1991

Biochemical And Cellular Characterization Of A Novel Cell Cycle Regulated Protein Kinase, P58(Gta).

Donald Edward Adams
University of Alabama at Birmingham

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Adams, Donald Edward, Ph.D.

University of Alabama at Birmingham, 1991

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BIOCHEMICAL AND CELLULAR CHARACTERIZATION OF A NOVEL CELL CYCLE REGULATED PROTEIN KINASE, $p58^{GTA}$

by

DONALD EDWARD ADAMS

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, in the Department of Microbiology in the Graduate School, The University of Alabama at Birmingham

BIRMINGHAM, ALABAMA

1991

ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

Degree Doctor of Philosophy Major Subject Microbiology

Name of Candidate Donald Edward Adams

Title Biochemical and Cellular Characterization of a Novel Cell Division Control Related Protein Kinase

A novel protein was identified through its association with an affinity purified preparation of ß1-4 galactosyltransferase (GalTase). This protein was named $p58^{GTA}$ based on its size on SDS-PAGE and its association with GalTase. A search of the GenBank and NBRF-PIR data bases revealed that p58 is a protein kinase and is related to the serine/threonine $p34^{CDC2/CDC28}$ cell division control protein kinases. The p58 kinase was also found to contain a 76 amino acid N-terminal regulatory region.

Biochemical analysis of the protein revealed that p58 was indeed a kinase and was present in both HeLa and yeast cell systems. It was found that overexpression of the kinase in CHO cells resulted in mitotic abnormalities and prolonged telophase and early G_1 phase of the cell cycle.

The effect of p58 on GalTase was then examined. It was found that overexpression of p58 in COS-1 cells resulted in an approximately three-fold increase in GalTase enzyme activity. Similar results were obtained by in-vitro analysis using purified enzymes. It was found that p58

phosphorylation of GalTase gave rise to the same isoenzyme forms seen in-vivo. It was also found that phosphorylation of GalTase by p58 gives rise to a six fold increase in the enzymes activity in-vitro.

Cell cycle analysis of the p58 protein showed that the kinase is regulated in a similar manner to p34. Its activity peaks in G_2/M phase of the cell cycle even though its protein levels remain constant. De novo protein synthesis occurs in G_2/M while mRNA synthesis occurs in G_1/S phase of the cycle. It was found that the levels of mRNA and protein activity were ten fold less than that of p34.

Abstract Approved by: Committee Chairman _____

Program Director _____

Date May 17, 1991 Dean of Graduate School _____

DEDICATION

For their unwavering love, support, and guidance,
I dedicate this manuscript
with all my love and appreciation,
to my parents.

George O. and Norma S. Adams

Thanks
Mom and Dad

ACKNOWLEDGEMENTS

I would like to start by thanking my mentor, Dr. Vincent J. Kidd, for allowing me the opportunity and privilege to work in his laboratory. The past few years in his laboratory has been a growing period both emotionally and intellectually. I am confident that in years to come I will have many opportunities to reflect back on what I have learned in his laboratory.

I would also like to thank those who sat on the various committees required for my academic advancement, especially those of my dissertation committee for taking time from their busy schedules to provide counsel and to critique my work. This also has provided me with the academic growth necessary for the achievement of my goals.

Then there is Skip Binder. He and the people in his laboratory have provided me with a wealth of knowledge, comradery, and scientific technology that has allowed me to finish this project.

Those who have interacted with me since I was a child are also responsible for the achievement of my goals. Be it either good friends or acquaintance each person has affected me in a way that has molded my personality and the way in which I view the world. For this reason I would like to thank "The Three Musketeers" Tom Ryan, Jeff Parkin,

and Joe Dillard for being such incredibly good friends. I wish you guys all the success in the world and I am confident that you each will attain all that you aspire.

I discovered over the years that I am very fortunate in having a family as close knit as my own. My parents of whom this manuscript is dedicated were not alone in their support of my success. My three older brothers George O., John W., Thomas J. and their families have also been a source of inspiration, support, and guidance since my early childhood. They will never know how much of a positive influence they have had on me. Thank you brothers for being there.

I owe a special thank you to my wife, Daria Ann Novekosky Adams. Since I met this girl nine years ago she has been a constant source of support and inspiration. Her attentive ear has always been there for me to talk out all of my aspirations and apprehensions. She has made my life complete and therefore the achievement of this degree is not a singular success but is instead a success for both of us. Thank you Daria.

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LIST OF ABBREVIATIONS

A	adenine
ATP	adenosine triphosphate
Asn	asparagine
Asp	aspartic acid
ß	beta
bp	basepairs
c	cellular
C	cytosine
Ca^{+2}	calcium
CAT	chloramphenicol acetyltransferase
cAMP	cyclic adenine monophosphate
CDC	cell division control
cDNA	complementary DNA
CHO	Chinese hamster ovary cell line
CIAP	calf intestine alkaline phosphatase
CLN	cyclin
COS	SV40 transformed African Green Monkey Kidney cell line
cpm	counts per minute
DHFR	dihydrofolate reductase
dITP	deoxyinosine triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
EGTA	Ethylenediaminetetracetic Acid

LIST OF ABBREVIATIONS (Continued)

FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
G	guanine
G_1	first growth stage of cell cycle
G_2	second growth stage of cell cycle
gal	galactose
GalTase	ß1-4 galactosyltransferase
GlcNAc	N-acetylglucosamine
Glu	glutamic acid
Gly	glycine
GTA	galactosyltransferase associated
h	human
hr	hour
hrs	hours
HeLa	human cervical carcinoma cell line
ile	isoleucine
kb	kilobases
kDa	kilodaltons
kV	kilovolt
LTR	long terminal repeat
Lys	lysine
M	mitosis, phase of cell cycle
mg	milligram
ml	milliliter
mM	millimolar
MMTV	mouse mammary tumor virus

LIST OF ABBREVIATIONS (Continued)

MPF	maturation promoting factor
mRNA	messenger RNA
N	amino terminal end of protein
NaCl	sodium chloride
NBRF-PIR	national biomedical research foundation-protein identification resource
nM	nanomolar
ORF	open reading frame
p	protein
P	phosphate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pp	phosphoprotein
RNA	ribonucleic acid
S	DNA synthesis phase of cell cycle
SDS	sodium dodecyl sulfate
SSC	saline sodium citrate
T	thymidine
TCA	trichloroacetic acid
Td	thymidine
U	uracil
UDP	uridine diphosphate
UTP	uridine triphosphate
μF	microfarad
μg	microgram
μl	microliter

INTRODUCTION

The research which will be discussed in this manuscript was made possible due to of years of dedicated work in two specific fields, cell division control and galactosyltransferase. The introduction has therefore been broken down into four parts. The first two parts are dedicated to giving background on the work done in the cell division control and the galactosyltransferase fields. The third part introduces a novel protein of which the remaining chapters study indepth. The last part of the introduction is a brief summary of the following chapters pointing out what to look forward to. This part of the introduction also recognizes the major co-authors for their contributions to the following text.

Cell Division Control Proteins

The cell division cycle can be broken down into two parts, S (synthesis, where DNA replication takes place), and M (mitosis, where the replicated DNA is separated and cytoplasmic division occurs [cytokinesis]). These two phases are further separated by two growth phases G_1 and G_2. This allows the observer to track the cell through a cycle starting at G_1 through S into G_2 to M and ending back in G_1. In most actively growing eukaryotic cells this cycle from G_1 to G_1 takes about 16 to 24 hours.

Mutations were identified in the budding yeast, Saccharomyces cerevisiae, (as well as in several other systems) that inhibit cell division when they were grown at restrictive temperatures (1). These temperature sensitive mutations resulted in the identification of over 35 different cell division control genes. Significant advances in the field of cell division control did not occur, however, until the mid-eighties when maturation promoting-factor (MPF) was first purified and characterized (2). MPF was known from earlier studies to be important in the transition from interphase to mitosis. It was responsible for chromosome condensation, nuclear membrane breakdown and the reorganization of the microtubule network representative of mitosis. Characterization of the components of MPF allowed the biochemical mechanisms of cell cycle regulation to be studied. It was found that MPF is composed of two major proteins $p34^{CDC2/CDC28}$ and cyclin B, in association with a few other proteins (3-7). These proteins appear to be modulated over the cell cycle with respect to both expression and activity as well as intracellular location (3-7). They were found to be so tightly regulated during the cell cycle that either an increase or decrease in their expression would adversely affect the cell division cycle (8,9). From these studies it was found that p34 was a protein kinase and was regulated by its phosphorylation state (3-9). Cyclin, as its name implies, is regulated by periodic accumulation and

destruction during the cell cycle. It was shown that synthesis of cyclin B may represent the entire protein synthetic requirement for cell cycle progression (5). However, even though cyclin is necessary for p34 activation it alone is not sufficient for cell cycle progression. It has been suggested that cyclin binds only unphosphorylated p34 and that it holds p34 in a position that allows p34 to be phosphorylated on residues tyrosine-15 and threonine-14 (these two residues threonine-14 and tyrosine-15 are within the ATP binding site of the p34 kinase)(5,10,11). The phosphorylation of these residues acts as a negative regulator for the kinase. It should be mentioned that the p34 component is phosphorylated on threonine, serine, and tyrosine residues distinct from residues 14 and 15, with no apparent regulatory role determined at this time. The negative regulatory event was proposed to be the result of the $p107^{wee1}$ protein kinase. The p107 kinase has significant homology to serine/threonine protein kinases (12) but was found to autophosphorylate itself on serine and tyrosine residues as well as phosphorylate p34 on tyrosine (13). This suggests that the wee1 kinase is a serine/tyrosine kinase, unique amongst protein kinases that have been characterized to date. The threonine phosphorylation of p34 is the result of an unidentified protein kinase.

The threonine/tyrosine phosphorylated form of the p34/cyclin complex is known as pre-MPF. Several methods of

activating this pre-MPF complex exist, however, it is not known whether they represent the same pathway. In cell free systems, active MPF can activate pre-MPF in the presence of cycloheximide, an inhibitor of protein synthesis, (14,15). Oakadaic acid, an inhibitor of phosphatase 1 and 2A, can also activate pre-MPF (16). These two results suggest that the activation of pre-MPF does not require de novo synthesis of another protein but requires the phosphorylation of some factor, most likely a kinase which activates the MPF specific phosphatase(s). Whatever the mechanism, the phosphorylated threonine-14 and tyrosine-15 residues of p34 must be dephosphorylated in order for MPF to be active (16,17). Threonine dephosphorylation other than at residue 14 may occur, but active p34 kinase retains phospho-threonine residues. A potential feedback mechanism is envisioned where active p34 would phosphorylate and activate a phospho-tryosine and a phospho-threonine phosphatase which would then further activate the pre-MPF complex (18). This model suggests that when MPF is then inactivated the phosphatase would no longer be phosphorylated and further activation of MPF would cease.

Two additional proteins associated with MPF are $p80^{cdc25}$ and $p13^{suc1}$. There is a 4-5 fold increase in $p80^{cdc25}$ concentration associated with p34 dephosphorylation as cells approach the G_2/M boundary (16,19). It has been shown that a mutation in p80 in yeast

can be rescued by a human T cell phosphatase, which can dephosphorylate tyrosine 15 in-vitro (20). This suggests that p80 is a phosphatase or an activator of a phosphatase. Since p80 has no sequence homology to any known phosphatase and has no phosphatase activity toward any of the known phosphatase substrates it is probably a phosphatase activator (13,21). p13 is neither a substrate or activator of p34, but was found to have a high affinity for p34 (5,10). These findings suggest p13 might interact closely with p80 or the phosphatase (21).

As little as 0.5% of unreplicated DNA was found to inhibit activation of MPF (22). This confirms the idea of a temporal control mechanism which would inhibit mitosis until DNA synthesis is finished. p80 was found to override the inhibitory control mechanism, which was correlated with dephosphorylation of tyrosine 15 on p34 (23). This action of p80 was blocked by addition of p13 (21). This suggests that p13 holds the pre-MPF complex in a conformation that prevents dephosphorylation of the sites in the ATP binding domain (21). Wheat germ agglutinin (an inhibitor of transport in and out of the nucleus) was shown to inhibit tyrosine dephosphorylation, but not phosphorylation (21). This suggests that the weel protein kinase and the threonine protein kinase both act in the cytoplasm while p80 and the phosphatase(s) act in the nucleus.

The transition from mitosis to G_1 is accompanied by the degradation of cyclin through a ubiquitin pathway at

the metaphase-anaphase transition (24). The question now is how does this ubiquitin pathway recognize cyclin at anaphase and not before? The answer is thought to arise from another protein kinase discussed below.

C-mos (pp30) a serine/threonine kinase uses cyclin as a substrate (25). It is therefore reasonable to assume that phosphorylation of cyclin may protect it from recognition by the ubiquitin pathway. Dephosphorylation of cyclin after metaphase would then lead to its recognition and degradation through a ubiquitinated pathway. p34 was also found to phosphorylate cyclin but on different sites than those seen by pp30 (Maller personnel communication). It appears at present that the only difference between the inactive pre-MPF complex and the active MPF complex is the phosphorylation of cyclin. Therefore the elegant pathway of phosphorylation/dephosphorylation of p34 may be only a secondary effect necessary to hold cyclin in a position which will allow it to be phosphorylated. The phosphorylated cyclin molecule would then place the MPF complex in a conformationally active state.

Three types of cyclins exist, A, B, and CLN. Cyclin A is associated with p34 primarily during G_1/S, and early G_2/M, cyclin B is associated with p34 primarily during G_2/M (16). The CLN cyclins are found in the G_1 phase of the cell cycle. The cyclin B molecule discussed above has been the most actively studied so far. The significance of the different cyclins is not known at present. One possibility

is to provide p34 altered substrate specificity over the cell cycle. The cyclin molecules also have a nuclear localization signal which provides p34 with a route into the nucleus.

Another cdc2 related protein kinase has been identified, $p32^{Eg1}$. This protein has several features which make it of interest. They include its perfectly conserved PSTAIR sequence (this sequence is conserved in p34 homologs). p32 also binds p13 and has both the threonine and tyrosine residues in the ATP binding domain (25). p32 cycles through the cell cycle similar to that of p34 but lacks the ability to bind any of the B type cyclins, but may bind to members of the CLN cyclins and possibly cyclin A (27,28). This suggests that this protein may function along a different pathway than that of the p34 protein.

Cell Surface Receptors

There are numerous cell surface receptors which are responsible for a number of cellular events. These events range from activating ion channels to increasing the synthesis of second messengers. In many cases the actions of these cell surface receptors results in altered cell growth or differentiation. It is expected that there would be some sort of a coupling between the actions of these receptors and the cell division control proteins discussed above. It is reasonable to assume that a unifying theme between these two groups of proteins would seem necessary

to potentially synchronize the events occurring on the cell surface with those inside.

It is with regard to this that several laboratories are interested in the actions of a cell surface form of the ß1-4 galactosyltransferase (GalTase) enzyme. Since the confirmation was made defining an isoenzyme form of GalTase being associated with cancer, (29-32), a number of investigators have been working out the intricacies of this enzymes activity. GalTase is probably best known for its trans Golgi activity where it functions in the biosynthesis of complex glycoconjugates by transferring galactose residues from UDP-gal to N-acetylglucosamine residues. The identification of GalTase on the cell surface of a number of different cell types (33-42) led to an intensive study on its mode of action, pathway of relocalization (which still remains illusive) and surface orientation (which found the transferase activity extracellularly orientated [42]). Roseman was among the first to report that GalTase may be involved in several aspects of cell adhesion and development (33). This initial observation opened the door for other studies which found GalTase to be present in great abundance on the surface of proliferating Balb/c3T3 cells, while dropping to undetectable levels in these same cells once they have reached confluency and become contact inhibited (34). Alternatively, when the noncontact inhibited Balb/c3T12 cells were used in the same type of experiment, GalTase enzyme activity was present at high

levels in both sparse and confluent cultures (34). This suggests the possibility that GalTase plays some role in the regulation of cell division. Further studies have implied that GalTase has an active role in cellular proliferation (35,36), malignant cell growth (37-39), fertility (40), cell migration (33,42-46), and morula compaction (41).

From these studies it was suggested that cell surface glycosyltransferases could participate in cellular interactions by recognizing and binding to their specific glycoconjugate substrates on adjacent cell surfaces and in the extracellular matrix (33,42). This type of surface receptor could endow cells with virtually unlimited specificity by displaying unique glycosyltransferase repertoires on their surface (42). The paradox to this research is that the nucleotide sugar donors required for the transferase have yet to be identified extracellularly. This suggests that a different unidentified sugar donor is utilized or a different mechanism, perhaps regulation by endocytosis, is at hand. Further work is needed in this area.

It is with respect to the above studies that this laboratory became interested in galactosyltransferase. In an early attempt to identify the cDNA for galactosyltransferase a cDNA for a previously unidentified protein was purified (35,47,48) and will be discussed below.

ß1-4 Galactosyltransferase Associated (GTA) Protein.

This protein has come to be called $p58^{GTA}$ based on its migration on SDS-PAGE and by its association with GalTase.

Translation of the cDNA gave a putative protein of 436 amino acids. When this putative protein was compared to the protein sequences published in the Genbank and the NBRF-PIR data bases, a region of 299 amino acids of the protein were found to be 68% homologous to the $p34^{cdc2/cdc28}$ protein kinases (Figure 1). This level of homology suggests that the proteins are evolutionarily related. In support of this it was found that p58 contains all 11 consensus protein kinase domains, as well as several other conserved amino acids thought to be involved, either directly or indirectly in serine/threonine kinase activity (49). p58 also contains regions that are unique to the p34 protein kinases. These regions include the conserved tyrosines found in and around the GXGXXG box, and a unique insertion region found between protein kinase subdomains X and XI (49-51).

The amino terminal region of the protein was found to contain 76 unique amino acids. This region is thought to play a role in the proteins localization, activity, and abundance because it contains three putative regulatory domains (see Figure 2 for schematic). The region contains a completely conserved calmodulin binding domain (52,53), a

Figure 1. Sequence homology between $p58^{GTA}$, $p34^{CDC2h}$, and $p34^{cdc28}$. Only the direct sequence homology between the proteins is shown, boxed areas.

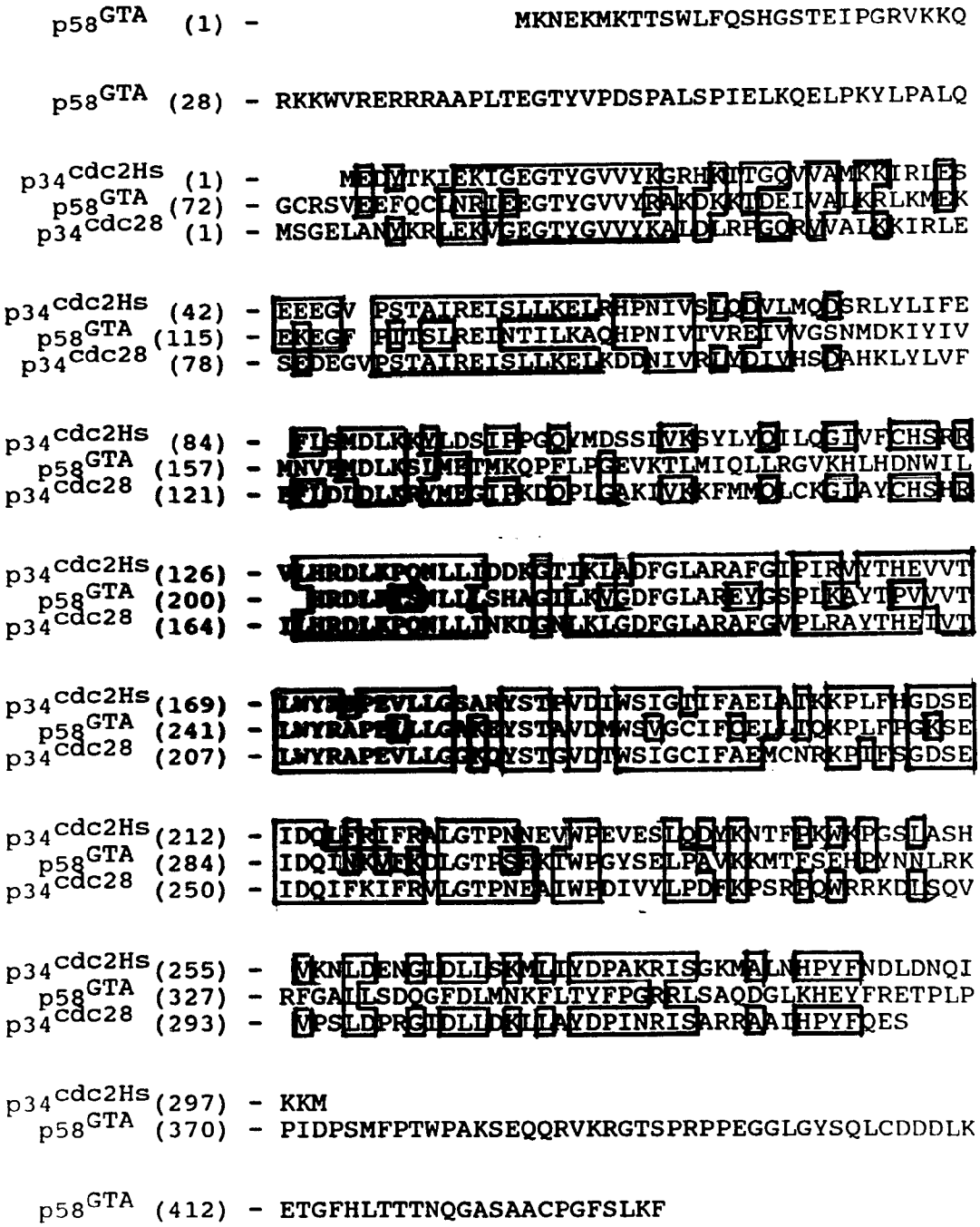
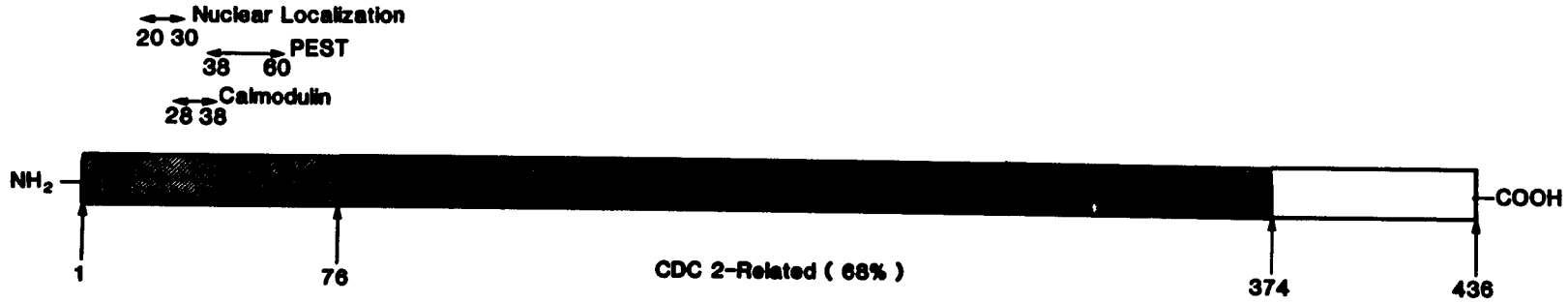

p58GTA (1) - MKNEKMKTTSWLFQSHGSTEIPGRVKKQ
p58GTA (28) - RKKWVRERRRAAPLTEGTYVPDSPALSPIELKQELPKYLPALQ
p34cdc2Hs (1) -
p58GTA (72) -
p34cdc28 (1) -
p34cdc2Hs (42) -
p58GTA (115) -
p34cdc28 (78) -
p34cdc2Hs (84) -
p58GTA (157) -
p34cdc28 (121) -
p34cdc2Hs (126) -
p58GTA (200) -
p34cdc28 (164) -
p34cdc2Hs (169) -
p58GTA (241) -
p34cdc28 (207) -
p34cdc2Hs (212) -
p58GTA (284) -
p34cdc28 (250) -
p34cdc2Hs (255) -
p58GTA (327) -
p34cdc28 (293) -
p34cdc2Hs (297) - KKM
p58GTA (370) - PIDPSMFPTWPAKSEQQRVKRGTSPRPPEGGLGYSQLCDDDLK
p58GTA (412) - ETGFHLTTTNQGASAACPGFSLKF

Figure 2. Structural domains of the p58 protein kinase.


