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A Novel Mammalian Protein, p55CDC, Present in Dividing Cells Is Associated with Protein Kinase Activity and Has Homology to the *Saccharomyces cerevisiae* Cell Division Cycle Proteins Cdc20 and Cdc4

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A novel protein, p55CDC, has been identified in cycling mammalian cells. This transcript is readily detectable in all exponentially growing cell lines but disappears when cells are chemically induced to fall out of the cell cycle and differentiate. The p55CDC protein appears to be essential for cell division, since transfection of antisense p55CDC cDNA into CHO cells resulted in isolation of only those cells which exhibited a compensatory increase in p55CDC transcripts in the sense orientation. Immunoprecipitation of p55CDC yielded protein complexes with kinase activity which fluctuated during the cell cycle. Since p55CDC does not have the conserved protein kinase domains, this activity must be due to one or more of the associated proteins in the immune complex. The highest levels of protein kinase activity were seen with α-casein and myelin basic protein as substrates and demonstrated a pattern of activity distinct from that described for the known cyclin-dependent cell division kinases. The p55CDC protein was also phosphorylated in dividing cells. The amino acid sequence of p55CDC contains seven repeats homologous to the β subunit of G proteins, and the highest degree of homology in these repeats was found with the *Saccharomyces cerevisiae* Cdc20 and Cdc4 proteins, which have been proposed to be involved in the formation of a functional bipolar mitotic spindle in yeast cells. The Gβ repeat has been postulated to mediate protein-protein interactions and, in p55CDC, may modulate its association with a unique cell cycle protein kinase. These findings suggest that p55CDC is a component of the mammalian cell cycle mechanism.

The eukaryotic cell cycle has a growth phase and a reproductive phase, the latter composed of the chromosome cycle and the centrosome cycle, which intersect in the establishment of the mitotic apparatus (for a review, see reference 47). The profound morphologic changes which result in mitosis are accompanied by a cascade of phosphorylation and dephosphorylation events. In mammalian cells, different complexes of kinases and their associated regulatory proteins control progression through discrete steps of the cell cycle (for a review, see references 59 and 68). While all eukaryotic cells use similar mechanisms to regulate progression through the stages of the cell cycle, it is clear that unique combinations of regulatory cyclins, kinases, and phosphatases are responsible for cell- and organism-specific patterns of cell division (16, 51, 52).

A variety of kinases which control the crucial transitions through the cell cycle have been identified. The best characterized are the cyclin-dependent kinases, such as $p34^{cdc2}$, which fluctuate in activity during the cell cycle (3, 18, 20, 28, 40–42, 48, 60, 78). The activity of many of these kinases is regulated by their association with one or more cyclins, which may be degraded at specific transition points in the cell cycle (44, 49, 59, 68). Other types of kinases have also been shown to vary in activity at different stages of the cell cycle and have been proposed to play a role in control of cell division, although they have little or no homology with $p34^{cdc2}$. These include the mitogen-activated protein (MAP) kinases and the MAP kinase (MEK) kinases, which regulate MAP kinase activity (for a review, see reference 11). In addition, a novel kinase has been identified in the fungus *Aspergillus nidulans*, the NIMA kinase, which is required to initiate mitosis (53–55). A mammalian kinase, Nek1, which has homology to the NIMA kinase, has been found in the mouse, in which it is expressed at high levels in gonadal tissues and may be required for meiosis (43).

Experiments in *Saccharomyces cerevisiae* have defined a number of other cell division cycle (Cdc) proteins which are also crucial for the orderly progression of the cell cycle, although the functions of many of these proteins have not been precisely defined (33). Two of these proteins, the products of the *CDC20* and *CDC4* genes, have been proposed to be elements of the mitotic spindle or segregational apparatus (34). *cdc20* temperature-sensitive mutants arrest in mitosis at the nonpermissive temperature after the formation of a complete short spindle and nuclear migration to the neck between the mother cell and a large bud (6). The Cdc20 protein is required for modulation of microtubule structure, either by promoting microtubule disassembly (1, 66) or by altering the surface of the microtubules, and is also required for microtubule-dependent processes other than mitosis (66).

The *CDC4* gene of *S. cerevisiae* (32) is essential for the initiation of DNA synthesis. Cells carrying a conditionally lethal temperature-sensitive mutation in *cdc4* arrest division at the nonpermissive temperature, and the cells have a termination phenotype of multiple buds, a single nucleus, and duplicated spindle pole bodies connected by a bridge structure (6). *CDC4* also appears to be required for karyogamy and sporulation (21, 69, 73). While the mechanism of action of the Cdc4

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protein is still unknown, subcellular localization studies with yeast cells have demonstrated that it is associated with the nucleoskeleton (7). The appearance of the duplicated spindle pole bodies has been proposed to indicate that the *CDC4* gene product is required for separation of the bodies and formation of the completed spindle (6, 77). It has recently been demonstrated that removal of the centrosome (the equivalent of the spindle pole body in higher eukaryotes) from mammalian cells uncouples the growth cycle from the reproductive cycle, indicating that cell division requires the presence of centrosomes to establish the bipolar mitotic spindle (45).

We have identified a novel mammalian protein, which we have termed p55CDC, which is present only in dividing cells. mRNA encoding p55CDC was ubiquitously present in all cell lines examined, as well as in embryonic tissue, placenta, and adult hematopoietic tissues, but was not detected in cells induced to differentiate and cease cell division. The amino acid sequence of p55CDC demonstrates regions of homology with the *S. cerevisiae* Cdc20 and Cdc4 proteins within the Gβ repeats found in the carboxy-terminal half of these three proteins. Expression of p55CDC appears to be crucial for cell division in mammalian cells. p55CDC is phosphorylated in cycling cells. Immune complexes precipitated by a polyclonal antiserum to p55CDC have a kinase activity which fluctuates during the cell cycle, although p55CDC itself does not appear to be an endogenous substrate of the kinase activity.

MATERIALS AND METHODS

RNA analysis. Total RNA was prepared from rat tissues, human thymus, and buffy coat by the method of Chomczynski and Sacchi (8). mRNA from human cell lines was prepared with the Fastrack kit (Invitrogen). Gel electrophoresis of total RNA (30 μg per lane) and Northern (RNA blot) hybridizations were performed as reported earlier (76). Radiolabeled probes were generated with the Amersham Multiprime DNA labeling system RPN.1601. mRNA size was determined by comparison with commercial RNA standards (Bethesda Research Laboratories). mRNA from other human tissues was purchased from Clontech, as was a multiple human tissue Northern blot.

To generate the riboprobes for the RNase protection assay, the gel-purified p55CDC cDNA fragment was subcloned into Bluescript (Stratagene) in both the sense and antisense orientations relative to the T7 promoter. All subsequent steps were performed as described previously (72). Briefly, cells (10^6/ml) were washed in phosphate-buffered saline (PBS) and lysed by incubation at room temperature for 20 min in 10 mM Tris (pH 8.0)–1 mM EDTA–20 mM dithiothreitol–100 μg of proteinase K per ml–0.2% sodium dodecyl sulfate (SDS). Lysed samples were added to hybridization mix with the labeled riboprobe and incubated at 84°C for 2 h. After RNase digestion for 20 min at 37°C with RNase A and RNase T_1, the sample was loaded onto a Sephacryl S200 Superfine gel filtration column (Sigma), and the radioactivity in the void volume fraction containing the protected probe was counted. The quantity of gene-specific RNA was calculated from comparison with a standard curve. All assays were performed in duplicate.

DNA analysis. Genomic Southern and restriction map analyses were performed by standard molecular biology techniques (64). Genomic DNA from various species was purchased from Clontech. Medium-stringency hybridizations were performed at 42°C in 40% formamide. All hybridizations were performed at a salt concentration of 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]). After overnight hybridizations, the filters were washed three times in 2× SSC–0.1% SDS at 50°C (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The final wash was done in 0.5× SSC–0.1% SDS for 30 min. The DNA sequence was determined by using Sequenase (U.S. Biochemical) and following the manufacturer's protocol. Sequencing was also performed on the Applied Biosystems 373A automated DNA sequencer with the Tag Dye Deoxy Terminator kit according to the suggested protocol. The comparative percent identity values between the genes carrying the Gβ motif were obtained by using the Genetics Computer Group BESTFIT program, with the gap weight set at 2.0 and the length weight set at 0.05.

cDNA cloning from newborn rat spleen. A rat genomic library made from a partial *Eco*RI digest ligated into Charon 4A (Clontech) was screened at low stringency (hybridizations performed in 43% formamide at 37°C) with a 435-bp cDNA probe encompassing the codons for amino acid residues 141 to 286 of the α-2,6-sialyltransferase gene (76). Restriction map analysis of the isolated genomic clone revealed a 2-kb *Bgl*II fragment that hybridized to the probe. This fragment was subcloned into a pUC vector, and further analysis narrowed the hybridizing region to a 0.26-kb *Pst*I fragment, which was used in all subsequent analyses.

Polyadenylated [poly(A)$^+$] RNA from newborn rat spleen was used to construct a cDNA library (Pharmacia kit), which was ligated into the λgt10 vector. This was packaged by using the Gigapack II Gold cloning kit (Stratagene). An initial packaging reaction gave 3.3×10^6 PFU, and 1×10^6 PFU were screened with the 0.26-kb *Pst*I fragment as the probe. The human HT1080 cell line cDNA library was constructed in the pSPORT-1 plasmid vector (BRL Life Technologies, Inc.). DNA from 44 pools of approximately 5,000 colonies each was linearized with *Not*I and screened by Southern blot with the rat p55CDC cDNA as a probe. Plaque and colony purification of the clones with the longest inserts were done by standard techniques (64).

