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Genetics

Increased expression of a 58-kDa protein kinase leads to changes in the CHO cell cycle

(checkpoints/cell division control/tubulin midbody/late telophase/Chinese hamster ovary cells)

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ABSTRACT **We have isolated and characterized cDNA encoding a human 58-kDa protein kinase that is homologous to the cell division control (CDC) protein kinases. This protein kinase also contains a unique N-terminal domain that may potentially regulate its function. Due to its relatedness to $p34^{CDC2}$, the human p58 cDNA was overexpressed in CHO cells to determine the effect on the cell cycle. Elevated expression of p58 in these cells resulted in prolonged late telophase and early G_1 phase of the cell cycle. These p58 overexpressors showed a significantly increased frequency of tubulin midbodies as well as significant increases in mitotic abnormalities. Thus, proper regulation of p58 protein kinase is essential for normal cell cycle progression in these cells.**

Protein kinases have been implicated in a number of biological responses, including the ability of eukaryotic cells to respond to external stimuli (for a review, see ref. 1). In excess of 100 distinct protein kinases have been identified, all with presumed specific cellular functions (2). A few of these enzymes have been purified sufficiently to determine amino acid sequences and biochemical properties (3–5), but most remain intractable to these techniques due to their low abundance. Many of these protein kinases have been isolated by molecular cloning utilizing the extremely conserved sequences of the catalytic domains (6). Conversely, some of these enzymes were discovered by association with proteins that may be involved with their function. In just such a manner we have identified a protein kinase that copurifies with a mammalian glycosyltransferase. The extensive sequence homology between this 58-kDa galactosyltransferase (GalTase)-associated (GTA) 58-kDa (p58) protein kinase and the CDC2/CDC28 cell-division-control protein kinases of yeast and humans suggests that it is ancestrally related to the CDC protein kinases. We previously showed that diminished levels of p58 mRNA and protein in CHO cells led to subtle changes in DNA replication (7). These observations, as well as conservation of a major portion of the p58 protein sequence with $p34^{CDC2}$, suggested that the GTA protein kinase might function in some manner during the cell cycle.

To test this possibility we overexpressed the p58 protein kinase in eukaryotic cells. Other groups have shown that overexpression of proteins necessary to execute basic cellular functions, such as β-tubulin and histones, can lead to lethal dominant effects (8, 9). If proper regulation of p58 is essential to normal cellular function, this type of analysis could provide clues to this function. Here we show that overexpression of the human p58 protein kinase in CHO cells affected cell cycle progression.¶

MATERIALS AND METHODS

Isolation and DNA Sequence Analysis of cDNA Clones. Human adult and fetal liver (Clontech) λgt11 cDNA libraries were screened using the λhp58-1 cDNA isolate and sequenced as described (10). A site-directed mutant cDNA encoding [Gly^{84}]p58 was made using an oligodeoxynucleotide (5′-AACAGGATCGGGGAGGGCACC-3′) and then sequenced to confirm mutagenesis by standard procedures (11).

***In Vitro* and *in Vivo* p58 Protein Analysis.** A 1.5-kilobase (kb) *Eco*RI restriction fragment from λhp58-5 containing the entire open reading frame of the cDNA (Fig. 1) was inserted in the vector pGEM-1 (Promega). A 1.7-kb human α_1-antitrypsin cDNA (12) was also inserted into pGEM-1. Transcription reactions using pGEM constructs were performed as described (13). *In vitro* translation used 1 μg of synthetic RNA in 25 μl of rabbit reticulocyte lysate (Stratagene) containing [^{35}S]methionine (Amersham) and was analyzed by SDS/10% PAGE. A synthetic 20-mer peptide (amino acids 22–41, Fig. 1; prepared by Multiple Peptide Systems, San Diego, CA) was coupled to keyhole limpet hemocyanin with glutaraldehyde and used to raise antiserum (14). *In vitro* translated p58 protein was immunoprecipitated using affinity-purified (15) p58-peptide antibodies [anti-p58-(22–41)]. Solid-phase immunoprecipitation (16) was performed in either the presence or the absence of 100 nM nonradioactive competing synthetic peptide. The immunoprecipitated [^{35}S]methionine-labeled protein was analyzed by SDS/10% PAGE. Western blot analysis used ^{125}I-labeled donkey anti-rabbit IgG (Amersham) as second antibody. Protein kinase activity was assessed by solid-phase immunoprecipitation (15), using purified p58 or preimmune antibodies, in the absence or presence of excess nonradioactive p58-(22–41) peptide. Bovine p58 was purified from bovine lactose synthetase/GalTase by Sephacryl S-200 (Pharmacia) chromatography followed by calmodulin-Sepharose chromatography.

