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Nuclear localization of vertebrate cyclin A correlates with its ability to form complexes with cdk catalytic subunits

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SUMMARY

Cyclins control the activities of cyclin-dependent protein kinases (cdks) and hence play a key role in cell cycle regulation. While B-type cyclins associate with $p34^{cdc2}$ to trigger entry into mitosis, progression through S phase requires cyclin A, presumably in association with $p33^{cdk2}$. Vertebrate A- and B-type cyclins display strikingly distinct subcellular localizations, but the mechanisms underlying these differential distributions are unknown. Here, we have begun to study the requirements for nuclear localization of cyclin A. We have isolated a cDNA coding for chicken cyclin A and constructed a series of deletion mutants. These were then transfected into HeLa cells, and the subcellular distribution of the mutant cyclin A proteins was determined by indirect immunofluorescence microscopy. In parallel, the cyclin A mutants were assayed for their ability to form complexes with cdk subunits. We found that deletion of more than 100 residues from the N terminus of cyclin A did not impair nuclear localization or cdk subunit binding and kinase activation. In contrast, removal of as few as 15 residues from the C terminus, or deletion of part of the internal cyclin box domain, abolished nuclear localization of cyclin A as well as its ability to bind to and activate cdk subunits. These results suggest that nuclear transport of cyclin A may depend on the formation of multiprotein complexes comprising cdk catalytic subunits.

Key words: cyclin, cdk, cyclin-dependent kinase, complex formation, nuclear transport

INTRODUCTION

Cyclin proteins determine the temporal pattern of activation of cyclin-dependent protein kinases (cdks) and thus play a key role in controlling cell cycle progression (for recent reviews see Hunt, 1991; Pines, 1992; Norbury and Nurse, 1992). The first members of the cyclin family, the A- and B-type cyclins, were identified in sea urchin eggs as proteins that are synthesized in each cell cycle and destroyed at mitosis (Evans et al., 1983; Swenson et al., 1986; Standart et al., 1987). As these cyclins are required for entry into mitosis, they have been referred to as 'mitotic cyclins'. More recently, additional cyclins have been identified, which appear to function at earlier stages of the cell cycle: a G_1 function is well established for the so-called CLNs in *Saccharomyces cerevisiae* (Cross, 1988; Nash et al., 1988; Hadwiger et al., 1989; Forsburg and Nurse, 1991), and similar functions may be carried out by the vertebrate 'G_1-cyclins' C, D and E (Koff et al., 1991; Lew et al., 1991; Matsushime et al., 1991; Motokura et al., 1991; Xiong et al., 1991; see also Léopold and O'Farrell, 1991).

The function of B-type cyclins is comparatively well understood. These proteins associate with $p34^{cdc2}$ to form MPF (M phase promoting factor), an evolutionarily conserved inducer of mitosis and meiosis (for reviews see Nurse, 1990; Draetta, 1990; Dorée, 1990; Maller, 1991). A-type cyclins also have a function at the G_2/M transition (Lehner and O'Farrell, 1990; Whitfield et al., 1990; Walker and Maller, 1991; Pagano et al., 1992a; Knoblich and Lehner, 1993), but in addition are required for progression through S phase (Girard et al., 1991; Pagano et al., 1992a; Zindy et al., 1992). Consistent with a dual role for A-type cyclins, these proteins associate not only with $p34^{cdc2}$, but also with the closely related kinase $p33^{cdk2}$ (Pines and Hunter, 1990; Tsai et al., 1991; Kobayashi et al., 1992; Clarke et al., 1992; Lees and Harlow, 1993). Furthermore, there is evidence that different cell cycle functions are executed by distinct cyclin A-cdk complexes (e.g. see Pagano et al., 1992a).

Deregulated expression or impaired function of cyclin A may contribute to carcinogenesis. This was suggested first by the identification of the cyclin A gene as the site of integration of hepatitis virus B in a case of hepatocellular carcinoma (Wang et al., 1990). Furthermore, cyclin A was identified as one of the cellular proteins that is specifically bound by E1A, the transforming protein of adenovirus (Pines and Hunter, 1990; Tsai et al., 1991), and both cyclin A and cdk2 were shown to form multiprotein complexes with p107, a protein related to the retinoblastoma gene product (Ewen et al., 1992; Faha et al., 1992). Interestingly, these latter complexes undergo cell cycle-dependent interactions with transcription factors of the E2F family (Ban-

dara et al., 1991; Mudryj et al., 1991; Shirodkar et al., 1992; Devoto et al., 1992; Pagano et al., 1992b; Cao et al., 1992).

In vertebrates, cyclin A accumulates in the nucleus as soon as it is synthesized (Pines and Hunter, 1991; Girard et al., 1991; Pagano et al., 1992a; Zindy et al., 1992). In contrast, B-type cyclins are predominantly cytoplasmic but undergo an abrupt translocation to the nucleus just prior to the onset of M phase (Pines and Hunter, 1991; Gallant and Nigg, 1992; Bailly et al., 1992; Ookata et al., 1992). In syncytial embryos of *Drosophila*, both A- and B-type cyclins were observed predominantly in the cytoplasm of interphase cells, but displayed clearly distinct distributions in early mitotic cells (Lehner and O'Farrell, 1989, 1990; Whitfield et al., 1990; Maldonado-Codina and Glover, 1992). Although the subcellular distribution of cyclin A in early *Drosophila* embryos appears to be different from that in vertebrate tissue culture cells, this difference is likely to reflect distinct cell cycle controls operating at different developmental stages, rather than fundamental differences between *Drosophila* and vertebrates. On the other hand, the cyclin B protein encoded by the cdc13 gene was reported to be constitutively nuclear in *S. pombe* (Booher et al., 1989); it is possible that this observation may relate to the fact that *Schizosaccharomyces pombe* undergoes a closed mitosis.

