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CELL CYCLE ASPECTS OF THE UNP UBIQUITIN PROTEASE

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ABSTRACT

The product of the proto-oncogene *Unp* (ubiquitous nuclear protein) was shown to be localized in the nuclear fraction of COS-1 cell lysates. *In situ* studies using indirect immunofluorescence indicated that, when overexpressed in COS-1 cells, the Unp polypeptide localized in a perinuclear fashion. Minimal association with the microtubular structures of the cell was deduced, however neither the mechanism involved or the extent of their association have been determined.

It has also been shown that the Unp protein follows a pattern of distribution, during the various phases of the cell cycle, similar to that exhibited by cyclin B1. During interphase both proteins appear perinuclear, however as the cell approaches mitosis they become 'diffuse' in their distribution. COS-1 cells expressing the Unp polypeptide displayed aberrant condensation of the chromosomes, whereas cells which did not express Unp exhibited the normal condensation pattern.Amino acid analysis of ^{32}Phosphorus labeled Unp polypeptide demonstrated that it was phosphorylated on a serine residue.

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LIST OF ABBREVIATIONS

ATP	Adenosine tri-phosphate
c-mos	Moloney murine sarcoma virus
CO_2	Carbon dioxide
^{o}C	Degrees Celcius
cdk	Cyclin dependent kinase
CAK	Cyclin activating kinase
cdc	Cell division control
cys	Cysteine
CMV	Cytomegalovirus
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
Doa	
DUB	Deubiquitinating enzyme
EDTA	Ethylene-diamine-tetraacetic acid
EGTA	Ethylenebis(oxyethylenenitrilo)-tetraacetic acid
FBS	Fetal bovine serum
HEPES	N-[2-hydroxyethyl] piperazine-N^1-[2ethanesulfonic acid]
his	Histidine
kb	Kilobase
kDa	KiloDalton
KH_2PO_4	Potassium dihydrogen orthophosphate
mA	Milliamps
MEM	Modified Eagle's medium
MPF	Mitosis promoting factor
Myc	Myelocytomatosis virus
Na_2HPO_4	Di-sodium hydrogen orthophosphate
NLS	Nuclear localization signal
ORF	Open reading frame
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PIPES	Piperazine-N,N^1bis[2-ethanesulfonic acid]
PMSF	Phenyl-methylsulphonyl fluoride
pRb	Retinoblastoma protein
pSer	Phospho-serine
pThr	Phospho-threonine
pTyr	Phospho-tyrosine
Rb	Retinoblastoma
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TBST	Tris buffered saline-Tween

TGF-β	Transforming growth factor-β
Tre-2	Transfection recombinant
Ub	Ubiquitin
Ubp	Ubiquitin specific protease
Unp	Ubiquitous nuclear protein
UTR	Untranslated region
V	Volts

OBJECTIVE

Ubiquitin specific protease (Unp) encodes a novel nuclear oncoprotein. When expressed from an active promoter, Unp has the ability to cause the tumourigenic transformation of NIH 3T3 cells injected into athymic mice (Gupta *et al.,* 1994). The potential of this protein product to form tumours may be a result of its ability to cause the de-regulation of cell cycle progression, a common feature of tumour cells.

The protease action of Unp enables it to remove the ubiquitin tag conjugated to proteins marked for degradation by the ubiquitin pathway (Baker and Gray, unpublished). Several cell cycle proteins, including cyclins, are known to be degraded via ubiquitination and may therefore be exposed to the protease action of Unp. The premature removal of the ubiqiutin tag from these proteins can result in arrest of the cell cycle (Seufert *et al.*, 1995) .

In order to elucidate the mechanism of Unp activation, *in situ* immunofluorescence was used to determine its location within the cell enabling its interaction with potential substrates to be investigated. This will contribute to our understanding of the mechanism by which Unp exerts its effect.

INTRODUCTION

It has been nearly two decades since there was conclusive proof that normal cells harbour cancer genes (Martin *et al.*, 1995). Normal cellular genes are known to have the potential to become oncogenic and this has lead the scientific world to believe that a defense against cancer requires an understanding of the 'normal' cell.

There has since been extensive studies on the cell, the nature of its growth signals and the system that transports these signals to the nucleus. These mechanisms in turn result in the activation of transcription of those genes involved in cell growth, differentiation and development (Forrester *et al.*, 1992). Cancer may develop when a cell releases itself from controls which exist over the mechanisms mentioned above suggesting that the phenotypic endpoint observed in neoplasia can be reached through several pathways, where each step may reflect the activation, mutation or loss of different genes (Klein and Klein, 1995).

Proto-oncogenes, one of the first group of genes with a proposed function in cancer, were first identified as cellular homologues of activated viral oncogenes that form a stable part of the genome of acutely transforming retroviruses (Cantley *et al.*, 1991). Two main observations implicate proto-oncogenes in the growth and development of normal cells; first, there is a high degree of conservation of proto-oncogenes throughout metazoan evolution and second, there is extensive involvement of mutant alleles of these genes in neoplasia as a whole (Klein, 1982). Indeed many proto-oncogenes have been shown to encode proteins that are involved in signaling pathways critical to the control of cell proliferation and differentiation, such as growth factors, their receptors, transducers of growth factor responses and transcription factors (Martin *et al.*, 1995).

There is evidence that a second group of genes, the tumour suppressors or 'anti-oncogenes', may be deleted or functionally disabled in cancer, thereby allowing the formation of a tumour (Muphree *et al.*, 1984, Benedict *et al.*, 1983). The most thoroughly studied tumour suppressor gene is Retinoblastoma, *Rb,* where both alleles are lost or inactivated in retinoblastoma tumour cells (Cavenee *et al.*, 1983).

The third and final group of genes implicated to influence neoplastic behaviour are the 'modulators'. These genes do not transform cells on their own , but rather modify the spread of tumour cells within the organism (De Baetselier *et al.*, 1980, Dano *et al.*, 1985). Two such examples are genes encoding the Major Histo-compatibility Complex (MHC) which may influence metatastic spread (Dano *et al.*, 1985), and genes involved in the control of proteolytic and homing mechanisms which may increase invasiveness of the neoplasia (Fenyo and Klein, 1976).

In order to decrease the frequency of genetic mutations, cells have developed mechanisms to conserve accuracy in the reading of their genetic material. Failure to repair DNA damage, entering mitosis with unreplicated DNA, or initiating anaphase before aligning the chromosomes correctly on the mitotic spindle, can result in cell death, aneuploid or mutant cells (Forsburg *et al.*, 1991). In uni-cellular organisms these errors diminish the reproductive capacity of the organism, whereas in multi-cellular organisms aneuploidy and mutation may produce the uncontrolled cell proliferation that gives rise to a majority of cancers (Vogelstein *et al.*, 1989).

In 1976, Nowell suggested that there could be mutations present within the cells which result in genetic variability (Nowell., 1976). This hypothesis has since been substantiated by several groups which has indicated that mutations in DNA repair genes (ie. mismatch

repair and excision repair) predispose carriers to cancer, presumably by increasing genomic instability (Fishel *et al.*, 1993).

Cells use three mechanisms to ensure accurate transmission of their genetic information (Nurse., 1990). First, there is the existence of repair mechanisms that correct spontaneous or environmentally induced errors in DNA replication and chromosome alignment. Second, delay mechanisms, or checkpoints, may detect these errors and arrest the cell cycle until the repairs are complete and finally, the damaged cells may be induced to die, a process known as apoptosis, thus preventing them from giving rise to mutant progeny (Nurse., 1990).

Recent work has identified a category of genes that when mutated increase genetic instability and accelerate cellular evolution (Hartwell *et al.*, 1994). These genes encode components of cell cycle checkpoints, implying that they may have a role in cancer cell evolution and thus have a potential impact on cancer prevention.

THE CELL CYCLE

Tumour cells, like normal dividing cells, execute an ordered set of steps called the cell cycle. Rapidly dividing human cells have a cell cycle that lasts approximately 24 hours which is divided into two sections (Murray, 1993) { Figure 1}.

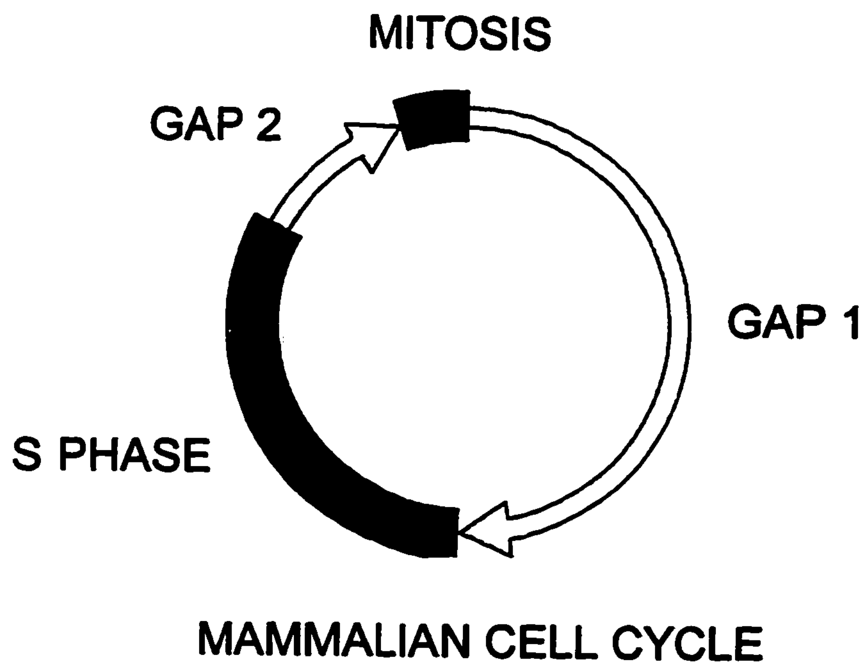
Interphase encompasses the majority of the cell cycle and can be further divided into two important sub-sections (Kamb, 1995). First are the continuous processes which occur

FIGURE 1

Stages of the cell cycle

Mitosis represents about 5% of the mammalian cell cycle, and there is a substantial G1 gap between mitosis and DNA synthesis (S), as well as a G2 gap between replication and mitosis.

Source; Murray, A. and Hunt, T. (1993) The cell cycle, an introduction. Oxford press.



MAMMALIAN CELL CYCLE

throughout interphase and include the synthesis of new ribosomes, membranes, mitochondria, endoplasmic reticulum and most cellular protein. The second sub-section, which occurs only once per cell cycle, is referred to as stepwise processes and involve chromosome replication which is restricted to the DNA synthesis or 'S' phase. The S phase itself occurs in the middle of interphase and is preceeded by a 'gap space' called G1 during which the cell prepares to synthesize its DNA. A second gap space, called G2, follows the S phase and represents the period when the cell prepares for division (Murray, 1993).

The second major section, called mitosis, lasts for approximately 30 minutes and ends with division of the cell (Dunphy, 1994). Mitosis can be described as 'a coordinated change in the architecture of the cell' which results in the segregation of the replicated chromosomes into two identical sets and initiates the division of the cell (Murray, 1993). {Figure 2}.

MITOSIS

The key feature of mitosis is the disappearance of the nucleus which occurs during the later stages of chromosomal condensation. The nuclear envelope is composed of inner and outer membranes, with a protein called lamin coating the nuclear side of the inner membrane. The nuclear membrane is punctuated with nuclear pores and these span the inner and outer membrane allowing communication between the nucleus and the cytoplasm. As cells enter mitosis the nuclear envelope breaks down, the filaments of lamina depolymerize and the nuclear pores vesiculate (Murray, 1993). The first sign that a cell is about to divide is that its chromosomes start to condense.

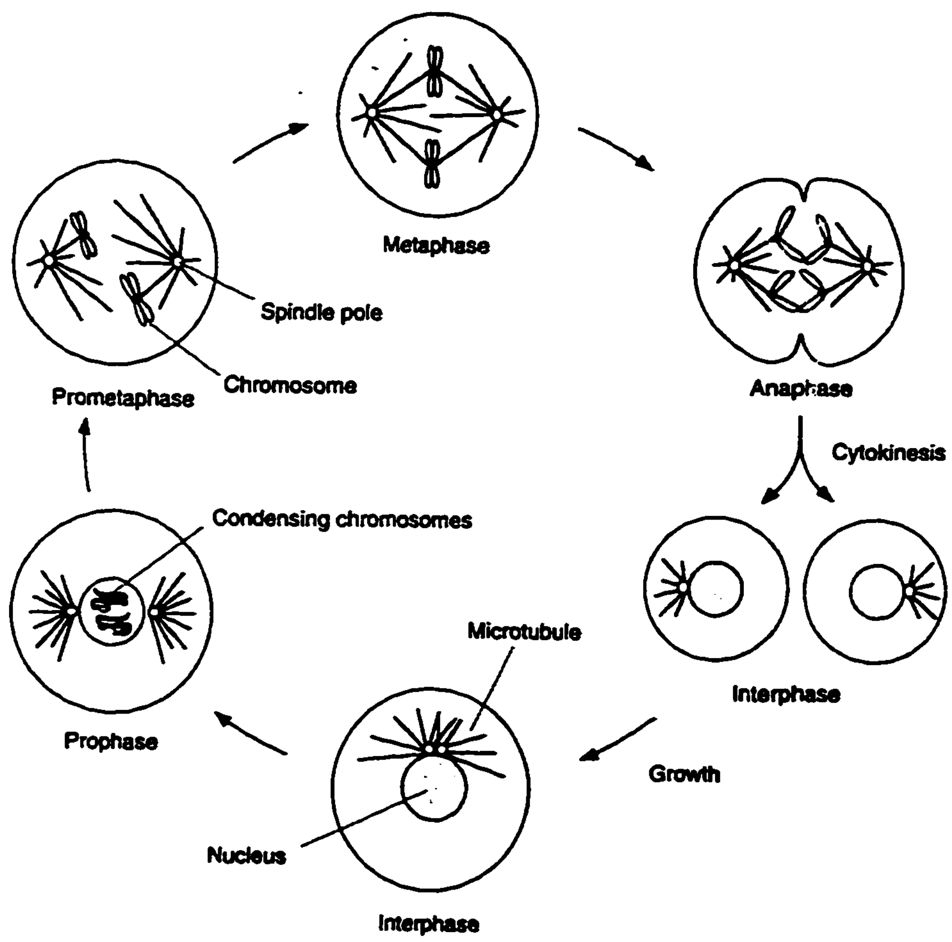
During interphase the DNA appears as a homogeneous cloud and as the cell is about to

FIGURE 2

Stages of mitosis

During prophase the centrosomes move to opposite sides of the nucleus, and the chromosomes condense. The nucleus breaks down, and as the cell progresses to metaphase, the chromosomes align on the centre of the spindle. At anaphase the linkage between sister chromatids is broken, and the sisters segregate to opposite poles. Cytokinesis will then generate two identical daughter cells.

Source; Murray, A. and Hunt, T. (1993) The cell cycle, an introduction. Oxford press.


Metaphase
Spindle pole
Chromosome
Prometaphase
Anaphase
Cytokinesis
Condensing chromosomes
Microtubule
Interphase
Prophase
Growth
Nucleus
Interphase

begin mitosis, it enters a stage called prophase. Here the chromosomes condense into a tangle of threads and are then resolved into sister chromatids. When the nuclear envelope breaks down, merging the nucleus and the cytoplasm, the cell leaves prophase. Meanwhile, the microtubules, which radiate from the spindle poles, undergo a variety of changes leading to the formation of the mitotic spindle. The two members of each chromatid pair attach to the microtubules originating from opposite poles and move along to the midway position. The cell, now in metaphase, destroys the links between the sister chromatids and the cell enters the final step of mitosis called anaphase. The chromatids continue to move along the microtubules and as they near the spindle poles cell division or cytokinesis begins (King *et al.,* 1994). The final outcome is the production of two identical daughter cells containing a full set of chromosomes.

Completion of the cell cycle requires the coordination of a large number of proteins many of which are regulated in a cell cycle dependent manner at the level of gene expression (Murray, 1992). Transitions between each phase of the eucaryotic cell cycle are controlled by the sequential activation of a series of cyclin dependent kinases (cdk's) (Murray, 1992). These enzymes are controlled by transient associations with cyclin regulatory subunits, binding of inhibitory polypeptides and reversible phosphorylation reactions (Nigg, 1995).

To promote progression of the cell towards DNA replication, cyclin/cdk complexes phosphorylate proteins required for the activation of genes involved in DNA synthesis, as well as components of the DNA replication machinery (Hartwell *et al.*, 1989). An alternative set of cyclin/cdk complexes trigger the phosphorylation of numerous proteins to

promote the structural changes observed as the cell enters mitosis (Nigg, 1995).

