CYTOLOGICAL, BIOCHEMICAL AND GENETICAL INVESTIGATION OF ACTIN AND TUBULIN IN FISSION YEASTS

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by

JAMES WARREN COPE

Department of Biology
Darwin Building
University College London
Gower Street
London
WC1E 6BT

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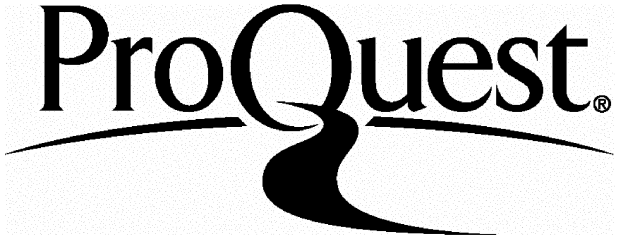

For Carol.

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ABSTRACT

A combined cytological, biochemical and genetical approach was applied to the distribution and composition of the microtubule and actin components of the fission yeast. Indirect immunofluorescence techniques were used to study the depolymerisation / polymerisation of microtubules in *Schizosaccharomyces pombe* and *Schizosaccharomyces japonicus* var. *versatilis* cells treated for 40 min at 1°C, subsequently re-warmed to 25°C. Microtubules depolymerised totally after 40 min at 1°C, but reappeared in 2 min at 25°C and at 30 min were indistinguishable from control cells. The effect of this treatment on actin distribution was examined. Actin was delocalised in cold treated cells, with some cells showing a ring of actin around the nucleus. Reformation of the normal actin array occurred even in cells in which microtubule polymerisation was blocked with 100μg ml^{-1} thiabendazole. Therefore polymerised microtubules are not required for the normal distribution of actin to be maintained.

One and two-dimensional gel electrophoresis and Western blotting using a monoclonal anti-actin antibody were carried out on block-released *cdc25.22* cells to determine whether actins' state changed throughout the cell cycle. Actin levels varied through the cell cycle. Attempts to determine whether any of the four species detected by two-dimensional gel electrophoresis were the result of post-translational modification gave equivocal results.

ABSTRACT

Classical genetic techniques were utilised to identify suppressors of the cold-sensitive β-tubulin mutant *nda3*-KM311 and thereby identify possible interacting proteins. Two approaches were employed: (i) 552 phenotypically revertant colonies were isolated after EMS-mutagenesis of *nda3*-KM311; these were further tested for temperature sensitivity at 36°C; (ii) three phenotypically revertant colonies were isolated by spontaneous mutation of *nda3*-KM311. Ten revertants from the chemically induced group (four temperature sensitive and six not) and the three spontaneous revertants were crossed to wild type cells and the resulting asci dissected, replica plated and scored for growth at 20°C. The cold sensitive phenotype did not segregate out, suggesting that all of the mutations were intragenic. This was confirmed by free spore analysis.

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CHAPTER 1

INTRODUCTION

1.1: The organism

Yeasts are used widely as models in which to study fundamental cellular processes such as secretion, signal transduction, cell division and the organisation and function of the cytoskeleton (Nasim et al., 1989; Pringle et al., 1986). Two members of the genus *Schizosaccharomyces* (Sipiczki, 1989) *Schizosaccharomyces pombe* and *Schizosaccharomyces japonicus* var. *versatilis* are used in this study. The genetics of *S.pombe* is well defined and many of the genes on its 14.3Mb genome (Kohli, 1987) have now been identified and mapped by a variety of techniques. These include classical genetics (Munz. et al., 1989), pulsed field gel electrophoresis (Smith et al., 1987) and more recently, yeast artificial chromosomes (Maier et al., 1992) and cosmid and phage libraries (Hoheisel et al., 1993). Both *S.pombe* and *S.japonicus* var. *versatilis* are cylindrically shaped cells with hemispherical ends, but *S.pombe* is considerably smaller than *S.japonicus* var. *versatilis* (7-14μm x 3μm and 7-20μm x 6μm; respectively, Alfa and Hyams, 1990). Both organisms grow by tip elongation only and so do not increase in girth during the cell cycle (Mitchison and Nurse, 1985). The larger size of *S.japonicus* var. *versatilis* enables greater resolution of subcellular organisation by light microscopy, especially of mitosis (Robinow and Hyams, 1989; Alfa and Hyams, 1990).

1.2: The cytoskeleton

The cytoskeleton of eukaryotic cells is responsible for many tasks including cell motility, cell division and intracellular organisation (Schliwa, 1986). Two of the major components of the cytoskeleton, microtubules and microfilaments are composed of polymeric proteins, tubulin and actin respectively, both of which are highly conserved throughout all eukaryotic cells. Both components are dynamic in nature and re-arrange during the cell cycle, most dramatically at the interphase - mitosis transition. The third major cytoskeletal component, intermediate filaments, has not so far been identified in yeast and will not be addressed here.

1.3: Microtubules

Microtubules are found in all eukaryotic cells and play similar roles in the maintenance of cell shape (Schliwa, 1986), the intracellular transport of vesicles and organelles (Schroer and Sheetz, 1991), chromosome movement at mitosis and meiosis (McIntosh, 1984) and the intracellular positioning of organelles such as the Golgi apparatus and endoplasmic reticulum (Schroer and Sheetz, 1991; Cole and Lippincott-Schwartz, 1995). Microtubules are also the main structural elements in cilia and flagella (Murray, 1994).

1.3.i: Microtubule structure

Structural analysis has shown that microtubules are 25nm diameter

hollow cylinders composed of thirteen protofilaments. Each protofilament is made up of tubulin heterodimers of one α and one β-tubulin subunit. These are arranged in a helical repetitive fashion around the microtubule (Amos, 1975). Such a subunit arrangement leads to an inherent structural polarity in the microtubule, with an α-tubulin subunit exposed at one end of the microtubule and a β-tubulin subunit at the other (Mandelkow and Mandelkow, 1995). This leads to asymmetric growth of the microtubule with a faster and slower growing end, known as the plus and minus ends respectively (Allen and Borisy, 1974). *In vitro* the addition and removal of subunits is biased to the plus end, the minus end usually being embedded in a microtubule organising centre (MTOC) and is therefore not free to exchange subunits. (Mitchison and Kirschner, 1984a). The plus end of the microtubule is thought to be "capped" with α-tubulin subunits while conversely the minus end is "capped" with β-tubulin subunits which interact with γ-tubulin at the microtubule organising centre (Oakley et al, 1990; Oakley, 1992; Song and Mandelkow; 1995, Mandelkow and Mandelkow, 1995). This interaction may not be direct but via microtubule associated proteins (MAPs) (Raff et al, 1993).

1.3.ii: Microtubule dynamics

Two distinct populations of microtubules have been shown to co-exist both *in vitro* and *in vivo*, a population which elongates and another that shrinks, either partially or completely (Mitchison and Kirschner, 1984a & b). It is thought that MAPs serve to stabilise microtubules so that they accumulate in the "growing phase" and therefore maintain a constant

length once reached (Horio and Hotani, 1986).

In interphase mammalian cells it has been shown that, in addition to the dynamic growing/shrinking microtubules, there is a subpopulation of relatively stable microtubules that resist the exchange of tubulin subunits (Schulze and Kirschner, 1987). Schulze and Kirschner (1987) have shown that these stable microtubules are post-translationally modified, by either acetylation (Piperno, 1987) or detyrosination (Gundersen et al., 1987a & b),or both and that dynamic microtubules in the same cells, are not so modified.

Interphase microtubules are longer and less dynamic than those in mitosis. This transition has been shown in cytoplasmic extracts of *Xenopus* to be controlled by phosphorylation, which is initiated by activation of the $p34^{cdc2}$ protein kinase (Verde et al., 1990). Long interphase-like microtubules persist when inhibitors of the $p34^{cdc2}$ kinase are added to mitotic cells (Picard et al., 1989).

1.3.iii: Microtubules in yeast

Microtubules in yeast are responsible for many tasks and are essential components of the mitotic and meiotic spindles that move chromosomes during cell division and of the machinery that moves nuclei towards one another during karyogamy (Cole and Lippincott-Scwartz, 1995). Yeast microtubules have also been implicated in the organisation of organelles such as the Golgi apparatus (Ayscough et al., 1993).

1.3.iv: Microtubules in *S.cerevisiae*

Microtubules in budding yeast are made up from three subunits, two α-tubulins and one β-tubulin. The genes encoding the two α-tubulin subunits in *S.cerevisiae* are *TUB1* and *TUB3* (Schatz et al., 1986a &b), whilst the single β-tubulin subunit is encoded by the *TUB2* gene (Neff et al., 1983). The recently discovered γ-tubulin gene in *S.cerevisiae* is designated *TUB4* (Marschall et al., 1995). Microtubules arise from a structure called the spindle pole body (SPB) that is embedded in the persistent nuclear envelope. Microtubules emanating from the cytoplasmic face of the SPB extend into the developing bud (Byers and Goetsch, 1975). Concurrent with bud emergence, the SPB duplicates and the daughter SPB's separate to establish the mitotic spindle. The mitotic spindle is present throughout bud expansion but only elongates late in the budding cycle, extending through the neck of the mother-bud junction and on into the bud, taking one set of chromosomes with it (Huffaker et al., 1988). The mother and bud cells then separate via the neck region being drawn closed. Cytoplasmic microtubules are also thought to be responsible for ensuring the proper orientation of nuclear division (Adams and Pringle; 1984, Palmer et al, 1993) and mediating the delivery of secretory vesicles to the bud (Byers and Goetsch, 1973; Byers and Goetsch, 1975; Byers, 1981), but are not essential for the formation and growth of buds (Huffaker et al., 1988; Jacobs et al., 1988).

1.3.v: Microtubules in *S.pombe*

In *S.pombe* as in *S.cerevisiae* , microtubules are also composed of three

subunits, two α's and one β. The genes encoding the α-tubulin subunits are *nda2*$^+$(Toda et al., 1984) and *atb2*$^+$ (Toda et al., 1984), while the β-tubulin subunit is encoded by the *nda3* gene (Hiraoka et al., 1984). γ-tubulin has been found in *S.pombe* and is encoded by the *gtb1*$^+$ gene (Horio et al., 1991). γ-tubulin is associated with both classes of fission yeast MTOC's, the spindle pole bodies and the equatorial MTOC's that initiate microtubule assembly post-mitosis (Zheng et al., 1991; Stearns et al., 1991; Horio et al., 1991). Less is generally known about microtubules in fission yeast, but Hagan and Hyams (1988) have described an interphase microtubule "basket" that stretches along the length of the interphase cell, disappears at the onset of mitosis to be replaced by a short mitotic spindle that runs between the duplicated SPB's. This subsequently elongates during anaphase to separate the chromosomes into the two daughter cells (Hagan and Hyams, 1988; Hagan et al., 1990; Alfa and Hyams, 1990). The role of microtubules in organelle integrity (Ayscough et al., 1993) has also been described with intact microtubules being found to be necessary for the stacking of the Golgi cisternae.

The most widely characterised post-translational modification of tubulin is the removal of the tyrosine residue at the C-terminus of α-tubulin, (tyr-tubulin) by a specific carboxypeptidase. This removal causes a novel type of α-tubulin that now terminates in a glutamate residue (glu-tubulin). This reaction can form a cycle with the tyrosine residue being re-added by the enzyme tubulin tyrosine ligase to re-form tyr-tubulin (Gundersen et al., 1987; Greer and Rosenbaum, 1989). The biological significance of this tyrosination - detyrosination cycle is at present unclear, but it is thought to be involved in the stabilisation of microtubules.

Alfa and Hyams (1990) investigated whether this tyrosination - detyrosination cycle was occurring in *S.pombe*, by the use of specific glu and tyr antibodies on tubulin extracts. Although both the α-tubulin's in *S.pombe* contain the C-terminal sequence -glu-glu-tyr, (Toda et al., 1984) no detyrosination of the tubulin could be detected (Alfa and Hyams, 1990). This is the first time that an organism that contains the C-terminal sequence (-glu-glu-tyr) has been reported that does not have the ability to detyrosinate it's tubulin (Alfa and Hyams, 1990).

1.3.vi: Comparison of microtubules from *S.cerevisiae* and *S.pombe*

Microtubules are highly conserved proteins and in both *S.pombe* and *S.cerevisiae* are composed of three tubulin subunits, two α's and one β (Alfa and Hyams, 1990, Schatz et al, 1986 a & b, Neff et al, 1983) encoded by two genes for α-tubulin and one for β-tubulin (Yanagida, 1987). The α and β-tubulin subunits that make up the polymeric microtubule are highly conserved and show greater than 70% sequence homology between species as diverse as yeasts and mammals (Yanagida, 1987). γ-tubulin in *S.pombe* and *S.cerevisiae* are quite diverse sharing only a 42% amino acid identity (Marschall et al., 1995), but both the *gtb1*$^{+}$ (Horio et al., 1991) and *TUB4* (Marschall et al., 1995) genes are essential and their respective gene products are localised to the spindle pole bodies.

1.4: Actin

Microfilaments are found in virtually all eukaryotes and are thought to be

involved in cell shape determination, cell motility, various contractile activities, endocytosis and secretion (Bretscher et al., 1994). Structural studies of actin filaments (F-actin) show that they are composed of globular monomers (G-actin) that polymerise to form an ordered, polarised structure (filamentous or F-actin) of 6-8nm in diameter (Schliwa, 1986). The monomers are thought to be wedge shaped, combining in the filamentous form to form a double stranded helix with approximately thirteen subunits between crossovers points on the helix (Schliwa, 1986). Filament formation occurs in three steps including monomer activation, nucleation and elongation (Schliwa, 1986). The elongation step requires a molecule of ATP to be hydrolysed for every additional monomer added to the growing filament. As for microtubule polymerisation the polymer grows preferentially at one end termed "the barbed end" (Schliwa, 1986).

1.4.i: *S.cerevisiae* actin

S.cerevisiae has a single actin gene, *ACT1* (Ng and Abelson, 1980) and three actin-related proteins encoded by *ACT2* (Schwob and Martin, 1992), *ACT3* (Harata et al., 1994) and *ACT5* (Muhua et al., 1994). *ACT3* is less related to *ACT1* than *ACT2*, but like *ACT1* is essential for viability (Harata et al., 1994). *ACT5* is more related to *ACT 1* than *ACT2*, with no information published on its relatedness to *ACT3* (Muhua et al., 1994). Recently Schroer and co-workers (1994) have determined a new classification system for the actin related proteins (arp's). Using this system *S.cerevisiae* would have two members in the arp1 family (*ACT3*

and *ACT5*) and one member in the arp2 family (*ACT2*).

Budding yeast actin has been extensively characterised biochemically, (Nefsky and Bretscher, 1992; Kron et al., 1992; Cook et al., 1992) and is 88% identical to mammalian γ-actins (Welch et al., 1994). In *S.cerevisiae,* actin can exist in two distinct forms, patches and filaments. Cortical actin patches are localised in areas of active growth and wall deposition within the bud (Kilmartin and Adams, 1984; Mulholland et al., 1994). Calmodulin has also been associated with these areas throughout the cell cycle (Brockerhoff and Davis, 1992), consequently actin is thought to be regulated by calcium directly (Welch et al., 1994) or via actin binding proteins, as is the case in motile cells (Stossel, 1993). Cofilin has also been localised to cortical actin patches which at the ultrastructural level are seen as plasma membrane invaginations that are thought to be areas of wall growth and osmotic regulation (Mulholland et al., 1994).

Actin appears at the cell surface of a mother cell, in a ring of patches marking the position from which the new bud will emerge . Subsequently, the patches become concentrated at the growing bud tips. Actin cables are localised within both the mother and bud along the mother-daughter cell axis (Mulholland et al., 1994). When bud expansion is complete the bud actin is delocalised but re-concentrates at the neck region of the dividing cells where the septum will form (Mullholland et al., 1994).

S.cerevisiae mutants defective for the single essential actin gene (*ACT1*) display problems in polarised cell surface growth, caused by defects in the late stages of the secretory pathway (Novick and Botstein, 1985), suggesting that actin may also function in the movement of secretory vesicles (Welch et al., 1994). Further work on proteins that bind to

budding yeast actin, has led to the production of myosin and tropomyosin mutants, although tropomyosin was discovered by using a affinity purified antibody to yeast tropomyosin to screen a genomic library in a λgt11 expression vector (Liu and Bretscher, 1989). Mutants in both genes accumulate membrane vesicles yet fail to display any major defects in secretion (Welch et al., 1994).

The low abundance of these proteins has precluded their biochemical analysis and localisation by immunofluorescence. However, *ACT5* is thought to be a component of the dynactin complex in *S.cerevisiae* together with cytoplasmic dynein, encoded by the DYN1 (Eshel et al., 1993) or DHC1 (Li et al., 1993) gene, the function of which includes the maintenance of correct spindle orientation (Palmer et al., 1992; Clark and Meyer, 1994; Muhua et al., 1994).

1.4.ii: Actin in *S.pombe*

S.pombe also has a single actin gene (*act1*$^+$, Mertins and Gallwitz, 1987) and, as yet, only one actin related protein, arp3, encoded by the *act2*$^+$gene (Lees-Miller et al., 1992; Schroer et al., 1994). In *S.pombe act1*$^+$and *act2*$^+$ share 35-40% identity at the peptide level and the predicted protein sequences show that they share several important structural regions and should therefore be able to perform similar roles. The *S.pombe act1* gene product (Mertins and Gallwitz, 1987) and has been shown to interact with several proteins, including tropomyosin (Balasubramanian et al., 1992), profilin (Balasubramanian et al., 1994) and myosin (May and Hyams,

personal communication).

The biochemistry of actin in fission yeast has been poorly characterised, but extensive cytological localisation has been carried out at both the light (Marks and Hyams, 1985; Marks et al., 1986; Robinow and Hyams, 1989; Alfa and Hyams, 1990; Alfa et al., 1993) and electron microscopic level (Kanbe et al., 1993; Kanbe et al., 1994). The distribution of F-actin in both *S.pombe* and *S.japonicus var versatilis* changes through the cell cycle (Marks and Hyams, 1985, Alfa and Hyams, 1990). Newly divided cells grow initially at one tip and then from both, actin distribution mirrors this growth pattern (Marks and Hyams, 1985). This change from single to dual end growth is called "new end take off" (NETO; Mitchison and Nurse, 1985). Cells then progress to mitosis at the end of which cytokinesis occurs, an actin ring precedes the formation of a septum which physically divides the duplicated cell organelles and DNA (Marks and Hyams, 1985).

