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Molecular cloning and characterization of the mRNA for cyclin from sea urchin eggs

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We have isolated a cDNA clone encoding sea urchin cyclin and determined its sequence. It contains a single open reading frame of 409 amino acids which shows homology with clam cyclins. RNA transcribed *in vitro* from this sequence was efficiently translated in reticulocyte lysates, yielding full-length cyclin. Injection of nanogram amounts of this synthetic mRNA into *Xenopus* oocytes caused them to mature more rapidly than with progesterone treatment. The sea urchin cyclin underwent two posttranslational modifications in the *Xenopus* oocytes during maturation. The first occurred at about the time that maturation became cycloheximide-resistant, when a small apparent increase in the molecular weight of cyclin was observed. The second modification involved destruction of the cyclin at about the time of white spot appearance, just as would have occurred at the metaphase/anaphase transition in the natural environment of a cleaving sea urchin embryo.

Key words: cyclin/sea urchin/cDNA sequence/transcription

Introduction

Cyclin is a protein found in fertilized eggs during cleavage and oocytes during meiotic maturation (Evans *et al.*, 1983; Swenson *et al.*, 1986; Standart *et al.*, 1987). [The name 'cyclin' has unfortunately been applied to two completely unrelated proteins. The cyclin described in this paper was first described by Evans *et al.* (1983) and should not be confused with the protein called cyclin by Almendral *et al.* (1987) which is also known as PCNA (proliferation-controlled nuclear antigen) (Prelich *et al.*, 1987).] In clams, sea urchins and starfish its mRNA is one of the most abundant messages laid down during oogenesis but not translated until after fertilization (Evans *et al.*, 1983; Rosenthal *et al.*, 1980, 1983). In the species we have studied, cyclin is one of the strongest [^{35}S]methionine-labelled polypeptides seen on a 1-dimensional SDS polyacrylamide gel within the first hour after fertilization. However, the intensity of the band oscillates due to destruction of essentially all the newly-made cyclin each time the cells pass through a meiotic or a mitotic division (Swenson *et al.*, 1986; Standart *et al.*, 1987). Thus, despite its high rate of synthesis, cyclin never accumulates in sufficient amounts to be seen as a Coomassie blue-stained band. The highly specific proteolysis of cyclin occurs during a period of about 5 min at the time of the metaphase/anaphase transition (Evans *et al.*, 1983; Swenson *et al.*, 1986). At other times in the cell cycle, cyclin is perfectly stable. We are working on the hypothesis that cyclin is one of the essential components for cells to enter mitosis, and that its destruction is necessary for exit from mitosis. This idea is based on the rapid rate of cyclin synthesis, its cell cycle-related oscillations, and the protein synthesis requirement for entry into mitosis (Hultin, 1961; Wilt *et al.*, 1967; Wagenaar, 1983; Newport and Kirschner, 1984). The hypothesis has recently received strong support from the demonstration that microinjection of clam cyclin A mRNA induces meiotic maturation in *Xenopus* oocytes (Swenson *et al.*, 1986), though it remains to be shown that cyclin can promote entry into mitosis in cycloheximide-arrested fertilized eggs.

This paper reports the amino acid sequence of sea urchin cyclin, based on the DNA sequence of cyclin cDNA clones isolated from *Arbacia punctulata*. Comparison of the clam cyclin A and urchin cyclin sequences, both of which are just over 400 residues long, shows strong homology limited to a sequence of about 36 amino acids in the middle of the proteins. Despite the considerable sequence divergence at both ends of the proteins, microinjection of sub-nanogram amounts of sea urchin cyclin mRNA also makes frog oocytes mature; furthermore its translation product in the oocytes is posttranslationally modified, and destroyed as the oocytes pass through meiosis. We also show that cyclin is the only sea urchin maternal mRNA capable of inducing frog oocyte maturation in this microinjection assay.

Results

Construction of a partial cDNA library and identification of a cyclin clone

When we began this work, clam cyclin clones had already been isolated by Rosenthal *et al.* (1983). We therefore screened a 'Northern blot' of clam and sea urchin mRNA with a nick-translated clam cyclin A clone (1T55), kindly supplied by Dr J.V.Ruderman. However, although clam maternal RNA gave a strong signal, sea urchin mRNA did not, even at very low stringency. Therefore this probe could not be used to screen sea urchin cDNA libraries. The reason for its failure to cross-hybridize became clear when we obtained the sea urchin sequence; there is hardly any homology between the nucleotide sequences. We therefore had to use a cell-free translation assay to identify a cDNA clone for cyclin. Messenger RNA was prepared from post-mitochondrial supernatants of egg homogenates of the sea urchin *A. punctulata* and fractionated on poly(U)–sepharose to select poly(A)$^+$ RNA and on sucrose gradients to enrich for cyclin mRNA by size. The fractions containing cyclin were identified by cell-free translation in the reticulocyte lysate (Figure 1). Cyclin mRNA sedimented close to 28S rRNA, suggesting that it was about 5 kb long, large compared to the minimum size of 1.5 kb necessary to encode a protein of cyclin's apparent M_r of 56 000. Double-stranded cDNA was synthesized by the method of Gubler and Hoffman (1983), cut with the restriction enzyme *Sau*3A and ligated with *Bam*HI-cut and phosphatase-treated M13mp8 replicative form DNA. The resulting plaques were screened in duplicate with alkali-treated [^{32}P]end-labelled poly(A)$^+$ RNA from eggs or from 14-h embryos. Cyclin mRNA levels are much lower at 14 h, judged by *in vitro* translation products (Evans *et al.*, 1983). Clones that hybridized strongly with egg RNA and weakly with 14-h embryo RNA were picked and grown up in

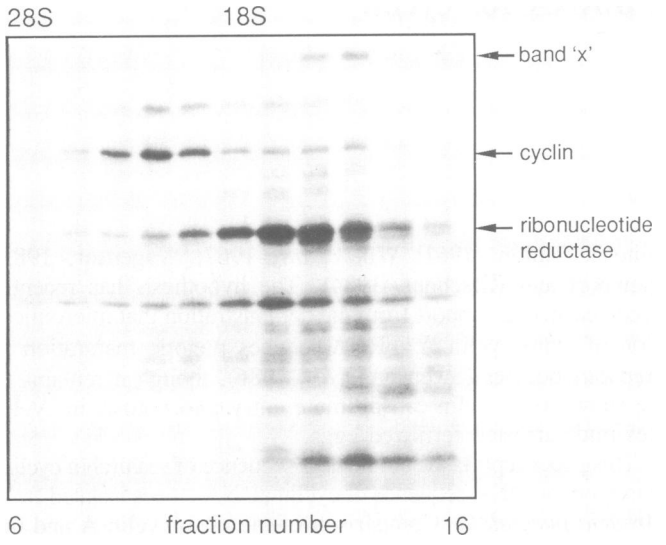


Fig. 1. Fractionation of sea urchin maternal mRNA on denaturing sucrose gradients. Poly(A)$^+$ RNA was prepared and fractionated on formamide/sucrose gradients as described in Materials and methods. This figure shows the autoradiograph of the translation assay of fractions 6–16. The positions of 18S and 28S ribosomal RNA (indicated above the panel) were determined from a parallel run of poly(A)$^-$ RNA. The positions of cyclin and the small subunit of ribonucleotide reductase are indicated on the right of the figure. Band 'x', a predominantly non-adenylated mRNA can be seen faintly in the top right-hand corner; its mobility gives a good indication of how large the other messages are compared to what would be expected.

