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# Cyclin: A Protein Specified by Maternal mRNA in Sea Urchin Eggs That Is Destroyed at Each Cleavage Division

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## Summary

**Cleavage in embryos of the sea urchin Arbacia punctulata consists of eight very rapid divisions that require continual protein synthesis to sustain them. This synthesis is programmed by stored maternal mRNAs, which code for three or four particularly abundant proteins whose synthesis is barely if at all detectable in the unfertilized egg. One of these proteins is destroyed every time the cells divide. Eggs of the sea urchin Lytechinus pictus and oocytes of the surf clam Spisula solidissima also contain proteins that only start to be made after fertilization and are destroyed at certain points in the cell division cycle. We propose to call these proteins the cyclins.**

## Introduction

Fertilization of eggs or meiotic maturation of oocytes in many organisms is accompanied by an increase in the rate of protein synthesis programmed by maternal mRNA (Woodland, 1982). In addition, changes in the pattern of protein synthesis are found in almost every case studied recently; the list includes starfish (Rosenthal et al., 1982), clams (Rosenthal et al., 1980), frogs (Woodland, 1982), and mice (Schultz and Wassarman, 1977; McGaughey and Van Blerkom, 1977; Braude et al., 1979). Sea urchins appear to be the exception, according to the careful studies of Brandhorst (1976). He used two-dimensional gels to analyze patterns of protein synthesis in eggs of Lytechinus pictus before and after fertilization, and found essentially no qualitative differences. However, more recent studies have shown that there is at least some qualitative translational regulation in urchins, for the mRNA for histones is apparently stored in the female pronucleus (Venezky et al., 1981) and not translated until after the first cleavage (Wells et al., 1981).

Neither the role of maternal mRNA nor the reasons for the existence of these striking examples of translational control are clear (Gross, 1968; Colman, 1983). However, inhibition of protein synthesis in fertilized sea urchin eggs by emetine or puromycin at $10^{-4}$ M (Hultin, 1961; Hogan and Gross, 1971) blocks development at the "streak" stage, in which the nucleus is particularly well marked and surrounded by stacked discs of membranes called annulate lamellae (Wagenaar and Mazia, 1978; Kessel, 1968). Such inhibitors permit normal fertilization, pronuclear fusion, and DNA replication, but completely prevent nuclear envelope breakdown, chromosome condensation, and formation of the mitotic spindle (Wagenaar and Mazia, 1978). If addition of emetine is delayed until about halfway through the cell cycle (about 30 min in Arbacia embryos developing at 19°C), the nuclear envelope breaks down normally, the chromosomes condense, the spindles form, and the cells divide, although they do not separate normally following cytokinesis. The simplest interpretation of these results is that one or more of the proteins whose synthesis is specified by maternal mRNA is absolutely required for cell division during cleavage.

In this paper we show that there are significant qualitative differences between the patterns of protein synthesis in eggs of Arbacia punctulata before and after activation by sperm or parthenogenetic agents. The same is true in eggs of Lytechinus pictus. Moreover, one or two of the proteins whose synthesis is strongly activated after fertilization appear to be destroyed more or less completely every time the embryos divide. Two proteins with similar properties are found in the clam Spisula solidissima.

## Results

Protein synthesis in sea urchin eggs can be activated by certain parthenogenetic treatments as well as by fertilization. For example, both 10 $\mu$M A23187 and 10 mM $NH_4Cl$ activate DNA and protein synthesis (von Ledebur-Villiger, 1972; Steinhardt and Epel, 1974) through $NH_4Cl$ raises the rate of protein synthesis to only about half the value it would attain after activation by A23187 or fertilization (Epel et al., 1974; Winkler et al., 1980). Neither ionophore-treated nor ammonia-activated eggs divide, unless they are given a further treatment, like brief exposure to hypertonic seawater or $D_2O$. Under these circumstances a proportion of the parthenogenetically activated embryos form functional asters and subsequently divide (Loeb, 1913; Dirksen, 1961; Kuriyama and Borisy, 1978; Mazia, 1978).

### New Proteins Are Made after Activation or Fertilization of Sea Urchin Eggs

Figure 1 compares the patterns of protein synthesis in unfertilized, fertilized, and parthenogenetically activated Arbacia eggs. $^{35}$S-methionine was added to suspensions of eggs, portions of which were then fertilized or activated. Samples were withdrawn at 10 min intervals for analysis on one-dimensional gels as described in Experimental Procedures.