In *S.japonicus* var. *versatilis* the distribution of F-actin throughout the cell cycle is different from that observed in *S.pombe* (Alfa and Hyams, 1990).Whilst in *S.pombe* the movement of actin from the cells tips to the middle occurs early in anaphase (Marks and Hyams, 1985), in *S.japonicus* var. *versatilis* it occurs only when anaphase is complete (Alfa and Hyams, 1990). Thus, unlike *S.pombe*, *S.japonicus* var. *versatilis* cells continue to grow during mitosis. Direct evidence for the involvement of actin in directing the deposition of cell wall material has been shown by Kobori et al (1989) and Osumi et al (1989) whose experiments involved co-staining regenerating protoplasts for cell wall material and actin, (with calcofluor

and phalloidin respectively). Co-localisation of actin with the appearance of new cell wall at certain points on the cell periphery was observed. These became elongated until the cell wall had totally regrown.

1.5: Molecular motors

Molecular motors that convert the chemical energy of nucleotide hydrolysis into movement along microtubules or microfilaments have been identified for some time in animal cells. These include kinesin, cytoplasmic dynein and myosin, which are involved in plus and minus directed movement along microtubules and movement along microfilaments respectively.

1.5.i: Yeast Microtubule motors

Proteins with homology to the motor domain of the kinesin heavy chain, have been reported in both *S.cerevisiae* (Meluh and Rose, 1990, Roof et al., 1992, Hoyt et al., 1992) and *S.pombe* (Hagan and Yanagida, 1990; 1992). These kinesin-related proteins share at least some functions in yeast as in multicellular organisms, in that they are involved in the sliding of antiparallel microtubules in the mitotic and meiotic spindles (Meluh and Rose, 1990, Hagan and Yanagida, 1990; 1992). The best characterised kinesin gene is KAR3 (Meluh and Rose, 1990), the kar3-1 mutant displays a thirty-fold lowering of karyogamy during mating, when crossed to wild type (Meluh and Rose, 1990). kar3-1 cells also grow more slowly than

wild type and 40% are nonviable with a distinctive single nucleus and short mitotic spindle, suggesting aberrant spindle elongation.

More recently other kinesin-related proteins have been described in *S.cerevisiae*. CIN8, (Hoyt et al., 1992 and KIP1 and KIP2, (Roof et al., 1992). All these kinesin-related proteins are similar to kinesin heavy chain, only in the motor domain and have divergent tails. The latter are thought to be responsible for the variety of roles that different kinesin-related proteins can play in cells (Goldstein, 1991). CIN8 and KIP1 were found to be functionally redundant, despite their tail sequences being dissimilar. All kinesin-related mutants are viable at the permissive temperature (26°C, Goldstein, 1993) and so could be thought of as non-essential, were it not for some interesting interactions between kinesin-like genes in multiple mutants (Roof et al., 1992; Goldstein, 1993).Triple KAR3 CIN8 KIP1 mutants are healthier than CIN8 KIP1 double mutants. KAR3 function is thought to be responsible for providing a counteracting force on the mitotic spindle to CIN8 and KIP1 (Saunders and Hoyt , 1992; Goldstein, 1993).

Cytoplasmic dynein has been found by two groups of researchers in *S.cerevisiae* (Eshel et al., 1993, Li et al., 1993). Both have implicated cytoplasmic dynein in nuclear segregation and mitotic spindle orientation in this yeast. Cytoplasmic dynein has recently been discovered in *S.pombe*, but no characterisation of the gene has yet been published.

1.5.ii: Yeast actin motors

In budding yeast, genes encoding members of myosin classes I (*MYO3* and *MYO5*; Goodson and Spudich., 1995; Goodson et al., 1995), II (*MYO1*, Watts et al., 1987) and V (*MYO2* and *MYO4*; Johnston et al., 1991; Haarer et al., 1994) have all been identified. Class I myosins are conventionally associated with membrane associated motility whereas the "unconventional" class V myosins are implicated in polarised vesicle movement. In non-muscle cells the "conventional" two-headed, filament forming myosin II's are typically associated with large-scale force production events such as cytokinesis (Cheney and Mooseker, 1992; Cheney et al., 1993). Genetic manipulation of the budding yeast myosins has indicated that myosin II does indeed play a role in cell division (Watts et al., 1987; Rodrigues and Patterson, 1990) whilst the myosin I's are concerned with the organisation of the cell surface (Goodson et al., 1995). The myosin V's function at a post-Golgi step in the secretory pathway (Govidan et al., 1991;1995). To date only a myosinII like motor has been found in *S.pombe* (May and Hyams, personal communication).

1.6: Aims of the project

The aims of this project were threefold: (1) to investigate the dynamics of microtubules and actin filaments and their possible interactions; (2) the investigation of the role of post-translational modification of actin through the cell division cycle and (3) to use a genetic screen to identify proteins interacting with β-tubulin.

CHAPTER 2

POLYMERISATION/DEPOLYMERISATION OF THE S.POMBE TUBULIN CYTOSKELETON AND ITS EFFECT ON THE ACTIN CYTOSKELETON

2.1: Introduction

Ultrastructural and cytological studies of the yeast cytoskeleton have been in progress for nearly thirty years (Robinow and Marak, 1966, McCully and Robinow, 1971, Adams and Pringle, 1984, Kilmartin and Adams, 1984, Marks and Hyams, 1985, Hagan and Hyams, 1988, Alfa and Hyams, 1990). Surprisingly, few attempts to depolymerise yeast microtubules *in vivo* have been reported. This may in some part be due to the insensitivity of *S.pombe* microtubules to the classic spindle poisons such as colchicine (Walker, 1982). However, some workers have attempted depolymerisation of the microtubule component of the fission yeast cytoskeleton by the use of drugs, such as benomyl (Yamamoto, 1980; Roy and Fantes, 1982) and thiabendazole (Ayscough et al., 1993). Similar attempts to depolymerise budding yeast microtubules with drugs such as; MBC (Quinlan et al., 1980), benomyl (Delgado and Conde, 1984) and nocodazole (Jochova-Svobodova et al., 1989). However, there is one description in the literature of attempts to depolymerise fission yeast microtubules *in vivo* by means of low temperature (Hagan and Yanagida, 1995). Depolymerisation of microtubules in homothermic animals with low temperature is a routine procedure (Vorobjev and Chentsov, 1983;

Gundersen et al., 1987; Baas et al., 1994), one that is usually achieved by cooling cells to 0°C with subsequent transfer to 37°C to allow microtubule recovery. Attempts to depolymerise the actin cytoskeleton in fission yeast have been attempted using cytochalasin A (Kanbe et al., 1993) one of the actin-capping group of drugs (Cooper, 1987).

In this chapter we describe an attempt to depolymerise the microtubule and actin cytoskeletons of fission yeast using low temperature alone (1°C), or in combination with the mitotic spindle poison TBZ, and it's subsequent repolymerisation on return to 25°C. These events were followed by means of immunofluorescence microscopy using antibodies to tubulin and actin and the F-actin specific probe phalloidin. Using these techniques we investigated the possible interactions between actin and tubulin components of the cytoskeleton of *S.pombe* at specific cell cycle points, using mutants that specifically block at G1/S (*cdc10-129*) and G2/M (*cdc25.22*).

2.2: Materials & Methods

2.2.i: Strains used

Wild type *S. pombe* 975h$^+$, 972h$^-$ and the temperature sensitive cell division cycle mutants; *cdc25-22h*$^-$ and *cdc10-129h*$^-$; *Schizosaccharomyces japonicus* var. *versatilis*, strain NCYC 419, (Wickerham and Duprat, 1945). Wild type strains (975h$^+$, 972h$^-$ and the cell division cycle mutant *cdc10-129h*$^-$) were all kindly provided by Dr. P

Nurse, while the other cell division cycle mutant (*cdc25-22h*$^{-}$) and double *act1*$^{+}$gene containing strain (*A16a*) were kindly provided by Dr. P Fantes. All other strains were obtained from the British National Collection of Yeast Cultures, Norwich.

2.2.ii:Growth of strains

S.pombe strains were grown on yeast extract (YE) medium (Gutz et al., 1974), containing 5g l^{-1} yeast extract (Difco) plus 30g l^{-1} glucose (BDH) and solidified with 20g l^{-1} bacto agar (Difco) for solid medium. For experiments using *S. japonicus* var. *versatilis*, YE medium was supplemented with 20g l^{-1} bacto peptone (Difco). Cell cycle mutants were grown / blocked in shaking water baths (New Brunswick Scientific) at 160 rpm in conical flasks at 25/36°C respectively.

2.2.iii: Cold treatment

Wild type cells of *S.pombe* and *S.japonicus* var. *versatilis* were grown to a density of approx. 5 x 10^6 cells ml^{-1}. Cell division cycle mutants (Nurse et al., 1976) were grown to a similar density and then arrested in cell cycle progress by incubation at the restrictive temperature (36°C) for two cell cycles (4.25 hours). Flasks containing the appropriate culture were placed in an ice / water mixture (1°C) on a shaker for times up to 60 min and samples removed at 10 minute intervals. Cells were then returned to

the shaking water bath at 25°C and samples removed for immunofluorescence microscopy at 0, 2, 5, 30 and 60 min. In some experiments the repolymerisation of microtubules was blocked by the addition of the microtubule inhibitor thiabendazole (TBZ) 10 min before removal from the ice / water incubation mixture to a final concentration of 100μg ml^{-1} (from a stock solution of 10mg ml^{-1} TBZ dissolved in DMSO, filter sterilised).

2.2.iv: Antibodies

Primary antibodies were N350 (monoclonal mouse anti-chicken gizzard actin antibody), (Amersham) and TAT1 polyclonal anti-tubulin antibody, kindly provided by Prof. K.Gull, (Woods et al., 1989). The secondary antibody was rhodamine conjugated rabbit anti-mouse (ICN Biomedicals)

2.2.v: Solutions for immunofluorescence microscopy

30% formaldehyde (w/v): 3g of *p*-formaldehyde dissolved in 8ml of PEM buffer was heated to 70°C in a water bath, 10M NaOH added until the solution cleared, made up to 10ml with PEM buffer then cooled to the temperature of the culture it was to be added to.

PM: 100mM Na-PIPES (piperazine-N,N'-bis(2-ethanesulfonic acid) (pH 6.9), 1mM $MgSO_4$.

PEM: PM containing 1mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N', N'-tetraacetic acid (EGTA).

PEMS: PEM containing 1M sorbitol.

PEMBAL: PEM containing 0.1M L-lysine, 1%(w/v) BSA (globulin free), 0.1% sodium azide.

PBS: 150mM NaCl, 1mM Na_2HPO_4 ($2H_2O$) (pH 7.2).

DAPI (4'6-diamidino-2-phenylindole) stock: $1mgml^{-1}$ in distilled H_2O.

Antifade (*p*-phenylenediamine): $10mgml^{-1}$ in PBS, pH 8.2

DAPI-antifade stock: 1µl of DAPI stock in 99µl of antifade stock.

2.2.vi: Fixation and permeabilisation

Two methods were used. Both are modified versions of the procedures described in Hagan & Hyams (1988) and Alfa et al., (1993).

(1) Methanol Fixation

Cultures were rapidly filtered onto 2.5cm diameter Millipore 3.0µm pore size filters. Cells were removed from the filter by washing in 10ml of -20°C methanol and incubated at -20°C for a minimum of 8 min. Fixed cells were harvested by centrifugation at 4°C for 5 min at 3,000 .g. The resulting pellet was washed three times in PEM buffer. Cell walls were removed by incubation in Novozym ($0.5mg\ ml^{-1}$) and zymolase 20T ($0.15mg\ ml^{-1}$) in PEMS buffer at 37°C. Once 10% of the cells were seen to have lost their cell walls (by an increase in phase contrast refractility), the enzymatic reaction was stopped by dilution of the enzyme/cell mixture with 4°C PEMS. Cells were washed twice with PEMS buffer (4°C), then incubated in PEMBAL for 40-60min at room temperature on a rotator

before being put into primary and, subsequently, secondary antibody.

(2) Formaldehyde Fixation

A fresh stock of 30% (w/v) formaldehyde was prepared as described earlier. The stock formaldehyde solution was added to growing cultures to a final concentration of 4% (w/v) for 30min. Cells were harvested and washed three times in PEM . Cells were protoplasted as described above. Following two washes in PEMS , cells were permeabilised using Triton-X-100 to a final concentration of 1% (v/v) for 1min at room temperature. Cells were subsequently washed twice with PEM then incubated in PEMBAL buffer for 40-60min at room temperature on a cell rotator before being put into primary and subsequently secondary antibody or into rhodamine-conjugated phalloidin.

2.2.vii: Antibody Incubation

Methanol fixed and permeabilised cells were incubated in 50-100µl of TAT1 anti-tubulin antibody (1 in 25 dilution) or N350 anti-actin antibody (1 in 100) made up in PEMBAL buffer, on a cell mixer for 12-16 hr at room temperature. Cells incubated in primary antibody were washed three times in PEMBAL buffer and incubated in 100-400µl of rhodamine conjugated secondary antibody diluted 1 in 50 in PEMBAL overnight in darkness on a rotator at room temperature.

2.2.viii: Phalloidin staining

Cells fixed by the formaldehyde method and subjected to gentle

permeabilisation were stained for F-actin using rhodamine-phalloidin (Marks and Hyams, 1985). A small pellet of cells (30-50μl) was resuspended in 100μl of rhodamine-conjugated phalloidin (Sigma) (20μg μl^{-1}) and incubated at room temperature on a rotator in the dark for 1 hr. Cells were then mounted as for antibody staining.

2.2.ix: Mounting cells

A monolayer of cells was achieved by applying a 50μl drop of a suspension of cells in PEMBAL to a round 13mm diameter, 0.155mm thick coverslip which had been treated by coating with a layer of poly-L-lysine (1mg ml^{-1}). Cells were dried onto the coverslip using a hairdrier. Coverslip covered in cells were placed, cell side down, onto a 3-4μl drop of PM buffer containing 4' , 6-diaminidino-2-phenylindole(DAPI, Sigma)/antifade (*p*-phenylenediamine, Sigma) both at a final concentration of 1mg ml^{-1}. Coverslips were sealed onto the slide with nail varnish and allowed to dry for at least 5min before viewing. Throughout this procedure exposure to light was avoided, to prevent bleaching of the rhodamine tag.

2.2.x: Microscopy

Mounted cells were viewed using a Zeiss Axiophot with epifluorescence optics and a Planapo 100 x 1.4 NA objective lens.

2.2.xi: Photography

Black and white photographs were taken on Kodak T-MAX 400 negative film, rated at 400 ASA. Colour photographs were taken on Fujichrome professional positive film, rated at 1600 ASA.

2.3: Results

2.3.i: Microtubule depolymerisation in wild type *S.pombe*

The distribution of microtubules in *S.pombe* has been extensively documented (Hagan and Hyams, 1988, Alfa and Hyams, 1990). A representative field of control wild type cells (no-cold treatment, no recovery, 0 / 0) *S.pombe* can be seen in Fig. 2.1A. Microtubules extend between the two poles of the cell, forming a corset that positions the nucleus. The time taken for complete microtubule depolymerisation in both *S.pombe* and *S.japonicus* var. *versatilis* was determined from a time course experiment to be 40 min. Cells subjected to low temperature (1°C) for up to 30 min still revealed persistent microtubule fragments. Cells that were cold plunged for 40 min and fixed immediately (40 / 0; Fig. 2.1B), exhibited diffuse staining throughout the whole cell, being brightest in the region over the nucleus. Cells that were allowed to recover from the cold treatment for 2 and 5 minutes respectively (40/2 and 40 / 5; Figs 2.1C and D) already showed extensive microtubule re-polymerisation, initially as short segments of fluorescence, mainly located at the mid-point of the cell. At the final time point (Figs 2.1E; 30 min

post-cold plunge) these short repolymerising microtubules were more extended and essentially indistinguishable from the control cells (Fig. 2.1A).

2.3.ii: Microtubule depolymerisation in *S.japonicus* var. *versatilis*

The distribution of microtubules in *S.japonicus* var. *versatilis* has been documented by Alfa and Hyams (1990). A representative field of control cells (no cold treatment, no recovery; 0/0) can be seen in Fig. 2.1A. As in *S.pombe,* microtubules extend between the two poles of the cell, forming a basket-like network. Cells cold plunged for 40 min and fixed immediately (40/0; Fig. 2.2B) again showed complete depolymerisation of the microtubule system and the appearance of diffuse staining throughout the cell. Recovery from cold treatment for 2 min (40/2; Fig. 2.2C) already showed extensive microtubule repolymerisation which was seen initially as either short segments of fluorescence, again mainly located at the mid-point of the cell, or two asters of staining depending whether the cell was in interphase or mitosis when it was subjected to cold treatment. Recovery from cold treatment for 5 min (40/5; Fig. 2.2D) showed considerably longer segments of fluorescence throughout the cell and in the final time point (Fig. 2.2E; 30 min post-cold plunge) the repolymerising microtubules could be seen to be extended, such that they were indistinguishable from the controls (Fig. 2.2A).

2.3.iii: Effects of low temperature on the actin cytoskeleton of *S.pombe*

Control cells (0 / 0) of *S.pombe* (Fig. 2.3A) showed the characteristic pattern of actin distribution described by Marks and Hyams (1985) and Alfa and Hyams (1990). That is, staining was in the form of dots at the growing tips in interphase cells and as an equatorial ring at the mid-point of cells preceding septum formation. Actin distribution in cells placed at 1°C for 40 min was variable (40 / 0, Fig. 2.3B). Actin staining was either completely absent or, a few small dim dots, or a smaller number of large bright dots persisted. Interestingly, a small proportion (< 1%) of *S.pombe* cells (Fig. 2.3B) showed a ring-like actin structure around the periphery of the nucleus. Wild type cells allowed to recover from the cold plunge treatment for 2 min revealed the return of control-like distribution of actin in some cells (Fig. 2.3C) while other cells displayed actin dots that were displaced from the tips of the cell. The majority of the wild type cells allowed to recover form cold plunge treatment for 5 min (Fig. 2.3D) displayed a control-like distribution of actin with a small proportion of cells revealing seemingly displaced actin throughout the cell. Wild type cells allowed to recover from cold-plunge treatment for 30 min (Fig. 2.3E) display a control-like distribution of actin dots indistinguishable from control cells (compare figs. 2.3A and 2.3E)

2.3. iv: Effects of low temperature on the actin cytoskeleton of *S.japonius* var. *versatilis*

Control cells of *S.japonicus* var. *versatilis* (0/0, Fig. 2.4A) showed the

pattern of actin distribution described by Alfa and Hyams (1990). That is staining was localised to the growing tips in interphase cells and at the mid-point of the cell preceding septum formation. Unlike *S.pombe* cells that were held at 1°C for 40 min, some *S.japonicus* var. *versatilis* cells revealed a normal actin distribution, but a proportion of cells showed delocalised actin with only a few bright or dim staining dots (Fig. 2.4B). *S.japonicus* var. *versatilis* cells allowed to recover from the cold plunge treatment for 2 min showed a normal actin distribution (Fig. 2.4C). Cells allowed to recover from cold plunge treatment for 5 min display a strange pattern of actin staining which seems localised to the periphery of the cell. These cells also seem bloated in comparison with cells from other time points (compare fig. 2.4D with figs. 2.4 A,B,C and E). A normal distribution of actin can be seen in the majority of cells allowed to recover from cold treatment for 30 min (Fig. 2.4E).