Cell culture, synchronization, and labeling. HL60 and K562 cells were grown in RPMI 1640 medium (Irvine Scientific) supplemented with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) and 15% fetal calf serum. Cells were seeded at a concentration of 0.2×10^6 cells per ml of medium. Cells treated with 1 mM sodium butyrate were grown in 75-cm^2 flasks for 3 days. Cells induced with phorbol ester were grown in the presence of 30 ng of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) per ml for 3 days. Cells were lysed with guanidine thiocyanate, and total RNA was prepared as described before (8). CHOd− cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, glutamine, nonessential amino acids, and hypoxanthine. Rat1 cells were maintained in DMEM containing 10% serum and glutamine, and HeLa cells were maintained in α minimal essential medium supplemented with 10% serum, glutamine, and nonessential amino acids.

HeLa cells were synchronized at the beginning of the S phase (G_1/S) by the double thymidine-aphidicolin block described by Heintz et al. (35). Cells harvested 4 h later were in the S phase (60). Synchronization at the G_2/M transition was achieved by growth in the presence of 0.5 μg of nocodazole per ml for 12 to 14 h. The medium was carefully suctioned off, and the nonadherent mitotic cells were harvested by gently pipetting buffer onto the monolayer. The adherent cells were washed with PBS and then lysed. This population is not mitotic and is predominantly in G_2 (36).

Asynchronous exponentially growing cells were grown in methionine- and cysteine-free medium containing 2% dialyzed serum for 1 h and then for 2 h in the same medium containing 100 μCi of ^{35}S Translabel (ICN Biomedicals) per ml. $^{32}P_i$ (ICN

Biomedicals) labeling was performed for 3 h after 1 h of incubation in phosphate-deficient medium.

Rat1 cells were growth arrested by rinsing the plates with PBS and then with medium containing 0.1% fetal calf serum. The cells were grown in the low-serum medium for 48 h to obtain a quiescent population. Labeling with ^{35}S-Translabel was performed as described above except that the dialyzed serum concentration was maintained at 0.1% and the label was incorporated over 1 h. For the exponentially growing population of Rat1 cells, the dialyzed serum concentration was maintained at 2% during the course of the labeling.

For flow cytometry analysis, 10^6 cells were washed in PBS and fixed in 70% ethanol–2.0% Triton X-100 for 1 h. Fixed cells were washed in PBS and stained in a solution of 50 μg of propidium iodide and 20 μg of RNase A per ml. The cells were analyzed for DNA content (fluorescence intensity) and cell size (forward scatter) with a FACScan (Becton Dickinson, Mountain View, Calif.).

Transfection of CHOd− cells. A 1.8-kb cDNA obtained from the newborn rat spleen library was cloned into the *Bam*HI site of the pMT010/A+ mammalian expression vector, which contains two dominant selectable markers, the bacterial *neo* gene and the mouse *DHFR* gene, driven by the simian virus 40 promoter (9). The cDNA was inserted downstream of the metallothionein promoter in both the sense (pMTp55s) and antisense (pMTp55as) orientations. These plasmids, as well as the vector alone as a control, were transfected into cells by using Lipofectin (BRL Life Technologies) and following the suggested protocol. Initial selection with geneticin at 400 μg/ml in medium without hypoxanthine was followed by stepwise amplification with methotrexate to a final concentration of 2 μM. Growth curves were determined in medium containing 0.05 mM zinc to induce the metallothionein promoter.

Antibody preparation. A p55CDC cDNA clone lacking the first 10 codons of p55CDC was inserted into the *Eco*RI site of the pGEX-3X vector (Pharmacia glutathione *S*-transferase gene fusion system). Competent XL-1 cells (Stratagene) were transformed, and a colony harboring the recombinant plasmid was isolated. Cultures were induced with isopropyl-β-D-thiogalactopyranoside (final concentration, 0.1 mM) for growth of the fusion protein. The 76-kDa insoluble fusion protein pellet was washed twice with PBS containing 1% Triton CF-54 and extracted with 10 M urea. The urea extract was dialyzed overnight against PBS and purified by SDS-polyacrylamide gel electrophoresis (PAGE). The electroeluted protein was mixed with Freund's complete adjuvant and used for immunization of rabbits.

To obtain an affinity column for purification of antiserum, the crude insoluble fusion protein pellet was resuspended in coupling buffer (0.1 M $NaHCO_3$ [pH 8.3], 0.5 M NaCl, 0.5% SDS) and coupled to cyanogen bromide-activated Sepharose according to the manufacturer's instructions (Pharmacia). The antiserum was first absorbed against an unrelated insoluble fusion protein to remove any antibodies reactive against glutathione *S*-transferase or contaminating *Escherichia coli* proteins. This partially purified antiserum was applied to the affinity column. The column was washed with five column volumes of PBS, and the affinity-purified antibodies were eluted with 3 M sodium thiocyanate. Pooled antibody fractions were immediately dialyzed against PBS and stored at −80°C. The flowthrough fraction from this column was used as adsorbed antiserum for control immunoprecipitations.

Immunoprecipitations and protein kinase assays. In vitro translation was performed with a nuclease-treated rabbit reticulocyte lysate (Promega) and [^{3}H]leucine (Amersham TRK683). The mRNA template was produced by using the Stratagene in vitro transcription kit and the p55CDC cDNA subcloned into the Bluescript vector as the substrate. Cell lysates were prepared as described before (60) after the plates had been rinsed twice with PBS. Cells were lysed in modified radioimmunoprecipitation assay (RIPA) buffer with additional proteases (150 mM NaCl, 1.0% Nonidet P-40, 1.0% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 6 mM Na_2HPO_4, 4 mM NaH_2PO_4, 50 mM NaF, 200 μM Na_3VO_4, 20 μg of aprotinin per ml, 1 μg of leupeptin per ml, 10 μg of soybean trypsin inhibitor per ml, and 50 μg of phenylmethylsulfonyl fluoride per ml). All protease inhibitors were purchased from Sigma. Protein concentrations were estimated by using the bicinchoninic acid reagent (Pierce). For 250 μg of lysate in a final volume of 700 μl of RIPA buffer, we used 7 μl of affinity-purified p55CDC antibodies (140 μg of protein per ml) or 12 μl of p55CDC adsorbed antiserum (700 μg of protein per ml), which gave an equivalent level of immunoglobulin (Ig) for both preparations. Immunoprecipitation of $p34^{cdc2}$ complexes was done with 10 μl of the $p34^{cdc2}$ mouse monoclonal antibody 17 (Santa Cruz Biotechnology). Other antibodies used in this study were Rb(1F8), a mouse monoclonal IgG antibody against an Rb–β-galactosidase fusion protein (Santa Cruz Biotechnology), and Rb(Ab-1), another monoclonal antibody against retinoblastoma (Rb) protein (Oncogene Science). The immune complexes were routinely incubated overnight on ice and collected next morning with 30 μl of a 50% slurry of protein G-Sepharose (Pharmacia). The washed pellets were assayed for histone H1 kinase activity as described before (60). All reactions were performed for 30 min at 30°C. Assays were also performed with a variety of kinase substrates at the concentrations indicated in the figure legends under the same assay conditions. Histone H1 was purchased from Boehringer Mannheim, and myelin basic protein, β-casein, and α-casein were all purchased from Sigma. The reaction products were quantitated by excising the stained bands from the dried gel and counting the radioactivity.

Nucleotide sequence accession number. The sequences of human and rat p55CDC have been assigned GenBank accession numbers U05340 and U05341, respectively.

RESULTS

p55CDC is expressed in neonatal and fetal rat and human tissues and has homology to the Cdc20 and Cdc4 proteins of *S. cerevisiae*. The gene encoding p55CDC was identified serendipitously during an attempt to identify novel glycosyltransferase enzymes by low-stringency screening of a rat genomic library with a cDNA encoding the rat α-2,6-sialyltransferase (57, 76). During one round of screening, a genomic clone was isolated. Restriction map analysis first narrowed the hybridizing region to a 2-kb *Bgl*II fragment. The cross-hybridizing region of this fragment was further narrowed to a 0.26-kb *Pst*I fragment, which was used for Northern analysis of various embryonic, neonatal, and adult rat tissues. Northern analysis revealed tissue-specific and developmentally regulated expression of a unique transcript (Fig. 1A). A 2-kb message was abundant in RNA from total rat embryo, and this transcript was enriched in embryonic rat liver. In 2-day-old neonatal rats, the level of message in the liver decreased precipitously, while the transcript was abundant in the spleen. In 16-day-old rats, the transcript was still abundant in the spleen and thymus but was barely detectable in the liver and kidney. The transcript was not detectable in any adult tissues, although a longer exposure of a blot containing more RNA did reveal a faint band in the spleen sample (data not shown). Ethidium bromide staining of the gel showed that equivalent amounts of RNA

