Journal of Cell Science 110, 157-168 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
JCS4352

γ-Tubulin in trypanosomes: molecular characterisation and localisation to multiple and diverse microtubule organising centres

V. Scott, T. Sherwin and K. Gull

School of Biological Sciences, University of Manchester, 2.205, Stopford Building, Oxford Road, Manchester, M13 9PT, UK

*Author for correspondence

SUMMARY

A genomic clone from *Trypanosoma brucei*, which contains a full length γ-tubulin gene, was isolated using degenerate oligonucleotide primers. The sequence of this clone predicts a protein of 447 amino acids having a high degree of homology with γ-tubulins from human and *Xenopus laevis* (67.2% amino acid identity) and only 57.7% identity with the *Plasmodium falciparum* γ-tubulin. Northern blot analysis of poly(A)$^+$ selected RNA from a procyclic culture detects a major transcript of approximately 2.2 kb plus a minor transcript of approximately 3.6 kb.

A fusion protein comprising almost the full length γ-tubulin gene product (amino acids 8-447) plus an amino-terminal histidine tag has been expressed and purified from *Escherichia coli* and used to raise a polyclonal antibody. Immunofluorescence, using this antibody, shows classical centrosomal localisation in mammalian cells. In *T. brucei* γ-tubulin is present in the basal bodies which subtend the flagellum and also at the anterior tip of the cell body where many minus ends of microtubules are located. Furthermore the antibody reveals a small subset of the sub-pellicular microtubules and a discrete dot within the nucleus which alters form with progression through the mitotic cycle. Evidence is also presented for discrete punctate staining within the microtubules of the cell body which may represent the presence of γ-tubulin on the ends of individual microtubules. Our results indicate that γ-tubulin is associated with diverse microtubule organising centres and structures in trypanosomes.

Key words: γ-Tubulin, Trypanosome, Cytoskeleton, MTOC

INTRODUCTION

The microtubule cytoskeleton plays a central role in cellular function both during interphase when it is crucial for maintaining shape, polarity and organelle distribution within the cell, and during mitosis when it is essential for correct chromosome segregation. The microtubule organising centres (MTOC) although morphologically diverse exhibit a conserved function in the orchestration of microtubule arrays and regulation of microtubule assembly. MTOCs (Pickett-Heaps, 1969) range from the structurally well characterised mammalian centrosome and fungal spindle pole body (SPB) to the less well defined acentriolar MTOC of the early mouse embryo. The most recently discovered third member of the tubulin superfamily, γ-tubulin (Oakley and Oakley, 1989) is now widely accepted as a marker of MTOCs (Oakley et al., 1990; Stearns et al., 1991; Horio et al., 1991; Gueth-Hallonet et al., 1993; Muresan et al., 1993). γ-Tubulins are nearly as similar to α- and β-tubulins (homologies of around 0.34) as α- and β-tubulin are to each other (homologies of around 0.43) (Burns, 1995a). However, unlike the abundant α- and β-tubulins which are the basic subunits of microtubules, γ-tubulin shows a much more restricted cellular distribution and lower abundance, estimated to be present at less than 1% the level of α- and β-tubulin (Stearns et al., 1991). γ-Tubulin was originally discovered in the filamentous fungus *Aspergillus nidulans* as the product of the *mip*A locus (Oakley and Oakley, 1989), of which mutant alleles were identified as extragenic suppressors of a heat-sensitive mutation in a β-tubulin gene.

γ-Tubulin has been shown to be an essential gene in studies of *A. nidulans* and *S. pombe*. It is a component of the spindle pole body, both during interphase and mitosis, and is required for microtubule function and nuclear division (Oakley et al., 1990; Horio et al., 1991). Mutant phenotypes in *A. nidulans* have been shown to arrest with fewer cytoplasmic microtubules than wild-type cells and virtually no mitotic apparatus (Oakley et al., 1990).

Since its discovery genes encoding γ-tubulin have been identified in a wide range of eukaryotes, from fungi, insects and mammalian cells to plants and protozoa, including *Plasmodium falciparum* and *Euplotes octocarinatus* (Stearns et al., 1991; Zheng et al., 1991; Horio et al., 1991; Fuchs et al., 1993; Maessen et al., 1993; Liang and Heckmann, 1993). The deduced amino acid sequences suggest that most γ-tubulin gene-products are highly conserved, exhibiting 57-67% amino acid identity. This conservation has recently been demonstrated experimentally by the functional complementation of a *Schizosaccharomyces pombe* γ-tubulin mutant transformed with a human (HeLa) γ-tubulin cDNA (Horio and Oakley, 1994).

The localisation of γ-tubulin to the MTOC in a wide variety of organisms where it is thought to bind the minus end of microtubules, has led to its postulated role in microtubule nucleation. Stearns et al. (1991) localised γ-tubulin to the peri-

centriolar material of the mammalian centrosome, the site of microtubule nucleation. Microinjection studies of mammalian cells with an antibody raised against γ-tubulin have provided in vivo evidence for a role for γ-tubulin in microtubule nucleation (Joshi et al., 1992). Cells subject to injection either just before or during mitosis are unable to assemble a functional spindle, while interphase cells subject to cold depolymerisation of their microtubules fail to reassemble their microtubule arrays in the presence of antibodies raised against γ-tubulin. Recent experiments attempting to reconstruct centrosome assembly in vitro (Felix et al., 1994; Stearns and Kirschner, 1994) have indicated that localisation of γ-tubulin to the centrosome is a pre-requisite for microtubule nucleation. γ-Tubulin is known to be present in animal cells as part of a large complex (Stearns and Kirschner, 1994). Also, in such animal cells there is evidence that within the centrosome γ-tubulin localises to ring structures at the base of nucleated microtubules (Moritz et al., 1995). However, it appears that localisation of γ-tubulin alone is not sufficient for MTOC-activity as the interphase SPB of *S. pombe* shows γ-tubulin staining by immunofluorescence localisation yet does not nucleate the cytoplasmic microtubule array (Masuda et al., 1992). Sunkel et al. (1995) have suggested that γ-tubulin may be required for both the structural organisation and function of MTOCs in *Drosophila* based on phenotypic studies of a mutant allele of the γ-tubulin gene. More recently, studies of the more divergent γ-tubulin Tub4 protein of *Saccharomyces cerevisiae* have shown that it is located at both cytoplasmic and nuclear faces of the spindle pole body (Sobel and Snyder, 1995; Spang et al., 1996; Marschall et al., 1996).

As γ-tubulin appears to be a marker for the MTOC in diverse eukaryotes we were interested to investigate possible homologues in the evolutionarily ancient organism *Trypanosoma brucei*. Procyclic cells of the African trypanosome comprise four major microtubule arrays; the sub-pellicular cortical microtubule array, the flagellar axoneme, basal body and the intranuclear mitotic spindle. The sub-pellicular corset of microtubules has been well characterised at the electron microscopy (EM) level and is present throughout the cell cycle (Sherwin and Gull, 1989a). Prior to cell division new microtubules must be inserted into the existing sub-pellicular microtubule array and basal body duplication in conjunction with the generation of a new flagellum must occur to ensure the correct distribution of microtubule structures to the daughter cells (Robinson et al., 1995). Little is known about the MTOCs responsible for orchestrating this cytoskeletal reorganisation. The mature basal body is known to subtend the flagellum which contains a classical 9 + 2 microtubule axoneme, however, the putative MTOCs for the intranuclear mitotic spindle and the sub-pellicular corset of cytoplasmic microtubules remain to be characterised. No discrete structure has been observed upon EM that might be correlated with these presumed sites of microtubule nucleation, therefore it is interesting to consider whether proteins which localise to the MTOCs of other eukaryotes, such as γ-tubulin, may exhibit a similar distribution in the trypanosome and provide an insight into the nature and distribution of the MTOCs of this organism. The α- and β-tubulin proteins are extremely abundant in *T. brucei* and have been shown to be encoded by long tandem repeats of alternating α- and β-tubulin genes (Seebeck et al., 1983; Thomashow et al., 1983) which are transcribed as a single polycistronic unit (Imboden et al., 1987). Whether or not a γ-tubulin gene is also present at this locus or an independent locus within the *T. brucei* genome is unknown. In an attempt to address some of the questions relating to the organisation of the microtubule cytoskeleton in the trypanosome we have cloned and sequenced the γ-tubulin gene from *T. brucei brucei* and examined the distribution of γ-tubulin within the cell.

MATERIALS AND METHODS

Culturing of *T. brucei* procyclic cells

Cells were maintained in SDM79 medium as described by Brun and Schönenberger (1979).

PCR cloning of a partial *T. brucei* γ-tubulin sequence

Standard molecular biology protocols were carried out according to the method of Sambrook et al. (1989) unless indicated otherwise.

Degenerate polymerase chain reaction (PCR) primers derived from conserved regions of the known γ-tubulin sequences were provided by B. Oakley. The sense primer spanned amino acids 48 to 53 (KDVFFY) of the *Aspergillus* γ-tubulin, which are conserved only in γ-tubulin sequences whereas the antisense primer spanned amino acids 143 to 148 (GGTGSG) of the *Aspergillus* γ-tubulin, a region which is highly conserved in all tubulin sequences, α, β and γ. Each oligonucleotide included a restriction site at its 5′ end to facilitate cloning. 100 ng of genomic DNA from T. *brucei rhodesiense* and *S. pombe* were used as templates for the PCR. Following digestion with the appropriate restriction enzymes the major PCR product was eluted from a 4% non-denaturing polyacrylamide gel and cloned into the pBluescript vector (Stratagene). PCR fragments were sequenced from double-stranded plasmid DNA by the dideoxy chain termination method (Sanger et al., 1977) using Sequenase DNA polymerase (USB).

