Identification of the Domains in Cyclin A Required for Binding to, and Activation of, p34^{cdc2} and p32^{cdk2} Protein Kinase Subunits

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The binding of cyclin A to p34^{cdc2} and p32^{cdk2} and the protein kinase activity of the complexes has been measured by cell-free translation of the corresponding mRNA in extracts of frog eggs, followed by immunoprecipitation. A variety of mutant cyclin A molecules have been constructed and tested in this assay. Small deletions and point mutations of highly conserved residues in the 100-residue "cyclin box" abolish binding and activation of both p34^{cdc2} and p32^{cdk2}. By contrast, large deletions at the N-terminus have no effect on kinase binding and activation, until they remove residues beyond 161, where the first conserved amino acids are found in all known examples of cyclin A. At the C-terminus, removal of 14 or more amino acids abolishes activity. We also demonstrate that deletion of, or point mutations, in the cyclin A homologue of the 10-residue "destruction box," previously described in cyclin B (Glutzer *et al.*, 1991) abolish cyclin proteolysis at the transition from M-phase to interphase.

INTRODUCTION

Cell cycle transitions require the activity of protein kinases that contain catalytic subunits encoded by members of the *cdc2* gene family together with a regulatory and activating subunit corresponding to a member of the cyclin family (reviewed by Nurse (1990) and Pines and Hunter (1990)). The association of a mitotic cyclin with p34^{cdc2} appears to be necessary to turn on its protein kinase activity (Solomon *et al.*, 1990; Desai *et al.*, 1992), and the destruction of cyclin turns off the protein kinase activity of p34^{cdc2} just before the onset of anaphase (Luca and Ruderman, 1989; Murray *et al.*, 1989; Luca *et al.*, 1991). It is now known that there is more than one *cdc2* gene in higher eukaryotes (Lehner and O'Farrell, 1990; Elledge and Spottswood, 1991; Paris *et al.*, 1991; Tsai *et al.*, 1991) and at least eight different kinds of cyclin (mitotic cyclins A, B1, and B2 in vertebrates; G1 cyclins CLN1/2 and CLN3 in budding yeast, and three cyclin homologues known as cyclins C, D (CYL1 or PRAD1) and E have recently been discovered

in humans (Hadwiger *et al.*, 1989; Léopold and O'Farrell, 1991; Lew *et al.*, 1991; Matsushime *et al.*, 1991; Motokura *et al.*, 1991; Xiong *et al.*, 1991). It is not yet clear what combinations of cyclin plus p34^{cdc2}-like subunits can associate to form active protein kinases or what special properties different cyclin subunits confer on their partners. Indeed, for some of the most recently discovered cyclins, particularly C and D, the evidence for association with protein kinase subunits is at present somewhat indirect. It is thought likely that cyclins are in some sense targeting subunits for p34^{cdc2} as well as controlling the timing of turning on and off its kinase activity (Minshull *et al.*, 1990; Solomon *et al.*, 1990). So far, most of the known substrates for cyclin/*cdc2* protein kinases are able to be phosphorylated by any combination of cyclin and *cdc2* subunit (Minshull *et al.*, 1990) provided their kinase activity has been turned on by the appropriate phosphorylation state of the *cdc2* subunit (Dunphy and Newport, 1989; Gould and Nurse, 1989; Morla *et al.*, 1989; Newport, 1989; Ducommun *et al.*, 1991; Featherstone and Russell, 1991; Gautier *et al.*, 1991; Gould *et al.*, 1991; Parker *et al.*, 1991, 1992; Strausfeld *et al.*, 1991; Kumagai and Dunphy, 1992; Millar and Russell, 1992) (for reviews see Fleig and Gould, 1991 and Maller, 1991). The cellular targets of

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these kinases are not well characterized, however, and at the relatively low concentrations of kinases and their target substrates found in cells, the specificity may be much higher.

The sequence homology between different members of the cyclin family is not extensive and is largely confined to a stretch of 100 residues in the middle of the linear sequence that is commonly known as the "cyclin box" (Nugent *et al.*, 1991). Even in this core, however, only five well-spaced residues (RDLKF) show complete conservation in all cyclins from yeast to man, and in cyclin C, which appears to be a yet more distant relative, the R and D are altered. If, as is believed, these proteins all associate with p34^{cdc2} or p34^{cdc2}-like proteins, it is likely that these conserved residues and the region spanned by them are important in the cyclin-p34^{cdc2} interaction, and if this is so, questions are raised about the function of the remaining 300 or so amino acids. One other clearly marked domain has been defined in the N-terminus of the mitotic cyclins A and B, which is required for their regulated sudden destruction at the metaphase-anaphase transition (Murray *et al.*, 1989; Glotzer *et al.*, 1991; Lorca *et al.*, 1992). As a first step to investigating structure-function relationships in cyclin A, we have made a number of N- and C-terminal deletions of *Xenopus* and bovine cyclin A1 and various small deletions and point mutations in the cyclin box. We have tested these constructs for their ability to promote *Xenopus* oocyte maturation, for their ability to bind and activate p34^{cdc2} and p32^{cdk2}, and for their ability to be destroyed. We find that the first 161 residues of cyclin A are dispensable for binding to and activation of p34^{cdc2}, but larger deletions, which encroach on the first conserved residues, cause complete loss of activity. By contrast, removal of as little as 14 amino acids at the C-terminus of cyclin A renders it completely inactive. Point mutations of the completely conserved R197 and D226 and deletions of two or more residues from the cyclin box cause serious or complete loss of activity. There is a perfect correlation between the ability of cyclin A to bind with p34^{cdc2} and its activity, both in vivo and in vitro. We also confirm that the N-terminal destruction box in cyclin A is necessary for programmed destruction triggered by addition of Ca²⁺ to an egg extract (Glotzer *et al.*, 1991; Luca *et al.*, 1991; Lorca *et al.*, 1992).

Finally, although we and others were previously unable to detect newly synthesized cyclin A associated with immunoprecipitates of p32^{cdk2} (Minshull *et al.*, 1990; Gabrielli *et al.*, 1992), we now report that when *Xenopus* cyclin A1 and p32^{cdk2} are capable of binding together to give an active histone kinase when their concentrations are increased by cell-free translation of added mRNA. By contrast, B-type cyclins do not appear to form complexes with p32^{cdk2} under the same conditions. The cyclin A mutations that abolish p34^{cdc2} binding also abolish p32^{cdk2} binding.

MATERIALS AND METHODS

Construction of Cyclin A Deletions

A full-length clone of *Xenopus* cyclin A1¹ in pGEM1 (clone XL4) (Minshull *et al.*, 1990) was digested at the unique EcoRV site. The linearized DNA (10 µg) was incubated with 5 U of Bal 31 nuclease (Boehringer, Mannheim, Germany) in a final volume of 100 µl in 20 mM tris-(hydroxymethyl)aminomethane (Tris)²-Cl, 100 mM NaCl, 5 mM CaCl₂, 5 mM MgCl₂, 1 mM EDTA, pH 8.0, at 30°. Samples of 15 µl were taken at 2-min intervals into an equal volume of 20 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), pH 7.3, to stop further digestion. The DNA was repaired with T4 DNA polymerase in the presence of 100 µM dNTPs, ligated with T4 DNA ligase, and introduced into *Escherichia coli* strain TG1 by standard Ca²⁺-mediated transformation. DNA was prepared from individual colonies and the size of deletion assessed by restriction enzyme analysis followed by agarose gel electrophoresis. Selected clones were transcribed (see below) and translated in the nuclease-treated rabbit reticulocyte lysate, and the [³⁵S]methionine-labeled products analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The clones that gave translation products of the expected size (as opposed to prematurely terminated products resulting from out-of-frame deletions) were selected for further analysis and were sequenced with an oligonucleotide with the sequence 5' ACAAGCT-GAAGTTTCC 3', corresponding to the amino acid sequence GKLQLV in *Xenopus* cyclin A1. Similar deletions were made from the Nsi I and Sty I sites (we thank Hashmat Sikder, who isolated and characterized Δ62). The junction sequences of the selected clones are listed in Table 1.

