of intact eggs and isolated yellow crescents for scanning electron microscopy have been described previously (Jeffery & Meier, 1983).

Analysis of proteins

The protein content of the YCF and the supernatant fraction was determined, and the proteins were separated by two-dimensional gel electrophoresis (O'Farrell, 1975). Prior to electrophoresis the samples were treated with a mixture of 50 $\mu g\,ml^{-1}$ DNase I and 5 $\mu g\,ml^{-1}$ RNase A (5 min at 4 °C), brought to 9 M-urea and 50 mM-lysine, and mixed with an equal volume of 5 % 2-mercaptoethanol, 2 % Nonadet P-40, 2 % ampholines (1·6 % pH range 5–7 and 0·4 % pH range 3·5–10), and 9·5 M-urea. The samples were applied to 4 % isoelectric focusing gels and, after electrophoresis at 400 V for 15 h, the gels were equilibrated for 1 h in 63 mM-Tris-HCl, 2·5 % SDS, 5 % 2-mercaptoethanol, and 10 % (w/v) glycerol (pH 6·8) and loaded onto 10 % polyacrylamide slab gels containing 1 % SDS. Electrophoresis in the second dimension was for 4·5 h at 80 V through the stacking gel and at 100 V through the separating gel. The gels were stained with silver nitrate (Wray, Boulikas, Wray & Hancock, 1981).

Isolation and analysis of RNA

The YCF was suspended in 2 vol of ice-cold 50 mM-Tris-HCl, 500 mM-NaCl, 10 mM-EDTA, 1 mM-EGTA, and 10 mM-VRC and the supernatant fraction was brought to 10 mM-VRC for RNA isolation. The mixtures were homogenized by five strokes of a Dounce homogenizer. The homogenate was centrifuged at 12 000 *g* (4 °C) for 10 min, and the supernatant was removed and

mixed with 0·5 vol of phenol (equilibrated with 100 mM-Tris-HCl, pH 8·0, 0·2% 2-mercaptoethanol, 0·1% 8-hydroxyquinoline) and 0·5 vol of 24:1 chloroform:isoamyl alcohol. The organic phase was extracted with an equal volume of 10 mM-Tris-HCl, 1 mM-EDTA (pH 7·6). The pooled aqueous phases were extracted twice with equal volumes of 1:1 phenol:chloroform containing 2% isoamyl alcohol (v/v). The aqueous phase was brought to 300 mM-sodium acetate (pH 6·0) and the RNA was precipitated with 3 vol of 95% ethanol (−20 °C). The RNA content was determined from the A_{260}. The poly(A) content was determined by hybridization with [^{3}H]poly(U) as described previously (Jeffery & Brawerman, 1974). VRC, which still contaminates RNA preparations after phenol extraction and ethanol precipitation and reduces the efficiency of *in vitro* translation, was removed by gel filtration. The RNA precipitate was resuspended in 50 mM-Tris-HCl, 10 mM-NaCl (pH 8·0) and loaded on a Sephadex G 50-300 column equilibrated with the same buffer. The RNA and VRC eluted from the column in separate peaks. The RNA peak was collected and precipitated with ethanol.

Subsaturating levels of total RNA from the YCF and the supernatant fraction were translated in an mRNA-dependent, wheat germ lysate (Morrison *et al.* 1979) supplemented with [^{35}S]methionine (1290 Ci micromole^{-1}, Amersham Radiochemicals, Arlington Heights, IL.). Two-dimensional gel electrophoresis of the translation mixtures was carried out as described above. The gels were dried and autoradiographed by exposure to Kodak X-Omat film for 2–10 weeks at −70 °C.