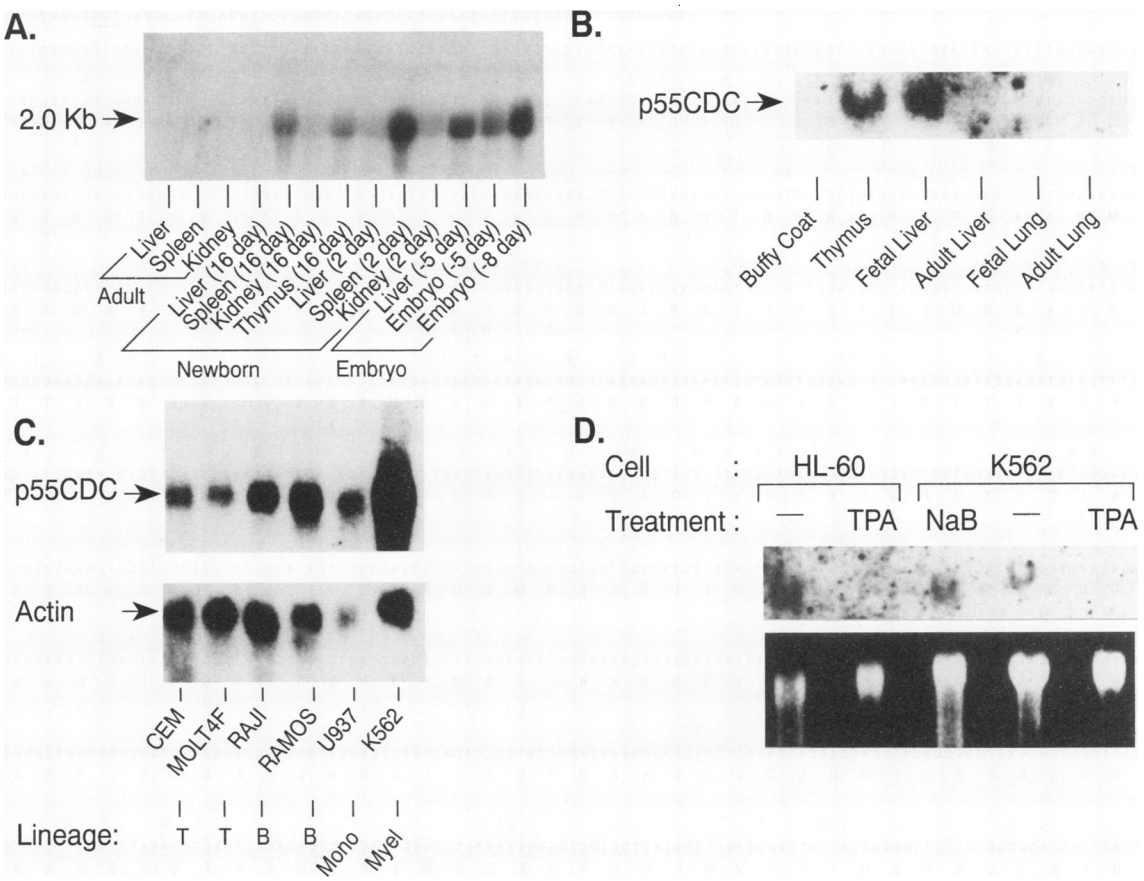

FIG. 1. Northern analysis of p55CDC. (A) Total RNA (30 μg) from a variety of rat tissues at different developmental stages was probed with a rat genomic 0.26-kb *Pst*I fragment. (B) Poly(A)$^+$ RNA (2.5 μg) from human tissues was probed with a ^{32}P-labeled p55CDC rat cDNA. (C) Poly(A)$^+$ RNA (2.5 μg) from human hematopoietic cell lines was analyzed with the same probe as in panel B. The signal obtained with a rat actin cDNA probe is shown for comparison. Mono, monocytic; myel, myeloerythroid; T, T cell; B, B cell. (D) Total RNA (30 μg) prepared from cell lines that were induced to differentiate as described in the text and from control cells was probed with the same probe as in panel B. The ethidium bromide stain for 28S RNA is shown for comparison. All details for RNA isolation and Northern blot hybridization are described in Materials and Methods.

had been loaded in all lanes (data not shown). The presence of the transcript in hematopoietic tissues, such as neonatal liver, thymus, and spleen, suggested that expression of this novel gene was highest in tissues in which cell proliferation was occurring.

To obtain a cDNA clone for sequencing, a cDNA library was constructed with poly(A)$^+$ RNA from 2-day-old rat spleen. With the *Pst*I genomic fragment as a probe, several positive plaques were identified, at a frequency of approximately 1:15,000. The two largest cDNA inserts were subcloned and sequenced. The nucleotide sequence (Fig. 2) coded for a protein of 499 amino acids, with a predicted molecular mass of 55 kDa. However, this sequence did not appear to encode a classical glycosyltransferase enzyme, since there was no evidence of an amino-terminal hydrophobic membrane-spanning signal anchor domain, which is essential for glycosyltransferases to be properly oriented in the Golgi (57). Nevertheless, the developmental pattern of expression of this transcript prompted further investigation.

A search of the GenBank/EMBL data base revealed that this protein had seven regions of homology with the WD-40 repeat of the β subunit of G proteins (27) (Fig. 3A) and with a number of proteins which contain this imperfect repeat motif (for a review, see references 12 and 74). These included the products of the *S. cerevisiae* genes *CDC20* (66) and *CDC4* (79), *TUP1/AER2* (80), *PRP4* (58), and *MSI1* (63) as well as the products of the *Drosophila melanogaster* gene *Espl*, the *Dictyostelium discoideum* gene *AAC3* (67), the *Arabidopsis thaliana* gene *COP1* (13), and the dTAF$_{II}$80 subunit of *Drosophila* TFIID (22). Two other mammalian proteins carrying the WD-40 repeat have also been identified (61, 71). The highest degree of homology, illustrated in Fig. 3B, was seen between p55CDC and two *S. cerevisiae* cell division cycle proteins, Cdc20 (519 amino acids) and Cdc4 (779 amino acids). BEST FIT analysis revealed a 45% identity between amino acids 172 to 407 of p55CDC and amino acids 249 to 479 of the Cdc20 protein, which increased to 59% when conservative substitutions were included. This was the only protein in which a high degree of similarity with the degenerate internal Gβ repeats in p55CDC was found. The Cdc4 protein was the only protein which showed strong homology with all seven repeats found in p55CDC (comparison using the first seven of the nine repeats found in the Cdc4 protein) (Fig. 3B). The alignment of the highly degenerate WD-40 repeats in these two proteins required the introduction of 16 gaps over 300 amino acid residues. This comparison indicated that 28% of the residues in this region were identical and 41% were identical or conservative substitutions. Notably, the *S. cerevisiae* Cdc20 and

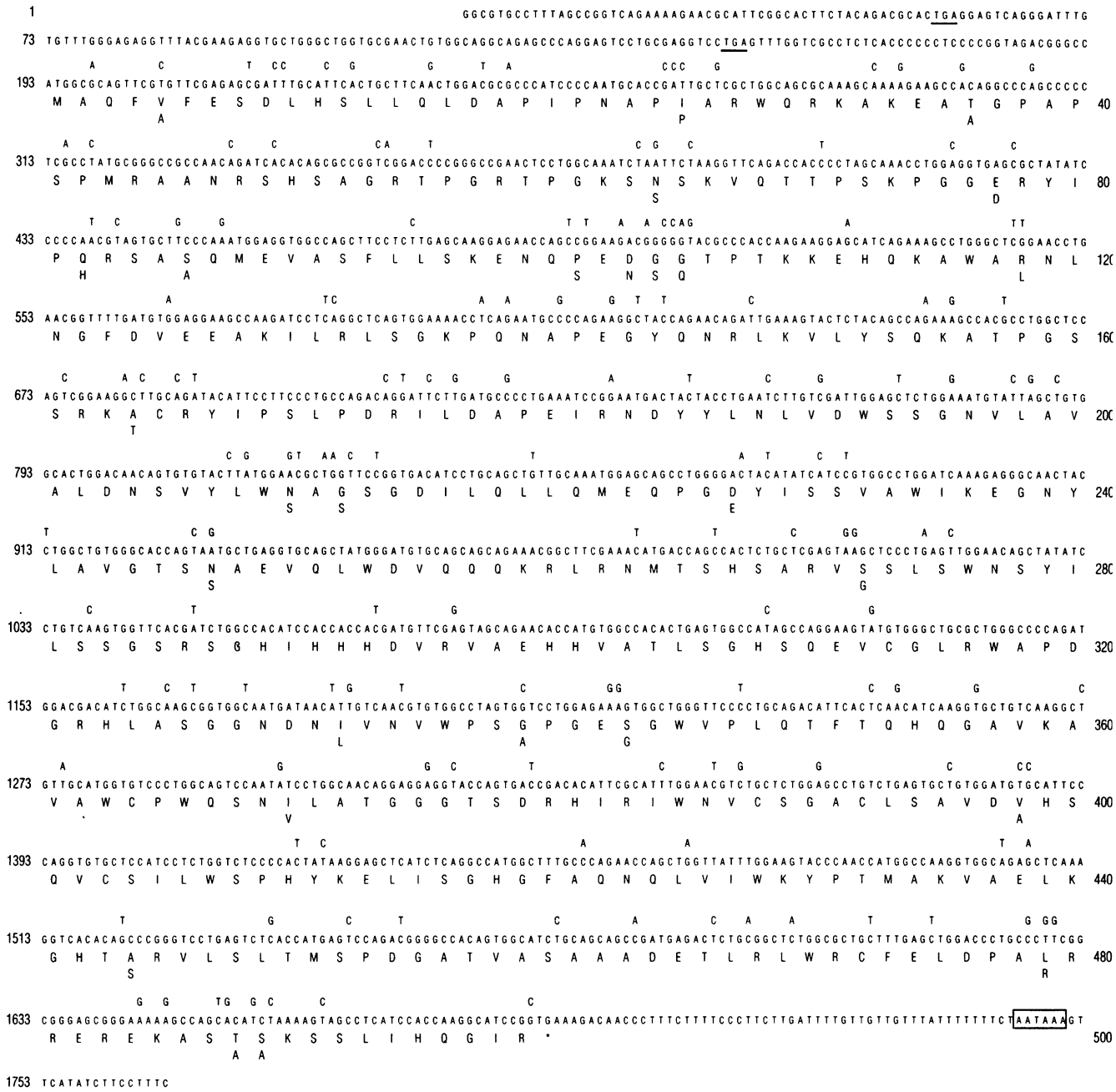

FIG. 2. Rat and human p55CDC sequences. The compiled sequence from two rat cDNA clones is shown. The open reading frame of the human cDNA is shown only where it differs from the rat sequence. Differences in the human nucleotide sequence are shown above and differences in the human amino acid sequence are shown below the rat sequence. Nucleotide base pair numbers are shown to the left, and amino acids, deduced from the nucleotides, are numbered at the right. Two in-frame stop codons upstream of the initiation methionine are underlined, and a polyadenylation signal downstream of the stop codon is boxed.