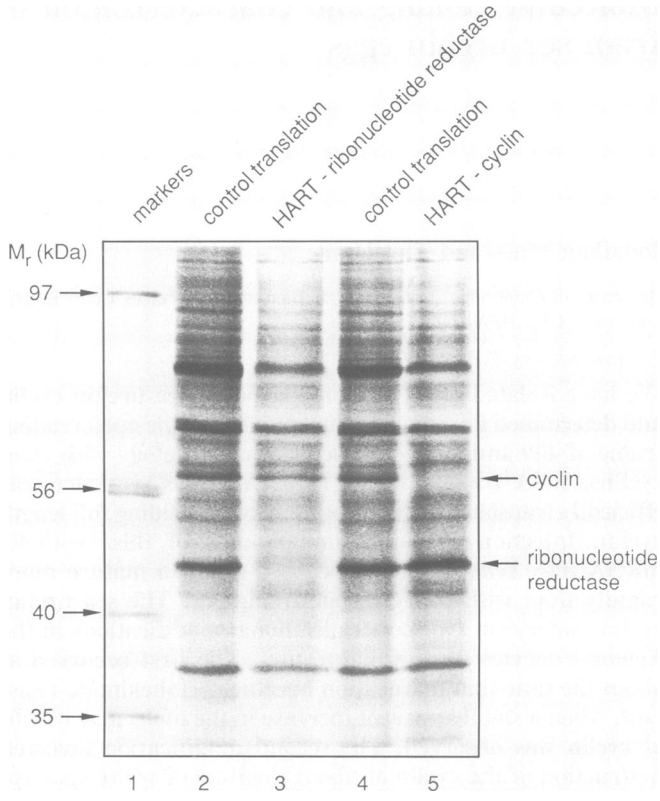


Fig. 2. Specific ablation of cyclin and ribonucleotide reductase by RNase H-mediated cDNA-directed digestion (hybrid arrest of translation). **Lane 1**, molecular weight markers; **lanes 2–5**, cell-free translation patterns specified by total *Arbacia* egg RNA after hybrid arrest of translation as described under methods; **lane 2**, no annealing or digestion; **lane 3** annealed with clone A18 (antisense ribonucleotide reductase); **lane 4**, control as lane 2; **lane 5**, annealed with clone E3 (antisense cyclin clone).

1.5 ml cultures. Phage DNA was prepared, digested with restriction enzyme *Hae*III and annealed with total egg RNA. The DNA:RNA hybrids were digested with RNase H as described by Minshull and Hunt (1986), and a sample of the digest was translated in the reticulocyte lysate and analyzed by SDS-polyacrylamide gel electrophoresis. Controls with wild-type M13mp8 DNA and with ribonucleotide reductase clone A18 (Standart *et al.*, 1985) were performed in parallel to check that the DNA-mediated ablation was working properly. The 39th clone tested, designated E3, gave the result shown in Figure 2; it caused the specific disappearance of the cyclin band from the translation products.

The DNA sequence of this clone showed limited homology to the coding region of the clam cyclin A protein (see Figures 5 and 6) and also revealed the presence of an internal *Sau*3A site, indicated on Figure 5. This could have arisen either from failure to digest the original cDNA to completion or from artifactual joining of two unrelated fragments during the ligation step. To test whether both parts of clone E3 corresponded to cyclin, the two parts of the clone were separately subcloned into M13mp8 and tested for their ability to specifically ablate cyclin synthesis when annealed with total egg RNA and digested with RNase H. In addition, oligonucleotides C1 and C2 (Figure 5) were synthesized and tested for their ability to prevent cyclin synthesis by RNase H-mediated hybrid arrest of translation. All these tests were positive, and clone E3 could thus be used as a probe to identify cyclin in full-length cDNA libraries.

Clone E3 was used to screen a Northern blot of RNA prepared from various stages of *Arbacia* development. Figure 3 shows that the concentration of the major cyclin mRNA falls quite rapidly about 5 or 6 h after fertilization to about 15–20% of its starting level. This lower level appears to be maintained for some time, and a signal is still detectable at 48 h of development (data not shown). This is in line with previous *in vivo* [^{35}S]methionine labelling experiments, showing a steady synthesis during the first 5 h after fertilization and a sudden reduction in the rate at around 6 h of development (see Figure 5 of Evans *et al.*, 1983; and T.H., unpublished data). The large size of cyclin mRNA was confirmed, since it migrated slower than 28S rRNA on denaturing gels. We estimate that cyclin mRNA is about 5.5 kb long.

Identification and sequence of full-length cyclin coding region cDNA clones

To obtain full-length cDNA clones for cyclin, cDNA prepared as described above was inserted into λgt10 using *Eco*RI linkers according to the protocols of Huynh *et al.* (1985). The largest clone obtained (cyc4) was only just over 2 kb long, but it was identified with a coding region probe and was therefore sure to contain some protein sequence. Fortunately it appears to contain the entire coding region. Its sequence was determined from subclones in M13 vectors as shown diagrammatically and listed in Figure 5. It contains an open reading frame of 409 amino acids, starting with an AUG codon in excellent context according to Kozak's rules (Kozak, 1982). There are no methionine codons upstream of the first one, and there are two stop codons (underlined) upstream in the same frame as this AUG. As described below, translation of a synthetic mRNA corresponding to this sequence makes full-length, active cyclin, so there is no doubt about where the coding region begins.

However, clone cyc4 is only 2 kb long, whereas the mRNA to which it corresponds is >5 kb long. Where is the missing sequence? Primer extension (Figure 4) showed that the 5′ end

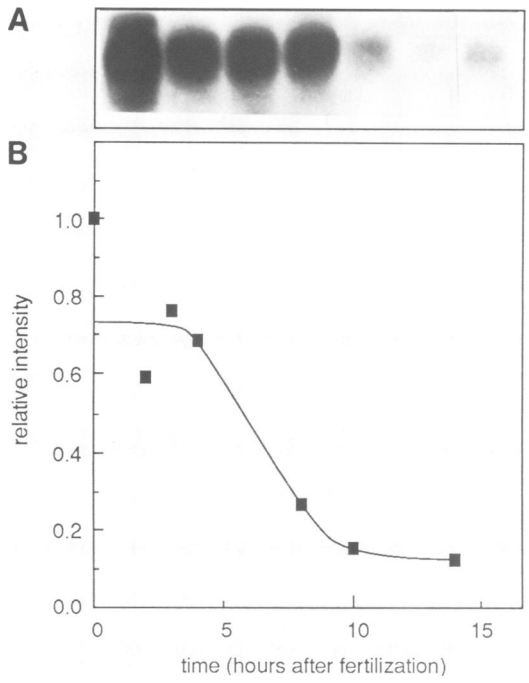


Fig. 3. Northern analysis of cyclin mRNA levels during early development. Total RNA (10 μg) from eggs (time zero) and embryos harvested at 2, 3, 4, 8, 10 and 14 h was fractionated on an agarose gel and probed with ^{32}P-labelled single-stranded cyclin DNA. **Panel A** shows the autoradiograph, and **panel B** the intensity of the cyclin band as determined by densitometry of a lighter exposure of the autoradiograph. The values are normalized to the intensity of the methylene blue-stained 28S RNA band on the filter as determined by scanning densitometry.

