Cyclin B2 Undergoes Cell Cycle–dependent Nuclear Translocation and, when Expressed as a Non-Destructible Mutant, Causes Mitotic Arrest in HeLa Cells

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Abstract. Cyclin proteins form complexes with members of the p34<sup>cdc2</sup> kinase family and they are essential components of the cell cycle regulatory machinery. They are thought to determine the timing of activation, the subcellular distribution, and/or the substrate specificity of cdc2-related kinases, but their precise mode of action remains to be elucidated. Here we report the cloning and sequencing of avian cyclin B2. Based on the use of monospecific antibodies raised against bacterially expressed protein, we also describe the subcellular distribution of cyclin B2 in chick embryo fibroblasts and in DU249 hepatoma cells. By indirect immunofluorescence microscopy we show that cyclin B2 is cytoplasmic during interphase of the cell cycle, but undergoes an abrupt translocation to the cell nucleus at the onset of mitotic prophase. Finally, we have examined the phenotypic consequences of expressing wild-type and mutated versions of avian cyclin B2 in HeLa cells. We found that expression of cyclin B2 carrying a mutation at arginine 32 (to serine) caused HeLa cells to arrest in a pseudomitotic state. Many of the arrested cells displayed multiple mitotic spindles, suggesting that the centrosome cycle had continued in spite of the cell cycle arrest.

The 34-kD protein kinase encoded by the homologs of the yeast cdc2<sup>+/CDC28</sup> gene plays a key role in controlling the cell cycle in all eukaryotes (for reviews see Nurse, 1990; Draetta, 1990; Pines and Hunter, 1990a). In yeasts, the p34<sup>cdc2</sup> kinase is essential for traverse of a G<sub>1</sub> control point (called Start) as well as entry into mitosis. In higher eukaryotes, its function is well established at the G2/M transition, but recent evidence suggests that the G1/S transition might be controlled by a related protein kinase called p33<sup>cdc2</sup> (Pines and Hunter, 1990b; Paris et al., 1991; Elledge and Spottswood, 1991; Tsai et al., 1991; Fang and Newport, 1991). The activity of p34<sup>cdc2</sup> during the cell cycle is regulated by phosphorylation–dephosphorylation reactions (e.g., Morla et al., 1989; Gould and Nurse, 1989; Krek and Nigg, 1991a,b; Norbury et al., 1991; Ducommun et al., 1991), and by complex formation between p34<sup>cdc2</sup> and other proteins, notably cyclins (Booher et al., 1989; Draetta et al., 1989; Giordano et al., 1989; Labbé et al., 1989; Meijer et al., 1989; Moreno et al., 1989; Pines and Hunter, 1989; Gautier et al., 1990). Cyclins were originally identified in marine invertebrates as proteins displaying a striking periodicity in synthesis and degradation during the cell cycle (Evans et al., 1983; Swenson et al., 1986). Subsequent studies carried out in many species, including yeasts, plants, and animals, led to the conclusion that the cyclin protein family comprises a large number of members. On the basis of sequence criteria and characteristic patterns of expression during the cell cycle, individual cyclins can be grouped into multiple classes (for review see Minshull et al., 1989a; Hunt, 1989; Reed, 1991; Xiong et al., 1991).

B-type cyclins are synthesized throughout interphase of the cell cycle, but abruptly destroyed at the metaphase–anaphase transition (Evans et al., 1983; Swenson et al., 1986; Pines and Hunter, 1989). They are components of the universal mitotic inducer MPF (M-phase–promoting factor) isolated from <i>Xenopus</i> and starfish oocytes (Draetta et al., 1989; Labbé et al., 1989; Meijer et al., 1989; Gautier et al., 1990), indicating that they function predominantly in M phase (reviewed in Hunt, 1989; see however Bueno et al., 1991). Synthesis of cyclin B is required for cycling <i>Xenopus</i> egg extracts to enter mitosis (Minshull et al., 1989b; Murray and Kirschner, 1989), and its destruction is necessary for inactivation of p34<sup>cdc2</sup> and exit from a mitotic state (Murray et al., 1989; Félix et al., 1990). Destruction of cyclin B occurs by ubiquitin–dependent proteolysis, and a motif of the structure RxLxxN (the "destruction box"), followed by a lysine-rich region in the NH<sub>2</sub> terminus of the protein, is essential for cell cycle–dependent degradation (Glotzer et al., 1991).

A-type cyclins are synthesized and destroyed slightly earlier than B-type cyclins, suggesting that they may carry out a different function (Whitfield et al., 1990; Minshull et al., 1990; Pines and Hunter, 1990b, 1991). Genetic evidence from <i>Drosophila</i> indicates that A-type cyclins are also required for mitosis (Lehner and O'Farrell, 1989, 1990b), but an additional role at earlier stages of the cell cycle, particu-
larly in S phase, is not excluded (d'Urso et al., 1990). It is intriguing in this context that A-type cyclins form complexes not only with p34<sup>60c2</sup>, but also with p33<sup>60c2</sup> (Tsai et al., 1991; see also Pines and Hunter, 1990b; Fang and Newport, 1991), and with transcription factors (Mudryi et al., 1991; Bandara et al., 1991).

The existence of cyclin-like proteins (CLN) with an essential function during G1 phase of the cell cycle has been convincingly documented in <i>Saccharomyces cerevisiae</i> (Nash et al., 1988; Cross, 1988; Richardson et al., 1989; Hadiwiger et al., 1989; Wittenberg et al., 1990; for review see Reed, 1991). A search for potential homologs in other species has led to the identification of several additional cyclins in vertebrates (Matsushima et al., 1991; Xiong et al., 1991; Motokura et al., 1991) as well as in fission yeast (Forsburg and Nurse, 1991). Although it is attractive to think that these novel cyclins may function at the G1/S transition, they are difficult to classify by structural criteria, and further studies will be required to definitively establish whether or not they represent functional homologs of the budding yeast CLNs.