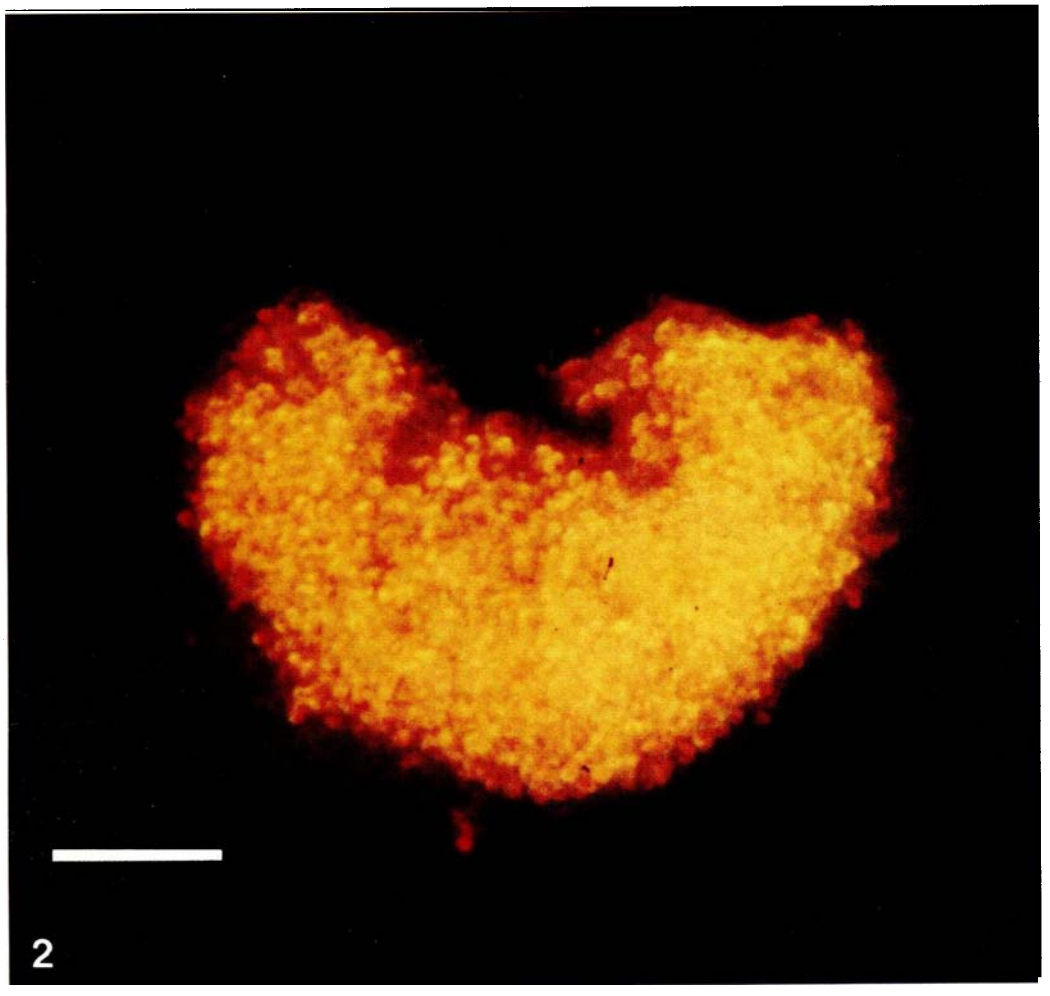
RESULTS AND DISCUSSION

Isolated yellow crescents

The structures recovered in the YCF were cytoplasmic fragments about the same size and shape as the yellow crescent region of intact zygotes (Figs 1–4). Like the yellow crescent *in vivo*, these fragments were highlighted by bright yellow

Fig. 1. A dark-field micrograph showing a field of isolated yellow crescents. Small clusters of yellow pigment granules that contaminate the YCF can also be discerned (arrow). Scale bar, 30 μm.

Fig. 2. A dark-field micrograph of an isolated yellow crescent. The yellow spheres within the crescent are pigment granules. Scale bar, 10 μm.