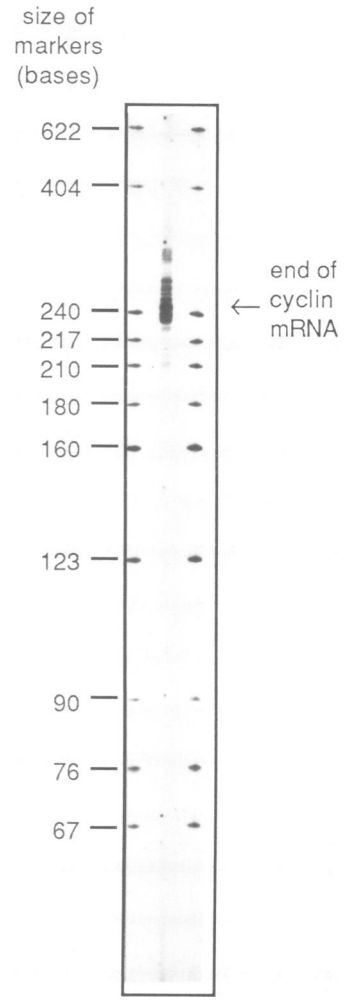


Fig. 4. Primer extension to locate the 5′ end of the mRNA. The oligonucleotide complementary to residues 41−65 of cyc4 was labelled with $^{32}PO_4$ at the 5′ end, and annealed to *A. punctulata* poly(A)$^+$ RNA. The primer was extended with reverse transcriptase and unlabelled dNTPs and analyzed on a DNA sequencing gel with *Msp*I digested pBR322 DNA size markers. The major product was slightly larger than the 238/242 doublet, and although there was marked heterogeneity, no bands shorter than about 220 nucleotides long were observed.

of the mRNA lay 250−290 nucleotides upstream of the initiator AUG (we do not know why there appears to be heterogeneity). The length of this leader is somewhat surprising for such an actively translated message, and may be related to the translational control of this mRNA; it will be interesting to determine its sequence. Clone cyc4 contains a stretch of ~40 A residues at its 3′ end (not shown) which probably served as the priming site for oligo(dT) in synthesis of the first strand of cDNA. However, most of the excess length of the mRNA must be at the 3′ end of the mRNA. Very long 3′ untranslated regions seem to be characteristic of maternal mRNA in sea urchins, since the sucrose gradient analysis shown in Figure 1 suggests that they are apt to be three or four times longer than they need be to encode their cognate proteins. One other defined mRNA we have studied, encoding the small subunit of ribonucleotide reductase, has a 3′ untranslated sequence ~2.2 kb long following a coding region of ~1.2 kb (J.Sleeman, J.P. and N.Standart, unpublished data).

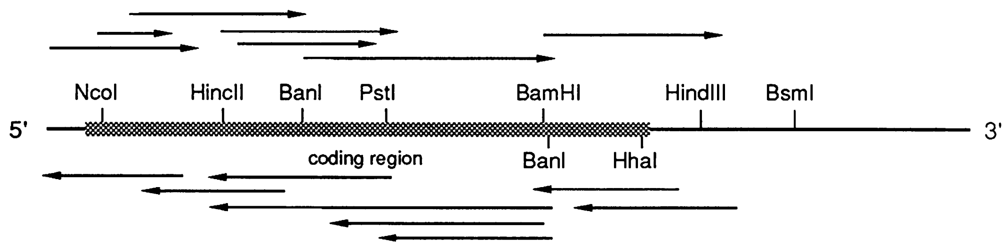
Comparison of the clam and sea urchin cyclin sequences

Comparison of the clam and sea urchin cyclin DNA and protein sequences are shown in the 'diagon' plots of Figure 6 (Staden, 1982). At the DNA level, the longest stretch of perfect homology is only 8 nucleotides, and with a score of 10/13 there is only a very short stretch of punctuated matches. The picture changes when the amino acid sequences are compared. Even at quite a stringent criterion the middle one third of the sequence matches well, and from position 239−273 of the clam sequence, 23/35 amino acids are identical and the changes are all very conservative. Other short stretches of high conservation are seen; a sequence RAALG at position 36 in the clam matches the same sequence at position 42 in the sea urchin, and the homology improves as the middle of the molecule is approached; DIY-YLR at position 166, MR-ILVDWLV at position 195, IDRFL at position 224, and Y-PS--AAAAI/L at position 334. Overall, the proteins show a low content of tryptophan and cysteine, and an absence of long hydrophobic stretches. Charged amino acids (D, E, H, K and R) comprise just over 25% of the residues with an overall charge close to neutral.

One curious feature of the sequence is the 5 occurrences of the dipeptide sequence KY. Four of these occur in similar places in both clam and urchin cyclin. There are also two occurrences each of KK and RR. These may be sites for proteolytic attack by trypsin-like enzymes.

Inspection of the sequence did not reveal homology with protein kinases, ATP or GTP-binding proteins, and no very convincing homology with any other proteins in the EMBL or GENBANK compilations were revealed using the program FASTP. It does not correspond to any of the yeast *cdc* genes that have been sequenced to date.

A



B

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                                                                                M   A   L   G   T   R   N   M   N   M   N   L
ACAAGTCTTTCCTGCTGATCCTGCTGACTGTTCAGTGTTTCTGTCGCGCACACACGTCATCAGGCTTCACTCGTCAATTTCATCATGGCTCTTGGAACAAGAAATATGAACATGAATCTC
        10    ↑ 20     ↑ 30      40      50      60      70      80      90     100     110     120
  termination codons    primer extension oligonucleotide

 H   G   E   S   K   H   T   F   N   N   E   N   V   S   A   R   L   G   G   K   S   I   A   V   Q   K   P   A   Q   R   A   A   L   G   N   I   S   N   V   V
CATGGTGAGAGCAAACACACATTCAACAATGAAAATGTCAGTGCAAGGCTCGGGGAAAGAGCATTGCTGTGCAAAAGCCAGCACAACGGGCAGCCCTTGGCAACATCAGTAATGTGGTT
       130     140     150     160     170     180     190     200     210     220     230     240

 R   T   A   Q   A   G   S   K   K   V   V   K   K   D   T   R   Q   K   A   M   T   K   T   K   A   T   S   S   L   H   A   V   V   G   L   P   V   E   D   L
CGAACTGCTCAGGCAGGAAGCAAGAAGGTTGTGAAAAAGGACACGAGACAAAAGGCTATGACCAAGACAAAGGCCACATCGTCTCTCCATGCTGTTGTTGGTCTCCCTGTAGAAGATCTC
       250     260     270     280     290     300     310     320     330     340     350     360