Recombinant DNA Constructs and Eukaryotic Cell Analysis. Two 1.5-kb *Eco*RI restriction fragments containing the coding region of the [Glu^{84}]p58 (wild-type) or [Gly^{84}]p58 cDNA were ligated into the *Sma* I site of the eukaryotic expression vector pMSG (17). Properly oriented pMSG-p58 constructs were linearized with *Pvu* I and introduced into *DHFR*$^-$ CHO cells (18) by electroporation as described below. The *DHFR*$^-$ CHO cells were cultured at 37°C in Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS). Prior to electroporation, the cells were grown to 40–50% confluency, pelleted, resuspended in 800 μl of sterile phosphate-buffered saline, and incubated on ice for 5 min. *Pvu* I-linearized pMSG-p58 DNA construct, or pMSG vector only, was then added to the cells for electroporation at 0.21 kV and 500 μF. The cells were allowed to grow in Ham's F-12/10% FBS for 24 hr and then changed to Dulbecco's modified Eagle's medium (DMEM) containing 5% FBS and selection drugs (17). After 21–26 days, colonies

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Abbreviations: GalTase, galactosyltransferase; GTA, GalTase-associated.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M37712).

were cloned in a 24-well plate with DMEM/5% FBS. Cell cycle analysis was performed using a Becton Dickinson FACStar. Doubling time was determined by determining the number of cells present at several times after initial plating. Indirect immunofluorescence analysis was essentially as described (19). The affinity-purified anti-p58 was used at a 1:50 dilution. Peptide competition was performed using 100 nM nonradioactive p58 peptide. Fluorescein-conjugated goat anti-rabbit IgG (Boehringer Mannheim) was used as second antibody. Anti-tubulin staining was carried out using Tu27B α-tubulin monoclonal antibody (19) at a 1:40 dilution and a biotin-conjugated anti-mouse second antibody (Boehringer Mannheim). Microscopy was performed on a Zeiss Axiophot microscope and all exposure times were identical.

RESULTS

Isolation and Sequence Analysis of p58 cDNA Clones. The original p58 (GTA) cDNA clone was isolated using a GalTase polyclonal antiserum that recognized p58 as well as a 48-kDa GalTase (10). Isolation of this clone may have functional implications for GalTase, as GalTase enzyme activity is influenced by its phosphorylation state (33) and others have reported that it is serine-phosphorylated (20). Detailed restriction maps were obtained for four clones, λhp58-2 to -5, obtained from a human fetal liver library. Complete analysis of the sequences of these cDNAs confirmed an open reading frame of 1302 base pairs (bp) that encodes a protein of 434 amino acids (Fig. 1). The λhp58-5 cDNA also contains 66 bp of 5′ untranslated sequence and 2136 bp of 3′ untranslated sequence. The 3′ untranslated sequence contains long runs of A and T nucleotides similar to those found in the 3′ untranslated regions of numerous protooncogenes, lymphokines, and growth factors (21). A consensus polyadenylylation signal (AATAAA) begins 18 bp from the start of the poly-(A)tail. The 5′ region contains two in-frame ATG codons, the first of which is designated position 1, as well as three in-frame termination codons located upstream of this ATG. The predicted p58 protein is ≈49.7 kDa, but *in vitro* translation and *in vivo* expression data presented here suggest that the protein migrates anomalously in SDS/PAGE.

The predicted protein sequence of p58 has a 299-amino acid region with 46% and 49% direct homology to the human CDC2Hs and yeast CDC28 protein kinases, respectively (data not shown) (22, 23). When conservative amino acid differences are considered in the comparisons, the level of homology rises to 68% between the p58 and p34^{CDC2} protein kinases. Comparison of the p58 sequence with all other protein kinases in the National Biomedical Research Foundation and GenBank data bases revealed that it is substantially less homologous, <30% identity, to all other known protein kinases (2). The blocks of homology within this region include all 11 of the predicted protein kinase subdomains, as well as the absolutely conserved amino acids within these regions (2).

In addition to the conserved CDC-related domain, p58 has a unique 74-amino acid N-terminal region. The possible function of this region is not known, but a portion of the N-terminal region contains the necessary amino acids for a calmodulin binding site (Fig. 1; ref. 24). In addition, three tandem putative PEST sequences (25) span amino acids 45–67. PEST sequences have been shown to be involved in the rapid degradation of proteins that contain them, including the eukaryotic cyclins (26). Finally, a potential nuclear localization sequence related to several similar viral and cellular oncogene sequences can be found between amino acids 20 and 30 of p58 (27). Interestingly, all three of these putative N-terminal regulatory domains overlap and are not found in any of the other CDC-related protein kinases that have been isolated and characterized.