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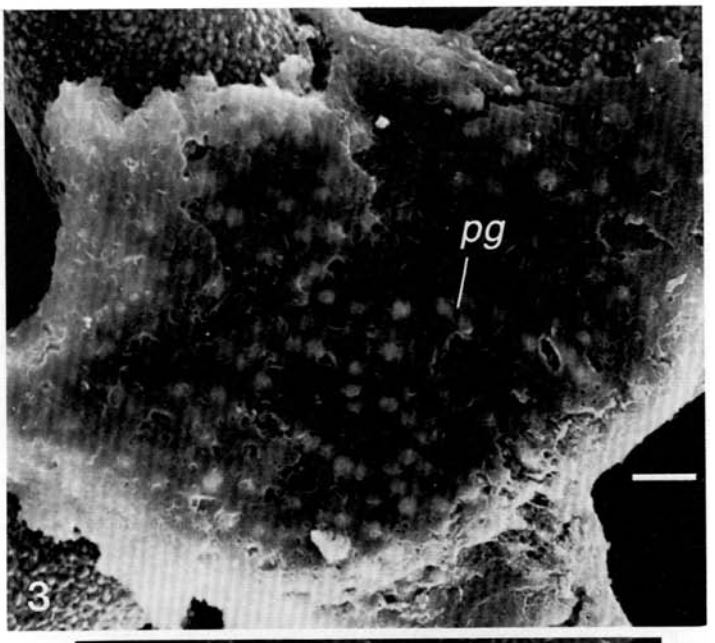
pigment granules embedded in an opaque matrix (Fig. 2). The YCF also contained small clusters of aggregated yellow pigment granules, presumably released from the crescents during the fractionation, but was depleted of yolk particles and pieces of chorion (Fig. 1).

The yield of yellow crescents was estimated by comparing the number of large yellow fragments in the YCF to the number of whole zygotes present at the beginning of the fractionation. In twelve experiments the yield ranged from 65 to 92%, but was frequently above 85%. These observations suggest that yellow crescents can be prepared in fairly high yields.

The fine structure of isolated yellow crescents was examined by transmission electron microscopy. *Styela* zygotes contain three major cytoplasmic regions (Conklin, 1905). The ectoplasm is located in the animal hemisphere, the myoplasm is located in the vegetal hemisphere, and the endoplasm fills the remainder of the cell. The ultrastructure of the cytoplasmic regions of *Styela* embryos has been described by Berg & Humphreys (1960). The myoplasm contains endoplasmic and abundant clusters of mitochondria which adhere to the surface of large pigment granules, the ectoplasm contains fine particulate material and is devoid of large granules, and the endoplasm is characterized by large numbers of membrane-bound yolk particles. Electron microscopy of the YCF showed the isolated yellow crescents to contain pigment granules, mitochondria, and endoplasmic reticulum embedded in a cytoplasmic ground substance (Fig. 5). The mitochondria adhered to the surface of the pigment granules in isolated crescents in a fashion similar to their spatial arrangement *in vivo* (Figs 5–6). Conspicuously absent from the isolated yellow crescents were areas containing large yolk granules, which would represent contamination by endoplasm, or areas devoid of large granules, which would represent contamination by ectoplasm. Whole mounts of isolated crescents examined by scanning electron microscopy showed that they also contained a portion of the plasma membrane (Fig. 3). Presumably this is the plasma membrane sector lying immediately above the yellow crescent region. These observations indicate that isolated yellow crescents contain the characteristic myoplasmic organelles and are not detectibly contaminated by ectoplasm or endoplasm.