While substantial progress has been made towards understanding biochemical aspects of cell cycle control, much less is presently known about the spatial organization of the cell cycle machinery. It is widely believed that cyclins may be important not only for the activation of cdk subunits, but also for their targeting to appropriate substrates (e.g. see Booher et al., 1989). Thus, it will be important to obtain information on the mechanisms that determine the subcellular localizations of different cyclins. Considering that these mechanisms appear to be subject to both cell cycle control and developmental regulation, they are likely to be complex.

Here, we present experiments designed to address this question. Specifically, we have cloned a cDNA for chicken cyclin A, and produced anti-cyclin A antibodies; these tools were then used to carry out a mutational analysis aimed at identifying the domains involved in the nuclear localization of cyclin A in somatic vertebrate cells. Using a standard protocol for the deletion mapping of nuclear localization signals (NLSs; e.g. see Kalderon et al., 1984), we were unable to identify any canonical NLS in cyclin A. Instead, we observed a strong correlation between the ability of cyclin A proteins to enter the nucleus and their ability to form complexes with cdk catalytic subunits. The most straightforward interpretation of these results is that transport of cyclin A to the nucleus requires complex formation with cdk subunits.

MATERIALS AND METHODS

Cloning and sequencing of a chicken cyclin A cDNA

A cDNA library, prepared from poly(A)$^+$ RNA of 10-day-old chicken embryos (Sap et al., 1986), was screened by plaque hybridization with a human cyclin A cDNA probe (Pines and Hunter, 1990). This probe was prepared using *Fok*I and *Sph*I to excise a fragment corresponding to residues 149-403 of the cyclin A coding sequence, and was labeled by random priming (Feinberg and Vogelstein, 1983). Screening of 220,000 plaques was done essentially as described previously (Gallant and Nigg, 1992). The first round of hybridization was carried out at low stringency. Filters were incubated with probe (240,000 cpm/ml) overnight at 30°C in 50% formamide, then washed for 3× 1 hour at room temperature in 2× SSC, 0.1% SDS. Thirty phages giving rise to hybridization signals were picked and subjected to two additional rounds of hybridization at higher stringency (hybridization overnight at 42°C, 50% formamide, washings for 3× 15 minutes at room temperature in 3× SSC, 0.1% SDS). Of 3 purified positive phages, inserts were excised by *Eco*RI and subcloned into the pGEM-3Zf(⁻) plasmid (Promega Biotech, Madison WI). From the longest insert (1922 bp), sequencing templates were prepared in both orientations, using *Xba*I and *Sph*I for plasmid linearization and *Exo*III (Promega Biotec, Madison WI) for the generation of nested deletions (Henikoff, 1984). Double-stranded plasmid sequencing (Chen and Seeburg, 1985) was carried out with Sequenase 2 (United States Biochemicals).

Construction and expression of cyclin A deletion mutants

Myc-tagged versions of wild-type and N-terminally truncated cyclin A cDNAs were constructed by cloning them into the *Eco*RI site of a pGEM2 plasmid containing a myc-tag (kindly provided by Dr T. Hunt, ICRF, London). (The myc-tag consists of the 15 amino acid sequence MEQKLISEEDLNMNS (Munro and Pelham, 1987), and the peptide EQKLISEED is recognized by the mouse monoclonal antibody 9E10 (Evan et al., 1985)). N-terminal deletions of 106 and 229 residues were created by excising cyclin A fragments from the original plasmid with *Eco*RI (cutting 3′ to the coding region) and *Nar*I or *Taq*I (cutting within the coding region), respectively. For expression in mammalian cells, the above cyclin A cDNAs were excised using *Hin*dIII and *Sna*BI, and blunt-end ligated into the *Hpa*I site of a pCMV vector (Krek and Nigg, 1991). An internal deletion within the cyclin box (residues 217 through 260) was created using *Hin*dIII and *Fok*I. C-terminally truncated cyclin A mutants were constructed from a wild-type cDNA cloned into the *Eco*RI site of a Bluescript plasmid (Stratagene) into which a myc-tag had been inserted by using *Hin*dIII and *Eco*RI. They were generated using *Cla*I (cutting 5′ of the myc-tag) in combination with *Hin*dIII (removing 179 residues), *Pst*I (−77 residues), *Mae*III (−53 residues) or *Dra*II (−36 residues). Mutants ΔC(−15) and ΔC(−49) were generated by *Exo*III deletion, whereas ΔC(−8) and ΔC(−4) were obtained by PCR. All C-terminal mutants were then inserted into the *Hpa*I site of pCMV, which had been modified by insertion (immediately downstream of the *Hpa*I site) of a double-stranded oligonucleotide (TTAATTAATTAA) encoding stop codons in all reading frames. All cloning steps were carried out according to standard procedures, and all reading frames were verified by direct sequencing.

Transient transfection experiments were carried out as described previously (Krek and Nigg, 1991), using the method of Chen and Okayama (1987). In brief, cells were grown in 3.5 cm dishes, transfected with 5 μg of pCMV plasmid and incubated for 16 hours at 3% CO_2. Following removal of the DNA-Ca^{2+} precipitate, they were incubated for an additional 8 hours at 7% CO_2, before they were fixed for indirect immunofluorescence microscopy.