Cdc4 proteins each displayed a greater degree of homology to the mammalian p55CDC protein than they did to each other.

The p55CDC expression pattern in human tissues was similar to that observed in rat tissues. Northern analysis of human tissues demonstrated high levels of expression in fetal liver and juvenile thymus, but no expression was seen in fetal lung, adult lung or liver, or adult buffy coat, which is composed primarily of nondividing leukocytes (Fig. 1B). A second Northern analysis (not shown) examining poly(A)$^+$ RNA from adult human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas demonstrated expression of p55CDC only in placenta, which contains actively dividing cells.

A number of human cell lines also expressed the p55CDC transcript. The transcript was abundant in all leukemia cell lines examined, including the T-cell lines MOLT4f and CEM, the B-cell lines Raji and Ramos, the monocytic cell line U937, and the myeloerythroid cell line K562 (Fig. 1C). Indeed, we observed expression of the p55CDC transcript in every cell line

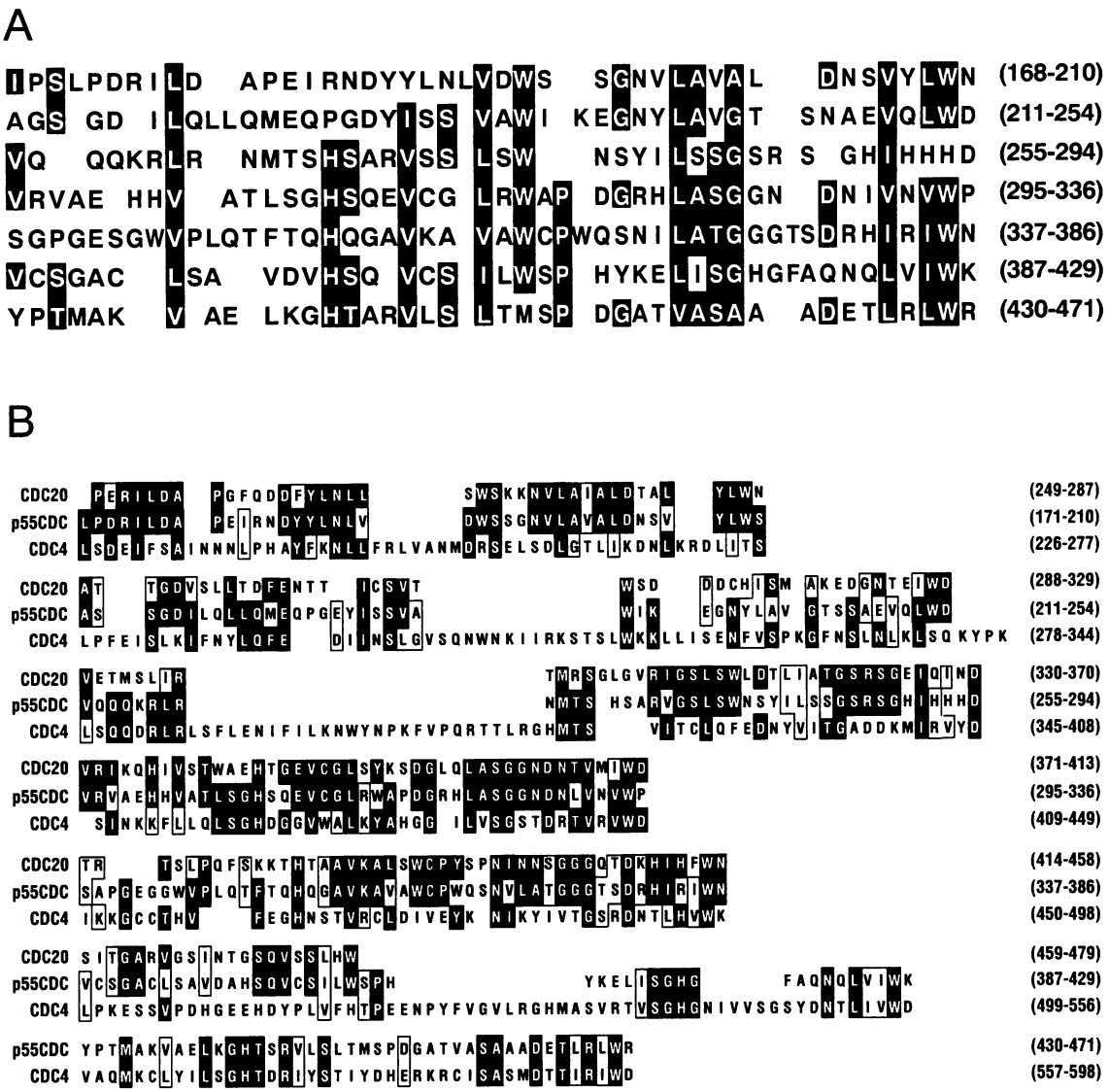

FIG. 3. p55CDC has seven Gβ repeats and shows homology to the *S. cerevisiae* Cdc20 and Cdc4 proteins. (A) Alignment of the seven rat p55CDC repeats was done manually after pairwise comparisons were made with BESTFIT program. Gaps were introduced to obtain optimal alignment and are represented by spaces. Identical residues and conservative substitutions which occur in four or more of the repeats are shown as white on black. Conservative substitutions are defined as I, L, or V; S or T; and A or G. (B) Alignment of the Gβ repeats of human p55CDC with the Cdc20 and Cdc4 repeats was done with the BESTFIT program and optimized visually. Gaps were introduced to obtain optimal alignment and are represented by spaces. Identical residues are shown as white on black, and conservative substitutions are boxed. Conservative substitutions are defined as I, L, or V; S or T; A or G; Y or F; D or E; and R, K, or H.

examined during the log phase of growth, regardless of lineage (data not shown).

To examine whether expression of the p55CDC transcript was related to the ability of cells to divide, we took advantage of the unique properties of two leukemia cell lines, K562 and HL60. K562 cells can be induced by treatment with sodium butyrate to undergo erythroid differentiation with no significant effect on growth rate (2). In contrast, treatment of K562 cells with the phorbol ester TPA causes monocytic differentiation accompanied by growth arrest (5). Treatment of HL60 cells with TPA also causes monocytic differentiation, with arrest of DNA synthesis and cell division (62). We examined the effects of these agents on the level of expression of p55CDC mRNA in these two cell lines (Fig. 1D). The p55 transcript was easily detectable in both mock-treated cell lines. For both K562 and HL60 cells, treatment with TPA resulted in loss of p55CDC mRNA expression. In the K562 cells treated with sodium butyrate, in which differentiation is not accompanied by growth arrest, the level of p55CDC transcript was roughly equal to that found in the mock-treated cells. These results indicate that p55CDC mRNA is synthesized only in dividing cells.

The human homolog of p55CDC was isolated from an HT1080 cell line cDNA library. A comparison of the open reading frames of the rat and human sequences showed 87% identity at the nucleotide level, which increased to 95% at the amino acid level (Fig. 2). Southern analysis demonstrated cross-hybridizing bands in rabbit, bovine, dog, and chicken genomic DNA, although no cross-hybridizing bands were seen in the lanes containing *S. cerevisiae* and *D. melanogaster* DNA

(data not shown). These results indicate that the gene encoding p55CDC is a single-copy gene, with no closely related genes in the species examined.

Effects of overexpression of p55CDC on cell proliferation. To explore the possible functions of the p55CDC protein, CHOd− cells were transfected with a plasmid containing the cDNA encoding rat p55CDC in either the sense (pMTp55s) or antisense (pMTp55as) orientation. Control cells were transfected with vector alone (pMT). After amplification with methotrexate, the three pools of cells were plated at a density of 0.5×10^6 cells per 60-mm plate in the presence of 0.05 mM zinc, and the growth profiles were plotted for 14 days (Fig. 4A). Pools of transfected cells were studied rather than individual clones to minimize the effect of any clonal variation in the CHOd− cells. Initially, little difference in the growth rates was observed among the three pools of transfected cells, although the pMTp55as-carrying cells were significantly larger than those carrying pMTp55s or the control cells, as shown by forward-scatter analysis determined by flow cytometry (data not shown) and visual observation under the microscope. Moreover, DNA content analysis, measured by propidium iodide uptake, showed that the pMTp55as-carrying cells had an increased amount of DNA per cell, indicating that these cells were hyperdiploid (Fig. 4B). As the cells began to reach confluence, dramatic differences in the growth profiles were observed. The pMTp55as-carrying cells reached confluence at a lower cell number, consistent with their larger size. After reaching confluence, the pMTp55as-carrying cells continued to divide slowly. The smaller pMTp55s-carrying cells continued to divide at a faster rate after reaching confluence. The pMTp55s-carrying cells reached a density of 24×10^6 cells per plate by day 14, compared with 6×10^6 cells per plate for the pMTp55as-carrying cells. The growth profile of the pMT-carrying cells fell midway between those of the cells carrying pMTp55s and those carrying pMTp55as.