The importance of cyclin proteins in cell cycle regulation is well established, but the large number of structurally distinct cyclin proteins expressed in any one cell type is bewildering. In particular, since cyclin proteins are thought to cooperate with catalytic subunits of the cdc2 kinase family to control different cell cycle transitions, the question arises what confers functional specialization to the different cyclin-like complexes. The timing of expression of individual cyclin proteins appears to almost certainly contributes to confer cell cycle specificity to kinase action. In addition, differences in the subcellular distribution of individual cyclins may play a role in determining the substrate specificity and/or the regulation of the associated kinase subunits. To understand the role of individual cyclin proteins at a molecular level, it will be important to determine their subcellular localization, and to study their functions by mutational analysis.

To approach these issues, we have begun to clone cDNAs encoding avian cyclin proteins, and to use monospecific antibodies for studying their expression and subcellular localization. Here we report a molecular characterization of chicken cyclin B2. We show that cyclin B2 is predominantly cytoplasmic for the most part of interphase, but undergoes an abrupt translocation to the nucleus at the onset of mitosis. Moreover, we have examined the phenotypic consequences of expressing wild-type and mutated chicken cyclin B2 in HeLa cells. We show that expression of a non-destructible cyclin B2 protein causes HeLa cells to arrest in a pseudomitotic state characterized by condensed chromatid, a disassembled nuclear envelope, and, in a high proportion of cells, multiple mitotic spindles.

**Materials and Methods**

**Cloning and Sequencing of a Chicken Cyclin B2 cDNA**

The Agt11 cDNA library used in this study was prepared from poly(A)<sup>+</sup> RNA of 10-d-old chicken embryos (Sap et al., 1986). For screening by plaque hybridization, nitrocellulose-bound phage DNA was first denatured for 2 min at room temperature in 0.5 M NaOH, 1.5 M NaCl, then placed for 90 s into a neutralizing solution (0.5 M Tris-HCl, pH 7.5, 1.5 M NaCl), washed for 90 s in 2× SSC and baked in a vacuum oven for 2 h at 80°C. The phages were blocked for 2 h in hybridization solution (5× Denhardt's, 50% formamide, 5× SSPE, 100 μg/ml of salmon sperm DNA) and hybridized for 13–19 h at 30°C with a 32P-labeled probe at 210,000 cpm/ml (see below). They were then washed three times for 60 min at room temperature in 2× SSC, 0.1% SDS, dried and exposed for 20–40 h using X-OMAT autoradiography film (Eastman Kodak Co., Rochester, NY) and intensifying screens.

To prepare a probe for hybridization, a 813 bp HindIII-BglII fragment was excised from a <i>Xenopus</i> cyclin B1 cDNA; this fragment codes for amino acids 70 to 340 of <i>Xenopus</i> cyclin B1, and thus spans the region that is most conserved among cyclin proteins (Minnshull et al., 1986b). After three rounds of phage purification, 28 phages (out of 240,000 plaques screened) showed strong hybridization with this probe. DNA was prepared from all 28 phages using Lambdaorb (Promega Biotec, Madison, WI), and subjected to restriction analysis. Several inserts were subcloned into the EcoRI site of the pGEM–32<sup>-</sup> plasmid (Promega Biotec). Nested deletions were generated using the Erase-A-Base kit (Promega Biotec), and double-stranded DNA was sequenced according to Chen and Seeburg (1985), using the Sequenase kit (Promega Biotec). One insert encoding part of a chicken cyclin protein closely related to Xenopus cyclin B2 was then used as a probe for Southern blot analysis of all 28 lambda phage DNA. Nine inserts hybridized strongly with the probe, the longest of which (1.5 kb) was subcloned into pGEM–32<sup>-</sup>(-) and sequentially in both orientations as described above. This plasmid, termed pGGcycB2, was found to contain the entire coding region of chicken cyclin B2.

**Preparation of Antibodies against Bacterially Expressed Chicken Cyclin B2**

To express full-length chicken cyclin B2 in E. coli, site-directed mutagenesis was used to introduce convenient restriction sites into pGGEcycB2. To this end, a 1,349-bp EcoRI/Mspl fragment was excised, filled in with Klenow polymerase, and cloned into the Smal site of M13mp8 (to give pM13cycB2). The orientation of the insert was chosen such that the single-stranded form of the phage represented the coding strand of the cyclin B2 cDNA. Using the Muta-Gen kit (Bio-Rad Laboratories, Cambridge, MA) for oligonucleotide-directed mutagenesis, the eight nucleotides immediately preceding the initiator ATG of cyclin B2 were changed to GTCGACAT, thereby introducing a SalI and a NdeI restriction site. The mutated cyclin B2 cDNA was then cloned back (as a SalI/XbaI fragment) into pGEM–32<sup>-</sup>(-), to give pGGcycB2(Nde). Finally, pGGcycB2(Nde) was partially digested with NdeI and BamHI, and the resulting 1,330-bp fragment, comprising the entire open reading frame of cyclin B2, was subcloned into pET-3a (Studier and Moffat, 1986; Studier et al., 1990), to give pETcycB2. Large quantities of cyclin B2 protein (about 20 mg/I of E. coli culture) could be obtained by growing DH5α transformed with pETcycB2 to OD<sub>600</sub> = 3.4, followed by 3 h of induction with phage XCE6 (at a multiplicity of infection >50% of total protein) was purified from the gel by electro-elution into 0.7 M sodium acetate, pH 5.0, 1% Triton X-100, 50 mM Tris-HCl, pH 8.0). Lyssates were incubated for 45 min on ice and sonicated four times for 30 s, using a Sonifier B-12 (Branson Cleaning Equipment Co., Shelton, CT). Proteins were pelleted by centrifugation for 30 min at 9,500 rpm in a rotor (model SS34; Sorvall Instruments, Newton, CT), and separated by SDS-PAGE. The cyclin B2 band (>50% of total protein) was purified from the gel by electro-elution into 40 ml Tris-HCl (pH 7.5), 2 mM EDTA, 0.2% SDS, as described previously (Krek et al., 1992).