There are clear differences between the patterns of proteins made before and after activation. The most strik-

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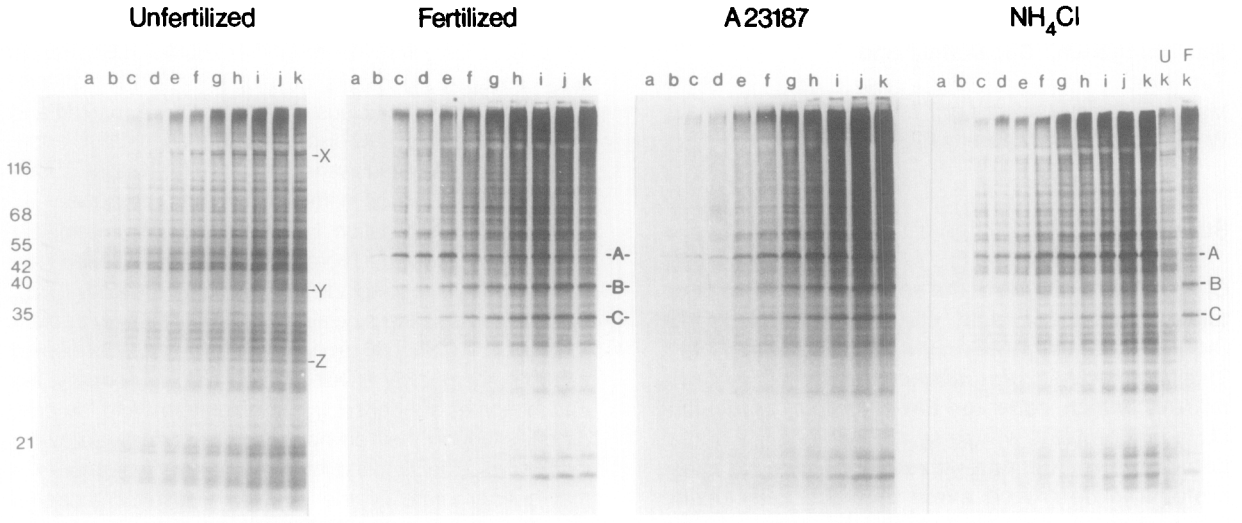


Figure 1. The Patterns of Protein Synthesis in Eggs before and after Activation

$^{35}$S-methionine was added at a final concentration of 30 μCi/ml to 15 ml of a suspension of 20,000 unfertilized eggs per milliliter. After 5 min, portions of this suspension were fertilized, or made 10 μM in A23187 by addition of 10 mM stock in dimethylsulfoxide, or made 10 mM in $NH_4$ Cl by addition of 1 M stock. Samples were taken for analysis at 10 min intervals starting at 25 min (lanes a) until 115 min (lanes j) after addition of the activator. Samples (lanes k) were taken at 127 min. The last two lanes labeled Uk and Fk are lighter exposures of the last unfertilized and fertilized lanes, respectively. The exposure of the autoradiograph in the unfertilized panel was four times longer than the other three to compensate for the low level of incorporation before fertilization. Bands X, Y, and Z are marked as examples of polypeptides whose synthesis is reduced after fertilization; and A, B, and C as the major nonhistone proteins whose synthesis is activated.

ing change is the appearance of the prominent new bands A, B, and C after fertilization or activation with A23187, much as happens in Spisula (Rosenthal et al., 1980). However, closer inspection reveals other interesting features, of which the most unexpected is the behavior of protein A, which we shall call "cyclin" henceforth. It is the most strongly labeled protein at early times after fertilization, but by 85 min after fertilization (lane g, fertilized) it has almost disappeared. It gets stronger again in lanes h and i, only to decline again in lane k. These oscillations in the level of cyclin are extremely reproducible, as can be seen in Figures 2, 3, and 6, which show similar behavior in different batches of fertilized Arbacia eggs.

### Cyclin Does Not Oscillate after Parthenogenetic Activation

Although cyclin synthesis is strongly induced by A23187 or $NH_4Cl$ treatment of unfertilized eggs, under neither of these circumstances does its level oscillate in the same way as in fertilized eggs. This is probably because the eggs do not divide, a point enlarged upon below.

### Ammonia Activation Does Not Turn On the Synthesis of All the Proteins

As mentioned above, activation of eggs with weak bases like $NH_4^+$ causes only about half the stimulation of protein synthesis that A23187 or fertilization gives. Is this because the synthesis of all the proteins is half turned on, or because half the proteins are fully turned on? Figure 1 shows that protein B did not appear after ammonia activation, though it is one of the most strongly labeled in fertilized and ionophore-activated eggs. For the most part, however, all the proteins are less strongly synthesized than in activations that involve release of intracellular calcium.

### The Synthesis of Some Proteins Stops after Fertilization

Several proteins, like the examples labeled X, Y, and Z on Figure 1, are made in the unfertilized egg but are very hard to detect after fertilization, though they continue to be made in ammonia-activated eggs. These comparisons are most easily made by examining the last three lanes of Figure 1, in which light exposures of the final lanes (k) from unfertilized (U) and fertilized (F) eggs are matched with the final ammonia-activated lane. The decline in synthesis in prefertilization bands is easier to see in Figure 7, which shows a similar experiment in Lytechinus pictus.

### Cyclin Is Destroyed Periodically at a Particular Point in the Cell Cycle

Figure 2 correlates the oscillations in the level of cyclin with the cleavage cycle. The experiment was conducted in the same way as the previous one, except that additional samples from the suspension of fertilized eggs were fixed in 1% glutaraldehyde for later examination under the microscope. The dashed line in Figure 2 denotes the cleavage index, measured as described in Experimental Procedures. The other two curves show the relative intensities of cyclin and protein B, as determined by densitometry of the autoradiographs. Label accumulates more or less linearly in protein B, whereas cyclin (band A) falls precipitously at the onset of cleavage, only to rise and fall again during the second cell cycle. Label in protein C also rose linearly, but the data have been omitted from this figure for clarity of presentation.