Since the cells transfected with the vector encoding an antisense transcript continued to survive, although with an altered phenotype, we examined the pools of transfected cells for the presence of sense and antisense p55CDC mRNA transcripts in an RNase protection assay (72). As shown in Table 1, the pMT-carrying cells had an average of 166 copies of sense mRNA per cell, while, as expected, the pMTp55s-carrying cells had an increased average of 734 copies of sense mRNA per cell. Surprisingly, the pMTp55as-carrying cells also had an increased number of copies of sense mRNA, with an average of 714 copies per cell. In addition, the pMTp55as-carrying cells had only a moderate amount of antisense mRNA, with an average of 205 copies per cell, despite having been transfected with cDNA encoding the antisense transcript.

The same pattern was observed when clonal cell lines isolated from the pools of cells were analyzed. Each of the four pMTp55as-carrying clonal lines made more of the sense transcript; in all lines, this amount was at least five times the amount of the antisense transcript. As expected, in the control pMT-carrying cells, the average number of copies of sense transcript per cell declined considerably in confluent cells. Genomic DNA analysis of six clonal cell lines demonstrated that the elevated expression of sense transcripts was not due to amplification of the endogenous gene.

The two clonal isolates expressing sense orientation transcripts were distinct from each other. In contrast, it is likely that all four of the pMTp55as-carrying clones that we isolated were derived from the expansion of only one transfected cell in the original pool of cells. Restriction map analyses of genomic DNA from the four clonal pMTp55as-carrying cell lines with two different restriction enzymes and two different probes, to detect either plasmid or p55CDC sequences, demonstrated identical banding patterns (data not shown). The results indicated that inhibition of p55CDC expression by antisense transcripts was compensated for by overexpression of sense transcripts. These data suggested that p55CDC was essential for maintenance of cell proliferation in culture.

Immune complexes detected by antibodies against p55CDC. Polyclonal rabbit antiserum was raised against a fusion protein consisting of p55CDC and glutathione *S*-transferase. Both the original antiserum and an affinity-purified antibody preparation precipitated a protein of 55 kDa from an in vitro transcription-translation reaction mix containing p55CDC cDNA (Fig. 5), consistent with the predicted mass of the polypeptide. Bands at 46 and 43 kDa were also precipitated by the antibody and may be proteins derived from translation starting at internal methionine residues or proteolytic fragments.

To examine the level of p55CDC production in the transfected cell lines, immunoprecipitations were performed on extracts of ^{35}S-labeled cells in log phase with the affinity-purified antibody. As shown in Fig. 6A, the cells carrying pMTp55s and pMTp55as had higher levels of p55CDC than the cells carrying pMT, consistent with the demonstration of increased numbers of transcripts encoding p55CDC in these cells. In the pMTp55s-carrying cells, there was a strong band of 31 kDa, which probably represents a degradation product of p55CDC, since this band was also detected on immunoblot analyses of cell extracts made with the polyclonal antibody preparation. This 31-kDa band was also observed when lysates from cells carrying either pMTp55s or pMTp55as were prepared without protease inhibitors, and no intact p55CDC was detected in the absence of protease inhibitors. This peptide was not $p34^{cdc2}$, since no $p34^{cdc2}$ protein was detected in any of the immune complexes.

Immunoprecipitates of p55CDC also contained a protein of 210 kDa (p210). The amount of p210 detected in the immunoprecipitates was roughly proportional to the amount of p55CDC. When this experiment was repeated on cells in the stationary phase, 7 days after plating, a significant decrease in the amounts of both p55CDC and p210 was observed (Fig. 6B); in Fig. 6B, a 1-week exposure of the autoradiogram was required to detect p55CDC, versus a 1-day exposure in Fig. 6A. These results, as well as those of the mRNA analyses shown in Fig. 1 and Table 1, indicated that production of p55CDC was highest in proliferating cells.

Kinase activity of p55CDC immune complexes. Since many events in the cell cycle are controlled by various kinases, it was of interest to determine whether p55CDC immune complexes had any kinase activity. All immune complexes examined for protein kinase activity were precipitated under conditions identical to those used in the experiment shown in Fig. 6. The immunoprecipitation buffer (1% Nonidet P-40, 1% deoxycholate, and 0.1% SDS) was formulated to minimize nonspecific protein association. A number of cell division kinases can phosphorylate histone H1, so this substrate was assayed first. As shown in Fig. 7A, immune complexes precipitated with p55CDC antibody phosphorylated histone H1. Immune complexes prepared from lysates of the pMT-, pMTp55s-, and pMTp55as-carrying cells all demonstrated kinase activity against histone H1. The highest levels of phosphorylation were seen in the cells carrying pMTp55s and pMTp55as, which show increased expression of p55CDC. In the negative controls, a small amount of residual activity was seen with adsorbed antiserum. In reactions performed without exogenous substrates, no phosphorylated proteins were detected, indicating that none of the proteins in the immune complex were endogenous substrates of the kinase activity. However, when

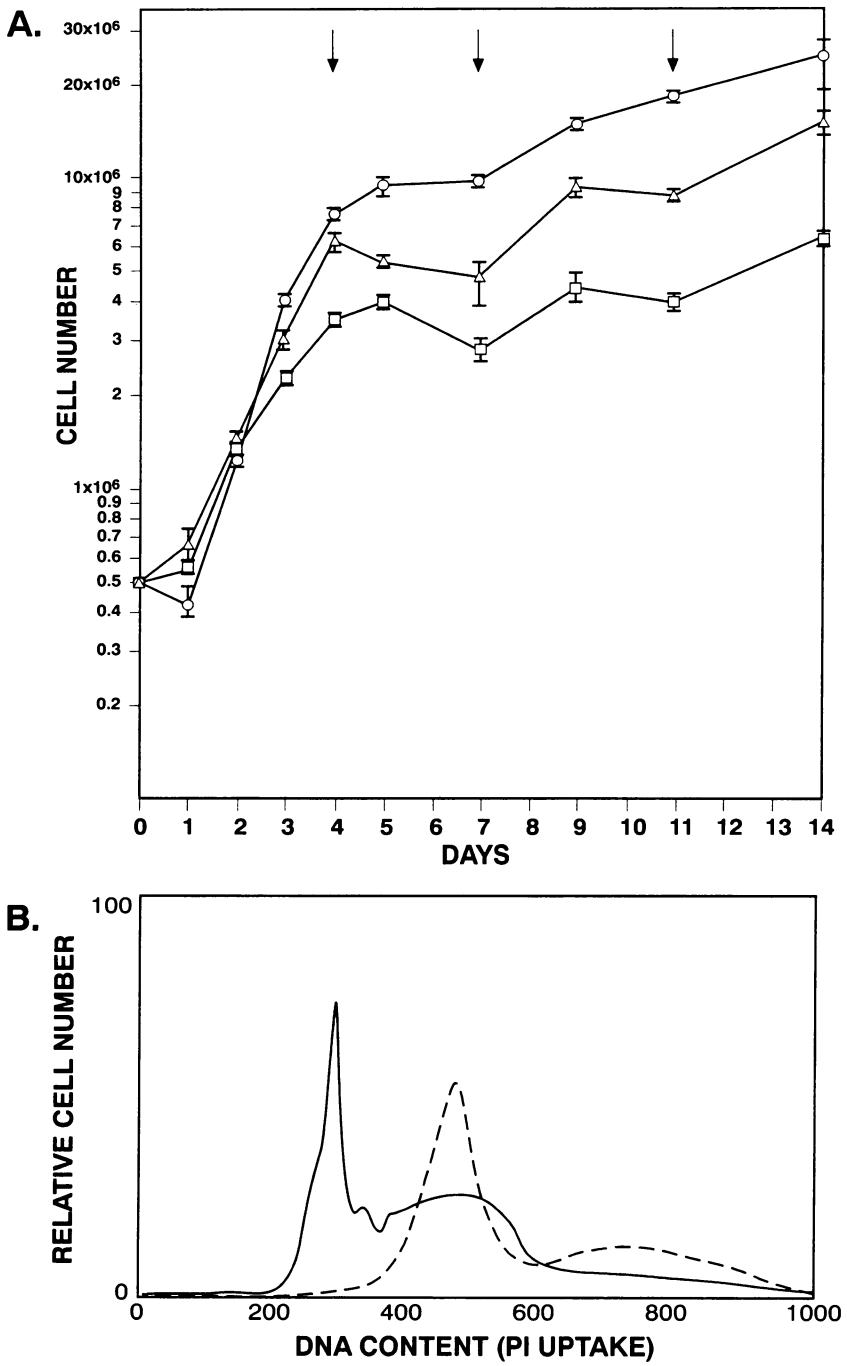

FIG. 4. Overexpression of p55CDC cDNA in the sense or antisense orientation in CHO cells alters growth profiles. (A) CHOd− cells were transfected with (Δ) pMT, (○) pMTp55s, or (□) pMTp55as DNA and amplified as described in Materials and Methods. Cells were plated at a starting density of 0.5×10^6 cells per 60-mm dish and counted on the days shown. Arrows indicate the days when the medium was changed. Each point represents the mean of duplicate counts from parallel cultures, which usually varied by 2 to 14% from the plotted mean. (B) Flow cytometry analysis of fixed and propidium iodide (PI) stained cells carrying pMTp55s (——) and pMTp55as (– – –) was performed as described in Materials and Methods.

all three pools of transfected cells were labeled with $^{32}P_i$ and p55CDC was immunoprecipitated, SDS-PAGE analysis revealed that p55CDC was phosphorylated (Fig. 7B). Thus, p55CDC is a substrate of another endogenous kinase in the CHO cells. In the pMTp55s-carrying cells, no ^{32}P-labeled 31-kDa band was detected (see Fig. 6A and B, lanes 8 and 10), indicating that the 31-kDa putative degradation fragment of p55CDC either is not phosphorylated or is dephosphorylated prior to degradation.