Nuclear Localization
20 30
PEST
38 60
Calmodulin
28 38
NH2
COOH
1
76
CDC 2-Related (68%)
374
436

nuclear localization signal (54), and three PEST sequences (55).

PEST sequences are associated with the rapid degradation of proteins which contain them (55). The model suggests that phosphorylation within PEST regions generates Ca^{+2} sequestrable sites. The Ca^{+2} activated calpain family of proteases are thought to recognize these Ca^{+2} binding sites, resulting in site specific proteolysis (55). Cleavage by the calpain family of proteases is thought to occur at a P_1-P_2-X consensus sequence, with cleavage between P_2 and X. P_1 can be either valine or leucine, and P_2 can be either tryptophane, methionine, or arginine (56). This type of consensus has been identified in the PEST region of the p58 kinase as valine-arginine-glutamic acid at residues 33-35.

Computer assisted structural analysis of the putative protein revealed that there are no N-linked glycosylation sites, or regions of hydrophobicity resembling a transmembrane spanning domain. Therefore, the protein is most likely cytoplasmically oriented. The analysis also revealed an abundance of proline rich turns present in the first 76 amino acids flanked by two alpha helices. However, there is no evidence suggesting that this helix-turn-helix motif resembles a DNA binding protein.

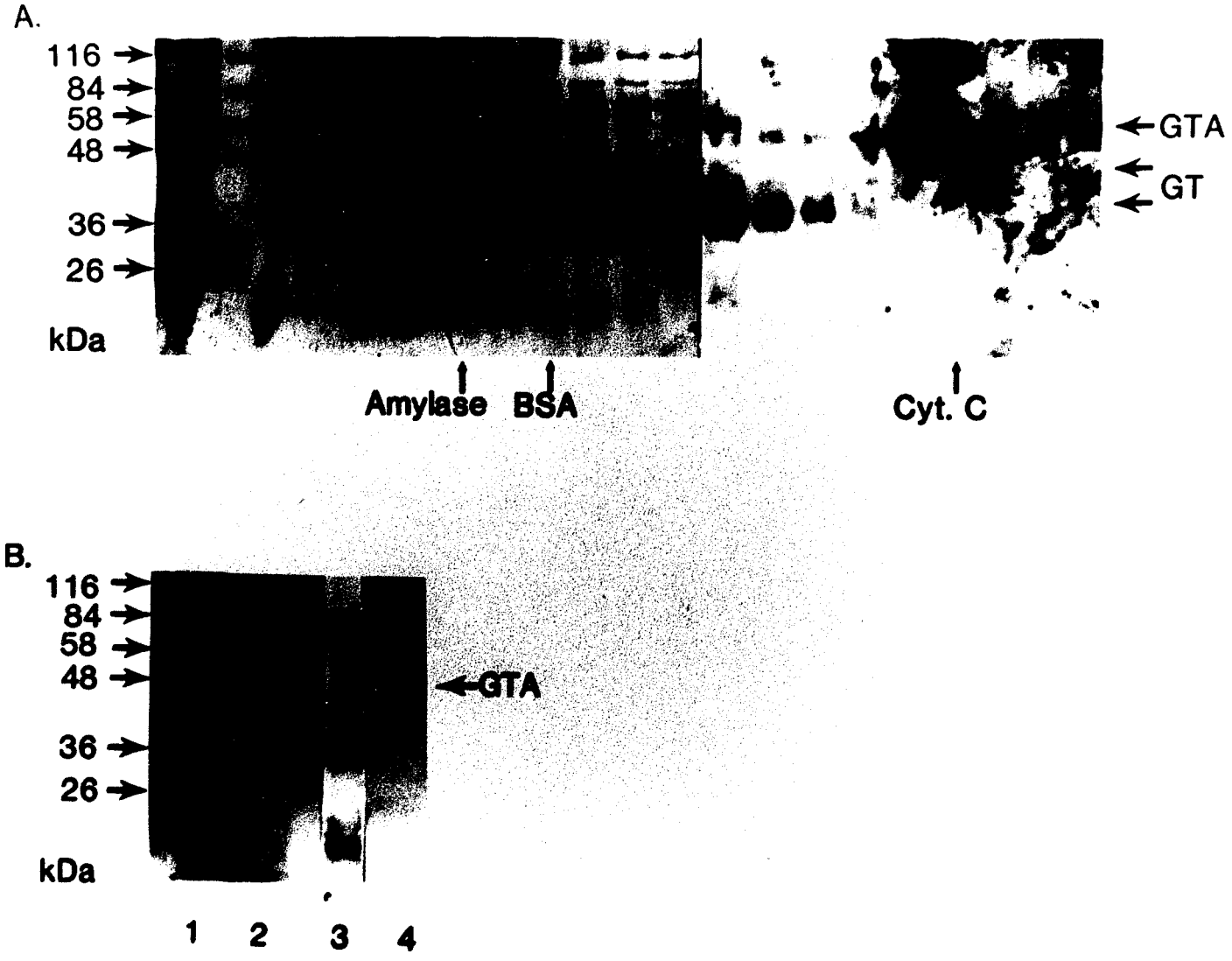
p58 was shown to be a component of a partially purified preparation of GalTase. This preparation of GalTase was, therefore, chosen as the starting material for

a two step purification of p58. First a sephacryl S-200 sizing column was used to crudely separate the proteins present in the commercially available preparation. Using a combination of 50 mM EGTA, and 50 mM NaCl in a 50 mM Tris buffer the association between GalTase and p58 was relieved (Figure 3, Panel A). As a second step purification and to show that p58 was able to bind calmodulin (A. Kraft and A. Means, personal communication), the sephacryl S-200 specific protein elutriate was passed over a calmodulin column (Figure 3, Panel B). By doing this two step purification the p58 kinase was purified to homogeneity, as determined by silver staining (Figure 3, Panel B).

The <u>in-vitro</u> kinase reactions found the kinase to require 10 mM Mg^{+2} and 1 mM ATP for activity. Results show that in addition to the GalTase proteins the kinase also uses casein and histone H1 as substrate. It was also found that the allosteric modifier of GalTase alpha lactalbumin has no effect on the phosphorylation of the GalTase proteins, but was itself phosphorylated by p58 (data not shown).

The purified kinase was found to be calmodulin independent suggesting two possibilities. First the calmodulin domain functions in another aspect of p58 regulation, maybe to block nuclear localization. The second possibility is that calmodulin may activate the kinase by binding and is then released. The purified

Figure 3. Purification of the p58 protein kinase. Panel A, is a silver stained gel of fractions from a sephacryl S-200 sizing column. Molecular weight is indicated to the left. Column standards are indicated along the bottom and galactosyltransferase (GT) and p58 (GTA) protein bands are indicated. Panel B, lanes 1-3 is a silver stained gel of, lane 1, molecular weight markers. Lane two, the EGTA fraction off a calmodulin sepharose column of the pooled p58 protein from the sizing column. Lane 3, silver stained gel of 5 ug of the semi-purified galactosyltransferase proteins. Lane 4, western blot of the material in lane 2, with the anti-$p58^{GTA}$ antibody.


A.
116
84
58
48
36
26
kDa
Amylase
BSA
Cyt. C
GTA
GT
B.
116
84
58
48
36
26
kDa
GTA
1
2
3
4

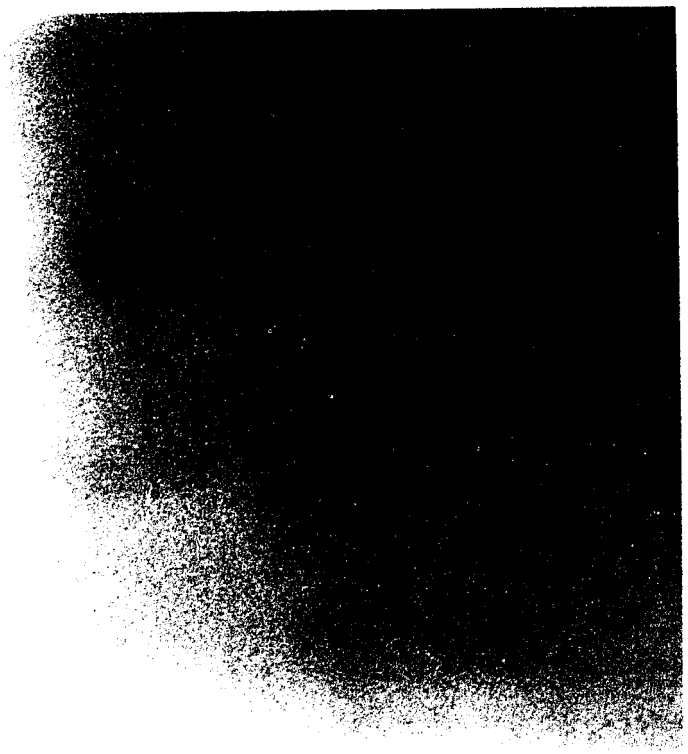
kinase may already have been activated and thus appear calmodulin independent.

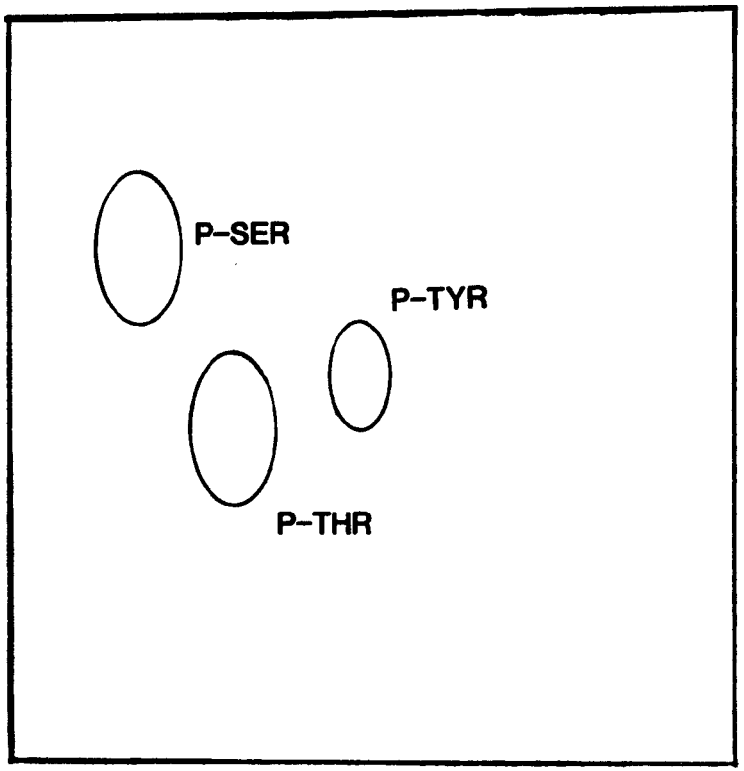
Phosphoamino acid analysis, using casein and histone H1 as substrates, revealed that p58 is a serine/threonine kinase, (Figure 4).

With this in-vitro biochemical data the in-vivo analysis was initiated. HeLa cells were found to express p58, and therefore were chosen as the model system for the in-vivo studies. These cells are well characterized and have been used in the study of a magnitude of different genes and gene products, including the CDC and CDC-related proteins, discussed above. Since these cells have been used to study the CDC phenomena, the study of p58 in these cells may be correlated to this work.

It was previously found that expression of the p58 gene is regulated via sequences within its 3' untranslated region (47). Within the 3' untranslated sequence are several AT rich putative regulatory regions similar to those found in c-Fos, c-Myc, as well as lymphokines and growth factors (57-59). Alterations in the level of endogenous p58 can affect the cell cycle; too little p58 allows cells to proceed through the cell cycle more rapidly. Too much p58 inhibits cell cycle progression, resulting in a sequestering of these cells at the late telophase/early G_1/S phase of the cell cycle as well as a 40-fold increase in mitotic abnormalities (48). These results suggested that the p58 kinase might function to

Figure 4. Two dimensional phosphoamino-acid analysis of histone H1 as a substrate for the p58 kinase. Left panel is an autoradiogram of the hydrolysed H1 protein indicating that the serine residue is compared. The panel on the right indicates the relative positions of phospho-ser, -thr, and -tyr residues.




P-SER
P-TYR
P-THR

control some aspect of cell cycle regulation, and that it may also act upon the GalTase proteins with which it copurifies.

Chapter organization

The specific purpose of the research was to characterize the p58 protein kinase. A two part study of GTA's protein activity, was proposed and executed. The first part was designed to characterize the activity of the protein in-vitro. Subsequently, the biology of the system was considered using in-vivo methods. The combination of these techniques has contributed to an understanding of how this protein is regulated. The following chapters are reprinted articles that characterize how this protein is regulated and how its activity effects the morphology and cell cycle progression of cells which express it.

The first chapter documents the sequencing of the cDNA and the initial observations that were made. From the cDNA sequence, computer assisted analysis was done which identified the putative protein as a serine/threonine protein kinase. It also allowed us to place the protein in the family of cell division control protein kinases. This computer analysis identified a unique stretch of amino acids in the N-terminal region of the putative protein. It was a 20 amino acid stretch within this region that was used to generate a polyclonal antibody.

Some of the initial biochemistry was done on the kinase in this reprint, in addition to microscopy which

identified the mitotic abnormalities associated with the overexpression of the kinase. The sequencing was mostly done by Bruce Bunnell and Lucie Heath, (a graduate student and a postdoctoral fellow respectively in Dr. Kidd's laboratory). Bruce Bunnell was also instrumental in the creation of and analysis of CHO cell overexpressers. My work consisted of the detailed computer search, in-vitro transcription/translation analysis, p58 protein purification and kinase assays, Western blot analysis with yeast, HeLa and GalTase proteins. I also generated and affinity purified the monospecific polyclonal antibody used in the following chapters and by several other international laboratories

The second chapter deals with more of the same but with respect to the actions of the kinase in relation to GalTase activity. Again Bruce Bunnell was instrumental in the creation of and analysis of kinase overexpressers, which showed that the phosphorylation state of GalTase increases with increasing amounts of the p58 kinase in the cell. The biochemistry of the system was my contribution, which illustrated that the phosphorylation state of the GalTase enzyme regulated its activity. The biochemistry also showed that the p58 kinase was capable of phosphorylating the GalTase enzyme creating isoenzyme forms identical to those previously published in-vivo (60). My antibodies were then used by Bruce in the last figure of this chapter to show that disrupting the association

between the p58 and GalTase proteins resulted in a decrease in GalTase activity.

The results from the above chapters suggest that the protein is related to the cell division control protein kinases, and its overexpression creates cell division mutants. The last chapter answers the very important question of: "How the p58 protein kinase is regulated over the cell cycle." We found that in addition to supporting the previous results the p58 kinase is regulated coordinately with the p34 protein kinase with which it shares homology. However, p58 is at least ten fold lower then p34 with respect to both mRNA expression and kinase activity. The phosphorylation state of the p58 kinase was also examined and it was found to be hyperphosphorylated in G_1/S phase of the cell cycle relative the its phosphorylation state at M phase of the cell cycle. This observation is also consistent with that found for p34, with the exception that p34 is hyperphosphorylated on threonine and tyrosine residues while p58 is hyperphosphorylated on serine residues.

ELEVATED EXPRESSION OF A p58 PROTEIN KINASE LEADS TO PROLONGED LATE TELOPHASE AND EARLY G_1-PHASE OF THE CELL CYCLE IN CHO CELLS

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ABSTRACT

We have isolated and characterized a 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.

INTRODUCTION

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 1). In excess of 100 distinct protein kinases have now 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 of these enzymes (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 new protein kinase that copurifies with a mammalian glycosyltransferase. The extensive degree of homology between this 58 kDa galactosyltransferase associated protein kinase (p58/GTA) and the CDC2/CDC28 cell division control protein kinases of yeast and man suggest that it is ancestrally related to the CDC protein kinases. We have previously shown that diminished levels of p58 mRNA and protein in CHO cells led to subtle changes in DNA replication (7). These observation, 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 ß-tublin 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. Adult human liver (10) and fetal human liver (Clontech) Lambda-gt11 cDNA libraries were screened using the Lambda-hp58-1 cDNA isolate and sequenced as previously 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) EcoRI restriction fragment from Lambda-hp58-5 containing the entire open reading frame (ORF) of the cDNA (Figure 1) was inserted in the vector pGEM-1 (Promega). A 1.7 kb human alpha-1-antitrypsin cDNA (12) was also inserted into the 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 (Strategene), containing [^{35}S]-methionine (Amersham) and was analyzed by SDS/10% PAGE. A synthetic 20-mer peptide (amino acids 22-41, Figure 1; prepared by Multiple Peptide Systems, San Diego, CA) was coupled to keyhole limpet hemocyanin with gluteraldehyde 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 absence of 100 nM nonradioactive competing

synthetic peptide. The immunoprecipitated [^{35}S]methionine labeled protein was analyzed by SDS/10% PAGE. Western blot analysis using ^{125}I-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/Galtosyltransferase by sephacryl S-200 (Pharmacia) chromatography followed by calmodulin-Sepharose chromatography.

Recombinant DNA Constructs and Eukaryotic Cell Analysis. Two 1.5 kb EcoRI restriction fragments containing the coding region of the [Glu^{84}]p58 (wild-type) or [Gly^{84}]p58 cDNA were ligated into the SmaI site of the eukaryotic expression vector pMSG (17). Properly oriented pMSG-p58 constructs were linearized with PvuI and introduced into DHFR$^-$ CHO cells (18) by electroporation as described below. The DHFR$^-$ CHO cells were cultured at 37^{o}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. PvuI 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 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 anti-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.