Isolation of a genomic *T. brucei* γ-tubulin clone

The *T. brucei* PCR fragment was radioactively labelled using the random hexanucleotide priming method of Feinberg and Vogelstein (1983) and used to screen an amplified genomic library from *T. brucei brucei* stock STIB 366 in the lambda EMBL4 vector (Schlaeppi et al., 1989). Three positive clones were obtained and analysed by restriction digestion and Southern blotting, using the original PCR fragment as probe. All three clones contained an approximately 6 kb *Sal*I fragment which was expected to contain the full-length γ-tubulin gene.

Nucleotide sequencing of the γ-tubulin gene

The full-length coding sequence of the *T. brucei brucei* γ-tubulin gene was determined by nucleotide sequencing of subclones of the 6 kb *Sal*I fragment in pBluescript as above. The extreme 5′ end of the sequence was obtained from a fragment cloned by RT-PCR. The PCR was carried out using first strand cDNA as template, with a 5′ splice leader primer and a pair of nested γ-tubulin primers. 30 rounds of PCR were carried out using the 5′ splice leader primer in conjunction with a *T. brucei* specific version of the original degenerate GGTGSG-primer. A tenth volume of this PCR was used as template for a further 30 rounds of amplification using the splice leader primer with a second *T. brucei* γ-tubulin-specific primer, which spanned amino acids 32 to 38 (RHDGVVE) of the *T. brucei* sequence. The *T. brucei* γ-tubulin gene was sequenced on both strands.

Northern blot hybridisation

Total RNA was prepared from 250 ml of a mid-log culture of *T. brucei* procyclic cells using the acid-phenol method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ selection was carried out using the polyA-tract mRNA isolation system (Promega). The entire poly(A)$^+$ fraction isolated from 1.3 mg total RNA was loaded onto a 1.2% formalde-

hyde gel and blotted onto Magnacharge membrane (MSI). Hybridisation was carried out in 50% formamide with a [^{32}P]UTP-labelled riboprobe derived from the original 310 bp γ-tubulin PCR fragment and the filter was washed in 1% SDS, 0.05 M $NaPO_4$, pH 7.2, at 55°C.

Expression of a *T. brucei* γ-tubulin fusion protein and antibody production

A 2.7 kb *Pst*I fragment encompassing all but the first 23 nucleotides of the coding sequence, plus endogenous stop codon and 3′ untranslated region of the *T. brucei* γ-tubulin gene was cloned in-frame into the *Pst*I site of the *E. coli* expression vector pTrchis (Invitrogen Corporation) to generate a fusion protein comprising a six histidine residue tag at the amino terminus and amino acids 8 to 447 of *T. brucei* γ-tubulin at the carboxy terminus. The expressed fusion protein was purified over a nickel column under denaturing conditions and eluted at pH 4.0 in the presence of 8 M urea. Following dialysis to remove the urea the insoluble fusion protein precipitated out and was recovered by centrifugation. The insoluble fraction was subject to SDS-PAGE (Laemmli, 1970), blotted onto nitrocellulose and ground-up in 200 μl PBS for use as an immunogen. Two rats received 5 injections of γ-tubulin fusion protein and the immune serum of one rat was used in all subsequent experiments.

Affinity selection of polyclonal antibody and immunoprobing of western blots

T. brucei γ-tubulin fusion protein was expressed in *E. coli* as previously described. A purified fraction of this was separated by 1-D-PAGE, transferred to nitrocellulose and the γ-tubulin band excised. The nitrocellulose strip was blocked overnight at 4°C in 5% (w/v) milk powder in PBS. The strip was then incubated for 3 hours at room temperature in 1 ml of the *T. brucei* γ-tubulin polyclonal antibody at a 1:10 dilution in PBS. After washing four times in PBS, bound antibody was eluted with 1 ml of 0.2 M glycine/1 mM EGTA, pH 2.5, for 10 minutes at room temperature with gentle agitation. The liquid was then decanted and neutralised with 1 M Tris-HCl, pH 8.2. BSA was added to a final concentration of 100 μg/ml, and the final solution dialysed against two changes of PBS at 4°C. The resulting affinity purified antibody solution was used for probing immunoblots as described by Sherwin et al. (1987).

Immunofluorescence of mammalian cells

Vero cells were cultured overnight on sterile glass coverslips, washed in PBS then fixed in –20°C methanol. Cells were rehydrated in PBS for 20 minutes at room temperature. The rat polyclonal antiserum raised against the *T. brucei* γ-tubulin fusion protein was diluted 1/50 in PBS for use on Vero cells and all washes were carried out in PBS-T (PBS plus 0.1% Tween-20). The second antibody was an anti-rat fluorescein isothiocyanate (FITC)-conjugate raised in rabbit (Dako). DNA was visualised using the DNA intercalating dye 4,6-diamidino-2-phenylindole (DAPI) and slides were mounted in Mowiol 4-88 containing 1 mg/ml *p*-phenylenediamine as antifade agent.

Immunofluorescence of *T. brucei* cytoskeletons

T. brucei cells were harvested in mid-log phase by centrifugation. The pellet was resuspended in half the original volume of 1% Nonidet P40 (Sigma) (v/v) in PEM (0.1 M Pipes, 2 mM EGTA, 1 mM $MgSO_4$). After incubation on ice for 5 minutes the cytoskeletons were harvested by centrifugation and resuspended in PEM alone. The cytoskeletons were settled onto poly-L-lysine coated slides and fixed in –20°C methanol for 20 minutes. Further processing for immunofluorescence was done essentially as described by Sherwin et al. (1987).

Capturing of digital images

Immunofluorescence was observed on a Leica DMRBE microscope and images were captured by a Hammamatsu CCD camera (trypanosomes) or a Hammamatsu SIT camera (mammalian cells) using NIH Image Macintosh based software (Lange et al., 1995).

Preparation of flagella and stable quadruplet of sub-pellicular microtubules

Cytoskeletons were prepared in solution, as described previously for immunofluorescence. The pellet of cytoskeletons was then resuspended in 1% NP40 in PEM containing 1 M NaCl and incubated on ice for 20 minutes. This preparation was then pelleted by centrifugation and resuspended in PEM alone.

Preparation of cells for thin section transmission EM (TEM)

Cells were prepared for thin section TEM as described by Sherwin and Gull (1989a).

Whole mount negative stain TEM

Flagella preparations were processed for whole mount negative stain TEM as described by Sherwin and Gull (1989a).

Immunogold labelling procedure

T. brucei cytoskeletons or flagella preparations were settled onto charged carbon coated Formvar grids and then processed according to the method of Sherwin and Gull (1989b).

RESULTS

Identification of the trypanosome γ-tubulin gene

Examination of existing published γ-tubulin sequences revealed two likely areas of sequence conservation suggesting a route for PCR cloning of a putative trypanosome γ-tubulin (Zheng et al., 1991; Stearns et al., 1991). The degenerate sense primer spanned amino acids 48 to 53 (KDVFFY) of the original *Aspergillus* γ-tubulin, which appear specific to γ-tubulin. The degenerate antisense primer spanned region 143 to 148 (GGTGSG) which appears to be highly conserved in all tubulin sequences. PCR amplification of trypanosome genomic DNA using these primers resulted in a clear single band of around 300 bp. These primers and conditions also produced a fragment of the expected size of approximately 400 bp when used with *S. pombe* DNA as a control (Horio et al., 1991), the larger size of the *S. pombe* fragment being explained by the presence of two introns within this region. The trypanosome PCR product was cloned and sequenced. The resulting sequence showed an excellent homology with existing γ-tubulin genes. This PCR product was then used to screen a genomic library and three positive clones were obtained. Analysis of these showed two to be identical but all to contain a 6 kb *Sal*I fragment which hybridised to the original PCR product and was therefore anticipated to contain the γ-tubulin gene. The full length coding sequence of the γ-tubulin gene was obtained by sub-cloning portions from this original *Sal*I fragment and by sequencing both strands. The sequence at the 5′ end was confirmed by analysis of a cDNA clone obtained using RT-PCR using internal γ-tubulin primers and a spliced leader primer specific to the *trans*-spliced leader sequence that is present on all *T. brucei* mRNAs.