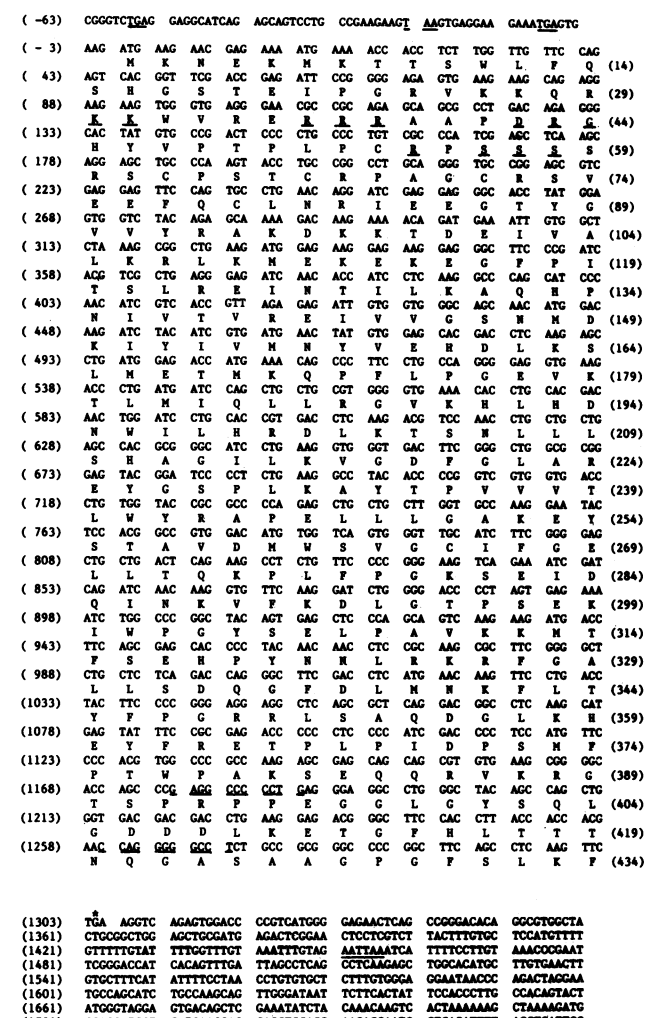

FIG. 1. Complete nucleotide sequence and predicted protein sequence for human p58. Nucleotide positions are shown in parentheses at left. In-frame termination codons in the 5′ untranslated region are underlined. The predicted amino acid sequence is shown below the nucleotide sequence and is numbered at right. The putative calmodulin-binding region is underlined. A perfect DNA stem–loop sequence is underlined in the C-terminal coding region. Potential polyadenylylation signals (AATAAA, AATTAA) are also underlined.

***In Vitro* Translation of the Human p58 cDNA, Identification of an Identical Protein in Bovine Lactose Synthetase, and Demonstration of Kinase Activity.** The capacity of this cDNA to make a functional protein product was examined *in vitro* using the 5′ 1.5-kb *Eco*RI fragment of λhp58-5 (Fig. 1). Similar experiments performed with the 3′, 2.0-kb *Eco*RI fragment resulted in no protein products (data not shown).

