A POU gene required for early cleavage and protein accumulation in the sea urchin embryo

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SUMMARY

SpOct is a POU gene expressed during oogenesis and early embryogenesis of the sea urchin, Strongylocentrotus purpuratus. In the first use of antisense technology in the sea urchin embryo, we report that disruption of SpOct gene function in 1-cell zygotes by the injection of antisense oligodeoxynucleotides arrests development prior to the first cell division. We show that single-stranded antisense oligodeoxynucleotides specifically block cleavage, and that injection of SpOct mRNA overcomes this block. The accumulation of [35S]methionine into zygotically synthesized protein is significantly reduced in antisense-injected embryos. DNA synthesis is also reduced by the antisense regimen as expected from the antisense inhibition of protein accumulation. That protein accumulation prior to the first cleavage is retarded by antisense targeting of a transcription factor is very surprising in light of classical work showing that the activation of protein synthesis does not require zygotic transcription. We conclude that either some new transcription is obligate for the accumulation of new protein, or that the SpOct gene plays a novel, non-transcriptional role in this process.

Key words: sea urchin, POU transcription factor, SpOct gene, cleavage, cell division

INTRODUCTION

The POU proteins, characterized by the highly conservative DNA-binding motif known as the POU domain, include the Oct and Pit families of transcription factors, and the Unc 86 gene product of Caenorhabditis elegans. Members of these gene families have been found in organisms ranging from mammals to arthropods (Rosenfeld, 1991; Verrijzer and Van der Vliet, 1993), and several are expressed in a developmental stage-specific (He et al., 1989; Billin et al., 1991; Dick et al., 1991) or tissue-specific manner (Clerc et al., 1988; Gerster et al., 1990; Tanaka and Herr, 1990). We recently isolated a sea urchin (Strongylocentrotus purpuratus) POU gene, designated SpOct, whose derived protein’s amino acid sequence places it in the Oct family, equidistant from mammalian Oct 1 and Oct 2 (Char et al., 1993). SpOct is expressed in oogenesis and early embryogenesis, and it encodes the predominant octamer-binding protein in early embryos.

There is little information on the function of POU genes in early development. Oct 1 and Oct 2 can stimulate the replication of adenovirus DNA (O’Neill et al., 1988; Verrijzer et al., 1990), suggesting that they may have a role in DNA replication. Injection of an excess of the octamer target DNA sequence into sea urchin zygotes results in embryonic arrest at various stages from blastula to pluteus (Franks et al., 1990). Although the identity of the proteins that bind the octamer element in this experiment is not known, the developmental arrest phenotype suggests that at least some targets of SpOct regulation are critically important for embryogenesis. One such target may be the α H2B histone gene: an octamer element is necessary for the zygotic expression of this gene, and the SpOct protein binds the α H2B octamer element avidly (Char et al., 1993; Bell et al., 1992).

We wished to examine further the function of the SpOct gene in sea urchin development. At the same time, we wished to test gene perturbation technologies that would allow direct analysis of gene function in the sea urchin embryo, a simple developmental system, well-suited to experimental embryology, but lacking genetics. One approach to reducing the activity of a specific gene in embryos is to microinject antisense oligonucleotides against a target mRNA into 1-cell zygotes or blastomeres of early embryos, thereby triggering the degradation of the mRNA by RNAse H (Minshull and Hunt, 1986; Dash et al., 1987). In the first use of this technology in the sea urchin embryo, we show that reduction of SpOct gene function in 1-cell zygotes by the injection of antisense oligodeoxynucleotides specifically blocks cleavage and retards the incorporation of [35S]methionine into protein. This effect on protein accumulation is surprising in light of classical work showing that protein synthesis prior to the first cleavage does not require zygotic transcription (reviewed in Davidson, 1977), and it suggests that the SpOct protein may play a novel, non-transcriptional role in this process.
MATERIALS AND METHODS

RNAse protection

RNAse protection analysis of α H2B histone mRNA was carried out as described (Colin et al., 1988; Bell et al., 1992). The probe was a 500 nt, 32P-labeled antisense RNA spanning the transcription start.