 P   T   E   M   R   S   T   S   P   D   V   L   D   A   M   E   V   D   Q   A   I   E   A   F   S   Q   Q   L   I   A   L   Q   V   E   D   I   D   K   D   D
CCTACAGAGATGAGGTCAACATCACCAGATGTCCTAGATGCTATGGAGGTTGATCAAGCAATTGAAGCCTTTTCGCAACAATTGATAGCGCTCCAGGTAGAGGACATTGACAAAGATGAT
       370     380     390     400     410 ↑   420     430     440     450     460     470     480
                                       Start of E3

 G   D   N   P   Q   L   C   S   E   Y   A   K   D   I   Y   L   Y   L   R   R   L   E   V   E   M   M   V   P   A   N   Y   L   D   R   Q   E   T   Q   I   T
GGGGATAACCCGCAACTGTGCAGCGAGTATGCCAAGGACATCTACCTGTACCTACGGAGGCTAGAGGTGGAGATGATGGTGCCTGCAAACTACCTGGACCGGCAGGAGACACAGATCACC
       490     500     510     520     530     540     550     560     570     580     590  ↑ 600
                        oligonucleotide C1                           internal Sau3A site of E3

 G   R   M   R   L   I   L   V   D   W   L   V   Q   V   H   L   R   F   H   L   L   Q   E   T   L   F   L   T   V   Q   L   I   D   R   F   L   A   E   H   S
GGGCGTATGCGGCTGATTCTTGTGGACTGGCTTGTCCAAGTGCACCTCCGCTTCCACCTCCTGCAAGAAACCCTGTTCCTCACCGTCCAGCTGATCGACAGATTTCTTGCTGAACATTCG
       610     620     630     640     650     660     670     680     690  ↑ 700     710     720
            oligonucleotide C2                                       end of E3

 V   S   K   G   K   L   Q   L   V   G   V   T   A   M   F   I   A   S   K   Y   E   E   M   Y   P   P   E   I   N   D   F   V   Y   I   T   D   N   A   Y   T
GTGTCGAAAGGAAAGCTGCAGCTTGTTGGAGTGACGGCTATGTTCATTGCCAGCAAATACGAAGAGATGTACCCTCCAGAAATCAACGACTTTGTCTACATCACAGACAATGCCTACACC
       730     740     750     760     770     780     790     800     810     820     830     840

 K   A   Q   I   R   Q   M   E   I   A   M   L   K   G   L   K   Y   K   L   G   K   P   L   C   L   H   F   L   R   R   N   S   K   A   A   G   V   D   A   Q
AAGGCCCAGATCAGGCAAATGGAAATTGCCATGCTCAAAGGACTCAAGTATAAGCTGGGAAAGCCGCTGTGCCTTCACTTCCTTCGACGCAACTCTAAAGCAGCTGGGGTGGATGCCCAG
       850     860     870     880     890     900     910     920     930     940     950     960

 K   H   T   L   A   K   Y   L   M   E   I   T   L   P   E   Y   S   M   V   Q   Y   S   P   S   E   I   A   A   A   A   I   Y   L   S   M   T   L   L   D   P
AAGCACACACTAGCCAAGTACCTAATGGAGATCACCCTTCCAGAGTACAGCATGGTGCAATACAGCCCTTCAGAGATTGCAGCAGCAGCCATCTACCTGTCCATGACGCTTCTGGATCCC
       970     980     990    1000    1010    1020    1030    1040    1050    1060    1070    1080

 E   T   H   S   S   W   C   P   K   M   T   H   Y   S   M   Y   S   E   D   H   L   R   P   I   V   Q   K   I   V   Q   I   L   L   R   D   D   S   A   S   Q
GAAACTCACAGCTCCTGGTGCCCCAAGATGACCCACTACAGCATGTACAGCGAGGATCACCTCAGGCCAATTGTGCAAAAGATTGTCCAGATTCTGCTCCGGGACGACTCTGCATCTCAG
      1090    1100    1110    1120    1130    1140    1150    1160    1170    1180    1190    1200

 K   Y   S   A   V   K   T   K   Y   G   S   S   K   F   M   K   I   S   G   I   A   Q   L   D   S   S   L   L   K   Q   I   A   Q   G   S   N   E   *
AAGTACTCGGCTGTGAAGACGAAATATGGCAGCAGCAAGTTCATGAAGATCAGTGGCATTGCACAGCTGGACTCATCTCTACTCAAACAAATAGCGCAAGGATCAAATGAGTGAAGAGAG
      1210    1220    1230    1240    1250    1260    1270    1280    1290    1300    1310    1320

GAAATCTCACTGATGGTCTGGACTAGTAATATTGCGTTAGTAATGCAATTGGCATGAATGGACTGCAATAGCAGCGTGGAGCTATTTCTTTTGTGTGTTGCAGCAAAGCTTACAAGAGGT
      1330    1340    1350    1360    1370    1380    1390    1400    1410    1420    1430    1440

CTCCAGATGTGTCTACATGAAGAGCGCCTAAATAACCATGTGCAGAAAACCTGCAATGATTTTATTAACTTTCCCTTCTAAATGCTTATATGAGCCTAGCTTTTGCAATGTGTGTTTATA
      1450    1460    1470    1480    1490    1500    1510    1520    1530    1540    1550    1560

CATATATAAAATCCTCGTGATTTATATCTGCAATGTCCAATGTCTTTAAAGATATCTTGTATACCATTAGACCA
      1570    1580    1590    1600    1610    1620    1630
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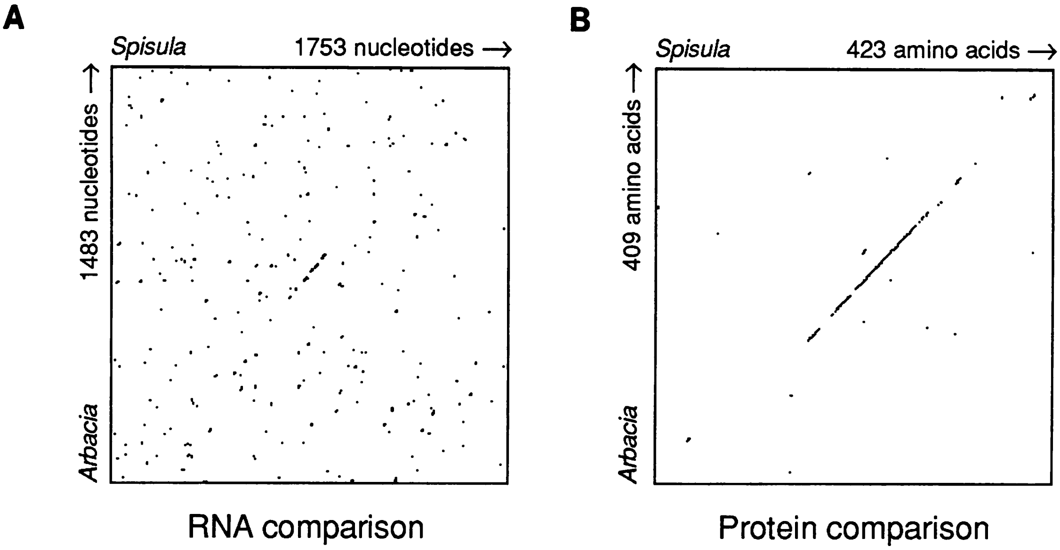
Fig. 5. Restriction map and nucleotide sequence of cyclin cDNA clone cyc4, starting at the 5′ end of the clone (as shown in Figure 4, the 5′ end of the mRNA appears to be about 220 nucleotides further upstream). Two TGA termination codons in the same frame as the putative initiator ATG are underlined. Also indicated are the start and finish of clone E3, the anti-cyclin M13 clone isolated by hybrid arrest of translation, and its internal *Sau*3A site. The longest conceptual open reading frame beginning at the first ATG shows homology with clam cyclin A as demonstrated in Figure 6.



