Cloning by differential screening of a *Xenopus* cDNA coding for a protein highly homologous to cdc2

*(cell cycle regulation/kinase cdc2/maturation-promoting factor/deadenylation)*

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ABSTRACT

Fertilization of *Xenopus laevis* eggs triggers a period of rapid cell division comprising 12 nearly synchronous mitoses. Protein synthesis is required for these divisions, and new proteins appear after fertilization. Others proteins, however, which are synthesized in the unfertilized egg, are no longer made in the early embryo. To identify such proteins, a differential screen of an egg cDNA library gave nine clones corresponding to mRNAs that are deadenylated soon after fertilization. The sequence of one of these clones (Eg1) revealed a high homology to p34cyc, the kinase subunit of maturation-promoting factor. Only 12 amino acids in the deduced amino acid sequence were unique to Eg1 when its sequence was compared to all other known examples of cdc2. Despite this strong similarity, however, Eg1 was unable to complement a yeast cdc2 mutant in *Schizosaccharomyces pombe* or a cdc28 mutant of *Saccharomyces cerevisiae*. Four Eg1 transcripts, two major and two minor, were found in *Xenopus* oocytes and early embryos. These RNAs appeared very early (stage I) in oogenesis and their level remained constant until the midblastula transition, at which time they declined. Eg1 RNA is found in the poly(A)* fraction of oocytes only between the time of meiotic maturation and fertilization—that is to say, in the unfertilized egg. At fertilization, the RNA loses its poly(A) tail and at the same time leaves the polyribosomes.

For most animals, the developmental period following fertilization is characterized by a period of very rapid cell division called cleavage (1). In the case of *Xenopus*, first cleavage takes place 1.5 h after fertilization and is followed by 11 almost synchronous cell divisions, which occur every 30 min (2). The onset of transcription is only clearly detected after the 12th cleavage at a stage called the midblastula transition (MBT). The MBT involves the coordinated desynchronization of cell divisions, the appearance of transcription and G1 and G2 phases, cell motility, and cell differentiation (2).

In the absence of actinomycin D embryos develop up to the MBT, whereas this development is blocked by puromycin or cycloheximide (3, 4). Similar results have been obtained for oocyte maturation, which is independent of new transcription but requires *de novo* translation (5). Qualitative analyses of the proteins synthesized in oocytes (stage VI), unfertilized eggs, and embryos have shown that during maturation and after fertilization new proteins appear but others are no longer synthesized (6–8). This suggests that the sequential synthesis of specific gene products necessary for maturation (first meiotic division), the metaphase block in the unfertilized egg and cleavages, is regulated at the translational level from the bulk of maternal mRNA. By differential screening of an egg cDNA library we have isolated 11 cDNA clones corresponding to discrete mRNAs that vary in adenylate and polysome recruitment after fertilization (9). Nine of these clones (denoted Eg1 to Eg9) are deadenylated after fertilization (9, 10). We have shown that, for at least one of these clones, deadenylation is associated with release from polysomes (9). These clones correspond therefore to mRNAs whose translation changes soon after fertilization during the very early phase of embryonic development. In the present paper we report the sequence analysis and the characterization at the RNA level of one of these clones (Eg1). It is highly homologous to p34cyc, an essential component of the maturation-promoting factor (MPF) (11, 12). A possible role of Eg1 that is unable to complement cdc2 *Schizosaccharomyces pombe* or cdc28 *Saccharomyces cerevisiae* mutants is discussed.

MATERIALS AND METHODS

Biological Materials. Ovaries were removed from anesthetized *Xenopus laevis* females, and the oocytes were isolated by treatment with dispase and collagenase, successively (13). The oocytes were sorted manually into the different oogenesis stages (14). Ovulated eggs were obtained from females following two injections of human chorionic gonadotrophin. Eggs were fertilized and embryos were cultured at 22°C in F1 modified medium (15).

DNA and RNA Preparation and RNase H Treatment. High molecular weight DNA from erythroblast nuclei was purified as described (16). Total RNA was prepared using the LiCl/urea procedure (17). Poly(A)* RNA was isolated by oligo(dT)-cellulose chromatography (18). Total RNA prepared from eggs or embryos (4 hr) was treated by RNase H in the presence or in the absence of (dT)12–18 (19).

DNA Sequencing. The restriction fragments indicated in Fig. 1 were purified by agarose gel electrophoresis and subcloned into the phagemid Bluestrip.* Single-strand DNA was prepared and sequenced by the dideoxy chain-termination method (20).

*Northerns and Southern Blots.* Samples of RNA were separated on agarose gels containing 6% formaldehyde and blotted onto nylon membranes (Hybond, Amersham). Purified inserts were 32P-labeled using random primer (21) to a specific activity of 5 × 106 cpm/μg. Hybridization was carried out in 50% formamide/1% SDS/10× Denhardt’s

Abbreviations: MBT, midblastula transition; MPF, maturation-promoting factor.