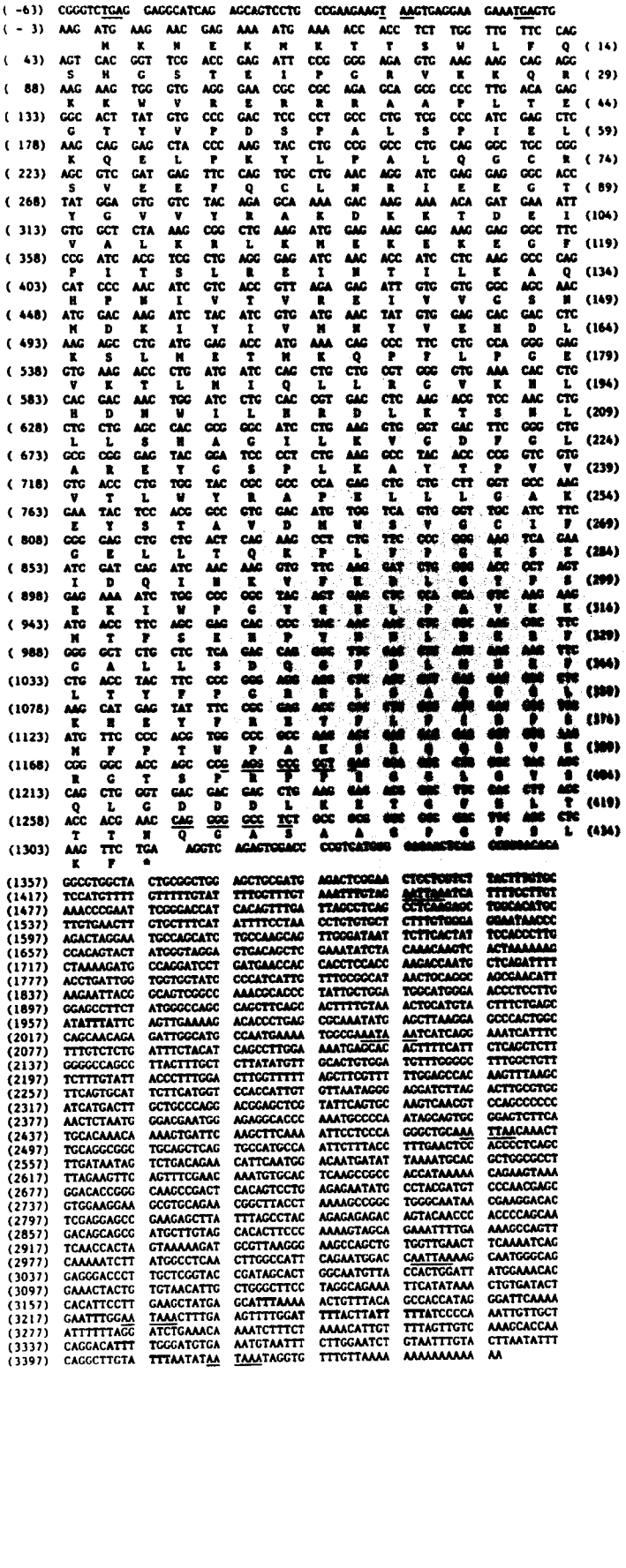
RESULTS

Isolation and DNA Sequence analysis of p58 cDNA Clones. The original p58 (GTA) cDNA clone was isolated using a GalTase polyclonal antiserum that recognized the p58 protein 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, Lambda-hp58-2 to -5, obtained from a human fetal liver library. Complete analysis of the sequences of these cDNAs confirmed an open

reading frame of 1308 base pairs (bp) that encodes a protein of 436 amino acids (Figure 1). The Lambda-hp58-5 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 polyadenylation 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 49.6 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

Figure 1. The 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 polyadenylation signals (AATAAA,AATTAA) are also underlined.



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 (Figure 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 amino-terminal regulatory domains overlap one another and are not found in any of the other CDC-related protein kinases that have been isolated and characterized.

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 isolate to make a functional protein product was examined in-vitro using the 5′ 1.5-kb EcoRI fragment of Lambda-hp-58-5 (Figure 1). Similar experiments were performed with the 3′ 2.0 kb EcoRI fragment resulted in no protein products (data not shown). When coding strand RNA

transcribed *in vitro* was added to a rabbit reticulocyte lysate system, a major protein band of approximately 55 kDa was observed (Figure 2A, lane 4). In contrast, synthetic noncoding RNA yielded no protein (Figure 2A, lane 3). A positive control consisting of human alpha-$_1$-antitrypsin cDNA, produced the expected 46-kDa protein (Figure 2A, lane 1). A negative control (no RNA) produced no protein (Figure 2A, lane 3).

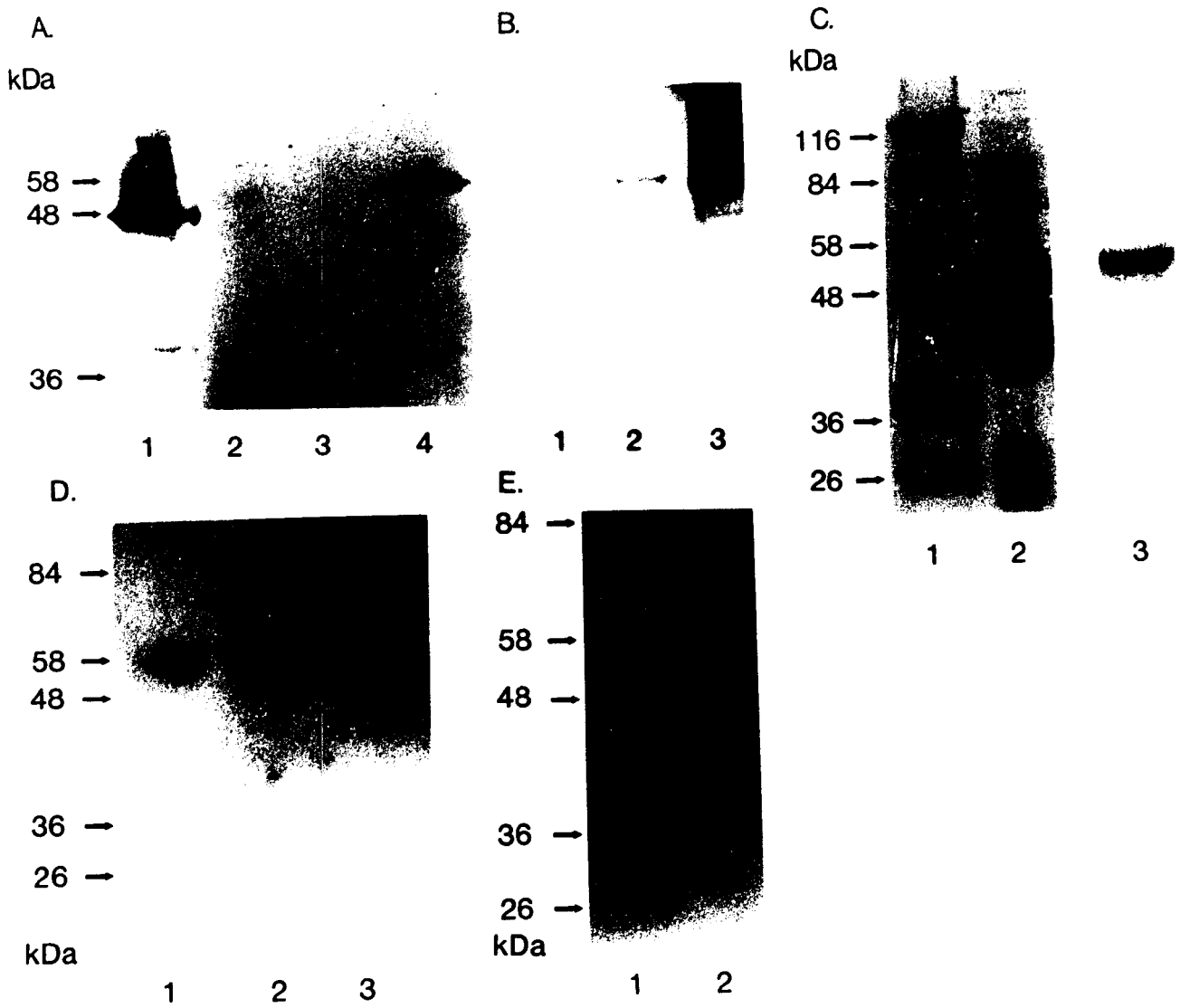
Next, we generated an antibody to a portion of p58, amino acids 22-41, that is not found in the $p34^{cdc2}$ protein kinases or other proteins. A high-titer polyclonal IgG antiserum was obtained and characterized by its ability to recognize the uncoupled synthetic peptide. Affinity-purified anti-p58-(22-41) 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* (Figure 2B, 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 (Figure 2b, lane 2) but not in the presence of an excess of the competitor (Figure 2B, lane 1). Bovine lactose synthetase was then resolved by SDS/PAGE and the proteins were either stained by Coomassie blue (Figure 2C, lane 2) or probed with anti-p58-(22-41), which detected a single 55- to 58 kDa protein (Figure 2C, lane 3). Western blots of cell lysates from *S. cerevisiae* (Figure 2D, lane 1) and from HeLa cells (Figure

2D, 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 <u>in-vitro</u> 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 (Figure 2E, 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 (Figure 2E, compare lanes 3 and 4). Additional <u>in-vitro</u> substrates for this kinase include casein and bovine GalTase, and all three substrates are phosphorylated equally well <u>in-vitro</u> (D.E.A., unpublished data).

<u>Cellular Phenotype Associated with Overexpression of p58.</u> 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

Figure 2. In-vitro and in-vivo p58 protein identification and kinase activity. (A) In-vitro translation products of synthetic RNA. Lanes:1, human alpha-$_1$-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 semi-purified 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 of 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.


A.
kDa
58
48
36
1
2
3
4
B.
1
2
3
C.
kDa
116
84
58
48
36
26
1
2
3
D.
84
58
48
36
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kDa
1
2
3
E.
84
58
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36
26
kDa
1
2

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 (Figure 3A). 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. Increase p58 protein expression was confirmed by Western blot analysis (figure 3B). The CHO cells containing the pMSG-p58 construct expressed approximately 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 (Figure 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 word). The doubling time for the

control cells (CHO and CHO/pMSG) was 14-16 hr, whereas that for the p58 overexpressors was 20-22 hr. The incorporation of [^{3}H] thymidine into the p58 overexpressors was 50-70% less than for either control cell group (Figure 5B). 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 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

Figure 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).

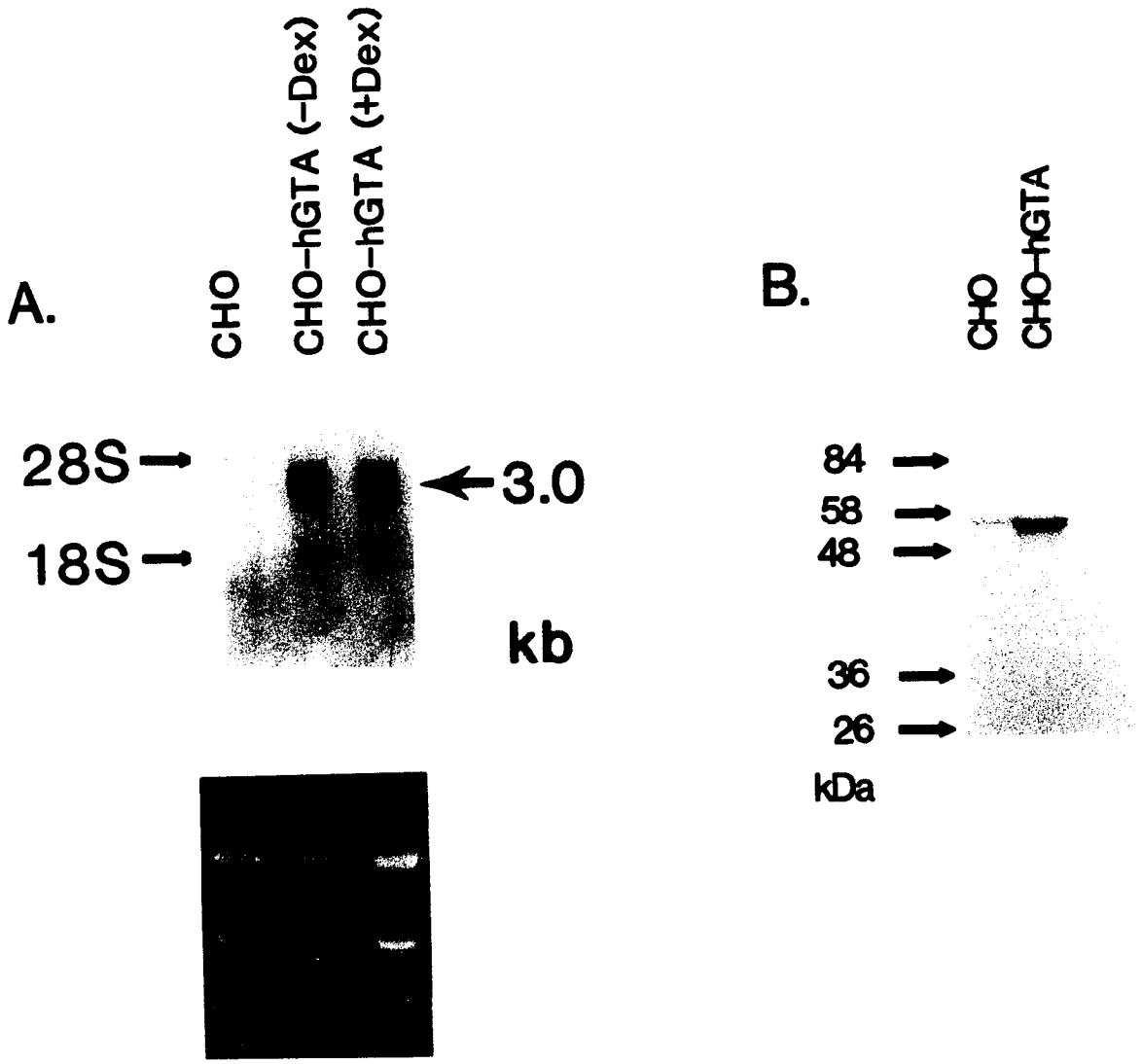

A.
CHO
CHO–hGTA (–Dex)
CHO–hGTA (+Dex)
28S
18S
3.0
kb
B.
CHO
CHO–hGTA
84
58
48
36
26
kDa

Figure 4. Phase-contrast microscopy of CHO/pMSG (Left) and CHO/pMSG-p58 (Right) cells. (x20).

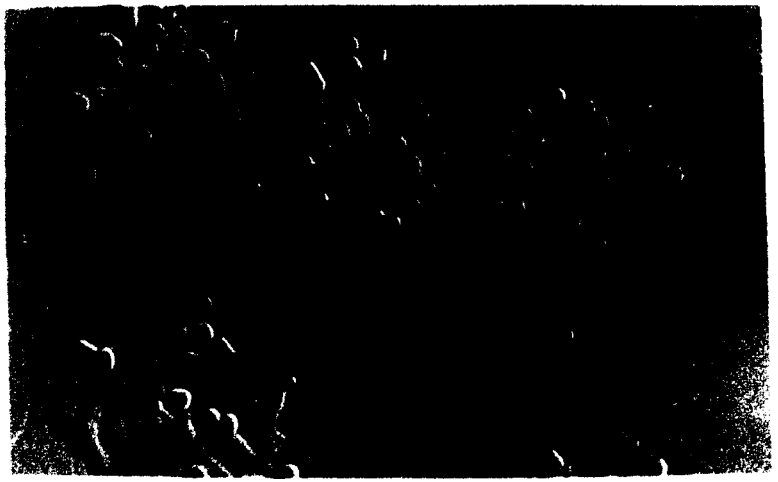
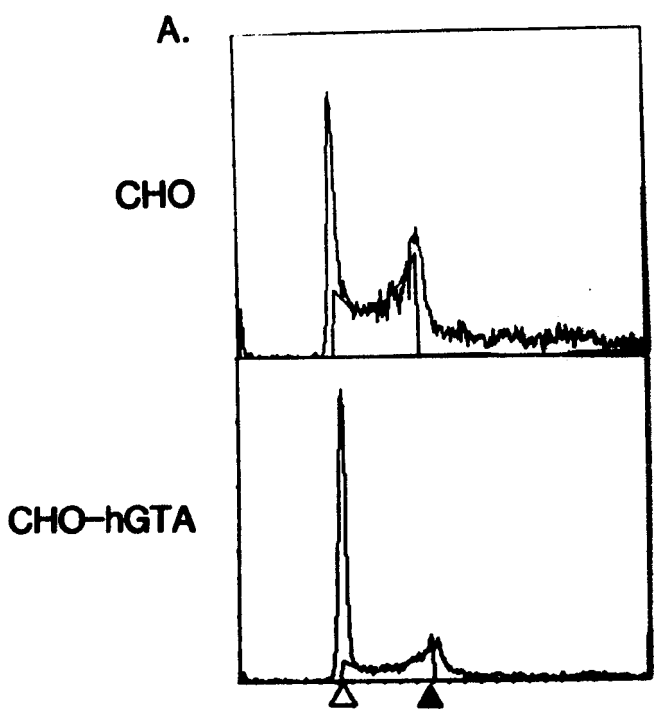


Figure 5. (A) Cell cycle analysis of identically grown subconfluent asynchronous cells. Open arrowhead, 2N DNA content; filled arrowhead, 4N DNA content. (B) [^{3}H]Thymidine incorporation in CHO cells (bar 1), CHO/pMSG cells (bar 2), and CHO/pMSG-p58 cells (bar 3).


A.
CHO
CHO–hGTA

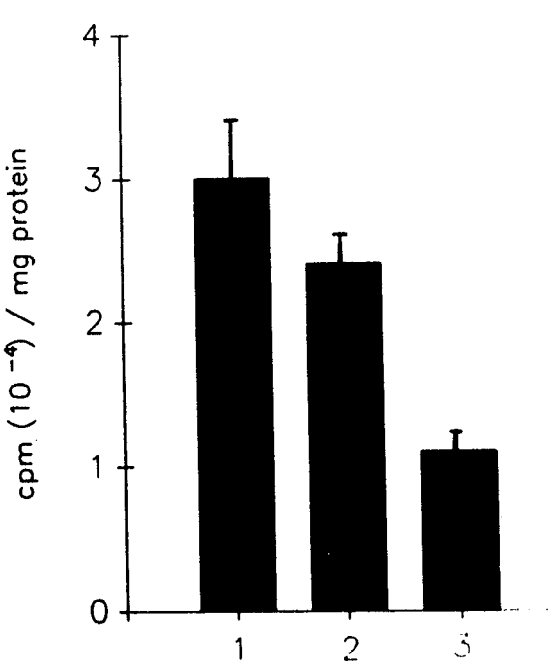
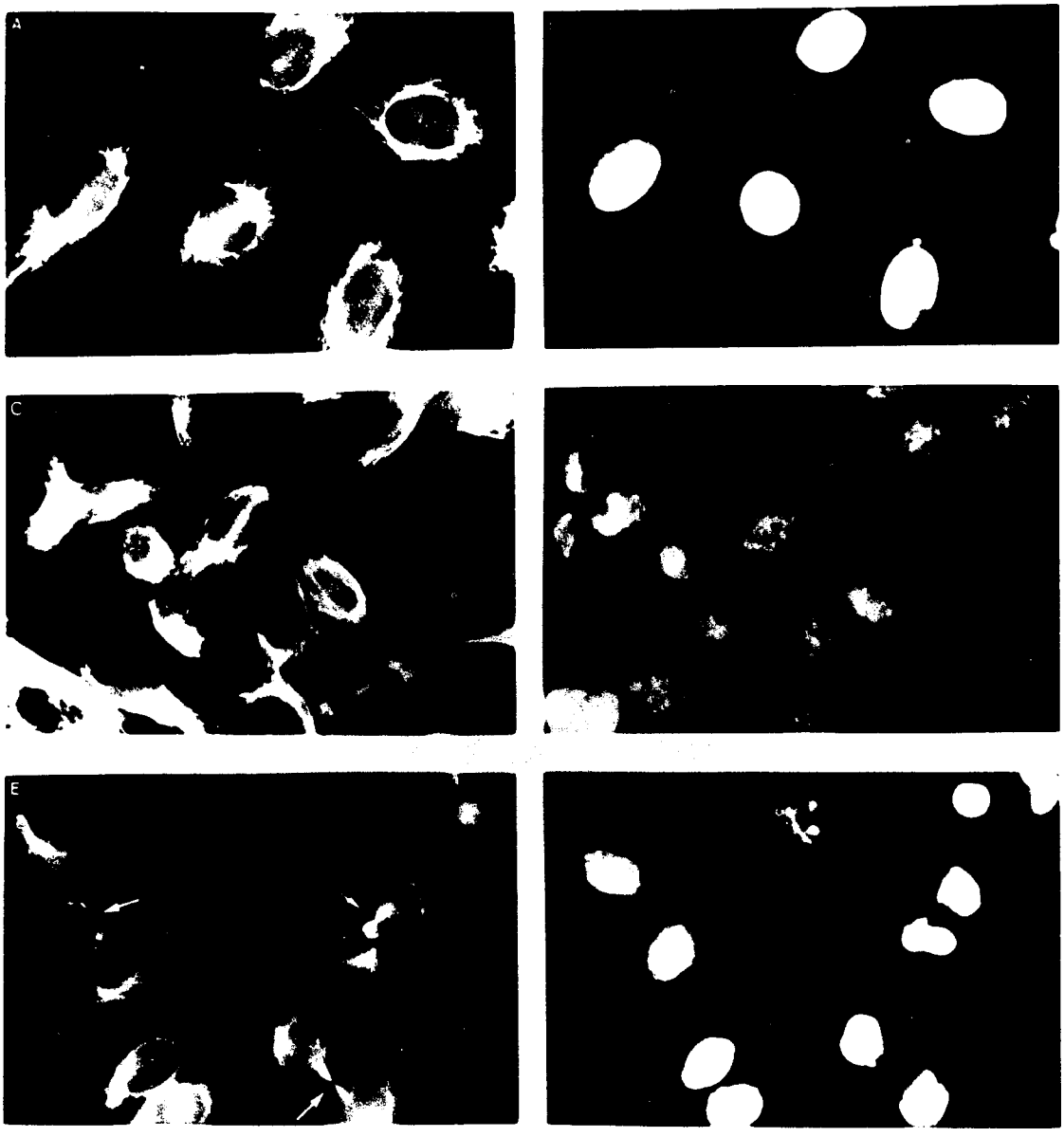