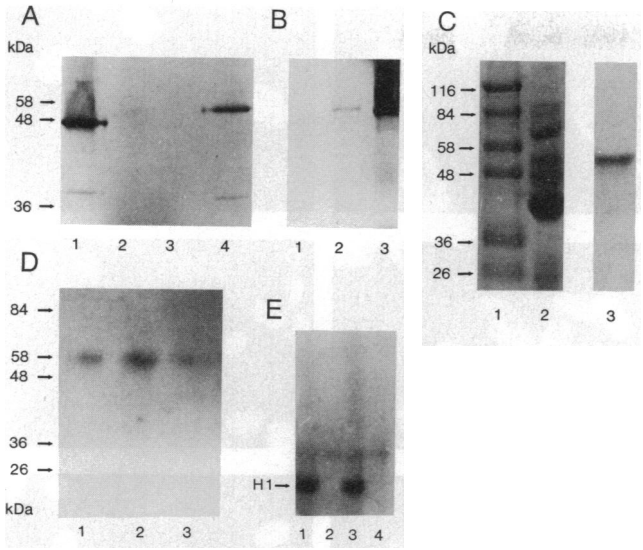

FIG. 2. *In vitro* and *in vivo* p58 protein identification and kinase activity. (*A*) *In vitro* translation products of synthetic RNA. Lanes: 1, human α_1-antitrypsin RNA; 2, no RNA; 3, antisense p58 RNA; 4, sense p58 RNA. (*B*) Lanes: 1, immunoprecipitation of the labeled p58 protein with anti-p58-(22–41) in the presence of competing peptide; 2, immunoprecipitation of the same protein in the absence of synthetic peptide; 3, the labeled p58 used for lanes 1 and 2. (*C*) Lanes: 1, molecular mass markers; 2, Coomassie blue-stained semipurified bovine lactose synthetase proteins; 3, Western blot of the proteins shown in lane 2 with anti-p58-(22–41). (*D*) Western blot analysis of yeast, human, and bovine proteins with anti-p58-(22–41). Lanes: 1, 100 μg of an S-100 fraction from *Saccharomyces cerevisiae*; 2, 100 μg of total HeLa cell protein; 3, 10 μg of the bovine lactose synthetase proteins. (*E*) *In vitro* histone H1 kinase activity. Lanes: 1, kinase activity in the pellet immunoprecipitated from p58 protein; 2, kinase activity in the supernatant from the immunoprecipitation; 3, kinase activity in the supernatant fraction from the immunoprecipitation of p58 with a preimmune antibody; 4, kinase activity in the pellet from immunoprecipitation of p58 with the preimmune antibody.

When coding-strand RNA transcribed *in vitro* was added to a rabbit reticulocyte lysate system, a major protein band at ≈55 kDa was observed (Fig. 2*A*, lane 4). In contrast, synthetic noncoding RNA yielded no protein (lane 3). A positive control, human α_1-antitrypsin cDNA, produced the expected 46-kDa protein (lane 1). A negative control (no RNA) produced no protein (lane 2).

Next, we generated an antibody to a portion of p58, amino acids 22–41, that is not found in the $p34^{CDC2}$ protein kinases or other proteins. A high-titer polyclonal IgG antiserum was obtained and characterized by its ability to recognize the uncoupled synthetic peptide. Affinity-purified anti-p58-(22–41) was shown to be specific for this sequence in the native protein by a number of criteria. Sense-strand p58 mRNA was translated *in vitro* (Fig. 2*B*, lane 3) and the product was used for immunoprecipitation. The affinity-purified antibody immunoprecipitated the *in vitro* translated human p58 protein in the absence of competing nonradioactive p58 peptide (Fig. 2*B*, lane 2) but not in the presence of an excess of the competitor (lane 1). Bovine lactose synthetase was then resolved by SDS/PAGE and the proteins were either stained by Coomassie blue (Fig. 2*C*, lane 2) or probed with anti-p58-(22–41), which detected a single 55- to 58-kDa protein (lane 3). Western blots of cell lysates from *S. cerevisiae* (Fig. 2*D*, lane 1) and from HeLa cells (lane 2) showed a single 55- to 58-kDa protein that reacted with the affinity-purified antibody. This signal was effectively competed by excess nonradioactive p58 peptide (data not shown).

To determine whether the p58 protein is a functional kinase, p58 was isolated from the bovine lactose synthetase/GalTase proteins. This affinity-purified preparation was then assayed for *in vitro* protein kinase activity by solid-phase immunoprecipitation. When p58 was immunoprecipitated with anti-p58-(22–41) and assayed using histone H1 as a substrate, the protein kinase activity was confined to the immunoprecipitated protein pellet (Fig. 2*E*, compare lanes 1 and 2). Conversely, when control preimmune antiserum was used to immunoprecipitate the purified bovine p58, only the supernatant fraction was capable of phosphorylating histone H1 (Fig. 2*E*, compare lanes 3 and 4). Additional *in vitro* substrates for this kinase include casein and bovine GalTase, and all three substrates are phosphorylated equally well *in vitro* (D.E.A., unpublished data).