Sequence of the *T. brucei* γ-tubulin

The full DNA sequence of the *T. brucei* γ-tubulin and its translation is shown in Fig. 1. The trypanosome γ-tubulin amino acid sequence showed around 67% identity with both the human and *Xenopus* sequences and 60% with *Aspergillus*. There was some variation in comparison with

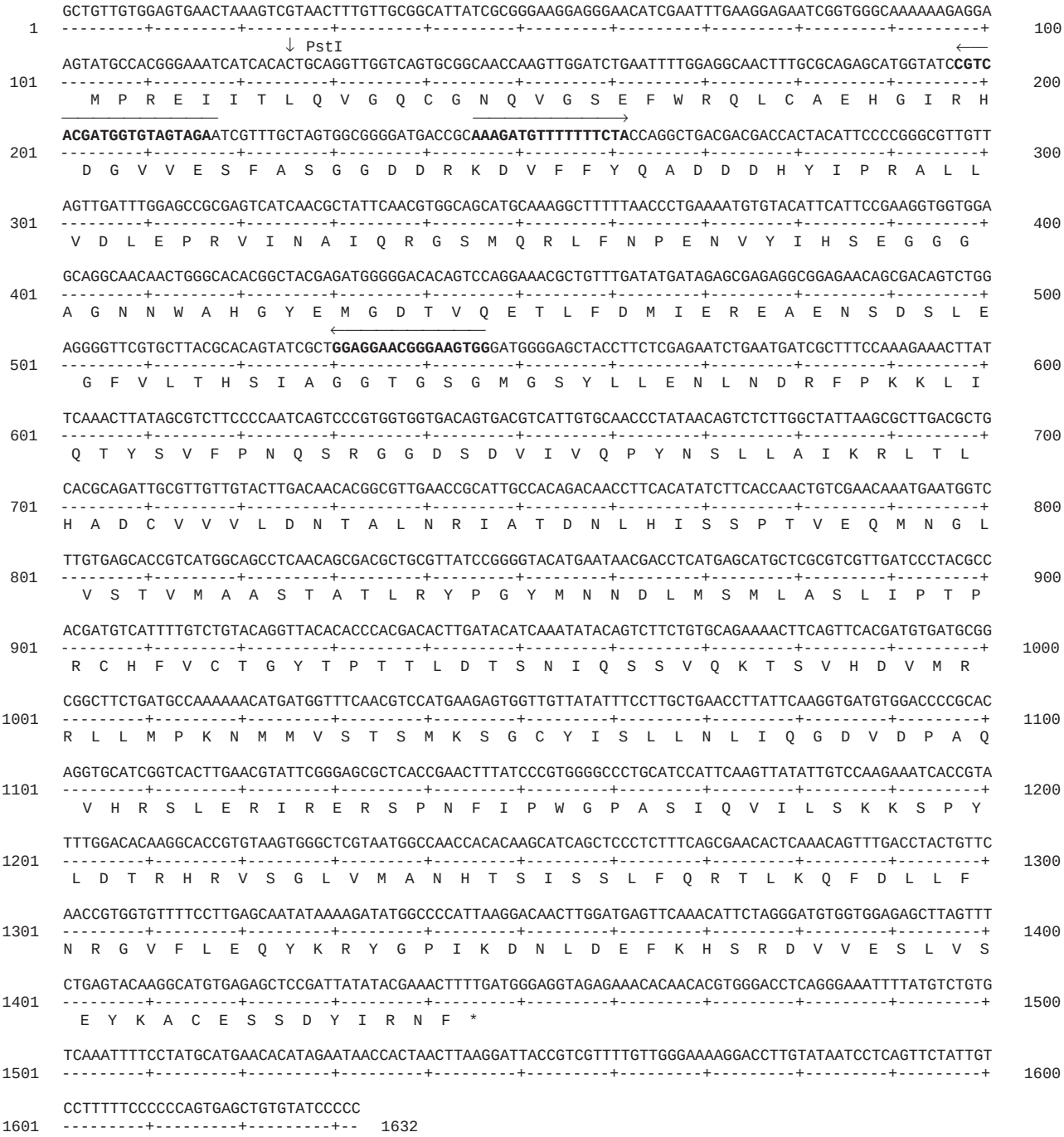

Fig. 1. Nucleotide sequence and deduced amino acid sequence of the *T. brucei brucei* γ-tubulin gene. The sequence derived from a genomic clone comprises the full-length coding sequence and partial 5′ and 3′-untranslated regions. Nucleotides indicated in bold represent the site of PCR primers used in the cloning and sequencing of the gene, the arrows above these sequences indicate sense (→) or antisense (←) primers. The single *Pst*I site, towards the 5′end of the coding sequence, is the site used to generate the histidine-tagged γ-tubulin fusion protein. This sequence has been submitted to the EMBL database, accession number Y07591.

other protozoal γ-tubulins including 57% identity with the malarial parasite, *Plasmodium falciparum*, 60% with *Euplotes* and only 41% with *Entamoeba* (Fig. 2). The trypanosome gene encodes a protein of 447 amino acids and comparison with the sequence from other evolutionarily diverse organisms shows high sequence conservation in the N terminal portion of the molecule. The C terminal portions are generally less well conserved and interestingly the *T. brucei* γ-tubulin gene encodes an aromatic C terminal residue (phenylalanine).

Expression of the γ-tubulin message

Trypanosome genes are often arranged as tandem duplications in the genome. However, restriction analysis of the large

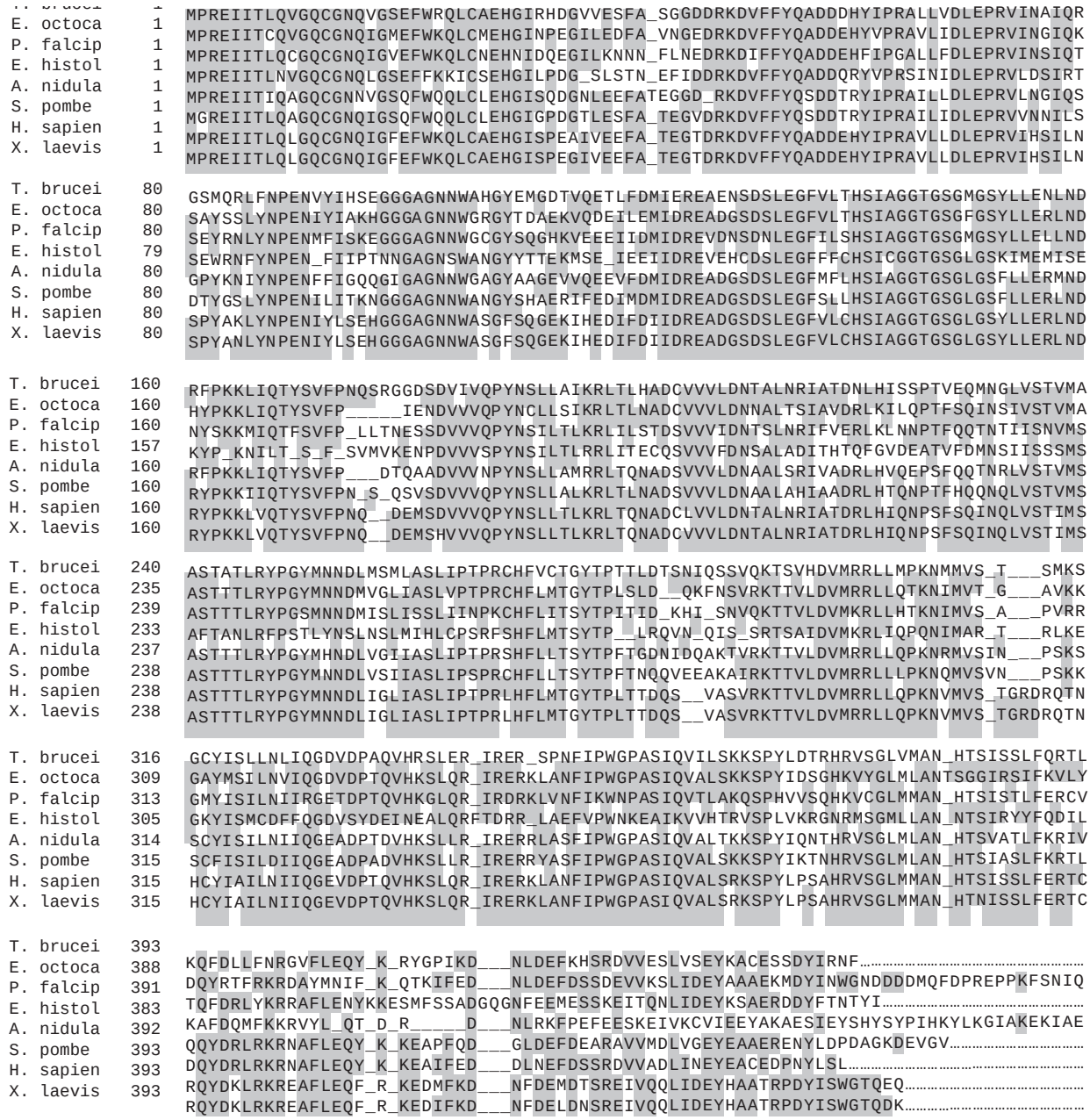

Fig. 2. Alignment of deduced amino acid sequences of γ-tubulins. Boxed sequences represent the consensus amino acid at each position. Nucleotide sequences were obtained from the GenBank and EMBL databases using the GCG program: *E. octocarinatus* (Liang and Heckmann, 1993), *P. falciparum* (Maessen et al., 1993), *Entamoeba histolytica* (accession no. U20322), *A. nidulans* (Oakley and Oakley, 1989), *S. pombe* (Stearns et al., 1991; Horio et al., 1991), *H. sapiens* (Zheng et al., 1991) and *X. laevis* (Stearns et al., 1991). This figure was compiled using MacMolly Tetra (Schoeneberg et al., 1994).

genomic clones containing the γ-tubulin gene suggested that it was present in only a single copy. Moreover, Southern blot analysis also indicated the presence of a single gene per haploid genome (data not shown). Northern blot analysis of poly(A)$^+$ RNA isolated from cultured procyclic (tsetse-gut form) trypanosomes indicated a major transcript of around 2.1 kb with a more minor band at 3.6 kb (Fig. 3). Even the major 2.1 kb transcript could not be detected in northern blots of total RNA indicating that the γ-tubulin mRNA is likely to be low abundance in comparison to those of the α- and β-tubulins of this organism. The minor 3.6 kb fragment does not represent cross-hybridisation with α- or β-tubulin mRNAs but may be explained as partially processed intermediates, given that trypanosomes possess polycistronic transcription. The African trypanosome life cycle contains a number of different cell types including the insect gut procyclic form and two major mammalian bloodstream phase cell types - short slender and stumpy cells. Using RT-PCR with a spliced leader and internal γ-tubulin sequence primers it was possible to obtain a diagnostic product from each of these stages of the life cycle (data not shown), indicative of the constitutive expression of the γ-tubulin gene in all the major stages of the parasites' life cycle.