B.
cpm (10 -6) / mg protein
4
3
2
1
0
1
2
3

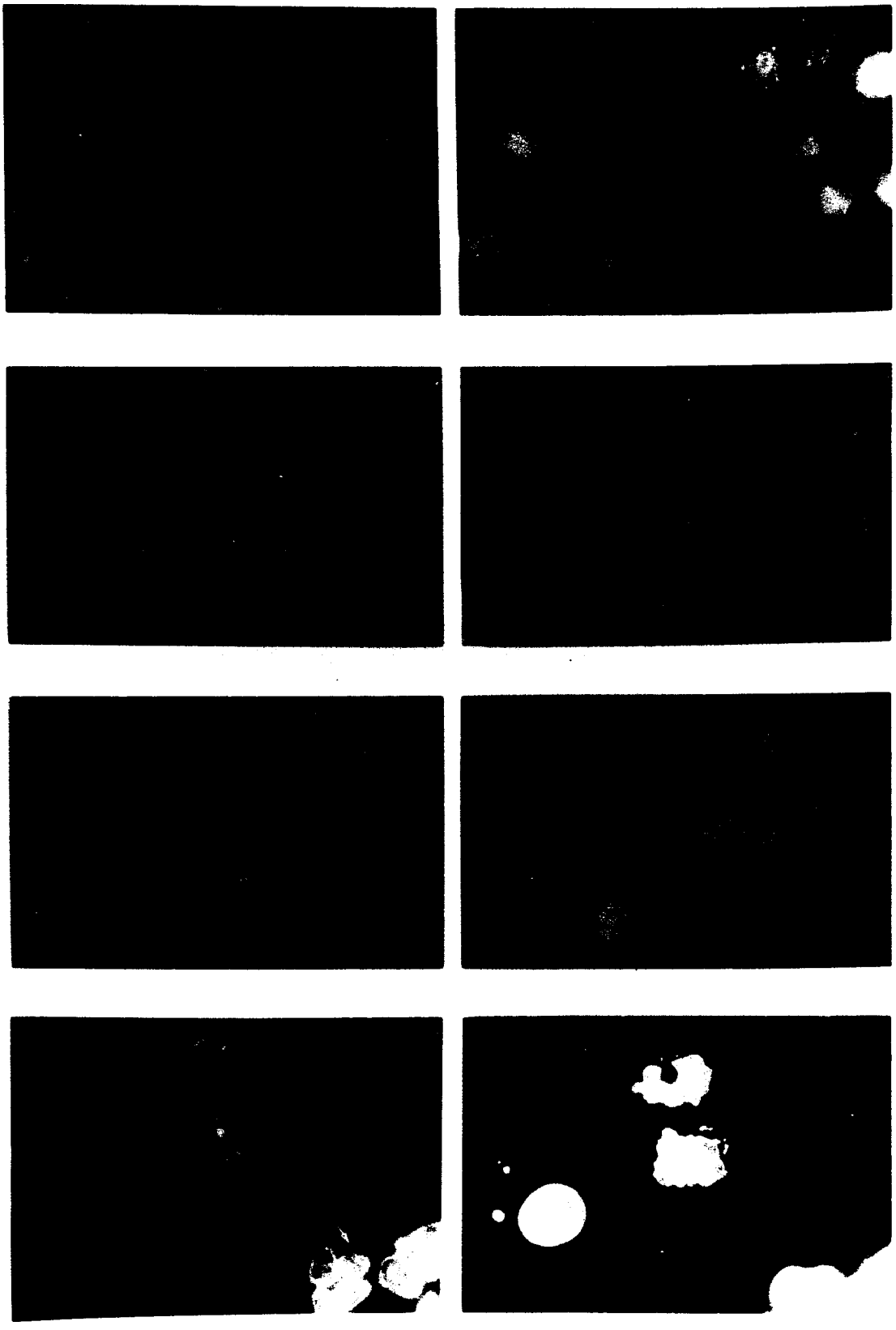
Figure 6. Cytoskeletal changes in p58 overexpressors. Anti-tubulin antibody staining is shown in A, C, and E. Corresponding Hoechst 33258 staining of nuclear DNA is shown in B, D, and F. The cells were CHO (A and B), CHO/pMSG (C and D), and CHO/pMSG-[Glu^{84}]p58 (E and F). Arrows in E indicate tubulin midbodies. (x50).


A
C
E

anti-tubulin antibodies. Both wild-type and vector-transfected cells appeared to be normal in cytoskeletal organization (Figure 6A and C). However, anti-tubulin staining of[Glu^{84}]p58, as well as [Gly^{84}]p58, clones revealed that many of these cells contained reforming cytoplasmic microtubule complexes as well as numerous tubulin midbodies (Figure 6E). Examination of multiple cell fields containing 400-600 cell 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 (Figure 7A), which was prevented effectively with excess p58 peptide competitor (Figure 7C). Affinity-purified preimmune antibody was used as a control (Figure 7E). p58 overexpressor cells with significant nuclear p58 staining (Figure 7G), but little cytoplasmic staining, contained numerous micronuclei (Figure 7H). Conversely, the smaller, rounded-up p58 overexpressors demonstrated significant cytoplasmic staining with the

Figure 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. (x75).



p58-peptide antiserum (Figure 7G). 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%).

DISCUSSION

We have isolated and characterized a 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 COOH-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 cDNA's (30-31), demonstration of protein kinase activity or effect of expression on cell cycle has not been shown.

Previously, we demonstrated 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 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 suggests 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 the $p13^{suc1}$ gene product, 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.

ACKNOWLEDGEMENTS

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TRANSIENT EXPRESSION OF A p58 PROTEIN KINASE cDNA ENHANCES MAMMALIAN GLYCOSYLTRANSFERASE ACTIVITY

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ABSTRACT

The effect of expression of a p58 protein kinase on mammalian ß1-4 galactosyltransferase enzyme activity was examined in-vitro and in-vivo. We found that p58 protein kinase expression enhanced galactosyltransferase (GalTase) enzyme activity approximately three-fold in vivo when compared to reporter gene activity. Galactosyltransferase enzyme activity was also substantially reduced in vitro when dephosphorylated, or when p58 specific antibodies were used to inhibit kinase activity. These results suggest that galactosyltransferase activity is influenced by phosphorylation, and that the p58 protein kinase may mediate this effect.

A new protein kinase, $p58^{GTA}$, was previously isolated using a non-specific ß1-4 galactosyltransferase (GalTase) polyclonal antibody (1,2). Molecular characterization of the $p58^{GTA}$ cDNA indicated that it encoded a protein of 436 amino acids, and that 299 amino acids of this sequence was 68% homologous to both yeast and human $p34^{cdc2}$ kinases (3). This protein also contains a unique amino-terminal domain with putative calmodulin binding, nuclear localization and PEST sequences (3). A specific antibody was made to a portion of this amino-terminal domain and was then used to demonstrate that $p58^{GTA}$ was a functional serine/threonine protein kinase, and that it co-purified with the bovine GalTase proteins. Elevated expression of the $p58^{GTA}$ kinase in CHO cells resulted in a sequestering of cells at the

late telophase/early G_1-phase cell cycle boundary, as well as a 40-fold increase in mitotic abnormalities (3). Conversely, diminished expression of the $p58^{GTA}$ kinase in CHO cells was found to enhance DNA replication and was accompanied by decreased GalTase enzyme activity (4). These results suggested that the $p58^{GTA}$ kinase might function to control some aspect of cell cycle regulation, and that it may also act upon the GalTase proteins with which it co-purifies.

Here, we report that the transient expression of the $p58^{GTA}$ kinase in COS cells results in a substantial increase in GalTase enzyme activity. This enhancement in GalTase activity is apparently not due to increased steady-state GalTase mRNA or protein levels, suggesting that the $p58^{GTA}$ kinase may post-translationally modify GalTase. Further support for this notion comes from evidence that dephosphorylation of the GalTase protein *in vitro* results in a substantial loss of enzyme activity.

MATERIALS AND METHODS

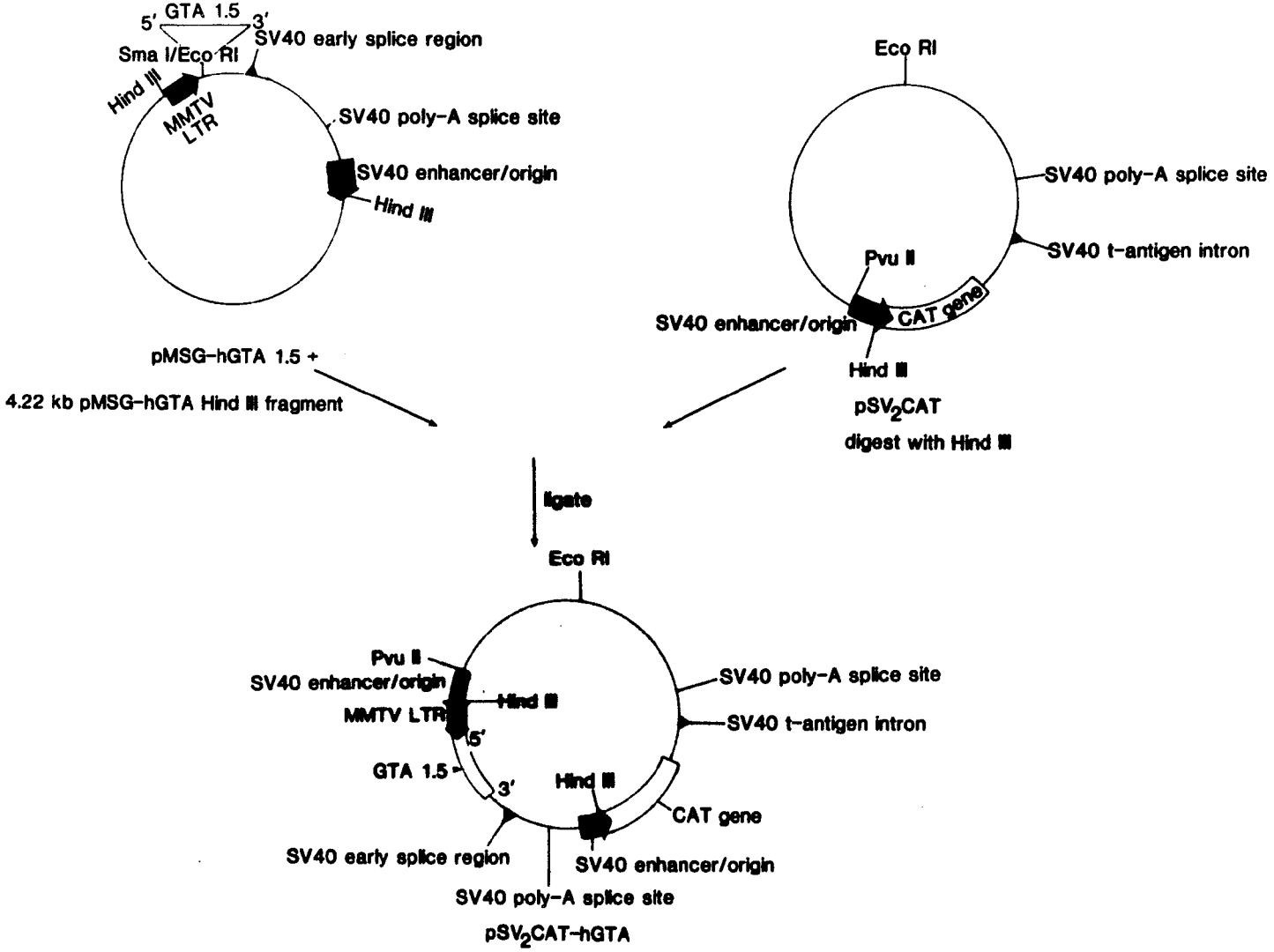
DNA Constructs and Expression Analysis. A 4.22 kb *Hind* III fragment from the pMSG-$p58^{GTA}$ construct (3), which contains the mouse mammary tumor (MMTV) LTR promoter, was ligated to *Hind* III digested pSV_2 CAT (5). Proper orientation of 4.22 kb pMSG-$p58^{GTA}$ fragment replaces the SV40 enhancer/origin sequence that was disrupted in pSV_2 CAT by *Hind* III digestion (Figure 1). The final construct, pSV_2CAT-hGTA, contains both the human $p58^{GTA}$ cDNA and the CAT gene, which

are expressed independently of one another, and as separate transcripts. The inclusion of the MMTV-LTR promoter allows induction of the $p58^{GTA}$ cDNA upon addition of 10^{-6}M dexamethasone (6). However, we and others have found that even in the absence of exogenous dexamethasone this promoter is active (3,7). Cos M-6 cells were electroporated as previously described (3). RNA and protein were extracted from the cells 60 hrs. post-electroporation for further analysis. Northern blots were performed as described previously (3) using human $p58^{GTA}$, murine GalTase, and human gamma-actin cDNA probes (3,4,8). Western blot analysis was performed as described previously using a rabbit anti-GalTase antibody (4).

Enzyme Activity Measurements. CAT activity was quantitated from 15 μg of total cellular protein from the electroporated COS cells essentially as described by others(5). GalTase enzyme assays were performed as described previously (3) using 10 μg of total cellular protein from the same cells examined above. All experiments were performed at least three different times.

In-vitro Phosphorylation, Dephosphorylation and Antibody Inhibition Studies. In-vitro kinase assays using purified bovine $p58^{GTA}$ kinase and GalTase were performed as described previously (3). Two dimensional gel electrophoresis and autoradiography was performed as described by Strous et al. (9). Bovine GalTase was treated with either 0.12 units of potato acid phosphatase for 15

Figure 1. Schematic diagram demonstrating the construction of the pSV_2CAT-hGTA expression plasmid from pMSG-hGTA and pSV_2CAT. Promoters are shown as filled arrowheads. The $p58^{GTA}$ cDNA and CAT gene are indicated. Additional features of the expression construct essential for function are also shown.


5′ GTA 1.5 3′
Sma I/Eco RI
SV40 early splice region
Hind III
MMTV LTR
SV40 poly-A splice site
SV40 enhancer/origin
Hind III
pMSG-hGTA 1.5 +
4.22 kb pMSG-hGTA Hind III fragment
Eco RI
SV40 poly-A splice site
SV40 t-antigen intron
Pvu II
SV40 enhancer/origin
CAT gene
Hind III
pSV2CAT
digest with Hind III
ligate
Eco RI
Pvu II
SV40 enhancer/origin
Hind III
MMTV LTR
5′
GTA 1.5
3′
Hind III
SV40 poly-A splice site
SV40 t-antigen intron
CAT gene
SV40 early splice region
SV40 enhancer/origin
SV40 poly-A splice site
pSV2CAT-hGTA

minutes at 37°C or 0.12 units of potato acid phosphatase plus 100 mM 4-nitrophenylphosphate prior to GalTase enzyme assay. For antibody inhibition studies, increasing amounts of affinity purified $p58^{GTA}$ peptide antibody (10 mg/ml) were incubated with 0.2 units of GalTase at 37°C for 30 minutes prior to GalTase enzyme assay. Additionally, increasing amounts of competing $p58^{GTA}$ peptide (starting at 100 nM and increasing in $\log_{10}$ increments) was incubated with 1 mg/ml of $p58^{GTA}$ antisera and 0.2 units of bovine GalTase at 37°C for 30 minutes prior to GalTase enzyme assay.

RESULTS AND DISCUSSION

Transient expression of the $p58^{GTA}$ cDNA and a reporter gene. To assess the possible influence of the $p58^{GTA}$ kinase *in vivo* on GalTase activity quantitatively, we designed an expression vector construct containing both the $p58^{GTA}$ cDNA and the *E. coli* chloramphenicol acetyltransferase (CAT) reporter gene (Figure 1). This vector allowed us to avoid potential problems with the efficiency of co-transfection of the two genes as separate plasmids.

Expression of the *E.coli* CAT gene and $p58^{GTA}$ cDNA was examined by functional assay and Northern blot analysis, respectively. In Figure 2, we demonstrate that the CAT gene contained in pSV_2CAT and pSV_2CAT-hGTA is functional in electroporated COS cells. CAT activity is indicated by the presence of both acetylated and di-acetylated

chloramphenicol in the pSV_2CAT positive controls and the pSV_2CAT-hGTA COS cells 60 hours post-electroporation, while no enzyme activity is present in negative control cells (Figure 2). The results of these experiments assured us that the reporter gene in our expression vector was functional, and that it would enable us to quantitate the effect of $p58^{GTA}$ expression on GalTase activity. Analysis of total RNA from COS cells electroporated with either pSV_2CAT or pSV_2CAT-hGTA plasmids was then performed by Northern blot using the human p58 cDNA probe. A hybrid 3.0 kb MMTV-$p58^{GTA}$ transcript, containing approximately 1,350 bp of the MMTV-LTR, 1,500 bp of the human $p58^{GTA}$ cDNA and 150 bp of SV40 derived sequences upstream from the poly(A) addition site was observed in the pSV_2CAT-hGTA electroporated COS cells (Figure 3, panel A, lane 2), but not in the pSV_2CAT electroporated cells (Figure 3A, lane 1). The 4.0 and 1.7 kb transcripts in both lanes are the endogenous African green monkey $p58^{GTA}$ transcripts. Equal loading of RNA samples was confirmed by hybridization with a gamma-actin cDNA probe (Figure 3, panel C). Previously, we have shown that this hybrid $p58^{GTA}$ mRNA leads to increased p58 protein expression by both Western blot and indirect immunofluorescence analysis (3).

Effects of $p58^{GTA}$ expression on endogenous GalTase enzyme activity. We next assayed GalTase enzyme activity in the various electroporated COS cells. Each electroporation experiment was performed on three different

Figure 2. Autoradiogram of a representative CAT assay. The origins and migration of chloramphenicol, acetylated chloramphenicol, and di-acetylated chloramphenicol are shown below. Two different pSV_2CAT-hGTA constructs were initially tested, however p/SV_2CAT-hGTA #48 was used in all subsequent experiments.