Cellular Phenotype Associated with Overexpression of p58. To examine the possible cellular function(s) of the p58 kinase, both the wild-type ([Glu^{84}]p58) and a site-directed mutant ([Gly^{84}]p58) cDNA were used since it was not clear initially whether Glu^{84} was normally encoded in the chromosomal gene. It has since been shown that [Glu^{84}]p58 cDNA is equivalent to the normal human chromosomal gene sequence. Proper integration and similar copy number of the linearized pMSG-p58 cDNA, as well as the pMSG vector only, were verified by analysis of genomic DNA (data not shown). A hybrid 3.0-kb p58 transcript was readily detected with the human cDNA probe in both uninduced and induced CHO cells (Fig. 3*A*). We, as well as others (28), have found that the mouse mammary tumor virus long terminal repeat (the promoter used in the pMSG vector) is active even in the absence of added glucocorticoid. Increased p58 protein expression was confirmed by Western blot analysis (Fig. 3*B*). The CHO cells containing the pMSG-p58 construct expressed ≈5-fold more p58 protein than wild-type CHO cells. Longer exposure of this Western blot revealed a much less abundant, slightly larger (66-kDa) protein that partitioned with nuclear proteins (B.A.B., unpublished data).

Overexpression of p58 in CHO cells resulted in cells that were rounded-up, small, and possibly paired (Fig. 4). Initially, we thought that cell surface changes in these cells might be mediating this physical change. Cells containing the constructs were plated onto various extracellular matrices, and no changes in the appearance of the cells were observed (data not shown). [CHO expression of kinase-defective p58 cDNAs constructed by site-directed mutagenesis (Lys^{106} to Asn or Asp^{219} to Asn) did not result in the phenotype seen in the p58 overexpressors described here (L.S.H., unpublished work).] The doubling time for the control cells (CHO and CHO/pMSG) was 14–16 hr, whereas that for the p58 over-

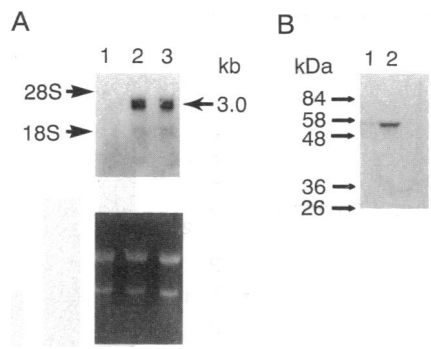

FIG. 3. (*A*) Northern blot analysis of 40 μg of total cytoplasmic RNA with the human p58 cDNA probe (*Upper*) and the same RNA samples visualized with ethidium bromide (*Lower*). Lanes: 1, nontransfected CHO cells; 2, uninduced CHO/pMSG-p58 cells; 3, CHO/pMSG-p58 cells induced with dexamethasone. (*B*) Western blot analysis of 50 μg of total protein from CHO cells (lane 1) and CHO/pMSG-p58 cells (lane 2) with anti-p58-(22–41).

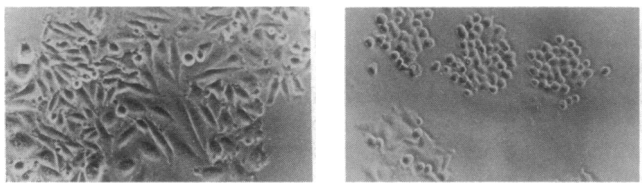

FIG. 4. Phase-contrast microscopy of CHO/pMSG (*Left*) and CHO/pMSG-p58 (*Right*) cells. (×20.)

expressors was 20–22 hr. The incorporation of [^{3}H]thymidine into the p58 overexpressors was 50–70% less than for either control cell group (Fig. 5*B*). Flow cytometric analysis of identically cultured subconfluent control (CHO) and p58 overexpressor (CHO/pMSG-p58) cells showed that 54% of the p58 overexpressors were in the G_1 phase of the cell cycle, whereas 56% of the control cells were in S phase and only 26% were in G_1. This analysis classified all paired late-telophase cells as G_1-phase cells. These results confirmed the previously observed differences in [^{3}H]thymidine incorporation and suggested that entry into S phase was inhibited in the p58 overexpressors.

Cytoplasmic Microtubule Organization of the p58 Overexpressors. The previous experiments indicated that overexpression of the p58 protein kinase in CHO cells limited the ability of these cells to enter S phase normally. Approximately 68 cells from a field of 300 p58 overexpressors had cytoplasmic bridges, indicative of paired daughter cells. This result suggested that the cells were not exiting, or were persisting in, a period of the cell cycle between late mitosis and G_1. We examined this possibility further by staining asynchronously growing populations of control cells and p58 overexpressors with anti-tubulin antibodies. Both wild-type and vector-transfected cells appeared to be normal in cytoskeletal organization (Fig. 6 *A* and *C*). However, anti-tubulin staining of [Glu^{84}]p58, as well as [Gly^{84}]p58, clones revealed that many of these cells contained re-forming cytoplasmic microtubule complexes as well as numerous tubulin midbodies (Fig. 6*E*). Examination of multiple cell fields containing 400–600 cells revealed that the p58 overexpressors contained a statistically significant increase in the number of midbodies (20.4%) versus either control cell population (2.5%). Tubulin midbodies are characteristic of paired daughter cells in late telophase during animal cell division (29). These results confirmed the results of the previous analyses.