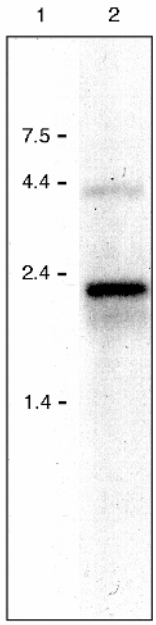

Fig. 3. Northern hybridisation of *T. brucei brucei* procyclic poly(A)$^+$ RNA with a *T. brucei* γ-tubulin probe. The size and position of migration of RNA standards (Gibco-BRL) is indicated in lane 1. A [^{32}P]UTP labelled riboprobe, derived from the original 310 bp γ-tubulin PCR fragment was used to probe a northern blot of poly(A)$^+$ RNA isolated from 1.3 mg total RNA. A major transcript of approximately 2.1 kb is detected, with a larger, minor hybridising band of approximately 3.6 kb (lane 2).

Anti-γ-tubulin antibodies

Early studies with monoclonal and polyclonal antibodies raised to heterologous γ-tubulins suggested that they were unable to recognise the trypanosome γ-tubulin. After sequencing of the gene this may be explained by small differences in the peptides used to raise such antibodies. Hence, we decided to raise a polyclonal sera to the trypanosome γ-tubulin that could be used in immunolocalisation studies. A 2.7 kb *Pst*I fragment encompassing almost all the coding sequence except for the first 23 nucleotides was cloned in frame into *E. coli* expression vector pTrchis. Expression of this plasmid in *E. coli* produced a fusion protein that was purified on a Nickel column. The purified γ-tubulin fusion protein was then run on SDS-PAGE, blotted to nitrocellulose and the major band excised and used as immunogen to raise specific anti-γ-tubulin antibodies in rats. Even though this polyclonal antibody was raised against the trypanosome γ-tubulin it showed good selectivity and cross reactivity for γ-tubulin in other species. Fig. 4 shows an example of this specificity in that the antibody clearly detects the centrosomal area of Vero cells both in interphase and in mitosis. The preimmune serum was negative and was used as a control in such experiments. The polyclonal antibody therefore recognises the now well established pattern of γ-tubulin localisation in mammalian cells.

The specificity of this polyclonal antibody for the *T. brucei* γ-tubulin protein was illustrated by immunoblotting using an affinity selected fraction of the serum against both the *E. coli* fusion protein and the endogenous trypanosome protein (Fig. 5). The antibody recognises a single band in extracts of *E. coli* which have been induced to express the γ-tubulin fusion protein (Fig. 5, lane 2). No such band is present in the uninduced *E. coli* extract (Fig. 5, lane 1). The antiserum also recognises a band of approximately 50 kDa in *T. brucei* cytoskeleton preparations (Fig. 5, lane 3). The spread of the band in lane 3 is the result of the extremely high protein loading needed to obtain this reaction, due to the very small amount of γ-tubulin in the extract.

Localisation of γ-tubulin in *T. brucei*

The trypanosome cell has a series of microtubule arrays and microtubule containing cell structures. These include a subpellicular corset of microtubules, the flagellum axoneme, the basal bodies and the intranuclear mitotic spindle (Sherwin and Gull, 1989a). Moreover, DAPI staining of the kinetoplast (mitochondrial DNA) and nucleus allows individual cells to be identified and positioned at particular points in the cell cycle (Woodward and Gull, 1990).

Immunofluorescence detection of γ-tubulin in cells showed a much more restricted localisation than is the case for α- and β-tubulin, as seen in Fig. 6. Here a series of images of the cell have been captured using phase (series 1), DAPI (series 4) and anti-γ-tubulin immunofluorescence (series 2, 3). Merged images are shown in series 5 (DAPI and fluorescence) and series 6 (phase and fluorescence). Cells throughout the population showed a general low level fluorescence, often punctate,

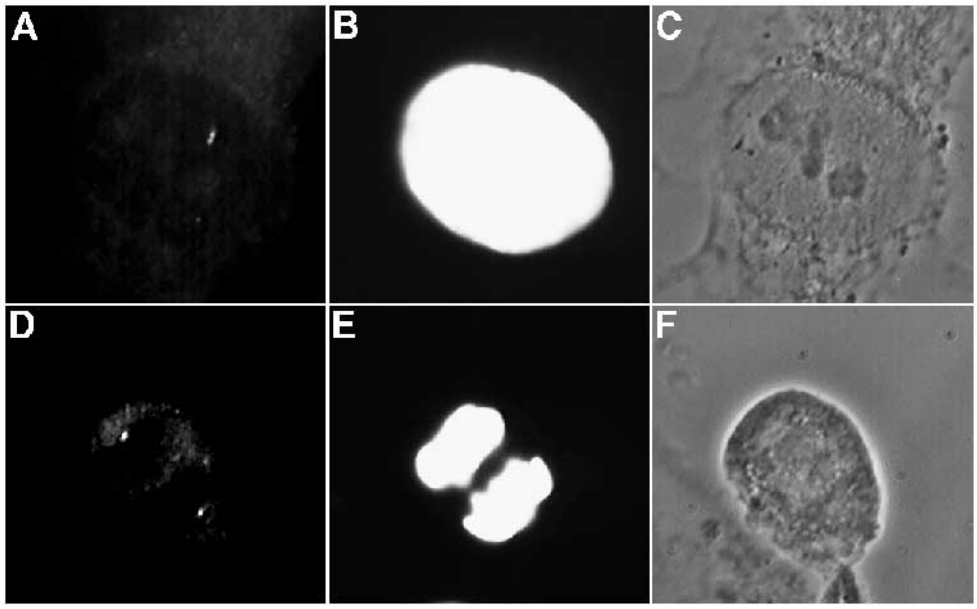

Fig. 4. Localisation of γ-tubulin to the centrosome of Vero cells using a polyclonal antiserum raised against a *T. brucei* γ-tubulin fusion protein. Anti-γ-tubulin staining was detected by indirect immunofluorescence using an FITC-conjugated second antibody. Use of the preimmune serum under the same conditions was negative (data not shown). (A) The anti-γ-tubulin staining of an interphase Vero cell, the corresponding DAPI and phase-contrast images are shown in B and C. (D, E and F) The characteristic anti-γ-tubulin staining pattern of a cell undergoing mitosis: (D) FITC-image showing γ-tubulin localisation, (E) DAPI-image showing the position of the chromosomes during anaphase, (F) phase-contrast image of the same cell.

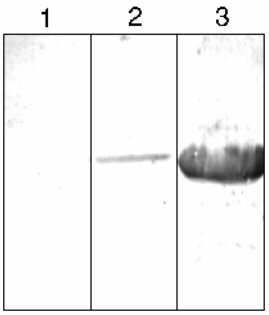

Fig. 5. The immunoblotting pattern revealed by the anti-*T. brucei* γ-tubulin polyclonal antibody. A whole cell extract of uninduced *E. coli* containing the *T. brucei* γ-tubulin gene is shown in lane 1, while lane 2 shows a whole cell sample of the same *E. coli* after induction for four hours. Lane 3 shows the immunostaining pattern obtained on cytoskeletal extracts of *T. brucei* procyclic cells.

over the cell body. Whilst at first sight this might be taken as background staining it was a regular occurrence and comparison with pre-immune and irrelevant sera indicated that although at a very low level and dispersed, the pattern may be significant. It is difficult to record this dispersed, low level, punctate fluorescence, however, it is illustrated in Fig. 6, series 2. This fluorescence is at the very extremes of detection in these very small cells but it is worth recording since it may reflect a very small distributed γ-tubulin component within the many individual microtubules of the sub-pellicular array. In addition to this pattern throughout the population, we also recorded