2.3.v: Effects of low temperature on the microtubules of *cdc10-129* and *cdc25-22*

We were interested in determining whether the actin ring seen in cold treated wild type *S.pombe* cells (Fig.2.3B) was actin collapsed from the tips of cells, or represented a displaced equatorial ring which is formed at the G2/M boundary of the cell cycle. We therefore repeated the cold plunge experiments in two cell cycle mutants that arrest at specific points in the cell cycle, *cdc10-129h*$^-$ and *cdc25-22h*$^-$ (Nurse, 1975), which block

at G1/S and G2/M respectively, when incubated at their restrictive temperature (36°C). The interphase microtubule cytoskeleton in *cdc25-22* and *cdc10-129* cells can be seen, as in wild type *S.pombe* (fig. 2.1A), to extend between the two ends of the cell (Figs. 2.5A and 2.6A). Cells treated with low temperature for 40 min revealed a depolymerised microtubule cytoskeleton with the appearance of diffuse tubulin staining throughout the cell being brightest in the region of the nucleus (40/0; fig. 2.5B, *cdc10-129* and fig. 2.6B, *cdc25-22*). Cells allowed to recover from cold treatment for 2 min (40 / 2, fig. 2.5C, *cdc10-129* and fig. 2.6C, *cdc25-22*) reveal microtubules that are starting to repolymerise (as for wild type, fig. 2.1C), and show small lengths of microtubule, mainly in the area over the nucleus, although some cells show small lengths of staining throughout the cell length. Again, as for wild type *S.pombe* (Fig. 2.1C-E) the more time the cells are allowed to recover from cold plunging, the longer the microtubules grow, until at 30 min (Fig. 2.5E,*cdc10-129* and Fig. 2.6E,*cdc25-22*) they are running along the entire length of the length of the cells, so that they are almost indistinguishable from control cells (Figs. 2.5A and 2.6A).

2.3.vi: Effects of low temperature treatment on actin in *cdc10-129* and *cdc25-22*

Control cells (0 / 0) of *cdc25-22* and *cdc10-129* (figs. 2.7A and 2.8A, respectively) showed normal distributions of actin indistinguishable from

wild type *S.pombe* (Fig. 2.3A). Cold treatment (1°C) of both *cdc10-129* and *cdc25-22* (Figs. 2.7B and 2.8B, respectively) for 40 min has similar effects on the actin as in wild type *S.pombe* (Fig. 2.3b), that is, delocalisation of the actin cytoskeleton. *cdc10-129* cells revealed many cables and large and small brightly and dimly staining dots of actin (Fig. 2.7B), while *cdc25-22* cells display very little actin staining except for a few dim dots. Many more of the cells (~10%) of both *cdc10-129* and *cdc25-22* revealed an actin ring surrounding the nucleus (Figs. 2.7B and 2.8B, respectively). In both *cdc10-129* and *cdc25-22* cells that had been allowed to recover from cold treatment for 2 min (Figs. 2.7C and 2.8C, respectively) the actin staining has returned to a control-like distribution in the majority of cells. *cdc10-129* cells that have been allowed to recover from cold treatment for 5 min (Fig. 2.7D) displayed a similar distribution of actin to control cells (Fig. 2.7A). *cdc25-22* cells that were allowed to recover for 5 min (Fig. 2.8D) displayed actin staining in the form of bright dots, mainly localised at the tips of the cells, but with some dots throughout the cell length together with cables of actin. After 30 min recovery from cold treatment (Fig.2.7E), *cdc10-129* cells closely resemble control cells (Fig. 2.7A), except that cables of actin could be seen in some cells. *cdc25-22* cells allowed to recover from cold treatment for 30 min (Fig. 2.8 E) displayed a similar localisation of actin to cells 5 min after recovery (Fig. 2.8D) with cables of actin staining running along the length of the cell from the tips.

2.3.vii: Dependence of actin distribution on microtubule recovery

In growing cells, there is a major re-organisation of both actin and tubulin at the G2/M boundary (Marks and Hyams, 1985; Hagan and Hyams, 1988). Cytoplasmic microtubules depolymerise and a short intranuclear spindle is formed. The dots of actin staining at the tips of cells disappear and a ring of actin is seen to form at the centre of the cell, predicting the position of the septum in the dividing cells (Marks and Hyams, 1985; Marks et al., 1986)

In order to assess whether these two events were interdependent, we investigated the reorganisation of the actin cytoskeleton, following depolymerisation of the microtubule cytoskeleton with low temperature (1°C) for 40 min with subsequent recovery in the presence of the microtubule polymerisation inhibitor TBZ. Cells that had TBZ added to stop the re-growth of microtubules post-cold treatment, showed a normal redistribution of actin within 5 min , (see summary diagrams, Figs. 2.9A & B). Hence, the reorganisation of actin following cold-depolymerisation is not microtubule dependent.

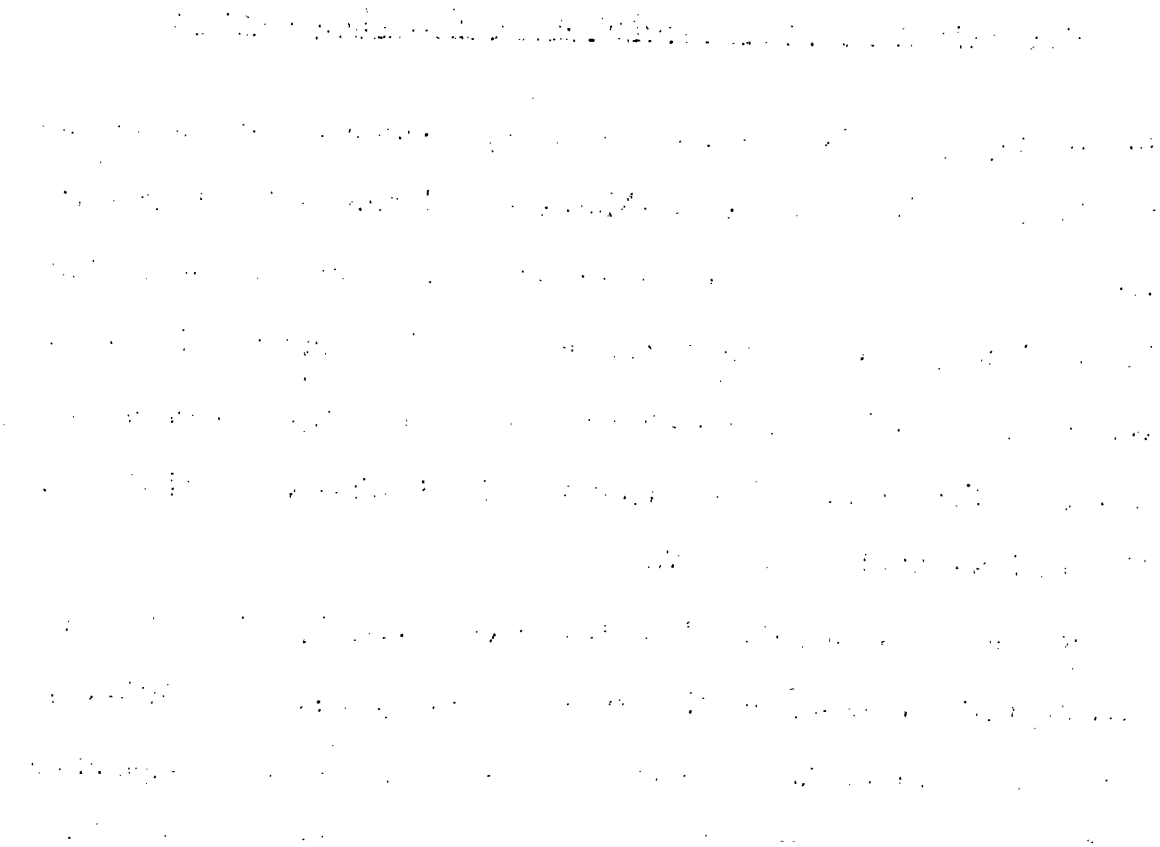


Figure 2.1 Tubulin staining of wild type *S.pombe* cold-plunged (1°C) and returned to 25°C

Figures 2.1, 2.2, 2.5 and 2.6 show cells stained the monoclonal anti-tubulin antibody (TAT1) after fixation by methanol, whilst figures 2.3, 2.4, 2.7 and 2.8 show cells stained with rhodamine phalloidin after fixation by formaldehyde.
All photographs were exposed unequally, allowing the background to appear and therefore staining of cells apears as bright as possible to aid analysis of the staining.
All experiments were carried out at least three times to determine the reproduciblity of the results shown.
(A) Control cells prior to cold-treatment, showing the tubulin cytoskeleton in cells, at stages throughout the cell cycle, the arrowed cell is in interphase with the characteristic "basket" of microtubules parallel with the cells long axis.. (B) Cells cold-plunged at 1°C for 40 min. (C-E) Recovery at 25°C for 2, 5 and 30 min before fixation and staining. Cells in (B) show a diffuse pattern of staining that is more dense over the centre of the cell, which is where the nucleus resides when stained with the DNA stain DAPI, (data not shown). Cells in (C) show short lengths of staining which elongate in (D) and (E) to form microtubules that span the length of the cell. Bar 10μm.

Figure 2.1

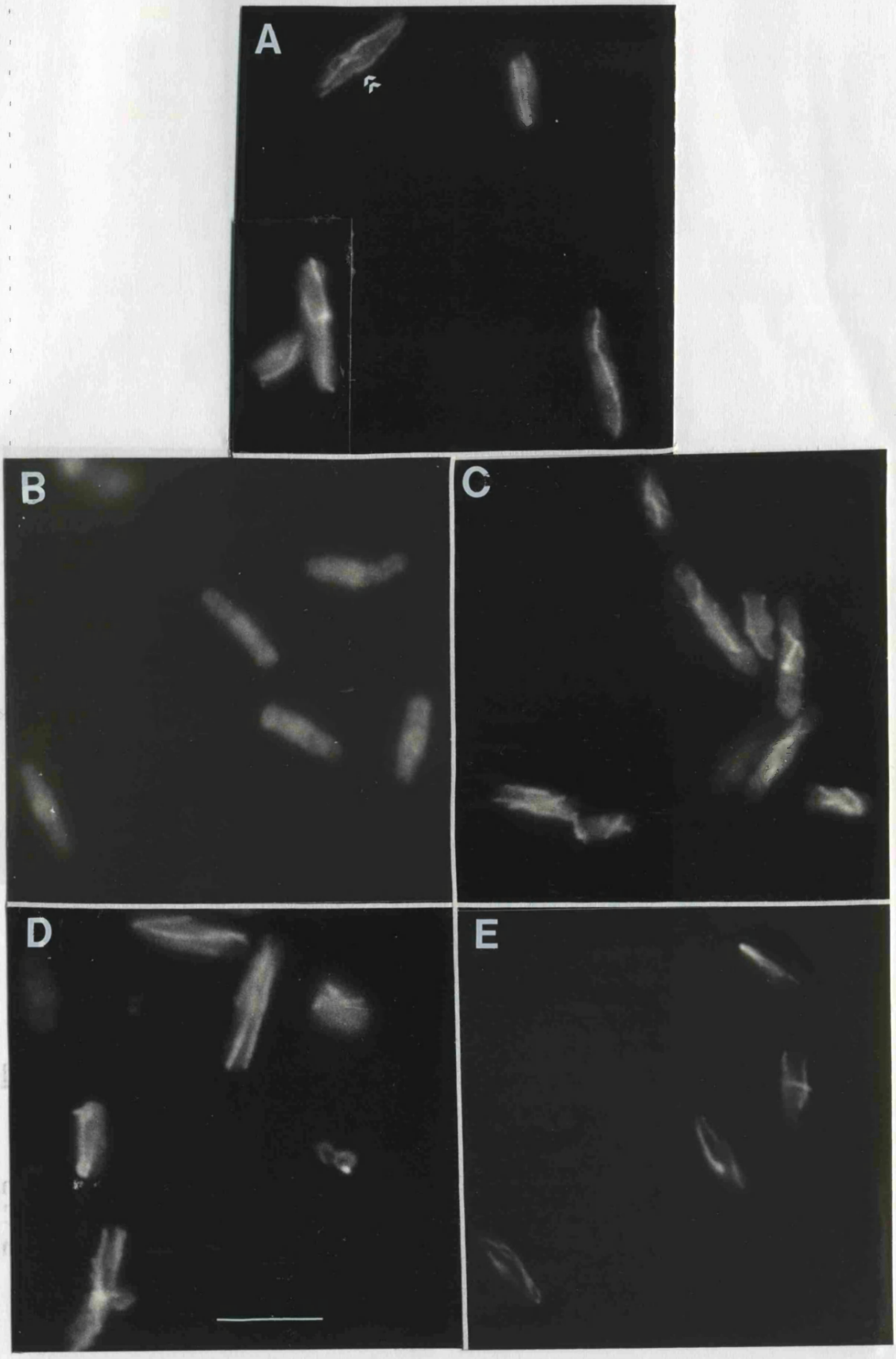


Figure 2.2 Tubulin staining of *S.japonicus* cold-plunged (1°C) and returned to 25°C

(A) Cells prior to cold-plunge treatment, showing the tubulin cytoskeleton at various stages throughout the cell cycle, with the cell arrowed displaying an anaphase spindle at the ends of which asters of microtubule staining can be seen. (B) Cells cold-plunged at 1°C for 40 min, displaying a diffuse staining pattern distributed throughout the cells with some cells showing a more dense staining pattern at the area of the nucleus, as determined by DAPI staining (data not shown). (C-E) Recovery at 25°C for 2, 5 and 30 min before fixation and staining, with some cells in (C, arrowed) displaying two discrete asters of microtubule staining. Cells in (D) and (E) show longer lengths of microtubule staining. Bar 10μm.

Figure 2.2

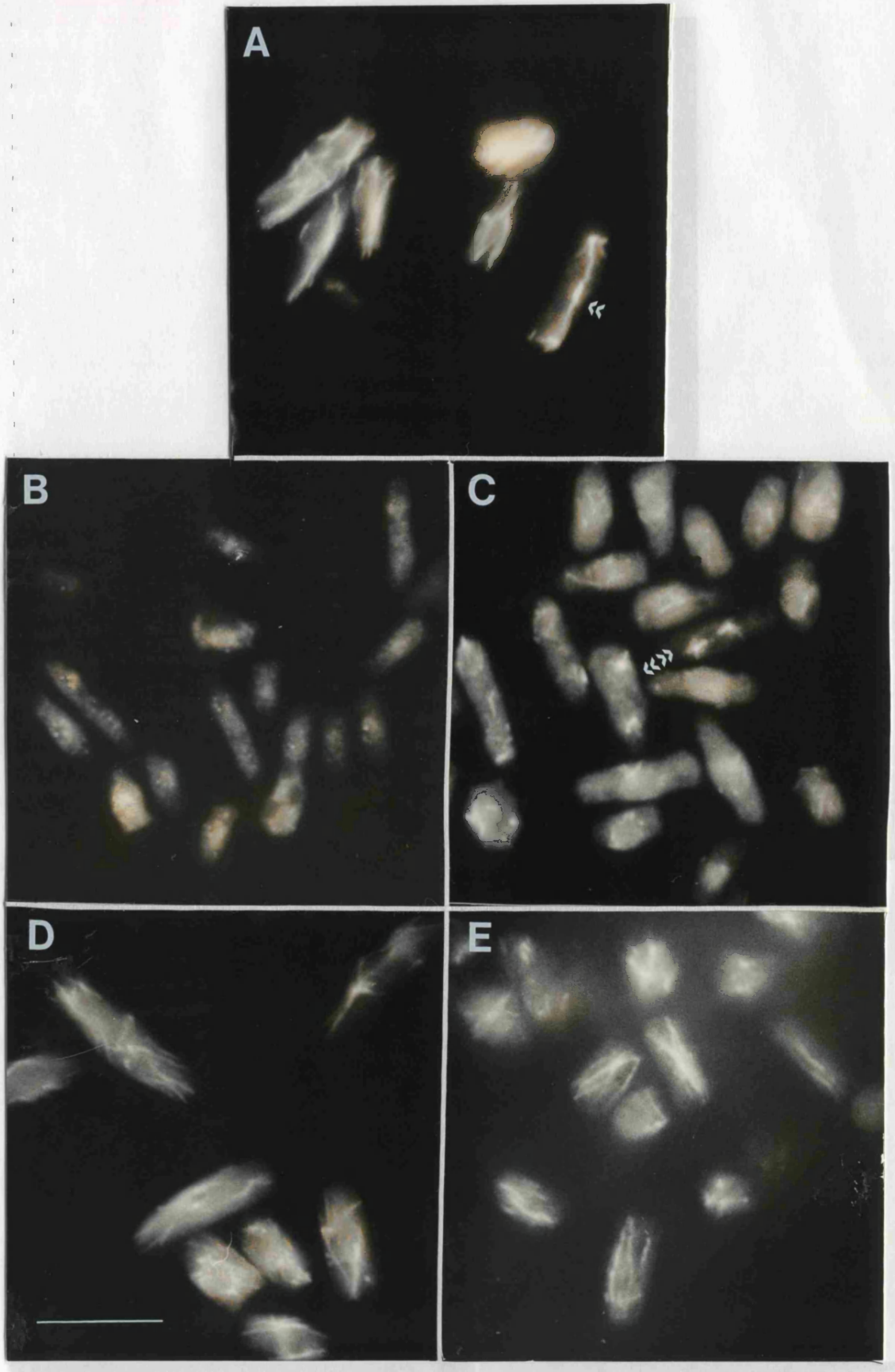


Figure 2.3 Actin staining of *S.pombe* cold-plunged (1°C) and returned to 25°C

(A) Cells prior to cold-plunge treatment, showing characteristic actin staining of the tips or mid-lines of cells depending whether the cells are growing from the tips of the laying down a septum (arrowed) prior to cytokinesis. (B) Cells cold-plunged for 40 min, with one cell (arrowed) showing a loop of actin stain around the periphery of the nucleus, as determined by DAPI staining (data not shown) and with the rest of the cells displayind disorganised actin staining. (C-E) Recovery at 25°C for 2, 5 and 30 min before fixation and staining, showing cells where the actin staining has largely returned to "normal", as in (A), some cells (arrowed) are undergoing cytokinesis, shown by the actin staining at their mid-points. Bar 10μm.