The procedure devised for yellow crescent fractionation is similar to that previously developed for the isolation of membrane caps from *Dictyostelium* cells (Condeelis, 1979). The fractionation of the yellow crescent, a cytoplasmic localization which is thought to be formed by a capping process (Jeffery, 1984*a*), probably depends on the existence of a unique cytoskeletal domain associated with a natural membrane cap (Jeffery & Meier, 1983). Certain cytoskeletal elements are known to be insoluble in high ionic strength media (Cooke, 1976); and it is suspected that this feature, along with a gelling of the surrounding cytoplasm, may be responsible for the resistance of the yellow crescent region to homogenization under conditions in which the remainder of the egg appears to be entirely dispersed. The presence of mitochondria, yellow pigment granules, membrane systems, and a cytoplasmic ground substance as well as cytoskeletal elements in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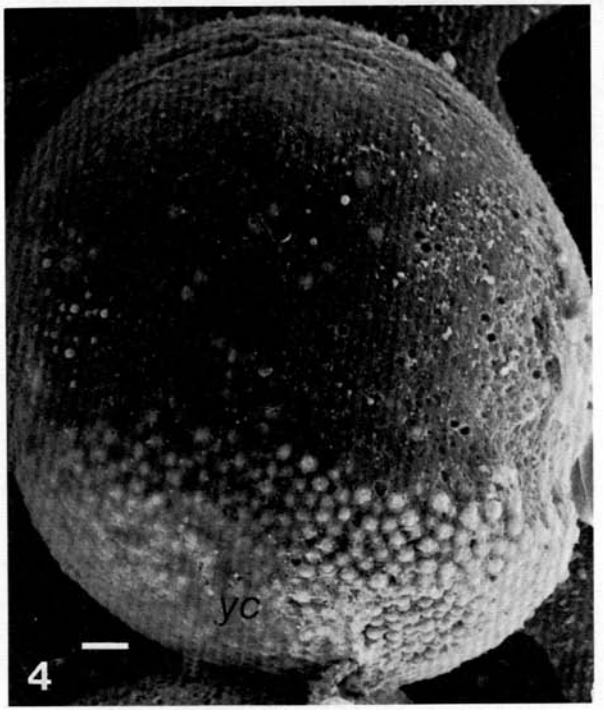
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isolated yellow crescents suggests that this fraction reflects the *in vivo* composition of the myoplasm.

Isolated yellow crescents contain unique proteins

The polypeptide composition of the YCF was examined and compared to that of the supernatant fraction to determine whether the yellow crescent contains a specific set of proteins. The YCF contained about 10% of the total egg protein. This value corresponds to the volume of egg cytoplasm which is occupied by the myoplasm (Jeffery *et al.* 1983). Two-dimensional gel electrophoresis was used to compare the proteins of the YCF and the supernatant fraction. In these experiments, and in the succeeding analyses of mRNA translation products, the pooled supernatant fractions obtained during the fractionation are assumed to represent the non-yellow crescent region of eggs. The contamination of the supernatant fraction by myoplasmic materials was minimized by restricting the analysis to fractionations in which the yield of yellow crescents was greater than 85%.

A number of differences were observed between the polypeptides of the YCF and supernatant fraction (Figs 7–8). Of 133 spots detected in silver-stained gels, 34 (26%) were shared in about the same amounts between the two fractions, 41 (31%) were depleted or absent in the YCF, 43 (32%) were enriched in the YCF, and 15 (11%) were detected only in the YCF (boxed polypeptides in Fig. 7). The yellow-crescent-restricted proteins were not evident in gels which contained three times more supernatant fraction protein than the YCF gels (Fig. 8) suggesting that these proteins are rare or absent in the remainder of the egg.

A number of proteins restricted to the yellow crescent are identical to components present in the underlying cytoskeletal domain. The 48×10^3 and 54×10^3 relative molecular mass yellow crescent proteins, along with actin (which is also present in isolated yellow crescents; Fig. 7), were previously identified as major constituents of Triton X-100-extracted *Styela* eggs (Jeffery & Meier, 1983). The 48×10^3 and 54×10^3 relative molecular mass polypeptides, which are similar in relative molecular mass and pI to vertebrate intermediate filament proteins (Jackson *et al.* 1980), may constitute part of the cytoskeletal matrix of the yellow crescent domain. Not all the yellow crescent proteins appear to be cytoskeletal, however. A 38×10^3 relative molecular mass yellow crescent polypeptide (starred in Fig. 7) was readily solubilized after eggs were treated with Triton X-100 (Jeffery

Fig. 3. A whole mount of an isolated yellow crescent observed by scanning electron microscopy. Note that a part of the plasma membrane (pocked with holes from the Triton X-100 permeabilization) can be seen above the layer of yellow crescent pigment granules (*pg*). Scale bar, 5 μm.

