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The role of intermediate filaments in early *Xenopus* development studied by antisense depletion of maternal mRNA

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Summary

The effects of depleting a maternal cytokeratin mRNA on the developing embryo are described. Cytokeratins are members of the intermediate filament family of cytoskeletal proteins, and are expressed in a cortical network of the superficial cytoplasm of the oocyte. After fertilisation, a new cortical network is built up, which comes to occupy only the most superficial cells of the blastula. The maternal cytokeratin mRNA is abundantly translated, both during oogenesis, and during oocyte maturation and after fertilisation. Depletion of the mRNA results in depletion of the cortical filaments at the blastula stage and leads to gastrulation abnormalities. We discuss the various possible control experiments required for antisense oligo depletion studies and the implications of these results for cytokeratin function.

Key words: maternal mRNA, antisense oligos, cytokeratin, *Xenopus*.

Introduction

During oogenesis, the *Xenopus* oocyte grows to a size of 1.2 mm and accumulates a large store of nutrients, mRNAs and proteins. It has long been recognized that this store is used during the early stages of development, particularly as zygotic transcripts are not expressed until after the mid-blastula transition (MBT), which occurs approximately at the 4000-cell stage. However, the precise roles of individual mRNAs and proteins are far from clear. The pre-MBT phase is a critical time in development, during which axes are established, adhesive differences appear and the earliest cell and tissue types are determined. Defining the functions of individual maternal proteins and mRNAs would be of considerable importance in understanding the mechanism of early development.

Since it is not at the moment possible to generate germline mutations in vertebrates, nor to carry out genetic manipulations in *Xenopus*, a number of strategies have been used to interfere with the function of individual proteins or mRNAs. One approach has been to inject antibodies specific for particular proteins into fertilized eggs or 2-cell-stage embryos (Warner et al., 1984; Wright et al., 1989, Klymkowsky 1992). This method may have a number of problems, including the accessibility of the protein, the likely mosaicism of effect due to the uneven distribution of the injected antibody and possibly the toxic nature of high local concentrations of immunoglobulins.

An alternative to the use of antibodies is to swamp the wild-type protein with excess of a mutated form (Herskowitz, 1987). This dominant negative strategy has been used successfully to study the function of fibroblast growth factor receptor in mesoderm induction (Amaya et al., 1991). Another strategy is to target and block the function of maternal mRNAs rather than proteins. This method will not work if the store of maternal protein is in itself sufficient for the needs of the embryo until the MBT. However, many gene products are stored both as mRNAs and proteins, suggesting that the protein levels have to be topped up by new translation as development proceeds. Attempts to deplete mRNAs in fertilized eggs by injecting antisense RNA were unsuccessful for endogenous transcripts, for reasons which remain obscure (Melton, 1985; Bass and Weintraub, 1987; Kimelman and Kirschner, 1989).

A more successful way of causing the degradation of specific mRNAs is by injecting antisense oligodeoxynucleotides (oligos) into oocytes or embryos. This approach was developed in oocytes by Dash et al. (1987), and tested in both oocytes and eggs by Shuttleworth et al. (1988). The optimum length of oligo both in terms of depletion activity and of depletion specificity was found to be a 14-18 mer. The method has now been quite widely used in oocytes to study the function of a variety of exogenous membrane proteins (Prives and Foukal, 1992). Antisense oligos have also proved successful tools in understanding the role of the proto-oncogene c-*mos* in meiotic maturation in oocytes (Sagata et al., 1988; Freeman et al., 1990), and of cyclins in mitosis in cell-free egg extracts (Minshull and Hunt, 1987).

In contrast, there have been relatively few functional studies using oligos injected into fertilized eggs (Dagle et al., 1990). This may be because egg injections present a

number of problems compared to oocytes. Oligos are unstable once injected and degrade more quickly in fertilized eggs compared to oocytes (Shuttleworth and Colman, 1988; Dagle et al., 1990). Furthermore, oligos and their degradation products are known to carry non-specific toxicity (Shuttleworth et al., 1988; Smith et al., 1990; Woolf et al., 1990) and fertilized eggs and embryos are more sensitive to this than oocytes (Woolf et al., 1990). One further problem of injections into eggs is that of mosaicism of effect due to the uneven distribution of the oligo as cleavage proceeds. Some of these problems may be avoided by injecting oligos into ovarian oocytes, allowing the target mRNA to be depleted and the oligo degraded, and then fertilizing them.