Figure 2.3

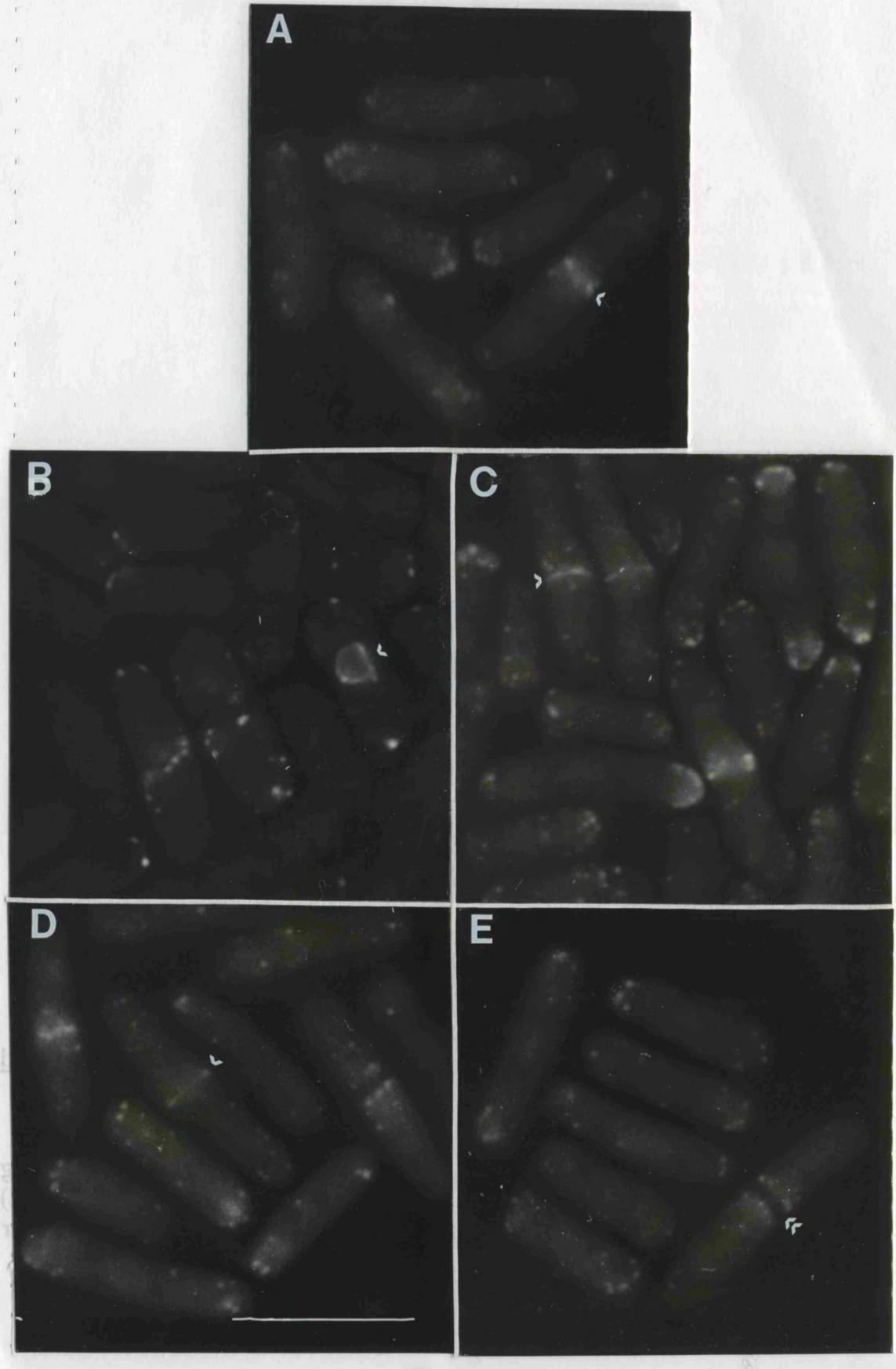


Figure 2.4 Actin staining of *S.japonicus* cold-plunged (1°C) and returned to 25°C

(A) Cells prior to cold-plunge treatment, showing characteristic staining of the tips or mid-lines of cells depending on whether they are in interphase of mitosis repectively. (B) Cells cold-plunged for 40 min with the majority of cells displaying a "control-like" staining pattern. (C-E) Recovery at 25°C for 2, 5 and 30 min before fixation and staining. Cells in (C) and (E) also show a "control-like" staining pattern with some cells undergoing cytokinesis as shown by the actin staining at the mid-points of the cells. However, some cells in (D, arrowed) are distorted in shape and the distribution of the actin staining is also disorganised, which is unsurprising as actin is thought to localise to areas of active cell wall deposition. Bar 10μm.

Figure 2.4

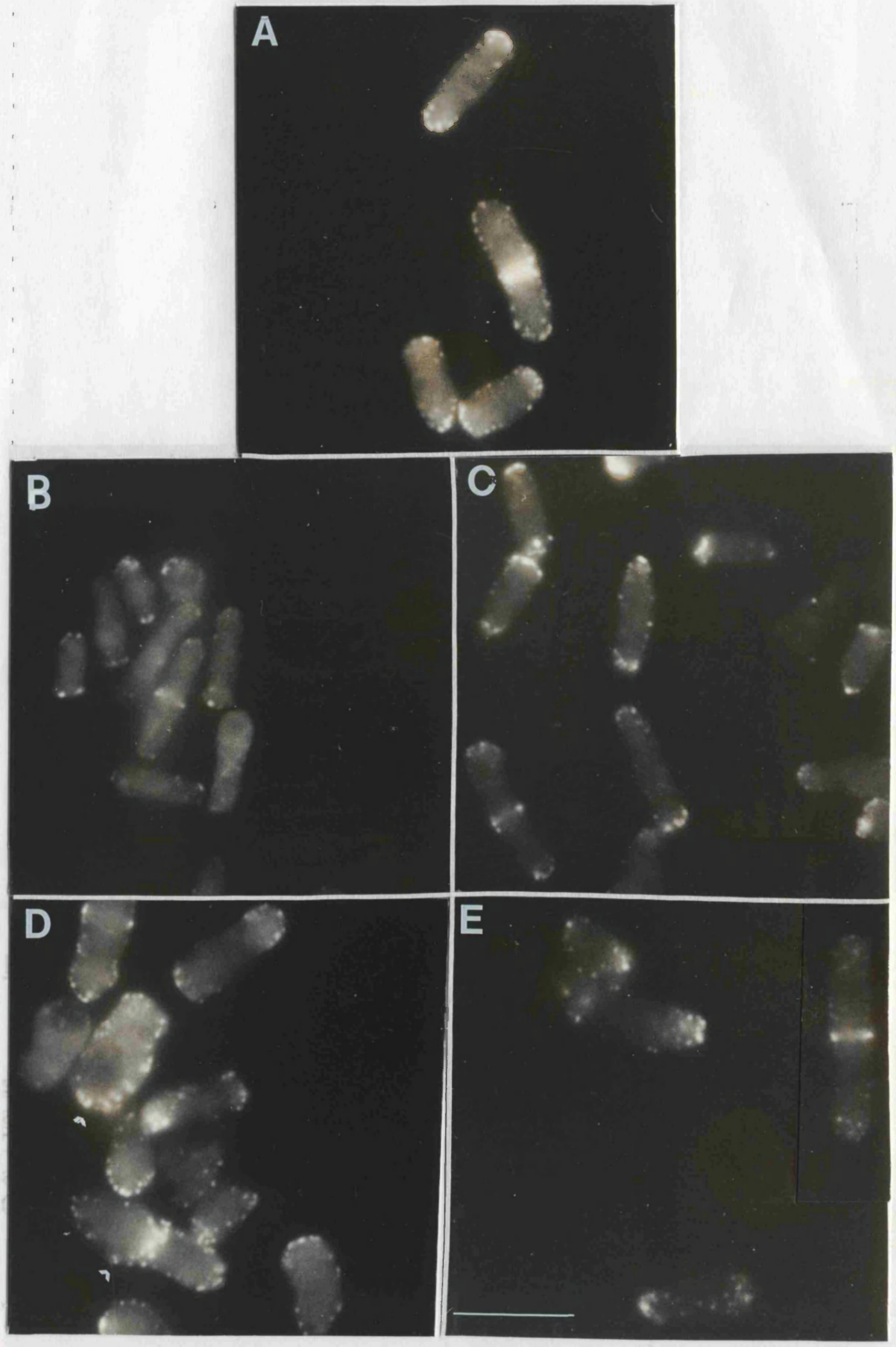


Figure 2.5 Tubulin staining of *cdc10-129h*$^-$ cold-plunged (1°C) and returned to 25°C

(A) Cells prior to cold-plunge treatment and temperature arrest, showing tubulin cytoskeleton in cells, at stages throughout the cell cycle, with the majority of cells in interphase, as characterised by the microtubule staining running from tip to tip along the cells long axis. (B) Cells temperature arrested at the G2/M transition point by incubation at 36°C for 4.25 hrs and then cold-plunged at 1°C for 40 min, showing a diffuse staining pattern with depolymerised microubules throughout the cells and a denser patch of staining in the area over the nucleus (arrowed), as judged by staining with the DNA specific dye (DAPI, data not shown). (C-E) Cells released from temperature block and re-incubated at 25°C for 2, 5 and 30 min. Short lengths of microtubule staining can be seen in (C) which lengthen as cells are left incubating at 25°C (D and E) to form full length microtubules that run the length of the cell. Bar 10μm.

Figure 2.5

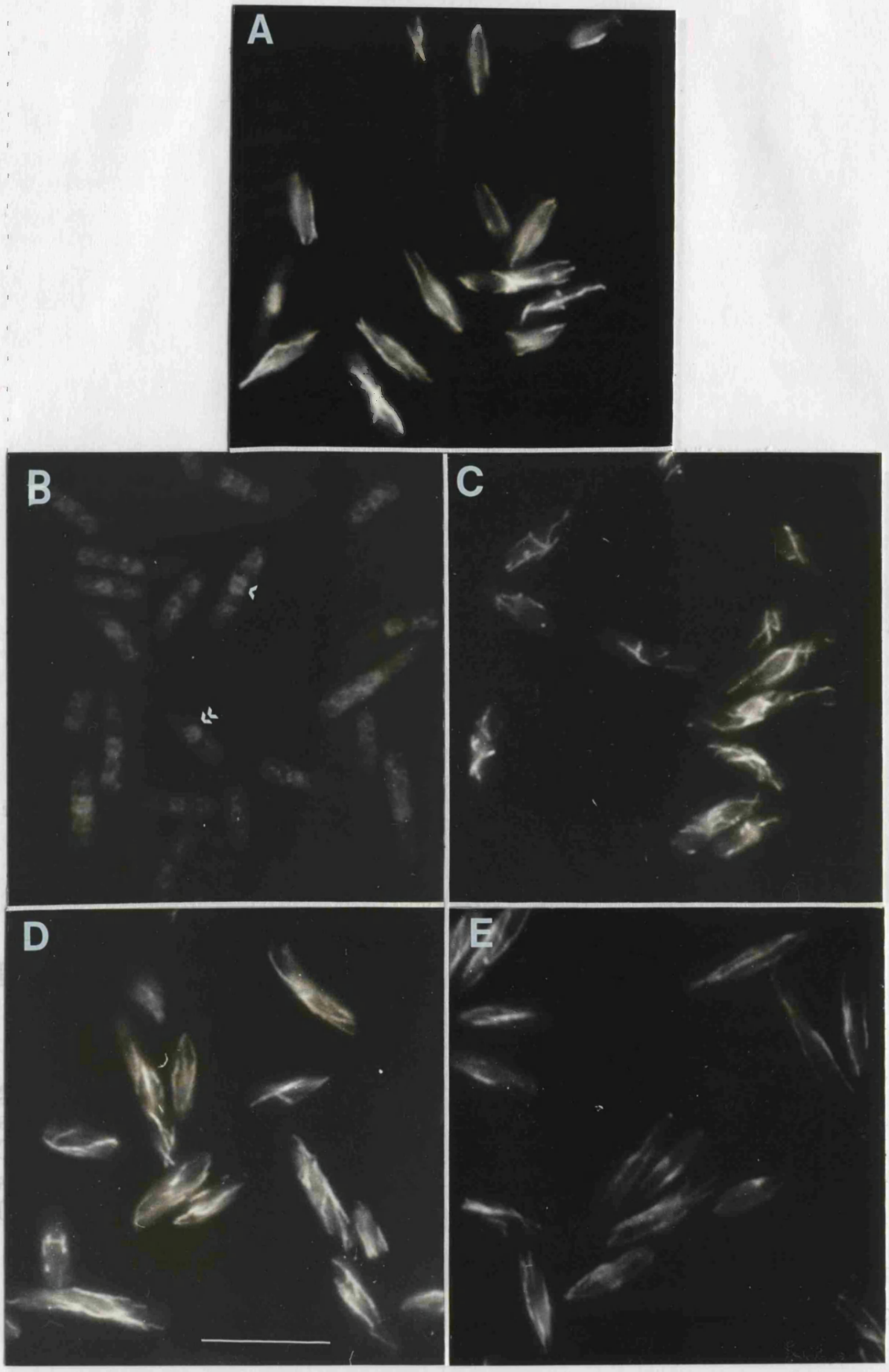


Figure 2.6 Tubulin staining of *cdc25-22h*$^{-}$ cold-plunged (1°C) and returned to 25°C

(A) Cells prior to cold-plunge treatment and temperature arrest, showing tubulin cytoskeleton in cells, at stages throughout the cell cycle with the majority of cells in interphase as shown by the tip to tip microtubule staining. (B) Cells temperature arrested at the G1/S transition point by incubation at 36°C for 4.25 hrs and then cold-plunged at 1°C for 40 min, a diffuse staining pattern can be seen with a denser pattern of staining in the area over the nucleus (arrowed), as determined by staining with the DNA stain (DAPI, data not shown). (C-E) Cells released from temperature block and re-incubated at 25°C for 2, 5 and 30 min. Cells in (C, arrowed) display short lengths of staining, mainly over the area of the nucleus. Cells in (D) and (E) show longer lengths of microtubule staining running from tip to tip, as seen in control cells (A). Bar 10μm.

Figure 2.6

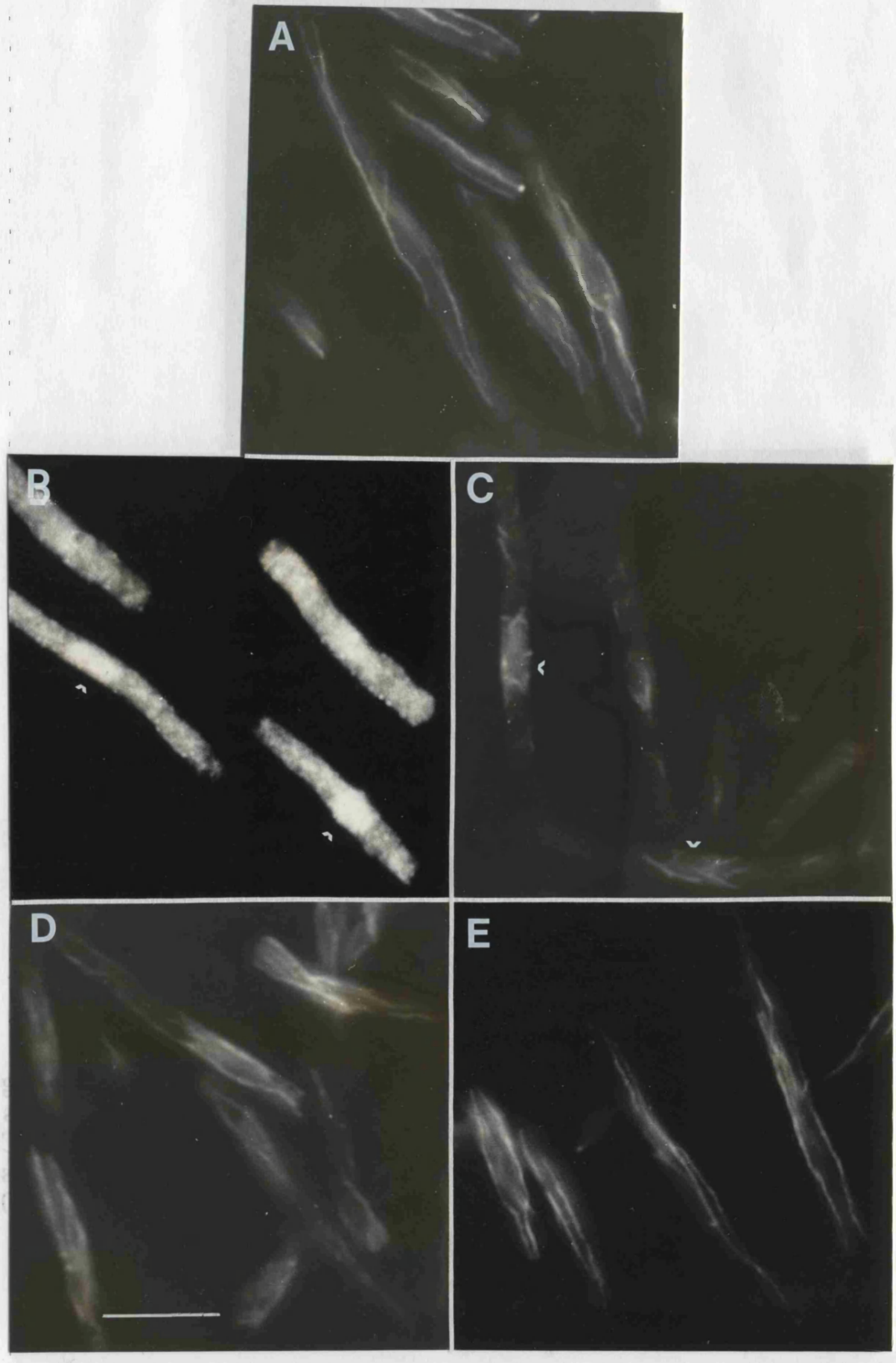


Figure 2.7 Actin staining of *cdc10-129h*$^-$ cold-plunged (1°C) and returned to 25°C

(A) Cells prior to cold-plunge treatment and temperature arrest, showing actin cytoskeleton in cells, at stages throughout the cell cycle characterised by staining at the tips or the mid-point of the cell depending whether the cells are in interphase or mitosis respectively. (B) Cells temperature arrested at the G1/S transition point by incubation at 36°C for 4.25 hrs and then cold-plunged at 1°C for 40 min, with some cells (arrowed) displaying a loop of actin staining around the periphery of the nucleus as detertermined by staining with the DNA specific dye (DAPI, data not shown). (C-E) Cells released from temperature block and re-incubated at 25°C for 2, 5 and 30 min, show staining comparable to that of control cells (A), some cells are undergoing cytokinesis (arrowed) as shown by the staining at the mid-point of the cells. Bar 10μm.