C-Terminal Cyclin Deletions

A series of C-terminal cyclin deletions, all of them including a terminator codon, were constructed by the polymerase chain reaction, with 3' primers containing a BamHI site, which was used to insert the truncated DNA between the HindIII and Bcl I sites of a modified cyclin A1 construct in which the first 21 residues of cyclin A1 were replaced with 15 amino acids of the c-myc epitope described by Evan *et al.* (1986), and the translational leader was derived from influenza virus NS protein as described by Dasso and Jackson (1989). The plasmid backbone was derived from pGEM2 (Promega, Madison, WI), modified by removal of the 252 bp EcoRI-Nae I fragment, in whose place was added an EcoRI-EcoRV fragment from a pET8 construct that included the T7 transcription terminator (the idea was to avoid the linearization step in the transcription protocol, but we found that there was too much readthrough, so we did not exploit this feature in practice). The sequence from the start of the T7 promoter to the start of the cyclin sequence was as follows

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TAATACGACTCACTATAGGGAGACCGGAAGCTAGCTTGGGCTGCAGGTCGACAGCAAAAGCAGGGTGAC
CAAGACATAATGGATCCCATGGAGCAAAAGCTCTTGAAGAAGATCTGAACAGTGCAATCCAGAA
M D P M E Q K L I S E E D L N S A F Q N
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The c-myc tag sequence is underlined. In this construct there is a unique EcoRI site at the 3' end of the cyclin 3' UTR (untranslated region), and the Nhe I, BstEII, BamHI, Nco I, and Bgl II in the sequence above are all unique in the plasmid (addition of the T7 terminator

¹ We refer to cyclin A1 to describe the form of *Xenopus* cyclin A used throughout this paper. *Xenopus* has at least one other cyclin A gene, which may have different properties (M. Howell and T.H., unpublished data).

² Abbreviations used: CSF, cytostatic factor; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; GVBD, germinal vesicle breakdown; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; IgG, immunoglobulin G; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; UTR, untranslated region of mRNA.

Table 1. Details of cyclin deletion constructs

Name	Length of deletion	Location of deletion	Junction sequence
Mutants of <i>Xenopus</i> cyclin A1			
Δ21	21	1–21	<u>MEQKLISEEDLN</u> *SAF
Δ62	39	24–62	ASSA/PAKS
Δ133	133	1–133	MDPM ^b EASP
Δ144	57	88–144	NPAP/QTSP
Δ147	58	90–147	APVA/PEDD
Δ158	57	102–158	VDEP/VAVS
Δ161	53	109–161	YSVE/SEYI
Δ169	69	101–169	YVDE/QYLR
Δ176	84	93–176	APKP/LKHR
Δ201	122	80–201	AFPG/M'DWL
Δ213	117	97–213	SFTV/HTET
Δ245	210	36–245	VEVQ/AILL
Δ232	2	231–232	FSLs/SVLR
Δ235	7	229–235	LDRF/RGKL
Δ237	10	228–237	YLDR/KLQL
Δ261	36	226–261	MNYL/DEFV
CΔ14	14	405–419	KTTKYM* ^d
CΔ24	24	395–419	QAQQAI*
CΔ50	50	369–419	AFTGYA*
CΔ79	79	340–419	KYVPSL*
CΔ200	200	219–419	LHTETL*
Mutants of Bovine cyclin A*			
Δ267	2	266–267	AEFV/S'DDT
Δ275	18	258–275	EEIY/QVLR
Δ278	24	255–278	SKFE/G*MEH
CΔ16	16	403–419	REKYKNS*

* Underlined sequence denote the c-myc tag. This leader was present in all the *Xenopus* C-terminal deletion series of constructs except CΔ200.

^b Italics indicate altered residues from vector sequences; the wild-type sequence is LDIS.

^c M replaces V.

^d An asterisk denotes a termination codon.

* The numbering system is that of the equivalent *Xenopus* sequences.

^f The S is a T in the wild-type sequence.

^g The G is an R in the wild-type sequence.

removed an *Nhe* I site in the vector), as are the internal *EcoRV*, *HindIII*, *Nsi* I, and *Bcl* I sites present in *Xenopus* cyclin A1.

The C-terminal deletion CΔ200 was derived from the *Nsi* I series of Bal 31 deletions. Closure of the deleted molecule formed a termination codon (Table 1).

Construction of Point Mutations

Mutagenesis of individual residues in cyclin A1 was either performed with the Amersham (Arlington Heights, IL) kit, or with a polymerase chain reaction-based strategy essentially as described by Horton and Pease (1991). The altered segments were checked by sequencing the double-stranded template DNA with the United States Biochemicals Sequenase kit (Cleveland, OH).

Construction and Expression of Bovine Cyclin A

A partial clone encoding all but the first 24 residues (compared with the sequence of human cyclin A) of bovine cyclin A was isolated from

a bovine lymphocyte cDNA library.³ The large *Sau*3AI fragment from this clone was inserted into the in-frame *Bam*HI site of a vector derived from pET3a (Studier *et al.*, 1990) encoding the IgG (immunoglobulin G) binding domain of staphylococcal protein A (derived by PCR from pRIT2T [Pharmacia, Uppsala, Sweden]). This construct encoded protein A at the N-terminus and bovine cyclin A at the C-terminus, minus the first 76 residues of the cyclin A (the junction sequence is FIQSLKDDPGNSRDLPINDEYVPVPP, cyclin residues underlined). The fusion protein comprised 620 amino acids and was soluble when induced with 0.1 mM isopropyl-β-D galactoside (IPTG) at an A₆₀₀ of 0.4 in *E. coli* BL21 (DE3) at 20° overnight. After a freeze-thaw cycle, the bacteria were digested with lysozyme, and the protein A-cyclin A constructs were purified from the 100 000 g supernatant by chromatography on IgG Sepharose as described by Solomon *et al.* (1990). Mutations were introduced into the construct by Bal 31 deletions at a unique *Acc* I site, and deletion of 16 residues at the C-terminus was performed by means of the polymerase chain reaction. The structures of these constructs are summarized in Table 1 and Figure 5.