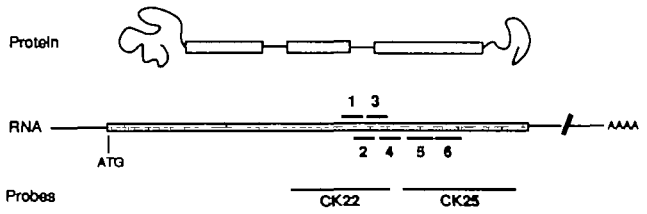
This review outlines the progress that we have made towards developing this method of analysis of maternal mRNA function. A similar approach (Kloc et al., 1989) showed that an asymmetrically distributed maternal mRNA xlgv7 was not required for normal development in *Xenopus*. We initially targetted mRNAs coding for intermediate filament proteins. The basic cytokeratin XCK1(8) is abundant in the oocyte and embryo, both as a protein (Franz et al., 1983; Gall et al., 1983; Godsave et al., 1984) and as an mRNA (Franz and Franke, 1986; Torpey et al., 1992a). Also the immunolocalization of XCK1(8) has been well described (Torpey et al., 1992b) and we have an antibody that recognizes this protein specifically (Torpey et al., 1992b). This keratin is synthesized abundantly both in the oocyte, and during maturation and early development (Torpey et al., 1992a), suggesting that the maternal store of protein may require supplementation by translation of the stored maternal mRNA. Finally the role of the intermediate filament proteins in early development is not clear, making this an important protein to target.

Injection of oligos into oocytes

Oocytes are manually defolliculated from freshly dissected ovary taken from an anaesthetized female. While collagenase treatment removes follicle cells more quickly than the manual method, such treatment also renders the oocytes unfertilizable.

For all the mRNAs that we have targetted, we needed to synthesize a panel of six or more oligos complementary to different parts of the RNA sequence, to find one that would deplete the mRNA. Fig. 1 shows an example of the panel prepared for cytokeratin XCK1(8) mRNA chosen randomly from the sequence. We have so far found no particular pattern of sequence similarity or position that correlates with the ability to cause RNA depletion. This variability of oligo effectiveness has been noted by others (Baker et al., 1990), and presumably reflects the secondary structure of the target RNA making only some parts of the sequence available for oligo hybridization.

Oligos are normally injected into the equatorial region of oocytes although we have not found a difference in effectiveness when injections are into the vegetal pole. Other authors have commented on variability of depletion of mRNAs according to the site of injection (Shuttleworth et al., 1988). Fig. 2A and B are RNAase protection assays



CK1	TGTCAGAAATCTGG
CK2	ACAACGGAAGTGTC
CK3	TCCAGGTCAAGGCT
CK4	AATGATGCCGTCCA
CK5	CCTTGTGGTGTAACGGGT
CK6	ATCTATTTCGGACTGCAGC

Fig. 1. The relative positions and sequences of the 6 oligos used to to try to deplete cytokeratin XCK1(8) mRNA. Oligos 1-4 are all within the linker1b/2 domain and oligos 5 and 6 are in coil 2. The relative positions of the two RNAase protection probes used in Fig. 2 are also shown

showing an experiment to compare the ability of six oligos complementary to the XCK1(8) mRNA. Only one of the six oligos (CK5) was effective in depleting the mRNA at doses of 10 ng. This amount of oligo is uncomfortably close to the 15-20 ng dose, which we and others (Woolf et al., 1990) have shown to cause non-specific toxicity in embryos. So to improve the efficiency and reduce the toxicity of oligo treatment, we made a modified form of CK5 (Integrated DNA Technology). This modification was suggested and tested by Dagle et al. (1990). It involves converting the first and last five phosphodiester bonds to methoxyethyl phosphoramidate linkages, which are not substrates for RNAase H. This leaves the central part of the oligo still available for cleavage, but the molecule is more resistant to exonuclease activity (Dagle et al., 1990). They showed that modified oligos are still detectable 40 minutes after injection into oocytes and 30 minutes after injection into fertilized eggs, in contrast to a 10 minute maximum for unmodified oligos. In our hands, such modified forms of oligos are 10-20 times more efficient than their unmodified counterparts, and can thus be injected in amounts ranging from 0.2 to 4 ng to cause substantial (greater than 95%) depletion of target mRNAs (Fig. 2). Once the mRNA in full-grown oocytes is depleted, it is not resynthesized over a period of 60 hours (Fig. 3).