Figure 2.7

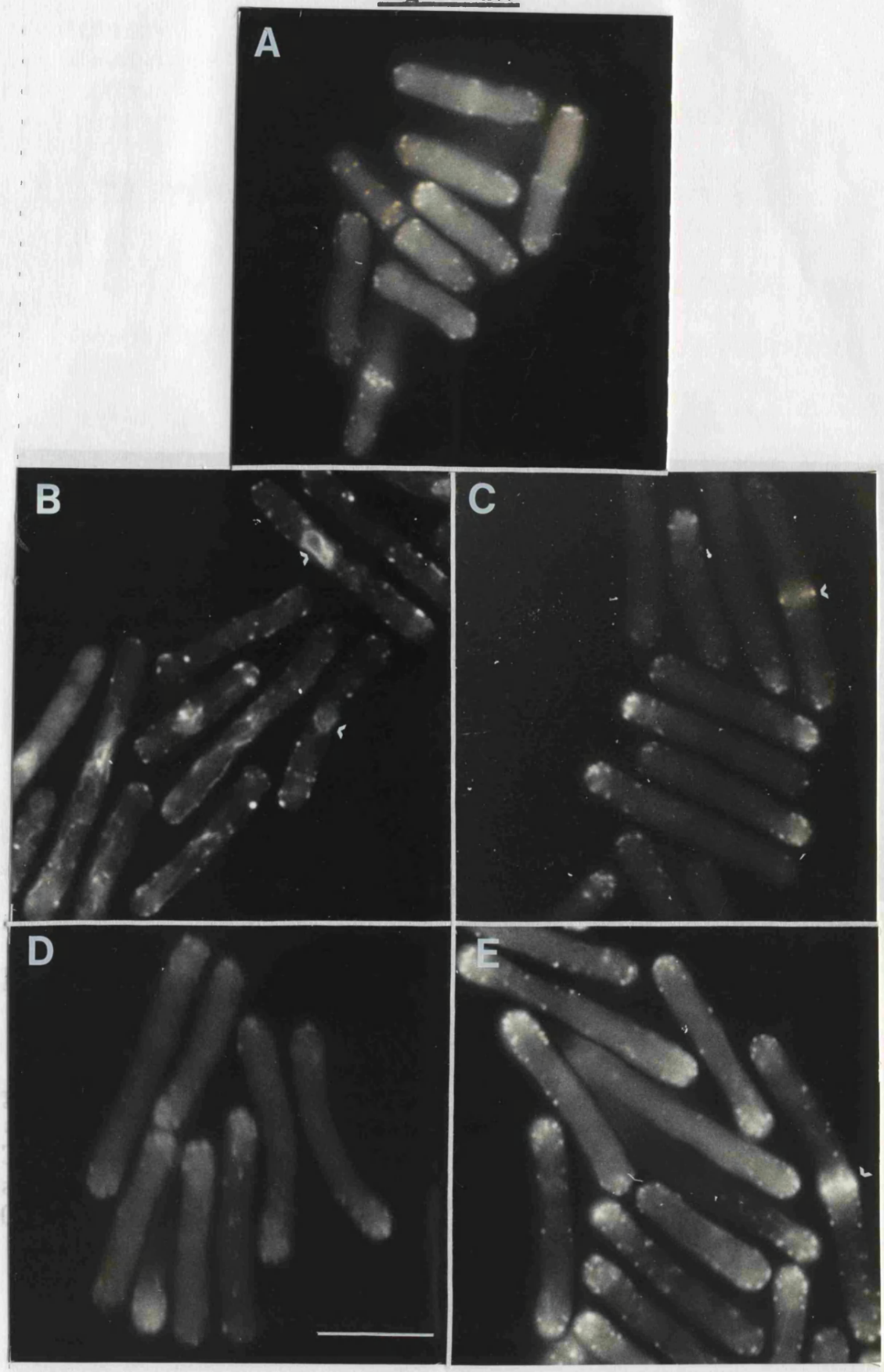


Figure 2.8 Actin staining of *cdc25-22h*$^{-}$ cold-plunged (1°C) and returned to 25°C

(A) Cells prior to cold plunge treatment and temperature arrest, showing actin cytoskeleton in cells, at stages throughout the cell cycle. (B) Cells temperature arrested at the G2/M transition point by incubation at 36°C for 4.25 hrs and then cold-plunged at 1°C for 40 min, with some cells (arrowed) showing a loop of actin staining around the periphery of the nucleus as determined by staining with the DNA specific dye (DAPI, data not shown). (C-E) Cells released from temperature block and re-incubated at 30°C for 2, 5 and 30 min, showing a "control-like" distribution of actin (A) with some cells undergoing cytokinesis (arrowed) shown by staining at the mid-point of the cell. Bar 10μm.

Figure 2.8

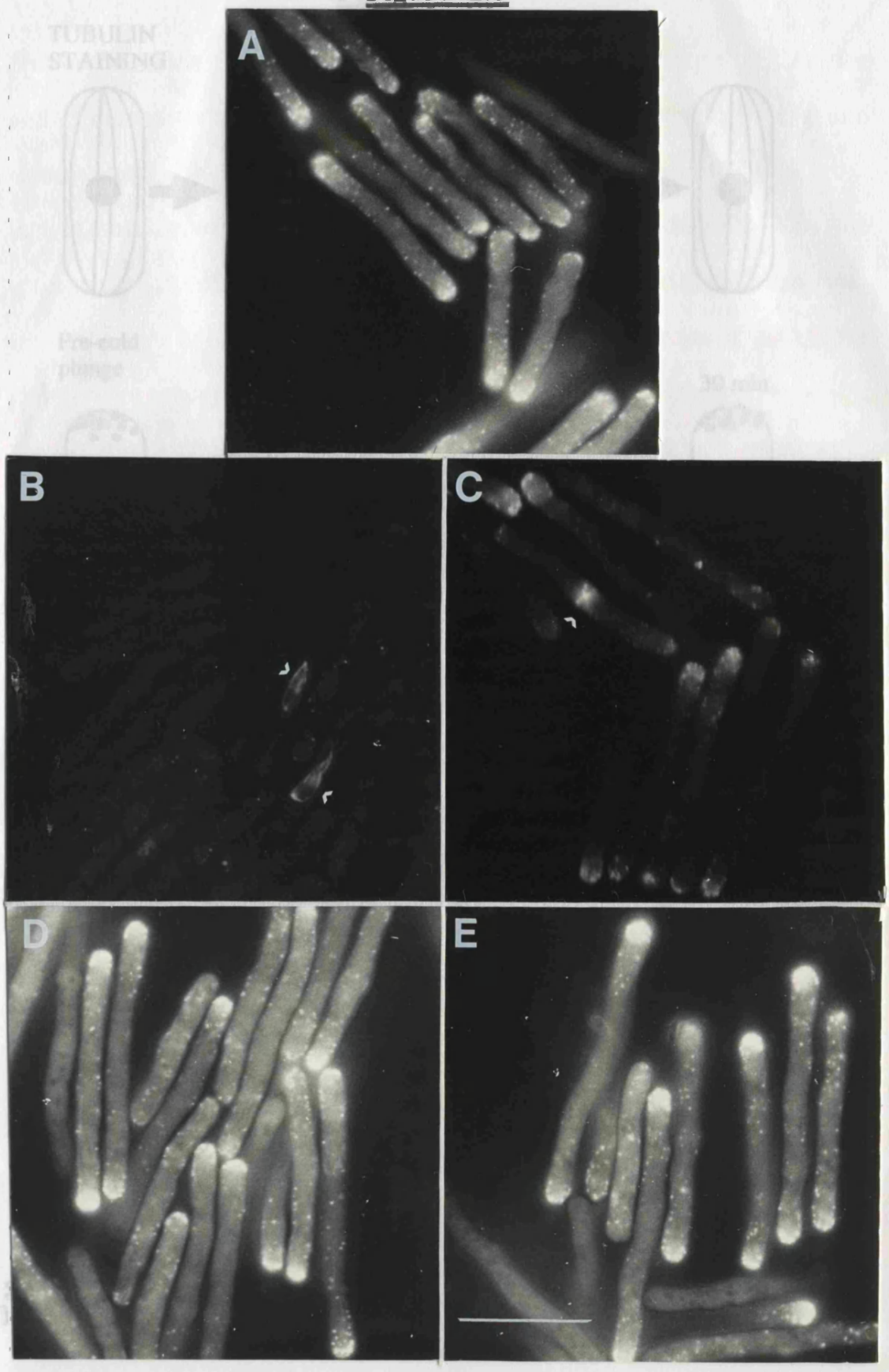


Figure 2.9 Summary diagrams of microtubule recovery experiments

(A) Microtubule and actin recovery at 25°C in *S.pombe* cells following cold treatment (1°C). (B) Actin recovery occurs in the absence of microtubule reassembly in TBZ treated cells.

Figure 2.9A

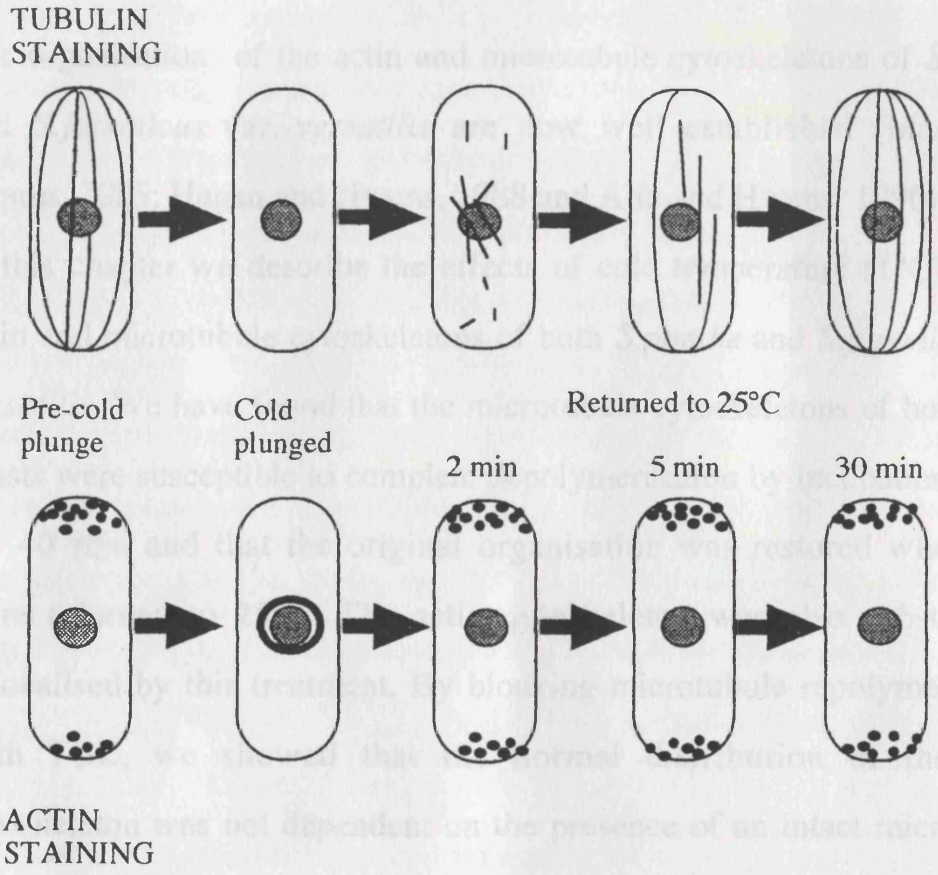
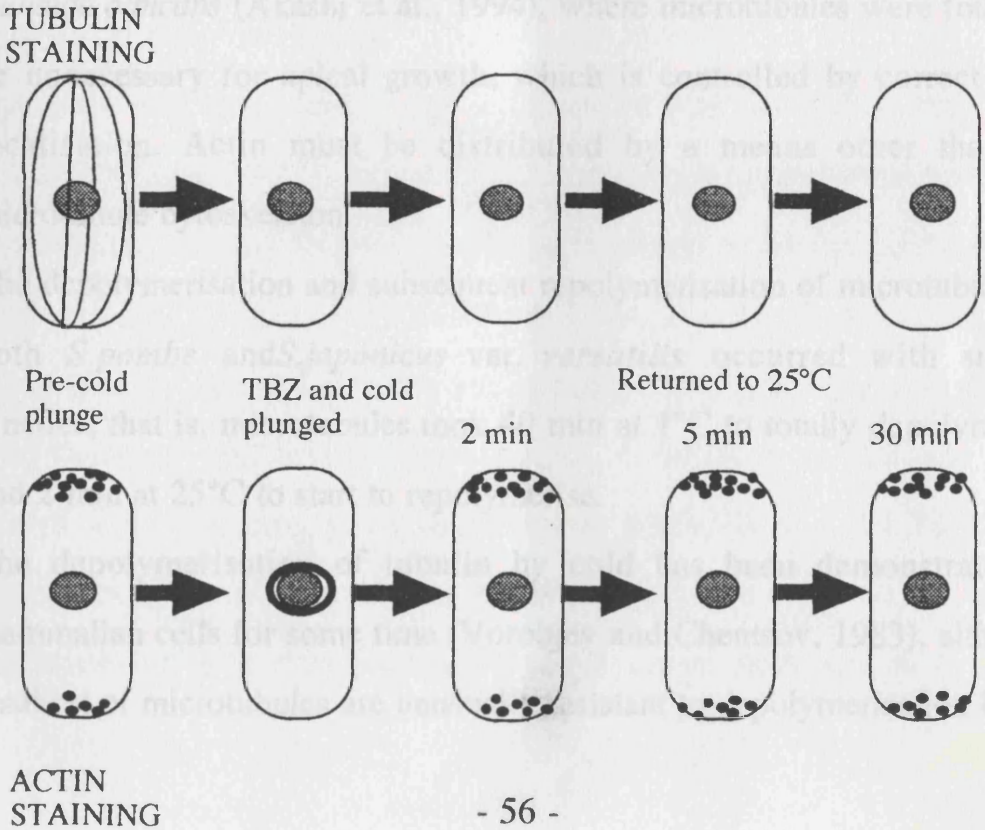


Figure 2.9B



2.4: Discussion

The organisation of the actin and microtubule cytoskeletons of *S.pombe* and *S.japonicus* var. *versatilis* are now well established (Marks and Hyams, 1985; Hagan and Hyams, 1988 and Alfa and Hyams, 1990).

In this chapter we describe the effects of cold temperature (1°C) on the actin and microtubule cytoskeletons of both *S.pombe* and *S.japonicus* var. *versatilis*. We have found that the microtubule cytoskeletons of both these yeasts were susceptible to complete depolymerisation by incubation at 1°C for 40 min and that the original organisation was restored when cells were returned to 25°C. The actin cytoskeleton was also substantially delocalised by this treatment. By blocking microtubule repolymerisation with TBZ, we showed that the normal distribution of the actin cytoskeleton was not dependent on the presence of an intact microtubule cytoskeleton. This theory is supported by work on the dimorphic yeast *Candida albicans* (Akashi et al., 1994), where microtubules were found to be unnecessary for apical growth, which is controlled by correct actin localisation. Actin must be distributed by a means other than the microtubule cytoskeleton.

The depolymerisation and subsequent repolymerisation of microtubules in both *S.pombe* and*S.japonicus* var. *versatilis* occurred with similar kinetics, that is, microtubules took 40 min at 1°C to totally depolymerise and 2 min at 25°C to start to repolymerise.

The depolymerisation of tubulin by cold has been demonstrated in mammalian cells for some time (Vorobjev and Chentsov, 1983), although a subset of microtubules are unusually resistant to depolymerisation by

low temperature. In other organisms, microtubules are completely resistant to depolymerisation by low temperatures. A modification of the β-tubulin subunit in the antarctic fish *Notothenia coriiceps neglecta* is thought to be responsible for microtubule stability at temperatures as low as -1.8°C. (Detrich and Parker, 1993).

In both fission yeasts, microtubules seemed to preferentially repolymerise at a site adjacent the nucleus, presumably from the spindle pole body. This is particularly clear in *S.japonicus* var. *versatilis* after 2 min recovery from cold treatment (Fig. 2.2 C) where two asters of microtubules can be seen in several cells. It is also clear from preliminary work depolymerising the microtubule cytoskeleton via low temperature incubation (1°C) in temperature arrested *cdc11* cells in which multiple nuclei form at the restrictive temperature. In this work asters of repolymerising microtubules can be seen over each nuclei when cells are allowed to recover from cold treatment at 25°C (Snell and Nurse, personal communication).