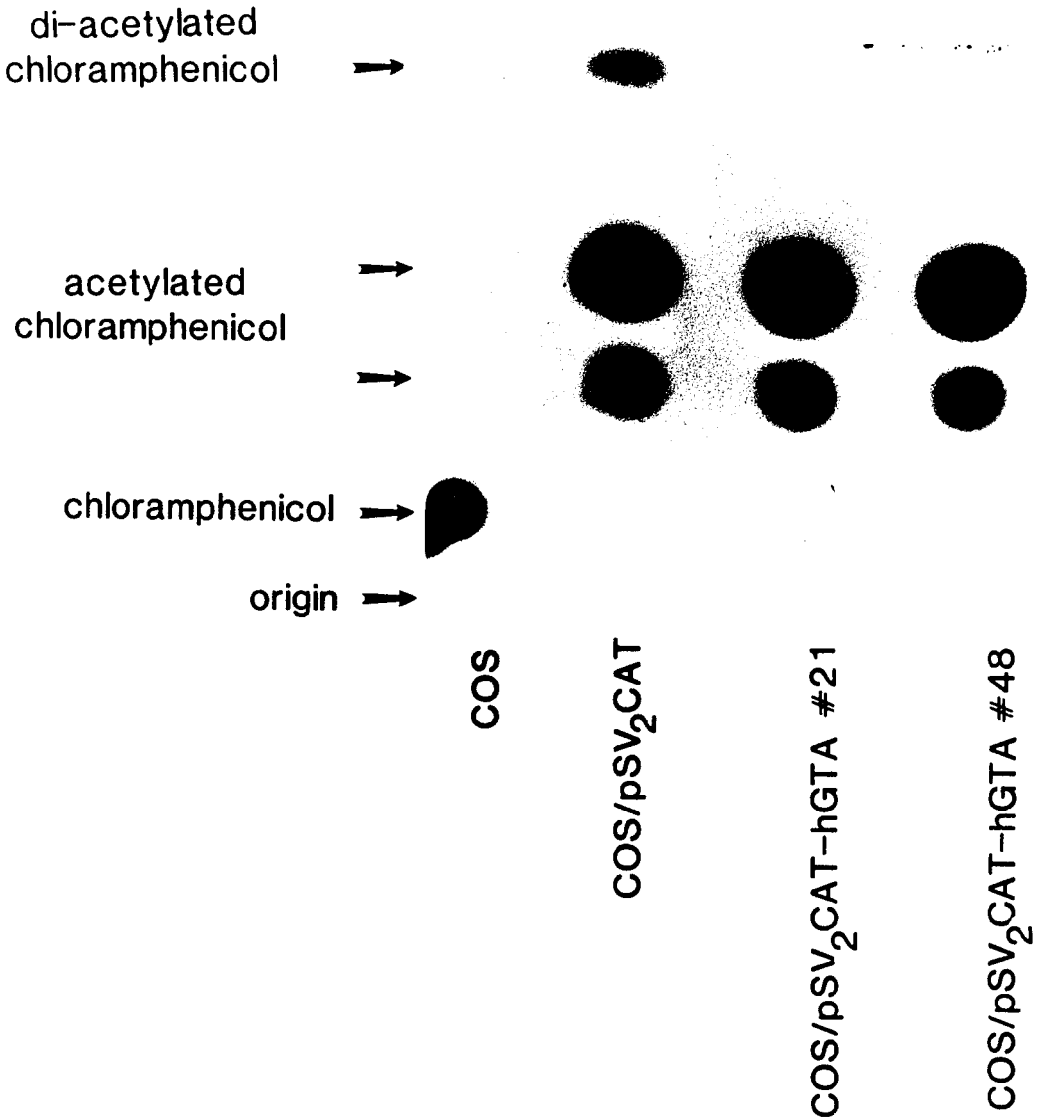
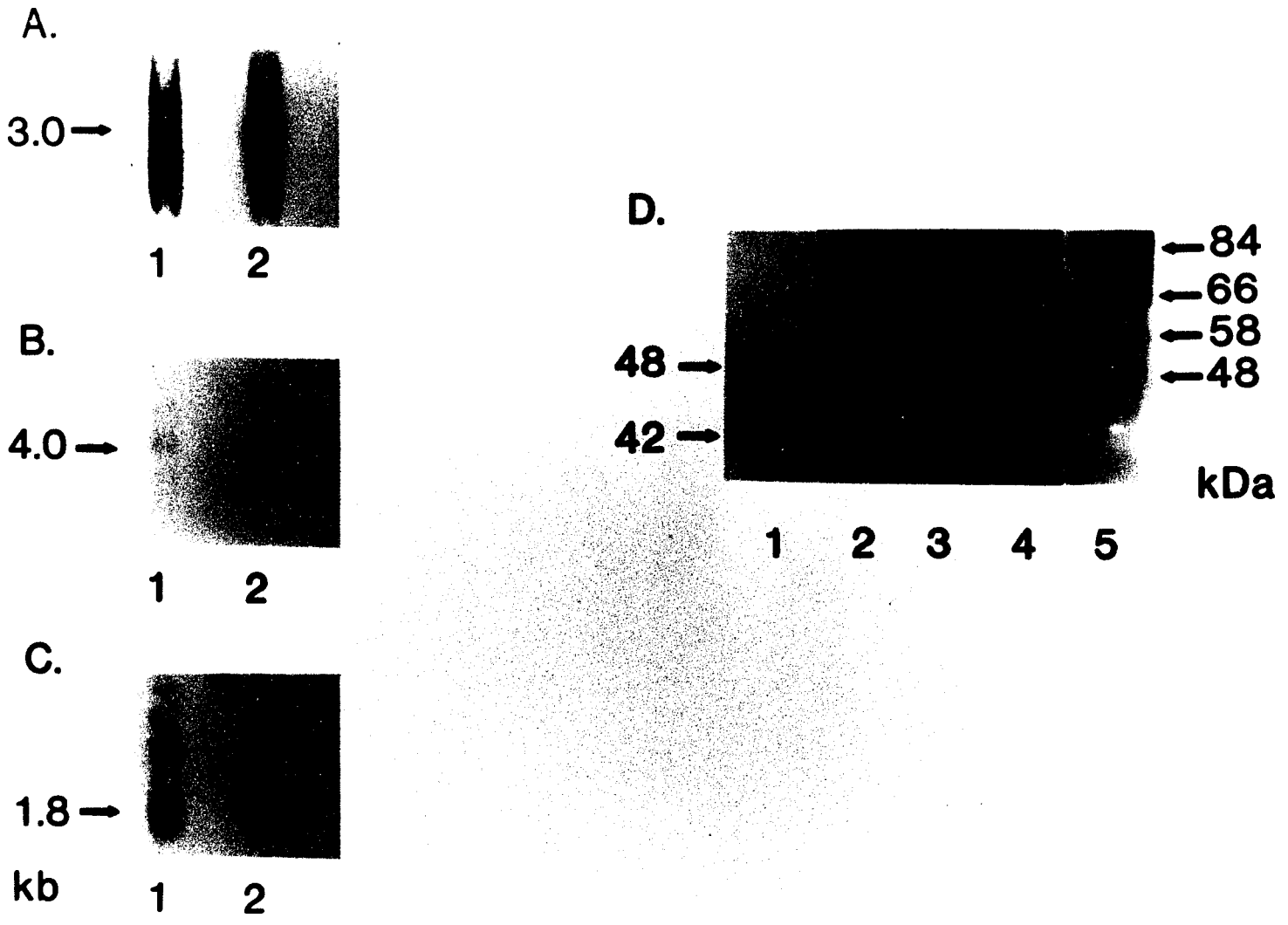

di-acetylated
chloramphenicol
acetylated
chloramphenicol
chloramphenicol
origin
COS
COS/pSV2CAT
COS/pSV2CAT-hGTA #21
COS/pSV2CAT-hGTA #48

Figure 3. Northern and Western blot analysis of cellular products. (Panel A) Northern blot analysis of 30 μg of total cellular RNA using the $p58^{GTA}$ cDNA (3) as probe. Lane 1, pSV_2CAT electroporated COS cells. Lane 2, pSV_2CAT-hGTA electroporated COS cells. (Panel B) Rehybridization of the same Northern blot shown in panel A with a murine GalTase cDNA probe (4). (Panel C) Rehybridization of the same Northern blot shown in panels A and B with a gamma-actin cDNA probe (8). (Panel D) Western blot analysis of cellular proteins and bovine GalTase using a polyclonal GalTase antibody. Lane 1, 0.1 μg of semi-purified bovine GalTase, demonstrating the ability of the GalTase antibody to recognize the 42 and 48 kDa bovine GalTase proteins. Lane 2, 50 μg of fetal calf serum proteins. Lane 3, 50 μg of total cellular protein from pSV_2CAT electroporated COS cells. Lane 4, 50 μg of total cellular protein from pSV_2CAT electroporated COS cells. Lane 5, molecular weight markers. The sizes of the molecular weights of the markers are shown on the right; the molecular weights of the two bovine GalTase proteins is shown on the left.


A.
3.0
1 2
B.
4.0
1 2
C.
1.8
kb
1 2
D.
84
66
58
48
48
42
kDa
1 2 3 4 5

occasions, using identical DNA constructs. The results of the CAT assays for each of the DNA constructs in column one of Table 1. The results of GalTase enzyme activity assays are shown as total cpm in column two of Table 1. To obtain an accurate quantitative difference we used the CAT activity values to establish a baseline value which was representative of transfection efficiency. This was done by normalizing the CAT values for all DNA constructs from each experiment to the lowest CAT assay value for that experiment, thereby generating a correction factor. The total cpm for the GalTase assays for the corresponding experiment was then corrected using this factor, and the values are shown in column three of Table 1. The relative-fold increase in GalTase activity in the pSV_2CAT-hGTA containing cells is shown in the final column of Table 1. The pSV_2CAT containing cells from each experiment were assigned a baseline value of one. These experiments demonstrate that expression of the $p58^{GTA}$ kinase in the absence of exogenous dexamethasone enhances GalTase activity approximately 2.5-fold, while in the presence of 10^{-6}M dexamethasone it is enhanced approximately 3.1-fold. Assays for changes in alpha-2, 6 sialocyltransferase enzyme activity from the same cell extracts demonstrated no substantial change (data not shown). Thus, either directly or indirectly, this kinase is capable of modulating GalTase activity.

TABLE 1

DNA Sample	CAT Assay (Total cpm)			4β-GT Assay (Total cpm)			4β-GT Assay (Corrected total cpm)*			Increase in 4β-GT Enzyme Activity#		
	1	2	3	1	2	3	1	2	3	1	2	3
COSM-6 pSV_2CAT	180,200	47,551	37,946	2,592	3,367	1,972	2,435	1,885	1,631	1.0	1.0	1.0
COSM-6 pSV2CAT-GTA1.5(+) (- Dex)	169,256	35,613	31,381	6,423	4,983	4,729	6,423	3,724	4,829	2.6	2.0	3.0
COSM-6 pSV2CAT-GTA1.5(+) (+ Dex)	175,688	26,622	64,638	7,908	4,873	11,894	7,619	4,873	5,947	3.1	2.6	3.6

* These 4β-GT values are corrected for the baseline CAT activity for each experiment shown in the first box. CAT activities were equalized to the lowest CAT value for each of the three experiments listed using each DNA construct (i.e., 169,256 for expt. 1, 26,622 for expt. 2, etc.) and the resulting factor(s) used to correct for variance in transfection and resulting GT enzyme activities in each experiment (4β-GT (total cpm) x corrected factor = 4β-GT (corrected cpm).

The relative fold-increase in 4β-GT activity is based on the pSV_2CAT vector containing cells equaling 1.0.

Finally, to crudely determine whether or not this effect was potentially post-transcriptional or post-translational, we analyzed the RNA and protein from these cells with specific GalTase cDNA and antibody probes (4,10). Rehybridization of the Northern blot in Figure 3 with a murine GalTase cDNA probe revealed that the steady-state level of GalTase mRNA was not affected by $p58^{GTA}$ expression (Figure 3, panel B, lanes 1 and 2). Similarly, the steady-state level of GTA protein in these cells was also invariable using a previously well characterized GalTase antibody (Figure 3, panel D). However, we detected two different molecular weight forms of GalTase, approximately 48 kDa and 52 kDa in size in the pSV_2CAT electroporated COS cells (Figure 3, panel D, lane 4). These sizes are consistent with the reported size of the GalTase proteins found in HeLa cells (11). The smaller 48 kDa molecular weight form of GalTase was not as abundant in the pSV_2CAT-hGTA electroporated cells, while the 52 kDa form was slightly enhanced (Figure 3, panel D, lane 3). The 42 kDa and 48 kDa bovine GalTase proteins seen by other GalTase antibodies (12) were also readily detected with this antibody (Figure 3, panel D, lane 1), while no immunoreactivity is seen when fetal calf serum is blotted (Figure 3, panel D, lane 2).

Bovine GalTase is Phosphorylated by $p58^{GTA}$ In-vitro and its Activity is Diminished by Dephosphorylation or $p58^{GTA}$ Antibody. The experiments shown above suggested that phosphorylation of GalTase might influence enzyme activity. This is not without precedent since Banerjee and colleagues have demonstrated that mannosylphosphodolichol synthase activity is enhanced by cAMP-mediated protein phosphorylation (13). Various isoenzyme forms of GalTase with acidic isoelectric focusing points have been described (14). Additionally, a serine phosphorylated form of GalTase has been isolated from both HeLa and HepG2 cells (9). Therefore, we examined the ability of the $p58^{GTA}$ kinase to phosphorylate bovine GalTase in-vitro. The phosphorylated 42 and 48 kDa GalTase proteins were examined by two-dimensional gel electrophoresis and autoradiography (Figure 4). We found that both the 42 and 48 kDa bovine GalTase proteins were phosphorylated by $p58^{GTA}$, and that the ^{32}P-labelled isoenzyme forms were identical to those published by Strous and Colleagues (9). Next, we treated bovine GalTase with potato acid phosphatase. In Figure 5 (lane 2) we show that in the presence of potato acid phosphatase GalTase activity is diminished approximately 6-fold as compared to native bovine GalTase (lane 1). To demonstrate that this effect was not due to proteolysis, the same experiment was performed in the presence of 100 mM phosphatase substrate, 4-nitrophenyl phosphate. There was no similar decrease in

Figure 4. Two dimensional gel analysis of the 42 and 48 kDa bovine GalTase proteins after phosphorylation by the purified bovine $p58^{GTA}$ kinase. The pH gradient was between 3.5 and 10. The position of bovine serum albumin (pH 4.7) is indicated by the arrow.

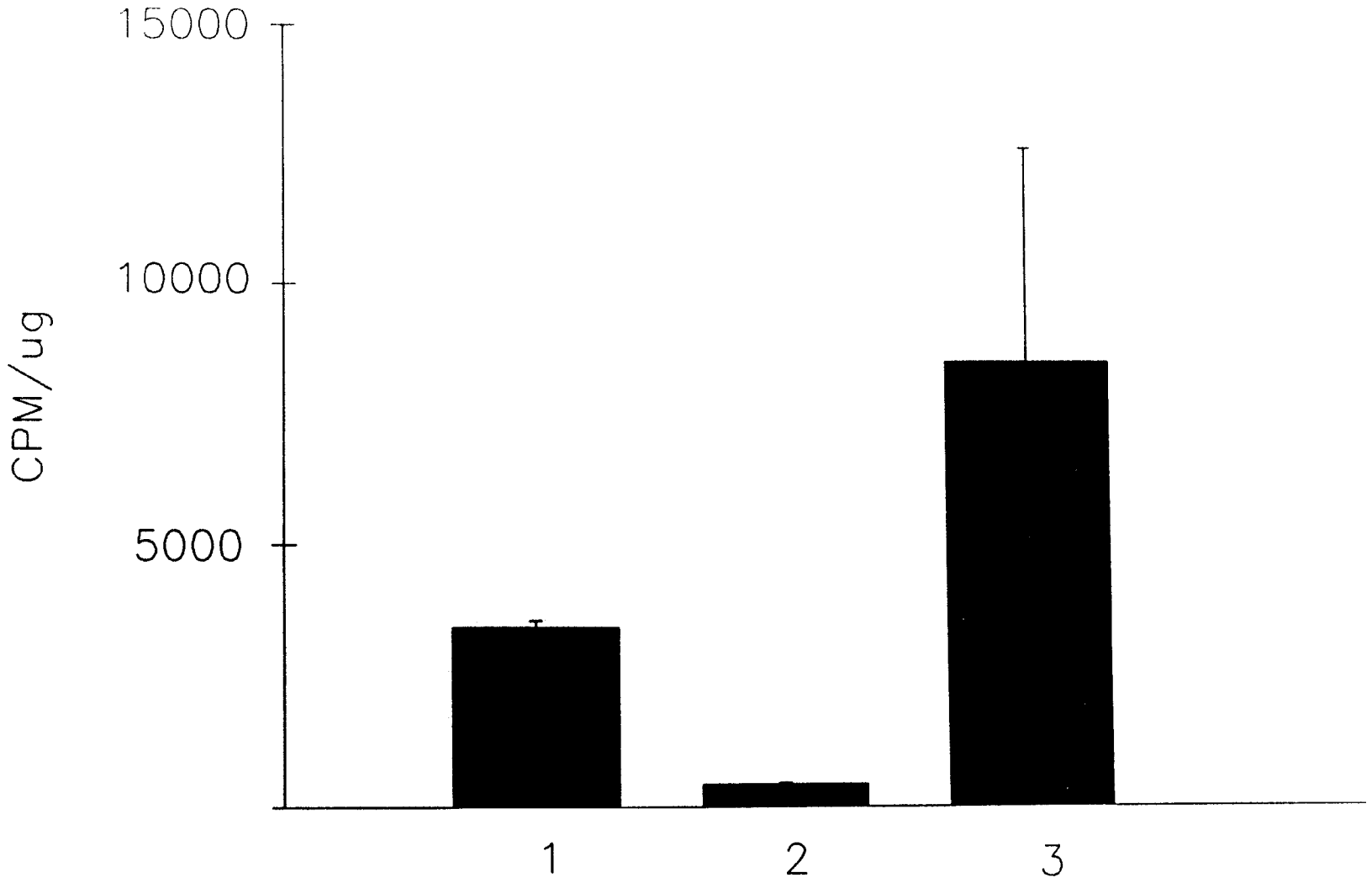
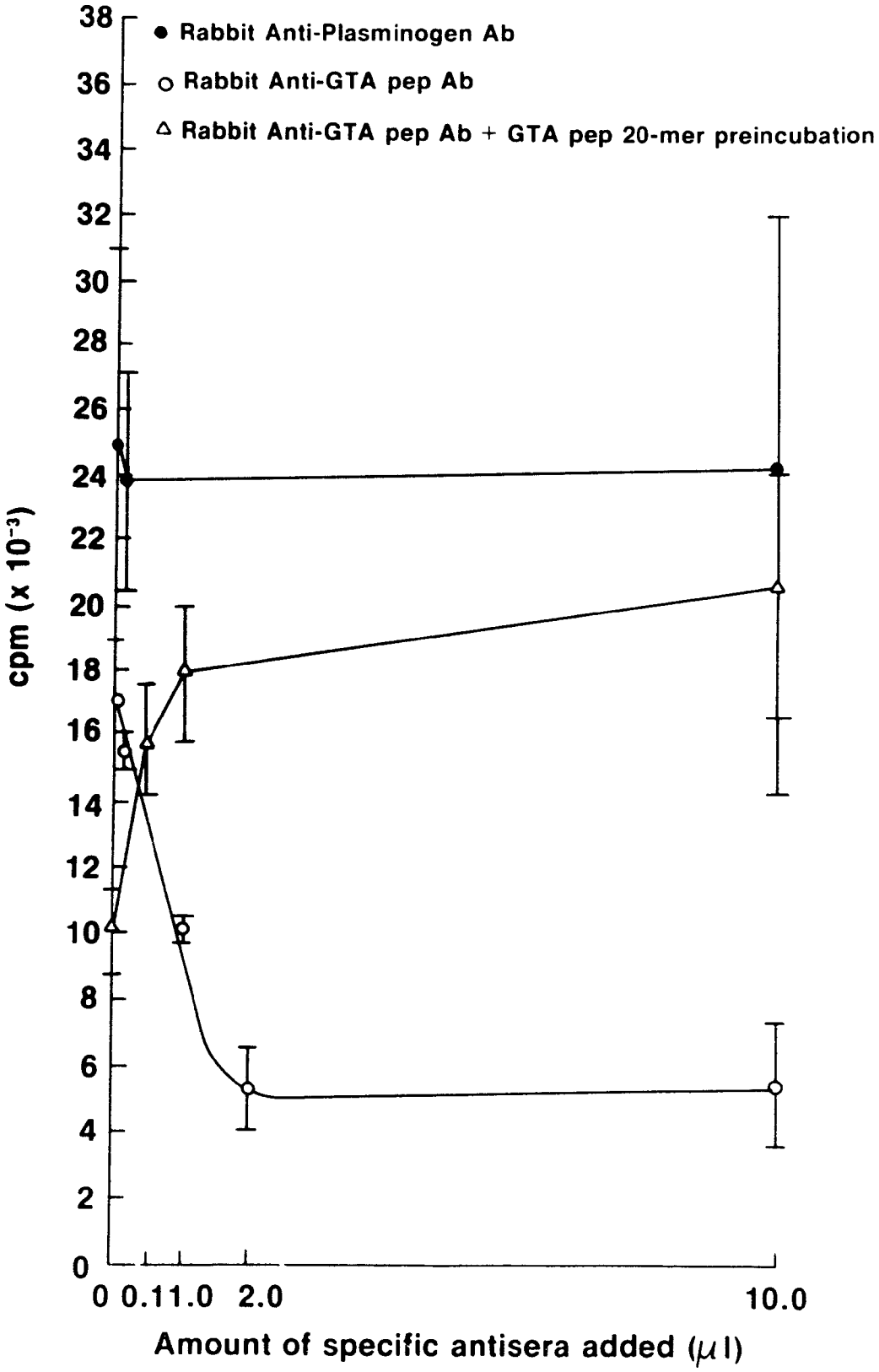

15000
10000
5000
CPM/ug
1
2
3

Figure 5. GalTase enzyme assays in the absence and presence of exogenously added potato acid phosphatase. Lane 1, 0.2 units of bovine GalTase. Lane 2, 0.2 units of bovine GalTase after treatment with potato acid phosphatase. Lane 3, same as lane 2 with the addition of 100 mM phosphatase substrate during the potato acid phosphatase incubation. All experiments were performed in triplicate. Standard deviations are indicated above each bar.


48
42
+
−

Figure 6. $p58^{GTA}$ antibody inhibition of GalTase enzyme activity. Increasing amounts of a $p58^{GTA}$ peptide antibody (open circles) were preincubated with bovine GalTase before enzyme assay analysis. Similarly, a fixed amount of $p58^{GTA}$ peptide antibody (1 mg/ml) was preincubated with increasing amounts of competing $p58^{GTA}$ peptide (100 nM, 1,000 nM and 10 mM) and bovine GalTase (triangle) before analysis. A nonspecific antibody control (filled circles) was also tested.

GalTase affected GalTase enzyme activity. We found that the $p58^{GTA}$ antibody decreased GalTase activity, and that this inhibition could be competed with the peptide to which the antibody was made (Figure 6). In addition, a nonspecific antibody control did not effect GalTase enzyme activity (Figure 6).


● Rabbit Anti-Plasminogen Ab
○ Rabbit Anti-GTA pep Ab
△ Rabbit Anti-GTA pep Ab + GTA pep 20-mer preincubation
cpm (x 10⁻³)
38
36
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
0 0.1 1.0 2.0 10.0
Amount of specific antisera added (μl)

GalTase activity (Figure 5, lane 3). The observed increase in GalTase activity in the presence of phosphatase substrate may reflect the presence of a contaminating phosphatase in the semi-purified bovine GalTase proteins. This was confirmed by incubating the bovine GalTase in the presence of 100 mM 4-nitrophenylphosphate alone (data not shown). Finally, we used a monospecific antibody to the $p58^{GTA}$ kinase (3,4) to determine whether pre-incubation of the antibody with bovine GalTase affected GalTase enzyme activity. We found that the $p58^{GTA}$ antibody decreased GalTase activity, and that this inhibition could be competed with the peptide to which the antibody was made (Figure 6). In addition, a nonspecific antibody control did not effect GalTase enzyme activity (Figure 6).

Our results suggest that GalTase activity is influenced by $p58^{GTA}$ kinase expression, possibly via phosphorylation of GalTase itself. Further, more definitive experiments are now underway using reconstituted *in vitro* systems and *in vivo* labeling to determine whether or not the $p58^{GTA}$ kinase functions to regulate cellular GalTase activity.