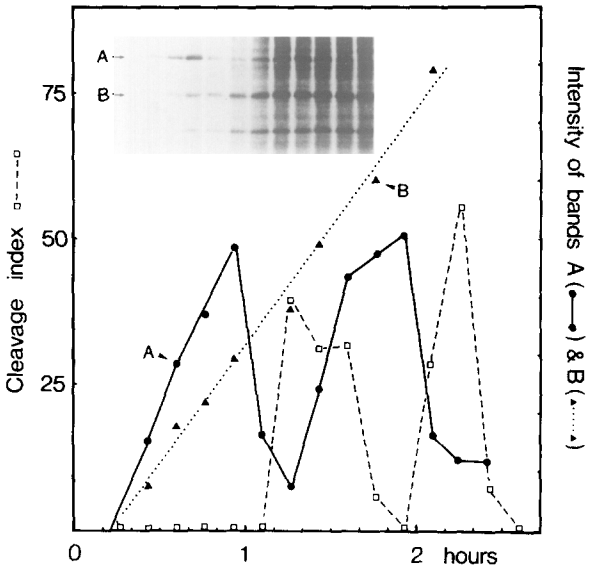


Figure 2. Correlation of the Level of Cyclin with the Cell Division Cycle

A suspension of eggs was fertilized, and after 6 min, $^{35}$S-methionine was added to a final concentration of 25 μCi/ml. Samples were taken for analysis on gels at 10 min intervals, starting at 16 min after fertilization. Samples were taken 20–30 sec later into 1% glutaraldehyde in calcium-free artificial seawater for later microscopic examination; the cleavage index is shown thus: □----□. The autoradiograph shown as an inset was scanned to yield the data plotted thus: cyclin, ●—●; protein B, ▲·····▲.

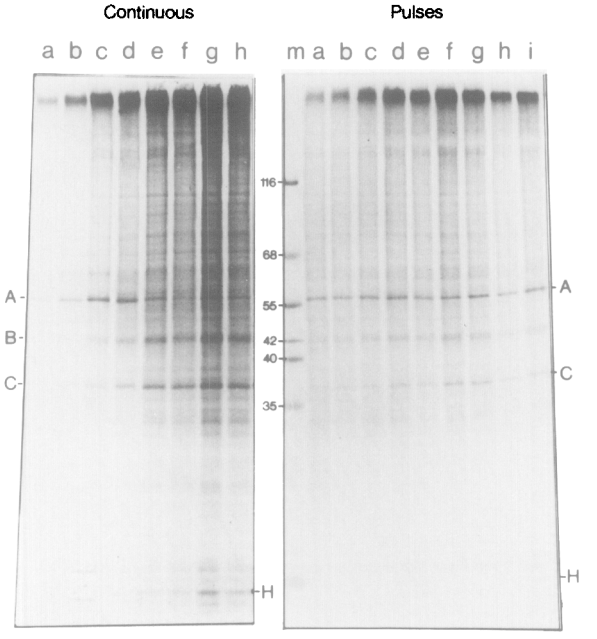


Figure 3. Comparison between Continuous and Pulse-Labeled Embryos

A batch of eggs was fertilized and split into two. The first 2 ml (30,000 eggs) had ½₀₀th volume of labeled methionine added 5 min after the sperm, and samples for analysis on gels were taken at 10 min intervals thereafter. The other batch was allowed to develop, and every 10 min a sample of 0.1 ml was added to a tube containing 1 μl of $^{35}$S-methionine, mixed, and incubated for 10 min before adding 0.2 ml 25% TCA. The letters above each lane correspond to the equivalent endpoints: (a) continuous label for 25 min, pulse from 15 to 25 min; (b) 35 min continuous, 25–35 min pulse; (c) 45; (d) 55; (e) 65; (f) 75; (g) 85; (h) 95; (i) 105 min. m: markers. Cleavage occurred between lanes e and f.

### The Cyclical Fluctuation in Cyclin Are Due to Periodic Destruction and Continuous Synthesis

It seemed most likely that the oscillation in the level of cyclin was achieved by continuous synthesis punctuated by destruction at a certain point in the cell cycle. To test this hypothesis, we examined the pattern of protein synthesis during successive 10 min pulses, at the same time performing a continuous labeling experiment in a parallel sample from the same batch of fertilized eggs. Figure 3 shows the continuous labeling experiment on the left, and the pulse-labeling experiment on the right; the lanes are related to the sampling time such that lane a represents a continuous 26 min label in the left panel and a pulse from 15 to 25 min in the right panel; lane b a continuous 36 min sample or a pulse from 25 to 35 min, and so on at 10 min intervals; the eggs started to divide at lane e. Whereas the continuous labeling shows the usual disappearance of cyclin just before cleavage, the pulse-labeling experiment shows the rate of synthesis of cyclin remaining essentially the same throughout the first cell cycle.