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. X14227).
solution (0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/10% dextran sulfate/1% sodium pyrophosphate/1% NaCl/0.05 M Tris-HCl, pH 8, at 4°C overnight. Filters were extensively washed in 0.3 M NaCl/30 mM sodium citrate/0.5% SDS at 65°C.

Ten micrograms of Xenopus genomic DNA was digested with EcoRI, HindIII, or BamHI. The samples were fractionated in agarose gels, transferred to nitrocellulose filters, and hybridized with the complete Egl probe. Hybridization and washing conditions were the same as those described for Northern blot analysis.

Polysonyal and Nonpolysonyal RNA Fractionation. One hundred eggs or embryos were homogenized in 5 ml of HKM buffer (20 mM Hepes, pH 7.4/300 mM KCl/10 mM MgCl2) containing 0.5% Nonidet P-40 and 20 μg of polyvinyl sulfate per ml and centrifuged through a 15–30% sucrose gradient for 11 hr at 27,000 rpm in a Beckman SW28 rotor (22). Gradients were monitored at 254 nm by pumping through an I.S.C.O. Ultramicrophotometer. The resulting gradient was fractionated into 10 fractions of 3 ml each.

For hybridization analysis, RNA samples were electrophoresed and blotted onto nitrocellulose filters. Filters were hybridized with [32P]dCTP-labeled cDNA probes. Hybridization conditions were the same as those described for Northern blot analysis.

Complementation Assays of S. cerevisiae cdc28 and S. pombe cdc2 Mutants. S. cerevisiae cells [strain OL128/3C (MATa, cdc28-6, leu2, ura3), a gift of M. Jacquet, Paris XI] were transformed by a modified protoplast method (23, 24) and selected at permissive temperature (26°C) for leucine prototrophy. A 1700-base-pair (bp) Sma I-Dra I fragment containing the entire open reading frame of the Egl gene was excised from the Bluescript plasmid and cloned into the Bgl II site of the expression vector pEMBLY302/2 (25). The resulting plasmid, pEMBL4, was used to transform a thermosensitive cdc28 mutant of S. cerevisiae [strain OL128/3C (MATa, cdc28-6, leu2, ura3)] by the protoplast method (23, 24). To test cdc28 suppression, transformed cells selected at permissive temperature (26°C) were plated on selective medium [0.17% YNB/0.5% ammonium sulfate/28 μg of uracil per ml/2% glucose (26)] and were grown either at permissive or at restrictive temperature (36°C).

Similarly, a haploid h- S. pombe strain carrying the temperature-sensitive cdc2 allele cdc2-33 (27), as well as the leu1-32 mutation, was transformed to leucine prototrophy at 25°C (the permissive temperature for cdc2-33) with plasmid pSM2-Egl and the control plasmids pSM2 and pIR22. The transformed colonies were selected at permissive temperature (26°C) and were grown (28). The transformant colonies were then replated to 35°C and examined microscopically after 24 hr. pSM2-Egl was constructed by blunt-end cloning a Pst I fragment containing the Egl cDNA into the polylinker Pvu II site of the expression vector pSM2 just downstream of the simian virus 40 promoter. This promoter has been shown to work well in the fission yeast (29). pIR22 contains the wild-type cdc2* gene.

RESULTS

Egl Sequence Analysis. The sequencing strategy, the nucleotide, and the predicted amino acid sequence of Egl cDNA are shown in Fig. 1. Egl cDNA is 1683 nucleotides long and contains 19 nucleotides of 5' flanking sequence, an open reading frame of 891 nucleotides, and a 3' flanking region of 773 nucleotides. The 3' untranslated region has several features: a 28-nucleotide direct repeat between nucleotides 961 and 1015 and a potential poly(A) signal, AUUAAA (nucleotides 1660–1665). In addition, several sequence motifs, UUUA(A)AU, similar to those shown to be necessary for maturation-specific adenylation of Xenopus mRNAs (30, 31), are also present (Fig. 1). The open reading frame codes for a 297-amino acid polypeptide (Mr, 35,948). In vitro translation of the mRNA transcribed in vitro from Egl cDNA produced a protein that migrates with a Mr of 32,000 on SDS/polyacrylamide gel (data not shown). Evidently, Egl is related to the protein kinase family and contains all of the amino acid motifs that other kinases have (32).