Expression of cyclin A in *E. coli* and production of antibodies

To raise antibodies against bacterially expressed cyclin A protein, an *Eco*RV-*Eco*RI fragment of the cyclin A cDNA was excised and subcloned into the *Bam*H1 site of pET3c (Novagen, Lugano,

Switzerland). The resulting plasmid codes for a protein containing 12 N-terminal amino acids derived from the expression vector, fused to the C-terminal 250 residues of cyclin A. The plasmid was transformed into *E. coli* (BL-21), bacteria were grown to an A_{600} of 0.4, and protein expression was induced by the addition for 3 hours of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. Bacteria were collected by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 2 mM DTT, 10% glycerol, 0.3% Triton X-100, 2 mM phenylmethylsulfonyl fluoride (PMSF), 0.7 mg/ml lysozyme) at 15% of the initial volume. After 15 minutes at room temperature the sample was frozen at −20°C, thawed and centrifuged for 20 minutes at 20,000 ***g*** (Sorvall, HB4 rotor). The pellet was washed once with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4; pH 7.4), resuspended in 10 ml of urea buffer (20 mM Tris-HCl, pH 8.0, 25 mM NaCl, 1 mM DTT, 1 mM PMSF, 6 M urea), and subjected to a brief sonication (1 minute, Branson sonifier 250; setting 2). Following a second centrifugation (20 minutes, 20,000 ***g*** in Sorvall HB4 rotor), the pellet was taken up in 2 ml of urea buffer. The bulk of cyclin A was recovered in this pellet, and constituted more than 95% of the total protein. For further purification, the sample was applied to a 12% SDS-polyacrylamide gel, and cyclin A was excised and electroeluted (100 V, 3 hours, 4°C) into dialysis tubing (elution buffer was Tris, 1.5 g/l; SDS, 0.5 g/l; glycine, 7.2 g/l). Using the above procedure, approximately 12 mg of cyclin A protein could be obtained from a 500 ml culture of bacteria.

For antibody production, purified cyclin A fusion protein was injected intramuscularly into New Zealand white rabbits. The first injection was done with cyclin A emulsified in complete Freund's adjuvant, three subsequent injections at 3- to 5-week intervals were done using incomplete adjuvant. Each time, 300 μg of cyclin A were injected. Rabbits were bled 10-14 days after the boosts.

In vitro translation and cdk binding assays

In vitro transcription-translation of pGEM plasmids containing myc-tagged cyclin A cDNAs was performed in the presence of [^{35}S]Met and [^{35}S]Cys (TranSlabel; ICN), using the TNT reticulocyte lysate kit (Promega). To assay the binding of these proteins to rabbit cdk subunits present in the reticulocyte lysate, translation mixtures were incubated for 30 minutes at 4°C with recombinant chicken p9^{suc1} (R. Golsteyn, P. Gallant, G. Maridor and E. A. Nigg, unpublished results) coupled to Sepharose beads. The p9^{suc1} beads were prepared according to Labbé et al. (1991), except that the heat denaturation step (to 90°C) was performed after gel filtration chromatography. Incubations were carried out in bead buffer containing protease inhibitors (Meijer et al., 1989). After four washings of the beads in bead buffer, samples were subjected to SDS-PAGE or washed once more in kinase assay buffer (Krek and Nigg, 1991) and used for histone H1 kinase assays.

Histone H1 kinase assays

Histone H1 kinase assays on p9^{suc1}-absorbed cyclin/cdk complexes were carried out in the presence of [γ-^{32}P]ATP and 0.5 μg/μl histone H1 (Boehringer Mannheim), as described previously (Krek and Nigg, 1991). Phosphorylated histone H1 was resolved on a 15% SDS-polyacrylamide gel and visualized by autoradiography.

Immunochemical techniques

The procedures for indirect immunofluorescence microscopy, metabolic labeling of cells, immunoprecipitation experiments and immunoblotting have been described previously (Krek and Nigg, 1991; Gallant and Nigg, 1992).

RESULTS

Cloning of a cDNA for chicken cyclin A and immunological characterization of the corresponding protein

Using a human cyclin A cDNA probe for low stringency hybridization, cDNA clones for chicken cyclin A were isolated from a λgt11 library. Following phage purification, the longest insert (1.9 kb) was subcloned into a pGEM plasmid and sequenced in both orientations. Fig. 1 shows the nucleotide sequence of the chicken cyclin A cDNA, and the corresponding translation product. The predicted chicken cyclin A has 395 amino acids and a molecular mass of 44,080; it displays all structural features characteristic of A-type cyclins (see legend to Fig. 1). There is no in-frame stop codon upstream of the initiating methionine shown in Fig. 1, but the flanking sequences of the presumed AUG initator codon fit the consensus for translation initation sites (Kozak, 1991), and the results shown below (see Fig. 2) indicate that this sequence most probably represents the entire protein.

Polyclonal rabbit antibodies were raised against the C-terminal 250 residues of cyclin A expressed in *E. coli*. These antibodies were tested by immunoblotting (Fig. 2A, upper panel) and immunoprecipitation experiments (Fig. 2B). Immunoblotting of whole cell extracts of chicken DU249 hepatoma cells (Fig. 2A, upper panel, lane 3) revealed a single protein of the expected molecular mass. This protein comigrated with the [^{35}S]methionine-labeled product obtained from the cloned cyclin A cDNA by in vitro transcription-translation, as shown by mixing of the samples (Fig. 2A, upper and lower panels, lanes 1 and 2). Likewise, a protein comigrating with in vitro-translated cyclin A could be immunoprecipitated from [^{35}S]methionine-labeled cells by immune serum (Fig. 2B, compare lanes 1 and 2), but not by pre-immune serum (Fig. 2B, lane 3). These results demonstrate the specificity of the anti-cyclin A antibodies, and they support the conclusion that the cDNA characterized here most probably encodes the entire chicken cyclin A protein.

Subcellular localization of wild-type chicken cyclin A and transfected cyclin A mutants

Staining of chicken DU249 cells with anti-cyclin A antibodies resulted in a strong nucleoplasmic fluorescence in most interphase cells (not shown), confirming and extending recent studies in other vertebrate species (Pines and Hunter, 1991; Girard et al., 1991; Pagano et al., 1992a; Zindy et al., 1992). This prompted us to examine the chicken cyclin A sequence for the presence of a potential nuclear localization signal (NLS). As illustrated by an alignment of chicken and human cyclin A (Fig. 3), neither protein contains a motif that would readily be considered as a typical NLS of either the monopartite or bipartite type (for review see Dingwall and Laskey, 1991). Importantly, however, this alignment shows that chicken cyclin A displays a high degree of sequence similarity to its human counterpart not only over the central cyclin box (marked by arrows) but throughout the C-terminal end domain. As shown below, sequence conservation of the C termini is functionally significant.