Oligonucleotide (ODN) preparation and sea urchin zygote injections

All ODNs were synthesized on an Applied Biosystems DNA synthesizer. ODNs were passed over a Sep-pak (C18) column (Waters), ethanol precipitated, and then purified on an Elutip column (S&S) followed by another ethanol precipitation. The nucleotide sequences of the ODNs were: α H2B histone antisense, 5′-TGGAGCCATGAT-GAA-3′ (spanning the translation start codon), its sense strand, 5′-TTTCACTGCGTCCA-3′; fibropellin 1 antisense ODN, 5′-GATCCAGGTGGTGTAGAGAA-3′; fibropellin 1 antisense cDNA, a3 (5′-TCATGGTCATGAGGACA-3′, the inverse complement of nucleotides 3200-3219 of the fibropellin 1 gene, Grimwade et al., 1991); SpOct antisense ODNs a1 (5′-TCCGGGGCTATTGTGAG-3′, the inverse complement of nucleotides 479-495 in the SpOct cDNA), a2 (5′-CACCCCTGGAAGT-AGCC-3′, the inverse complement of nucleotides 479-495 in the SpOct cDNA), a3 (5′-TCCATGGTCATGAGGACA-3′, the inverse complement of −10 to +11 in the SpOct cDNA), and a4 (5′-ATCCTGGCAGCTGGAGATGAGG3′ complementary to −56 to −36 in the SpOct cDNA); and control ODNs c2 (5′-ACACACATTAGGATACG-3′; nucleotides 1722-1739 in the SpOct cDNA), c1 (5′-CAAGCCGAGCAGGAGG-G-3′, a fragment of 5′ non-translated sequence from the mouse Msx2 gene) and c3 (5′-GCTGAGCAAGGATGAGAC-3′, a randomized version of a3).

ODNs were injected at a concentration of 1 mg/ml in 25% sterile glycerol, except when otherwise indicated. Injections were performed immediately after eggs were fertilized as described (Bell et al., 1992). 30-50 embryos were injected per dish, with both antisense and control ODNs usually injected into the same batch of eggs. Injected embryos were incubated at 15°C. Experiments were considered valid only if 70% or more of the embryos survived the injection. Only embryos that appeared viable under the light microscope through 8 hours were counted; those damaged during the injection process were discarded. Non-injected, fertilized embryos were also followed over the course of each experiment (data not shown).

In vitro transcription of injected mRNAs

Full-length SpOct (Char et al., 1993) and a genomic clone of the Spurpurata SpMsx gene (S. Dobias and R. Maxson, unpublished data) were transcribed using the Megascript kit (Ambion). Transcripts were capped at their 5′ ends with m7G5′ppp5′G (Ambion) and co-injected with ODNs into 1-cell zygotes at a concentration of 1.5 mg/ml.

Measurement of DNA synthesis

Following injection, embryos were incubated at 15°C in filtered sea water containing 50 mCi/ml of [3H]thymidine. After 2.5 hours, embryos were collected and washed three times in sea water and lysed in 0.5% SDS. 20 mg of yeast RNA was added to each sample and DNA was precipitated with ice-cold TCA at a final concentration of 10%. Samples were collected on GF/C filters and washed four times with 5 ml of 10% TCA, and three times with 5 ml of cold 95% ethanol. Filters were air-dried, treated with Protosol and counted in Omnifluor (New England Nuclear).

Measurement of protein synthesis

Embryos were incubated at 15°C in filtered sea water containing 50-100 mCi/ml of [35S]methionine (Amersham) immediately after injection. They were collected by centrifugation, washed 3× with sea water, lysed in 20 ml of NETS-EGTA (Bell et al., 1992) and precipitated with ice-cold 10% TCA. The precipitates were collected by centrifugation, resuspended in 2× SDS sample buffer and either dried on a GF/C filter (Whatman) for counting, or loaded on an 8.5% acrylamide-SDS gel.