Preparation of mRNA for Translation

Plasmid DNA was linearized at the end of the 3' UTR with the appropriate restriction enzyme (*Bam*HI for the untagged cyclin A constructs, *Eco*RI for the tagged ones) and used as templates for transcription in vitro using T7 RNA polymerase essentially according to Nielsen and Shapiro (1986). Capped mRNAs were made in 50-μl reactions that contained 4–5 μg of DNA, 40 mM Tris-Cl, pH 8.0, 15 mM MgCl₂, 5 mM dithiothreitol (DTT), 1 mM each of ATP, cytidine triphosphate (CTP), and uridine triphosphate (UTP), 0.1 mM guanosine triphosphate (GTP), 0.5 mM m⁷G(5')ppp(5')G (New England Biolabs, Beverly, MA), 50 units of RNasin (Boehringer), and 25 units of T7 RNA polymerase (purified from *E. coli* BL21 harboring pAR1219 according to a protocol supplied by Dr. J.J. Dunn, Brookhaven National Laboratory, Upton, New York; [see Studier *et al.*, 1990] or purchased from New England Biolabs). After 30 min at 37°, the GTP concentration was increased to 1 mM and incubation continued for a further 60 min. The reactions were extracted twice with phenol/chloroform, and the RNA was recovered by ethanol precipitation. The RNA pellets were resuspended in 0.2 mM EDTA, pH 7.0, at a concentration of 1–2 μg/μl.

Microinjection of mRNA into *Xenopus* Oocytes

Stage VI oocytes were obtained from female *Xenopus laevis*, with the use of mild digestion with collagenase as described by Colman (1984). We injected ~50 nl of 1 ng/nl mRNA into each oocyte, which were kept at 22° in modified Barth's medium. Maturation was assessed by the appearance of the characteristic white spot on the animal pole and checked in doubtful cases by dissection.

Translation in Cell-Free Extracts of *Xenopus* Eggs

For cell-free translation of mRNA, we prepared "cytostatic factor (CSF)-arrested" extracts from unactivated *Xenopus* eggs according to the protocol of Murray (1991). Sucrose was added to the extracts to a final concentration of 200 mM, and small aliquots were frozen in liquid nitrogen. For translation, mRNA was added at 100 μg/ml and [³⁵S]methionine to 0.5 mCi/ml in a reaction of 10 μl, which was incubated at 23° for 1 h. Part of the reaction mix was used for SDS-PAGE and autoradiography (typically, the equivalent of 0.2–0.5 μl was analyzed on one gel lane) and another part for immunoprecipitation or p13^{suc} affinity chromatography as described below. In some cases, the frog cell extract was mixed with an equal volume of nuclease-treated rabbit reticulocyte lysate, which stimulated translation of certain added mRNAs, some of which (for example the mRNAs for p34^{cdc2}

³ The sequence of this bovine cyclin A clone has been deposited in the GENBANK/EMBL database with accession number X68321.

and p32^{cdk2}) were translated extremely poorly in CSF extracts. We found that placing the coding regions of such mRNAs within the 5' and 3' untranslated regions of cyclin A1 mRNA improved their translatability. Conversely, removing the 3' UTR from cyclin A1 mRNA strongly reduced its translation in the *Xenopus* cell-free system, although it did not significantly reduce its translation in the reticulocyte system.

We estimated the concentration of proteins produced by cell-free translation of added mRNA in pure reticulocyte lysate in two independent ways: by measuring the radioactivity in the protein, knowing the specific activity of the methionine pool, and also by immunoblotting and comparison with bacterial cyclin A standards. The two methods gave estimates of 0.4–1 µg/ml for wild-type cyclin A after 60 min translation. We did not perform these measurements in frog extracts.

Affinity Chromatography on p13^{suc1}-Sepharose

The affinity matrix for p34^{cdc2}, p13^{suc1}-Sepharose, was prepared by modifications of the procedure described by Brizuela *et al.* (1987). The p13^{suc1} was purified from *E. coli* BL21(DE3) carrying the intronless p13^{suc1} gene in the modified pET3 vector pRK172, grown in 2xTY medium and induced with 0.5 mM IPTG. The bacteria were lysed on ice in 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 2 mM EDTA, 1 mM DTT, and 1 mM PMSF, pH 7.2, by addition of 2 mg/ml lysozyme followed by sonication. The extract was clarified by centrifugation for 20 min at 10 000 rpm in the Sorvall SS34 rotor (Sorvall Instruments, Newton, CT), dialyzed against the lysis buffer, and loaded on a DEAE-Sepharose column. The column was developed with a 0–500 mM NaCl gradient; p13^{suc1} eluted between 30 and 60 mM NaCl, well ahead of most of the other proteins. The pooled fractions containing the p13^{suc1} were concentrated with 65% saturated ammonium sulfate, and the pellet was dissolved in coupling buffer: 500 mM NaCl, 100 mM Na₂CO₃. This solution was then further purified by gel filtration on AcA 34 (IBF Biotechnics, Villeneuve-la-Garenne, France) equilibrated with the NaCl/carbonate coupling buffer. This p13^{suc1} appeared extremely pure by SDS-PAGE, and the peak column fractions were used directly for coupling to the beads.

For analysis of translation reactions, 5 µl were diluted with 400 µl of 'bead buffer' (Dunphy *et al.*, 1988): 50 mM Tris-Cl, pH 7.4, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% (vol/vol) NP40, 5 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml soybean trypsin inhibitor, 100 µM benzamidin and were spun in an Eppendorf (Hamburg, Germany) centrifuge for 5 min. The supernatant was added to 10 µl of p13^{suc1}-beads equilibrated with bead buffer. The tubes were rotated gently for 1 h at 4°. The beads were harvested by centrifugation and washed three times with bead buffer before extraction with 20 µl of SDS sample buffer and analysis by SDS-PAGE and autoradiography.

Immunoprecipitations and Immunoblotting

Mouse anti-p34^{cdc2} polyclonal antisera and monoclonal antibodies were raised against the C-terminal two-thirds of *Xenopus* p34^{cdc2}, starting at an internal *Nco* I site whose ATG encodes M 85. This clone was kindly provided by John Newport; see Milarski *et al.* (1991). The *Nco* I-BamHI fragment was subcloned into pET8c and expressed (as insoluble inclusion bodies) in *E. coli* BL21(DE3) according to Studier *et al.* (1990). Monoclonal antibody A17 reacted with p34^{cdc2} and not with p32^{cdk2} and was capable of immunoprecipitating cyclins that were associated with p34^{cdc2} (many we obtained only recognised free p34^{cdc2}). We typically added 100 µl of crude culture medium from the myeloma to 5 µl of translation mix and then recovered the immune complexes with 10–20 µl of protein A-Sepharose. The procedure for polyclonal antisera was slightly different. The antisera (typically 2 µl) were first incubated with 20 µl of protein A-Sepharose suspension (Pharmacia) for 15 min at 4° with gentle mixing. These antibody-loaded beads were washed three times in phosphate-buffered saline, and then mixed with 5 µl of the [³⁵S]methionine-labeled translation

reactions diluted to a final volume of 400 µl with bead buffer. In both cases, the bound proteins were eluted with SDS gel sample buffer and analyzed by SDS-PAGE. Anti-cyclin A1 antibodies were the same as described by Kobayashi *et al.* (1991a). The anti-cdk2 antibody was raised in rabbits against full-length cdk2 expressed in pET3. The anti-c-myc epitope monoclonal (Evan *et al.*, 1986) was obtained from the ICRF monoclonal production unit.

Proteins were transferred electrophoretically onto Immobilon filters (Millipore, Bedford, MA) and PSTAIRE-positive polypeptides were detected with the monoclonal antibody of Yamashita *et al.*, (1992), with the Amersham (Aylesbury, England) ECL detection system.