Increased Aneuploidy in p58 Overexpressors. We examined CHO control cells and p58 overexpressors by indirect immunofluorescence using the affinity-purified anti-p58-(22–41) and the DNA-staining dye Hoechst 33258. Control CHO cells showed both diffuse cytoplasmic and punctate nuclear staining (Fig. 7*A*), which was prevented effectively with excess p58 peptide competitor (Fig. 7*C*). Affinity-purified preimmune antibody was used as a control (Fig. 7*E*). p58 overexpressor cells with significant nuclear p58 staining (Fig. 7*G*), but little cytoplasmic staining, contained numerous micronuclei (Fig. 7*H*). Conversely, the smaller, rounded-up p58 overexpressors demonstrated significant cytoplasmic staining with the p58-peptide antiserum (Fig. 7*G*). When numerous large cell fields (400–600 cells) were analyzed from both of the control cell populations and the p58 overexpressors, we found a statistically significant increase in both micronucleated and giant cells in the p58 overexpressors (40.5%) versus the controls (4.7%).

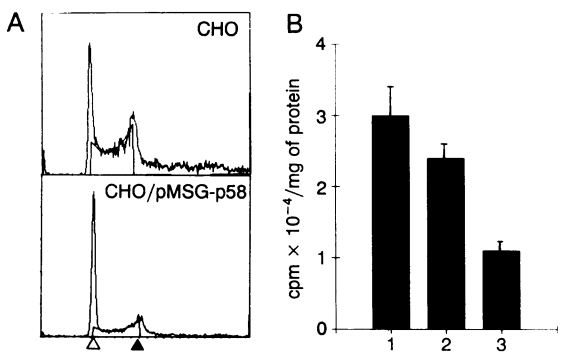

FIG. 5. (*A*) Cell cycle analysis of identically grown subconfluent asynchronous cells. Open arrowhead, 2*N* DNA content; filled arrowhead, 4*N* DNA content. (*B*) [^{3}H]Thymidine incorporation in CHO cells (bar 1), CHO/pMSG cells (bar 2), and CHO/pMSG-p58 cells (bar 3).

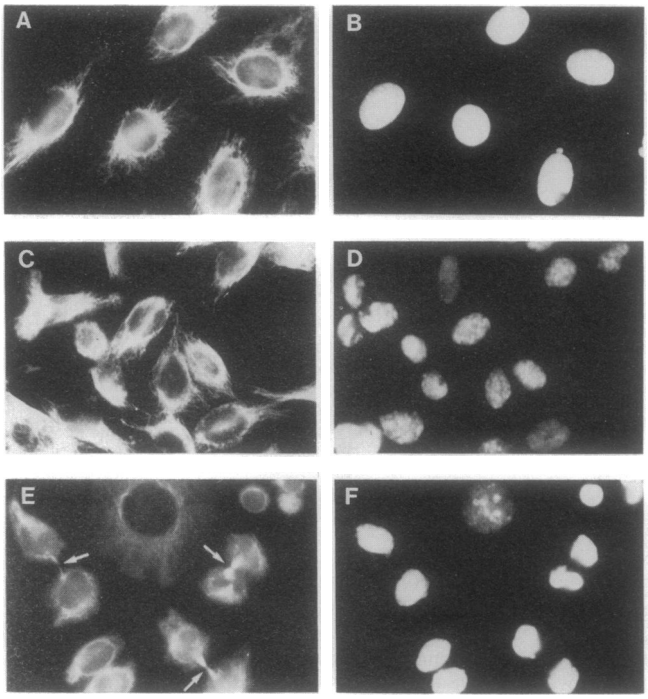

FIG. 6. Cytoskeletal changes in p58 overexpressors. Anti-tubulin antibody staining is shown in *A*, *C*, and *E*. Corresponding Hoechst 33258 staining of nuclear DNA is shown in *B*, *D*, and *F*. The cells were CHO (*A* and *B*), CHO/pMSG (*C* and *D*), and CHO/pMSG-[Glu^{84}]p58 (*E* and *F*). Arrows in *E* indicate tubulin midbodies. (×50.)