RESULTS

To test the applicability of the antisense approach to the sea urchin embryo, we first asked whether injection of antisense oligodeoxynucleotides against a maternal mRNA caused its degradation. We chose as the test subject the abundant maternal message encoding α H2B histone (Maxson et al., 1983) rather than the rare and thus difficult to quantitate SpOct mRNA (Char et al., 1993). Antisense α H2B and control ODNs were injected into fertilized eggs, and RNA was harvested and analyzed for α H2B mRNA by RNAse protection. The amount of α H2B mRNA was substantially reduced in the antisense injected eggs (Fig. 1A). We tested the specificity of the antisense-mediated degradation of mRNA by injecting an antisense ODN against SpOct and monitoring α H2B histone mRNA levels. SpOct ODN-injected embryos contained levels of α H2B mRNA identical to control-injected embryos (Fig. 1B). The data presented in Fig. 1 thus demonstrate that, in the sea urchin embryo, as in Xenopus, antisense ODNs specifically cause the degradation of their target mRNAs.

We next asked whether the injection of ODNs directed against the SpOct mRNA affected embryonic development. We used three different antisense and corresponding control ODNs synthesized at different times, thus minimizing the possibility that antisense-induced phenotypes were the result of contaminants in the ODNs. Control ODNs were an SpOct sense-strand, a random sequence ODN, or an antisense ODN for an unrelated gene (For ODNs sequences see Materials and Methods). Injection of ODN a2 resulted in the cleavage-arrest of 69% of the embryos (Fig. 2A). Injection of a1 (Fig. 2B) or a3 (data not shown) resulted in the cleavage arrest of approximately 30% of the embryos and a retardation in the rate of development of a large proportion of injected zygotes. Pooled data from experiments carried out with a1, a2 and a3, as well as control ODNs c1, c2 and c3 are shown in Fig. 2C. Upon injection of antisense ODNs, 74±11% of the embryos failed to undergo the first embryonic cleavage at the normal time while only about 10±3% of control ODN-injected embryos remained uncleaved.

That cleavage arrest was caused by three different antisense ODNs and not by control ODNs strongly suggested that this effect was specific. Nevertheless, to rule out decisively the possibility that contaminants caused the arrest in development, we injected 1-cell zygotes with a double-stranded ODN composed of the a1 antisense ODN annealed with its complement. In parallel, ODN a1 was mixed with an irrelevant ODN, c1, and injected into embryos. Only 13±2% of the embryos injected with the antisense-irrelevant ODN mixture cleaved by 2 hours, whereas 85±2% of the double-stranded ODN-injected embryos did so (Fig. 3A). At 4 hours, cleavage had occurred in 25% of antisense-irrelevant ODN-injected embryos and 100% of double-stranded ODN-injected embryos (data not shown). Thus, the cleavage-arrest phenotype was not caused by a cytotoxic contaminant in the antisense ODN solution.

If single-stranded antisense ODNs cause developmental
arrest by promoting the degradation of SpOct mRNA, then microinjection of in vitro-synthesized SpOct mRNA into antisense SpOct ODN-injected embryos should rescue them, while microinjection of an irrelevant mRNA should not. We injected an antisense ODN against the SpOct 5' non-coding sequence (ODN a4) into 1-cell zygotes along with either SpOct mRNA or an irrelevant homeobox mRNA encoding a sea urchin Msx-class protein (S. Dobias and R. Maxson, unpublished data). The co-injected SpOct mRNA was transcribed from a cDNA lacking the 5' non-coding sequence to which the ODN was complementary. Co-injection of SpOct mRNA enabled 64±3% of the embryos to cleave by 2 hours,
whereas 12±3% of the control-injected embryos did so (Fig. 3B). By 4 hours, cleavage had occurred in 87±3% of $SpOct$ mRNA-injected embryos and 36±5% of control mRNA-injected embryos (data not shown). We conclude that injection of synthetic $SpOct$ mRNA can rescue embryos in which endogenous $SpOct$ gene function has been disrupted, demonstrating both that antisense $SpOct$ ODNs act sequence-specifically to cause cleavage arrest, and that their target is $SpOct$ mRNA.