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CELL CYCLE REGULATION OF THE EXPRESSION AND ACTIVITY OF A p58 PROTEIN KINASE

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SUMMARY

A new human serine/threonine protein kinase, p58, containing a kinase domain that is 46% identical to human $p34^{cdc2}$ was previously isolated and characterized. Minimal overexpression of the p58 kinase in CHO cells resulted in a delay of the normal cell cycle in late telophase, as well as dramatically increased mitotic abnormalities. Here we report that p58 protein kinase expression and activity are coordinately regulated with $p34^{cdc2}$ during the cell cycle. Namely, p58 protein kinase activity peaks during mitosis and during late G_1- and early S- phase, while steady-state levels of the p58 protein remain constant. From studies presented in this report it appears that the in vivo regulation of the p58 kinase may be due to differential levels of phosphorylation of serine residues on the protein during the cell cycle. At the G_1/S- phase boundary p58 is heavily serine phosphorylated, while it is only minimally serine phosphophorylated during mitosis. In addition, serine phosphorylation of the p58 protein kinase is essential for phosphorylation of substrates in vitro. Finally, the potential relationship between numerous $p34^{cdc2}$ phosphorylation sites in the p58 sequence, its coordinate regulation with $p34^{cdc2}$ during the cell cycle, and its differential phosphorylation during the cell cycle are discussed.

INTRODUCTION

A great deal of progress has been made in the past few years towards an understanding of the molecular mechanisms responsible for cell cycle control (for reviews see Nurse, 1990 and Lewin, 1990). A major component of both the G_2- to M- and G_1- to S-phase transitions is the serine/threonine protein kinase $p34^{cdc2}$. Elegant studies have demonstrated that this protein kinase is the catalytic subunit of starfish, clam and Xenopus oocyte maturation promoting factor (MPF) (Arion et al., 1988; Draetta et al., 1989; Dunphy et al., 1988; Gautier et al., 1988) and its somatic cell mitotic counterpart (Draetta and Beach, 1988; Langan et al., 1989; Brizuela et al., 1989). When $p34^{cdc2}$ is associated with a cyclin regulatory subunit the $p34^{cdc2}$/cyclin protein kinase complex phosphorylates a number of cellular substrates that play crucial roles in regulating the physical changes that occur in an orderly fashion during the cell cycle (Moreno et al., 1989; Norbury, and Nurse, 1989). It has been suggested that the $p34^{cdc2}$ protein kinase is, in fact, both the S- and M-phase kinase in mammalian cells, and that its specificity is regulated by distinct post-translational modifications of the protein that occur during the cell cycle (Pines and Hunter, 1990; Pondaven et al., 1990; Moreno et al., 1989; Broek et al., 1991). Broek and colleagues have found that cell mutants of the yeast S. pombe capable of replicating their DNA twice without mitosis encode mutated alleles of

the cdc2 protein kinase (Broek et al., 1991). These results strongly suggest that distinct post-translational modifications of cdc2 demark whether or not the protein functions during the S-phase or M-phase transition. In further support of this notion, Gould and Nurse (1989) found that phosphorylation of tyrosine residue 15 of the mitotic form of S. pombe cdc2 inhibited kinase activity. They also found that inhibition of $p34^{cdc2}$ kinase activity prevented the completion of mitosis. This inhibition was relieved by the action of an unknown phosphatase, subsequently activating the cdc2/cyclin complex which allowed normal progression through mitosis. More recently, several $p34^{cdc2}$-related molecules have been isolated from various sources (Courchesne et al., 1989; Elion et al., 1990; Pines and Hunter, 1990; Paris et al., 1991). The exact functions of these cdc2-related proteins are not precisely known. One mammalian protein, $p32^{cdc2}$, has been found to be preferentially associated with cyclin A during S-phase (Pines and Hunter, 1990). The yeast cdc2-related protein KSS1 has been found to overcome pheromone-induced arrest of cell cycling, while the yeast cdc2-related protein FUS3 leads to the arrest of cells in G_1 and mating (Courchesne et al., 1989; Elion et al., 1990). A function for the Eg-1 protein, which is more highly related to $p34^{cdc2}$ than either KSS1 or FUS3, remains to be shown. However, based on the regulation of Eg-1 expression in Xenopus oocytes and actively proliferating Xenopus adult

kidney and testis cells, it has been proposed that Eg-1 might be involved in the G_1- to S- phase transition (Paris et al., 1991).

A new, more distantly cdc2 structurally related protein kinase, p58, has also recently been described (Bunnell et al., 1990a, b; Kidd et al., 1991). This p58 protein kinase contains three distinct structural domains: an NH_2-terminal putative regulatory domain, a 299 amino acid cdc2-related domain, and a 61 amino acid COOH-terminal domain. However, one of the hallmarks of $p34^{cdc2}$-related protein kinase, the conserved "PSTAIR" motif found immediately downstream of the proposed ATP-binding domain (Nurse, 1990), is not found in the p58 protein kinase. Other regulatory features, such as threonine 14, tyrosine 15 and threonine 167, are completely conserved at equivalent positions in the p58 protein kinase. A major portion of the p58 protein kinase appears to be localized to the nucleolus, while smaller amounts are found in the cytoplasm. When this p58 protein kinase is overexpressed minimally a unique phenotype results; namely, a major portion of an asynchronously growing cell population is delayed in late telophase and growth of these cells is slowed. Furthermore, approximately half of the cells delayed at late telophase proceed to form numerous micronuclei due to abnormal mitoses. The p58 protein kinase has also been shown to be well conserved evolutionarily, expressed at early time points during

murine embryogenesis, but not at birth. It is then reexpressed in all terminally-differentiated adult tissues examined thus far (Kidd et al., 1991). These results suggested that the p58 protein kinase might function during the cell cycle. Therefore, a systematic study was undertaken to biochemically characterize the p58 protein kinase as a function of the cell cycle in mammalian cells. In this report we find that p58 gene expression and kinase activity are coordinately regulated with, but distinct from, $p34^{cdc2}$ kinase regulation as a function of cell cycle. We also find that the p58 protein kinase is a phosphoprotein, containing primarily phosphoserine, that its phosphorylation state changes during the cell cycle, and that changes in p58 phosphorylation affect kinase activity in vitro. The implications of these results relative to the previously observed subcellular localization and altered phenotype due to minimal overexpression will be discussed, as will its coordinate regulation with the $p34^{cdc2}$ protein kinase.

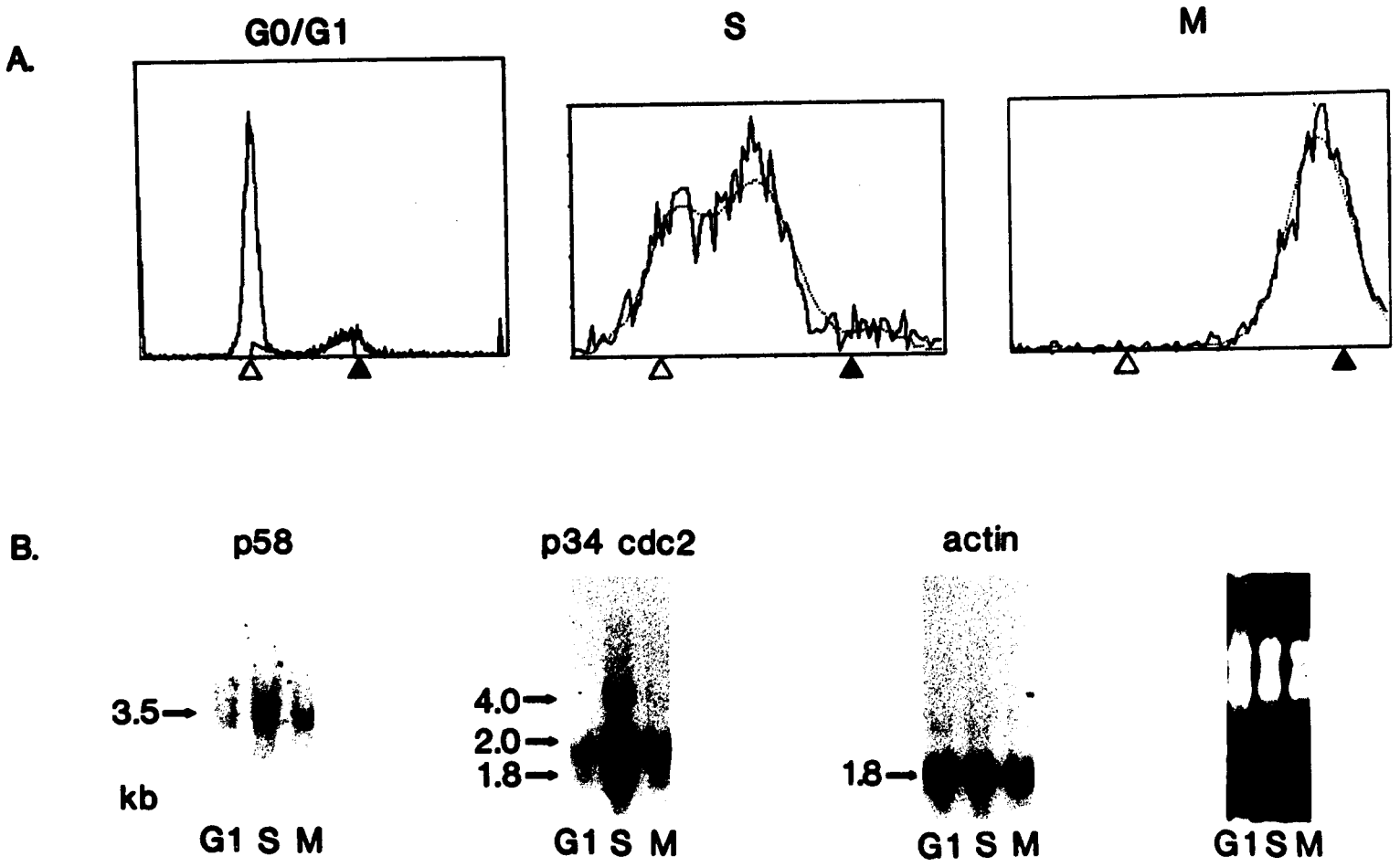
RESULTS

Expression of the p58 protein kinase mRNA during the cell cycle.

Experiments by McGowan et al (1990) have demonstrated that $p34^{cdc2}$ gene expression is regulated in a periodic manner during the HeLa cell cycle. We undertook similar, but more abbreviated studies, of p58 gene expression. HeLa cells were blocked in G_0/G_1-, G_1/S- or G_2/M- phase by

treatment with low serum, thymidine/aphidicolin or nocodazole, respectively. Aliquots of cells were then either analyzed by flow cytometry (Figure 1, panel A) or total RNA was extracted for Northern blot analysis. The Northern blot was sequentially hybridized with the human p58 cDNA, human $p34^{cdc2}$ cDNA or human gamma-actin cDNA probes (Figure 1, panel B). Steady-state levels of p58 mRNA peaked during S-phase, (Figure 1, panel B, lane 2), were substantially lower during G_2- and M- phase (Figure 1, panel B, lane 3), and were negligible in $G0/G_1$- phase cells (Figure 1, panel B, lane 1). Similarly, steady-state $p34^{cdc2}$ mRNA levels were highest in S- phase and lowest in G_0/G_1- phase cells (Figure 1, panel B), which is in accordance with data presented by others (McGowan et al., 1990). Identical loadings of total RNA was confirmed by hybridization with a gamma-actin cDNA probe as well as visualization of the ethidium bromide stained RNA samples (Figure 1, panel B). It is worth noting that p58 mRNA levels are approximately 10-20% of the level of $p34^{cdc2}$ steady-state mRNA levels in HeLa cells (Figure 1, panel B; confirmed by laser densitometry, and accounting for differences in specific activities of the various probes and exposure times, data not shown).

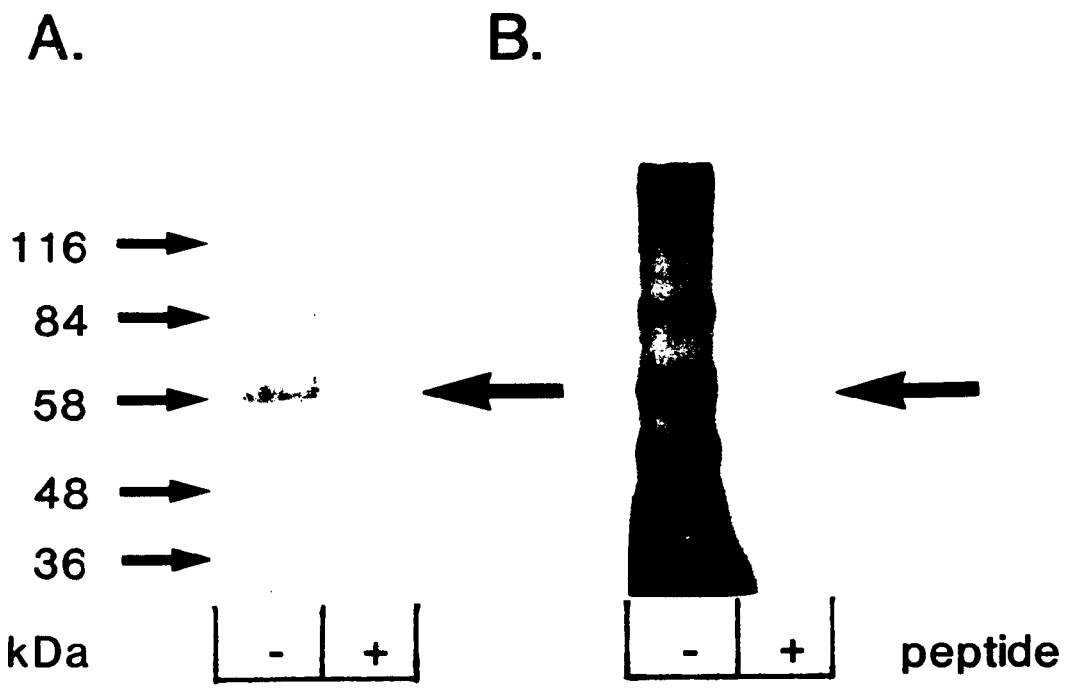
Figure 1. Cell cycle analysis of steady-state mRNA for p58, $p34^{cdc2}$, and gamma-actin. (Panel A) HeLa cells were blocked in either G_0/G_1-, G_1/S-, or G_2/M-phase and an aliquot of these cells analyzed by flow cytometry as described in experimental procedures. The results are shown for each cell fraction. Open arrowheads equal 2N DNA content while filled arrowheads equal 4N DNA content. (Panel B) Total RNA extracted from the cells described in panel A was electrophoresed on a 1.5% agarose-formaldehyde gel and transfered to a nylon-nitrocellulose membrane for hybridization. The same blot was sequentially hybridized to the indicated probes. A corresponding ethidium bromide stained gel of identical RNAs is also shown. The phase of the cell cycle corresponding to the RNA samples is shown directly below each lane. Molecular weights (in kilobases) is shown to the left of the various transcripts.

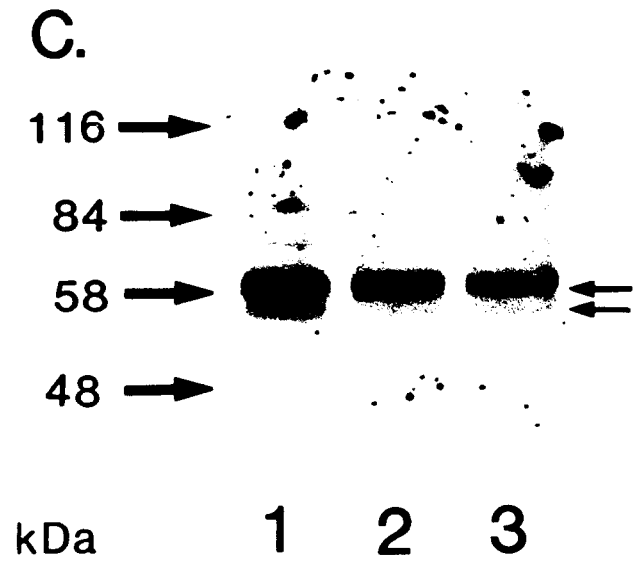

A.
G0/G1
S
M
B.
p58
p34 cdc2
actin
3.5
kb
4.0
2.0
1.8
1.8
G1 S M
G1 S M
G1 S M
G1 S M

Analyses of p58 immunoprecipitates from metabolically labeled cells.

We have previously reported the characterization of an affinity-purified p58 peptide antisera (Bunnell et al., 1990a). To assess the ability of this antisera to specifically immunoprecipitate the p58 protein kinase from HeLa cell RIPA lysates we metabolically labeled cells with Tran-^{35}S label and then immunoprecipitated p58 protein either in the absence or presence of competing cold peptide (Figure 2, panel A). A broad, predominant 58 kDa band, as well as lesser amounts of a larger 80-90 kDa band, was seen in the absence of competing peptide but not in the presence of 100 nM competing peptide (Figure 2, panel A). Prolonged exposures of this gel did not reveal any other significant protein bands (data not shown). The additional 80-90 kDa protein band most likely represents a polypeptide that may form a complex with p58, since it too is competed by the addition of the cold peptide. These results are not unlike those seen with immunoprecipitates of the p34^{cdc2} protein kinase (Draetta and Beach, 1988). The broad nature of the p58 protein band suggested that the protein might be post-translationally modified. Based on the similarity to p34^{cdc2}, one of the most obvious modifications could be phosphorylation of tyrosine, threonine or serine residues of the p58 protein. We, therefore, metabolically labeled HeLa cells with ^{32}P-orthophosphate and performed an identical p58 immunoprecipitation (Figure 2, panel B).

Figure 2. Immunological detection of the p58 protein kinase from metabolically labeled cells. (Panel A) Immunoprecipitation of [^{35}S]methionine, cysteine labeled HeLa cell lysate in the absence (-) or presence (+) of a 100 nM concentration of cold p58 peptide to which the antisera is made. Molecular weight markers are shown to the left. The large arrow on the right indicates the position of the p58 protein. (Panel B) Immunoprecipitation of [^{32}P] orthophosphate labeled HeLa cell lysate in the absence (-) or presence (+) of a 100 nM concentration of cold p58 peptide. The molecular weight markers co-migrate with those shown in panel A. The large arrow on the right indicates the position of the p58 protein. (Panel C) Immunoprecipitation of [^{35}S] methionine, cysteine pulse-chase labeled HeLa cell lysate with the affinity-purified p58 antisera. Cells were labeled for 4 hours in the presence of [^{35}S] methionine, cysteine and then chased by growth in media containing cold methionine and cysteine for 30 minutes (lane 1), 60 minutes (lane 2), or 2 hours (lane 3). Molecular weight markers are shown on the left. The two arrows of the right indicate the position of the two different migrating forms of p58.


A.
B.
116
84
58
48
36
kDa
-
+
-
+
peptide


C.
116
84
58
48
kDa
1
2
3

We found that p58 was, indeed, a phosphoprotein as was the 80-90 kDa polypeptide seen previously, and that both polypeptides were competed by addition of a 100 nM concentration of the cold peptide (Figure 2, panel B). In addition, smaller 30-36 kDa and 50 kDa phosphoproteins were observed that were not seen when tran-^{35}S label was used to metabolically label the cells (compare Figure 2, panels A and B). Immunoblots of the p58 anti-peptide antisera immunoprecipitations from HeLa cells using p34^{cdc2} "PSTAIR" antisera did not demonstrate the presence of p34^{cdc2} in these immunoprecipitates (data not shown). Therefore, under the conditions used in these experiments the presence of p34^{cdc2} was ruled out. This is supported further by p13^{suc1} depletion experiments described below. Finally, to examine p58 protein stability, as well as the potential nature of the broad band seen in the first experiment, a pulse-chase experiment was performed using Tran-^{35}S label and a higher percentage protein gel in an attempt to resolve the p58 region (Figure 2, panel C). We found that there were at least two apparent p58 polypeptides that differed slightly in mobility at early time points (Figure 2, panel C, lane 1), and that the faster migrating band disappeared within two hours of the chase (Figure 2, panel C, lane 3). Additionally, the overall amount of p58 protein decreased slightly over this same period of time. Treatment of the slower migrating form of p58 with phosphatase resulted in significant conversion to the

faster migrating form (data not shown). Further details pertaining to the phosphorylation state of p58 during the cell cycle are presented below.

Regulation of p58 protein kinase activity, de novo protein synthesis, and steady-state protein levels in thymidine/nocodazole blocked and released cells.