We wished to examine whether p55CDC in different cell lines was associated with other proteins in immune complexes and whether these complexes also had kinase activity. The Rat1 fibroblast and HeLa cell lines were chosen for this analysis. Proliferating Rat1 and HeLa cells were lysed, and

TABLE 1. mRNA copy number[a]

Plasmid	Cell line or clone	mRNA copies/cell			
		48 h		7 days	
		Sense	Antisense	Sense	Antisense
pMT	CHOd−	166 ± 6	—[b]	4 ± 7	—
pMTp55s	CHOd−	734 ± 40	21 ± 7	240 ± 9	16 ± 5
pMTp55as	CHOd−	714 ± 10	205 ± 10	263 ± 10	96 ± 10
pMTp55s	A_2	771 ± 12	2	ND[c]	ND
pMTp55s	B_6	4,136 ± 66	117 ± 4	ND	ND
pMTp55as	B_{12}	706 ± 20	126 ± 3	ND	ND
pMTp55as	G_6	1,176 ± 10	213 ± 14	ND	ND
pMTp55as	H_5	928 ± 17	157 ± 2	ND	ND
pMTp55as	H_{11}	1,149 ± 21	128 ± 7	ND	ND

[a] The number of copies of p55CDC mRNA expressed by cells carrying plasmids in which the p55CDC-encoding cDNA was inserted in either the sense (pMTp55s) or antisense (pMTp55as) orientation was determined. The vector (pMT) alone was used as a control.
[b] —, same as background.
[c] ND, not determined.

immune complexes were precipitated with the affinity-purified p55CDC antibody (Fig. 8A, lanes 2 and 4). SDS-PAGE analysis of the immune complexes did not reveal the 210-kDa band seen in CHO cells but did reveal other discrete bands which appeared to be cell specific. In the Rat1 cells, a protein of 110 kDa was present in the p55CDC immune complexes, while a protein of 100 kDa was seen in p55CDC immune complexes in HeLa cells.

The presence in the p55CDC immune complexes of associated proteins of 110 kDa in Rat1 cells and 100 kDa in HeLa cells suggested that the Rb protein might be associated with p55CDC in these cells. The Rb protein has been found in association with a variety of cell cycle proteins (15, 24, 25), including members of the E2F family of proteins, which have molecular masses of 50 to 57 kDa (29, 37, 50). However, parallel immunoprecipitations of cell lysates with the p55CDC antiserum and two different pRb antibodies demonstrated no comigrating bands (Fig. 8A, lanes 5 and 6). In addition, double immunoprecipitation experiments failed to reveal any association of pRb with p55CDC (data not shown), indicating that neither the 100-kDa nor the 110-kDa protein was the Rb protein.

The p55CDC immune complexes from HeLa cells were examined for kinase activity against a number of different substrates (Fig. 8B). Kinase activity was detected with histone H1, myelin basic protein, and α-casein; maximal activity was detected with myelin basic protein. The level of kinase activity correlated with p55CDC concentration, since increasing the amount of antibody used for the immunoprecipitation resulted in increased phosphorylation of myelin basic protein (Fig. 8C).

To determine whether the p55CDC-associated kinase activity fluctuated during the cell cycle, as has been described for the cyclin-dependent kinases, cells were arrested at various points in the cell cycle and immune complexes precipitated from cell lysates were examined for kinase activity. A cell cycle-related fluctuation in kinase activity was detected with only one of the three substrates examined, α-casein (Fig. 8D and E). Kinase activity against α-casein was present in asynchronous HeLa cells and in cells blocked in G_1 by serum starvation. The level of activity against α-casein dropped approximately fourfold in cells arrested at G_1/S and returned to the higher levels in cells harvested during S phase. This activity remained constant in cells in the G_2 stage of the cell cycle and decreased sixfold in cells at the G_2/M transition.

Kinase activity against histone H1 by p55CDC immune complexes was stable throughout the cell cycle (Fig. 8E). Background kinase activity against H1 in the G_2/M samples (Fig. 8D) was most likely due to residual $p34^{cdc2}$ in these samples. Kinase activity against myelin basic protein was also relatively constant throughout the cell cycle except at the G_2/M transition, when a twofold decrease in activity was observed. The amount of p55CDC present in cells, detected by immunoblotting, did not appear to fluctuate during the cell cycle, in contrast to the fluctuation in kinase activity observed with p55CDC immune complexes (data not shown).

The expression of p55CDC and the associated kinase activity in growing and quiescent populations of cells was compared by exploiting the ability of Rat1 cells to arrest growth under limiting serum conditions. As shown in Fig. 9A, exponentially growing Rat1 cells actively synthesized labeled p55CDC (lanes 3 to 6), while the quiescent population showed minimal production of p55CDC within the 1-h labeling period (lanes 9 to 11). To rule out the possibility that the kinase activity that we observed was precipitated nonspecifically from the cell lysates, increasing amounts of p55CDC antibody were used in the immunoprecipitations. As shown in Fig. 9A, lanes 3 to 6, increasing the amount of p55CDC antibody resulted in the precipitation of increasing levels of p55CDC. This result is consistent with the result shown in Fig. 8C, in which increasing the amount of antibody used for precipitation increased the

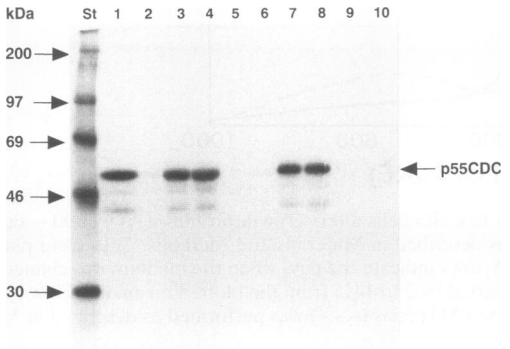

FIG. 5. Immunoprecipitations of in vitro-translated p55CDC. The labeled p55CDC peptide was produced by in vitro translation with [^{3}H]leucine. Aliquots of the in vitro translation reaction mix were immunoprecipitated as described in Materials and Methods. Lanes: 1, total reaction mix; 2, no antibody; 3 and 4, 20 and 40 μg of p55CDC antiserum, respectively; 5 and 6, 40 and 80 μg of preimmune serum, respectively; 7 and 8, 0.7 and 1.4 μg of affinity-purified p55CDC antibodies, respectively; 9 and 10, 35 and 70 μg of p55CDC adsorbed antiserum, respectively; st, size standards (arrows).

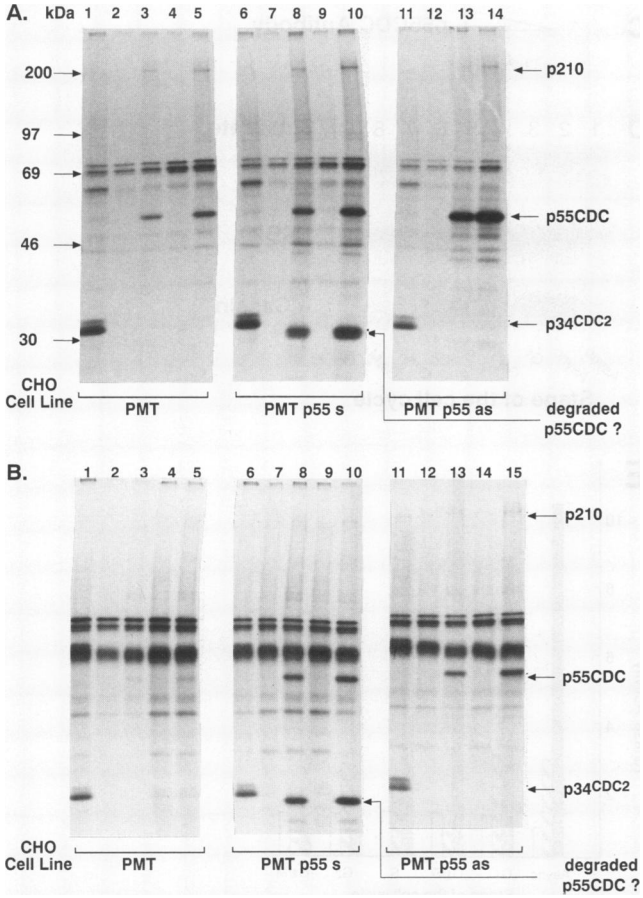

FIG. 6. Immune complexes detected by antibodies against p55CDC. (A) Cell lysates from ^{35}S-labeled cells in log phase (250 μg in lanes 1, 2, 3, 6, 7, 8, 11, 12, and 13; 500 μg in lanes 4, 5, 9, 10, and 14) were immunoprecipitated with various antibodies. Immune complexes obtained with 10 μl of monoclonal antibody against p34^{cdc2} (lanes 1, 6, and 11), p55CDC adsorbed antiserum (8.4 μg per lane; lanes 2, 4, 7, 9, and 12), and affinity-purified p55CDC antiserum (1 μg per lane; lanes 3, 5, 8, 10, 13, and 14) were analyzed on SDS–10% PAGE gels. The dried gel was exposed for autoradiography for 1 day. (B) Cell lysates from ^{35}S-labeled cells in stationary phase (250 μg in lanes 1, 2, 3, 6, 7, 8, 11, 12, and 13; 500 μg in lanes 4, 5, 9, 10, 14, and 15) were immunoprecipitated with various antibodies. Immune complexes obtained with 10 μl of monoclonal antibody against p34^{cdc2} (lanes 1, 6, and 11), p55CDC adsorbed antiserum (8.4 μg per lane; lanes 2, 4, 7, 9, 12, and 14), or affinity-purified p55CDC antibody (1 μg per lane; lanes 3, 5, 8, 10, 13, and 15) were analyzed on SDS–10% PAGE gels. Autoradiography was performed for 1 week.