At several points during the cell cycle, the cell is subject to controls, some of which reflect the action of extracellular growth factors and hormones, and these serve to integrate cell proliferation with growth and differentiation (Nigg, 1995). A second major control mechanism of cell growth are the checkpoint controls and these verify the integrity of the replication process (Hartwell *et al.*, 1989, Murray *et al.*, 1992).

THE CDK CATALYTIC SUBUNIT

Cdks have been shown to be closely related in both size, being approximately 35-40 kDa, and sequence, with greater than 40% homology existing between kinase types (Morgan, 1995). Many protein kinases have been shown to be closely related to the cdks, however, few possess their cyclin dependent activity. Our understanding of cdk structure and function has been based mainly on studies performed on the cdks of budding and fission yeast, however, vertebrate studies are revealing many functional similarities with the yeast model.

The cdk catalytic subunit contains a 300 amino acid core which is inactive when present as a monomer and unphosphorylated. The primary regulator of cdk activity is the cyclin subunit and as has been mentioned, each cdk interacts with a specific subset of cyclins although the size of the subset may vary. Cyclins are thought to contain regions that target the cdk to specific subcellular locations where they interact with select substrates found within the cell (Morgan, 1995). In addition to activating cdks by binding through their cyclin box, they may also promote association with other cell cycle substrates (Hoffman *et al.*, 1993).

Cdk activation also requires phosphorylation at a conserved threonine residue , whose position varies depending on the cdk. The enzyme responsible for phosphorylating the threonine of cdc 2 (identified as number 161), and cdk 2 has been identified as cyclin activating kinase (CAK), which is a multi subunit enzyme with a highly conserved catalytic subunit called cdk 7 (Fisher *et al.*, 1994). Cdk 7, like its kinase substrates, also contains a potential phosphorylation site indicating that its activation may also require phosphorylation (Fisher *et al.*, 1994). A second major subunit of CAK has been identified and is called cyclin H demonstrating that CAK belongs to the family of cdk-cyclin complexes (Tassan *et al.*, 1994).

In the normal vertebrate cell cycle, phosphorylation of the threonine correlates with cyclin binding (Krek and Nigg, 1991), therefore changes in the phosphorylation state of cdks may not be due to changes in the activity of CAK alone, which does not alter during the cell cycle. Instead, the observed changes may reflect the ability of cyclin binding to stimulate cdk phosphorylation (Morgan, 1995).

THE CDK/CYCLIN INTERACTION

Cyclins have been shown to represent a family of structurally related proteins conserved through evolution (Wang *et al.*, 1990, Koff *et al.*, 1991). They can be distinguished on the basis of conserved sequence motifs, patterns of appearance and functional roles during specific phases of the cell cycle (Hunt, 1991). Homology among the cyclins is limited to a 100 amino acid conserved domain called the 'cyclin box' which is responsible for cdk binding and activation (Glotzer *et al.*, 1991, Pines *et al.*, 1993){ Figure 3}.

G1 cyclins, first described in *Sacchromyces cerevisiae*, are the rate limiting step in the progression of the cell from G1 to S phase (Reed *et al.*, 1990). Several putative G1 cyclins exist in higher eucaryotes, namely cyclin E, D (subtypes1-3) and C, and there is evidence that they all regulate G1-phase progression or S-phase commitment in the cell (Rozenberg *et al.*, 1995). As yet, very little is known about the function of cyclin C and its corresponding cdk partner. Cyclin E forms preferential complexes with the kinase cdk 2 in human cells (Dulic *et al.*, 1992, Koff *et al.*, 1992). The activity of cdk 2 peaks prior to S-phase entry, while the expression of its partner, cyclin E, fluctuates periodically through the cell cycle, being maximal at G1 (Lew *et al.*, 1991, Ohtsubo *et al.*, 1993).

The three D-type cyclins are induced earlier than cyclin E and are able to complex with various cdks, including cdk 2, 4, 5 and 6 (Matsushime *et al.*, 1992, Xiong *et al.*, 1992, Bates *et al.*, 1994, Meyerson *et al.*, 1994). Cyclin D1 is the most studied of the three and has been shown to be both rate limiting and necessary for G1 progression (Rozenberg *et al.*, 1995).

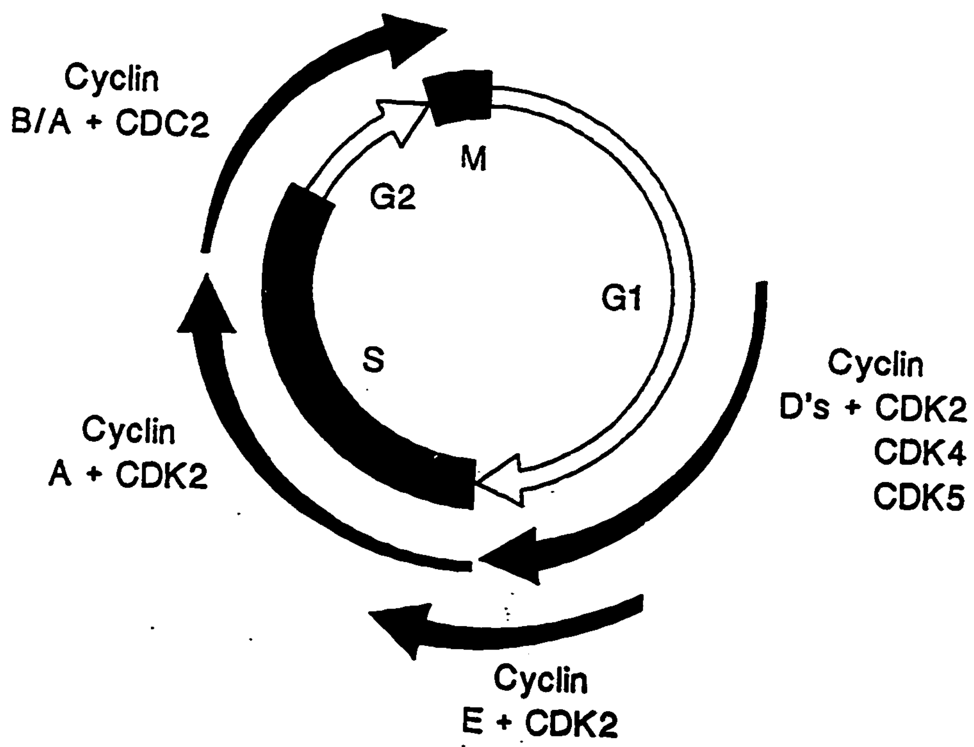
Cyclin A is required at more than one phase in the eucaryotic cell cycle and forms a complex with cell division cycle (cdc) 2 (cdk 1) and cdk 2 (Pagano *et al.*, 1991, Rozenberg *et al.*, 1995). Expression of both cyclin A and cdk 2 begins in late G1 shortly prior to S phase entry (Carbonaro-Hall *et al.*, 1993). The importance of cdk 2 in the G1/S transition has been demonstrated, however, it remains unclear whether the requirement for cdk 2 at this point in the cycle is due to its association with cyclin A and/or cyclin E.(Van den Heuvel *et al.*, 1993),. The expression of cyclin A at the G1/S transition is a target of signals that control cell proliferation such as adhesion dependent signals (Guadagno *et al.*,

FIGURE 3

Interactions of cdk/cyclin complexes during particular cell cycle transitions

The schematic omits cdk7/cyclin H (CAK), a complex that is required for multiple transitions in the mammalian cell cycle.

Adapted from; Sherr, C.J. (1993) Mammalian G1 cyclins *Cell* **73**: 1059-1065


Cyclin
B/A + CDC2
M
G2
G1
S
Cyclin
D's + CDK2
CDK4
CDK5
Cyclin
A + CDK2
Cyclin
E + CDK2

1993). The cyclin A/ cdk 2 complex has been shown to have an involvement in S phase (Rozenberg *et al.*, 1995). A link between both cyclin A and cdk 2 with the DNA replication machinery has been reinforced by the observation that both are specifically localized at sub-nuclear sites of ongoing DNA replication, much like the replication protein, proliferating cell nuclear antigen (PCNA) (Cardoso *et al.*, 1993). This suggests that cyclin A is required for initiation and/or progression of the cell through DNA replication.

Cyclin A function is required in a second phase of the cell cycle, G2 (Rozenberg *et al.*, 1995). It has been suggested that the cyclin A/cdc 2 complex may be involved in the activation of the M-phase cyclin B/cdc 2 (MPF) complex which in turn is necessary for entry into mitosis (Rozenberg *et al.*, 1995).

The mitotic or B type cyclins (1 and 2) have been shown to associate with the kinase cdc- 2 resulting in the formation of a complex called mitosis promoting factor (MPF) which regulates both mitotic entry and exit in the cell (Nurse, 1994). Cyclin B1 and B2 levels are known to be low in G1, while they increase during S and G2 and peak at mitosis (Jackman *et al.*, 1995). Activity of both B cyclins and the associated kinase appear when the cell enters mitosis and disappears as the cyclins are degraded in anaphase (Nigg, 1995).

Human cyclin B1 and B2 differ dramatically in their subcellular localization, with B1 co-localizing with the microtubules and B2 associating primarily with the Golgi region of the cell (Jackman *et al.*, 1995). The different locations of the B type cyclins implicates them in the organization of different aspects of the cellular architecture at mitosis, however the mitotic cyclin/kinase complexes may also have distinct roles in the cell cycle (Jackman *et al.*, 1995).

REGULATION OF THE CDK/CYCLIN COMPLEX

Regulation of the cyclin/cdk complex occurs through a series of phosphorylation /dephosphorylation reactions and the importance of these mechanisms has been demonstrated in both cdk 2 and cdc 2 respectively (Morgan, 1995).

At the beginning of interphase cdc 2 is in an unphosphorylated state rendering it inactive (Morgan, 1995). As the cell approaches mitosis the levels of cyclin B rise, and it associates with cdc 2 {Figure 4}. The association of cdc 2 with cyclin B induces phosphorylation of cdc 2 at three residues, namely tyrosine 15, threonine 14 and threonine 161 thereby maintaining the cyclin B/cdc 2 complex in an inactive state (Solomon *et al.*, 1990). In cdk 2, the side chains of these residues hang from the ATP binding site and are thus in a position to affect kinase activity when phosphorylated (DeBont, 1993). The mechanism of inhibition is as yet unknown however it has been proposed that cyclin binding may allow the residues to become accesible to phosphorylation reactions (Solomon *et al.*, 1990). Dephosphorylation of tyrosine 15 and threonine 14 by a phosphatase at the end of G2 activates the cyclin B/cdc 2 complex (Morgan, 1995).

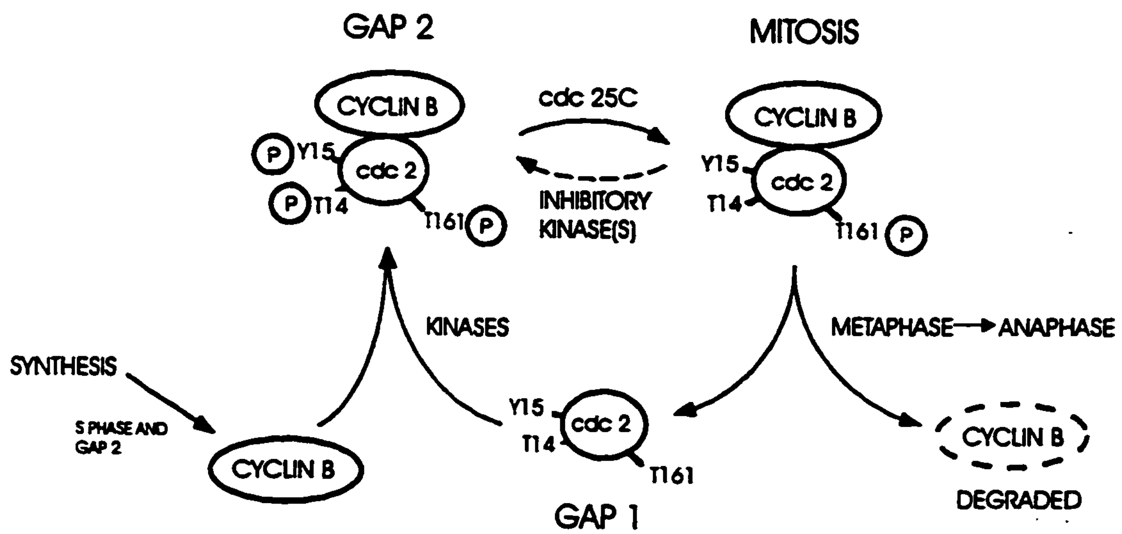
Cdc activating complex (CAK) is involved in the phosphorylation of threonine 161 in cdc 2 (Parker *et* . ˉ, 1992). *Wee 1*, the dual specificity kinase, has been identified as the gene encoding the enzyme responsible for the phosphorylation of tyrosine 15 on cdc2, however the kinase responsible for phosphorylation of threonine 14 has not yet been identified (Parker *et al.*, 1992). The dephosphorylation of both residues is undertaken by a

FIGURE 4

Cell cycle regulation of cdc2/cyclin B complexes

The complex of these two proteins forms in late S phase and is maintained in a pre-active state by inhibitory phosphorylations at two sites within the ATP-binding domain of cdc2 (threonine-14, tyrosine-15). The formation of a complex between cdc2 and cyclin B is ensured by phosphorylation of the cdc2 at threonine-161. Inhibitory phosphorylations are removed by the protein phosphatase cdc25C to produce an active cdc2/cyclin B complex which promotes the G2/M transition. Exit from mitosis requires the destruction of cyclin B and dephosphorylation of threonine-161, leading to inactivation of cdc2.

Source; O'Connor, P.M. and Kohn, K.W. (1992) A fundamental role for cell cycle regulation in the chemosensitivity of cancer cells? *Sem. Cancer Biol.* Vol 3: 409-416


GAP 2
MITOSIS
CYCLIN B
P Y15
cdc 2
P T14
T161 P
cdc 25C
INHIBITORY
KINASE(S)
CYCLIN B
Y15
cdc 2
T14
T161 P
KINASES
METAPHASE→ANAPHASE
SYNTHESIS
S PHASE AND
GAP 2
CYCLIN B
Y15
cdc 2
T14
T161
GAP 1
CYCLIN B
DEGRADED

dual specificity phosphatase called *cdc 25* (Solomon *et al.*, 1990).The rise in levels of active MPF switches on a degradation pathway resulting in the destruction of cyclin B and thus exit of the cell from mitosis (Morgan, 1995).

As has been outlined, cyclin function is primarily controlled by changes in its levels and these oscillations in concentration are produced mainly by changes in the rate of cyclin degradation (Morgan, 1995). Constant synthesis of a particular cyclin, for example the mitotic cyclin B, during the cell cycle results in a linear increase in the cyclin concentration. This will continue until the onset of mitosis when the rate of cyclin degradation increases abruptly producing a rapid decline in the cyclin levels (Glotzer *et al.*, 1991). Cyclin degradation has been shown to involve ubiquitin dependent proteolytic machinery and this will now be discussed.

THE UBIQUITIN-DEPENDENT PROTEOLYTIC PATHWAY

The passsage of cells from one stage to another of the cell cycle is tightly regulated by a number of controls or checkpoints, which involve the activation or inactivation of specific proteins. Modification of many kinase subunits can be carried out either by phosphorylation or degradation of the cyclin proteins.

At the onset of the cell cycle, there is a relatively small amount of a particular cyclin protein in the cell. This amount rises until it reaches a threshold at which point a protein kinase is activated. The kinase phosphorylates a number of substrates starting a cascade of events that ends in cell division. Once division starts, the cyclins are tagged with a 'degradation signal' and are degraded immediately to the component amino acids; the process will then be repeated.

Cyclin degradation is one of the key steps governing exit from mitosis and progress into the next cell cycle. Studies have indicated that when a region in the N-terminus of a cyclin is fused to a particular protein tag it produces a hybrid protein susceptible to proteolysis during mitosis. This process is mediated by Ubiquitin (Glotzer *et al.*, 1991, Varchavsky, 1992).

Ubiquitin (Ub) is an abundant, 76 residue protein whose amino acid sequence is conserved among animal species (Ozkaynak *et al.*, 1987). The protein has two well-characterised functional sites which are required for its activity. The first is a lysine at position 48 which serves as an acceptor site for other Ub molecules leading to the formation of long chain molecules. Second, there is a site in the C-terminus of the protein which mediates the formation of isopeptide bonds with lysine amino groups on acceptor proteins (Ciechanover *et al.*, 1994).

The process whereby acceptor proteins become joined through their lysine amino groups to a carboxyl group within Ub is called ubiquitination. Ub signals have been identified in the turnover of a variety of proteins and are therefore essential to many cellular processes, including DNA repair, heat shock response and cell cycle control (Finley *et al.*, 1991) { Figure 5}.