This result is confirmed in Figure 4, which shows the quantitation of the intensity of the bands in the pulse-labeling experiment. One would expect most of the proteins to behave like protein C, and show a linear increase in the rate of synthesis with time. Likewise, the behavior of band H, which is almost certainly a histone, is in accord with the findings of Wells et al. (1981); its synthesis is barely detectable before first cleavage, and rises rapidly at the two-cell stage. The behavior of cyclin (band A) shows a third way that mRNA can be recruited, for its rate of synthesis rises very rapidly after fertilization, with only a relatively small rise thereafter. There does not appear to be any significant variation in its rate of synthesis correlated with the cell division cycle. This result suggests that the variations in the intensity of cyclin revealed by a continuous label are due to its destruction by periodic proteolysis, and not to periodic synthesis coupled with continuous rapid turnover.

The fact that there is no sign of periodic variations in the extent of labeling of cyclin in short pulses suggests that newly synthesized cyclin may have to participate in some sort of maturation or assembly process before it can be destroyed.

The possibility that cyclin is labeled by methionine by some process other than normal protein synthesis is excluded by two kinds of experiments. First, its synthesis can be programmed in a reticulocyte lysate by mRNA extracted from unfertilized or fertilized eggs (data not shown). Second, its labeling is inhibited by emetine, as is shown below.

### The Rate of Cyclin Synthesis Declines at the End of Cleavage

Figure 5 shows that cyclin continues to be synthesized at a high rate for at least 5 h after fertilization at 20°C (morula

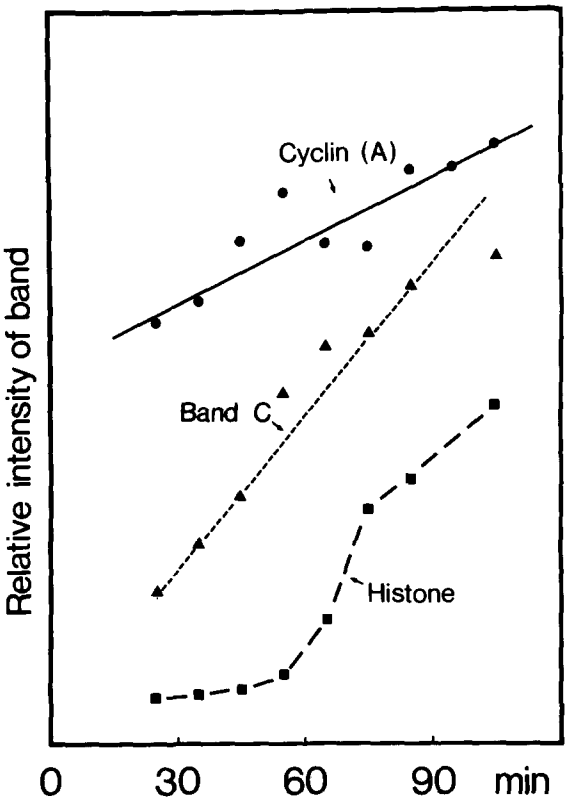


Figure 4. Rates of Synthesis of Three Proteins during the First 2 h of Cleavage

The relative intensities of cyclin, protein C, and the putative histone band were determined from the autoradiograph shown in the right panel of Figure 3 by densitometry.

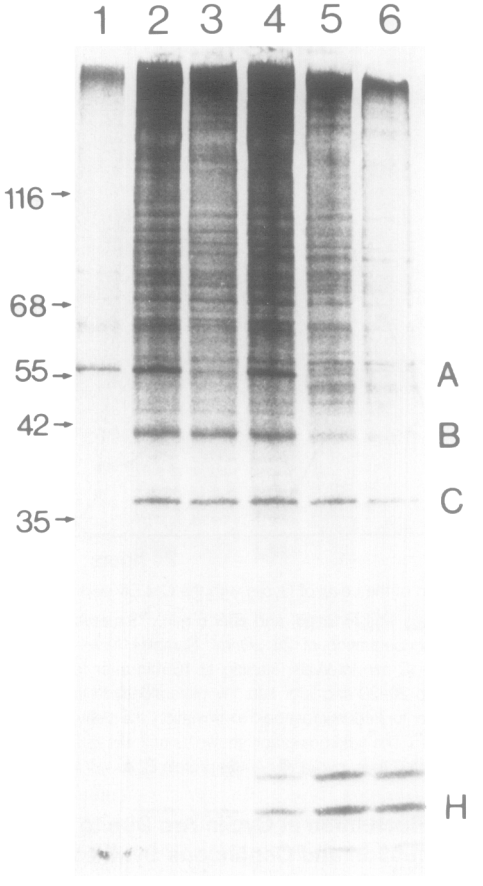


Figure 5. Patterns of Synthesis at Later Stages of Development, Showing the Disappearance of Cyclin after Adding Emetine