What cellular processes are affected by the antisense $SpOct$ regimen? In $S. purpuratus$, the rate of protein synthesis increases dramatically 5-10 minutes after fertilization, following a change in membrane potential, release of $Ca^{2+}$ from internal compartments, and an increase in intracellular pH (Whitaker and Steinhardt, 1982). This activation of protein synthesis and the accumulation of new protein during the first few hours of development is mediated by maternal mRNAs (Dube et al., 1985). When fertilized eggs are treated with the protein synthesis inhibitors, DNA synthesis in the first cell cycle occurs normally, but development is arrested early in the first mitosis, and cell division fails to occur (Wilt et al., 1967; Wagenaar, 1983). This phenotype is similar to that observed in embryos injected with antisense $SpOct$ ODNs. We thus hypothesized that the $SpOct$ antisense treatment affected protein synthesis. As a test of this hypothesis, we measured $[^{35}S]$methionine incorporation into newly synthesized proteins in antisense $SpOct$ ODN-injected and control ODN-injected embryos. Following injection of ODNs, zygotes were incubated with $[^{35}S]$methionine for 2 hours. In experiments in which antisense ODNs a3 or a1 were injected, incorporation of $[^{35}S]$methionine was reduced to 31±6% as compared to ODN c2-injected embryos (Fig. 4). Size-separation of $[^{35}S]$methionine-labeled protein on one-dimensional SDS-PAGE gels failed to provide evidence for a selective effect on any size class of proteins (data not shown). These results indicate that the injection of antisense $SpOct$ ODNs causes a significant, general inhibition in protein accumulation. This inhibition is not likely to be due to a general effect of antisense ODNs on protein accumulation or mRNA templates, since antisense ODNs against two maternal mRNAs other than $SpOct$, $\alpha$H2B histone mRNA and fibropellin 1 ($SpEGF 1$) mRNA (Grimwade et al., 1991), did not reduce methionine incorporation into protein (Fig. 4). Neither did injection of $SpOct$ antisense ODNs reduce levels of $\alpha$H2B histone mRNA (Fig. 1).

The role of Oct-1 in the replication of adenovirus DNA (O’Neill et al., 1988; Verrijzer et al., 1990) suggested that SpOct may participate in DNA replication in cleaving sea urchin blastomeres. Since the first S-phase is insensitive to inhibitors of protein synthesis (Wilt et al., 1967; Wagenaar,
the translation of SpOct mRNA is not required for the first S phase (though maternal SpOct protein might be). The question of whether SpOct is required for the second S phase, approximately 120 minutes after fertilization, is confounded by the fact that the second S phase does require new protein synthesis. Based on protein synthesis inhibitor studies, a 3-fold reduction in protein accumulation, at the threshold of the amount required to affect DNA replication, should cause a slight to moderate effect on [$^{3}$$\text{H}$]thymidine incorporation into DNA during the second S phase (F.H. Wilt, personal communication). Consistent with this prediction, we found that [$^{3}$$\text{H}$]thymidine incorporation into DNA in antisense-treated embryos in a 2.5 hour interval after fertilization was 77±13% of control ODN-injected embryos (Fig. 5). Essentially all of this incorporation was aphidicolin-sensitive (data not shown) and thus represented incorporation into nuclear DNA (Huberman, 1981). In such a continuous labeling regimen comprising both the first and second S phases, approximately two thirds of the [$^{3}$$\text{H}$]thymidine should have been incorporated in the second S phase. Thus we would expect a partial inhibition of [$^{3}$$\text{H}$]thymidine incorporation in the second S phase to result in 33% incorporation relative to controls. Our finding of 77% incorporation is therefore consistent with a partial inhibition of DNA replication during the second S-phase as expected from a 3-fold reduction of protein accumulation during the first 2 hours of development.