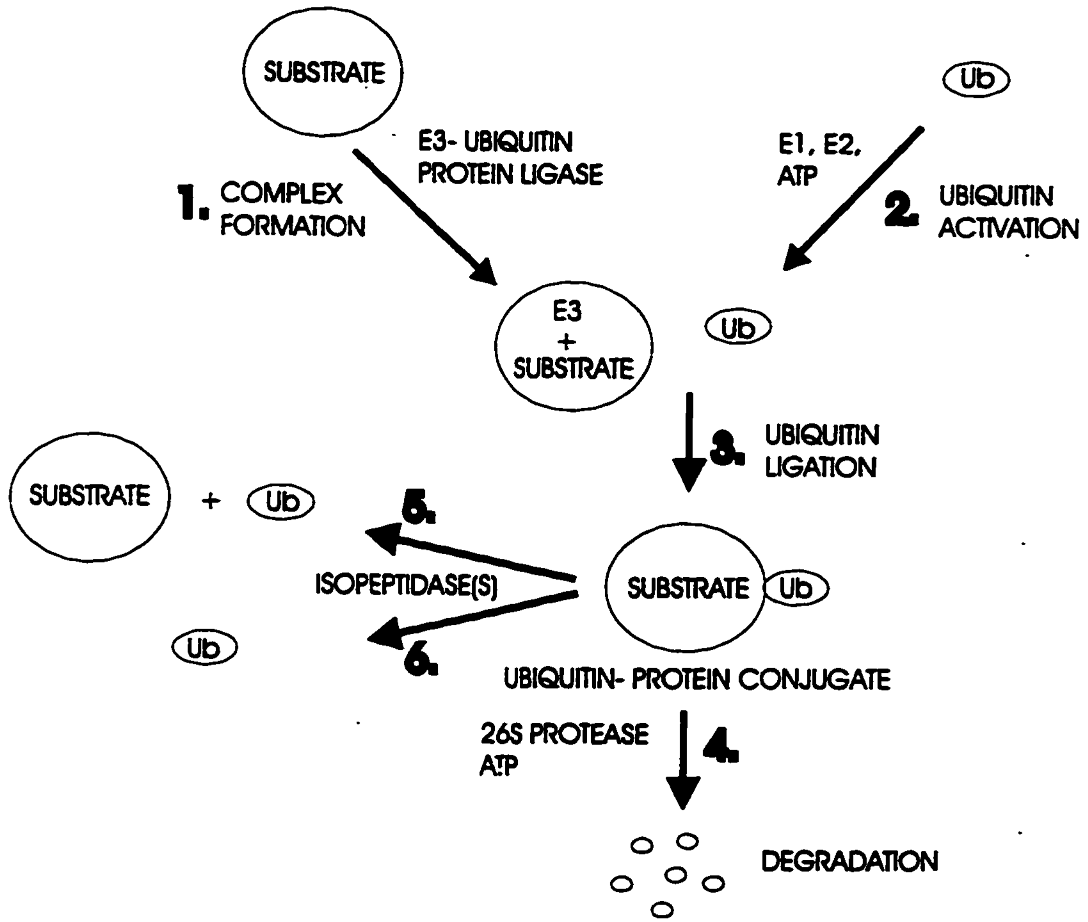
The C-terminal glycine of Ub is activated by the hydrolysis of ATP to form a high energy thiol ester intermediate in a reaction catalyzed by the ubiquitin-activating enzyme E1. The 'activated' Ub molecule is subsequently 'transferred' to the thiol group on a carrier protein called a ubiquitin-carrier enzyme or E2. The E2 enzyme, either with or without the

FIGURE 5

Overview of ubiquitin conjugation and degradation pathway

1. Complex formation of the protein substrate and E3. **2.** The activation of ubiquitin by E1 and E2. **3.** Conjugation of ubiquitin(s) to the protein substrate. **4.** ATP-dependent degradation of ubiquitin protein conjugates to peptides and free amino acids, mediated by the 26S protease complex. **5.** Release of reutilizable ubiquitin and protein substrates via isopeptidase(s). **6.** Release of reutilizable ubiquitin via isopeptidase(s) during proteolysis of the protein substrates to peptides and free amino acids.

Adapted from; Ciechanover, A.J. and Schwartz, A.L. (1994) Cellular proteolytic systems. Wiley-Liss Publ.



SUBSTRATE
Ub
E3- UBIQUITIN
PROTEIN LIGASE
E1, E2,
ATP
1. COMPLEX
FORMATION
2. UBIQUITIN
ACTIVATION
E3
+
SUBSTRATE
Ub
3. UBIQUITIN
LIGATION
SUBSTRATE
+
Ub
5.
ISOPEPTIDASE(S)
SUBSTRATE
Ub
Ub
6.
UBIQUITIN- PROTEIN CONJUGATE
26S PROTEASE
ATP
4.
DEGRADATION

aid of a substrate recognition factor (E3), then catalyses the formation of an isopeptide bond between the activated carboxy-terminal glycine residue of Ub and the ε-amino groups of a lysine residue in the protein substrate (Ozkaynak *et al.*, 1987, Hershko *et al.*,1992, Jentsch 1992a and 1992b, Ciechanover *et al.*, 1994).

Previous work had predicted the existence of "N-end-recognizing" factors that select potential proteolytic substrates by binding to their amino terminal residues . These proteins were later identified as the E3 proteins that were previously shown to bind proteolytic substrates prior to their ubiquitination by the E2 enzymes (Ciechanover and Schwartz, 1989).

Ubiquinated proteins are in a dynamic state being subject to either further rounds of Ub addition, removal of their Ub tags by de-ubiquitinating enzymes or degradation by a specific ATP dependent protease complex called the 26S proteasome into free amino acids and free and reutilizable *Ub* (Rechsteiner *et al.*, 1993).

In the case of cell cycle control, cyclins have been shown to be degraded via the ubiquitin pathway. Mitotic cyclins are the principle subunit of the Mitosis Promoting Factor (MPF) whose activation has been shown to induce mitosis (Ciechanover *et al.*, 1994). The end of mitosis, marked by the transition from metaphase to anaphase, is induced by the degradation of cyclin, leading to the inactivation of MPF. Cyclin degradation ceases as the cell enters interphase resulting in an increase in cyclin levels, the activation of MPF and the start of a second round of mitosis. During interphase the cyclin remains ubiquitinated, however due to the low multiplicity of the ubiquitin, degradation is not incurred (Glotzer *et al.*, 1991).

Two conserved regions in mitotic cyclins, between residues 13-66, were shown to be required for their ubiquitin mediated degradation (Glotzer *et al.*, 1991). One termed the degradation box consists of a motif from residues 42-50 (RXXLXXIXN) and this is the proposed recognition site for the E2 enzymes. The second motif consists of four lysine residues spanning residues 60-66, and this is thought to contain the ubiquitin ligation site (Finley *et al.*, 1991). These two motifs are sufficient to target heterologous mitotic proteins for ubiquitin mediated proteolysis (Bartel *et al.*, 1990).

UBIQUITIN NUCLEAR PROTEASE (*UNP*)

While investigating retroviral insertional mutagenesis, the technique where exogeneous DNA is introduced into the germ line of mammalian cells (Gridley *et al.*, 1987), Gray *et al.* characterized the mouse mutant Mpv 20. This mutation conveys an early embryonic lethal phenotype where mice homozygous for Mpv 20 die at the eight cell stage (Gray, unpublished).

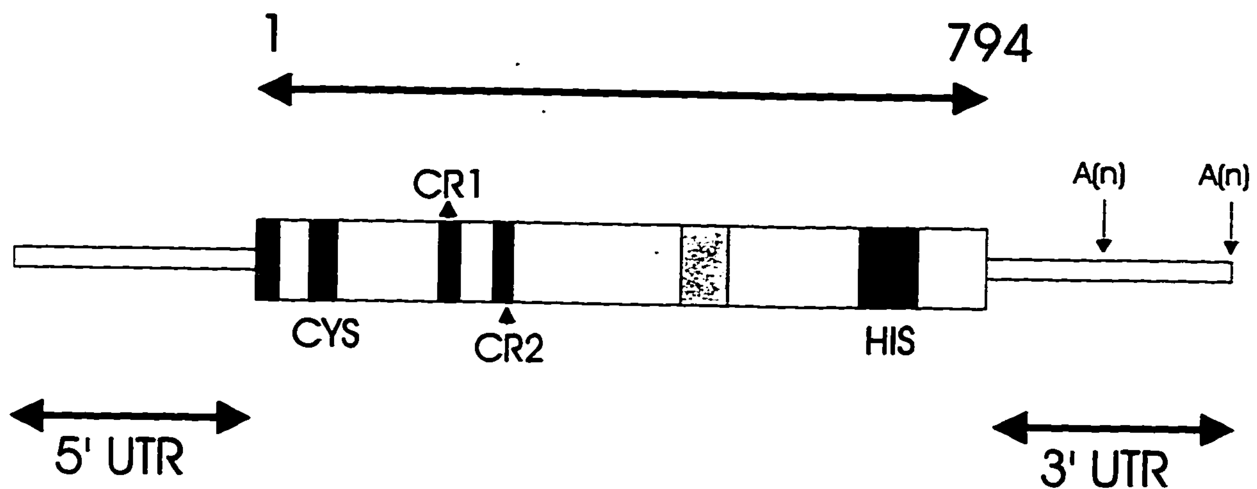
During an effort to clone and identify the disrupted gene(s), two mouse cDNA's corresponding to a novel gene named Ubiquitous Nuclear Protease (*Unp*) were isolated. {Figure 6}. When the sequence of these clones was analyzed a single common open reading frame (ORF) was identified. The ORF was preceeded by a relatively large 5' untranslated region (UTR) containing 11 short ORF's, each with an initiation and termination codon. The Kozak scanning model for translation dictates that the first AUG codon with proper consensus (GCCGCCA/GCCAUGG) serves as the initiation codon (Kozak, 1989). However, contrary to this model, it has been observed in a subset of mRNA's encoding growth regulatory molecules such as transforming growth factor-β

FIGURE 6

Schematic of the *Unp* cDNA

A schematic representation of the Unp cDNA and functional motifs found in the predicted amino acid sequence. All abbreviations are listed below figure.

Adapted from; Gupta, K. (1994) Cloning and characterization of *Unp* a novel nuclear proto-oncogene. PhD Thesis.



■ p300 binding consensus

■ Cysteine/histidine residues-
Ubiquitin protease consensus

■ CR1 (LHE) & CR2 (LXCXE)-
pRb binding consensus

▤ Nuclear localization signal (PQKKKK)

(TGF-β), that the translation machinery ignores these preceeding initiation codons (Collins *et al.*, 1985, Park *et al.*, 1987, Garvin *et al.*, 1988, Arricke *et al.*, 1991). Studies are currently underway to determine the actual initiation site used in *Unp* mRNA translation. Removal of the upstream ORF's from TGF-β was shown to lead to an increase in the translation efficiency of the mRNA (Marth *et al.*, 1988; Arrick *et al.*, 1991). Following the removal of the 5' UTR from the full length *Unp*, the levels of *Unp* expression increased 5 fold (Gray, unpublished) suggesting that the 5' leader is indeed influencing mRNA translation.

Sequence similarity of *Unp* to the oncogene *Tre-2* led to subsequent investigation of the tumour forming potential of *Unp* (Nakamura *et al.*, 1992). Gupta *et al.*, showed that when athymic CD1 mice were injected with NIH 3T3 cells, expressing both the full length *Unp* cDNA (794 amino acids) or a *Myc* tagged truncated cDNA encoding amino acids 234-794, tumours developed while those mice receiving control untransfected 3T3 cells showed no tumour formation (Gupta *et al.*, 1994).

When the two cDNA's obtained were fully sequenced it was found that even though the 3' region of both clones were polyadenylated, one had an additional 300 base pairs beyond. Neither cDNA possessed the consensus (AATAAA) polyadenylation signal (Proudfoot, 1991), however both contained degenerate signals upstream of the poly(A) tails (Gupta Ph.D. thesis, 1994).

The *Unp* gene is transcribed into two types of mRNA species (3.5 and 3.7 kb) each differing at the 3' end (Gupta Ph.D. thesis, 1994). Both polyadenylation signals were determined to be utilized and ignored at the same frequency thereby giving rise to equal

expression of both transcripts (Gupta Ph.D. thesis, 1994). Such polyadenylation sites have been observed in other eucaryotic systems (Birnstiel *et al.*, 1985), however it is unknown if their presence in the *Unp* gene conveys some regulatory function.

Protein analysis of the *Unp* ORF predicted a 794 amino acid protein having a molecular mass of approximately 98 kilo-daltons (kDa). However, when rabbit antisera was raised to a multiple antigen peptide corresponding to positions 764-777 of the predicted amino acid sequence of *Unp*, it was observed that the antibody detected an 180 kDa endogeneous species in the nuclear fraction of NIH 3T3 cell lysates (Gupta *et al.*, 1994). This large aberration from the predicted size may be due in part to some post-translational modification of the *Unp* protein such as phosphorylation, a hypothesis that was further investigated in this study.

Although the amino acid sequence has not revealed the function of *Unp*, several known motifs have been identified (Gupta *et al.*, 1994){Figure 6}. A putative nuclear localization signal (NLS), similar to that found in the tumour suppressor protein p53 (Shaulsky *et al.*, 1990) has been identified, however its function in the localization of *Unp* is undetermined.

A bipartite binding consensus sequence for the retinoblastoma protein (pRB), designated LHE X_n LXCXE , where X is any amino acid and X_n is a variable spacer region, has been identified in *Unp*. This motif has been shown to be highly conserved in proteins that physically associate with pRB (Hu *et al.*, 1990), suggesting that *Unp* may be a cellular pRB binding protein (Gupta Ph.D. thesis, 1994). P300, a pRB related protein has a consensus binding motif of MRKXXXL, where X is any amino acid (Wang *et al.*, 1993), and this sequence has also been identified in *Unp* sequence, leading us to believe that *Unp* may have the potential to associate with p300. Any type of physical interaction of *Unp*

with either of these proteins has yet to be shown.

UNP POSSESSES UBIQUITIN SPECIFIC PROTEASE ACTIVITY

The *Unp* ORF has been shown to contain cysteine (cys) and histidine (his) centred domains which are conserved among the ubiquitin-specific proteases of *Saccharomyces cerevisiae* (Tobias *et al.*, 1991, Baker *et al.*, 1992). These proteases are members of a family of enzymes which cleave ubiquitin tags from substrates targeted for proteolytic degradation (Varshavasky, 1992). The above two regions alone have been shown to comprise all the active sites of the protease (Bartel *et al*,. 1990).

The *Unp* protein was shown to possess ubiquitin protease activity in various cleavage assays (Baker and Gray, unpublished). These assays were based on the N-end rule degradation signal which states that " the metabolic stability of a protein can be related to the identity of its amino-terminal residue" and variants on this rule have been shown to operate in yeast, bacteria and eucaryotes (Bartel *et al.*, 1990). The 20 amino acids which can be present at the N-terminus were divided into two groups; stabilizing and destabilizing residues with respect to the half lives they confer on different proteins ("N-end rule").

Based on this knowledge the following assay was performed to determine if Unp did possess cleavage activity. In brief, the full length *Unp* cDNA was transformed into two strains of the bacteria *E. coli,* one containing the Ub-methionine- βgal fusion protein and the other Ub-arginine-βgal. In the absence of exogeneous ubiquitin cleavage activity, both these strains produce blue colonies since bacterial cells lack ubiquitin protease activity. Based on the N-end rule, when cleavage activity is present, bacteria containing the

methionine fusion protein will still produce blue colonies while those possessing the arginine fusion construct will produce white colonies (Varshavasky, 1992).

It was found that in the presence of *Unp*, Ub-arginine-βgal containing bacteria did in fact produce white colonies confirming that *Unp* does code for a ubiquitin specific protease (Baker and Gray, unpublished). These results were verified extracts from the Unp expressing E. coli were shown to be capable of cleaving a purified ubiquitin-glutathione-S-transferase fusion protein (Baker and Gray, unpublished).

Additional experiments have shown that mutation of the conserved cysteine domain in *Unp* eliminated its cleavage activity, confirming the importance of the cysteine domain in the protease activity of *Unp* (Gupta, 1994).

RATIONALE FOR STUDY OF MITOTIC CYCLINS

Cyclins are known to be degraded through the ubiquitin pathway, in particular the B type cyclins, which are vital for the both the transition into and exit from mitosis (Glotzer *et al.*, 1991, Varchavsky, 1992). In yeast, prevention of cyclin B degradation prevents cell cycle progression at the G2 or early M phase (Seufert *et al.,* 1995), and it has been shown that injection of antibodies to cyclin A during G2 prevents the cell undergoing mitosis (Rozenberg *et al.*, 1995).