We have also described a ring of actin in both wild type and, more frequently in *cdc10-129* and *cdc25-22* mutants of *S.pombe* that have been incubated at 1°C for 40 min. This ring is unlikely to be the result of actin at the cell tips collapsing towards the nucleus, as it was found to be a two-dimensional ring and not a ball surrounding the entire nuclear surface. A cold sensitive, benomyl resistant mutant, *ben4* (Roy and Fantes, 1982) shows a similar ring of actin staining when held at its restrictive temperature (Schroeder-Lorenz et al., 1987). *Ben4* is an extragenic suppressors of actin mutants, and conversely, some mutations that

suppress the *ben4* phenotype, map to the *S.pombe* actin (*act1*)$^{+}$ gene (Schroeder-Lorenz et al., 1987).

CHAPTER 3

ONE AND TWO DIMENSIONAL GEL ELECTROPHORESIS AND WESTERN BLOTTING OF ACTIN FROM S. POMBE

3.1: Introduction

The actin cytoskeleton of *S.pombe* has been widely studied cytologically (Marks and Hyams, 1985, Alfa and Hyams, 1990; Kanbe et al., 1993; 1994), genetically (Mertins and Gallwitz, 1987, Lees-Miller et al., 1992), but little biochemical work has been done to study this important protein in this organism (Scordilis et al., 1991). Considerably more work has been undertaken on the budding yeast actin cytoskeleton (Drubin, 1990, Johannes and Gallwitz, 1991, Palmer et al., 1992, Chowdhury and Gustin, 1992, Drubin et al., 1993, Adams et al., 1993). It has been recently shown that *S.pombe* has two genes encoding members of the actin family; *act1*$^+$(Mertins and Gallwitz, 1987) and *act2*$^+$(Lees-Miller et al., 1992). These genes encode proteins of 374 and 427 amino acids respectively and show only 35-40% identity to each other. *act1*$^+$encodes a protein with a relative molecular mass (M_r) of 42,000, while *act2*$^+$is predicted to encode a 47,376 M_r protein. They have similar pI's of 5.2 and 5.7 respectively. These are values similar to those published by Scordilis et al (1991) where the biochemical purification of actin from *S.pombe* resulted in pellet and supernatant fractions having Mr's of 46,800 and 47,800 respectively. Furthermore, the pI's were also similar,

5.6 (pellet) and 5.2 (supernatant). These differences are thought to be due to a high proportion of non-polymerised G-actin in the supernatant fractions. *Staphylococcus aureus* V8 protease maps separated by SDS-PAGE of these two actin isoforms, were identical, leading Scordilis et al. (1991) to conclude that the two isoforms were the result of post-translational modification(s) of the *act1*$^+$encoded protein. However, the more recent work by Lees-Miller et al (1992) has demonstrated that *S.pombe* has another actin-related gene called *act2*$^+$. Lees-Miller et al (1992) have estimated the abundance of *act2*$^+$through codon usage analysis and calculate "that *act2*$^+$is unlikely to represent a large portion of the actin-based structures detected in yeast by phalloidin or by anti-actin antibodies." Using the terminology of Schroer et al (1994), the *act2*$^+$ protein is now designated Arp3.

In this chapter we describe our biochemical study of actin in *S.pombe* using both one and two-dimensional electrophoretic separation with subsequent Western blotting , probed with a monoclonal anti actin antibody. We also show the changes that actin undergoes through the cell cycle via the use of a cell division cycle mutant with subsequent one-dimensional electrophoresis and Western blotting.

3.2: Materials & Methods

3.2.i: Strains used

Wild type *Schizosaccharomyces pombe 972h⁻*, the cell division cycle mutant *cdc25-22 h-* and the double act1 mutant *A16a* (Schroeder- Lorenz., et al, 1987) were maintained and described as in Chapter 2.

3.2.ii: Protein extraction

Cytosolic extracts were prepared using the following protocol: cells at a density of 5-8 x 10^6 cells ml^{-1} were harvested in a Beckman GS-6 benchtop centrifuge at 3, 000 x g for 5 minutes at 4°C. The supernatant was removed and the pellet mixed with an equal volume of 425-500μm diameter acid washed glass beads (Sigma). An equal volume of extraction buffer (25mM Tris pH7.5, 80mM β-glycerophosphate, 15mM *p*-nitrophenylphosphate, 20mM 1,2-di (2-aminoethoxy) ethane-*NNN'N'*-tetra-acetic acid (EGTA), 15mM $MgCl_2$, 1mM DL-dithiothreitol (DTT), 0.1M sodium orthovanadate, 0.1mM sodium fluoride, 0.1% (v/v) Nonidet P-40, 1mM phenylmethylsulfonyl fluoride (PMSF), 5mg ml^{-1} of leupeptin, N-tosyl-L-phenylalanine chloromethyl (TPCK) and pepstatin A), was then added and mixed for 5-10 minutes on a Janke & Kunkel

eppendorf shaker at 4°C . Cell debris and unbroken cells were removed by centrifugation at 13,000 x g for 5 minutes (Micro Centaur) at 4°C. The resulting supernatant was removed and an aliquot used for calculation of protein content (Bradford,1976). The remaining supernatant was mixed with half the volume of sample buffer (125mM Tris, pH6.8; 2% (w/v) SDS; 10% (v/v) glycerol; 5% (v/v) 2-mercaptoethanol; 0.01% (w/v) bromophenol blue) and boiled for 2 minutes. Samples thus prepared were stored at 4°C until use.

3.2.iii: Alkaline phosphatase dephosphorylation of protein extracts

Calf intestinal alkaline phosphatase (100 units, Sigma) was added to *S.pombe* protein extracts after treatment with extraction buffer and glass beads (see above). Calf intestinal alkaline phosphatase was also added (100units) to *S.pombe* extracts after treatment with extraction buffer (minus protease inhibitors) and glass beads.

3.2.iv: One Dimensional Gel Electrophoresis and Immunoblotting

SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli (1970), using a Hoeffer "Mighty small II" mini-gel system. 1.5mm thick 10 x 9cm separating gels were prepared to the following recipe: 375mM Tris (Tris (hydroxymethyl) amino-methane), pH 8.9; 0.2% (w/v) SDS; 12.5% (w/v) acrylamide; 0.33% (w/v) bis-acrylamide;

0.1% (w/v) ammonium persulphate; 0.001% (v/v) TEMED (N N N' N'-tetramethylethylene-diamine). After polymerisation 1.5cm deep stacking gels of the following recipe were added: 82.5mM Tris, pH6.7; 0.2% SDS; 5% acrylamide; 0.13% bis-acrylamide; 0.05% ammonium persulphate; 0.001% TEMED. Samples were separated in running buffer containing 25mM Tris, pH 8.3; 192mM glycine; 0.1% (w/v) SDS, at a constant current of 40mA, using a LKB Macrodrive 1 model 2301 power supply, until the dye front had reached the bottom of the gel.

Proteins were transferred to Whatman Immobilon membrane for 40 minutes at a constant 25V, using a Millipore Graphite Electroblotter I semi-dry blotting system and LKB Macrodrive 1 model 2031 power supply. The membrane was then transferred to blocking solution (0.15M NaCl, 0.01M Na_2HPO_4 ($2H_2O$) pH7.2 and 1% (w/v) Marvel non-fat dried milk) for 40 minutes. After blocking, the membrane was incubated in primary antibody (Amersham monoclonal mouse anti-chicken gizzard actin) diluted 1 in 833-1000 in PBS-Tween, i.e. PBS (0.15M NaCl, 0.01M Na_2HPO_4 ($2H_2O$) pH 7.2), 0.5% (v/v) Tween 20, 0.1% (w/v) Marvel and 0.1% (w/v) sodium azide overnight on a rotator (Luckham cell mixer). The membrane was then washed in PBS-Tween 10 minutes and PBS for 10 minutes. The membrane was incubated in secondary antibody (Sigma, rat anti-mouse peroxidase conjugate, diluted 1 in 1000, buffer as for primary antibody but omitting the sodium azide), for 1 hour at room temperature. The membrane was then washed twice for 10 minutes in PBS. The membrane could now be developed using the Amersham ECL system. 0.5ml of detection solutions 1 and 2 were mixed in a petri dish

and the membrane was immersed into the mixture for 60 seconds with constant agitation. Excess detection solutions were removed by blotting the edge of the membrane with a tissue and then wrapped in Saran wrap avoiding creases and bubbles. The membrane could now be applied to X-ray film (Fuji HR) with the protein side facing the film in complete darkness. Exposure times varied from 20 seconds to 4 minutes, so a range of exposures was undertaken and the time taken for some of the background to appear was normally used. X-ray film was processed with a Fuji X-100 automatic processor.

3.2.v: Molecular weight determination

Sigma pre-prepared molecular weight markers were separated on both one-dimensional and two-dimensional gel electrophoresis and their relative mobilities plotted on a graph. The resulting curve allowed molecular weights of unknown proteins to be determined.

3.2.vi: pI determination

Bio-Rad pI markers were separated by two-dimensional gel electrophoresis and silver stained. Unknown sample protein pI's were determined by comparison with the graph plotted of the markers relative mobilities.

3.2.vii: Two Dimensional Gel Electrophoresis and Immunoblotting

Using the Bio-Rad Mini-protean II, two dimensional gel electrophoresis system, 8cm long 2mm in diameter first dimensional tube gels were prepared from the following recipe: 9.2M urea, 4% (w/v) acrylamide, 0.2% (w/v) bis-acrylamide, 2% (v/v) Triton X-100, 2% (v/v) Bio-Lyte 5-7 ampholyte, 0.01% (w/v) ammonium persulphate and 0.1 % (v/v) TEMED.

First dimension tube gels were pre-focused at 200V for 10 minutes, 300V for 15 minutes and 400V for 15 minutes to enable better resolution. Thoroughly degassed upper chamber buffer (20mM NaOH) and lower chamber buffer (10mM H_3PO_4) were added prior to pre-focussing.

Samples were loaded dissolved in first dimension sample buffer: 9.5M (w/v) Urea, 2% (v/v) Triton X-100, 5% (v/v) 2-mercaptoethanol, 2% (v/v) Bio-Lyte 5-7 ampholyte and overlayed with 20-30µl of first dimension sample overlay buffer: 9M (w/v) Urea, 1% (v/v) Bio-Lyte 5-7 ampholyte, 0.0004% (w/v) bromophenol blue. The samples were run at 500V for 10 minutes and then 750V for 3.5 hours. The tube gels were extracted and gently placed in the well of a Bio-Rad mini-SDS polyacrylamide gel system and run as for one dimension gels (above). Gels were then Western blotted and developed as for one dimensional gels (above).

3.2.viii: Silver staining

(Silver staining was carried out after the method of Heukeshoven and Dernick (1988). All steps were carried out on a shaker (Stovall). Gels were immersed in fixative (40% (v/v) ethanol, 10% (v/v) glacial acetic acid and 50% (v/v) distilled water), for 40 minutes at room temperature, then transferred into incubation solution (3mM sodium thiosulphate, 0.52% (v/v) gluteraldehyde, 125mM sodium acetate and 30% (v/v) ethanol), for 30 minutes at room temperature. Gels were then washed three times in distilled water for 15 minutes in total. Gels were now stained for 40 minutes at room temperature in silver solution (1.5mM silver nitrate and 0.05% (v/v) formaldehyde, added just before use). Gels were developed in prechilled (4°C) developing solution (30mM sodium carbonate with 0.025% (v/v) formaldehyde added just before use). Cooling the developer allowed a more precise control over development times. Development continued until no more bands appeared but before the background became too dark. Staining was then preserved by immersion in stop solution (15mM sodium EGTA), for at least 15 minutes. Gels were washed twice for 10 minutes in total in distilled water and kept in preservation solution (10% (v/v) glycerol in distilled water) at 4°C.

3.3: Results

3.3.i: One and two-dimensional Western blots of actin

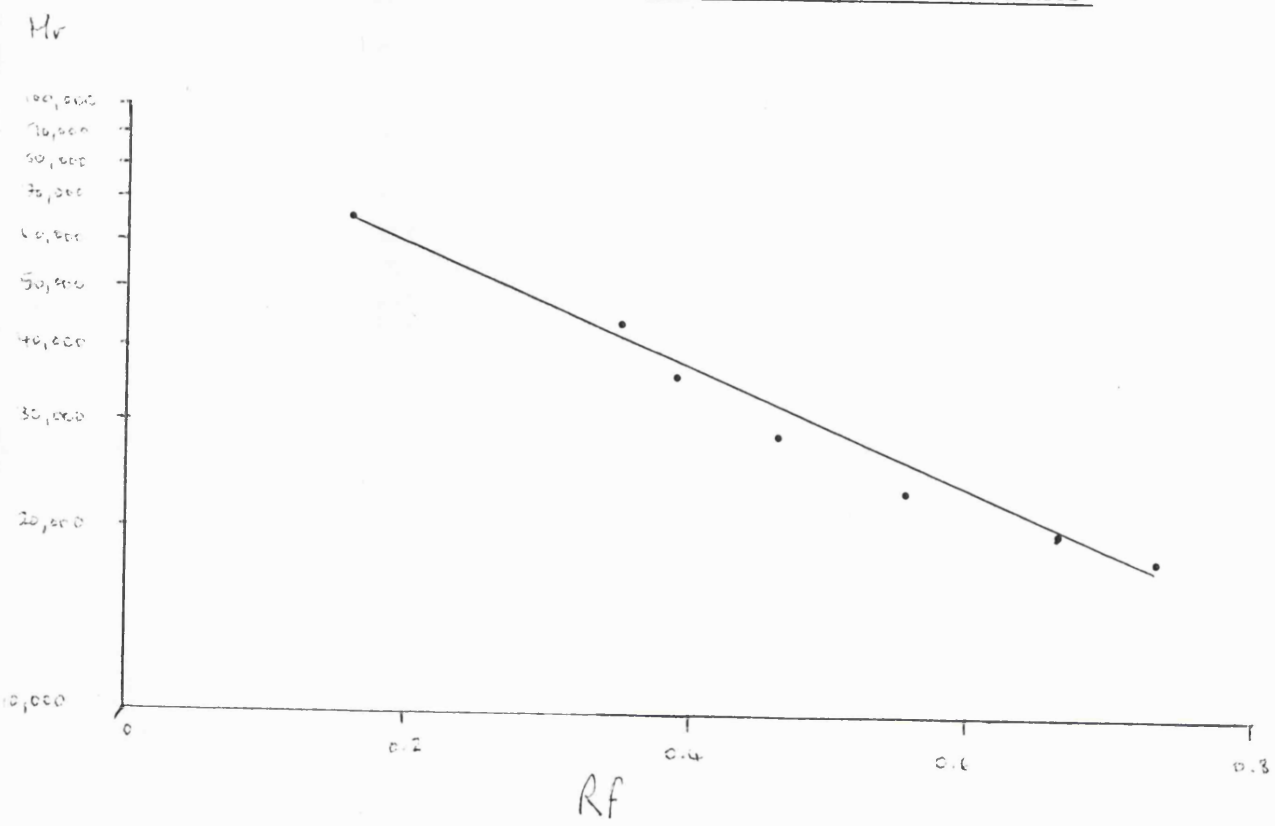
Pellet and supernatant fractions of protein extracts from wild type cells and from the mutants *cdc25-22h*$^-$ and *A16a* were Western blotted and subsequently probed with the monoclonal anti-actin antibody N-350. Only the pellet fraction displayed bands (Fig. 3.1)

The wild type extract displayed two bands when the pellet fraction was separated using one-dimensional SDS-PAGE, (Fig. 3.2, lane B). The Mr's of these bands were 50,000 and 48,000. Protein extracts from both *A16a* and *cdc25-22h*$^-$ also revealed two bands with the same Mr's (Fig. 3.2, lanes A and C, respectively).

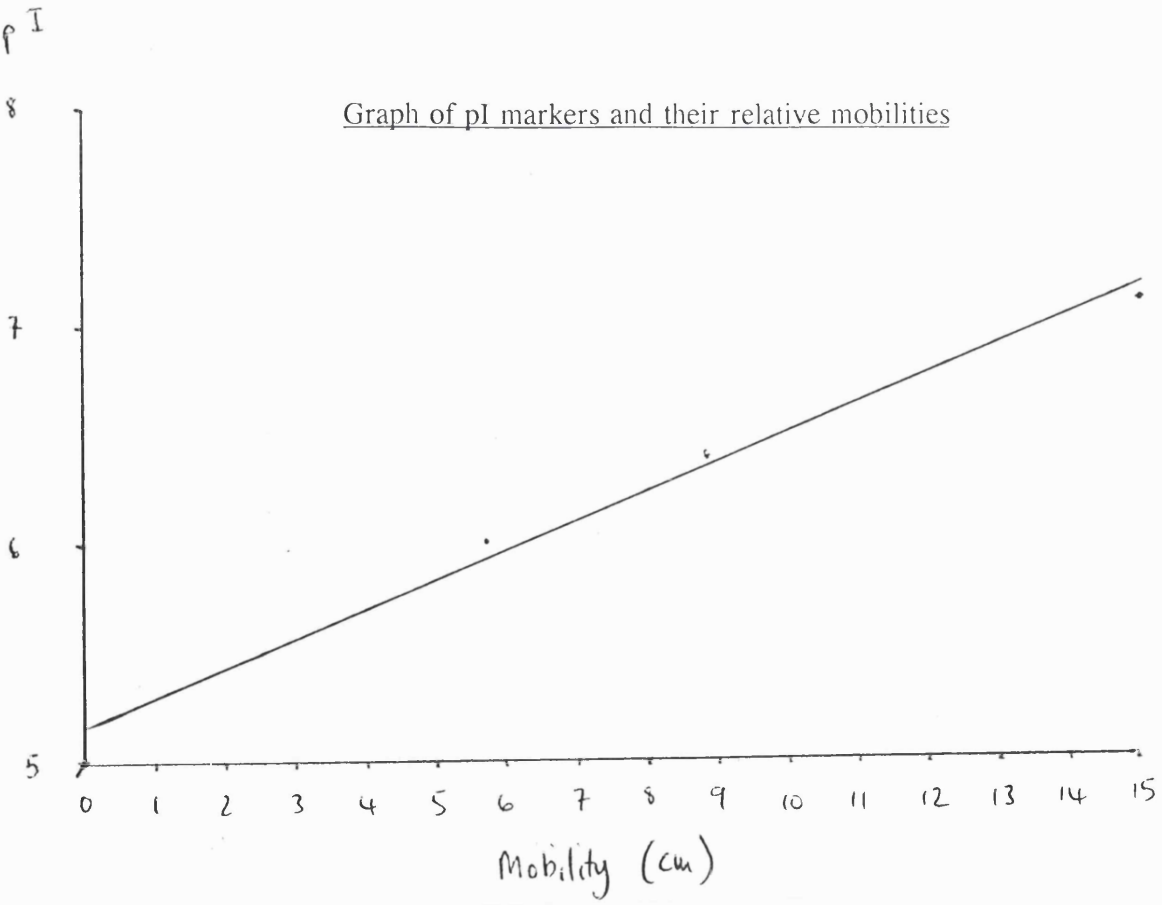
Using two-dimensional gel electrophoresis to determine whether any other bands existed in the *cdc25-22h*$^-$ extract, that could not be separated soley on weight (Fig. 3.3) , four distinct spots could be seen. These spots have pI's of 5.45 (a), 5.53 (b), 5.92 (c) and 5.4 (d). The Mr's of three of the spots (a,b and c) being 50,000 and the fourth spot (d) being 48,000.