DISCUSSION

We have demonstrated that injection of antisense ODNs against the SpOct mRNA both retards cleavage and reduces protein accumulation in sea urchin zygotes. Several lines of evidence support the view that these antisense effects on embryonic cleavage and protein accumulation are a specific consequence of the SpOct ODNs. First, injection of antisense, sense, or duplex sense-antisense ODNs rules out a dominant, non-specific inhibitory contaminant in the antisense ODN preparation as the cause of the antisense phenotype. Second, injection of SpOct mRNA rescues the cleavage arrest phenotype, demonstrating that it is a specific result of the targeting of SpOct mRNA. Third, antisense ODNs against two other maternal mRNAs, $\alpha$ H2B histone and fibropellin 1, do not affect either cleavage or protein synthesis. Therefore, neither the cleavage-arrest nor the protein accumulation phenotypes can be attributed to a general effect of ODN-mRNA hybrids. Our results thus demonstrate that antisense ODNs offer an effective means of targeting maternal templates in sea urchin embryos. Since we do not know the half-life of injected ODNs, we do not yet know if they will prove effective against zygotic mRNAs that accumulate after early cleavage.

Antisense ODNs have been shown to affect transcript levels, translation or both (Minshull and Hunt, 1986; Dash et al., 1987; Stein and Cheng, 1993). Our analysis of a model template, $\alpha$ H2B histone mRNA, suggests an effect on mRNA amount, probably via an RNAse H-mediated cleavage of the mRNA template. However, since $\alpha$ H2B histone mRNA is sequestered

Fig. 4. Incorporation of [$^{35}$S]methionine into protein in SpOct antisense ODN-injected embryos. Antisense and control SpOct, fibropellin 1 and $\alpha$ H2B histone ODNs were injected into 1-cell zygotes and the incorporation of [$^{35}$S]methionine into newly synthesized protein was measured over a 2 hour time course. SpOct antisense ODNs a1 and a3 were compared to control ODN c2. The columns refer to percentage incorporation of [$^{35}$S]methionine relative to control-ODN injected embryos ± s.e.m. of 5 experiments with SpOct ODNs and 3 experiments with fibropellin (SpEGF) 1 and $\alpha$ H2B histone ODNs. Approximately 50-60 embryos were injected for each ODN per experiment. Total [$^{35}$S] incorporated for control-injected embryos was 6000-8000 counts/minute.

Fig. 5. Incorporation of [$^{3}$$\text{H}$]thymidine into DNA in SpOct antisense ODN-injected embryos. Approximately 60 embryos were injected with antisense ODN a1 or a3 in five separate experiments. A similar number were injected with control ODNs. Following injection, embryos were incubated at 15°C in filtered sea water containing [$^{3}$$\text{H}$]thymidine. After 2.5 hours, embryos were collected, lysed in buffer containing SDS and the DNA precipitated with TCA. TCA-insoluble radioactivity was determined by scintillation counting. The columns represent the relative incorporation of [$^{3}$$\text{H}$]thymidine ± s.e.m. Total [$^{3}$$\text{H}$] incorporated for control-injected embryos was 978-3988 counts/minute.
in the nucleus in the 1-cell zygote until approximately 80 minutes after fertilization (Showman et al., 1982; DeLeon et al., 1982), antisense ODNs may affect it differently from other sea urchin maternal mRNAs. The analysis of additional maternal templates will clarify this point.