Fig. 4. A whole mount of an intact egg observed by scanning electron microscopy showing the yellow crescent (*yc*) region (lower portion). The intact egg was briefly treated with 0·5% Triton X-100 to highlight the yellow pigment granules. Scale bar, 5 μm.

& Meier, 1983) suggesting that it is a non-cytoskeletal protein. The finding of specific polypeptides in the yellow crescent of *Styela* eggs confirms the cases of

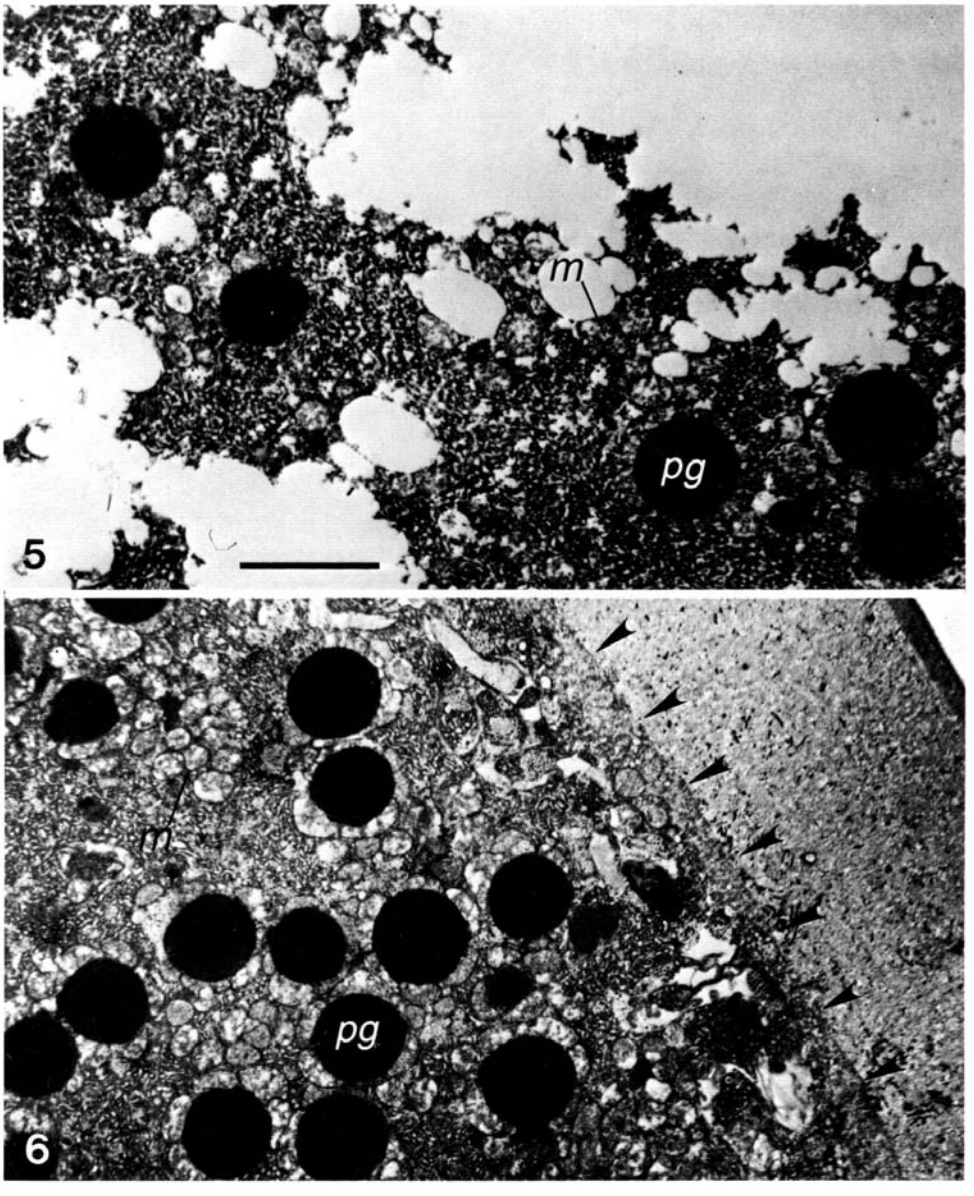

Fig. 5. An electron micrograph of a thin section through an isolated yellow crescent. Note the presence of mitochondria (*m*), pigment granules (*pg*) and endoplasmic reticulum in similar spatial arrangements as found in the yellow crescent region of the intact egg (see Fig. 6). Scale bar, 2 μm.