Fertilization of ovarian oocytes

Two methods have been described to fertilize ovarian oocytes (Heasman et al., 1992) neither of which is easy but both of which have given some success in fertilizing oligo-injected oocytes (Kloc et al., 1989; Torpey et al., 1992a). We routinely use the oocyte transfer technique described previously (Holwill et al., 1987). Briefly, this involves maturing the cultured and injected oocytes with progesterone (1 μM) for 8 hours and then vital staining control

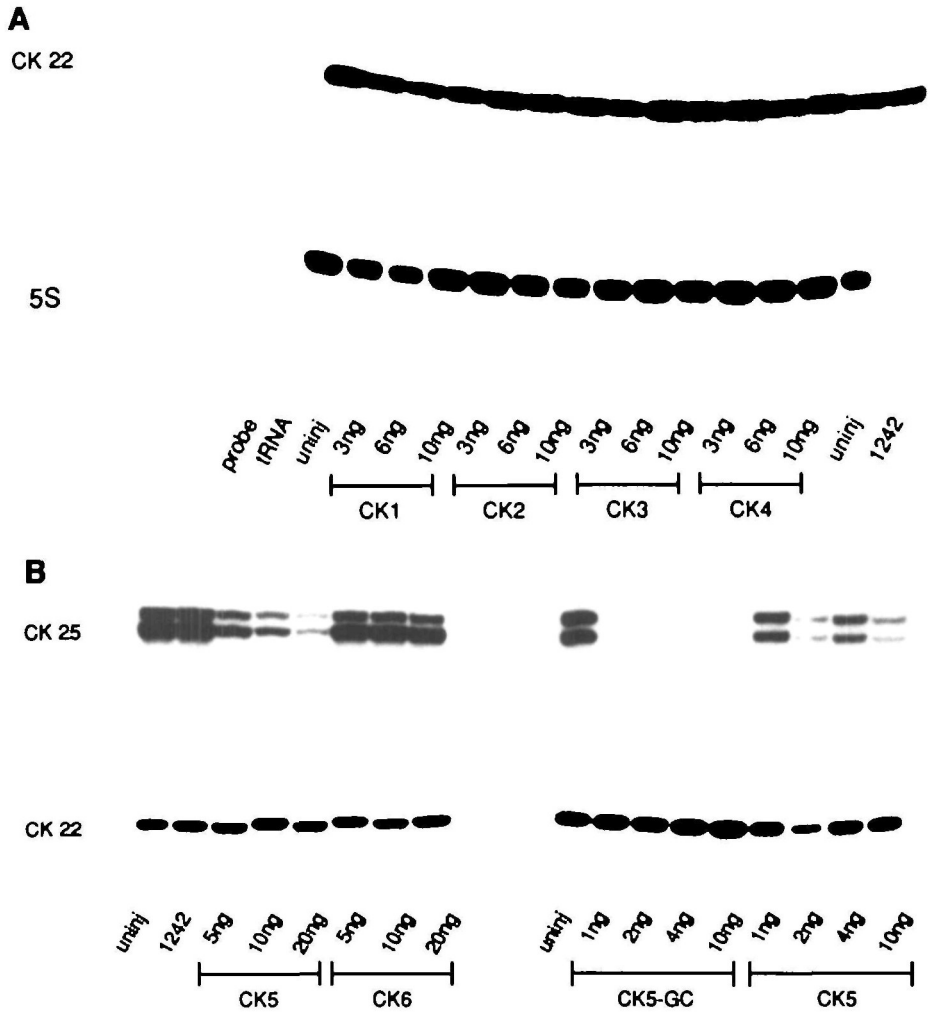


Fig. 2. The effects of oligos on cytokeratin XCK1(8) mRNA. (A) RNAase protection assay using the CK22 probe on RNA extracted from oocytes that had been injected with oligos 1-4 of Fig. 1. The oligos are ineffective at depleting the XCK1(8) mRNA. A small sample of the RNA extracted from each batch was removed and analysed with a 5s RNA probe to control for RNA recovery. (B) RNAase protection assay using the CK25 probe (which spans the target site of the oligos) on oocytes that were injected either with unmodified oligos 5 (CK5) and 6, or with the phosphoramidate-modified form of CK5 (CK5-GC). Oligo 6 does not deplete the mRNA for XCK1(8), but oligo 5 is effective at doses of 10-20 ng. The modified form of CK5 is effective in doses of 1 ng. The CK 22 probe does not span the target sites of these oligos but a position towards the 5′ end of the mRNA. Note that there is very little degradation of the 5′ fragment of the cleaved mRNA.

and experimental groups with vital dyes. The coloured oocytes are then transferred into an anaesthetized host which has been stimulated with human chorionic gonadotrophin to lay eggs. The host female recovers in anaesthetic-free water and lays the coloured eggs 3-5 hours later. The eggs are then fertilized using a sperm suspension.