C

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1        10        20        30        40        50        60        70        80        90       100       110       120
MSQPFALHHDGENNGLQMQRRGKMNTRSNQVSGQKRAALGVITKPKNQQVRIQPSRAATKKSSEFNIQDENAFSVFNAKTFGQQPSQFPTSVDPTPAAPVQKAQRVHVTDIPAALTTLCL
                         ||                                           |      |                   |       |
                     MALGTRNMNMNLHGESKHTFNNENVSARLGGKSIAVQKPAQRAALGNISNVVRTAQAGSKKVVKKDTRQKAMTKTKATSSLHAVVGLPVEDLPTEMRST
                     1        10        20        30        40        50        60        70        80        90

       130       140       150       160       170       180       190       200       210       220       230       240
EPLTEVPGSPDIIEEEDSMESPMILDLPPEYKPLDREAVILTVEEDIYNYLRQAEMKNRAKPGYMKRQ-TDITTSMRCILVDWLVEVSEEYKLHRETLFLGVNYIDRFLSKISVLRGKLQ
 |        |   |  |                |          ||| |||  |        |  || | ||  || ||||||| |     |  ||||| |  |||||   ||  ||||
SPDVLDAMEVDQAIEAFSQQLIALQVEDIDKDDGDNPQLCSEYAKDIYLYLRRLEVEMMVPANYLDRQETQITGRMRLIILVDWLVQVHLRFHLLQETLFLTVQLIDRFLAEHSVSKGKLQ
         110       120       130       140       150       160       170       180       190       200       210

       250       260       270       280       290       300       310       320       330       340       350       360
LVGAASMFLAAKYEEIYPPDVKEFAYITDDTYTSQQVLRMEHLILKVLTFDVAVPTTNWFCEDFLKSCDADDKLKSLTMFLTELTLIDMDAYLKYVPSITAAAALCLARYSLGMEPWPQN
|||   || | |||| |||    | ||||  ||  |   ||   || |      |    |          |     |   | | ||        | ||  ||||  |    |  |
LVGVTAMFIASKYEEMYPPEINDFVYITDNAYTKAQIRQMEIAMLKGLKYKLGKPLCLHFLRRNSKAAGVDAQKHTLAKYLMEITLPEYSMVQ-YSPSEIAAAAIYLSMTLLDPETHSSW
         230       240       250       260       270       280       290       300       310       320       330

       370       380       390       400       410       420
LVKKTGYEIGHFVDCLKDLHKTSLGAESHQQQAVQEKYKQDKYHQVSDFSKNPVPHNLALLAL             Clam A
  | | |                                  ||                 |
CPKMTHYSMYSEDHLRPIVQKIVQILLRDDSASQKYSAVKTKYGSSKFMKISGIAQLDSSLLKQIAQGSNE     Urchin
         350       360       370       380       390       400                409
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Fig. 6. Comparisons of *A. punctulata* cyclin and *S. solidissima* cyclin A RNA and protein sequences. **Panel A**, 'diagon' comparison of mRNA sequences at a score of 10/13. **Panel B**, protein sequence comparisons at a score of 135/11 (see Staden, 1982). **Panel C** shows a manual alignment of the two protein sequences, clam on the top line and urchin below it. The symbol '|' denotes an exact match, and dashes (-) indicate the location of two 1-residue gaps introduced to obtain better alignment.

Sea urchin cyclin mRNA causes rapid maturation of frog oocytes

The *Eco*RI fragment of clone cyc4 was inserted into the transcription vector pGEM 2, containing both SP6 and T7 RNA polymerase-specific promoters. A clone was isolated which made cyclin mRNA when transcribed by T7 RNA polymerase. Messenger RNA made from this DNA was translated in the reticulocyte lysate and compared with the translation products of authentic mRNA from *A. punctulata* as well as polypeptides synthesized by intact fertilized eggs of *A. lixula*. Figure 7 shows that a protein with precisely the mobility of *Arbacia* cyclin was the only product of the synthetic mRNA. As a final test, the RNA was injected into stage 6 *Xenopus* oocytes. Table I shows that all the injected oocytes underwent germinal vesicle breakdown and white spot formation. The response was seen at the lowest dose of RNA injected, ~0.3 ng per oocyte, although as Figure 8 shows, the lower the dose of RNA the longer it took for the oocytes to mature. Injection of equivalent amounts of the antisense version of cyclin mRNA had no visible effect on the oocytes. Labelling with [^{35}S]methionine showed that a 56 kd protein corresponding to sea urchin cyclin was synthesized in these oocytes and specifically destroyed as they began to show the white spots that announce the formation of the meiotic metaphase spindle (Figure 9). This strong band was not seen in uninjected controls (data not shown). A slight reduction in gel mobility of the cyclin occurred after 1 h. The nature of this modification is not known, though very preliminary studes of *in vitro* synthesized cyclin suggest that it is due to phosphorylation (it requires ATP and is promoted by NaF; A.A.Stewart and T.H., unpublished observations). Cycloheximide prevented both progesterone- and cyclin mRNA-induced oocyte maturation when added at zero time (Table II). However, when addition of cycloheximide was delayed, by 90 min in the RNA-injected and 150 min in the progesterone-treated oocytes, maturation was no longer inhibited. The times at which maturation in half the oocytes was no longer inhibited by cycloheximide occurred at about 60 min for the mRNA-injected cells and 120 min in the case of progesterone treatment.