Histone H1 Kinase Assay

To assay the histone H1 kinase activity associated with cyclin A translated in vitro, the CSF extracts were made mRNA-dependent essentially as described by Murray (1991). In brief, the CSF extracts were treated with 0.5 µg/ml RNase A (Boehringer) for 10 min at 10° followed by one-fortieth volume of 50% (vol/vol) RNasin (Boehringer) for 10 min at 10°. Next, 0.4 mM CaCl₂, 200 mM sucrose, and 50 µg/ml calf liver tRNA (Boehringer) were added and the reaction incubated for 15 min at 23°. Aliquots were frozen in liquid nitrogen and stored at –80° until use.

Aliquots of these nuclease-treated extracts were incubated with 100 µg/ml added cyclin mRNA and [³⁵S]methionine, and at 30-min intervals, samples were taken for affinity absorption on p13^{suc1} beads as described above, except that the buffer in this case was 80 mM Na β-glycerophosphate, pH 7.3, 20 mM EGTA, 15 mM MgCl₂, and 1 mM DTT. The beads were assayed in 10 µl of H1 kinase buffer (50 mM Na β-glycerophosphate, pH 7.3, 0.3 mM EDTA, 15 mM MgCl₂, 2 mM DTT [Cicirelli *et al.*, 1988]) with 25 µM ATP, 0.25 µCi/µl γ-[³²P]ATP (Amersham PB 10218), and 275 µg/ml histone H1 (Boehringer). After 30 min at 23°, the reaction was terminated by addition of 25 µl of SDS sample buffer. The samples were analyzed for cyclin synthesis and histone phosphorylation by SDS-PAGE and autoradiography.

Activation of H1 kinase by protein A-bovine cyclin A was achieved by addition of 0.4 µg of wild-type or mutant protein to 25 µl of interphase *Xenopus* egg extract. After incubation for 10 min at 21°, 250 µl of H1 kinase buffer was added, followed by 20 µl of IgG-Sepharose slurry (Pharmacia). The samples were rotated at 4° for 1 h, the beads recovered, washed, and assayed for kinase activity as described above.

Xenopus Cultured Cells

A line of *Xenopus* fibroblasts (WAK) were obtained from R.A. Laskey (Cambridge) and propagated in 75% (vol/vol) E4 medium supplemented with 10% (vol/vol) fetal calf serum at 27°. At a density of ~5 × 10⁶ cells/14-cm plate, the cells were washed with serum-free medium and lysed on ice with buffer containing 50 mM Tris-Cl, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1% NP40, 10 µg/ml cytochalasin B, 1 mM PMSF, 1 µg/ml leupeptin, 2 µg/ml aprotinin, 10 µg/ml soybean trypsin inhibitor, 15 µg/ml benzamidin, and 10 µg/ml pepstatin, pH 7.5. The cells were scraped off and debris removed by centrifugation at 17 000 g for 30 min. The supernatant was used for immunoblotting.

Cyclin Destruction Assays

To measure the ability of cyclin A mutants to undergo programmed proteolysis, the test mRNA was translated in "CSF extracts" as described above. Translation was allowed to proceed for 1 h in the presence of [³⁵S]methionine at which point 0.1 mM cycloheximide was added to block further protein synthesis. The sample was divided in two, and one sample was made 0.4 mM in CaCl₂, which triggers the destruction of cyclin after a few minutes' lag (Lohka and Maller, 1985; Murray *et al.*, 1989; Shamu and Murray, 1992). Samples were taken for analysis on SDS-PAGE at intervals after adding the CaCl₂.

The Conservation Plot for Comparing Cyclin A Sequences

Figure 1 contains a plot that compares the degree of polypeptide sequence conservation in examples of cyclin A from a variety of species: chicken, clam, cow, frog, fruit fly, hamster, human, limpet, mouse, and sea urchin (Swenson *et al.*, 1986; Lehner and O'Farrell, 1989; Minshull *et al.*, 1990; Wang *et al.*, 1990; van Loon *et al.*, 1991), and see ACKNOWLEDGMENTS). The sequences were aligned by the Intelligenetics Geneworks® (Mountain View, CA) protein alignment module and adjusted by eye. At each position in the sequence, the number of different amino acids was counted. Thus at position 197, all 10 examples of cyclin A have arginine, so the score is 1. At position 204, where the other nine sequences have an L, limpet cyclin A has an M. This position therefore scored 2. These scores were then converted (arbitrarily) as follows: 1 remained 1, 2 became 0.8, 3 became 0.6, 4 became 0.4, and lower scores were transformed into their reciprocal. These numbers were plotted against residue number, and the conservation axis was relabeled as 1, 2 etc. In this way, peaks of conservation and troughs of nonconservation in the sequence comparisons are easily visualised. One additional adjustment was made. The peaks produced by the "destruction box" and the conserved

FXXXVDE sequence do not appear when cyclin A sequences are strictly aligned because of slight differences in the spacing of these elements with respect to the cyclin box and each other. When locally aligned, they give the values shown in Figure 1. Their positions in the plot are those found in frog cyclin A1.

RESULTS

The cDNA for cyclin A1 from *Xenopus* contains unique *Sty I*, *EcoRV*, *HindIII*, and *Nsi I* sites as shown in Figure 1, which, with Table 1, summarizes most of the deletions and mutations we have made. Figure 1 shows how these mutations map in relation to the sequence conservation shown by a variety of examples of cyclin A. For example, mutant $\Delta 133$ removed all of the N-terminal 133 residues of cyclin A1 by fusing the efficient translational start site of influenza virus NS protein to the *HindIII* site (Dasso and Jackson, 1989). Other mutants contained internal deletions of varying size and retained some

Figure 1. Summary of cyclin A deletions. Full length cyclin A1 (wild type) contains 419 amino acids. Solid lines denote the open reading frame of cyclin A, the cyclin box is a black rectangle, and the destruction domain is an open box. The activity of the mutant polypeptides in binding to p34^{cdc2} (all constructs), activating histone H1 kinase in vitro (wild-type, $\Delta 21$, $\Delta 161$, and 169), or promoting oocyte maturation (see Table 2) is indicated at the right side of each construct. The diagram below represents sequence conservation in the cyclin A family determined and plotted as described in MATERIALS AND METHODS. Peaks indicate conserved residues and troughs lack of conservation at the numbered residues.

portion of the correct N-terminus. They are named for their most C-terminal missing residue. The C-terminal deletion series was constructed with the 9E10 epitope from human *c-myc* replacing the N-terminal 21 amino acids of cyclin A1. All the constructs contained a T7 promoter upstream to allow cell-free synthesis of capped mRNA, and all ended with a termination codon, as indicated in Table 1.

The activity of cyclin A mutant mRNAs were tested by microinjection into stage VI *Xenopus* oocytes, which were incubated and scored for their ability to undergo meiotic maturation. Controls of untreated, progesterone-treated and wild-type cyclin A mRNA-injected oocytes were included in every experiment. The results of this test are given in Table 2. Wild-type cyclin mRNA typically caused maturation in all oocytes in a sample of ≥ 20 , starting at 2–3 h and completing by 5–6 h. Deletions up to and including $\Delta 161$ were as active as full-length cyclin A1, whereas all deletions from $\Delta 169$ onwards were completely inactive. As shown in Figure 1, the first conserved residue in all known examples of cyclin A is Y 164, closely followed by I 168. These residues are conserved in the majority of known mitotic cyclin sequences, including *cdc13* of *Schizosaccharomyces pombe* and the *CLB* genes of budding yeast. $\Delta 161$ preserves both these residues, whereas they are deleted from $\Delta 169$. We conclude that these residues are necessary for the activity of cyclin A, although we have not tested this point by making point mutations.