DISCUSSION

We have isolated and characterized cDNA encoding human p58 GTA protein kinase. The protein consists of three domains: a unique 74-amino acid N-terminal domain followed by a 299-amino acid CDC-related domain and a 61-amino acid C-terminal domain. Characterization of the purified bovine p58 protein has shown that it is, indeed, a protein kinase. While others have recently identified additional p34^{CDC2}-related cDNAs (30, 31), demonstration of protein kinase activity or effect of expression on the cell cycle has not been shown.

Previously, we found that expression of the p58 gene is regulated via sequences within its 3′ untranslated region and that diminished p58 mRNA and protein results in apparent changes in DNA replication (7). Here we have shown that a severalfold increase in the expression of the p58 kinase alters normal progression of these cells through the cell cycle and dramatically increases the frequency of mitotic abnormalities. This altered phenotype is not the result of a dramatic change in total cellular p58 kinase levels or subcellular localization. Thus, we suggest that alterations in the level of endogenous p58 can affect the cell cycle: too little p58 allows

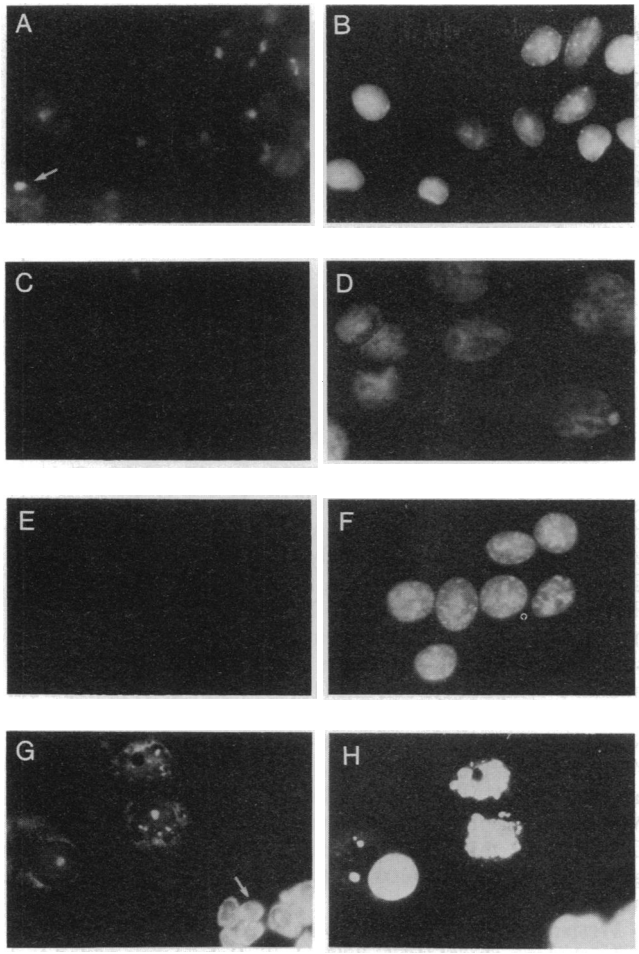

FIG. 7. CHO and CHO/pMSG-[Glu^{84}]p58 cells were analyzed by indirect immunofluorescence with anti-p58-(22–41) and Hoechst 33258. *A*, *C*, and *G* are fluorescence micrographs of cells stained with the p58-peptide antibody. *E* shows staining with preimmune antibody. *B*, *D*, *H*, and *F* are the corresponding cells stained with Hoechst 33258. (*A*) Wild-type CHO cells. (*C*) Wild-type CHO cells stained in the presence of competing peptide. (*E*) Wild-type CHO cells stained with preimmune antibody. (*G*) p58 overexpressors stained with the p58 antibody. Arrow in *A* shows the punctate nuclear and diffuse cytoplasmic p58 staining in wild-type cells; arrow in *G* shows bright cytoplasmic staining for p58 in the rounded-up cells. (×75.)

cells to proceed through the cell cycle more rapidly, whereas too much p58 inhibits cell cycle progression. These results are consistent with the possibility that the p58 kinase acts as a negative regulator of some component of the cell cycle pathway. The altered cell cycle phenotype and mitotic abnormalities associated with these overexpressors suggest that elevated expression of the p58 kinase interferes with the late events associated with normal mitosis. Similar mitotic abnormalities have been found in rat fibroblasts microinjected with either purified protein or antibodies corresponding to p13^{suc1}, a protein that is found complexed with the p34^{CDC2} kinase but whose function is unknown (32). However, no similar sequestering of cells at the late telophase/early G_1 boundary has been associated with abnormal expression of cell cycle gene products. Our results indicate that this CDC-related protein kinase can adversely affect the cell cycle when inappropriately expressed.