This figure shows the continuation of the experiment described in Figure 3; lane 1 is in fact a second sample of lane c of that figure, 45 min after fertilization and 40 min with $^{35}$S-methionine. Lane 2 was sampled 3 hr 21 min after fertilization, with 55 min exposure to label; lane 3 represents the same batch of embryos 30 min later, having been exposed to $10^{-4}$ M emetine during that time. The next sample was taken 4 hr 51 min after fertilization, the last 37 min of that time with label present. Lane 5 and 6 were sampled at 10 hr 14 min and 10 hr 44 min, respectively, after 45 min and 75 min exposures to $^{35}$S-methionine. The last 30 min of lane 6 incubation had $10^{-4}$ M emetine present.

stage). After the first three divisions, the synchrony of cell division is lost (Harvey, 1956), and continuous labeling did not show the oscillations in cyclin levels seen during the first three cleavage divisions (data not shown). However, it seemed likely that cyclin was still being periodically destroyed. To detect this, we blocked protein synthesis with emetine, and took samples 30 min later for analysis on gels. Cyclin disappeared from embryos exposed to the inhibitor, as comparison of lanes 2 and 3 shows. Cyclin is still synthesized at a high rate at 5 h (lane 4), more than halfway through cleavage, but is hard to detect at 10 h, by which time the rate of cell division has slowed considerably and the embryo is at the midblastula transition (Newport and Kirschner, 1982; Wilson, 1896; Hinegardner, 1967; O'Melia, 1983). Careful inspection of lanes 5 and 6 is required to detect the synthesis of cyclin at this stage, for the band is only faintly visible. However, it disappears after exposure to emetine, showing that it is still being made and cycling. In contrast, proteins B and C are still being synthesized and appear quite strongly at this time.

### Cytochalasin, Colchicine, and Taxol All Slow Down the Disappearance of Cyclin

We have already seen that cyclin does not cycle normally when protein synthesis has been activated by A23187 or $NH_4Cl$, suggesting that cell division is necessary for its disappearance. Figure 6 shows that when eggs are fertilized, but cell division is blocked by cytochalasin D or colchicine, the pattern of disappearance of cyclin is altered, so that instead of its intensity suddenly declining at 60–70 min, as in the controls, it disappears much later and much more slowly. The significance of this slow degradation is unclear. Both drugs allowed the normal activation of protein synthesis. Taxol had exactly the same effect (data not shown). It seems that the rapid disappearance of cyclin depends on some aspect of normal cleavage. Whether the converse is true—i.e., that disappearance of cyclin is essential for cell division—we cannot say.

### There Are Two Cyclins in Lytechinus pictus

We have looked for cyclin-like proteins in two other marine organisms; Lytechinus pictus, a California sea urchin that

diverged from Arbacia punctulata about 30 million years ago; and a mollusc, the clam Spisula solidissima, which is phylogenetically very distant from the echinoderms. Figure 7 shows an experiment with Lytechinus performed along the same lines as Figure 1. In general terms, the results resemble those from Arbacia, in that a striking change in the pattern of protein synthesis occurs after fertilization, and that some of the newly made proteins are cyclically destroyed. But there are also significant differences from Arbacia. First, Lytechinus has not one but two prominent cyclins, and they oscillate in different phases. Moreover, the lower molecular weight of the two cyclins shows oscillatory behavior after ammonia activation, something we did not see in Arbacia.

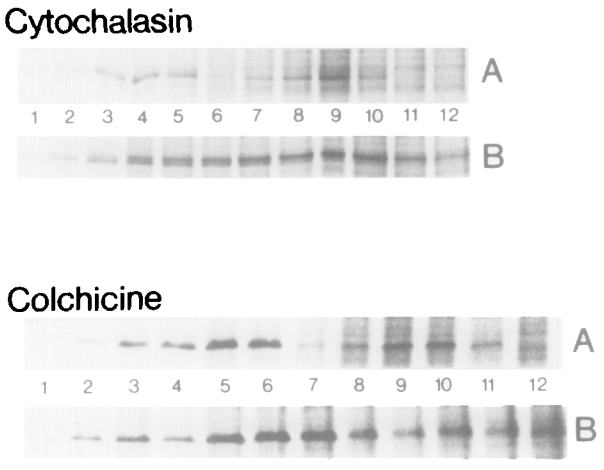


Figure 6. The Effects of Cleavage Inhibitors on Cyclin Breakdown

Two separate experiments are shown here, using different batches of fertilized eggs. In each case, a suspension of eggs was split in two after fertilization and addition of $^{35}$S-methionine. One lot was allowed to develop normally, while the other contained either cytochalasin B (2 μg/ml, 1/1000 vol of 2 mg/ml stock in DMSO) or 100 μM colchicine (1/1000 vol of 40 mg/ml aqueous stock solution). The first samples were taken at 15 min after fertilization, and subsequent samples at 10 min intervals. By sample 12, the control embryos (A) had all reached the four-cell stage, whereas the embryos containing the inhibitors (B) had not divided.

### Proteins A and B in Spisula Are Cyclins

As in Lytechinus, two proteins in Spisula showed oscillations correlated with the cell cycle (Figure 8); they are the proteins referred to as A and B by Rosenthal et al. (1980). These proteins are scarce in unfertilized oocytes, and their synthesis increases very abruptly after fertilization; virtually all of the mRNA for protein A becomes associated with polysomes by first cleavage (E. T. R., unpublished observations).