Unfractionated maternal RNA from *A. punctulata* was injected into *Xenopus* oocytes and, like pure cyclin mRNA, caused maturation. In order to see whether cyclin mRNA was the com-

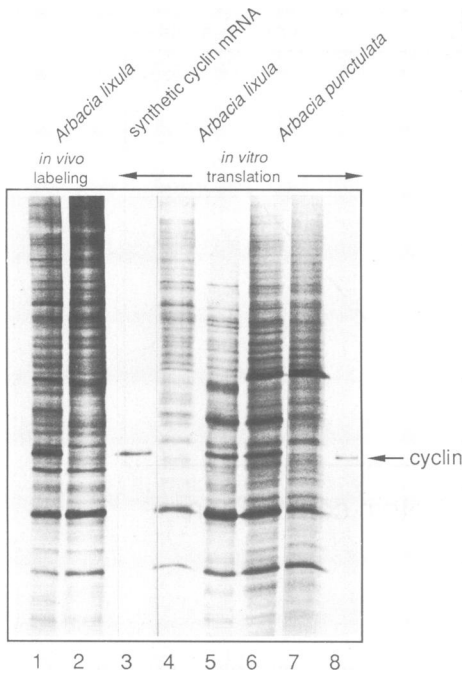

Fig. 7. The translation product of synthetic cyclin mRNA matches cyclin made in fertilized *A. lixula* eggs and the product specified *in vitro* by *A. punctulata* unfractionated maternal mRNA. **Lanes 1** and **2**: fertilized eggs of *A. lixula* were labelled with [^{35}S]methionine, and samples taken for analysis on gels after 30 min (**lane 1**) and 75 min (**lane 2**) at the time of first cleavage. **Lanes 3** and **8**: *in vitro* translation product of synthetic cyclin mRNA derived from a subclone of cyc4 in pGEM2; **lanes 4** and **5**: *in vitro* translation of total RNA from *A. lixula* eggs, ablated with anti-cyclin DNA in lane 4; **lanes 6** and **7**: *in vitro* translation products of total RNA from *A. punctulata* eggs, ablated with anti-cyclin DNA in lane 7.

Table I. Effect of sea urchin RNA on oocyte maturation

	Amount of RNA injected (ng/oocyte)	Number of oocytes injected	%GVBD
Experiment 1			
(A) *A. punctulata* total RNA	245	8	100
	100	5	80
	50	5	80
	25	5	80
	12.5	5	80
	6.3	5	0
(B) Cyclin ablated total RNA	245	7	0
	100	5	0
	50	5	0
(C) T7 transcript of cyclin mRNA	25	5	100
	12.5	5	100
	6.3	5	100
	3.1	5	100
	1.6	5	100
	0.7	5	100
	0.3	5	100
(D) Anti-sense cyclin mRNA	25	10	0
	17.5	11	0
Experiment 2			
Progesterone		7	57
Control (uninjected)		8	0
Total RNA (245 ng)		9	22
Ribonucleotide reductase ablated (245 ng)		12	25
Cyclin ablated RNA (245 ng)		11	0
Pure cyclin mRNA (17.5 ng)		8	62

Sea urchin egg RNA makes frog oocytes mature. **Experiment 1** used manually defolliculated oocytes, and was done in collaboration with Mike Wu. (**A**) Total RNA from *A. punctulata* eggs was injected into *Xenopus* oocytes in a volume of 50 nl, and the appearance of the white spot scored after 5–6 h. The temperature varied between 21 and 24°C. (**B**) A sample of the same RNA as used in (**A**) was subjected to RNase H-mediated digestion after annealing with single-stranded DNA of clone E3 (shown here). Controls with clone A18 (anti-ribonucleotide reductase; Standart *et al.*, 1985) did not affect the maturation-promoting activity of the RNA, and oocytes injected with the cyclin-ablated RNA matured at the same rate as uninjected controls when incubated with progesterone. (**C**) Oocytes received the indicated amounts of synthetic cyclin mRNA in a volume of 50 nl, or (**D**), the complementary transcript from the same plasmid. **Experiment 2** was performed in collaboration with John Gurdon using oocytes still surrounded by their follicles. This batch of oocytes did not respond to progesterone as well as the oocytes in experiment 1; indeed, pure cyclin mRNA was slightly more effective than progesterone in this series. Two other experiments gave similar results.

ponent responsible for this effect, a portion of the maternal RNA was annealed with single-stranded DNA of clone E3 and digested with RNase H. This completely abolished the oocyte maturation response. When the RNA was annealed with ribonucleotide reductase clone A18 and digested with RNase H, the maturation response was unaffected. Dose responses for these RNAs are shown in experiment 2 of Table I.

These results show that cyclin mRNA is sufficient to initiate *Xenopus* oocyte maturation, and extend the results of Swenson *et al.* (1986) by showing that maternal mRNA probably contains no other sequences capable of promoting oocyte maturation, at least as detectable by this assay.

Discussion

This paper describes the isolation and sequencing of clones for cyclin from the sea urchin *A. punctulata*. Although cyclin mRNA is abundant in sea urchin eggs, its cloning presented problems. Sea urchin maternal mRNA is difficult to purify in good yield because it binds poorly to oligo(dT) cellulose; unlike clam and starfish mRNA its poly(A) tails do not appear to get longer after fertilization (Rosenthal *et al.*, 1983; Rosenthal and Wilt, 1986). In addition, most of the maternal messages in sea urchins, including cyclin, have unusually long 3′ untranslated regions, making it hard to obtain full-length cDNA extending into the coding region. The only assay for cyclin mRNA was *in vitro* translation, which required individual screening of potential clones by hybrid selection or hybrid arrest of translation. Fortunately, cyclin synthesis can easily be detected by 1-dimensional SDS gel electrophoresis, and the procedure for hybrid arrest of translation in the reticulocyte lysate we have developed is reasonably fast and in our hands more reliable than hybrid selection. The procedure uses relatively little RNA. Hybrid arrest of translation in reticulocyte lysates using RNase H may be useful in other cases where the assay of translation products is the only way to identify a gene.

The sequence of cyclin does not reveal obvious clues to what it does. The only known proteins it resembles are the two clam cyclins A and B. The comparison between the urchin cyclin and clam cyclin A shows only a short central portion of the sequences with almost complete identity. Presumably this region is functionally important. There is somewhat better homology, particularly at the C-terminus, between urchin cyclin and clam cyclin B (J.Westerdorf and J.Ruderman, personal communication), but this is not a great help since we do not know what cyclin B does

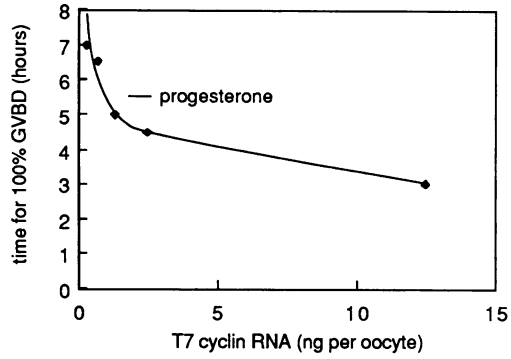

Fig. 8. Time course of frog oocyte maturation in response to pure cyclin mRNA. Serial dilutions of synthetic cyclin mRNA made from the pGEM2 cyc4 subclone were injected into groups of five *Xenopus* oocytes in a volume of 50 nl. Oocytes injected with the same volume of water or antisense transcript failed to mature. The time taken for 'white-spot formation' was noted and averaged for each group. The time taken for progesterone-induced maturation in a parallel uninjected group of oocytes is indicated by the line and caption 'progesterone'.