Fig. 6. An electron micrograph of a thin section through the yellow crescent region of an intact egg. Note the spatial arrangement of mitochondria (*m*) and pigment granules (*pg*). The plasma membrane is indicated by the series of arrowheads.

qualitative protein localization previously reported in different cytoplasmic regions of other eggs (Moen & Namenwirth, 1977; Gutzeit & Gehring, 1980; Schmidt, 1980; Jäckle & Eagleson, 1980).

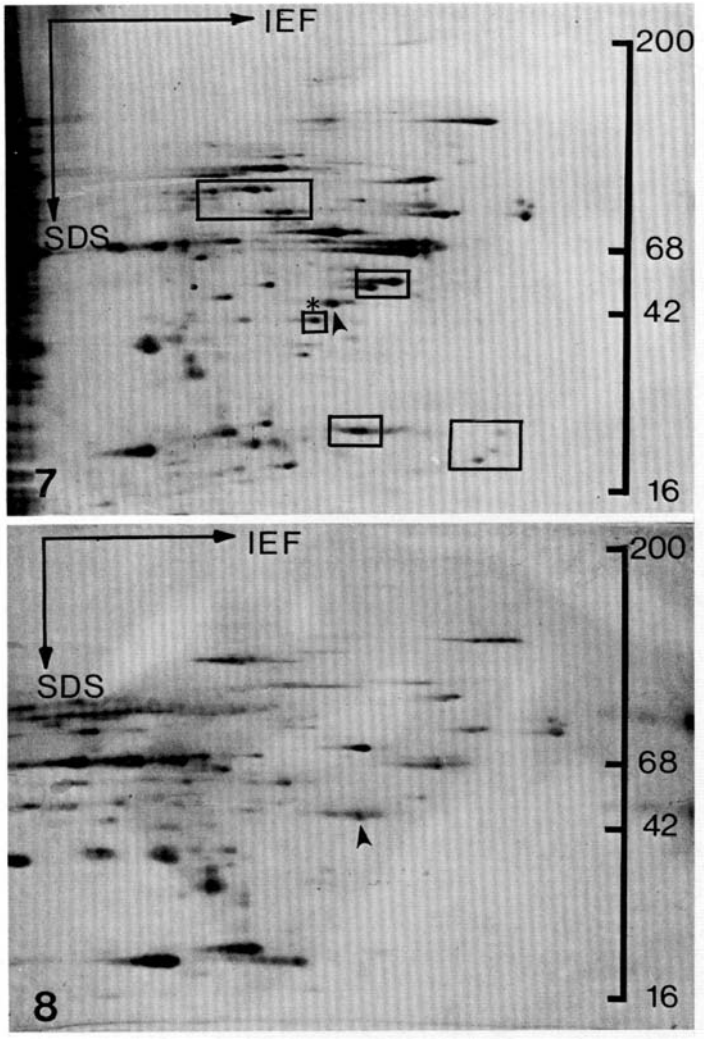

Fig. 7. Two-dimensional gel electrophoresis of polypeptides present in the YCF. The arrowhead indicates the position of actin. Boxed polypeptides or groups of polypeptides represent some of the species restricted to or greatly enriched in the YCF relative to the supernatant fraction (see Fig. 8). The right-hand ordinate is relative molecular mass $\times 10^{-3}$. The starred box defines the position of the 38×10^{-3} relative molecular mass yellow crescent polypeptide. 20 μg of total protein was applied to the gel.