This method of fertilization is very variable in its success rate, the main variability being in the status of the oocytes themselves. It is essential to use full-grown stage-6 oocytes which have not become over-ripe, but this is generally difficult to assess. However, a high-yield experiment will result in groups of 30-40 fertilized eggs for each experimental batch.

The merits of injection of oligos into oocytes rather than into fertilized eggs

Given the difficulty of fertilizing ovarian oocytes, we wondered if phosphoramidate-modified oligos might be sufficiently efficient in denaturing mRNAs to allow effective depletion without toxicity after injection of small quantities into fertilized eggs. We were encouraged by the result that 10 ng of anti-cyclin oligo had a specific and non-toxic effect in 2-cell-stage embryos (Dagle et al., 1990). In a series of experiments, we monitored the effect of different doses of a modified CK5 injected into 2- and 4-cell-stage embryos both on mRNA levels (Fig. 4) and on embryonic development (Fig. 5). We found that the modified oligos were very efficient. For example, 0.8 ng of modified CK5 oligo injected into 2 cells of the 4-cell-stage embryo reduces the levels of cytokeratin mRNA to 10-20% of control levels, while 0.4 ng is sufficient to produce the same effect when injected into fertilized eggs (Fig. 4). Unfortunately this oligo, together with other unrelated modified oligos (specific for MyoD and vimentin mRNA) injected at the same concentrations, cause similar abnormalities (Fig. 5). On this basis, we judge that the phenotype (irregular cell size due to abnormal cleavages, patchy pigment and lack of adhesion leading to pregastrula arrest) is due to non-specific effects. A similar conclusion was drawn in a recent study using phosphorothioate-modified oligos (Woolf et al., 1990). Eggs developing from modified oligo-injected oocytes in the same dose range (1-5 ng) do not show this phenotype. Since oocytes seemed so much less liable to non-specific effects, we concentrated on these in our mRNA depletion studies.

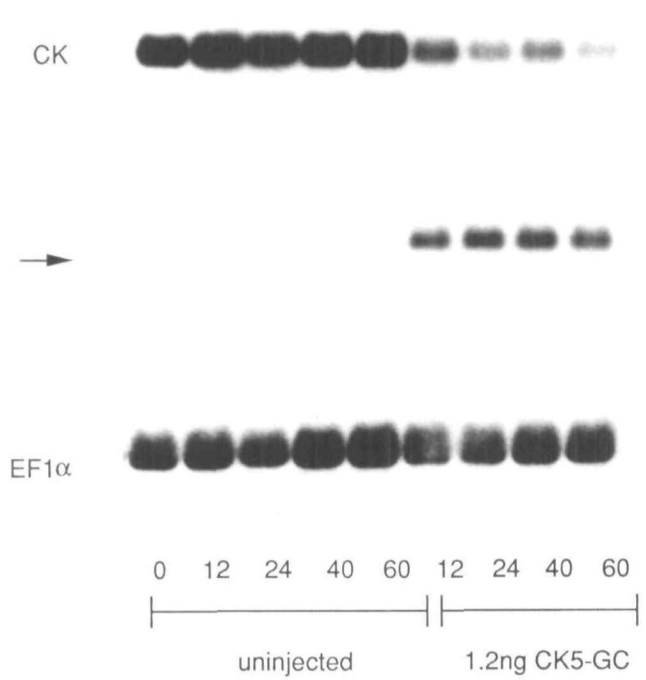


Fig. 3. A northern blot of RNA extracted from oocytes injected with 1.2 ng CK5-GC and frozen at intervals. The blot was probed with a random primed probe for XCK1(8). A group of control uninjected oocytes were analysed in parallel. Arrow indicates the stable 5′ fragment of the cleaved XCK1(8) mRNA.