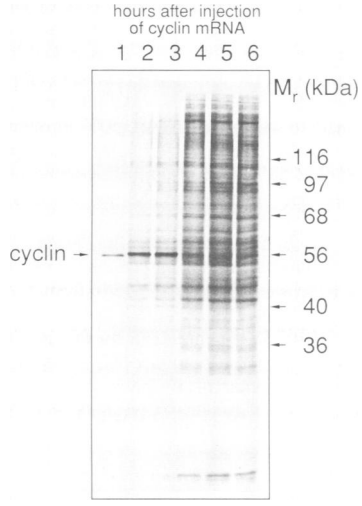

Fig. 9. Sea urchin cyclin is modified and destroyed in frog oocytes. A group of 14 oocytes were injected with 25 ng of synthetic cyclin mRNA and allowed to mature in the presence of 10 μCi of [^{35}S]methionine. Oocytes were removed at intervals of 1 h and processed for analysis on acrylamide gels as described in Materials and methods. Each track here represents about one eighth of an oocyte.

(or why clams have two cyclins, whereas urchins only have one). Neither the clam nor the urchin cyclin sequences shown in this paper contain obvious homologies to known enzymes.

It takes very little cyclin mRNA to make frog oocytes mature. How does it do it? *Xenopus* oocytes are arrested in first meiotic prophase, poised at the G2-M transition of the cell cycle. They are induced to enter 'M-phase' by exposure to progesterone produced by surrounding follicle cells (Schuetz, 1967). Protein synthesis is essential during the early stages of progesterone-induced maturation but a little over halfway to the appearance of the white spot that signals formation of the meiotic metaphase spindle, protein synthesis inhibitors no longer block maturation. This 'point of no return' is almost exactly coincident with the appearance of MPF (maturation or M-phase promoting factor) activity (Wasserman and Masui, 1976; Masui and Clarke, 1979). MPF is present at high levels in *Xenopus* eggs, and is prepared by crushing them in a centrifugal field and taking the supernatant.

Table II. Effect of cycloheximide on maturation induced by cyclin mRNA and progesterone

	Time of cycloheximide addition (min)						
	0	30	60	90	120	150	180
Oocytes matured with mRNA	0	0	3/5	5/5	4/5	4/5	3/3
Oocytes matured with progesterone	0	0	0	0	2/5	5/5	3/3

Manually defolliculated oocytes were either injected with 25 ng of pure cyclin mRNA or incubated with 2 μg/ml progesterone. Groups of five were placed in 100 μg/ml cycloheximide at 30 min intervals up to 3 h, and formation of white spots (the figures presented in the table) scored at 7 h. In the mRNA-injected oocytes, the first signs of maturation were seen at 90 min, whereas in the progesterone-treated oocytes, similar changes began at 120 min. No spontaneous maturation occurred in any of the oocytes used in these experiments.

The active principle seems to be a phosphoprotein of ~100 000 daltons (Gerhart *et al.*, 1985).

Microinjection of MPF causes oocytes to mature much more rapidly than progesterone and MPF-induced maturation does not require protein synthesis. Thus it is generally agreed that MPF is the agent directly responsible for catalysing entry into meiosis and mitosis (Newport and Kirschner, 1984; Ford, 1985). How is MPF activated? Injection of small amounts of MPF into oocytes rapidly causes the appearance of endogenous MPF by activation of an inert 'pro-MPF' precursor. The activation of pro-MPF to MPF almost certainly involves phosphorylation (Gerhart *et al.*, 1985) and maturation is accompanied by a general increase in protein kinase activity as well as increases in specific protein kinases, such as the lamin kinase that is associated with nuclear envelope breakdown (Maller *et al.*, 1977; Karsenti *et al.*, 1987; Newport, 1987). However, in the natural course of events the first traces of MPF to initiate its autoactivation are not supplied by microinjection, but by the synthesis of some new protein under the influence of progesterone. The sequence of events in oocyte maturation may be schematically represented thus:

Progesterone → synthesis of protein(s) X↑ → MPF activated → protein kinase activated → maturation

Protein X is a hypothetical entity responsible for activating the first traces of MPF to initiate the presumed autophosphorylation cascade. Since MPF is thought to be activated by phosphorylation, one might suppose that 'protein X' corresponds to MPF kinase. Cyclin would fit into this scheme as protein X except that it does not show homology with known protein kinases. Thus, if cyclin does correspond to 'protein X' and activates MPF, we prefer to think that it does so by inactivating a protein phosphatase or some such negative regulator of MPF activation. When cyclin is destroyed, the protein phosphatase is once more active, and MPF converted back to pro-MPF.

The crucial test of this hypothesis will be the injection of cyclin protein into frog oocytes, rather than cyclin mRNA. If progesterone acts by turning on cyclin synthesis, and cyclin turns on MPF, injection of cyclin protein ought to promote maturation even in the presence of cycloheximide, just as MPF itself does. If it were to have this property, cyclin would be indistinguishable from MPF by the oocyte maturation assay, and the next question would be whether cyclin is sufficient to promote mitosis in a cycloheximide-arrested cleaving embryo. The oocyte activation test is ambiguous in the sense that it scores for agents that activate pro-MPF as well as for MPF itself.

If this account is correct, *Xenopus* oocytes should contain their own version of cyclin mRNA. The homologies between clam

and urchin cyclins encouraged us to screen *Xenopus* egg and oocyte cDNA libraries with oligonucleotides corresponding to the conserved regions, and partial frog cyclin clones have recently been isolated (J.Minshull, J.P. and T.H., unpublished observations). The DNA sequence of one of these clones shows high homology to the central region of clam and sea urchin cyclin. In addition, *Xenopus* egg mRNA (but not oocyte poly(A)$^+$ RNA) causes maturation of frog oocytes, exactly like clam and sea urchin RNA (T.H., M.Wu., E.T.Rosenthal and J.C.Gerhart, in preparation). This is compatible with the idea that *Xenopus* oocyte maturation is caused by activating the translation of endogenous maternal cyclin mRNA. We are currently testing this hypothesis.

Materials and methods

Sea urchin eggs

Eggs and sperm of *A. punctulata* were obtained by electrical stimulation from animals collected by the Department of Marine Resources of the Marine Biological Laboratory, Woods Hole, MA, USA. *A. lixula* were collected at the Station Marine, Villefranche sur Mer, France.

Extraction of RNA

Preparation of sea urchin egg and embryo extracts was performed as described by Standart *et al.*, 1985. Post-mitochondrial supernatants were frozen and stored as 2-ml aliquots in liquid nitrogen. RNA was prepared from the extracts as described by Minshull and Hunt (1986).

Isolation of poly(A)$^+$ RNA

Poly(A)$^+$ RNA was prepared by fractionating total egg or embryo RNA on poly(U)−sepharose 4B columns with a bed volume of 1 ml (approximate binding capacity of 150 μg of mRNA). Columns were washed with 10 volumes of elution buffer and equilibrated with loading buffer before use. RNA was heated to 60°C for 5 min and loaded in 0.3 M NaCl, 50 mM Tris−Cl pH 7.8, 10 mM EDTA. Poly(A)$^+$ RNA was eluted in 2−3 column volumes of 90% v/v formamide, 10 mM Hepes pH 7.5, 1 mM EDTA, and ethanol precipitated. Poly(A)$^+$ RNA was dissolved in 1 mM EDTA pH 7.5 at a concentration of ~1 mg/ml. It usually represented about 1% of the total RNA. RNA fractions were assayed by translation in the reticulocyte lysate and analysis on 15% acrylamide gels according to Jackson and Hunt (1983).