It is conceivable that our consistent failure to obtain cells which stably over-express the Unp protein is due to the disruption of the mitotic cyclins' cellular function, perhaps through alterations in their ubiquitin mediated proteolysis. I therefore sought to first ascertain if the subcellular localization of Unp was consistent with this hypothesis and second, determine if the phenotype of cells transiently transfected with Unp resembles that

of yeast cells possessing mutations in mitotic cyclins.

MATERIALS AND METHODS

SECTION 1

1.1 CONSTRUCTION OF THE pDG50 PLASMID

For *in vitro* transcription and translation of the *Unp* protein, *Unp* complementary DNA (cDNA) minus the 5' untranslated region (UTR) was cloned in frame 5' to the 6x *Myc* tag using Polymerase Chain Reaction (PCR) (Gray, unpublished).

The resultant 3.2 kilobase (kb) fragment was purified on low melt agarose using the Geneclean System (Bio101) and cloned into the Bam *H1*/ Not *1* site of the plasmid *p*cDNA3 (Invitrogen). Here *Unp* expression was under control of the Cytomegalovirus (CMV) promoter in mammalian cells and under the T7 promoter for *in vitro* transcription using T7 polymerase {Figure 7}.

The ligation product was transformed into competent TG-1 *E. coli* by electroporation and the resultant ampicillin resistant bacterial clones were analyzed for the presence and orientation of the *Unp* sequence. The construct was then confirmed by double stranded sequencing.

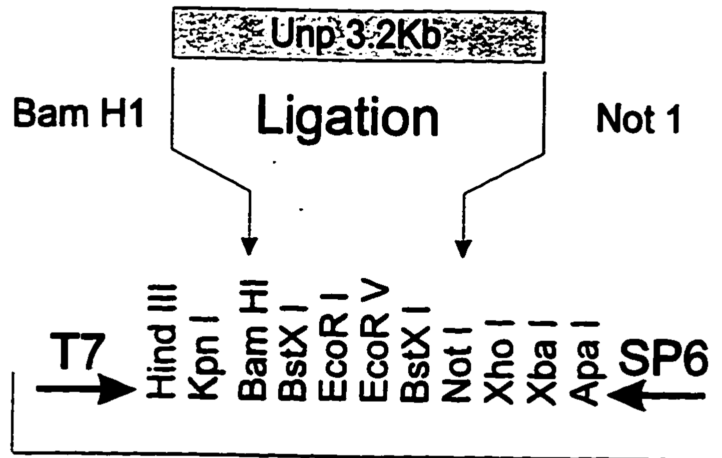
1.2 TRANSIENT TRANSFECTION OF THE *UNP* CONSTRUCT INTO COS-1 CELLS

DNA used in transfections was isolated using cesium chloride density gradient centrifugation. All constructs were then confirmed by restriction digest prior to use. An African Green Monkey kidney cell line (COS -1) was grown on a monolayer and

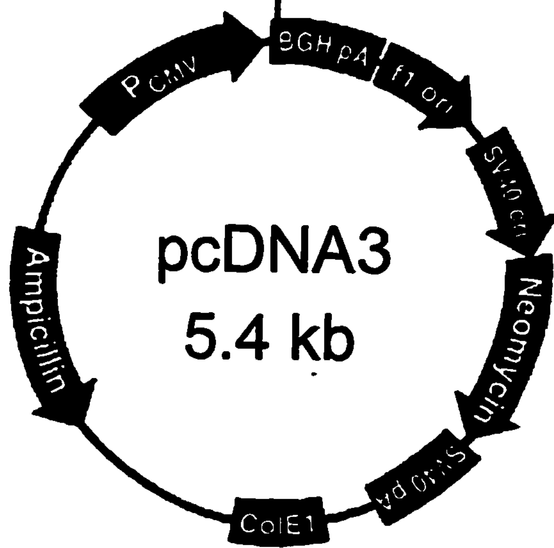
FIGURE 7

Schematic of pDG 50 construct

pDG 50 is the construct which contains the Unp cDNA, minus the 5' UTR cloned in frame to the 6x *Myc* epitope. Expression is driven by the CMV promoter from the pcDNA 3 vector (Invitrogen).



Unp 3.2Kb
Bam H1
Ligation
Not 1
T7
Hind III
Kpn I
Bam HI
BstX I
EcoR I
EcoR V
BstX I
Not I
Xho I
Xba I
Apa I
SP6



P CMV
BGH pA
f1 ori
Neomycin
SV40 pA
ColE1
Ampicillin
pcDNA3
5.4 kb

maintained in Modified Eagle's Media (α-MEM) (Gibco-BRL) containing 10% heat inactivated fetal bovine serum (FBS) (Gibco-BRL) at 37°C in 5% CO_2. Transfections were performed using an electroporation method. In brief, cells were harvested in a solution of 0.25% Trypsin/ 1mM EDTA in 1x Phosphate Buffered Saline (PBS)(150mM NaCl, 16mM Na_2HPO_4, 4mM KH_2PO_4, 16mM KCl), washed twice in 1x PBS and re-suspended in 5ml α-MEM. The cells were then counted on a Coulter counter (Coulter Electronics) and 2 x 10^6 cells re-suspended in 500 μl α-MEM. 10 μg of the appropriate construct plasmid DNA suspended in Tris/EDTA buffer (TE) (10mM Tris-HCl pH 8.0, 1mM EDTA) was added to the cells and the mixture incubated on ice for 10 minutes. The transfection mix was shocked using 220V (Bio-Rad Gene Pulser) at 960 μF capacitance, with a resultant time constant of approximately 25 milliseconds. The cells were allowed to recover at room temperature for 20 minutes and subsequently plated on 100 mm tissue culture dishes. All transfected cells, unless otherwise stated, were incubated for a minimum of 24 hours at 37°C in 5% CO_2 prior to protein isolation.

Untransfected COS-1 cell stocks were stored at -80°C in freezing medium consisting of α-MEM containing 10% serum and 10% Dimethylsulphoxide (DMSO). Cells were harvested in a solution of 0.25% Trypsin/ 1mM EDTA in 1x PBS, washed twice in 1x PBS and re-suspended in 900μl α-MEM containing 100μl DMSO. Prior to storage at -80°C the cells were quickly frozen on dry ice for 10 minutes.

1.3 IMMUNOBLOT ANALYSIS

Whole cell extracts or membrane extracts were dissolved in sodium dodecyl sulphate

(SDS) sample buffer (2% SDS, 20% glycerol, 50mM Tris pH 6.8, 5% β-mercaptoethanol, 0.01% bromophenol blue). Prior to resolution using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (8% polyacrylamide), all samples were boiled for 10 minutes and allowed to cool on ice. The samples were then transferred to a nitrocellulose membrane (Hybond C-extra), previously soaked in Transfer Buffer (0.2 M Glycine, 0.02M Tris-HCl, 6% Methanol) for 15 minutes at room temperature. Blots were electrophoresed at 0.8 milliamps (mA) for 2 hours at 4°C. The membrane was then blocked in Tris Buffered Saline Tween (TBST)(180mM NaCl, 10mM Tris-HCl, 0.05% Tween 20) supplemented with 5% Skimmed Carnation milk. Blots were probed with either a monoclonal antibody 9E10 directed against 6X *Myc* tag at a dilution of 1:100) (Evan *et al.*, 1985)(gift of Dr. John Bell) or a polyclonal antibody directed against Actin (1:3000) which was used to determine the presence of cytoplasmic proteins. Visualization of the blots was carried out using horse radish peroxidase-coupled goat anti-mouse secondary (Boehringer Mannheim). The antibody was detected by allowing 500μl of each Enhanced Luminol Reagent and Oxidizing agent from the NEN Renaissance Kit (NEN) to react with the blot for 1 minute at room temperature. The membrane was exposed to autoradiography film (Reflection-Dupont) for varying time periods.

1.4 NUCLEAR AND CYTOPLASMIC FRACTIONATION OF UNP TRANSFECTED COS-1 CELLS

2 x 10^6 COS-1 cells, transfected as outlined previously, were incubated at 37°C for 24

hours after which the cells were washed 3 times in 1x PBS. To harvest the cells 100 μl Hypotonic buffer (10mM HEPES, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 10mM KCl, 200 μg/ml Phenyl-methylsulphonylfluoride (PMSF), 2 μg/ml Apropinin, 5 μg/ml Leupeptin per 100mm plate) was added and the plate incubated on ice for 5 minutes. The cells were collected by scraping, ruptured using 12 strokes of a 1ml Dounce Homogenizer and incubated on ice for 30 minutes. To separate the nuclear pellet from cytoplasmic fraction, the lysate was then centrifuged at 1500 rpm for 5 minutes at 4°C. The fraction retained after a further centrifugation step of the supernatant at 30,000 rpm for 5 minutes at 4°C was classed as the cytoplasmic fraction. The pellet from the initial spin was resuspended in Lysis buffer (20mM Tris-HCl pH 7.5, 150mM NaCl, 2mM EDTA, 1% Triton X-100, 200 μg/ml PMSF, 2 μg/ml Apropinin, 5 μg/ml Leupeptin) centrifuged at 30,000 rpm for 5 minutes at 4°C and the supernatant represented nuclear fraction 1. The resultant pellet was resuspended in 100 μl Lysis buffer and was classed as nuclear fraction 2. A portion of each sample was resuspended in 2x SDS sample buffer, resolved on SDS-PAGE and subjected to autoradiography.

SECTION 2

2.1 PREPARATION OF COVERSLIPS FOR IMMUNOFLUORESCENCE

Glass cover-slips were flamed in concentrated ethanol and placed in sterile 35 mm tissue culture dishes. 0.15% gelatin in 1x PBS was placed on the cover slip surface and allowed to dry overnight at room temperature. 1x 10^5 transfected COS-1 cells were applied to each gelatin coated slide and left to recover for 24 hours at 37°C before treatment. Prior to fixing, the cells were washed 1x PBS to remove cellular debris.

2.2 METHANOL FIXATION PROCEDURES FOR DETECTION OF UNP AND CYCLIN B1

Methanol (-20ºC) was carefully applied to the surface of the cover-slip which was then placed at room temperature for 5 minutes and subsequently washed twice with 1x PBS for 5 minutes. The cover-slips were then labeled with the appropriate antibody combination or stored at 4°C in 1x PBS for a maximum of 7 days.

2.3 DETECTION OF MYC-TAGGED UNP AND CYCLIN B1

Cells were incubated with 1:500 primary anti c-*Myc* rabbit polyclonal IgG (Santa Cruz) in 1x PBS containing 0.3% Triton X-100 for 1 hour at 37ºC in a humidity chamber. Unbound primary was removed by washing 3 times with 1x PBS for 5 minute intervals after which the cells were treated with 1:50 secondary anti-rabbit IgG Fluorescein linked antibody (Amersham) in 1x PBS containing 0.3% Triton X-100 and subject to the same

conditions as above for 45 minutes. The cells were then washed 3 times with 1x PBS for 5 minute intervals and the coverslips were subsequently mounted using "Slow-fade Antifade" (Molecular probes). Fluorescent cells were detected by Zeiss (ZF) microscopy and photographs were taken using a Zeiss camera and 400 Elite film (Kodak).

Detection of Cyclin B1 was performed as outlined above except cells were incubated with 1:25 primary anti-Cyclin B1 mouse polyclonal IgG (Santa Cruz) and 1:100 secondary anti-mouse IgG Rhodamine B conjugate (TAGO Inc.)

2.4 SIMULTANEOUS FIXATION/ EXTRACTION (FIX/EX) FOR DETECTION OF MICROTUBULES

Transfected COS-1 cells adhered to gelatin coated coverslips were washed 3 times with 1x PEM buffer (80mM PIPES, 5mM EGTA, 1mM $MgCl_2$). Fix/Ex (0.5% Triton X-100, 0.25% Glutaraldehyde, 3% Formalin in 1x PEM buffer) was added to the coverslips and left at room temperature for 10 minutes. Samples were then washed 3 times with 1x PEM and stored in 1x PBS at 4°C until use.

Detection method was the same as previously described for detection of *Myc*-tagged Unp except cells were incubated with 1:200 primary anti β-Tubulin antibody (Yol1/34) and 1:150 secondary anti-rat IgG Fluorescein linked antibody (both antibodies a gift from Dr. Micheline Paulin-Lavasseur).

SECTION 3

3.1 CELL CYCLE ARREST STUDIES

All experiments were performed in duplicate with COS-1 cells transfected as outlined in Section 1.2 and allowed to recover on 100mm tissue culture dishes in α-MEM containing 10% FBS at 37°C / 5% CO_2 for 24 hours. Following the treatment outlined below, the cells were harvested with 0.25% trypsin/EDTA and prepared for Flow cytometric analysis (protocol outlined in Section 3.5).

COS-1 cells, transfected as previously described, were placed on gelatin coated cover slips and subject to the same treatments as the 100mm tissue culture plates above. However, following the treatments, these cells were fixed with methanol and labeled for the presence of Cyclin B1 and Unp as described above.

3.2 G1 PHASE ARREST

Media on each 100mm plate was replaced with serum-free α-MEM and the cells placed at 37°C for 12 hours and was again replaced with α-MEM containing 10% FBS after which the cells were incubated for a further 12 hours at 37°C. The percentage of cells in G1 phase was calculated by flow cytometry analysis.

3.3 S PHASE ARREST

Hydroxyurea, an agent that causes cell cycle arrest in the S phase prior to DNA replication, was added to the transfected cells to a final concentration of 10mM/ml and the cells placed at 37°C for a further 30 hours. The percentage of cells in G1/S phase was

confirmed by flow cytometry. Exposure of the cells to longer periods of treatment with hydroxyurea resulted in cell death.

3.4 MITOTIC ARREST

Colcemid, an agent that causes cellular arrest at prometaphase/metaphase by disrupting the microtubules, was added to a final concentration of 0.06 μg/ml and the cells placed at 37°C for 12 hours. A minority of cells were observed to have developed the metaphase plate indicating late metaphase. Mitotic phase arrest was confirmed by flow cytometry.

3.5 FLOW CYTOMETRY ANALYSIS

Unp expressing COS cells (2×10^6) were harvested by trypsinization as described in section 1.2 and resuspended in 5ml 1x PBS. 100 μl was removed and counted using a Coulter Counter (Coulter Electronics). The cells were then washed 2 times with cold 1x PBS and resuspended in 1ml 70% Ethanol in 1x PBS per 1×10^6 cells. Cells were incubated on ice for 15 minutes and then stored at -20°C prior to staining with Propidium iodide (gift from Dr.M. McBurney) and analysis using Coulter Epics XL-MCL profile II flow cytometer. The software used was Coulter Epics XL Flow Cytometry workstation version 1.5 (1993) (Coulter Corporation) and the single parameter cell cycle histograms were further analyzed by "Multicycle AV" advanced version cell cycle analysis version 3.11 (1993) (Phoenix Flow Systems, San Diego, CA).

SECTION 4

4.1 ^{32}P ORTHOPHOSPHATE LABELLING OF COS-1 CELLS

Prior to incubation with the radiolabel, a plate of COS-1 cells, transfected as previously described with *Myc*-Unp, was placed in Dulbeccos Modified Eagle's media, in the absence of Glutamine and Sodium phosphate, and supplemented with 1x Glutamine (Glutamax-Sigma) and incubated for 30 to 60 minutes at 37°C. Filter sterilized sodium ortho-vanadate was added to a final concentration of 500 μM to prevent the de-phosphorylation of translated proteins. 200 μCi /ml ^{32}P Ortho-phosphate (Amersham) was then added and the cells incubated at 37°C for 4 hours in a chamber gassed with 5% CO_2. The cells were washed 5 times in 1x PBS after which they were harvested in 1 ml Lysis A buffer (Lysis buffer with the addition of 10mM sodium fluoride and 4mM sodium pyrophosphate), centrifuged at 13,000 rpm for 5 minutes at 4°C and the supernatant retained. To reduce extraneous background the supernatant was pre-cleared with Protein A Sepharose CL-4B beads (Pharmacia). In brief, 50 μl pre-washed protein A sepharose beads in 1:1 Lysis A buffer (supplemented as above) was added to the lysate and rotated at 4°C for 30 minutes. The mixture was then centrifuged at 13,000 rpm for 5 minutes at 4°C and the supernatant retained. This procedure was repeated 3 times.