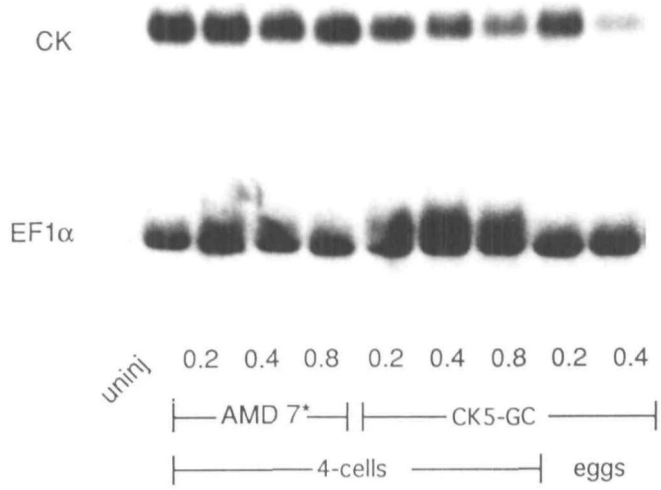


Fig. 4. A northern blot of RNA extracted from 4-cell-stage embryos or fertilized eggs injected either with various doses of modified CK5 oligo (CK5-GC) or with a modified oligo which depletes the mRNA for an unrelated protein MyoD (AMD7*). The blot was probed with a random primed probe for XCK1(8).

The effects of depleting mRNA in ovarian oocytes

One very good indication of the effectiveness of an oligo knock-out of a specific mRNA is to show that once the RNA has been degraded there is an effect on the total amount of target protein and on the levels of new protein synthesis compared to other proteins. We have shown this in the case of XCK1(8) mRNA in a number of ways.

Firstly, we cultured modified CK5-injected oocytes for increasing lengths of time, and the effect of the depletion of XCK1(8) mRNA on cytokeratin protein was monitored in two ways, immunocytochemically, using an XCK1(8) specific antibody, and by 2-dimensional fluorography (Torpey et al., 1992a). Keratin filaments are normally most abundant in the cortices of oocytes where they maintain a cortical lattice for at least 3 days in culture. In contrast, oocytes that have been injected with CK5 oligo and cultured for 60 hours show no visible network. Interestingly, these cytokeratin filaments are obligate heteropolymers of acidic (XLK3a/19) and basic (XCK 1(8)) keratin subunits. We wondered if the oligo was also depleting the acidic cytokeratin mRNA. However, on reprobing a northern blot of CK5 injected-oocyte RNA with an acidic keratin-specific probe, we found that the XLK3a/19 m RNA level was unaffected. When we compared the amounts of newly synthesized XCK1(8) and other proteins by 2-dimensional fluorography of uninjected and CK5-injected oocytes, we found that a protein in the correct position to be the acidic partner of XCK1(8) also showed reduced synthesis after CK5 oligo injection. Therefore, it seems likely that the loss of the basic cytokeratin protein destabilizes the acidic protein, as has been described in other systems for a number of cases of keratin heteropolymers (Domenjoud et al., 1988; Knapp and Franke, 1989; Lu and Lane, 1991).

A second strategy, which is proving successful in showing that depleting mRNA levels has a specific effect on the protein product, is to metabolically label oligo-injected and control oocytes with [^{35}S]methionine for 24 hours and then immunoprecipitate the protein with an appropriate specific antibody.

The phenotypes of maternal mRNA-depleted embryos: how can we know they are specific?

The denoument of these experiments is to fertilize a batch of embryos that has had a target mRNA-depleted as oocytes, and to look for developmental abnormalities. In the light of the known potential toxicity of oligos and the inherent variability between batches of oocytes, it is essential to know that any effect is specifically the result of the absence of the target mRNA and its protein product. This can be achieved by a combination of controls.