Fractionation of RNA on sucrose density gradients

The RNA from poly(U)−sepharose was fractionated on 5−20% w/v linear sucrose gradients in 100 mM LiCl, 10 mM Tris−Cl pH 7.5, 5 mM EDTA, 0.2% SDS and 50% v/v formamide spun for 5 h at 50 000 r.p.m., 15°C, in a Beckman SW 50.1 rotor. Samples were denatured by heating for 5 min at 37°C in 80% v/v formamide and sucrose gradient buffer, diluted with an equal volume of buffer and loaded onto the gradient. Up to 100 μg of RNA was loaded per gradient. Fractions were ethanol precipitated and resuspended in 11 μl of water. One microliter was assayed by translation in the reticulocyte lysate.

cDNA library constructions

First and second strand cDNA synthesis reactions were carried out according to Watson and Jackson, 1985, except that the first strand buffer was 140 mM KOAc, 50 mM Tris−acetate pH 8.2 at 41°C, 8 mM MgOAc, 4 mM DTT, 0.5 mM dATP and dGTP and TTP, 250 μM unlabelled dCTP, 10 μCi of α[^{32}P]-dCTP, 20 units RNasin and 50 μg/ml actinomycin D. For the library constructed in M13mp8, the cDNA was size fractionated immediately after second strand synthesis on a 1% agarose gel. Molecules of greater than 1.5 kb were absorbed onto positively charged membrane (Schleicher and Schuell NA45), cut with *Sau*3A and ligated into *Bam*HI cut M13mp8 RF. This was introduced into *E. coli* strain TG 1 by the 'standard transformation' method of Hanahan (1985).

For the library constructed in λgt10 the cDNA was methylated, and *Eco*RI linkers added, digested and fractionated by gel filtration according to Huynh *et al.*, 1985. The *Eco*RI linkers were phosphorylated according to Maxam and Gilbert, 1980. Packaging into λgt10 using 'Gigapack' packaging mix was carried out according to the manufacturers instructions (Stratagene, formerly Vector Cloning Systems). The library was amplified through *E. coli* C600 *hfl*.

Screening the cDNA libraries

Filters (0.45 μm nitrocellulose) were prepared according to Mason and Williams, 1985. The M13 library was screened with [γ-^{32}P]ATP 5′ end-labelled RNA in 6 × SSC, 4% w/v skimmed milk, 50% formamide and 100 μg/ml boiled herring sperm DNA at 42°C overnight. Filters were washed with 1 × SSC, 0.5% SDS at 55°C. The λgt10 library was screened using a [α-^{32}P]dCTP labelled 'primed-cut' probe in 6 × SSC, 4% w/v skimmed milk, and 100 μg/ml boiled herring sperm DNA at 62°C overnight. Filters were washed with 0.1 × SSC, 0.5% SDS at 55°C.

DNA sequencing

The insert from the λgt10 clone cyc4 was subcloned into pUC8. Restriction fragments of the purified insert were subcloned into M13 vectors (as indicated in Figure 5) for sequencing by the chain termination method of Sanger *et al.* (1977), as detailed by Bankier and Barrell (1983). In order to obtain overlaps, deletions were made with *Bal*31 nuclease, and the oligonucleotides indicated in Figure 5 were used as primers. Most of the sequence was read independently at least three times, and all of the sequence was determined in both directions.

Hybrid arrest of translation

Putative positive clones from the M13 library were screened by hybrid arrest of translation of *A. punctulata* RNA in a reticulocyte lysate cell-free translation system as described by Minsull and Hunt (1986). Typically, 7 μg of unfractionated [not poly(A)$^+$] *Arbacia* egg RNA was annealed with 1 μg of *Hae*III-digested ssDNA for 20 min at 60°C and digested with RNase H for 30 min at 37°C in a volume of 3 μl. One microliter of this mixture was translated in 10 μl of reticulocyte lysate mix, with 0.5 mCi/ml [^{35}S]methionine. The reaction was stopped with 6 μl of 100 μg/ml RNase A in 10 mM EDTA pH 7.5, and diluted 15 min later with 50 μl of SDS-gel sample buffer. Fifteen microliters of this mixture were loaded onto acrylamide gels with 4 × 0.8 mm slots and run for ~2 h at 150 V.

Northern blots

RNA samples were treated with glyoxal and run on phosphate-buffered agarose gels as described by Maniatis *et al.* (1982). The RNA was transferred onto nylon membrane after electrophoresis and the RNA cross-linked to the membrane by exposure to light at 302 nm. Filters were hybridized to an [α-^{32}P]dCTP labelled 'primed-cut' probe in 6 × SSC, 4% w/v skimmed milk (treated with 0.1% v/v diethylpyrocarbonate to inactivate RNase), 50% formamide and 100 μg/ml boiled herring sperm DNA at 42°C overnight. Filters were washed with 0.1 × SSC, 0.5% SDS at 55°C. After autoradiography the membranes were stained with methylene blue to detect the 28S and 18S RNA bands.

Synthesis of synthetic cyclin mRNA with T7 RNA polymerase

Cloned *A. punctulata* cyclin was inserted into the *Eco*RI site of the vector pGEM 2 (Promega Biotech). RNA was synthesized *in vitro* from 4 μg of template using the following conditions: 40 mM Tris−Cl, pH 8.0, 15 mM $MgCl_2$, 1 mM ATP, CTP, UTP, 5 μCi [α-^{32}P]UTP, 0.1 mM GTP for the first 30 min increased to 1.1 mM for the next hour, 0.5 mM m^{7}GpppG, 5 mM DTT, 0.5 μg BSA, 100 units RNasin and 25−50 units of T7 RNA polymerase in a total volume of 100 μl. After phenol extraction and ethanol precipitation the RNA was dissolved in water at ~0.5 mg/ml.

Micro-injection of mRNA into Xenopus laevis oocytes

Xenopus oocytes were manually removed from surrounding follicle tissue and microinjected with 50 nl of RNA solution according to Wu and Gerhart (1980). Oocytes were incubated at 20°C until all the oocytes incubated in progesterone had undergone germinal vesicle breakdown (GVBD) as indicated by the appearance of a white spot at the animal pole. In doubtful cases oocytes were soaked in 8% TCA for 10 min, dissected with fine forceps and examined under a binocular microscope to determine whether they had undergone GVBD.

Labelling Xenopus oocytes with [^{35}S]methionine and analysis on acrylamide gels

Oocytes injected with synthetic cyclin mRNA were incubated in 1 mCi/ml of [^{35}S]methionine in modified Ringers solution. Two oocytes were removed at each time point in about 10 μl of buffer and immediately frozen on dry ice. For analysis on SDS−acrylamide gels, the oocytes were homogenized in 10 μl of 40 mM Na β-glycerophosphate, pH 7.2, 10 mM EDTA, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, and 1% v/v 2-mercaptoethanol. The homogenate was centrifuged 5 min in a microfuge at 4°C and the supernatant mixed with an equal volume of SDS-gel sample buffer. A sample of 5 μl was analysed on a 15% acrylamide gel.

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