The sample was then divided into two microcentrifuge tubes and each diluted to 1 ml with Lysis buffer. To one of the samples 10 μg of precipitating *Myc* 9E10 antibody was added and to the other 10 μg rabbit anti-mouse serum, the binding antibody for Protein A sepharose, was added as a control. The lysate was rotated for 1 hour at 4°C at which point

50 μl Protein A sepharose beads, previously incubated for 1 hour at 4°C with rabbit anti-mouse serum to enable the *Myc* tagged proteins to bind effectively, was added and the sample rotated at 4°C for a further hour. The beads were washed 3 times with Lysis buffer, resuspended in 2x SDS sample buffer, resolved on SDS-PAGE and subjected to autoradiography.

4.2 PHOSPHO-AMINO ACID ANALYSIS

Phosphorylated proteins were immunoprecipitated as previously outlined, resolved on SDS-PAGE, and transferred to Immobilon-P membranes (Millipore). Radioactive bands corresponding to the immunoprecipitated proteins were excised from the membrane and hydrolysed directly in 6 N double distilled HCl for 75 minutes at 110°C. The hydrolysate was lyophilized, mixed with authentic phospho-amino acids, spotted onto Cell type-100 thin layer chromatography plates (Sigma) and resolved by two dimensional thin-layer electrophoresis. In the first dimension the samples migrated at pH 3.5 (Pyridine, acetic acid, double distilled water, 1:10:189) for 15 minutes at 1500 volts. The thin layer was then dried, rotated 90° and resolved at pH 1.9 (88% formic acid, acetic acid, double distilled water, 1:3:40) for 30 minutes at 1500 volts. Phospho-amino acid standards were detected by 2% ninhydrin/acetone staining of the thin layer plates and radioactive phospho-amino acids were detected by autoradiography.

RESULTS

CHAPTER 1

SUB-CELLULAR LOCALIZATION OF THE UNP POLYPEPTIDE

Analysis of the predicted amino acid translation product of *Unp*, demonstrated that a putative nuclear localization signal resided within the Unp polypeptide. Its identification was based on its similarity to the NLS-1 domain found in the tumour suppressor protein p53. NLS-1 is the main nuclear localization signal found in p53 and is responsible for directing p53 as well as heterologous proteins to the nucleus (Shaulsky *et al.*, 1990). Based on this knowledge cell fractionation studies were performed and Unp was reported as a nuclear protein (Gupta *et al.*, 1994).

The initial objective of subsequent research was to identify potential substrates of Unp, therefore it was necessary to verify the primary location of Unp within the cell. To facilitate these *in vitro* studies in the absence of a working antibody, the full length *Unp* cDNA was fused to a 6 X *Myc* epitope to which a commercial antibody, anti-*Myc* 9E10, is available (Evan *et al.*, 1985). The resultant fusion was ligated into the pcDNA 3 mammalian expression vector to create pDG 50 which was then transfected into NIH 3T3 cells. Attempts at producing a stable Unp expressing 3T3 cell line were unsuccessful, therefore transient transfections were performed into COS-1 cells. These cells allow the expression of the Unp protein in large quantities and permit easier detection in subsequent *in situ* localization studies.

EXPRESSION OF THE UNP POLYPEPTIDE

Western analysis was performed on COS-1 lysates from cells transfected with pDG 50 to determine if the construct was expressing the Unp protein. Detection of the *Myc*-tagged Unp by using anti-*Myc* antibody revealed a protein doublet migrating at approximately 129 and 140 kDa {Figure 8, lanes 4-6}. The nuclear protein kinase Sty (Duncan *et al.*, 1995), also tagged with the 6x *Myc* epitope, was used as an antibody control {Figure 8, lanes 1-3}.

The Unp doublet migrates at a mass slower than that predicted from the amino acid sequence coupled with the *Myc* epitope tag (approximately 105 kDa). This mobility shift was thought to be due to a post-translational modification such as phosphorylation which was subsequently investigated and will be discussed later in this thesis.

FRACTIONATION STUDIES ON UNP TRANSFECTED COS-1 CELLS

Previous fractionation studies have shown that the majority of endogenous Unp is localized in the nuclear fraction of NIH 3T3 cells (Gupta *et al.*, 1994). In order to identify the sub-localization of Unp within the nucleus, fractionation of COS-1 cells which overexpress the *Myc* tagged Unp was performed. This resulted in the production of three fractions, cytoplasmic, nuclear 1 and nuclear 2. Nuclear 1 contained the soluble proteins within the nucleus, while nuclear 2 was comprised of insoluble proteins such as components of the nuclear matrix. To ensure fractionation was complete, aliquots of both nuclear and the cytoplasmic fractions from the initial centrifugation step performed at 1500 rpm (refer to Section 1.4, Materials and Methods) were analysed under light microscopy. It was seen from the resultant slides that all the nuclear fractions {Figure 9A,

FIGURE 8

Western blot analysis of Unp expression in COS-1 cells

COS-1 cells were transfected with either pDG 50 (lanes 4-6) or pECE-*Myc*-Sty (lanes 1-3). Expression of Unp was determined by immuno-blotting with the anti-*Myc* antibody and enhanced chemiluminescence.
Arrows indicate the position of the Unp protein doublet.

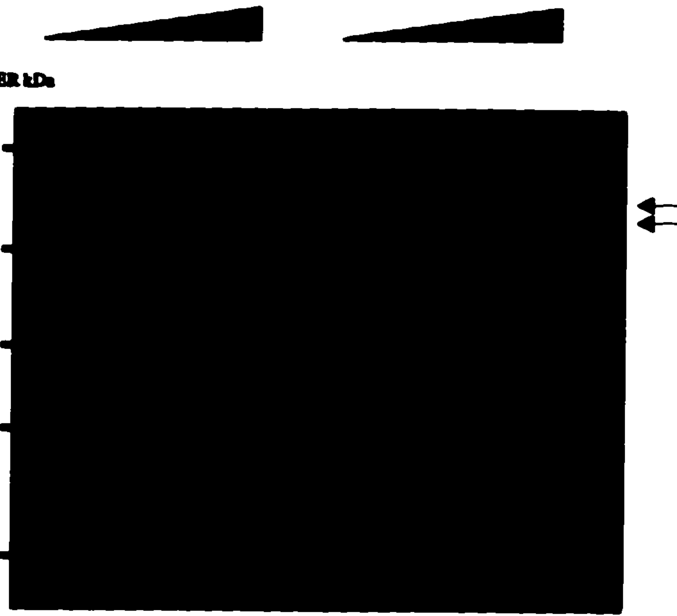
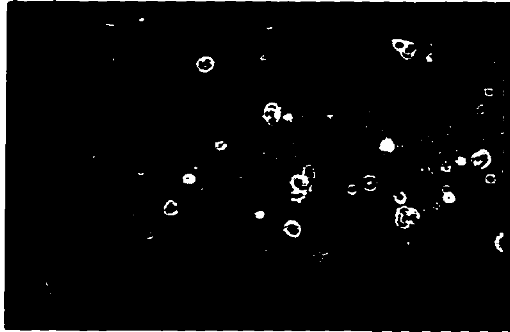

pECE MYC-STY
pDG50
MARKER kDa
175
83
62
47.5
32.5
1
2
3
4
5
6

FIGURE 9

Phase microscopy of fractionated COS-1 cells, transfected with the Unp polypeptide

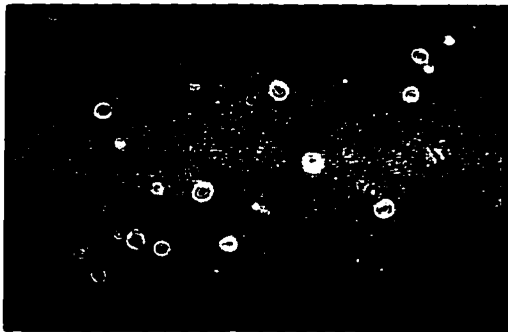
COS-1 cells were transfected with pcDNA 3 **(A and B)**, pDG 50 **(C and D)** or pECE-Myc-Sty **(E and F)** fusion constructs. The lysates were fractionated and aliquots removed after the first centrifugation step. These were placed on glass slides and examined under phase microscopy at a magnification of x20.
Nuclear **(A, C and E)** and cytoplasmic fractions **(B, D and F)**.

A



B

C



D

E

F

C and E} contained isolated nuclei which were absent from the cytoplasmic fraction, {Figure 9B, D and F}, confirming that the fractionation procedure was adequate.

Half of each fraction was resolved on SDS-PAGE and transferred to a nylon membrane. The resultant western blot was exposed to the anti-*Myc* and anti-actin antibodies and results confirmed that Unp was found predominately in the nuclear fractions {Figure 10, lanes 4 and 7}. As expected, Sty was localized to nuclear fractions 1 and 2 {Figure 10, lanes 5 and 8}. The Actin protein was used to indicate the presence of cytoplasmic proteins {Figure 10, lanes 1-8}.

We conclude that the Unp polypeptide is indeed primarily localized within the nuclear fraction as was previously shown.

LOCALIZATION STUDIES OF UNP BY INDIRECT IMMUNO-FLUORESCENCE

To further localize Unp within the nucleus visualization of Unp *in situ* was performed by indirect imunofluorescence using the anti-*Myc* antibody.

COS-1 cells were transiently transfected with pDG 50 and the cells allowed to adhere to gelatin coated coverslips. The following day, the cells were fixed and labeled with anti-*Myc* antibody which was detected using a secondary antibody conjugated to fluorescein. These studies revealed that Unp was localized in the perinuclear region of the cell with a low level detectable in the nucleoplasm {Figure 11A and B}. Similar transfections with the Sty-Myc fusion construct showed exclusive nuclear staining as expected {Duncan *et al.*, 1995, and Figure 11C}.

The distribution of Unp appeared similar to that observed by others for cyclin B1 (Jackman *et al.*, 1995). Cyclin B1 is known to translocate during the transition of the cell

FIGURE 10

Detection of Unp in the nuclear fraction of COS-1 cells

COS-1 cells were transfected with pcDNA 3, pDG 50 or pECE-Myc-Sty fusion constructs. The lysates were fractionated and the proteins separated on SDS-PAGE, transferred to membrane and exposed to anti-*Myc* antibody.
Western blot analysis of COS-1 cell lysates fractionated into cytoplasmic (lanes 1 and 2), nuclear 1 (lanes 3-5) and nuclear 2 (lanes 6-8) fractions.
Arrows indicate the Unp protein doublet. * indicates actin control.

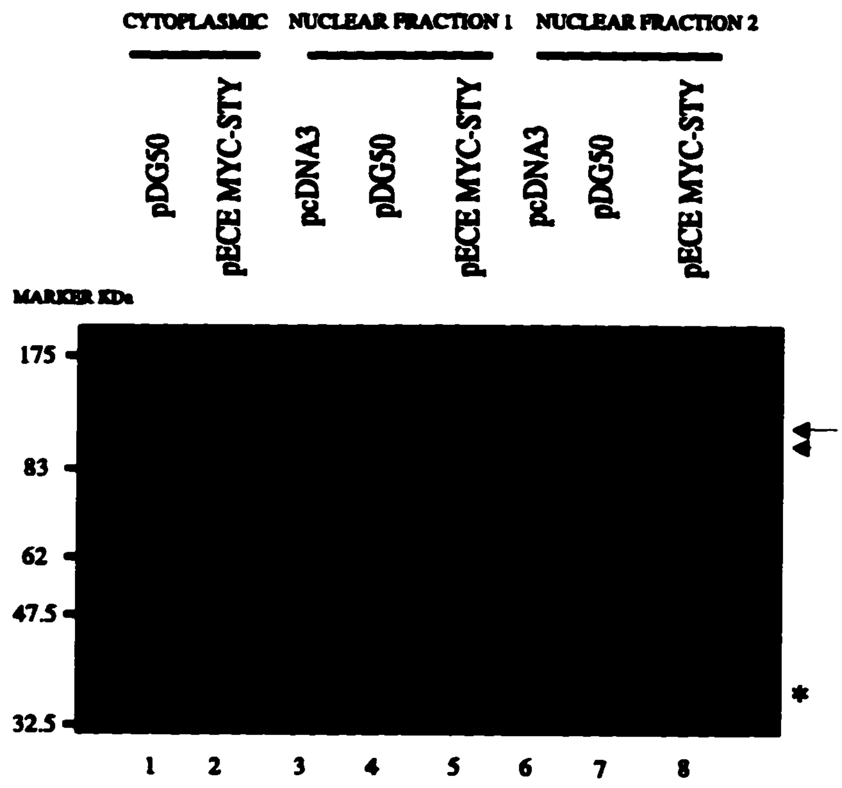

CYTOPLASMIC
NUCLEAR FRACTION 1
NUCLEAR FRACTION 2
pDG50
pECE MYC-STY
pcDNA3
pDG50
pECE MYC-STY
pcDNA3
pDG50
pECE MYC-STY
MARKER KDa
175
83
62
47.5
32.5
*
1
2
3
4
5
6
7
8

FIGURE 11

Perinuclear localization of Unp

Indirect immunofluorescent staining of Unp transfected COS-1 cells using the anti-*Myc* antibody.

A & B: pDG 50 transfected cells exhibiting nucleoplasmic distribution of Unp, magnification x40 **C**: pECE-*Myc-Sty* transfected cells showing the nuclear staining of Sty, magnification x63. **D**: anti-*Myc* antibody alone. **E**: Control, anti-mouse antibody conjugated to fluorescein in absence of primary anti-*Myc* antibody, magnification x40.

A

B

C

D

E

from G1 to mitosis. During interphase human cyclin B1 co-localizes with micro-tubules in a perinuclear fashion, however as the cell enters prophase, prior to the break down of the nuclear envelope, cyclin B1 translocates to the nucleus where it binds to both the spindle apparatus and chromosomes (Pines and Hunter, 1991)

To investigate the possibilty that Unp and cyclin B1 may co-localize and thus interact, in the cell, double labeling of Unp transfected COS-1 cells with both cyclin B1 and Unp was performed. Initially, to confirm that cyclin B1 and micro-tubules co-localize, untransfected COS-1 cells were labeled with anti- β Tubulin antibody (YOL 1/34), which was detected using fluorescein conjugated secondary antibody. These cells displayed the perinuclear network characteristic of microtubules {Figure 12A}. Subsequent labeling of untransfected COS-1 cells with anti-cyclin B1 showed a similar perinuclear distribution to that observed for the microtubules {Figure 12B}. Confocal microscopy of COS-1 cells labeled with anti-cyclin B1 and anti-β Tubulin antibodies showed a clear co-localization of the two (data not shown) confirming the results cited in published literature (Jackman *et al.*, 1995).

Double labeling experiments were then performed on Unp transfected COS-1 cells using both the anti-β Tubulin and anti-*Myc* antibodies. Confocal microscopy showed that although Unp does associate with the microtubules to some degree, this association is not to the extent observed for cyclin B1 {Figure 13A, B and C}. The Sty protein was again used as a nuclear control and minimal association was seen with the microtubules {Figure 13D, E and F}

FIGURE 12

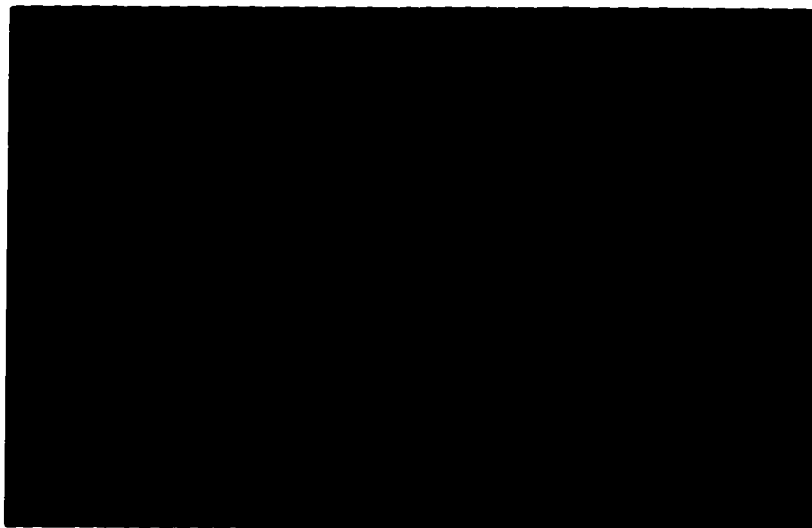
Comparison of cyclin B1 and microtubule distribution in COS-1 cells

Indirect immunofluorescent staining of untransfected COS-1 cells using the anti-cyclin B1 and anti-β Tubulin antibodies.

Untransfected COS-1 cells labeled with anti-β Tubulin **(A)**, anti-cyclin B1 **(B)**, secondary antibody for the detection of anti-β Tubulin alone **(C)**.
Magnification x40.