Rabbits were injected intramuscularly with 200 μg of protein in 0.5 ml of PBS, emulsified in an equal volume of Freund's complete adjuvant for the first injection, and incomplete adjuvant for all subsequent injections (at 2-wk intervals). The appearance of cyclin B2 immunoreactivity in the sera was monitored by immunoblotting. Antibodies were affinity-purified on bacterially expressed cyclin B2 that had been blotted onto nitrocellulose strips, as described by Nigg et al. (1985). For antigen pre-adsorption experiments, affinity-purified antibodies were incubated for 2 h at room temperature with bacterially expressed cyclin B2 blotted to a nitrocellulose filter.

**Protein Techniques**

Chick embryo fibroblasts and HeLa cells were cultured as described previously (Krek and Nigg, 1991b). Chicken hepatoma DU249 were cultured...
and synchronized during the cell cycle as described in Krek and Nigg (1991a). Protein extracts were prepared by lysing the DU249 cells in gel sample buffer, and immunoblotting was carried out as described previously (Krek and Nigg, 1989). Labeling of DU249 cells with [35S]Met (Amer sham Corp., Arlington Heights, IL) or a mixture of [35S]Met and [35S]Cy5 (Tran 35S-label; ICN Biomedicals, Inc., Irvine, CA), and immunoprecipitation experiments were done as described by Krek and Nigg (1991a), with the following additions to the lysis buffer: aprotinin (1 μg/ml), chymostatin (10 μg/ml), E-64 (10 μg/ml), leupeptin (10 μg/ml), pepstatin A (10 μg/ml), PMSF (200 μg/ml), RNase A (30 μg/ml), DNase I (30 μg/ml). For immunoprecipitations, 4 μl of anti-cyclin B2 immune serum R18 (or the corresponding pre-immune serum) were used per 10-cm culture dish.

To prepare radiolabeled chicken cyclin B2, PGGcy5c2 was linearized with XbaI and transcribed in vitro using T7 polymerase (Riboprobe system; Promega Biotec). The resulting RNA was then translated in a reticulocyte lysate in the presence of [35S]Met, as described by the manufacturer (Promega Biotec).

In vitro kinase assays in the presence or absence of 500 μM of histone H1 (Sigma Chemical Co., St. Louis, MO) were performed as described by Krek and Nigg (1991a).

**Indirect Immunofluorescence Microscopy**

For formaldehyde fixation, cells were treated for 10 min with 3% paraformaldehyde/2% sucrose in PBS. Then, they were processed as described in Krek and Nigg (1991b). Fixations with methanol/aceton and paraformaldehyde/methanol (for stabilization of microtubules) have been also described (Nigg et al., 1985; Krek and Nigg, 1991b). Incubations with primary antibodies were for 30 min at room temperature. For double immunofluorescence microscopy, incubations with the two primary antibodies were carried out sequentially. Secondary reagents were mixed and applied to the cells for 15 min at room temperature. Cells were then washed and mounted in 90% glycerol/10% 1 M Tris-HCl (pH 9.0) and viewed with a Polivar fluorescence microscope, using a 100x oil immersion objective.

For visualization of microtubules, the hybridoma supernatant of the mAb TAT1 was used at a 1:5 dilution (Woods et al., 1989). The lamina was stained with the mAb E-3 (acetics diluted 1:1,000; Lehnert et al., 1986). Secondary reagents were affinity-purified TRITC-coupled goat anti-rabbit IgG (Pierce Chemical Co., Rockford, IL; used 1:1,000), and FITC-coupled goat anti-mouse IgG (Sigma Chemical Co.; used 1:500). Hoechst dye 33258 for DNA staining (used 1:1,000) was added to the cells together with secondary antibodies.

**Mutagenesis and Transfections**

For transient expression in HeLa cells, the plasmid pCMVcycB2 was prepared by filling in a XbaI/EcoRI fragment of pMI3cycB2 with Klenow polymerase, and cloning it into the Hpal site of pCMVneo (see Fig. 5 A, and Krek and Nigg, 1991b). Transfections were carried out as described by Krek and Nigg (1991b), using the method of Chen and Okayama (1987); 10 μg of pCMVcycB2 DNA was used per 3.5-cm tissue culture dish. Time 0 for transfection was washed off the cells. The mutant chicken cyclin B2 was constructed by changing the codon for Arg32 from AGG to AGC (coding for a serine), by direct biochemical evidence. To this end, DU249 cells were synchronized in the cell cycle by releasing them for various periods from nocodazole-induced M-phase arrest, as described previously (Krek and Nigg, 1991a). Then, whole cell lysates were prepared, equalized for protein content, and probed by immunoblotting for the abundance of cyclin B2 (Fig. 3 E). Cyclin B2 was present at high levels in pseudo-metaphase-arrested cells (Fig. 3 E, lane 2), but completely absent from G1 phase cells (Fig. 3 E, lane 2). It gradually re-accumulated as cells progressed through S phase into G2 (Fig. 3 E, lanes 3–5). We note that the electrophoretic mobility of mitotic cyclin B2 (Fig. 3 E, lane 3) is slightly reduced when compared to the form present in interphase cells (Fig. 3 E, lanes 3–5). A similar shift in mobility of the mitotic form of Xenopus cyclin B2 was recently shown to be a result of phosphorylation on serine 90 (Izumi and Maller, 1991).