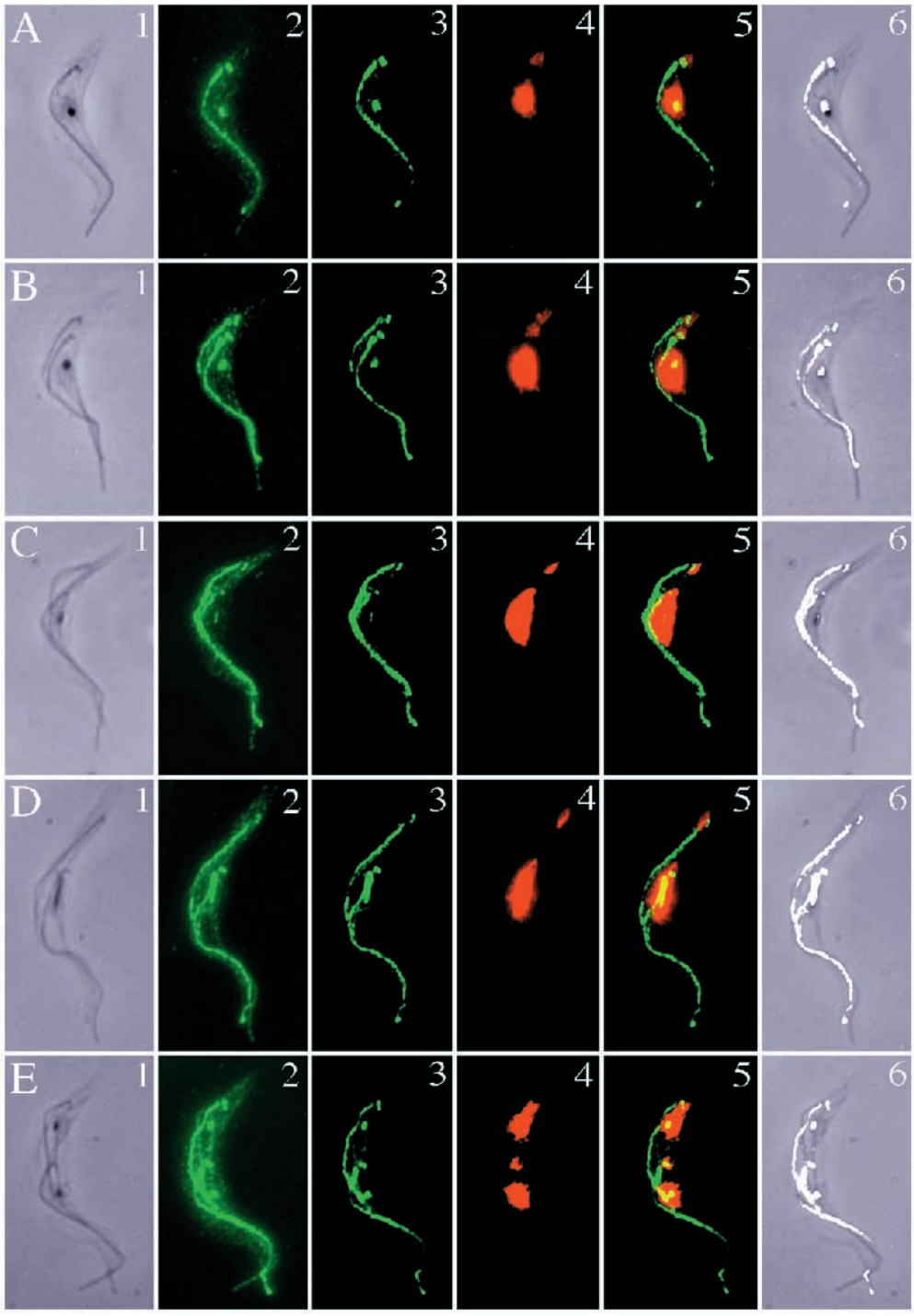

Fig. 6. The results of immunofluorescence on *T. brucei* cytoskeletons using the anti-γ-tubulin polyclonal antibody. The panel consists of a series of cytoskeletons which are at progressing stages of the cell cycle: (A) a cell early in the cell cycle, (B) the basal bodies have duplicated and a short new flagellum is present, (C) a cell entering mitosis, (D) a cell in mitosis, (E) a cell in late mitosis. Several views of each cytoskeleton are shown to illustrate various points. Series 1 shows the phase contrast of the cytoskeleton while series 2 shows an unadjusted image of the γ-tubulin staining pattern (green). Series 3 represents a refined image to show the major staining pattern of the γ-tubulin polyclonal antibody (also green). Series 4 shows the DNA content of the cytoskeletons (red). The γ-tubulin staining pattern (green) and the DNA content (red) are shown merged together in series 5 to show the relative positioning. Finally the γ-tubulin staining pattern (white) is shown superimposed on the phase contrast image in series 6.

localised patterns of fluorescence that varied with the stage of the cell cycle (Fig. 6A-E). Cells in the early period of the cell cycle showed 4 major sites of anti-γ-tubulin staining: a dot in the nucleus closely associated with the nucleolus, the basal bodies, the anterior tip of the cell and a punctate line following the region of the flagellum attachment zone. Such a pattern is seen in the cell in Fig. 6A. The merged images (series 5, 6) clearly show the bright dot is located at the anterior tip of the cell and not the flagellum tip. Moreover, such image merging techniques show that the punctate line follows the flagellum attachment zone in the cell rather than the flagellum axoneme itself. Also of note is a distinct gap in staining between the basal bodies and the flagellum attachment zone. After S phase the replicated kinetoplast DNA segregates and can be seen closely associated with the basal bodies of both the old and new flagellum (Robinson and Gull, 1991). In such cells (Fig. 6B) anti-γ-tubulin staining is associated with both sets of basal bodies and both flagellum attachment zones. Moreover, when the cell enters mitosis there appears to be a two dot staining pattern in the nucleus which then elongates to form a bar-shaped region in immunofluorescence corresponding to the developing mitotic spindle (Fig. 6C,D). In late mitotic cells the anti-γ-tubulin immunofluorescence pattern in the cytoplasmic regions is essentially the same as that in the earlier stages of the cell cycle. However, in such cells there are now 2 dots in the elongated nucleus which appear to localise with the poles of the extended intranuclear spindle (Fig. 6E).

The specific position of the punctate line of fluorescence along the flagellum attachment zone and the low level dispersed fluorescence over the cell body was studied further by immuno electron microscopy (Fig. 7). Studies of mild detergent extracted cytoskeletons revealed that areas of the sub-pellicular array of microtubules showed small but discrete localised aggregates of gold particles (Fig. 7D), indicating that these represented the ultrastructural equivalents of the low level cell body fluorescence seen at the light microscope level and