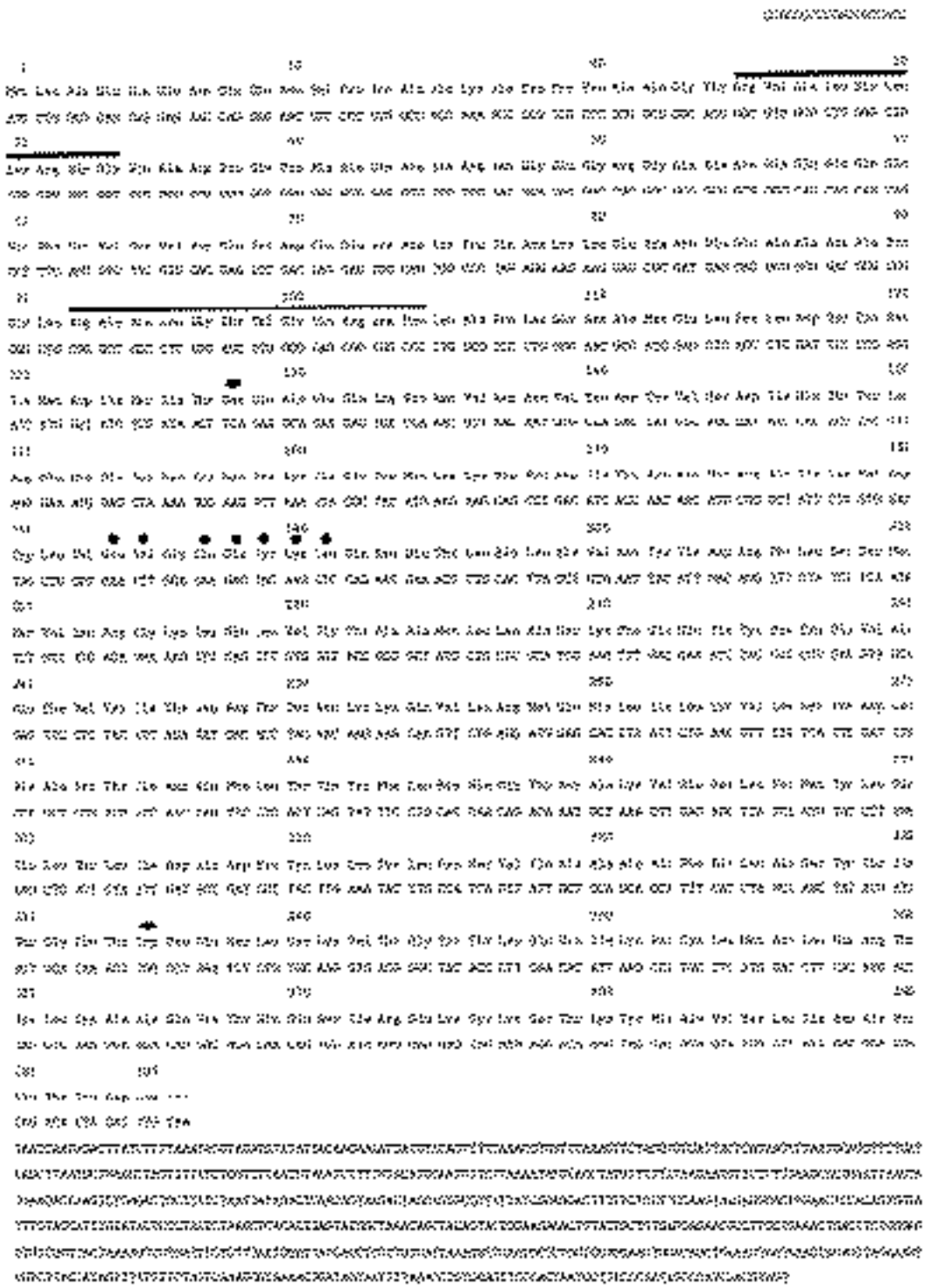

Fig. 1. cDNA sequence and deduced amino acid sequence of chicken cyclin A. The cDNA sequence shown contains 1922 nucleotides (EMBL database accession number X72892). The largest open reading frame extends from nucleotide 26 through 1210 and codes for a protein of 395 amino acids. The approximate boundaries of the centrally located ‘cyclin box’ (residues 128-335) are indicated by arrows, and filled dots mark residues characteristic of A-type cyclins (Minshull et al., 1989; Nugent et al., 1991; Hunt, 1991). Interestingly, the N-terminal domain of chicken cyclin A contains two motifs that qualify as potential destruction boxes (residues 25-34 and 92-104; denoted by solid lines); such motifs were shown to be required for cell cycle-dependent degradation of A- and B-type cyclins (Glotzer et al., 1991; Luca et al., 1991; Gallant and Nigg, 1992; Lorca et al., 1992; Kobayashi et al., 1992). Whether or not both candidate motifs in cyclin A are functional remains to be determined.