Fig. 8. Two-dimensional gel electrophoresis of polypeptides present in the supernatant fraction. The details and labelling of this figure are the same as those in Fig. 7. 60 μg of total protein was applied to the gel.

Prevalent yellow crescent mRNAs also exist in other regions of the egg

The RNA composition of the YCF was compared to that of the supernatant fraction. Isolated yellow crescents were found to contain about 8% of the total egg RNA, again fairly consistent with the relative egg volume devoted to this region (Jeffery *et al.* 1983). Solution hybridization with [H^3]poly(U) showed the YCF to contain only 1–3% of the total egg poly(A). The low level of poly(A) detected in the yellow crescent is consistent with *in situ* hybridization results showing that this region contains less than 5% of the egg poly(A) (Jeffery *et al.* 1983). These studies suggest that the yellow crescent contains either low levels of poly(A)$^+$RNA or poly(A)$^+$RNA molecules with relatively short poly(A) tracts.

To determine the distribution of mRNA species between the myoplasm and the remainder of the egg, RNA extracted from the YCF and the supernatant fraction was translated *in vitro* and the [^{35}S]methionine-labelled polypeptide products were compared by two-dimensional gel electrophoresis and autoradiography. About 125 polypeptides, presumably those encoded by the most prevalent egg messages, could be detected in the gels (Figs 9–10). Although there were quantitative differences between these two fractions, the translation products encoded by mRNA from the YCF and the supernatant fraction were qualitatively identical.

One of the translation products seen in the gels corresponds to the position of the 38×10^3 relative molecular mass polypeptide which is restricted to the yellow crescent (starred in Fig. 7). The message encoding this polypeptide appears to be present in both the YCF and the supernatant fraction (downward-pointing arrowhead in Figs 9–10). A translation product corresponding to the 54×10^3 relative molecular mass myoplasmic polypeptide was also encoded by a message present in both fractions (downward-pointing arrowhead in Figs 9–10). The results suggest that messages coding for some yellow-crescent-restricted polypeptides are also present in other parts of the egg. Unfortunately, none of the other yellow crescent translation products could be correlated with polypeptides present in the silver-stained gels. The messages encoding these polypeptides may be rare in zygotes or may not be processed correctly after *in vitro* translation.

These results, which suggest that the same prevalent egg mRNAs are located in the yellow crescent as in the other cytoplasmic regions of *Styela* eggs, are subject to the following qualifications. First, since the two-dimensional gel system only resolves acidic proteins, it is possible that there could be spatial differences in the distribution of messages coding for neutral or basic proteins. Second, some mRNAs could be restricted to the yellow crescent but, because of structural peculiarities (Richter, Smith, Anderson & Davidson, 1984), may not be detectable by *in vitro* translation. Third, although the isolated yellow crescents appear to be structurally intact and maternal mRNA is known to be tenaciously associated with a cytoskeletal framework in *Styela* eggs (Jeffery, 1984*b*), localized yellow crescent mRNAs could have been released into the supernatant fraction during the isolation.