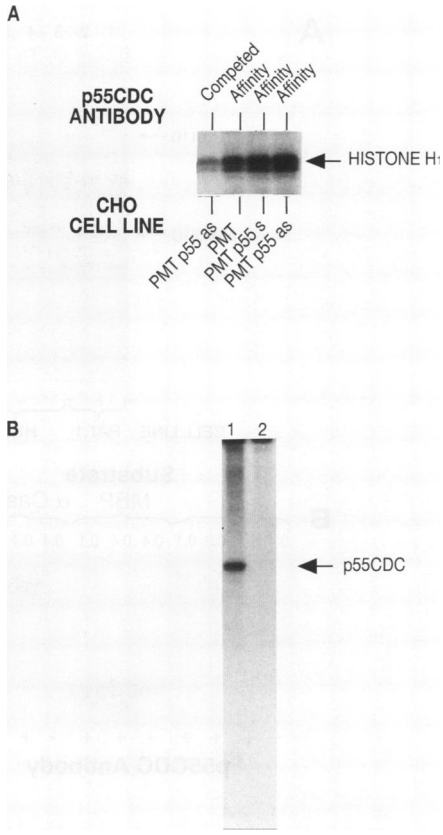

FIG. 7. Histone H1 kinase activity of p55CDC immune complexes and phosphorylation of p55CDC. (A) Lysates of CHO cell lines transfected with vector alone (pMT), vector expressing the sense transcript (pMTp55s), or vector expressing the antisense transcript (pMTp55as) were immunoprecipitated with affinity-purified p55CDC antibody or p55CDC adsorbed antiserum (lane labeled Competed). Immune complexes were assayed for histone H1 kinase activity as described in Materials and Methods. (B) CHO cells were labeled with $^{32}P_i$ as detailed in Materials and Methods. Immune complexes obtained from 900 μg of lysate precipitated with 1 μg of affinity-purified p55CDC antibody (lane 1) or 28 μg of p55CDC adsorbed antiserum (lane 2) were analyzed by SDS-PAGE.

level of p55CDC kinase activity detected. The production of labeled p34^{cdc2} was also substantially reduced in the quiescent population (Fig. 9A, compare lanes 1 and 7), although the total amount of p34^{cdc2} in the two samples was virtually equivalent, as detected on Coomassie blue-stained gels of immunoprecipitated material (data not shown).

We also examined the p55CDC-associated kinase activity in growing and quiescent cell populations and compared it with that observed for p34^{cdc2} immune complexes as a control. A higher level of activity was observed with the p55CDC complexes with α-casein as a substrate, since α-casein is a poor substrate for the p34^{cdc2} kinase (Fig. 9B). Both the p34^{cdc2} kinase and the p55CDC-associated kinase showed a decrease in activity in the quiescent cells. As seen in the HeLa cells, no significant change in the p55CDC-associated kinase activity was observed when myelin basic protein was used as a substrate (data not shown).

DISCUSSION

We have identified a novel mammalian protein which is ubiquitously expressed in proliferating cells. The transcript encoding the p55CDC protein was present in all cell lines we examined, as well as in embryonic tissues and adult hematopoietic tissues. The presence of the transcript in human fetal liver, a hematopoietic tissue, but not in human fetal lung may reflect the relative rates of cell division of these two tissues. The p55CDC transcript was not detected in other adult tissues, including peripheral blood leukocytes, a population composed primarily of terminally differentiated cells which do not undergo further cell division in the circulation. Moreover, human hematopoietic cell lines which were induced to differentiate with chemical agents also demonstrated loss of the p55CDC transcript as cell division ceased.

The expression of p55CDC appears to be essential for cell division, since when we attempted to create stably transfected cells expressing antisense transcripts, only those cells which

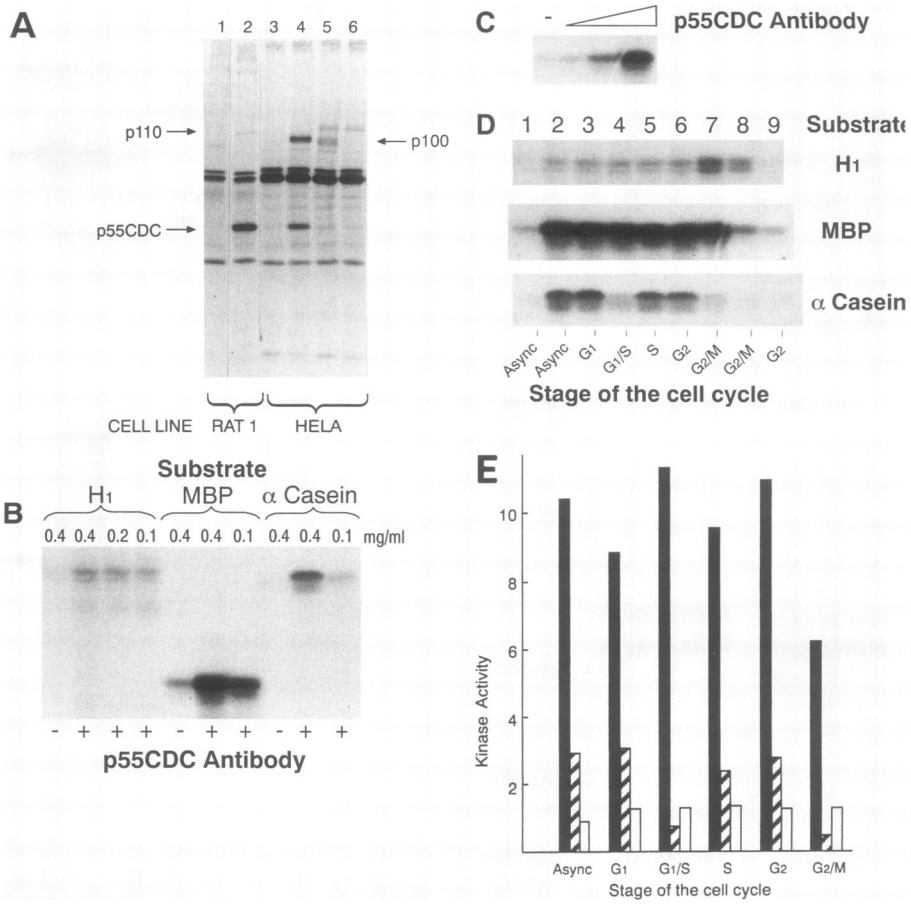

FIG. 8. Immune complexes detected by p55CDC antibodies in Rat1 and HeLa cells and their kinase activity against a variety of substrates at different stages of the cell cycle. (A) ^{35}S-labeled cell lysates (250 μg) from exponentially growing Rat1 and HeLa cells were immunoprecipitated with p55CDC adsorbed antiserum (lanes 1 and 3), affinity-purified p55CDC antibody (lanes 2 and 4), and two different monoclonal antibodies against Rb protein (lanes 5 and 6). (B) Unlabeled lysates (200 μg) from HeLa cells were immunoprecipitated with either control p55CDC adsorbed antiserum (first lane for each substrate) or with affinity-purified p55CDC antibody (remaining lanes). The immunoprecipitates were assayed for kinase activity with histone H1, myelin basic protein (MBP), or α-casein as the substrate; the histone H1 concentrations in these assays were 0.4, 0.2, and 0.1 mg/ml, respectively. MBP and α-casein concentrations decreased from 0.4 to 0.1 mg/ml. (C) Unlabeled lysates (200 μg) prepared from HeLa cells were immunoprecipitated with increasing amounts of affinity-purified p55CDC antibody (0.07, 0.28, and 1.12 μg). The negative control was done with 4.2 μg of the p55CDC adsorbed antiserum (lane —). Kinase assays were performed as described in Materials and Methods with 0.4 mg of MBP per ml as the substrate. (D) Unlabeled lysates (200 μg) were prepared from HeLa cells at various stages of the cell cycle as described in Materials and Methods and immunoprecipitated with either 8.4 μg of p55CDC adsorbed antiserum (lanes 1, 8, and 9) or 1.0 μg of affinity-purified p55CDC antibody (lanes 2 to 7). Kinase assays were performed with 0.4 mg of H1, MBP, or α-casein per ml as exogenous substrates. Async, asynchronous. (E) The radioactivity in the excised bands from the dried gel shown in panel D was counted. The control values (panel D, lanes 1, 8, and 9) were subtracted from the experimental values (panel D, lanes 2 to 7), and the results were graphed. Solid bars, MBP; hatched bars, α-casein; open bars, histone H1. Kinase activity values are 10^3 cpm.

were overproducing sense transcripts, apparently to compensate for the loss of p55CDC mRNA, survived. It is clear that those cells which made p55CDC mRNA had a selective advantage during the multiple rounds of drug selection and amplification, since we did not identify any pMTp55as-carrying clones which did not make an excess of sense orientation transcripts.