To demonstrate that cyclin B2 associates with p34<sup>cdc2</sup> pro-

**Characterization of Anti-cyclin B2 Antibodies**

To study the chicken cyclin B2 protein, polyclonal rabbit antibodies were raised against the full-length protein expressed in *E. coli* (see Materials and Methods). The specificity of these antibodies is illustrated in Fig. 3, A and B. When assayed by immunoprecipitation, the immune-serum (R18) brought down one major protein from metabolically labeled DU249 cells (Fig. 3 A, lane 1, arrow). The apparent molecular weight of this protein (45 kD) is in agreement with the predicted molecular weight of cyclin B2, as well as with the apparent molecular weight of cyclin B2 translated in vitro from a cDNA-derived mRNA template (data not shown). The arrowhead in Fig. 3 A points to a co-precipitating protein of ~34 kD; as indicated by comparative peptide mapping, this protein corresponds to p34<sup>cdc2</sup> (not shown; see also Draetta et al., 1989; Pines and Hunter, 1989; Meijer et al., 1989; Gautier et al., 1990). Pre-immune serum did not precipitate p45<sup>cyc10</sup>B2, nor the 34-kD protein (Fig. 3 A, lane 2). When tested by immunoblotting on DU249 whole cell lysates, the antibody R18 reacted predominantly with p45<sup>cyc10</sup>B2 (Fig. 3 B, lane 1, arrow), but a minor cross-reactive band could also be detected (Fig. 3 B, lane 1, dot). Neither of these proteins was recognized by pre-immune serum (Fig. 3 B, lane 2). Because of the observed cross-reactivity, the R18 serum was affinity-purified on bacterially produced cyclin B2 (see Materials and Methods). The resulting affinity-purified reagent recognized exclusively p45<sup>cyc10</sup>B2 (Fig. 3 B, lane 3), and pre-incubation of this antibody with bacterially expressed cyclin B2 completely abolished immunoreactivity (Fig. 3 B, lane 4). In the subsequent studies, total serum was used for biochemical assays, whereas affinity-purified antibodies were used for immunofluorescence experiments.

**Cell Cycle–dependent Expression and Kinase Association of Cyclin B2**

Although sequence alignments strongly suggested that the avian cyclin protein identified here represents a B-type cyclin, we considered it important to corroborate this notion by direct biochemical evidence. To this end, DU249 cells were synchronized in the cell cycle by releasing them for various periods from a nocodazole-induced M-phase arrest, as described previously (Krek and Nigg, 1991a). Then, whole cell lysates were prepared, equalized for protein content, and probed by immunoblotting for the abundance of cyclin B2 (Fig. 3 E). Cyclin B2 was present at high levels in pseudo-metaphase-arrested cells (Fig. 3 E, lane 2), but completely absent from G1 phase cells (Fig. 3 E, lane 2). It gradually re-accumulated as cells progressed through S phase into G2 (Fig. 3 E, lanes 3–5). We note that the electrophoretic mobility of mitotic cyclin B2 (Fig. 3 E, lane 3) is slightly reduced when compared to the form present in interphase cells (Fig. 3 E, lanes 3–5). A similar shift in mobility of the mitotic form of Xenopus cyclin B2 was recently shown to be a result of phosphorylation on serine 90 (Izumi and Maller, 1991).
tein kinase, it was immunoprecipitated from interphase and (nocodazole-arrested) M-phase DU249 cells, and the washed immune complexes were subjected to in vitro kinase assays; these were carried out in either the presence (Fig. 3 D) or absence (Fig. 3 C) of histone H1 as an exogenous substrate. No protein kinase activity could be immunoprecipitated from interphase cells (Fig. 3, C and D, lanes 4), but an active kinase was readily detected in the cyclin B2 immunoprecipitates prepared from M-phase cells (Fig. 3, C and D, lanes 2). This kinase efficiently phosphorylated cyclin B2 (Fig. 3 C) as well as histone H1 (Fig. 3 D), as expected for p34cdc2 (Bonher et al., 1989; Draetta et al., 1989; Giordano et al., 1989; Labbd et al., 1989; Meijer et al., 1989; Moreno et al., 1989; Pines and Hunter, 1989; Gautier et al., 1990). Attesting to the specificity of the observed co-precipitation, no kinase activity could be detected in immunoprecipitates prepared with pre-immune serum from either interphase (Fig. 3, C and D, lanes 3) or M-phase cells (Fig. 3, C and D, lanes 1).

Using p13'-beads for isolation of mitotic histone H1 kinases (e.g., Brizuela et al., 1987), we found that the cyclin B2-p34cdc2 complex accounts for ~60% of the total kinase activity (not shown). The remaining activity may probably be ascribed to complexes between p34cdc2 (or related kinases) and other cyclins (e.g., cyclin B1).

**Subcellular Localization of Cyclin B2**

Fig. 3 F shows the subcellular distribution of chicken cyclin B2 in formaldehyde-fixed, detergent-permeabilized DU249 hepatoma cells, as determined by indirect immunofluorescence microscopy. Virtually identical results were obtained when analyzing chick embryo fibroblasts, staining could be completely abolished by pre-absorption of the antibodies.
Destruction