## Discussion

### The Qualitative Changes in Protein Synthesis after Fertilization

In Arbacia three polypeptides, and in Lytechinus four polypeptides, show a much larger proportional increase in synthesis after fertilization than the majority of maternally coded proteins. This is contrary to previous interpretations based on the results of Brandhorst (1976), but is more consistent with recent findings in a wide variety of other organisms, as mentioned in the Introduction. In all the marine invertebrates studied, three or four very abundant newly synthesized proteins appear after activation, while a large number of others show a quantitative increase in synthesis. However, there is an almost complete lack of correspondence between the patterns of labeled proteins and the pattern of stained bands on the gels, implying that

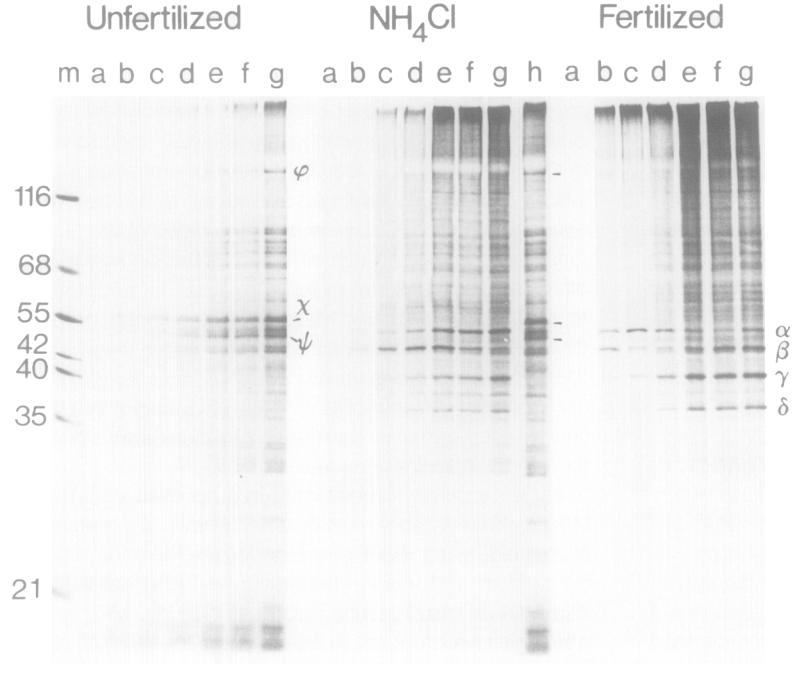


Figure 7. Patterns of Protein Synthesis in Lytechinus pictus Eggs and Embryos

A 1% suspension of Lytechinus eggs was labeled with 50 μCi/ml $^{35}$S-methionine and divided in three. One lot had no further additions; one had 10 mM $NH_4Cl$ (final) added, and one had a 10,000-fold dilution of "dry" sperm. The first sample was taken for analysis at 15 min, and subsequent samples at 10 min intervals. The fertilized embryos underwent first cleavage at lane f. Phi, chi, and psi: proteins in the unfertilized sample whose synthesis declines after activation. Alpha, beta, gamma, and delta: strongly labeled proteins that appear after activation. Lane h: further sample of unfertilized eggs taken 10 min after sample g. Lane m: marker. Exposure of the "unfertilized" autoradiograph was four times longer than that of the other two. Densitometry of the bands in the region 70–100 kilodaltons, where there do not appear to be significant changes in the pattern of protein synthesis, showed that fertilization caused a 7-fold increase in their rate of labeling.

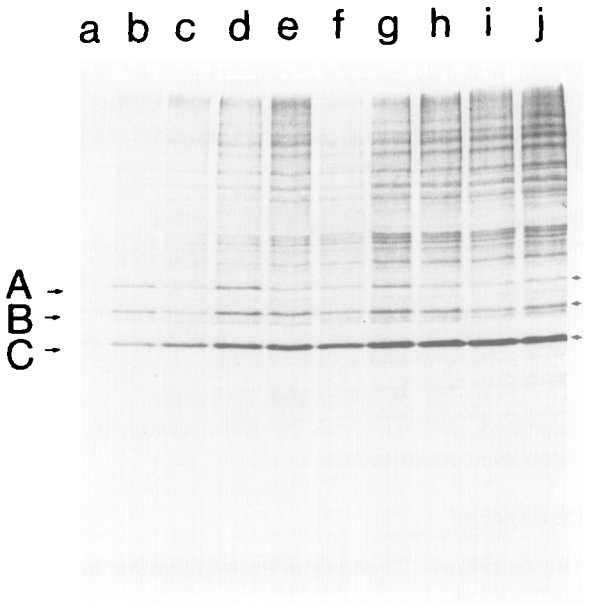


Figure 8. Proteins A and B in Spisula Are Cyclins

Spisula oocytes were fertilized and labeled with $^{35}$S-methionine, and samples were taken for analysis on gels in exactly the same way as the sea urchin experiments. Lane a shows a sample taken 45 min after fertilization, and there are 10 min between each lane (lane j is thus 135 min after fertilization). First cleavage occurred at about lane c.

protein synthesis during early development results in a considerable remodeling of embryonic proteins.