The homology of p55CDC with the *S. cerevisiae* Cdc20 and Cdc4 proteins raises the intriguing possibility that p55CDC may have a role in the centrosome cycle. The centrosome cycle (38) remains enigmatic, since few of the molecules that drive and regulate this cycle have been identified. The primary role of the centrosome/spindle pole body is to function as the microtubule-organizing center in eukaryotic cells. Centrosome division precedes nuclear division, since the duplicated centrosome is essential for establishing the bipolar array of microtubules that constitute the mitotic spindle (47). The Cdc20 protein has been implicated in microtubule function at many stages of the *S. cerevisiae* life cycle, including mitosis (34, 56, 66). The Cdc4 protein plays a vital role in the centrosome/spindle pole body cycle, since mutations in the *CDC4* gene result in arrest of cell division and aberrant formation of the mitotic apparatus due to duplicated but unseparated spindle pole bodies (6). The Cdc4 protein has been localized to the entire nucleus (77) and specifically to the nucleoskeleton (7). Preliminary immunohistochemical studies with affinity-purified p55CDC antibody in interphase HeLa and CHO cells have demonstrated staining of the cytoskeleton and the nucleus, with concentrated staining visible at the nuclear envelope (75). This pattern of staining may be related to that seen for the Cdc4 protein, since the mitotic spindle of yeast cells is formed within an intact nuclear envelope, whereas in mammalian cells the mitotic apparatus is assembled after breakdown of the nuclear envelope in prophase.

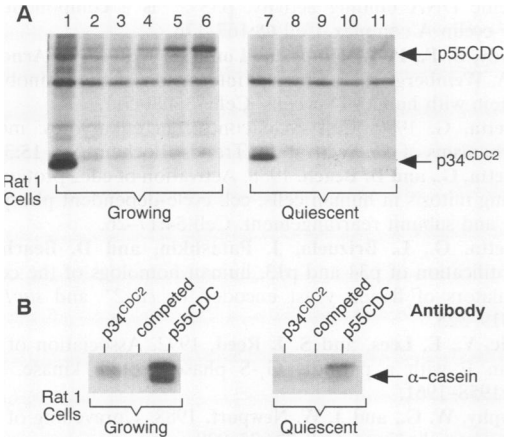

FIG. 9. Cycling cells actively translate p55CDC and show higher levels of associated α-casein kinase activity than quiescent cells. (A) Growing and quiescent Rat1 cells were labeled for 1 h with ^{35}S-Translabel as described in Materials and Methods. Lysates (100 μg) were immunoprecipitated with various antibodies. Immune complexes obtained with 10 μl of monoclonal antibody against p34^{cdc2} (lanes 1 and 7), p55CDC adsorbed antiserum (8.4 μg per lane; lanes 2 and 8), and affinity-purified p55CDC antibody at 0.035 μg (lane 3), 0.14 μg (lanes 4 and 9), 0.56 μg (lanes 5 and 10), or 1.12 μg (lanes 6 and 11) per lane were analyzed by SDS-PAGE. (B) Lysates (100 μg) were prepared from growing and quiescent Rat1 cells as described in Materials and Methods. Immune complexes were obtained with 10 μl of monoclonal antibody against p34^{cdc2}, 8.4 μg of p55CDC adsorbed antiserum (lanes labeled competed), and 1.12 μg of affinity-purified p55CDC antibody. Kinase assays were performed as described in Materials and Methods with 0.4 mg of α-casein per ml as the substrate.

The degree of homology between human p55CDC and *S. cerevisiae* Cdc20 (45% identity over 236 amino acid residues with only four gaps) appears to be highly significant. Although the Cdc20 protein has not been demonstrated to associate with a protein kinase, *CDC20* overexpression can suppress the *cdc15* mutation (1), and the *CDC15* gene product has been identified as a protein kinase (65). This implies some interaction between the Cdc20 protein and a protein kinase. It will be of considerable interest to determine whether p55CDC can substitute for Cdc20 protein function in yeast cells. Recently, a clone isolated from a *Xenopus* oocyte cDNA library by virtue of its ability to suppress the temperature-sensitive defect of the *S. cerevisiae cdc15* mutation was shown to encode a protein of 518 amino acids that has seven Gβ repeats in its carboxy-terminal half (70). This protein, called βTrCP (β-transducin repeat-containing protein), was not a functional homolog of Cdc20, though overexpression of the genes for both of these proteins can suppress the *cdc15* mutation (1, 70). Both βTrCP and p55CDC have seven Gβ repeats and show 24% identity over this region.

The homology of p55CDC with the Cdc20 and Cdc4 proteins is found in the Gβ subunit repeats (12, 27, 74). In the Cdc4 protein, the repeats depicted in Fig. 3 extend from amino acids 226 to 598. In addition, the first 95 amino acids of the Cdc4 protein have been shown not to be required for activity (79), implying that the region containing the Gβ subunit repeats is essential for the function of the Cdc4 protein. While other proteins also demonstrated homology with p55CDC in this region, the homology with the Cdc20 protein was the highest and the homology with the Cdc4 protein was the most extensive, encompassing all seven repeats of this domain in p55CDC. This motif has been associated with the formation of stable protein-protein complexes, in G proteins as well as in other proteins identified in the homology search. Some of these proteins interact with proteins containing the tetratricopeptide repeat (for a review, see reference 30). Thus, while identification of the Gβ repeat motif in p55CDC does not indicate a precise function for this novel protein, it is consistent with our finding that immunoprecipitation of p55CDC from various cell lines resulted in the immunoprecipitation of associated proteins.

The immune complexes precipitated by the p55CDC antiserum from three different cell lines displayed kinase activity against a variety of substrates. The p55CDC protein does not appear to be a kinase, since the sequence of p55CDC contains no regions of significant homology with any known kinases (31). Thus, one or more of the associated proteins in the p55CDC immune complexes is a kinase, the activity of which may be regulated by association with p55CDC. In dividing cells, p55CDC is phosphorylated (Fig. 7B), although it is not a substrate for the kinase in the immune complex. While the amount of p55CDC protein in the cell does not appear to fluctuate during the cell cycle, the phosphorylation state of p55CDC may either control the subcellular localization of the protein or affect protein-protein interactions. In p55CDC, there are four potential p34^{cdc2} phosphorylation sites (16) between amino acids 41 and 71; the amino acid sequence of the Cdc4 protein also reveals four potential p34^{cdc2} phosphorylation sites in the region upstream of the Gβ subunit repeats, between amino acids 185 and 223. The presence of a putative 31-kDa breakdown product of p55CDC in the pMTp55s-carrying cells (Fig. 6A and B) may reflect degradation of p55CDC produced constitutively from the metallothionein promoter in the plasmid. The presence of this peptide may indicate a requirement by the cells to control the level of newly synthesized p55CDC protein at a specific stage in the cell cycle.

The p55CDC-associated kinase activity is novel in two respects. First, in the synchronized HeLa cells, a decrease in kinase activity towards α-casein was seen during thymidine-aphidicolin-induced arrest at the beginning of S phase (G_1/S) and also at the pseudo-metaphase arrest caused by nocodazole treatment (Fig. 8D and E). This decrease during M phase does not appear to be related simply to the potential toxicity of nocodazole, since the G_2 cells, which had relatively high levels of activity, underwent the same treatment. This profile of kinase activity has not been described for any other cell cycle-associated kinase (14, 17, 19, 26, 39, 46, 60). Second, the p55CDC immune complexes had kinase activity against a number of different substrates, including histone H1, myelin basic protein, and α-casein (Fig. 8B). This is in contrast to the specificities of other known cell cycle-associated kinases (4, 10, 23, 43, 49, 53, 54). Moreover, no other kinases have been found to associate with a 55-kDa protein resembling p55CDC.

Figure 9A shows that translation of new p55CDC molecules can be observed only in a dividing population of cells. This result is similar to that shown in Fig. 6, in which an exponentially growing population of CHOd− cells (Fig. 6A, lanes 3 and 5) produced significantly more p55CDC than a confluent culture (Fig. 6B, lanes 3 and 5). The kinase activity results (Fig. 8 and 9) are more complex, since a fluctuation in activity was observed when α-casein was used as a substrate but no fluctuation was seen when myelin basic protein was used. This result may reflect the possibility that more than one kinase activity is associated with p55CDC, since p55CDC may exist in different phosphorylation states and at several subcellular locations. However, under the harsh cell lysis conditions used in these experiments, a pool of free p55CDC as well as p55CDC associated with a variety of proteins would be present

in immunoprecipitates, so that subtle distinctions in substrate specificity are difficult to analyze in these samples. Moreover, it is probable that the affinity-purified antibody has a preference for free p55CDC over p55CDC associated with other proteins. This may explain the lack of a linear correlation between different levels of p55CDC observed in the cell lines (Fig. 6A) and the level of kinase activity observed in them (Fig. 7A). Further analysis with subcellular fractions obtained at defined stages of the cell cycle may discriminate among distinct kinase activities associated with p55CDC in dividing cells.

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