Figure 2. Amino acid sequence alignment of chickencyclin B2 withXenopusB-typecyclins, and consensussequences for A- and B-typecyclins. Thesequences are presented using the single-letter code. Amino acids of theXenopuscyclins are indicated only where they diverge from the chicken sequence; identical residues are indicated by dashes. Points mark the positions ... (this paper);Xenopuscyclins B1 and B2 (Minshull et al., 1989b); human cyclin B (Pines and Hunter, 1989); Drosophila cyclin B (Lehner and O'Farrell, 1990b; Whitfield et al., 1990); clam cyclin B (Westendorf et al., 1989); starfish cyclin B (Labbé et al., 1989); sea urchin cyclin B (Pines and Hunt, 1987); fission yeastcdc13(Hagan et al., 1988; Booher and Beach, 1988); clam cyclin A (Swenson et al., 1986); Drosophila cyclin A (Lehner and O'Farrell, 1989); Xenopus cyclin A1 (Minshull et al., 1990); human cyclin A (Wang et al., 1990); chicken cyclin A (Maridor, G., P. Gallant, and E. A. Nigg, unpublished results).

with bacterially expressed cyclin B2 protein (not shown), and results were independent of the procedures used for cell fixation and permeabilization (see Fig. 4 below). Consistent with the data demonstrating cell cycle dependent expression of cyclin B2 (Fig. 3 E), we found that anti-cyclin B2 antibodies did not stain all cells present in a population of exponentially growing DU249 cells (compare cyclin B2 staining in Fig. 3 F with the DNA staining of the same cells in Fig. 3 G). Based on the results shown in Fig. 3 E, it seems reasonable to conclude that the cyclin B2-negative cells were in G1 phase, while the cyclin B2-positive cells correspond to cells at later stages of the cell cycle (i.e., S or G2 phase). In the positive staining interphase cells, cyclin B2 was located predominantly in the cytoplasm, but prominent labeling of a small round organelle (usually one per cell, but occasionally two; see Fig. 4 a) could also be seen (Fig. 3 F). These organelles were most frequently located in a perinuclear position, although they were sometimes observed above or below the cell nucleus. The most likely interpretation of this observation is that the labeled dotlike structures correspond to interphase centrosomes.

The cells shown in Fig. 3 F are representative of the vast majority of cells present in exponentially growing cultures. However, a small number of the cells stained with anti-cyclin B2 antibodies displayed a striking nuclear fluorescence. As judged by phase contrast microscopy, most of these latter cells showed early signs of chromosome condensation and thus were about to enter mitosis. To follow up on this observation, we examined the distribution of cyclin B2 at various stages of mitosis. Fig. 4 summarizes the results obtained with methanol/acetone-fixed and -permeabilized chick embryo fibroblasts. In interphase fibroblasts, cyclin B2 was either undetectable (not shown) or cytoplasmic (Fig. 4 a; notice the staining of a putative duplicated centrosome indicated by arrows), consistent with the results obtained with DU249 cells (Fig. 3 F). In all prophase cells, however, cyclin B2 was predominantly nuclear (Fig. 4 b). Whereas these cells displayed partially condensed chromosomes, as visual-
Figure 3. Characterization of cell cycle properties of cyclin B2, using specific antibodies. (A) Immunoprecipitations from whole cell extracts of \(^{[35S]}\)methionine-labeled DU249 cells, using either anti-cyclin B2 immune serum R18 (lane 1) or pre-immune serum (lane 2). The arrow points to p45\(^{\text{cyclin B2}}\), the arrowhead to p34\(^{\text{cyclin B2}}\) co-precipitating with cyclin B2. Molecular weight standards are indicated (from top to bottom: phosphorylase B, 92 kD; BSA, 66 kD; ovalbumin, 43 kD; carbonic anhydrase, 31 kD). (B) DU249 whole cell extracts were analyzed by immunoblotting, using anti-cyclin B2 antibodies as primary reagents, followed by \(^{125}\)I-labeled secondary antibodies. Lane 1, immune serum R18 (1:500); lane 2, pre-immune serum (1:500); lane 3, affinity-purified antibodies (1:50); lane 4, affinity-purified antibodies after pre-adsorption to cyclin B2 protein (1:50). Affinity-purification of anti-cyclin B2 antibodies on cyclin B2 antigen bound to nitrocellulose strips was carried out as described in Materials and Methods. The arrow points to p45\(^{\text{cyclin B2}}\), whereas the dot indicates a crossreactive band seen with whole serum (lane 1), but not with affinity-purified antibodies (lane 3). (C and D) In vitro kinase assays, using cyclin B2 immunoprecipitates from either mitotic or interphase DU249 cells. The protein kinase activity co-precipitating with cyclin B2 was assayed in the presence (D) or absence (C) of 0.5 mg/ml of histone H1 as an exogenous substrate. After incubation with \(^{32}\)P-\(\gamma\)-ATP, samples were separated on 10\% SDS-polyacrylamide gels, and phosphorylations were visualized by autoradiography. C and D show the relevant parts of the autoradiographs, illustrating phosphorylation of p45\(^{\text{cyclin B2}}\) and histone H1, respectively. Lanes 1 and 2, immunoprecipitations from mitotic cells, using anti-cyclin B2 immune serum (lane 2) or pre-immune serum (lane 1). Lanes 3 and 4, immunoprecipitations from interphase cells, using anti-cyclin B2 immune serum (lane 4) or pre-immune serum (lane 3). (E) Cell cycle-dependent expression of chicken cyclin B2. DU249 cells were released from a nocodazole-induced block at pseudometaphase; at different times after release, whole cell extracts were prepared, equalized for protein content, and subjected to immunoblotting, using anti-cyclin B2 serum, followed by \(^{125}\)I-labeled secondary antibodies; only the relevant part of the autoradiograph is shown. Lanes 1–5, samples were prepared at 0, 4, 8, 12, and 16 h after release from the nocodazole block. Under these conditions, cells pass synchronously through G1 phase (4 h sample), S phase (8 and 12 h samples), and G2 phase (16 h sample) (Krek and Nigg, 1991b). (F) Immunofluorescence localization of cyclin B2 in exponentially growing DU249 cells. These were fixed and permeabilized using paraformaldehyde/Triton X-100, and incubated with affinity-purified anti-cyclin B2 antibodies, followed by rhodamine-conjugated goat anti-rabbit IgG antibodies. (G) shows DNA staining of the same cells by Hoechst dye 33258. The arrowheads point to two cells which do not contain any detectable cyclin B2, and thus are presumed to be in G1 phase of the cell cycle. Bar, 15 \(\mu\)m.