(1) Non-specific toxicity

There is clear evidence from this and other work that oligos both in their modified and unmodified forms are toxic above certain concentrations. The toxic phenotype varies from complete arrest at fertilization, to a slowing and abnormal pattern of cleavage, combined with changes in pigment distribution and loss of adhesion, culminating in arrest, generally before gastrulation. Interestingly oligos very seldom

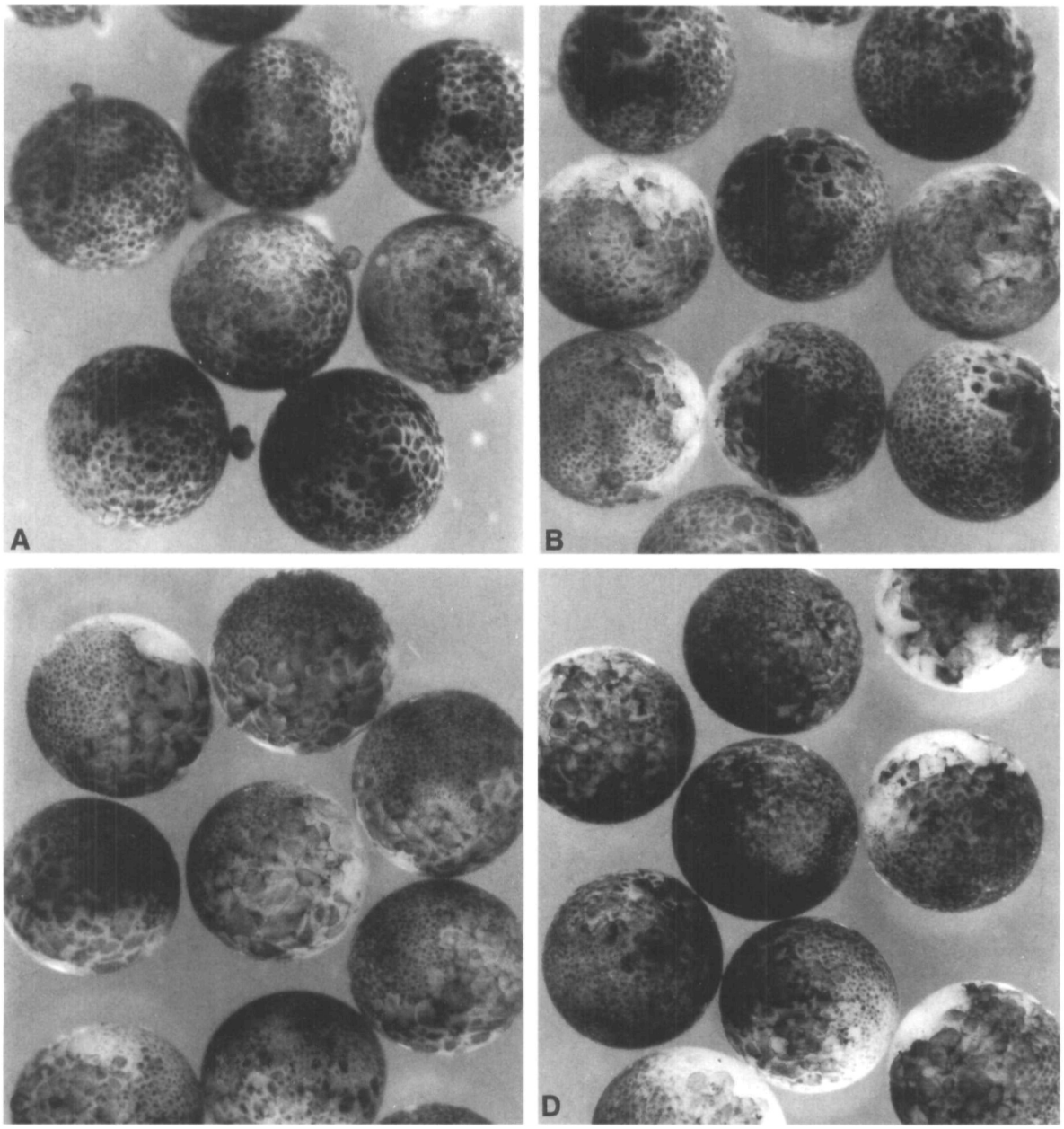


Fig. 5. Injection of modified oligos into embryos causes non-specific abnormalities. Embryos were injected with modified oligos at the 4-cell-stage (2 of 4 cells injected, 0.4 ng each blastomere), and photographed at the late blastula stage. Oligos used were: (A) AMD7*, a modified oligo that depletes MyoD mRNA, (B) Oct, an irrelevent modified oligo, (C) 1242, a modified oligo that depletes vimentin mRNA and (D) CK5-GC, which depletes CK5 mRNA. Patches of cells showing slowed or arrested cleavage and abnormal pigmentation are visible in all cases, although they are most obvious in 1242 injected embryos. Uninjected or water-injected embryos do not show this effect.