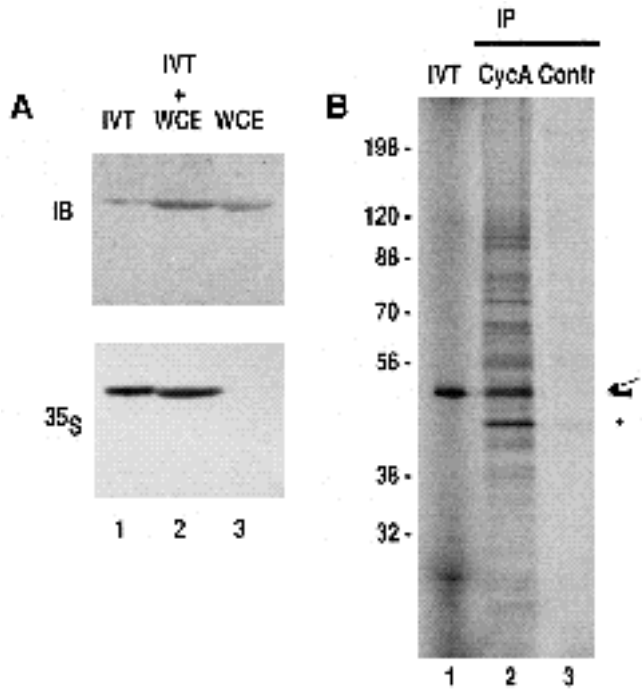

Fig. 2. Immunochemical identification of chicken cyclin A. Antibodies were raised against bacterially expressed cyclin A and used for immunoblotting (A) and immunoprecipitation experiments (B). For comparison, cyclin A was synthesized in vitro, using the cloned cDNA in a coupled transcription-translation system. (A) In vitro-translated [^{35}S]methionine-labeled cyclin A (IVT) or whole chicken DU249 cell extract (WCE) were resolved by SDS-PAGE on a 12% gel, either individually (lanes 1 and 3, respectively) or after mixing (lane 2). The migration of cyclin A was then visualized by both immunoblotting (upper panel) and autoradiography (lower panel). Note that in vitro-translated cyclin A comigrates with the immunoreactive protein in whole cell extract (compared to lane 1, the immunoreactive proteins visible in lanes 2 and 3 are slightly shifted; this is most likely due to a compression caused by the large amounts of cellular proteins present in these latter lanes). (B) Cyclin A was immunoprecipitated (IP) from [^{35}S]methionine-labeled chicken DU249 cells and resolved by SDS-PAGE on a 12% gel (lane 2). For comparison, in vitro-translated [^{35}S]methionine-labeled cyclin A was analyzed on the same gel (lane 1). Note that the major immunoprecipitated protein comigrates exactly with the in vitro-translated cyclin A (arrowhead). We presume that the protein displaying a faster electrophoretic mobility (star) represents a degradation product of cyclin A; the minor band visible slightly above cyclin A (arrow) may represent a phosphorylated form. Cyclin A was not immunoprecipitated by pre-immune serum (lane 3). Size standards (kDa) are shown on the left.

Fig. 3. Sequence alignment between chicken and human cyclin A. The amino acid sequence of chicken cyclin A (GGCYCA) is represented using the single-letter code. The sequence of the human cyclin A (HSCYCA; Wang et al., 1990) is indicated only at positions where it diverges from the chicken sequence; identical residues are indicated by dashes, and dots mark the positions where gaps were introduced to optimize the alignment. Arrowheads mark the approximate boundaries of the 'cyclin box', while the only clustered basic residues (with potential NLS function) are printed in bold in chicken cyclin A. Note that high sequence conservation between chicken and human cyclin A is not limited to the cyclin boxes (88% identity, 92% similarity over 208 residues), but extends throughout the C termini (75% identity, 81% similarity over 60 residues).

To determine the structural requirements for nuclear localization of cyclin A, several deletion mutants were constructed (for a schematic illustration see Fig. 4A). These were then transfected into HeLa cells, and the subcellular distribution of the corresponding cyclin A proteins was determined by indirect immunofluorescence microscopy (Fig. 5; for a summary of results see Fig. 4B). Although only the N terminus of cyclin A contains small clusters of basic residues that might conceivably have been considered as loose matches to a NLS consensus (see Fig. 3), a mutant protein lacking almost the entire N-terminal end domain (ΔN(−106)) was localized to the nucleus (Fig. 5b) as efficiently as wild-type cyclin A (Fig. 5a). In contrast, a cyclin A protein lacking part of its cyclin box (Fig. 5c) was located in the cytoplasm, and the same localization was seen for all C-terminal deletion mutants lacking 15 or more residues (Fig. 5d-g). Deletion of 8 C-terminal residues reduced efficiency of nuclear transport slightly (Fig. 5h), and deletion of only 4 residues was without effect (Fig. 5i). When either the N- or C-terminal half of cyclin A was deleted, the resulting small size proteins, ΔN(−229) of 20 kDa and ΔC(−179) of 26 kDa, were distributed about equally between nucleus and cytoplasm (data not shown). These latter results were expected since the nuclear pore complex provides a diffusion channel with a diameter of about 9 nm (Peters, 1986), and hence allows free diffusion of proteins smaller than approximately 40 kDa (Paine and Horowitz, 1980).

Nuclear localization of cyclin A correlates with binding to cdk subunits

The above results show that the nuclear localization of cyclin A requires the presence of the extreme C terminus as well as sequences within the central cyclin box domain. This argues against the existence of a typical, short NLS motif within cyclin A, and instead suggested to us that nuclear transport of cyclin A might depend on interactions with other proteins. As cdk subunits are the most prominent complex partners of cyclin A, all cyclin A mutants were tested for their ability to interact with cdk subunits (Fig. 6). For this purpose, we took advantage of the fact that rabbit reticulocyte lysates contain endogenous cdk sub-