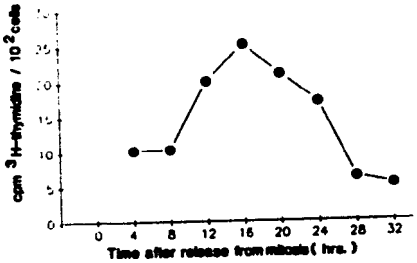
To monitor the abundance and activity of the p58 protein kinase during the cell cycle, cells were blocked with thymidine and then the microtubule destabilizing drug nocodazole and then released and monitored for a period of 32 hours. Treatment of cells with nocodazole produces a reversible metaphase arrest (Zieve et al., 1980). Determination of a successful cell cycle block was assessed by flow cytometry as described above, as well as the ability of the released cells to continue proliferation and growth as assessed by measuring the rate of DNA replication (Nilsen and Baglioni, 1979). Flow cytometry demonstrated that greater than 90% of the cells were blocked at the expected position in cell cycle (data not shown). Further demonstration of cell synchrony and viability was shown by the ability of these cells to continue progression through the cell cycle normally when released from the cycle block, as judged by [^{3}H]thymidine incorporation into DNA (Figure 3, panel A). We then determined the level of H1 kinase activity associated with RIPA cell extracts by incubation with the $p13^{suc1}$ protein coupled to sepharose beads (Pondaven et al., 1990). It has been demonstrated that all

known cdc2 and cdc2-like kinases bind to the $p13^{suc1}$ protein, and this methodology is routinely used to assay H1 kinase activity from cycling cells or oocytes (Dunphy et al., 1988; Dunphy and Newport, 1989; Arion et al., 1988; Draetta et al., 1989; Meijer et al., 1989; Brizuela et al, 1989; Paris et al., 1991). Extracts from the mitotically blocked and released HeLa cells demonstrated the character-istic H1 kinase cycling pattern (Figure 3, panels B and C). These same HeLa cell extracts were depleted a second time with $p13^{suc1}$ beads and then used for direct immunoprecipitation ofthe p58 protein kinase. The purpose of the $p13^{suc1}$ depletion was two-fold; 1) to demonstrate the synchrony of the HeLa cell extracts with regard to all H1 kinase activities, and 2) to deplete any $p13^{suc1}$-binding proteins before p58 immuno-precipitation. Earlier experiments have demonstrated that $p13^{suc1}$ beads do not deplete any portion of the p58 protein kinase from either HeLa cell extracts (Adams and Kidd, data not shown) or from sea urchin oocytes (Meijer and Kidd, unpublished).

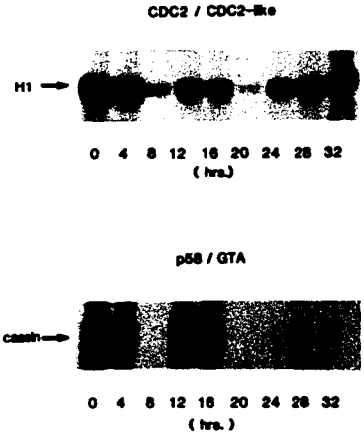
The $p13^{suc1}$-depleted HeLa cell extracts were then immunoprecipitated with the previously characterized affinity-purified p58 peptide antisera (Bunnell et al., 1990a). This antisera was made to a portion of the putative NH_2-terminal regulatory domain of p58, and shares no sequence homology with the $p34^{cdc2}$ protein kinases or any other reported protein kinase sequence (Bunnell et al.,

Figure 3. Cell cycle regulation of p58 after a block in mitosis. (Panel A) [^{3}H]Thymidine (Td) incorporation in HeLa cells that were blocked in mitosis with thymidine/nocodazole and subsequently released for continued growth. The peak in DNA replication at 16 hours post-nocodazole block corresponded to maximal levels of HeLa cells (>90%) in S-phase as judged by flow cytometry (data not shown). The time after release from the cell cycle block is shown in hours on the x-axis, while the level of [^{3}H]-Td incorporation (cpm/100 cells) is shown on the y-axis. (Panel B) Protein kinase assays for histone H1 kinase activity and p58 kinase activity from lysates of HeLa cells corresponding to the cells described in panel A. Time points were normalized for equal amounts of protein before either p13^{suc1}-Sepharose depletion (H1 kinase) or subsequent anti-peptide precipitation (p58). The p58 immunoprecipitation was performed on lysates that were first depleted of H1 kinase activity by p13^{suc1}-Sepharose incubation. These results are representative of four different experiments. (Panel C) The labeled histone H1 or casein bands shown in the autoradiograms in panel B were excised from the dried gels and the Cherenkov counts determined as described in experimental methods. (Panel D) p58 anti-peptide antisera immunoprecipitation of cells labeled for 4 hrs with [^{35}S]-methionine, cysteine prior to collection and lysis. The cell lysates were normalized for equal cpm before precipitation. The arrow on the right indicates the position of the p58 protein. (Panel E) Immunoblot of 100 μg of lysate from each time point, probed with the p58 anti-peptide antisera using a second antibody detected by alkaline phosphatase.

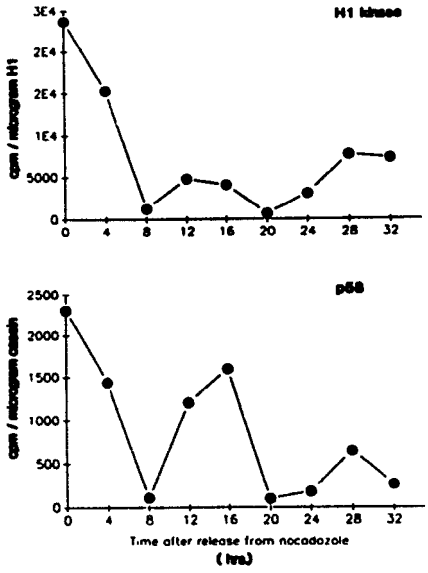
A.


cpm ^{3}H-thymidine / 10^2 cells
0 4 8 12 16 20 24 28 32
Time after release from mitosis (hrs.)

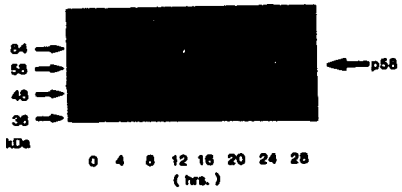
B.


CDC2 / CDC2-like
H1
0 4 8 12 16 20 24 28 32
(hrs.)
p58 / GTA
casein
0 4 8 12 16 20 24 28 32
(hrs.)

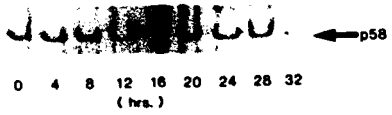
C.


H1 kinase
cpm / nanogram H1
3E4
2E4
2E4
1E4
5000
0
0 4 8 12 16 20 24 28 32
p58
cpm / nanogram casein
2500
2000
1500
1000
500
0
0 4 8 12 16 20 24 28 32
Time after release from nocadozole
(hrs.)

D.


84
58
48
36
kDa
p58
0 4 8 12 16 20 24 28
(hrs.)

E.


p58
0 4 8 12 16 20 24 28 32
(hrs.)

1990a). We found that p58 protein kinase activity varied during the cell cycle, with a major peak of activity coinciding with G_2/M- phase and a second smaller peak of activity coinciding with the G_1/S- phase boundary (Figure 3, panels B and C). Synchrony of this activity was maintained to approximately 32 hours post-nocodazole release, which also correlated with the timing of a second mitosis in these cells.

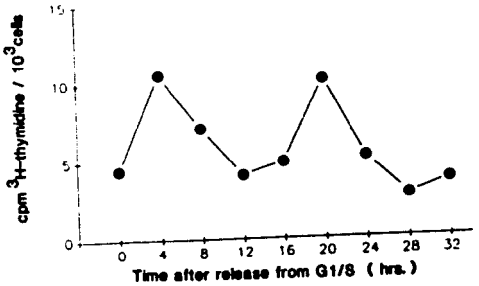
We then repeated the experiment as described above, only this time the cells were metabolically labeled with Tran-^{35}S label to monitor <u>de novo</u> p58 protein synthesis as a function of cell cycle. Others have shown that even though steady-state $p34^{cdc2}$ protein levels are constant during the cell cycle, periodic <u>de novo</u> biosynthesis of $p34^{cdc2}$ occurs (McGowan, 1990). Similarly, p58 <u>de novo</u> protein synthesis appears to be periodic, peaking with mitotic activity (time = 0 hrs.) (Figure 3, panel D). However, like $p34^{cdc2}$, steady-state p58 protein levels did not vary appreciably during the cell cycle (Figure 3, panel E). Equal loadings of total cellular protein for each time point was verified by Coomassie blue staining of these proteins (data not shown). It should be noted that in all of the experiments described above, p58 protein kinase activity and steady-state levels were found to be approximately 10% that of H1 kinase activity bound to $p13^{suc1}$, which is comparable to the differences in $p34^{cdc2}$ and p58 steady-state mRNA levels.

Regulation of p58 protein kinase activity, de novo protein synthesis, and steady-state protein levels in thymidine/aphidicolin blocked and released cells.

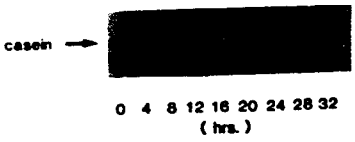
The experiments described in the previous section strongly suggested that p58 kinase activity peaked at two different points in the cell cycle; namely, a large peak of activity associated with the G_2/M- phase boundary, and a somewhat smaller peak of activity associated with the G_1/S-phase boundary. Since these experiments were performed with a microtubule destabilizing drug, nocodazole, we decided to perform an identical series of experiments using a second, and distinct, methodology for cell cycle synchronization to verify these results. Others have shown that sequential treatment of HeLa cells with thymidine/aphidicolin is a viable methodology for cell synchronization of HeLa cells at the G_1/S-phase boundary (Pines and Hunter, 1989). Therefore, HeLa cells were blocked and released by this method and cell synchrony determined by flow cytometry and by measuring the ability of these cells to continue to replicate DNA. Once again, flow cytometry demonstrated that greater than 90% of the cells were blocked at the appropriate point in the cell cycle (data not shown), and further demonstration of cell synchrony and viability after release from the block was shown by [^{3}H]thymidine incorporation into DNA (Figure 4, panel A). The p58 protein kinase was then immunoprecipitated from RIPA lysates of these cells and the

Figure 4. Cell cycle regulation of p58 after a block at the G_1/S-phase boundary. (Panel A) [^{3}H]-Td incorporation in HeLa cells that were blocked at the G^1/S-phase boundary by sequential treatment with thymidine/aphidicoline and subsequently released for continued growth. The peaks in DNA replication observed at 4 and 20 hours post release corresponded to large numbers (> 90%, 4 hrs; > 70%, 20 hrs.) of cells in S-phase as determined by flow cytometry while, the dip in DNA replication at 12 hrs. post release corresponds to large numbers (> 90%) of cells in mitosis (data not shown). (Panel B) p58 protein kinase assays from lysates of HeLa cells corresponding to the cells described in panel A. Time points were normalized for equal amounts of protein before immunoprecipitation with the p58 anti-peptide antisera. These results are representative of four different experiments. (Panel C) The labeled casein bands were excised from the dried gel shown in panel B and the Cherenkov counts determined. (Panel D) p58 anti-peptide antisera immunoprecipitation of cells labeled for 4 hours with [^{35}S]-methionine, cysteine prior to collection and lysis. The cell lysates were normalized for equal cpm before precipitation. The arrow on the right indicates the position of the p58 protein. (Panel E) Immunoblot of 70 μg of lysate from each time point, probed with the p58 anti-peptide antisera using a secondary antibody detected by alkaline phosphatase.

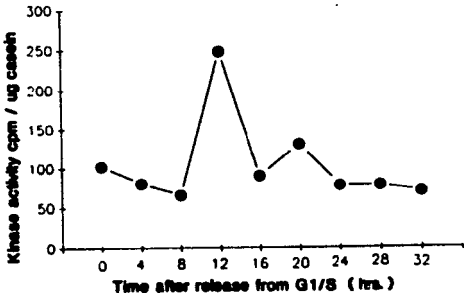
A.


cpm ^{3}H-thymidine / 10^3cells
15
10
5
0
0 4 8 12 16 20 24 28 32
Time after release from G1/S (hrs.)

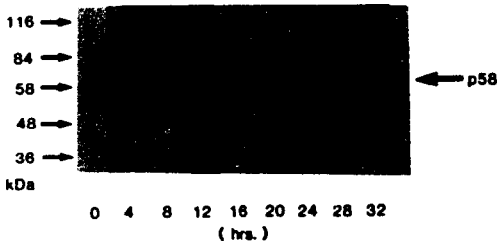
B.


casein
0 4 8 12 16 20 24 28 32
(hrs.)

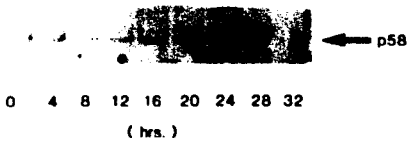
C.


Kinase activity cpm / ug casein
300
250
200
150
100
50
0
0 4 8 12 16 20 24 28 32
Time after release from G1/S (hrs.)

D.


116
84
58
48
36
kDa
p58
0 4 8 12 16 20 24 28 32
(hrs.)

E.


p58
0 4 8 12 16 20 24 28 32
(hrs.)

associated protein kinase activity determined (Figure 4, panels B and C). Distinct peaks in p58 protein kinase activity were found associated with cells at the G_2/M-phase boundary. In addition, the magnitude of p58 protein kinase activity was highest at G_2/M, and somewhat lower at G_1/S (Figure 4, panel C). These results are identical to those obtained using the mitotic block and release with regard to timing and magnitude during the cell cycle.

This same experiment was repeated as described above and the cells were metabolically labeled with Tran-^{35}S label to monitor de novo p58 protein synthesis. We found that p58 is most actively synthesized during late S- and G_2-phase (time= 12 hrs.) (Figure 4, panel D). This is in agreement with the results obtained in the previous experiments using thymidine/nocodozale. When we examined steady-state p58 protein levels from these cells we found that the levels did not vary appreciably during the cell cycle (Figure 4, panel E), once again in agreement with the results presented earlier.

Phosphorylation status of p58 during the cell cycle.

In this study we have shown that the p58 protein kinase is a phosphoprotein and that metabolically labeled cells rapidly (0.5-2.0 hrs.) convert a faster migrating form of the protein to a slightly slower migrating form. To examine the potential differences in the phosphorylation state of the p58 protein, during the cell cycle equivalent numbers of HeLa cells were either blocked at the G_1/S- or

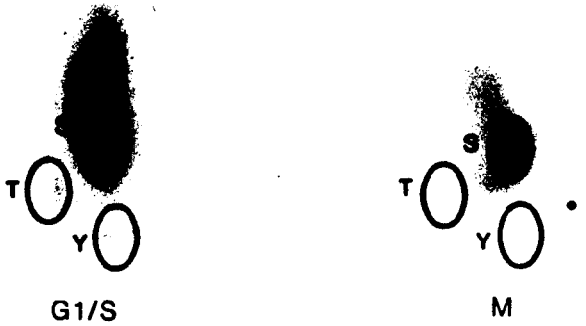
G_2/M- phase boundary and metabolically labeled with orthophosphate. Total cell protein was then isolated and the level of ^{32}P-incorporation quantitated. G_2/M- phase blocked cells incorporated approximately 5-fold more phosphate than G_1/S- phase cells (see Experimental Procedures). The p58 protein kinase was then immunoprecipitated from these samples as described previously. Surprisingly, the p58 protein kinase was more heavily phosphorylated in the G_1/S- phase cells (Figure 5, panel A). To examine the nature of this phosphorylated p58 protein more closely, the bands were excised from the dried gel and subjected to phosphoamino acid analysis. We found that the p58 protein kinase was heavily phosphorylated on serine, and to a much lesser extent on threonine and possibly tyrosine, in G_1/S- phase cells (Figure 5, panel B). Cells blocked in G_2/M- phase contained p58 that was phosphorylated at much lower levels on serine, and to no detectable level on either threonine or tyrosine (Figure 5, panel B). These results are quite distinct from the *in vivo* phosphoamino acid analysis of $p34^{cdc2}$ from HeLa cells, which is primarily phosphorylated on threonine and tyrosine, and to a lesser extent serine (Draetta et al., 1988).

Previously, we reported that active bovine p58 protein kinase could be purified to apparent homogeneity (Bunnell et al., 1990a). We therefore decided to examine the effects of specific phosphatase treatment on the activity

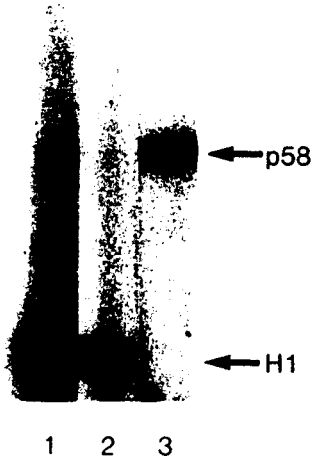
Figure 5. Phosphoamino-acid analysis of the p58 protein kinase from G_1/S-phase and G_2/M-phase HeLa cells. (Panel A) Autoradiograph of p58 immunoprecipitated from [^{32}P] orthophosphate labeled cell lysates corresponding to either the G_1/S-phase or G_2/M-phase boundary. Identical numbers of HeLa cells ($1X10^6$) were used for lysis and immunoprecipitation of p58 procedures (for details see experimental procedures). (Panel B) 2-D-phosphoamino-acid analysis of the excised p58 bands shown in panel A. The G_1/S-phase ^{32}P-labeled p58 panel was exposed for 1 week. The G_2/M-phase ^{32}P-labeled p58 panel was exposed for 3 weeks. (Panel C) In vitro kinase reactions using p58 anti-peptide antisera immunoprecipitated bovine p58 protein kinase (which had been purified to apparent homogeneity beforehand). Histone H1 was used as a substrate in all three reactions shown. Lane 1, p58, untreated; lane 2, p58 treated with 0.1 units CIAP in the presence of excess phosphatase substrate; lane 3, p58 treated with 0.1 units CIAP in the absence of phosphatase substrate. The relative positions of phosphorylated histone H1 and p58 are indicated on the right.

A.

B.



C.



of the purified bovine p58 protein kinase. Calf intestine alkaline phosphatase (CIAP) is known to dephosphorylate phosphoserine residues but not phosphothreonine residues (McVey et al., 1989), and it is easily inactivated by brief exposure to high temperature. We had previously determined that the p58 protein kinase was stable at 68°C for up to 10 minutes (Adams, unpublished data). Equivalent amounts of purified active bovine p58 protein kinase were immunoprecipitated with the affinity-purified p58 peptide antibody and then either left untreated, incubated in the presence of CIAP and excess phosphatase substrate, or incubated with CIAP (and the CIAP subsequently heat inactivated). Following these treatments the p58 protein kinase was assayed *in vitro* using histone H1 as a substrate. We found that, as previously reported (Bunnell et al., 1990a), the untreated p58 protein kinase phosphorylated histone H1 (Figure 5, panel C, lane 1). In the presence of CIAP and excess phosphatase substrate histone H1 phosphorylation was slightly diminished, but not destroyed (Figure 5, panel C, lane 2). While p58 protein kinase treated only with the CIAP lost its ability to phosphorylate histone H1 entirely, it appeared to undergo significant autophosphorylation (Figure 5, panel C, lane 3). These results suggest that some degree of serine phosphorylation of the kinase is necessary for activity and are in agreement with the *in vivo* phosphoamino acid analysis of p58. They further suggest that once the p58

kinase has been dephosphorylated by CIAP it is capable of undergoing autophosphorylation. However, autophosphorylation of p58 is not sufficient to restore full kinase activity using histone H1 as a substrate. Further experiments are now underway to specifically map the phosphorylation sites of the p58 protein kinase. Despite repeated attempts, we were not able to reproducibly demonstrate changes in tyrosine phosphorylation of p58 during the cell cycle with antiphosphotyrosine antibodies (data not shown).

DISCUSSION

In this study we have shown that the serine/threonine protein kinase p58, which contains a protein kinase domain that is distantly related to $p34^{cdc2}$, is itself regulated during the cell cycle in mammalian cells. This was demonstrated by use of two distinct methodologies to block and release HeLa cells in the cell cycle, and the subsequent analyses of the p58 gene product. First, the expression of steady-state p58 mRNA closely mimics the periodic expression of steady-state $p34^{cdc2}$ mRNA (McGowan et al., 1990). Secondly, p58 protein kinase activity fluctuates during the cell cycle in a periodic manner that resembles the activity of the histone H1 kinase in HeLa cells, and the timing of that activity is identical in HeLa cells synchronized and released by two different methodologies. Thirdly, de novo synthesis of the p58 protein kinase fluctuates during the cell cycle, peaking

during late S- and G_2- phase, while the steady-state level of p58 protein remains relatively constant using the two separate methods for blocking cells. Once again, these characteristics of p58 expression closely mimic the periodic biosynthesis and steady-state levels of the $p34^{cdc2}$ protein kinase during the HeLa cell cycle (Draetta and Beach, 1988; McGowan et al., 1990). Finally, the p58 protein kinase is a phosphoprotein that is also subject to dynamic change during the cell cycle. Thus, the p58 protein kinase is not only structurally related to $p34^{cdc2}$, but functionally as well. However, unlike the human $p34^{cdc2}$ protein kinase which is primarily phosphorylated on threonine and tyrosine (Draetta and Beach, 1988; Draetta et al., 1988), the human p58 protein kinase is primarily phosphorylated on serine.

The phosphorylation state of the p58 protein kinase may be of significance with regard to its regulation during the cell cycle. Examination of the human and murine p58 protein kinase sequences reveals seven potential $p34^{cdc2}$ phosphorylation sites at Ser 51, Ser 55, Ser 230, Thr 236, Ser/Thr 297, Thr 367 and Ser 393. Four of these serine residues are absolutely conserved between the two species (Kidd et al., 1991). Among these putative phosphorylation sites there is a perfect consensus S-P-L-K $p34^{cdc2}$ phosphorylation site which lies within a protein kinase subdomain that may be important for protein kinase catalytic function (Hanks et al., 1988).

Autophosphorylation of residues within this subdomain may increase catalytic activity of tyrosine protein kinases by inducing changes in protein conformation (Hanks et al., 1988). In addition, four of the six potential $p34^{cdc2}$ phosphorylation sites are found outside of the conserved kinase domain, two in the putative NH_2-terminal regulatory domain and two in the unique COOH-terminal domain. The coordinate regulation of p58 protein kinase activity with $p34^{cdc2}$ activity during the cell cycle, the observation that the p58 protein kinase is serine phosphorylated in vivo, and the observation that serine phosphorylation of p58 is essential for in vitro kinase activity may suggest a role for these putative $p34^{cdc2}$ phosphorylation sites in p58 function.