prevent maturation or activation. The basis of this toxicity is unknown, but may be due to the high levels of deoxynucleotides released by degradation of the oligo, or by secondary structures that interfere with normal metabolic reactions. When used in very high amounts (greater than 20 ng) oligos also suppress general levels of protein synthesis. This can be checked for by ^{35}S labelling and avoided by using small amounts of modified oligos. We have also ruled out the possibility that a contaminant of the oligo synthesis is the cause of toxicity in the following way: when sense and antisense oligos are synthesized in the same way and injected together into oocytes there is no increase in abnormal phenotype. Indeed for CK5 oligo directed against the cytokeratin mRNA the phenotype is rescued, suggesting the specificty of the knock-out. The potential problem of oligo toxicity is avoided in our experiments by using oocytes rather than fertilized eggs for injections, and by keeping the oligo dose as low as possible by using phosphoramidate-modified oligos. Using this regime, none of the oligos show non-specific toxicity at doses of 0.2-4 ng.

(2) Non-specific RNA knock-out

It seems quite feasible that one oligo complementary to a target mRNA may also by chance hybridize to a totally different mRNA. This can best be controlled for by having more than one oligo of different sequences complementary to different parts of the target mRNA. If they all cause the same phenotype then the chance of them all also recognizing the same non-target species is vanishingly small. Similarly, irrelevant modified oligos particularly ones that target other mRNAs are also used to show the sequence specificity of the phenotype. Also reprobing blots with probes for related mRNAs to the target mRNA can be reassuring about the specificity of a knock-out. Finally, a good test of a specific effect is to show that depletion of the oligo results in a reduction of the target protein levels in the developing embryos. This can be achieved by immunocytochemistry (Torpey et al., 1992a).

Oocyte variability

The age-old cure for oocyte variability is to repeat the experiment on many batches with large numbers. Every oligo experiment is accompanied by a northern blot to show the mRNA is depleted in each case, and great care is taken to ensure that no oocytes miss injection.The final test of the specificity of a phenotype is to rescue it by injecting the full-length mRNA back into the oocyte. This in itself is not without difficulty, particularly when modified oligos have been used, which remain sufficiently active to degrade injected mRNA some time after their (the oligos') introduction.

Phenotypes and conclusions

One reassuring outcome of these experiments is that oligos targetted on different maternal mRNAs cause different phenotypes in development. Preliminary results suggest that vimentin mRNA is required in the early cleavage stages, while EP cadherin mRNA is needed to top up the adhesive protein levels by the mid-blastula stage (our unpublished observations). In contrast, cytokeratin mRNA-depleted oocytes, which have a large store of protein do not show a visible phenotype until the late blastula stage (Torpey et al., 1992a), although immunocytochemical studies show that keratin filaments are much shorter and disorganized by the mid-blastula stage. At the beginning of stage 9 (late blastula) in the control batches, the cortical cells flatten and compact over the embryonic surface in a movement that may mark the beginning of epiboly, and resembles compaction in mouse embryos. In the XCK1(8) depleted embryos, all the surface cells remained raised up, giving a 'cobblestone' appearance. The cell division rate and pigmentation pattern remained unaffected. A second feature of the phenotype is that experimental embryos lose the ability to heal a wounded surface, a feat that is usually completed within an hour for a small cut in the surface of a control oocyte. Finally the XCK1(8) depleted embryos also have a defect at gastrulation. If the vitelline membrane is left in place, the cells that normally invaginate, evaginate as a sheet advancing over the animal cap. If the vitelline membrane is removed, the embryos form classic exogastrulae. This effect is not seen when irrelevant oligos are injected and is almost completely rescued by co-injection of sense and antisense CK5.

These results point to a role for the cytokeratin filaments in producing and maintaining an integrated cortical shell around the embryo. Without them the epithelial surface is destabilized and cannot support normal gastrulation movements. A rather similar result has come from work on the mouse and human cytokeratin 14. Several pieces of work have shown that lack of this keratin, either in transgenic mice (Vassar et al., 1991) or in human hereditary conditions (Coulombe et al., 1991; Lane et al., 1992), results in loss of mechanical strength in the skin.

In conclusion, there are now several means of depleting or functionally perturbing the mRNAs and/or proteins inherited from the oocyte by the early embryo. There are many obvious candidates for targetting, including the cytoskeleton, cell surface and the signalling molecules responsible for early cell specification.

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