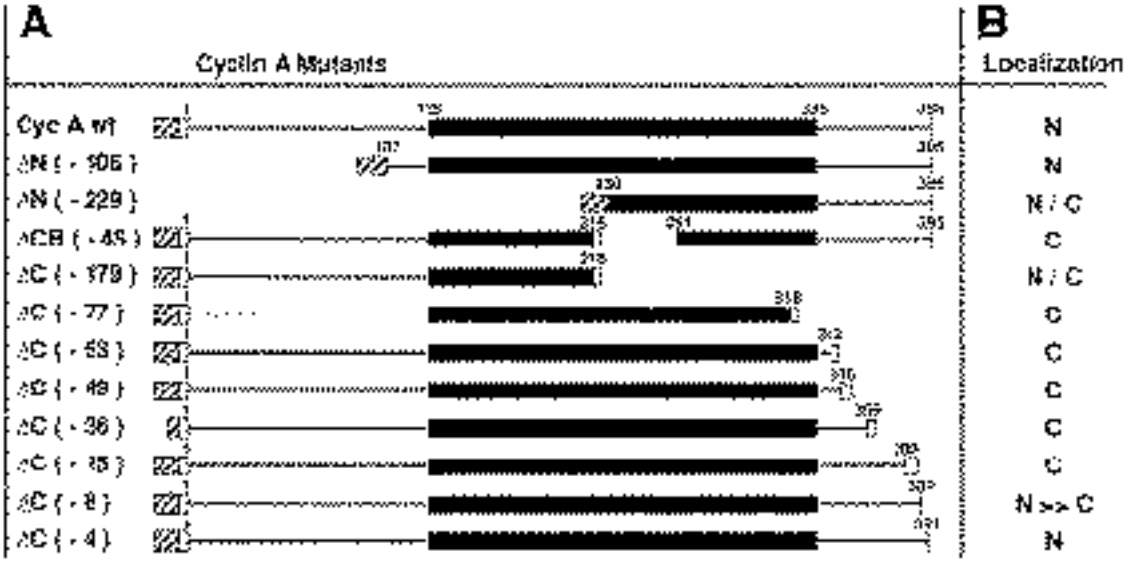

Fig. 4. (A) Schematic representation of cyclin A mutants. The black bar denotes the highly conserved central cyclin box. The hatched bar marks the myc-tag, and the white bars indicate additional amino acids that arose from the construction of the various pCMV expression plasmids. Note that the myc-tag was destroyed in the ΔC(−36) mutant, because of an internal *Dra*II site. (B) Summary of subcellular localizations of cyclin A mutants. N, nuclear; C, cytoplasmic; N/C, equal distribution between nucleus and cytoplasm; N>>C: nuclear in 80% of the cells, cytoplasmic in 20% of the cells.

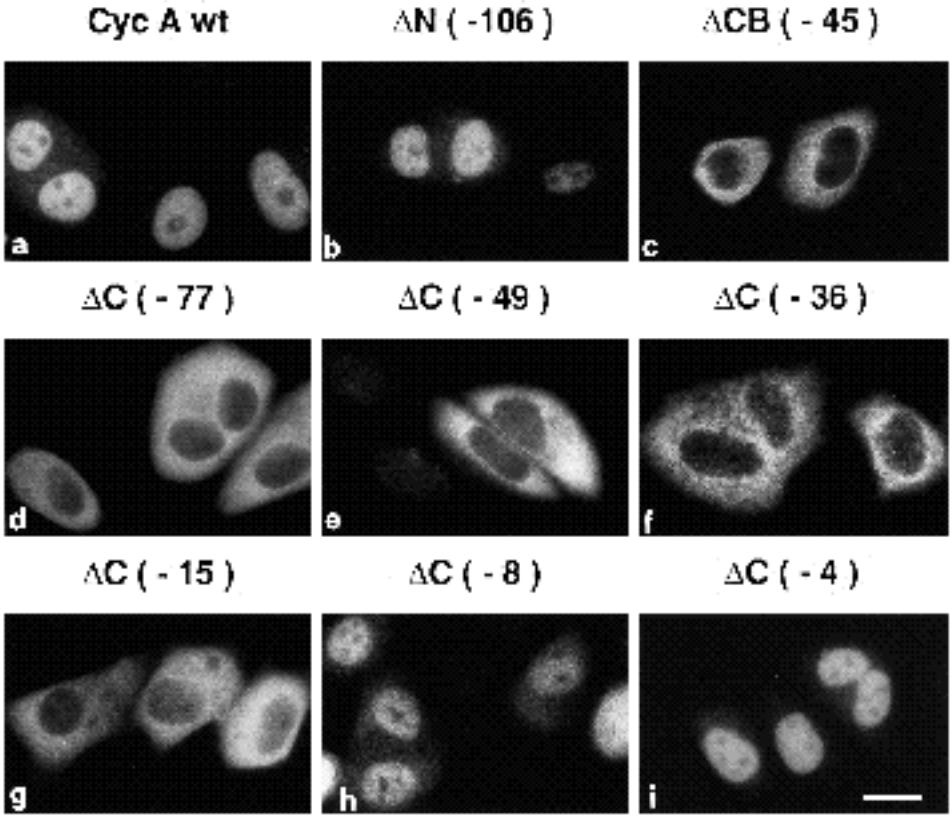

Fig. 5. Subcellular localization of wild-type and mutant cyclin A. Cyclin A proteins were transiently expressed in HeLa cells. Eight hours after transfection, cells were fixed and permeabilized with paraformaldehyde and Triton X-100 (a-e, g-i) or methanol/acetone (f). Then, the subcellular distribution of cyclin A was examined by immunofluorescence microscopy, using either the monoclonal antibody 9E10 (Evan et al., 1985) against the myc-tag (a-e, g-i), or affinity-purified rabbit anti-cyclin A antibodies (f). Note that the latter antibodies were raised against chicken cyclin A; they did not recognize human cyclin A in immunofluorescence experiments. Anti-cyclin A antibodies were used for detection of the ΔC(−36) mutant, because the myc-tag had been destroyed during construction of this mutant (see legend to Fig. 4). Cyclin A mutants are designated according to Fig. 4A. Bar in i, 10 μm.