A

B



C

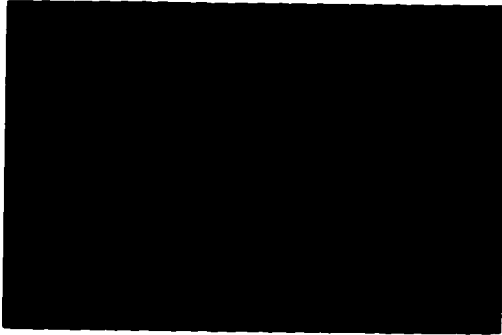
FIGURE 13

Comparison of Unp and microtubule distribution in COS-1 cells

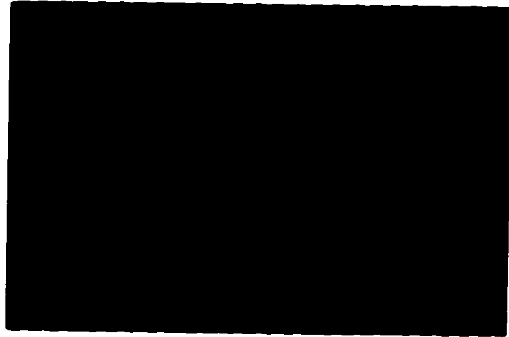
Indirect immunofluorescent staining of Unp transfected COS-1 cells using the anti-*Myc* antibody and anti-β Tubulin antibody.

pDG 50 transfected COS-1 cells labeled with anti-β Tubulin **(A)** and anti-*Myc* antibody **(B)**, **C**: overlay of the two signals.
pECE-Myc-Sty transfected COS-1 cells labeled with anti-β Tubulin **(D)** and anti-*Myc* antibody **(E)**, **F**: overlay of the two signals.
Magnification x40. Alignment of red and green signals appears yellow.

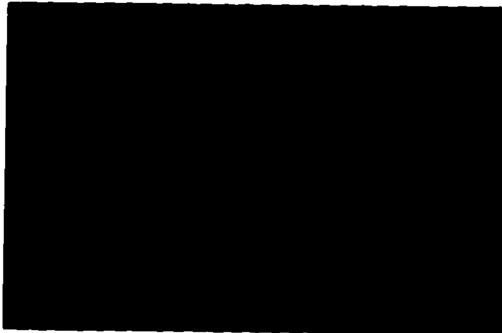
A



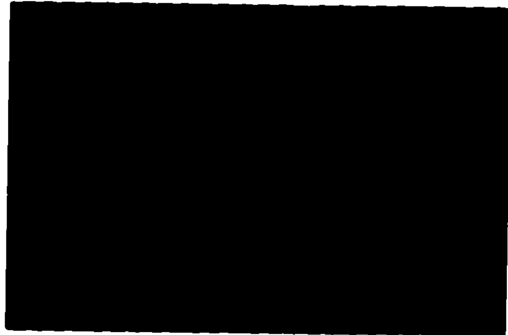
B



C



D



E

F

We conclude that even though Unp displays a perinuclear distribution similar to that of cyclin B1, it does not associate with the microtubule network to the same degree. This suggests that if there is interaction between Unp and the microtubules, it may not occur to the same extent or be via the same mechanism as for cyclin B1.

CHAPTER 2

UNP DISTRIBUTION ALTERS DURING THE CELL CYCLE

The observation that Unp and cyclin B1 possess similar, though not overlapping, distribution patterns suggests that Unp may also behave in a cell cycle dependent manner similar to that seen for cyclin B1 (Pines and Hunter, 1991). It is known that during interphase human cyclin B1 colocalizes with the microtubules (Jackman *et al.*, 1995). At the onset of mitosis cyclin B1 translocates to the nucleus where it binds to the spindle apparatus and the chromosomes (Jackman *et al.*, 1995).

It was observed in preliminary labeling studies that, while the majority of COS-1 cells possessed perinuclear distribution of cyclin B1 indicating they were in interphase, some cells were 'rounding up; and displayed 'diffuse' staining of cyclin B1 {Figure 14A}. Though this observation does not confirm the translocation of cyclin B1 into the nucleus it suggests that the cells are preparating for the onset on mitotic events (Murray, 1993).

Subsequent Hoechst staining of the nuclei of these cells revealed condensation of their chromosomes which is indicative of the cells entry into mitosis {Figure 14B}. This stage of mitosis is known to be the first visual sign that the cell is about to undergo division as the chromosomes form distinct thread like structures prior to their resolution into sister chromatids (Murray, 1993).

Double labeling experiments were performed to determine if the same 'diffuse' distribution pattern could be seen in cells expressing the Unp polypeptide. The majority of cells possessed perinuclear staining for both Unp and cyclin B1 and exhibited chromosome

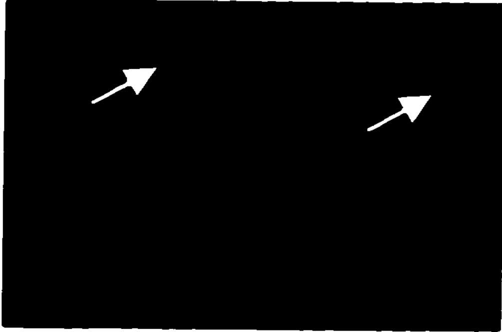
FIGURE 14

Chromosomal condensation observed in random mitotic cells

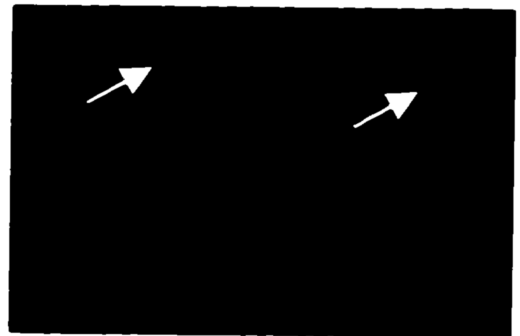
Indirect immunofluorescent staining of transfected COS-1 cells using the anti-cyclin B1 antibody.

pDG 50 transfected COS-1 cells labeled with anti-cyclin B1 **(A)**, and Hoechst DNA stain **(B)**. Control, anti-goat antibody conjugated to rhodamine in absence of anti-cyclin B1 antibody **(C)**.
Magnification x40. Arrows indicate cells displaying chromosome condensation.

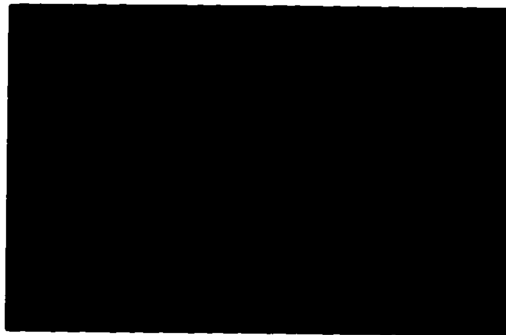
A



B



C



patterns indicative of interphase, (ie. they were not visible as distinct structures but formed a diffuse nucleoplasmic stain). A low number of cells were observed to possess 'diffuse' staining for both Unp and cyclin B1, which is consistent with Unp following a similar cell cycle pattern as cyclin B1, though again it does not confirm translocation of both into the nucleus. Subsequent Hoechst staining of the nuclei of these cells revealed that chromosome condensation had not occurred (Data not shown).

CELL CYCLE ARREST

The low transfection efficiency of Unp, resulted in an unappreciable number of transfected cells in varying stages of the cell cycle. This made it difficult to assess whether the absence of chromosome condensation in those cells possessing Unp was a phenotype of Unp overexpression.

To enhance the number, and so possibilty of observing Unp in specific phases of the cell cycle, arrest studies were performed. In order to define the stages at which the cells were arrested we assessed them by both Flow cytometry and Hoechst DNA staining.

MITOTIC ARREST OF COS-1 CELLS

Transfected COS-1 cells were exposed to the compound Colcemid for a maximum of 24 hours. Colcemid causes cellular arrest at the prometaphase / metaphase boundary by disrupting the stability of the micro-tubules. Flow cytometry analysis determined the percentage of cells exhibiting mitotic arrest (Table 1).

Cells positive for 'diffuse' staining of cyclin B1 were observed to have condensed

TABLE 1- SUMMARY OF FLOW CYTOMETRY ANALYSIS

Mean data from two sample sets of transfected COS-1 cells exposed to treatments inducing cell cycle arrest at various phases. Raw data can be found in Appendix 1.

CELL TREATMENT	% G1	% G2/M	% S PHASE
Non treated cells	41.4	19.15	39.45
Exposure to colcemid	1.8	77.9	20.3
Exposure to hydroxyurea	59.55	14.25	26.2

chromosomes {Figure 15B and 15C}. Cells displaying 'diffuse' staining with both the anti-*Myc* and anti-cyclin B1 antibodies possessed chromosomes that were in a non condensed state,and further examination of these cells, by Hoechst stain, revealed there were apparent aberrations in the condensation of the chromosomes.

Hoechst staining of a number of cells exhibiting 'diffuse' cyclin B1 and Unp confirmed that the cells initiated prophase as the stain was dispersed throughout the cell to a lesser degree than previously observed in interphase cells {Figure 16A and B}.

A cell count was performed to compare the incidence of abnormal nuclear staining with the frequency of Unp presence (Table 2a). Analysis of 41 Unp transfected cells in mitotic arrest revealed that 35 cells with 'diffuse' staining for both Unp and cyclin B1, possessed non-condensed chromosomes. 30 of those cells also exhibited aberrations in their nuclear DNA. The chromosomes were partially condensed and did not appear to form the tight condensed threads expected of the cells entry into mitosis. This was compared to the number of pcDNA 3 transfected COS-1 cells in Colcemid arrest, displaying diffuse cyclin B1 staining (Table 2b). It was seen that of the 110 cells displaying diffuse cyclin B1, 109 possessed chromosomes that were condensed (Table 2b).

This indicated a dramatic increase in the frequency of non-condensed chromosomes when the cells were transfected with the Unp polypeptide. This data suggests that the over-expression of the Unp polypeptide in COS-1 cells interferes with the mechanism of normal mitotic progression.

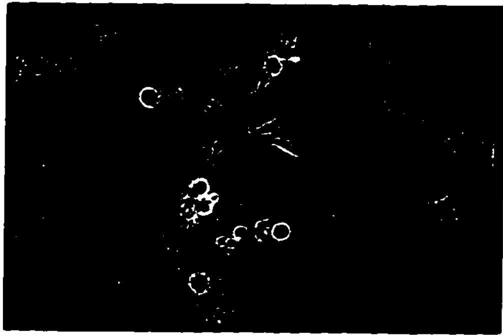
FIGURE 15

Mitotic arrest of COS-1 cells enriches for cells with condensed chromosomes.

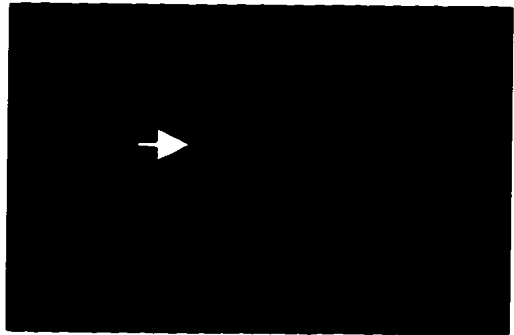
Indirect immunofluorescent staining of transfected COS-1 cells treated with colcemid for 24 hours, using the anti-cyclin B1 antibody.

pDG 50 transfected COS-1 cells treated with colcemid, representative field under phase contrast **(A)**, labeled with anti-cyclin B1 antibody,**(B)** and Hoechst DNA stain **(C)**. Magnification x40. Arrows indicate nuclei exhibiting chromosomal condensation.

A



B



C

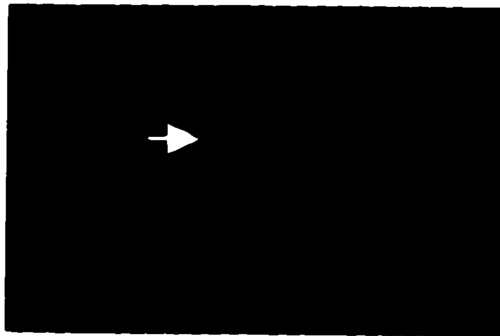


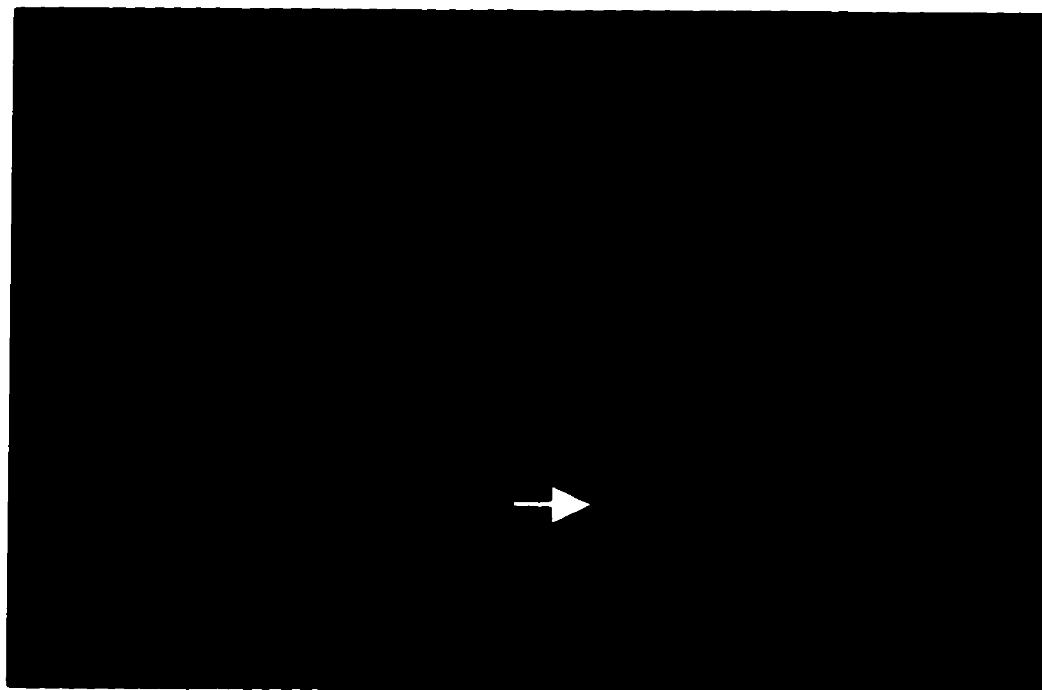
FIGURE 16

Representative abnormal nuclei observed in Unp transfected COS-1 cells

Indirect immunofluorescent staining of transfected COS-1 cells treated with colcemid for 24 hours, using the anti-*Myc* antibody.

pDG 50 transfected COS-1 cells labeled with anti-*Myc* antibody **(A)** and Hoechst DNA stain **(B)**. Arrows indicate nuclei exhibiting abnormal chromosome staining. Magnification x40.