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1. Hunter, T. & Cooper, J. A. (1986) in *The Enzymes*, eds. Boyer, P. D. & Krebs, E. G. (Academic, Orlando, FL), Vol. 17, pp. 191–246.
2. Hanks, S. K., Quinn, A. M. & Hunter, T. (1988) *Science* **241**, 42–52.
3. Takio, K., Wade, R. D., Smith, S. B., Krebs, E. G., Walsh, K. A. & Titani, K. (1984) *Biochemistry* **23**, 4207–4218.
4. Takio, K., Blumenthal, D. K., Edelman, A. M., Walsh, K. A., Krebs, E. G. & Titani, K. (1985) *Biochemistry* **24**, 6028–6037.
5. Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M. & Ramachandran, J. (1985) *Nature (London)* **313**, 756–761.
6. Hanks, S. K. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 388–392.
7. Bunnell, B., Fillmore, H., Gregory, P. & Kidd, V. J. (1990) *Somat. Cell Mol. Genet.* **16**, 151–162.
8. Meeks-Wagner, D. & Hartwell, L. H. (1986) *Cell* **44**, 43–52.
9. Burke, D., Gasdaska, P. & Hartwell, L. (1989) *Mol. Cell. Biol.* **9**, 1049–1059.
10. Humphreys-Beher, M. G., Bunnell, B., vanTuinen, P., Ledbetter, D. & Kidd, V. J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 8918–8922.
11. Zoller, M. J. & Smith, M. (1983) *Methods Enzymol.* **100**, 468–500.
12. Long, G. L., Chandra, T., Woo, S. L. C., Davie, E. W. & Kurachi, K. (1984) *Biochemistry* **23**, 4828–4837.
13. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. & Green, M. R. (1984) *Nucleic Acids Res.* **12**, 7035–7056.
14. Wood, J. N. (1984) in *Proteins*, ed. Walker, J. M. (Humana, Clifton, NJ), Vol. 1, pp. 261–270.
15. Draetta, G. & Beach, D. (1988) *Cell* **54**, 17–26.
16. Chen, C. H., Sowder, J. T., Lahti, J. M., Cihak, J., Losch, V. & Cooper, M. D. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 2351–2355.
17. Klessig, D. F., Brough, D. E. & Cleghon, V. (1984) *Mol. Cell. Biol.* **4**, 1354–1362.
18. Urlaub, G. & Chasin, L. A. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4216–4220.
19. Brinkley, B. R., Zinkowski, R. P., Mollon, W. L., Davis, F. M., Pisegna, M. A., Pershouse, M. & Rao, P. N. (1988) *Nature (London)* **336**, 251–254.
20. Strous, G. J., VanKerkhof, P., Fallon, R. J. & Schwartz, A. L. (1987) *Eur. J. Biochem.* **169**, 307–311.
21. Shaw, G. & Kamen, R. (1986) *Cell* **46**, 659–667.
22. Lee, M. G. & Nurse, P. (1987) *Nature (London)* **327**, 31–35.
23. Lorincz, A. T. & Reed, S. I. (1984) *Nature (London)* **307**, 183–185.
24. Kemp, B. E., Pearson, R. B., Guerriero, V., Bagchi, I. C. & Means, A. R. (1987) *J. Biol. Chem.* **262**, 2542–2548.
25. Rogers, S., Wells, R. & Rechsteiner, M. (1986) *Science* **234**, 364–368.
26. Hadwiger, J. A., Wittenberg, C., Richardson, H. E., DeBarros Lopez, M. & Reed, S. I. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6255–6259.
27. Kalderon, D., Roberts, B. L., Richardson, W. D. & Smith, A. E. (1984) *Cell* **39**, 499–509.
28. Saitoh, T., Sundsmo, M., Roch, J.-M., Kimura, N., Cole, G., Schubert, D., Olterdorf, T. & Schenk, D. B. (1989) *Cell* **58**, 615–622.
29. Fuller, G. M., Brinkley, B. R. & Boughter, J. M. (1975) *Science* **187**, 948–950.
30. Courchesne, W. E., Kunisawa, R. & Thorner, J. (1989) *Cell* **58**, 1107–1119.
31. Elion, E. A., Grisafi, P. L. & Fink, G. R. (1990) *Cell* **60**, 649–664.
32. Riabowol, K., Draetta, G., Brizuela, L., Vandre, D. & Beach, D. (1989) *Cell* **57**, 393–401.
33. Bunnell, B. A., Adams, D. A. & Kidd, V. J. (1990) *Biochem. Biophys. Res. Commun.*, in press.