units that are competent to interact with translated cyclins (Kobayashi et al., 1992). Following translation of wild-type and mutant cyclin A proteins (Fig. 6, top panel), cyclin A/cdk complexes were isolated using chicken $p9^{suc1}$ protein coupled to Sepharose beads, and the cdk-bound cyclins were visualized by SDS-PAGE and fluorography (Fig. 6, central panel). Furthermore, the isolated cyclin A/cdk complexes were assayed for kinase activity, using histone H1 as a substrate (Fig. 6, bottom panel). Remarkably, cdk binding as well as generation of high levels of kinase activity was observed for all forms of cyclin A that were capable of localizing to the nucleus (i.e. wild-type, ΔN(−106),

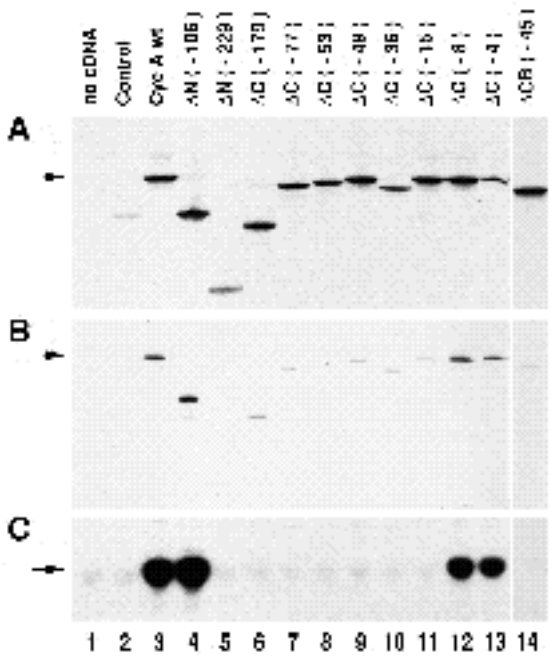

Fig. 6. Activation of rabbit reticulocyte cdk subunits by in vitro-translated cyclin A. Myc-tagged cyclin A cDNAs (Fig. 4A) were translated in rabbit reticulocyte lysates, in the presence of [^{35}S]Met and [^{35}S]Cys. One tenth of each sample was analyzed on a 12% SDS-polyacrylamide gel (A), the remainder was incubated for 30 minutes at 4°C with chicken p9^{suc1} coupled to Sepharose beads. After washing of the beads, one aliquot of the cyclin A/cdk complexes was analyzed by SDS-PAGE (B). The remaining samples were used for histone H1 kinase assays, as described previously (Krek and Nigg, 1991). Phosphorylated histone H1 was resolved on a 15% SDS-polyacrylamide gel and visualized by autoradiography (C). (A) Visualization of ^{35}S-labeled in vitro-synthesized cyclin A by electrophoresis and fluorography. For control, transcription-translation reactions were carried out in the absence of cDNA (lane 1), or the presence of a cDNA encoding the nucleolar protein No38 (lane 2). Arrowhead indicates the migration of wild-type cyclin A (lane 3). Mutant cyclin A proteins (lanes 4-14) are designated according to Fig. 4A. Note that ΔC(−36) migrates anomalously, due to destruction of the myc-tag by *Dra*II (see legend to Fig. 4A). (B) Binding of ^{35}S-labeled translation products (shown in A) to rabbit cdk subunits. Arrowhead indicates the migration of wild-type cyclin A (lane 3). Note that significant binding to p9^{suc1} beads occurred only with wild-type cyclin A and the ΔN(−106), ΔC(−8) and ΔC(−4) mutants. (C) Histone H1 kinase activity associated with the cyclin A/cdk complexes isolated from reticulocyte lysates (as shown in A). Arrow points to ^{32}P-labeled histone H1, as detected by autoradiography.

ΔC(−8) and ΔC(−4)). Conversely, none of the cytoplasmically located cyclin A mutants were capable of either binding or activating cdk subunits (Fig. 6). Taken together, these results reveal a strong correlation between nuclear localization of cyclin A and its ability to bind to cdk subunits.

There are currently no antibodies available with known specificities for individual cdk subunits of the rabbit, and isolation of cdk/cyclin complexes with p9^{suc1} beads does not allow identification of individual cdk members. Thus, we have no direct information on the molecular identity of the cdk subunits that are activated by cyclin A in the reticulocyte lysate. However, in view of the in vivo specificity of cyclin A (e.g. see Pines and Hunter, 1990; Tsai et al., 1991), they most likely correspond to p34^{cdc2} and/or p33^{cdk2}.

DISCUSSION

In vertebrates, cyclins A and B display strikingly different subcellular distributions. Specifically, cyclin A accumulates in the nucleus immediately after its synthesis (Pines and Hunter, 1991; Girard et al., 1991; Pagano et al., 1992a; Zindy et al., 1992), while cyclins B1 and B2 remain cytoplasmic until shortly before the onset of mitosis (Pines and Hunter, 1991; Gallant and Nigg, 1992; Ookata et al., 1992; Bailly et al., 1992). A- and B-type cyclins differ also in their abilities to form complexes with other proteins: B-type cyclins bind predominantly, if not exclusively, to p34^{cdc2}, while A-type cyclins interact with both p34^{cdc2} and p33^{cdk2} (Pines and Hunter, 1990; Tsai et al., 1991). Furthermore, only cyclin A and cdk2 are found in DNA-binding multiprotein complexes with p107 and the transcription factor E2F (Ewen et al., 1992; Faha et al., 1992; Shirodkar et al., 1992; Devoto et al., 1992; Cao et al., 1992; Pagano et al., 1992b).