A



B

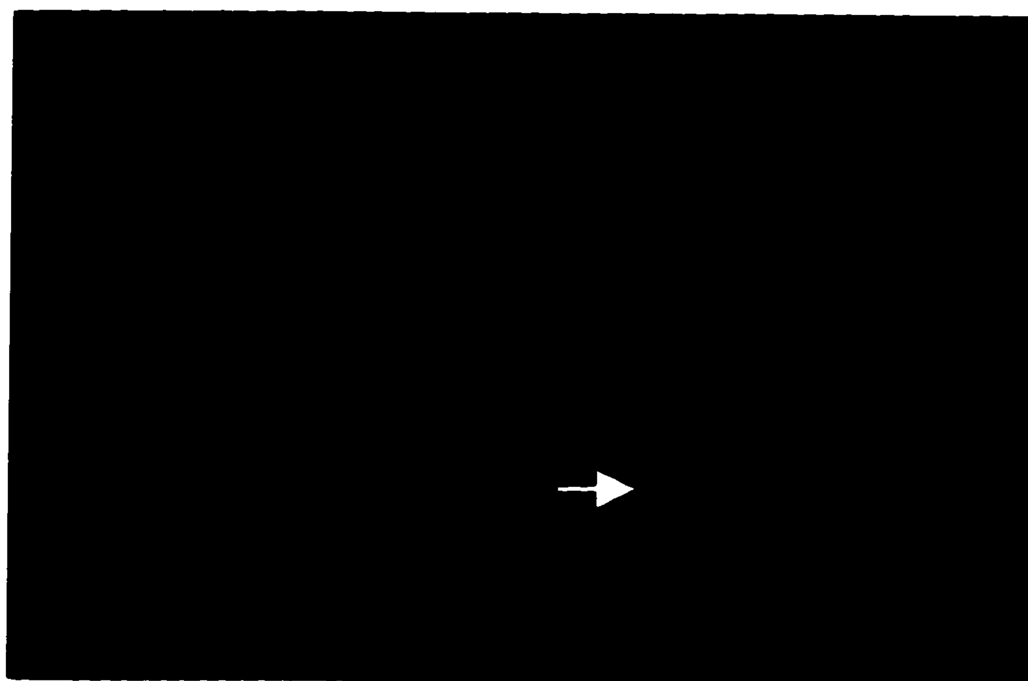


TABLE 2- SUMMARY OF CELL COUNTS

Data are based on original counts found in Appendix 2

TABLE 2(a)

COS-1 cells, transfected with Unp and possessing diffuse staining for both cyclin B1 and Unp were examined for abnormal chromosome condensation.

pDG 50	TOTAL # DIFFUSE	# CONDENSED	# NON CONDENSED	# ABNORMAL
UN-TREATED	5	0	5	5
COLCEMID EXPOSURE	41	6	35	30

TABLE 2(b)

COS-1 cells, transfected with pcDNA 3 and possessing diffuse staining for cyclin B1 were examined for abnormal chromosome condensation.

pcDNA 3	TOTAL # DIFFUSE	# CONDENSED	# NON CONDENSED	# ABNORMAL
UN-TREATED	24	23	1	0
COLCEMID EXPOSURE	110	109	1	0

G1/S PHASE ARREST OF COS-1 CELLS

To address whether Unp overexpression had an effect at cell cycle stages other than mitosis, COS-1 cells were arrested in G1/S phase by exposure to hydroxyurea. The percentage of cells in each phase was determined using flow cytometry (Table 1) which confirmed 85.75% of the cells were in G1/S phase arrest. Indirect immunofluorescence analysis was performed with anti-*Myc* and anti-cyclin B1 antibodies. No detectable change in the perinuclear distribution of either cyclin B1 and Unp was observed {Figure 17B and D}. Inspection of the nuclei via Hoechst staining revealed the chromosomes were in a form indicative of interphase and no abnormalities were observed {Figure 17C and E}. We can therefore deduce that the effect that exerts on chromosome condensation is phase dependent and is restricted via our method of analysis, to mitosis.

FIGURE 17

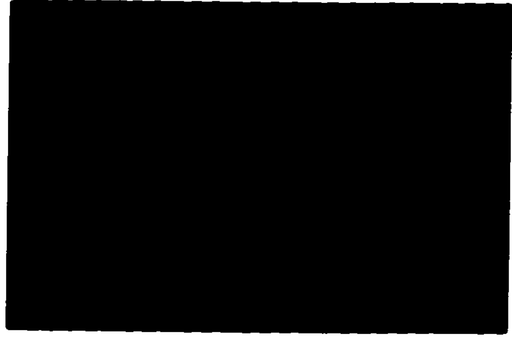
G1/S phase arrest of COS-1 cells

Indirect immunofluorescent staining of transfected COS-1 cells, treated with hydroxyurea for 30 hours, using the anti-*Myc* and anti-cyclin B1 antibody.

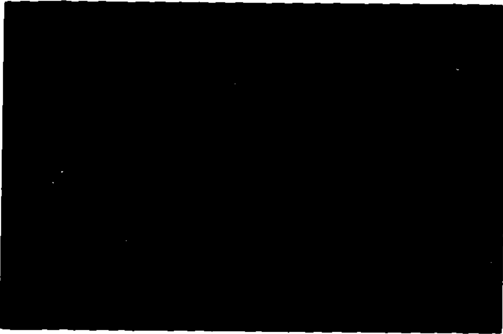
pDG 50 transfected COS-1 cells treated with hydroxyurea. Representative field under phase contrast , magnification x20 **(A)**. Different fields labeled with anti-cyclin B1 antibody **(B)**, anti-*Myc* antibody **(D)**, and Hoechst DNA stain **(C and E)**.
Magnification x40.

A

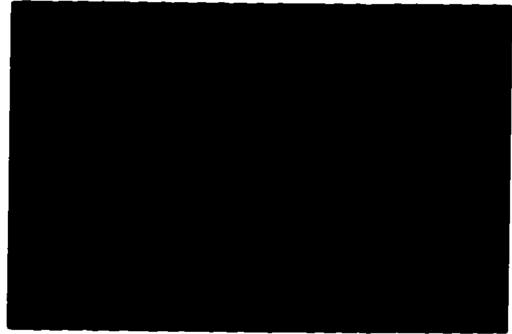
B



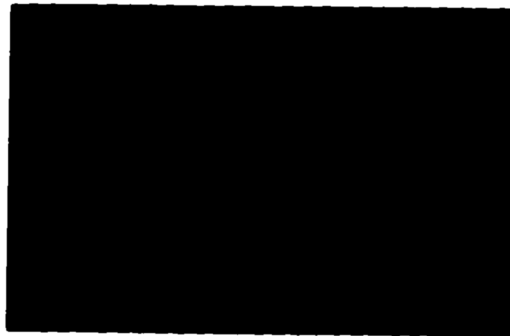
C



D



E



CHAPTER 3

UNP IS PHOSPHORYLATED ON A SERINE RESIDUE

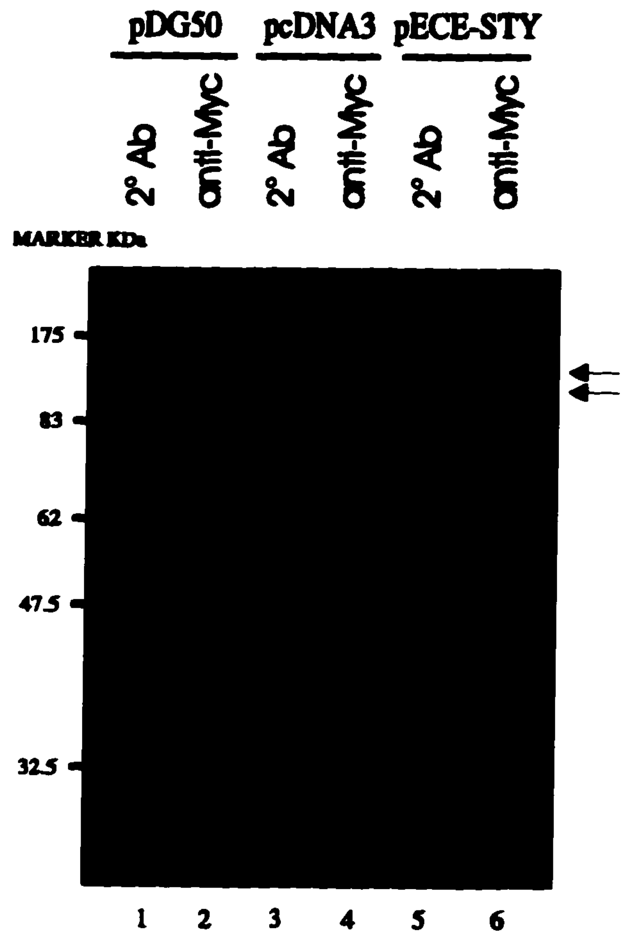
Unp was observed to migrate as a doublet in Unp expressing COS-1 protein lysates. The differences in the mobility of Unp may be due to post-translational modification such as phosphorylation. To address this question, pDG 50 transfected COS-1 cells were *in vivo* labelled with ^{32}Phosphorous after which the Unp polypeptide was immunoprecipitated with anti-*Myc* antibody and the labeled phospho-proteins separated by SDS-PAGE. The resultant western blot was transferred to membrane and exposed to autoradiograph film. A doublet was observed migrating at an identical mass to that observed in immuno-blotting analysis {Figure 8 and Figure 18(a)}. The Unp phospho-protein was excised and subjected to phospho-amino acid analysis which confirmed that Unp was indeed phosphorylated at one or more serine residues {Figure 18(b)}. Extended exposure of the blot to film did not reveal any tyrosine or threonine phosphorylation.

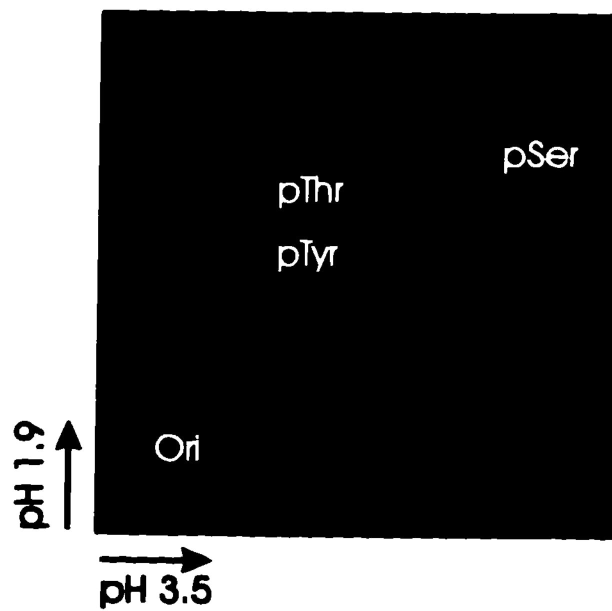
FIGURE 18

Unp is a phospho-protein

COS-1 cells transfected with pcDNA 3expression vector, pDG 50 or pECE-Myc-Sty were in vivo labeled with [^{32}P] orthophosphate. *Myc* tagged proteins were immuno-precipitated with the Myc antibody, resolved by SDS-PAGE, transferred to membrane and exposed to autoradiography (a). Phospho-amino acid analysis of phosphorylated Unp (b).

Positions of the amino acid standards are shown pSer=phosphoserine, pThr= phosphothreonine, pTyr=phosphotyrosine


pDG50
pcDNA3
pECE-STY
2° Ab
anti-Myc
2° Ab
anti-Myc
2° Ab
anti-Myc
MARKER KDa
175
83
62
47.5
32.5
1
2
3
4
5
6


pSer
pThr
pTyr
Ori
pH 1.9
pH 3.5

DISCUSSION

Tumour formation arises as a consequence of alterations in the control of cell proliferation and disorders in the interaction between cells and their surroundings (Murray, 1993). A disruption in the relationship between increase in cell number and withdrawal from the cell cycle due to differentiation and apoptosis, results in breakdown of the controls over cellular proliferation. Control over cell multiplication is a combination of both positive and negative signals which affect these two processes and tumourigenicity can result when genetic changes influence these control points.

It is now possible to identify the genetic changes that contribute to the occurrence of malignancy and this has greatly advanced our understanding of the mechanisms involved in tumour formation. It has become increasingly clear that many of the genes whose function is to inhibit cell proliferation may act through the inhibition of the expression of a proto-oncogene or the activity of its protein.

Unp was suspected to posses tumourigenic potetial due to similarity in its amino acid sequence to the *tre-2* oncoprotein (Nakamura *et al.*, 1992). This hypothesis was investigated and it was observed that when NIH 3T3 cells expressing the Unp protein were injected into athymic mice, tumours resulted in all the test animals (Gupta *et al.*, 1994), suggesting that Unp had the potential to disrupt programmed cell cycle events and checkpoints. The question of the mechanism involved remains unanswered, however the amino acid sequence of Unp contains several conserved motifs whose function in other genes has been established. This provided us with a clue to begin the investigation into the role of Unp in the cell.

PERINUCLEAR DISTRBUTION OF THE UNP POLYPEPTIDE

A motif, coding for a putative nuclear localization signal (NLS), was shown to be present in the amino acid sequence of Unp. To determine if the Unp polypeptide was distributed in the nucleus perhaps as a result of this signal, fractionation studies were performed in NIH 3T3 cells where the endogeneous polypeptide was analysed. These studies revealed that the majority of Unp protein was found in the nuclear fraction (Gupta *et al.*, 1994).

Fractionation studies were also carried out using transfected COS-1 cells, overexpressing the *Myc* epitope tagged Unp polypeptide. Western analysis of the fractionated lysates confirmed that Unp was localized predominately in the nuclear fraction {Figure 10}. The distribution of the epitope tagged Unp was further examined *in situ* by indirect immunoflourescence. A low level of Unp was observed in the nucleoplasm, however the majority was localized in a perinuclear fashion outside the nucleus {Figure 11}.

Although these results appear conflicting, the fractionation procedure may not be of sufficient stringency to permit the isolation of proteins tightly associated with the nuclear envelope, resulting in proteins found within this region fractionating along with those found in the nucleoplasm. This distribution pattern suggests that Unp may be tightly associated with some component of the nuclear membrane.

THE UNP POLYPEPTIDE IS PHOSPHORYLATED

As previously discussed the key components of cell cycle progression are regulated by phosphorylation {Figure 4}(Morgan., 1995). This includes the phosphorylation/de-phosphorylation of the catalytic cyclin dependent kinases (cdks), phosphatases and the cyclins. We propose that if Unp had the ability to interact with any of the cell cycle proteins as suggested, Unp activity and this interaction may also be regulated via phosphorylation. The Unp polypeptide was seen by SDS-PAGE to migrate as a doublet {Figure 18(a)} suggesting that phosphorylation may be present. Such migration patterns are reminiscent of proteins exhibiting both hypo- and hyper-phosphorylated forms (Gotoh *et al.* 1991, Tang *et al.* 1993).

It is known that the cdks are serine/threonine kinases and indeed it has been demonstrated that cyclin D1 is a nuclear phospho-protein which is phosphorylated on three serine residues, one of which can be located to the cyclin box (Sewing *et al.*, 1994).
It is therefore clear that serine phosphorylation is an important regulatory mechanism in the control of the cell cycle. Although the nature of Unp phosphorylation remains unknown, we speculate that it may be a regulatory factor required for the catalytic activity, substrate binding or sub-cellular localization of the Unp protein.

DEREGULATION OF THE CELL CYCLE BY UNP

The open reading frame of Unp was seen to contain two cysteine and histidine centred domains which are known to be conserved among ubiquitin specific proteases (Baker *et*

al., 1992). These proteases have the potential to cleave the ubiquitin tag associated with intracellular proteins by cleaving the amide linkage between the C-terminus of ubiquitin and either the α-amino or ε-amino groups of the substrate (Varshavasky, 1992). A large family of genes encoding these deubiquitinating enzymes has been identified with several members being implicated in growth and development, including the oncogene *tre-2* (Papa *et al*., 1993, Zhu *et al*., 1996).

The ability of Unp to cleave the ubiquitin degradation tag from protein substrates (Baker and Gray, unpublished) led us to hypothesize that Unp may have a substrate which is a cell cycle protein as several cell cycle regulators are known to be degraded via the ubiquitin pathway. The product of the proto-oncogene *c-mos*, and many of the cyclins, such as cyclin A and B, are well studied examples (Ciechanover *et al*., 1994). The degradation of cyclins is a key element in the regulation of the eucaryotic cell cycle. Ubiquitin-mediated degradation of mitotic cyclins has been extensively studied and was shown to be vital to both the activation and inactivation of MPF and the cells' entry to and exit from mitosis (Glotzer *et al*., 1991, Varchavasky, 1992).

The levels of cyclin A are known to rise during S phase reaching a threshhold during G2 and then sharply decreasing prior to the entry of the cell into mitosis (Sherr, C.J, 1993). In contrast, cyclin B rises gradually through the progression of G2, reaching threshold levels at mitosis and remaining constant until its rapid decrease at the end of mitosis (Jackman *et al*., 1995).

Cyclin A was initially classified, along with cyclin B, as a mitotic cyclin which was essential to the G2/M transition (Pagano *et al*., 1992). Both of the above interact with cdc

2, and the degradation of both cyclins is required for the exit from mitosis (Roy *et al.*, 1991). Cyclin A is destroyed before nuclear envelope breakdown, however micro-injection of antibodies to cyclin A into cells in G2 phase resulting in the premature inhibition of cyclin A activity, inhibits entry into mitosis (Pagano *et al.*, 1992).

PROPOSED MODELS FOR UNP ACTION

Deubiquitinating enzymes studied in yeast have multiple functions with some, such as *Ubp 2*, having the ability to remove ubiquitin from ubiquitin conjugated substrates prior to proteasome substrate binding, thereby slowing the turnover of the proteins (Baker *et al.*, 1992). Others, such as *Doa 4* may remove ubiquitin from proteasome bound degradation products allowing recycling of ubiquitin and proteasomes, thereby promoting further protein degradation (Papa *et al.*, 1993).