In an attempt to determine whether the observed actin pattern changed through the cell division cycle, *cdc25-22h*$^-$ cells were synchronised by cell cycle arrest at G2/M and subsequent release. Samples were taken through one cell cycle and separated by one-dimensional gel electrophoresis followed by Western blotting with the actin antibody. At the zero time point it was possible to resolve a single major actin band

Graph of molecular weight markers relative mobilities



Graph of pI markers and their relative mobilities



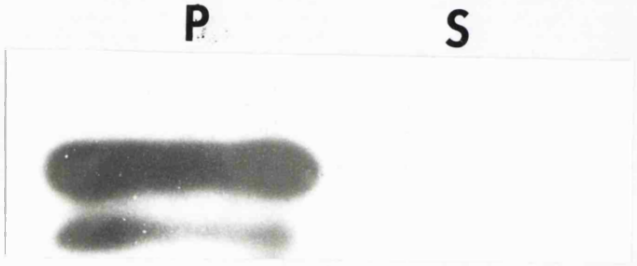


Figure 3.1 One-dimensional gel of pellet and supernatant fractions from wild type (972h-) *S.pombe*, Western blotted with monoclonal anti-actin antibody.

(P) Contains pellet fraction resulting from a 13, 000 x g spin of glass bead ground cells, displaying two bands with Mr's of 50,000 and 48,000.
(S) Contains supernatant fraction resulting from identical treatment to lane (P), displaying no bands.

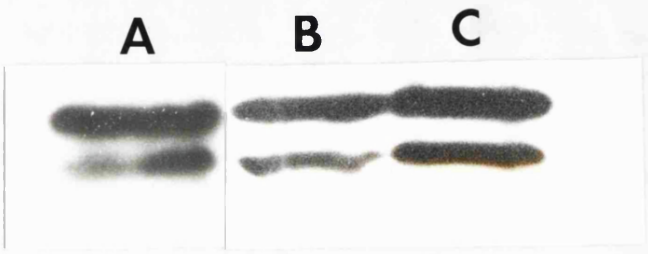
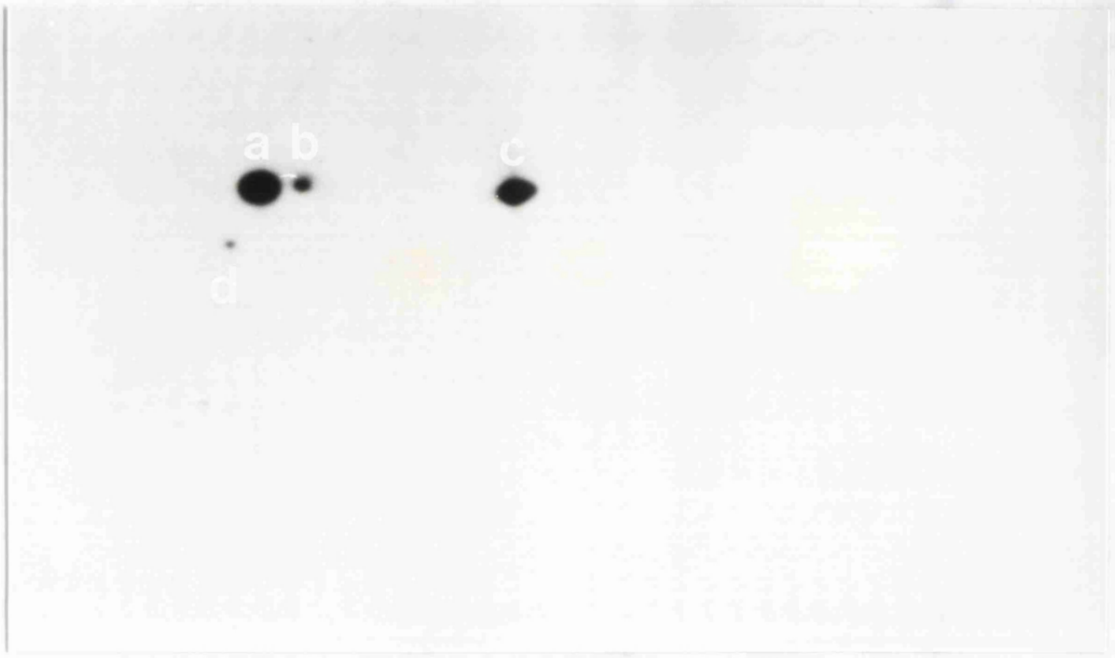


Figure 3.2 One-dimensional gel of total protein extracts from wild type (*972h-*), *cdc25-22h*$^{-}$ and *A16a*, Western blotted with monoclonal anti-actin antibody.

(A) Contains total protein extract from the double actin mutant (*A16a*), displaying two bands with Mr's of 50,000 and 48,000.

(B) Contains total protein extract from wild type (972h^{-}) *S.pombe*, displaying two bands with Mr's of 50,000 and 48,000.

(C) Contains total protein extract from *cdc25-22h*$^{-}$, displaying two bands with Mr's of 50,000 and 48,000.



5 pI 7

Figure 3.3 Two-dimensional gel electrophoretic separation of total protein extract from *cdc25-22h*$^{-}$, Western blotted with monoclonal anti-actin antibody.

Four spots with Mr's ranging from 48,000 to 50,000, with spots a, b and c having the same Mr of 50,000. The spots have differing pI' of 5.4 (spot d), 5.45 (spot a), 5.53 (spot b) and 5.92 (spot c).

with an Mr 48,000 with minor bands at 50,000 and 39,000 (Fig.3.4b). as cells progressed into mitosis, as judged by the rise in septation index (Fig.3.4a), the 50,000 Mr band increased in intensity, as did a band with an Mr of 33,500. At the completion of mitosis, a dramatic change in the actin pattern was evident, namely, the abrupt decline of both the 50,000 Mr band and the two lower bands.

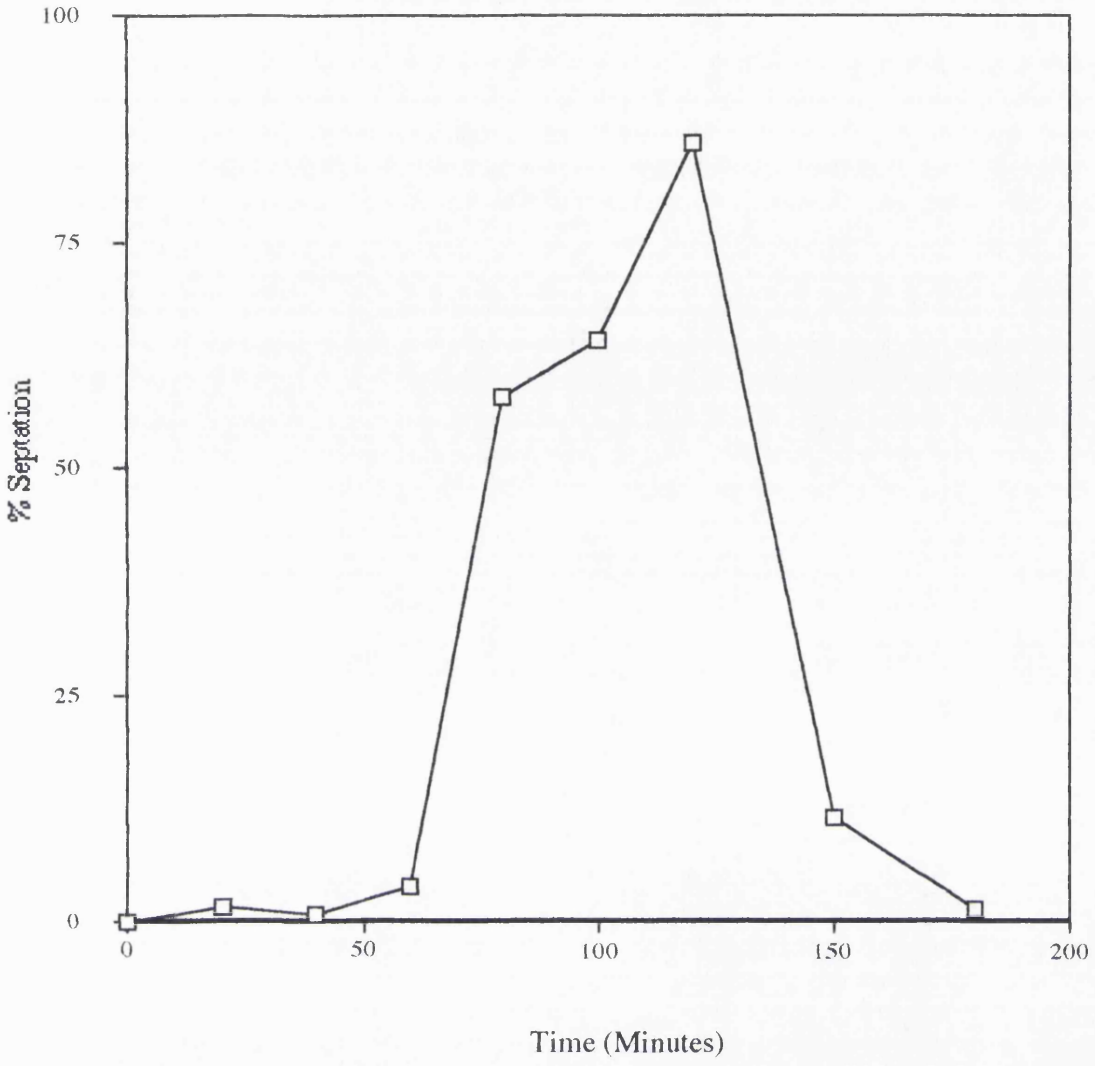
Attempts to determine whether the observed changes were the result of phosphorylation were undertaken by exposing cytosolic extracts to calf intestinal alkaline phosphatase. Unfortunately, the activity of the alkaline phosphatase was inhibited by the protease inhibitors added to the extraction buffer. Removal of the resulted in the degradation of sample and this important point remains unresolved.

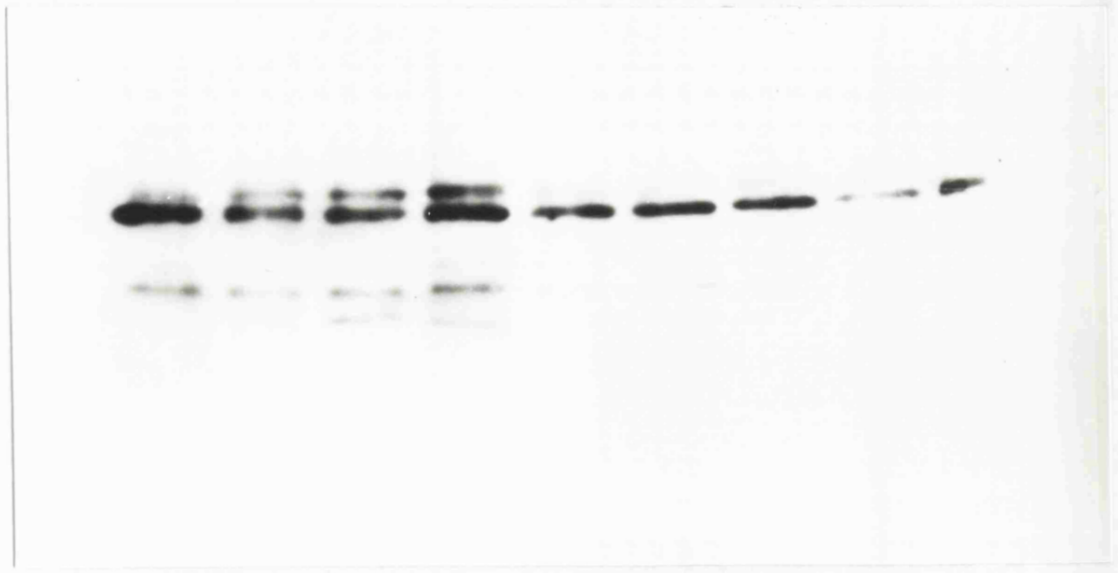
3.4: Discussion

Only the pellet fraction of wild type cell homogenates revealed actin bands on Western blots probed with the monoclonal anti-actin antibody N-350. Taken at face value, this result suggests that all actin in *S.pombe* is in a particulate form, that is, there is no pool of soluble or g-actin. Some support for this view comes from the similarity of the fluorescence staining patterns obtained with phalloidin (specific for F-actin) and actin antibody (which should recognise both G and F forms). However, Marks (1988) was able to adsorb G-actin from *S.pombe* cytosolic extracts onto a DNAse I column and Scordilis et al. (1991) have reported the purification of actin from the same starting material. It is possible that the

Figure 3.4 Western blot and corresponding graph of cdc25-22h- synchronised and subsequently released in the cell cycle.

One dimensional gel electrophoretic separation and subsequent Western blotting and probing with a monoclonal anti-actin antibody of total protein extract from *cdc 25-22h*$^{-}$ that has been synchronised with temperature arrest (36°C) and released (25°C), with samples collected at time points throughout the first cell cycle and up to 200 min. A septation peak can clearly be seen on the graph, with the corresponding Western blot showing a loss of the upper band (Mr 50,000).


100
75
50
25
0
% Septation
0
50
100
150
200
Time (Minutes)



fractionation conditions employed in this study preserved actin in its (presumably native) particulate state, presumably the 'dots' or 'patches' associated with the growing tips of the cell. If this is true, it should be possible to isolate actin in this form and identify those proteins associated with it *in vivo*. Protein extracts of the mutants *A16a* and *cdc25-22h*$^{-}$ when separated by one dimensional gel electrophoresis, each contained bands which comigrated with those in the wild type extract (compare lanes A, B and C, Fig. 3.2). Two-dimensional gel electrophoresis of the *cdc25-22* pellet fraction resolved four spots that reacted with the actin antibody; two major spots at Mr 50,000 having pI's of 5.45 and 5.92 respectively, and two minor spots, one of Mr 50,000 with a pI of 5.53 and one of Mr 48,000 with a pI of 5.4. Scordilis et al., (1991) identified two actin isoforms in *S.pombe*; a form that was capable of polymerisation (Mr= 46,800; pI= 5.6) and a form that was not (Mr= 47,800; pI= 5.2). Neither of these values compare precisely with that expected from a 'typical' actin which has a Mr of 42,000 and a pI of 5.2. Neither do they compare with that of the product of the *act2*$^{+}$ gene, Arp3 (Lees-Miller et al., 1992) which has a calculated Mr of 47,376 and a pI of 5.7.

Given the relatively low levels of sequence identity between *act1*$^{+}$ and *act2*$^{+}$ (35-40%) and the estimated low abundance of the Act2 (Arp3) protein (Lees-Miller et al., 1992), it is unlikely that any of the four spots represents this species. To attempt to address this point further, we examined a strain, *A16a*, carrying an additional copy of the *act1*$^{+}$ gene (Schroeder-Lorenz et al., 1987). The relationship would be altered in *A16a*. In the event, the actin pattern from this strain was very much as in

wild type although this point needs further examination using two-dimensional gel electrophoresis and a strain in which the *act2*$^{+}$ gene is deleted. More likely, the different forms of actin resolved here represent post-translational modifications as have previously been described in *Dictyostelium* (Schweiger et al., 1992). Tyrosine phosphorylation of actin in *Dictyostelium* has recently been implicated in reversible rearrangements in the actin cytoskeleton through the cell cycle (Jungbluth et al., 1994). Our attempts to determine directly whether *S.pombe* actin was phosphorylated were unsuccessful. However, the fact that the changes we observe are cell cycle dependent, makes this explanation a likely one. Synchronised cells passing through the G2/M boundary of the cell cycle, a point at which actin undergoes a dramatic reorganisation, was accompanied by a major reduction of the intensity of the Mr 50,000 species, consistent with it being dephosphorylated at this stage of the cell cycle.

CHAPTER 4

ISOLATION AND ANALYSIS OF REVERTANTS OF THE COLD SENSITIVE, β-TUBULIN MUTANT *nda3*-KM311

4.1: Introduction

The fission yeast *Schizosaccharomyces pombe* has a cytoskeleton composed of microtubules and microfilaments plus their associated proteins (Chapter 1). Microtubules are the polymerisation products of two different tubulin subunits denoted α and β. In *S.pombe* and the distantly related yeast *S.cerevisiae,* there are three tubulin genes, and approximately 70% homology is shared between the same genes in each organism (Yanagida, 1987). The two α-tubulin genes in *S.pombe* are, *nda2*$^+$ (Toda et al., 1984) and *atb2*$^+$ (Hirano and Yanagida, 1988) and in *S.cerevisiae TUB1* and *TUB3* (Schatz et al., 1986a & b). The single β-tubulin gene is designated *nda3*$^+$ in *S.pombe* (Toda, et al., 1983) and *TUB2* in *S.cerevisiae* (Neff, et al., 1983). The *S.pombe nda2*$^+$ gene has been sequenced, (Hirano and Yanagida, 1988) and contains a single 89 base pair intron at codon 18 and encodes 455 amino acids which gives the protein a molecular mass of 51,200. The *atb2*$^+$ gene has also been sequenced (Hirano and Yanagida, 1988). It has no introns and encodes 449 amino acids, resulting in a protein having a predicted molecular mass of 50,600. The two α-tubulin genes in *S.pombe* share 89% homology

(Yanagida, 1987). The *nda3*$^+$ gene encodes 448 amino acids and has five short introns that result in a protein with a molecular mass of 49,400. Only *nda2*$^+$ and *nda3*$^+$ are essential genes in *S.pombe* (Yanagida, 1987).