Comparison of the predicted Xenopus Egl protein sequence with sequences in the EMBL/Genbank data base showed that Egl was highly homologous to the cdc2 protein sequences of S. pombe (33), chicken (34), mouse (35), and human (36) and to the cdc28 protein of S. cerevisiae (37): 65.3%, 60.6%, 66.3%, 63.6%, and 64%, respectively (Fig. 2). Therefore, the similarity between Xenopus Egl protein and the various cdc2 proteins or cdc28 protein is almost the same as the similarity between S. pombe cdc2 and S. cerevisiae cdc28 (69.6%) or between S. cerevisiae cdc28 and human cdc2 (64.5%). Moreover, specific parts of the cdc2 protein sequence, such as the
cdc2 antibodies and also cdc2.

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mologs from Northern SC CDC28, also only signals, diagram hybridized Egl protein labeled were MBT constant about cDNA of total 3A). Expression of Egl mRNA stages RNA. Therefore, this in Fig. to the tissues observed in 30-fold, this in Fig. I to the LDL-PGQ-QR ------ES-D-- RSNC-R--- IL-A-S I-K--V- --A--NNEV-E-ESLQ--N---KPGSLASH-KN---N-L-- --DDK-V------IP--VY- that cdc28 (37), the budding yeast homolog of 16-residue "PSTAIR" motif, are present in Egl, and in vitro synthesized Egl protein was immunoprecipitated by anti-PSTAIR antibodies and also binds to p13 nucle (data not shown).

Expression of Egl mRNA During Oogenesis and Early Development. Total RNAs extracted from oocytes (stage I to stage VI), eggs, and pre-MBT embryos were subjected to Northern analysis using a 32P-labeled Egl cDNA probe (Fig. 3A). This probe, made using the entire Egl cDNA, essentially hybridized to two major transcripts of about 2 and 3 kilobases (kb) in all samples. In fact, this probe also hybridized to additional transcripts that are less abundant (Fig. 3B).

Between stages II and VI of oogenesis total RNA increases about 20- to 30-fold, mainly due to an accumulation of a large amount of rRNA (1). Therefore, the decrease in the autoradiogram signals, observed in Fig. 3A for the 2- and 3-kb mRNAs, reflects the dilution of these transcripts in the pool of total RNA. Taking this in account, quantification of autoradiograms from these Northern analyses confirmed that the number of these transcripts per oocyte or embryo was approximately constant from stage II of oogenesis to the MBT in developing embryos. Total RNAs extracted from several adult tissues (brain, liver, skin, kidney, and testis) were also subjected to Northern analysis using the 32P-labeled probe to the entire Egl cDNA. Egl transcripts were only detected in testis RNA (data not shown). However, this probe also visualized several transcripts in RNA extracted from exponentially growing Xenopus kidney A6 cells (see Fig. 3B).

Egl Gene Family. We have shown that the cloned Egl cDNA hybridizes to several RNAs of different sizes (Fig. 3B). This suggests that the mRNA corresponding to the Egl cDNA may be transcribed from one of several genes coding for proteins of closely related sequence/function. In this case partial cDNA probes corresponding to the coding sequence should visualize all or most of these transcripts, whereas probes to the 3' untranslated region, generally less well conserved among related genes, should only hybridize with the homologous mRNA. Therefore subclones of Egl cDNA, corresponding to the main 5' coding region (nucleotides 1-213) or to the 3' untranslated region (nucleotides 1083-1683), were used to produce 32P-labeled probes. Northern analysis of total RNA from oocyte stage VI, eggs, and embryos (6 hr) showed that the 3' probe visualized a total of five transcripts in oocytes and embryos (109, 28, 3, 3, and 3.6 kb) (Fig. 4A). With the 3' probe, however, only the 2-kb mRNA was clearly detected, although faint signals persisted from the other transcripts. These results show, therefore, that the coding regions of the Egl transcripts are closely related but their 3' untranslated regions are different. In addition, this comparative Northern analysis with the 5' and 3' probes shows that the cloned Egl cDNA corresponds uniquely to the 2-kb mRNA. To evaluate the number of Egl-related genes, total Xenopus genomic DNA was digested separately with EcoRI, BamHI, and HindIII and subjected to Southern analysis using a 32P-labeled probe (Fig. 4B). Quantification of the autoradiogram signals, relative to that for Egl cDNA, showed that at least four Egl-related genes may exist in the haploid Xenopus genome.