Ectopic Expression of Chicken Cyclin B2 in Human Cells

In a next series of experiments, avian cyclin B2 protein was transiently expressed in mammalian cells. For this purpose, the eukaryotic expression plasmid pCMVcyclB2 (Fig. 5 A) was constructed and introduced into HeLa cells, using calcium-phosphate–mediated transfection (Chen and Oka-yma, 1987; Krek and Nigg, 1991b). The ectopic expression of chicken cyclin B2 was then monitored by immunofluorescence microscopy, using antibodies against avian cyclin B2. These experiments provide a further control for antibody
specificity, and they set the stage for a mutational analysis of the function of cyclin B2.

At 24 h after transfection, the percentage of cells positive for chicken cyclin B2 was in the range of 3 to 10%. However, these numbers are likely to represent an underestimate of the transfection efficiency: assuming that exogenous cyclin B2 was efficiently degraded during mitosis (see below), cells having gone through mitosis would be expected to contain no or very little chicken cyclin B2, and thus score negative despite being transfected. As expected in view of the species-preference of the anti-chicken cyclin B2 antibodies, most HeLa cells in the population displayed negligible fluorescence (compare the cyclin B2 staining in Fig. 5B with the DNA staining of the same cells in Fig. 5C). Among the fluorescence-positive cells, the majority displayed cytoplasmic staining with concentrated fluorescence at the presumed centrosome(s) (Fig. 5B, lower right corner, black arrowheads). As observed with DU249 cells and chick embryo fibroblasts (see above), occasional transfected cells displayed a pronounced nuclear staining (Fig. 5B, lower left corner, white arrowhead); virtually all of these cells were in prophase (see the DNA staining in Fig. 5C). In metaphase, the transfected chicken cyclin distributed throughout the cell, and no fluorescent staining was seen in any of the anaphase cells examined (not shown). From these results we conclude that the subcellular distribution of ectopically expressed chicken cyclin B2 in HeLa cells is a faithful reflection of that observed in chicken cells.

A Mutant Cyclin B2 Causes Mitotic Arrest in HeLa Cells

Studies based on a cell-free cycling Xenopus egg extract led to the conclusion that destruction of B-type cyclins is required for exit from a mitotic state (Murray et al., 1989; Félix et al., 1990), a notion supported also by recent studies in yeast (Ghiara et al., 1991). On the other hand, complete destruction of cyclin B does not appear to be required for M-phase exit during the rapid early cell cycles in Drosophila syncytial embryos (Lehner and O'Farrell, 1990b; Whitfield et al., 1990), nor during the transition from meiosis I to meiosis II in clams and frogs (Westendorf et al., 1989; Kobayashi et al., 1991). It was of interest, therefore, to examine the consequences of expressing a nondestructible B-type
Figure 5. Ectopic expression of chicken cyclin B2 in HeLa cells. (A) Diagram of the chicken cyclin B2 expression plasmid (pCMVcycB2) used in this study. Wild-type and mutant (Arg32 changed to serine) chicken cyclin B2 cDNAs were placed under the control of the cytomegalovirus promoter/enhancer element (CMV P/E). A polyadenylation signal (poly A) is provided in this plasmid by the long terminal repeat (LTR) of the original mouse retroviral vector (Bender et al., 1987). ψ denotes the presence of a retroviral packaging signal. (B) Subcellular localization of chicken cyclin B2 in transfected HeLa cells. 24 h after transfection, cells were fixed and permeabilized using paraformaldehyde/Triton X-100. They were then incubated with affinity-purified anti-cyclin B2 antibody, followed by rhodamine-conjugated goat anti-rabbit IgG antibodies. C shows the same cells stained with Hoechst dye 33258. The black arrowheads in B point to centrosome-like structures decorated by anti-cyclin B2 antibodies. The white arrowheads in B and C identify a prophase cell. Bar, 15 μm.

cyclin mutant in somatic vertebrate cells. To this end, a point mutation was introduced into chicken cyclin B2, resulting in a change of arginine 32 to serine (Arg32 to Ser). Since a corresponding mutation in sea urchin cyclin B (arginine 42 to cysteine) had previously been shown to prevent mitotic destruction of the protein in a cycling Xenopus extract (Glotzer et al., 1991), the Arg32 to Ser mutation was expected to render cyclin B2 nondestructible. The mutant cyclin B2 was transfected into HeLa cells, using the expression vector described above, and its phenotypic effects were compared to those induced by wild-type cyclin B2. Cells were fixed at various times after transfection, and stained with anti-cyclin B2 antibodies. Compared to the transfection efficiency of the plasmid encoding the wild-type cyclin B2 (3–10%), transfections with the plasmid encoding the mutant protein were somewhat more efficient (10–20% transfected cells). Up to 24 h after transfection, mutant and wild-type cyclin B2 proteins were expressed to similar levels, as indicated by comparable fluorescence intensities. At 36 and 48 h after transfection, however, an increasing number of brightly stained cells could be detected in cultures expressing mutant cyclin B2, suggestive of increased stability of the mutant protein (not shown).