The second α-tubulin gene (*atb2*$^+$) seems to be non-essential for normal growth and conjugation (Adachi et al,. 1986). A similar situation exists in *S.cerevisiae* (Schatz et al., 1986; 1987). Gel electrophoresis has been used to confirm the presence of three tubulin subunits in *S.pombe* (Alfa and Hyams, 1990).

The third member of the tubulin superfamily, γ-tubulin, is encoded by the *gtb1*$^+$ gene in *S.pombe* and the *TUB4* gene in *S.cerevisiae*. γ-tubulin is located soley at the MTOC's (Horio et al., 1991; Stearns et al., 1991) and in *S.pombe* this localisation is independent of microtubules (Stearns et al., 1991). *gtb1*$^+$ is an essential gene that contains six short introns. It encodes a protein with a predicted amino acid length of 446, resulting in a relative molecular mass of 49,912 (Stearns et al., 1991, Horio et al., 1991). The human γ-tubulin gene can substitute for the endogenous *S.pombe* gene, demonstrating the conservation of γ-tubulins through evolution (Horio and Oakley, 1994).

There is little information on the interactions of yeast tubulin with other cellular proteins. Two different biochemical approaches have been utilised to identify proteins that interact with microtubules: firstly biochemical analysis of proteins which co-purify with tubulin through cycles of *in-vitro* assembly-disassembly (Olmsted and Borisy, 1975), secondly, identification of proteins that bind to taxol stabilised, MAP-

depleted brain tubulin (Vallee, 1982) *In-vitro* assembly has been successfully carried out in *S.cerevisiae* (Kilmartin, 1981, Barnes et al., 1992, Davies et al., 1993), but not yet in *S.pombe*. Taxol has no effect on yeast tubulin and attempts to bind putative yeast MAPs to taxol-stabilised, MAP-free bovine microtubules have been unsuccessful (Alfa, 1989). An alternative strategy that is available in a genetically manipulable organism such as a yeast, is suppressor analysis (Jarvik and Botstein, 1975) and unlinked noncomplementation (Stearns and Botstein, 1988). These approaches have been successfully carried out in *S.cerevisiae* to identify proteins that interact with subunits of tubulin (Pasqualone and Huffaker, 1994) and novel conditional lethal mutations, in each of the tubulin genes (Stearns and Botstein, 1988).

We have selected for extragenic suppressors that revert the cold-sensitive phenotype of the β-tubulin gene *nda3*-KM311. If such extragenic mutations could be identified, it is likely that they will encode proteins that interact with, or replace in function, the mutated protein.

4.2: Materials and Methods

4.2.i: Strains used

Wild type *S.pombe* , *972h*$^{-}$ and *975h*$^{+}$, and both mating types of the cold sensitive β-tubulin mutant *nda3* -KM311(Toda et al., 1983).

4.2.ii: Medium used

Yeast extract (YE) medium was prepared as described in Chapter 2. Yeast extract peptone (YEP) medium was YE medium containing 20g l^{-1} of bacto-peptone. Malt extract medium (ME) contained 5g l^{-1} of malt extract (Difco) and 30 g l^{-1} of D-glucose. Agar containing TBZ was prepared by adding the correct amount of TBZ stock (10mg ml^{-1} in DMSO) to growth medium following autoclaving, but just before the agar set.

4.2.iii: Mutagenesis

10ml of*nda3h*$^{+}$ cells were grown in YE medium to a density of 1 x 10^{7} cells ml^{-1}, and 200μl of the chemical mutagen ethylmethanesulphonate (EMS, Sigma) added. Aliquots of cells were removed at hourly intervals, washed in fresh medium and plated in duplicate onto YE .

4.2.iv: Selection of EMS-induced revertants

Mutagenised cells were plated onto YE agar and incubated at 20°C (the non-permissive temperature for *nda3*). Colonies were counted, picked and re-patched onto fresh YE plates to be reselected at 20°C. Ten of the 502 recoverable revertants were selected at random using a random number generator (Casio). These were then retested for growth at 20, 30 and 36°C. Ten randomly selected revertants (numbers 81, 86, 173, 194, 211, 216, 271, 307, 386 and 497) were also tested for sensitivity to TBZ at 20, 30 and 36°C on solid media. Cells were streaked onto plates containing different concentrations of TBZ : 0; 5; 10; 20; 35 and 50μg ml^{-1} and placed at 20, 30 and 36°C and allowed to grow for 4 to 10 days, (cells at lower temperatures require longer to grow). These ten revertants were also tested for TBZ sensitivity at 20, 30 and 36°C in liquid medium. Cells were inoculated from a stationary culture (1 x 10^7 cells ml^{-1}) into 50ml of prewarmed YEP media to a final concentration of $1x10^6$ cells ml^{-1}. 15μl samples were removed at 30 minute intervals and counted using a haemocytometer and an Olympus CH3 microscope with a 40x phase contrast objective lens.

4.2.v: Selection of spontaneous revertants

nda3h$^+$ and *h*$^-$ cells were grown overnight at 30°C to a final cell density of approx. $5x10^6$ cells ml^{-1}. Approximately 1.4 x 10^7 *nda3h*$^+$ and

$4.85x10^7$ *h*$^-$ cells were spread onto YEP plates and incubated at 20°C for 10 days. Colony counts were then performed.

4.2.vi: Random spore analysis

All of the ten EMS generated revertants were mixed with wild type of the opposite mating type and a little sterile distilled water on ME plates and allowed to form asci at 27°C. The presence of asci was checked visually using phase contrast optics. Asci and dead cells were scraped from the plates with a sterile inoculating loop and resuspended in 1ml of sterile distilled water. The asci/water mixture was gently vortexed to distribute the asci evenly, 50µl of glucuronidase added and incubated overnight at 37°C to digest the asci walls and kill any vegetative cells. A drop of the resulting spore suspension was aseptically removed and phase contrast optics used to confirm the absence of vegetative cells that may have survived the glucuronidase treatment. Spores were counted with the aid of a haemocytometer and approximately 200 spores per plate were spread onto YEP with a glass spreader. Plates were sealed with Nescofilm and incubated at 30°C for 5 days. These plates were subsequently replica plated onto YEP and incubated at 20°C for 10 days. Pairs of plates incubated at the different temperatures (30 and 20°C) were then aligned and viewed with the aid of a light box to check for colonies that had grown on the plate incubated at 30°C, but not on the corresponding plate incubated at 20°C.

4.2.vii: Tetrad analysis

Cells of opposite mating types were mixed together in a little distilled water on ME plates. Cells were allowed to conjugate at 26°C for one- two days. Conjugation and meiosis were judged to have occurred by phase contrast microscopy. A loop of the conjugation mixture was diluted in 1ml of distilled water and inoculated onto a YE plate. The tetrads were then dissected using a Singer MSM system micromanipulator. Spores were allowed to grow at 30°C and then replica plated to YE plates to be incubated at 20 and 36°C. The number of spores able to grow at 20 and 36°C were recorded (Table4.1), only tetrads that produced 4 viable spores at 30°C were included in these counts.

4.3: Results

Chemical mutagenesis resulted in 552 colonies that were able to grow at the restrictive temperature for *nda3*-KM311 (20°C), giving a mutation rate of approx. 1.8×10^{-5}. Only 502 of these were recoverable after storage at -70°C and each was assigned a number. Spontaneous mutation resulted in three h^+ colonies that were able to form colonies at 20°C, giving a mutation rate of approx. 4.6×10^{-6}. These were assigned the numbers 503; 504 and 505. The latter three isolates, plus ten chosen randomly from the EMS experiments, were selected for further study.

All 13 strains proved to be cs^+ (growth at 20°C) following re-isolation from single cells. Three strains (81, 211 and 386) failed to grow at 36°C, indicating that reversion of the cs^- phenotype also resulted in the appearance of temperature sensitivity. Tetrads dissected from backcrossing these strains to wild type are shown in Tables 4.1 and 4.2. Crosses between all of the revertants to wild type yielded asci, in the majority of cases, containing the full complement of four spores. The exceptions were revertant numbers 81 and 86 which yielded few complete tetrads and therefore it was possible to analyse only a limited number of these crosses. In all cases, in both the EMS-induced and spontaneous revertants, crosses to wild type failed to segregate cold-sensitive progeny, that is, all four progeny were cs^+ (Table 4.1). The most likely explanation for this result is that all 13 revertants were intragenic. However, to eliminate the possibility of an extragenic, but tightly linked mutation, random spore analysis was performed. Again, no cold sensitive colonies were recovered from approximately 1000 spores analysed for each of the ten EMS-induced or the three spontaneous revertant/wild type crosses. In isolates 81, 173, 211 and 386, crossing to wild type produced 2:2 segregation of the ts^- phenotype. The cold sensitive phenotype of *nda3h*$^+$ segregated 2:2 when crossed to wild type ($972h^-$) for all 11 tetrads dissected.

Table 4.1 Genetic characterisation of EMS generated revertants

Strain:	Phenotype:	Incomplete tetrads:	Segregation of temperature-sensitive phenotype: ts^- : ts^+	Segregation of cold-sensitive phenotype: cs^-: cs^+
81	ts- cs+	Many	2:2 (5)	0:4 (5)
86	ts+ cs+	Many		0:4 (7)
173	ts- cs+	Few	2:2 (26)	0:4 (26)
194	ts+ cs+	Few		0:4 (17)
211	ts- cs+	Few	2:2 (28)	0:4 (28)
216	ts+ cs+	Few		0:4 (15)
271	ts+ cs+	Few		0:4 (12)
307	ts+ cs+	Few		0:4 (15)
386	ts- cs+	Few	2:2 (17)	0:4 (17)
497	ts+ cs+	Few		0:4 (11)

N.B. The figures in brackets denote the number of tetrads dissected.

Table 4.2 Genetic characterisation of spontaneous revertants

Revertant number:	Phenotype:	Incomplete tetrads:	Segregation of cold-sensitive phenotype: cs^-: cs^+
503	ts+ cs+	Few	0:4 (11)
504	ts+ cs+	Few	0:4 (14)
505	ts+ cs+	Few	0:4 (13)

N.B. The figures in brackets denote the numbers of tetrads dissected.

4.3.i: Sensitivity of revertants to TBZ on solid medium

We examined whether reversion of the cold-sensitive phenotype of *nda3*-KM311 was accompanied by a change in sensitivity to the anti-microtubule drug TBZ. Wild type cells formed colonies up to 10 μg ml^{-1} TBZ at 20°C, rising to 20μg ml^{-1} at 30°C and 36°C (Table 4.3). *nda3*-KM311 cells formed colonies up to 10μg ml^{-1} at 30°C rising to 50μg ml^{-1}, the highest concentration tested, at 36°C. Revertant number 86 exhibited resistance to 50μg ml^{-1} TBZ at all temperatures tested. However, revertant number 81 showed increased resistance to TBZ at 20 and 30°C (35μg ml^{-1}). This strain is temperature-sensitive for growth at 36°C. Revertant number 386 exhibited sensitivity to TBZ at 20 and 30°C, being unable to form colonies on plates containing 10μg ml^{-1}. Revertant 386 was also temperature sensitive and so could not form colonies at 36°C. All revertants, except numbers 216 and 386 exhibited increased resistance to TBZ at 30°C when compared to the strain they were derived from, that is *nda3*-KM311. All revertants, except number 386 exhibited increased resistance to TBZ at 20°C when compared to wild type cells (Table 4.3).

Table 4.3 Sensitivity of *nda3* revertants to TBZ on solid medium

Strains:	TBZ concentration (μg ml^{-1})					
	0	5	10	20	35	50
(20°C)						
wild type (972h$^-$)	+	+	+	-	-	-
nda3h$^+$	-	-	-	-	-	-
81	+	+	+	+	+	-
86	+	+	+	+	+	+
173	+	+	+	+	-	-
194	+	+	-	-	-	-
211	+	+	+	+	-	-
216	+	+	-	-	-	-
271	+	+	+	+	+	-
307	+	+	+	+	-	-
386	+	+	-	-	-	-
497	+	+	+	-	-	-
(30°C)						
wild type (972h$^-$)	+	+	+	+	-	-
nda3h$^+$	+	+	+	-	-	-
81	+	+	+	+	+	-
86	+	+	+	+	+	+
173	+	+	+	+	-	-
194	+	+	+	+	+	-
211	+	+	+	+	-	-
216	+	+	+	-	-	-
271	+	+	+	+	+	-
307	+	+	+	+	+	+
386	+	+	-	-	-	-
497	+	+	+	+	+	+
(36°C)						
wild type (972h$^-$)	+	+	+	+	-	-
nda3h$^+$	+	+	+	+	+	+
81	-	-	-	-	-	-
86	+	+	+	+	+	+
173	-	-	-	-	-	-
194	+	+	+	+	+	+
211	-	-	-	-	-	-
216	+	+	+	+	-	-
271	+	+	+	-	-	-
307	+	+	+	+	+	+
386	-	-	-	-	-	-
497	+	+	+	+	+	+

NB. + denotes growth and - denotes no growth.

4.3.ii: Sensitivity of revertants to TBZ in liquid medium

Cells inoculated into liquid medium clumped badly and thus were impossible to be count. No results were obtained concerning the sensitivity of cells in liquid medium.

4.4: Discussion

The isolation of allele-specific extragenic suppressors of mutant genes, is a powerful tool in yeast genetics (Huffaker et al., 1987). We have generated 502 revertants of the cold sensitive β-tubulin mutant *nda3*-KM311 by chemical mutagenesis, plus 3 spontaneous revertants of this mutant. Of the ten of the chemically-generated revertants tested, four were also temperature sensitive for growth. The temperature sensitive phenotype segregated 2:2 in the crosses to wild type cells (Table 4.1). By contrast, the cold sensitive phenotype of the original mutant *nda3*-KM311 did not segregate (Table 4.1). In both tetrad analysis and free spore analysis no cold sensitive segregants were recovered (Table 4.1). This suggests that all the mutations were intragenic. Thus the reversion of cold sensitivity and the acquisition of temperature sensitivity in isolates 81, 173, 211 and 386 appear to be the results of different genetic events. The mutant *ben1* isolated on the basis of its resistance to the fungicide benomyl (Yamamoto, 1980), was subsequently shown to be allelic to *nda3* (Toda et al., 1983). All 13 revertants were tested for sensitivity to a range of concentrations of TBZ in solid media. As controls, the sensitivity of the wild type (*972h-*) and the original mutant (*nda3*-KM311) strains were

also tested. In wild type cells, TBZ sensitivity was temperature dependent; cells were able to form colonies on plates containing 10μg ml^{-1} TBZ at 20°C, but this increased to 20μg ml^{-1} at 36°C. This is an expected consequence of the cold-lability of microtubules. Plates containing 35μg ml^{-1} TBZ that were incubated for several days longer showed colony growth, probably due to spontaneous mutants that had become resistant to TBZ over time. The sensitivity values for wild type cells over the range of temperatures tested is in accordance with those published by other workers (Yamamoto, 1980; Umensono et al., 1983; Toda et al., 1983). *nda3* -KM311 was resistant to high concentrations (50μg ml^{-1}) of TBZ at 36°C, but was more sensitive at 30°C (Table 4.3). This is contrary to work by Toda and co-workers (1983) where *nda3*-KM311 was reported to be unable to grow on plates containing 50μg ml^{-1} of TBZ at 37°C. This discrepancy is possibly due to differences in the growth media used.

These studies have therefore failed to identify an extragenic suppressor for a mutation in the fission yeast β-tubulin gene. It would be interesting to repeat this analysis with another cold-sensitive allele of *nda3* , were such available. An alternative strategy might be to isolate suppressors of *ben1*, or to directly isolate mutants having altered sensitivity to TBZ and related drugs. However, the latter approach need not lead to the identity of microtubule-related genes. For instance, isolation of methylbenzyl-carbamylate (MBC) sensitive mutants has yielded the gene for the fission yeast 26S protease subunit (Gordon et al., 1993) and a gene isolated on its supersensitvity to isopropyl N-3-chlorophenyl carbamate (CIPC) revealed

no homology to any other when database searching was undertaken (Ishiguro et al., 1993).

CHAPTER 5

SUMMARY

This thesis represents the first study of the cytoskeleton incorporating three distinct approaches: 1) Cytology, using immunofluorescence microscopy combined with *in vivo* depolymerisation/repolymerisation of microtubules; 2) Biochemistry, using one and two-dimensional separation of proteins with subsequent specific staining by Western blotting and a monoclonal actin antibody and 3) Genetics, using extragenic suppressor analysis, to investigate proteins that co-opt with the β-tubulin subunit of microtubules.

5.1 Summary of findings

We have described the depolymerisation of microtubules in the fission yeasts *S.pombe* and *S.japonicus var versatilis* by cold treatment (1°C for 40 min) and the subsequent repolymerisation of the microtubules, which commences in 2 min on subsequent return of the cells to 25°C. During this procedure we have described the disarray of the actin cytoskeleton in a proportion of cells after low temperature treatment. Actin returns with faster kinetics than that displayed by microtubules. A minority of cells of the cell cycle mutants *cdc10-129* and *cdc25-22*, treated with low temperature in the above way, formed actin rings with an aberrant location, that is, twisted through 90° in the longitudinal plane of the cell. Wild type cells also displayed these actin rings but at a much lower

frequency (1% in wild type compared to 10% in *cdc10-129* and *cdc25-22* cells).

We have described two species of actin in wild type, *A16a* (double actin containing) and *cdc25-22* mutants, when separated by one-dimensional gel electrophoresis with subsequent Western blotting probed with a monoclonal anti-actin antibody and further describe changes in levels of actin throughout the cell cycle using the cell cycle mutant *cdc25-22* which has been previously blocked at the G2 / M boundary. We describe the possibility of chemically modified forms of actin which appear as four spots on two-dimensional gels of *S.pombe* cytosolic protein extracts, when probed with a monoclonal anti-actin antibody.

By the use of EMS mutagenesis we have isolated 552 revertants of the cold sensitive β-tubulin encoding gene (*nda3*-KM311). Spontaneous mutagenesis of the mutant *nda3*-KM311 yielded a further three revertants of the *nda3* gene. Upon further characterisation of ten randomly selected EMS induced revertants and the three spontaneous revertants, we found that the original cold sensitive phenotype of the *nda3*-KM311 mutant could not be recovered. This limited our study of these revertants as they were all highly likely to be intragenic reversions of the original *nda3* mutation.

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