Our data demonstrate that $SpOct$ antisense ODNs inhibit protein accumulation over a 2 hour interval following fertilization. We have not performed a kinetic analysis of the antisense effect; thus we do not know whether the effect is at the level of protein synthesis, decay, or both. Whichever process is affected, it is extremely surprising that $SpOct$, a member of the well-studied family of Oct transcription factors (Char et al., 1993), is required for the accumulation of new protein during early cleavage, a process that does not depend on new transcription. Classical experiments have shown that embryos reared in the presence of the transcription inhibitor actinomycin D accumulate radioactive amino acids into new protein during the first few hours of development in a manner indistinguishable from control embryos (Gross and Cousineau, 1964). Moreover, enucleated merogones, when parthenogenetically activated, synthesize protein at a near-normal rate (Brachet et al., 1963; Denny and Tyler, 1964; Craig and Piatigorsky, 1971). These data have long been interpreted as evidence that egg activation, including the activation of protein synthesis and early accumulation of protein, is independent of zygotic transcription (e.g., Davidson, 1977).

Why then is $SpOct$ mRNA, and presumably $SpOct$ protein, required for protein accumulation during early development? Our results are consistent with two possibilities, each requiring revision of a currently accepted paradigm. First, in spite of the actinomycin D data (Gross and Cousineau, 1964), some new transcription may be required for the early accumulation of protein, and $SpOct$ may play a role in such transcription. In the actinomycin D experiments, a small amount of zygotic transcription could have escaped the drug blockade, allowing the synthesis of a sufficient amount of a critical factor to activate translation and accumulate new protein normally. The merogone experiments (Brachet et al., 1963; Denny and Tyler, 1964; Craig and Piatigorsky, 1971) pose a more significant challenge to the proposition that zygotic transcription is required for events in egg activation and early cleavage. It is possible that, although enucleated merogones initiate protein synthesis in a quantitatively normal way upon parthenogenetic activation, they do so via a non-physiological mechanism that is distinct from that of normal egg activation.

A second, and we think more likely explanation of the $SpOct$ antisense phenotype is that $SpOct$ has a novel, non-transcriptional function in protein accumulation during early cleavage. It may, for example, participate in the mobilization of maternal mRNA for translation; it may have a direct role in translation; or it may be a component of the signal transduction pathway entrained by the interaction of the sperm with the cell membrane and culminating in the activation of protein synthesis.

The $SpOct$ antisense effect on protein accumulation can explain why antisense-treated embryos cleave at an abnormally slow rate. The general inhibition of protein synthesis probably prevents the normal accumulation of proteins required for the process of cell division, likely including cyclins (Rosenthal et al., 1980). In the sea urchin, cyclins are encoded by maternal mRNAs (Evans et al., 1983). Synthesis of these proteins increases linearly after fertilization in parallel with general protein synthesis. They are then destroyed during cell division and are resynthesized in the following cell cycle from stored templates (Swenson et al., 1986; Standart et al., 1987). There is a narrow time window during which the protein synthesis inhibitor emetine causes cleavage arrest. This window coincides with the synthesis of B-type cyclins, which serve as a regulatory subunit of MPF, a protein complex composed of the p34cdc2 protein kinase and cyclin B (Draetta et al., 1989). By preventing the normal activation of protein synthesis, injection of antisense $SpOct$ ODNs probably interferes with the activity of MPF, and consequently prevents or retards cell division.

We point out, however, that, although an effect on MPF is an attractive explanation of the retardation of cleavage, it is possible that this phenotype is unrelated to the effect of the $SpOct$ antisense ODNs on protein synthesis. $SpOct$ may regulate the activity of genes other than cyclin B-p34cdc2 that are required for progression through the cell cycle. Although there are no obvious candidates for such genes, we can be fairly certain that histone genes are not among them. Even if inhibition of $SpOct$ gene activity reduced the level of $SpOct$-dependent transcription of a $H2B$ histone genes, such a reduction probably would not cause early developmental arrest since sea urchin eggs are endowed with a sufficiently large store of histone mRNA and protein to meet the demands of DNA synthesis at least to the midblastula stage (Poccia, 1986).

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