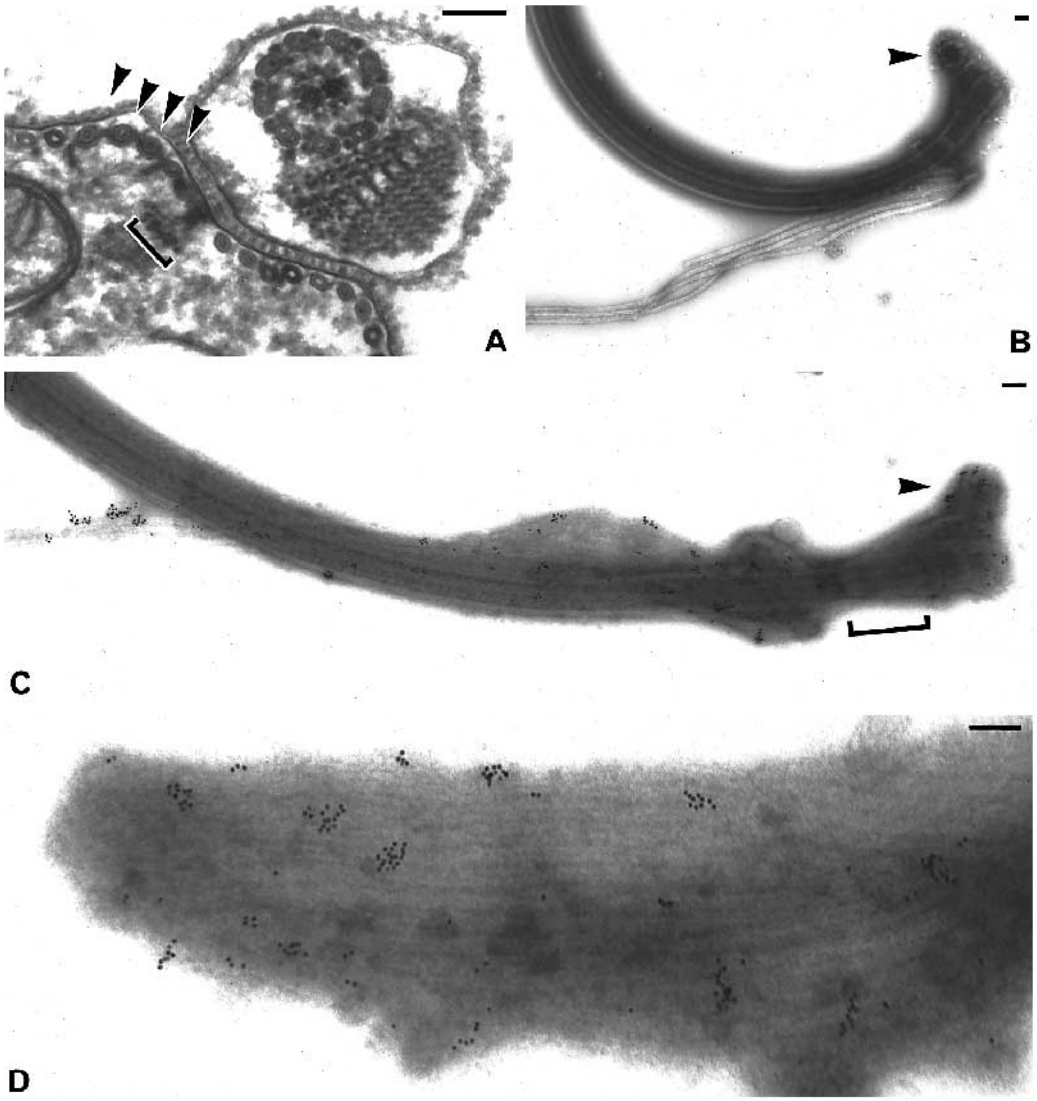

Fig. 7. Electron microscope studies of the four specialised sub-pellicular microtubules. A thin section TEM micrograph of the flagellum area in whole cells is depicted in A. The 9 + 2 axoneme is clearly visible together with the paraflagellar rod (PFR) structure which make up the flagellum. The flagellum is adhered to the cell body via a filamentous structure which emanates from the PFR, crosses the flagellum and cell body membranes and is clearly visible in the cell body (square bracket) where it interrupts the regular spacing of the sub-pellicular microtubules. Four of these microtubules, immediately left of the filamentous structure when viewed from the posterior end of the cell, are morphologically distinct in that they are bound by a portion of the ER (arrowheads). These four microtubules can be isolated together with the flagellum and the pro-basal body (arrowhead), shown by whole mount negative stain EM in B. Thus the four microtubules are biochemically different. Immunogold EM studies of such a preparation (C) shows that the anti-γ-tubulin polyclonal antibody labels both the pro-basal body (arrowhead) and the mature basal body subtending the flagellum. Notice that only the proximal end of the mature basal body is labelled and no further labelling is seen down to the basal plate (square bracket). The four microtubules are also clearly labelled. A portion of the anterior tip of the cytoskeleton is shown in D, after immunogold labelling with the anti-γ-tubulin polyclonal antibody. The labelling occurs in very discretely localised groups which may represent the punctate pattern seen on the cell body by immunofluorescence. Bars, 0.1 μm.

suggestive that γ-tubulin may indeed be localised to specific punctate sites within this array. The rather intense line of fluorescence along the flagellum attachment zone (FAZ) is intriguing. We know from our earlier studies that this consists of a filament system plus four very specific microtubules (Sherwin and Gull, 1989a; Bastin et al., 1996). These four microtubules are seen in a cross section in Fig. 7A, they have smooth endoplasmic reticulum attached to them and are invariably positioned on the immediate left-hand side of the FAZ filament when viewed from the posterior of the cell. This position can be concluded by using the orientation of the dynein arms of the flagellum axoneme as a marker. These four microtubules have a different origin to others within the subpellicullar array in that they emerge from the region of the basal bodies in the flagellum pocket area. When all of the membranes are removed during the preparation of a cytoskeleton, it is impossible to then ascertain the precise identity of the four microtubules. However, if such trypanosome cytoskeletons are then treated with high salt then the sub-pellicular microtubules are removed leaving the flagellum axoneme and under certain conditions these four specific microtubules. Fig. 7B shows a negatively stained image of such a preparation. When we used such preparations in our immunoelectron microscopy studies with the anti-γ-tubulin polyclonal, Fig. 7C, we found that these four microtubules were decorated with gold particles indicating that the line of fluorescence along the cell was a reflection of labelling of this FAZ associated set of differentiated microtubules. The labelling of the pro-basal body and the mature basal body is also cleary visible. The staining on the mature basal body subtending the flagellum is restricted to the proximal end of the basal body. There is a clear gap in labelling from the proximal end of the basal body down to the basal plate, equivalent to the gap seen in immunofluorescence between the basal bodies and the four microtubule subset. Fig. 7D shows a portion of the anterior end of a cytoskeleton. The immunogold label appears in discrete clusters over the cytoskeleton which may be analogous to the punctate immunofluorescence staining pattern on the sub-pellicular microtubule array.

DISCUSSION

The γ-tubulin gene of *T. brucei* appears to be present as a single copy per haploid genome and is transcribed in all of the life cycle stages studied (tsetse midgut and two mammalian blood stream stages). The gene copy number contrasts with the other two members of the tubulin superfamily, α- and β-tubulin, whose genes occur as tandem (α, β) repeats with approximately 15 copies of these genes in the genome (Seebeck et al., 1983; Thomashow et al., 1983). This disparity in gene copy number is reflected in the relative abundance of the α, β and γ-tubulin transcripts. While α- and β-tubulin mRNA is relatively abundant, a γ-tubulin transcript can only be detected by northern analysis in a poly(A)$^+$-selected fraction of RNA. In other eukaryotes it is estimated that the γ-tubulin gene product is only present at approximately 1% of the level of α- and β-tubulin (Stearns et al., 1991). This is reflected in the localisation of γ-tubulin at discrete sites (MTOCs) compared with the presence of α- and β-tubulin throughout the microtubule cytoskeleton.

Comparison of the deduced amino acid sequence of the *T. brucei* γ-tubulin gene with other γ-tubulin sequences shows that in general it maintains the amino acids previously determined to be conserved between all tubulins, as well as those sites thought to be involved in GTP binding (Burns, 1995a). There are unique residues in the *T. brucei* γ-tubulin and these are distributed throughout the sequence. There is a high degree of conservation within the γ-tubulin family and *T. brucei* exhibits blocks of sequence that are often conserved between γ-tubulins including the KDVFFY sequence used in the original PCR amplification. However, the *T. brucei* sequence follows the pattern that there appears to be greater sequence conservation at the N terminus than the C terminus and this is seen in the heterogeneity in length of γ-tubulin gene products amongst different organisms, accounted for mainly by the C-terminal extensions. Unlike the α- and β-tubulins which have an acidic C terminus, this is not the case for γ-tubulins. The *T. brucei* γ-tubulin does not appear to show a major pattern of homology with other protozoal γ-tubulins relative to those of other organisms, the overall degree of amino acid identity being greatest with the *Xenopus* and human sequences. Finally, it is worth noting that the consensus peptide RKRnAF which Burns and Surrridge (1994) have hypothesised to be involved in TCP1α chaperonin mediated folding is rather unusual in *T. brucei* (FNRGVF).

It is of interest that the *T. brucei* γ-tubulin gene is the only γ-tubulin sequence to date to encode a C-terminal aromatic residue (a phenylalanine). The α- and β-tubulins of *T. brucei* both encode a tyrosine residue at this position, however, only the α-tubulin is able to enter the detryrosination and tyrosination cycle in which the C-terminal tyrosine is a substrate for tubulin carboxypeptidase and can be returned by tubulin tyrosine ligase. The presence of tyrosinated α-tubulin is a marker for newly formed microtubules in the *T. brucei* cytoskeleton (Sherwin et al., 1987; Sherwin and Gull, 1989b). Whether or not γ-tubulin has the potential to enter a comparable pathway is unknown and the low level of γ-tubulin expression makes it a difficult problem to address. However, there is no great similarity between the C terminus of trypanosome α- and γ-tubulins and hence since this sequence seems to be involved in substrate recognition by the tyrosination cycle enzymes (Ersfeld et al., 1993) it then seems likely that this post translational modification is restricted to α-tubulin in trypanosomes.

The *T. brucei* γ-tubulin gene product shows a complex localisation pattern as determined by immunofluorescence and immunogold electron microscopy. There are 5 sites within the cell showing varying levels of γ-tubulin signal: the sub-pellicular array, the anterior tip of the cell body, the intra nuclear dot/spindle poles, the basal bodies and the 4 stable microtubules of the flagellum attachment zone.

Location in, or very close to, the basal body (the microtubule organising centre for the flagellum) is reminiscent of previous reports of γ-tubulin in mouse sperm basal bodies (Palacios et al., 1993) and ciliated epithelial cells (Muresan et al., 1993). Stearns and Kirschner (1994) did not detect γ-tubulin in basal bodies of *Tetrahymena*, however, Dibbayawan et al. (1995) were able to show that γ-tubulin was associated with centrioles in HeLa cells and the basal body complex in *Chlamydomonas.* There is good evidence for the association of γ-tubulin with the ends of microtubules in centrosomes of early *Drosophila*

embryos, these centrosomes contain only partially formed centrioles. Moudjou et al. (1996) have again recently pointed out the close association of γ-tubulin with centrioles as well as the fact that in mammalian cells over 80% of total γ-tubulin may reside as cytosolic forms. The proximal region of the basal body represents the minus ends of the triplet microtubules. It may be significant therefore, that γ-tubulin is associated with the pro-basal body and only the proximal end of the mature basal body. As the pro-basal body is formed first and then, at a later point, elongates to form the mature basal body, this suggests that no further association of γ-tubulin along the basal body is required during elongation to produce a mature functional basal body. Such an association with the proximal end of centrioles in animal cells has also been reported (Fuller et al., 1995; Lange and Gull, 1996).