This remodeling involves the selectively timed recruitment of maternal mRNA. Most mRNAs are recruited gradually, so that the rate of synthesis of the proteins they code for rises linearly over the first few hours of development, until about the 200-cell stage (Goustin and Wilt, 1981; Hinegardner, 1967; O'Melia, 1983). The intracellular concentration of these proteins therefore rises exponentially; an excellent example of this general class is ribonucleotide reductase (Noronha et al., 1972).

Not all proteins follow this pattern. As mentioned in the Introduction, the translation of maternal histone mRNA is delayed until after first cleavage (Wells et al., 1981).

A third type of message recruitment is shown by cyclin mRNA, which appears to be very rapidly and almost completely loaded onto ribosomes shortly after fertilization (Figure 4). Although the rate of synthesis of cyclin remains constant, which should lead to its linear accumulation, the fact that this protein is regularly destroyed means that its cytoplasmic concentration follows a kind of saw-tooth pattern.

### Proteins Specified by Maternal Messages and Mitotis

Previous studies of the mitotic spindle from cleaving sea urchin embryos suggested that proteins specified by maternal mRNA are preferentially associated with the mitotic apparatus (Stafford and Iverson, 1964), and although the proteins in question have never been fully characterized or unambiguously identified (Wilt et al., 1967; Raff et al., 1972), it seems to have become accepted that tubulin is one of the more abundant maternally coded proteins (Davidson, 1976; Fulton and Simpson, 1979; Davidson et al., 1982). Certainly the mobility of the major cyclin in Arbacia is very close to that of alpha and beta tubulin, and at first we thought it probably was one of these proteins. However, by comparison with the appropriate standards, cyclin was clearly resolved from tubulins. Nor did cyclin bind to a tubulin antibody-affinity column (with the rat monoclonal antibody isolated by Kilmartin et al., 1982), although Arbacia tubulin and, to our surprise, protein B were retained quantitatively by it (unpublished observations). This suggests that protein B may be associated with the spindle apparatus via tubulin, but more experiments are required to clarify this point. However, these observations show that previous claims to the effect that tubulin is a significant product of maternal mRNA were mistaken (Raff et al., 1975). This raises in even more acute form the question of why protein synthesis is so absolutely required for even the first cleavage division.

### "Periodic Proteins"

It is difficult to believe that the behavior of the cyclins is not connected with processes involved in cell division, but at this stage we have no direct evidence that it is. The synthesis and periodic destruction of proteins have long been proposed to account for various features of the cell cycle (Mitchison, 1971; John et al., 1981). Mitotic spindles come and go; chromosomes condense and extend; the nuclear envelope dissolves and reforms (Mazia, 1961). To induce the de novo synthesis of an enzyme and subsequently destroy it is one way of turning its activity on and off. While one would have thought it improbable that such a mechanism would be used by cells, since more questions are raised than solved by its existence, we find it difficult to interpret the behavior of cyclin in any other way.

Unfortunately, we have no direct evidence as to the physiological role of cyclin, but one of its more plausible roles is promoting either directly or indirectly the breakdown of the nuclear envelope, since failure to dissolve the nuclear membrane is the most obvious consequence of inhibition of protein synthesis in cleaving embryos (Wagenaar and Mazia, 1978). Moreover, the fact that the nuclear membrane breaks down after ammonia activation and never reforms despite multiple rounds of chromosome replication (Paweletz and Mazia, 1979) correlates precisely with the failure of cyclin to disappear under these conditions. However, this is very speculative, and tests such as microinjection of ammonia-activated cytoplasm into emetine-arrested eggs will be required to elucidate the role of the various proteins involved.

Such experiments performed in amphibian oocytes revealed the existence of the entity known as maturation promoting factor (MPF), which causes germinal vesicle breakdown and meiotic maturation in these cells (Masui and Markert, 1971; Smith and Ecker, 1971). MPF undergoes cyclic fluctuations in level during subsequent cleavage cycles, which are prevented by colchicine (Wasser-

man and Smith, 1978). Furthermore, MPF requires protein synthesis for its formation, and presumably is a protein (Gerhart, 1980). The parallels between the behavior of MPF and cyclin are striking, but whether there is a direct correspondence between the physiological entity and the chemical one remains to be determined.

## Experimental Procedures

### Materials

Arbacia punctulata and Spisula solidissima were obtained from the Department of Marine Resources of the Marine Biological Laboratory, Woods Hole, and kept in running seawater at ambient temperature (about 20°C), whereas Lytechinus pictus were obtained from Pacific Biomarine, Inc, Venice, California, and kept in chilled seawater tanks at about 15°C. Gametes were obtained from Arbacia by 12 V AC electrical stimulation, from Spisula by dissection, and from Lytechinus by injection of 0.5 M KCl.

$^{35}$S-methionine was the generous gift of Amersham Corp. It was their SJ204, which contains 25 mM K acetate and 14 mM 2-mercaptoethanol. It was diluted with water to a concentration of 5 mCi/ml and stored as small aliquots at −70°C.