Deletion Mutants of Cyclin A That Fail to Promote Oocyte Maturation Cannot Bind to p13^{suc1}-Sepharose or to p34^{cdc2}

We next translated several of the cyclin constructs described in Table 1 in the *Xenopus* cell-free system (Figure 2A), and tested which of them could bind to p13^{suc1}-Sepharose, an affinity resin for p34^{cdc2} and associated cyclins (Brizuela *et al.*, 1987; Draetta *et al.*, 1989; Labbé *et al.*, 1989; Pondaven *et al.*, 1990). Figure 2B shows that the cyclin A deletion mutants that were active in the oocyte maturation assay bound to p13^{suc1} beads, whereas the inactive mutants failed to bind. It is still not completely clear how p34^{cdc2} binds to p13^{suc1}, however, and it was possible that the mutant cyclins might form stable complexes with p34^{cdc2} that were no longer capable of associating with p13^{suc1}. Figure 2D shows that this was not the case, because when we used a monoclonal anti-p34^{cdc2} antibody to immunoprecipitate the various cyclin A constructs, the results exactly followed those of the activity and p13^{suc1}-binding assays. Constructs $\Delta 133$, $\Delta 144$, and $\Delta 161$ bound to p34^{cdc2}, whereas $\Delta 169$ and deletions extending beyond the start of the cyclin box did not bind.

The C-Terminus of Cyclin A is Required for Binding to p34^{cdc2}

To define the C-terminal end of cyclin A that is necessary for binding to p34^{cdc2}, we constructed a series of

Table 2. Oocyte maturation promoted by N- and C-terminal deletions of cyclin A1

	Microinjection		% GVBD
	Total	GVBD	
Wild-type cyclin A1	23	22	96
$\Delta 21$ (<i>myc</i> tagged)	24	23	96
$\Delta 133$	25	24	96
$\Delta 144$	12	12	100
$\Delta 147$	13	12	92
$\Delta 158$	23	22	96
$\Delta 161$	23	23	100
$\Delta 169$	21	1	5
$\Delta 176$	29	0	0
$\Delta 201$	14	0	0
$\Delta 213$	10	0	0
$\Delta 245$	19	0	0
C $\Delta 14$	32	2	6
C $\Delta 25$	26	4	15
C $\Delta 50$	36	0	0
C $\Delta 200$	22	0	0

Stage VI oocytes were injected with mRNA corresponding to the constructs shown in Figure 1 and scored for maturation as described in MATERIALS AND METHODS. The ability to promote maturation is lost between residues 161 and 169, and the first 133 residues are wholly dispensable in this assay.

C-terminal deletion mutants (see MATERIALS AND METHODS). The ability of these constructs to bind with companion kinase subunits was tested in the same way as the experiment shown in Figure 2. Immunoprecipitation was performed with the A17 monoclonal anti-p34^{cdc2} antibody. Figure 3 shows that the shortest C-terminal deletion, lacking only 14 amino acids from its C-terminus, failed to bind to p34^{cdc2}, and removal of 24 and 50 residues did not restore binding. Larger deletions of 79, 139, and 200 residues were similarly inactive. We also made a construct that deleted the 16 C-terminal residues of bovine cyclin A (see Figure 5), which showed no detectable *cdc2* binding activity in experiments like the one shown in Figure 3, or histone H1 kinase activity, as Figure 6 shows. When these C-terminal deletions were tested by the oocyte maturation assay (Table 2), we found that C $\Delta 14$ and C $\Delta 24$ had slight residual activity; $\sim 10\%$ of the oocytes matured with normal kinetics. This may indicate that the *in vivo* assay is capable of detecting weaker interactions between p34^{cdc2} and cyclin A than the *in vitro* binding assay. Clearly, however, these C-terminally deleted constructs are seriously impaired in activity.

Activation of Histone H1 Kinase by Mutants of Cyclin A

The ability of full-length cyclin A1 and the two mutants $\Delta 161$ and $\Delta 169$ were further tested for their ability to activate histone kinase activity in interphase extracts of

Figure 2. Association of cyclin mutants with p34^{cdc2}. mRNAs corresponding to the indicated cyclin A mutants were translated in Ca²⁺-treated CSF extract with [³⁵S]methionine. (A) Analysis of the complete reaction by SDS-PAGE and autoradiography. (B) Analysis by adsorption to p13^{suc1} Sepharose. B-type cyclins running at ~55 kDa (right) provided an internal positive control for recovery. (C) Translation of selected mutants in a different extract and (D) Analysis of the binding of these constructs to a monoclonal anti-p34^{cdc2} antibody A17.

Xenopus eggs. Figure 4 shows that there was a gradual increase in histone H1 kinase activity associated with p13^{suc1}-Sepharose beads prepared from extracts that were incubated with mRNA encoding wild-type cyclin A1 or Δ161, but no increase in kinase activity was observed when Δ169 or no mRNA was added to the

reactions. Similar results were obtained when immunoprecipitates of *myc*-tagged cyclin A1 produced by cell-free translation were assayed for their associated histone kinase activity. Wild-type cyclin A1 displayed activity (for example, see Figure 9), whereas C-terminal truncations completely lacked it.

Figure 3. C-terminal deletions abolish cyclin A binding to p34^{cdc2}. Cyclin A Δ21 and C-terminally truncated derivatives were translated in *Xenopus* egg extract (A) and immunoprecipitated with monoclonal anti-p34^{cdc2} (B).

Small Deletions in the Cyclin Box Reduce or Abolish Activity of Cyclin A

We made another set of internal deletions in *Xenopus* cyclin A1 with its unique *Nsi* I site or in bovine cyclin A with its unique *Acc* I site, as indicated in Figure 5. Removal of two or more amino acids at the *Nsi* I site completely inactivated frog cyclin A, as measured by the oocyte maturation assay (Table 3) or by the in vitro binding assay. The deletion of two amino acids further down the sequence in bovine cyclin A changed the sequence VYITD in the italicized sequence below to VSD, i.e., loss of YI and changing the next T to S: ASKFEEL-YIPPEVAEFVYITDDTYTKQVLRME (underlined letters indicate the residues conserved in cyclin A from clam, fly, frog, and man). This construct (Δ267) gave ~10% residual kinase activity when the construct was added to an interphase extract derived from *Xenopus* eggs (Figure 6), which we interpret as showing that weak

Figure 4. Histone H1 kinase activity associated with wild type or mutant Δ161 and Δ169 cyclin A. Mutant or wild-type cyclin A mRNAs were translated in nuclease-treated activated egg extracts, and the histone H1 kinase activity associated with p13^{suc1} beads was measured at various times after the start of the incubation as indicated below each lane.

Figure 5. The structure of cyclin box mutants in frog and cow cyclin A. The position of the alanine substitutions and internal deletions in the cyclin box are shown to scale. Protein A-cyclin A is missing the first 76 residues of bovine cyclin A and has 264 residues of protein A at the N-terminus that are not shown here. Activity indicated in the right margin comes from binding to p34^{cdc2} and the ability to activate the histone H1 kinase of p34^{cdc2}.

binding to p34^{cdc2} gives weak activation. Longer deletions in this location completely abolished the activity of bovine cyclin A. It is noteworthy that so far we have not found a mutant cyclin A that can bind to p34^{cdc2} but not activate it as a protein kinase. It thus appears that, if cyclin A can bind to p34^{cdc2}, it turns on the kinase activity of the complex.