The most striking effect of expressing mutant cyclin B2 was to increase the number of cells displaying a mitotic phenotype (Fig. 6). This phenotype was monitored by staining cells with anti-tubulin antibodies to visualize mitotic spindles (Fig. 6 A, top), and with Hoechst dye to demonstrate chromosome condensation (Fig. 6 A, bottom). Cells expressing avian cyclin B2 proteins were distinguished from non-expressing cells by staining with anti-cyclin B2 antibodies (Fig. 6 A, middle). Fig. 6 B presents a semi-quantitative analysis of the frequency of cells displaying a mitotic phenotype in response to expression of wild-type (open bars) or mutant cyclin B2 (hatched bars). Up to 36 h after transfection, the percentage of mitotic cells expressing wild-type and mutant cyclin B2 was found to increase steadily with time. However, the effect produced by mutant cyclin B2 was considerably more severe than that produced by the wild-type protein. By 36 h, 63% of the cells expressing mutant cyclin B2 were in a mitotic state, while only 13% of the cells expressing the wild-type protein displayed a comparable phenotype. Since mitotic cells adhere poorly to glass cover-slips, and thus are easily lost during preparation of the samples for immunofluorescent staining, the values shown in Fig. 6 B are likely to represent an underestimate of the true percentage of cyclin-expressing mitotic cells. The problem of cell loss became progressively more severe with increasing time after transfection, indicating that cells were arrested in a mitotic state and eventually died. This phenomenon explains why the relative proportion of mitotic cells expressing mutant cyclin B2 appeared to have decreased by 48 h after transfection (Fig. 6 B).

Compared to mitotic cells expressing no avian cyclin protein (Fig. 6 A, control lane), the mitotic cells expressing avian cyclin B2 frequently appeared abnormal: they often displayed multiple spindle poles (up to four; Fig. 6 A, top), and the condensed chromosomes were not properly aligned.
Figure 6. Effect of ectopic expression of mutant cyclin B2 on cell cycle progression. HeLa cells were transfected with 10 μg of either wild-type or mutant (R32S) chicken cyclin B2 expression plasmids. At different times after transfection (32 h for A; as indicated for B) the cells were fixed and permeabilized using either paraformaldehyde/Triton X-100 (B) or paraformaldehyde/methanol (A). Cells shown in A were then incubated sequentially with anti-cyclin B2 antibodies (A, middle) and mouse monoclonal anti-tubulin antibody TAT1 (A, top), followed by simultaneous incubation with rhodamine-conjugated goat anti-rabbit IgG antibodies, fluorescein-conjugated goat anti-mouse IgG antibodies, and Hoechst dye 33258 (A, bottom). Representative examples of individual cells with spindle structures typical of mitotically arrested, mutant cyclin B2 transfected HeLa cells are shown. For comparison, a single mitotic cell from a plate transfected with the expression vector pCMV alone is shown under “control.” (B) Cells were incubated with anti-chicken cyclin B2 antibodies, followed by rhodamine-conjugated goat anti-rabbit IgG antibodies and Hoechst dye 33258. For every time point, 70-200 cells expressing chicken cyclin B2 were counted, and cells containing condensed DNA (as shown in A) were scored as mitotic. Bar, 10 μm.

Discussion

We have cloned and sequenced a cDNA coding for chicken cyclin B2, and used monospecific antibodies to determine the subcellular localization of the corresponding protein. The most striking conclusion emerging from these studies is that cyclin B2 undergoes a dramatic cell cycle-dependent redistribution from the cytoplasm to the nucleus. This nuclear translocation occurs at the onset of mitosis, i.e., just before nuclear envelope breakdown or lamina disassembly are detectable. These results raise the possibility that the timed transition of cyclin B2 from the cytoplasm to the nucleus may represent an important element in regulating the onset of mitosis. We have also examined the phenotypic consequences of expressing wild-type and mutant avian cyclin B2 in HeLa cells. We found that expression of a protein carrying a point mutation in its amino-terminus (Arg32 to Ser) causes HeLa cells to arrest in a pseudomitotic state. Interestingly, many of these arrested cells displayed multiple mitotic spindles, suggesting that the centrosome cycle had been uncoupled from the nuclear cell cycle.

Cell Cycle-dependent Nuclear Translocation of Cyclin B2

The subcellular localization of chicken cyclin B2 was studied in primary chick embryo fibroblasts and in virally (MC-29) transformed chicken hepatoma cells, with virtually identical results. In exponentially growing cultures, the intensity of...
immunofluorescent staining for cyclin B2 was variable, consistent with the cell cycle–dependent synthesis and destruction pattern of the protein. While some interphase cells were virtually unstained and thus presumed to be in early G1 phase, others, presumed to be in S or G2 phase, displayed specific staining of the cytoplasm. In the latter cells, pronounced labeling of small dotlike structures was also visible. In view of the perinuclear location and the number of these structures (one or two per cell), they almost certainly correspond to interphase centrosomes. This notion is supported by the finding that p34^{cd2} is also associated with interphase centrosomes (Bailly et al., 1989; Krek and Nigg, 1991b).

Most strikingly, cyclin B2 was found to undergo a cell cycle–dependent redistribution from the cytoplasm to the nucleus. In the vast majority of the cells displaying nuclear staining for cyclin B2, early signs of chromosome condensation could be detected, indicating that these cells were about to enter mitosis. The translocation of cyclin B2 from the cytoplasm to the nucleus is likely to be a rapid event, since we never saw any cells displaying uniform labeling of cytoplasm and nucleus.

In Schizosaccharomyces pombe, the B-type cyclin encoded by the cdc13 gene was reported to be nuclear at all cell cycle stages (Booher et al., 1989; Alfa et al., 1989, 1990), at variance with the results reported here. If this difference is physiologically meaningful, it may be related to the fact that S. pombe undergoes a closed mitosis. In Drosophila, cyclin B was found to be predominantly cytoplasmatic during interphase, but associated with condensing chromatin in early mitotic cells (Lehner and O'Farrel, 1990b). Recently, the subcellular distribution of human cyclins A and B1 has also been reported (Pines and Hunter, 1991). While cyclin A was nuclear, cyclin B1 displayed a cell cycle–dependent relocalization from the cytoplasm to the nucleus, in excellent agreement with the data shown here for cyclin B2. It seems, therefore, that cyclins B1 and B2 display at most minor differences in subcellular distribution, in spite of significant differences in primary structure.