Rat monoclonal anti-yeast tubulin antibody YOL1/34 coupled to Sepharose 4B was the generous gift of John Kilmartin (Kilmartin et al., 1982).

Radioactively labeled proteins for molecular weight standards were prepared from a mixture, each at 1 mg/ml, of purified proteins dissolved in water: cytochrome c, RNAase A, soybean trypsin inhibitor, carbonic anhydrase, glyceraldehyde 3 phosphate dehydrogenase, creatine kinase, actin, glutamate dehydrogenase, bovine serum albumin, and beta galactosidase. This mixture was incubated at 50°C with 0.16 M Tris-Cl, 5 mM dithioerythritol (pH 8.8) for 2 min and then precipitated with 12.5% w/v tricholoroacetic acid. The precipitate was taken up in 1 M HEPES, 2 mM EDTA, 1.5 mM $^{14}$C-N-ethylmaleimide, specific activity 23.7 mCi/mmol (New England Nuclear), pH 7.4, and incubated at 20°C for 30 min. It was then diluted in SDS gel sample buffer to a concentration of about 0.1 mg/ml for each component (the exact dilution was judged by running serial dilutions on a gel) and stored at −70°C in small aliquots. A parallel sample was incubated directly with $^{14}$C-N-ethylmaleimide (i.e., without the reductant and without TCA precipitation) at pH 7.4. This gave good labeling of all proteins except for RNAase and soybean trypsin inhibitor, which do not have free-SH groups. Carbonic anhydrase appears not to contain any reactive groups at all. The labeled markers in the figures of this paper contained a mixture of these two preparations.

### Preparation and Incubation of Eggs and Embryos

Eggs were washed by repeated settling through Millipore-filtered seawater. They were suspended at a final density of 15,000–20,000 eggs/ml, and incubated in 20 ml glass scintillation vials standing in shallow dishes of water perched in Styrofoam boxes to provide a constant temperature environment at 20°C; ice was added occasionally if necessary. The vials usually contained 2 ml of egg or embryo suspension at the start, and were shaken gently before sampling. More than 90% of the fertilized eggs raised fertilization membranes and showed normal cleavage in all the experiments described in this paper.

### The Cleavage Index

To correlate the time of cleavage with changes in the pattern of incorporation, samples of the embryos were taken into seawater containing 1% glutaraldehyde. The suspensions were photographed under the microscope, and the fraction of cells that had divided was counted. The cleavage index shown in Figure 2 is derived by determining the fraction of cells that divided between one sample and the next.

### Fertilization and Labeling

"Dry" sperm was diluted 100-fold with Millipore-filtered seawater and allowed to stand approximately 5 min before a further 200-fold dilution into the suspension of eggs. Labeled methionine was added at a final concentration of 1 vol of 5 mCi/ml stock to 200 vol of suspension—about 25 μCi/ml final, $1.8 \times 10^{-8}$ M. We noticed that even at this relatively high dilution of the added label, the fertilized samples containing label always took about 15 min longer to undergo first cleavage than unlabeled controls. In one experiment not shown here we tried to use $^{3}$H-lysine, which completely blocked cleavage. Evidently one should be careful of the label in these experiments; we do not know whether the delay in division caused by the methionine was due to radiation damage (to which eggs are known to be highly susceptible) or to the acetate and mercaptoethanol in the solution. A preliminary test suggested that neither of these unlabeled compounds alone slowed division at the concentrations resulting from their presence in the labeled solution as supplied, but that the combination might be damaging.

It should also be mentioned that the overall kinetics of incorporation of methionine into TCA-precipitable material were not linear, owing to the fact that by 2 hr of incubation well over 50% of the label had been incorporated into protein. This is very like the situation reported by Ecker and Smith (1966) in frogs' eggs; such eggs rely on stored yolk proteins for amino acids, whose pools are very small and constantly replenished from the stores. This means that the specific activity of the methionine pool declines during the course of the experiments. We do not think this affects the interpretation of our results, but it should be borne in mind.

### Sample Preparation and Analysis

Samples of 50 μl were withdrawn from the incubation vessels with a Gilson Pipetman whose tips were cut back about 1 mm to enlarge the holes. The samples were delivered into tubes containing 100 μl of 25% w/v TCA. The precipitates were harvested by centrifugation, washed twice with acetone, dried under vacuum, and dissolved in 30 μl of SDS-gel sample buffer.

Acrylamide gels 0.8 mm thick were run according to the formulas of Anderson et al. (1973). Each lane 2–3 mm wide, was loaded with 10–15 μl, corresponding to about 100–200 embryos.

The gels were stained with 0.5% Coomassie blue R250 in 45% methanol, 45% water, 10% acetic acid; destained in 20% methanol, 75% water, 5% acetic acid; and dried onto Whatman 3MM paper. The dried gels were autoradiographed with Fuji Rx film.

The autoradiographs were scanned with a Transidyne 2955 scanning densitometer connected via an Interactive Microware Inc. 12-bit analog-digital converter to an Apple II microcomputer. This permitted integration of peaks by a procedure formally analogous to cutting and weighing.

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