Single Point Mutations of Conserved Residues in the Cyclin Box Abolish the Ability of Cyclin A1 to Bind and Activate p34^{cdc2}

Five residues in the cyclin box are conserved in essentially all known cyclins, including the yeast CLN genes (the major exception is cyclin C). They are (in *Xenopus* numbering) R197, D226, L241, K252, and E281. So far we have constructed, confirmed by sequencing, and tested R197 → A and D226 → A (Figure 5). Both of these point mutations caused complete loss of ability of

Table 3. Small deletions within the cyclin box abolish oocyte maturation promoted by cyclin A1 mRNA

	Microinjection		
	Total	GVBD	% GVBD
Progesterone	30	26	87
Wild-type cyclin A1	22	20	91
Δ232	24	0	0
Δ235	28	0	0
Δ237	27	0	0
Δ261	25	0	0

Stage VI oocytes were injected with mRNA encoding the mutants of *Nsi* I series whose structures are shown in Figure 5 and Table 1.

Figure 6. Histone H1 kinase activity associated with protein A-cyclin A mutants. Wild-type protein A-cyclin A or the mutant constructs shown in Table 1 and Figure 5 were added to CSF extract that had been activated by addition of Ca^{2+} . After 10 min, the cyclin- p34^{cdc2} complexes were recovered on IgG beads and assayed for histone H1 kinase as described in MATERIALS AND METHODS.

the mutant cyclin A1 to form complexes with p34^{cdc2} in the CSF-extract translation and immunoprecipitation assay.

Xenopus Cyclin A1 Can Bind Both p34^{cdc2} and p32^{cdk2}

Xenopus eggs contain two well-characterized members of the cdc2 family, p34^{cdc2} , and p32^{cdk2} , the latter originally known as Eg1, because its mRNA was polyadenylated and translated only during the time of progesterone-induced maturation until the time of fertilization (Paris *et al.*, 1991). The sequences of these two polypeptides are very similar in the regions where all examples of 'cdc2' proteins are conserved and differ in

the regions in between. Although it was originally found that p32^{cdk2} did not substitute for cdc2 in *S. pombe* (Paris *et al.*, 1991), more recent evidence indicates that the human homologue of Eg1, now known as cdk2 (cyclin dependent kinase) is capable of such substitution, particularly when certain other mutations were present in the tester strains (Elledge and Spottswood, 1991; Meyerson *et al.*, 1992a).

To test whether *Xenopus* cyclin A1 could associate with p32^{cdk2} as well as p34^{cdc2} , we co-translated mRNA encoding myc-tagged cyclin A1 with cdc2 or cdk2 mRNA in the CSF extract. At the end of the incubation with [^{35}S]methionine, antibodies against cyclin A were added and the immunoprecipitates collected on protein A-Sepharose beads. Figure 7 shows that both p34^{cdc2} and p32^{cdk2} were immunoprecipitated with anti-myc or anti-cyclin A antiserum when full length myc-tagged cyclin A1 ($\Delta 21$) mRNA or the mRNA for the active $\Delta 161$ construct was present. But when mRNAs encoding the C-terminally truncated cyclin A construct $\text{C}\Delta 14$ or the inactive $\Delta 169$ mRNA were used, the binding of the kinase subunits was the same as background reactions in which no cyclin A mRNA was added. Thus mutants that fail to associate with p34^{cdc2} also cannot bind p32^{cdk2} .

We conclude that under the conditions of these assays, when the levels of both cyclin A and p32^{cdk2} have been increased by translation of added mRNA, cyclin A can bind to either p34^{cdc2} or to p32^{cdk2} . We should stress that we stand by our previous data (Minshull *et al.*, 1990), which has been confirmed by Fang and Newport (1991) and by Maller and his colleagues (Gabrielli *et al.*, 1992), that in frog egg extracts, no cyclin A1 can be detected in association with p32^{cdk2} . Presumably this is partly a matter of relative concentrations

Figure 7. Cyclin A mutants that fail to bind to p34^{cdc2} cannot bind to p32^{cdk2} . Cyclin A1 mRNA was co-translated with mRNA for either p34^{cdc2} or p32^{cdk2} in a cell-free translation system composed of equal volumes of *Xenopus* CSF extract and nuclease-treated rabbit reticulocyte lysate. Reactions were immunoprecipitated with rabbit polyclonal anti-cyclin A antiserum. (A) Complete translation reactions. (B) Immunoprecipitates.

because as Figure 8A shows, the levels of $p34^{cdc2}$ are 10–20 times higher than those of the $p32^{cdk2}$ in normal frog eggs, in agreement with Gabrielli *et al.*, (1992). By contrast, extracts of somatic *Xenopus* cells (WAK fibroblasts) contain roughly equal concentrations of $p34^{cdc2}$ and $p32^{cdk2}$. To get an idea of the relative affinities of $p34^{cdc2}$ and $p32^{cdk2}$ subunits for cyclin A, soluble protein A-bovine cyclin A protein produced by expression in bacteria was added to a frog egg extract in which the mRNA for $p32^{cdk2}$ or $p34^{cdc2}$ had been translated with added [35 S]methionine, and the cyclin (together with any bound proteins) recovered by incubation with IgG-Sepharose beads. Figure 8B shows that both $p34^{cdc2}$ and $p32^{cdk2}$ bound to the beads, but considerably more of the labeled $p34^{cdc2}$ was retained. Apart from the background of labeled polypeptides produced by translation of endogenous mRNAs, we included negative controls of a truncated cyclin B2 mRNA and a *Xenopus* PCTAIRE clone (Figure 8, lanes 3 and 4). Compared with $p32^{cdk2}$ and $p34^{cdc2}$, these did not bind significantly to the protein A-cyclin A. Because the amount of label in each kinase subunit was adjusted to be the same, this means that the specific activity of the $p34^{cdc2}$ was roughly one-tenth of that of the $p32^{cdk2}$. Thus even though the cyclin A was added in excess, there was a strong tendency for $p34^{cdc2}$ to bind in preference to $p32^{cdk2}$ in the crude egg extract, which may explain why it is normally impossible to find cyclin A associated with $p32^{cdk2}$ in these extracts.

Cyclin A- $p32^{cdk2}$ Complexes Possess Histone H1 Kinase Activity

To measure the protein kinase activity of $p32^{cdk2}$ with histone H1 as the substrate, we used a C-terminally *c-myc*-tagged version (Kobayashi *et al.*, 1991a). The mRNA for this tagged $p32^{cdk2}$ was translated in the *Xenopus* egg/reticulocyte lysate mixture either in the presence, or the absence, of wild-type cyclin A1 mRNA (Figure 9A, lanes 2 and 3), probably associated with its activation (Gu *et al.*, 1992). This shift was not observed when wild-type *cdk2* mRNA was used (Figure 7A).