The detection of the nuclear location of γ-tubulin in *T. brucei* is interesting. In trypanosomes the mitotic spindle poles are not organised by discrete identifiable structures such as the SPBs of yeasts and other fungi. Whilst in yeasts the SPB is even identifiable in interphase, in trypanosomes it is difficult to identify any such polar structure. The spindle poles appear to terminate in the nucleoplasm close to the nuclear envelope. Thus, the detection of γ-tubulin in the spindle and at the poles suggest the existence of an, as yet, unidentified polar structure. In the interphase nuclei in Fig. 6, series A and B, the γ-tubulin staining coincides with a dark structure within the extracted nucleus, which probably represents the nucleolar remnant. In other organisms, such as *Physarum* plasmodia, which form an intranuclear spindle without discrete spindle pole structures both immunofluorescence and electron microscopy studies have shown that initially spindle MTOCs seem to be localised immediately adjacent to the nucleolus (Havercroft and Gull, 1983; Schedl et al., 1984). The fact that the γ-tubulin dot is seen in the nucleus even during interphase is reminiscent of the *S. pombe* SPB where γ-tubulin can be detected at all stages of the cell cycle yet microtubules are only subtended during mitosis. This suggests that in *T. brucei*, as in *S. pombe,* it is likely that location of γ-tubulin in a structure is not sufficient for microtubule nucleation and some activation is necessary (Horio et al., 1991; Masuda, et al., 1992). The intranuclear localisation of some form of γ-tubulin in *T. brucei* parallels the recent description of the location of γ-tubulin on both intranuclear and cytoplasmic plaques of the *Saccharomyces cerevisiae* SPB (Spang et al., 1996).

The sub-pellicular corset of microtubules surrounding the *T. brucei* cell lacks any obvious MTOC and we have previously shown that new microtubules are intercalated into this two-dimensional lattice between old microtubules (Sherwin and Gull, 1989b). γ-Tubulin, in other systems, has been shown to locate to the minus ends of microtubules and we have recently shown that the microtubules of the *T. brucei* sub-pellicular corset are of varying lengths and thus have staggered ends within the array. Their polarity is such that their minus ends point towards the anterior end of the cell (Robinson et al., 1995). Thus, one explanation for the low level punctate staining over the cell body and the bright dot at the anterior end of the cell would be that small amounts of γ-tubulin are located at the minus ends of each sub-pellicular microtubule. The absolute tapered anterior tip of the cell is therefore the only position in the corset where more than a few microtubules end. Thus, this would lead to a more concentrated γ-tubulin signal. In this scenario γ-tubulin might be marking the ends of individual microtubules rather than marking the site of a discrete MTOC for this complex. Our immunogold and immunofluorescence labelling suggests that the γ-tubulin signal, although at a low level in this sub-pellicular corset, is significant. However, it is not of sufficient resolution to determine whether the signal is actually located at the ends of individual microtubules or at other sites. Visualisation of the ends of separated microtubules requires heavy detergent treatment to remove all membranes and then stringent aldehyde fixation and stabilisation of partly opened microtubule sheets, plus critical point drying. Our attempts to use such protocols which require more extensive aldehyde fixations have not been successful with our present antibodies. Novákova et al. (1996) have also noted that a range of other anti-γ-tubulin antibodies do not work well after aldehyde fixation of cells and cytoskeletons.

The labelling of the 4 stable microtubules of the flagellum attachment zone is intriguing and unusual. These microtubules, although present within the sub-pellicular corset, are distinctly different from the others of that complex. They are always associated with smooth endoplasmic reticulum and since they emerge from a distinct site close to the basal body in the flagellum pocket area it appears likely that they have a different polarity to all the other microtubules of the corset (Robinson et al., 1995). Moreover, they are very stable to conditions such as high salt which depolymerise the other corset microtubules. The pattern of labelling with our anti-γ-tubulin antibody is rather suggestive of the fact that γ-tubulin is associated with the microtubules rather than being an integral component and we do know that we can label these microtubules with anti-α-tubulin antibodies. Therefore, even though it is known that γ-tubulin can form tubular structures when overexpressed in some cells (Shu and Joshi, 1995) we do not, at present, favour the idea that these 4 microtubules actually contain γ-tubulin. Although much recent work on γ-tubulin suggests that it is located mainly at the minus ends of microtubules it is interesting that immunofluorescence and immunogold labelling along microtubules has been seen in some plant cells (Liu et al., 1993; Hoffman et al., 1994), some particular microtubules within the spindle of mammalian cells (Lajoie-Mazenc et al., 1994) and in the spindle of the micronucleus of *Euplotes octocarinatus* (Liang et al., 1996). These and other reports raise the general difficulty of immunodetection of low abundance proteins or dispersed amounts of a protein such as γ-tubulin. Detection at ‘centrosomal’ or SPB-body types of MTOC where there is a concentration of microtubule minus ends is well established. However, determining whether low level signals seen along certain types of microtubules represent significant signals is much more difficult. However, it is worth noting that even in the very earliest reports of γ-tubulin detection Oakley et al. (1990) did not rule out the significance of a very low level of fluorescence along the spindle in *Aspergillus*.

We do see this signal along the 4 microtubules in *T. brucei* as significant since it correlates extremely well with their distinct position, structural organisation and stability in contrast to immediately adjacent microtubules. Also, the fact that the many other immediately adjacent microtubules of the sub-pellicular corset do not label is of useful internal control. Therefore, these 4 specialised microtubules in *T. brucei* may just represent more highly defined and extreme examples of specific microtubules that have a form of associated γ-tubulin

located not only at their minus ends. Alternatively, a more radical possibility, is that this antibody is also detecting a so far uncharacterised form of tubulin which is more closely related to γ-tubulin than to α- or β-tubulin (Burns, 1995b). Thus, the pattern of γ-tubulin localisation in *T. brucei* cells is extremely interesting. It has allowed a better definition of the intranuclear spindle pole and basal body MTOCs. Its role and significance both in these structures and in the other areas of the cell such as the 4 specialised microtubules is now best approached by studies of the phenotypes produced by perturbing γ-tubulin expression. These molecular genetic approaches are now becoming possible in trypanosomes and coupled with the very precise pattern of the microtubule cytoskeleton should provide for some interesting analyses of γ-tubulin function in a cell expressing many different forms of microtubule structure.

This work was supported by a Wellcome Trust Programme and Wellcome Trust Equipment Grants. We thank Liz and Berl Oakley for early privileged information and for generous discussions. We thank Roy Burns for discussions of interesting aspects of γ-tubulin sequence homologies and other members of our lab. for assistance and helpful conversations. This work benefited from the use of the SEQNET facility, at CCL, Daresbury laboratory.

REFERENCES

Bastin, P., Matthews, K. R. and Gull, K. (1996). The paraflagellar rod of kinetoplastida: Solved and unsolved questions. *Parasitol. Today* **12**, 302-307.

Brun, R. and Schönenburger, M. (1979). Cultivation and in vitro cloning of procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. *Acta Tropica* **36**, 289-292.

Burns, R. G. and Surridge, C. D. (1994). Functional role of a consensus peptide which is common to α-, β- and γ-tubulin, to actin and centractin, to phytochrome A, and to the TCP1α chaperonin protein. *FEBS Lett.* **347**, 105-111.

Burns, R. G. (1995a). Analysis of the γ-tubulin sequences: implications for the functional properties of γ-tubulin. *J. Cell Sci.* **108**, 2123-2130.

Burns, R. G. (1995b). Identification of two new members of the tubulin family. *Cell Motil. Cytoskel.* **31**, 255-258.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Dibbayawan, T. P., Harper, J. D. I., Elliot, J. E., Gunning, B. E. S. and Marc, J. (1995). A γ-tubulin that associates specifically with centrioles in HeLa cells and the basal body complex in *Chlamydomonas*. *Cell Biol. Int.* **19**, 559-567.

Ersfeld, K., Wehland, J., Plessmann, U., Dodemont, H., Gerke, V. and Weber, K. (1993). Characterisation of the tubulin tyrosine ligase. *J. Cell Biol.* **120**, 725-732.

Feinberg, A. P. and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**, 6-13.

Felix, M.-A., Antony, C., Wright, M. and Maro, B. (1994). Centrosome assembly in vitro: role of γ-tubulin recruitment in *Xenopus* sperm aster formation. *J. Cell Biol.* **124**, 19-31.

Fuchs, U., Moepps, B., Maucher, H. P. and Schraudolf, H. (1993). Isolation, characterisation and sequence of a cDNA encoding γ-tubulin protein from the fern *Anemia phyllitidis* L. Sw. *Plant Mol. Biol.* **23**, 595-603.

Fuller, S. D., Gowen, B. E., Reinsch, S., Sawyer, A., Buendia, B., Wepf, R. and Karsenti, E. (1995). The core of the mammalian centriole contains gamma-tubulin. *Curr. Biol.* **5**, 1384-1393.

Gueth-Hallonet, C., Antony, C., Aghion, J., Santa-Maria, A., Lajoie-Mazenc, I., Wright, M. and Maro, B. (1993). γ-Tubulin is present in acentriolar MTOCs during early mouse development. *J. Cell Sci.* **105**, 157-166.

Havercroft, J. C. and Gull, K. (1983). Demonstration of different patterns of microtubule organization in Physarum polycephalum myxamoebae and plasmodia using immunofluorescence microscopy. *Eur. J. Cell Biol.* **32**, 67-74.

Hoffman, J. C., Vaughn, K. C. and Joshi, H. C. (1994). Structural and immunocytochemical characterization of microtubule-organizing centers in Pteridophyte spermatogenous cells. *Protoplasma* **179**, 46-60.