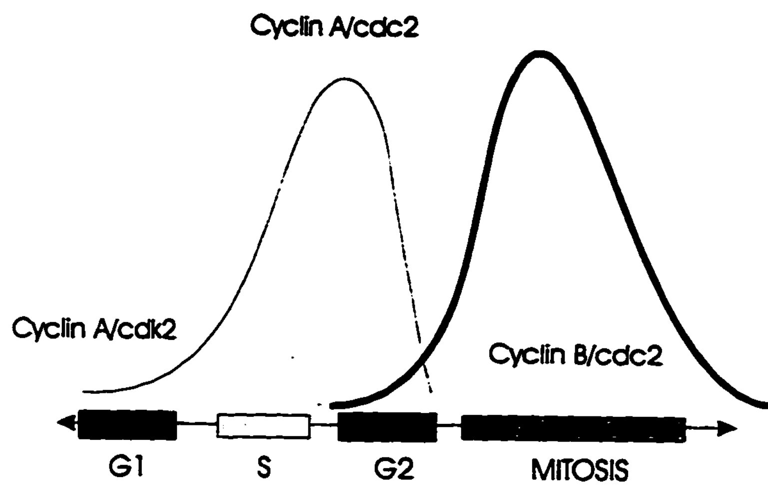
Unp, as a deubiquitinating enzyme may therefore act through two mechanisms. First, Unp may cleave the Ub degradation tag from the N-terminus of the cyclin A. This will prevent the recognition of cyclin A by the proteasome complex thus cyclin A levels will not be decreased. Cyclin A is in complex with cdc 2 and as a result of its stabilization free cdc 2 will be unavailable for binding to cyclin B. Therefore, the MPF complex which is required for the progression of mitosis, will no longer be produced (Nurse, 1994). This will result in a delayed arrest of the cell at mitosis, perhaps allowing the early stages, such as chromosome condensation, to be initiated but not completed, thus preventing the cell continuing cell division {Figure 19}.

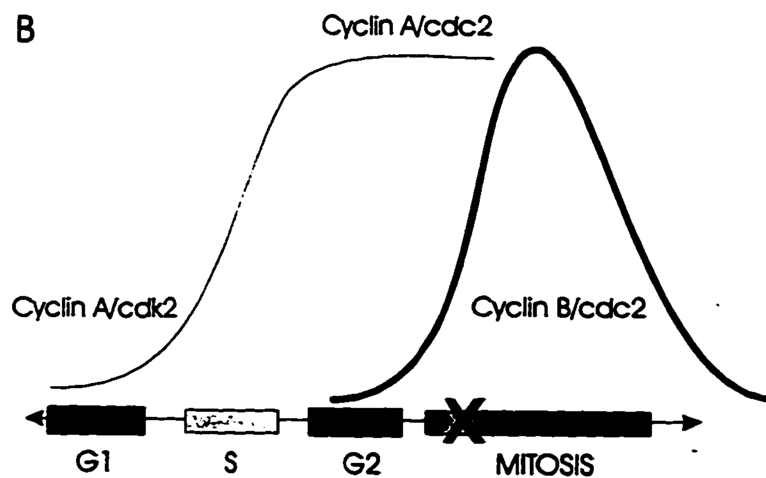
Second, overexpression of Unp may result in the increased turnover in cyclin A

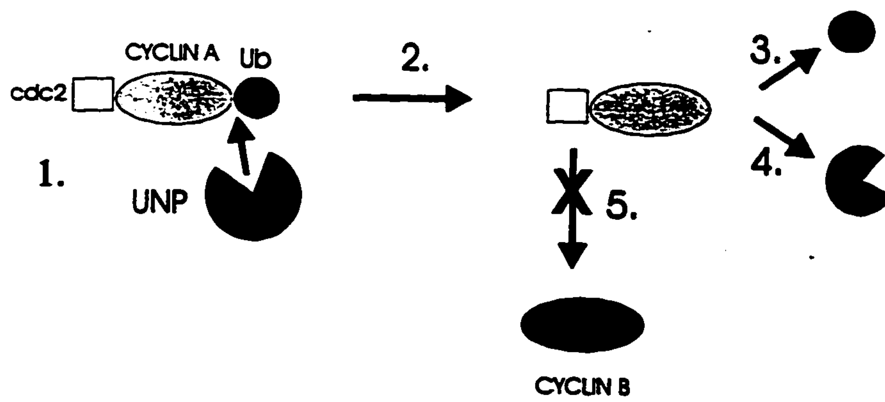
FIGURE 19

Unp protease action results in the stabilization of cyclin A

A: Oscillations of both cyclin A/cdc 2 and cyclin B/cdc2 during normal cell cycle progression.
B: **1.** The protease activity of Unp removes the ubiquitin tag from cyclin A, which is in complex with the kinase cdc 2. **2.** Cyclin A is therefore no longer targeted for degradation via the Ubiquitin mediated pathway. **3.** Reutilizable ubiquitin is released. **4.** The Unp polypeptide is available to act on remaining cyclin A molecules. **5.** Cdc 2 is sequestered by the presence of cyclin A and is not available to bind to and form an active complex with cyclin B. The cell is unable to proceed through mitosis due to the absence of an active MPF complex.


A
Cyclin A/cdc2
Cyclin A/cdk2
Cyclin B/cdc2
G1
S
G2
MITOSIS


B
Cyclin A/cdc2
Cyclin A/cdk2
Cyclin B/cdc2
G1
S
G2
MITOSIS


CYCLIN A
Ub
cdc2
1.
UNP
2.
3.
4.
5.
CYCLIN B

through a mechanism similar to that of *Doa 4*. We propose that as the levels of cyclin A rise during S and G2, its degradation, prior to the entry of the cell into mitosis, will be prevented by the protease action of Unp. The Ub tagged cyclin A will reach the proteasome complex and upon binding to the 19S subunit will have the Ub cleaved by the protease action of Unp. This will allow cyclin A to be processed by the proteasome into free amino acids

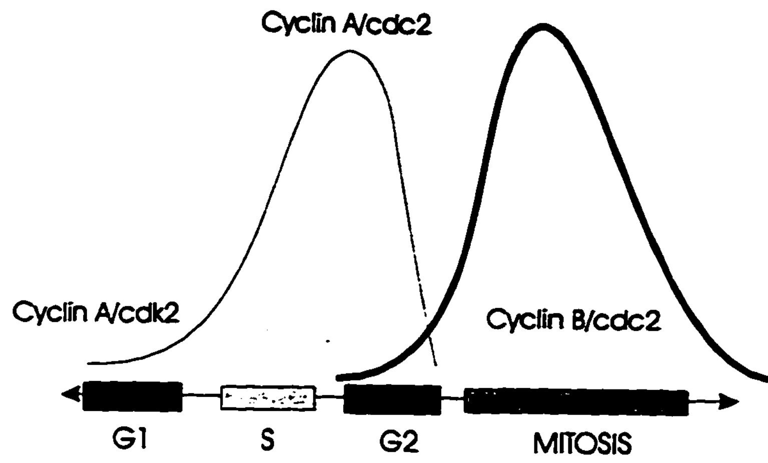
Cyclin A and its associated catalytic kinase cdc 2 are involved in the activation of the M phase cdc 2- cyclin B complex (Pagano *et al.*, 1992). The increased turnover of cyclin A by the action of Unp may prevent levels of the cyclin A/cdc 2 complex reaching the threshhold required for the activation of cyclin B, thus the cell will arrest prior to mitosis but may begin the intial stages of chromosome condensation {Figure 20}.

FIGURE 20

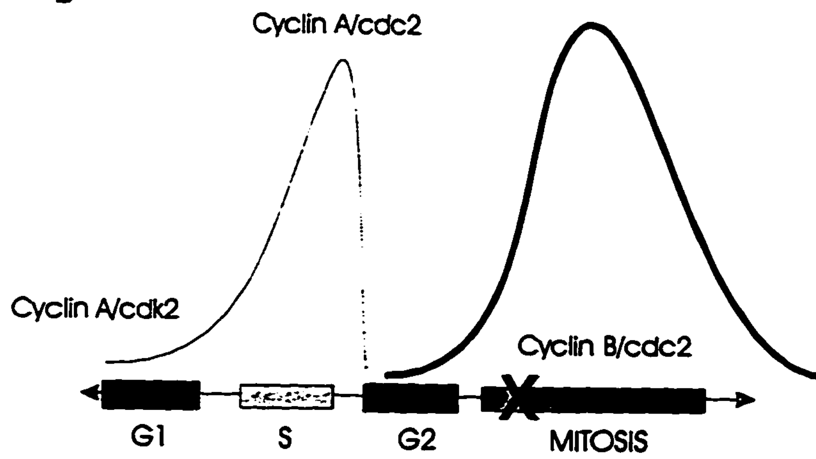
The protease action of Unp results in the increased turnover of cyclin A

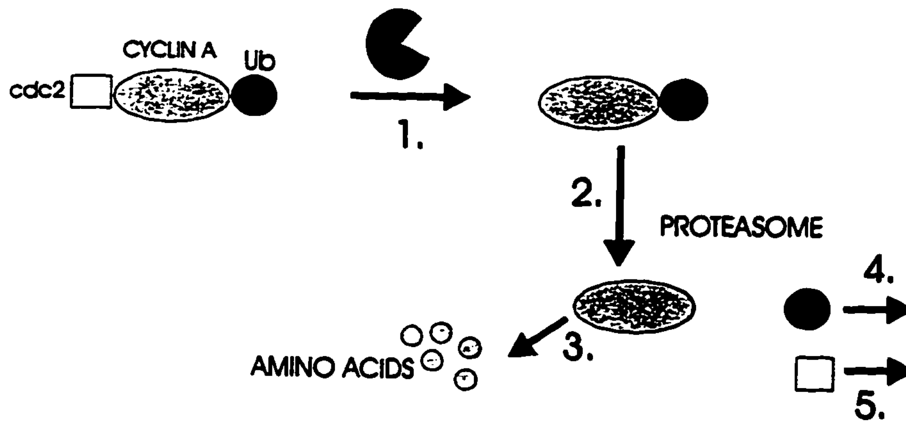
A: Oscillations of both cyclin A/cdc 2 and cyclin B/cdc2 during normal cell cycle progression.
B: 1. The ubiquitin tagged cyclin A is targeted for degradation by the proteasome complex. 2. Cyclin A will be transported to the proteasome where it will bind to the 19S complex in preparation for degradation. 3. Unp will then cleave the ubiquitin tag from cyclin A allowing it to be processed by the proteasome into free amino acids. 4. Reutilizable ubiquitin is released. 5. Increased turnover of cyclin A will prevent the required threshold levels of the cyclin A/cdc 2 complex ocurring, thus the activation of the cyclin B/cdc 2 (MPF) complex is prevented. The activation of MPF is required for the transition of the cell from G2 to mitosis, therefore the cells will arrest in the late stages of G2, early stages of mitosis.

A



B





CONCLUSION

The evidence presented in this thesis suggests that overexpression of the Unp cDNA results in cell cycle arrest in the early stages of mitosis prior to the completion of chromosome condensation. This implies that Unp may be affecting cell cycle proteins that regulate the cells entry or passage through the initial stages of mitosis. It is known that two main complexes are required for the successful entry of the cell into M phase, cyclin A /cdc 2 and cyclin B/cdc 2. Both cyclins have been shown to be degraded via the ubiquitin pathway and are therefore candidates for the protease action of Unp. Indirect immunofluorescence of cyclin B1 showed no qualitative reduction in its levels in cells overexpressing Unp and so we propose that Unp may be interfering with cyclin A.

The observed aberration in chromosome condensation observed in COS-1 cells may be the resultant phenotype when the Unp cDNA is overexpressed. Unp overexpression in NIH 3T3 cells leads to tumours in nude mice indicating a loss of cell cycle control but not viability (Gupta *et al.,* 1994) . When overexpressed in COS-1 cells, Unp leads to loss of cell cycle control (ie. arrest in mitosis) and a presumed loss of cell viability as cells expressing Unp, when left in culture, die. The high degree of amplification of plasmids permitted by COS-1 cell transfection may produce Unp protein at a level resulting in deregulation of chromosome condensation eventually causing cell death. In contrast, the levels of Unp found in stably transfected NIH 3T3 cells, which is considerably lower than that in COS-1 cells, resulted in the formation of tumours (Gupta *et al.,* 1994). Thus deregulated expression levels of the Unp polypeptide may dictate the fate of the cell, with lower levels of Unp resulting in the formation of tumours and higher levels resulting mitotic arrest and cell death.

An alternative explanation for this observation may be that the action or activity of Unp varies from one cell type to another. Precedence for such effects has been observed with overexpression of the DUB-1 protein, which is also a ubiquitin protease (Zhu *et al.* 1996). Overexpression of DUB-1 in haematopoietic cells leads to cell cycle arrest in G1 without loss of cell viability, however, overexpression in NIH 3T3 cells had no observable effect. Therefore, the phenotype observed in the transient transfection of Unp into COS-1 cells may be the result of Unp levels which are adequate to cause a perturbance in the interplay between cyclin A and B thus affecting the cells transition from G2 to M phase resulting in the deregulation of cell proliferation.

FUTURE OBJECTIVES

The association of Unp and cyclin A remains to be clarified. Co-localizing studies of Unp and cyclin A *in situ* would allow comparison of their distribution patterns, while additional cell cycle analysis may identify the cell cycle phase where the change of locale of both Unp and cyclin A occurs . As has been outlined, Cyclin A is degraded via the ubiquitin pathway and may be a substrate of Unp. Purification of the cyclin A protein may permit deubiquitination studies to be performed in vitro. Removal of the ubiquitin tag from cyclin A by the protease action of the Unp polypeptide could then be monitored.

The phenotype observed in COS-1 cells overexpressing Unp does not permit the deduction of the normal situation where Unp is expressed at basal levels. The production of an inducible Unp construct may allow the normal function of Unp in the cell to be determined. It is then possible to deduce the regulatory mechanisms involved in

maintaining basal levels of Unp in the cell, what triggers its overexpression and thus mitotic arrest.

Unp's activity and/or interaction with other proteins may be regulated by phosphorylation, as has been observed in many of the cell cycle proteins. Identification of phosphorylation sites within the Unp amino acid sequence would go a long way in identifying the importance of this post-translational modification in the regulation of Unp action.

The migration of Unp as a doublet may not be due entirely, if at all, to phosphorylation. Other post-translational modifications such as ubiquitination or glycosylation may also be involved. These studies may lead to the deduction of Unp action in the cell.

APPENDIX 1- RAW DATA FROM FLOW CYTOMETRY ANALYSIS

CELL TREATMENT	% G1	% G2/M	% S PHASE
Untreated cells	40.7 42.1	17.3 21	42.8 36.3
Exposure to colcemid	1.1 2.5	81.4 74.4	17.6 23.0
Exposure to hydroxyurea	60.4 58.7	13.5 15.0	26.1 264.

APPENDIX 2- RAW DATA FROM CELL COUNTS

1. COS-1 cells were transfected with either the pcDNA3 vector alone or pDG 50, labeled with cyclin B1 and then classed into two groups based on the distribution of cyclin B1. The two groups, diffuse and peripheral, were examined at the DNA level and further classed on their chromosomal state.

NON-TREATED CELLS	PERIPHERAL		DIFFUSE	
	Non condensed	Condensed	Non condensed	Condensed
COS-1 cells transfected with pcDNA3	70	15	1	14
	79	12	0	9
COS-1 cells transfected with pDG50	56	1	0	3
	54	0	0	1
	62	1	0	2
	53	1	0	5
	70	1	0	4
	61	0	0	4
	58	1	0	1

2. COS-1 cells were transfected with either the pcDNA3 vector alone or pDG 50, treated for 24 hours with 0.06ug/ml colcemid and then labeled with cyclin B. The cells were classed into two groups based on the distribution of cyclin B1. The two groups, diffuse and peripheral, were examined at the DNA level and further classed on their chromosomal state.

CELLS EXPOSED TO COLCEMID FOR 24 HOURS	PERIPHERAL		DIFFUSE	
	Non condensed	Condensed	Non condensed	Condensed
COS-1 cells transfected with pcDNA3	43	3	0	54
	43	1	1	55
COS-1 cells transfected with pDG50	14	0	1	46
	11	1	0	33
	21	0	4	30
	8	0	0	52
	14	2	0	44
	24	3	0	31
	13	0	0	45

APPENDIX 2 (contd)

3. Cells labeled with cyclin B1 and divided into diffuse and peripheral stained groups were examined for the successful transfection of the Unp polypeptide.

COS-1 cells transfected with pDG 50	PERIPHERAL		DIFFUSE	
	TRANSFECTED	N/T	TRANSFECTED	N/T
Non treated	4	53	1	2
	9	45	0	1
	8	55	0	2
	11	43	1	4
	6	65	0	4
	10	51	0	4
	0	59	0	1
Exposure to colcemid for 24 hours	1	13	5	42
	2	10	11	22
	1	20	8	26
	0	8	0	52
	2	14	7	37
	3	24	4	27
	0	13	6	39

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ADDENDUM

Subsequent to the completion of the work described in this thesis, it has come to light that the construct pDG 50 lacks sequences that may extend the authentic amino terminus of the Unp protein. It is currently not known whether the longer protein is made in cells or whether this addition alters any of the results herein.