The observed cell cycle–dependent nuclear translocation of B-type cyclins raises two important questions. First, how is the redistribution to the cell nucleus controlled, and second, what is its physiological significance? Since nuclear entry of cyclin B2 occurred very rapidly and before any visible alterations of the nuclear envelope, it appears most likely that nuclear uptake of B-type cyclins involves an active transport mechanism. In principle, one could argue that B-type cyclins contain an intrinsic nuclear localization signal (NLS), but that this signal is prevented from functioning for most of interphase of the cell cycle, due to either masking of the NLS or binding of the protein to some cytoplasmic anchoring structure (for discussion see Nigg, 1990; Nigg et al., 1991). Cell cycle–specific modification of either cyclin B or the masking/anchoring protein might then trigger activation of the NLS at the G2/M transition. Our observation that the subcellular distribution of cyclin B2 was correctly regulated when the avian protein was overexpressed in HeLa cells argues against the existence of a saturable NLS-masking or -anchoring protein. It appears more likely, therefore, that an NLS might be created on cyclin B2 at the G2/M transition, or that cyclin B2 undergoes piggyback transport to the nucleus.

The major complex partner of cyclin B2, p34^{cd2}, is located predominantly in the cell nucleus, although a significant cytoplasmic pool has also been detected (Bailly et al., 1989; Riabowol et al., 1989; Krek and Nigg, 1991b). No evidence has been obtained for a cell cycle–dependent redistribution of p34^{cd2}. However, it is intriguing that p34^{cd2} is activated through phosphorylation on Thr4 and Tyr15 at about the time when B-type cyclins redistribute from the cytoplasm to the nucleus (Krek and Nigg, 1991a,b). Since complex formation between p34^{cd2} and B-type cyclins appears to be required for phosphorylation of p34^{cd2} on Thr4 and Tyr15 (Solomon et al., 1990; Meijer et al., 1991; Parker et al., 1991), it seems that newly synthesized cyclin B associates with p34^{cd2} in the cytoplasm, and that this complex is kept inactive through phosphorylation of p34^{cd2} (on Thr4 and Tyr15) by cytoplasmic kinases. What remains to be determined, is where in the cell dephosphorylation of p34^{cd2} on Thr4 and Tyr15 occurs. If dephosphorylation occurs in the cytoplasm, then the concomitant activation of the p34^{cd2} kinase might play a role in inducing nuclear translocation of the p34^{cd2}–cyclin B complex, thereby allowing appropriate kinase–substrate interactions to occur. Conversely, if dephosphorylation occurs in the nucleus, then nuclear translocation may represent a prerequisite for activation of the complex, and hence play an important regulatory role.

**Ectopic Expression of a Non-destructible Cyclin B2 Induces Mitotic Arrest in HeLa Cells**

By mutating Arg32 of chicken cyclin B2 to Ser, we have constructed a cyclin B2 protein that we expected to be resistant to the ubiquitin-dependent mitotic degradation system described in Xenopus egg extracts (Glotzer et al., 1991). Since >60% of the HeLa cells transfected with the mutant cyclin B2 arrested in mitosis within 36 h, we conclude that somatic cells most probably contain a cell cycle–specific proteolytic degradation system similar to that described in Xenopus egg extracts, and that the destruction of B-type cyclins is essential for somatic cells to exit from mitosis. In parallel experiments carried out with wild-type cyclin B2, ~13% of the transfected cells arrested in a mitotic state, suggesting that the mitotically activated degradation system is saturable. We also note that a significant percentage of (wild-type and mutant) cyclin B-positive cells displayed an interphase phenotype even after prolonged culturing of transfected cells (up to 72 h). Thus, overexpression of B-type cyclins influences not only mitotic events, but may also slow down or inhibit other cell cycle transitions, possibly due to unscheduled complex formation with p34^{cd2} (or related kinases) during the G1/S transition.

Little if any effects on cell cycle progression were observed at early times after transfection, indicating that ectopically expressed cyclin B2 did not induce cells to enter mitosis prematurely, but merely arrested them once they had entered mitosis spontaneously. This conclusion is supported by the observation that the mitotically arrested cells displayed mitotic spindles and fully condensed chromosomes (Fig. 6A). In striking contrast, we have shown previously that transfection of HeLa cells with a p34^{cd2} protein carrying mutations in the inhibitory phosphorylation sites (Thr4 and Tyr15) rapidly promoted a large proportion of cells to enter mitosis.
prematurely. The pseudomitotic cells expressing the mutant p34\(^{\text{acc}}\) protein were characterized by abnormally condensed chromatin, and they lacked mitotic spindles. Remarkably, many of the mitotically arrested transfected cells contained up to four spindle poles. Concomitantly, the chromosomes were not aligned in either a metaphase plate or an anaphase configuration, but arranged as if the cells had attempted to segregate their chromosomes in multiple directions (Fig. 6A). These observations suggest that in these cyclin B2-expressing cells the centrosome cycle had continued despite the arrest of the nuclear division cycle. A similar uncoupling of nuclear cell cycle and centrosome cycle has been observed previously in Drosophila embryos treated with aphidicolin (Glover et al., 1989), in sea urchin eggs treated with emetine/anisomycin (Sluder et al., 1990) or aphidicolin (Glover et al., 1989), in sea urchin egg treated cyclin: a protein specified by maternal mRNA in sea urchin egg that is destroyed at each cell division. Cell. 33: 389-396.

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