Extensive studies on the nuclear import of many viral and cellular proteins have revealed the existence of comparatively short sequence motifs that are able to function as NLSs. These motifs are generally identified by deletion analyses and domain-transfer experiments; by definition, they are both necessary and sufficient for nuclear transport of a protein (for recent reviews see Garcia-Bustos et al., 1991; Silver, 1991; Dingwall and Laskey, 1991). Using a standard deletion analysis, we were unable to uncover a typical NLS in cyclin A. Although deletion of only 15 C-terminal residues completely abolished nuclear localization, the C terminus of cyclin A cannot be considered as an NLS, since its presence was not sufficient to confer nuclear localization to a mutant carrying a deletion within the cyclin box. Deletion of more than a hundred N-terminal residues was without significant effect on the subcellular distribution of cyclin A. It appears, therefore, that the nuclear localization of cyclin A depends on a structure that requires both elements of the central cyclin box and the C terminus. Interestingly, the very same domains also were found to be required for the formation of functional complexes with cdk catalytic subunits.

There are two caveats associated with the interpretation of all mutational analyses. The first is that mutations may cause generalized misfolding of a protein. A priori, it seems rather unlikely that cyclin A would snap into a completely non-functional conformation simply because of terminating a few amino acids too early (as in the ΔC(−15) mutant), while, conversely, it can clearly fold in a functional way in the complete absence of translation of the first 106 amino acids (as in the ΔN(−106) mutant). Although the possibility of misfolding cannot be rigorously excluded, we note that Lees and Harlow (1993) reported residual biological activity of C-terminally deleted cyclin A proteins (see below). The second caveat is that even strong correlations can only suggest but never prove causal relationships. To

further substantiate the model proposed here, it may therefore be necessary in future studies to analyze the nuclear transport of individual cyclin A and cdk subunits in a cell-free transport system.

Recently, two laboratories have used recombinant proteins to examine the structural requirements for cyclin A binding to $p34^{cdc2}$, with somewhat conflicting results (Kobayashi et al., 1992; Lees and Harlow, 1993). While Kobayashi and coworkers also found an absolute requirement of the C terminus of cyclin A for cdk binding, the study by Lees and Harlow suggests that the presence of the C terminus may not be strictly necessary. Our present results are in better agreement with those of Kobayashi et al. (1992), but the data by Lees and Harlow (1993) may be based on more sensitive assays; in any event, they provide evidence against the argument that deletion of the C terminus might create completely non-functional cyclin A proteins. Neither Kobayashi et al. (1992) nor Lees and Harlow (1993) have studied the subcellular localization of cyclin A.

Despite the caveats discussed above, we believe that the most straightforward interpretation of our results is that cyclin A may enter the nucleus only when bound to cdk subunits. This conclusion is in apparent contrast to earlier studies showing that the cdc13 cyclin B protein accumulates in nuclei of *S. pombe* cells even in the absence of the cdc2 gene product (Booher et al., 1989). Whether this reflects a difference between A- and B-type cyclins or between yeast and vertebrates remains to be investigated. Also, we do not presently know whether all cyclin A/cdk complexes are competent to localize to the nucleus, but note that in all vertebrate somatic cells studied so far the bulk of cyclin A appears to be nuclear (Pines and Hunter, 1991; Girard et al., 1991; Pagano et al., 1992a; Zindy et al., 1992; this study). On the other hand, it is unlikely that binding to cdks would be sufficient to explain the nuclear localization of cyclin A. Neither $p34^{cdc2}$ nor $p33^{cdk2}$ contain any obvious candidate NLSs, and complexes between $p34^{cdc2}$ and cyclin B clearly occur in the cytoplasm. One might argue that an unusual type of NLS might be created at the interface between cyclin A and cdk subunits, or be generated on one of these proteins via a conformational change induced by binding of the other, but there is no precedent for such a mechanism of nuclear protein transport. Thus, it appears more likely that cyclin A/cdk complexes enter the nucleus in association with yet other proteins, via a 'piggyback' transport mechanism.

At present, we can only speculate on the identity of the protein(s) that might be involved in carrying cyclin A/cdk complexes to the nucleus. Cyclin A and $p33^{cdk2}$ are known to bind specifically to p107, a nuclear protein related to the retinoblastoma gene product pRb (Ewen et al., 1992; Faha et al., 1992; Shirodkar et al., 1992; Devoto et al., 1992; Pagano et al., 1992b; Cao et al., 1992), and complexes of cyclin A with $p34^{cdc2}$ were reported to associate with pRb (Williams et al., 1992). Thus, one should not, a priori, exclude the possibility that binding to proteins of the pRb/p107-family might play a role in determining the subcellular localization of A-type cyclins. Consistent with this idea, we found a perfect correlation between the ability of cyclin A proteins to localize to the nucleus in vivo, and their ability to bind to GST-p107 fusion proteins in vitro (S. Dayan, P. Gallant, G. Maridor, M. Ewen and E. A. Nigg, unpublished results).

Elucidation of the mechanism(s) involved in targeting cyclin A/cdk complexes to the nucleus will most probably require the establishment of cell-free transport systems. Although several such systems have been previously developed (e.g. see Newmeyer et al., 1986; Adam et al., 1990), identification of functional carrier(s) for cyclin A/cdk complexes will not be a trivial task. This is illustrated best by considering that no receptors or carriers have so far been molecularly characterized even for proteins with canonical NLS motifs, despite intensive research over the last several years (for reviews see Silver, 1991; Yamasaki and Lanford, 1992; Dingwall and Laskey, 1992).

In summary, our mutant analysis has revealed a strong correlation between structural features of cyclin A required for entering the nucleus and those involved in complex formation with cdk catalytic subunits. Although other explanations cannot be excluded, the most straightforward interpretation of these results is that nuclear localization of cyclin A requires complex formation with cdk subunits. If this interpretation is correct, it implies that the information required for reaching a particular subcellular compartment may not in all cases be encoded within cyclin polypeptides per se, but may be generated upon binding of cdk subunits. Differences in the subcellular localizations of various cyclins might then be the consequence rather than the cause of specific interactions between cyclin/cdk complexes and other proteins.

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