When no mRNA was added (the negative control), no kinase activity was associated with the 9E10 anti-*c-myc* antibody (Figure 9C, lane 1) and only very low activity with polyclonal anti-*cdk2* antibody (Figure 9C, lane 3). But as Figure 9C, lane 4 shows, the anti-*c-myc* antibody carried strong histone H1 kinase activity when $p32^{cdk2}$ -*c-myc* was translated together with wild-type cyclin A1. When *c-myc*-cyclin A was translated with no other added mRNA (in which case, the newly translated cyclin A can associate with the endogenous, unlabeled $p34^{cdc2}$), strong histone H1 kinase activity was present in the 9E10 immunoprecipitate (Figure 9C, lane

Figure 8. Relative concentrations of $p34^{cdc2}$ and $p32^{cdk2}$ and their affinity for cyclin A. (A) an immunoblot of equal protein loadings of *Xenopus* fibroblast (WAK cells) and egg extracts analyzed by SDS-PAGE in adjacent lanes probed with an anti-PSTAIRE monoclonal antibody (Yamashita *et al.*, 1992) as described in MATERIALS AND METHODS. (B) various mRNAs translated in a 4:1 vol/vol mixture of reticulocyte lysate: *Xenopus* egg extract. Lane 1, *cdc2*; lane 2, *cdk2*; lane 3, PCTAIRE (a *cdc2*-related protein [Meyerson *et al.*, 1992]); lane 4, truncated cyclin B2 Δ93; lane 5, no added mRNA. The translated products of lanes 1–4 were mixed to give equal loading of each encoded polypeptide (lane 6). Protein A-cyclin A was added to this mixture at the indicated concentrations and recovered on IgG-Sepharose, which was eluted with SDS gel sample buffer and analyzed in lanes 7–9.

5). The low kinase activity detected when *cdk2* mRNA was translated without added cyclin A mRNA suggests that there cannot be a large concentration of a free, unlabeled (and unidentified) companion subunit of *cdk2*

Figure 9. Cyclin A can activate the H1 kinase activity of both p34^{cdk2} and p32^{cdk2}. Four translation reactions were set up in a 1:1 mixture of reticulocyte lysate and frog egg extract. (A) Lane 1, no mRNA; lane 2, c-myc tagged cdk2; lane 3, mixture of wild-type cyclin A and c-myc tagged cdk2; lane 4, c-myc tagged cyclin A ($\Delta 21$). (B) Immunoprecipitates with anti-c-myc antibody 9E10 in lanes 1, 2, 4, and 5, and rabbit polyclonal anti-p32^{cdk2} antiserum in lane 3. (C) Histone H1 kinase activity of these immunoprecipitates.

in these extracts. Only when cdk2 mRNA was co-translated with cyclin A mRNA did the p32^{cdk2} display kinase activity. Figure 9B, lane 2, shows that cyclin B derived from translation of endogenous mRNA could not be detected binding to p32^{cdk2} in this experiment (or in other similar experiments not shown), whereas endogenous B-type cyclins did bind to anti-p34^{cdk2} antibodies (see Figure 2, B and D, and Figure 3B). We have not tried increasing the level of cyclin B translation with added mRNA, which might force some cdk2 binding.

The Sequence RTVLGVIGD is Required for Programmed Cyclin Proteolysis

The most peculiar property of mitotic cyclins is their destruction at the onset of anaphase. It was previously shown by Glotzer *et al.* (1991) that the short conserved N-terminal sequence in sea urchin cyclin B, RAALGNISN, was required for this proteolysis. More recently, Luca *et al.* (1991) showed that removal of the N-terminal 60 amino acids from clam cyclin A had a similarly stabilizing effect on the protein. We tested three constructs to check that the destruction of *Xenopus* cyclin A1 required the closely related peptide motif RTVLGVIGD at positions 41–50. The first construct was $\Delta 133$ in which the N-terminal 133 amino acids are completely deleted; in the second construct, a unique *Sty* I site at the N-terminus of *Xenopus* cyclin A1 was used to make

Bal 31 deletions. One of the in-frame mutants, $\Delta 62$ (Figure 10A), deleted the whole of the putative destruction box. Finally, we altered the conserved R and L residues of the destruction box to alanine by site-directed mutagenesis. Destruction was tested in unactivated frog egg extracts in which cyclin proteolysis and concomitant loss of histone H1 kinase activity can be triggered by addition of 0.4 mM Ca²⁺. Figure 10 shows that all these mutants were stable, so we conclude that the N-terminal RTVLGVIGD motif is necessary for the destruction of cyclin A1. A similar conclusion has recently been reported for limpet cyclin A by Lorca *et al.* (1992), who mutated the R of the related RSALGTITNQ sequence to C. We will report elsewhere on the stability of the rest of the mutants described in this paper, all of which have an intact destruction box. Their story is somewhat more complicated, for several of them are unexpectedly stable.

DISCUSSION

As discussed in the INTRODUCTION, the cyclin family of proteins share a common region of about 100 residues known as the cyclin box, which contains five very highly conserved residues. The first important data in this paper show that point mutations of the first two of these residues completely abolishes the ability of cyclin A to bind to p34^{cdk2}. Similarly, deletions of only two residues in

Figure 10. Identification of a destruction domain in cyclin A. Wild-type cyclin A or the mutants indicated in A were translated in CSF extract for 1 h at 21°. At this point, CaCl_2 was added to 0.4 mM, cycloheximide to 100 μM , and samples taken for analysis by SDS-PAGE at the indicated times. Left panel of experiments B–D shows wild-type cyclin A destruction; right panel shows mutants as indicated. B–D represent different experiments. Disappearance of the endogenous B-type cyclins provides an internal positive control.

two different parts of the cyclin box in one case completely inactivated and in the other 90% inactivated the ability of the cyclin to bind to p34^{cdc2} or to activate its kinase activity. Thus, both the composition and exact

spacing of residues in the cyclin box are implicated in interactions with the companion kinase subunit. Together with sequence comparison data, these results strongly suggest that the cyclin box represents the heart of the p34^{cdc2} binding domain in the cyclin family.

Comparison of the sequences of members of the cyclin A family (Figure 1) suggests that with the exception of two small conserved islands, the destruction domain at residues 41–50 and the motif FXXXVDE at positions 91–100, there is practically no sequence conservation in the N-terminal 163 residues. We show here that complete removal of the first 133 residues, or an internal deletion of 53 residues from 109 to 161, has no detectable effect on the ability of the cyclin to bind to and activate p34^{cdc2} . The larger deletion, removing 69 residues from positions 101 to 169, is completely inactive and deletes the first two residues that are found in all known examples of cyclin A. The cyclin box proper starts with the conserved M 196, and it is not clear what role residues 164–195 play in the function of cyclin A. It is noteworthy that for the most part, the equivalent residues in the B-type cyclin family (which bind p34^{cdc2}) are different from those in the cyclin A family, but the equivalents of EY 163–4 and I 168 are present in *Xenopus* cyclins B1 and B2 and also in *Saccharomyces cerevisiae* CLN1 and 2. CLN3 has EY, but not the I. Our data suggest that these residues are crucial for binding to members of the cdc2 family of kinases. This line of reasoning leads to a problem, however: if this is so, how is it possible for cyclin E, which lack these residues and shows essentially no similarity with other cyclins until the MRAIL sequence, to bind to p32^{cdc2} ? Only structural information can clarify this puzzle.

Whereas large stretches of the N-terminus of both cyclins A and B can be lost without losing p34^{cdc2} binding, the C-terminus is indispensable for reasons that are hard to understand, given the lack of sequence similarity between cyclins in this region. Even within the cyclin A family, the homology plot in Figure 1 suggests that the C-terminal 120 residues represents a separate structural domain, with a region of great diversity between positions 300–315 (the position where B-type cyclins have their characteristic FLRRXSK). Considering how much various cyclins differ from one another in the sequences that flank the cyclin box, the intolerance of cyclin A to changes well outside the central conserved cyclin box is strikingly odd. It may be that these outlying motifs represent built-in safety checks, so to speak, which serve to prevent fragments or other altered forms of cyclins from binding to cdc2, and thereby make it more difficult to generate “plain vanilla” forms of the kinase, whose activity would probably be dangerous if they were able to be formed. It would be most helpful to know the structure of the molecule, to see how cyclin binds to cdc2.