Because of these qualifications and the fact that only the most prevalent mRNAs are detectable by *in vitro* translation, the results of this investigation do not exclude the possibility that specific mRNA molecules are qualitatively localized in the myoplasm of *Styela* eggs. However, they do suggest that messages coding for some

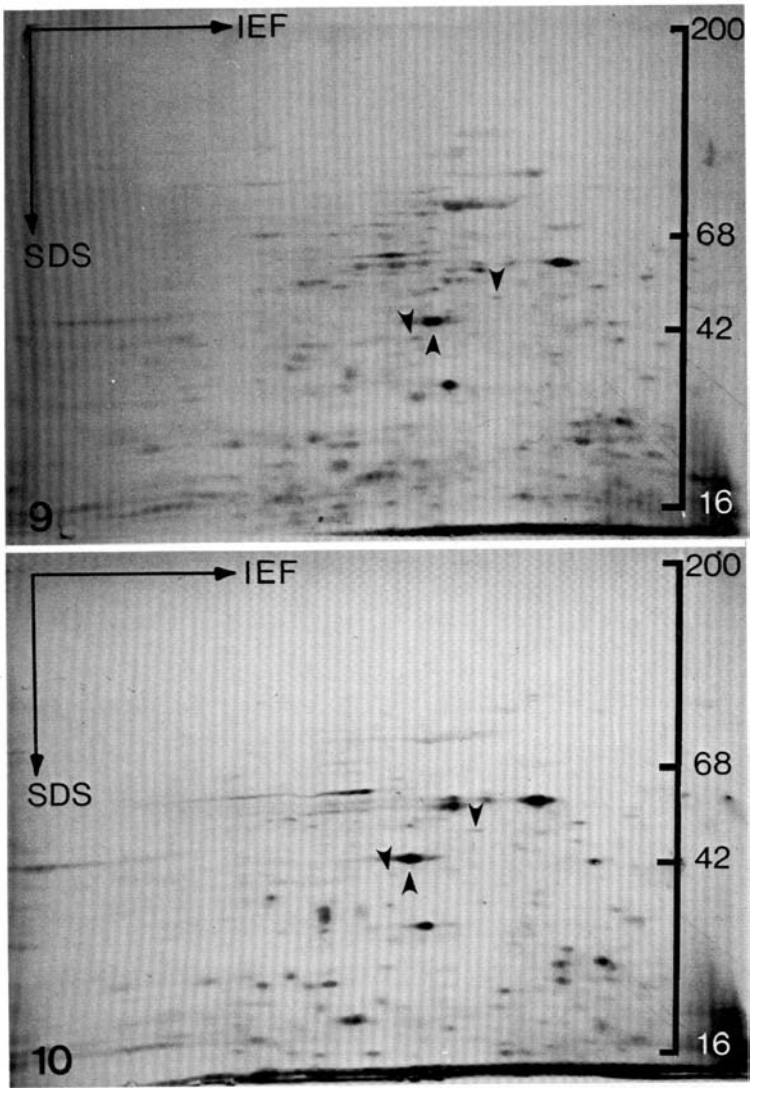

Fig. 9. Two-dimensional gel electrophoresis of *in vitro* translation products directed by RNA isolated from the YCF. The upward-pointing arrowhead indicates the position of actin. The downward-pointing arrowheads indicate the position of translation products corresponding to the 38×10^{-3} and 54×10^{-3} relative molecular mass yellow crescent proteins.

Fig. 10. Two-dimensional gel electrophoresis of *in vitro* translation products directed by RNA isolated from the supernatant fraction. Other details are the same as those in Fig. 9.

of the localized yellow crescent proteins (i.e. the 38×10^3 and 54×10^3 relative molecular mass myoplasmic proteins) are not restricted to this region of the egg. Therefore, assuming these mRNAs are translated throughout the egg cytoplasm, the process by which their protein products become localized in the yellow crescent must be dependent on post-translational events.

CONCLUSIONS

The development of a method for the isolation of yellow crescents has provided a means of comparing the spatial distribution of macromolecules between the myoplasm and other cytoplasmic regions of ascidian eggs. Using this method the yellow crescent has been shown to contain more than a dozen proteins that are rare or absent in other regions of the egg. Two of the yellow crescent proteins are coded for by mRNA species that are not restricted to the myoplasm. The yellow crescent fractionation method described in this investigation may eventually provide a source for cytoplasmic factors that can be used to develop a functional assay for the identification of ascidian muscle cell determinants.

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