Previously we have shown that the p58 protein kinase appears to be localized to both the cytoplasm and the nucleolus, and that limited overexpression of this protein kinase blocked cell cycle progression during late telophase (Bunnell et al., 1990a). The nucleolar protein nucleolin is normally phosphorylated during mitosis on threonine residues contained in a $p34^{cdc2}$ consensus phosphorylation sequence which is repeated nine times in the NH_2-terminal region of nucleolin (Belenguer et al., 1990). Phosphorylation of these threonine residues on nucleolin by the $p34^{cdc2}$ protein kinase in vitro was shown to match the in vivo phosphorylated threonine residues of the same protein (Peter et al., 1990; Belenguer et al., 1990).

However, due to the nature of *in vitro* and *in vivo* phosphorylation studies the assignment of a protein kinase responsible for *in vivo* phosphorylation of nucleolin during mitosis can only be inferred. It has also been suggested that nucleolin phosphorylation during mitosis could modulate the process of chromatin condensation-decondensation as well as its interactions with rRNA transcripts or with rDNA templates (Belenguer et al., 1990; Peter et al., 1990). Hyperphos-phorylation of these substrates might prevent normal cell cycle progression and result in a cycle delay during late telophase, as well as potential chromosomal changes that could lead to subsequent mitotic abnormalities such as micronuclei. These events were observed when the p58 protein kinase was overexpressed in CHO cells (Bunnell et al., 1990a). We are now examining the ability of the p58 protein kinase to phosphorylate nucleolin, and, if so, determine whether or not these sites are identical to those phosphorylated during mitosis *in vivo*. The apparent nucleolar sub-cellular localization, as well as the mitotic activity of the p58 protein kinase supports the possibility that this kinase may function to phosphorylate nucleolar substrates during mitosis. Given these results, it will now be of interest to determine whether or not the p58 protein kinase is another potential cellular substrate of the $p34^{cdc2}$ protein kinase.

EXPERIMENTAL METHODS

Cell Culture, Labeling and Synchronization.

HeLa cells (obtained from Dr. B. Brinkley, UAB) were cultured in flasks at 37^0C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1X pen-strep.

Proteins were labeled with ^{35}S by first incubating cells in methionine- and cysteine- free DMEM containing 10% dialyzed FBS for 4 hours, and then adding 1mCi/ml of Tran [^{35}S] label ([^{35}S] methionine and [^{35}S] cysteine, > 1,000 Ci/mmol; ICN Radiochemicals, Irvine, CA) for 2-4 hrs. at 37^0C, as indicated. For ^{32}P labeling, cells were incubated in phosphate-free DMEM containing 10% dialyzed FBS and 1mCi/ml of carrier-free [^{32}P] orthophosphate (ICN) for 16 hrs. at 37^0C (Pines and Hunter, 1989).

Cells were synchronized in G_0/G_1 by growing sub-confluent cells on DMEM plus 0.5% FBS for 48 hrs. Cells were synchronized at the G_1/S boundary by sequential thymidine (Sigma, St. Louis, MO) and aphidicoline (Sigma) treatment according to Heintz et al (1983). Cells were synchronized in metaphase by addition of 0.04 μg/ml nocodazole (Sigma) after initially treating the cells with thymidine (Sigma) as described by Zieve et al. (1980). Cells were re-seeded after drug blocks as described by Pines and Hunter (1989). Equal aliquots of cells (1 X 10^6 cells) were used to isolate cellular protein using either a modified RIPA buffer (see below) or SDS sample buffer.

Viability and synchrony of cells released from drug or serum blocks was assessed by both flow cytometry and [^{3}H]-thymidine (Amersham) incorporation as described by Nilsen and Baglioni (1979). Flow cytometry analyses of cellular DNA content was accomplished by using a Becton Dickinson FACStar cytometer. For this analysis ethanol-fixed cells were treated with a solution of 40 μg/ml propidium iodide and 40 μg/ml RNAse A.

RNA Extraction and Northern Blotting

Total RNA was extracted from synchronized HeLa cells as described by Chirgwin et al. (1979). Forty micrograms of RNA was denatured by treatment with formaldehyde and formamide, then electrophoresed on 1.5% agarose-formaldehyde gels as previously described (Kidd et al., 1991). The RNA was then transferred to a Duralose (Stratagene) filter membrane, UV-crosslinked, pre-hybridized and hybridized with [^{32}P]-labeled cDNA probes as previously described (Bunnell et al., 1990a; Kidd et al., 1991). The human p34^{cdc2} cDNA clone was isolated by screening 300,000 plaques from a human fetal liver lambda-gt11 cDNA library (CloneTech) with the following oligonucleotide:

5′- ATGGAAGATTATACCAAAATAGAG-3′; corresponding to nucleotides 141-165 of the human p34^{cdc2} cDNA reported by Lee and Nurse (1987). The oligonucleotide was labeled with [^{32}P]-gamma-ATP as previously described (Kidd et al., 1983). The 1.9 kb insert of this cDNA clone was sequenced

in its entirety as described (Bunnell et al., 1990a; Kidd et al., 1991), and it was found to be identical to the coding sequence reported by Lee and Nurse (1987). The human p58 cDNA (Bunnell et al., 1990a) and gamma-actin cDNA (Gunning et al., 1983) were previously reported. Exposure times for the Northern blot to XAR-5 film at -80^{0}C were as follows: human p58, 3 days; human p34^{cdc2}, 12 hours; human gamma-actin, 2 hours.

Immunoprecipitation and In Vitro Kinase Reactions

Cell Lysis Buffer. Cell lysis buffer consists of 60 mM ß-glycerol phosphate (Sigma), 0.1% NP40, 25 mM MOPS pH 7.2, 15 mM EGTA, 15 mM MgCl2, 1 mM DTT, 0.1 mM NaVanadate, 0.1 mM NaF, 10 ?g/ml soybean trypsin inhibitor (Sigma), 15 mM p-nitrophenyl phosphate (Sigma).

Bead Buffer. The bead buffer consists of 5 mM NaF, 50 mM Tris-HCl pH 7.4, 250 mM NaCl, 5 mM EGTA, 5 mM EDTA, 0.1% NP40, 10 µg/ml aprotinin, 10 µg/ml soybean trypsin inhibitor and 16 µg/ml benzamidine.

Kinase Buffer. The kinase buffer contained 10 mM MgCl2, 10 µM ATP, 1 µg/ml casein (Sigma) or histone H1 (Boehringer-Mannheim) and 10 µCi of [^{32}P]-gamma-ATP (100 mCi/ml, Amersham).

1X 10^6 cells were lysed with 100 µl of lysis buffer. To this, 10 µg of affinity-purified p58 peptide antibody (Bunnell et al., 1990a) was added and the mixture was incubated 2-12 hrs. at 0=0C. 30 µl of protein-A agarose (Pharmacia) was then added and incubated by rotation at 4^{0}C

for 30 minutes. The protein-A agarose/p58 antibody beads were then pelleted in a microfuge at 12K for 1 min. The pelleted beads were then washed 2-3 times with bead buffer (1 ml each wash). The washed beads are then pelleted and resuspended in 40 μl of kinase buffer. This reaction is incubated for 30 minutes at 37^0C, mixing by inversion every 5 minutes. The beads are then pelleted by centrifugation in a microfuge at 12K for 2 minutes and the supernate removed. The protein in the supernate was then precipitated with 9 volumes of cold acetone, centrifuged, and the pellet resuspended in SDS-PAGE disruption buffer to a concentration of 1μg/μl. This sample was then analyzed on 10% SDS-PAGE, the gel dried and autoradiographed for 8-12 hours at room temperature. After autoradiography the casein or histone H1 bands were excised from the dried gel and Cherenkov radiation determined (Draetta and Beach, 1988; Draetta et al., 1988). A total of four separate experiments were performed for each of the cell cycle assays. Similar results were obtained in all cases.

H1 Kinase Purification and Assays

The purification of H1 kinase from HeLa cells using $p13^{suc1}$-Sepharose beads was performed as described by Pondaven et al (1990) using the cell lysis buffer and bead buffer described earlier. The H1 kinase assay was performed as described by Pondaven et al. (1990) and the products analyzed by 10% SDS-PAGE as described by Draetta and Beach (1988).

Phosphoamino Acid Analysis and Phosphatase Treatment of Purified p58 Kinase.

Cell culture, labeling with [^{32}P] orthophosphate and immunoprecipitation with the affinity purified p58 peptide antisera was performed as described earlier. 1X10^{6} cells were blocked at either the G_1/S-phase with thymidine/aphidicolin or G_2/M-phase boundary with thymidine/-nocodazole as described above. For the phosphoamino acid analysis ^{32}P-labeled p58 protein bands were excised from the dried polyacrylamide gels and the protein extracted as described (Bunnell et al., 1990b). Phosphoamino-acid analysis of the ^{32}P-labeled samples was then performed as previously described (Bunnell et al., 1990b). The thin layer chromato-graphy plates were then stained with ninhydrin to develop the positions of the unlabeled standards, and the plates autoradiographed by exposing to Kodak X-AR5 film at -80^{0}C for 1-3 weeks.

Purification, immunoprecipitation and kinase assay of the active bovine p58 kinase was performed as previously described (Bunnell et al., 1990a). The immunoprecipitated p58 protein kinase was treated with 0.1 units of CIAP in the presence or absence of 100 mM 4-nitrophenyl phosphate for 15 minutes. The CIAP was subsequently inactivated by treatment at 68^{0}C for 5 minutes. The purified p58 kinase was then assayed for activity using histone H1 as a substrate and assayed by 10% SDS-PAGE as described earlier. The ^{32}P-labeled products were visualized by autoradiography

of the dried acrylamide gel at room temperature for 8 hours using Kodak X-AR5 film.

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CONCLUSIONS

This research reveals several important observations about a novel cell cycle regulated protein kinase. The manner in which the kinase was discovered was a bit fortuitous, but has allowed us to identify what appears to be another pathway for the regulation of cell division. The initial discovery of the sequence homology between this kinase and that of the previously reported p34 kinase was very exciting. It allowed us to immediately place the p58 protein in a kinase family and pursue investigations that would illustrate its cell cycle regulation.

The existence of a unique 76 amino acid N-terminal regulatory domain was equally interesting. The presence of this domain suggests that the p58 kinase may be under a higher level control process than those of the previously published p34 and related kinases. For instance the nuclear localization signal present on the p58 kinase would allow this protein to be transported through the nuclear membrane in the absence of a second shuttling protein like cyclin, which is required by the p34 kinase for nuclear localization. The PEST sites could contribute to the destabilization of this protein and as such contribute to another aspect of its regulation which is different from that of p34. The last component of this regulatory region,

the calmodulin binding domain, may play a number of potential roles. Its association with the kinase could potentially mask the nuclear localization signal sequences thereby preventing the kinase from entering the nucleus. Calmodulin binding could also mask the PEST sites thus preventing the kinase from being degraded. Calmodulin could contribute to the activation of the kinase or even potentially hold the kinase in a conformation necessary for its interaction with other proteins. It is interesting to note that calmodulin also cycles over the cell cycle peaking at the G_1/S boundary (59). Pharmacological agents which selectively antagonize calmodulin were found to block cell cycle progression both at the G_1/S boundary and during G_2/M (61). The role played by this regulatory region and the effects of calmodulin on it were however beyond the scope of this study. This study was dedicated to the identification of a novel protein kinase and determining its regulation of over the cell cycle.

The first chapter reports the initial sequence and protein biochemistry. The use of an <u>in-vitro</u> transcription/translation system showed that the cDNA was capable of encoding a protein kinase and showed that the protein migrates larger than its predicted sequence on SDS-PAGE. This observation was not that unusual due to the proline rich content of the N-terminus of the kinase (62). These initial studies also allowed the characterization of a high titer polyclonal antibody which was made to a highly

antigenic site within the N-terminal region. It was observed that the kinase activity of p58 was not inhibited by precipitation with this antibody.

Two dimensional phosphoamino acid analysis confirmed that p58 was a serine/threonine kinase. The ability of p58 to use casein and histone H1 as substrate was not as important as the observation that it could also use GalTase as a substrate. This observation was very important because it allowed us to show (in chapter two) that the phosphorylation of the GalTase enzyme by the p58 kinase actually increased its activity. The report by Strous (60) that the GalTase enzyme was phosphorylated in-vivo on serine residues added support to our in-vitro observations. These two pieces of data were very important because they show that the kinase does have a potential cellular substrate and their interactions affect the activity of this substrate.

In chapter one we also show that overexpression of the p58 kinase has an adverse effect on the cells division cycle in addition to its morphology. Cells overexpressing the p58 kinase were found to lose their matrix adhesive properties and would round up. This was found to be true even when incubated on several different extracellular matrices. Indirect immunoflouresence was done using an anti-tubulin antibody and showed that cells overexpressing p58 were paired and had a significant increase in the number of midbodies and micronuclei as compared to normal

cells, much like the effects seen when p13 was injected into cells (9). It was as if these cells were stuck in a late telophase early G_1 phase of the cell cycle. This assumption was proven correct by several observations. The cells had a doubling time twice that of normal cells. FACs analysis then showed that the cells were in the G_1 phase of the cell cycle, and classified the paired daughter cells into this G_1 phase. Further studies with [^{3}H]thymidine showed that the p58 overexpressing cells appeared to be inhibited from entering S phase of the cell cycle. These observations are significant considering the temporal control mechanisms mentioned in the introduction.

Another significant observation seen in the first chapter is the presence of the p58 kinase in the nucleus. This confirms that the kinase does enter the nucleus and was found specifically in the nucleolar region. Its exact function there is not known at present. There are several proteins in the nucleolar region which are necessary for ribosome biosynthesis as well as nuclear organization which may be targets for the kinase. Two examples are nucleolin and NO38 both of which are phosphoproteins (63-65). Nucleolin is believed to function in the transcription and processing of ribosomal RNA in addition to maintaining nucleolar chromatin organization (63-65). Nucleolin was found to remain associated with mitotic chromosomes and is a substrate <u>in-vitro</u> for p34 (16,64,65). It is thought that nucleolin undergoes a phosphorylation/

dephosphorylation cycle that regulates nucleolar chromatin organization in addition to transcription and processing of ribosomal RNA. Its believed that p34 may phosphorylate nucleolin thereby arresting ribosome synthesis and allowing mitotic condensation of the nucleolar chromatin as well as allowing disaggregation of the nucleolus (16,63). NO38 is a nucleolar protein implicated in the packaging and transport of preribosomal particles (64,65). It was found to diffuse away from the nucleolar region upon entrance to mitosis (64). It also undergoes a phosphorylation/ dephosphorylation cycle and is a possible substrate for p34 which could lead to similar results seen with nucleolin (65). The phosphorylation of these proteins is precedent for the possibility that p58 may also function in the regulation of nucleolar organization.

These initial studies lead us into the cell cycle studies of which the last chapter is dedicated. HeLa cells were chosen in these studies because they have been used for the characterization of other cell cycle proteins. It was thought that the studies could therefore be correlated to those studies. Our initial observation that the mRNA levels of p58 are coordinately regulated with that of p34 was very exciting, and confirmed previously published reports of p34 mRNA expression (66). We then showed that the p58 protein was stable for at least two hours, and decided to study the p58 kinase activity at four hour intervals after release from two cell cycle blocks, G_1/S

and G_2/M. After insuring that the cells were cycling through the cycle correctly by both FACs analysis and [^{3}H]-thymidine incorporation the study of kinase activity was undertaken. The results showed that the p58 and p34 proteins had activity that was coordinately regulated, yet distinct from one another. The p58 kinase activity was, however, 10 fold lower than that of p34. This was not surprizing due to the 10 fold lower levels of mRNA seen on the Northern blot analysis. The kinase activity was exactly as was published previously for p34 suggesting that the cell are cycling correctly (5,66-68). De novo synthesis of the p58 protein was also found to correspond to p34, occurring in G_2/M phase of the cell cycle (66). This protein synthesis occurs even though the levels of total cellular p58 protein remains constant throughout the cell cycle. These studies are again in complete agreement with what has been found for p34 (5,66). A model was suggested for p34 that old and new pools of protein mix at G_2/M, during de novo synthesis of the proteins. During this period some of the protein is degraded by an undetermined pathway (66). It is thought that p58 levels are being regulated in a similar manner possibly through the use of the PEST sequences in the N-terminal region. Finally phosphorylation studies show that the kinase is hyperphosphorylated in G_1/S as compared to mitotic protein. These results are consistent with what has previously been

reported for p34 which is hyperphosphorylated in the G_1/S boundary as compared to mitosis (68).

It was also determined that serine phosphorylation was required for the p58 kinase to be active. There is also at least one absolutely conserved p34 phosphorylation site in the kinase domain of p58. These two observations suggest that p34 may phosphorylate p58 thus regulating its activity. Studies regarding this possibility are currently under investigation in the laboratory.

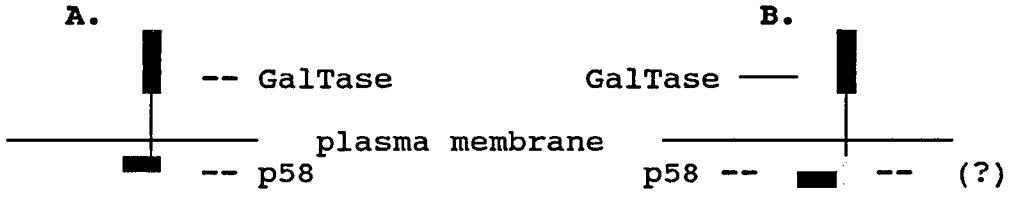
Potential intracellular substrates for the p58 kinase other than GalTase can only be speculated. p58 was found to have some activity towards histone H1. This protein was previously reported to be phosphorylated by p34 (5). Phosphorylation of histone H1 is known to cause chromatin condensation. Further studies are needed to determine if p34 is the intracellular kinase which phosphorylates histone H1 or if it is the target for some other kinase, maybe p58. The localization of p58 to the nucleolar region also presents some interesting possibilities. However, p34 has been implicated as the kinase which phosphorylates all of the major proteins in this region. Studies are underway in the laboratory to determine if p58 can phosphorylate any of these potential nucleolar substrates.

The very close homology in addition to the coordinate regulation between the two kinases is intriguing. It adds support to the possibility of a protein kinase cascade which pyramids out from one or two initiation points to

regulate the activity of a magnitude of separate proteins, and ultimately control cell division.

The triggers that regulate a cell's entrance into mitosis has as yet been unidentified in the cell division control field. The appearance of the p58 kinase associated with a cell surface receptor reported as having a role in cell division is very exciting. It is as yet the only such cell cycle regulated protein known to have such an interaction. The fact that the kinase was found to influence the activity of GalTase in addition to its subcellular localization could be a very significant contribution to the understanding of the triggering events required for cell cycle progression. It is reasonable to assume with regard to what has already been published in the GalTase field that the transferase could in some way regulate the activity and/or localization of the p58 kinase to initiate mitosis.

Several pathways can be envisioned here on how the GalTase, p58 and p34 proteins could potentially interact. p58 may bind directly with the GalTase protein and thereby regulate its activity, panel A. On the other hand, the



interaction between these two proteins could be an indirect one, panel B. In this case a third protein, possibly calmodulin, could facilitate the interaction of the proteins either by acting as the cement to hold them together or by holding the proteins in a conformation suited for their interaction. In either case a signaling event on the outside of the cell would then be conveyed through the cell surface by the GalTase enzyme to the p58 protein. The most reasonable method of transfer would be through some sort of a conformational change of the GalTase enzyme. This conformational change would allow the p58 protein to be release from the GalTase enzyme and enter into its function either in the cytoplasm or the nucleus as discussed above. The p58 protein may interact with the p34 protein. Due to the number of potential p34 phosphorylation sites on the p58 kinase this possibility can not be ignored. The interactions between these two proteins can be envisioned in several different ways. The p34 protein may both may use p58 as one of its substrates and thus activate a branch point in the regulation of cell division by p34. A second possibility is that the p34 protein may dead end by activating the p58 protein and the active p58 protein may extend the p34 pathway.

This work has opened the door to a number of studies regarding the utilization of other cellular substrates, determining its consensus phosphorylation site in addition to resolving what kinases regulate p58s activity.

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APPENDIX

A few additional observations were made with respect to the activation of the GalTase enzyme by the p58 protein kinase. GalTase has been found to exist in milk, however, the mechanism by which it passes through the plasma membrane still remains illusive. In milk, GalTase is found associated with alpha lactalbumin, an allosteric modifier of the GalTase enzyme. When alpha lactalbumin is associated with the GalTase enzyme, it is known as Lactose synthetase because the complex transfers galactose to glucose molecules and thus makes the milk sugar lactose. I found that in addition to phosphorylating the GalTase protein, the p58 kinase also phosphorylated alpha lactalbumin, and as we found in chapter two, the lactose synthetase enzymatic activity increased five to six fold. These observations are significant to the canned milk people because by adding more active kinase to the milk they could increase the nutritional value of that milk by increasing the production of lactose. This work was left unfinished and is reported for the first time here.

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Name of Candidate Donald E. Adams

Major Subject Microbiology

Title of Dissertation Biochemical and Cellular Characterization of a Novel Cell Cycle Regulated Protein Kinase, $p58^{GTA}$

Dissertation Committee:

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Director of Graduate Program _____

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