We have confirmed that the homologue of the cyclin B destruction box in cyclin A is indeed necessary for

programmed proteolysis at the end of mitosis, both by removing relatively large domains including this sequence and also by point mutations that change QRTVLGVLD to QATVAGVID.

It seems improbable that the only function of the first 161 residues is to provide a correctly spaced and presented destruction signal. For one thing, there is another small region of conservation at positions 91–100, which has some echoes in cyclin E, but not in cyclins B or D. The assays that we have used in these studies do not necessarily measure all the cellular functions of cyclin A; the ability to bind to p34^{cdc2} or p32^{cdk2} represents the minimum possible activity of cyclin A and does not measure the more subtle properties of subcellular targeting of the complex to specific cellular compartments and particular substrates. It will be important to test these properties in the future.

In this regard, it is important to note that the oocytes that were induced to mature by injected cyclin A1 mRNA underwent abnormal maturation. Unlike what happens in response to progesterone, the white spot stage was transient even when indestructible forms of cyclin were injected. The stimulated oocytes generally took on a marbled appearance, as though they were unable to arrest stably at meiosis II, and after several hours, there were often signs of necrosis. This deserves further study.

How Does Cyclin A mRNA Lead to Oocyte Maturation?

Ever since Swenson *et al.* (1986) showed that clam cyclin A mRNA could cause GVBD (germinal vesicle breakdown) and white spot formation in *Xenopus* oocytes, there has been a question of how it acts. This property of cyclin A mRNA (and more recently, protein [Roy *et al.*, 1991]) has become even more puzzling as we learn more about the state of *Xenopus* oocytes before maturation and as claims emerge to suggest that cyclin A is normally involved in the control of S-phase (Fang and Newport, 1991; Girard *et al.*, 1991; Pagano *et al.*, 1992). Stage VI *Xenopus* oocytes do not contain detectable amounts of cyclin A protein, and the translation of the stockpile of maternal mRNA for cyclin A does not begin until about the time of GVBD (Kobayashi *et al.*, 1991b). Moreover, oocyte maturation can occur completely normally after >95% of the endogenous cyclin A mRNA has been ablated with antisense oligonucleotides (Minshull *et al.*, 1991). Assuming that maturation requires active p34^{cdc2} (an unexamined point, although it would be exceedingly heretical to question it), we argued that the preformed store of cyclin B2 and p34^{cdc2} must be adequate to perform the whole process. So, how does cyclin A promote maturation?

We show here that mutant cyclin A molecules that cannot bind to p34^{cdc2} fail to promote maturation. This makes it highly probable that cyclin A acts by combining

with free p34^{cdc2} and activating its protein kinase activity. How is it that newly synthesized cyclin A can activate p34^{cdc2}, whereas the maternal stockpile of cyclin B/cdc2 is devoid of activity? We suppose that cyclin A directly activates free p34^{cdc2} in the oocytes, which then tips the balance of a positive feedback loop, leading to activation of the endogenous cyclin B2-p34^{cdc2}. Precisely how this occurs is not yet clear. As recently discussed in some detail by Devault *et al.*, (1992), it is presumably necessary either to activate the tyrosine 15 phosphatase encoded by cdc25, or to inhibit the tyrosine 15 kinase encoded by a wee1-like enzyme, or both, to activate preformed cyclin B-p34^{cdc2} protein kinase (Dunphy and Newport, 1989; Dunphy and Kumagai, 1991; Gautier *et al.*, 1991). Further work is necessary to discover what happens in frog oocytes.

Why Did Previous Studies Fail to Detect Cyclin A-p32^{cdk2} Association in Xenopus Extracts?

We were at first surprised to find that *Xenopus* p32^{cdk2} could form complexes with cyclin A, considering that studies in three other laboratories besides our own failed to detect newly synthesized cyclin A bound to p32^{cdk2} (Minshull *et al.*, 1990; Fang and Newport, 1991; Paris *et al.*, 1991; Gabrielli *et al.*, 1992). More recently, data from mammalian cells has shown that cyclin A is often found predominantly associated with cdk2 (Tsai *et al.*, 1991; Desai *et al.*, 1992). The data in this paper clearly show that *Xenopus* cyclin A and p32^{cdk2} can, like their mammalian counterparts, bind tightly together and produce active histone H1 kinase. However, there are potentially important differences in the way our more recent experiments were performed and the situation in intact *Xenopus* eggs and embryos. First, to obtain high level translation of cdk2 mRNA, we found it necessary to add reticulocyte lysate to the *Xenopus* extract; it is thus possible that the presence of reticulocyte lysate may promote the interaction in some way, although it does not do so in the case of inactive mutants, like CΔ14. More relevant, perhaps, is that the concentration of p32^{cdk2} is normally much less (perhaps ~5–10%) of p34^{cdc2} in frog extract (see Figure 8), and the concentration of cyclin A is probably lower still. In the cell-free translation experiments, the concentrations of both components are much increased, which tends to promote interaction. There is very little cyclin A in normal eggs, and they contain a large excess of p34^{cdc2}, so it is very likely that most of the cyclin is 'mopped up' by the p34^{cdc2}. The experiment shown in Figure 8 suggests that in the frog egg extract, the relative affinities of p34^{cdc2} for cyclin A is higher than that of p32^{cdk2} for cyclin A. These results by no means exclude the possibility that p32^{cdk2} has other, as yet unidentified, cyclin or cyclin-like subunits. What is perhaps more puzzling is that in human fibroblasts, cyclin A is mainly found associated with p32^{cdk2} and not with p34^{cdc2} (Tsai *et al.*,

1991). One possible explanation is that the cyclin A found in eggs is different from that found in somatic cells, and we have preliminary evidence that this is true. It will be necessary to design experiments to compare the relative affinities of cyclins A1 (the oocyte cyclin A) and A2 (the presumptive somatic cyclin A) for various kinase subunits.

Do Some Mutations Cause Misfolding?

Both the reviewers of the submitted version of this paper raised the following question: how do we know that proteins with greatly altered sequences fold up correctly? Could nonbinding of cyclin A to p34^{cdc2} be caused by gross misfolding, rather than deletion of, or alterations in key interacting residues in the subunit interface domain? We did not discuss this question because we thought it was very difficult, if not impossible, to answer in the absence of structural assays that are independent of functional ones. In the case of point mutations in the cyclin box, we would argue that single alanine replacements are generally well tolerated (see Gibbs and Zoller, 1991), but deletions of two amino acids might, we imagine, cause large changes in conformation. At the same time, large deletions of up to 133 amino acids in the N-terminal 161 residues of cyclin A are perfectly tolerated and presumably do not induce misfolding, because they are able to bind and to activate p34^{cdc2} and p32^{cdk2}. We are more puzzled by the failure of the short C-terminal truncations of cyclin A to bind to p34^{cdc2}, given the variations in sequence between different members of the cyclin family in this region of the molecule. We are exploring the use of mild protease digestion, which may provide a way to detect gross structural alterations. In truth, however, solving the crystal structure of cyclin A-p34^{cdc2} kinase is the only sure way to obtain precise, accurate and detailed information.

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