Horio, T., Uzawa, S., Jung, M. K., Oakley, B. R., Tanaka, K. and Yanagida, M. (1991). The fission yeast γ-tubulin is essential for mitosis and is localised at microtubule organising centres. *J. Cell Sci.* **99**, 693-700.

Horio, T. and Oakley, B. R. (1994). Human γ-tubulin functions in fission yeast. *J. Cell Biol.* **126**, 1465-1473.

Imboden, M. A., Laird, P. W., Affolter, M. and Seebeck, T. (1987). Transcription of the intergenic regions of the tubulin cluster of *Trypanosoma brucei:* evidence for a polycistronic transcription unit in a eukaryote. *Nucl. Acids Res.* **15**, 7357-7368.

Joshi, H. C., Palacios, M. J., McNamara, L. and Cleveland, D. W. (1992). γ-Tubulin is a centrosomal protein required for cell cycle-dependent microtubule nucleation. *Nature* **356**, 80-83.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lajoie-Mazenc, I., Tollon, Y., Detraves, C., Julian, M., Moisand, A., Gueth-Hallonet, C., Debec, A., Salles-Passador, I., Puget, A., Mazarguil, H., Raynaud-Messina, B. and Wright, M. (1994). Recruitment of antigenic gamma-tubulin during mitosis in animal cells: presence of gamma-tubulin in the mitotic spindle. *J. Cell Sci.* **107**, 2825-2837.

Lange, B. M. H., Sherwin, T., Hagan, I. M. and Gull, K. (1995). The basics of immunofluorescence video microscopy for mammalian and microbial systems. *Trends Cell Biol.* **5**, 328-332.

Lange, B. M. H. and Gull, K. (1996). Structure and function of the centriole in animal cells: progress and questions. *Trends Cell Biol.* **6**, 348-352.

Liang, A. and Heckmann, K. (1993). The macronuclear γ-tubulin-encoding gene of *Euplotes octocarinatus* contains two introns and an in-frame TGA. *Gene* **136**, 319-322.

Liang, A., Ruiz, F., Heckmann, K., Klotz, C., Tollon, Y., Beisson, J. and Wright, M. (1996). Gamma-tubulin is permanently associated with basal bodies in ciliates. *Eur. J. Cell Biol.* **70**, 331-338.

Liu, B., Marc, J., Joshi, H. C. and Palevitz, B. A. (1993). A γ-tubulin-related protein associated with the microtubule arrays of higher plants in a cell cycle-dependent manner. *J. Cell Sci.* **104**, 1217-1228.

Maessen, S., Wesseling, J. G., Smits, M. A., Konings, R. N. H. and Schoenmakers J. G. G. (1993). The γ-tubulin gene of the malaria parasite *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **60**, 27-36.

Marschall, L. G., Jeng, R. L., Mulholland, J. and Stearns, T. (1996). Analysis of Tub4p, a yeast γ-tubulin-like protein: Implications for microtubule-organizing center function. *J. Cell Biol.* **134**, 443-454.

Masuda, H., Sevik, M. and Cande, W. Z. (1992). In vitro microtubule-nucleating activity of spindle pole bodies in fission yeast *Schizosaccharomyces pombe*: cell cycle-dependent activation in *Xenopus* cell-free extracts. *J. Cell Biol.* **117**, 1055-1066.

Moritz, M., Braunfeld, M. B., Sedat, J. W., Alberts, B. and Agard, D. A. (1995). Microtubule nucleation by γ-tubulin-containing rings in the centrosome. *Nature* **378**, 638-640.

Moudjou, M., Bordes, N., Paintrand, M. and Bornens, M. (1996). γ-Tubulin in mammalian cells: the centrosomal and the cytosolic forms. *J. Cell Sci.* **109**, 875-887.

Muresan, V., Joshi, H. C. and Besharse, J. C. (1993). γ-Tubulin in differentiated cell types: localisation in the vicinity of basal bodies in retinal photoreceptors and ciliated epithelia. *J. Cell Sci.* **104**, 1229-1237.

Nováková, M., Dráberová, E., Schürmann, W., Czihak, G., Viklicky, V. and Dráber, P. (1996). γ-Tubulin redistribution in taxol-treated mitotic cells probed by monoclonal antibodies. *Cell Motil. Cytoskel.* **33**, 38-51.

Oakley, C. E. and Oakley, B. R. (1989). Identification of γ-tubulin, a new member of the tubulin superfamily encoded by *mip*A gene of *Aspergillus nidulans*. *Nature* **338**, 662-664.

Oakley, B. R., Oakley, C. E., Yoon, Y. and Jung, M. K. (1990). γ-Tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*. *Cell* **61**, 1289-1301.

Palacios, M. J., Joshi, H. C., Simerly, C. and Schatten, G. (1993). γ-Tubulin reorganisation during mouse fertilisation and early development. *J. Cell Sci.* **104**, 383-389.

Pickett-Heaps, J. D. (1969). The evolution of the mitotic apparatus: An attempt at comparative ultrastructural cytology in dividing plant cells. *Cytobios* **3**, 257-280.

Robinson, D. R. and Gull, K. (1991). Basal body movement as a mechanism

for mitochondrial genome segregation in the trypanosome cell cycle. *Nature* **352**, 731-734.

Robinson, D. R., Sherwin, T., Ploubidou, A., Byard, E. H. and Gull, K. (1995). Microtubule polarity and dynamics in the control of organelle positioning, segregation and cytokinesis in the trypanosome cell cycle. *J. Cell Biol.* **128**, 1163-1172.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. 2nd edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain terminating inhibitors. *Proc. Nat. Acad. Sci. USA* **74**, 5463-5467.

Schedl, T., Burland, T. G., Gull, K. and Dove, W. F. (1984). Cell cycle regulation of tubulin RNA level, tubulin protein synthesis, and assembly of microtubules in *Physarum*. *J. Cell Biol.* **99**, 155-165.

Schlaeppi, K., Deflorin, J. and Seebeck, T. (1989). The major component of the paraflagellar rod of *Trypanosoma brucei* is a helical protein that is encoded by two identical, tandemly linked genes. *J. Cell Biol.* **109**, 1695-1709.

Schoeneberg, U., Vahrson, W., Priedemuth, U. and Wittig, B. (1994). Analysis and interpretation of DNA and protein sequences using MacMolly Tetra. 3rd. Edition, KAROI-Verl. Bornemann.

Seebeck, T., Whittaker, P. A., Imboden, M. A., Hardman, N. and Braun, R. (1983). Tubulin genes of *Trypanosoma brucei*: a tightly clustered family of alternating genes. *Proc. Nat. Acad. Sci. USA* **80**, 4634-4638.

Sherwin, T., Schneider, A., Sasse, R., Seebeck, T. and Gull, K. (1987). Distinct localization and cell cycle dependence of COOH terminally tyrosinolated α-tubulin in the microtubules of *Trypanosoma brucei brucei*. *J. Cell Biol.* **104**, 439-446.

Sherwin, T. and Gull, K. (1989a). The cell division cycle of *Trypanosoma brucei brucei*: timing of event markers and cytoskeletal modulations. *Phil. Trans. Roy. Soc. Lond. B.* **323**, 573-588.

Sherwin, T. and Gull, K. (1989b). Visualization of detyrosination along single microtubules reveals novel methods of assembly during cytoskeletal duplication in trypanosomes. *Cell* **57**, 211-221.

Shu, H.-B. and Joshi, H. C. (1995). γ-Tubulin can both nucleate microtubule assembly and self-assemble into novel tubular structures in mammalian cells. *J. Cell Biol.* **130**, 1137-1147.

Sobel, S. G. and Snyder, M. (1995). A highly divergent γ-tubulin gene is essential for cell growth and proper microtubule organization in *Saccharomyces cerevisiae*. *J. Cell Biol.* **131**, 1775-1788.

Spang, A., Geissler, S., Grein, K. and Schiebel, E. (1996). γ-Tubulin-like Tub4p of *Saccharomyces cerevisiae* is associated with the spindle pole body substructures that organise microtubules and is required for mitotic spindle function. *J. Cell Biol.* **134**, 429-441.

Stearns, T., Evans, L. and Kirschner, M. (1991). γ-Tubulin is a highly conserved component of the centrosome. *Cell* **65**, 825-836.

Stearns, T. and Kirschner, M. (1994). In vitro reconstruction of centrosome assembly and function: the central role of γ-tubulin. *Cell* **76**, 623-637.

Sunkel, C. E., Gomes, R., Sampaio, P., Perdigao, J. and Gonzalez, C. (1995). γ-Tubulin is required for the structure and function of the microtubule organizing center in *Drosophila* neuroblasts. *EMBO J.* **14**, 28-36.

Thomashow, L. S., Milhausen, M., Rutter, W. J. and Agabian, N. (1983). Tubulin genes are tandemly linked and clustered in the genome of *Trypanosoma brucei*. *Cell* **32**, 35-43.

Woodward, R. and Gull, K. (1990). Timing of nuclear and kinetoplast DNA replication and early morphological events in the cell cycle of *Trypanosoma brucei*. *J. Cell Sci.* **95**, 49-57.

Zheng, Y., Jung, M. K. and Oakley, B. R. (1991). γ-Tubulin is present in *Drosophila melanogaster* and *Homo sapiens* and is associated with the centrosome. *Cell* **65**, 817-823.

(Received 9 September 1996 - Accepted 31 October 1996)