Posttranscriptional Modifications of Egl mRNA. Closer examination of the data in Fig. 3A shows that the 2-kb transcript appears to be larger in eggs than in either oocytes or embryos. We have previously shown that the Egl cDNA was selected by differential screening as an mRNA that is deadenylated after fertilization (9). To ascertain whether the size differences observed for the 2-kb transcript corresponded to adenylated or deadenylated states of the same mRNA, RNAs from eggs and embryos were fractionated by chromatography on oligo(dT)-cellulose. Northern analysis of these poly(A)+ and poly(A)− fractions from the different samples showed that the slightly larger 2-kb transcript was only present in the poly(A)+ fraction prepared from the eggs (Fig. 5A). After fertilization the amount of this larger 2-kb transcript decreased in the poly(A)+ fraction, and there was a concomitant increase of the smaller 2-kb transcript in the poly(A)− fraction. It should be noted that the 3-kb transcript also passed from the poly(A)+ to the poly(A)− fractions after fertilization, indicative of a similar postfertilization deadenylation of this mRNA (Fig. 5A). Further evidence that the size difference in the 2-kb transcript was due to a change in the polyadenylation was obtained by incubating total RNA extracted from eggs and embryos with (dT)12-18 in the presence or absence of RNase H. Northern analysis of these samples showed that the size differences of the 2-kb mRNA between the egg and embryo samples were abolished by removal of the poly(A) tract (Fig. 5B).
polysomal fractions were prepared from eggs and 6-hr embryos, and the RNA extracted from these fractions was subjected to Northern analysis. The results showed that the 2-kb mRNA visualized by the Egl cDNA probe was present in the polysomal fraction obtained from eggs but was completely absent from this fraction obtained from 6-hr embryos (Fig. 5C). When polysomal and non-polysomal fractions were prepared in the presence of EDTA, this transcript was always found in the nonpolysomal fraction (data not shown). Therefore, the postfertilization deadenylation of this 2-kb mRNA is associated with its release from polysomes.

**Biological Role of Egl.** The sequence analysis described above suggests that Egl is a protein whose function may be similar to that of cdc2 or cdc28—i.e., a regulator of G2/M and G1/S transition during the cell cycle (11, 12). The different analyses at the RNA level that we have performed suggest that this mRNA is translated in the eggs, a time when important changes in the cell cycle status occur. We therefore postulated that Egl could be the *Xenopus* counterpart of cdc2. To test this postulate, we tried to complement *S. cerevisiae cdc28* and *S. pombe cdc2* mutants with Egl. Cells harboring a thermosensitive cdc28 mutation (strain OL128/3C) were transformed with plasmid pEMBLYe30/2, a replicative plasmid harboring the LEU2 gene and the phosphoglycerate kinase (PGK) promoter and terminator (25), or with pEMBL4, a pEMBLYe30/2-derived plasmid in which expression of the Egl gene is under control of the PGK promoter. OL128/3C cells were also transformed with a Yep13 plasmid containing the cdc28 gene. As expected cells containing pEMBLYe30/2 were not able to grow at restrictive temperature (36°C). On the contrary, cells containing the cdc28 gene on Yep13 (38) were able to grow at 36°C. Finally, cells containing pEMBL4 did not grow at 36°C. Thus the presence of Egl did not suppress the cdc28 mutation.

Likewise, fission yeast cells carrying the cdc2-33 mutation could not be rescued by expression of the Egl protein from the plasmid pSM2-Egl. This was shown by transforming a cdc2-33 leu1-32 haploid strain with pSM2-Egl and with the control vector pSM2 and replica plating the resulting leu+ transformants to the restrictive temperature for cdc2-33 (see *Materials and Methods*). At the restrictive temperature the cells underwent cell cycle arrest, becoming highly elongated; in contrast, cells transformed with plasmid pRT22, which contains the wild-type cdc2+ gene, were able to grow and divide normally at the high temperature.

**DISCUSSION**

In the present paper we report the sequence analysis and the characterization at the RNA level of a clone (Egl1) selected by differential hybridization. Its protein sequence is highly homologous to known cdc2 protein sequences (33–37). Egl1 contains the 16-residue PSTAIR region and specifically the tyrosine in position 15, which has been shown to play a key role in mitosis entry through phosphorylation and dephosphorylation processes (11, 12). Only 12 of a total of 297 amino acids were found to be specific to Egl1 when compared to the other published cdc2 sequences. It should be pointed out that 4 of the amino acids that are missing in Egl1 are tyrosines. Since Egl cDNA was unable to complement either cdc2 S. pombe or cdc28 S. cerevisiae mutants, we can postulate that at least some of these amino acid changes are important for the function of the protein. *A priori* this negative result in the completion experiments did not exclude that Egl1 could be the *Xenopus* counterpart of S. pombe cdc2. But, by using specific antibodies against Egl1 protein, it has been found that the protein is not a component of purified MPF and does not form complexes with mitotic cyclins (39). Immunoprecipitates of Egl protein possess histone kinase activity and the protein also binds to p13mel affinity columns (39). There-
fore, although Eg1 does not appear to be functionally equivalent to cdc2, the properties of this protein indicate the need for caution in using p13 beads or PSTAIR antibodies as tools for studying specifically the p34\textsuperscript{cdk2} protein.

In this study we showed that Eg1 RNA is specifically found in the poly(A)\textsuperscript{+} fraction in the eggs. After fertilization the RNA is adenylated and removed from the polysomes. Other studies show that during maturation polysome den-

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